Expression of TRB3 promotes epithelial-mesenchymal transition of MLE-12 murine alveolar type II epithelial cells through the TGF-β1/Smad3 signaling pathway

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Abstract. The aim of the present study was to investigate whether the expression of tribbles pseudokinase 3 (TRB3) is involved in pulmonary interstitial fibrosis and to examine the possible mechanisms. The expression of TRB3 in murine alveolar type II epithelial cells (MLE-12 cells) following transforming growth factor \u03b31 (TGF-\u03b31) stimulation was assessed using various techniques, including western blot and reverse transcription-quantitative polymerase chain reaction assays. TRB3 overexpression and downregulation models were used to evaluate the impact of TRB3 on the TGF-\u00b31-induced epithelial-mesenchymal transition (EMT) of MLE-12 cells. The downregulation of TRB3 was induced by RNA interference. The expression of TRB3 was significantly increased in MLE-12 cells following the activation of TGF-β1 (P<0.05). The overexpression of TRB3 was found to promote activation of the TGF-β1/Smad3 signaling pathway, EMT, and the upregulated expression of β-catenin and EMT-related genes and proteins (P<0.05), whereas the downregulation of TRB3 attenuated the promoting effect on EMT induced by TGF-\beta1. In addition, the overexpression of TRB3 inhibited MLE-12 cell proliferation by stimulating apoptosis, leading to the formation of pulmonary fibrosis (PF). The positive feedback loop demonstrated that TGF-\u00b31 induced the expression of TRB3, and TRB3, in turn, stimulated EMT and promoted the onset of PF through activation of the TGF-\u00b31/Smad3 signaling pathway. Therefore, TRB3 may promote the formation of PF through the TGF-β1/Smad3 signaling pathway.

Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive, interstitial lung disease, with a poor prognosis and a mechanism of pathogenesis that remains to be elucidated. The median survival rate of patients with IPF is only 3-5 years, in which the main pathological feature is the abnormal deposition of extracellular matrix (ECM). The current therapeutic approaches for IPF are ineffective (1,2), as the pathogenesis of IPF remains to be fully elucidated. Multiple signaling pathways and various factors have been reported to have meaningful roles in pulmonary fibrosis (PF), among which transforming growth factor-\u03b31 (TGF-\u03b31) is considered a key factor during the onset of PF. TGF-\beta1 has been demonstrated to promote PF by enhancing the EMT of alveolar type II epithelial cells (3,4). The transcription factor Smad3 is an important downstream signaling factor of the TGF-\u00b31 signaling pathway, which is involved in various physiological and pathological processes. TGF-\beta1 binds to its specific receptor, stimulating Smad3-regulated and fibrosis-related gene transcription and initiating fibrosis. Tribbles pseudokinase 3 (TRB3) is one member of the mammalian kinase-like tribbles homologues belonging to the pseudokinase family. It is involved in regulating various signaling pathways, including mitogen-activated protein kinase, Notch, and Wnt, and in modulating various processes, including cell proliferation, differentiation, apoptosis, and carcinogenesis (5,6). TRB3 can react with Smad3 by enhancing the regulatory activity of Smad3-induced transcription and targeting gene expression, leading to activation of the TGF-\u03b31/Smad3 signaling pathway. Studies have indicated that the expression of TRB3 is upregulated in renal fibrosis, myocardial fibrosis and tumor tissue when its expression level positively correlated with the fibrosis; however, silencing TRB3 inhibits fibrosis (7-9). The role of TRB3 in PF remains to be fully elucidated. Therefore, in the present study, novel targets for IPF treatment were investigated by demonstrating the role of TRB3 in the pathogenesis of IPF.

Materials and methods

Adenovirus vector construction. Primers were designed based on the TRB3 sequence published by NCBI GenBank

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(https://www.ncbi.nlm.nih.gov/nuccore?db=nucleotide; ref. no. MGI:3562334). The target gene was amplified using TRB3 cDNA as a template, of which the product was constructed into a vector for the TRB3 recombinant plasmid (Ad-TRB3). The TRB3-specific short hairpin (sh)RNA was designed as follows: mmu-TRB3-small interfering RNA1 (5'-GAAGAA ACCGTTGGAGTTTG-3') was constructed for the recombinant plasmid Ad-TRB3-shRNA. The plasmids were cultured and purified, and adenovirus packaging was performed. The infectious titer of the recombinant adenovirus was evaluated using a TCID50 assay, which was 1.1x10¹¹ (PFU/ml).

