Abstract. SRY-related high-mobility group box 9 (SOX9) is an important transcriptional factor that regulates diverse genes involved in development and stemness. Dysregulation of SOX9 encourages carcinogenesis in various types of cancer, including breast cancer. The present study aimed to explore the role of SOX9 in triple-negative breast cancer (TNBC). SOX9 expression was significantly upregulated in the TNBC MDA-MB-231, MDA-MB-436 and MDA-MB-468 cell lines compared with that in BT-549 cells. Based on a lentivirus assay, SOX9 inhibition in MDA-MB-231 and MDA-MB-436 cells suppressed cell proliferation and colony formation. Apoptosis was increased and the cell cycle was arrested at the G₀/G₁ phase in SOX9-knockdown cells. Transwell and wound-healing assays demonstrated that SOX9 inhibition decreased the migration and invasion of MDA-MB-231 and MDA-MB-436 cells. RNA sequencing identified that numerous genes were regulated by SOX9, including nucleophosmin, thioredoxin reductase 1, succinate dehydrogenase complex subunit D, nuclear receptor binding SET domain protein 2, eukaryotic translation initiation factor 4γ1 and glycogen phosphorylase L. Overall, the current study suggested that SOX9 acted as an oncogene in TNBC.

Introduction

Breast cancer is one of the most common malignant tumors that endanger the health of women; its incidence rate ranked first and its mortality rate ranked second among female malignancies worldwide in 2018 (1). Triple-negative breast cancer (TNBC) refers to breast cancer that is negative for the estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2), and accounts for 15-20% of basal-like breast cancer cases (2,3). The endocrine drug tamoxifen and the HER2 monoclonal antibody trastuzumab have been successful in the treatment of ER+/HER2- or HER2+ patients with breast cancer (4,5). However, since patients with TNBC do not express these receptors, endocrine and targeted drugs are not effective for these patients (6). Understanding the underlying molecular mechanisms may help identify biomarkers for diagnosis and drug targets.

SRY-related high-mobility group box (SOX) family members are a cluster of transcription factors that regulate various cellular processes, including stemness, proliferation, differentiation and survival (7). Increasing evidence has demonstrated that dysregulation of SOX9 serves a pivotal role in cancer development. SOX9 expression is upregulated in various types of cancer, including malignant pancreatic neoplasms (8), non-small cell lung carcinoma (9) and esophageal squamous cell carcinoma (10). However, low SOX9 expression predicts a poor survival in patients with gastric cancer (11). Hepatitis B virus (HBV) transcriptionally activates SOX9 in hepatocellular carcinoma cells, whereas SOX9 upregulation represses the replication of HBV (12). In breast cancer, SOX9 activates FXYD domain containing ion transport regulator 3 expression, which promotes the formation of activated complex by interacting with Src and ERα, thereby enhancing the stemness of breast cancer cells (13). However, the involvement of SOX9 in TNBC remains to be determined.

The present study aimed to investigate the role of SOX9 by knocking it down in TNBC cells. Cell Counting Kit-8 (CCK-8) and colony formation assays were performed to study the effect of SOX9 silencing on cell proliferation. Whether SOX9-knockdown regulated the migration and invasion of TNBC cells was also explored. Finally, RNA sequencing was applied to explore the downstream effectors of SOX9 in TNBC.

Materials and methods

Cell culture. Human TNBC BT-549, MDA-MB-231, MDA-MB-436 and MDA-MB-468 cells were obtained from the American Type Culture Collection. 293T cells were purchased from Thermo Fisher Scientific, Inc. and 1% penicillin and streptomycin solution (Corning, Inc.).
were cultured in DMEM (HyClone; Cytiva) supplemented with 10% FBS and 1% penicillin and streptomycin solution. Cell culture was maintained in a 37°C incubator with 5% CO₂.

