Single Cell Proteomics Profiling Reveals That Embryo-Secreted TNF-α Plays a Critical Role During Embryo Implantation to the Endometrium

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Received: 21 January 2021 / Accepted: 14 December 2021 / Published online: 27 January 2022
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Abstract
It has been long-known that endometrium-secreted cytokines play a critical role during embryo implantation. However, whether cytokines secreted from the embryo are relevant to the process of embryo implantation remains unclear. The concentration of cytokines in embryo culture medium was tested using a newly developed, high-sensitivity single-cell proteomic platform and evaluated in comparison to embryo quality and clinical outcome. The effect of TNF-α on embryo and endometrium Ishikawa cells was investigated using immunofluorescence staining, CCK-8 assay, TUNEL staining, and RT-qPCR. Of the 10 cytokines measured, only TNF-α concentration was significantly higher in the group with embryo implantation failure. Immunofluorescence staining showed that the expression of TNF-α was unevenly distributed in blastocysts, and the expression level was significantly correlated with the blastocyst inner cell mass (ICM) quality score. Gene profiling showed that addition of TNF-α led to increased expression of tumor necrosis factor receptor 1 (TNFR1) and apoptosis-related genes and that this could be inhibited by the TNF-α receptor inhibitor etanercept (ETA). In addition, an increased expression of water and ion channels, including AQP3, CFTR, ENaCA, and CRISP2 was also observed which could also be inhibited by ETA. Our results show that higher embryo-secreted TNF-α levels are associated with implantation failure through activation of TNF-α receptor, and TNF-α may be an independent predictor for pre-transfer assessment of the embryo development potential in IVF patients.

Keywords Culture medium · Embryo quality · TNF-α · IVF outcome

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Background

Embryo implantation is a highly complex process orchestrated through close interaction between the embryo and endometrium. During this process, the invasion of the embryo is accompanied by the release of cytokines, free DNA, and metabolites, which constitute the major molecular determinants critical for embryo invasion [1, 2]. Cytokine release, especially TNF-α secreted from the endometrium, has long been shown to be critical to embryo invasion as is essentially a highly controlled inflammation process [3]. A recent study has shown that embryos can also actively express immune molecules during development that play roles in the interaction with the endometrium. Using a mouse embryo activation model, He et al. found that several cytokines, including TNF-α, are involved in blastocyst activation and play roles in X-chromosome reactivation [4]. Dysregulation of these factors could therefore potentially disrupt normal interactions between the embryo and endometrium, leading to implantation failure.

Implantation failure is a major cause of unsuccessful in vitro fertilization (IVF) in the clinic [5]. Repeated implantation failure accounts for at least 50% of IVF failure cases [6]. The cause of embryo implantation failure can be a consequence of uterine, embryonic, and male factors [7]; therefore, it is vital to identify biomarkers which can be used for early detection of possible implantation complications.

Embryonic factors are also critical for proper embryo implantation process during in vitro culture in IVF. For example, it is well-known that embryos secrete a wide variety of proteins and other factors to guide successful implantation in the endometrium [8]. Although cytokines have been extensively studied and long used as biomarkers in many settings including for preterm labor and preeclampsia [9], the quantitative analysis of cytokines in embryo culture medium has always been a challenge due to low volume, low concentration, and possible contamination from exosomes and microvesicles in the medium itself. Recent advances in single molecule microfluidic analysis have greatly improved detection sensitivity; however, these techniques have yet to be applied to embryo implantation [10].

Single-cell analysis has revolutionized biomedical research [11, 12]. Single-cell transcriptome analysis, for example, has been well-established to decipher gene expression and epigenetic regulation in both gametes and pre-implantation embryos in mice and humans [11, 13]. Single-cell proteomics, on the other hand, has lagged behind due to additional technical challenges. Nevertheless, single-cell analysis of immune and other cells has proceeded very rapidly. We previously showed that single-cell analysis has great potential in immune cell subtyping and cancer cell heterogeneity analysis [3, 14]. However, until now, no study has been used for embryo quality analysis.

Ishikawa cell is a human endometrial cell line, which widely used to study embryo implantation in vitro [15–17]. Herein, we used single-cell proteomics to understand the role of cytokines in the relationship between embryo quality and implantation and used the Ishikawa cell line to study the effect of cytokines on the endometrium. We show that the cytokine levels in the embryo culture medium differ from those in the embryo. Furthermore, we demonstrate the important difference in TNF-α secretion between embryos of different quality. Using immunofluorescence staining, we found differences in the embryonic localization of TNF-α. In vitro cell culture model, TNF-α promotes endometrial cell apoptosis and changes in expression of water and centrifugal channels. These results demonstrate that TNF-α expressed in the embryo plays a critical role in the regulation of embryo implantation. Understanding this relationship has great potential in assessing the successful of embryo implantation during IVF.

