

Aprepitant inhibits the progression of esophageal squamous cancer by blocking the truncated neurokinin-1 receptor

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Abstract. Increasing evidence showed that the substance P (SP)/neurokinin-1 receptor (NK1R) complex is involved in the development of several cancers. However, little is known about the mechanisms by which SP/NK1R complex plays a role in esophageal squamous cell carcinoma (ESCC) progression. RT-qPCR, CCK-8, Transwell, western blotting, immunohistochemical, immunofluorescence, ELISA and analysis of apoptosis were employed in the present study. It was aimed to investigate the function and therapeutic potential of the SP/tr-NK1R system in human ESCC progression. The results revealed that both SP and tr-NK1R were highly expressed in ESCC cell lines and specimens. In ESCC tissues, SP was mainly derived from ESCC cells and M2 macrophages. The NK1R antagonist aprepitant inhibited the SP-induced proliferation of human ESCC cell lines. Aprepitant inhibited cell migration and invasion and induced apoptosis of ESCC cells by downregulating the PI3K/AKT/mTOR signaling pathways. Animal experiments revealed that aprepitant inhibited tumor progression of ESCC in xenograft mice. In conclusion, high expression of SP plus tr-NK1R indicated poor prognosis in ESCC, suggesting that aprepitant has a potential application in ESCC. To the best of our knowledge, high SP and tr-NK1R expression in ESCC cell lines was reported for the first time in the present study. These findings provided evidence for a novel therapeutic strategy for patients with ESCC.

Introduction

Esophageal cancer is a common malignant digestive tumor, ranking only second to gastric cancer in incidence (1). Globally, ~450,000 people are affected by esophageal cancer each year and this incidence is growing in 2018 (2). Esophageal squamous cell carcinoma (ESCC) is a major histopathological subtype

of esophageal cancer with high prevalence in China (2). Even though the clinical diagnosis and therapeutic strategies have improved over the past two decades, the 5-year survival rate of ESCC patients remains unfavorable. Therefore, novel therapeutic modalities are urgently needed to improve prognosis of ESCC.

Substance P (SP) is a member of the tachykinin family, and is an extensively distributed neurotransmitter. After specifically binding to the neurokinin-1 receptor (NK1R), SP performs multiple functions in cells. Importantly, the potential mitogenic role of SP has been found in several different cancer types (3-5). NK1R has 2 subtypes, full length-NK1R (fl-NK1R) and truncated-NK1RT (tr-NK1R). The fl-NK1R has 407 amino acids, while the tr-NK1R subtype only has 311 amino acids, with this deficiency located at the C-terminus (6). Tr-NK1R can also bind to G proteins but is less efficient than fl-NK1R in internalization and desensitization (7). Tr-NK1R is highly overexpressed in hepatoblastoma, whereas fl-NK1R is expressed in negligible quantities (8). The SP/NK1R complex is an essential component of cancer cells and tumor microenvironment, and plays an oncogenic role in cell proliferation, migration and angiogenesis in hepatoblastoma and gallbladder cancer (8,9). Therefore, the application of the NK1R antagonist as an innovative anticancer drug warrants further study.

NK1R antagonists can be divided into peptide and non-peptide types. Aprepitant is a member of the non-peptide group that can cross the blood-brain barrier. It is mainly used to prevent acute and delayed nausea and vomiting during the initial and succeeding cycles treatment of antitumor chemotherapy (10,11). Importantly, only mild, transient, and tolerable side effects and no significant toxic side effects have been observed thus far for this drug, even in high doses (12).

The present study aimed to investigate the functions and therapeutic potential of the r-NK1R complex in human ESCC progression. It was hypothesized that aprepitant could inhibit the progression of ESCC by competitively binding tr-NK1R with SP. Towards this goal, SP and tr-NK1R expression was detected in ESCC cell lines and specimens, and the functions and molecular mechanisms of the NK1R antagonist aprepitant in ESCC were investigated.

Materials and methods

Patient's samples. ESCC tissue and corresponding normal esophageal tissue samples were collected from 25 patients

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(17 men and 8 women; age range, 50-72 years) with ESCC who underwent esophagectomy surgery in the Fourth Hospital of Hebei Medical University (Shijiazhuang, China) between January 2019 and April 2019. In addition, 84 samples (61 men and 23 women; age range, 48-80 years) of ESCC tissues were also collected from patients with ESCC who underwent esophagectomy surgery in the Fourth Hospital of Hebei Medical University between October 2016 and January 2017. All patients did not undergo preoperative adjuvant chemotherapy and radiotherapy. However, patients received radiotherapy or chemotherapy postoperatively, which may have had an impact on survival.

The present study was approved (approval no. 2020KY227) by the Medical Ethics Committee of the Fourth Hospital of Hebei Medical University (Shijiazhuang, China) and was conducted according to the tents of the Helsinki Declaration (seventh revision, 2013). Written informed consent was provided by all patients.

Cell culture. The human ESCC cell lines TE1, KYSE-150, and KYSE-170 were cultured in Dulbecco's Modified Eagle Medium (DMEM Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal calf serum and 1% penicillin/streptomycin. Cells were cultured at 37°C in a water-saturated atmosphere of 5% CO₂ in air. Human fibroblasts (Human fetal lung fibroblast, HLF1, cat. no. CL-0106) were purchased from Procell Life Science & Technology Co., Ltd., and cultured in Ham's F-12K (Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal calf serum and 1% penicillin/streptomycin.

Drugs. The PI3K inhibitor, Pictilisib (cat. no. HY-50094) and AKT inhibitor, Capivasertib (cat. no. HY-15431) were purchased from MedChemExpress.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from the cells or tissues by using a TRIzol[®] solution (Promega Corporation). Reverse transcription reactions were performed with reverse transcriptase (Go Script Reverse Transcription; Promega Corporation). RT-qPCR was conducted by using an SYBR Green PCR Kit (Promega Corporation) with a real-time PCR System (ABI 7500). The thermocycling conditions of qPCR were as follows: Initial denaturation, 70°C for 5 min; annealing, 25°C for 5 min; extension, 42°C for 60 min; and denaturation, 70°C for 15 min. The gene-specific RT-qPCR primers were as follows: fl-NK1R (specific primers were designed for >NM_001058.4; forward, 5'-GTTCCGTCTGGGCTTCAA-3' and reverse, 5'-CCAGGCGGCTGACTTTGT-3'); tr-NK1R (specific primers were designed for >NM_015727.3; forward, 5'-GGGCCACAAGACCATCTACA-3' and reverse, 5'-AAGTTAGCTGCAGTCCCCAC-3'); Arg-1 forward, 5'-GCAAGGTGATGGAAGAA-3' and reverse, 5'-CTGGTGTAAGATGGGT-3'; CD206 forward, 5'-CGTGTGCACCTACCTCAAGA-3' and reverse, 5'-AAGGACAGACCACTAATTCAGT-3'; IL-10 forward, 5'-GGAGAACCTGAAGACCT-3' and reverse, 5'-GGCTTTGTAGATGCCTTTC-3'; CCL22 forward, 5'-GCCTACTCTGATGACCGTGG-3' and reverse, 5'-AGAGAGTTGGCACAGGCTTC-3'; Class A macrophage scavenger receptor (SR) forward, 5'-GCAGGGCCCTCTTAAGATCA-3' and reverse, 5'-AACACGGGAACC

AAAGTCAT-3'; and GAPDH forward, 5'-AGAAGGCTGGGCTCATTTG-3' and reverse, 5'-GCAGGAGGCATTGCTGATGAT-3'. RNA expression levels were normalized to GAPDH expression levels. Relative expression levels were calculated using the 2^{-ΔΔC_q} method (13).

Western blot analysis. The total protein was prepared by using RIPA buffer (Beyotime Institute of Biotechnology). Protein concentrations were detected by using a BCA protein assay kit (Thermo Fisher Scientific, Inc.). Protein (40 μg/lane) was separated by electrophoresis on 6-15% SDS-polyacrylamide gels and transferred onto polyvinylidene fluoride membranes. Membranes were cut and incubated at 37°C in 5% BSA (Beijing Solarbio Science & Technology Co., Ltd.) for 1 h, and then incubated at 4°C overnight with primary antibodies at a 1:1,000 dilution. The primary antibodies against cleaved Caspase-3 (cat. no. 19677-1-AP), cleaved poly (ADP-ribose) polymerase (PARP, cat. no. 13371-1-AP) and β-actin (cat. no. 20536-1-AP) were purchased from Proteintech Group, Inc. Meanwhile, PI3k-p110α (cat. no. 4255), total AKT (cat. no. 9272), phospho-AKT (cat. no. 4060), total mTOR (cat. no. 2983), phospho-mTOR (cat. no. 5536), total 4EBP1 (cat. no. 9644), phospho-4EBP1 (cat. no. 9451), total p70S6K (cat. no. 2708) and phospho-p70S6K (cat. no. 9234) were purchased from Cell Signaling Technology, Inc. Membranes were then washed with tris-buffered saline Tween (1% Tween-20) and incubated with secondary HRP-conjugated antibodies (1:10,000) for 2 h at room temperature. The secondary antibodies were purchased from Proteintech Group, Inc (cat. no. PR30012 and PR30012). The antibody was detected by enhanced chemiluminescence reaction, visualization was performed using an ECL kit (Thermo Fisher Scientific, Inc.). ImageJ software (version 1.8.0_172; National Institutes of Health) was used to analyze the gray value of the western blot.

