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Original Research Article

LncRNA PFAL suppresses TNF-α-induced inflammation by upregulating miR-18a in WI-38 cells

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Abstract

Purpose: Pneumonia is a serious respiratory disease among children with high mortality and morbidity all over the world. Long non-coding RNAs have been proven to play a vital role in many inflammatory diseases including pneumonia. In the present study, the protective impact of lncRNA PFAL on cell viability, cell apoptosis and secretion of inflammatory cytokines, as well as the underlying molecular mechanism in TNF-α-induced inflammatory injury model of pneumonia were investigated.
Methods: WI-38 cell line was treated with 20 ng/ml TNF-α to establish an inflammatory injury model of pneumonia. LncRNA PFAL or miR-18a was up- or down-regulated in the WI-38 cells by transfection procedure. Cell viability was assessed using CCK-8 assay, while the rate of cell apoptosis was measured by utilizing flow cytometry. The mRNA expression levels of lncRNA PFAL, miR-18a, apoptosis-related and JNK pathway genes were determined with RT-qPCR. Moreover, the production of inflammatory cytokines such as IL-6 and MCP-1 were detected by using Western blot analysis.
Results: The results indicated that cell viability was significantly ($P<0.05$) reduced, while the rate of cell apoptosis was increased in the TNF-α-induced WI-38 cells. Also, TNF-α treatment enhanced the expression of inflammatory cytokines that included IL-6 and MCP-1 in WI-38 cells. Overexpression of PFAL suppressed the injury induced by TNF-α and miR-18a was positively regulated by PFAL. Moreover, the inhibition of miR-18a weakens the effect of PFAL overexpression in TNF-α-induced cell injury. Furthermore, PFAL and miR-18a were involved in the regulation of JNK pathway.
Conclusion: Overexpression of PFAL suppresses TNF-α-induced WI-38 cell injury by up-regulating miR-18a via the inactivation of JNK signaling pathway.

Keywords: Inflammation, JNK pathway, miR-18a, PFAL, Pneumonia, TNF-α

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INTRODUCTION

Pneumonia is an acute respiratory disease which is the major cause of morbidity and mortality worldwide and is found particularly in children [1]. Mycoplasma, viruses and bacteria are the common pathogens of pneumonia among children [2]. The main symptoms of pneumonia include shortness of breath, fever, fixed wet lung sounds, chest pain and dry cough [3]. During acute attack of pneumonia, if not cured in time, it can cause severe injury to the respiratory and other systems in the body, leading to dysfunction of many organs [4]. Previous studies have revealed that unnecessary inflammatory and immune reactions might be significant causes of pneumonia [5]. In spite of several researches on the diagnosis and treatment of pneumonia, the underlying mechanism of pneumonia pathogenesis still remains unclear.

Long non-coding RNAs are the RNAs that are >200 nucleotides long and lack protein coding ability, which play a vital role in gene expression and RNA translation [6]. It has been extensively studied that dysregulation of lncRNAs is associated with many inflammation-related diseases, including lung cancer [7], osteoarthritis [8], and pneumonia [9]. A number of examples have shown the aberrant expressions of LncRNAs; such as lncRNA CRNDE [1], lncRNA GAS5 [11] and lncRNA NKILA [12]. A recent study revealed that overexpression of lncRNA PFAL enhanced cell proliferation and migration and promoted fibrogenesis and activation of fibroblast in both *in vivo* and *in vitro* models of lung fibrosis via regulating miR-18a expressions [13]. However, whether lncRNA PFAL exert its function in pathogenesis of pneumonia is still unknown.

MicroRNAs (miRNAs) are type of non-coding RNAs with about 22 nucleotides in length. They play an important role in the regulation of gene expression by binding to the 3'-UTR of their target genes and are involved in the various pathological processes including pneumonia [14]. Among these RNAs are miR-18a, which is a new emerging miRNA that plays a vital role in inflammatory diseases and several cancers [15]. A recent study revealed that the response of T17 in airway inflammation was significantly enhanced by the down-regulation of miR-18a expression *in vivo* [16]. Another study demonstrated that the expression of miR-18a was down-regulated during liver fibrosis *in vitro* and *in vivo* [13]. However, the underlying mechanism of miR-18a in the pathogenesis of pneumonia is yet to be examined. The present study was aimed at investigating the impacts of lncRNA PFAL on cell viability, cell apoptosis and secretion of inflammatory cytokines, as well as the underlying molecular mechanism in TNF-α-induced inflammatory injury model of pneumonia.

