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ANXA10 induction by interaction with tumor-associated macrophages promotes the growth of esophageal squamous cell carcinoma

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Abbreviations:
ANXA10, annexin A10; CXCL8, C-X-C chemokine ligand 8; ESCC, esophageal squamous cell carcinoma; IFN, interferon; IL, interleukin; M-CSF, macrophage colony stimulating factor; PBMC, peripheral blood mononuclear cell; PBMo, peripheral blood monocyte; TAM, tumor-associated macrophage; TECM, conditioned media of TE series esophageal squamous cell carcinoma cell line

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Tumor-associated macrophages (TAMs) have important roles in the growth, angiogenesis and progression of various tumors. Although we have demonstrated the association of an increased number of infiltrating CD204$^+$ TAMs with poor prognosis in esophageal squamous cell carcinomas (ESCCs), the roles of TAMs in ESCC remain unclear. Here, to study the effects of TAMs on the tumor microenvironment of ESCCs, we established a co-culture assay using a human ESCC cell line and TAM-like peripheral blood monocyte-derived macrophages and performed a cDNA microarray analysis between monocultured and co-cultured ESCC cell lines. Our qRT-PCR confirmed that in the co-cultured ESCC cell lines, *CYP1A1*, *DHRS3*, *ANXA10*, *KLK6* and *CYP1B1* mRNA were highly up-regulated; *AMTN* and *IGFL1* mRNA were down-regulated. We observed that the high expression of a calcium-dependent phospholipid-binding protein ANXA10 was closely associated with the depth of invasion and high numbers of infiltrating CD68$^+$ and CD204$^+$ TAMs and poor disease-free survival ($P = 0.0216$). We also found ANXA10 promoted the cell growth of ESCC cell lines *via* the phosphorylation of Akt and Erk1/2 pathways *in vitro*. These results suggest that ANXA10 induced by the interaction with TAMs in the tumor microenvironment is associated with cell growth and poor prognosis in human ESCC tissues.

KEYWORDS
ANXA10, cell growth, esophageal squamous cell carcinoma, tumor-associated macrophage, tumor microenvironment

Esophageal cancer is the sixth most common cause of cancer-related deaths with an estimated 455,800 new cases and 400,200 deaths in 2012 worldwide.[1] The overall 5-year survival of patients with esophageal cancer ranges from 15% to 25%.[2] Esophageal squamous cell carcinoma (ESCC) is one of the fatal diseases because of the rapid progression and the lack of early detection. ESCC is characterized by early lymphatic metastasis, which is the main cause of the poor prognosis in patients with this malignancy.[3] Even in superficial esophageal cancers invading no further than the submucosa, 30%–40% of the cases have regional lymph node metastasis.[4] Tobacco use and alcohol consumption are known to contribute to a high risk of ESCC and genetic polymorphisms including *ALDH2*, *CCND1*, *PTEN* and *PD-L1* have been identified as risks for ESCC.[5–8]

Tumor microenvironments are composed of non-tumor cells and their stroma such as fibroblasts, vascular cells, glial cells, smooth muscle cells, endothelial cells, fat cells and cells of the immune system. Macrophages are the most abundant cancer stromal cells involved in the host immune system. Macrophages have two different phenotypes; classically activated macrophage (M1) and alternatively activated macrophage (M2). M1 macrophages are induced by interferon-gamma (IFN-γ), whereas M2 macrophages are induced by interleukin (IL)-4 and IL-13.[9] Tumor-associated macrophages (TAMs) have differentiated into the M2 phenotype and contribute to the progression of disease. The protein CD163 and CD204 have been used as markers of M2 macrophages.[10,11] TAMs induce angiogenesis in the tumor microenvironment, suppress antitumor immunity and directly stimulate tumor cell proliferation. TAMs also participate in the formation of a cancer stem cell niche and pre-metastatic niche to promote tumor progression.[12]

We demonstrated an association between an increased number of infiltrating $CD204^+$ TAMs and the poor prognosis of ESCCs.[13] $CD204^+$ TAMs have been reported to correlate with poor prognosis in many cancers, including lung cancer, bladder cancer, breast cancer, ovarian cancer and uterine cervical adenocarcinoma.[14–18] However, the pathological mechanisms of TAMs on the tumor microenvironment of ESCC remain unclear. In the present study, we established a co-culture assay using human ESCC cell lines and TAM-like peripheral blood monocyte (PBMo)-derived macrophages to investigate the pathological roles of TAMs in ESCC. We further investigated the cancer-associated gene expression profile in ESCC cell lines co-cultured with TAM-like PBMo-derived macrophages by a cDNA microarray analysis.

MATERIALS AND METHODS

Cell lines and cell culture

Three human ESCC cell lines (TE-8, TE-9 and TE-15) were obtained from the RIKEN BioResource Center (Tsukuba, Japan). A short tandem repeat analysis of TE series ESCC cell lines was conducted at RIKEN and the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). We routinely propagated and prepared the conditioned media of TE series ESCC cell lines (TECM) as described elsewhere.[13]

Co-culture assay

Peripheral blood mononuclear cells (PBMCs) were obtained from healthy volunteer donors who provided informed consent. Purification of $CD14^+$ PBMo from the PBMCs, macrophage differentiation and induction of TAM-like polarization were performed as previously described.[19] A co-culture assay was established using ESCC cells (TE-8 and TE-9) in 6-well plate and TAM-like PBMo-derived macrophages in 0.4 μm pore membrane insert (BD Falcon, Lincoln Park, NY, USA). The ESCC cells were plated on the bottom chamber at 2.0×10^5 cells/well in serum-free medium 1 day prior to co-culture assay. We washed TAM-like PBMo-derived macrophages in inserts three times with serum-free medium and co-cultured with ESCC cells in serum-free condition for 2 days.

Cell proliferation assay

The TE cells were seeded in 24 plates at 1.0×10^4/well with serum-free medium and incubated at 37°C in 5% CO_2. After 24or 48 h, CellTiter 96 Aqueous One Solution Reagent (Promega, Madison, WI, USA) was added to each well. The absorbance at 492 nm measured using an Infinite 200 PRO microplate reader (Tecan, Mannedorf, Switzerland).

cDNA microarray analysis

Total RNA was extracted from two monocultured and co-cultured ESCC cell lines (TE-8 and TE-9) with TAM-like PBMo-derived macrophages using the RNeasy kit (Qiagen, Hilden, Germany). A cDNA microarray was performed using the GeneChip Human Gene 2.0 ST Array (Affymetrix, Santa Clara, CA, USA), containing 53617 probes characterized human genes and expressed sequence tags. A company (Filgen, Nagoya, Japan) conducted the DNA microarray

analysis. We deposited the data to the Gene Expression Omnibus database (GSE118642).