To distinguish between the tr-NK1R and fl-NK1R on western blot analysis, two specific antibodies were used to detect the C- and N-terminus: one antibody bound to an epitope at the N-terminus (cat. no. NB300-119; Novus Biologicals, LLC), and one antibody bound to an epitope at the C-terminus (cat. no. S8305; Sigma-Aldrich; Merck KGaA).

Cell Counting Kit-8 (CCK-8) assay. Cell proliferation was evaluated by using the Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc.). First, the cell suspension was inoculated (100 μl/well) in a 96-well plate, and the plate was incubated in a humidified incubator. Second, 10 μl of the CCK-8 solution was added to each well of the plate, and the plate was incubated at 37°C for 1.5 h in the incubator. Finally, the absorbance was measured at 450 nm by using a microplate reader.

Transwell migration and invasion assays. Transwell migration assay was performed by using chambers (24-well insert, Corning, Inc.) with 8-μm pore size polycarbonate filters coated without Matrigel (Beyotime Institute of Biotechnology) on the upper side. Meanwhile, the invasion assay was conducted by using the chambers with Matrigel on the upper side. Transwell membranes were precoated with Matrigel for 1 h at 37°C. The chambers were placed into a 24-well plate, and the lower chamber was filled with DMEM containing 20% fetal bovine

serum (FBS, Gibco; Thermo Fisher Scientific, Inc.). The ESCC cell suspension was inoculated (3×10^4 /well) in the upper chamber with and without aprepitant, and the lower chamber was filled with DMEM containing 20% FBS. The 24-well plate was then incubated at 37°C for 24 h. The cells that passed through the filter and attached to the lower compartment of the filter were identified by crystal violet staining. The number of transmembrane cells in different groups was counted using a light microscope.

Wound healing experiments. The cells (1×10^6) were inoculated in a six-well plate. After 12 h, when the cell fusion rate reached ~90%, the cells were lined with a pipette tip at the same angle and force. To ensure the detection in the same location, the back of the six-well plate was marked. The medium was replaced with serum-free medium with different concentrations of aprepitant. Phase-contrast images were recorded randomly with an inverted light microscope at the time of wounding and after 12 and 24 h. The experiment was repeated 3 times to ensure the accuracy of the results.

Analysis of apoptosis. Apoptosis was assessed by TUNEL staining. Briefly, after treatment with the different concentrations of aprepitant, the cells (3×10^3) were fixed in 4% paraformaldehyde for 30 min. This was followed by incubation with 0.1% Triton X-100 for 15 min. Then, 50 μ l of TUNEL incubation buffer was added to every well for 2 h. The number of apoptotic cells was recorded by fluorescence microscopy. The number of apoptotic cells was determined from five randomly selected fields. Apoptosis was also monitored with Annexin V-FITC/PI staining using Cell Apoptosis kit with Annexin V-FITC and PI (cat. no. 40302ES20; Shanghai Yeasen Biotechnology Co., Ltd.). According to the instructions, 5 μ l of Annexin V-FITC and 10 μ l of PI staining solution were added to the cells and gently mixed, and incubated for 10-15 min at room temperature and protected from light. Fluorescence microscopy was used to observe and capture images. The Annexin V-FITC fluorescence signal is green and the PI fluorescence signal is red.

Xenograft experiments. A total of 10 four-week old male BALB/c immunocompromised mice weighing about 20 g were obtained from the Vital River Laboratory Animal Technology Co. Ltd., Beijing. The animals were housed at 22-25°C, 40-60% humidity, 12/12-h dark/light cycles, and free access to food and water. First, 5×10^6 KYSE-170 cells in 200 μ l phosphate-buffered saline (PBS) were injected under the skin of the right flank. Mice were weighed, and the tumor was measured every other day. When the tumor volume reached 100 mm³, the mice were randomly divided into two groups. The treatment group was treated daily with an 0.3 mg/kg aprepitant by intraperitoneal injection every other day. The control group received 200 μ l of solvent. After 2 weeks of administration, the mice were sacrificed (Tail vein injection of pentobarbital sodium, 200 mg/kg), and the tumor volume and weight were measured. Tumor volume was calculated using the following formula: mm³=0.5 x length x width². A tissue section was fixed at 4°C for 16 h with 4% formaldehyde for immunohistochemical analyses, and another section was used to extract RNA for molecular analyses.

All animal experiments were performed at the Animal Laboratory Center of the Fourth Hospital of Hebei Medical University. All animal experiments were approved (approval no. 20190008) by the Animal Care Committee of the Fourth Hospital of Hebei Medical University (Shijiazhuang, China).

Immunohistochemistry. Paraffin-embedded slides (5 μ M) were deparaffinized in xylene, rehydrated using a decreasing alcohol gradient and washed with 1X PBS three times for 5 min. The sections were then heated in a microwave oven for 5 min in 10 mmol/l Na-citrate buffer (pH 6.0) for antigen retrieval and washed again with 1X PBS. The sections were immersed in 0.3% hydrogen peroxide in methanol for 20 min to suppress endogenous peroxidase activity. After further washing with 1X PBS, the sections were incubated in 10% normal goat serum (Proteintech Group, Inc.) at room temperature in a humidified chamber for 30 min to prevent non-specific immunoglobulin binding. The sections were then treated with the 1:100-diluted antibodies at 4°C overnight. The primary antibodies against CD68 (cat. no. 66231-2-Ig), CD163 (cat. no. 16646-1-AP), Ki67 (cat. no. 27309-1-AP), MMP-9 (cat. no. 10375-2-AP) and CD31 (cat. no. 11265-1-AP) were purchased from Proteintech Group, Inc. Normal IgG (cat. no. ab172730; Abcam) instead of the primary antibody served as the negative control. A streptavidin-biotinylated HRP-based detection system was used to reveal specific binding. The sections were counterstained with hematoxylin for light microscopic review and evaluation. The expression was ranked on the sum of intensity and area from 0 to 7: 0-2, negative expression; 3-7, positive expression (3-4, weak positive expression; 5-7, strong positive expression). Staining intensity was graded as follows: 0, no staining; 1, mild staining; 2, moderate staining; and 3, intense staining. The staining area was scored as follows: 0, no staining; 1, 1-25% area; 2, 26-50% area; 3, 51-75% area; and 4, 76-100% area.

Immunofluorescence. The steps before blocking were the same as those aforementioned in the Immunohistochemistry section. The sections were incubated in 10% BSA at 37°C in a humidified chamber for 30 min to block non-specific immunoglobulin binding. The sections were then treated with the 1:100-diluted antibodies SP (cat. no. S1542; MilliporeSigma) and CD163 (cat. no. ab156769; Abcam) at 4°C overnight. Fluorescein (FITC)-conjugated Affinipure Goat Anti-Mouse IgG (H+L) (cat. no. SA00003-1) and Rhodamine (TRITC)-conjugated Goat Anti-Rabbit IgG (H+L) (cat. no. SA00007-2) were used to detect different fluorescence signal. Cell nuclei were stained with 0.2 mg DAPI/ml PBS for 10 min. The sections were sealed by anti-fluorescence quenching sealed tablets after PBS three times washing. Then images were captured using a fluorescent microscope.

Enzyme-linked immunosorbent assay (ELISA). Cell supernatants were collected for ELISA. ELISA assays were performed in 96-well ELISA plates using an SP ELISA kit (cat. no. ab133029; Abcam), according to the manufacturer's instructions.

Small interfering RNA (siRNA) transfection. TE1, KYSE-150 and KYSE-170 cells were transfected with a double siRNA (Guangzhou RiboBio Co., Ltd.) with Hi-perfect Transfection

Reagent (Qiagen GmbH) according to the manufacturer's instructions. The final concentration of siRNA was 50 nM. After 48 h of transfection, the knockdown gene effect was measured using western blot analysis and RT-qPCR. Specific siRNAs were designed for fl-NK1R derived from transcript >NM_001058.4 and tr-NK1R derived from transcript >NM_015727.3. The sequences were as follows: fl-NK1R sense, 5'-CCACCAUCUCCACAGUGGU-3' and antisense, 5'-ACCACUGUGGAGAUGGUGG-3'; tr-NK1R sense, 5'-ACCCAGCUGUGAGACAAGA-3' and antisense, 5'-UCUUGUCUCACAGCUGGGU-3'; and si-NC sense, 5'-UUCUCCGAACGUGUCACGUTT-3' and antisense, 5'-ACGUGACACGUUCGGAGAATT-3'.

