Effects of zinc finger protein 403 on the proliferation, migration and invasion abilities of prostate cancer cells

XINTONG XU1*, ZHIHUI ZHU1*, YIPENG XU2, SHASHA TIAN1, YINGJUN JIANG2,3 and HUAJUN ZHAO1

1College of Pharmaceutical Sciences, Zhejiang Chinese Medical University, Hangzhou, Zhejiang 311402; 2Institute of Cancer Research and Basic Medical Sciences of Chinese Academy of Sciences, Cancer Hospital of University of Chinese Academy of Sciences, Zhejiang Cancer Hospital, Hangzhou, Zhejiang 310022; 3The Second Clinical Medical College, Zhejiang Chinese Medical University, Hangzhou, Zhejiang 310053, P.R. China

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Abstract. Zinc finger protein 403 (ZFP403), located on human chromosome 17q12-21, is closely associated with the development of cancer. However, to date, there are a limited number of studies on the biological functions of this gene, particularly in prostate cancer (PCa). The results of the present study demonstrated that compared with normal tissues, the expression of ZFP403 was markedly lower in PCa tissues, as shown by the evaluation of the Gene Expression Profiling Interactive Analysis 2 database. The decreased expression of ZFP403 in PCa clinical tissues and cell lines was confirmed by immunohistochemistry, reverse transcription-quantitative PCR and western blot analysis. Using short hairpin (sh)RNA inhibition, stably-silenced ZFP403 cell lines were then constructed by lentiviral transfection (LV-PC3-shRNA-1 and 2; LV-DU145-shRNA-1 and 2). The results revealed that the knockdown of ZFP403 in PCa cells promoted cellular proliferation, colony formation, migration and invasiveness in vitro. Moreover, the levels of tumor growth- and motility-related proteins were significantly altered after ZFP403-knockdown. A xenograft tumor model using nude mice was established to elucidate the role of ZFP403 in tumorigenesis in vivo. Tumor growth was significantly increased in mice injected with ZFP403-knockdown cells compared with the control mice. Overall, the findings of the present study demonstrate that ZFP403 functions as a tumor suppressor gene in PCa by affecting the proliferation, migration and invasiveness of PCa cells, suggesting its potential use as a clinical diagnostic marker.

Introduction

Prostate cancer (PCa) is one of the most common malignant tumors of the male genitourinary system. According to pathological characteristics, the World Health Organization classifies PCa into five categories: i) adenocarcinoma (acinar adenocarcinoma); ii) ductal adenocarcinoma; iii) urothelial carcinoma; iv) squamous cell carcinoma; and v) and adenosquamous carcinoma, of which adenocarcinomas account for >95% of cases. Current data indicate that PCa ranks second in incidence and fifth in mortality rate among male malignant tumors. Heterogeneity is one of the characteristics of PCa, and it can be inert or highly invasive (1). In almost 90% of cases, PCa is confined to organs or locally advanced (2,3). However, PCa is aggressive and metastasizes through the blood and lymphatic systems, making it highly prone to recurrence in 10-15% of cases (4). Radiotherapy or radical prostatectomy are the preferred treatment methods for early-stage PCa, and are administered according to clinical stage and prostate-specific antigen levels (5). In highly metastatic PCa, antiandrogen therapy or androgen deprivation therapy (ADT) can reduce the levels of circulating testosterone by surgery or chemical castration (3,6). However, these effects are short-lived, with the majority of patients becoming resistant to ADT after 18-36 months, and gradually developing castration-resistant PCa (CRPC) (2,7). Clinically, CRPC often exhibits a high degree of invasiveness and is associated with a poor response to treatment (8), and misdiagnosis leads to the unnecessary suffering of patients due to further treatments. Therefore, the identification of novel biomarkers that can be used to predict disease outcomes and promote the effective treatment of PCa, is urgently required.

Zinc finger protein 403 (ZFP403), located in the 17q12-q21.1 region in humans, is highly conserved between Drosophila and humans. Mice and humans share 87% homology in their ZFP403 nucleotide sequences, and 96% homology in their
amino acid sequences (9). In humans, ZFP403 has two known transcript types: full-length transcripts encoding a protein composed of 696 amino acids, known as gametogenetin binding protein 2 (GGNBP2), and short transcripts encoding a protein consisting of 288 amino acids, termed laryngeal cancer-related gene 1 (10-12).

