Effects of vascular endothelial growth factor and insulin growth factor-1 on proliferation, migration, osteogenesis and vascularization of human carious dental pulp stem cells

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Abstract. The present study aimed to investigate the effects of vascular endothelial growth factor (VEGF) and insulin-like growth factor-1 (IGF-1) on the proliferation, migration and differentiation of human carious dental pulp stem cells (hCDPSCs), and to elucidate the underlying mechanism(s). Cell counting kit-8 assay was used to detect the effect of different concentrations of IGF-1 and VEGF on the proliferation of hCDPSCs. Transwell assay was used to detect the migratory ability of the hCDPSCs. Alizarin red and alkaline phosphatase (ALP) staining were used to detect the osteogenic ability of hCDPSCs, whereas the angiogenic ability of the hCDPSCs was tested by tube formation assay. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blotting were used to detect the expression levels of associated genes and proteins. IGF-1 (100 ng/ml) or VEGF (25 ng/ml) alone were revealed to be able to promote proliferation and migration of hCDPSCs; however, the combined use of IGF-1 and VEGF enhanced this effect when compared with the use of either agent in isolation. Alizarin red and ALP staining revealed that the use of either VEGF or IGF-1 alone did not result in any significant effects, whereas their use in combination promoted the osteogenic differentiation of hCDPSCs. In addition, the RT-qPCR and western blotting analyses revealed that the expression levels of Runx-related transcription factor 2 (RUNX2), bone sialoprotein (BSP) and ALP were increased upon combined treatment of the cells with VEGF and IGF-1. The expression levels of VEGF and platelet-derived growth factor (PDGF) in hCDPSCs were enhanced upon treatment with either VEGF or IGF-1 in isolation, with greater effects observed when VEGF and IGF-1 were added in combination, indicating that VEGF and IGF-1 may exert a synergistic role in these events. Further experiments revealed that the combination of VEGF and IGF-1 led to an activation of the AKT signaling pathway. The proliferation and angiogenesis of hCDPSCs were also shown to be more effective compared with treatment with either VEGF or IGF-1 in isolation. Taken together, the present study has demonstrated that the combined use of VEGF and IGF-1 leads to an increase in the proliferation, migration, osteogenesis and angiogenesis of hCDPSCs and, furthermore, these signaling molecules may mediate their effects via activation of the AKT signaling pathway.

Introduction

Human dental pulp stem cells (DPSCs) possess the ability to proliferate and to differentiate into odontoblasts, which enables them to participate in the reconstruction and repair of diseased pulp tissue (1-3). At present, dental caries, and the subsequent inflammation caused by dental pathogens, are common and nonnegligible clinical problems (4,5). Human carious dental pulp stem cells (hCDPSCs) have attracted increasing levels of attention (6). Compared with normal DPSCs, hCDPSCs have a number of advantages, such as a high proliferation rate and easy availability (6-9). hCDPSCs are considered to be ideal ‘seed’ cells in tissue engineering, although their characteristics have yet to be fully elucidated.

Growth factors (GFs) are types of polypeptide or glycoprotein that possess the biological properties of promoting cell proliferation, differentiation and locomotion (10-12). Among them, vascular endothelial growth factor (VEGF) and insulin-like growth factor-1 (IGF-1) are two important GFs with unique functions (13-15). VEGF has the capacity to trigger newly forming vessels in order to provide a blood supply for bone regeneration (16). IGF-1, a member of the insulin-like peptide family, is a ubiquitous and important peptide hormone and anti-apoptotic factor that exerts important roles in organ apoptosis and differentiation (17). It has been reported that IGF-1 may promote the proliferation of dental pulp stem cells (DPSCs) and periodontal ligament stem cells (PDLSCs), and induce their osteogenic/odontogenic differentiation (18,19). It has been shown, that when either VEGF (25 ng/ml) or IGF-1 (100 ng/ml) was used, mesenchymal stem cells (MSCs) exhibited the strongest proliferative capacity (19-21). Furthermore, it was reported that the combined use of VEGF and IGF-1 enhanced osteogenic differentiation of
periosteum-derived progenitor cells (PDPCs) and skin-derived MSCs (S-MSCs) (22). However, to the best of our knowledge, the effects of VEGF and IGF-1, either alone or in combination, on hCDPSCs has yet to be elucidated.

