The QseB/QseC two-component system contributes to virulence of *Actinobacillus pleuropneumoniae* by downregulating *apf* gene cluster transcription

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**Abstract**

*Actinobacillus pleuropneumoniae* (APP) is the major pathogen of porcine contagious pleuropneumonia (PCP). The QseB/QseC two-component system (TCS) consists of the regulator QseB and the kinase QseC, which relates to quorum sensing (QS) and virulence in some bacteria. Here, we investigated the role of QseB/QseC in *apf* gene cluster (apfABCD) expression of APP. Our results have showed that QseB/QseC TCS can potentially regulate the expression of *apf* gene cluster. The ΔqseBC, ΔapfA, ΔapfB, ΔapfC and ΔapfD strains are more sensitive to acidic and osmotic stressful conditions, and exhibit lower biofilm formation ability than wild-type (WT) strain, whereas the complemented strains show similar phenotype to the WT strain. In addition, the mutants have defective anti-phagocytosis, adhesion and invasion when they come into contact with the host cells. In experimental animal models of infection, mice infected with ΔqseBC, ΔapfA, ΔapfB, ΔapfC and ΔapfD strains showed lower mortality and bacterial loads in the lung and the blood than those infected with WT strain. In conclusion, our results suggest that QseB/QseC TCS contributes to stress resistance, biofilm formation, phagocytosis, adhesion, invasion and virulence by downregulating expression of *apf* gene cluster in *A. pleuropneumoniae*.

**Keywords:** *A. pleuropneumoniae*, QseB/QseC, Transcriptional regulation, *apf* gene cluster, Virulence

**Background**

Porcine contagious pleuropneumonia (PCP) is a highly infectious porcine respiratory disease, which is caused by *Actinobacillus pleuropneumoniae* (APP). This disease is widespread in many countries and has brought great loss to farming and animal husbandry enterprises (González et al. 2017). Clinically, PCP is mainly characterized by acute fibrinous hemorrhagic pleuropneumonia and chronic fibrinous necrotizing pleuropneumonia (Sassu et al. 2018). So far, APP is divided into 2 biotypes and 19 serovars (Bosse et al. 2018; Sassu et al. 2018; To et al. 2021; Stringer et al. 2021). Some factors associated with virulence such as toxins, lipopolysaccharide, adhesion molecules, and outer membrane proteins contribute to the pathogenicity (Chiers et al. 2010).

To improve the adaptability of bacteria, their changing environment can be sensed and responded by the two-component systems (TCSs) (Jacob-Dubuisson et al. 2018). The bacterial TCS consists of a histidine kinase (HK) and a response regulator (RR) (Buelow and Raivio 2010). When the HK transfers a phosphate group to the RR, the RR can directly or indirectly regulate the transcriptional expression of the target gene (Chiers et al. 2010).
The qseBC and apf gene cluster contribute to the resistance to phagocytosis, adhesion and invasion of APP to host cells

To investigate whether the qseBC and apf gene cluster contribute to the resistance to phagocytosis, adhesion and invasion, ΔqseBC, ΔapfA, ΔapfB, ΔapfC, ΔapfD and the complemented strains were examined for their phagocytosis of RAW264.7, adhesion and invasion to NPtTr cells, and the WT strain was used as a control. It was found that the ΔqseBC, ΔapfA, ΔapfB, ΔapfC and ΔapfD mutants had defective anti-phagocytosis of RAW264.7 macrophage (Fig. 6a) and the abilities...
adhesion (Fig. 6b) and invasion to NPTr cells (Fig. 6c) were lower than those of WT and the complemented strains.

Virulence of the ΔqseBC and Δapf gene cluster mutants in mice
The virulence of ΔqseBC and Δapf gene cluster mutants was investigated by using Balb/c mouse models of APP in vivo (Xie et al. 2016). We found that the Survival rates of mice at 120 h were 16.67, 16.67, 83.33, 66.67, 66.67, 66.67, 0, 0, 0, 16.67 and 0 for WT, ΔqseBC, ΔapfA, ΔapfB, ΔapfC, ΔapfD, CΔqseBC, CΔapfA, CΔapfB, CΔapfC and CΔapfD-infected groups, respectively (Fig. 7a). Although the survival of mice at 120 h were equal in WT and ΔqseBC groups, it was interesting that the survival of mice infected with ΔqseBC (83.33) was significantly higher than that of WT (50.00) at 6 h post-infection. Furthermore, the bacterial loads of the ΔqseBC, ΔapfA, ΔapfB, ΔapfC and ΔapfD strains in the lung (Fig. 7b) and the blood (Fig. 7c) were lower than those by WT and the complemented strains at 6 h post-infection (P < 0.05). Taken together, our results suggest that QseB/QseC TCS and apf gene cluster may contribute to the virulence in the early stage of infection of A. pleuropneumoniae.