Methods

Patients

Infertile couples undergoing in vitro fertilization-intracytoplasmic sperm injection (IVF-ICSI) treatment with fresh cleavage embryo transfer at our center during July, 2018, were included in this study. The indications for assisted reproductive technology (ART) intervention were tubal factor infertility, anovulation, endometriosis, male factor infertility, or unexplained. In cases of male subfertility, immunologic factors, or unexplained fertilization failure in a previous IVF cycle, we performed ICSI. No patient selection or exclusion criteria were used. Our study was approved by the ethics committee of the Reproduction & Women–Children Hospital of Chengdu University of Traditional Chinese Medicine, and all patients provided informed consent.

Human Ovarian Stimulation Protocols and Oocyte Retrieval

Conventional ovarian stimulation for IVF-ICSI was performed. Patients were treated with a standard gonadotropin stimulation protocol using a short-acting gonadotropin-releasing hormone agonist (GnRH-α, Ferring Pharmaceuticals, Saint Prex, Switzerland), and GnRH-α was administered at 0.1 mg/d from the middle luteal phase of the last menstrual cycle to desensitize the pituitary gland. With
the long-acting gonadotropin-releasing hormone agonist (GnRH-α; Diphereline, Ipsen, Paris, France), patients were administered 3.75 mg/d GnRH-α during the menstrual phase. For the antagonist protocol, patients were treated with 0.25 mg/d of gonadotropin-releasing hormone antagonist (GnRH-A; Cetrotide, Merck Serono, Darmstadt, Germany). Ovarian stimulation was initiated with doses of recombinant follicle-stimulating hormone (recombinant FSH, Gonal-f; Merck Serono) or human menopausal gonadotropin (Lixzon Pharmaceutical Group, Zuhai, China) varying from 150 to 300 IU/d according to the patient’s age and ovarian reserve. The dose of recombinant FSH and human menopausal gonadotropin was adjusted and individualized for each patient based on follicular growth. Ovarian stimulation was monitored by transvaginal ultrasound and measurement of plasma estradiol levels. An intramuscular injection of human chorionic gonadotropin (hCG, Lixzon) at 5000–10,000 U was administered during the night when follicles ≥ 18 mm in diameter accounted for more than half of those ≥ 14 mm, and the average estradiol level of the follicles ≥ 14 mm in diameter was not less than 200 pg/mL for the GnRH-α protocol. The same doses of hCG and time were administered for the GnRH-A protocol when either follicles ≥ 18 mm in diameter accounted for more than two or ≥ 17 mm in diameter accounted for more than three. Ultrasound-guided puncture was conducted for oocyte retrieval approximately 36 h later. All oocyte retrieval and embryo transplantation surgeries were performed by the same experienced surgeon, and embryos were scored as described by Gardner et al. [18]. In general, one embryo was transplanted in each instance, which was culture in vitro for 3 days. An intramuscular injection of progesterone (80 mg/d; Zhejiang Xiangju Pharmaceutical Co., Ltd, Taizhou, China) was administered for luteal support.

Single Cell Proteomic Cytokine Measurement

We collected culture medium on day three of embryo in vitro culture and measured cytokine levels. Each independent culture embryo can collect 20–30 μL of medium for detection. Twenty-three samples were used for detection. Clinical data showed that 9 cases were pregnancy and 14 cases were non pregnancy. The detailed methods of single cell proteomic assay have been described in a previous study [3, 19]. Briefly, Polydimethylsiloxane (PDMS) microchips with parallel microchannels (100 μm width) were assembled with a poly L-lysine-coated glass slide. Differentcapture antibodies (1.5 μL for each antibody) were injected into the individual microchannels for overnight coating to form antibody stripes for multiplexed protein detection. The microchannels were then washed with 1% BSA 3–5 times and blocked with 3% BSA for 10 min. After blocking, the PDMS microchip was removed, and the antibody-coated glass slide was washed with Dulbecco’s phosphate-buffered saline (DPBS), 50/50 DPBS/distilled (DI) water, and DI water sequentially before blowing dry with N2. Another PDMS microchip with parallel microchannels (100 μm width) was made hydrophilic by treating with O2 plasma for 2 min before being aligned perpendicularly with antibody stripes on the glass slide. The microchannels were blocked with 3% BSA for 1 h to reduce nonspecific adsorption. Each sample (1–2 μL) was injected into the individual microchannel and incubated overnight. Afterward, a detection antibody mixture and streptavidin-APC were added sequentially for 1 h and 0.5 h, respectively. The fluorescence results were scanned and analyzed with a GenePix 4300A scanner and its corresponding software (Molecular Devices, San Jose, CA, USA).