Statistical analysis. Data were presented as the mean \pm standard deviation (SD) and compared using paired Student's t-test and Mann-Whitney U test. The chi-square test was used to analyze the association between protein expression and clinicopathological parameters. Kaplan-Meier method was used for survival analysis and comparison among groups was conducted using the log-rank test. All statistical analyses were performed using SPSS 22.0 software (IBM Corp.). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

tr-NK1R is highly expressed in human ESCC specimens. To detect the expression of NK1R in ESCC cell lines, two sets of primers to detect two types of NK-1 receptors in ESCC cells were first designed: fl-NK1R and tr-NK-1R. The results showed downregulated fl-NK1R expressed in ESCC cells with human fibroblasts as the negative control (Fig. 1A). By contrast, tr-NK-1R expression was higher in ESCC cells than in human fibroblasts. For protein analysis, because there was no antibody specifically manufactured for tr-NK-1R commercially available, the C-terminus and N-terminus of the NK1R protein was detected to distinguish the expression of these two subtypes. The antibody used for the N-terminus can detect both fl-NK1R and tr-NK-1R, but antibody for the C-terminus can only detect fl-NK1R expression. As demonstrated in Fig. 1B, only tr-NK-1R was upregulated at the protein level in ESCC cell lines, consistent with the mRNA expression.

To further investigate the role of the SP/NK1R system in ESCC, SP and NK1R expression was detected in 25 pairs of ESCC and normal esophageal tissues. At the mRNA level, tr-NK1R overexpression was found in 19 samples (Fig. 1C) and fl-NK1R was found in only 4 samples (Fig. 1D). Overall, tr-NK1R expression was significantly higher in ESCC tissues (Fig. 1E). At the protein level, both NK1R and SP expression were increased in the ESCC tissue compared with normal esophageal tissue (Fig. 1F). Collectively, these results revealed that tr-NK1R is highly expressed in human ESCC lines and specimens.

In normal tissues, SP is mainly expressed in human immune cells, including monocytes, macrophages, lymphocytes, microglia, dendritic cells and bone marrow stem cells (14). Therefore, SP expression was also detected in immune cells of ESCC tissues using immunofluorescence. SP was also highly expressed in M2 polarized macrophages labeled with CD163 (Fig. 1G). As the most abundant the immune cells in the tumor

microenvironment, M2 polarized macrophages play crucial roles in tumor progression (14). The infiltration of M2 polarized macrophages labeled with CD68 and CD163 was increased in ESCC tissues (Fig. 1H). Furthermore, when human myeloid leukemia mononuclear THP-1 cells were polarized into M2 macrophages with IL-4 and IL-13, SP was also significantly increased to the similar level of ESCC cells (Fig. 1I and J). This suggested that ESCC cells and M2 macrophages are two important sources of SP in ESCC tissues.

NK1R antagonist aprepitant inhibits SP-induced proliferation of human ESCC cell lines. TE1, KYSE-150 and KYSE-170 cells were stimulated with increasing concentrations of SP, and cell growth of the cells after 48 h was observed. Cell growth was most pronounced at concentrations of 10^{-7} M (Fig. 2A). When the NK1R antagonist aprepitant was added to ESCC cell lines, CCK-8 proliferation assay showed significant growth inhibition (Fig. 2B). However, compared with ESCC cell lines, fibroblasts that expressed less NK1R, were less sensitive to this treatment. These results suggested that human fibroblast cells are resistant to the proliferation inhibition effect of aprepitant due to the lower expression of tr-NK1R compared with ESCC cell lines. However, SP blockage of the anti-SP antibody significantly reduced the growth of these 3 cell strains (Fig. 2C), suggesting that ESCC cells can promote their growth by autocrine secretion of SP. However, after treatment with a sublethal concentration of aprepitant, the addition of SP reversed the anti-proliferation effect of aprepitant (Fig. 2D). In summary, ESCC cell lines expressed higher tr-NK1R and were more sensitive to treatment than human fibroblasts. This result clearly indicated that the treatment effect of aprepitant in ESCC cells is specifically triggered via the SP/NK1R complex, and not via drug toxicity.

To further explore the role of different subtypes of NK1R, fl-NK1R and tr-NK1R levels were decreased by transfection with specific siRNA in TE1, KYSE-150 and KYSE-170 cells. Si-tr-NK1R downregulated both tr-NK1R and fl-NK1R. However, tr-NK1R decreased significantly, while fl-NK1R decreased slightly, and tr-NK1R was substantially more expressed than fl-NK1R in ESCC. It is considered that si-tr-NK1R still acts mainly on tr-NK1R, and the western blot results supported this (Fig. 2E). Compared with the untreated ESCC cells, downregulated tr-NK1R expression suppressed proliferation, while downregulated of fl-NK1R expression did not alter cell proliferation (Fig. 2F). When sublethal dose of aprepitant was applied to transfected ESCC cells, downregulated tr-NK1R expression attenuated the inhibition rate of aprepitant in all three ESCC cell lines (Fig. 2G). These results suggested that tr-NK1R, not fl-NK1R, plays a major role in ESCC proliferation. When tr-NK1R was downregulated, the effect of aprepitant was weakened, indicating that aprepitant mainly played an anti-proliferative role by binding to tr-NK1R.

Aprepitant inhibits migration and invasion in human ESCC cells. To clarify the effect of aprepitant on cell migration and invasion, wound healing and Transwell assays were used to detect the migration and invasion of ESCC cell lines *in vitro*. In the wound healing assay, the scratch healing rate significantly declined in the aprepitant group (Fig. 3A), indicating that aprepitant inhibited the migration in ESCC cells. In the

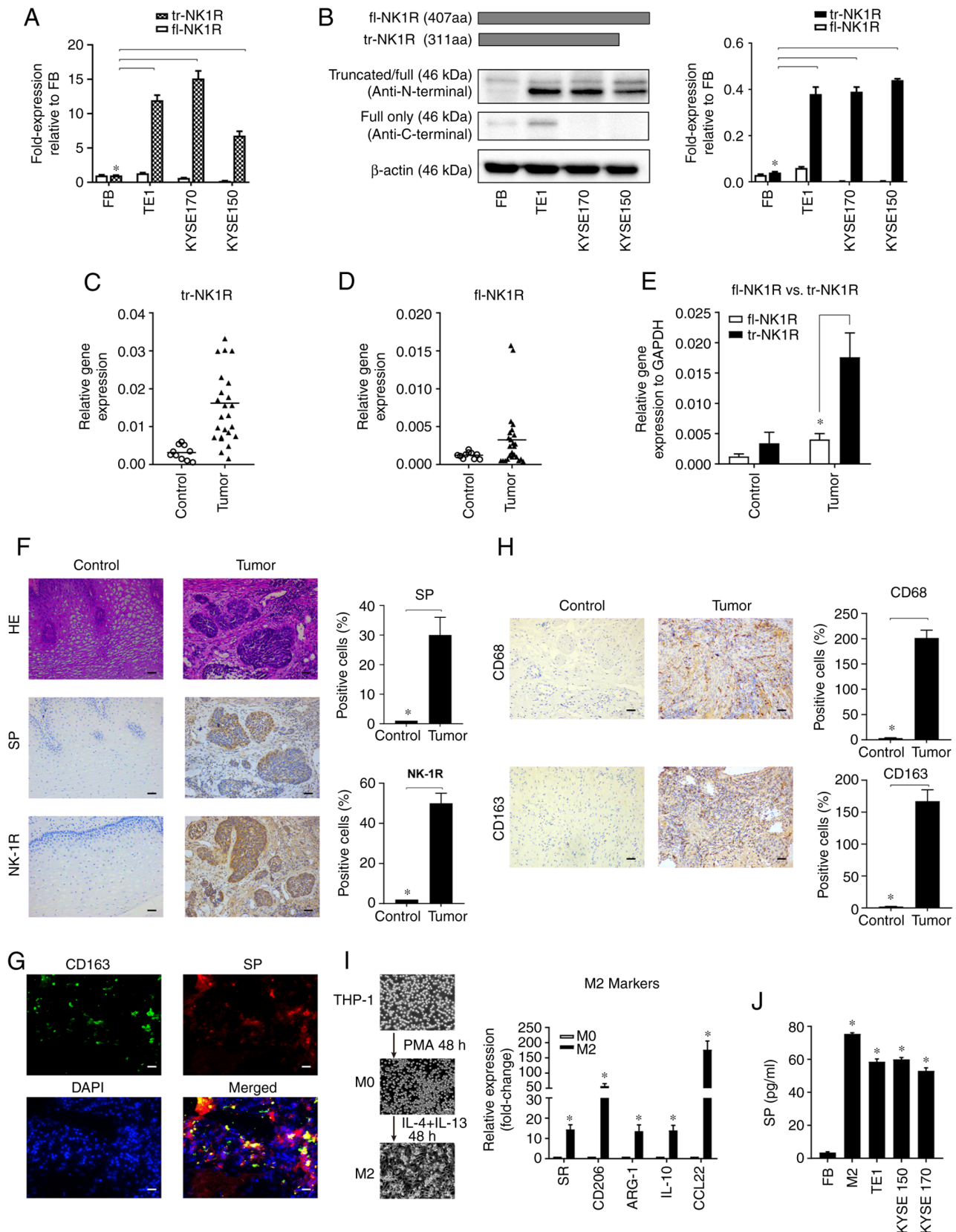


Figure 1. Expression patterns of NK1R in human ESCC cell lines and human tumor specimens. (A) mRNA expression of NK1R is shown as fold expression relative to human FB. (B) Western blot analysis for specific antibodies binding to either fl-NK1R or both tr- and fl-NK1R. (C and D) mRNA expression of (C) tr-NK1R and (D) fl-NK1R in normal esophageal and ESCC specimens. (E) Relative mRNA expression of tr-NK1R and fl-NK1R in ESCC. (F) H&E staining from human ESCC tissue and IHC staining for NK1R and SP are shown at higher magnification for both tumor and normal tissues. (G) CD163 and SP expression in ESCC tissues detected by immunofluorescence. Green: CD163; Red: SP; Blue: DAPI. (H) Cells were stained with antibodies against CD68 and CD163. Representative IHC images of ESCC and normal tissues with statistics of the corresponding expression levels are shown. (I) Microscopic images showing the differentiated progression from M0 to M2 and expression of M2 markers in THP-1 cells treated with IL-4 and IL-13. (J) SP secretion from FB, M2 macrophage and ESCC cells detected by ELISA. Scale bars, 100 μ m. * P <0.05. NK1R, neurokinin-1 receptor; ESCC, esophageal squamous cell carcinoma; FB, fibroblasts; fl, full length; tr, truncated; SP, substance P; IHC, immunohistochemical.