Discussion
The QseB/QseC TCS is considered as a regulatory system that relates to quorum sensing. Previous studies have revealed that QseB/QseC TCS contributes to inter boundary signal transduction and regulation of virulence gene expression, as well as toxin production (Clarke...
et al. 2006). Khajanchi et al. have demonstrated that QseB/QseC TCS can regulate virulence of APP in vitro and in vivo (Khajanchi et al. 2012). It has been reported that the inactivation of QseB/QseC leads to a decrease in biofilm formation of E. coli (Li et al. 2020). Bearson and Bearson have also found that QseB/QseC is involved in S. typhimurium colonization of swine (Bearson and Bearson 2008).

In this study, the EMSAs were performed to detect the regulatory relationships between QseB and apf gene cluster. Initially, purified QseB protein was directly used for EMSAs, but it was found that QseB could not bind to the promoter of apf gene cluster and pilM (positive control). Yan et al. demonstrated that phosphorylated CpxR protein could bind to the promoter of wecA in APP (Yan et al. 2020). Then we phosphorylated the QseB protein and found that phosphorylated QseB could bind to the promoter of and apf gene cluster and pilM (positive control). The results suggest that phosphorylated QseB might regulate the transcription of apf gene cluster.

Our results presented here demonstrate that the ΔqseBC and Δapf gene cluster mutants are more sensitive to acidic and osmotic stressful conditions than WT and complemented strains, which are consistent with earlier studies (Li et al. 2020). The ΔqseBC and Δapf gene cluster mutants also exhibit lower biofilm formation ability than WT strain and the complemented mutants. Biofilm is an extracellular polymer formed on the surface of bacterial colonies, which can cause self-agglutination and adhesion of bacteria. Biofilms of many strains of APP have been detected and are thought to relate to bacterial colonization (Kaplan et al. 2004; Kaplan and Mulks 2005). Taken together, our results suggest that QseB/QseC affects stress resistance and biofilm formation by regulating the expression of apf gene cluster.

In addition, this research suggests that the virulence of ΔqseBC and Δapf gene cluster mutants were more attenuated than that of WT and the complemented strains in the mouse infection models. Liu et al. used a pig infection model to evaluate the virulence of ΔqseBC of A. pleuropneumonia, and measured the clinical signs such as appetite, dyspnea, lethargy and fever of the infected pigs at 12, 24, 36, 48 and 60 h. They found that there was no significant difference between the clinical scores of the pigs inoculated with ΔqseBC mutant and WT, indicating that QseB/QseC had no significant effect on the virulence
of APP (Liu et al. 2015). We also found that the survival rates were similar in mice infected with WT or the \( \Delta qseBC \), however, it was interesting that the survival rate of mice infected with \( \Delta qseBC \) (83.33) was significantly higher than that of WT (50.00) at 6 h. In order to further verify our result, we analyzed the bacterial colonization ability in mice tissues, and found that the amount of colonization by the \( \Delta qseBC \) in the lung and the blood were both lower than those by the WT at 6 h post-infection. These results suggested that QseB/QseC TCS might contribute to the virulence in the early stages of infection.

Besides, the \( \Delta qseBC \) and \( \Delta apf \) gene cluster mutants contribute to the resistance to phagocytosis. Bacteria are phagocytic and form phagosomes in phagocytes. Lysosomes fuse with phagosomes to form phagolysosomes. A variety of bactericidal substances and hydrolases in lysosomes kill and digest bacteria. The thallus residue is expelled from the cell (Cao et al. 2019). By knocking out the \( apf \) genes, the mutant strains could not synthesize the Tfp assembly protein normally and the virulence of these mutant strains were significantly weakened, which made the mutants more easier to be phagocytic by macrophage.