Cell culturing. The MLE-12 murine alveolar type II epithelial cells, from Fuxiang Biotechnology Co., Ltd. (Shanghai, China) were cultured in a 5% CO_2 atmosphere at 37°C with saturated humidity.

Overexpression and downregulation of TRB3. The cells were seeded (the density was $1.2x10^6$ cells/ml) in a six-well plate and divided into five groups randomly: i) control group (Group C); ii) TGF- β 1 group (Group T); iii) TGF- β 1 + Ad-GFP group (Group T + G); iv) TGF- β 1 + Ad-TRB3 group (Group T + TRB3); and v) TGF- β 1 + Ad-TRB3-shRNA group (Group T + TRB3-shRNA). The cells were infected with the corresponding adenovirus vectors when at 60% confluency, MLE-12 cells in logarithmic growth phase were infected with multiple types of virus. Each virus was infected at three multiplicities of infection: 50, 100 and 200 for Ad-GFP; 200, 400 and 800 for Ad-TRB3; 100, 200 and 400 for Ad-TRB3-shRNA. The transduction time was 48 h. Subsequently, the cells were harvested, and total protein and RNA were extracted from the cells in each group.

Fluorogenic reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was extracted from the MLE-12 cells using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Reverse transcription was performed using the PrimeScript RT reagent kit (Takara Bio, Inc., Otsu, Japan) and incubated at 37°C for 15 min and at 85°C for 5 sec. The target genes were then amplified using the SYBR Premix Ex Taq kit (Takara Bio, Inc.). Thermocycling conditions were as follows: Initial denaturation at 94°C for 4 min, followed by 40 cycles of 94°C for 30 sec, 58°C for 30 sec and 72°C for 30 sec. The results were analyzed using the software of the ABI 7500 system (version 2.0.6; Applied Biosystems; Thermo Fisher Scientific, Inc.). The primers for target genes are listed in Table I. β-actin was used as an internal control, and values for each target gene were normalized using the expression level of β -actin (10).

Western blot analysis. Total cell protein was extracted using cell lysis buffer (Beyotime Institute of Biotechnology, Haimen, China), according to the manufacturer's protocol. A total of 5 μ g protein was loaded in each lane. SDS-PAGE (10%) was performed for each group, and the proteins were transferred from the gel onto PVDF membranes. Following blocking for 2 h using 1X Bovine lacto transfer technique optimizer (Thermo Fisher Scientific, Inc.), the membranes were incubated with anti-TRB3 (cat. no. sc-390242; Santa Cruz Biotechnology

Inc., Dallas, TX, USA), anti-phosphorylated (p-)Smad3 (cat. no. 9523S; Cell Signaling Technology, Inc., Danvers, MA, USA), anti-E-cadherin (cat. no. wl01482; Wanleibio Co., Ltd., Shanghai, China), anti-Vimentin (cat. no. wl00742; Wanleibio Co., Ltd.), anti-Fibronectin (cat. no. sc-69681; Santa Cruz Biotechnology, Inc.) and anti-GAPDH (cat. no. sc-47724; Santa Cruz Biotechnology, Inc.) antibodies at 4°C, overnight. The concentration of each primary antibody was 1:1,000. Subsequently, the membranes were incubated with secondary antibody labeled with horseradish peroxidase (1:10,000; cat. no. sc-2004; Santa Cruz Biotechnology, Inc.) at room temperature for 1.5 h. Following incubation with the secondary antibody, all membranes were washed, incubated in the Western Lightningä Chemiluminescence reagent (PerkinElmer, Inc., Waltham, MA USA), and photographic films were exposed to the membrane in a darkroom. Finally, images of the membranes were captured using the gel imaging system from LabWorksä 4.5 (UVP, LLC, Phoenix, AZ, USA), and the band brightness of each group was calculated.

