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Original Research

TIE2-high cervical cancer cells promote tumor angiogenesis by upregulating TIE2 and VEGFR2 in endothelial cells

Shuran Tan [a,1], Yuanyuan Chen [a,1], Shi Du [a], Wenhan Li [a], Pan Liu [a], Jing Zhao [a], Ping Yang [b], Jing Cai [a], Rui Gao [a,*], Zehua Wang [a,*]

[a] Department of Obstetrics and Gynecology, Wuhan Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, 1277 Jiefang Avenue, Wuhan, Hubei 430022, China

[b] Department of Obstetrics and Gynecology, First Affiliated Hospital, School of Medicine, Shihezi University, Shihezi, China

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ABSTRACT

Tyrosine kinase with immunoglobulin and epidermal growth factor homology domains 2 (TIE2), the receptor for angiopoietins, has been found highly expressed in cervical cancer and associated with poor prognosis. However, the potential role of tumoral TIE2 in cervical cancer angiogenesis and the underlying mechanisms remain unexplored. Here, based on multicolor immunofluorescence of 64 cervical cancer tissues, we found that TIE2 level in cervical cancer cells was positively related to shorter survival and higher microvessel density in tumor. In vitro and in vivo experiments verified that TIE2-high cervical cancer cells could promote tumor angiogenesis. TIE2-high tumor cells induced an amplified expression of TIE2 and vascular endothelial growth factor receptor 2 (VEGFR2) in HUVECs to promote angiogenesis via TIE2 -AKT/MAPK signals, which could be reversed or partially reversed by TIE2, AKT or MAPK inhibitors and activated by angiopoietin-1 and angiopoietin-2. In conclusion, TIE2-high cervical cancer cells promote tumor angiogenesis by upregulating TIE2 and VEGFR2 in endothelial cells via TIE2-AKT/MAPK axis inside tumor cells.

Introduction

Cervical cancer is the fourth most common malignant disease in women, accounting for more than 300,000 cancer-related deaths worldwide each year [1]. Although patients with early-stage cervical cancer have a favorable prognosis with a 5-year overall survival over 85%, those diagnosed with locally advanced or advanced cervical cancer still suffer from underdiagnosis despite improved concomitant chemoradiotherapy [2], thus indicating the need to deepen our understanding of the mechanisms underlying tumor progression that may spawn new therapeutic strategies. Angiogenesis is essential for tumor growth and metastasis and has been considered an effective therapeutic target in solid tumors [3]. Recently, anti-vascular endothelial growth factor drugs, such as the human vascular endothelial growth factor (VEGF)--specific monoclonal antibody bevacizumab, have been approved to treat metastatic or recurrent cervical cancer, but the clinical response and benefits are limited [4–6]. Thus, further investigation of the mechanism underlying tumor angiogenesis is urgently needed.

Tyrosine kinase with immunoglobulin and epidermal growth factor homology domains 2 (TIE2), encoded by the *TEK* gene, is a receptor for angiopoietins (human ANG1, ANG2 and ANG4) that are predominantly expressed on the surface of endothelial cells and regulate endothelial cell survival, proliferation, migration, adhesion and spreading [7,8]. TIE2 signaling is modulated by its ligands. ANG1 is a full agonist, while ANG2 is a context-dependent antagonist; the coordination between ANG1 and ANG2 determines the activation level of TIE2 [9,10]. In collaboration with the VEGF pathway, the ANG-TIE2 system regulates vascular development, maintains vascular homeostasis and is involved in the angiogenic switch [9]. Intriguingly, TIE2 is expressed not only on endothelial cells but also on hematopoietic stem cells, osteoblasts,

Abbreviations: TIE2, Tyrosine kinase with immunoglobulin and epidermal growth factor homology domains 2; VEGFR2, vascular endothelial growth factor receptor 2; HUVEC, Human Umbilical Vein Endothelial Cells; VEGF, vascular endothelial growth factor; ANG, Angiogenin; OS, Overall survival; DFS, Disease-free survival; Ep-CAM, Epithelial cell adhesion molecule; CD31, Platelet endothelial cell adhesion molecule-1; WB, Western blotting; qRT-PCR, Real-time quantitative PCR; CM, Conditioned medium; IHC, Immunohistochemistry; MVD, Micro-vessel density.

* Corresponding authors.
E-mail addresses: gaorui_zzu@163.com (R. Gao), zehuawang@163.net (Z. Wang).

[1] These authors contributed equally to this work.

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macrophages and tumor cells [11–14]. Tumor cell-expressed TIE2 has been found to exert various biological functions, such as regulating cell stemness, cell adhesion to the extracellular matrix, and angiogenesis [11,15,16]. Our previous study revealed that TIE2 expression was increased in cervical cancer tissues in both malignant epithelia and tumor-associated endothelia compared to their normal counterparts, and a high TIE2 level in tumor cells predicted shorter progression-free survival and overall survival in patients. Moreover, ANG1 and ANG1 could improve the migration and invasion properties of cervical cancer cells *in vitro* and sustain xenograft growth in mice [17]. However, the role of TIE2 in cervical cancer angiogenesis remains unclear.

Here, by using multiplexed immunofluorescence staining, we demonstrate a positive correlation between TIE2 levels in cervical cancer cells and microvessel density in tumors. We found that TIE2 upregulation in cervical cancer cells could enhance angiogenesis *in vitro* and *in vivo*, which may be attributable to the increased expression of TIE2 and VEGFR2 and angiopoietins in endothelial cells and tumor cells, respectively, in response to TIE2 signaling in tumor cells. We revealed the functional involvement of tumor cell-expressed TIE2 in angiogenesis in cervical cancer, suggesting that anti-TIE2 therapy may offer a double hit to angiogenesis by affecting the TIE2 and VEGF pathways simultaneously in TIE2-high cervical cancers.

Materials and methods

Patients' histological specimens

In the present study, paraffin-embedded surgical specimens from 64 patients who underwent surgery without prior radiotherapy, chemotherapy or anti-angiogenic therapy at our hospital from 2012 to 2018 were analyzed. All patients were pathologically diagnosed with primary cervical cancer and did not have other malignant diseases or conditions associated with abnormal immunity or hemopoietic function. The pathological characteristics of these cervical cancer patients are summarized in Table 1. Patients with a follow-up period of more than six months were included in the survival analysis. DFS and OS were defined as the time interval from surgery to first tumor recurrence and death, respectively, or to the last follow-up when no event occurred.

Multiplexed immunofluorescence staining

Immunofluorescence assays for epithelial cell adhesion molecule (Ep-CAM), TIE2 and CD31 (an endothelial maker) were performed with tissue microarrays. Briefly, after being deparaffinized and dehydrated, the slides were immersed in EDTA antigen retrieval buffer (pH 8.0), and heat-induced antigen retrieval was performed. To block endogenous peroxidases and antigens, 3% H_2O_2 and 3% bovine serum albumin (BSA) were used, respectively. The slides were then sequentially incubated with anti-Ep-CAM, anti-TIE2 and anti-CD31 antibodies (Table S1). A TSA kit (Wuhan Servicebio Technology Co., Ltd., China) was used for signal detection, and the nuclei were counterstained with *DAPI*. The slides were scanned by a slice scanner (Pannoramic MIDI: 3Dhistech, Hungary), and the images were analysed via HALO 2.0 Area Quantification algorithm (Indica Labs; Corrales, NM) at Nanjing Freethinking Biotechnology Co., Ltd. (China). The Ep-CAM+TIE2+ and $CD31^+$ cells, representing TIE2-expressing cervical cancer cells and vascular endothelial cells, respectively, were automatically identified and counted with HALO 2.0 software (Indica Labs; Corrales, NM), and the ratios of Ep-CAM^+TIE2^+ cells (Ep-CAM^+TIE2^+%) and $CD31^+$ cells ($CD31^+$%) to total cells (DAPI) were calculated.