ZFP403 is closely associated with the occurrence and development of several types of cancer (13). Studies have demonstrated that full-length ZFP403 may serve as a potential tumor suppressor, and that ZFP403 is downregulated in various malignant tumors, such as breast (9), ovarian cancer (14) and glioma (15). Furthermore, the absence of ZFP403 can promote cellular proliferation (16) and tumorigenesis. However, the role of ZFP403 in PCa has not been fully investigated.

The aim of the present study was to determine the effect of ZFP403 on the carcinogenesis and progression of PCa, and to support the role of ZFP403 as a potential target for PCa by investigating its underlying mechanisms and effects on cell proliferation, migration and invasion therein.

Materials and methods

Differential expression analysis using the Gene Expression Profiling Interactive Analysis (GEPIA) database. Data from the GEPIA database (http://geopia.cancer-pku.cn/) were used to determine the association between ZFP403 and PCa. A gene symbol (ZFP403) was entered into the ‘Enter gene name’ field. The ‘GoPIA!’ button was clicked to generate the expression profile of the input gene across all tumor and normal tissues, in the form of dot plots or body maps. The ‘Boxplot’ tab in the ‘Expression DIY’ was clicked, and the ‘PRAD’ option was selected under ‘Cancer name’; the results were then presented in box plots.

Tissue samples. PCa and adjacent tissues were collected from 19 male patients (aged 55 to 75 years old) who underwent treatment at the Department of Urology, Cancer Hospital of the University of Chinese Academy of Sciences (Hangzhou, China) between November 2013 and July 2015. The study was approved by the Hospital’s Committee for the Protection of Human Subjects. All patients signed written informed consent forms in accordance with the Declaration of Helsinki.

Immunohistochemistry (IHC). IHC was used to determine the level of ZFP403 expression in clinical PCa tissues. The experiment was performed as previously described (14). The data were obtained by semi-quantitative analysis and finally presented as cut-off values. The cut-off values were determined by the multiplication of the staining intensity score and positive area score. Staining intensity scores were defined as follows: 0, negative; 1, weak; 2, moderate; and 3, strong. Positive area scores were defined as follows: 0, <25%; 1, 26-50%; 2, 51-75%; and 3, >75%.

Cells and cell culture. The LNCaP, PC3, DU145, 22RV1 and RWPE-1 cell lines were purchased from the Chinese Academy of Sciences. Between December 5th and 12th, 2019, these cell lines were authenticated by the analysis of 21 autosomal short tandem repeat loci (Biover Applied Biotechnology Co. Ltd.). RWPE-1 cells were cultured in K-SFM medium (Gibco; Thermo Fisher Scientific, Inc.), and the other cell lines were maintained in DMEM (Gibco; Thermo Fisher Scientific, Inc.). DMEM contained 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), and the cells were maintained at 37°C in a humidified atmosphere (5% CO2).

Lentivirus infection and establishment of ZFP40-knockdown cells. Lentiviruses were purchased from Shanghai GeneChem. Short hairpin (sh)RNA sequences specifically targeting ZFP403 (shRNA1, 5'-GAGCAUAACCAGUCCUAAU-3'; shRNA2, 5'-GGUAAUGACGAGUUGAA-3') were cloned into the hU6-MCS-CMV-Puromycin vector. An empty vector was used as the negative control. The cells were seeded (1x10⁴) into a 24-well plate (Wuxi NEST Biotechnology Co., Ltd.) and cultured at 37°C overnight. Lentivirus (1x10⁵ TU/ml, 10 µl) was mixed with Opti-MEM (500 µl; Gibco; Thermo Fisher Scientific, Inc.) and polybrene (5 µg/ml; Shanghai GeneChem), and then used to infect the cells. The medium was replaced after 12 h, and complete medium containing 1 µg/ml puromycin (Sigma-Aldrich; Merck KGaA) was used to establish cells in which ZFP403 had been stably silenced. After a week, the transfection efficiency was assessed by reverse transcription-quantitative PCR (RT-qPCR) and western blot.

