Apatinib inhibits gastric carcinoma development by regulating the expression levels of IL-17 via the Bax/Bcl-2 signaling pathway

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Abstract. Gastric carcinoma is a common type of gastrointestinal tumor with high morbidity and mortality rates. IL-17 is a newly discovered cytokine that has been reported to serve an important role in the development of gastric carcinoma. The potential effect of apatinib on IL-17 expression levels in the development of gastric carcinoma has been rarely reported. The present study aimed to investigate the potential mechanism of IL-17 and apatinib in the development of gastric carcinoma. A total of 30 tumor and para-carcinoma tissues were collected from 30 patients with gastric carcinoma between January 2019 and December 2019 and the expression levels of IL-17 in the tissues were analyzed by reverse transcription-quantitative PCR and western blotting. An in vitro model of gastric carcinoma was also established using the HGC-27 cell line, in which the cells were divided into control, IL-17, IL-17-apatinib and apatinib groups. The expression levels of IL-17, Bax, Bcl-2 and caspase-3 were analyzed using reverse transcription-quantitative PCR and western blotting. An MTT assay and flow cytometry were used to analyze the proliferation and apoptosis of HGC-27 cells, respectively, and a Transwell assay was used to analyze the invasive ability of HGC-27 cells. The results revealed that the expression levels of IL-17 were significantly upregulated in the gastric carcinoma tissues compared with the para-carcinoma tissues. In vitro, IL-17 treatment promoted the proliferation and invasive ability of HGC-27 cells, but inhibited the apoptosis with the significantly downregulated expression levels of Bax and caspase-3, and the upregulated expression levels of Bcl-2 than control group. Conversely, apatinib treatment significantly inhibited the proliferative and invasive abilities of HGC-27 cells, but promoted cell apoptosis in the IL-17 and IL-17-apatinib groups. Collectively, the present results suggested that the upregulation of IL-17 may be associated with the occurrence and development of gastric carcinoma. The findings indicated that apatinib may inhibit gastric carcinoma development by regulating IL-17 expression via the Bax/Bcl-2 signaling pathway. Therefore, the present findings may enhance the current knowledge of the effect of apatinib on gastric carcinoma cells.

Introduction

Gastric carcinoma is a common type of malignant tumor worldwide and it is estimated that newly diagnosed cases in China annually account for 43% of the global cases, with ~400,000 cases (1). Due to its high rate of mortality, gastric carcinoma poses a serious threat to human health and life (2-4). The etiology of gastric carcinoma is complicated, and the vast majority of gastric carcinomas are diagnosed in the advanced stages because only a small percentage of patients can be diagnosed and treated at an early stage (5). Previous studies have reported that the occurrence of the majority of gastric carcinoma cases were the result of the combined action of multiple environmental factors, such as dietary, lifestyle and environmental influential factors, and tumor susceptibility factors, such as the influencing factors of blood vessel invasion (6,7). It was previously identified that gastric carcinoma caused by Helicobacter pylori was closely associated with the immune response of the body (8,9). Moreover, cellular immunity dominated by cytokines was discovered to be involved in the occurrence of gastric carcinoma (10-12).

Cytokines, such as ILs, IFNs, colony stimulating factor, chemokines and growth factors, not only serve important roles in immune regulation, but also participate in the occurrence and development of various types of carcinoma (13,14). Liu et al (13) demonstrated that the potency of IL-34, macrophage colony stimulating factor, tumour-associated macrophages (TAMs) and the combination of IL-34/TAMs as novel biological markers for gastric carcinoma. ILs are mainly involved in the activation and regulation of immune cells in the body (15). IL-17 consists of six protein members (16-18), and has been revealed to serve a vital role in the development

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of numerous types of malignant cancer, including colorectal cancer (19). Furthermore, the upregulation of IL-17 was observed in various types of tumor tissue, such as breast carcinoma and gastric carcinoma (20, 21), which suggested that IL-17 may be associated with the development of tumors. 

