Abstract

Background: LNK adaptor protein is a crucial regulator of normal hematopoiesis, which down-regulates activated tyrosine kinases at the cell surface resulting in an antitumor effect. To date, little studies have examined activities of LNK in solid tumors except ovarian cancer.

Methods: Clinical tissue chips were obtained from 16 clinical patients after surgery. Western blotting assay and quantitative real time PCR was performed to measure the expression of LNK. We investigate the in vivo and vitro effect of LNK in Triple Negative Breast Cancer by using cell proliferation migration assays and an in vivo murine xenograft model. Western blotting assay was performed to investigate the mechanism of LNK in triple negative breast cancer.

Results: We found that the levels of LNK expression were elevated in high grade triple-negative breast cancer through Clinical tissue chips. Remarkably, overexpression of LNK can promote breast cancer cell proliferation and migration in vivo and vitro, while silencing of LNK show the opposite phenomenon. We also found that LNK can promote breast cancer cell to proliferate and migrate via activating JAK/STAT3 and ERK1/2 pathway.

Conclusions: Our results suggest that the adaptor protein LNK acts as a positive signal transduction modulator in TNBC.

Keywords: LNK, Triple-negative breast cancer, p-ERK, JAK/STAT3

Background

Breast cancer is one of the most high incidence and mortality rate disease in the world [1], which is a heterogeneous disease and there are multiple ways by which to classify breast cancer into its subtypes [2]. Clinically, the primary diagnosis remains the histopathology report of the tumor which assesses the presence or absence of hormone receptors for estrogen (ER), progesterone (PR), and the human epidermal growth factor receptor-2 (HER2) [3]. The expression of these receptors is required to determine the patient’s suitability for endocrine therapies such as Tamoxifen, Anastrozole, and Trastuzumab [4]. The majority of breast cancers are receptor positive (77%) [5] and targeted treatment has proven efficacy. However, in the case of breast cancers that are negative for all three receptors (triple negative breast cancers, TNBC) there is, as yet, no targeted treatment available. Therefore, TNBC is more difficult to treat than target-specific breast cancer in clinical treatment [6–8]. And the only available treatments are chemotherapy and surgery [9]. Until now, numerous of trials with PARP inhibitors, angiogenesis inhibitors, EGFR inhibitors, SRC kinase inhibitors, and androgen receptor inhibitors have been used for therapy of TNBC, but none of them displayed significant improvements in all TNBC cases because of the heterogeneity of disease [9, 10]. Therefore, TNBC has a poor prognosis, which is associated with an increased number and earlier
Our studies showed that LNK was abnormally expressed in TNBC, thus we can speculate that the LNK may have a certain correlation with TNBC, which indicated that LNK protein may have a certain relationship with the function of TNBC. If indeed LNK functions as a tumor suppressor or promotor in TNBC, possibly it can be a therapeutic target, which can promote us to make progress in TNBC. Therefore, these studies mainly investigated the effects of LNK on TNBC in vivo and in vitro, and further explored the mechanism mediated the effect of LNK on TNBC.

**Materials and methods**

**Antibodies and reagents**

The following antibodies were used in this study: β-actin (proteintech), LNK (Abcam), p-ERK/ERK (Abcam), p-AKT/AKT (Abcam), p-STAT3/STAT3 (CST), p-STAT5/STAT5 (proteintech).

**Cell lines and cell culture**

TNBC cell lines MDA-MB-468, MDA-MB-231 (bought by ATCC) which were maintained in DF12 containing 10% fetal bovine serum (FBS). Cells were grown at 37 °C with 5% CO₂ in humidified air and stable cell line generation.

**Establishment of NOD-SCID mouse breast in situ fat pad model**

All animal procedures were performed under standard conditions and in accordance with protocols approved by the Experimental Animal Care Commission at China Pharmaceutical University. We divided our group into non-specific control group (NC), the silenced LNK group (SH1), SH2 and OE MDA-MB-231 cell suspensions (1 × 10⁶) were injected into the breast in situ fat pad and sutured the wound. Tumor growth was directly measured using a caliper. Each group included six mice. When the tumor volume growed into 1000 mm³, the mice were sacrificed and the fat pads, tumors, and fat pads containing tumors were removed and embedded in paraffin after 48 h of fixation in buffered formalin.