The phosphoinositide 3-kinase (PI3K)/Akt signaling pathway is an important signal transduction pathway, which tightly controls cell proliferation, migration, differentiation and self-renewal (23,24). It has been reported that activation of the Akt signaling pathway led to an improvement in the proliferative and differentiation abilities of MSCs, thereby enhancing stemness (25).

In the present study, IGF-1 and VEGF, when applied either alone or in combination, were observed to exert an effect on the proliferation, migration and differentiation of hCDPSCs in vitro, and these effects were found to be associated with the Akt signaling pathway. These findings add to our knowledge of the cellular biological foundation for clinical application of hCDPSCs in the treatment of dental pulp disease.

Materials and methods

Isolation and culture of hCDPSCs. hCDPSCs were obtained from the carious pulp tissues, which were collected from teeth diagnosed with deep caries. The diagnosis of deep caries was determined by endodontic specialists on the basis of clinical assessment (26). A total of 20 patients (28-30 years of age; 10 male and 10 female patients) were informed about the nature of this research project, agreed to participate, and signed informed consent forms for scientific experiments involving human subjects. All patients were diagnosed with deep caries. The diagnosis of deep caries was made by trained endodontists based on clinical and radiographic criteria. The patients were recruited from the dental clinic of the University of Medicine and Dentistry of New Jersey (UMDNJ). The study was performed in accordance with the guidelines of the Institutional Review Board of UMDNJ (protocol no. 2010002924).

The study protocol was performed according to a standard protocol approved by the Ethics Committee of the Southern Medical University. The pulp was carefully collected (1/3 of the apical pulp was discarded), cut into small pieces, and digested with collagenase (3 mg/ml)/dispase enzyme (4 mg/ml; Sigma-Aldrich; Merck KGaA) for 30 min. After 7-10 days, the primary cultured hCDPSCs were observed to be able to climb out along the edge of the tissue blocks. When the confluence of hCDPSCs reached ~80%, the cells were subcultured in new flasks. Cells were cultured with HyClone™ α-MEM; Thermo Fisher Scientific, Inc.) containing 10% Gibco fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin (all from Gibco; Thermo Fisher Scientific, Inc.). For all incubations in the methods section, unless specified otherwise, cells were cultured at 37°C in an atmosphere of 5% CO₂.

Phenotypic identification of hCDPSCs. Passage 3 hCDPSCs were stained in 300 µl PBS. Fc blocking reagent was used to block the non-specific detection of the Fc component of all antibodies. Flow cytometry (BD Accuri C6, Becton Dickinson Biosciences) was used to detect the positive rate of stem cell surface markers. All data were analyzed by FlowJo Software (v10.0; FlowJo LLC).

Cell monoclonal assay and osteogenic induction. hCDPSCs (2,000 cells) were inoculated into a 10 cm culture dish at 37°C in an atmosphere of 5% CO₂. After 14 days, hCDPSCs were fixed by 4% paraformaldehyde for 30 mins and stained with Giemsa for 10 mins in 37°C. Cells with more than 50 cells were counted as one unit.

Passage 3 hCDPSCs were cultured with osteogenic medium (10% FBS, 10 mmol/l sodium β-glycerophosphate, 50 mg/l ascorbic acid and 0.1 µmol/l dexamethasone in α-MEM). After 7 days of induction, hCDPSCs were fixed for 15 min with 4% paraformaldehyde, and subsequently incubated with ALP stain for 10 min at room temperature. After 21 days of induction, hCDPSCs were stained with Alizarin red for 10 min at room temperature following fixation with 4% paraformaldehyde. After a thorough rinse, the cells were observed and counted under a microscope a light microscope with x100 magnification.

