A novel oncolytic herpes simplex virus armed with the carboxyl-terminus of murine MyD116 has enhanced anti-tumour efficacy against human breast cancer cells

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Abstract. Oncolytic herpes simplex virus-1 (oHSV-1) vectors are promising therapeutic agents for cancer. The deletion of the γ34.5 gene eliminates the neurovirulence but attenuates virus replication at the same time. The carboxyl-terminus of protein phosphatase 1 regulatory subunit 15A (also known as MyD116/GADD34) is homologous to that of γ34.5; hence, it may substitute for γ34.5 to enhance the replication and cytotoxicity of the virus. To investigate whether the C-terminus of MyD116 can enhance the anti-tumour efficacy of G47Δ on human breast cancer cells, a GDI16 mutant was constructed by inserting a γ34.5-MyD116 chimaera into the G47Δ genome using a bacterial artificial chromosome and two recombinase systems (Cre/loxP and FLPE/FRT). A GD-empty mutant containing only the cytomegalovirus sequence was also created as a control using the same method. Next, the replication and cytotoxicity of these two virus vectors were evaluated in breast cancer cells. Compared with the GD-empty vector, GD116 possessed an enhanced replication capability and oncolytic activity in MCF-7 and MDA-MB-231 cells. On the fifth day after infection with GD116 at MOIs of 0.01 and 0.1, 49.2 and 82.8% of MCF-7 cells, respectively, were killed, with 35.0 and 50.2% of MDA-MB-231 cells, respectively, killed by GD116 at MOIs of 0.1 and 0.3. Additionally, the insertion of the γ34.5-MyD116 chimaera into G47Δ genome further increased replication in MDA-MB-468 at 48 h after infection, although no increased cytotoxic effect was observed. The findings of the present study indicate that the C terminus of the MyD116 gene can be substituted for the corresponding domain of the γ34.5 gene of oHSV-1 to promote the replication of the virus in infected cells. Furthermore, the novel virus mutant GD116 armed with a γ34.5-MyD116 chimaera has enhanced anti-tumour efficacy against human breast cancer cells in vitro.

Introduction

Breast cancer is the most common malignancy and the leading cause of cancer-associated mortality in women worldwide (1,2). Each year, >1.5 million new cases of breast cancer are reported around the world (3), and it has been estimated that 10-12% of women will develop breast cancer over the course of their lives (4). Although relapse-free survival of patients with breast cancer has been improved by modern chemo-, endocrine and targeted therapies, improved therapeutic options for advanced disease are urgently required (5).

Oncolytic herpes simplex virus-1 (oHSV-1) is a potential therapeutic vector for human cancer (6,7). Several oncolytic HSV mutants have already been used in clinical trials for various solid tumours (7-9). Talimogene laherparepvec (T-VEC), an oHSV expressing granulocyte-macrophage colony-stimulating factor, was approved by the U.S. Food and Drug Administration for the treatment of melanoma in 2015 (10). G47Δ is a third-generation oHSV-1 that lacks the γ34.5 neurovirulence gene and possesses an additional deletion of the α47 gene (11,12). Previous studies have demonstrated that G47Δ can effectively target breast cancer cells in vitro and in vivo (13-16). However, certain breast cancer cell lines remain insensitive to G47Δ (data not shown). Infected cell protein 34.5 (ICP34.5), encoded by the γ34.5 gene, has diverse activities: It is critical for the replication and neurovirulence of oHSV-1 in humans and in animal models (17). oHSV-1 mutants without γ34.5 were highly attenuated for neurovirulence, although deletion of γ34.5 also compromises the replication of the virus (18). An early report found that a stretch of 64 amino acids at the C-terminus of the γ34.5 gene was homologous to the C termini of murine protein phosphatase 1 regulatory subunit 15A (also known as MyD116/GADD34) (19). In addition, the C terminus of MyD116 can substitute for the corresponding domain of the

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γ34.5 gene of oHSV-1 to preclude the premature shutoff of total protein synthesis in infected human cells (19). Therefore, we hypothesised that the insertion of the MyD116 C-terminus might enhance the replication and virulence of G47Δ.