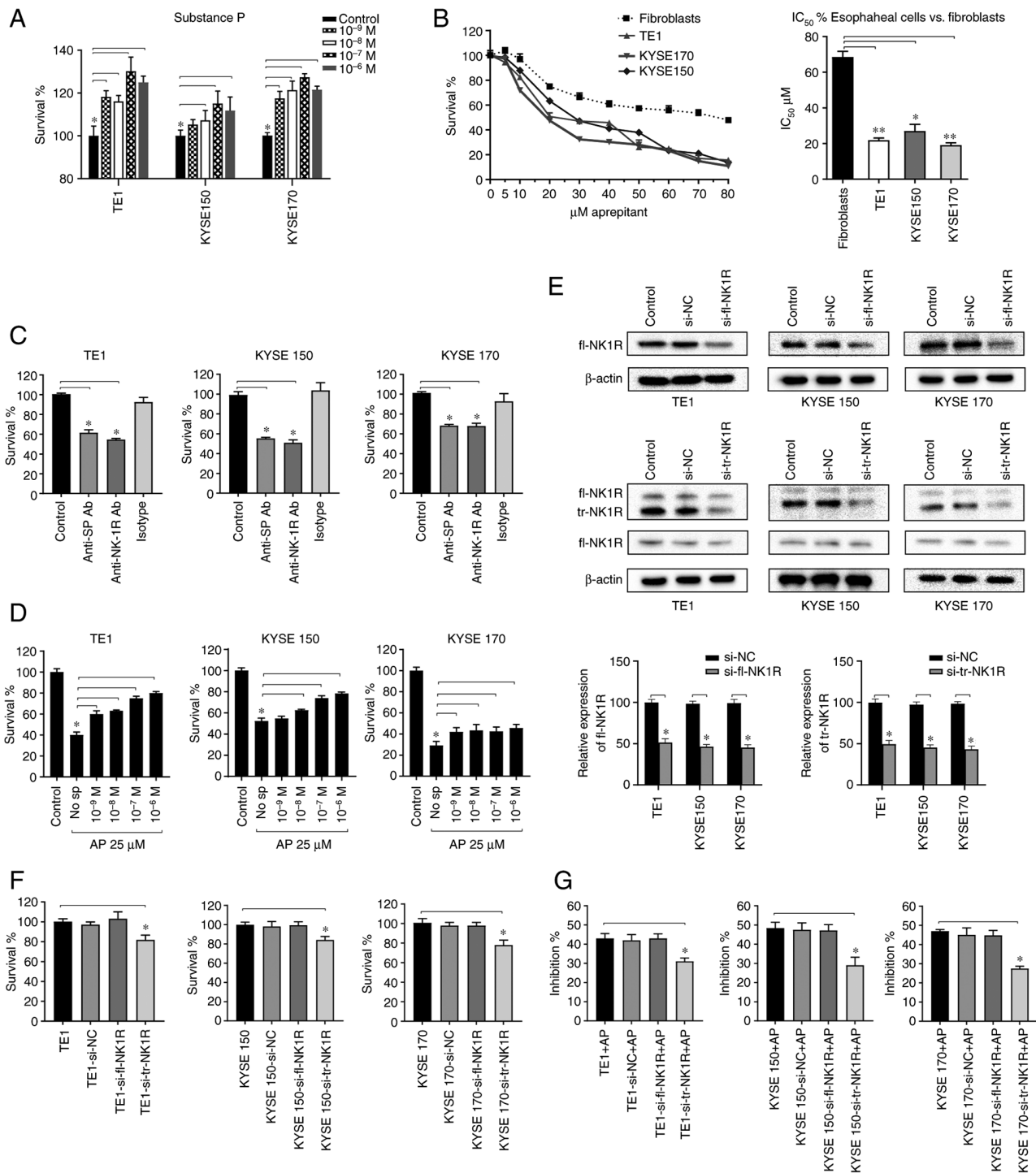


Figure 2. AP inhibits the SP-induced proliferation of human ESCC cell lines. (A) TE1, KYSE-150, and KYSE-170 cells were stimulated with different concentrations of SP to clarify its mitogenic potential in ESCC cells. (B) CCK-8 assays determining cell survival after treatment with Aprepitant for 48 h are shown for the cell lines TE1, KYSE-150 and KYSE-170, and for human fibroblasts. Based on these data, IC₅₀ (μM) was calculated and compared with fibroblasts for statistical analysis. (C) TE1, KYSE-150 and KYSE-170 cells were treated with anti-SP, anti-NK1R and isotype antibodies at a final concentration of 1:100. The effects were analyzed with CCK-8 proliferation assays. (D) All three ESCC cell lines were treated with different concentrations of SP and 25 μM Aprepitant, and survival effects were detected by CCK-8 assay. (E) The knockdown efficiency of fl-NK1R and tr-NK1R detected by Western blot analysis in TE1, KYSE-150 and KYSE-170 cells. (F) The proliferation of TE1, KYSE-150 and KYSE170 cells, respectively, after fl-NK1R and tr-NK1R were knocked down. (G) After fl-NK1R and tr-NK1R were knocked down respectively in TE1, KYSE-150 and KYSE-170 cells, IC₅₀ of Aprepitant was applied to observe the inhibitory rate. *P<0.05 and **P<0.01. AP, Aprepitant; SP, substance P; ESCC, esophageal squamous cell carcinoma; CCK-8, Cell Counting Kit-8; IC₅₀, half maximal inhibitory concentration; NK1R, neurokinin-1 receptor; fl, full length; tr, truncated; si-, small interfering; NC, negative control.

Transwell migration assay, the number of cells passing through the basement membrane of the chamber was observed under an inverted microscope. Compared with the control group, the Aprepitant treatment group showed a significantly lower

number of migratory cells in all three esophageal cancer cell lines (Fig. 3B). For the invasion assay, the number of cells that transferred to the Matrigel and reached the lower surface was significantly reduced after treatment with Aprepitant (Fig. 3C).

