• Basic Research •

Nintedanib induces apoptosis in human pterygium cells through the FGFR2-ERK signalling pathway

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Abstract

● **AIM:** To investigate whether nintedanib can inhibit pterygium cells through the fibroblast growth factor receptor 2 (FGFR2)/extracellular-signal-regulated kinase (ERK) pathway.

● **METHODS:** Human primary pterygium cells were cultured *in vitro*. After treatment with nintedanib, the cell morphology was observed under microscopy, the morphological changes of the nucleus were observed after DAPI staining, apoptosis was analyzed by Annexin-V FITC/PI double staining, and the changes of apoptosis-associated proteins were detected by Western blot. The binding ability of nintedanib to FGFR2 was predicted by molecular docking. Finally, by silencing FGFR2, we explored whether nintedanib inhibited FGFR2/ERK pathway.

● **RESULTS:** The results showed that nintedanib inhibited the growth of pterygium cells and caused nuclear pyknosis. The results of Annexin-VFITC/PI double staining showed that nintedanib was able to induce early and late apoptosis of pterygium cells, significantly increasing the expression of apoptosis-associated proteins Bax and cleaved-Caspase3 ($P<0.05$), and reducing the expression of Bcl-2 ($P<0.05$). In addition, nintedanib significantly inhibited ERK1/2 phosphorylation through FGFR2 ($P<0.05$). After silencing the expression of FGFR2, there was no significant difference in the inhibition of ERK1/2 phosphorylation by nintedanib ($P>0.05$).

● **CONCLUSION:** Nintedanib induces apoptosis of pterygium cells by inhibiting FGFR2/ERK pathway.

● **KEYWORDS:** pterygium; nintedanib; fibroblast growth factor receptor 2; extracellular-signal-regulated kinase; apoptosis

INTRODUCTION

Pterygium is a typical ocular surface lesion characterized by aggressive growth of fibro conjunctival tissue extending on the corneal surface, leading to decreased visual acuity, and severe patients may also develop tumours[1]. The aetiology of pterygium is related to chronic irritation caused by ultraviolet light and dust, among others, and patients affected by severe pterygium eventually lead to visual loss[2]. Numerous studies have provided evidence that various molecules, for example, growth factors, matrix metalloproteinases, and interleukins, are closely related to angiogenesis, fibrosis, proliferation, and inflammation that constitute pterygium pathology[3-4]. Several medical methods have been developed to prevent surgical excision of pterygium[5]. However, the potential ocular side effects limit the application of the above treatment methods. Elevated fibroblast growth factor (FGF) levels have been found to correlate with the formation and recurrence of pterygium[6]. FGF binding to its receptor, fibroblast growth factor receptor (FGFR), triggers a conformational change in FGFR that leads to dimerization and activation of FGFR[7]. The occurrence of pterygium is related to the expression of proliferation-related factors[8]. Blockade of the FGF/FGFR signalling has been found to result in the down-regulation of FGF, slowing cell proliferation and colony formation, decreasing invasion and drug resistance, and increasing apoptosis of cells[9]. Therefore, blockade of the FGFR signalling pathway has shown potential applications in treating pterygium.

Nintedanib, a selective small-molecule FGFR inhibitor, has recently been approved to treat lung adenocarcinoma and small-cell lung cancer[10-11] and has been used to block angiogenesis-related signalling and proliferation of cells[12-13]. In addition, it has been shown that abnormal differentiation of pterygium epithelium is associated with the extracellular signal-regulated kinase (ERK) signalling pathway *in vitro*[14].

Therefore, this study focused on whether nintedanib could inhibit pterygium proliferation and induces apoptosis and whether this phenomenon is associated with inhibition of the FGFR2/ERK pathway and angiogenesis-related signalling, thus providing an experimental basis for pharmacological treatment of pterygium.

SUBJECTS AND METHODS

Ethical Approval The Ethics Committee approved the study protocol for Human Research of the Ningbo Eye Hospital [No.2020-20(K)-C1]. The study protocol adhered to the tenets of the Declaration of Helsinki[15]. Informed consent was obtained from all patients before surgery.

Cell Culture Pterygium tissue was removed from the patient, which was cut into 1 mm^3 tissue pieces under aseptic conditions and digested with 0.025% collagenase type II for 20min at 37°C. Trypsin 0.05% digestion for another 10min, incompletely digested tissue pieces are filtered out through 70 μm nylon mesh. Cell suspensions were centrifugation at 1500 rpm for 10min. The collected cells were cultured in DMEM/F12 medium (GIBCO) containing 10% fetal bovine serum. Pterygium epithelial cells were purified by mechanical scraping and density gradient centrifugation. The purity of pterygium epithelial cells was determined by flow cytometry as follows. Cell suspensions were first incubated with irrelevant immunoglobulins, and it could block Fc receptors for non-specific binding. Then cells were resuspended in 250 μL of fixation/permeabilization (BD) solution and incubated for 20min at 4°C. The cells were then incubated in the dark for 30min at 4°C with FITC-labeled MUC1 (559774, BD) and PE-labeled K10 (orb485164, Biorbyt) antibodies. The cells were washed twice (1200 g, 5min) with 1 mL of 1% phosphate-buffered saline-bovine serum albumin (PBS-BSA), resuspension in 500 μL of PBS containing 1% p-formaldehyde, and the cell fluorescence was measured.