In the present study, a TNF-α-induced inflammatory injury of WI-38 cells in pneumonia was established and the impact of lncRNA PFAL and miR-18a on cell viability, cell apoptosis and secretion of inflammatory cytokines were detected by using CCK-8 assay, flow cytometry and Western blot analysis in an *in vitro* model of pneumonia. Moreover, the effect of lncRNA PFAL in the regulation of JNK signaling pathway was also investigated.

EXPERIMENTAL

Cell culture

WI-38 cell line was purchased from ATCC, VA, USA and cultured in a DMEM (Sigma Aldrich, MO, USA) with 10 % FBS and 5 % CO_2 at 37 ^oC. The culture of WI-38 cells were treated with TNF-α (20 ng/ml; Sigma Aldrich, MO, USA) to establish an inflammatory injury model [17].

Cell transfection

MiR-18a inhibitors and negative control (NC) were obtained from (GenePharma, Shanghai, China) and were transfected into WI-38 cells by using Lipofectamine 2000 (Thermo Fisher, NJ, USA). The full length of PFAL was inserted into pcDNA3.1 vector and named as pc-PFAL, and also transfected into WI-38 cells by using Lipofectamine 2000 according to instructions of the manufacturer.

CCK-8 assay

WI-38 cells ($1x10^3$ cells/well) were seeded in 96-well plates. After the cell reached 85 % confluence, CCK-8 solution was added to each well and incubated for 2 h with 5 % CO_2 at 37 ^0C. A microplate reader (LabX, ON, Canada) was used to measure absorbance at 450 nm.

Flow cytometry

Annexin V-FITC/PI kit (BioVision, CA, USA) was used to measure cell apoptosis. Cells were rinsed with PBS three times and collected with Trypsin (Biocompare, CA, USA). The cell suspension was mixed with 500 µl of 1X Binding Buffer followed by the addition of 5 µl of propidium iodide (PI) and 5 µl of Annexin V-FITC, followed by incubation for 5 min in the dark at room temperature. A microplate detector (LabX, ON, Canada) was used to measure the

Table 1: Forward and backward nucleotide sequences of RT-qPCR primers

Target gene	Direction	Nucleotide sequence
IL-6	Forward	5'-ATCCTTTGGAGGCAAGACAT-3'
	Backward	5'-TCCTGTTCCTTCTGGAGTTG-3'
MCP-1	Forward	5'-ATCCTTTGGAGGCAAGACAT-3'
	Backward	5'-TCCTGTTCCTTCTGGAGTTG-3'
Bax	Forward	5'-GGCCTGAGTCCAGCTCTTTA-3'
	Backward	5'- G TCCTGGAGACAGGGACATC-3'
Bcl-2	Forward	5'-CCTCGCTGCACAAATACTCC-3'
	Backward	5'-T GGAGAGAATGTTGGCGTCT -3'
β-actin	Forward	5'-TCAGGTCATCACTATCGG-3'
	Backward	5'-AAAGAAAGGGTGTAAAACGCA-3'

absorbance at 530 nm and the level of apoptosis was detected by using flow cytometry (Biocompare, CA, USA).

RT-qPCR

RT-qPCR kit protocol was followed to perform expression analysis. Total RNAs were extracted from WI-38 cells by using TRIzol reagent (Thermo Fisher, NJ, USA). The extracted total RNAs were reverse transcribed into cDNAs by using a cDNA reverse transcription kit (BIORAD, CA, USA) and EmeraldAmo PCR Master Mix Kit (TakaraBio, Goteburg, Sweden) was used to perform RT-qPCR. The expressions of miR-18a and PFAL were detected by SYBER Select Master Mix (SigmaAldrich, MO, USA) and the amplification was carried out on an ABI 7300-fast REAl Time PCR system (Thermo Fisher, NJ, USA). The primer sequences used were given in Table 1. GAPDH and U6 were considered as internal control genes for lncRNA and miRNA, respectively. Relative expressions were determined by using $2^{-\triangle\triangle Ct}$ method.

