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Diverse effects of mycobacterial proline-proline-glutamic acid proteins upon interaction with host macrophages

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One sentence summary: Diverse effects of PPE proteins on Mtb–macrophage interaction.

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ABSTRACT

The proline-proline-glutamic acid (PPE) family proteins are abundant only in pathogenic Mycobacteria, but their general functions are far from unveiled. To investigate their roles in how Mycobacterium tuberculosis (Mtb) resists killing by the host, 25 PPE recombinant Mycobacterium smegmatis strains that overexpress Mtb PPE proteins were constructed. During phagocytosis, a similar amount of intracellular bacteria was observed at 2 h post-infection (hpi) for 24 PPE recombinants, while a 50% reduction of entrance was observed for the PPE29 recombinant. In addition, we found that 20 ppe genes significantly influenced the survival of mycobacteria within macrophage cells. Mycobacterial survival was promoted by overexpression of 18 of these genes and inhibited by the other two. Highest survival was observed for the PPE27 recombinant. We also measured the levels of proinflammatory cytokines tumor necrosis factor-alpha and interleukin-6 secreted by macrophages. The overall effects varied among the different PPE recombinants. Moreover, we also found that various PPE recombinants exhibited increased resistance against oxidative, acidic and sodium dodecyl sulfate stresses that could be encountered in vivo. Together, our results indicate that PPE proteins play distinct roles in mycobacterial survival in macrophages. The findings described here broaden our understanding of mycobacterial pathogenicity.

Keywords: Mycobacterium; PPE protein; macrophage; virulence

INTRODUCTION

About 8% of the coding capability of the Mycobacterium tuberculosis (Mt) H37Rv genome is annotated as a unique class of proteins named as proline-glutamic acid (PE)/proline-proline-glutamic acid (PPE) proteins (Cole, Brosch and Parkhill 1998), so named based on a preserved PPE-containing motif at 180 amino acids, from the N-terminus of the proteins (Cole, Brosch and Parkhill 1998). The ppe genes are unique in the mycobacteria genus and in particular, widely distributed in pathogenic mycobacteria. For example, a total 69 ppe genes are annotated in the genome of Mt H37Rv, and 106 ppe genes are annotated in the genome of Mycobacterium marinum (M. marinum) (Cole, Brosch and Parkhill 1998; Stinear, Seemann and Harrison 2008). In contrast, only two PPE proteins are annotated in the non-pathogenic Mycobacterium smegmatis (M. smegmatis) (Sampson et al. 2006). According to the homology and characteristic motifs in their C-terminal domains, PPE proteins can be divided into five sublineages: PPE-SVP (with a GxxSVPxxW motif), PPE-MPTR...
genes were then incubated with the appropriate ppe mutant was \( \theta \) gene has been applied in previous studies on the function of sinusoidal proteins such as PPE41 and PPE57 were found in inclusion bodies; PPE2 inhibits nitric oxide generation of macrophages (Bhat et al. 2011), and M. avium PPE25 is expressed post-bacterial invasion into macrophages, which could efficiently inhibit phagosome acidification (Li et al. 2005). Our previous study on the \( M. \ marium \) ppe38 gene has demonstrated that the invasion ability of the ppe38 mutant was significantly reduced compared to that of the wild-type strain (Dong et al. 2012). Furthermore, certain PPE proteins influence the cytokine secretion of macrophages, such as tumor necrosis factor-alpha (TNF-\( \alpha \)) and interleukin-6 (IL-6), through binding to the Toll-like receptor 2 (Nair et al. 2009; Daim, Kawamura and Tsuchiya 2011; Wang et al. 2011; Khubai et al. 2016; Mi et al. 2016; Udgata, Qureshi and Mukhopadhyay 2016). Based on these data, PPE proteins are speculated to be utilized by pathogenic mycobacteria to resist the unique microenvironment that they inhabit, and mycobacterial PPE proteins are thought to interfere with the immunity mediated by macrophages (Bets et al. 2002; Voskuil et al. 2004; Fontan et al. 2008; Mohareer, Tundup and Hasnain 2011; Soldini et al. 2011; Akhter et al. 2012; Kohli et al. 2012). Therefore, a comprehensive study of the ppe genes could improve our understanding of the complex interaction between Mtb and macrophages.