Quantitative reverse transcription-PCR (qRT-PCR) and RT-PCR

Quantitative RT-PCR amplifications of *CYP1A1*, *DHRS3*, *ANXA10*, *KLK6*, *CYP1B1*, *AMTN*, *IGFL1* and the control gene *GAPDH* were conducted using the ABI Step One Real-time PCR system (Applied Biosystems, Foster City, CA, USA). The primers for those genes were from TaqMan Gene Expression Assays (Applied Biosystems). The primers were as follows: *CYP1A1* (Hs01054797_g1), *DHRS3* (Hs00191073_m1), *ANXA10* (Hs01105012_m1), *KLK6* (Hs00160519_m1), *CYP1B1* (Hs02382916_s1), *AMTN* (Hs00418384_m1), *IGFL1* (Hs01651089_g1) and *GAPDH* (Hs02786624_g1).

We performed RT-PCR amplifications of *ANXA10* and the control gene *GAPDH*. The primer sequences used for RT-PCR were: *ANXA10*, forward, 5′-GCAATTCATGAC TTTGGTTT-3′ reverse 5′-TTTTCCATATCGCTCTTTGT-3′; *GAPDH*, forward 5′-ACCACAGTCCATGCCATCAC-3′, reverse 5′-TCCACCACCCTGTTGCTGTA-3′.

Tissue samples

A total of 68 primary ESCC tissues were obtained from patients whose ESCCs were treated surgically at Kobe University Hospital (Kobe, Japan) from 2005 to 2010. None had received any chemotherapy or radiotherapy before surgery. Histological and clinical-pathological evaluations were determined according to the Japanese Classification of Esophageal Cancer proposed by the Japan Esophageal Society along with the TNM classification of the Union for International Cancer Control.[20,21]

Immunohistochemistry (IHC)

Immunohistochemistry was performed on 4-μm sections of paraffin-embedded specimens. After deparaffinization and hydration, antigen retrieval was conducted. The endogenous peroxidase activity was quenched by 15 min in 3% hydrogen peroxide and then 15 min in bovine serum albumin at room temperature. The tissue sections were then incubated with anti-ANXA10 rabbit polyclonal antibody (1:200, NBP190156SS, Novus Biologicals, Littleton, CO, USA), anti-CYB1B1 rabbit polyclonal antibody (1:50, sc-32882, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-KLK6 mouse monoclonal antibody (1:50, sc-374564, Santa Cruz) at 4°C in a moist chamber overnight. The next day, the slides were incubated with second antibodies (Envision Dual Link; DakoCytomation, Glostrup, Denmark) for 30 minutes at room temperature. The slides were then colored with 3,3'-diaminobenzidine tetrahydrochloride (Muto Pure Chemicals, Tokyo, Japan) for 15 min. ANXA10 immunoreactivity in ESCC tissue was scored based on the intensity of positive cancer cells seen in the invasive area compared with that of the corresponding normal esophageal epithelium as a negative internal control. The staining intensity of ANXA10 was evaluated as low (negative to weak immunoreaction) and high (moderate to intense immunoreactivity). The evaluation and scoring were performed by three independent pathologists (authors H.K., Y.K. and H.Y.).

Western blotting

Cells were lysed in cell lysis buffer (50 mM Tris-HCl pH 7.5, 125 mM NaCl, 5 mM EDTA and 0.1% Triton X-100) containing both 1% protease inhibitor and 1% phosphatase inhibitor cocktail (Sigma-Aldrich, St Louis, MO, USA). Equal amounts of protein were separated on 10% SDS-PAGE and then transferred to polyvinylidene fluoride membranes with an iBlot® 2 Gel Transfer Stack (Thermo Fisher Scientific). The membranes were incubated with primary antibodies at 4°C overnight. The primary rabbit antibodies used were anti-ANXA10 antibody (1:500, NBP1-90156SS, Novus Biologicals), anti-phosphorylated Akt (Ser473) (/1:500, #4060, Cell Signaling Technology, Beverly, MA), anti-phosphorylated Akt (Thr308) (1:500, #2965, Cell Signaling), anti-Akt (1:1000, #9272, Cell Signaling), anti-phosphorylated Erk1/2 antibody (Thr202/Tyr204) (1:500, #9101, Cell Signaling), anti-Erk1/2 antibody (1:1000, #9102, Cell Signaling), anti-β-actin antibody (1:1000, #4970, Cell Signaling). The membranes were then probed with secondary antibodies for 90 min at room temperature. The secondary antibody was horseradish peroxidase-conjugated donkey anti-rabbit IgG (1:1000, NA934V, GE Healthcare, Little Chalfont, Buckinghamshire, UK).

Cell transfection

Cells of the ESCC lines TE-8 and TE-9 were transfected with 20nM small interfering RNA (siRNA) targeting human ANXA10 (sc-72503, Santa Cruz) and negative control siRNA (Sigma-Aldrich) for 2 days.

We used plasmid DNA that encoded *ANXA10* and *Myc-DDK*-tagged ORF clone ANXA10 (NM_007193, OriGene Technologies, Rockville, MD) as transfection ready DNA for overexpressing ANXA10 in the ESCC cell line TE-15. The plasmid DNA were transfected into TE-15 cells using Lipofectamine® 3000 reagent (Thermo Fisher Scientific). After gene transfection 2 days, the complete medium

containing 300μg/mL Geneticin Thermo Fisher Scientific® (G418, Thermo Fisher Scientific) was added for selection. Empty pCMV vectors (OriGene) were designated as control vectors. At 4 weeks later, the resistant colonies were obtained.

Statistical analysis

Statistical significance was analyzed by two-sided Student's *t*-test and one way analysis of variance (ANOVA). Relations between clinical-pathological features and immunohistochemical results were analyzed by the χ^2-test. Survival curves were estimated by the Kaplan–Meier method and evaluated using log-rank test. The experimental *in vitro* data represent three or four independent experiments. A *P*-value of <0.05 was considered statistically significant. Statistical analyses were carried out using the SPSS Statistics ver. 22 program (IBM, Chicago, IL, USA).