Western blot analysis

Total extraction sample kit (Intron Biotechnology, Gyeonggi, Korea) was used to extract total proteins and the proteins were quantified using BCA method (Thermo Fisher, NJ, USA). Concentrated and separation gels were prepared and 20 µL of protein sample was loaded into each well. Eighty (80) V was used for the electrophoresis and the set up was stopped when the mophenol blue reached the edge of the concentrated gel. Transfer onto nitrocellulose membranes (Thermo Fisher, NJ, USA) was conducted for 2 h at 3 mA followed by the blocking of the membranes with 5 % FBS (Biocompare, CA, USA) for 2 h at room temperature. The following primary antibodies which include: anti-IL-6 (ab6672; 1:500; Abcam, MA, USA) and anti-MCP-1 (ab21396; 1:5000; Abcam, MA, USA) were incubated at 4 ^{o}C overnight. These primary antibodies were then re-incubated with Goat Anti-Rabbit IgG H&L (HRP; ab205718; 1: 2000; Abcam, MA, USA) secondary antibody at room temperature for 1 h. After ECL chemiluminescence (Abcam, MA, USA) for 30 min, the protein content was analyzed by using Image-Pro Plus (Media Cybernetics, MD, USA).

Statistical analysis

SPSS 18.0 software (SPSS, IL, USA) was used to analyze the data collected and all the data were presented as mean ± standard deviation (SD). GraphPad Prism 7.0 (GraphPad Software, CA, USA) was used for statistical analysis. ANOVA was employed for comparison among different groups while student's t-test was utilized for comparison between two groups. P less than 0.05 was considered statistically significant.

RESULTS

The TNF-α inflammatory injury model was established

Cell viability and apoptosis were detected by using CCK-8 assay and flow cytometry, respectively. The results of cell viability and apoptosis using CCK-8 assay and flow cytometry, respectively (Figure 1A & B) demonstrated that cell viability was significantly reduced and the rate of apoptosis was enhanced in the TNF-α-induced WI-38 cells compared to that of the control group. Moreover, mRNA expressions of apoptotic-related genes such as Bax and Bcl-2 were measured by using RT-qPCR and the results showed that the expression of Bax was increased while that of Bcl-2 decreased in the TNF-α-induced WI-38 cells compared to that of the control group (Figure 1C). Furthermore, the level of inflammatory cytokines was assessed by using Western blot analysis and the results (Figure 1D) revealed that the level of inflammatory cytokines such as IL-6 and MCP-1 was significantly enhanced in TNF-α-induced WI-38 cells compared with control group. These results
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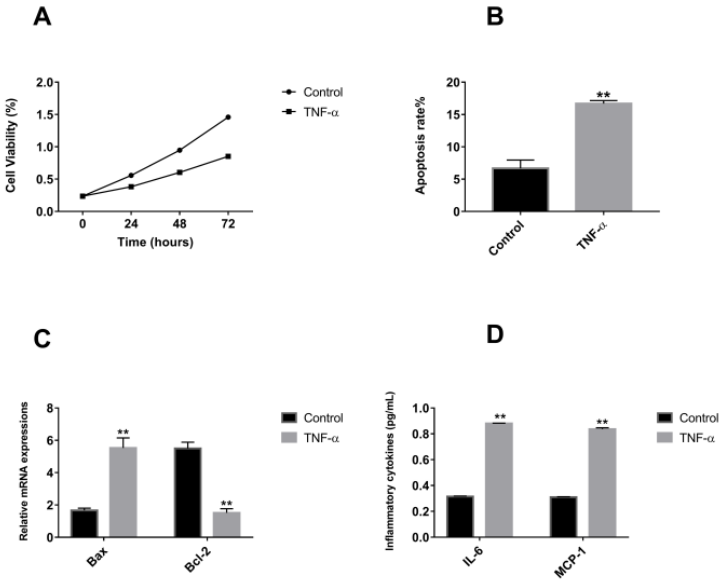

Figure 1: TNF-α-induced inflammatory injury in WI-38 cells. *(A) CCK-8 assay was used to measure cell viability in TNF-α-induced WI-38 cells. (B) Apoptotic rate was determined by using flow cytometry in TNF-α-induced WI-38 cells. (C) RT-qPCR was utilized to measure mRNA expression level of apoptotic-related genes Bax and Bcl-2. (D) Level of inflammatory cytokines were determined by using Western blot analysis. P < 0.05*