Embryo Immunofluorescence Stain Methods

The day five embryo was fixed with 4% PFA for 15 min at room temperature and 0.3% Triton X-100 in PBS was added for 20 min to permeabilize the cell membrane. Then, the embryo was incubated with primary antibodies for TNF-α (1:100, Protein Tech Inc., Rosemont, IL, USA) and CDX2 (1:100, Abcam, Inc., Cambridge, MA, USA) at 4 °C overnight. Subsequently, the embryo was incubated with Alexa Fluor 594-conjugated goat anti-mouse IgG (H+L), (1:1000, Invitrogen, Inc., Carlsbad, CA, USA) and Alexa Fluor 488-conjugated goat anti-rabbit IgG (H+L) (1:1000, Invitrogen, Inc.) for 60 min at room temperature in the dark, followed by mounting with antifade mounting medium with DAPI (Beyotime, Inc., Shanghai, China). The analysis was conducted using a confocal laser scanning microscope (Olympus FV1000, Olympus Corporation, Waltham, MA, USA) (Nikon N-STORM & A1, Nikon Corporation, Tokyo, Japan). The six embryos used for staining originated from the same volunteer.

Ishikawa Cell Culture

The human endometrial cancer cell line Ishikawa was purchased from Biovector NTCC (Beijing, China). Cells were cultured in Eagle’s minimum essential medium (EMEM; ATCC; Manassas, VA, USA) supplemented with 10% (v/v) FBS (Gibco; ThermoFisher Scientific, Inc., Waltham, MA, USA) and 100 nM penicillin/streptomycin in a 5% CO2 incubator at 37 °C.

Cell Counting Kit-8 Analysis

In each well of a 96-well plate, 1 × 10⁴ Ishikawa cells were cultured as attached monolayers overnight. Varying doses of TNF-α (R&D Systems, Minneapolis, MN, USA) and ETA were then added. After 48 h of treatment, photos were taken using an inverted microscope (Olympus IX71, Olympus.
Corporation), and cells were processed with the Cell Counting Kit-8 from Dojindo (Shanghai, China). Quantitative analysis was performed using an automatic microplate reader (Varioskan Flash, Fisher Scientific, Inc., Waltham, MA, USA). Each test had two replicates (n = 3).

**Apoptosis Measurement with TUNEL Staining**

Ishikawa cells were grown on 14 mm glass coverslips at a density of 2 × 10^5 cells per well in a 24-well plate. Ten nanograms per milliliter TNF-α and 25 μg/mL ETA were used to treat the cell line. After 48 h of treatment, the cells were analyzed with the TUNEL BrightGreen Apoptosis Detection Kit (Vazyme, Inc., Nanjing, China), followed by mounting with antifade mounting medium with DAPI (Beyotime, Inc.) and β-actin, forward, 5′- CAC ACT TCA GT -3′; LIF, forward, 5′- CCA ACG TGA CGG ACT TCC C -3′ and reverse, 5′- TAC ACG ACT ATG CGG TAC AGC -3′; CRYBB2, forward, 5′- CAA CTT CAC CGG GAA GAT G -3′ and reverse, 5′- CGG GGT ACT GTG AGC CAA C -3′, and β-actin, forward, 5′- CAT GTA CGT TGC TAT CCA GGC -3′ and reverse, 5′- CTC CTT AAT GTC ACG CAC GAT -3′. The following PCR conditions were used for PCR: initial denaturation for 1 min at 95 °C; 40 cycles of 95 °C for 10 s and 60 °C for 20 s.

**Statistical Analysis**

All results are presented as the mean ± standard error of the mean. Statistical analysis was performed using GraphPad Prism 7 Project software (GraphPad Software Inc., San Diego, CA, USA). Quantitative analysis was performed using Image-Pro Plus 6.0 software (Media Cybernetics, Rockville, MD, USA). Differences were analyzed using one-way analysis of variance followed by the Newman-Keuls post hoc test. P < 0.05 was considered to indicate a statistically significant difference.