However, understanding comprehensive picture of the Mtb-macrophage interaction has been limited to date, perhaps due to the following reasons. First, the highly homologous sequences and high GC content (up to 80%) of the ppe genes make it difficult to obtain individual ppe knockout mutants. Second, ppe genes may function redundantly, making it difficult to delineate the phenotype of a singly-mutated strain. Third, numerous PPE proteins such as PPE41 and PPE57 were found in inclusion bodies during protein expression in Escherichia coli (Choudhary et al. 2004; Zhang et al. 2007), posing a big challenge in protein purification for in vitro studies.

Here, we applied a compatible model in which PPE proteins are overexpressed in \( M. \ smegmatis \) individually. This approach has been applied in previous studies on the function of single ppe genes, such as PPE37 and PPE17 (Daim, Kawamura and Tsuchiya 2011; Dona et al. 2013). A set of the \( M. \ smegmatis \) recombinants expressing each of 25 PPE proteins were constructed and the responses of \( R. \ avium \) macrophage cells to the infecting strains were observed. Two major parameters representative of the macrophage response were evaluated: phagocytosis and survival of the recombinants in macrophage cells. In addition, the levels of the cytokines TNF-\( \alpha \) and IL-6 secreted by infected macrophage cells were also measured at 24 h post-infection (hpi) with PPE recombinants. The survival rate of each PPE recombinant was also evaluated under environmental stress conditions such as low pH and sodium dodecyl sulfate (SDS). Through these analyses, various effects of PPE proteins were evaluated on the resistance of the Mtb strains to elimination by macrophages.

**METHODS**

**Bacterial strains and media**

The \( M. \ smegmatis \) mc2\( \text{155} \) strain was a gift from Dr Liu J (University of Toronto, Canada). The genomic DNA was extracted from \( M. \ h37r v \) in our laboratory. The murine macrophage cell line \( R. \ avium \) 264.7 was obtained from Shang-hai Institute of Biochemistry and Cell Biology (SIBCB, China). Escherichia coli DH5\( \alpha \) was routinely maintained in lysogeny broth (LB) medium (Sigma-Aldrich, USA) for DNA cloning. \( M. \ smegmatis \) mc2\( \text{155} \) was grown in Middlebrook 7H9 broth or on Middlebrook 7H10 agar (Difco, BD Difco\( \text{TM} \), USA) supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC), 0.5% glycerol, and 0.05% Tween 80 at 37°C. Hygromycin (150 \( \mu \)g/ml) was added when necessary. All reagents are persue from Sigma-Aldrich, USA.

**Plasmids and construction of \( M. \ smegmatis \) recombinants**

The primers used to amplify \( M. \ h37r v \) ppe genes are listed in Table S1 (Supporting Information). Briefly, the full-length ppe genes were PCR-amplified from the genomic DNA of \( M. \ h37r v \). Amplified ppe genes were then incubated with the appropriate restriction enzymes, and the restriction fragments were cloned into the pSMT3LxEGFP vector (Snawin et al. 1999). Additionally, the ppe16 gene was amplified without its termination codon and ligated to pSMT3LxEGFP vector after digestion of amplified product with Bgl\( \text{II} \) and NdeI (Fermentas, Thermo Fisher, USA). The plasmids were transformed into \( M. \ smegmatis \) mc2\( \text{155} \) via electroporation.

**Macrophage infections**

The \( R. \ avium \) 264.7 cells was maintained at 37°C in an atmosphere of 5% \( \text{CO}_2 \) in DMEM medium (Gibco) supplemented with 10% FBS. Suspension cultures of \( R. \ avium \) 264.7 were seeded at 3 \( \times \) 10\( \text{4} \) cells per well in 96-well tissue culture plates prior to experimentation. After 24 h, cells were infected with PPE recombinants at a multiplicity of infection (MOI) of 10:1 (bacteria-to-macrophage cell ratio). The initial infection dosage was confirmed by plating bacteria and determining colony-forming units (CFUs) in each individual experiment. The initial infection was allowed to proceed for 2 h. For measuring the phagocytosis of PPE recombinants into macrophages, extracellular bacteria were removed following the infection after 2 h by washing with warm DMEM three times. Triton X-100 (Sigma-Aldrich, USA) was then added to 0.01% (w/v) and the bacteria released from lysed macrophages were plated on Middlebrook 7H10 agar plates. For the intracellular survival assay, 100 \( \mu \)L of 200 \( \mu \)M gentamycin (Sigma-Aldrich, USA) was added at 2 hpi to kill extracellular bacteria for 1 h. C-DMEM (10% FBS+ 100 mM HEPES) (Gibco, Thermo Fisher, USA) and gentamycin (100 \( \mu \)M) were then added. At 24 and 48 hpi, the macrophages were lysed and bacteria were spread on Middlebrook 7H10 agar plates. The CFUs were determined as a measure of the intracellular survival of PPE recombinants.