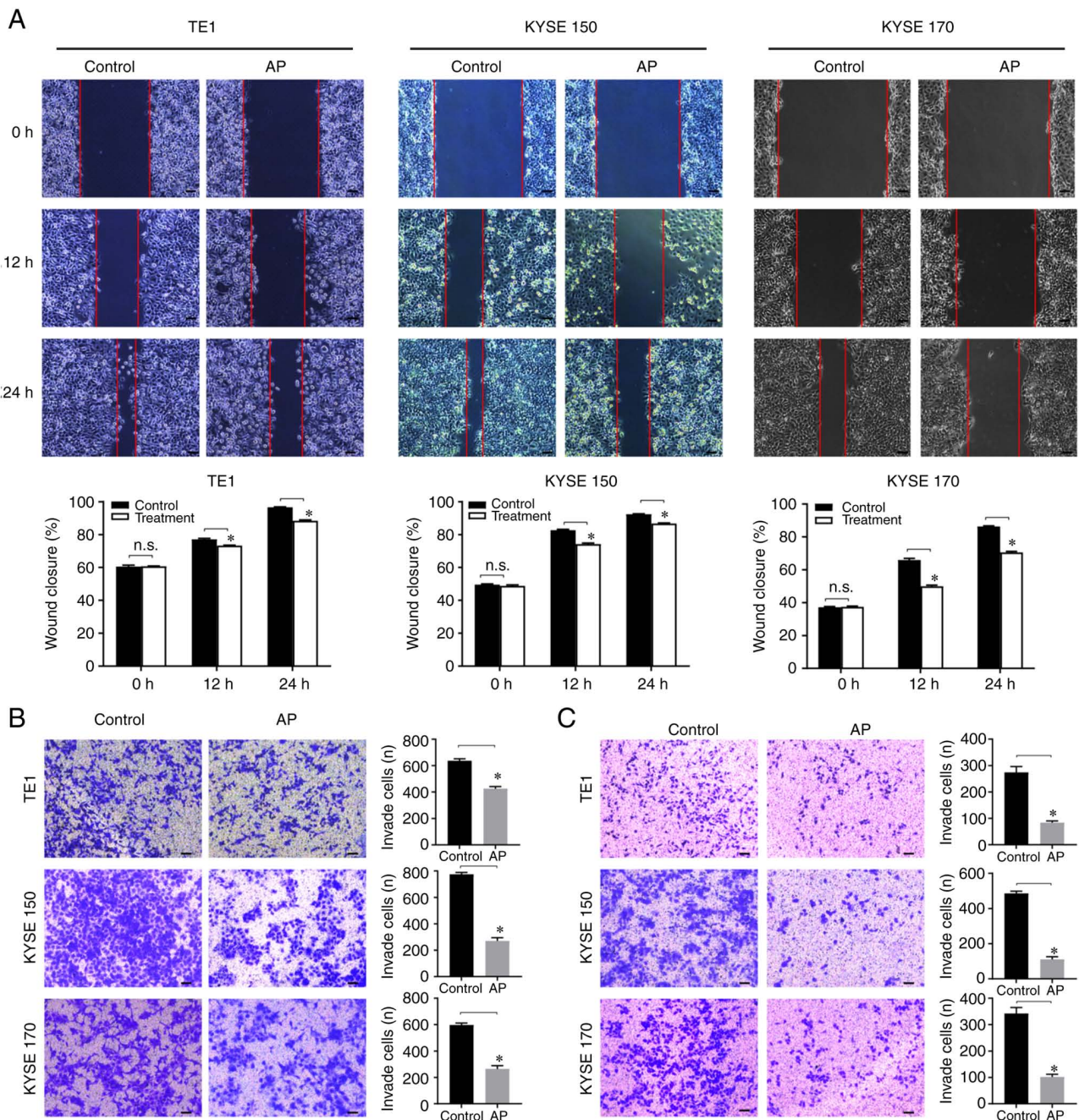


Figure 3. AP inhibits the invasion and migration in human ESCC cells. (A) The wound healing assay showed the effect of aprepitant on mobility. (B and C) The effect after treatment with aprepitant on (B) migration and (C) invasion of ESCC cells was investigated by using Transwell and Matrigel assay, respectively. Average counts were collected from 5 random microscopic fields. Scale bars, 100 μ m. * P <0.05. AP, aprepitant; ESCC, esophageal squamous cell carcinoma.

Aprepitant induces apoptosis in human ESCC cells. Blocking NK1R has been reported to induce apoptosis *in vitro* and *in vivo* via increase of mitochondrial reactive oxygen species (15). Therefore, it was detected whether aprepitant could affect the apoptosis in ESCC cell lines. TUNEL staining results revealed that aprepitant induced ESCC cell apoptosis in a dose-dependent manner after 24-h treatment (Fig. 4A). To analyze apoptosis in more detail, apoptotic markers for poly (ADP-ribose) polymerase (PARP) and caspase-3 were analyzed at the protein level by western blotting. Compared with the negative control, both the cleaved PARP and the cleaved caspase-3 were increased in a dose-dependent manner after aprepitant treatment for 24 h (Fig. 4B). The changes in

apoptotic markers showed that the late apoptotic mechanism was activated. Additionally, these cells were stained with anti-Annexin V-FITC antibody and propidium iodide to assess apoptosis by fluorescence microscopy (Fig. 4C), which showed a dose-dependent increase of late apoptotic cells.

Aprepitant affects ESCC cells function by downregulating PI3K/AKT/mTOR signaling pathways. When PI3K and AKT inhibitors (Pictilisib and Capivasertib, respectively) were applied to ESCC cells, cell proliferation was significantly reduced (Fig. 5A and B), suggesting that the PI3K/AKT signaling pathway was involved in the development of ESCC. Previous studies reported new evidence of a positive cross-relationship

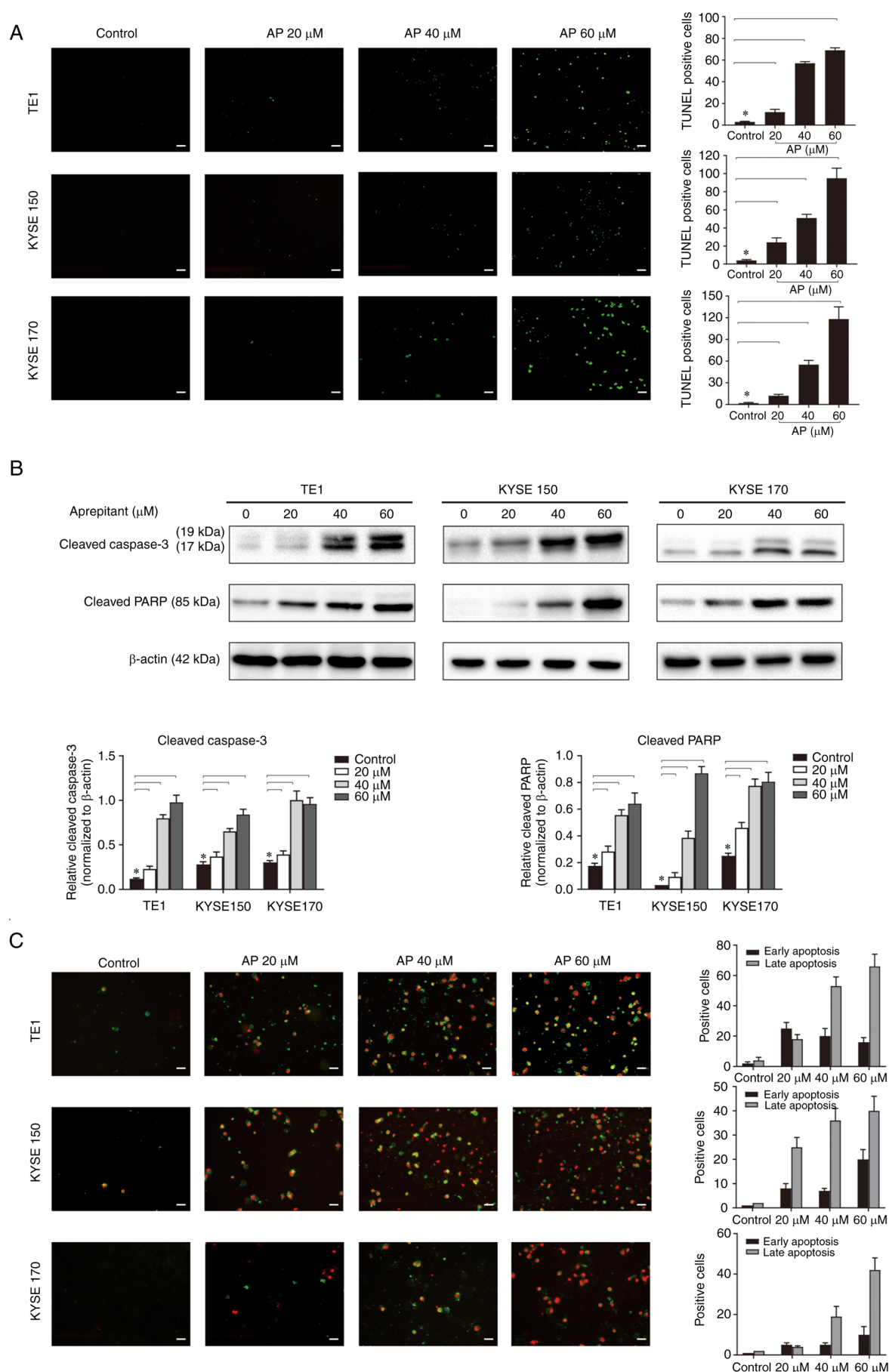


Figure 4. AP induces apoptosis in esophageal squamous cell carcinoma cells. (A) TUNEL staining 24 h after treatment with increasing doses of aprepitant was carried out for TE1, KYSE-150 and KYSE-170 cells. (B and C) TE1, KYSE-150, and KYSE-170 cells were treated with increasing doses of aprepitant. (B) Western blot analysis and (C) TUNEL assay were performed for the apoptotic markers of cleaved PARP and Caspase-3. * $P < 0.05$. AP, aprepitant.