Cell culture and transfection

The human cervical cancer cell lines SiHa (squamous cell carcinoma) and HeLa (adenocarcinoma) and HUVECs (human umbilical vein endothelial cells) were obtained from American Type Culture Collection and were cultured in RPMI 1640 medium supplemented with 10% FBS (Gibco). All the cell lines were cultured in a humidified incubator at 37 °C in 5% CO_2, authenticated by short tandem repeat (STR) profiling and confirmed to be mycoplasma-negative. Lentiviruses harboring *TEK*-expressing vector and no-load lentivirus vectors (LV-NC) were constructed by GenePharma Co., Ltd. (Shanghai, China) and used to individually transfect HeLa and SiHa cells to generate HeLa-NC, HeLa-*TEK*, SiHa-NC and SiHa-*TEK* sublines according to the manufacturer's instructions. Approximately 48 h after transduction, the stable cells were selected by exposure to puromycin (2 μg/ml).

Western blot analysis

Cells were harvested and lysed in RIPA buffer with protease inhibitor cocktail and protein phosphorylase inhibitors A and B for 15 min on ice. After centrifugation at 12,500 g at 4°C for 15 min, the protein concentration was measured with a bicinchoninic acid (BCA) assay. Then, the samples were separated by 10% sodium dodecyl sulfate-PAGE (SDS–PAGE) and transferred onto a 0.45 μm PVDF membrane. The membrane was blocked with 5% nonfat milk in Tris-buffered saline with Tween-20 (TBST) for 1.5 h at room temperature and incubated with primary antibodies at 4°C overnight. Horseradish peroxidase-conjugated anti-rabbit, anti-mouse or anti-goat secondary antibodies were subsequently used to incubate with membrane. Protein bands were detected with an enhanced chemiluminescence kit (Pierce, Thermo Scientific, Waltham, MA, USA) in a Molecular Imager® ChemiDocTM XRS+ with Image LabTM Software (BioRad Laboratories, Hercules, CA, USA). Details about the antibodies used are shown in the Table S1. Then the gray value of protein bands was analyzed using ImageJ software. The experiment was repeated three times.

RNA extraction and qRT–PCR

Total RNA was extracted from cells using TRIzol reagent (TaKaRa, Japan) according to the manufacturer's instructions. The RNA concentration was measured with a Nanodrop 2000. Reverse transcription was carried out with HiScript®III RT SuperMix (Vazyme, China) according to the manufacturer's instructions. The two-step PCR program was as follows: 95°C for 30 s, 40 cycles at 95°C for 10 s, 60°C for 30 s, and 70°C for 10 s. All primers were designed based on NCBI reference sequences and synthesized by Tsingke Biotechnology Co. Ltd. (China); their sequences are listed in Table S2. Quantitative real-time PCR was performed on an Applied Biosystem StepOne Plus PCR system (ABI) using AceQ qPCR SYBR Green Master Mix (Vazyme, China). The 2-ΔΔCt method was used to calculate the expression of mRNAs at the same time using ACTIN as internal controls. The experiment was repeated three times.

Preparation of cervical cancer cell-derived conditioned medium (CM)

HeLa-NC, HeLa-*TEK*, SiHa-NC and SiHa-*TEK* cells and HeLa-*TEK* and SiHa-*TEK* cells treated with 10 mg/ml bevacizumab (Roche Diagnostics GmbH, Mannheim, Germany), 5 μM TIE2 kinase inhibitor (Selleck Chemicals, Houston, TX, USA), 10 μM MK-2206 2HCl (Akt inhibitor, Selleck Chemicals, Houston, TX, USA), 5 μM Adezmapimod (MAPK inhibitor, Selleck Chemicals, Houston, TX, USA), or 500 ng/ml recombinant human ANG2 and ANG1 protein purchased from R&D Systems reached 80% confluence. The cells were flushed in PBS three times and cultured with serum-free RPMI 1640 medium overnight. The supernatant was collected, and the cell fragments were removed through five minutes of centrifugation at 12,000 g. Then, HUVECs were cultured with conditioned medium described above for 48 h for subsequent experiments.