**Breast cancer tissue chip analysis**

The microarray analysis of breast cancer was provided by the Zhongda Hospital affiliated to Southeast University of the hospital. The correlation between LNK and TNBC patients’ gender, age, tumor size, stage, classification and metastasis was analyzed, and the correlation of adverse prognosis was confirmed.
Construction of LNK stable silencing or overexpressing cell lines

MDA-MB-468 or MDA-MB-231 cells are spotted in dishes, after the cells are attached for 24 h, the cells density reaches 50–60%. The medium was changed to fresh DF12 medium containing 10 μg/ml polybrene, and the lentivirus suspension was separately added and incubated in a 37 °C incubator for 24 h; After non-specific control group (NC) and silent (or) overexpressing LNK virus solution for 48 h, replace it with normal Fresh DF12 medium was further cultured for 24 h; Transfected cells were screened by the addition of puromycin (final concentration 10 μM).

Apoptosis detection

Adherent cells were collected by trypsin digestion without EDTA. Then wash the cells twice with PBS and use centrifugation at 2000 rpm for 5 min to collect 1–5 × 10⁶ cells; Add 5 μL of 7-AAD dye solution to 50 μL of Binding Buffer and mix. Add the above 7-AAD dye solution to the collected cells and mix; room temperature, protection from light, reaction for 5 to 15 min. After the reaction, add 450 μL of Binding Buffer and mix; Add 1 μL of Annexin V-PE to mix; room temperature, protection from light, reaction 5–15 min. After the reaction, add 400 μL PI staining and mix, avoiding light for 1–5 min.

Flow cytometry detection, excitation wavelength Ex = 488 nm; emission wave length Em = 578 nm; Annexin V-PE orange red fluorescence is recommended to use FL2 channel detection; excitation wavelength Ex = 546 nm; emission wave length Em = 647 nm, 7-AAD Red Fluorescence recommends using FL3 channel detection.

Cell cycle detection

Wash the cells once with PBS (centrifugation 2000 rpm, 5 min), collect and adjust the cell concentration to 1 × 10⁶/mL, and take 1 mL of single cell suspension; After preparing the single cell suspension, the supernatant is removed, 500 μL of an equal volume of 70% cold ethanol is added to the cells, and fixed at 4 °C overnight, and the fixing solution is washed away with PBS before dyeing; Add 100 μL of RNAaseA in a 37 °C water bath for 30 min; further add 400 μL PI staining and mix, avoiding light for 30 min at 4 °C; On-machine detection, recording red fluorescence at 488 nm wave length of excitation light.

Transwell migration experiment

The SH1/2 and NC group cells of MDA-MB-231/ MDA-MB-468 and the cells of OE and NC groups were collected and counted; the Transwell migration chamber was placed in a 24-well plate, and added 500 μL of medium containing 10% FBS to the outer chamber. Then 200 μL of cell suspension containing 5 × 10⁴ cells was added to the inner chamber, and after 12 h of incubation in the incubator, the stains I in the Diff stain solution box was fixed for 10 min, and the stains II and III were washed for 10 min, respectively, and washed. Photographed on a microscope and used Image J software to calculate the number of migrated cells.

Wound healing experiment

The SH1/SH2 and NC group cells of MDA-MB-231/ MDA-MB-468 and the cells of OE and NC groups were collected, counted, and added to a six-well plate at 1 × 10⁶ cells per well; the incubator was placed overnight until the cells were attached. After that, use the sterilized white gun head to draw the word “well” in each hole; wash it in PBS twice, remove the residual cell debris; place it in the incubator for constant temperature culture; shoot the same “cross” area of the cells at different time periods, observe cell scratch healing ability.

Plateclone formation experiment

The SH1/SH2 and NC group cells of MDA-MB-231/ MDA-MB-468 and the OE and NC group cells were collected, digested, centrifuged, and the cell suspension was counted and diluted to 1–2 × 10⁵ cells/mL. A corresponding volume of the cell suspension was taken in complete medium, and after pipetting and mixing, the cell suspension was added to a 6-well plate. 2 mL of cell suspension per well contains 1000 cells. Incubation was continued in the incubator until at least 50 cells in a single cell mass, and the culture was terminated. The 6-well plate was removed; the supernatant was discarded, and washed twice with PBS. Add 1 mL of the chamber R1 stain solution to each well and fix it at room temperature for 15 min. After the end, the fixing solution was removed, and 1 mL of crystal violet dye solution was added to each well, and the mixture was allowed to stand at room temperature for 15–20 min. Finally, rinse the bottom of the board with a small amount of tap water to wash away the excess crystal violet. Take pictures after the 6-well plate is dry.