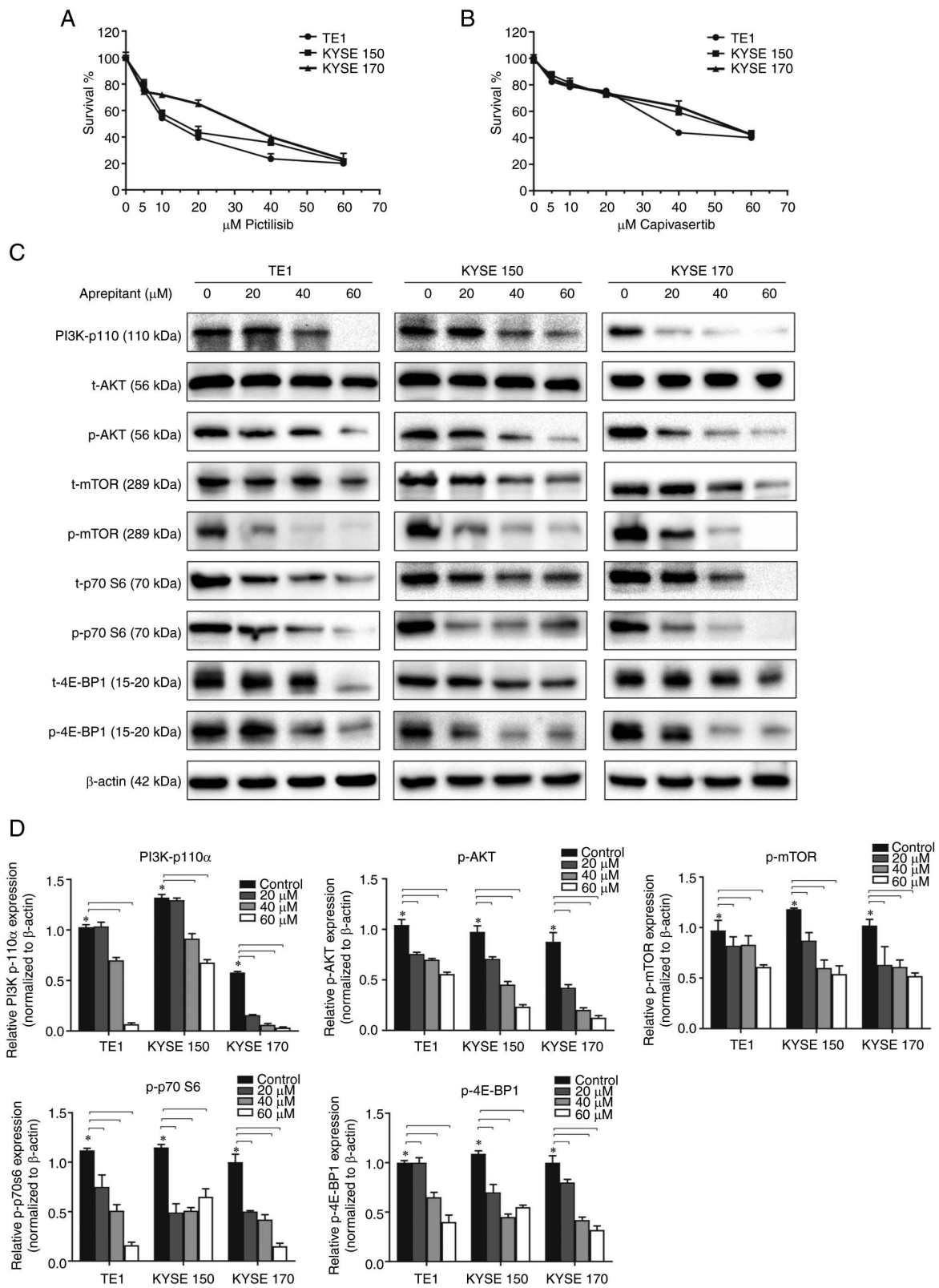


Figure 5. Aprepitant inhibition leads to downregulation of the PI3K/AKT signaling pathways. (A and B) TE1, KYSE-150 and KYSE-170 cells were stimulated with different concentrations of (A) Pictilisib or (B) Capivasertib, and cell survival was detected by Cell Counting Kit-8. (C and D) TE1, KYSE-150 and KYSE-170 cells were treated with increasing doses of aprepitant for 12 h, and protein lysates were probed for PI3K-p110α, total and (p)-AKT, total and (p)-mTOR, total and (p)-4EBP-1 and total and (p)-p70S6K. *P<0.05. p-, phosphorylated.

between NK1R overexpression and PI3K/AKT-mediated cell proliferation (16). However, this cross-relationship in ESCC remains unclear. To investigate the PI3K/AKT signaling

pathways when NK1R was antagonized, ESCC was treated with gradually increasing doses of aprepitant for 24 h. Western blot analysis for PI3K, AKT, mTOR, 4E-BP1, and p70S6K

was then performed in their total and phosphorylated form. A robust decrease was observed in PI3K-p110 α . Although both the total and phosphorylated AKT mTOR, 4E-BP1, and p70S6K forms were downregulated, the phosphorylated form was more significantly decreased. This resulted in a sharply decreased phosphorylation to total protein ratios. The result of western blotting indicated a strong downregulation of the PI3K/AKT/mTOR signaling pathway at the protein level by NK1R inhibition with aprepitant (Fig. 5C and D).

Aprepitant inhibits tumor progression in ESCC xenograft mice. To investigate the effect of aprepitant on ESCC progression *in vivo*, human KYSE-170 cells were subcutaneously implanted in the right flank of nude mice, and aprepitant was intraperitoneally injected when the tumor volume reached 100 mm³. There was no significant difference in weight or health status between the two groups throughout the whole treatment period (Fig. 6A). The results revealed that the tumor volume in nude mice was significantly reduced in the aprepitant-treated group on day 4 (Fig. 6B). After 14 days of treatment, tumor weight was significantly decreased in the aprepitant-treated group (Fig. 6C). None of the treated animals showed adverse effects, and there was no significant difference in morphology and structure of the important organs between the two groups (Fig. S1). Immunohistochemical staining revealed a high expression of total NK1R in both the control and treatment groups, and there was no significant difference in staining between these two groups (Fig. S2A). In addition, there was no difference in the expression of SP between these two groups (Fig. S2B).

To detect tumor-associated migration, matrix metalloproteinase 9 (MMP-9) was measured using immunohistochemical evaluation. The results showed that the number of positive cells were significantly reduced in the experimental group (Fig. 6D). Ki-67 staining in tumor cells exhibited a significantly decreased proliferation rate in the treatment group (Fig. 6E). Angiogenesis *in vivo* was further investigated by immunohistochemical analysis with CD31. The results revealed that both the microvascular density and the vascularized area were significantly reduced in the treatment group (Fig. 6F). These data suggested that aprepitant inhibited tumor progression in ESCC xenograft mice.

High expression of SP plus tr-NK1R indicates poor prognosis in ESCC patients. To further explore the clinical significance of SP and tr-NK1R in ESCC progression, SP and tr-NK1R expression were analyzed using serial sections in 84 samples of ESCC tissues (Fig. 7A). High expression of SP and tr-NK1R were correlated with poor clinical stage (Fig. 7B). The results demonstrated that high expression levels of SP plus tr-NK1R indicate poor prognosis of ESCC, but high expression of SP or tr-NK1R alone did not affect the outcome of patients with ESCC (Fig. 7C).

Discussion

The SP/NK1R complex is involved in various types of cancer (9,17,18). However, there are only few studies on the expression and function of the SP/NK1R complex in ESCC. The present study found that tr-NK1R is overexpressed in

ESCC cell lines and tissues. In ESCC tissues, SP mainly originates from cancer cells and M2 macrophages. Blocking NK1R with its antagonist aprepitant induced tumor suppression (Fig. 8), both *in vitro* and *in vivo*. Furthermore, it was identified that high expression levels of SP plus tr-NK1R indicate poor prognosis of ESCC patients without aprepitant therapy. Collectively, these results indicated that the SP/NK1R complex may be a novel therapeutic target for patients with ESCC. To the best of our knowledge, the present study is the first to report tr-NK1R overexpression in ESCC cells.

SP, a member of the tachykinin family, is widely distributed in nerve fibers (19). It performs a series of biological functions after specific binding to NK1R. Previous studies have shown that SP plays an essential role in tumor development. Cancer cells can secrete SP to promote growth, invasion, migration, angiogenesis and inhibit apoptosis (20-22). Cancer cells can secrete SP and promote their growth. Mohammadi *et al* (23) reported that SP could accelerate the progression of human ESCC growth via MMP-2, MMP-9, VEGF-A and VEGFR1 overexpression. Specific SP monoclonal antibody treatment was also found to impair cell proliferation and increase cell apoptosis in breast cancer cell lines (24). The current study found a dose-dependent proliferation of ESCC cells under SP treatment, whereas cell proliferation was inhibited after anti-SP and anti-NK1R antibodies were added. In addition, ESCC cell supernatant was detected by ELISA, and the results revealed the presence of SP. Collectively, these findings support that ESCC cells promote their own growth by auto-stimulatory SP production.

The tumor microenvironment is formed by cancer cells and extracellular matrix, which affects tumor growth, drug resistance and metastasis (25). As a vital component of the tumor microenvironment, macrophages have been reported to produce SP under inflammatory conditions (26). The results of the present study revealed that in ESCC tissues, most infiltrating macrophages were M2 type macrophages, and these M2 macrophages could produce SP.

The results of the present study present a novel promising antitumor method via antagonism of NK1R. To the best of our knowledge, this is the first study to identify that tr-NK1R was overexpressed in ESCC cell lines, and proliferation, invasion and migration were significantly inhibited after blockage of tr-NK1R. Further, SP can induce ESCC proliferation, and a certain amount of SP can reverse the inhibitory effect of aprepitant. Overall, it was observed that SP and its receptor antagonist aprepitant competitively interact with NK1R, and the combination is reversible.