RESULTS

Up-regulated and down-regulated genes in TE-8 and TE-9 ESCC cells co-cultured with TAM-like PBMo-derived macrophages

We first constructed an *in vitro* system of PBMo-derived macrophages for their differentiation into TAM-like PBMo-derived macrophages. We then used a co-culture assay

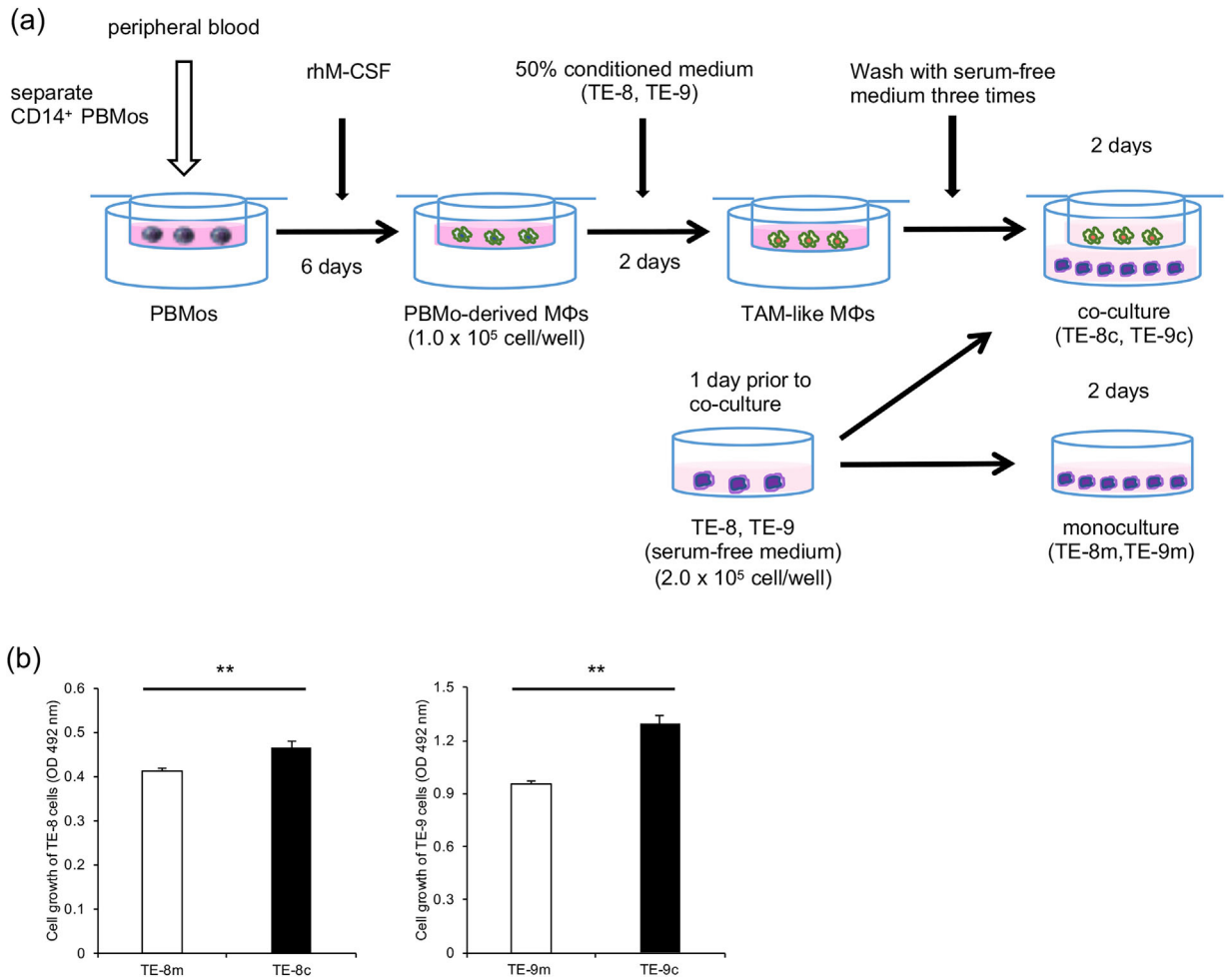

Figure 1 Co-culture assay of ESCC cells with TAM-like PBMo-derived macrophages. (**a**) Experiment outline of the co-culture assay of the two ESCC cell lines (TE-8, TE-9) with TAM-like PBMo-derived macrophages. $CD14^+$ PBMo were cultured at 1.0×10^5 cells/insert with 25 ng/mL M-CSF for 6 days to induce macrophages. We exchanged with 50% TECM (TE-8 or TE-9) in inserts to induce TAM-like PBMo-derived macrophages. TE-8 and TE-9 ESCC cells were seeded on the bottom chamber of 6-well plates at 2.0×10^5 cells/well in serum-free medium 1 day prior to co-culture assay. We washed TAM-like PBMo-derived macrophages in inserts three times with serum-free medium and co-cultured with ESCC cells in serum-free condition for 2 days. (**b**) The cell growth activity was increased in both ESCC cell lines (TE-8, TE-9) co-cultured with TAM-like PBMo-derived macrophages compared to the monocultured ESCC cell lines. Cells were seeded on 24-well plates at 1.0×10^4 cells/well in serum-free RPMI-1640 with monocultured or co-cultured ESCC cell lines. After 48 hours, the cell growth activities were determined by an MTS assay.

with TAM-like PBMo-derived macrophages and ESCC cell lines to study the effects of TAMs on the ESCC tumor microenvironment (Fig. 1a). We observed increased cell growth in TE-8 and TE-9 ESCC cells co-cultured with TAM-like PBMo-derived macrophages compared to the growth of monocultured cancer cells (Fig. 1b).

We subsequently performed a cDNA microarray analysis using total RNAs from the TE-8 and TE-9 ESCC cells monocultured and co-cultured with TAM-like PBMo-derived macrophages. We identified 316 up-regulated (ratio $\geq$ 2.0) and 293 down-regulated (ratio $\leq$ 0.5) genes in TE-8 cells and 619 up-regulated and 520 down-regulated genes in TE-9 cells (Table S1). We focused on commonly up-regulated (*CYP1A1*, *DHRS3*, *ANXA10*, *KLK6* and *CYP1B1*) and down-regulated (*AMTN* and *IGFL1*) genes in both co-cultured ESCC cell lines (Table 1). The results of the cDNA microarray analysis were confirmed by qRT-PCR (Fig. 2a–g).