Edu proliferation experiment

The SH1/SH2 and NC group cells of MDA-MB-231/ MDA-MB-468 and the cells of OE and NC groups were collected, digested, centrifuged, and the cell suspension was counted and diluted to 5 × 10⁴ cells/mL to be seeded in 12 wells. Plate cell slides were placed in a 37 °C constant temperature, 5% CO₂ incubator for 24 h; the previous medium was discarded, fresh complete medium containing 40 μM EDU was added, and cultured at 37 °C constant temperature, 5% CO₂ Incubate the box for 2 h.
and mark the proliferating cells with EDU; remove the medium, add 1 mL of 4% neutral formaldehyde per well, and incubate for 15 min at room temperature; remove the fixative and prepare 3% BSA in 1 mL PBS per well. Wash the cells twice with washing solution; remove the washing solution, add 0.5 mL Triton X-100 in 1 mL PBS per well, incubate for 20 min at room temperature; remove the permeation solution, and wash the 3% BSA solution in 1 mL PBS per well. Wash twice; remove the washing solution; add 0.5 mL of 1× Click-iT reaction mixture to each well, gently shake the plate to ensure that the reaction mixture is evenly covered with the slides, incubate for 30 min at room temperature in the dark; remove the reaction mixture and add to each well. The cells were washed twice with 3% BSA in 1 mL PBS. Discard the old washing solution, wash once with 1 mL of PBS, remove the washing solution; dilute Hoechst 33342 staining solution with PBS to a final concentration of 5 μg/mL, and add 1 mL of 1× Hoechst 33342 dilution to each well. Incubate for 30 min at room temperature on a shaker in the dark; remove the Hoechst 33342 solution and wash once with 1 mL of PBS per well to remove the wash. Fix the cells and take a photo under a fluorescence microscope.

**Western blot assay**

Cellular or tissue proteins were extracted and analyzed by western blot. The total cell lysates were extracted from the cells, adding phosphatase and protease inhibitors. Total proteins (20 μg) were separated via 8–15% SDS-PAGE and transferred onto PVDF membrane (Millipore, USA). Next, the membranes were blocked with 5% BSA and incubated with primary anti-bodies (dilution in 5% BSA-TBST) for overnight at 4 °C. Then, probed it with secondary antibody for 1 h at room temperature. Subsequently, the expression of the target proteins was detected by Chemiluminescent HRP Sub-strate (Millipore, USA).

**Quantitative real time PCR**

The total cellular RNA was isolated using the TRIzol Reagent (Vazyme, Nanjing, China) and reverse transcribed with the HiScript QRT SuperMix for qPCR (Vazyme). The mRNA levels were measured using the SYBR Green master mix (Vazyme).

**Results**

**High expression of LNK in triple-negative breast cancer**

Previous studies showed that overexpression of LNK inhibited cell growth and caused cell death in many leukemia cell lines, but similar experiments performed in several solid tumor cell lines had little effect on their proliferation, suggesting that LNK might have a different role in solid tumor cells compared to those of the hematopoietic system. Clinical tissue chips studies have indicated that LNK is abnormally expressed in breast cancer, which indicates that LNK may have a certain effect on breast cancer. We firstly compared the expression of LNK in non-TNBC to TNBC and found that LNK was higher expression in triple-negative breast cancer than in non-negative breast cancer (Fig. 1a). Subsequently, we investigated the expression of LNK in several breast cancer cell lines by RT-PCR and western blots.

Unexpectedly, we found that LNK were significantly upregulated (Fig. 1b, c) during TNBC cell lines (MDA-MB-468, MDA-MB-231, HCC1937, BT549), compared to those of non-triple negative breast cancer cell lines (MCF7, SKBR3, BT474), when the cells were cultivated to complete medium containing 10% FBS. Therefore, in the following studies, we mainly investigate the roles of TNBC cell lines (MDA-MB-468 and MDA-MB-231).