Effect of VEGF and IGF-1 on proliferation and migration of hCDPSCs. Passage 3 hCDPSCs in the exponential growth phase were collected by 0.25% Trypsin and centrifuged at 1,500 x g for 1 min at 37°C, and inoculated (1,500 cells) into 96-well plates, and induced by the addition of no agent (blank control), IGF-1 (100 ng/ml), VEGF (25 mg/ml), or VEGF (25 mg/ml) + IGF-1 (100 ng/ml), respectively. Cell proliferation was analyzed using a Cell Counting Kit-8 (CCK-8) assay (Beyotime Institute of Biotechnology). According to the manufacturer’s protocol, on days 1, 3, 5, 7 and 9, the absorbance at 490 nm wavelength was measured following incubation with CCK8 reagent for 3 h. Similarly, after the cells were cultured in the different experimental group set-ups, migration assay was performed in a Transwell chamber. Serum-free medium was added in the upper chamber, and 5x10⁴ cells were inoculated in each well. 10% FBS medium was added into the lower chamber. The cells were incubated at 37°C in an atmosphere of 5% CO₂ for 24 h. Subsequently, the supernatant was discarded, the cells were fixed in 4% paraformaldehyde for 30 min and stained by 0.1% crystal violet in 37°C.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from cells in the different groups using TRIzol®, and total RNA was reverse-transcribed into cDNA according to the instructions of the RT kit employed (Qiagen GmbH). The content of RNA was quantified (Nanodrop; Thermo Fisher Scientific, Inc.). SYBR Green was purchased from Qiagen GmbH and the sequences of the RT-PCR primers (Thermo Fisher Scientific, Inc.) are given in Table I. GAPDH was used as reference gene. The following thermocycling conditions were used: 95°C for 20 sec, annealing at 60°C for 30 sec, and extension at 72°C for 30 sec (40 cycles). Each assay was performed in triplicate, and quantification was performed using the 2^{-ΔΔCq} method (27).
Western blot analysis. Passage 3 hCDPSCs from the different groups were collected by 0.25% Trypsin and centrifuged at 1,500 x g for 1 min at 37˚C, and subsequently the total protein was extracted using RIPA buffer (Beyotime Institute of Biotechnology) (28). Aliquots of 40 µg protein from each sample were subjected to SDS-PAGE (10% gels), and the proteins were subsequently transferred on to PVDF membranes (Life Sciences, Ann Arbor, MI, USA). The membranes were subsequently blocked with TBST containing 5% defatted milk powder at room temperature for 1 h, and then incubated overnight with primary antibodies against RUNX2 (1:500, cat. no. 12556), BSP (1:500, cat. no. 5468S), ALP (1:500, cat. no. 8681), VEGF (1:500, cat. no. 2463), PdGF (1:500, cat. no. 3169), AKT (1:500, cat. no. 4685), phospho-AKT (p-AKT) (1:500, cat. no. 9614) and cyclin D1 (1:500, cat. no. 2978; all antibodies from Cell Signaling Technology, Danvers, MA, USA) at 4˚C overnight. The membranes were subsequently washed with PBS three times. Peroxidase-conjugated goat anti-rabbit or goat anti-mouse was used as the secondary antibody (1:500, cat. no. 4414 or 4410S, respectively; 1:1,000 dilution; Cell Signaling Technology), and the membranes were incubated with secondary antibody for 1 h at room temperature, prior to subsequent exposure to an Odyssey 2-colour infrared laser imaging system (LI-COR Biosciences, Lincoln, NE, USA). The semi-quantitative results for each western blot were measured using the Image-Pro Plus 6.0 program (Media Cybernetics, Inc., Rockville, MD, USA).

In vitro Matrigel™ tube formation assay. Passage 3 hCDPSCs in the exponential growth phase were collected by 0.25% Trypsin and centrifuged at 1,500 x g for 1 min at 37˚C, and cell suspensions (1x10^5 cells) were prepared. Matrigel™ was coated onto 12-well plates according to the manufacturer’s protocol (29,30). The cells were gently mixed, inoculated on to the aggregated Matrigel™, and subsequently were incubated in 37˚C in an atmosphere of 5% CO₂ for 7 h. The condition of the vessels was observed under a light microscope, and the density of vessels was determined using Image-Pro Plus 6.0 program (Media Cybernetics, Inc.).

