Efficacy of gefitinib-celecoxib combination therapy in docetaxel-resistant prostate cancer

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Abstract. Resistance to docetaxel is a major clinical problem in castration-resistant prostate cancer (CRPC). We have previously reported that the combined inhibition of epidermal growth factor receptor (EGFR) and cyclooxygenase-2 (COX-2) led to an increased antitumor activity of docetaxel in CRPC. In the present study, we explored the efficacy of the combination of EGFR inhibition (by gefitinib) and COX‑2 inhibition (by celecoxib) as a potential treatment for docetaxel-resistant CRPC. We established two docetaxel-resistant prostate cancer cell lines, PC3/DR and DU145/DR, by culturing PC3 and DU145 cells in docetaxel in a dose-escalating manner. The EGFR and COX-2 protein expression levels were determined. The effects of gefitinib and celecoxib on cell proliferation, apoptosis and invasion in vitro and in vivo were evaluated. In vitro changes in Bcl-2, FOXM1 and ABCB1 expression were analyzed. The expression of Ki-67 and cleaved-caspase-3 was also examined in DU145/DR tumor tissue. The enhanced expression of EGFR and COX-2 was observed in docetaxel-resistant CRPC relative to the parental cell lines. MTT, clone formation and fluorescence-activated cell sorting (FACS) analyses demonstrated that gefitinib and celecoxib in combination decreased cell viability and enhanced the rate of apoptosis when compared with either drug used alone. Additionally, the combination treatment was superior in inhibiting cell invasion and induced significant decreases in Bcl-2, FOXM1 and ABCB1 expression levels. Furthermore, the gefitinib-celecoxib combination inhibited DU145/DR tumor growth to a greater extent than either treatment used individually. The expression of Ki-67 was reduced, whereas cleaved-caspase-3 protein expression was increased in the tumors from the combination therapy group. In conclusion, the combined inhibition of EGFR and COX-2 by gefitinib and celecoxib may overcome docetaxel resistance in human CRPC. These findings provided a molecular basis for the clinical application of a novel combination therapy for docetaxel-resistant CRPC.

Introduction

Prostate cancer (PCa) remains among the most frequently diagnosed solid tumors in men, and is the second-leading cause of cancer-associated mortalities. The vast majority of PCa patients treated with androgen ablation therapy eventually develop castration-resistant PCa (CRPC) and bone metastasis. While taxane-based chemotherapy regimens such as docetaxel and cabazitaxel are widely used as first-line treatments for CRPC, the associated improvement in survival is moderate (~2 months) and patients typically experience significant side-effects (1). Multiple clinical trials have attempted to improve the survival benefit of docetaxel treatment in CRPC by combining it with other agents, however these attempts have so far been unsuccessful. The tested agents include lenalidomide (2), calcitriol (3), dasatinib (4), vascular endothelial growth factor (VEGF) inhibitors (5,6) and endothelin receptor antagonists (7,8) as well as others (9-12). In addition, docetaxel is only suitable for chemotherapy-naïve patients, and resistance develops over time (13). While cabazitaxel is currently used to treat these resistant patients, no chemotherapy regimens have been successfully established for CRPC patients who have developed tumor resistance to both docetaxel and cabazitaxel in randomized clinical trials. Therefore, alternative therapeutic strategies with greater long-term health benefits are required for CRPC patients.
Accumulating evidence has indicated that targeting epidermal growth factor receptor (EGFR) (14,15) and cyclooxygenase-2 (COX-2) (16,17) could be a promising strategy for preventing or delaying docetaxel resistance. Furthermore, a direct interaction between EGFR signaling and COX-2 activity has been suggested to occur in many types of cancer (18,19). Our previous study also indicated that the combination of COX-2 and EGFR inhibitors could significantly improve the therapeutic effects of docetaxel in CRPC, with lower toxicity (20). However, the extent of their therapeutic effect and their mechanism in docetaxel-resistant CRPC remain elusive.

In the present study, we determined the antitumor efficacy of the tyrosine phosphorylated (p-)EGFR-selective inhibitor gefitinib, and the COX-2 inhibitor celecoxib combination therapy on two established docetaxel-resistant CRPC cell lines (PC3/DR and DU145/DR) in vitro and in vivo. We also studied the effect of this novel regimen on tumor regulating proteins.