Conjunctival cells were obtained from small pieces of the patient's normal conjunctiva, added mixed digestive juice to the minced tissue for 10min, add the culture medium to terminate, filtered and centrifuged at 1000 g/min. Centrifuged for 6min, then discard the supernatant, gently blow and beat to blow the cells well and inoculate them into culture flasks at $1×10^4$/mL. After the cells were confluent and formed into membranes, the culture medium was sucked off, rinsed once with D-Hanks solution, digested with 0.25% trypsin and 0.06% ethylene diamine tetraacetic acid (EDTA) for 5min, centrifuged, and gently blown well. Fetal bovine serum, ampicillin, and streptomycin used in cell culture were purchased from Life Technologies. Pterygium cells were cultured in DMEM/F12 medium containing 10% fetal bovine serum at 37°C and 5% CO_2, and the cell morphology was observed regularly.

Detection of CCK-8 Cell Activity The human pterygium epithelial cells and conjunctival cells in good condition and logarithmic growth phase were collected, and the cell concentration was adjusted to $(5.0-6.0)×10^6$ cells. The 100 μL cell suspension was added to each well, which was seeded into a 96-well cell culture plate, incubated with 5% CO_2 in an incubator at 37°C overnight. Nintedanib purchased from Merck (SML2848, Merck, US), dissolved in PBS (initial concentration 200 μmol/L, the final concentration of 0.25, 0.5, 1, 2.5, 5, and 10 μmol/L), was added to each well at 100 μL and in triplicate wells. Cells were cultured in 5% CO_2, 37°C for 24h, and the status of human pterygium cells was observed under an inverted microscope. After 24h of pterygium cell culture, 20 μL of CCK-8 solution was added to each well, and the culture was continued for 4h, followed by low-speed shaking on a shaker for 5min. The absorbance was measured at an OD 450 nm on a microplate reader. A control well (without drug group) was also set up to calculate the inhibition rate (%) = [(OD of the control group − OD of the experimental group)/OD of the control group]×100%.

Immunofluorescence Cells were inoculated on 6-well plates containing coverslips. After treatment with different concentrations of nintedanib, coverslips were removed and fixed with 4% paraformaldehyde. After permeabilization with 0.5% Triton X-100, cells were blocked with 1% BSA at room temperature for 30min. The primary antibody was added and incubated overnight at 4°C, followed by the addition of the FITC-labelled secondary antibody and incubation for another 1h. DAPI solution 1 μg/mL was added to stain cell nuclei for 10min. Coverslips were removed for observation under a fluorescent microscope and photographed.

Western Blot Human pterygium epithelial cells treated with nintedanib at 0, 2.5, 5, and 10 μmol/L for 48h were collected and lysed in RIPA cell lysate for 10min on ice and centrifuged at 12 000 g for 15min at 4°C. The supernatant was collected and quantified using BCA. After separation by 10% SDS-PAGE, the proteins were transferred to PVDF membranes, blocked with 5% skimmed milk for 2h at room temperature, added first antibody phospho-ERK (Cell Signaling, #4370), ERK (Cell Signaling, #4695), FGF2 (ab208687, Abcam), phospho FGFR2 (#12503, SAB), FGFR2 (ab109372, Abcam), Bax (ab32503, Abcam), Bcl-2 (ab182858, Abcam), cleaved-Caspase 3 (ab2302, Abcam), caspase 3 (ab32351, Abcam), respectively, and incubate overnight at 4°C. After the PVDF membrane was washed three times with TBST, an HRP-labeled secondary antibody was added and incubated at room temperature for 2h. The membrane was then washed three times with TBST. Finally, development detection was performed by ECL kit. Observations were performed using the UVP gel imaging system, and the gray levels of the bands were

quantified with Image J software. GAPDH (ab8245, Abcam) and β-actin (ab8226, Abcam) were used as internal reference proteins.

Molecular Docking The 3D structure of the compound nintedanib was downloaded from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/). The 30 structure of FGFR2 protein (PDB ID: 7KIA) was downloaded from the RCSB Protein Data Bank (http://www.rcsb.org/). Water molecules were removed, hydrogen atoms were added by PyMol, and protein structures were saved in PDBQT format. Autodock vina 1.1.2 was used for molecular docking. We determined the protein active site coordinates based on the protein-ligand location, using default values for all other parameters. Finally, the conformations with the lowest docking binding energy were analyzed with PyMol for docking binding mode analysis.