At present, the standard therapy for gastric carcinoma includes surgical intervention followed by combination chemotherapy (22). While chemotherapy has become the primary treatment option for advanced gastric carcinoma (23), the effect of traditional chemotherapy is not ideal. Targeted therapy is a novel cancer treatment at the cellular or molecular level (24), in which specially designed drugs can target and act on the specific genes or proteins necessary for tumor growth. The adverse reactions of targeted therapy in patients are usually more easily tolerated compared with those of chemotherapy (25). Therefore, targeted therapies for various types of solid tumor have gained increased attention. As a popular anti angiogenic targeting drug, apatinib can block the formation of new blood vessels in tumor tissue and thus, inhibit the progression of tumors (26). Notably, apatinib has previously demonstrated efficacy in patients with metastatic gastric carcinoma (27).

Although both IL-17 and apatinib have been closely associated with the process of tumor angiogenesis, to the best of our knowledge, the potential effect of apatinib on IL-17 expression levels in the development of gastric carcinoma has been rarely reported. In the present study, the effects of IL-17 and apatinib on gastric carcinoma were investigated. Overall, the present study may be of great significance to provide novel specific targets for the treatment of gastric carcinoma.

Materials and methods

Clinical sample collection. A total of 30 tumor and para-carcinoma tissues were obtained from 30 patients with gastric carcinoma (aged between 55 and 73 years, who received surgery at Tianjin Nankai Hospital (Tianjin, China) between January 2019 and December 2019 (Table I). Tissue samples were immediately stored in liquid nitrogen at -80°C after resection. Written informed consent was obtained from each participant before surgery. The study conformed to the Declaration of Helsinki and was approved by Tianjin Nankai Hospital ethics committee (approval no. NKYY_YXKT_IRB_2019_101_01).

Cell culture and treatment. The human gastric carcinoma cell line, HGC-27, was obtained from the American Type Culture Collection. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (HyClone; Cytiva), 100 U/ml penicillin and 100 U/ml streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.) in an incubator with 5% CO₂ at 37°C. Cell propagation was performed every 24 h. The cells in the logarithmic phase were used for further analysis.

After cells were fully adhered to the well, they were cultured at 37°C at a density of 2×10⁵ cells/cm² and divided into the following groups: i) Control group; ii) IL-17 group; iii) IL-17-apatinib group; and iv) apatinib group. Cells in the IL-17 group were treated with 10 ng/ml IL-17 (Invitrogen; Thermo Fisher Scientific, Inc.) for 48 h. The apatinib group was treated with 50 ng/ml apatinib (cat. no. S7297, Selleck Chemicals) for 48 h. For the IL-17-apatinib group, cells were cultured with 10 ng/ml IL-17 and 50 ng/ml apatinib for 48 h. Cells in the Control group were treated with same volume of DMEM. All treatments were performed at 37°C.

Reverse transcription-quantitative PCR (RT-qPCR). The expression levels of IL-17 in the para-carcinoma and carcinoma tissues and the expression levels of Bax, Bcl-2 and caspase-3 in HGC-27 cells among the four different groups were analyzed using RT-qPCR. Total RNA was extracted from the cells and tissues using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Total RNA was reverse transcribed into cDNA using the PrimeScript™ One Step RT-PCR kit (Takara Biotechnology Co., Ltd.) at 37°C. qPCR was subsequently performed using the SYBR® Premix Dimmer Eraser kit (Takara Biotechnology Co., Ltd.). The following thermocycling conditions were used for the qPCR: Initial denaturation at 94°C for 5 min; followed by 35 cycles at 94°C for 30 sec, 57°C for 30 sec and 72°C for 30 sec; and a final cycle at 72°C for 5 min. The following primers pairs were used for the qPCR: IL-17 forward, 5'-CTGGACATCCCGGGGCTGC-3' and reverse, 5'-GTCTGTCCGCTGAACACAGTCT-3'; Bcl-2 forward, 5'-TGGGATGCCTTTGTGGAAC-3' and reverse, 5'-CATATTTGTGTTGGGCAGTT-3'; Bax forward, 5'-TCCGAGTGGCAGCTGAGATGT-3' and reverse, 5'-TGCTGGCAAAGTGAAGAGGGCA-3'; caspase-3 forward, 5'-GCAACCTCAGGAAAACTT-3' and reverse, 5'-TTTTCAGGCTCAACACAGGTTCA-3'; and GAPDH forward, 5'-GGAAAGCTGTGGCGTGAT-3' and reverse, 5'-AGGTTGAAGAATTGGGAGTT-3'. The relative expression levels of the target genes were quantified using the 2^(-ΔΔCq) method (28). Each experiment was repeated ≥3 times. GAPDH was used as the internal loading control. The target expressions were normalized using the expression levels of GAPDH as a reference. All kits were used according to the manufacturer's protocols.