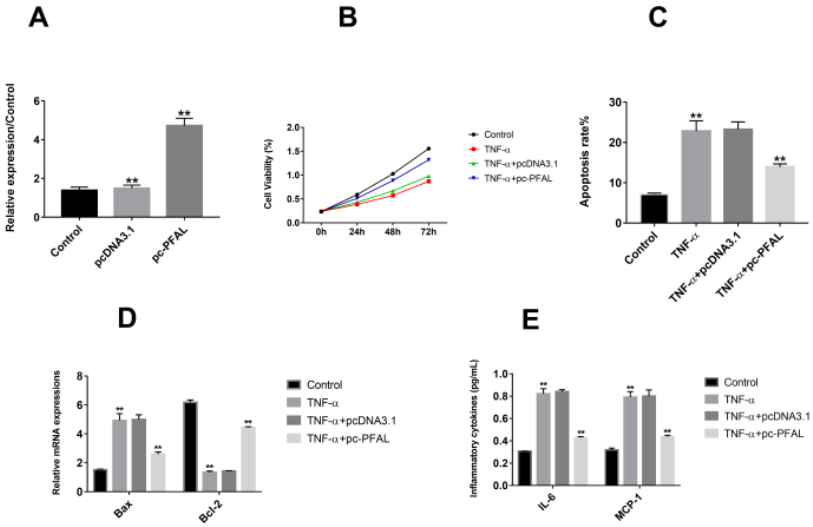

Figure 2: Overexpression of PFAL suppressed TNF-α-induced inflammatory injury in WI-38 cells. *(A) Expression of PFAL was measured by using RT-qPCR. (B) Cell viability (C) Cell apoptosis (D) Apoptosis-related mRNAs of Bax and Bcl-2 genes. (E) Level of inflammatory cytokines. P < 0.05*

verified a successful establishment of TNF-α-induced inflammatory model.

Overexpression of PFAL suppressed TNF-α-induced injury

The impact of PFAL on TNF-α-induced injury was determined by overexpressing PFAL in WI-38 cells. The results showed that the expression of PFAL was up-regulated when pc-PFAL was transfected into WI-38 cells (Figure 2A). Also, cell viability was significantly enhanced and the rate of apoptosis was decreased in TNF-α-induced WI-38 cells after PFAL overexpression as shown in Figure 2B-C. Moreover, expression of Bcl-2 was markedly enhanced while that of Bax was considerably decreased in the TNF-α-induced WI-38 cells after overexpression of PFAL (Figure 2D). Furthermore, the results (Figure 2E) of the Western blot analysis showed that expression of inflammatory cytokines of IL-6 and MCP-1 was markedly down-regulated.

Therefore, over-expression of PFAL suppressed TNF-α-induced injury in WI-38 cells.

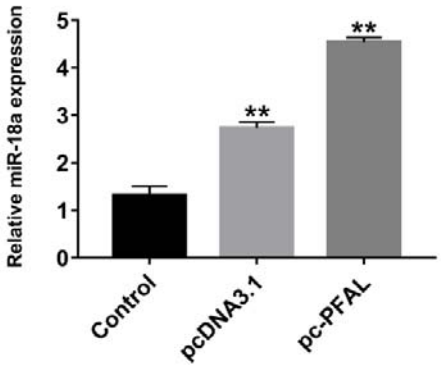

Figure 3: PFAL overexpression up-regulated miR-18a expression. *The miR-18a expression was measured by using RT-qPCR. P < 0.05*

PFAL overexpression-induced protective effects weakened by miR-18a inhibition

Furthermore, miR-18a inhibitor was transfected into WI-38 cells and the results (Figure 4A) showed that expression of miR-18a was markedly decreased in the transfected cells. Moreover, miR-18a inhibitor was transfected into the cells overexpressed with PFAL and the results revealed that PFAL overexpression-triggered effects were significantly (P<0.05) reduced on cell viability and apoptosis by the inhibition of miR-18a expression (Figure 4B-C). Also, expression of Bax was significantly enhanced while that of Bcl-2 was decreased after miR-18a inhibition (Figure 4D). The results (Figure 4E) from this study also showed that expression levels of inflammatory cytokines IL-6 and MCP-1 were considerably up-regulated after miR-18a inhibition. These results elucidated that the overexpression-induced protective impact of PFAL was weakened by the inhibition of miR-18a.