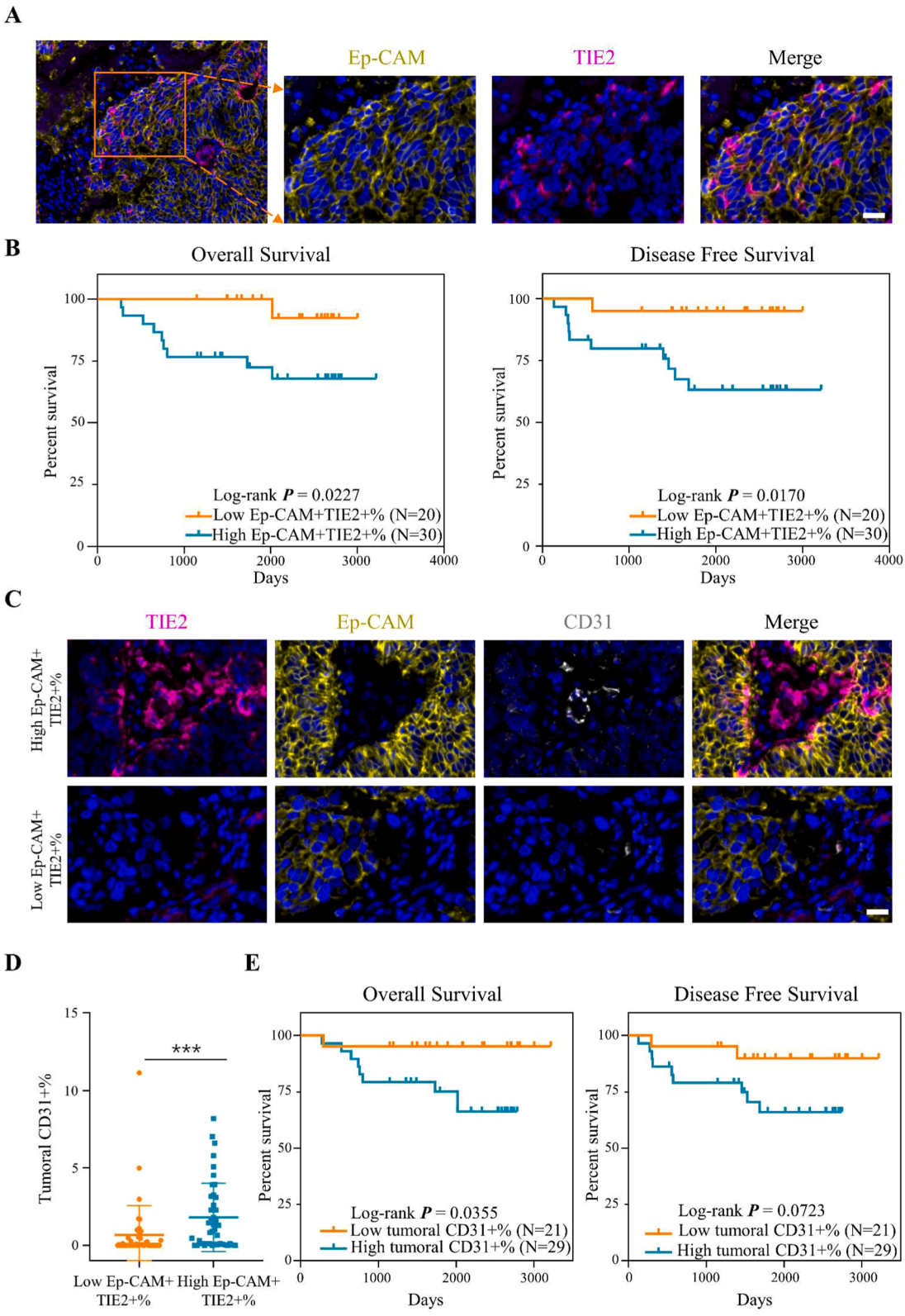

Fig. 1. TIE2 is correlated with poorer prognosis and angiogenesis in cervical cancer. A, Representative images of multiplexed immunofluorescence staining of cervical cancer tissue show that TIE2 (magenta) is expressed on tumor cells. Scale bar: 20 μm. **B**, Patients with high expression of TIE2 in tumor cells presented poorer OS (left) and DFS (right) than patients with low expression of TIE2 in tumor cells. Yellow lines: 20 patients with low expression of TIE2 in tumor cells; blue lines: 30 patients with high expression of TIE2 in tumor cells. **C**, Representative images of multiplexed immunofluorescence staining of cervical cancer tissues. **D**, The statistical graph shows the density of tumoral CD31 (CD31%) in tumors with high and low Ep-CAM+TIE2+ density. TIE2, magenta; Ep-CAM, gold; CD31, white. Scale bar: 20 μm. **E**, Patients with a high density of CD31 in the tumor present poorer OS (left) and DFS (right) than patients with a low density of CD31 in the tumor. Yellow lines: 21 patients with a low density of CD31 in tumors; blue lines: 29 patients with a high density of CD31 in tumors. (All experiments were repeated in triplicate or more. Data with error bars are presented to indicate the mean ± SEM values. ns $P > 0.05$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$)

Table 1
The relation of EPCAM+TIE2+ cells and CD31+ cells to clinical pathological paraments in cervical cancer.

Clinicopathological parameters	N (%)	Ep-CAM+TIE2+ cells% Median (Range)	Mann-Whitney u-Test P value	CD31+ cells% Median (Range)	Mann-Whitney u-Test P value
Total patients	64 (100)	0.17 (0.00-25.78)		0.20 (0.00-11.13)	
Age (years)					
<45	24 (37.5)	0.3051 (0.00-25.78)	0.8545	0.305 (0.00-11.13)	0.1797
≥45	40 (62.5)	0.1381 (0.00-22.32)		0.11 (0.00-8.18)	
FIGO*					
IA1-IB1	44 (68.8)	0.1181 (0.00-16.76)	0.0243	0.115 (0.00-7.02)	0.0381
IB2-IIB	20 (31.2)	0.3200 (0.00-25.78)		0.645 (0.00-11.13)	
Histological type					
Squamous cell carcinoma	54 (84.4)	0.2047 (0.00-25.78)	0.5483	0.27 (0.00-11.13)	0.4517
Adenocarcinoma	10 (15.6)	0.115 (0.00-22.32)		0.02 (0.00-7.02)	
Histologic grade					
Well/Modrately differentiated	53 (82.8)	0.15 (0.00-25.78)	0.3278	0.17 (0.00-11.13)	0.5191
Poorly differentiated	11 (17.2)	0.3002 (0.00-16.76)		0.65 (0.00-5.08)	
Lymph-vascular space invasion					
Negative	38 (59.4)	0.2550 (0.00-16.76)	0.7928	0.235 (0.00-8.18)	0.4717
Positive	26 (40.6)	0.15 (0.00-25.78)		0.085 (0.00-11.13)	
Lymph node metastasis					
Negative	42 (65.6)	0.275 (0.00-16.76)	0.8907	0.145 (0.00-8.18)	0.8618
Positive	22 (34.4)	0.145 (0.00-25.78)		0.395 (0.00-11.13)	

* FIGO: the International Federation of Gynecology and Obstetrics 2009 staging criteria.

EdU (5-ethynyl-2'-deoxyuridine) proliferation assay

HUVECs treated with conditioned medium were seeded into 96-well plates with 10^4 cells per well. An EdU proliferation assay was also used to investigate the proliferation of cells according to the manufacturer's instructions for the EdU proliferation kit (RiboBio, China). After the cells were attached to the wall, the medium was changed to EdU solution for two hours, and then, the cells were fixed with 4% paraformaldehyde for 30 min. After treatment with 0.5% Triton for 10 min, Apollo staining reaction solution and 1 × Hoechst 33342 reaction solution were used for EdU staining and DNA staining for 30 min in sequence. Images were taken under a fluorescence inverted microscope within 48 h after staining and counted with ImageJ software (ImageJ software, National Institutes of Health, Bethesda, MD, USA). The proportion of EdU-bound cells was the proportion of proliferating cells. The experiment was repeated three times.

Migration and invasion assays

A total of 4 × 10^4 HUVECs treated with conditioned medium for 48 h were plated in 24-well Transwell plates with inserts (8 μm in diameter, Corning) with or without Matrigel. The medium in the inserts was free of FBS, whereas the medium outside the inserts was supplemented with 10% FBS. After 24 h of incubation, the cell inserts were fixed with 4% paraformaldehyde for 30 min, and 0.1% crystal violet was used for staining for 30 min. Cells were counted by photographing through the microscope. The experiment was repeated three times.

Tube formation assay

HUVECs were precultured in serum-free RPMI 1640 media for 24 h. A 96-well plate was coated with Matrigel (50 μl). Thereafter, HUVECs were seeded into wells (10,000/well), and 200 μl of conditioned medium from primary cancer cells or cancer cells pretreated with inhibitors or recombinant human angiopoietins described above was added with or without 100 mg/ml bevacizumab (Roche Diagnostics GmbH, Mannheim, Germany) or 5 μM TIE2 kinase inhibitor (Selleck Chemicals, Houston, TX, USA). After incubation for 12 h, the cells were stained with calcein-AM for 10 min, and images were captured by fluorescence microscopy. Tube formation was quantified using ImageJ. The experiment was repeated three times.

In vivo xenograft models

Four-week-old female nude mice were purchased from Beijing Hua Fukang Biological Polytron Technologies, Inc. All mice were housed in the experimental animal center of Tongji Medical College, Huazhong University of Science and Technology. All procedures followed the operational specifications of the animal management committee. Twelve mice were randomly divided into two groups and received subcutaneous injections of 2 × 10^6 SiHa-NC or SiHa-*TEK* cells. The tumor size and weight was monitored every 3 days, and the animals were sacrificed by cervical dislocation with the largest subcutaneous tumor volume reaching 150 mm^3. After sacrifice, the tumors were removed for size and weight measurements. The volume of the tumor was calculated with the following formula: length × width × width/2 (mm^3).

Immunohistochemistry (IHC) and assessment of microvessel density (MVD)

Paraffin slides were dehydrated three times with TO clarifier for biological tissue slicing and dehydrated by sequential washing with anhydrous ethanol, 95% ethanol, 75% ethanol, 50% ethanol and PBS. Antigen retrieval was performed for 20 min in citric acid antigen repair buffer (pH 6.0) at 95°C. After antigen retrieval, hydrogen peroxide