Statistical analysis. SPSS 17.0 was used for statistical analysis. Data are expressed as the mean ± standard deviation. Comparisons among groups was performed using one-way ANOVA, and post-hoc comparisons were performed by the least significant difference (LSD) t test. P<0.05 was considered to indicate a statistically significant difference.

Results

Identification of hCDPSCs. Passage 3 hCDPSCs were collected and cultured for stem cell identification. The cells exhibited the property of monoclonal formation, as well as osteogenic differentiation and mineralization (Fig. 1A-C). The flow cytometry experiments revealed positive expression of CD29, CD44, CD90 and CD105, and negative expression of CD45 and CD133 (Fig. 1D). According to the criteria for defining multipotent MSCs, the isolated human carious dental pulp cells were identified as MSCs.

Effects of VEGF and IGF-1 on proliferation and migration of hCDPSCs. CCK8 assay revealed that the proliferation of hCDPSCs could be stimulated by adding either VEGF or IGF-1 alone, whereas the addition of VEGF and IGF-1 in combination led to a further enhancement in the proliferation rate of the hCDPSCs (Fig. 2A).

Transwell assay was subsequently used to detect the migratory ability of hCDPSCs upon treatment with VEGF and IGF-1, either separately or in combination. The results demonstrated that there was a synergistic effect of VEGF/IGF-1 treatment on cell migration, and the effect of combined treatment was greater than that of either of these two GFs when added in isolation (Fig. 2B).

Effects of VEGF and IGF-1 on osteogenic differentiation of hCDPSCs in vitro. For these experiments, five experimental groups were established: Blank control (Con), ordinary osteogenic medium (OM), osteogenic medium plus VEGF (VEGF + OM), osteogenic medium plus IGF-1 (IGF-1 + OM), and osteogenic medium plus VEGF and IGF-1 (VEGF + IGF-1 + OM). Alizarin red staining and ALP staining revealed that, compared with the OM group, the osteogenic ability of the VEGF + IGF-1 + OM group revealed a significant enhancement in the staining intensity (Fig. 3A-C). The corresponding RT-qPCR and western blotting results revealed that the mRNA and protein expression levels of the osteogenesissassociated genes (i.e., Runx2, BSP and ALP) increased significantly in the VEGF + OM and IGF-1 + OM groups, whereas the largest increase was observed for the VEGF + IGF-1 + OM group (Fig. 4A-C).

Effects of VEGF and IGF-1 on angiogenic differentiation of hCDPSCs in vitro. Tube formation assays revealed that the number of tubules could be increased by adding either VEGF or IGF-1 alone, whereas the addition of VEGF and IGF in combination led to a further increase in the angiogenic ability of hCDPSCs (Fig. 5B and C). The RT-qPCR and

<table>
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<tr>
<th>Gene</th>
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<tr>
<td>RUNX2</td>
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</tr>
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<td></td>
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<td></td>
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RUNX2, Runt-related transcription factor 2; BSP, bone sialoprotein; ALP, alkaline phosphatase; VEGF, vascular endothelial growth factor; PdGF, platelet-derived growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
western blotting results exhibited a similar trend to that of the osteogenesis-induction assay: The expression of vessel formation-associated genes and proteins (i.e., VEGF and PDGF) increased significantly in the OM + VEGF and OM + IGF-1 groups, whereas the VEGF + IGF-1 + OM group exhibited the greatest enhancement (Fig. 5A, D and E).

Effect of VEGF and IGF-1 on the AKT signaling pathway in hCDPSCs in vitro. Compared with the control group, the expression levels of p-AKT and cyclin D1 were increased upon addition of either VEGF or IGF-1 alone, whereas the expression of these two proteins was further increased by adding VEGF and IGF-1 in combination to activate the AKT signaling pathway of the hCDPSCs (Fig. 6). These results demonstrated that the combination of VEGF and IGF-1 elicited a synergistic effect (Fig. 6).

Discussion
Currently, hDPSCs are commonly used as ‘seed’ cells in bone tissue engineering due to their high self-renewal capacity and
stemness (3,31). hCDPSCs are a unique type of dental stem cell, since they are derived from the pulp in deep carious teeth (7,8). This special environmental stimulus enables the stronger proliferation and osteogenic differentiation capability of hCDPSCs, and therefore they are now recognized as a potential source for regenerative medicine (7,8).