**SOX9-knockdown by lentivirus.** To silence SOX9 in MDA-MB-231 and MDA-MB-468 cells, the lentivirus vector pGCSIL-GFP was constructed, which contained a short hairpin (shRNA) sequence targeting SOX9. shRNA sequences were as follows: Negative control (shNC), 5'-TTTCCCGAA CGTGTACAGT-3'; shSOX9-1, 5'-GCATCCCTCAATTTC TGTATA-3'; shSOX9-2, 5'-GCGAGAAGTCCGGTGA AAAAA-3'; and shSOX9-3, 5'-CTCCACCTCACCATTCA GAA-3'. For lentivirus packaging, the expression plasmid (pGCSIL-GFP; 20 mg; Shanghai GeneChem Co., Ltd.) with two helper plasmids (pHelper 1, 15 mg and pHelper 2, 10 mg; Shanghai GeneChem Co., Ltd.) were co-transfected into 293T cells using Lipofectamine™ 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) in 10-cm plates. After 48 and 72 h of transfection, lentiviruses were harvested. Following purification and titration, the harvested virus particles were used to infect MDA-MB-231 and MDA-MB-468 cells using a multiplicity of infection of 20. After 72 h, cells were harvested for knockdown efficiency examination when green fluorescence was visible.

**CCK-8 assay.** CCK-8 reagent was purchased from Sigma-Aldrich (Merck KGaA) and used according to the manufacturer's instructions. MDA-MB-231 and MDA-MB-468 cells were trypsinized and seeded in triplicate in 96-well plates at a concentration of 2,000 cells/well. Each well contained 100 µl culture medium. After 6, 12, 24, 36, 48 and 72 h, 10 µl CCK-8 reagent was added to each well. The plates were maintained in a 37°C incubator for 3 h. The absorbance at 450 nm was measured using a microplate reader (Thermo Fisher Scientific, Inc) after agitation for 30 sec.

**Colony formation assay.** shNC- and shSOX9-transfected MDA-MB-231 and MDA-MB-468 cells were seeded into 6-well plates at concentrations of 50, 100, 200, 250, 500 and 1,000 cells/well. The culture medium was replaced by fresh medium every 3 days. The colonies were formed after culturing for 14 days at 37°C. The colonies were washed three times with PBS, fixed with 4% paraformaldehyde (Wuhan Servicebio Technology Co., Ltd.) for 20 min at room temperature and stained with Glemsa (Sigma-Aldrich; Merck KGaA) staining solution for 20 min at room temperature. A colony was defined as >50 cells. The colony formation rate was calculated by colony number/seeding number.

**Transwell assay.** Transwell assay was performed to assess migration and invasion. For invasion, Matrigel® (Corning, Inc.) was mixed with serum-free medium in a 1:8 ratio. A total of 100 µl of the mixture was added onto the upper surface of the migration chambers (Corning, Inc.; 8.0-µm filter) and incubated at 37°C for 30 min. MDA-MB-231 cells were trypsinized and washed with serum-free medium. A total of 1x10⁴ cells in 100 µl serum-free medium were seeded onto the upper surface of the chambers, while the lower chambers were filled with medium with 10% FBS. After 24 h at 37°C, the culture medium and the cells attached on the upper surface were removed. Cells attached on the lower surface were fixed with methanol for 30 min at room temperature and stained with 0.1% crystal violet for 30 min at room temperature. The images of migratory and invasive cells were collected using a light microscope (Olympus Corporation; magnification, x100).

**Wound-healing assay.** MDA-MB-231 cells were seeded into 6-well plates and cultured overnight. The cells were grown to ~100% confluence in the wells. Linear scratch wounds were developed using 10-µl pipette tips. The floating cells were washed five times with PBS. Serum-free medium was used to maintain the cells. The images were captured at 0, 6, 12, 18 and 24 h using an inverted light microscope (Olympus Corporation; magnification, x100). The wound-healing percentage was calculated using the following formula: (Wound area at 0 h-wound area of indicated time)/wound area at 0 h) x100. Wound area was analyzed using ImageJ (v4.0; National Institute of Health).