Materials and methods

Cell culture. LNCaP, PC-3 and DU-145 human CRPC cell lines were purchased from the Chinese Academy of Sciences, Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). These were maintained at 37˚C with 5% CO₂ in an F12 and RPMI-1640 culture medium containing 10% fetal bovine serum (FBS; all from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 26 mmol/l NaH₂CO₃ (pH 7.4), 1% L-glutamine and antibiotics. PC3/DR and DU145/DR cells were established as described in our previous study (21).

Cell proliferation assay. Cells (2,000/well) were plated in triplicate in a 96-well plate. The anti-proliferative effect of treatment with gefitinib and celecoxib (MedchemExpress, Monmouth Junction, NJ, USA) on the cells was determined by an MTT assay. In brief, 2 mg/ml MTT in PBS solution was added at 50 µl/well, and the cells were incubated at 37˚C for 2 h. Dimethyl sulfoxide (100 µl) was then added to each well. The absorbance was measured at a wavelength of 490 nm with a microplate reader (BioTek Instruments, Inc. Winooski, VT, USA).

Clone formation assay. Cells were seeded in 6-well plates at a density of 500 cells/well and incubated for 24 h. Then the cells were treated with gefitinib, celecoxib or a combination of both. The cells were incubated for 14 days, resulting in the formation of visible clonal colonies. The colonies were fixed with 4% formaldehyde for 10 min and dyed with 5 ml 0.5% crystal violet (Nantong Chem-Base Co., Ltd., Jiangsu, China). These were maintained at 37˚C with 5% CO₂ for 15 min. The number of colonies/well was then counted.

Flow cytometric analysis. The cells were treated with gefitinib, celecoxib or a combination of both. Following 24 h of incubation, the growth medium was removed and the cells were harvested. Supernatants were discarded and pellets were resuspended in 400 µl propidium iodide (PI) solution (50 µg/ml PI, 0.1% Triton X-100 and 0.1% sodium citrate in PBS). Samples were then incubated at 4˚C in the dark, before being subjected to flow cytometric analysis to determine the proportion of apoptotic and necrotic cells. Analyses were performed using a FACSCalibur™ flow cytometer (Becton Dickinson, San Jose, CA, USA).

In vitro invasion assays. The invasive potential of the PCa cells was assessed by their ability to penetrate a Boyden chamber with an 8-µm pore polyethylene terephthalate insert overlaid with a thin layer of Matrigel. Cells were untreated (control), or pretreated with gefitinib, celecoxib or both, for 24 h. For each condition, 5x10⁷/ml cells/well were loaded into the top of either the BD BioCoat Matrigel cell invasion chamber (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. After incubation for 24 h at 37˚C, the invasive cells reaching the lower chamber were stained with crystal violet and counted under phase-contrast microscopy (Leica IX51 microscope; Leica Microsystems, Wetzlar, Germany). Cell invasion was expressed as the mean number of invading cells in five random fields of view.

Western blot analysis. Cells were treated with gefitinib, celecoxib or a combination of both. The cells were harvested and lysed, following which lysates were extracted with T-PER tissue protein extraction reagent (Pierce, Rockford, IL, USA). A BCA Protein Assay kit was used to determine total protein concentration, and lysates containing equal amounts of protein (20 µg) were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes using a Bio-Rad SemiDry apparatus. After blocking with closed liquid containing 5% skimmed milk powder at room temperature for 2 h, the membranes were incubated with the following primary antibodies: EGFR (cat. no. sc-71033), COX-2 (cat. no. sc-166475), ABCB1 (cat. no. sc-55510) (all from Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and Bcl-2 (cat. no. ab59348) and FOXM1 (cat. no. ab180710) (both from Abcam, Cambridge, UK) and GAPDH (cat. no. MB0077; BioWorld, Dublin, OH, USA). All primary antibodies were diluted with QuickBlock™ Antibody Dilution Buffer (cat. no. P0256FT; Shanghai, China) before use. Blots were incubated with anti-rabbit IgG secondary antibody (cat. no. BS10650; Bioworld, Dublin, OH, USA) diluted in skimmed milk powder for 1 h at room temperature. The protein bands were detected with a chemiluminescence detection system (Thermo Fisher Scientific, Inc., MA, USA). All western blotting was performed in triplicate and quantified by densitometry using Gel-Pro 5.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