Figure 3. Osteogenic differentiation of hCDPSCs upon treatment with VEGF and IGF-1. (A) The osteogenic differentiation capability of hCDPSCs was examined using Alizarin red staining, with (B) subsequent quantification of the results. *P<0.01 vs. control. (C) The results from ALP staining are shown. For the description of the experimental groups (Control, OM, VEGF+OM, IGF-1+OM and VEGF+IGF-1+OM), see the Results section. Scale bar, 200 µm. hCDPSCs, human carious dental pulp stem cells; IGF-1, insulin-like growth factor 1; VEGF, vascular endothelial growth factor; ALP, alkaline phosphatase.

Figure 4. Analysis of the osteogenesis-associated genes and proteins of hCDPSCs upon treatment with VEGF and IGF-1. (A) The mRNA expression levels of the RUNX2, BSP and ALP genes were examined by RT-qPCR. (B) The protein expression levels of RUNX2, BSP and ALP were examined by western blotting, and (C) the relative protein expression levels were normalized against GAPDH. *P<0.05, **P<0.01 vs. control. For the description of the experimental groups (Control, OM, VEGF+OM, IGF-1+OM and VEGF+IGF-1+OM), see the Results section. hCDPSCs, human carious dental pulp stem cells; IGF-1, insulin-like growth factor 1; VEGF, vascular endothelial growth factor; RUNX2, Runt-related transcription factor 2; BSP, bone sialoprotein; ALP, alkaline phosphatase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
Figure 5. Angiogenic differentiation of hCDPSCs upon treatment with VEGF and IGF-1. (A) mRNA expression levels of VEGF and PDGF were examined by RT-qPCR. (B and C) Tube-formation ability was examined in the different groups. Magnification, x200. (D) The protein expression levels of VEGF and PDGF were detected by western blotting, and (E) the relative protein expression level was normalized against GAPDH. *P<0.05, **P<0.01 vs. control. For the description of the experimental groups (control, VEGF, IGF-1 and VEGF+IGF-1), see the Results section. hCDPSCs, human carious dental pulp stem cells; IGF-1, insulin-like growth factor 1; VEGF, vascular endothelial growth factor; PDGF, platelet-derived growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Figure 6. Effect of AKT signaling pathway in hCDPSCs upon treatment with VEGF and IGF-1. The protein expression levels of P-AKT, T-AKT and cyclin D1 were detected by western blotting, and the relative protein expression levels were calculated. **P<0.01 vs. control. For the description of the experimental groups (Control, VEGF, IGF-1 and VEGF+IGF-1), see the Results section. hCDPSCs, human carious dental pulp stem cells; IGF-1, insulin-like growth factor 1; VEGF, vascular endothelial growth factor; P-AKT, phosphorylated AKT; T-AKT, total AKT.
GFs are critical in tissue regeneration, as they have important roles in regulating cell functions (32,33). Previous studies have reported that the combination of IGF-1 and other GFs (such as PDGF-B or BMP-2) may affect cell proliferation, promote osteogenesis, and help the reconstruction of tooth-supporting tissues (34-36). IGF-1 has been identified to stimulate osteogenic differentiation via the mitogen-activated protein kinase signaling pathway, suggesting that IGF-1 has a role in regulation of the periodontal tissue (37). VEGF has the potential of triggering neovascularization, which is able to regulate both normal and pathological conditions, and provides blood supply for tissue regeneration (38,39). Angiogenesis is essential for bone reconstruction, as new bone formation relies on a suitable blood supply to provide cells and nutrients (40). Therefore, the selection of suitable GFs has become an important research focus in tissue engineering therapy.