The expression levels of ANXA10 were closely correlated with clinical-pathological features and the prognosis of the ESCC patients

To check the clinical-pathological features of the up-regulated genes extracted by the cDNA microarray analysis, we performed immunohistochemistry of ANXA10, KLK6 and CYP1B1 whose specific antibodies were available on 68 cases of ESCC tissues (Figs. 3a, S1, S2 and Tables S2, S3). Among the three molecules, the expression levels of ANXA10 in cancer cells were significantly correlated with the depth of invasion ($P = 0.031$) in the ESCC patients (Table 2). We also observed that a high number of infiltrating $CD68^+$ ($P = 0.046$) or $CD204^+$ ($P = 0.006$) macrophages was significantly correlated with high expressions of ANXA10 in cancer cells (Table 2). The disease-free survival of the patients with a high expression of ANXA10 was significantly shorter compared to that of the patients with low ANXA10 by log-rank test ($P = 0.0216$) (Fig. 3b). The overall survival of the patients and cancer-related survival was not significantly different between the low and high ANXA10 expression groups (Fig. 3b).

ANXA10 knockdown suppressed the growth of TE-8 and TE-9 ESCC cells by inhibiting Akt and Erk1/2 phosphorylation

The up-regulations of ANXA10 mRNA (Fig. 4a) and protein (Fig. 4b) were confirmed in TE-8 and TE-9 cells co-cultured with TAM-like PBMo-derived macrophages compared to the monocultured cells by both RT-PCR and western blotting. We then attempted to knockdown ANXA10 in TE-8 and TE-9 cells to determine the biological significance of ANXA10 in the growth of ESCC cells. The levels of ANXA10 mRNA and protein were significantly decreased in siRNA-transfected TE-8 and TE-9 cells (Fig. 4c, d, f). ANXA10 knockdown significantly inhibited the growth of both of these ESCC cell lines (Fig. 4e). ANXA10 knockdown suppressed the phosphorylation status of p-Akt (Ser473 and Thr308) and p-Erk1/2 (Thr202/Tyr204) and inhibit the growth of the ESCC cell lines (Fig. 4f).

Overexpression of ANXA10 induced the growth of TE-15 cells by triggering Akt and Erk1/2 phosphorylation

TE-15 cells demonstrated lower ANXA10 mRNA and protein expression compared to the TE-8 and TE-9 cells (Fig. 5a, b). To examine the effect of ANXA10 overexpression, we introduced ANXA10 expression vector into TE-15 cells. We confirmed that the ANXA10 mRNA and protein expressions were increased in transfected TE-15 cells (Fig. 5c, d, f). ANXA10 overexpression significantly induced

Table 1 Up-regulated and down-regulated genes in co-cultured TE-8 and TE-9 ESCC cell lines

			Fold change (co-culture/monoculture)	
Accession number	**Gene description**	**Gene symbol**	**TE-8**	**TE-9**
Up-regulated genes (ratio $\geq$ 2.0)				
NM_000499	*Cytochrome P450, family 1, subfamily A, polypeptide*	*CYP1A1*	13.866687	26.960763
NM_004573	*Dehydrogenase/reductase (SDR family) member 3*	*DHRS3*	3.6046498	3.6668914
NM_007193	*Annexin A10*	*ANXA10*	3.1650218	2.4714345
NM_001012964	*Kallikrein-related peptidase 6*	*KLK6*	2.7406091	3.2696303
NM_000104	*Cytochrome P450, family 1, subfamily B, polypeptide*	*CYP1B1*	2.4083839	14.074274
Down-regulated genes (ratio $\leq$ 0.5)				
NM_002989	*amelotin*	*AMTN*	0.39392439	0.46568208
NM_006419	*IGF-like family member 1*	*IGFL1*	0.49403735	0.13806771