Immunofluorescence (IF). The cells were seeded onto coverslips and fixed using paraformaldehyde. Following this, rabbit TRB3 antibody (1:1,000; sc-390242; Santa Cruz Biotechnology, Inc.) was used as the primary antibody and was incubated at 4°C for 24 h. Donkey anti-rabbit immunoglobulin G labeled with FITC (1:1,000; cat. no. sab4600003; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was used as the secondary antibody and was incubated at 37° for 1.5 h. The nucleus was stained with DAPI (Sigma; Merck KGaA). Finally, the coverslip was placed onto the object slide, examined under a confocal laser scanning microscope (CLSM, FV1000, Olympus Corporation, Tokyo, Japan), and images were captured.

Flow cytometry. The MLE-12 cells were plated into a six-well plate and infected according to the grouping. The cells were harvested and resuspended 48 h after infection and stained following the protocols of the ApoScreen Annexin V Apoptosis kit (SouthernBiotech, Birmingham, AL, USA) and Propidium Iodide kit (Roche Diagnostics, Basel, Switzerland). Following staining for 1 h, the cells were examined and analyzed using CellQuest software (version 6.0; Becton, Dickinson and Company, Franklin Lakes, NJ, USA).

Cell Counting Kit-8 (CCK-8). The MLE-12 cells were cultured in 96-well plates and infected according to the grouping. After 48 h, 10 μ l CCK-8 solution was pipetted into each well, and the incubation was continued for 2 h. The absorbance was measured at 450 nm using a microplate reader. The OD value was in proportion to the living cell number.

Statistical analysis. Using data from the present study, a database was established using SPSS19.0 (IBM Corp., Armonk, NY, USA). All data were consistent with normal distribution and are presented as the mean \pm standard deviation. Differences between two means were compared using a t-test, while that among multiple groups using analysis of

Table I. Primers for each murine gene.

Gene name	Sequence (5'-3')	Product length (bp)
CollaI forward	GAGACAGGCGAACAAGGTGA	399
CollaI reverse	CTCAAGGTCACGGTCACGAA	
α -SMA forward	GTACCCAGGCATTGCTGACA	271
α-SMA reverse	GAGGCGCTGATCCACAAAAC	
TRB3 forward	GGAACCTTCAGAGCGACTT	341
TRB3 reverse	TGGCACTCAGGGAGCATC	
β-actin forward	CCACCATGTACCCAGGCATT	189
β-actin reverse	CGGACTCATCGTACTCCTGC	

 α -SMA, α -smooth muscle actin; CollaI, collagen type I.

Table II. mRNA and protein expression levels of TRB3 in each group.

Group	TRB3mRNA/ (2 ^{-ΔΔCq})	TRB3 protein/ (TRB3/GAPDH)
C	1.0000±0.0070	1.0000±0.0120
Т	2.0250±0.0750ª	1.6820±0.2110 ^a
T+G	1.5400±0.1500 ^b	1.6520±0.0310 ^b
T+TRB3	4.1250±0.3150°	2.8520±0.2110°
T+TRB3-shRNA	0.0190±0.0010°	0.3220±0.0020°

GAPDH served as a loading control. Results are presented as the fold of the control mean \pm standard deviation. ^aP<0.05 compared with Group C; ^bP>0.05 compared with Group T; ^cP<0.05 compared with Group T+G. C, control; T/TRB3, tribbles pseudokinase 3; G, GFP; shRNA, short hairpin RNA.

variance. P<0.05 was considered to indicate a statistically significant difference.

Results

mRNA and protein expression levels of TRB3 increase in TGF- β 1-stimulated MLE-12 cells. The mRNA and protein expression levels of TRB3 were low in normal MLE-12 cells but were significantly higher in the TGF- β 1-stimulated cells, particularly in Group T + TRB3. When TRB3-shRNA was added, the mRNA and protein levels of TRB3 were the lowest (P<0.05; Table II). Furthermore, the IF assay suggested that the expression of TRB3 in the nucleus was increased in the TGF- β 1-stimulated MLE-12 cells (Fig. 1).

TRB3 stimulates the activation of the TGF- β 1/Smad3 signaling pathway. The protein expression level of p-Smad3 in the MLE-12 cells was assessed when TRB3 was upregulated or downregulated, respectively (P<0.05). The results demonstrated that, when TRB3 was overexpressed, the protein expression of p-Smad3 increased (P<0.05). By contrast, the

Table III. Protein expression level of p-Smad3 in each group.