**RNA extraction and reverse transcription-quantitative PCR (RT-qPCR).** Total RNA in MDA-MB-231 and MDA-MB-468 cells was extracted using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). RNA was reverse transcribed to cDNA using the Reverse transcription system kit (Promega Corporation; cat. no. A3500), according to the manufacturer's instructions. qPCR analysis was performed using SYBR Master Mixture (Takara Bio, Inc.) on the Agilent MX3000p Real Time PCR system (Agilent Technologies, Inc.) as follows: 1 cycle at 95°C for 30 sec; 40 cycles at 95°C for 5 sec and 60°C for 20 sec; 1 cycle at 65°C for 15 sec. qPCR primers were as follows: SOX9 forward, 5'-AGCGACAGCATCAAGAC-3' and reverse, 5'-CTGTAACCGATCGTGTTGGG-3'; and GAPDH forward, 5'-CTGATCCGCATCGTGTTGGG-3' and reverse, 5'-CACCTGTGCTGCTAGCCCCA-3'. mRNA expression was analyzed using the 2^ΔΔCq method (14).

**Western blotting.** Western blotting was used to determine the protein expression levels in TNBC cells. Total protein (40 µg) was extracted from MDA-MB-231 and MDA-MB-468 cells using RIPA lysis buffer (Beyotime Institute of Biotechnology), supplemented with protease and phosphatase inhibitors cocktail (Roche Diagnostics). The protein concentration was detected using a BCA Protein Assay kit. A total of 40 µg of protein was loaded and separated via 12% SDS-PAGE, followed by transfer to PVDF membranes (EMD Millipore). Membranes were blocked with 5% skimmed milk dissolved in PBS-Tween (0.1% Tween) at room temperature for 1.5 h and incubated with primary antibodies overnight at 4°C. After incubating with peroxidase-conjugated secondary antibodies for 2 h at room temperature, the protein abundance was analyzed using an enhanced chemiluminescence system (EMD Millipore). Primary antibodies against SOX9 (1:1,000; cat. no. ab815966) and GAPDH (1:1,000; cat. no. ab8245) were purchased from Abcam. HRP-conjugated anti-mouse (1:5,000; cat. no. sc-2005) or anti-rabbit (1:5,000; cat. no. sc-2004) secondary antibodies were from Santa Cruz Biotechnology, Inc.

**Apoptosis analysis.** Apoptosis was analyzed using an annexin V/PI apoptosis detection kit according to the manufacturer's protocol (cat. no. 88-8007-72; Invitrogen; Thermo Fisher Scientific Corporation; cat. no. A3500), according to the manufacturer's instructions. qPCR analysis was performed using SYBR Master Mixture (Takara Bio, Inc.) on the Agilent MX3000p Real Time PCR system (Agilent Technologies, Inc.) as follows: 1 cycle at 95°C for 30 sec; 40 cycles at 95°C for 5 sec and 60°C for 20 sec; 1 cycle at 65°C for 15 sec. qPCR primers were as follows: SOX9 forward, 5'-AGCGACAGCATCAAGAC-3' and reverse, 5'-CTGTAACCGATCGTGTTGGG-3'; and GAPDH forward, 5'-CTGATCCGCATCGTGTTGGG-3' and reverse, 5'-CACCTGTGCTGCTAGCCCCA-3'. mRNA expression was analyzed using the 2^ΔΔCq method (14).
Scientific, Inc.). MDA-MB-231 and MDA-MB-468 cells were trypsinized with EDTA-free 0.25% Trypsin (Corning, Inc.). The cells and cell supernatants were collected by centrifugation at 1,000 x g for 5 min at room temperature. The cell pellet was washed using ice-cold D-Hanks (pH 7.2-7.4) buffer and 1X binding buffer. The cell pellet was resuspended in 200 µl 1X binding buffer. After incubating with 10 µl Annexin V-APC for 10-15 min in the dark at room temperature, cells were stained with PI staining buffer (5 µl) for 10-15 min on ice at room temperature. A total of 400-800 µl 1X binding buffer was added and apoptosis was analyzed on a Guava easyCyte HT system (EMD Millipore). The data was analyzed using the guavasoft software (v2.7; Merck KGaA).

**Cell cycle analysis.** PI staining was used to analyze the cell cycle. When MDA-MB-231 and MDA-MB-468 cells reached 80% confluence, they were trypsinized and centrifuged at 1,000 x g for 5 min at room temperature. The cell pellet was washed using ice-cold D-Hanks (pH 7.2-7.4) buffer and fixed with ice-cold 75% ethanol for ≥1 h at -20°C. The cells were centrifuged at 1,000 x g for 5 min at room temperature and the pellet was washed twice with D-Hanks buffer. Finally, the cell pellet was resuspended in 0.6-1 ml staining buffer [40X PI solution (2 mg/ml); 100X RNase solution (10 mg/ml); 1X D-Hanks; 25:10:1,000] at room temperature for 30 min and subjected to cell cycle analysis using a Guava easyCyte HT system (EMD Millipore). The data was analyzed using the guavasoft software (v2.7; Merck KGaA).