**LNK did not play roles on cell cycle and apoptosis of TNBC**

To explore the function of LNK in TNBC, we choose MDA-MB-231 and MDA-MB-468 to construct LNK stably silenced and overexpressed cell lines, which were verified by Western blot (Fig. 2a). In addition, MDA-MB-231 had a significant silencing efficiency, while MDA-MB-468 had no obvious silencing efficiency (Fig. 2a). This is mainly related to the low expression of LNK in MDA-MB-468 cells (Fig. 1b, c). Therefore, to explore the functional importance of this finding in TNBC, we mainly studied both forced-expression and silencing of LNK in MDA-MB-231 and MDA-MB-468 for the subsequent experiments.

Firstly, we examined the effect of silencing and overexpressing LNK on the cell cycle of breast cancer by PI staining. Our study shows that the silencing and overexpressing of LNK had no significant effect on cell cycle in MDA-MB-231 (Fig. 2b–d). Then, we investigated the effect of LNK on cell apoptosis in MDA-MB-231 cells and MDA-MB-468 cells, the results showed that the silencing and overexpressing of LNK had no significant effect on apoptosis in MDA-MB-231 cells or MDA-MB-468 cells (Fig. 3a–c). Therefore, these results suggested LNK have no effect on cell cycle and apoptosis of breast cancer cells, which is similar to the previous studies in ovarian cancer [15].

**LNK promoted the proliferation and migration ability in vitro**

In order to verify the effect of LNK on proliferation of TNBC cells, we performed cell colony formation assay and Edu staining assay. The results showed that numbers of TNBC cells in the medium were significantly inhibited after silencing of LNK in MDA-MB-231, which indicated that LNK might affect the proliferation of TNBC cells.
(Fig. 4a). On the other hand, when the MDA-MB-231 and MDA-MB-468 were forced expressing LNK, the number of TNBC cells were significantly increased (Fig. 4a, b), which reversely showed that LNK can promote the growth of TNBC cells. Therefore, the above results preliminarily suggested that LNK could promote TNBC cells to proliferate.

Furtherly, we used Edu staining to verify the proliferative role of LNK in TNBC. The results showed that the MDA-MB-231 was forced silenced LNK, the number of breast cancer cells in the medium were reduced, and on the other hand, the MDA-MB-468 were forced expressing LNK, the number of breast cancer cells were significantly increased (Fig. 4c–e), which was consistent with the results of the colony formation assay, which furtherly confirmed that LNK can promote TNBC proliferation.

Cell migration was measured by using the wound healing assay (Fig. 5a–c). After 12 h of the wound scratch, silencing LNK MDA-MB-231 cells maintained a nearly intact gap. In contrast, the control cells nearly closed the healing gap, indicating that LNK may indeed play a role in regulation of cell migration. On the other
Fig. 2 The effect of LNK on cell cycle in TNBC cells. 

a According to the expression of LNK at the protein level, breast cancer cells MDA-MB-231 and MDA-MB-468 with expression of LNK were selected to construct LNK stably silenced and overexpressed cell lines, and the efficiency was verified by Western blot.

b–d The effect of silencing and over-expressing LNK on the cell cycle of TNBC cell lines MDA-MB-231 and MDA-MB-468 were investigated by using cell cycle detection kit.
hand, overexpressing LNK MDA-MB-231 cells closed the healing gap nearly, compared to the control group, which indicated that LNK may promote TNBC cells to migrate. But the forced-expressing LNK MDA-MB-468 cells didn't show the similar results (Fig. 5c). In addition, the transwell experiments found that

**Fig. 3** The effect of LNK on cell apoptosis in TNBC cells lines. a–c The effect of silencing and over-expressing LNK on the cell cycle of TNBC cell lines MDA-MB-231 and MDA-MB-468 were investigated by using Apoptosis kit
silencing LNK of MDA-MB-231 (Fig. 5d, e) migrated significantly less than the control group. Similarly overexpressing LNK of MDA-MB-231 (Fig. 5e) and MDA-MB-468 (Fig. 5f) migrated more. It indicates that the LNK may have a function of promoting cell migration in TNBC cells, which is different from the studies in the ovarian cancer [14].
LNK promotes tumor growth and migration in an in vivo murine xenograft model

To verify the effect of LNK on TNBC in vivo, we investigated the effect of LNK on TNBC growth via the in vivo xenograft tumor experiment. Firstly we used NOD-SCID female mice to inoculate MDA-MB-231-NC, MDA-MB-231-SH1/2 and MDA-MB-231-OE cells in situ with a mammary fat pad. And when the tumor volume reached 100 mm³, we measured the tumor volume three times a week, observed the activity of the mice and recorded the death of the mice. When the tumor volume reached 1000 mm³, the mice were sacrificed and we observed the Lymph node and lung migration of the xenograft tumor mice in vivo.