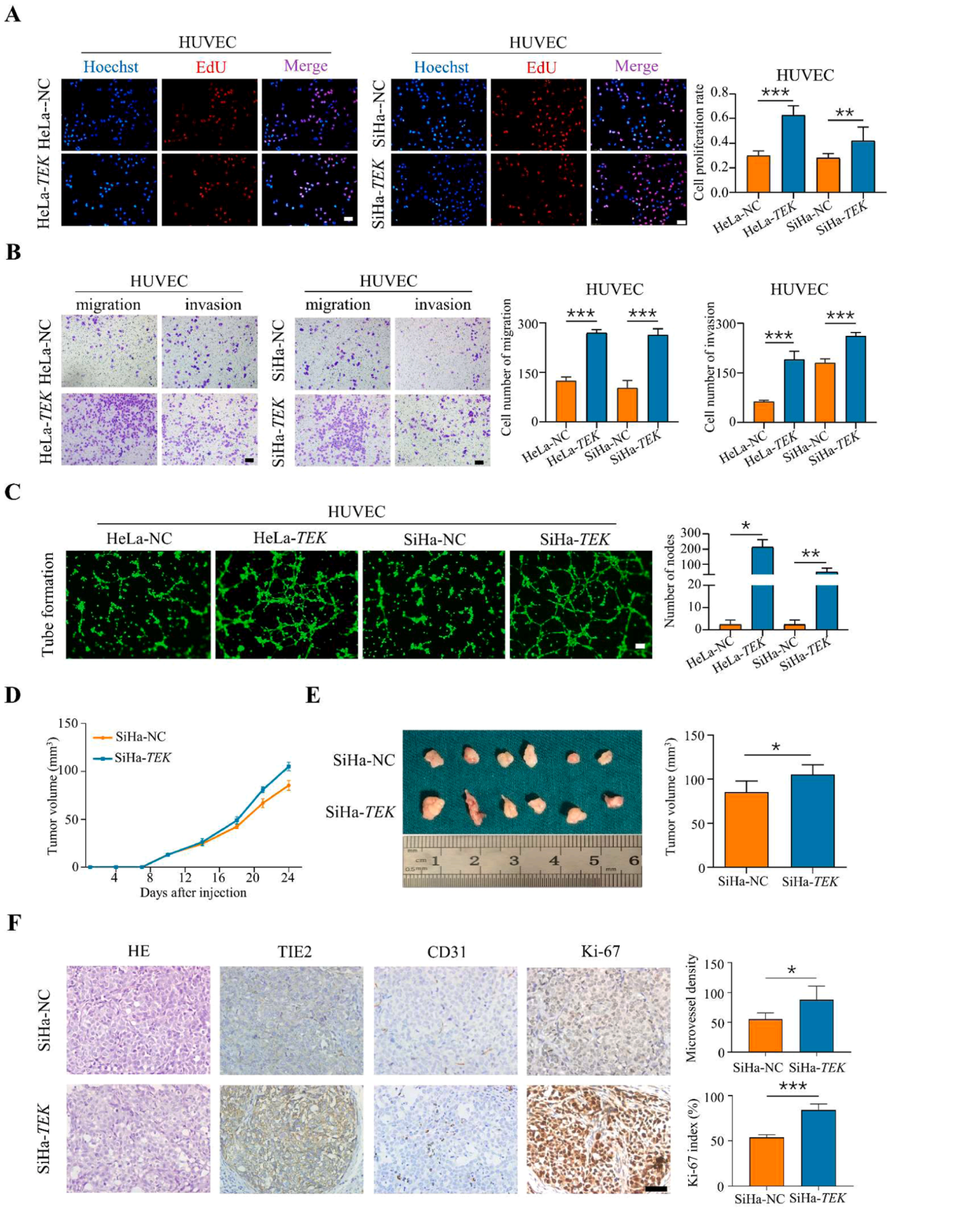

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Fig. 2. TIE2 promotes tumor angiogenesis in cervical cancer *in vitro and in vivo*. **A**, Representative images of EdU assays show the proliferation ability of HUVECs stimulated with CM from HeLa-NC, HeLa-*TEK,* SiHa-NC and SiHa-*TEK cells*. The histograms show the proliferation cell ratio of the EdU assays. Scale bar: 100 μm. **B**, Representative images of Transwell assays show the migration and invasion ability of HUVECs stimulated with CM from HeLa-NC, HeLa-*TEK,* SiHa-NC and SiHa-*TEK cells.* The histograms show the number of migrating and invading HUVECs. Scale bar: 100 μm. **C**, Representative images of tube formation assays show the tube formation ability of HUVECs stimulated with CM from HeLa-NC, HeLa-*TEK*, SiHa-NC and SiHa-*TEK cells*. The histograms show the number of nodes of the tube formation assays. Scale bar: 100 μm. **D-F**, SiHa-NC and SiHa-*TEK* cells were injected subcutaneously into nude mice (BALB/C, n = 6 for each group). **D**, Tumor growth curves of each group of nude mice. **E**, Images of subcutaneous xenografts and tumors removed from mice (left); the histograms show the tumor growth volume of each group of nude mice (right). **F**, IHC staining of anti-TIE2, anti-CD31 and anti-Ki-67 shows elevated expression of TIE2, MVD and Ki-67 in the subcutaneous xenografts with SiHa-*TEK* cells compared with SiHa-NC cells. (left); the histograms show the MVD and Ki-67 index (right). Scale bar: 50 μm. (All experiments were repeated in triplicate or more. Data with error bars are presented to indicate the mean ± SEM values. ns $P > 0.05$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$)

solution was used to inactivate endogenous peroxidase. Then, 3% H2O2 and 10% normal goat serum were used to quench endogenous peroxidase activity and block the antigen, respectively. The tissues were incubated with primary antibodies at 4°C overnight. Next, the sections were incubated at room temperature in corresponding biotinylated secondary antibodies for 30 min and streptavidin with horseradish peroxidase. Immunostaining was performed with diaminobenzidine (DAB) and hematoxylin. Finally, the sections were dehydrated by sequential washing with PBS, 50% ethanol, 75% ethanol, 95% ethanol and anhydrous ethanol and mounted. The tissue slices were then sealed and dried in an oven, followed by photo observation and score analysis. The antibodies used are listed in the Table S1.

Vascular hot spots were determined by scanning anti-CD31-stained slides at × 100 fields, and MVD was counted in the most vascularized areas. Four selected hot spots in × 200 fields were counted, and any endothelial cell cluster, even without a lumen, was counted. The median values for MVD were calculated[18]. The percentage of Ki-67+ cells was calculated by averaging five randomly selected fields of view. The expression of VEGFR2 was measured in 5 randomly selected areas for each tumor, and the average values were calculated. The expression of p-TIE2 p-AKT and p-MAPK was determined by the IHC score. The IHC results were scored according to the following formula: staining intensity (none = 0, weak = 1, moderate = 2, strong = 3) and staining area (none = 0, less than 30% = 1, between 30 and 60% = 2, more than 60% = 3).

Gene expression profiling interactive analysis (GEPIA) database

GEPIA (http://gepia.cancer-pku.cn/) analysis based on The Cancer Genome Atlas (TCGA) and Genotype Tissue Expression (GTEx) was conducted to examine the correlation of *TEK* or *ANGPT1* or *ANGPT2* and *VEGFR2*[19].

Statistical analysis

Statistical analysis was performed using GraphPad Prism 8.0.1 software (GraphPad, Bethesda, MD, USA). Student's t-test was used to evaluate the difference between two groups. Survival analysis was performed by the log-rank/Mantel–Cox test. The ratio of Ep-CAM+ TIE2+ cells to clinical pathological parameters was analysed by the Mann–Whitney U test. $P<0.05$ was considered significant.

Results

TIE2 is correlated with poorer prognosis and angiogenesis in cervical cancer

First, the densities of TIE2-expressing tumor cells (Ep-CAM$^+$TIE2$^+$%) and endothelia (CD31$^+$%) in cervical cancers were evaluated by multi-immunofluorescence assays in 64 human cervical cancer specimens. TIE2 signals could be observed in both Ep-CAM$^+$ and Ep-CAM$^-$ cells (Fig. 1A). The Ep-CAM$^+$TIE2$^+$% varied between tumors, ranging from 0 to 25.78% with a median of 0.17%, indicating inter- and intratumoral heterogeneity of the TIE2 expression pattern. More importantly, a high Ep-CAM$^+$TIE2$^+$ density (Ep-CAM$^+$TIE2$^+$% > 0.11%) was significantly associated with poorer overall survival (OS) (P =0.0227) and disease-free survival (DFS) (P =0.0170) in patients (Fig. 1B) and advanced FIGO stage (P =0.0243) (Table 1). Similar to TIE2, the CD31+% was altered between tumors, ranging from 0 to 11.13% with a median of 0.20%, and tumors with high Ep-CAM$^+$TIE2$^+$ density exhibited higher CD31+ density (Fig. 1C, D), indicating that tumor-expressing TIE2 was related to tumor angiogenesis. Furthermore, a high CD31+ density (CD31+% > 0.12%) was notably associated with poorer OS (P=0.0355) but not DFS in patients (Fig. 1E) and advanced FIGO stage (P=0.0381) (Table 1). These results suggest that TIE2-high tumor cells may play a role in tumor angiogenesis and progression.