At the same time, the abilities of the \( \Delta qseBC \) and \( \Delta apf \) gene cluster strains to adhesion and invasion of cells were lower than that of the WT strain. Adhesion colonization is a key step for pathogen infection and pathogenesis after pathogen invasion. APP specifically colonizes the lower respiratory tract of pigs, adhering to bronchial cilia and alveolar epithelial cells (Dom et al. 1994). The results suggest that the QseB/QseC TCS can affect the expression of \( apf \) gene clusters, mediating the adhesion and invasion of APP, and thus establishing infection. To sum up, this research indicate that QseB/QseC TCS and \( apf \) gene cluster could contribute to virulence in the early stage of infection of APP in vivo. The data in this study will provide theoretical basis for the prevention of infection with APP.

**Conclusions**

In summary, we confirm that QseB/QseC TCS contributes to the stress resistance, biofilm formation, phagocytosis, adhesion, invasion and virulence of APP by downregulating the expression of \( apf \) gene cluster.
Methods

Strains, plasmids, primers and culture conditions
The experimental materials are listed in Tables 1 and 2. S4074 was used as WT strain of *A. pleuropneumoniae* (Donà and Perreten 2018). APP strains were inoculated on tryptic soy agar (TSA; Difco Laboratories, USA) containing 10% (v/v) inactivated newborn bovine serum and 10 µg/mL nicotinamide adenine dinucleotide (NAD; Solarbio, China), then, a single colony was selected and inoculated into tryptic soy broth (TSB; Difco Laboratories, USA) (Yuan et al. 2014). Chloramphenicol was added to the culture medium as needed, where the final concentration was 25 µg/mL for *E. coli* screening, 4 µg/mL for APP transformants screening, and 2 µg/mL for APP complemented strains screening. All strains were cultured in Luria-Bertani (LB; Haibo, China), and the cultivation of *E. coli* J2155 requires the addition of diaminopimelic acid (dapA; Sigma-Aldrich, USA) (Yuan et al. 2014). Chloramphenicol was added to the culture medium as needed, where the final concentration was 25 µg/mL for *E. coli* screening.

Construction of mutant and complemented strains
The mutant strains ΔqseBC, ΔapfA, ΔapfB, ΔapfC, ΔapfD and the complemented strains CΔqseBC, CΔapfA, CΔapfB, CΔapfC and CΔapfD were constructed as described earlier (Li et al. 2018). In Brief, the upstream and downstream fragments of qseBC, apfA, apfB, apfC and apfD were amplified, respectively. And the fragments were combined via overlapping polymerase chain reaction (PCR). These products were purified and cloned into the vector pEMOC2 (Oswald et al. 1999) to generate the recombinant plasmids of pEΔqseBC, pEΔapfA, pEΔapfB, pEΔapfC and pEΔapfD, respectively. These plasmids were used to construct ΔqseBC, ΔapfA, ΔapfB, ΔapfC and ΔapfD mutants by conjugal transfer. The qseBC and apf gene cluster were amplified and PCR products were cloned into vector pJFF224-XN (Frey 1992), respectively. Then, the plasmids pJFF-qseBC, pJFF-DapfA, pJFF-DapfB, pJFF-DapfC and pJFF-DapfD were transferred into the corresponding mutant strains by electric transformation (2.5 KV, 25 µFD, 800 Ω). These mutants were screened on TSA (supplemented with chloramphenicol, NAD, and bovine serum) and verified by PCR and DNA sequencing (data not shown).

RNA extraction and RT-qPCR
WT and ΔqseBC strains were cultured in TSB (supplemented with NAD and bovine serum) overnight, then diluted with fresh medium at a ratio of 1:100 and grown to the OD₆₀₀ of 0.6. The Bacteria Total RNA Isolation Kit (Sangon Biotech, China) was used to extract total RNA. The HiScript II Q RT SuperMix (+gDNA wiper) (Vazyme, China) was used to synthesize the first strand cDNA. AceQ qPCR SYBR Green Master Mix (Vazyme, China) was used for quantitative PCR (qPCR), which performed by a one-step reaction (Walters et al. 2006). The inverted cDNA and 16S rRNA gene were used as template and endogenous control, respectively. Specific procedure, reaction system and conditions were as instructed by these kits. Then, we used the 2⁻ΔΔCt method to quantitatively analyze the expression level of target genes (Livak and Schmittgen 2001).