Reverse transcription-quantitative (RT-q) PCR. The mRNA levels of ZFP403 in PCa cells were analyzed by RT-qPCR. According to the manufacturer’s instructions, total RNA was extracted using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) and then reverse transcribed into cDNA using the PrimeScript™RT reagent Kit (Takara Bio, Inc.). The concentration of nucleic acid was quantified using a cell Imaging Multi-mode Reader (BioTek Instruments, Inc.). Subsequently, qPCR was performed with SYBR-Green (Bio-Rad Laboratories, Inc.) using the CFX96 Real-Time system (Bio-Rad Laboratories, Inc.). The thermocycling parameters were as follows: 95°C for 2 min; 40 cycles at 95°C for 20 sec, 58°C for 20 sec, and 72°C for 15 sec; from 65°C to 95°C, to rise 0.5°C every 5 sec. The relative expression level of ZFP403 was quantified using the 2−∆∆Cq method (17). The specific primer sequences were as previously described: ZFP403 forward, 5'-ACAGGGATTAGACAGATTGGAAC-3' and reverse, 5'-TCATTGAACAATATTCTCACA-3'; and GAPDH forward, 5'-AGAAGGCTGGGCCTATTGG-3' and reverse, 5'-AGGGGCCATCCACAGTTCCTT-3' (14).

Western blot analysis. Total protein was extracted from PCa cells using RIPA lysis buffer (Beyotime Institute of Biotechnology) and the concentration of protein was determined using a BCA Protein Assay kit (Beyotime Institute of Biotechnology). The samples (50 µg per lane) were separated by 8 or 10% SDS-PAGE and then transferred onto PVDF membranes (EMD Millipore). After blocking with TBST containing 5% skim milk at room temperature for 1 h, the membranes were incubated overnight at 4°C with the following primary antibodies as appropriate: GGNBP2 (1:250; cat. no. ab203104, Sigma-Aldrich; Merck KGaA); cdc2 (cat. no. 9116), cyclin B1 (cat. no. 12231), β-catenin (cat. no. 9562), MMP-2 (cat. no. 4022) and β-actin (cat. no. 4970) (all 1:1,000; all from Cell Signaling Technology, Inc.); cdc25K (1:500; cat. no. sc-13138; Santa Cruz Biotechnology, Inc.); E-cadherin (cat. no. 1702-1) and...
vimentin (cat. no. 2862-1) (1:1,000; both from Epitomics, Inc.); and heparanase (1:500; cat. no. ab42817; Abcam). Goat anti-rabbit IgG (H + L)-HRP and goat anti-mouse IgG (H + L)-HRP (1:3,000; Bio-Rad Laboratories, Inc.) were used as secondary antibodies and incubated with the membrane at room temperature for 2 h. Protein bands were visualized using Clarity™ Western ECL Substrate (Bio-Rad Laboratories, Inc.) (18).

**Colonies formation assay.** In vitro colony formation ability and tumorigenicity were investigated by plate and soft-agar colony formation assays. For plate colony formation assays, 500 cells per well were seeded into 24-well plates and cultured at 37°C. After 14 days, the cells were fixed with 4% paraformaldehyde for 15 min, and then stained with freshly prepared crystal violet for 20 min (both at room temperature). For the soft-agar colony formation assay, cells (1x10^3 per well) were plated in 0.35% low melting point agarose in a 24-well plate. The cells were cultured at 37°C and the medium was changed twice a week. After 28 days, 1 mg/ml iodonitrotetrazolium chloride (Sigma-Aldrich; Merck KGaA) was added to each well and the plate was incubated for a further 24 h. The colonies were counted under a Leica DM500 microscope (magnification, x40; Leica Microsystems, Inc.) (14).

**Cell cycle analysis.** The cells were stained using the PI/RNase staining kit (BD Pharmingen; BD Biosciences), and the distribution of the cell cycle was analyzed by flow cytometry using the Guava easyCyte 6HT-2L (Merck KGaA) (19). The results were analyzed using ModFit LT™ software (Windows version 4.0; Verity Software House).