TIE2-high cervical cancer cells promote tumor angiogenesis in vitro and in vivo

To further investigate the role of TIE2-high tumor cells in cervical cancer angiogenesis, Considering the low TIE2 level in SiHa and HeLa cell lines, we infected HeLa and SiHa cells with *TEK*-overexpressing lentivirus (HeLa-*TEK* and SiHa-*TEK*) to establish TIE2-high sublines; cells infected with negative control vector lentivirus (HeLa-NC and SiHa-NC) served as controls (Fig. S1A, B). Then, HUVECs were incubated with CM from these cervical cancer cells, and we found that, compared with the NC cells, the CM from HeLa-*TEK* and SiHa-*TEK* cells notably promoted the cell proliferation, migration, invasion and tube formation abilities of HUVECs *in vitro* (Figs. 2A-C, S1C). To verify the proangiogenic function of Tie2-high cervical cancer cells *in vivo*, SiHa-NC and SiHa-*TEK* cells were injected subcutaneously into nude mice. As shown in Fig. 2D-E, subcutaneous xenografts of SiHa-*TEK* exhibited more rapid growth and larger volumes. Consistently, the proliferation marker Ki-67 was more highly expressed in the SiHa-*TEK* group than in the SiHa-NC group, indicating that TIE2 promoted the progression of cervical

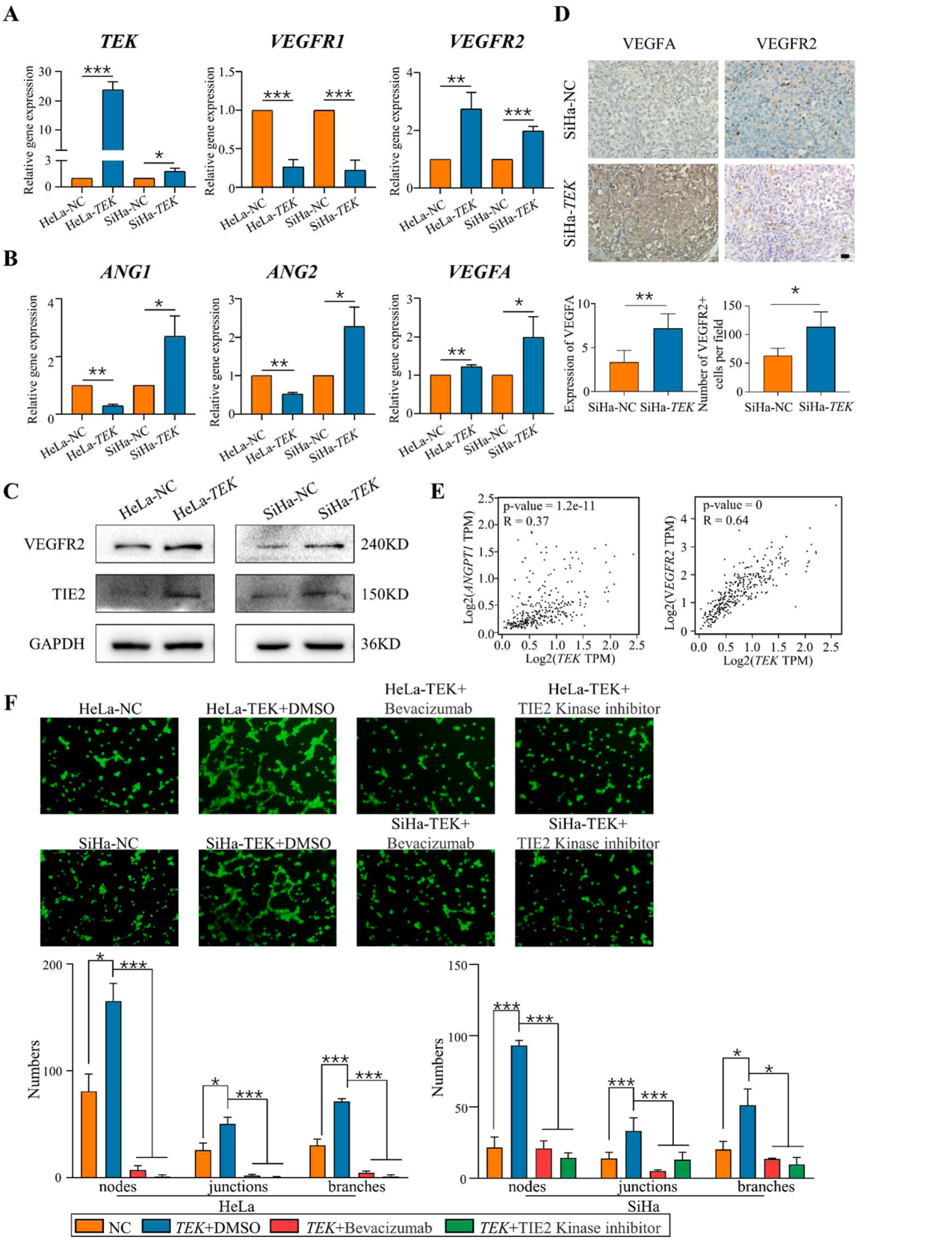

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Fig. 3. TIE2-high cervical cancer cells promote HUVEC angiogenesis by upregulating TIE2 and VEGFR2 in HUVECs. A, The histograms of qRT–PCR results show the expression of *TEK, VEGFR1* and *VEGFR2* in HUVECs stimulated with CM from HeLa-NC, HeLa-*TEK*, SiHa-NC and SiHa-*TEK cells.* **B**, The histograms of qRT–PCR results show the relative expression of *ANGPT1, ANGPT2* and *VEGFA* in HeLa-NC, HeLa-*TEK*, SiHa-NC and SiHa-*TEK* cells. **C**, Western blotting images show the expression of VEGFR2, TIE2 and GAPDH in HUVECs stimulated with CM from HeLa-NC, HeLa-*TEK*, SiHa-NC and SiHa-*TEK cells.* **D**, IHC staining of anti-VEGFA and anti-VEGFR2 shows elevated expression of VEGFA and VEGFR2 in the subcutaneous xenografts with SiHa-*TEK* cells compared with SiHa-NC cells. (up); The histograms show the expression of VEGFA and VEGFR2. Scale bar: 50 μm. **E**, Pairwise gene correlation analysis between *TEK* and *ANGPT1* and *VEGFR2* was performed by using *GEPIA*. **F**, Representative images of tube formation assays show the tube formation ability of HUVECs stimulated with CM from HeLa-NC, HeLa-*TEK*, SiHa-NC and SiHa-*TEK cells* with or without bevacizumab and TIE2 kinase inhibitor. The histograms show the number of nodes, junctions and branches of the tube formation assays. HUVECs were stimulated with CM from HeLa-NC, HeLa-*TEK*, SiHa-NC and SiHa-*TEK cells* with or without bevacizumab and TIE2 kinase inhibitor. Scale bar: 50 μm. (All experiments were repeated in triplicate or more. Data with error bars are presented to indicate the mean ± SEM values. ns $P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$)

cancer. To detect angiogenesis, we examined the expression of CD31 in tumors by IHC. Indeed, SiHa-*TEK* subcutaneous xenografts exhibited higher microvessel density than the control group, which was in line with the *in vitro* results (Fig. 2F). These findings indicate that TIE2 enhances the angiogenesis-inducing ability of cervical cancer cells.