Table I. Clinicopathological information of patients with gastric carcinoma used in the present study (n=30, mean ± SD).

| Variable                      | Parameters          |
|-------------------------------|---------------------|
| Age, years                    | 65.32±9.33          |
| Number of male patients (%)   | 17 (56.67)          |
| BMI, kg/m²                    | 25.30±4.66          |
| Urea nitrogen, mmol/l         | 5.62±1.53           |
| Creatinine, µmol/l            | 79.3±8.21           |
| Low density lipoprotein, mmol/l| 3.63±1.38          |
| Triglyceride, mmol/l          | 1.96±0.74           |
| Total cholesterol, mmol/l     | 4.77±1.24           |
| Number of diabetic patients (%)| 20 (66.67)         |
| Number of patients with hypertension (%) | 11 (36.67) |
| Number of patients with a smoking history (%) | 19 (63.33) |

Continuous variables are expressed as mean ± SD and categorical data are expressed as numbers and percentages.
Western blotting. The protein expression levels of IL-17, Bax, Bcl-2 and caspase-3 in HGC-27 cells were analyzed using western blotting. Cells were collected and total protein was extracted using RIPA lysis buffer (Beyotime Institute of Biotechnology) on ice. The supernatant was retained to detect protein concentration of IL-17, Bax, Bcl-2 and caspase-3 after high-speed centrifugation (10,000 x g, 4°C, 60 min) using BCA. Equivalent amounts of protein/lane (30 µg/lane) in RIPA lysis buffer was separated via 10% SDS-PAGE (Sigma-Aldrich; Merck KGaA). The separated proteins were subsequently transferred onto a PVDF membrane (Sigma-Aldrich; Merck KGaA) and blocked with 5% milk in Tris-buffered saline for 1 h at 25°C. Then, the membranes were incubated with the following primary antibodies overnight at 4°C: Anti-Bax (1:1,000; cat. no. B3428 Sigma-Aldrich; Merck KGaA), anti-Bcl-2 (1:1,000; cat. no. B3170, Sigma-Aldrich; Merck KGaA), anti-caspase-3 (1:1,000; cat. no. ABC495, Sigma-Aldrich; Merck KGaA), anti-IL-17 (1:1,000; cat. no. PRS4887, Sigma-Aldrich; Merck KGaA) and anti-GAPDH (1:1,000; cat. no. G8795, Sigma-Aldrich; Merck KGaA). Following the primary antibody incubation, the membranes were incubated with a secondary antibody (1:5,000; cat. no. ZB-2301; goat anti-rabbit; Beijing Zhongshan Jinqiao Biotechnology Co., Ltd.) for 1 h. The protein bands were visualized using an ECL kit (Beijing Solarbio Science & Technology Co., Ltd.). The band intensity was semi-quantified using Image-Pro Plus 6.0 analysis software (Media Cybernetics, Inc.). Blots were repeated ≥ 3 times for every condition.

MTT assay. Cell proliferation assay was performed using MTT reagent (Shanghai Macklin Biochemical Co., Ltd.). HGC-27 cells in the logarithmic phase were seeded into 96-well plates at a density of 5x10⁴ cells/well and incubated for 24 h. After the cells had fully adhered to the well, cell suspension (100 µl/well) of IL-17, apatinib or IL-17-apatinib were added into the test wells. Following 12, 24 or 48 h incubation at 37°C, 20 µl MTT (5 mg/ml) solution was added to each well and incubated for another 4 h. After removing the supernatant, 100 µl per well dimethyl sulfoxide (DMSO) (Shanghai Macklin Biochemical Co., Ltd.) was used to dissolve the formazan crystals. The absorbance values of each sample were measured with a microplate reader at 490 nm. Experiments were repeated ≥3 times and data are expressed as the mean ± standard error of the mean.

