Abstract. The aim of the study was to investigate the effects of lactic acid on the phenotypic polarization and immune function of macrophages. The human monocyte/macrophage cell line, THP-1, was selected and treated with lactic acid. Immunofluorescence staining, laser confocal microscopy, reverse-transcription polymerase chain reaction (RT-PCR), western blot, siRNA, and ELISA analyses were used to observe changes in the levels of cluster of differentiation (CD)68, CD163, hypoxia inducible factor (HIF)-1α, and programmed death ligand -1 (PD-L1) as well as those of cytokines, tumor necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-12, and IL-10. THP-1 macrophages and T cells were co-cultured in vitro to observe the changes in proliferation and apoptosis of T cells. The results showed that, lactic acid (15 mmol/l) significantly upregulated the expression of the macrophage M2 marker CD163 (P<0.05), cytokines, IFN-γ and IL-10, secreted by M2-Tumor-associated macrophages (TAM, P<0.05), and HIF-1α and PD-L1 (P<0.05), and down-regulated the expression of cytokines, TNF-α and IL-12, secreted by M1-TAM (P<0.05). Redistribution of M2-TAM subsets and PD-L1 expression was reversed after further transfection of THP-1 cells with HIF-1α siRNA (P<0.05). After co-culturing, T-cell proliferation was inhibited and apoptosis was promoted. In summary, modulation of lactic acid level can redistribute M2-TAM subsets and upregulate PD-L1 to assist tumor immune escape. The HIF-1α signaling pathway may participate in this process, revealing that macrophages, as ‘checkpoints’ in organisms, are links that connect the immune status and tumor evolution, and can be used as a target in tumor treatment.

Introduction

The development and progression of a tumor depends not only on its proliferation, but also on its interactions with the local microenvironment (1). Tumors have hypoxic stress microenvironments (2). An acidic microenvironment [pH (6.5-6.8) vs. (7.4)] caused by a large amount of anaerobic glycolysis is an important characteristic of malignant tumors, and an important factor in inducing their occurrence, metastasis, and drug resistance (3).

Tumor-associated macrophages (TAMs) are the most important cell subsets in the aforementioned environment, and their numbers and functions are affected by many factors related to the host and tumor (4). TAMs are primarily divided into the following two subtypes; M1-TAM is a part of the classical activation pathway, and M2-TAM is a part of the alternative activation pathway. M1-TAM shows a high expression of interleukin (IL)-12, low expression of IL-10, and plays an important role in antitumor activity (4). M2-TAM has low expression of IL-12, high expression of IL-10, and exerts a tumor-promoting effect. Experiments have shown that TAMs can play an important positive regulatory role in the development and progression of tumors (5). In recent years, with a more thorough understanding of acidic microenvironments, these are now considered a new target for tumor diagnosis and treatment, especially in regulating TAM immune escape (6). Previous studies have shown that lactic acid can maintain the immunosuppressive tumor microenvironment by regulating the transformation of macrophages to the M2 type and reducing the cytotoxic function of natural killer...
cells (7,8). Previous findings showed that TAMs can also combine with programmed death-1 (PD-1) expressed on the surface of T cells through programmed death-ligand 1 (PD-L1) and mediate the immune escape of tumor cells through the PD-L1/PD-1 pathway to promote tumor progression (9,10). However, the authenticity of this mechanism is unclear at present.

In this study, by investigating the effects of lactic acid on macrophage redistribution and immune function, the possible mechanism by which tumor cells, including pancreatic cancer cells, release lactic acid through glycolysis to promote immune escape was explored, providing a foundation for new treatment directions.

Materials and methods

Cell cultures and treatments. The human monocyte/macrophage cell line THP-1, human T lymphocyte cell line H9, and BxPC-3 human pancreatic cancer cell lines (American Tissue Type Collection) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), 0.1 mM nonessential amino acids, 0.2 mM glutamine, 1 mM pyruvate, and 10% heat-inactivated fetal bovine serum (FBS) and were incubated in 5% CO₂ humidified atmosphere at 37°C. Cells were grown to 80% confluency prior to treatment.