PFAL overexpression inhibited JNK pathway by modulating miR-18a expression

The effect of PFAL on JNK pathway was observed and the results (Figure 5) obtained indicated that the ratio of p/t-JNK and p/t-c-Jun was considerably enhanced when WI-38 cells were treated with TNF-α. On the other hand, the ratio of p/t-JNK and p/t-c-Jun was markedly reduced after the transfection of pc-PFAL into TNF-α-induced WI-38 cells. Moreover, the inhibition of miR-18a surprisingly improved the overexpression-triggered effects of PFAL, suggesting that the overexpression of PFAL inhibited JNK pathways via regulating miR-18a expression.

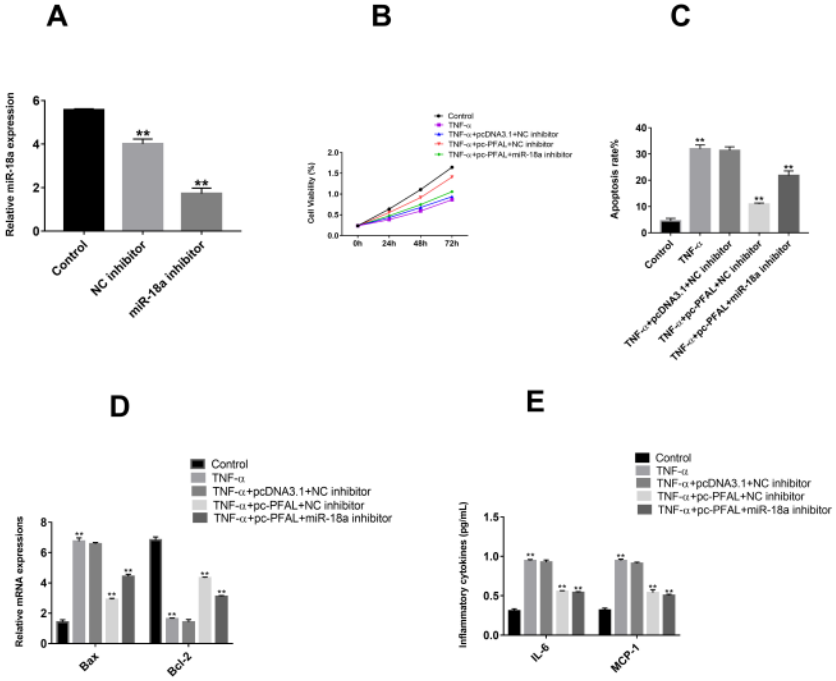

Figure 4: PFAL overexpression-induced protective effects weakened by miR-18a inhibition. *(A) Level of miR-18a mRNA was measured by using RT-qPCR. (B) Cell viability (C) Cell apoptosis (D) Apoptosis-related Bax and Bcl-2 mRNA levels (E) Levels of inflammatory cytokines. P < 0.05*

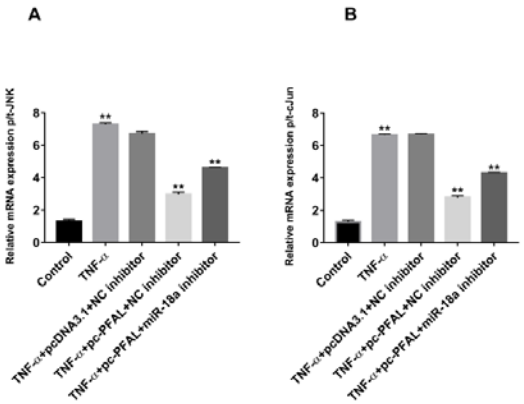

Figure 5: PFAL overexpression inhibited JNK pathway by modulating miR-18a expression. *(A-B) The relative mRNA expression levels of p/t-JNK and p/t-c-Jun were detected by using RT-qPCR in TNF-α-induced WI-38 cells. P < 0.05*