Flow cytometric analysis of apoptosis. HGC-27 cells in the four groups were collected (1,000 x g, 5 min, 4°C) and resuspended in 0.5 ml binding solution (Annexin V-FITC apoptosis detection kit; Beyotime Institute of Biotechnology) at a density of 5x10⁵ cells/ml. The cells were transferred to a flow tube (100 µl/tube) and then incubated with 5 µl Annexin-V allophycocyanin and 5 µl 7-Aminoactinomycin D at 4°C for 5 min. At the end of the incubation, the cells were washed with ice-cold PBS three times and the fluorescence intensity was measured using a FACSCalibur flow cytometer (BD Biosciences) with FlowJo software (version 7.6, Tree Star, Inc.) The apoptotic cells were combined with Annexin V labeled with FITC, and the percentage of early and late apoptotic cells was calculated.

Transwell invasion assays. Transwell assays were performed with 8-µm pore Transwell plates (EMD Millipore). Briefly, 5x10⁴ HGC-27 cells in 100 µl DMEM (Gibco; Thermo Fisher Scientific, Inc.) medium were seeded into the upper chamber of the Transwell plate in serum-free DMEM. The lower chamber was filled with DMEM supplemented with 10% FBS. The cells were allowed to migrate across a polycarbonate filter precoated with Matrigel. Following 24 h of incubation at 37°C, the invasive cells were in the lower chamber were fixed in 5% glutaraldehyde for 10 min at 4°C, stained using 0.5% crystal violet solution for 15 min at 37°C and counted with an inverted phase-contrast microscope (magnification, x200; Nikon TE-2000U, Nikon Corporation). Each experiment was repeated ≥ 3 times.

Statistical analysis. All data were analyzed using SPSS 18.0 software (SPSS, Inc.). Continuous variables were expressed as mean ± SD and categorical data were expressed as numbers and percentages. A paired Student's t-test (two-tailed) or one-way ANOVA followed by a Tukey's post hoc test were used as appropriate to determine the statistical differences between groups. Each experiment was repeated ≥ 3 times. P<0.05 was considered to indicate a statistically significant difference.

Results

Analysis of the data from patients with gastric carcinoma. The basic information of the patients with gastric carcinoma, including age, sex, BMI, smoking and hypertension history, and biochemical indexes, are presented in Table I. The patients had a mean age of 65.32±9.33 years and a mean BMI of 25.30±4.66 kg/m². In total, 56.67% of the patients were male. Gastric carcinoma and para-carcinoma tissues were obtained from the 30 patients.

Expression levels of IL-17 in the gastric carcinoma tissues. The expression levels of IL-17 in the gastric carcinoma and para-carcinoma tissues were analyzed using RT-qPCR (Fig. 1A) and western blotting (Fig. 1B). The results of the analyses revealed that IL-17 expression levels were upregulated in gastric carcinoma tissues compared with para-carcinoma tissues. Thus, IL-17 was suggested to be upregulated in patients with gastric carcinoma.

Effect of apatinib and IL-17 on the proliferative ability of HGC-27 cells. To determine the effect of apatinib and IL-17 on the proliferation of HGC-27 cells, an MTT assay was performed (Fig. 2). Following incubation for 48 h, IL-17 stimulation significantly promoted the proliferation in the IL-17 group compared with the control group, while apatinib treatment significantly inhibited the proliferation of HGC-27 cells compared with the control group, particularly in the apatinib group following incubation for 48 h. Compared with the IL-17 group, the absorbance values were significantly decreased in IL-17-apatinib group, most notable in the apatinib group following incubation for 48 h. These results suggested that IL-17 may promote cell proliferation, while apatinib may effectively suppress the proliferation of HGC-27 cells.
Effect of apatinib and IL‑17 on the apoptosis of HGC‑27 cells.
The effect of apatinib and IL‑17 on the apoptosis of HGC‑27 cells was further investigated (Fig. 3). The results revealed that IL‑17 significantly inhibited the apoptotic rate of HGC‑27 cells in the IL‑17 group compared with the control group (Fig. 3A, B and E). Moreover, apatinib reversed the inhibitory effect of IL‑17 and promoted the apoptosis of HGC‑27 cells. Compared with in the IL‑17 group, the cell apoptotic rate was significantly increased in the IL‑17‑apatinib group (Fig. 3B, C and E). Compared with the IL‑17‑apatinib group, the cell apoptotic rate in the apatinib group was significantly increased (Fig. 3C‑E).