DU145/DR xenografts in nude mice. BALB/C nu/nu male mice (4-6 weeks old) weighing 18-22 g were obtained from the Shanghai SLAC Animal Laboratory (Shanghai, China). The mice were maintained in a well-ventilated enclosed system under controlled temperature (20-25˚C) and humidity (40-60%), with precautions to prevent pathogenic infestation. They were housed under constant 12-h light/dark cycles (light duration from 7:00 to 19:00) with food and water available ad libitum. Animal experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH, Bethesda, MD, USA), and were approved by the local Ethics Committee at Nanjing BenQ Hospital, Affiliated to Nanjing Medical University (Nanjing, China). DU145/DR cells were resuspended in PBS to a final density of 2x10⁷ cells/ml, and 100 µl cell suspension...
Figure 1. Anti-proliferative effect of gefitinib and celecoxib in PC3/DR and DU145/DR cell lines as evaluated by MTT and clone formation assays. (A) The expression of EGFR and COX-2 protein in androgen-sensitive LNCaP cells, castration-resistant PC3 and DU145 cells, and their resistant sublines PC3/DR and DU145/DR, as determined by western blot assays. Representative results of three similar separate experiments are shown. Protein levels were normalized to the respective GAPDH level. (B) MTT assay results. (C and D) IC$_{50}$ values for gefitinib and celecoxib. Results are presented as the mean of 3-4 independent experiments. (E, F and G) MTT and clone formation assays after drug treatment of the docetaxel-resistant cells: Doses of 0.625 µmol/l gefitinib and 5 µmol/l celecoxib were selected for PC3/DR cells, and doses of 0.312 µmol/l gefitinib and 10 µmol/l celecoxib were selected for DU145/DR cells for further experiments. Results shown are from 3-4 independent experiments. *P<0.05.

Figure 2. FACS analyses of the apoptotic effect induced by gefitinib and celecoxib alone, or in combination, on PC3/DR and DU145/DR cell lines. The induction of apoptosis and necrosis in PC3/DR and DU145/DR cells treated with gefitinib, celecoxib or a combination of both agents, as evaluated by flow cytometric analysis. The experiments were performed in triplicate. *P<0.05.
was transferred into the dorsal flank of each mouse using a 27-gauge needle. The resulting tumor volumes were determined with digital calipers and calculated according to the equation $V = \frac{L \times W^2}{2}$, where $V$ is the volume, $L$ is the length and $W$ is the width. When well-established tumors of $\sim 0.4 \text{ cm}^3$ were detected, the mice were randomly allocated into four groups (n=5 per group) and administered the following treatment for a period of 10 weeks: i) control (PBS treatment); ii) gefitinib (100 mg/kg/day); iii) celecoxib (4 mg/kg/day); iv) gefitinib (100 mg/kg/day) plus celecoxib (4 mg/kg/day). At week 10, the mice were sacrificed by cervical dislocation after the last dosage and subcutaneous tumors were harvested.

Immunohistochemistry (IHC). The expression levels of Ki-67 and cleaved caspase-3, indices for proliferation and apoptosis, were determined in the mouse tumor tissue by IHC. A section of each tumor was fixed in 10% formalin. Antibodies against Ki-67 (dilution, 1:500; cat. no. ab15580) and caspase-3 (dilution, 1:300; cat. no. ab2302; both from Abcam) were used to stain 4-µm sections according to the manufacturer's protocols. The sections were examined for positive staining and vessel density that was quantified as previously described (22,23). Representative fields were imaged under an x200 magnification.