**Migration and invasion assays.** The migratory and invasive abilities of PCa cells were investigated using a Transwell chamber (Corning, Inc.). For invasion assays, Matrigel (BD Biosciences) was thawed at 4°C overnight and diluted in serum-free medium. Then, 50 μl diluted Matrigel was added to the upper chamber and incubated at 37°C for 20 min. Cells (1x10^5) were seeded into the upper chamber with serum-free medium containing 0.5% BSA, while the lower chamber was filled with 600 μl medium containing 10% FBS. After incubation for 24 h at 37°C, the cells were fixed with 4% polyoxymethylene for 15 min at room temperature, and then stained using the Hematoxylin and Eosin Staining Kit (Beyotime Institute of Biotechnology). The cells that had not passed through the polycarbonate membrane were removed with a cotton swab, and the invasive cells were visualized using a Leica DM500 microscope and counted in five randomly selected fields (magnification, x100). For migration assays, all conditions were consistent, except for the use of Matrigel (20).

**Xenograft tumor study.** Male BALB/c nude mice (n=12) were obtained from the Shanghai Laboratory Animal Center (CAS), and were used at 5 weeks of age. The mice were housed and maintained in specific pathogen-free conditions under a 12 h light-dark cycle at 25°C, with free access to food and water. The tumor xenograft experiments were approved and conducted according to the guidelines provided by the Experimental Animal Center of Zhejiang Chinese Medical University (no. SYXX-2018-0012). PC3 cells with or without ZFP403-knockdown were harvested, resuspended in serum-free DMEM medium (2x10^6) and subcutaneously injected into the dorsa of the mice (21). Animal health and behavior were monitored daily. When the tumor burden had reached ~50 mm³, the sizes were measured every 3 days and the tumor volume was calculated using the following formula: \( V = \frac{1}{2} \times (\text{length} \times \text{width}^2) \). After 40 days, the mice were sacrificed by cervical dislocation. Death was verified by the absence of a heartbeat and the onset of rigor mortis.

**Statistical analysis.** The data are presented as the mean ± SD. Comparison between multiple groups was performed using one-way ANOVA followed by Tukey's post hoc test, while a paired Student's t-test was used for comparisons between 2 groups. All data analyses were performed using GraphPad Prism version 5 (GraphPad Software, Inc.), and \( P<0.05 \) was considered to indicate a statistically significant difference.

**Results**

**ZFP403 is downregulated in human PCa.** Data from the GEPIA2 database were used to determine the association between ZFP403 and PCa. Differential gene expression analysis revealed that ZFP403 expression was lower in PCa tissues than in normal tissues (Fig. 1A). To further examine the expression level of ZFP403 in PCa, 19 groups of PCa and corresponding adjacent tissues were analyzed using IHC. The results revealed that the expression of ZFP403 in PCa tissues was significantly lower than that in adjacent normal tissues (\( P<0.01; \) Fig. 1B and C). RT-qPCR and western blot analysis were then used to detect the ZFP403 level in normal prostatic epithelial cells (RWPE-1) and PCa cell lines (LNCaP, PC3, DU145 and 22RV1). As shown in Fig. 1D and E, the expression levels of ZFP403 were lower in cancer cells than in RWPE-1 cells (\( P<0.01 \)). These data indicate that ZFP403 may function as a tumor suppressor in PCa.

**ZFP403-knockdown promotes the proliferation of PCa cells.** To evaluate the role of ZFP403 in the proliferation PCa cells, human PCa cell lines (PC3 and DU145), which are insensitive to androgens, were selected for transfection with ZFP403-shRNA lentivirus. The results revealed that the expression of ZFP403 was markedly decreased in the ZFP403-knockdown cells compared with that in the negative control (NC) cells, though to greater degree in cells transfected with ZFP403-shRNA-1 (Fig. 2A and B). Plate colony formation assays were performed to determine the effects of ZFP403 on colony formation. The results demonstrated that knocking down ZFP403 significantly increased PC3 and DU145 cell colony numbers (Fig. 2C). Furthermore, in the soft-agar colony formation assay, ZFP403-knockdown resulted in a marked increase in the number of colonies (Fig. 2D), indicating that ZFP403 inhibits tumorigenicity in vitro.

In order to further examine the effect of ZFP403 on the proliferation of PCa cells, the cell cycle distribution was detected by flow cytometry. The results revealed that the number of cells transitioning from the S phase to the G0/M phase was significantly increased in PCa cells in which
**ZFP403** was knocked down (Fig. 3A and B). On this basis, western blot analysis was performed to detect the expression of G2/M phase-related proteins. The results demonstrated that knocking down **ZFP403** upregulated the expression of cyclin B1, cdc2 and cdc25C (Fig. 3C). These data indicate that silencing **ZFP403** retains the viability and promotes the proliferation of PCa cells.