FGFR2 Silencing Referring to the method of Serge *et al*[16] and Pu *et al*[17], FGFR2 knockdown experiments were performed with On-Target Plus Smartpool siRNA (Dharmacon RNA Technologies, Lafayette, CO, USA). siRNA was diluted in Opti-MEM (Thermo Fisher Scientific, Waltham, MA, USA) and combined with RNAiMAX (Invitrogen, Carlsbad, CA, USA) to a final concentration of 40 nmol/L oligonucleotide. The oligomer- RNAiMAX complex combined with or without nintedanib was added to cells. After 48h, cell lysates were subjected to Western blot to verify the downstream proteins.

Annexin-VFITC/PI Double Staining Human pterygium cell apoptosis was determined by AnnexinV/PI apoptosis detection kit (No.556547, BD, USA). After treatment with nintedanib for 24h, the cells were trypsinized and suspended in a binding buffer. Then 5 µL of AnnexinV and 5 µL of PI solution were added to the cells, respectively, and incubated for 15min at room temperature in the dark. Then cells were subject to flow cytometry, and data were analyzed using Flowjo software (FlowJo™ v10.7).

Statistical Analysis Statistical analysis was performed using SPSS 20.0 statistical software. The results were expressed as mean±standard deviation. The data of the two groups were compared by *t*-test. The differences in multiple groups were compared by one-way analysis of variance (ANOVA), and Tukey was used for post-inspection. *P*<0.05 was considered statistically significant.

RESULTS

Effect of Nintedanib on Cell Morphology in Human Pterygium Epithelial Cells We successfully established primary cultures of pterygium by digestion plus explant, with a mixture of different cell types in the primary cultures (Figure 1A). After mechanical scraping and density gradient centrifugation, the cell morphology tended to homogenise at the eighth generation (Figure 1B). Then pterygium epithelial cell purity was measured by flow cytometry, as

shown in Figure 1C. The proportion of epithelial cells in primary cultures reached 72% (MUC1 and K10 double-positive cells). Nintedanib significantly inhibited pterygium cells at concentrations greater than 0.5 µmol/L (*P*<0.05), while there was no significant difference in the inhibition rate of normal conjunctival cells (Figure 2A). Microscopic observation of pterygium epithelial cell morphology after treatment with different concentrations of nintedanib revealed that with increasing drug concentration, cell morphology was disrupted, some tiny bubbles appeared, and the number of cells also decreased (Figure 2B). However, nintedanib had no morphological effect on conjunctival cells (Figure 2C). In addition, with condensation of nuclei and cell morphology evident by DAPI staining, nintedanib significantly induced nuclear apoptosis in pterygium epithelia, and there was no significant effect on the conjunctival nucleus (Figure 2D, 2E). Therefore, nintedanib induced apoptosis in pterygium epithelial cells.

FGFR2 Expressed in Pterygium Epithelial Cells FGFR2 was evenly distributed in the pterygium cells (Figure 3A) but did not change significantly with increasing nintedanib drug concentration. However, we found that the expression of FGFR2 was lower in normal conjunctival cells than in pterygium cells, and nintedanib had no effect on conjunctival cells (Figure 3B).

Nintedanib Induces Apoptosis of Pterygium Epithelium Apoptosis was detected by flow cytometry. Nintedanib significantly induced apoptosis, and the apoptosis rate increased with the increase in concentration (Figure 4A). After treatment with 10 µmol/L nintedanib for 24h, the late apoptosis of human pterygium epithelial cells was (7.54±0.65)%, and the early apoptosis was (7.99±0.76)%. Compared with the control group, the late apoptosis of the control group was (0.10±0.01)%, and the early apoptosis was (1.20±0.54)%, and the difference was statistically significant (*P*<0.05). As seen from the immunoblotting results (Figure 4B), apoptosis-related proteins (cleaved-caspase-3 and Bax) were significantly increased (*P*<0.05), and Bcl-2 was significantly decreased (*P*<0.05) in human pterygium epithelial cells in response to nintedanib. At 10 µmol/L nintedanib, the difference was more pronounced (*P*<0.01), indicating that nintedanib promotes apoptosis.