Compared to the non-specific control (NC) group, the knockdown (SH1/2) and the overexpression (OE) group had no significant effect on the body weight of the mice (Fig. 6a, f). However, compared to the NC group, the tumor volume of the knockdown group was significantly reduced (Fig. 6b); while the OE group tumor growth rate was lower than the NC group in the first 20 days. And 20 days later, the growth rate is accelerated, which resulted in the OE group tumor volume becoming bigger than the NC group (Fig. 6g). And the mice tumor figure of the knockdown and overexpression group showed the similar results (Fig. 6c, h). Our results showed that lung metastasis was significantly less in the knockdown group than NC group and the tumor weight of the knockdown
group was significantly lower than that of the control group (Fig. 6d, e), and at the same time, the lung metastasis and the tumor weight of the OE group was significantly increased (Fig. 6i, j). The above results indicate that the LNK can also promote the proliferation and migration of breast cancer cells in vivo, which is consistent with our results in vitro.
Mechanism of LNK promoting breast cancer proliferation or migration

Previous studies showed that LNK interacts closely with JAK2-STAT, PI3K/AKT, and ERK1/2 signaling pathway-related proteins [21, 30, 31]. It is speculated that LNK may affect the proliferation and migration of breast cancer cells through these key signaling pathways. Therefore, we verify this signaling pathways through the Western blot analysis (Fig. 7a, b) and found that when MDA-MB-231 silenced LNK, p-ERK1/2 and p-STAT5 signaling pathways were significantly inhibited, while p-AKT and p-STAT3 were also inhibited to some extent (Fig. 7a). Conversely, when MDA-MB-231 overexpressed LNK, the p-ERK1/2 and p-STAT5 pathways were significantly activated (Fig. 7a). At the same time, MDA-MB-468 cells overexpressed LNK, p-ERK1/2 and p-STAT3 were significantly activated (Fig. 7b). In addition, the p-AKT pathway was slightly activated, while the p-STAT5 pathway was not significantly altered (Fig. 7b). These results confirmed that LNK mainly activates the p-ERK1/2 pathway, and in addition, have a certain degree of activation with the p-STAT5, p-AKT and p-STAT3 pathways, thereby promoting breast cancer cell proliferation and migration ability, which is similar to the studies in Ovarian cancer [14].

Discussion

LNK (SH2B3) is an adaptor protein studied extensively in normal and malignant hematopoietic cells, which plays an important role in Multiple blood diseases, such as Fanconi anemia (FA), Acute lymphoblastic leukemia (ALL), Aortic dissection (AD) and so on [31–33]. And research also shows that LNK mutations have recently been found in patients with myeloproliferative neoplasms (MPNs), early T cell acute lymphoblastic leukemia, Ph-like acute lymphoblastic leukemia, B-precursor acute lymphoblastic leukemia, and Down syndrome-related myeloid disorders [29, 31, 32, 34, 35]. Though the clinical sample chip analysis, we found that the LNK is abnormally expressed in breast cancer, which may play a different role in breast cancer. Furtherly, our research showed that the expression of LNK in TNBC is higher than that in breast cancer. Besides, previous studies showed that LNK has few studies on solid tumors except for ovarian cancer [14]. On the other hand, clinical studies proved that LNK has a high surface state in TNBC which prompted us to study whether LNK also has a certain functional relevance to TNBC.

It is important to realize that our groups and others have previously demonstrated that LNK might play an alternate role in solid tumor cells in hematopoietic and lymphoid cancers [36, 37], while it showed few effects on proliferation and migration in some solid tumor cell lines [35], and
regarded as a negative inhibitor in tumor [38]. In this study, we found that when LNK was over-expressed in MAD-MB-468 and MDA-MB-231 cells, it makes the proliferative capacity significantly enhanced. On the contrary, we found that when the LNK was silenced, the TNBC proliferative capacity significantly reduced. It was initially proved that the LNK may have a certain promoted effect on the proliferation of TNBC cells in vitro. It was initially showed that LNK may have a certain promoted effect on the proliferation of TNBC cells, which is similar to the ovarian cancer [14] but different from Multiple blood diseases, like, Fanconi anemia (FA), Acute lymphoblastic leukemia (ALL), Aortic dissection (AD) and so on [21, 31–33].

