Mitofusin 2 inhibits bladder cancer cell proliferation and invasion via the Wnt/β-catenin pathway

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Abstract. The present study aimed to investigate the biological role of the mitochondrial GTPase mitofusin-2 (MFN2) in bladder cancer. MFN2 mRNA expression in tumor and paired adjacent non-tumor tissues from 8 patients was investigated using reverse transcription-quantitative polymerase chain reaction analysis. Immunohistochemistry was used to investigate MFN2 expression in 117 bladder cancer specimens. The associations of MFN2 expression with clinicopathological parameters were evaluated statistically. In addition, the biological role of MFN2 in the proliferation, migration and invasion of bladder cancer cells was examined. It was identified that MFN2 expression was significantly downregulated in bladder cancer tissues compared with normal tissues. MFN2 expression was associated with tumor stage, tumor grade and lymph node status. Furthermore, patients with low MFN2 expression demonstrated a shorter overall survival time (P=0.025). MFN2 knockdown by small interfering RNA promoted cancer cell proliferation, migration and invasion in vitro, and enhanced tumor progression in vivo. Mechanistically, MFN2 was revealed to be involved in Wnt/β-catenin signaling. In conclusion, MFN2 may serve as a potential therapeutic target in the treatment of bladder cancer, and the progress of bladder cancer may be delayed by regulating MFN2 expression.

Introduction

Bladder cancer is the most common malignancy in the human urinary system (1). Histologically, bladder cancer can be divided into the following types: Non-muscle invasive bladder cancer (NMIBC) and muscle invasive bladder cancer (MIBC) (2). A total of 70-80% of all cases are diagnosed as NMIBC and ~20% of patients have locally advanced or metastatic disease at the time of diagnosis (3). The long-term disease-free survival rate of patients with advanced stage or metastatic disease is only 15% (4). Therefore, it is critical to identify novel predictive markers for tumor metastasis to inform the clinical prognosis of patients with bladder cancer.

The mitochondrial GTPase mitofusin-2 (MFN2) is a GTPase embedded in the outer membrane of mitochondria. The MFN2 gene is located at chromosome 1p36.22 and is composed of 757 amino acids (5). MFN2 participates in mitochondrial fusion and contributes to the maintenance and operation of the mitochondrial network (6). Additionally, MFN2 has been demonstrated to participate in numerous physiological processes, including mitochondrial fusion, cardiac metabolism and the mitochondria-mediated apoptotic pathway (7). Further studies have indicated that MFN2 is involved in the regulation of vascular smooth muscle cell proliferation, and it may serve a role in the pathophysiology of obesity and Charcot-Marie-Tooth disease (8,9). MFN2 has been implicated in a number of human cancer types to serve a role in driving proliferation and metastasis. For example, Xu et al (10) demonstrated that MFN2 suppresses breast cancer cell progression by inhibiting mTOR complex 2 (mTORC2)/Akt signaling. Zhang et al (11) revealed that MFN2 overexpression can suppress gastric cancer cell proliferation and colony formation, weaken the invasion and migratory ability of cancer cells by downregulating matrix metalloproteinase (MMP)-2 and MMP-9, inhibit the cell cycle and induce apoptosis. However, the biological roles of MFN2 in bladder cancer remain unknown.

The current study evaluated the expression of MFN2 in bladder cancer tissues and cells. Subsequently, the associations between MFN2 expression, clinical pathological factors and prognosis of patients with bladder cancer were evaluated. Furthermore, the role of MFN2 in bladder cancer cell proliferation, migration and invasion was investigated. In summary, the results of the present study suggested that MFN2 serves a crucial role in bladder cancer development and progression.

Materials and methods

Patients. For the present retrospective study, 117 patients (55 male and 62 female patients aged from 42-72 years) with MIBC were recruited at Zhuhai People's Hospital (Zhuhai, China) between January 2007 and June 2012. Patients who had received chemotherapy prior to surgery were excluded from the present study. Tumor stages were determined according to
the criteria of the World Health Organization (12). In addition, eight pairs of snap-frozen bladder cancer and normal adjacent-noncancerous tissues (5 male and 3 female patients aged from 45-67 years) for reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis were obtained from Zhuhai People's Hospital between January 2012 and June 2012. The present study was approved by the Institutional Review Board of Zhuhai People's Hospital. Written informed consent was obtained from all participants in the study.