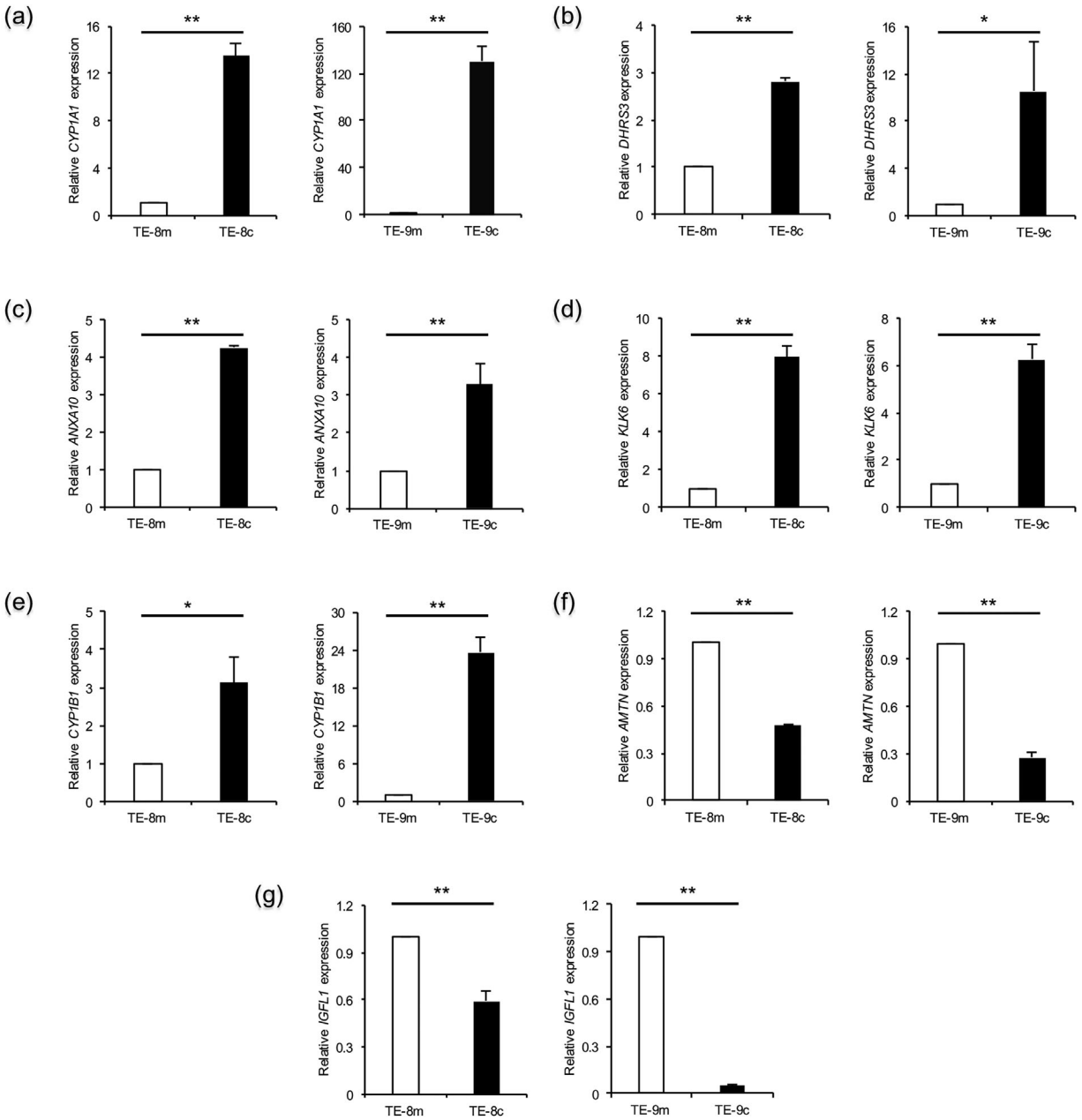

Figure 2 The mRNA levels of up-regulated or down-regulated genes in ESCC cell lines monocultured and co-cultured with TAM-like PBMo-derived macrophages by quantitative RT-PCR. (**a–e**) The mRNA levels of *CYP1A1*, *DHRS3*, *ANXA10*, *KLK6* and *CYP1B1* were up-regulated and (**f, g**) *AMTN* and *IGFL1* were down-regulated in co-cultured TE-8 and TE-9 cells compared to the respective monocultured ESCC cell lines. *GAPDH* was used as a control. Data are mean ± SEM in triplicate. $^{**}P < 0.01$, Student's *t*-test.

cell growth of TE-15 cells (Fig. 5e). ANXA10 overexpression increased the phosphorylation status of p-Akt (Ser473 and Thr308) and p-Erk1/2 (Thr202/Tyr204) and increased the growth induction of the ESCC cells (Fig. 5f).

DISCUSSION

Our prior study demonstrated that PBMo-derived macrophages stimulated with TECM acquire M2-like characteristics and up-regulated GDF15 and CXCL8 expression.[19,22] GDF15 and CXCL8 are associated with $CD204^{+}$ macrophage infiltration in human ESCC and it promoted the growth of ESCC cells *via* the phosphorylation of Akt and Erk1/2.[19,22] TAMs have been reported to promote tumor progression in pancreatic cancer, breast cancer and hepatocellular carcinoma by a cancer cell-macrophage co-culture system.[23–25] In the present study, we newly established a co-culture system with human ESCC cell lines and TAM-like PBMo-derived macrophages to investigate the biological effects of

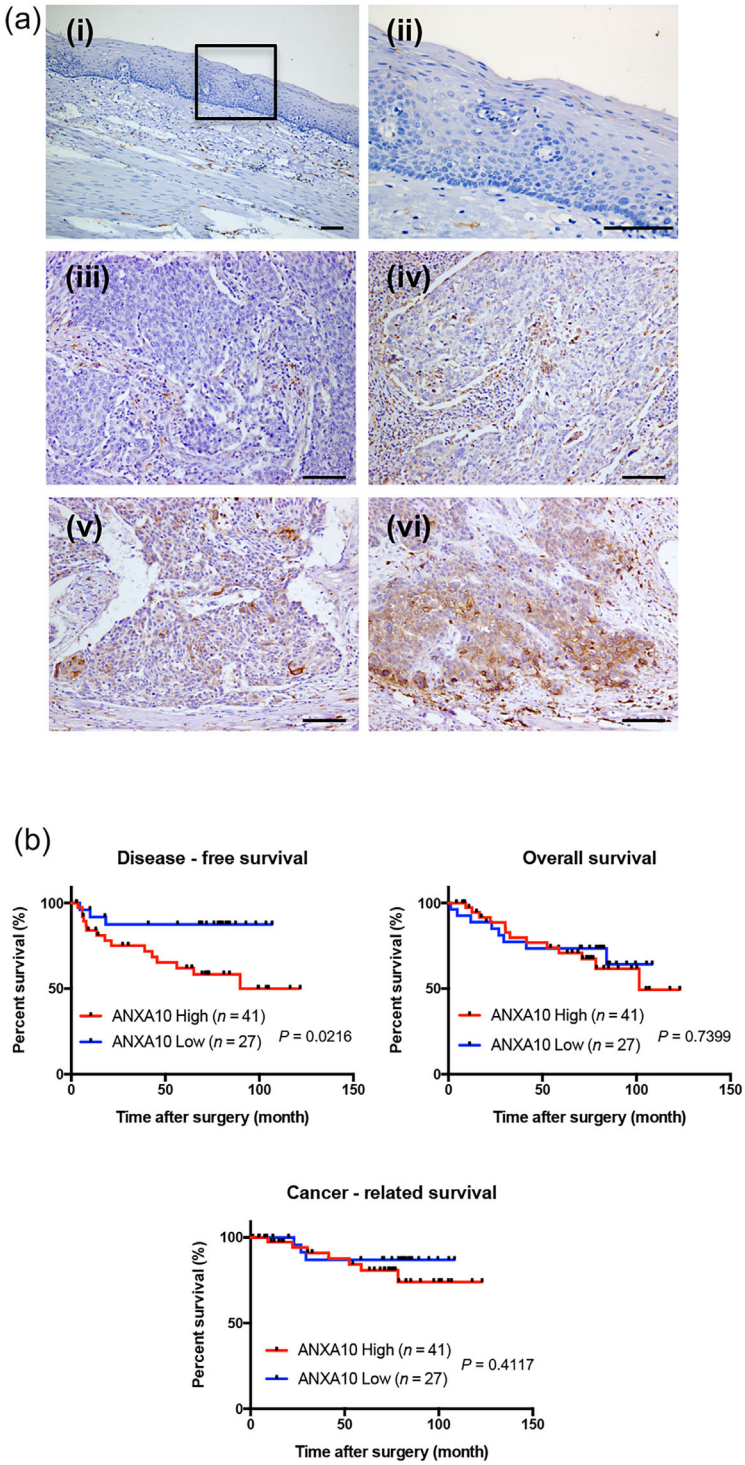

Figure 3 A high expression of ANXA10 in human ESCC tissue was associated with the poor prognosis of ESCC patients. (**a**) Representative images of immunohistochemical staining for ANXA10 in human ESCC tissue. (i) Normal esophageal squamous epithelium with negative ANXA10 immunoreactivity. (ii) Higher power view of ANXA10 immunoreaction in the normal esophageal squamous epithelium (square, i). Representative images of negative (iii), weak (iv), moderate (v) and intense (vi) ANXA10 immunoreactivities. ANXA10 expression was seen in cancer cells and the stromal or inflammatory cells. We assessed the levels of immunoreaction in cancer cells around the invasive front of ESCC and divided the cases into low (negative and weak) and high (moderate and intense) groups. Scale bars: 100 μm. (**b**) Kaplan–Meier analyses of the disease-free survival, overall survival and cancer-related survival of ESCC patients divided into high- and low-expression groups based their expression levels of ANXA10. The data were analyzed by log-rank test.