Primary human pancreatic cells were isolated from the same pancreatic site in pancreatic cancer and non-cancerous partial pancreatotomy specimens, respectively. Tissue samples obtained from areas within 2.0 cm around the tumor were obtained from 45 patients undergoing partial pancreate-duodenectomy (Whipple resection) for pancreatic cancer at the Department of Hepatobiliary and Pancreatic Surgery, Affiliated Hospital of the Xi’an Jiaotong University from January 2016 to December 2018. Of the 45 patients, there were 30 men and 15 women. The median age at the time of surgery was 60.5 years (range 42-77 years). All the patients were obtained from 45 patients undergoing partial pancreatomy specimens, respectively, tissue samples were collected and transferred to the laboratory. After several washings with sterile phosphate-buffered saline (PBS), 1 cm² was removed from the tumor and incubated for 10 min. Terminal deoxynucleotidyl transferases (TdT) were used for the incorporation of DNA strand breaks in situ for 1 h at 37°C in a humidified box. Positive control slides were treated with DNase and negative control slides were treated with phosphate-buffered saline (PBS) instead of TdT. DNA fragments were stained using 3,3'-diaminobenzidine (DAB) as a substrate for peroxidase, and hematoxylin was used as a counterstain. The apoptotic index was calculated as a ratio of the number of apoptotic cells to the total number of tumor cells on each slide (10 of fields analyzed per sample).

Immunofluorescence assay. Exponentially growing cells were seeded on 25 mm square glass coverslips placed in 35 mm diameter culture dishes. After treatment, the cells were fixed with 4% formaldehyde for 5 min at 25°C, permeabilized with 0.2% solution of Triton X-100 in PBS, and blocked with 2% bovine serum albumin-PBS for 30 min at 25°C. Slides were incubated with anti-CD68 and anti-CD163 overnight. Fluorescent imaging was conducted with a confocal laser scanning microscope (Carl Zeiss MicroImaging, Inc.).

Reverse-transcription polymerase chain reaction. Total RNA was isolated using TRIzol® reagent (GIBCO BRL), and the quantities were determined spectrophotometrically. First-strand cDNA was synthesized from 2 µg of total RNA using a RevertAid Kit (Fermentas MBI). The polymerase chain reaction (PCR) primer sets were designed 1) for PD-L1, forward: 5'-CGACATGTCGATCGATCTCTGGTCTTC-3' and reverse: 5'-CCCTCGAGCGCGCCGCTATCCCTTTC-3'; 2) for CD68, forward: 5'-CACGCAGCACGAGTGGACATTCTT-3' and reverse: 5'-TGGGCAAGAGAACCATTGTGTC-3'; 3) for CD163, forward: 5'-AGCATGGAAGCGGTCTCTGTGATT-3' and reverse: 5'-AGCTGACTCATTCCTCACTCAGAAGAAG-3'; and 4) for β-actin, forward: 5'-ATCCTGGTGATACATGATTCAAG-3' and reverse: 5'-AGGAGAGAAGGGCTGAGAAGTG-3'. The PCR conditions included an initial cDNA synthesis reaction at 42°C for 1 h using a RevertAid Kit (Fermentas MBI) followed by a denaturation step for 5 min at 94°C and 22 cycles: 30 sec at 94°C, 30 sec at 55°C, and 30 sec at 72°C.
55°C, and 30 sec at 72°C. After the last cycle, a final extension was performed at 72°C for 10 min. The housekeeping gene β-actin was used as an internal control.

Western blotting. Briefly, 5x10^5 cells were incubated on ice for 30 min in 0.5 ml of ice-cold whole-cell lysate buffer. Debris were removed by centrifugation at 23 x g, for 10 min at 4°C. The protein content of the cells was determined, and the cellular lysates were separated by 10% SDS-PAGE and electro-transferred onto nitrocellulose membranes. After being blocked with 5% non-fat milk in TBST, the membranes were incubated with primary antibodies CD68 (1:500, sc-20060), CD163 (1:500, sc-20066), HIF-1α (1:500, sc-13515), PD-L1 (1:500, sc-293425) at 4°C overnight, followed by 1:2,000 horseradish peroxidase-conjugated secondary antibody (Santa Cruz, sc-51602) for 2 h. Immunoreactive bands were visualized using an Enhanced Chemiluminescence Kit (Amersham Pharmacia Biotech). The western blot signals were quantified by densitometric analysis using Total Lab Nonlinear Dynamic Image Analysis software (Nonlinear).