Aprepitant is a novel and promising compound that is currently approved by the Food and Drug Administration for preventing nausea and vomiting caused by oral chemotherapy (27). Based on the positive results in earlier controlled studies of aprepitant, clinicians have also used it as a treatment for major depressive disorder, pain and migraine (28). As numerous patients with cancer experience cancer pain and post-chemotherapy nausea, it can be assumed that blocking NK1R can suppress the effect of SP, making aprepitant particularly useful for tumor treatment (29). In addition, aprepitant may have a therapeutic antitumor response effect and could alleviate certain of

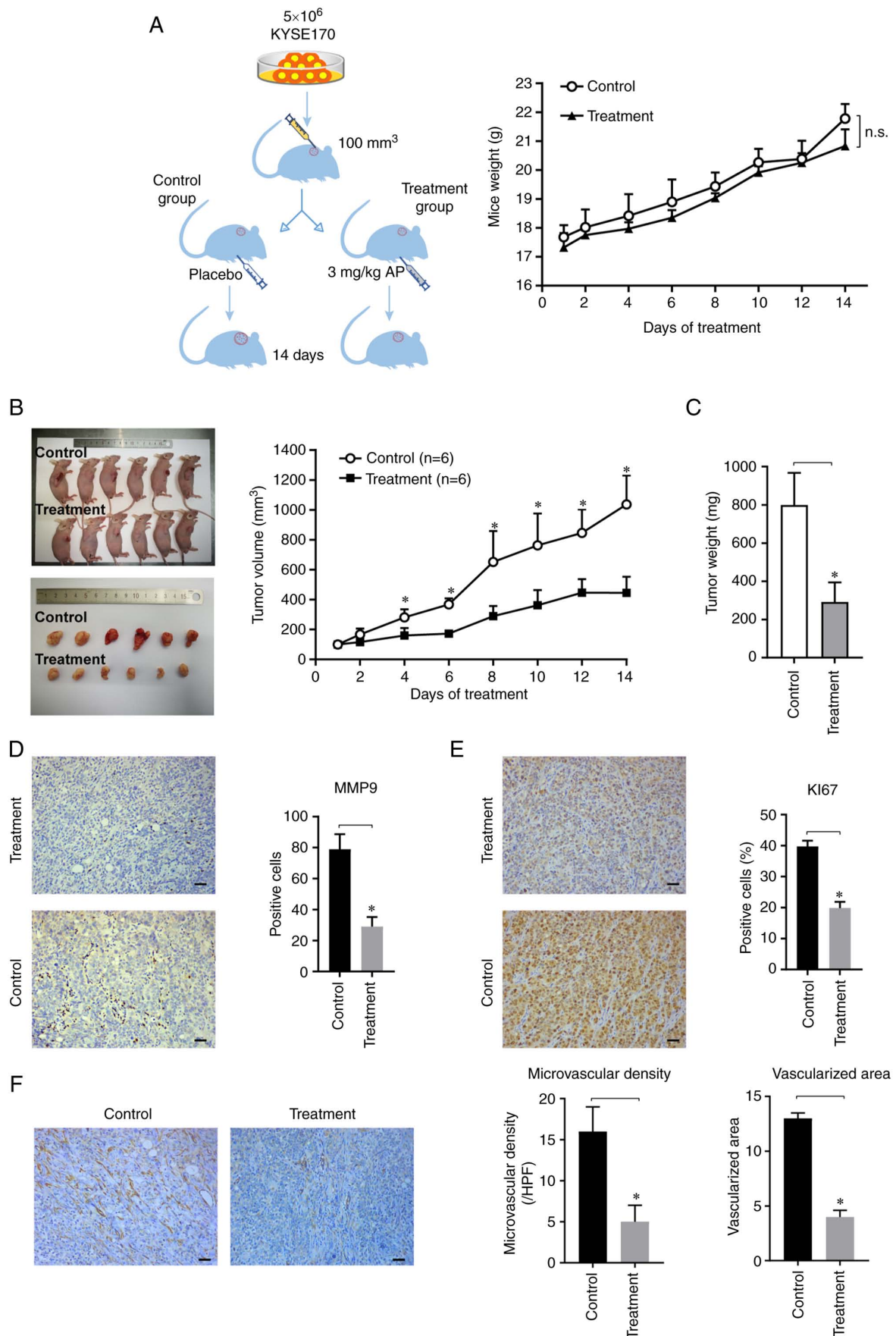


Figure 6. *In vivo* treatment of KYSE-170 xenografted mice with aprepitant. (A) Nude mice were xenografted subcutaneously with human KYSE-170 cells and randomly divided into 2 groups (control group n=6; treatment group n=6). Aprepitant was administered with intraperitoneal injection every other day when tumors reached 100 mm³ until day 14. (B) The tumor volume was measured with a caliper and depicted as mm³ at the indicated time points. (C) The tumor weight after 14 days was shown. (D and E) The immunohistochemical staining for MMP-9 and Ki-67 is shown. (F) Microvascular density and vascularized areas are depicted, additionally, *in vivo* angiogenesis was analyzed in mice by CD31 immunohistochemistry. Scale bars, 100 μ m. *P<0.05. n.s., not significant.

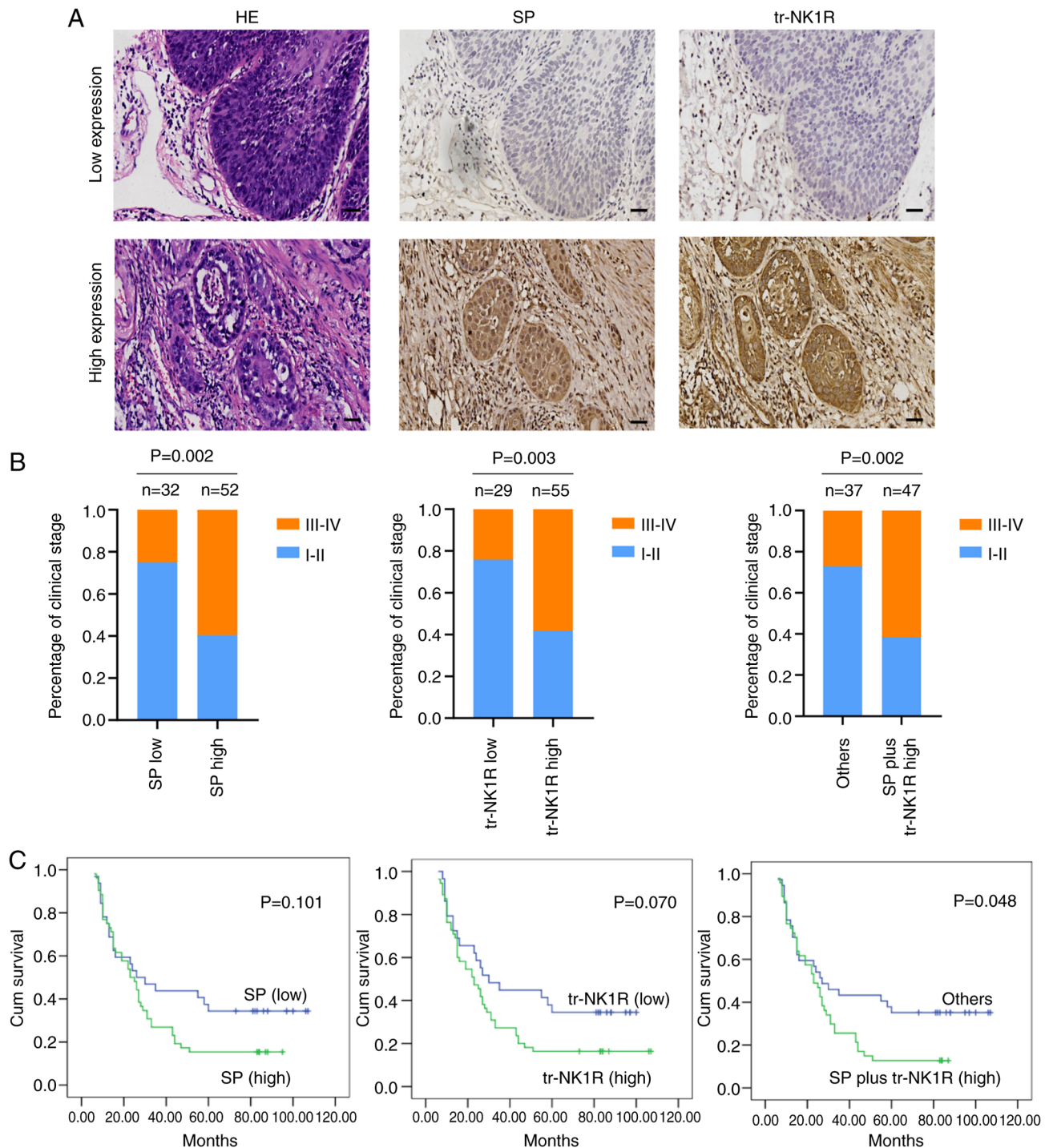


Figure 7. High expression of SP plus tr-NK1R indicates poor prognosis of patients with ESCC. (A) SP and tr-NK1R expression in serial ESCC tissues detected by immunohistochemistry. (B) The correlation between SP/tr-NK1R expression and the clinical stage of ESCC. (C) Significance of SP and tr-NK1R expression in the poor prognosis of patients with ESCC. SP, substance P; tr-, truncated; NK1R, neurokinin-1 receptor; ESCC, esophageal squamous cell carcinoma.

the adverse symptoms of cancer and its therapy (30). In the present study, wound healing and Transwell assays showed a significantly decreased migration and invasion abilities after aprepitant administration. Furthermore, aprepitant induced apoptosis in human ESCC cells. Javid *et al* (19) also reported that aprepitant promoted caspase-dependent apoptotic cell death and G2/M arrest in cancer stem-like ESCC spheres. Aprepitant appears to be a promising treatment modality for ESCC as a single agent or in conjunction with other chemotherapeutic drugs.