**RNA sequencing.** Firstly, total RNA was extracted from shNC- and shSOX9-transfected MDA-MB-231 cells using TRIzol reagent. Subsequently, RNA sequencing was performed by Beijing Aoweise Gene Technology Co., Ltd. Briefly, the total RNA integrity was evaluated using an Agilent Bioanalyzer 2100 (Agilent Technologies, Inc.). The RNA Clean XP kit (cat. no. A63987; Beckman Coulter, Inc.) and RNase-free DNase Set (cat. no. 79254; Qiagen GmbH) were used for RNA clean-up and DNA removing, respectively. A total of 1 µg purified RNA was used for cDNA library generation using a VAHTSTM mRNA-seq v2 library Prep kit (cat. no. NR612-01; Vazyme Biotech Co., Ltd.). The quality of constructed cDNA libraries were evaluated with an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.) according to the manufacturer's protocol. The products were sequenced using an Illumina NovaSeq 6000 SP Reagent kit v1.5 (300 cycles; cat. no. 200284000; Illumina, Inc.) with a paired-end read length of 150 bp. A total of 1 nM of the final library was used for sequencing. For the analysis of these three shRNAs downregulated SOX9 expression in MDA-MB-231 cells, while shSOX9-3 did not significantly inhibit SOX9 mRNA expression, with shSOX9-2 being the most efficient (Fig. 2A). Western blotting results revealed that all of these three shRNAs downregulated SOX9 expression (Fig. 2A). In MDA-MB-468 cells, all three shRNAs significantly suppressed SOX9 mRNA expression, with shSOX9-2 being the most efficient (Fig. 2B). Western blotting results were consistent with the RT-qPCR results (Fig. 2B). Since shSOX9-2 exhibited the highest knockdown efficiency, it was used for subsequent experiments.

**Results**

SOX9 is highly expressed in TNBC cells. SOX9 is a pivotal modulator of stemness and contributes to cancer development (17). To explore the relevance of SOX9 in TNBC, SOX9 expression was detected in BT-549, MDA-MB-231, MDA-MB-436 and MDA-MB-468 cells. SOX9 mRNA expression was lowest in BT-549 cells, while MDA-MB-436 and MDA-MB-468 exhibited the highest SOX9 mRNA expression (Fig. 1A). Western blotting results revealed that SOX9 was highly expressed in MDA-MB-231, MDA-MB-468 and MDA-MB-436 cells, while a marginal signal band was observed in BT-549 cells (Fig. 1B).

Establishment of SOX9-knockdown in MDA-MB-231 and MDA-MB-468 cells. Due to SOX9 was higher expression in both MDA-MB-231 and MDA-MB-468 cells than other TNBC cells MDA-MB-436 and BT-549. MDA-MB-231 and MDA-MB-468 cells were chosen for subsequent experiments to study the role of SOX9 in TNBC. Three shRNA sequences were used to inhibit SOX9 expression in the cells. Compared with shNC, shSOX9-1 and shSOX9-2 significantly decreased SOX9 mRNA expression in MDA-MB-231 cells, while shSOX9-3 did not significantly inhibit SOX9 mRNA expression (Fig. 2A). Western blotting results revealed that all of these three shRNAs downregulated SOX9 expression (Fig. 2A). In MDA-MB-468 cells, all three shRNAs significantly suppressed SOX9 mRNA expression, with shSOX9-2 being the most efficient (Fig. 2B). Western blotting results were consistent with the RT-qPCR results (Fig. 2B). Since shSOX9-2 exhibited the highest knockdown efficiency, it was used for subsequent experiments.