**Gene set enrichment analysis (GSEA).** Data were obtained from the cancer genome atlas hub (https://tcga.xenahubs.net.data set ID: TGGA, BLCA.sampleMap/ HiSeqV2_PANCAN). GSEA was performed using the GSEA platform (version 2.0.9; http://www. broadinstitute.org/gsea) to assess the association between MFN2 expression and Wnt signaling pathway signatures.

**Cell lines and transfection.** The bladder cancer cell lines, T24 and J82, were obtained from The Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The bladder cancer cell lines T24 and J82 were grown in RPMI-1640 medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; HyClone; GE Healthcare Life Sciences, Logan, UT, USA) at 37°C in 5% CO₂. The control scramble small interfering (si)RNA and the si-MFN2 were synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). Cells were transfected with control scramble siRNA or the si-MFN2 isoforms at a final concentration of 1 mg/ml and 100 nM, respectively, using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C in 5% CO₂, 10% fetal bovine serum (FBS; HyClone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; HyClone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; HyClone; GE Healthcare Life Sciences, Logan, UT, USA) at 37°C in 5% CO₂, followed by 40 cycles at 95°C for 5 sec, 55°C for 30 sec, and 72°C for 34 sec. The transcripts were amplified with an initial denaturation at 95°C for 30 sec, followed by 40 cycles at 95°C for 5 sec, 55°C for 30 sec and 72°C for 34 sec. MFN2 expression data were normalized to GAPDH expression. Relative mRNA expression levels were calculated using the 2^(-ΔΔCq) method (13). The map for the Wnt/β-catenin genes was generated using MeV 4.9.0 software (14).

**Western blot analysis.** Both cell lines were lysed using radioimmunoprecipitation assay lysis buffer (Shanghai Biyunyuan Bio-Tech Co., Ltd., Shanghai, China) at 4°C for 1 h. The protein concentration of each lysate was determined using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc.) Protein samples (30 µg/lane) were electrophoresed using 8-15% SDS-PAGE, followed by transfer to polyvinylidene difluoride membranes. Following blocking of non-specific binding for 1 h using 5% non-fat milk at room temperature., the membranes were incubated overnight on ice with primary antibodies against MFN2 (1:2,000; cat. no. ab124773; Abcam, Cambridge, UK), β-catenin (1:4,000; cat. no. ab32572; Abcam), GAPDH (1:2,500; cat. no. ab70699; Abcam) and p84 (1:2,500; cat. no. ab125019; Abcam). Subsequently, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:4,000; cat. no. ab6721; Abcam) for 2 h at room temperature. A membrane-enhanced chemiluminescence reagent ECL kit (GE Healthcare Life Sciences) was used to visualize the immunoreactive bands.

**Immunohistochemical (IHC) analysis.** Tissues were fixed in 4% paraformaldehyde solution at room temperature for 24 h and embedded in paraffin and serial sections (4 µm thick) were prepared. Sections were de-paraffinized in dimethyl benzene I, II and IIII solutions, each for 5 min, rehydrated with a gradient of alcohol solutions (100, 95, 75 and 50%, each for 5 min) and the endogenous peroxidase activity was blocked using 3% hydrogen peroxide for 15 min. The section was pre-treated using pressure cooker (100°C) heat-mediated antigen retrieval with sodium citrate buffer (pH 6.0) for 30 min. Non-specific immunoglobulin binding was blocked by incubation with 10% normal goat serum (cat. no. ab7481, Abcam) for 15 min. Following washing with phosphate-buffered saline (PBS), the slides were incubated with a primary