TIE2-high cervical cancer cells promote angiogenesis by upregulating TIE2 and VEGFR2 expression in HUVECs

To explore the potential mechanism by which TIE2-high cervical cancer cells promote angiogenesis, we tested the changes in the expression of angiogenic receptors *TEK, VEGFR1*, and *VEGFR2* in HUVECs exposed to tumor cell CM and their ligands *ANG1, ANG2*, and *VEGFA* in tumor cells after upregulating *TEK* expression in HeLa and SiHa cells by qRT–PCR. Compared with NC HUVECs, the expression of *TEK* and *VEGFR2* in HUVECs stimulated with CM from HeLa-*TEK* and SiHa-*TEK* cells was significantly increased, whereas *VEGFR1* expression was decreased (Fig. 3A). In tumor cells, the upregulation of *TEK* elevated *VEGFA* expression in both HeLa and SiHa cells, while *ANG1* and *ANG2* were decreased in HeLa-*TEK* cells but increased in SiHa-*TEK* cells compared with NC cells (Fig. 3B). Western blot analysis confirmed the elevated VEGFR2 and TIE2 levels in HUVECs cultured with CM from HeLa-*TEK* and SiHa-*TEK* cells (Figs. 3C, S2A), and IHC staining of SiHa-NC and SiHa-*TEK* subcutaneous tumors revealed higher VEFGA and VEGFR2 signals in the SiHa-*TEK* group (Fig. 3D). Moreover, the results of pairwise gene correlation analysis of GEPIA data showed that the expression of VEGFA, VEGFR2, ANGPT1 and ANGPT2 was positively related to *TEK* expression in cervical cancers (Figs. 3E, S2B). Similar to the results described above, CM from *TEK* cells significantly enhanced the tube formation abilities of HUVECs compared to NC but was reversed by the use of bevacizumab or TIE2 kinase inhibitor in CM, indicating the critical role of VEGF and TIE2 signals in TIE2-high tumor cells promoting angiogenesis (Fig. 3F). Overall, these results illustrate that TIE2-high cervical cancer cells may promote the angiogenesis of HUVECs by upregulating TIE2 and VEGFR2.

TIE2-high cervical cancer cells promote angiogenesis dependent on TIE2-AKT/MAPK signaling

As a tyrosine kinase receptor, TIE2 can bind and activate downstream AKT and MAPK signaling to carry out signal transduction in endothelial cells[20]. Intriguingly, the TIE2 pathway was activated in TIE2-high cervical cancer cells, and p-TIE2, p-AKT and p-MAPK were upregulated in HeLa-*TEK* and SiHa-*TEK cells* compared to control cells (Figs. 4A, S3A). IHC staining of p-TIE2, p-AKT and p-MAPK in subcutaneous tumors verified the *in vitro* results (Fig. 4B). The additional use of a TIE2 kinase inhibitor reversed the upregulation of p-TIE2, p-AKT and p-MAPK in HeLa-*TEK* and SiHa-*TEK* cells (Figs. 4C, S3B-C), and VEGFR2 was downregulated in HUVECs incubated with CM from *TEK* cells pretreated with the above inhibitors (Figs. 4D, S3D). Consistent with the immunoblotting results, the enhancing ability of TIE2-high tumor cells to promote tube formation was reversed by the use of inhibitors, indicating the involvement of TIE2-AKT/MAPK signaling within TIIE2-high tumor cells (Fig. 4E).

Then, we demonstrated that human recombinant ANG1 and ANG2 stimulation increased the expression of phosphorylated TIE2 as well as the downstream kinases p-AKT and p-MAPK within tumor cells compared with unstimulated cells (Figs. 5A, S4A-B). The VEGFR2 expression and tube formation ability of HUVECs were also enhanced (Figs. 5B-C, S4C). The results of pairwise gene correlation analysis of GEPIA data showed that the expression of *VEGFR2* was positively related to *ANGPT1* and *ANGPT2* expression in cervical cancers (Fig. 5D). These data indicate that the TIE2-AKT/MAPK axis is crucial for TIE2-high cervical cancer in promoting angiogenesis and can be activated by angiopoietins.

Discussion

In the present study, we focused on the function of tumor cells expressing TIE2 and the potential mechanism by which TIE2-high tumor cells promote angiogenesis. We demonstrated that TIE2-high cervical cancer cells can promote angiogenesis by upregulating TIE2 and VEGFR2 expression in endothelial cells. Furthermore, we revealed that TIE2 could activate downstream AKT and MAPK signaling to promote VEGFR2 expression in HUVECs and that angiopoietins could activate the TIE2-AKT/MAPK axis to promote angiogenesis.

Diversified molecular mechanisms and cellular processes contribute to tumor angiogenesis, which relies on multiple interactions between different cells, particularly between tumor cells and endothelial cells, in which VEGF and TIE2 signals have been widely studied [7]. Although TIE2 is mainly expressed in HUVECs, many studies have suggested that TIE2 is expressed in nonstromal cells in cancers such as breast and thyroid cancers [16,21]. TIE2 was downregulated and negatively related to tumor metastasis in oral squamous cell carcinoma and in gastric and breast cancers, TIE2 was overexpressed and positively related to tumor angiogenesis, indicating that TIE2 plays varied roles in different tumors [16,22–24]. And in cervical cancer, tumor cell-derived exosomes deliver TIE2 protein to macrophages to promote angiogenesis in cervical cancer [25]. Our previous study reported that TIE2 was highly expressed in cervical cancer cells and that its expression in tumor cells predicted