Nintedanib Inhibits FGFR2/ERK pathway Nintedanib binds to FGFR2 with a binding affinity of -8 kcal/mol based on docking with FGFR2 (Figure 5A). And it forms hydrogen bonds with amino acid residues Gly490 and Gly570 of FGFR2, had van der Waals forces with amino acid residues Gly490, Gly488, Glu489, Asn571, Tyr566, Ala567, and Lys517, and has hydrophobic interactions with Leu487, Leu633, Ala643, Ile548, Phe564, Val495, and Ala515.
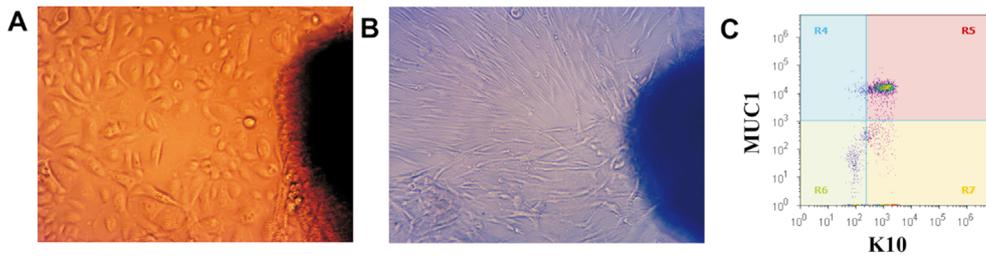
Figure 1 Identification of primary pterygium cells A: Phase contrast microscopy of primary human pterygium-derived cells; B: Phase contrast microscopy of passaged human pterygium-derived cells (Generation 8); C: The results showed that the purity of pterygium epithelial cells accounted for up to 74%. MUC1: transmembrane glycoprotein mucin 1; K10: Keratin 10.
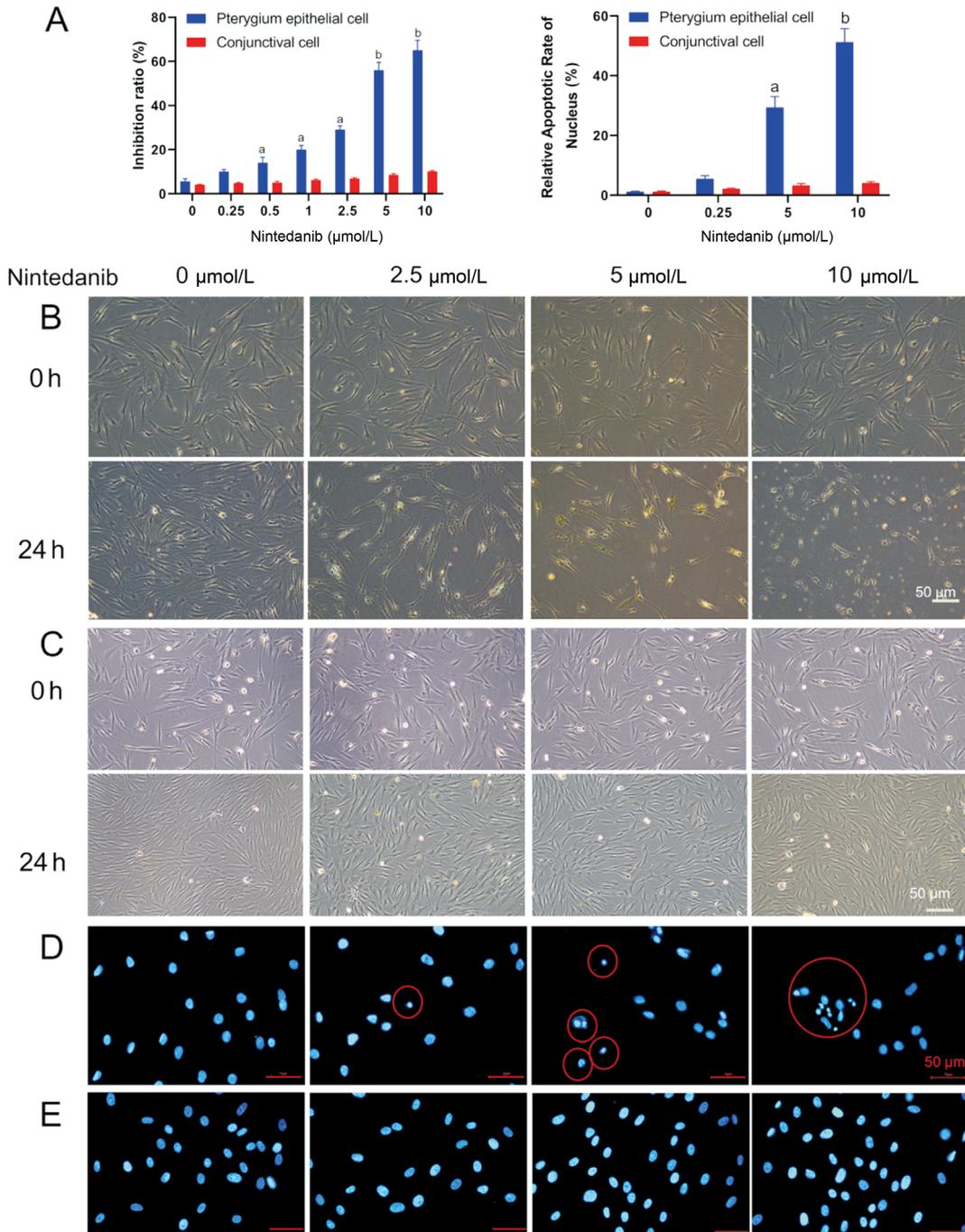