Table 2 Expression levels of ANXA10 in ESCC and their correlation with clinical-pathological features and infiltrating macrophages phenotype

		Expression of ANXA10		
	Number of cases	Low ($n = 27$)	High ($n = 41$)	*P*-value[†]
Age				
<65	32	13	19	1.000
≥65	36	14	22	
Histological grade[‡]				
HGIEN + WDSCC	15	8	7	0.246
MDSCC + PDSCC	53	19	34	
Depth of invasion[‡]				
T1	47	23	24	0.031*
T2 + T3	21	4	17	
Lymphatic vessel invasion[‡]				
Negative	37	18	19	0.137
Positive	31	9	22	
Blood vessel invasion[‡]				
Negative	43	18	25	1.000
Positive	25	9	16	
Lymph node metastasis[‡]				
Negative	42	18	24	0.310
Positive	26	9	17	
Stage[§]				
0 + I	38	16	22	0.455
II + III + IV	30	11	19	
$CD68^+$cells[¶]				
Low	34	17	17	0.046*
High	34	10	24	
$CD163^+$cells[¶]				
Low	33	16	17	0.082
High	35	11	24	
$CD204^+$cells[¶]				
Low	33	18	15	0.006**
High	35	9	26	

[†]Data were analyzed by χ^2-test. $P < 0.05$ was considered statistically significant: $^*P < 0.05$, $^{**}P < 0.01$.

[‡]According to the Japanese Classification of Esophageal Cancer. HGIEN, high-grade intraepithelial neoplasia; WDSCC, well-differentiated squamous cell carcinoma; MDSCC, moderately differentiated squamous cell carcinoma; PDSCC, poorly differentiated squamous cell carcinoma. T1, tumor invades mucosa and submucosa; T2, tumor invades muscularis propria; T3, tumor invades adventitia.[20]

[§]According to the 7th edition of the TNM classification.[21]

[¶]The patients were divided into low- and high-groups based on median number of infiltrating $CD68^+$, $CD163^+$ or $CD204^+$ macrophage in tumor cell nests and the tumor stroma.

TAMs on ESCC cells. We observed an increased proliferation of ESCC cells co-cultured with TAM-like PBMo-derived macrophages compared to the proliferation of monocultured cancer cells. We then extracted up-regulated and down-regulated genes in ESCC cells co-cultured with TAM-like PBMo-derived macrophages in a comparison with monocultured cancer cells by cDNA microarray analysis. We focused on *KLK6*, *CYP1B1* and *ANXA10* among the up-regulated genes.

KLK6 belongs to a large family of Kallikrein-related peptidases that secrete serine proteases and are aberrantly expressed in many cancers.[26] KLK6 is correlated with tumor progression in gastric cancer, ovarian cancer and melanoma.[27–29] However, the roles of KLK6 in ESCCs have not been elucidated. Our present findings revealed that high expression levels of KLK6 were negatively correlated with the number of infiltrating $CD68^+$ ($P = 0.023$), $CD163^+$ ($P = 0.011$) and $CD204^+$ ($P = 0.011$) macrophages