siRNA assay. To inhibit the expression of HIF-1α, siRNA oligos were used (Qiagen). HIF-1α siRNA target sequence was: 5'-AGGAAGAACTATGAACTACA-3', and the sequence of the control siRNA was: 5'-UUCUCCGAACGU GUCAGT-3'; this selection was based on the results of our previous study (11). The cells (n=2x10^6) were transfected with siRNA targeted against HIF-1α (100 nm/l) or a control siRNA (Qiagen) using Lipofectamine 2000 (Invitrogen). Cells were covered overnight prior to starvation. This was then followed by treatment with lactic acid (15 mmol/l) for 24 h. Finally, the cells were harvested for reverse-transcription polymerase chain reaction (RT-PCR) and western blotting.

Indirect co-culture model. The T cells were added to Petri dishes at a density of 1.5x10^5/ml; after 24 h, TAM-CM was added, and the cells were cultured for 48 h. Cells in PBS or serum-free medium were used as controls. ELISA and TUNEL assays were used to observe the factor and apoptosis of T cells in each Petri dish. Proteins were extracted from the cells. The co-culture of pancreatic cells, THP-1 or pancreatic cancer cells was performed in the same manner.

Statistical analysis. Each experiment was performed at least three times. Data are provided as means ± standard deviation and differences were evaluated using Student's t-test and a one-way ANOVA (when the variance was homogeneous, Tukey's test was used as the post hoc test; when the variance was uneven, tamhane test was used as the post hoc test). Homogeneity of variance was determined with Fisher test. P<0.05 was considered statistically significant. All statistical analyses were performed using SPSS Version 13.0 statistical software (SPSS).

Results

Changes in the proliferation of THP-1 macrophages with different concentrations of lactic acid. We first examined the effects of different concentrations of lactic acid (0, 5, 10, 15, and 20 mmol/l) on the proliferation of THP-1 cells by administering different concentrations for 48 h (7). Cell proliferation did not change significantly after treatment with lactic acid concentrations <15 mmol/l (P>0.05), whereas it was inhibited at 20 mmol/l, indicating drug toxicity. Therefore, lactic acid concentration of 15 mmol/l was selected for subsequent experiments (Fig. 1).

Lactic acid upregulated the expression of macrophage marker molecules in M2 macrophages. It has previously been reported that CD68 is a specific molecular marker for macrophages, and CD163 expression is a marker for M2-TAMs (4). The ratio of CD163:CD68, thus, represents the proportion of M2-TAMs. To clarify whether lactic acid can redistribute M2-TAMs, THP-1 macrophages were treated with 15 mmol/l for 48 h. Immunofluorescence analysis revealed that the fluorescence intensity of CD163 in the lactic acid-treated group was significantly higher than that in the blank control group; by contrast, the fluorescence intensity of CD68 did not change considerably (Fig. 2A). RT-PCR and western blot analysis revealed that the ratio of CD163:CD68 in the lactic acid group was significantly higher than that in the blank control group (Fig. 2B and C). Similarly, the levels of cytokines, IFN-γ and IL-10, secreted in the supernatant by M2-TAMs of the lactic acid-treated group were higher than those secreted by M1-TAMs of the blank control group (P<0.05). Additionally, the levels of cytokines, TNF-α and IL-12, secreted by M1-TAMs decreased (P<0.05; Fig. 2D). These results suggested that lactic acid can redistribute M2-TAM subsets.