Group	p-Smad3/GAPDH	t	
С	1.0000±0.0112		
Т	1.7830±0.0085	55.81ª	
T+G	1.7610 ± 0.0048	2.239 ^b	
T+TRB3	3.8550±0.0293	71.24°	
T+TRB3-shRNA	0.5481±0.0289	41.49°	

GAPDH served as a loading control. Results are presented as the fold of the control mean ± standard deviation. ^aP<0.05, compared with Group C; ^bP>0.05 compared with Group T; ^cP<0.05 compared with Group T+G. p-Smad3, phosphorylated Smad; C, control; T/TRB3, tribbles pseudokinase 3; G, GFP; shRNA, short hairpin RNA.



Figure 1. TRB3 is increased in TGF- β 1-stimulated MLE-12 cells. GAPDH served as a loading control. The cells were stained with anti-TRB3. (A) Group T, after pure TGF- β 1 stimulation. The immunofluorescence showed an increase in the number of TRB3-positive cells in the nuclei of the MLE-12 cells. (B) Group C, GAPDH (control). The TRB3-positive cells decreased. (C) Group T + TRB3, after transfecting TGF- β 1 with TRB3 gene virus to stimulate the MLE-12 cells. The number of TRB3-positive cells in the nuclei increased significantly. (D) Group T + TRB3 shRNA, after transfecting TGF- β 1 with TRB3 silenced gene virus to stimulate the MLE-12 cells. The TRB3-positive cells in the nuclei ncreased significantly. (D) Group T + TRB3 shRNA, after transfecting TGF- β 1 with TRB3 silenced gene virus to stimulate the MLE-12 cells. The TRB3-positive cells in the nuclei decreased. There was upregulation of TRB3 staining in the nucleus of MLE-12 cells (TGF- β 1) when compared with the control (magnification, x200). TRB3, tribbles pseudokinase 3; TGF- β 1, transforming growth factor- β 1.

downregulation of TRB3 led to decreased protein expression of p-Smad3 (P<0.05; Table III).

Overexpression of TRB3 promotes the EMT of TGF- β 1stimulated MLE-12 cells, which is inhibited by the downregulation of TRB3. Further investigation found that, compared with the TGF- β 1 stimulation group, the overexpression of TRB3 enhanced the mRNA expression levels of α -smooth muscle actin (α -SMA) and collagen type I (CollaI), two fibroblast markers, in MLE-12 cells (P<0.05; Table IV). In addition, the results of the western blot analysis indicated that the expression of E-cadherin (epithelial marker) decreased,

Group	CollaI	t	α-SMA	t
С	1.0100±0.0130		1.0025±0.0007	
Т	2.2700±0.2300	5.522ª	2.4700±0.3900	17.911ª
T+G	1.2400 ± 0.1700	3.601 ^b	2.3300±0.2500	0.302 ^b
T+TRB3	6.2301±0.7300	6.657°	6.2500±0.2500	11.092°
T+TRB3-shRNA	0.2450 ± 0.0450	5.658°	0.4800 ± 0.1200	6.671°

Table IV. mRNA expression of α -SMA and CollaI in MLE-12 cells in each group.

Results are presented as $2^{-\Delta\Delta Cq}$ mean ± standard deviation. ^aP<0.05 compared with Group C; ^bP>0.05 compared with Group T; ^cP<0.05 compared with Group T+G. α -SMA, α -smooth muscle actin; Colla I, collagen type I; C, control; T/TRB3, tribbles pseudokinase 3; G, GFP; shRNA, short hairpin RNA.



Figure 2. Overexpression of TRB3 promotes EMT and silencing TRB3 ameliorates EMT of MLE-12 cells. The western blot gels respectively show the expression of E-cadherin protein, Vimentin protein and Fibronectin protein. GAPDH served as a loading control. The results are presented as the fold of the control. EMT, epithelial-mesenchymal transition; TRB3, tribbles pseudokinase 3; TGF- β 1/T, transforming growth factor- β 1; shRNA, short hairpin RNA.

whereas the expression levels of Vimentin and Fibronectin (mesenchymal markers) increased (P<0.05; Table V; Fig. 2). When the expression of TRB3 was downregulated by RNA interference, the mRNA expression levels of α -SMA and CollaI were also downregulated (P<0.05; Table IV). Furthermore, the expression of E-cadherin increased, and the expression levels of Vimentin and Fibronectin decreased (P<0.05) (Table V; Fig. 2). Compared with Group C, the morphology of cells in Group T changed from polygonal or rectangular to spindle-shaped (Fig. 3A and B).