The current study investigated changes in signaling pathways after treatment with aprepitant in ESCC cells. After NK1R was antagonized by aprepitant, the phosphorylation levels of both AKT and mTOR were significantly decreased. To the best of our knowledge, these changes have not been described in ESCC cell lines thus far. PI3K-p110 α , p-AKT and p-mTOR were inhibited in a dose-dependent manner. Ge *et al* (15) reported that the NK1R antagonists aprepitant and SR140333 could induce apoptosis in myeloid leukemia through oxidative stress. They also examined the PI3K/AKT/mTOR

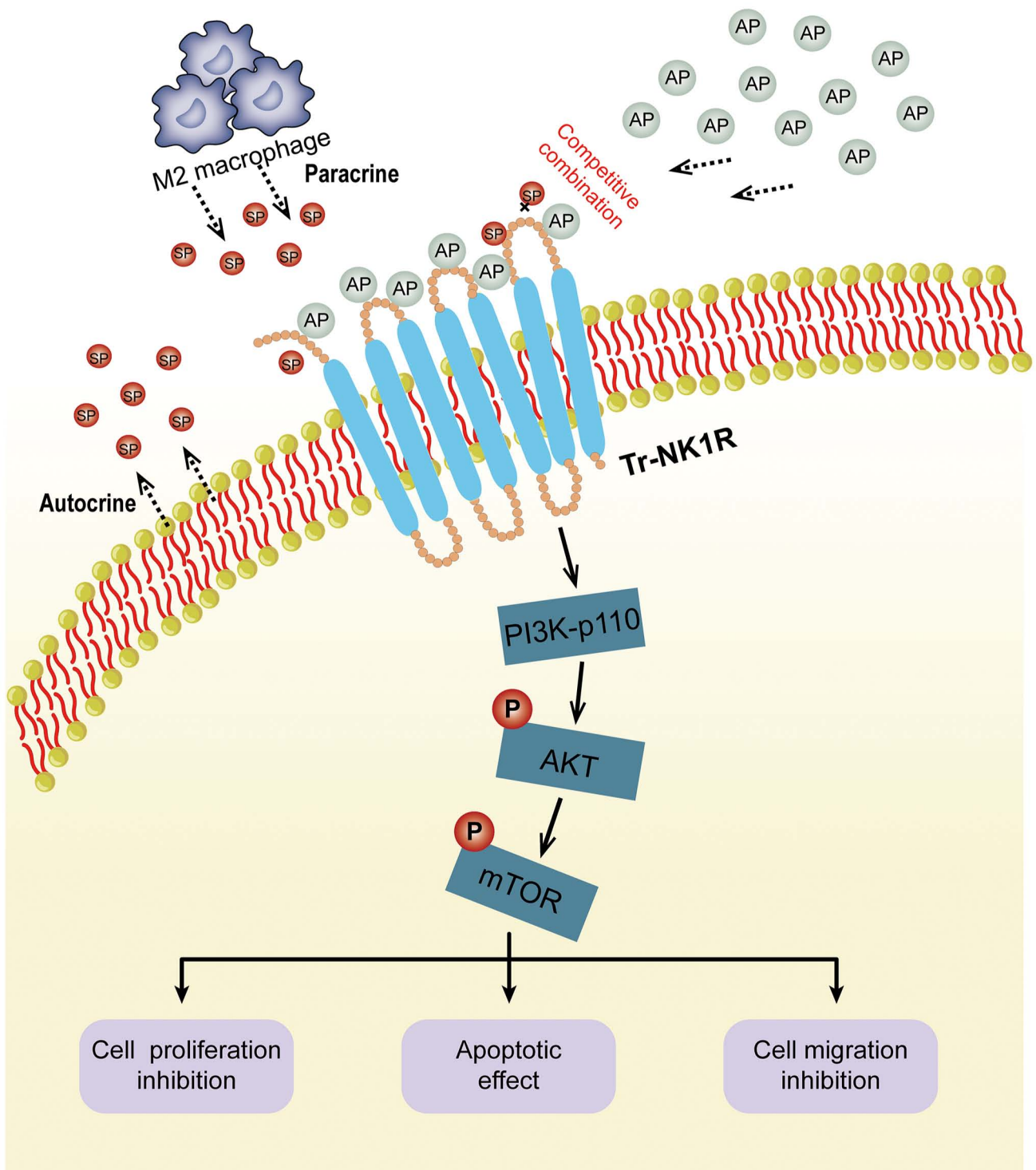


Figure 8. Diagram of molecular mechanism demonstrating how AP inhibits the progression of esophageal squamous cancer by blocking the tr-NK1R. tr-, truncated; NK1R, neurokinin-1 receptor; SP, substance P; AP, aprepitant.

signaling pathways and found that inhibition of these pathways no noticeable effect on the proliferation of myeloid leukemia cells. By contrast, the current study revealed that the PI3K/AKT/mTOR signaling pathway was significantly inhibited after treatment with the NK1R antagonist aprepitant for 48 h, which may be due to the different sources of tumor tissue and the longer duration of treatment. To further explore the role of aprepitant in the PI3K/AKT/mTOR pathway, the PI3K inhibitor Pictilisib was used to block the effects of aprepitant.

It was found that PI3Kp110, phosphorylated AKT and mTOR were decreased after using Pictilisib. However, aprepitant also exerts an inhibiting effect on ESCC progression by inhibiting the PI3K/AKT/mTOR signaling pathway. Currently, the PI3K/AKT/mTOR signaling pathway inhibitors cannot not be used to block aprepitant to validate the critical role of this pathway. Therefore, the PI3K/AKT/mTOR signaling pathway activator could be included in the study to further the effect of aprepitant in the PI3K/AKT/mTOR signaling pathway. In

future studies, the critical role of the aprepitant in this pathway will be verified by investigating whether the PI3K/AKT/mTOR signaling pathway activators can block the action of aprepitant. The present study provided a new idea for studying the mechanism of NK1R antagonists and pointed out a new direction for researching the role of NK1R antagonists in ESCC.

The current evaluation of tumorigenesis in nude mice demonstrated that aprepitant has a prominent antitumor effect, consistent with previous findings (8,24,31). Bigioni *et al* (32) reported that NK1R targeting in breast carcinoma cell lines in xenografted mice had a similar *in vivo* effect. Another study described a therapeutic effect in hepatoblastoma cells of xenografted mice (8). Unlike the aforementioned two studies that used an intravenous or oral NK1R antagonist, the NK1R antagonist was administered peritoneally. Therapeutic outcomes were achieved in all administration methods. Experiments with higher doses of 40 mg/kg/day orally, which is markedly higher than the intraperitoneal dose and also showed a significant therapeutic effect. In clinical samples, the high expression levels of SP plus tr-NK1R indicated poor prognosis of ESCC, but high expression of SP or tr-NK1R alone had no prognostic impact.

Although the current study revealed certain effects of the SP/NK1R complex, tr-NK1R was overexpressed in ESCC cell lines, whereas fl-NK1R was expressed at extremely low levels. This expression pattern of two different splice variants at the mRNA level was in accordance with that found at the cellular level. Further studies are needed to clarify why T tr-NK1R but not fl-NK1R is overexpressed in ESCC.

The SP/NK1R complex is expressed in various types of cancer, but the expression of its NK1R splicing variant has not been reported in ESCC. The present observations indicated that only tr-NK1R is highly expressed in ESCC cell lines, and this provides relevant evidence for targeted treatment in ESCC. Importantly, it was found that human ESCC cells overexpress NK1R, particularly the truncated type, and its antagonist aprepitant had significant inhibitory effects both *in vivo* and *in vitro*. These results support the SP/NK1R complex as a new therapeutic target in human ESCC. There are certain limitations to the present study; further studies are needed to explore the usefulness of NK1R antagonists as an anticancer strategy against ESCC.

In conclusion, in ESCC tissues, SP is mainly derived from ESCC cells and M2 macrophages. The NK1R antagonist aprepitant inhibited the SP-induced proliferation, migration and invasion, and induced the apoptosis of human ESCC cells through downregulating the PI3K/AKT/mTOR signaling pathways. Furthermore, aprepitant inhibited tumor progression in ESCC xenograft mice. In human ESCC tissues, high expression of SP plus tr-NK1R indicated poor prognosis, suggesting the strong potential of aprepitant as an anticancer treatment modality in ESCC.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

YZ, MS and BS conceived and designed the study. YZ wrote the manuscript. YZ, JL and YW performed experiments. FL and LG. contributed to data interpretation and statistical analysis. All the authors reviewed the manuscript. YZ and MS confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved (approval no. 2020KY227) by the Medical Ethics Committee of the Fourth Hospital of Hebei Medical University (Shijiazhuang, China) and was conducted according to the tents of the Helsinki Declaration. Written informed consent was provided by all patients. All animal experiments were approved (approval no. 20190008) by the Animal Care Committee of the Fourth Hospital of Hebei Medical University (Shijiazhuang, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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