**Determination of sensitivity to stress conditions**

PPE recombinant strains were prepared and their sensitivity toward different stress conditions were measured as described previously (Singh et al. 2016). Briefly, bacterial cultures were collected and adjusted to an optical density at 600 nm of 0.02. To measure the sensitivity of recombinant PPE to stressors,
including hydrogen peroxide (5 mM \( \text{H}_2\text{O}_2 \)), 0.1% SDS and low pH (pH 5.5), the bacteria were collected after incubation at 37°C for 24 h and then plated by serial dilution to determine CFUs. The stress agents were all purchased from Sigma-Aldrich, USA.

**Assay for cytokine production**

As described previously, RAW264.7 cells were infected with PPE recombinants and cell-free supernatants were collected at 3 and 24 hpi. Cytokine levels of TNF-\( \alpha \) and IL-6 were determined using enzyme-linked immunosorbent assays (ELISAs) according to the manufacturer’s protocols (BD, USA).

**Phylogenetic analysis**

The full-length protein sequences corresponding to 24 PPE family genes were downloaded from Tuberculist (http://tuberculist.epfl.ch). The complete alignment of the amino acid sequences of the 24 PPE proteins was performed using the ClustalX program at the European Bioinformatics Institute (http://www.ebi.ac.uk) and a neighbor-joining phylogeny tree using the default parameters was generated by MEGA5 (Tamura et al. 2011).

**Statistical analysis**

Data from all experiments was analyzed using the Student’s two-tailed t-test. Statistical significance was defined as a \( P \)-value <0.05. Error bars are representative of standard deviation (SD) values.

**RESULTS**

**The effects of PPE proteins on the phagocytosis of *Mycobacterium smegmatis***

In order to investigate the role of ppe genes in Mtb pathogenesis, we constructed 25 PPE recombinants (listed in Table 1). Quantitative reverse transcription (qRT)-PCR analysis was used to evaluate the transcription of ppe genes in *M. smegmatis* (Msmeg-PPEx; Fig. S1A, Supporting Information). Moreover, the Msmeg-PPE16EGFP strain was constructed and the PPE16-GFP fusion protein was detected using a fluorescence microscope; the fluorescence data showed the successful expression of the ppe16 gene in *M. smegmatis* (Fig. S1B, Supporting Information).

**Survival of PPE recombinants in macrophages**

Next, we sought to identify the ppe genes that are necessary for mycobacteria to escape the bactericidal effects of macrophages. The survival of each PPE recombinant in RAW264.7 cells was investigated. Infection with the PPE recombinants was performed at an initial MOI of 1:10 and the survival of each strain was measured by enumerating the CFUs of intracellular bacteria at 3, 24 and 48 hpi. Based on the phagocytosis assay results, the initial bacterial load of PPE29 recombinant was doubled and a similar CFU value for all PPE recombinants at 3 hpi was observed (data not shown), indicating that the infections yielded comparable intracellular bacterial loads. Significantly enhanced survival was observed in 12 out of 25 PPE recombinants at 24 hpi (Fig. 2A). At 48 hpi, 15 PPE recombinants displayed higher survival (Fig. 2B). Mycobacterium smegmatis expressing PPE27 showed the highest bacterial counts at both 24 and 48 hpi. Six PPE recombinants, including Msmeg-PPE68, displayed a better survival, but only at 48 hpi. Moreover, Msmeg-PPE10 and Msmeg-PPE24 recombinants were more susceptible to macrophage-mediated killing compared to the vector control at 48 hpi.