**Results**

**TNF-α Is Significantly Increased in the Non-Pregnancy Group**

The clinical characteristics of the groups recruited in this study are shown in Table 1. The median age of the non-pregnancy group was older than that of the live birth group (P < 0.05), and the duration of infertility was higher in the non-pregnancy group. The other parameters showed no significant difference. In this study, we investigated

| Characteristic | Outcome | Nonpregnancy | Pregnancy |
|---------------|---------|--------------|------------|
| Age (year), median | 36.5 | 28 |
| Duration of infertility, median (IQR) | 7.0 (4–11.25) | 4.0 (2.75–4.5) |
| Primary infertility | 6 (50) | 6 (66.67) |
| Cause of infertility | 6 (50) | 1 (0.11) |
| > 1 cause | 4 (33.33) | 6 (66.67) |
| Anovulatory | 0 | 0 |
| Tubal | 0 | 0 |
| Male factor | 2 (0.17) | 2 (22.22) |
| Endometriosis | 0 | 0 |
| Unexplained | 0 | 0 |
the major Th1/Th2-related cytokines from the spent IVF medium. We analyzed 10 cytokines — IL-1β, IL-6, IL-8, IL-10, MCP1, MIP-1α, MIP-1β, RANTES, GM-CSF, and TNF-α. In the implantation failure group, only TNF-α levels were significantly increased compared with those in the control group (Fig. 1A–J). The other cytokines showed no significant difference between implantation and implantation failure groups, indicating that TNF-α is the most significantly affected cytokine in comparing these two conditions. The TNF-α ROC showed that the area under the curve was 0.8095 (Fig. 1K). Interestingly, the association analysis indicated that the level of TNF-α is significantly associated with age (Fig. 1L). Therefore, our results indicate that advanced age could contribute to higher TNF-α concentration in

Fig. 1 A–J Ten cytokines in human embryo culture medium from IVF center were tested by the single cell cytokine array. **P < 0.01. Not all samples had signals at the time of testing. K The ROC curve of TNF-α. L Correlation analysis of age and TNF-α expression in culture medium

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embryo culture medium, and higher TNF-α may be relevant to the implantation failure of the embryo.

In addition, we analyzed the relationship between embryo quality and cytokine expression in embryo culture medium (supplementary Fig. 4, Good: good quality embryo, n = 9; Non: non quality embryo, n = 14). Only MCP1 levels were increased compared with control group \((**P < 0.05)\). However, there was no difference in the expression of TNF-α between the two groups. Just as embryo quality cannot predict embryo implantation clinically.

Immunofluorescence Staining of TNF-α Indicates Polarized Localization in Early Human Embryos

We found that TNF-α is the most significantly upregulated cytokine in the spent culture medium, which correlates with previous research indicating that TNF-α is vital to embryo implantation [20]. Considering that the embryo is attached to the endometrium in a specific orientation, we used immunofluorescence staining to detect whether TNF-α expression in the embryo exhibits a similar patterning.

Our results show that TNF-α is indeed expressed in both the blastocyst inner cell mass (ICM) and the trophectoderm (TM) of the embryo (Fig. 2A). Advanced, we found that low-quality embryos have a typically dispersed distribution (Supplementary Fig. 1). Clinical symptoms show that poor quality embryos are one of the major causes for implantation failure. According to our results, we found that the fluorescence intensity of stained TNF-α was negatively correlated with embryo quality factors, especially the ICM score (Fig. 2B and C). Further, the same results were obtained on embryos which from different volunteers (Supplementary Figs. 2 and 3). This indicates that TNF-α expression is correlated to embryo quality and may interfere with the implantation process.

TNF-α Treatment Leads to Inhibited Proliferation, Increased Apoptosis in the Ishikawa Cell Line

To further delineate whether increased TNF-α is detrimental for embryo implantation, we examined whether adding TNF-α could affect endometrium receptivity. Increased apoptosis and decreased proliferation are the
two significant features related to endometrium receptivity. We examined whether TNF-α affects apoptosis of the endometrium cells. Ishikawa cells were treated with 0, 1, 2, 5, 10, and 20 ng/mL of TNF-α independently for 48 h (Fig. 3C). Ten nanograms per milliliter TNF-α can cause cell damage, but not too much. We chose 10 ng/mL TNF-α for all subsequent experiments. Our results show that adding 10 ng/mL of TNF-α led to increased apoptosis in the Ishikawa cell line, which could be inhibited by ETA, a well-known inhibitor TNF-α inhibitor (Fig. 3A and B). After treatment with ETA, the cell proliferation was also significantly rescued compared to TNF-α treatment alone (Fig. 3C, D).