Figure 2 Effects of nintedanib on pterygium epithelial cells and conjunctival cell A: Effect of different concentrations of nintedanib on the inhibition rate of pterygium epithelial cells and conjunctival cells; B: Nintedanib caused pterygium epithelial cell shrinkage after 24h; C: Nintedanib had no significant effect on conjunctival cell morphology after 24h; D: Nintedanib induced nuclear apoptosis in pterygium epithelium; D: Nintedanib had no significant effect on conjunctival nucleus morphology. [a]$P<0.05$, [b]$P<0.01$, compared with 0 μmol/L group.
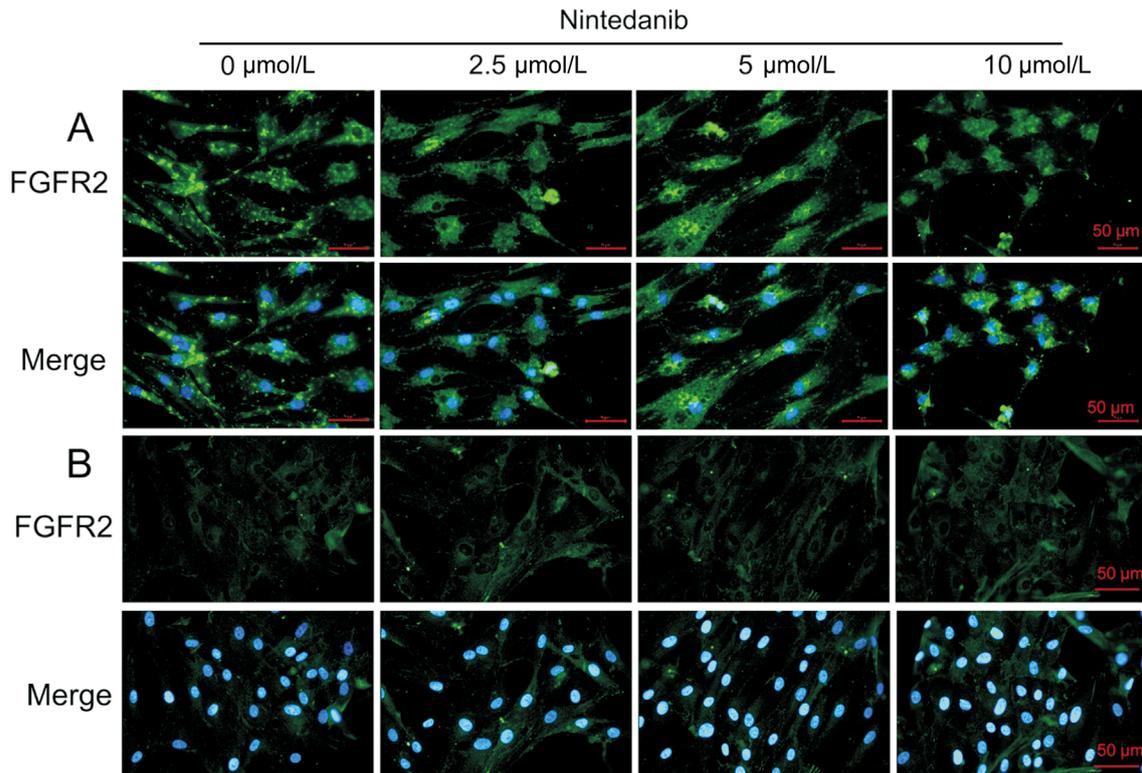
Figure 3 FGFR2 is highly expressed in pterygium epithelial cells A: Immunofluorescence results showed FGFR2 was highly expressed in pterygium epithelial cells, and nintedanib had no significant effect on FGFR2; B: FGFR2 expression was low in normal conjunctival cells, and there is no significant effect of nintedanib on conjunctival cells.

FGFR2 was silenced in pterygium epithelial cells to explore the mechanism of nintedanib further. After treatment with nintedanib, the expression of FGFR2 and its downstream proteins were detected. The effect of nintedanib on the FGFR2/ERK pathway was first investigated in control and experimental groups treated with different concentrations of nintedanib (Figure 5B). In the control group, as the concentration of nintedanib increased, the ratio of p-ERK(1/2)/ERK(1/2) gradually decreased ($P<0.05$), nintedanib reduced the degree of phosphorylated FGFR2 ($P<0.05$), and there was no significant difference in the corresponding change of total protein expression. In contrast, in FGFR2-silenced cells, the evolution of phosphorylated ERK1/2 was not significant ($P>0.05$), and the small-interfering non-specific control (Si-NC) group showed the same trend as the control group. While in FGFR2 silenced group, nintedanib in pterygium epithelial cells was found to have no significant effect on the expression of FGFR2 ($P>0.05$). This result suggests that FGFR2 was one of the pathways that activated ERK1/2. Nintedanib can indeed target FGFR2 to inhibit human pterygium epithelial cell proliferation. Inhibiting FGFR2 could effectively reduce the activation of downstream ERK1/2.