DISCUSSION

Pneumonia is one of the most common respiratory infection prevalent all over the world, especially in young children. It is caused by the inflammation of the lower respiratory tract [18]. The most important pro-inflammatory cytokines present in the body is TNF-α, which is responsible for the regulation of systematic and local inflammatory responses and immune functions of the cell [19]. In the present study, a TNF-α-induced inflammatory model of pneumonia was first established and the role of PFAL and miR-18a on the pathogenesis of disease was investigated. After the WI-38 cells were treated with TNF-α to establish an inflammatory injury model; CCK-8 assay, flow cytometry and RT-qPCR were applied for analysis. The results indicated that cell viability was significantly (P<0.05) reduced, apoptosis enhanced, mRNA expression level of Bax was increased while that of Bcl-2 decreased, indicating a successful establishment of TNF-α-induced inflammatory injury model. In order to investigate the functional role of PFAL, transfection procedure was used to overexpress PFAL in WI-38 cells and the outcome revealed that the overexpression of PFAL suppressed TNF-α-induced injury in treated cells. It was also discovered that PFAL and miR-18a play a vital role in cell viability, cell apoptosis and level of inflammatory cytokines.

A number of evidences have shown that lncRNAs are involved in the pathogenesis of many diseases such as organ fibrosis [20]. Liver fibrosis-related lncRNA1 stimulated liver fibrosis and hepatic stellate cell activation via Notch and TGF-β signaling pathways [21]. Another study revealed that lncRNA MALAT1 enhanced cell apoptosis and released inflammatory cytokines in LPS-induced WI-38 cells via down-regulating NF-κB signaling pathway [22]. A recent study demonstrated that the expression of PFAL was up-regulated in BLM-mediated pulmonary fibrosis and silencing of PFAL mitigated the progression of IPF [13]. However, the molecular mechanism of lncRNA PFAL in pneumonia is still unknown. Therefore, in the present study, the impact of lncRNA PFAL on TNF-α-induced inflammation injury in WI-38 cells was investigated and the results elucidated that pc-PFAL significantly (P<0.05) enhanced cell viability and decreased cell apoptosis in TNF-α-induced WI-38 cells. Moreover, the mRNA expression of apoptotic-related proteins Bcl-2 was enhanced while that of Bax was decreased. Furthermore, the expression levels of inflammatory cytokines of IL-1β and IL-6 were remarkably down-regulated in the TNF-α-induced WI-38 cells. These findings demonstrated that PFAL exerts a protective effect against TNF-α-induced inflammation injury in pneumonia.

Additionally, miRNAs have been widely used in the regulation of various inflammatory diseases including pneumonia [23]. Among these miRNAs, miR-18a plays a vital role in various lung diseases. A recent study has shown that a high glucose-mediated endothelium-mesenchymal transition was inhibited by miR-18a via Notch [24]. Another study revealed that poor prognosis in non-small cell lung cancer patients was due to the high circulation of miR-18a [25]. However, the underlying mechanism of miR-18a in pneumonia still remains unclear. More so, previous studies have shown that JNK pathway plays an important role in the regulation of immune response in Raw 264.7 infection caused by *S. pneumonia* [26]. Furthermore, the inflammatory injury caused by bacterial infection was regulated by miR-18a via regulating JNK pathways in lung fibrosis [27]. However, whether miR-18a regulate JNK pathway in pneumonia is yet to be investigated. In the present study, miR-18a exerted its inhibitory function against the TNF-α-induced inflammation in WI-38 cells. Moreover, the findings from our investigation revealed that PFAL positively regulated miR-18a while inhibition of miR-18a inhibited the PFAL-induced protection. Furthermore, pc-PFAL inhibited the TNF-α induced phosphorylation of JNK pathway, while miR-18a inhibitor surprisingly improved pc-PFAL-induced phosphorylation.

CONCLUSION

The results from this study elucidated the

protective impact of PFAL against TNF-α-induced inflammatory-injury of pneumonia on WI-38 cells by inhibiting cell apoptosis and levels of inflammatory cytokines via miR-18a and JNK pathway. Therefore, our findings suggest a novel therapeutic target for the diagnosis and treatment of pneumonia, possibly among children.

DECLARATIONS

Acknowledgement

None provided

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the author(s) named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. All authors read and approved the manuscript for publication. BZ and TL contributed equally to this work.

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