SOX9-knockdown suppresses the proliferation and colony formation in TNBC cells. Since SOX9 was efficiently silenced by shSOX9-2, shNC- and shSOX9-2-transfected MDA-MB-231 and MDA-MB-468 cells were subjected to cell proliferation analysis. CCK-8 assay was performed to analyze cell proliferation. The results indicated that, compared with the shNC, SOX9-knockdown significantly decreased the proliferation of MDA-MB-231 and MDA-MB-468 cells (Fig. 3A). To validate the results, colony formation was evaluated in shNC- and shSOX9-2-transfected MDA-MB-231 and MDA-MB-468 cells. Compared with shNC cells, shSOX9-2 cells exhibited significantly decreased colony formation (Fig. 3B). The current results suggested that SOX9-knockdown suppressed the proliferation and colony formation of TNBC cells.

SOX9 regulates apoptosis and the cell cycle in TNBC cells. Decreased apoptosis and accelerated cell cycle progression are common features of cancer cells (18). Whether SOX9
regulated apoptosis and the cell cycle was explored by staining the cells with PI/Annexin V-APC and PI, respectively. In MDA-MB-231 and MDA-MB-468 cells, SOX9-knockdown significantly increased apoptosis (Fig. 4A and B). Additionally, SOX9-knockdown resulted in significantly increased G1 cell cycle phase in both cell lines, compared with the shNC (Fig. 4C and D). However, the S phase in MDA-MB-231 cells remained unchanged, while it was significantly decreased in
MDA-MB-468 cells after SOX9-knockdown, compared with that in the shNC-transfected cells (Fig. 4C and D). The G_{1} phase was significantly decreased in shSOX9-2-transfected MDA-MB-231 cells compared with that in the shNC-transfected cells, but unchanged in shSOX9-2-transfected MDA-MB-468 cells (Fig. 4C and D). Overall, SOX9 silencing caused cell cycle arrest at the G_{1} phase.

Downregulation of SOX9 inhibits migration and invasion of TNBC cells. TNBC is a malignant tumor with a high metastasis rate (19). It has been reported that SOX9 contributes to the migration and invasion of various cancer cells (17,20,21). Therefore, the present study explored whether SOX9-knockdown regulated the migration of TNBC cells using wound-healing assays. The results revealed that cell migration was significantly repressed by SOX9-knockdown at 12, 18 and 24 h (Fig. 5A and B). To validate the effect of SOX9 on TNBC cell migration and invasion, Transwell assays without or with Matrigel-coated chambers, respectively, were performed. The results indicated that, compared with the shNC, SOX9-knockdown significantly decreased the migration and invasion of TNBC cells (Fig. 5C and D). The results suggested that SOX9 may be essential for TNBC cell migration and invasion.

RNA sequencing of dysregulated genes after SOX9-knockdown in TNBC cells. To elucidate the downstream factors regulated by SOX9, RNA sequencing was performed in control and SOX9-knockdown cells. The results revealed that SOX9-knockdown led to the upregulation and downregulation of numerous genes (Table S1). The top three upregulated genes included nucleophosmin (NPM1), thioredoxin reductase 1 (TXNRD1) and succinate dehydrogenase complex subunit D (SDHD), while the most downregulated genes were nuclear receptor binding SET domain protein 2 (NSD2), eukaryotic translation initiation factor 4γ (EIF4G1) and glycogen phosphorylase L (PYGL) (Table SII). The expression levels of these genes in breast invasive carcinoma (BRCA) tissues were analyzed based on data from TCGA database. The results indicated that the expression levels of NPM1, TXNRD1, NSD2
Figure 4. SOX9 regulates apoptosis and the cell cycle in triple-negative breast cancer cells. Apoptosis was detected using PI/Annexin V-APC staining and flow cytometry analysis in shNC- and shSOX9-2-transfected (A) MDA-MB-231 and (B) MDA-MB-468 cells. The cell cycle was measured using PI staining and flow cytometry analysis in shNC- and shSOX9-2-transfected (C) MDA-MB-231 and (D) MDA-MB-468 cells. *P<0.05, **P<0.01 and ***P<0.001 vs. shNC. sh, short hairpin RNA; NC, negative control; SOX9, SRY-related high-mobility group box 9.