In addition, as shown in Figure 5C, the nintedanib showed little effect on apoptosis-related proteins in the siFGFR2 group ($P>0.05$), and upregulated pro-apoptotic proteins in the Si-NC group and the control group. Based on the above results, it was postulated that nintedanib could promote apoptosis of pterygium epithelial cells *via* FGFR2, as seen from the schematic diagram of the relevant pathways (Figure 6).

DISCUSSION
FGF2 is one of the most critical factors used to promote cell growth and is widely expressed in normal tissues, organs and many cancer tissues[18]. In recent years, many studies have shown that FGF2 plays a vital role in the maintenance and spread of tumours and maintains normal stem cells' characteristics[19] and active epithelial-mesenchymal transition[20]. And blockade of FGF2/FGFR2 signalling leads to down-regulation of FGF2, slows cell proliferation and colony formation, decreases invasion and drug resistance and increases apoptosis of cells[9]. Pterygium has tumour-like features, such as proliferation and invasion, and may develop into tumours. FGF2 is involved in angiogenesis, wound healing and various endocrine signalling pathways, and increased expression of FGF2 is found in infiltrating mast cells, epithelium and blood vessels of pterygium[21]. Wang *et al*[22] showed that the vascular density of pterygium was significantly negatively correlated with the course of the disease. It is worth thinking that they did not detect the expression level of FGFR. The relationship between FGFR and pterygium angiogenesis still needs further study.
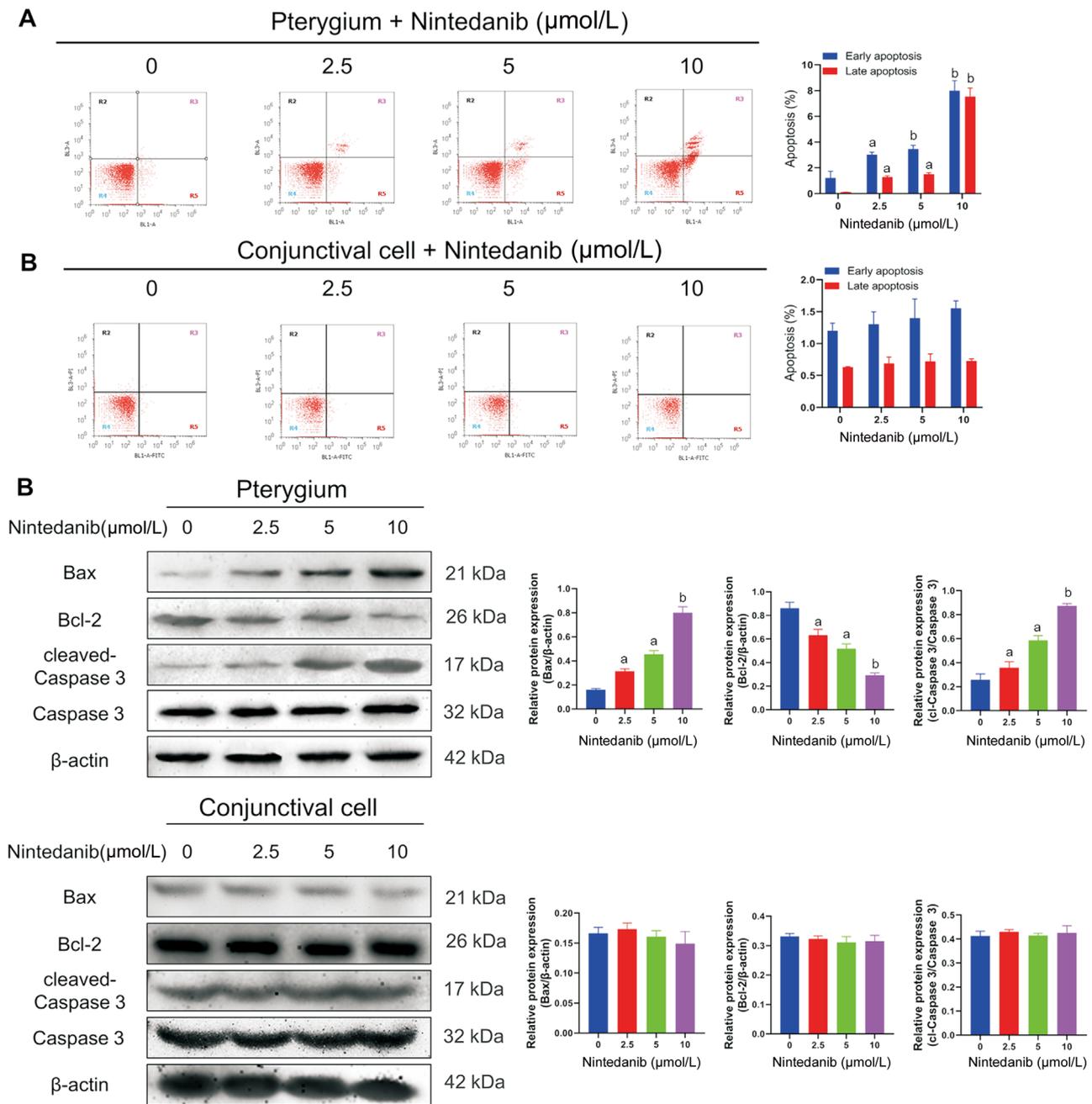
Figure 4 Nintedanib induced apoptosis of pterygium epithelial cells A: Flow cytometry results. B: Nintedanib promoted apoptosis-related proteins. [a]$P<0.05$, [b]$P<0.01$, compared with 0 µmol/L group.