To examine whether PPE proteins affect the phagocytosis of macrophage cells, the intracellular bacterial load of each PPE recombinant at 2 hpi was counted. The phagocytosis of 24 PPE recombinants did not show significant differences, except for the PPE29 recombinant, which had a reduced CFU compared with the vector control (Fig. S2, Supporting Information). The PPE38 recombinant (Msmeg-PPE38) was also evaluated, as our previous study showed that it could assist the invasion of *M. marinum* into macrophages (Dong et al. 2012). Consistent with the data from the previous study, the phagocytosis of Msmeg-PPE38 by macrophage cells was also enhanced (Fig. 1). The invasion of Msmeg-PPE29 and Msmeg-PPE38 was further tested in another cell infection model using non-phagocytic HeLa cells, and the outcome from either strain did not differ from that of the vector control in these cells (data not shown).

**Table 1.** The list of ppe genes of *Mycobacterium tuberculosis* tested in this study.

<table>
<thead>
<tr>
<th>Sublineage</th>
<th>Gene name</th>
<th>Tested/total(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (PPW superfamily)</td>
<td>ppe68</td>
<td>1/1</td>
</tr>
<tr>
<td>II (PPW superfamily)</td>
<td>ppe11, ppe37, ppe2, ppe5, ppe20</td>
<td>5/10</td>
</tr>
<tr>
<td>III</td>
<td>ppe29, ppe21, ppe14, ppe18, ppe19, ppe26, ppe25, ppe27, ppe50, ppe51</td>
<td>1/6</td>
</tr>
<tr>
<td>IV (SVP superfamily)</td>
<td>ppe29, ppe21, ppe14, ppe18, ppe19, ppe26, ppe25, ppe27, ppe50, ppe51</td>
<td>10/26</td>
</tr>
<tr>
<td>V (MPTR)</td>
<td>ppe10, ppe12, ppe13, ppe16, ppe24, ppe35</td>
<td>6/19</td>
</tr>
<tr>
<td>Else(^b)</td>
<td>ppe7, ppe39</td>
<td>2/7</td>
</tr>
</tbody>
</table>

\( ^a \)The number of tested PPE recombinants belonging to each sublineage is listed. 
\( ^b \) Undivided PPE proteins.

Next, we sought to identify the ppe genes that are necessary for mycobacteria to escape the bactericidal effects of macrophages. The survival of each PPE recombinant in RAW264.7 cells was investigated. Infection with the PPE recombinants was performed at an initial MOI of 1:10 and the survival of each strain was measured by enumerating the CFUs of intracellular bacteria at 3, 24 and 48 hpi. Based on the phagocytosis assay results, the initial bacterial load of PPE29 recombinant was doubled and a similar CFU value for all PPE recombinants at 3 hpi was observed (data not shown), indicating that the infections yielded comparable intracellular bacterial loads. Significantly enhanced survival was observed in 12 out of 25 PPE recombinants at 24 hpi (Fig. 2A). At 48 hpi, 15 PPE recombinants displayed higher survival (Fig. 2B). Mycobacterium smegmatis expressing PPE27 showed the highest bacterial counts at both 24 and 48 hpi. Six PPE recombinants, including Msmeg-PPE68, displayed a better survival, but only at 48 hpi. Moreover, Msmeg-PPE10 and Msmeg-PPE24 recombinants were more susceptible to macrophage-mediated killing compared to the vector control at 48 hpi.
phylogenetic reconstructions, ppe genes were divided into five sublineages, expanding with the esx gene clusters (Sampson et al. 2006). We speculated that the functionally distinct roles of ppe genes might correlate with the division of the sublineages. However, our data did not reveal any clear patterns of association between the phylogenetic sublineages and ppe gene effects (Fig. S3, Supporting Information). Taken together, our data show enhanced/reduced survival of 20 PPE recombinants, indicating a distinct role of these PPE proteins in mycobacterial survival.