Effect of apatinib and IL‑17 on the expression levels of Bax, Bcl‑2 and caspase‑3 in HGC‑27 cells. The expression levels of the apoptosis‑related factors Bax, caspase‑3 and Bcl‑2 were also analyzed using RT‑qPCR and western blotting (Fig. 4). The RT‑qPCR results revealed that the expression levels of the proapoptotic genes Bax and caspase‑3 were significantly downregulated in IL‑17 group compared with the control group (Fig. 4A and C). However, the expression levels of the anti‑apoptotic gene Bcl‑2 in the IL‑17 group were significantly upregulated compared with the control group (Fig. 4B). Moreover, the expression levels of Bax and caspase‑3 were significantly upregulated, while the expression levels of Bcl‑2 were significantly downregulated, in the IL‑17‑apatinib and apatinib groups compared with the IL‑17 and control groups. Compared with the IL‑17‑apatinib group, the apatinib group revealed the upregulated expression levels of Bax and caspase‑3 and the downregulated expression levels of Bcl‑2. Western blotting demonstrated similar results to the RT‑qPCR results (Fig. 4D). Therefore, it these findings suggested that apatinib and IL‑17 may serve important roles during the progression of cell apoptosis.

Invasive abilities of HGC‑27 cells. A Transwell assay was performed to evaluate the invasive ability of HGC‑27 cells (Fig. 5). A significant increase in the invasive cell number was observed in the IL‑17 group compared with the control group (Fig. 5A, B and E), which indicated that IL‑17 may promote the HGC‑27 cell invasive ability. In addition, the invasive ability of HGC‑27 cells was significantly impaired in the apatinib group compared with the control group (Fig. 5A, D and E). Moreover, the invasive cell number in the apatinib group was reduced compared with the IL‑17 and IL‑17‑apatinib groups (Fig. 5B‑E). Notably, compared with the IL‑17 group, the IL‑17‑apatinib groups also showed a significantly decreased number of invasive cell (Fig. 5B and C).

Discussion
IL‑17 is mainly produced by CD4+ T helper 17 cells (29). Previous studies have reported that CD8+ T cells also produced IL‑17, which performed similar functions to the IL‑17 produced by CD4+ cells (30,31). Compared with healthy tissues, significantly upregulated expression levels of IL‑17 in gastric carcinoma tissues were discovered to be associated with the lymphatic vascular invasion of tumors, suggesting that IL‑17 may promote the progression of tumors (20,32). However, Iida et al (33) revealed that the survival time of patients with
high IL‑17 mRNA expression levels was significantly longer compared with those with low IL‑17 mRNA expression levels by detecting the IL‑17 mRNA expression levels in the peritoneal lavage of patients with gastric carcinoma following surgery. Previous studies have also demonstrated that tumor growth and distant metastasis in IL‑17 deficient mice were significantly increased compared with those in the control group, suggesting that IL‑17 could enhance the antitumor immunity of local T cells in tumors (34,35).

The present study investigated the effects of IL‑17 in gastric carcinoma. The expression levels of IL‑17 were analyzed between gastric carcinoma tissues and para‑carcinoma tissues. IL‑17 was originally identified as a proinflammatory cytokine that induced inflammatory cells and factors, and exerted crucial roles in promoting and maintaining tumor activities by regulating the promotion of angiogenesis and tumor cell migration (32). The current results revealed that the expression levels of IL‑17 were upregulated in gastric carcinoma tissues compared with paired para‑carcinoma tissues. Thus, it was indicated that IL‑17 may promote the occurrence and be associated with the development of gastric carcinoma.

Apatinib is a popular anti‑angiogenic targeting drug (36,37) and a small molecule tyrosine kinase inhibitor of VEGFR (38). Apatinib has been reported to inhibit the progression of tumors by blocking the formation of new blood vessels in tumor tissues, and in the past, it has been used as a combination drug for the treatment of gastric carcinoma (39). A previous study reported that apatinib improved the efficacy of fluorouracil and paclitaxel both in vitro and in vivo, suggesting that apatinib may be an efficient and acceptably safe treatment for late‑stage gastric carcinoma (40). The clinical efficacy of apatinib in treating metastatic gastric carcinoma was also confirmed (27). Thus, the present study...
further investigated the effect of apatinib on IL-17 and the potential mechanism during the development of gastric carcinoma using an in vitro model of gastric carcinoma with HGC-27 cells.