Overexpression of TRB3 reduces MLE-12 cell proliferation by promoting TGF- β 1-induced cellular apoptosis. The results of the flow cytometry and CCK assays demonstrated that, following TGF- β 1 stimulation, MLE-12 cell apoptosis increased (P<0.05) and proliferation decreased (P<0.05). The overexpression of TRB3 promoted the cell apoptosis induced by TGF- β 1, leading to the significant inhibition of cell proliferation. Furthermore, the downregulated expression of TRB3 reduced the cell apoptosis induced by TGF- β 1 (P<0.05), thereby attenuating the inhibitory effect of TGF- β 1 on cell proliferation (Fig. 4, Table VI).

Discussion

The main pathological feature of PF is the abnormal deposition of ECM. The dynamic process of ECM deposition, remodeling, and reabsorption regulates normal tissue growth, development and wound healing. It also maintains internal homeostasis, with malfunctioning processes giving rise to the pathological remodeling of tissues and organs, including tissue and organ fibrosis. Studies have indicated that during PF, alveolar type II epithelial cells trans-differentiate into fibroblasts and myofibroblasts through EMT (11-13) and secrete excessive collagen and other proteins, resulting in excessive ECM deposition (1). The TGF-β1/Smad3 signaling pathway is an important regulatory pathway of EMT. During PF, the expression of TGF-β1 increases, which inhibits alveolar epithelial cell proliferation by transducing signals through plasma membrane receptors, and it promotes EMT and the differentiation of fibroblasts into myofibroblasts, finally resulting in the stimulation of fibrosis. The expression of TRB3 has been found to be upregulated in tissues of various fibrotic diseases; the overexpression of TRB3 has also been demonstrated to stimulate the EMT of tumor cells and other types of epithelial cells (5,6,8,9). Silencing TRB3 alleviated collagen and fibronectin deposition in tissues with pathological fibrosis; however, the role of TRB3 in fibrotic lung tissue has remained unclear. In the present study, it was hypothesized that TRB3 promotes the EMT of mouse alveolar type II epithelial cells by interacting with the TGF-\beta1/Smad3 signaling pathway.

The results of the present study demonstrated that, following TGF-β1 stimulation, the mRNA and protein expression levels of TRB3 in the MLE-12 cells increased significantly. The expression levels of TRB3 were modulated using adenovirus vectors in cells. When TRB3 was overexpressed, TGF-β1/Smad3 signaling pathway activation was enhanced, p-Smad3 accumulation was induced, fibrosis-related gene transcription was promoted, and finally the EMT of MLE-12 cells and ECM deposition were stimulated. By contrast, the downregulation of TRB3 was demonstrated to inhibit TGF-β1/Smad3 signaling pathway activation, reduce the phosphorylation of Smad3, suppress the EMT of MLE-12 cells, and attenuate ECM deposition. In tumor tissue, a positive feedback loop was found, which facilitated the onset of EMT of tumor cells. On one hand, TRB3 was involved in maintaining the level of p-Smad3; on the other hand, it interacted with Smad3

Group	E-cadherin/GAPDH	t	Vimentin/GAPDH	t	Fibronectin/GAPDH	t
C	1.0000±0.0560		1.0000±0.0577		1.0000±0.0231	
Т	0.5258±0.0145	7.967ª	1.3050±0.0579	3.728ª	1.4060±0.1156	3.443ª
T+G	0.5624±0.1179	1.958 ^b	1.3640±0.0878	0.565 ^b	1.6270±0.0145	1.892 ^b
T+TRB3	0.2576±0.0043	24.272°	1.9410±0.0231	6.353°	3.2750±0.0380	40.572°
T+TRB3-shRNA	0.8910±0.0059	24.973°	0.8043±0.0579	5.326°	0.4606 ± 0.0580	74.581°

Table V. Protein expression levels of E-cadherin, Vimentin and Fibronectin in MLE-12 cells in each group, as measured by densitometry analysis.