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**Table I. Reverse transcription-quantitative polymerase chain reaction primer sequences.**

| Gene   | Primers (5'→3')       |
|--------|------------------------|
| MFN2   | F: CCCCTTTGTCTTTATGCAGTTT  <br> R: TTTGGAGAGGAGTGTGCTTTTTT |
| CCND1  | F: CAATGACCCCGCAAGCTTTTTC  <br> R: CATGGAGGGGCGATTTGGAAG |
| CTLA4  | F: CATGATGGGGAAATGTTGACCTAC  <br> R: TCAGTCTTGGATAGTGGTTGTC |
| c-myc  | F: GGCTCTGGCAAAAGGTCAAAAGGT  <br> R: CTGCGTATGTTGCTGATGTTGTC |
| c-Jun  | F: TCCAAGTGCCGAAAAAAGGAAG  <br> R: CGAGTTCTAGCTTTCAAGGT |
| LEF1   | F: ATGTCAACTTACCAACAAAAGCA  <br> R: CCGGAGCAGAAAGGTGAAAG |
| TCF1   | F: TTGATGCTAGTCTTCTGGTTGAC  <br> R: CTTTGAGACTCTTCTGGTIC |
| AXIN2  | F: ACTGCCCCACACGATAAGGAG  <br> R: CTGCGTATGTTGCTTGGAC |
| GAPDH  | F: TCAAGAAGTGGTGTAAGCAG  <br> R: CGTCAAGGTTGGAGGAGG |

MFN2, mitofusin 2; CCND1, cyclin D1; CTLA4, cytotoxic T-lymphocyte associated protein 4; lymphoid enhancer binding factor 1; TCF1, T-cell factor 1.
antibody against MFN2 (1:200; cat. no. ab124773; Abcam) or Ki67 (1:200; cat. no. ab15580; Abcam) for 60 min at room temperature, followed by a biotin-conjugated secondary antibody (1:1,000; cat. no. ab6720, Abcam) for 30 min at room temperature. Slides were observed under a light microscope (magnification, x40). The intensity of MFN2 staining was scored as follows: 0, negative staining; 1, weakly positive; 2, moderately positive; and 3, strongly positive. The percentage of staining was scored as follows: 0, 0% immunoreactive cells; 1, ≤5% immunoreactive cells; 2, 5-50% immunoreactive cells; 3, 51-80% immunoreactive cells; and 4, >80% immunoreactive cells. The IHC score was multiplied by the intensity percentage of staining. Scores of 0-4 were considered to indicate ‘low’ expression and those of 6-12 were considered to indicate ‘high’ expression.

Bromodeoxyuridine labeling. A total of 5x10⁴ cells (T24 or J82) were grown on coverslips at 70% confluence were incubated with 5-bromodeoxyuridine (BrdU) for 1 h and stained with anti-BrdU antibody (1:250; cat. no. BRDUAPCMOB-1; Upstate Biotechnology, Inc., Lake Placid, NY, USA) at room temperature, according to the manufacturer’s protocol. Cells were stained with DAPI (1 µg/ml; Sigma-Aldrich; Merck KGaA) for 20 min at room temperature. All images were captured via fluorescence microscopy (magnification, x20).

Wound healing assay. Both cell lines were plated in 24-well plates and serum-starved for 24 h prior to scratching with a pipette tip. Following scratching, the cells were washed with PBS, images were obtained using a light microscope (magnification, x20) and the cells were placed into medium with 1% FBS to prevent proliferation. After incubating the cells at 37°C in a 5% CO₂ atmosphere. Wound closure was calculated by examining the distance between the opposite edges of the wound and normalizing the width to the starting width using a light microscope (magnification, x20). Each sample was assayed in triplicate and ImageJ 1.47 was used to analyze the resulting images.

Cell invasion and migration assay. Cell invasion and migration ability was detected using a Transwell system (EMD Millipore, Billerica, MA, USA). Following transfection with MFN2-specific siRNA, 1x10⁴ of both cell lines in 200 µl serum-free Dulbecco’s modified Eagle’s medium (DMEM; HyClone; GE Healthcare, Chicago, IL, USA) were seeded in the upper chamber of a Transwell and the bottom of the chamber was filled with 600 µl DMEM containing 10% FBS. For the invasion assay, Matrigel (1:5; Becton, Dickinson and ImageJ 1.47 (National Institutes of Health, Bethesda, MD, USA) was used to analyze the resulting images.
Company, Franklin Lakes, NJ, USA) was added to pre-coat the Transwell chambers and incubated at 37°C for 3 h. Cells on the upper side of the filter were removed after incubating the cells at room temperature for 24 h. The filter membrane was stained with crystal violet for 15 min at 25°C and the number of the cells that remained adherent to the underside of the membrane were counted using an inverted light microscope (magnification, x20).