Expression of QseB
Primers PqseB-F and PqseB-R were used to amplify qseB gene for PCR, and plasmid pET-qseB was transferred
into E. coli BL21 (DE3) competent cells and grown in LB to OD$_{600}$ of 0.6. QseB protein was induced with 1.00 mM isopropyl-β-D-thiogalactoside (IPTG) at 25 °C for 4 h. After suspension, cells were crushed by high-pressure cell crusher and centrifuged at 4 °C. QseB was then purified by the Ni-NTA resin affinity chromatography. The purified QseB protein was analyzed by SDS-PAGE and Western Blot, then stored at −80 °C.

**Electrophoretic mobility shift assays**

Primers were used to amplify DNA probes containing apf, pilM and glpK promoter region for PCR,
Table 1 Strains, plasmids and protein used in this study

| Strains or plasmids | Characteristics |
|---------------------|-----------------|
| S4074               | A. pleuropneumoniae reference strain of serovar 1; WT strain |
| ΔqseBC              | A. pleuropneumoniae S4074 qseBC-deletion mutant |
| ΔapfA               | A. pleuropneumoniae S4074 apfA-deletion mutant |
| ΔapfB               | A. pleuropneumoniae S4074 apfB-deletion mutant |
| ΔapfC               | A. pleuropneumoniae S4074 apfC-deletion mutant |
| ΔapfD               | A. pleuropneumoniae S4074 apfD-deletion mutant |
| CΔqseBC             | Complemented strain of ΔqseBC; Cm^r |
| CΔapfA              | Complemented strain of ΔapfA; Cm^r |
| CΔapfB              | Complemented strain of ΔapfB; Cm^r |
| CΔapfC              | Complemented strain of ΔapfC; Cm^r |
| CΔapfD              | Complemented strain of ΔapfD; Cm^r |

**Plasmids**

- pEMOC2: Transconjugation vector: ColE1 ori mob RP4 sacB, Amp^r, Cm^r
- pETqseBC: Up- and down-stream arms of qseBC were ligated sequentially into pEMOC2, and used as the transconjugation vector for qseBC gene deletion
- pETapfA: Up- and down-stream arms of apfA were ligated sequentially into pEMOC2, and used as the transconjugation vector for apfA gene deletion
- pETapfB: Up- and down-stream arms of apfB were ligated sequentially into pEMOC2, and used as the transconjugation vector for apfB gene deletion
- pETapfC: Up- and down-stream arms of apfC were ligated sequentially into pEMOC2, and used as the transconjugation vector for apfC gene deletion
- pETapfD: Up- and down-stream arms of apfD were ligated sequentially into pEMOC2, and used as the transconjugation vector for apfD gene deletion
- pJFF224-XN: E. coli-APP shuttle vector: RSF1010 replicon; mob oriV, Cm^r
- pJFF-qseBC: pJFF224-XN carrying the intact qseBC
- pJFF-apfA: pJFF224-XN carrying the intact apfA
- pJFF-apfB: pJFF224-XN carrying the intact apfB
- pJFF-apfC: pJFF224-XN carrying the intact apfC
- pJFF-apfD: pJFF224-XN carrying the intact apfD
- pET-30a: Expression vector; Kan^r
- pET-qseB: pET-30a carrying qseB gene

Cm^r, chloramphenicol resistance, Amp^r, ampicillin resistance, Kan^r, kanamycin resistance

respectively. After the PCR products were purified, biotin labeling of EMSA probes were carried out using the EMSA Probe Biotin Labeling Kit (Beyotime, China). QseB protein was phosphorylated by Sigma Acetate Kinase from E. coli (Sigma, USA). The pilM probe that can bind to QseB protein was used as a positive control, and glpK probe that can not bind to QseB protein was used as a negative control (Liu et al. 2015). EMSAs were performed with the Chemiluminescent EMSA Kit (Beyotime, China).

**Acidic and osmotic stress resistance assays**

WT, ΔqseBC, ΔapfA, ΔapfB, ΔapfC and ΔapfD strains were cultured in TSB (supplemented with NAD and bovine serum) overnight, then diluted with fresh medium at a ratio of 1:100 and grown to the mid-logarithmic phase. All strains were resuspended in TSB (supplemented with NAD and bovine serum) containing 0.02 M HCl (Rode et al. 2010) and 0.50 M KCl (Yin and Mimura 2020), respectively, and incubated for 3 h, followed by acidic and osmotic stress resistance assays. Bacteria cultured in TSB without any addition were used as control. The incubated samples were serially diluted and selected the appropriate dilution gradient samples to culture in TSA (supplemented with NAD and bovine serum). The bacterial survival rate of each group was determined by dividing CFU of the experimental group by that of the control group.