Statistical analysis. Data were expressed as the mean ± standard deviation (SD). All experimental data were compared using a Student's t-test. Statistical analyses were performed using GraphPad Prism 5.01 software (GraphPad Software, Inc., La Jolla, CA, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

**Gefitinib and celecoxib combination therapy result in a greater cytotoxic effect in docetaxel-resistant PCa cell lines.** Two docetaxel-resistant PCa cell lines, PC3/DR and DU145/DR, were established by culturing PC3 and DU145 cells in docetaxel in a dose-escalating manner. Our previous study revealed that resistant cell lines exhibited $\sim 20$- and $\sim 200$-fold higher resistance, respectively to docetaxel, compared with the respective parental cells (21). The EGFR and COX-2 protein levels were higher in the resistant cell lines than in
An MTT assay demonstrated that the PC3/DR and DU145/DR cells were sensitive to gefitinib and celecoxib treatment, with half-maximal inhibitory concentration (IC₅₀) values of 4.118 and 5.009 µM (gefitinib) and 36.364 and 24.485 µM (celecoxib), respectively (Fig. 1C and D). Concentrations of gefitinib and celecoxib that produced ~30% growth inhibition were selected for further experiments (0.625 and 5 µmol/l for PC3/DR cells, and 0.312 and 10 µmol/l for DU145/DR cells). As displayed in Fig. 1E-G, based on the data from MTT and clone formation assays, gefitinib or celecoxib monotherapy induced mild cell growth inhibition (P<0.05). However, co-treatment with both drugs resulted in a supra-additive tumor cell growth inhibition which was stronger than when either drug was used alone (P<0.05).

The exposure of PC3/DR and DU145/DR cells to either gefitinib or celecoxib alone for 24 h significantly enhanced the rate of apoptosis and necrosis compared with the untreated cells (P<0.05). However, when both drugs were used in combination, an even greater rate of cell death was observed than that of either drug used alone (P<0.05) (Fig. 2).

Cell invasive ability is reduced by combined treatment with gefitinib and celecoxib in PC3/DR and DU145/DR cells. Analysis of the invasive potential of PC3/DR and DU145/DR cells revealed that following treatment with gefitinib or celecoxib, the invasive ability of the cells was significantly inhibited (P<0.05) when compared with the untreated control. Furthermore, when both drugs were used in combination, a supra-additive inhibitory effect on cell invasion ability was observed, with greater potency than when either drug was used alone (P<0.05) (Fig. 3).

Changes in the expression of Bcl-2, FOXM1 and ABCB1 (MDR1) are induced by gefitinib and celecoxib treatment of the parental lines (Fig. 1A and B). An MTT assay demonstrated that the PC3/DR and DU145/DR cells were sensitive to gefitinib and celecoxib treatment, with half-maximal inhibitory concentration (IC₅₀) values of 4.118 and 5.009 µM (gefitinib) and 36.364 and 24.485 µM (celecoxib), respectively (Fig. 1C and D). Concentrations of gefitinib and celecoxib that produced ~30% growth inhibition were selected for further experiments (0.625 and 5 µmol/l for PC3/DR cells, and 0.312 and 10 µmol/l for DU145/DR cells). As displayed in Fig. 1E-G, based on the data from MTT and clone formation assays, gefitinib or celecoxib monotherapy induced mild cell growth inhibition (P<0.05). However, co-treatment with both drugs resulted in a supra-additive tumor cell growth inhibition which was stronger than when either drug was used alone (P<0.05).

Gefitinib and celecoxib combination therapy result in a greater induction of apoptosis and necrosis in PC3/DR and DU145/DR cells. The exposure of PC3/DR and DU145/DR cells to either gefitinib or celecoxib alone for 24 h significantly enhanced the rate of apoptosis and necrosis compared with the untreated cells (P<0.05). However, when both drugs were used in combination, an even greater rate of cell death was observed than that of either drug used alone (P<0.05) (Fig. 2).

Cell invasive ability is reduced by combined treatment with gefitinib and celecoxib in PC3/DR and DU145/DR cells. Analysis of the invasive potential of PC3/DR and DU145/DR cells revealed that following treatment with gefitinib or celecoxib, the invasive ability of the cells was significantly inhibited (P<0.05) when compared with the untreated control. Furthermore, when both drugs were used in combination, a supra-additive inhibitory effect on cell invasion ability was observed, with greater potency than when either drug was used alone (P<0.05) (Fig. 3).