Furthermore, Previous studies have shown that LNK affects cell adhesion, ECM interaction and so on, which is consistent with the result that overexpression of LNK inhibited cell migration in normal endothelial cells [24, 37]. Additionally, LNK was recently found to play an important role in the regulation of focal adhesion complexes [14, 34]. In this study, we found that the overexpressing LNK can promote tumor migration, which is contrary to previous studies. When we silenced LNK, we found that the migration ability of breast cancer cells was opposite to that of ovarian cancer [14]. In this study, we found that over-expression of LNK can promote breast cancer migration. Our research also showed that LNK had no significant effect on the cell cycle and apoptosis of TNBC, which has also not been reported in other cancer.

The in vivo xenograft experiments indicated that LNK can promote cell growth and generate bigger tumor. Then, why does LNK appear different in leukemia cells verses TNBC cell? LNK does not have enzymatic activity, whose function is totally dependent on its binding partners [36, 38]. And we also find that the other two members [SH2B1 and APS (SH2B2)] of this family of proteins share similar sequence homologies in leukemia cells, whose stimulatory and inhibitory roles also appear to be cell-type and pathway dependent [35, 39]. Many hematopoietic malignancies including MPN, act as classical “activated kinase diseases” driven by a mutant activated receptor tyrosine kinase (e.g. FLT3-ITD, mutant c-KIT) or the downstream kinase. Besides participating in cell adhesion and ECM-interaction, pathway analysis suggested a possible role of LNK in the JAK2-STAT and p-ERK1/2 signaling pathways. LNK is a lymphocyte-specific adaptor protein that plays, which has an essential role part in the JAK-STAT signaling pathway via negative regulation of normal hematopoiesis. LNK is capable of coordinating with additional oncogenic pathways likes the JAK/STAT signaling, which suggests recommending a suppressor role for LNK in MPN development [30, 39]. LNK was appeared to tie both wild-type and MPN mutant types of the MPL receptor (MPLW515L) and to antagonize the activation of JAK2, STAT3, ERK, and AKT [30, 36]. Our study proved that when TNBC was silenced LNK, p-ERK1/2 and p-STAT5 signaling pathways were significantly inhibited, and p-AKT and p-STAT3 were also inhibited, which confirmed that LNK mainly activated the p-ERK1/2 pathway. In addition, the p-STAT5, p-AKT and p-STAT3 pathways also have a certain degree of activation, thereby promoting breast cancer cell proliferation and migration ability. While, in ovarian cancers LNK mainly activated the p-AKT and p-MAPK pathways, improved cell adhesion, moderated cell migration, and advanced in vivo tumor growth in a murine xenograft model [14], which is similar to TNBC. In contrast to detection in hematologic malignancies, the adaptor protein LNK acts as a positive signal transduction modulator in solid tumors [14].

Conclusion
In summary, our data firstly identified several unique functions of LNK in TNBC cells. LNK augmented the p-AKT, p-MAPK and ERK1/2 pathways, promoted cell migration, enhanced cell adhesion, and promoted the in vivo tumor growth and migration in vivo xenograft tumor model. Our results suggest that the adaptor protein LNK acts as a positive signal transduction modulator in TNBC which is similar to the ovarian cancer, and it is in contrast to the findings in hematologic malignancies, We believe that our observations are novel and open a new area of inquiry for this important adaptor protein, which may be regarded as a new drug targets in the following studies.

Abbreviations
TNBC: Triple negative breast cancers; ER: Estrogen; PR: Progesterone; HER2: Human epidermal growth factor receptor-2; PH: Pleckstrin homology; SCF: Stem cell factor; MPL: Myeloproliferative leukemia protein; EPO: Erythropoietin; PDGF: Platelet-derived growth factor; TNF: Tumor necrosis factor; MPN: Myeloproliferative neoplasms; RT-PCR: Quantitative real time PCR.

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Authors’ contributions
JL participated in the conception and design of the study. WY contributed the cell culture and treatment for the study. JL, WY and YZ performed the data collection and analysis. XC, LH, HH and CW contributed the critical revision of the manuscript for important intellectual content. JL and WY interpreted the data and produced the draft of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
This study was approved by the Zhongda Hospital under the supervision of the Ethics Committee, and all patients signed the informed consent forms.
Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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