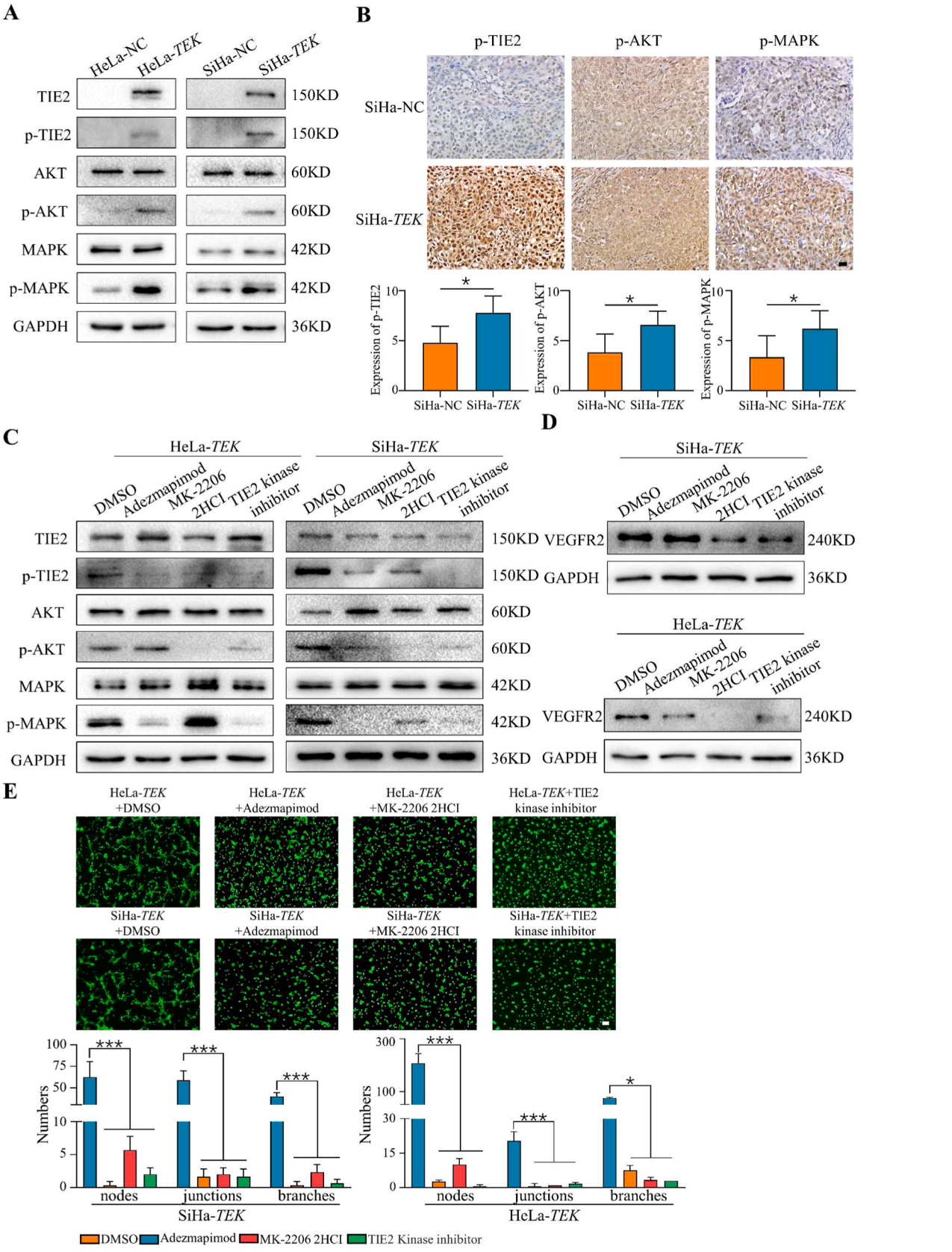

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Fig. 4. The role of TIE2-MAPK/AKT signaling in TIE2-mediated upregulation of VEGFR2 and angiogenesis in HUVECs. A, Western blotting images show the expression of TIE2, p-TIE2, AKT, p-AKT, MAPK, p-MAPK and GAPDH in HeLa-NC, HeLa-*TEK*, SiHa-NC and SiHa-*TEK cells.* **B**, IHC staining of anti-p-TIE2, anti-p-AKT and anti-p-MAPK shows elevated expression of p-TIE2, p-AKT and p-MAPK in the subcutaneous xenografts with SiHa-*TEK* cells compared with SiHa-NC cells (up). The histograms show the expression of p-TIE2, p-AKT and p-MAPK. Scale bar: 50 μm. **C**, Western blotting images show the expression of TIE2, p-TIE2, AKT, p-AKT, MAPK, p-MAPK and GAPDH in HeLa-*TEK* and SiHa-*TEK* cells treated with DMSO, Adezmapimod (MAPK inhibitor), MK-2206 2HCI (AKT inhibitor) or TIE2 kinase inhibitor. **D**, Western blotting images show the expression of VEGFR2 and GAPDH in HUVECs stimulated with CM from HeLa-*TEK* and SiHa-*TEK* cells pretreated with DMSO, Adezmapimod, MK-2206 2HCI or TIE2 kinase inhibitor. **E**, Representative images of tube formation assays show the tube formation ability of HUVECs treated with CM from HeLa-*TEK* and SiHa-*TEK* cells pretreated with DMSO, Adezmapimod, MK-2206 2HCI or TIE2 kinase inhibitor. The histograms show the number of nodes, junctions and branches of the tube formation assays. HUVECs were treated with CM from SiHa-*TEK* cells pretreated with DMSO, Adezmapimod, MK-2206 2HCI or TIE2 kinase inhibitor. Scale bar: 100 μm. (All experiments were repeated in triplicate or more. Data with error bars are presented to indicate the mean ± SEM values. ns $P > 0.05$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$)

poorer prognosis, implying that TIE2 may participate in the development and progression of cervical cancer [17]. However, the association between TIE2 within tumor cells and cancer angiogenesis remains unclear.

In our study, we found that the expression of TIE2 in cervical cancer cells was positively associated with the MVD of cervical cancer with endothelial CD31 labeling and risks for recurrence and death in patients. Next, we explored the angiogenesis-inducing ability of TIE2 in cervical cancer. It was found that TIE2-high cervical cancer cells promoted HUVEC proliferation, migration, invasion and tube formation, which were correlated with endothelial angiogenesis. *In vivo* subcutaneous xenografts of SiHa-*TEK* exhibited a larger volume, more rapid growth and higher microvessel density. Angiogenesis is a complex process that is precisely regulated by specific factors, including angiogenic factors and their receptors [7]. For example, in cervical and ovarian cancers, OVA66 overexpression increases VEGF production by cancer cells to enhance angiogenesis [26]. Here, we found that VEGFR2 and TIE2 were upregulated in HUVECs and that VEGFA was overexpressed in TIE2-high cancer cells. For angiopoietins, ANG1 and ANG2 were upregulated in SiHa-*TEK* cells but not in HeLa-*TEK* cells. Considering the different genetic backgrounds of these cell lines, the role of TIE2 in HeLa cells requires further investigation. In endothelial cells, VEGFR2 is the major mediator of cell proliferation, and the angiogenic effects of VEGFA and VEGFR1 remain controversial, which could not only bind with VEGFA but also prevent the binding of VEGFA and VEGFR2 [27]. Therefore, in our study, VEGFR1 downregulation in HUVECs did not hinder the angiogenic effects of VEGFA and VEGFR2 upregulation.

Many studies have proven that TIE2 can activate downstream AKT and MAPK pathways in endothelial cells [21]. Whether TIE2 could also activate downstream AKT and MAPK pathways in tumor cells was not detected. It has only been demonstrated that TIE2 induces papillary thyroid carcinoma proliferation and migration via the PI3K/AKT pathway, but whether TIE2-AKT signaling in tumor cells affects angiogenesis has not been confirmed to our knowledge. Notably, as TIE2 functions in HUVECs, TIE2 within tumor cells could activate downstream AKT and MAPK signals in tumor cells, and the inhibition of TIE2, AKT or MAPK in TIE2-high cancer cells rescued the upregulation of VEGFR2 and the enhanced tube formation ability of HUVECs. Moreover, we found that the use of different inhibitors causes the variable expression of TIE2. Inhibitions of AKT or MAPK pathways may reduce TIE2 phosphorylation and cause MAPK or AKT signals compensation, which may be the complicated signals crosstalk. Given the different pathological types, HPV status and genetic characteristics, it will be quite possible that there are differences of the role of MAPK pathway between SiHa-TEK and HeLa-TEK cells in the induction of VEGFR2 expression in HUVECs. In addition, TIE2 may also regulate other biological processes of cervical cancer cells, as AKT and MAPK signals have been reported to play important roles in tumor growth, invasion and metastasis [28,29]. Therefore, TIE2 within cancer cells may regulate the progression of cervical cancer through a variety of biological mechanisms in addition to angiogenesis. In addition to AKT and MAPK pathways, it has been reported that TIE2 can also activate downstream DOKR/Nck/PAK signals to promote endothelial cell proliferation and migration [30]. For example, Xia et al. identified that the Nck1/Rac1/PAK1/MMP2 cascade is critical for angiogenesis in CSCC [31]. However, whether TIE2 has an impact on the DOKR/Nck/PAK pathway in cervical cancer cells needs further investigation. Although some basic pathways are understood, the complexity of signaling will affect our understanding of the underlying mechanism. Phosphatases play an important role in signaling. Gene arrays and phosphoproteomics are being widely used, such as a nano-LC-MS/MS based quantitative phosphotyrosine screening approach, by which could identify more kinases associated with mutations, providing broader opportunities for the use of inhibitors as well as targets for new therapeutic strategies [32-34].

In endothelial cells, TIE2 acts as a receptor that interacts with angiopoietins to regulate angiogenesis. In our study, TIE2-high cervical cancer cells stimulated with ANG1, a full agonist of TIE2 and ANG2, a partial agonist of TIE2 when ANG1 is absent, displayed a stronger ability to induce angiogenesis than those treated with BSA. Similarly, in gliomas, Ang1 enhanced the adhesion of TIE2-expressing glioma cells and brain tumor cells to endothelial cells, which illustrated that TIE2 within tumor cells could be activated by angiopoietins [15]. Our previous results confirmed that Ang1 and Ang2 were highly expressed in cervical cancer and promoted tumor metastasis [17]. Combined with what was described before, there may be an autocrine Ang-TIE2 action to promote tumor metastasis and angiogenesis, which may be essential to tumor angiogenesis and an underlying therapeutic target but remains to be explored.