In addition, Park et al[23] showed that FGF2 could induce the expression of cyclooxygenase 2 (COX-2), which is absent in normal conjunctiva. However, it is present in human pterygium[24]. COX-2 is a critical enzyme in inflammatory cytokine-induced angiogenesis, so it is tempting to speculate that FGF2 could induce inflammation and angiogenesis in pterygium. In the future, we will consider the clinical use of nintedanib to verify whether it can inhibit inflammation and angiogenesis in pterygium epithelial cells. By knocking down the expression of FGF2, it can block the signalling of FGF2/FGFR2, reduce the proliferation, invasion and drug resistance of cells, and increase the apoptosis of cells[25]. Liu et al[26]

showed that inhibition of FGF2 expression impedes ERK1/2 activation, reduces IL-6 secretion, decreases phosphorylation of STAT3, decreases levels of downstream molecules Cyclin D1 and Bcl-xl, induces mitochondria-associated apoptosis, and inhibits cell proliferation. The above studies imply that inhibition of FGF2-related pathways is one option for treating pterygium. At the same time, it has essential prospects in the treatment of pterygium by inhibiting the expression of ERK1/2 phosphorylation.

Nintedanib is a novel triple angiokinase inhibitor that blocks intracellular signalling essential for the proliferation and survival of angiogenesis-related cells[27-28]. There are few
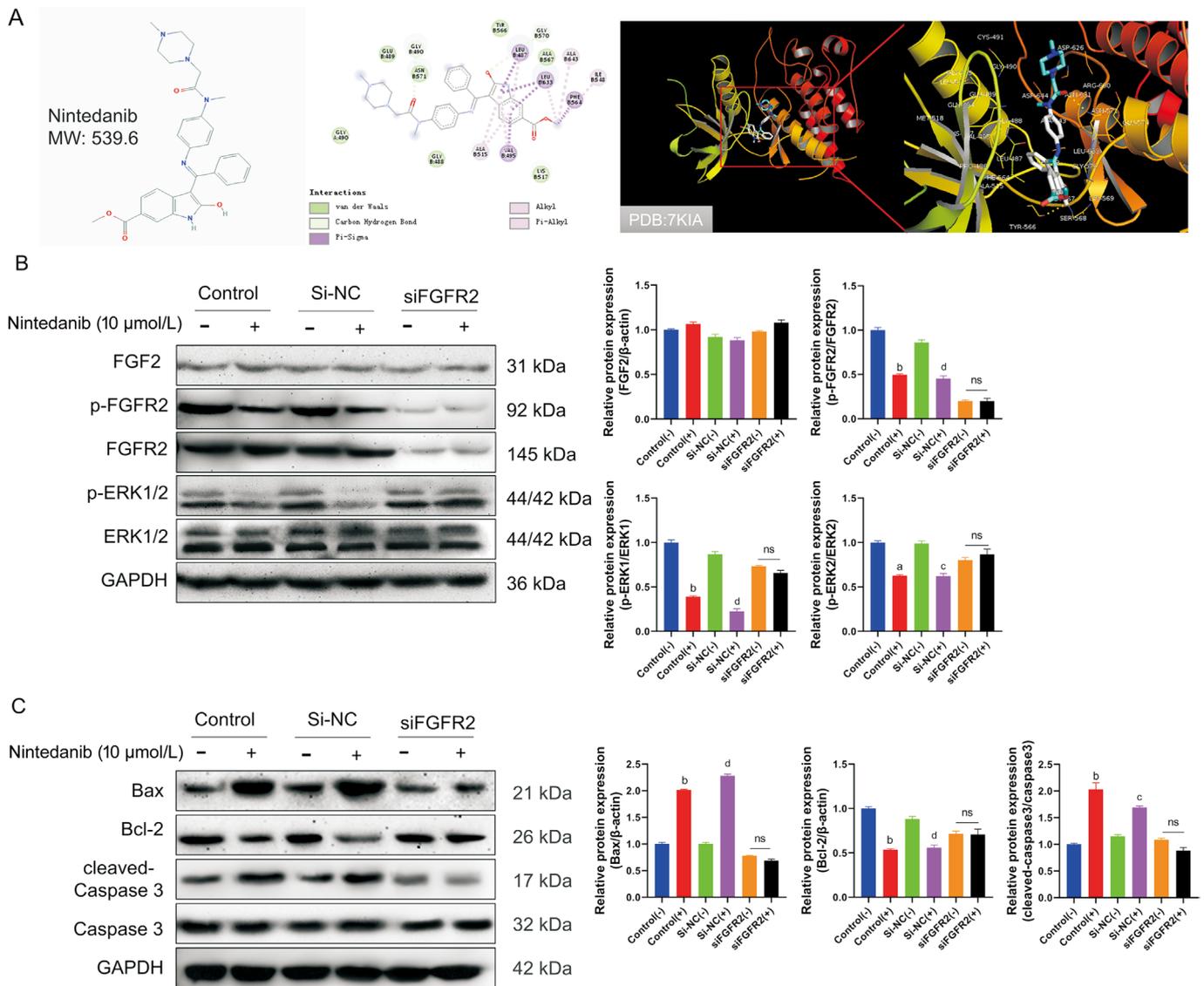
Figure 5 Nintedanib inhibited FGFR2/ERK pathway A: Structure of nintedanib and schematic presentation for docking to EGFR2; B: Nintedanib inhibited ERK1/2 phosphorylation through FGFR2; C: Nintedanib promoted apoptosis in pterygium *via* the FGFR2/ERK pathway. [a]$P<0.05$, [b]$P<0.01$, compared with Control(-) group, [c]$P<0.05$, [d]$P<0.01$, compared with Si-NC(-) group. Si-NC: Small interfering non-specific control.
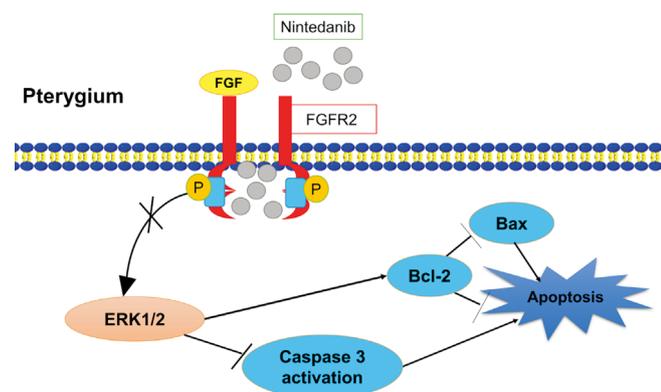