Figure 5. SOX9-knockdown decreases the migration and invasion of triple-negative breast cancer cells. (A and B) shNC- and shSOX9-2-transfected MDA-MB-231 cells were subjected to a wound-healing assay. Scale bar, 100 µm. (C and D) shNC- and shSOX9-2-transfected MDA-MB-231 cells were subjected to Transwell analysis of migration and invasion. Scale bar, 100 µm. *P<0.05, **P<0.01 and ***P<0.001 vs. shNC. sh, short hairpin RNA; NC, negative control; SOX9, SRY-related high-mobility group box 9.
and EIF4G1 were upregulated in BRCA tissues, while PYGL and SDHD was downregulated in BRCA tissues compared with in normal tissues (Fig. 6).

Discussion

Breast cancer poses the greatest threat to the health of women worldwide. Breast cancer has become the second leading cause of death in the United States, with 1,150,000 new cases and 370,000 deaths each year (1). Although TNBC is not the most common type of breast cancer, it is highly malignant and no effective drugs are available against this deadly disease (19). Understanding the molecular events driving this malignancy may help to gain insight into the pathological process of the disease. In the present study, it was revealed that SOX9 expression was higher in TNBC MDA-MB-231 and MDA-MB-468 cells compared with in TNBC BT-549 and MDA-MB-436 cells. SOX9 expression was critical for the proliferation and migration of TNBC cells, including MDA-MB-231 and MDA-MB-436 cells. SOX9-knockdown suppressed the proliferation, colony formation, cell cycle, migration and invasion, and induced apoptosis of TNBC cells.

SOX9 is a member of the SOX family, which controls the expression of numerous genes, such as VGF nerve growth factor inducible (22,23). Since it is a transcriptional factor, drugs to inhibit SOX may have highly toxic side effects (22). Based on the current study, SOX9 functions as an oncogene in TNBC. The proliferation, colony formation, migration and invasion of MDA-MB-231 and MDA-MB-436 cells were significantly suppressed by SOX9-knockdown. However, knowing the function may not help in our understanding of the molecular events downstream of SOX9 in TNBC. Therefore, RNA sequencing was performed in the present study to illustrate the downstream targets of SOX9. Several newly reported genes regulated by SOX9 were identified, including NPM1, TXNRD1 and SDHD, which were upregulated, and NSD2, EIF4G1 and PYGL, which were downregulated by SOX9-knockdown. Mutations in NPM1 serve an important role in acute myeloid leukemia (24,25). NPM1 expression is upregulated in patients with TNBC, and NPM1-knockdown suppresses TNBC cell proliferation (26), suggesting that NPM1 acts as an oncogene in TNBC. This may imply that inhibition of TNBC cell proliferation by SOX9-knockdown may not act via the regulation of NPM1 expression. TXNRD1 and EIF4G1 expression is upregulated in distinct malignancies, such hepatocellular carcinoma and lung cancer (27-29). NSD2 upregulation promotes renal cancer growth by activating Akt/Erk signaling (30). However, the function of SDHD and PYGL remains unclear in cancer. Based on TCGA database, the present study revealed that the expression levels of EIF4G1, NPM1, NSD2 and TXNRD1 were upregulated, whereas those of PYGL and SDHD were downregulated in breast cancer tissues compared with those in normal tissues. Nevertheless, the expression levels of these genes were analyzed in all breast cancer types from TCGA; therefore, the expression profile among TNBC tissues and
other types of breast cancer requires further investigation. Therefore, SOX9 may promote TNBC cell proliferation and migration via regulation of one of the aforementioned genes. Nevertheless, future studies should be performed to elucidate the downstream effectors of SOX9 in TNBC. Future studies may be important for the treatment of patients with TNBC with SOX9 upregulation.

In summary, the present study revealed that SOX9 was critical for the proliferation, colony formation, migration and invasion of TNBC cells. Mechanistically, NPM1, TXNRD1, SDHD, NSD2, EIF4G1 or PYGL may be downstream effectors of SOX9 in TNBC. However, further experiments should be performed to clarify the molecular mechanisms by which SOX9 promotes TNBC cell proliferation and migration.

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Availability of data and materials

The datasets generated and/or analyzed during the current study are available in the Gene Expression Omnibus repository, under the accession number PRJNA721654.

Authors’ contributions

YFW and YXT designed the study, performed the experiments, and drafted and revised the manuscript. HFD, XL, QQW and CG conducted some experiments and revised the manuscript. YFW and YXT confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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