**Biofilm assay**

All strains were cultured overnight, then diluted with fresh brain heart infusion broth (BHI; Oxoid Ltd., UK) (supplemented with NAD) at a ratio of 1:100. Totally 100 μL of the inocula was added to 96-well microtiter plates (Corning, USA) in triplicat. After incubated for 72 h, the bacterial inocula was sucked away with a syringe, and then removed unattached bacteria. Placed the plates in a warm oven to dry and added 100 μL 0.1% (v/v) crystal violet into the well. The plates were carefully washed with tap water. After drying, 33% (v/v) glacial acetic acid was used to dissolve the biofilm. Each well of the plates was measured OD_{590} with a Multi-Detection Microplate Reader (BMG Labtech, Germany).

**Cell phagocytosis assay**

RAW264.7 cells were cultured in 24-well plates with DMEM (supplemented with FBS) to analyze the phagocytosis ability (Carreras-Gonzalez et al. 2019). Briefly, all strains were added to RAW264.7 cells in the plates at the multiplicity of infection (MOI) of 100, respectively. After incubation for 2 h, the mixture were treated with 100 μg/mL of gentamicin to kill any extracellular bacteria. Following an incubation for 45 min, 1 mL pre-cooled 0.025% (v/v) Triton X-100 was used to lyse those
| Primers used in this study | Sequence (5’-3’)* |
|---------------------------|------------------|
| qseBC-up/F/R              | TTCGCAGCATTGCTTTAATCCTGCCG | For mutant construction |
| qseBC-down/F/R            | GGAAATTTAAAGCGGCAATGCCG | |
| apfA-up/F/R               | CTCGCCATGTTATCGGCAAAGAAGC | |
| apfA-down/F/R             | GTTCTGTGGGATATTCGCAATGC | |
| apfB-up/F/R               | CGTGCGCTGCTTACCTGCACG | |
| apfB-down/F/R             | GCTCTGAGCGATGTTACGCAATGC | |
| apfC-up/F/R               | TGCCGGAGGAGATTTGCCTATATG | |
| apfC-down/F/R             | CGGTGCAATGCTTACGCAATGC | |
| apfD-up/F/R               | CGCTCAGGCTGCAACGTTATACG | |
| apfD-down/F/R             | CCGGAGGCTGCAACGTTATACG | |
| apfA-qPCR-F/R             | CTGTGTGGAATTTGCTATACG | For complement construction |
| apfB-qPCR-F/R             | CGGTGCAATGCTTACGCAATGC | |
| apfC-qPCR-F/R             | CGGTGCAATGCTTACGCAATGC | |
| apfD-qPCR-F/R             | CGGTGCAATGCTTACGCAATGC | |

| Primers used in this study (Continued) | Sequence (5’-3’)* |
|----------------------------------------|------------------|
| qseBC-exterior/F/R                     | CGCCCATGAGCGCAGTTTTCG | For RT-qPCR |
| apfD-exterior/F/R                      | CGCCCATGAGCGCAGTTTTCG | |
| apfD-interior/F/R                      | CGCCCATGAGCGCAGTTTTCG | |
| PIIL-EMSA-F/R                          | CGCCCATGAGCGCAGTTTTCG | For EMSAs |
| glpK-EMSA-F/R                          | CGCCCATGAGCGCAGTTTTCG | |

*Restriction sites are underlined
cells for 10 min at 4°C or on ice. The lysates were ser-ially diluted and selected the appropriate dilution gradient cells to plate on TSA (supplemented with NAD and bovine serum) overnight to determine bacterial counts.

**Cell adhesion and invasion assays**

NPTr cells were used to investigate the abilities of adhesion and invasion (Zhou et al. 2013). Briefly, all strains were added to NPTr cells at the MOI of 100 and incubated for 2 h. For the adhesion assays, each well was lysed by using 0.025% (v/v) Triton X-100 after the culture supernatant removed. The cell lysates were serially diluted to determine bacterial counts, which may contain adherent and invasive cells. For invasion assays, gentamicin was also added to each well after washing with PBS and further cultured for 45 min. Then, the cells were lysed and diluted in the appropriate dilution gradient for bacterial count.