Dual-luciferase reporter assay. Both cell lines were plated in triplicate wells of 24-well plates and allowed to settle for 12 h. Subsequently, 100 ng of reporter plasmids (100 ng; EMD Millipore, Billerica, MA, USA) containing TOP-Flash or FOP-Flash luciferase reporter plus 1 ng pRL-TK Renilla plasmid were co-transfected using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Firefly and Renilla luciferase activity were measured 48 h following transfection using a Dual Luciferase Reporter assay kit (Promega Cooperation, Madison, WI, USA), according to manufacturer’s protocol.

Tumor models. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Zhuhai People’s Hospital. Male five-week-old BALB/c nude mice (14-16 g) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). The mice were maintained in a pathogen-free environment with free access to food/water, on a 10/14 h light/dark cycle, with an ambient temperature between 18 and 29°C and a daily temperature difference ≤3°C, relative humidity 40-70% and the air ventilated 10 times/h. For the tumor formation assay, 5x10^6 cells of either cell line in suspension were subcutaneously injected into the flank of each mouse. Tumor volume was calculated with the following formula: Length x width^2/2.

To assess the biological roles of MFN2 in bladder cancer and corresponding adjacent non-tumor tissues (Fig. 1A). Representative immunostaining of MFN2 in bladder cancer tissues is presented in Fig. 1B.

Results

Expression of MFN2 in bladder cancer tissues. MFN2 mRNA expression levels were determined in eight pairs of bladder cancer and corresponding adjacent non-tumor tissues by RT-qPCR. The results demonstrated that MFN2 mRNA expression was significantly lower in bladder cancer compared with non-tumor hepatic tissues (Fig. 1A). Representative immunostaining of MFN2 in bladder cancer tissues is summarized in Table II. Associations between MFN2 expression and clinicopathological parameters of patients with bladder cancer.

| Variable                  | MFN2 expression | P-value |
|---------------------------|-----------------|---------|
| Age, years                | Low (n=56)      |         |
| <50                       | 22              | 0.357   |
| ≥50                       | 34              |         |
| Sex                       |                 |         |
| Male                      | 30              | 0.173   |
| Female                    | 26              |         |
| Tumor stage               |                 | 0.006   |
| I-II                      | 18              |         |
| III-IV                    | 38              |         |
| Tumor size, cm            |                 | 0.072   |
| ≤3                        | 21              |         |
| >3                        | 35              |         |
| Tumor grade               |                 | 0.037   |
| Low                       | 27              |         |
| High                      | 29              |         |
| Histopathological type    |                 | 0.814   |
| TCC                       | 44              |         |
| Other                     | 12              |         |
| Lymph node status         |                 | 0.002   |
| Negative                  | 39              |         |
| Positive                  | 17              |         |
| MFN2, mitofusin 2; TCC, transitional cell carcinoma. |

Associations between MFN2 expression and clinicopathological factors. The clinical significance of the MFN2 expression level in patients with bladder cancer is summarized in Table II. Tumor stage (P=0.006), tumor grade (P=0.037) and lymph node status (P=0.002) were identified to be significantly associated with MFN2 expression, however, no associations were revealed for age, sex, tumor size or histological type. Furthermore, Kaplan-Meier survival curves demonstrated that patients with a low MFN2 expression level exhibited a markedly worse overall survival time compared with patients with high MFN2 expression (P=0.025). However, MFN2 expression did not affect the recurrence-free survival time (P=0.255; Fig. 1C). Multivariate analysis using the Cox proportional hazards model also indicated that MFN2 expression was an independent predictor for patients with bladder cancer (P=0.017; Table III).