Changes in the expression of Bcl-2, FOXM1 and ABCB1 (MDR1) are induced by gefitinib and celecoxib treatment of
Efficacy of gefitinib-celecoxib co-therapy in docetaxel resistance

**PC3/DR and DU145/DR cells.** The results of western blotting demonstrated that gefitinib or celecoxib monotherapy reduced the expression of Bcl-2, FOXM1 and ABCB1 (MDR1) in the resistant cell lines (Fig. 4). Their levels were further decreased when the cells were subjected to combination therapy (P<0.05).

**Gefitinib and celecoxib combination therapy inhibits DU145/DR tumor growth in vivo.** The antitumor activities of the control, gefitinib, celecoxib and gefitinib in combination with celecoxib were analyzed. A linear mixed model with random intersects was fitted to the log-transformed data to compare tumor growth over time in the different treatment groups. The results indicated that although gefitinib and celecoxib alone moderately inhibited tumor growth, no significant differences were found compared with the control (P>0.05). However, the combination of gefitinib with celecoxib significantly inhibited tumor growth when compared with the control or monotherapies (P<0.05) (Fig. 5). In addition, co-treatment with both drugs was well tolerated, as no weight loss or other signs of acute or delayed toxicity were observed.

Ki-67 and caspase-3 immunostaining of treated xenograft tumors. As displayed in Fig. 6A, the tumor Ki-67 index was significantly decreased in the gefitinib and celecoxib monotherapy groups compared with the control group. However, the tumor Ki-67 index of the combination therapy group with both drugs was significantly lower than that of either monotherapy group (P<0.05). In addition, the cleaved-caspase-3 levels of the tumors from the combination group were significantly higher than in the other groups (P<0.05) (Fig. 6B).
Discussion

Taxane-based chemotherapy regimens remain the first-line treatment for CRPC as they are associated with the highest tumor regression and prostate-specific antigen (PSA) response of the existing regimens. The survival benefit is also superior to other chemotherapies currently in clinical use. Docetaxel is primarily useful for patients who have received no prior chemotherapy and is administered with prednisone to minimize the side-effects. However, docetaxel resistance ultimately develops, prompting the switch to a cabazitaxel-prednisone regimen (24). However, the survival benefit of this regimen is also moderate, as resistance develops rapidly. In addition, numerous side-effects are associated with this regimen, including treatment-related mortality. The present study explored the efficacy of an alternative treatment for docetaxel-resistant CRPC.

It has been suggested that EGFR and COX-2 play an important role in the development of docetaxel resistance in PCa. Their inhibition enhances the efficacy of docetaxel treatment (14-17,20). However, these studies were conducted in docetaxel-sensitive CRPC cells, rather than docetaxel-resistant. In the present study, we established two docetaxel-resistant CRPC cell lines (PC3/DR and DU145/DR) by culturing PC3 and DU145 cells in docetaxel with an escalating dose, and found that EGFR and COX-2 expression was significantly elevated in the resistant cells compared with the parental cell lines, indicating their possible role in docetaxel resistance. Therefore, we hypothesized that EGFR and COX-2 inhibition could have therapeutic potential in docetaxel-resistant CRPC.

Gefitinib and celecoxib are specific inhibitors for EGFR and COX-2, respectively. Gefitinib inhibits the phosphorylation of EGFR and has been applied for the treatment of advanced lung cancer. Previous studies have shown that gefitinib treatment can inhibit EGFR activity in CRPC cells to enhance their sensitivity to docetaxel (14,15). Celecoxib is a COX-2-selective nonsteroidal anti-inflammatory drug used to treat pain and inflammation in osteoarthritis and rheumatoid arthritis. It was demonstrated that celecoxib significantly increased chemotherapeutic drug-induced apoptosis in PCa cells (16) and increased the efficacy of androgen withdrawal in vivo (25). However, to provide a curative benefit, such an inhibitor must be used long-term or at a high dosage, which can lead to an increase in dose-related side-effects. Consequently, dose reduction strategies to provide similar therapeutic benefit through the appropriate combination with other drugs, may be more clinically viable. In the present study, we explored the effect of targeting EGFR and COX-2 with gefitinib and celecoxib, respectively, in docetaxel-resistant PCa cells.