First-generation anti-angiogenic agents, such as the human vascular endothelial growth factor (VEGF)-specific monoclonal antibody bevacizumab, have been approved to treat metastatic or recurrent cervical cancer, but the clinical response and benefits are limited [4–6]. Recently, angiopoietins and RTKs have been attractive targets for improved antiangiogenic tumor therapy, emerging second-generation anti-angiogenic agents. Though huge progress has been made in anti-angiogenic therapy, bevacizumab is the only agent approved for the treatment of metastatic or recurrent cervical cancer [35]. Thus, it's urgent to develop new therapies for cervical cancer. In our study, we found TIE2-high cervical cancer cells could promote tumor angiogenesis and tumor growth by inducing TIE2 and VEGFR2 expression in endothelial cell. Anti-TIE2 therapy may offer a double hit to angiogenesis by affecting the TIE2 and VEGF pathways simultaneously in TIE2-high

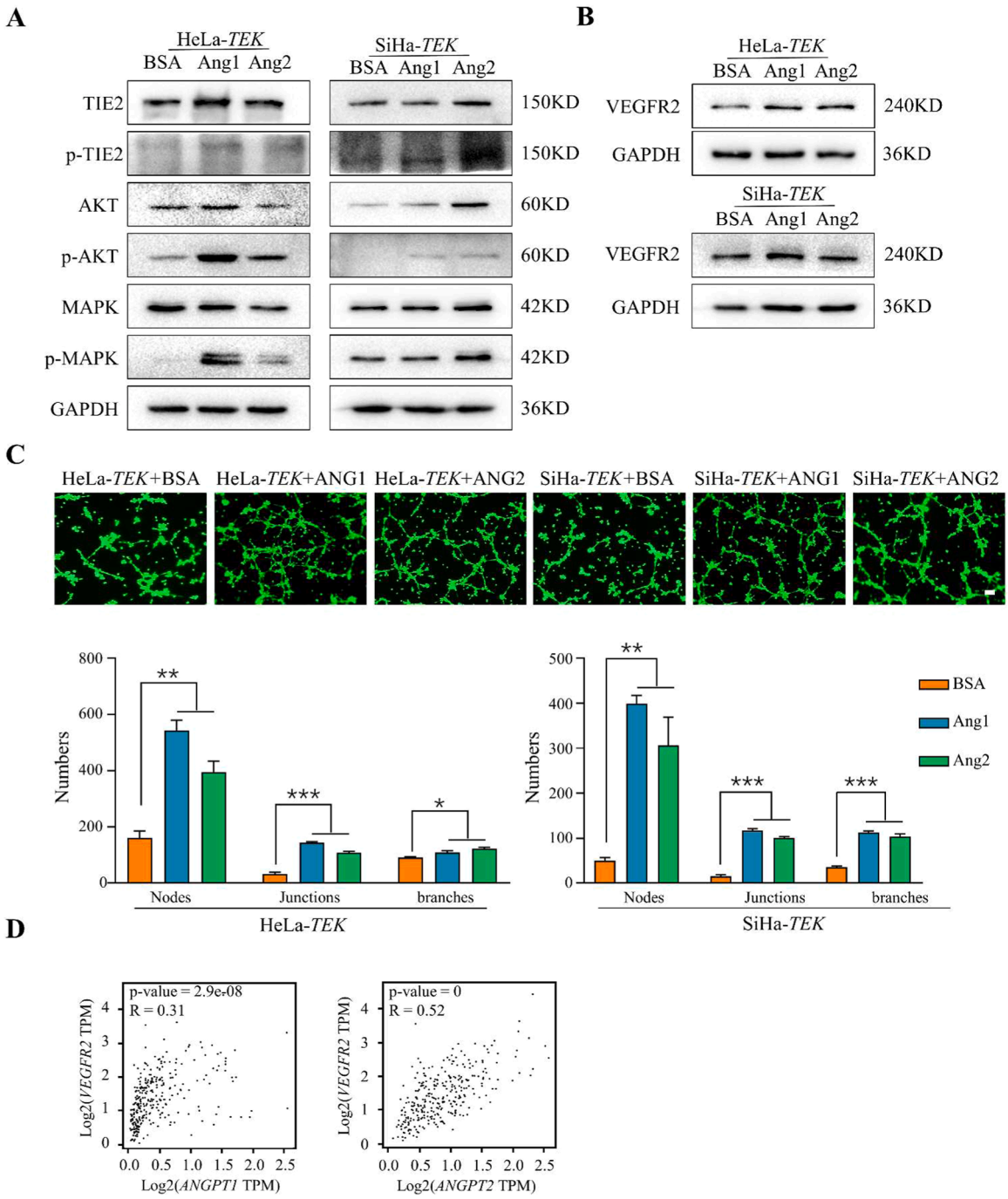

Fig. 5. TIE2-high cervical cancer cells can be activated by ANG1 and ANG2 to promote angiogenesis. A, Western blotting images show the expression of TIE2, p-TIE2, AKT, p-AKT, MAPK, p-MAPK and GAPDH in HeLa-*TEK* and SiHa-*TEK* cells stimulated with BSA, ANG1 or ANG2. **B**, Western blotting images show the expression of VEGFR2 and GAPDH in HUVECs stimulated with CM from HeLa-*TEK* and SiHa-*TEK* cells stimulated with BSA, ANG1 or ANG2. **C**, The histograms show the number of nodes, junctions and branches of the tube formation assays. HUVECs were treated with CM from SiHa-*TEK* cells stimulated with BSA, ANG1 or ANG2. **D**, Pairwise gene correlation analysis between *VEGFR2* and *ANGPT1* and *ANGPT2* was performed by using *GEPIA*. (All experiments were repeated in triplicate or more. Data with error bars are presented to indicate the mean ± SEM values. ns $P > 0.05$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$)

cervical cancers, providing more evidence for new anti-angiogenic therapies for cervical cancer. A clinical trial has been reported that bispecific Ang1/Ang2 antibody, AMG780, offered superiority in inhibiting tumor growth, viable tumor fraction, and tumor EC proliferation for advanced breast tumors [36]. There are also several small-molecule tyrosine kinase inhibitor (TKI) drugs that inhibit Tie2, many of which also inhibit VEGFR2. Regorafenib and Ripretinib have been approved as a salvage therapy for metastatic colorectal cancer and mutant c-kit expressing gastrointestinal stromal tumors respectively [37-39]. With the deepening of the understanding of the Ang-TIE2 system, it is possible to use these drugs in combination with anti-VEGF therapies. Indeed, targeting both pathways shows superior anti-angiogenic and antitumor effects compared to treatments alone [40,41].

Conclusion

In conclusion, we identified that TIE2-high tumor cells upregulate TIE2 and VEGFR2 expression on endothelial cells by activating the TIE2-

AKT/MAPK axis to promote angiogenesis. These findings provide new insight into tumor angiogenesis and antiangiogenic therapy.

Availability of data and materials

The datasets generated during and/or analyzed during the present study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The protocol for the human study was reviewed and approved by the Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China) (IORG No: IORG0003571), and informed consent was obtained from all patients. All animal experiments were approved by the Animal Management Committee of Tongji Medical College, Huazhong University of Science and Technology (IACUC Number:645).

Consent for publication

Not applicable.

CRediT authorship contribution statement

Shuran Tan: Methodology, Software, Validation, Formal analysis, Investigation, Writing – original draft, Visualization. **Yuanyuan Chen:** Methodology, Software, Validation, Formal analysis. **Shi Du:** Software, Validation, Formal analysis. **Wenhan Li:** Formal analysis, Investigation. **Pan Liu:** Investigation. **Jing Zhao:** Investigation. **Ping Yang:** Resources. **Jing Cai:** Funding acquisition, Resources. **Rui Gao:** Resources, Supervision, Project administration. **Zehua Wang:** Funding acquisition, Conceptualization, Resources, Writing – review & editing, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2022.101539.

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