**ZFP403-knockdown promotes the migration and invasion abilities of PCa cells.** To determine the potential role of **ZFP403** in the metastasis of PCa, the effects of **ZFP403** on migration and invasion were evaluated using Transwell chamber migration and invasion assays. The results revealed that the cell migratory and invasive abilities were significantly promoted following **ZFP403-knockdown** (Fig. 4A and B).

Further experiments demonstrated that **ZFP403-knockdown** in PC3 and DU145 cells resulted in downregulation of the epithelial cell marker E-cadherin, and the upregulation of the interstitial cell marker vimentin. In addition, β-catenin, heparanase and matrix metalloproteinase 2 (MMP2) were also upregulated, as shown in Fig. 4C. Taken together, these results suggest that silencing **ZFP403** promotes PCa metastasis by enhancing cell migration and invasiveness.

**Antitumor effect of ZFP403 on tumor xenografts in vivo.** In order to examine the effect of **ZFP403** on tumor development and progression in vivo, a tumor xenograft model of PCa cells was established by subcutaneous inoculation of BALB/c nude mice. Body weight and tumor volume were measured every 3 days for 40 days. The growth rate of subcutaneous tumors in the **ZFP403-knockdown** group was significantly higher than that in the NC group. However, no significant difference in body weight was observed between the groups (Fig. 5A-D). In addition, compared with the
NC group, the mRNA and protein levels of ZFP403 were decreased in the ZFP403-knockdown group, as shown by RT-qPCR and western blot analysis, respectively (Fig. 5E-F). These data suggest that decreased ZFP403 expression is...
closely associated with the tumorigenicity and development of PCa in vivo.

Discussion

Due to the aging of the population and changes in lifestyle, PCa has gradually become one of the most common cancer types among men worldwide (22, 23). Currently, radical prostatectomy, radiotherapy and hormone therapy are effective options for the treatment of early-stage PCa; however, there are still challenges for the clinical treatment of advanced PCa (24). Anti-androgen therapy is usually effective in 80-90% of patients with advanced or aggressive PCa, and has become the standard treatment of choice (25). However, ADT is only effective against androgen-dependent PCa, and does not eliminate androgen-sensitive or androgen-independent cancer. Therefore, the majority of patients enter a hormone-insensitive state after a few years of hormone therapy, and eventually fail to respond to hormone therapy (26, 27). In addition, radiotherapy and chemotherapy are largely ineffective in patients with advanced PCa, and may even result in severe toxicity and side-effects (28, 29).

With the development of molecular biological technologies, and further understanding of the mechanisms of tumorigenesis at the cellular and molecular levels, the targeted molecular therapy of tumors has entered a new era. Worldwide, >60% of ongoing clinical trials of molecular targeted therapy are for cancer, including brain, lung, breast, pancreatic, liver, colorectal, bladder, head and neck, skin, ovarian and kidney cancer, as well as PCa (30). Targeted anticancer drugs commonly used in clinical practice include Herceptin, Glivec, Iressa and Tagrisso (31). However, few of these drugs are applicable for the treatment of PCa, thus the identification of novel potential targets for PCa is critical.

In the present study, in order to further determine the progression of patients with advanced and castration-resistant PCa, androgen-independent PCa cells (PC3 and DU145) (32) were used to investigate the functions of ZFP403. The expression of ZFP403 in PCa tissues was significantly lower than that in adjacent tissues. Furthermore, the expression of ZFP403 at
both the protein and mRNA level in different PCA cells was lower than that in normal epithelial prostate cells, which was consistent with the aforementioned IHC results. The decreased expression of ZFP403 maintained the growth of PCA, indicating that ZFP403 may be involved in the progression of PCA as a tumor suppressor gene. shRNA interference and lentivirus packaging technology were then used to silence ZFP403 for further analysis.