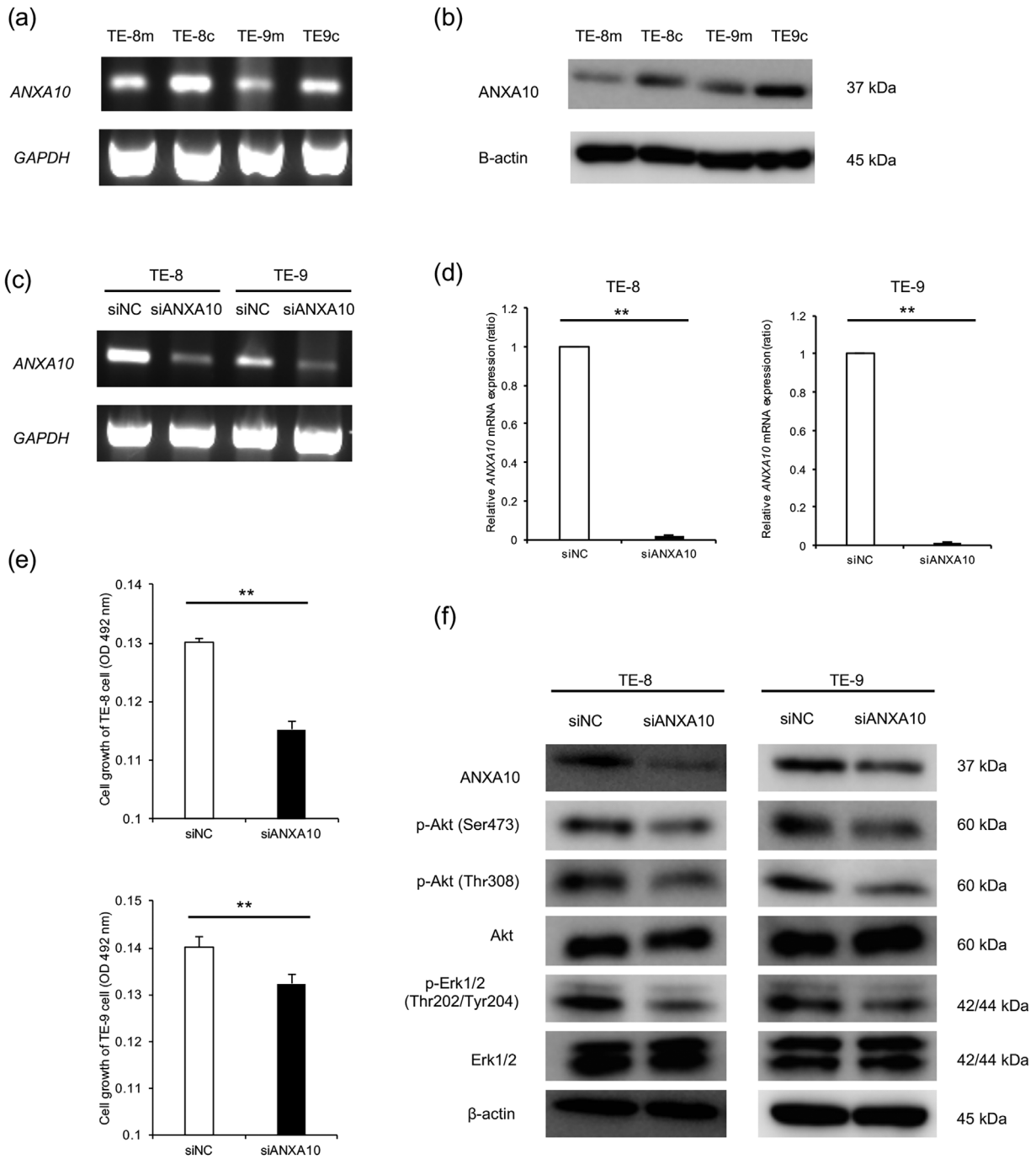

Figure 4 Effects of ANXA10 knockdown on TE-8 and TE-9 cells. (**a**) The mRNA levels of *ANXA10* in monocultured and co-cultured TE-8 and TE-9 cells with TAM-like PBMo-derived macrophages were analyzed by RT-PCR. *GAPDH* was used as a control. (**b**) The protein levels of ANXA10 in monocultured and co-cultured TE-8 and TE-9 cells with TAM-like PBMo-derived macrophages were confirmed by western blotting. β-actin was used as a control. (**c, d**) The effects of the knockdown of *ANXA10* expression in TE-8 and TE-9 transfected cells were confirmed. The mRNA level of the knockdown of *ANXA10* was confirmed by RT-PCR and qRT-PCR. *GAPDH* was used as a control. Data are mean ± SEM in triplicate. $^{**}P < 0.01$, Student's *t*-test. (**e**) The effects of ANXA10 knockdown on the cell growth of TE-8 and TE-9 cells were confirmed by MTS assay. Cells were seeded on 24-well plates at 1.0×10^4 cells/well in serum-free RPMI-1640 with or without siANXA10-transfected cells. The cell growth activity was determined 24 h after siANXA10 transfection by an MTS assay. The cellular growth of siANXA10-transfected TE-8 and TE-9 cells was significantly inhibited. Data are mean ± SEM in triplicate. $^{**}P < 0.01$, Student's *t*-test. (**f**) Western blotting was performed on the total protein of the ESCC cell lines using specific antibodies against Akt, p-Akt (Ser473), p-Akt (Thr308), p-Erk1/2 (Thr202/Tyr204), Erk1/2 and β-actin in the siANXA10 transfected cells compared with the control. The expression levels of p-Akt and p-Erk were decreased in the siANXA10-transfected cells compared to the control.