**TNF-α Treatment Could Activate TNF-α Receptor and Apoptosis Related Genes in Ishikawa Cell Line**

If excessive TNF-α is detrimental for endometrium receptivity through regulation of apoptosis and proliferation, it may elicit these changes through TNF-α receptor-mediated apoptosis in the endometrium. We next measured the expression of the TNF-α receptor TNFR1 as well as apoptosis-related

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**Figure 3**

A and B TUNEL staining assay measuring cell apoptosis after 48 h incubation. Ten nanograms per milliliter TNF-α and 25 μg/ml ETA were used in the test. Green, TUNEL stained nuclei of apoptosis cells; blue, all nuclei counterstained with DAPI. Magnification, x200.

***P < 0.001 vs. the control group, ##P < 0.01 vs. the TNF-α group.

C and D Add TNF-α lead to dose dependent inhibit cell proliferation in the Ishikawa cell after 48 h incubation, while adding ETA can recover the inhibition caused by TNF-α in the Ishikawa cell model, tested by CCK-8 assay. Each group was compared with the TNF-α (0 ng/ml) ETA (0 μg/ml) group, and that group was regarded as “1” for data statistics. Magnification, x200.
genes. Our results show that TNFR1 and Caspase-8 expression was significantly increased. In addition, water and ion channels, including AQP3, CFTR, ENaCA, and CRISP2 were significantly upregulated as well. These channels have important functions in regulating the endometrium, which has been shown to be critical for endometrium receptivity (Fig. 4). These results demonstrate that adding TNF-α leads to upregulation of ion channel gene expression. Other endometrium receptivity markers such as LIF also show increased expression upon the addition of TNF-α.

If TNF-α can be secreted from the embryo, can the addition of the TNF receptor inhibitor ETA attenuate the effect of secreted TNF-α on the endometrium and restore efficient embryo implantation? Our results show that ETA significantly inhibited gene expression and apoptosis in cells compared with the TNF-α-only group. This indicates that blocking TNFR1/2 can significantly inhibit the apoptosis caused by TNF-α (Fig. 3A and B). Together, these results show that excessive TNF-α is detrimental to endometrium receptivity through activation of the TNF-α receptor and downstream pathways.

Discussion

In our study, we have shown that TNF-α is secreted by the embryo, and its secretion is significantly increased in the embryo culture medium in embryo implantation failure patients compared with control. The higher levels of TNF-α in the spent medium from implantation failure embryos show that TNF-α levels are important determinants of implantation potential.

The source of TNF-α in the embryo is not entirely clear. TNF-α is either secreted from the embryo itself, or it can be expressed from the sperm or oocyte [21]. In our database, we found that one subgroup showed expression of TNF-α. However, there was no expression in the oocyte database. Therefore, the exact source of TNF-α remains to be investigated further. We did not find any expression in the embryo database; however, the expression of embryo-induced genes, including TNFR1, is increased. These results indicate that TNF-α expression in normal embryos is quite low. One interesting observation is that an association study showed a significant correlation between age and the amount of expressed TNF-α. This indicates that understanding the impact of age on embryo quality could help to further investigate the source of higher TNF-α secretion in implantation failure embryos.

In a previous study, it was shown that some cytokines, such as IL-8, show differential expression between implanted and non-implanted embryos [22]. However, the method used and the detection thread were low in this study. Although our method is semi-quantitative and cannot perform absolute quantification using the current method, our method can be applied to absolute quantification with a caliber with revision.

One interesting observation is that, in our study, some cytokines, such as GM-CSF and IL-1, showed increased expression in non-implanted embryos. GM-CSF is also important for embryo implantation, and recent observations have shown that uterine infusion of cytokines, including GM-CSF, showed successful outcomes in the treatment of repeated miscarriage patients [23]. Another potential implication is that recent studies have shown that cytokines are beneficial for the growth of poor-quality embryos. Taken together, this indicates that the exact mechanism of cytokine regulation of embryo implantation requires further investigation.
The non-invasive measurement of biomarkers shows great promise and is currently under intensive study. Examples of such biomarkers include proteins, nucleic acids, and other metabolites [24, 25]. These and other methods could also be used to predict the implantation potential of the embryo [6, 26]. It is hopeful that future studies would provide more reliable biomarkers during the IVF process.