**PPE proteins play different roles in Mycobacterium smegmatis defense against stress**

To further delineate the role of the 25 ppe genes on bacterial survival in macrophages, we mimicked the intracellular environment of macrophages infected with Mtb by treating the PPE recombinants with acidic stress (pH 5.5) and hydrogen peroxide (H$_2$O$_2$) in vitro. When cultured in acidic 7H9 medium for 24 h, four PPE recombinants (Msmeg-PPE18, Msmeg-PPE3, Msmeg-PPE12 and Msmeg-PPE25) were more resistant than the parental control, while Msmeg-PPE29, Msmeg-PPE35 and Msmeg-PPE39 were more sensitive than the parental strain (Fig. 3A). After exposure to 5 mM H$_2$O$_2$ for 24 h, survival of Msmeg-PPE18, Msmeg-PPE1, Msmeg-PPE26 and Msmeg-PPE11 was significantly higher than that of the control Msmeg-P (Fig. 3B). Based on these data, we conclude that certain PPE proteins confer resistance to environmental stressors.

The distinct cell wall structure of Mtb is known to be pivotal in protection of the bacteria against harmful conditions such as oxidative damage and antibiotics. Therefore, we also investigated the ability of the PPE recombinants to resist SDS (0.1%), a detergent that is often used to collapse the bacterial cell wall. Fourteen PPE recombinants, including Msmeg-PPE18 and Msmeg-PPE27, were more resistant to SDS, while six PPE recombinants were more susceptible (Fig. 4). Taken together, the results indicate that PPE proteins may play an important role in maintaining cell wall structure, which, in turn, would promote the intracellular survival of Mtb.

**PPE proteins affect TNF-α and IL-6 secretion**

Previous studies showed that PPE proteins are capable of modulating proinflammatory cytokine secretion by macrophages (Nair, Pandey and Mukhopadhyay 2011; Dong et al. 2012). We infected RAW264.7 macrophage cells with several PPE recombinants and monitored the amount of secreted TNF-α and IL-6 at both 3 and 24 hpi using ELISAs. A total of 11 PPE recombinants were selected based on the results from the survival assay, including 8 with enhanced, 1 with reduced and 2 with unaltered survival. Neither TNF-α nor IL-6 was detectable at 3 hpi in RAW264.7 cells infected with any of the PPE recombinant (data not shown). At 24 hpi, the secretion of TNF-α and IL-6 varied in different PPE recombinants (Fig. 5A and B). Significantly lower TNF-α and IL-6 levels were detected in...
RAW264.7 macrophages infected with Msmeg-PPE25, Msmeg-PPE7 or Msmeg-PPE12 recombinants. Enhanced TNF-α secretion was observed only from macrophages infected with the Msmeg-PPE19 recombinant.

**DISCUSSION**

Despite the annotation and periodic updating of the genome of Mtb since 1998 (Cole, Brosch and Parkhill 1998), the functions of many Mtb PPE proteins remain uncharacterized. As speculated in a recent review (Fishbein et al. 2015), PPE proteins might play crucial roles in mycobacterial pathogenesis by modulating various host immune responses. In this study, the functions of 25 PPE proteins of Mtb H37Rv were investigated. Among them, PPE29 protein significantly reduced macrophage phagocytosis; 18 PPE proteins enhanced and 2 reduced intracellular bacterial survival. Moreover, PPE19 expressed in recombinant Mycobacterium smegmatis increased the production of TNF-α, while PPE7, PPE12 and PPE25 downregulated the secretion of both TNF-α and IL-6.

Phagocytosis is the first step of the Mycobacterium–host interaction, and may provide an opportunity for Mtb to manipulate the host environment (Pieters 2008; Jayachandran, Scherr and Pieters 2012). Our earlier study showed that PPE38 assists the invasion of M. marinum into macrophages (Dong et al. 2012). However, overexpression of PPE29 reduced the phagocytosis of M. smegmatis in this study. Therefore, we surmise that certain PPE proteins play distinct roles in mediating phagocytosis. To further explore the cause of such differences between PPE38 and PPE29, the HeLa cell infection model was utilized, since it has been illustrated that peptides of a mycobacterial virulent factor, Mce4F, could inhibit invasion of both macrophage cells and epithelial cells by Mtb (Rodriguez et al. 2014). No difference was observed in the invasion of HeLa cells by either the PPE29 or PPE38 recombinant (data not shown). Thus, the exact mechanism that drives the contradictory effects of PPE29 and PPE38 on phagocytosis remains to be clarified in further studies.