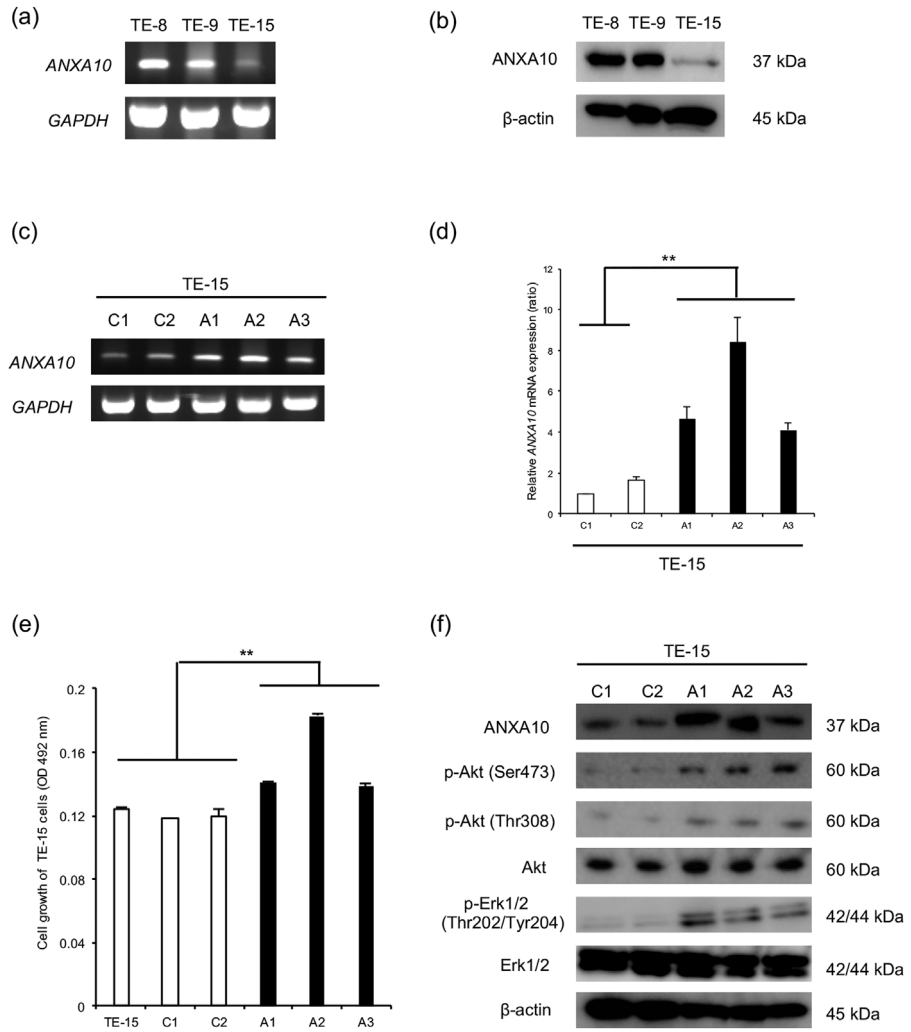

Figure 5 Effects of ANXA10 overexpression in TE-15 cells. (**a, b**) The expression of ANXA10 in ESCC cell lines (TE-8, TE-9 and TE-15) were confirmed by RT-PCR (**a**) and western blotting (**b**). The expression of ANXA10 in the TE-15 cells was significantly lower than that of TE-8 and TE-9 cells. (**c, d**) The effects of ANXA10 vector transfection in ANXA10 expression in TE-15 cells (three clones: A1, A2, A3) were evaluated. Increased *ANXA10* mRNA expression in the transfected cells compared to control cells (two clones: C1, C2) was confirmed by RT-PCR (**c**) and qRT-PCR (**d**). *GAPDH* was used as an internal control. Data are mean ± SEM in triplicate. $^{**}P < 0.01$, Student's *t*-test. (**e**) The effects of ANXA10 overexpression on the cell growth of TE-15 cells was confirmed by MTS assay. Cells were seeded on 24-well plates at 1.0×10^4 cells/well in serum-free RPMI-1640. After 24 h, cell growth activities were determined by an MTS assay. The cellular growth of ANXA10 vector-transfected TE-15 cells was promoted. Data are mean ± SEM in triplicate. $^{**}P < 0.01$, Student's *t*-test. (**f**) Western blotting was performed on the total protein of ESCC cell lines using specific antibodies against Akt, p-Akt (Ser473), p-Akt (Thr308), p-Erk1/2 (Thr202/Tyr204) and Erk1/2 in the ANXA10 vector-transfected cells compared with controls. β-actin was used as a control.

in ESCCs. We also observed that a high expression of KLK6 was not correlated with the poor prognosis of ESCC patients. CYP1B1 is a recently cloned dioxin-inducible form of the cytochrome P450 family of xenobiotic metabolizing enzymes.[30] Recent studies reported that polymorphism of *CYP1B1* which plays a role in the metabolism of xenobiotics was not associated with ESCC risk.[31,32] Our present clinical-pathological findings suggest that a high expression of CYP1B1 is associated with the depth of invasion and disease-free survival but not with infiltrated the number of infiltrated TAMs.

Annexins are a family of Ca^{2+}/lipid-binding proteins that differ from most other Ca^{2+}-binding proteins in their Ca^{2+}-binding sites.[33,34] ANXA10 is a member of the annexin family and the cDNA of human *ANXA10* located on chromosome 4q33.[35] ANXA10 is expressed in the foveolar cells and glandular cells of the body or antrum- type gastric mucosa, Brunner gland cells of the duodenum, and urothelium of urinary bladder.[36] ANXA10 is currently a subject of interest as a marker for the colorectal serrated neoplasia pathway. The expression of ANXA10 was reported to be increased in sessile serrated adenomas/polyps (SSA/Ps)

and traditional serrated adenomas compared to normal colonic epithelia, hyperplastic polyps, tubulovillous adenomas and tubular adenomas.[37,38] Regarding ANXA10 and cancer, ANXA10 was first reported on hepatocellular carcinoma (HCC), as chromosome 4q is one of the most common chromosomal regions with a high frequency of allelic loss in HCC. In HCC, down-regulation of ANXA10 is correlated with vascular invasion, early recurrence and poor prognosis.[39] A correlation of ANXA10 down-regulation and poor prognosis has also been reported in gastric cancer, bladder cancer and prostate cancer.[40–43]

On the other hand, it was reported that ANXA10 overexpression was correlated with a poor prognosis and associated with poor prognostic molecular features such as CIMP and the BRAF mutation in colorectal carcinomas.[44] ANXA10 was significantly overexpressed in pancreatic intraepithelial neoplasia, intraductal papillary mucinous neoplasm and pancreatic adenocarcinoma and the co-expression of ANXA10 and CD24 was associated with disease progression, while ANXA10 was not expressed in normal pancreatic epithelia.[45] In oral squamous cell carcinoma (OSCC), it was reported that ANXA10 overexpression was associated with tumoral progression.[46]

In esophageal cancer, it was reported that compared to normal squamous esophagus epithelium, ANXA10 was highly expressed in esophageal adenocarcinoma and squamous cell carcinoma.[47] However, the expression of ANXA10 in ESCC tissues and its correlation to clinicalpathological features have not been elucidated. Our present analysis demonstrated that a high expression of ANXA10 in ESCC cells was significantly correlated with the depth of invasion, high numbers of infiltrating $CD68^+$ and $CD204^+$ TAMs and poor disease-free survival. This strongly implies that ANXA10 plays an important role in the progression of ESCCs. Our present findings also indicate the possibility that ANXA10 expression in cancer cells is provoked by an interaction with TAMs in the ESCC microenvironment.

It has been reported that the silencing of ANXA10 resulted in decreased cellular proliferation by the inactivation of ERK in OSCC.[46] ANXA10 might affect intracellular Ca^{2+} homeostasis and directly or indirectly interact with the activation of MEK/ERK signaling pathways.[46] To determine whether ANXA10 function is relevant to ESCC progression, we performed functional studies using siRNA. The results showed that ANXA10 knockdown significantly decreased cell growth by inhibiting Akt and Erk1/2 signaling pathways. We also introduced ANXA10 expression vector into ESCC cells with a low expression of ANXA10 and we observed that ANXA10 significantly increased cell growth by activating Akt and Erk1/2 signaling pathways. Our results are the first to indicate that ANXA10 might play an important role in the growth of ESCCs by activating both the Akt andErk1/2 signaling pathways.

In conclusion, our findings revealed that a high expression of ANXA10 is closely correlated with the depth of invasion, high numbers of infiltrating $CD68^+$ and $CD204^+$ TAMs and a poor prognosis of ESCC patients. Our findings also demonstrated that ANXA10 promoted the cell growth of ESCC cells *via* the phosphorylation of Akt and Erk1/2 pathways *in vitro*. ANXA10 might be a new biomarker and therapeutic target for the progression of ESCC.

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DISCLOSURE STATEMENT

None declared.

AUTHOR CONTRIBUTIONS

HK and YK conceived and carried out the experiments. MH and KS prepared the tissue samples and provided cells. NH, MN and MS supervised the work and analyzed the data. HK and HY drafted the manuscript or figures. All the authors had final approval of the submitted and published manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website.

Figure S1. The evaluation of the KLK6 expression in human ESCC tissue and the prognoses of the ESCC patients.

Figure S2. The evaluation of the CYP1B1 expression in human ESCC tissue and the prognoses of the ESCC patients.

Table S1. The gene expression profile of up-regulated and down-regulated genes in co-cultured TE-8 and TE-9 ESCC cell lines that demonstrated a ratio $\geq$ 2.0 or $\leq$0.5 difference.

Table S2. The expression levels of KLK6 in ESCC and its correlation with clinical-pathological features and the phenotype of infiltrating macrophages.

Table S3. The expression levels of CYP1B1 in ESCC and its correlation with clinical-pathological features and the phenotype of infiltrating macrophages.