How increased TNF-α contributes to implantation failure is not clear. One possibility is the increased TNF-α level could induce apoptosis through binding to its cognate receptor [26]. Second, increased secretion may be harmful to the endometrium. Other potential mechanisms also contribute to the secretion of TNF-α, such as a recent study which demonstrated that ATP is involved in the secretion of TNF-α from the membrane [8, 27]. Third, the downstream target of TNF-α warrants further clarification. Secretion of excessive TNF-α increased the expression of the pyroptosis marker, Caspase-8, which may indicate the possibility that excessive TNF-α leads to pyroptosis of the embryo and endometrium, leading to the demise of the embryo during implantation window [28]. Interestingly, our study showed that TNF-α could also increase the expression of other implantation markers such as LIF [29].

TNF-α inhibitors such as ETA have been widely used for the prevention of miscarriage. ETA has been widely used as a clinical TNF-α inhibitor in autoimmune diseases such as rheumatoid arthritis [30]. ETA showed excellent ability to scavenge TNF-α molecules in vitro as well as in vivo, and our previous work showed that it could activate the NF-κB signaling pathway in the ovary during PCOS pathogenesis [31]. The underlying mechanism of how ETA could promote embryo implantation requires further investigation. We believe the work presented here helps guide future work on both the underlying mechanism of cytokine function in endometrium receptivity and on the development of clinical interventions in IVF treatment.

**Conclusion**

In this study, we found that increased secretion of TNF-α is related to implantation failure, and we showed that blocking of the TNF-α pathway could be a potential effective prophylactic treatment for this condition.

**Abbreviations**  TNF-α: tumor necrosis factor alpha; ICM: blastocysts inner cell mass; TM: trophoderm of the embryo; ETA: etanercept; TNFR1: tumor necrosis factor receptor 1; TNFR2: tumor necrosis factor receptor 2; CFTR: cystic fibrosis transmembrane conductance regulator; ENaC: epithelial Na (+) channel subunit alpha; AQP3: aquaporin-3; CRISP2: cysteine-rich secretory protein 2; LIF: leukemia inhibitory factor; CRYBB2: beta-crystallin B2; TH1/2: helper T cells 1/2; IL-1β: interleukin-1 beta; IL-6: interleukin-6; IL-8: interleukin-8; IL-10: interleukin-10; MCP1: monocyte chemoattractant protein 1; MIP-1α: macrophage inflammatory protein 1-alpha; MIP-1β: macrophage inflammatory protein 1-beta; RANTES: C-C motif chemokine 5; T-cell-specific protein; GM-CSF: granulocyte-macrophage colony-stimulating factor; NF-κB: nuclear factor of kappa light polypeptide gene enhancer in B-cells 3; IVF: in vitro fertilization; ICs1: intracytoplasmic sperm injection; ART: assisted reproductive technology; ROC: receiver operating characteristic curve; PCOS: polycystic ovary syndrome

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s43032-021-00833-7.

**Acknowledgements** We thank AIVFO (Inc., Chengdu, China) for providing technical support. Especially, we grateful for the technical assistance provided by Zhang Jie and Bo Lin in Histology and Imaging Platform, Core Facilities of West China Hospital, Sichuan University.

**Author Contribution** WX and MZ developed the concept of the study; all authors contributed to data accumulation; JL and XS contributed to data analysis; all authors contributed to data interpretation. JL and WX wrote the manuscript. All authors contributed to revisions of the manuscript and approved the final submission. JL had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

**Funding** This work was supported by the National Key Research and Development Program of China (2018YFC1002804), Scientific and Translational Research Fund of Sichuan Provincial Department of Science and Technology (2017YSZH0032), the Key Research and Development Support Program of Chengdu (2019-YF05-00017-SN), and the Natural Sciences Foundation of China (81901435).

**Availability of Supporting Data** The dataset used and/or analyzed during the current study is available from the corresponding author upon reasonable request. Registry data are available publicly (see references).

**Declarations**

**Ethics Approval and Consent to Participate** The Second Affiliated Hospital of Chengdu University of Traditional Chinese Medicine Ethics Committee; approval number: 2017–01. Participants provided consent to participate in this study.

**Consent for Publication** Not applicable.

**Conflict of Interest** The authors declare no competing interests.

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