The intracellular survival of Mycobacterium is the outcome of the battle between the bacilli and macrophages. In this study, 18 out of 25 PPE proteins were found to be beneficial for Mycobacterium, promoting their resistance to macrophage-mediated killing. These data suggest a general function of the majority of PPE proteins in promoting resistance to macrophage-mediated immune responses. Among the 18 PPE proteins investigated in this study, PPE25 and PPE18 have been previously reported as virulence factors in different Mycobacterium spp. (Li et al. 2005; Bhat et al. 2012). In addition, ppe31, ppe68, ppe24 and ppe50 have been proposed to be essential genes in vivo (Lamichhane et al. 2003; Sassetti and Rubin 2003; Sassetti, Boyd and Rubin 2003). Other proteins identified in this study have not been reported before, but their importance is supported by a report that many ppe genes are differentially upregulated at different infection stages in a mouse model (Kruh et al. 2010). We also investigated the survival of the PPE recombinants after exposure to environmental stressors like H$_2$O$_2$, low pH and 0.1% SDS that mimic stressors commonly encountered by Mtb within macrophages. Msmeg-PPE27 was found to be more resistant, whereas Msmeg-PPE10 was more sensitive, to SDS treatment, which might explain its different survival pattern in macrophages. Besides, a higher survival rate of Msmeg-PPE18 was observed under the three stress conditions, indicating its key role in defending Mtb against macrophage-mediated elimination.

Proinflammatory cytokines play significant roles in controlling mycobacterial infections. TNF-α, a key cytokine, is mainly secreted by activated macrophages and modulates both the innate and the adaptive immune response against tuberculosis (Flynn et al. 1995; Bean et al. 1999; Kaneko et al. 1999; Gardam et al. 2003; Tobin, Roca and Oh 2012). TNF-α and IL-6 secretion was attenuated in RAW264.7 cells infected with either Msmeg-PPE12, Msmeg-PPE7 or Msmeg-PPE25, indicating that certain PPE proteins might circumvent host sterilization processes by inhibiting proinflammatory cytokine secretion following infection. Taken together, our data suggest that PPE proteins play distinct
Figure 5. Cytokines secretion of macrophage cells infected by PPE recombinants. After infection of macrophages by Msmeg-PPE and Msmeg-V strains at an MOI = 10 for 24 h, supernatants of infected cells were collected and the amount of secreted (A–F) TNF-α and (G–L) IL-6 was measured by ELISA. Data represented were from three technique repeats. Similar results were obtained in three independent experiments (*, P < 0.05, determined by Student’s two-tailed t-test).

and important roles, which mostly benefit Mtb residence in host cells.

To investigate whether the distinct functions of PPE proteins are correlated with their phylogenetic classification, a phylogenetic tree of the tested PPE proteins was constructed. However, no clear pattern of association between their classification into sublineages and their functions could be identified. Various functions of the ppe genes were observed in sublineage V, which contains ppe genes that enhanced or reduced Mtb survival. Interestingly, we noticed that all eight ppe genes that are present only in the Mtb genome, but not in M. marinum or M. leprae, participated in enhancement/reduction of the survival of M. smegmatis.

In this study, we took advantage of the overexpression model, since it allows ready identification of the effects of individual PPE proteins, and helps circumvent the redundancy-mediated limitations of gene knockout studies (Sampson 2011). In this study, several PPE proteins did not display obvious effects, but we cannot rule out their possible importance in other pathogenic functions such as antigen presentation. In addition, only a few pe genes have been annotated in M. smegmatis, and some ppe genes are co-transcribed with pe genes (Cole, Brosch and Parkhill 1998). These genes could be functionally related (Betts et al. 2002; Abdallah, Verboom and Hannes 2006; Majumdar et al. 2012). Further research is needed to fully delineate the mechanism of how these 20 ppe genes influence the survival of mycobacteria within macrophages. In conclusion, our study has identified important functions of ppe genes in the interaction between Mycobacterium and macrophage cells, and provides new insights into mycobacterial pathogenesis.

SUPPLEMENTARY DATA
Supplementary data are available at FEMSLE online.

AUTHOR CONTRIBUTIONS
LM and QG designed experiments. LM and JFT performed experiments. LM, WQL, CN, and QG analyzed data. LM, CN, and QG wrote the manuscript. QG conceived and supervised the study. All authors reviewed and approved the final manuscript.

FINDING
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Conflict of interest. None declared.