MFN2 knockdown promotes bladder cancer cell proliferation and migration. To assess the biological roles of MFN2 in
Figure 2. Knockdown of MFN2 promotes the proliferation, migration and invasion of cancer cells. (A) The transfection efficiency of si-MFN2 was analyzed by measuring transcript levels in T24 and J82 cells using reverse transcription-polymerase chain reaction and western blotting. (B) Representative micrographs and quantification (right panel) of BrdU incorporation in the indicated bladder cancer cells. Magnification, x200. (C) The effect of MFN2 knockdown in cancer cell migration was determined by wound healing assay and the uncovered areas in the wound healing assays were quantified as a percentage of the original wound area (right panel). (D) Transwell cell migration assays and Matrigel invasion assays of cancer cells revealed an increased migration and invasive ability of T24 and J82 cells following transfection with si-MFN2. Magnification, x200. Quantification of the migratory and invasive cells of each cell line are presented as proportions of their vector controls (right panels). Data are presented as the mean ± standard deviation of three independent experiments *P<0.05 vs. respective vector group. MFN2, mitofusin 2; si, small interfering; BrdU, 5-bromodeoxyuridine.
PANG et al.: MFN2 INHIBITS THE PROLIFERATION AND INVASION OF BLADDER CANCER

bladder cancer cells, the expression of MFN2 was knocked down using specific siRNA in T24 and J82 cell lines (Fig. 2A). The BrdU incorporation assay demonstrated that knockdown of MFN2 significantly increased cell proliferation in both cell lines (Fig. 2B). Furthermore, wound healing and Transwell assays revealed that MFN2 silencing significantly increased cell migration and invasion in bladder cancer cells (Fig. 2C and D).

MFN2 knockdown facilitates the tumorigenicity of bladder cancer cells in vivo. To examine the role of MFN2 in
the tumorigenicity of bladder cancer cells \textit{in vivo}, subcutaneous xenograft tumor models were generated using MFN2-knockdown T24 and J82 cells. The results demonstrated that tumors formed from MFN2-knockdown cells were larger and significantly heavier compared with the control (Fig. 3A-C). In addition, IHC staining revealed a significantly higher expression level of Ki67 and an increased proliferation index in the MFN2-knockdown groups compared with the control group (Fig. 3D and E). In summary, the \textit{in vivo} studies indicated that suppressed expression of MFN2 promotes tumorigenicity.

\textit{MFN2 regulates bladder cancer cell proliferation and migration via the Wnt/\(\beta\)-catenin signaling pathway}. The molecular mechanisms of MFN2-inhibited bladder cancer cell proliferation and migration were also investigated. Publicly
available gene expression array data for bladder cancer was analyzed by GSEA and it was observed that MFN2 expression was negatively associated with the activation of the Wnt/β-catenin pathway (Fig. 4A). Furthermore, knockdown of MFN2 significantly increased the transactivation activity of β-catenin/TCF in both T24 and J82 cells, which was revealed by luciferase reporter assay (Fig. 4B). Furthermore, the expression levels of downstream genes of Wnt/β-catenin, including cyclin D1, cytopotoxic T-lymphocyte associated protein 4, c-myc, c-Jun, lymphoid enhancer binding factor 1, T-cell factor 1 and Axin2, were markedly higher in MFN2-knockdown bladder cancer cells compared with control cells (Fig. 4C). Notably, knockdown of MFN2 increased the translocation of β-catenin into the nucleus, as determined by immunoblotting of cytoplasmic and nuclear protein extracts (Fig. 4D).

Discussion

The present study investigated the clinical significance and biological role of MFN2 in bladder cancer pathogenesis. First, the expression level of MFN2 was revealed to be significantly lower in bladder cancer tissue compared with adjacent non-tumor tissues. Second, MFN2 expression level was identified to be associated with tumor stage, lymph node metastasis and poor prognosis in patients with bladder cancer. Third, silencing of MFN2 promoted bladder cancer proliferation and metastasis via regulation of the Wnt/β-catenin signaling pathway. These results indicated that MFN2 is a candidate tumor suppressor in bladder cancer, and may be exploited as a target for potential clinical treatments for bladder cancer.