GAPDH served as a loading control. Results are presented as the fold of the control mean ± standard deviation. ^aP<0.05, compared with Group C; ^bP>0.05, compared with Group T; ^cP<0.05 compared with Group T+G. T/TRB3, tribbles pseudokinase 3; G, GFP; shRNA, short hairpin RNA.

Table VI. Cell apoptosis and proliferation assessment in each group.

Group	Apoptotic rate	t	OD value	t
С	1.5730±0.0145		0.4387±0.0025	
Т	6.4872±0.1017	47.820ª	0.3537±0.0189	7.720ª
T+G	5.800±0.2930	2.214 ^b	0.3600 ± 0.0240	0.359 ^b
T+TRB3	10.3201±0.0727	14.963°	0.2340 ± 0.0102	8.369°
T+TRB3-shRNA	3.8172±0.0035	6.721°	0.4213±0.0114	3.997°

^aP<0.05 compared with Group C; ^bP>0.05 compared with Group T; ^cP<0.05 compared with Group T+G. T/TRB3, tribbles pseudokinase 3; G, GFP; shRNA, short hairpin RNA.



Figure 3. Morphological changes of cells in Group C and Group T(x200). (A) Cells in Group C were polygonal or rectangular (red arrows). (B) Morphology of cells in Group T stimulated with TGF- β 1 changed to spindle-shaped (white arrows). C, control; TGF- β 1/T, transforming growth factor- β 1.



Figure 4. Potential signal pathway. TRB3, tribbles pseudokinase 3; TGF- β 1/T, transforming growth factor- β 1; α -SMA, smooth muscle actin; CollaI, collagen type I; EMT, epithelial-mesenchymal transition.

directly. In turn, the overexpression of Smad3 promoted the transcription of TRB3 (5), which is consistent with the present results.

Alveolar type II epithelial cells are the dominant cells in lung tissue, which secrete multiple surface proteins and other components of the ECM. Injury or apoptosis of cells influence lung fibrosis, which may be the promoting factor of IPF onset (14-17). The present study demonstrated that the overexpression of TRB3 stimulated TGF- β 1-induced cell apoptosis, aggravating the inhibition of cell proliferation, whereas the downregulation of TRB3 resulted in reduced apoptosis, alleviating the inhibition of proliferation, which confirmed our hypothesis.

TGF- β 1 is a key factor that stimulates fibrosis not only through the classic TGF- β 1/Smad3 signaling pathway, but also in the redox reaction to modulate epithelial cell apoptosis, contributing to lung fibrosis (18,19). Several studies have indicated that in numerous diseases, including fibrosis, TRB3 stimulates the apoptosis of various cell types, including myocardial and renal tubular cells, through the p53 pathway, oxidative stress and endoplasmic reticulum stress (20-24), whereas silencing TRB3 led to significantly reduced cell apoptosis. Until now, the effect of TRB3 on alveolar type II epithelial cell apoptosis has not been evaluated. The present study demonstrated that the downregulation of TRB3 attenuated the inhibitory effect of TGF- β 1 on cell proliferation, as TRB3 can stimulate TGF- β 1-induced alveolar type II epithelial cell apoptosis.

Therefore, it was hypothesized that during the early stages of lung fibrosis, the expression of TRB3 is upregulated, which interacts with the TGF- β 1/Smad3 signaling pathway to promote EMT, inhibit the proliferation of alveolar type II epithelial cells by stimulating their apoptosis, and finally modulate the onset of fibrosis. Interfering with the expression of TRB3 can regulate the development of fibrosis by inhibiting alveolar type II epithelial cell apoptosis. Additional investigation is required to answer questions on additional components and elucidate more detailed mechanisms within the regulatory network of the development of fibrosis. Attention is also required on the difference between *in vitro* and *in vivo* studies.

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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

WY, WC and LM made substantial contributions to the conception and design of the present study. WY, WC and JT collected, analyzed and interpreted the data. WC and WY drafted the manuscript. WY critically revised the manuscript. WY has given final approval of the version to be published and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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