To further investigate the biological functions of ZFP403, the effects of ZFP403-knockdown on the proliferation and metastasis of PCA cells were evaluated. The results revealed that in vitro cell colony formation ability and tumorigenicity were enhanced following the knockdown of ZFP403. The cell cycle is one of the key modes of regulating cell growth, thus the effect of ZFP403 on cell cycle distribution was examined by flow cytometry. The results demonstrated that
the number of cells transitioning from the S to the G2/M phase was increased in ZFP403-knockdown cells compared with NC cells.

Cell cycle progression is regulated by cyclin-dependent kinase, whose activity is strictly regulated by cyclin. The ectopic expression of cyclin is associated with the progression of a number of malignant tumors (33). Studies have indicated that the expression of cyclin B1 plays a key role in the G2/M-phase transition of human PCa cells (34,35). Studies using the transgenic PCa mouse model have also demonstrated that the expression of cyclin B1 is increased in poorly-differentiated androgen-independent PCa (36). Thus, in the present study, cyclin-related proteins were assessed in the G2/M phase before and after ZFP403-knockdown. The results revealed that the expression levels of cdc2 and cyclin B1 were upregulated, while the expression of cdc25C was downregulated, suggesting that ZFP403-knockdown accelerates the transition to the G2/M phase and promotes cancer cell proliferation. However, the presence of phosphorylated cdc25C could not be detected (data not shown). This result is consistent with the
ZFP403 is a classical Cys2His2 (C2H2)-type zinc finger protein, which is encoded by 2% of human genes (37), constituting the largest sequence-specific DNA binding protein family (38). Numerous studies have demonstrated that C2H2-type zinc finger proteins can regulate the transcription of downstream genes involved in cellular proliferation and metastasis. At the same time, C2H2-type zinc finger proteins act as recruiters of chromatin modifiers or structural proteins, regulating the migration and invasion abilities of cancer cells (39,40). Therefore, it was hypothesized that ZFP403 may be involved in the migration and invasiveness of PCa. Indeed, the results of Transwell migration and invasion assays confirmed this hypothesis, where ZFP403-knockdown significantly enhanced the migration and invasion capacities of PCa cells. Further experiments also demonstrated that ZFP403-knockdown promoted metastasis by regulating the expression of epithelial markers, mesenchymal markers, MMP2, heparanase and β-catenin. β-catenin and E-cadherin are usually present as an E-cadherin/β-catenin complex located in cell-cell adherent junctions in the cell membrane. However, the loss of E-cadherin leads to epithelial-mesenchymal transition (EMT), accompanied by the deregulation of the Wnt signaling pathway. In addition, as a key component of the Wnt pathway, β-catenin plays an important role in the negative regulation of E-cadherin and EMT (41,42). Research has indicated that disassociation of the E-cadherin/β-catenin complex leads to the suppression of E-cadherin and the nuclear translocation of β-catenin, which enhances the invasive and migratory potential of tumors (42).

In order to improve our understanding of the function of ZFP403 in tumor development and progression in vivo, a xenograft model was used in the present study. ZFP403-knockdown induced the formation and development of transplanted tumors in nude mice, suggesting that ZFP403 may be a potential tumor suppressor in PCa.

In conclusion, the effect of ZFP403 in PCa was preliminarily examined in the present study. The results demonstrate that ZFP403-knockdown promotes the progression of PCa by enhancing proliferation, migration and invasiveness. Furthermore, ZFP403 was confirmed to function as a tumor suppressor in PCa, and this finding is consistent with the results of the overexpression of ZFP403 in PC3 (43). Future studies will aim to further investigate the functions of ZFP403 in PCa, in order to establish a novel therapeutic target for patients with advanced metastatic and hormone-independent PCa.

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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.

Authors’ contributions
XX and ZZ performed the experiments and contributed to the study design, as well as the acquisition, analysis and interpretation of the data. YX and YJ collected and analyzed the clinical samples. ST contributed to the acquisition, analysis and interpretation of the data. HZ contributed to study conception and revised the manuscript critically. All authors have read and approved the final manuscript.

Ethics approval and consent to participate
The present study was approved by the Ethics Committee of Zhejiang Cancer Hospital (Hangzhou, China), and all enrolled patients provided written informed consent. The animal experiments were conducted with the approval of the Experimental Animal Ethical Committee of Zhejiang Chinese Medical University (SYXK20180012).

Patient consent for publication
No applicable.

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

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