In the present study, apatinib demonstrated a significant suppressive effect on the proliferation of HGC-27 cells in the IL-17-apatinib and apatinib groups compared with the IL-17 and control groups, which suggested that apatinib may help to suppress the development of gastric carcinoma. Moreover, the results of the flow cytometric analysis demonstrated that apatinib promoted the apoptosis of HGC-27 cells. The expression levels of Bax and caspase-3 in the IL-17-apatinib group were significantly upregulated, while Bcl-2 expression levels were significantly downregulated compared with the control group, which indicated that the use of apatinib may promote cell apoptosis in gastric carcinoma. In addition, the results of the Transwell assay demonstrated that IL-17 promoted the invasive ability of HGC-27 cells, while apatinib inhibited the invasive ability of HGC-27 cells. Apatinib presented an antagonistic effect with IL-17, which effectively helped to suppress the invasive ability of HGC-27 cells and thus, by extension, can be hypothesized to inhibit the development of gastric carcinoma. Therefore, apatinib was suggested to serve an important role in suppressing the development of gastric carcinoma by promoting apoptosis and inhibiting the proliferative and invasive abilities of gastric carcinoma cells.

Nonetheless, there are some limitations to the current study. For instance, the present study initially analyzed the expression levels of IL-17 in 30 paired gastric carcinoma and para-carcinoma tissues from 30 patients with gastric carcinoma; this sample size is relatively small, which may cause bias to the results. Therefore, studies with larger cohorts are required to verify the findings of the present study. Secondly, the present study only examined the potential mechanism of IL-17 and apatinib in the development of gastric carcinoma in an in vitro model of HGC-27 cells, and thus additional in vivo research is required. Finally, the mechanism of apatinib in inhibiting the progression of gastric carcinoma via the Bax/Bcl-2 signaling pathway remains to be fully elucidated. Therefore, how the Bax/Bcl-2 signaling pathway functions in gastric carcinoma and how apatinib regulates the expression of IL-17 should be further studied. Nonetheless, the present research expanded the current knowledge of the effect of apatinib on gastric carcinoma cells stimulated by IL-17.

In conclusion, gastric carcinoma tissues were revealed to have upregulated IL-17 expression levels compared with para-carcinoma tissues. IL-17 was identified to promote the proliferative and invasive abilities of HGC-27 cells, and
inhibit cell apoptosis, by significantly downregulating the expression levels of Bax and caspase-3 and upregulating the expression levels of Bcl-2. Therefore, the current findings suggested that IL-17 may be closely associated with the occurrence and development of gastric carcinoma. Conversely, apatinib, used as a treatment drug, inhibited the proliferative and invasive abilities of HGC-27 cells and promoted cell apoptosis. Thus, it was suggested that apatinib may inhibit cell proliferation and invasion, and promote apoptosis by regulating IL-17 expression via the Bax/Bcl-2 signaling pathway and may serve a vital role in the development of gastric carcinoma.

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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

JZ conceived and designed the study. TW and LC performed the experiments. TW, LC and JZ analyzed and interpreted the data. TW and JZ wrote the manuscript. JZ, TW and LC confirmed the authenticity of all the raw data. All authors read and approved the final manuscript.

Figure 5. Apatinib inhibits the invasive ability of the HGC-27 cell line. A Transwell assay was used to determine the invasive abilities of HGC-27 cells in the (A) control group, (B) IL-17 group, (C) IL-17-apatinib group and (D) apatinib group. Scale bar, 100-µm. (E) Semi-quantification of the invasive cells number calculated from parts (A-D). Data are presented as the mean ± SD (n=3). A one-way ANOVA followed by a Tukey's post hoc test was used for the statistical analysis. *P<0.01.
Ethics approval and consent to participate

The present study conformed to the Declaration of Helsinki and was approved by Tianjin Nankai Hospital ethics committee (approval no. NKY_YXKT_IRB_2019_010_D1). Written informed consent was obtained from each participant before surgery.

Patent consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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