**Bacterial virulence in vivo**

Six-week-old female Balb/c mice were purchased from Experimental Animal Center of Three Gorges University (Yichang, China). The animal experiments were performed as described previously, with some modifications (Li et al. 2018). To determine the survival rates, mice were randomly divided into 11 groups (6 per group): WT, ΔqseBC, CΔqseBC, ΔapfA, CΔapfA, ΔapfB, CΔapfB, ΔapfC, CΔapfC, ΔapfD and CΔapfD. Briefly, all strains were grown to the OD600 of 0.6. Each mouse was inoculated with 5.00 × 10^6 CFU by intraperitoneal injection. Clinical symptoms and mortality rates of mice were observed and recorded every day. The surviving mice were euthanized a week later. To determine the bacterial colonization ability of mice tissues, each mice was inoculated with 1.00 × 10^8 CFU by intraperitoneal injection. At 6 h post-infection, blood samples were collected and anticoagulated by heparin. Then the mice were humanely-euthanized and lung tissue samples were taken out about 0.1 g for homogenization by using a Tissue lyser (Jingxin, China). 100 μL of each blood and lung sample was used for gradient dilution. CFU was calculated by appropriate dilution gradient tissue fluid cultured on TSA (supplemented with NAD and bovine serum).

**Statistical analysis**

Statistical analysis was performed via GraphPad Prism 7 software (San Diego, USA). The results were presented as mean ± SD. The survival rate of mice was analyzed by log-rank (Mantel-Cox) test. The bacterial load in mouse tissues was analyzed by two-tail Mann-Whitney test. Student’s t test was used to compare differences between groups, where P < 0.05 was considered significant.

**Abbreviations**

APP: Actinobacillus pleuropneumoniae; BHI: Brain heart infusion; bp: Base pair; cDNA: Complementary DNA; CFU: Colony forming units; DAP: 2,6-Diaminopimelic acid; ddH2O: Double distilled H2O; DME: Dulbecco’s modified eagle medium; DNA: Deoxyribonucleic acid; EMSA: Electrophoretic mobility shift assay; FBS: Foetal bovine serum; g: Gram; h: Hour; IPTG: Isopropyl-β-D-thiogalactopyranoside; kDa: Kilodalton; LB: Luria bertani; min: Minute; MOI: Multiplicity of infection; mol: Mole; NAD: Nicotinamide adenine dinucleotide; OD: Optical density; PAGE: Polyacrylamide gel electrophoresis; PBS: Phosphate buffered saline; PCP: Porcine contagious pleuropneumonia; PCR: Polymerase chain reaction; RT-qPCR: Quantitative reverse transcription PCR; r/min: Rotation per minute; RNA: Ribonucleic acid; sec: Second; SDS: Sodium dodecylsulphate; SPF: Specific pathogen free; TCS: Two-component system; TSA: Tryptic soy agar; TSB: Tryptic soy broth; μL: Microliter; °C: Degree centigrade

**Acknowledgements**

We appreciated Prof. Hongbo Zhou at Huazhong Agricultural University for providing NPTr cells. At the same time, we thank Mr. Jinlin Liu, Ms. Beibei Dou, Ms. Linlin Hu and Ms. Dan Yang for providing warm help.

**Authors’ contributions**

BD and WB conceived of the study, and participated in its design and coordination. BD and WP constructed the mutant and complemented strains. BD, JT and FenY participated in the animal assays. BD, KY and FL performed the statistical analysis. BD drafted the manuscript. WB, FanY and HC directed the project. All authors have read and approved the final version of the manuscript.

**Availability of data and materials**

Data will be shared upon request by the readers.

**Declarations**

**Ethics approval and consent to participate**

All animal assays were performed according to the guidelines of the Laboratory Animal Monitoring Committee of Huazhong Agricultural University (HZAUMO-2020-083).

**Consent for publication**

Not applicable.

**Competing interests**

Author Huanchun Chen was not involved in the journal’s review or decisions related to this manuscript. The authors declare no other conflict of interest.

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**Received:** 22 November 2021  **Accepted:** 4 January 2022  **Published online:** 27 January 2022

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