To select appropriate concentrations for the in vitro experiments, in our preliminary experiments, we established concentration-response curves for both drugs to determine the individual concentrations of each drug that yielded ~30% growth inhibition in the PC3/DR and DU145/DR cells. Following this, to reasonably evaluate the combined effect of these two drugs, based on their concentration-response curves, we determined their relative appropriate concentrations for the subsequent combination experiments as follows: 0.625 µmol/l (gefitinib) and 5 µmol/l (celecoxib) in PC3/DR cells, and 0.312 µmol/l (gefitinib) and 10 µmol/l (celecoxib) in DU145/DR cells. We took into account two main considerations for determining drug dosages: Whether the dosages were able to effectively inhibit cell growth and whether the effects of a combination of the two drugs result in possible lethal inhibition. Based on the aforementioned lines of enquiry, drug concentrations that led to 30% growth inhibition were deemed appropriate. If dosages that led to 40 or 50% growth inhibition were selected for each drug, a combination of the two drugs may have resulted in lethal growth inhibition, which would have led to an ineffective evaluation of the additive effects of the drugs. We observed a greater effect on cell growth inhibition when EGFR and COX-2 were simultaneously inhibited by the combination of gefitinib and celecoxib, rather than individually. Furthermore, our results regarding the induction of apoptosis and inhibition of cell invasion by combination therapy were consistent with the MTT and clone formation assay results. In the apoptosis experiment, treatment with gefitinib and celecoxib alone or a combination of the two drugs did not result in a high apoptosis rate. In other words, most of the cells still maintained their own activity. Although we did not completely exclude the effects of apoptosis from the invasion assay, the effect of such concentrations of different drug treatments on cell invasiveness is reasonably expected. A significant decrease in tumor volume was also observed in mice treated with the combination of both drugs compared with each monotherapy group. Based on the aforementioned results, it is possible that this novel combination may be clinically effective in preventing prostate tumor growth and metastasis.

The upregulation of the multidrug resistance protein ABCB1 (also known as MDR1) has been verified as a mechanism underlying docetaxel resistance in PCa. Its synthesis may be induced by docetaxel treatment, and it diminishes the efficacy of docetaxel by actively removing it from cells across the membrane (26,27). Similarly, FOXM1 was revealed to mediate resistance to docetaxel in gastric and lung cancers, and its inhibition enhanced the docetaxel sensitivity of docetaxel-resistant cells (28-30). Bcl-2 expression was also revealed to be associated with docetaxel resistance in PCa (31-33). In our study, we found that the antitumor effects of gefitinib and celecoxib combination therapy may stem from the inhibition of ABCB1, FOXM1 and Bcl-2 expression. Western blotting data from both the mono- and combination therapy experiments demonstrated that the expression levels of these proteins were significantly reduced. Furthermore, we also used IHC to detect the expression of Ki-67 and cleaved-caspase-3 in vivo, as is widely performed to assess the proliferative and apoptotic potential of a tumor, respectively (34-36). Our findings revealed that EGFR and COX-2 inhibitor combination therapy had a beneficial effect on these factors as well, as Ki-67 was downregulated, and cleaved caspase-3 was upregulated. However, further studies are warranted to evaluate the exact and detailed mechanism of these factors in the context of CRPC pathogenesis and therapy. Future studies are also warranted to determine whether inhibiting EGFR and COX-2 improves the efficacy of docetaxel in treating docetaxel-resistant CRPC by e.g. studying the effect of the combination of their inhibition and docetaxel.

In conclusion, our study revealed, for the first time, the application of the combination of gefitinib and celecoxib therapy in
docetaxel-resistant CRPC, confirming their improved tumor inhibitory effect relative to gefitinib or celecoxib monotherapy. The inhibition of the EGFR and COX-2 pathways by gefitinib-celecoxib co-therapy represents a potential treatment for docetaxel-resistant CRPC.

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Availability of data and material
The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions
JZL, ZX and ZYR conceived and designed the study. JH and YY analyzed the results and were major contributors in writing the manuscript. JZL and JGZ performed the experiments. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participation
The animal experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH, Bethesda, MD, USA), and approved by the local Ethics Committee of Nanjing BenQ Hospital, Affiliated to Nanjing Medical University, China.

Patient consent for publication
Not applicable.

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

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