In the present study, the CCK8 assay results demonstrated that the combination of VEGF and IGF-1 exerted a synergistic effect on proliferation. Transwell assay revealed that the migratory ability of hCDPSCs upon combined treatment of VEGF and IGF-1 was markedly stronger compared with that of treating with VEGF or IGF-1 alone. This finding indicated that there was a synergistic effect resulting from treating the cells with VEGF and IGF-1 in combination. It has been previously shown that, when either VEGF (25 ng/ml) or IGF-1 (100 ng/ml) were used, MSCs exhibited the strongest proliferative capacity (19-21). Furthermore, it was reported that the combined use of VEGF and IGF-1 enhanced osteogenic PDPCs and S-MSCs (22). Therefore, based on the findings of these previous studies, a combination of IGF-1 (100 ng/ml) and VEGF (25 ng/ml) was used in the present study to investigate the effects of IGF-1 and VEGF on proliferation, migration, osteogenesis and vascularization of hCDPSCs.

RUNX2 is a highly conserved transcription factor, known as the most important regulator in osteoblast and odontoblast differentiation (41). Runx2 activates bone-associated genes and promotes mineralization in the early stage of osteoblast differentiation (41,42). ALP is the enzyme that is predominantly involved in both bone and tooth mineralization, and elevated levels of ALP may be considered as an early marker of odontoblast differentiation and dentin formation (43). BSP is an important product during osteogenic differentiation, which may clearly indicate the level of ALP (44). The results of RT-PCR and western blotting in the present study demonstrated that VEGF and IGF-1 exerted a synergistic effect on bone formation, which was more effective than using either of the drugs alone. It has been reported that the combined use of VEGF and IGF-1 could induce the differentiation of S-MSCs into osteoblasts, and that the IGF-1 and insulin signaling pathways may function as important mediators in terms of Runx2 activity (45).

The results from the alizarin red and ALP staining experiments in the present study revealed that the combined use of VEGF and IGF-1 could promote the osteogenesis of hCDPSCs. VEGF has been widely studied as a major regulator in angiogenesis-associated processes, in which the molecule exerts both direct and indirect functions (46). VEGF stimulates cell proliferation and migration, whereas on the other hand, it increases the permeability of blood vessels and allows plasma proteins to leak out of blood vessels, a process that serves an important role in remodeling the extracellular matrix to adapt to angiogenesis (46,47). The addition of VEGF during the early stage of bone repair may lead to an improvement in the blood supply of the microenvironment and the formation of new bone (47). The results of the present study showed that the single application of either VEGF or IGF-1 was sufficient to promote vascular differentiation of hCDPSCs, whereas the combined use of VEGF and IGF-1 elicited a synergistic effect.

The PI3K/Akt pathway is a tyrosine kinase receptor-mediated signaling system, which exists widely in various types of cell (23). It is an important signal-transduction pathway involved in the regulation of cell growth, proliferation and differentiation (24). Activated Akt can induce changes in a series of downstream factors [including mammalian target of rapamycin, (mTOR), glycogen synthase kinase 3 (GSK3), Bax, NF-κB, caspases, etc.] and participate in the regulation of cell growth, differentiation, division and migration (24). The phosphorylation level of Akt may also reflect the activity of whole signaling pathway (48). It has been shown that IGF-1 promotes the vascular differentiation of adipose-derived stem cells and endothelial cells via the PI3K/AKT signaling pathway (48). The activation of autophagic activity is able to enhance the osteogenic differentiation of hBMSCs, thereby providing a novel therapeutic target for osteoporosis treatment (49). Therefore, it is possible to surmise that activation of the PI3K-AKT pathway may give rise to the observed effects associated with the mTOR pathway and autophagy activity during the osteogenic differentiation of hCDPSCs.

In conclusion, the present study has identified that the AKT signaling pathway may be activated by the use of VEGF or IGF-1 alone, whereas the AKT signaling pathway can be further activated by the combined use of VEGF and IGF-1. This suggests that the combined use of VEGF and IGF-1 may have the additive effects through AKT signaling pathway.

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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

BW and WL designed the study. WL, WX and JL guided the experiments. WL, YC and YP performed the experiments. WL and WX collected and processed the clinical data. JL, YC and YP analyzed and interpreted the patient data. BW and WL wrote the paper, and BW reviewed and edited the manuscript. All authors read and approved the final manuscript.
Ethics approval and consent to participate

The study protocol was performed according to a standard protocol approved by the Ethics Committee of the Southern Medical University. All patients signed written informed consent forms.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References