Figure 6 Nintedanib inhibited the proliferation of pterygium epithelial cells by inhibiting FGFR2/ERK and promoted the expression of apoptosis-related proteins.

studies on the effect of the FGF2/FGFR2 pathway in pterygium epithelial cells. In this study, we found that nintedanib could

induce apoptosis in pterygium epithelial cells and inhibited ERK1/2 phosphorylation by inhibiting FGFR2, accompanied by an increase in apoptosis proteins Bax and cleaved caspase3, and it is a new finding. The study by Tan *et al*[29] found that the development of pterygium was due to the disruption of the normal process of conjunctival cell apoptosis. And the expression of the anti-apoptotic protein Bcl-2 may be associated with pterygium recurrence, and it also plays a vital role in the anti-apoptotic process of pterygium cells[30]. Significant expression of Bcl-2 was observed in the basal epithelial layer of all pterygium epithelial cells, while normal conjunctiva did not show high expression of Bcl-2. This study verified that nintedanib could induce apoptosis in pterygium cells by inhibiting Bcl-2. When FGFR2 was silenced, there was no significant difference in the expression of apoptosis-related proteins. Showed that nintedanib leads to apoptosis in

pterygium epithelial cells through the FGFR2/ERK pathway. In addition, studies have demonstrated that autophagy and apoptosis play an essential role in the pathogenesis of human pterygium. One of the reasons why pterygium continuously erodes is that its normal autophagic process is inhibited[31-32]. Therefore, the role of autophagy in the pathogenesis of pterygium can be considered in the future.

In summary, this study showed that nintedanib could target FGFR2/REK pathway and inhibit ERK1/2 phosphorylation, thereby inducing autophagic death of pterygium cells. This provides an experimental basis for the future use of nintedanib in treating pterygium.

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