MFN2, also termed the hyperplasia suppressor gene, was originally identified in vascular smooth muscle cells from spontaneously hypertensive rats (15). Overexpression of MFN2 induces the formation of mitochondrial networks and may involve a major rearrangement of the coiled coil domains (16). It is also involved in the clearance of damaged mitochondria via selective autophagy and the regulation of the unfolded protein response upon estrogen receptor stress (17).

Notably, MFN2 functions as a mitochondrial receptor for Parkin protein (PARK2) and is required for PARK2 recruitment to dysfunctional mitochondria (18). A previous study revealed the antitumor effect of MFN2 in cancer cells (19). Further studies confirmed this anti-proliferative effect in a number of tumor cell lines, including hepatocellular carcinoma cells (20), breast cancer cells (10) and lung adenocarcinoma cells (21). However, to the best of our knowledge, the expression of MFN2 in bladder cancer tissues, and its potential clinical and biological significance, have not been described. The present study identified that MFN2 expression was significantly downregulated in bladder cancer tissues and cells compared with the control groups, which indicated that MFN2 may function as a tumor suppressor in bladder cancer.

A number of studies have demonstrated that MFN2 expression is tightly associated with the clinical prognosis of patients with cancer. Wang et al (20) reported that patients with hepatocellular carcinoma with low MFN2 expression exhibited a poorer prognosis compared with patients with high MFN2 expression. In support of these findings, Feng et al (22) demonstrated that semaphorin 3C and MFN2 co-expression has value for the prognosis of patients with hepatocellular carcinoma following hepatectomy. Fang et al (23) observed that patients with gastric cancer with high expression levels of MFN2 have a significantly higher overall survival rate and disease-free survival rate compared with those with low expression levels. The current study demonstrated that the MFN2 expression level was significantly associated with overall and recurrence-free survival of patients with bladder cancer. In summary, these studies suggest that MFN2 may serve as a prognostic factor for predicting the clinical outcome of patients with cancer.

In addition to a direct role in predicting clinical prognosis, studies have demonstrated that MFN2 exhibits a role in regulating tumor cell growth. Xu et al (10) demonstrated that MFN2 suppresses breast cancer cell growth and metastasis through inhibition of mTORC2/Akt signaling. By contrast, inhibition of MFN2 can effectively repress tumor growth and metastasis (24). Similarly, Ma et al (25) reported that MFN2 mediates MCF-7 cell proliferation and apoptosis via the phosphoinositide 3-kinase/Akt signaling pathway. The current results demonstrated that MFN2 can regulate bladder cancer cell proliferation, migration and invasion through the Wnt/β-catenin signaling pathway. However, the detailed cellular signaling mechanisms between MFN2 and Wnt signaling require further investigation. In addition, it was identified that low MFN2 expression was predictive of lymph node metastasis in patients with bladder cancer. Therefore, the present results are consistent with previous findings that suggest MFN2 may serve an important role in promoting cancer migration and progression. However, further studies are required to investigate the precise role of MFN2 in the metastatic process of bladder cancer.

The present study possessed several limitations that should be acknowledged. First, it was a small retrospective review at a single center, which included only 117 patients with MIBC. Second, only the effects of MFN2 knockdown in bladder cancer cells were examined and the effects of MFN2 overexpression in other cell lines were not investigated.

In conclusion, MFN2 may act as an independent marker for the prognosis of patients with bladder cancer. Silencing of MFN2 can promote cell proliferation, migration and invasion. Therefore, MFN2 may be used as a potential therapeutic target for bladder cancer in the future.

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Availability of data and materials

All data analyzed during this study are included in this manuscript.

Authors' contributions

GP and QX conducted the experiments and analyzed the results. GP and JY designed the study and wrote the manuscript. All
authors reviewed the results and approved the final version 2of the manuscript.

Ethics approval and consent to participate

The present study was approved by the Institutional Review Board of Zhuhai People's Hospital (Zhuhai, China). Written informed consent was obtained from all participants. All animal care and experiments were approved by the Institutional Animal Care and Use Committee of Zhuhai People's Hospital.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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