Lactic acid upregulated the expression of PD-L1 protein in M2-TAMs. To determine whether lactic acid can affect PD-L1 expression in macrophages, 15 mmol/l lactic acid was used to treat THP-1 macrophages for 48 h. RT-PCR showed that PD-L1 mRNA expression significantly increased in the lactic acid-treated group compared to that in the blank control group (P<0.05; Fig. 3A and B). Similarly, western blot analysis revealed that the expression of PD-L1 protein was significantly upregulated in the lactic acid-treated
Figure 2. Lactic acid upregulated the expression of M2 macrophage marker molecules in THP-1 induced by lactic acid. (A) CD68 and CD163 protein expression in THP-1 was induced by lactic acid stained with FITC-labeled IgG antibody and analyzed by confocal microscopy. The CD163 fluorescence signal in THP-1 cells induced by lactic acid was higher than that in the control; the fluorescence intensity of CD68 did not change considerably in the two groups (magnification, x400). (B) mRNA expression and quantification of CD68 and CD163 in THP-1 cells induced by lactic acid via RT-PCR. (C) The protein expression and quantification of CD68 and CD163 in THP-1 cells induced by lactic acid were assessed by western blotting. (D) The levels of cytokines IFN-γ, IL-10, TNF-α, and IL-12 secreted in THP-1 cells induced by lactic acid were assessed by ELISA. Data from at least three independent experiments with duplicate determinations were expressed as means ± SD. *P<0.05 was considered statistically significant. NS, control group; Lactate, lactic acid group.
group compared to that in the blank control group (P<0.05; Fig. 3C and D), suggesting that lactic acid may affect tumor immune escape through the PD-L1/PD-1 pathway, after redistributing M2-TAM subsets.

Effect of the lactic acid-induced redistribution of M2-TAM subsets on the biological behaviors of T cells. PD-L1 and PD-1 are negative costimulatory molecules that can induce apoptosis of specific cytotoxic T lymphocytes (CTLs) and reduce the sensitivity of tumors to the cytotoxic effect of CTLs, thereby assisting tumor immune escape. To determine whether M2-TAMs overexpressing PD-L1 following treatment with lactic acid affected the proliferation and apoptosis of T cells, T cells and macrophages were first co-cultured indirectly, then lactic acid/PBS was added to the co-culture (Fig. 4A). M2-TAMs in the lactic acid treatment group had a significantly lower ability to induce activation and proliferation of T cells than those co-cultured in the PBS control group (Fig. 4B). T-cell apoptosis in the co-cultured group was higher in the lactic acid treatment group than that in the PBS control group (Fig. 4C; Table I), suggesting that M2-TAMs reduce the cytotoxic effect of T cells.

HIF-1α signaling pathway may be the key regulatory mechanism. To determine whether the HIF-1α signaling pathway was involved in the lactic acid-induced redistribution of M2-TAM subsets and PD-L1 overexpression, a western blot was used to detect HIF-1α protein expression in THP-1 macrophages treated with lactic acid for 48 h. HIF-1α expression was significantly increased in the lactic acid-treated group as compared to that in the blank control group (Fig. 5A and D). This suggests that HIF-1α plays a regulatory role in the redistribution of M2-TAM subsets and PD-L1 overexpression. Successful transfection with HIF-1α siRNA was verified using western blotting (Fig. 5B and E). To further verify the effect of HIF-1α on M2-TAM subsets and PD-L1 overexpression, we pre-treated macrophages with HIF-1α siRNA and treated them with lactic acid (Fig. 5C and F). There was no significant difference in the distribution of M2-TAM subsets and PD-L1 expression between the HIF-1α siRNA-treated group and the

Figure 3. PD-L1 mRNA and protein expression in THP-1 cells induced by lactic acid was evaluated by RT-PCR and western blotting. (A and B) The mRNA expression and quantification of PD-L1 in THP-1 cells induced by lactic acid was evaluated by RT-PCR. (C and D) The protein expression and quantification of PD-L1 in THP-1 cells induced by lactic acid was evaluated by western blotting. Data from at least three independent experiments with duplicate determinations are expressed as means ± SD. *P<0.05, statistically significant. NS, control group; Lactate, lactic acid group.
control group (Fig. 5G; Table II). These data indicated that the HIF-1α signaling pathway participates to some extent in the lactic acid-induced redistribution of M₂-TAM subsets and PD-L1 overexpression.

**Redistribution of M₂-TAM subsets and effects on T cell apoptosis after the co-culture of pancreatic cancer cells, macrophages, and T cells.** To clarify whether lactic acid released by pancreatic cancer cells redistributes M₂-TAM subsets and regulates immune function, we first detected the lactic acid concentration in the supernatant after the co-culturing of pancreatic cancer cells, macrophages, and T cells using a direct or indirect triple-cell culture model (Fig. 6A). Compared with the normal pancreatic cell culture group, the lactic acid concentration in the pancreatic cancer cell co-culture group significantly increased (P<0.05; Fig. 6D). To determine whether lactic acid released by pancreatic cancer cells redistributed M₂-TAMs, the co-cultured cells were collected to detect CD163/CD68 mRNA expression. RT-PCR results showed that the ratio of CD163/CD68 mRNA was significantly upregulated in the pancreatic cancer cell co-culture group compared with the control group (Fig. 6C).
that in the normal pancreatic cell culture group (P<0.05) (Fig. 6E and G). Similarly, RT-PCR results showed that PD-L1 mRNA expression was significantly upregulated in the pancreatic cancer cell co-culture group as compared to that in the normal pancreatic cell culture group (P<0.05) (Fig. 6B and C). Compared with the normal pancreatic cell culture group, the pancreatic cancer cell co-culture group significantly induced T cell apoptosis (P<0.05; Fig. 6H; Table III). The results of the triple-cell culture suggested that lactic acid released by pancreatic cancer cells can redistribute M2-TAM subsets and regulate immune escape.

**Discussion**

Tumor cells consist of an acidic microenvironment (2). Lactic acid, a glycolytic metabolite, is believed to be a key factor in
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redistributing macrophage polarization in the tumor microenvironment (3). Previously, it was shown that a high-lactic acid environment in pancreatic cancer tissue samples is consistent with the distribution of M2-TAM subsets (7). The results of this in vitro experiment confirmed that lactic acid could redistribute M2-TAM subsets (7), which is consistent with the results of the current study. This suggests that lactic acid may be an initiating factor for M2-TAMs to promote tumor progression by their transformation from a ‘good’ macrophage into a ‘bad’ macrophage.

The development and progression of solid tumors are immune regulation processes (12). Various factors in the tumor microenvironment interact with each other, interfering with the balance of the immune system, resulting in the immune escape of tumors (13). According to previous findings, macrophages act as checkpoints by determining whether to initiate an immune response or induce immune tolerance, thus, serving as a bridge between the immune state and tumor evolution (4). The mechanism underlying the M2-TAM modulation of immune escape has not yet been fully elucidated, and previous findings have shown that PD-L1 may be involved in this process (9). PD-L1 belongs to the B7 family and contains IgV-like, IgC-like, and transmembrane regions, and a cytoplasmic tail (14). The latter is involved in intracellular signal transduction, while the IgV and IgC regions are involved in intercellular signal transduction. The binding of PD-L1 to PD-1 on T cells promotes the phosphorylation of tyrosine in the

Table III. T-cell apoptosis was higher in the pancreatic cancer cell co-culture group compared with the normal pancreatic cell culture group (mean ± SD).

| Group         | TUNEL-positive cells (apoptotic index) (%) |
|---------------|--------------------------------------------|
| Normal cell   | 1.07±0.62                                   |
| Cancer cell   | 7.00±0.52<sup>a</sup>                      |

<sup>a</sup>P<0.05 vs. normal cell group.

Figure 6. Redistribution of M2-TAM subsets and effects on T-cell apoptosis after the triple-cell indirect culture model of pancreatic cancer cells, macrophages, and T cells. (A) Schematic representation of the technique of the triple-cell culture model. (B and C) RT-PCR analysis shows that the PD-L1 mRNA expression in THP-1 cells in the pancreatic cancer cell co-culture group was significantly upregulated compared to that in the normal pancreatic cell co-culture group. (D) ELISA results showed that lactate concentration in the pancreatic cancer cell co-culture group was significantly upregulated compared with that in the normal pancreatic cell co-culture group. (E-G) RT-PCR show that the CD163/CD68 mRNA expression of THP-1 in the pancreatic cancer cell co-culture group was significantly upregulated compared to that in the normal pancreatic cell co-culture group. (H) TUNEL assays show that T-cell apoptosis was higher in the pancreatic cancer cell co-culture group than in the normal pancreatic cell co-culture group. Data from at least three independent experiments with duplicate determinations are expressed as means ± SD. *P<0.05 was considered statistically significant.
ITSM domain of PD-1, causes the dephosphorylation of downstream Syk kinase and phosphoinositide 3-kinase, inhibits the activation of downstream AKT, extracellular signal-activated kinase, and other pathways, and ultimately inhibits the transcription and translation of the genes and cytokines required for T-cell activation (14). These activities play a role in negatively regulating T-cell activity. For example, Dong et al (15), based on the results of an in vitro experiment utilizing mouse models, reported that activation of the PD-L1/PD-1 signaling pathway may induce the specific apoptosis of CTLs, which reduces the sensitivity of the CTL cytotoxic killing effect and promotes the immune escape of tumor cells. Zhou et al (16), found that in patients with liver cancer, TAM also inhibits functions of tumor-infiltrating lymphocytes through the highly expressed PD-L1 on its surface, leading to the escape of tumor cells from immune cell attack. Consistent with this finding, we observed that the high expression of PD-L1 in the TAM of pancreatic cancer was consistent with CD163. Moreover, correlation analysis of clinical factors revealed that the overexpression of PD-L1 in TAM was positively associated with lymph node metastasis and tumor staging. In the analysis of prognostic factors of pancreatic cancer, PD-L1 in TAM may serve as an independent risk factor. Patients with PD-L1 overexpression had a significantly worse prognosis than patients with low expression, suggesting that the PD-L1/PD-1 signaling pathway is involved in the mechanism underlying the M2-TAM regulation of tumor immune escape (17). However, results of this study still need to be verified through relevant in vitro experiments.

The abovementioned results show that modulation of lactic acid level may initiate the polarization of macrophages. Previous findings regarding this mechanism have reported that lactic acid is absorbed and oxidized by macrophage mitochondria through MCT1 into pyruvate (18). The latter inhibits prolyl hydroxylase domain (PHD) activity through direct competition with the PHD substrate, leading to the stable accumulation of HIF-1α, and subsequently, inducing the transformation of the M2-TAM subsets (19). In the present study, we found that the expression of HIF-1α protein significantly increased after the treatment of THP-1 macrophages with lactic acid. There was no significant difference in the distribution of M2-TAM cell subsets and PD-L1 expression after macrophages were pretreated with HIF-1α siRNA then treated with lactic acid. This indicates that the HIF-1α signaling pathway participates to some extent in the redistributing of M2-TAM subsets by lactic acid and PD-L1 overexpression, which is consistent with the finding that HIF-1α is involved in the redistribution of M2-TAM subsets and regulation of the immune function. Moreover, we used a triple-cell culture model consisting of pancreatic cancer cells, macrophages, and T cells to simulate the real microenvironment of pancreatic cancer, and again verified that lactic acid released by glycolysis in pancreatic cancer cells could redistribute M2-TAMs and further regulate PD-L1/PD-1 signals to mediate T-cell apoptosis, thereby assisting immune escape. Results of the present study demonstrated the significance of the involvement of lactic acid, a key metabolite, in tumor progression. siRNA is an important tool for genetic analysis of mammalian cells. siRNA-induced gene silencing in mammalian cells can effectively inhibit the expression of specific genes. In this experiment, single siRNA of HIF-1α corresponding to target genes were prepared and detected. Further research with more siRNAs of HIF-1α may be useful to obtain more reliable results.

There are some limitations to our study. First, lactic acid alone (without macrophage co-culture) may affect T-cell proliferation and apoptosis, and lactic acid alone was not applied as a control group to exclude this bias. Second, we performed TUNEL assays to access T-cell apoptosis. However, the results could be further verified if other methods such as annexin-V staining could also be used. Third, we did not evaluate the mechanism for reshaping and immune function of M2-TAM subsets. This remains to be studied in further experiments.

In conclusion, this in vitro experiment further explored the hypothesis that tumor cells use lactic acid, a glycolysis product, as a weapon to activate M2-TAMs via the bystander effect and, thus, further regulate PD-L1/PD-1 signal-mediated immune escape. These results suggest that lactic acid, macrophages, and PD-L1 are intrinsically linked, and provide an important theoretical basis for the treatment of pancreatic cancer.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Authors' contributions
TS, SC and XC conceived and designed the experiments. TS, SC, TW and WL performed the experiments. XC, SL, JZ and YY analyzed the data. JM, WL, XC, WL and YK contributed to the conducting the experiments. TS wrote the paper and XC, WL and YK revised the article. All authors approved the final version of the manuscript.

Ethics approval and consent to participate
Ethics approval was obtained from the Human Subjects Committee of the Xi'an Jiaotong University, China. All the patients in the study provided signed informed consent.

Patient consent for publication
Not applicable.
Competing interests

The authors declare that they have no competing interests.

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