To test this hypothesis, a bacterial artificial chromosome (BAC) and two recombinase systems (Cre/loxP and FLPE/FRT) were used to reconstruct a novel oHSV-1 mutant, GD116, in which a chimeric gene (γ34.5-MyD116) consisting of the N-terminus of the γ34.5 gene and the C-terminus of the MyD116 gene was inserted (19). In this study, the novel vector, GD116, exhibited enhanced anti-tumour effects on breast cancer in vitro.

Materials and methods

Cells. The human breast cancer cell lines MCF-7, SK-BR-3 and MDA-MB-231 were provided by Dr Musheng Zeng (State Key Laboratory of Oncology in Southern China, Sun Yat-sen University Cancer Center, Guangzhou, China), and the MDA-MB-468 cell line was obtained from Dr Xiaoming Xie (Sun Yat-sen University Cancer Center, Guangzhou, China). The Vero cell line (Cercopithecus aethiops kidney cells) was purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). All cell types were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), which was supplemented with 4.5 g/l glucose and 10% foetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) at 37°C in an atmosphere containing 5% CO₂.

Plasmids. The plasmid pRB4871, containing the γ34.5-MyD116 chimaera was kindly provided by Professor Bernard Roizman (Marjorie B. Kovler Viral Oncology Laboratories, University of Chicago, Chicago, IL, USA) (19). The pG47Δ-BAC backbone plasmid and the pVec91 shuttle plasmid were obtained from Professor Samuel D. Rabkin (Molecular Neurosurgery Laboratory, Massachusetts General Hospital, Boston, MA, USA), and the pG47A-BAC backbone plasmid was created by a two-step replacement procedure, in which G47Δ DNA was cloned into a BAC vector for propagation in Escherichia coli, as described previously (20). The γ34.5-MyD116 chimaera fragment from pRB4871 was ligated into the BamHI-Stul site of pVec91 [containing LacZ,loxP, and FRT sites; multiple cloning sites; lambda stuffer sequences; and a cytomegalovirus (CMV) promoter] to generate pVec91-116.

Virus construction. The recombinant oHSV-1 vectors were constructed using a BAC and Cre/loxP and FLP/FRT recombinase systems, as reported previously (20,21). Mixtures of the pG47Δ-BAC plasmid (2 µg) and pVec91-116 or pVec91 (200 ng) were incubated with Cre recombinase (New England BioLabs, Ipswich, MA, USA) at 37°C for 30 min and then electropropored into E. coli DH10B using a Gene Pulser Xcell™ (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The bacteria were streaked onto LB plates containing chloramphenicol (15 µg/ml; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and kanamycin (30 µg/ml; Sigma-Aldrich; Merck KGaA), and positive clones containing the pG47Δ-BAC-116 recombinant and pG47A-BAC-empty plasmids were selected. Next, the recombinant plasmids were digested with HindIII and separated by electrophoresis on 0.75% agarose gels in TBE buffer (Tris-base, boric acid and EDTA) at 2.5 cm/V for 12 h with a 1 kb DNA extension ladder (Invitrogen; Thermo Fisher Scientific, Inc.). Co-transfection of pG47Δ-BAC-116 or pG47Δ-BAC-empty and a FLP recombinase expression plasmid, pOG44 (Invitrogen; Thermo Fisher Scientific, Inc.), into Vero cells was performed with Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.). Following this, the removal of the FRT-flanked BAC sequences resulted in the generation of recombinant viruses. The progeny viruses were further selected by limiting dilution and were finally designated 'GD116' and 'GD-empty'.

Analyses of viral DNA. Viral DNA was isolated using the Hirt Supernatant DNA Purification kit (GenMed Sciences Inc., Shanghai, China), and polymerase chain reaction (PCR) was performed using recombinant Taq DNA polymerase (Takara Bio, Inc., Otsu, Japan). Primers (CMV forward 5’-ACTGCTTACTGGTATCAG-3’ and reverse, 5’-GTC TAACCTCGTGCTCTC-3’) were used to amplify a specific 113 bp fragment for detection of the CMV sequence. Primers (γ34.5-MyD116 forward, 5’-GCCGCTCATATTGCTCA-3’ and γ34.5-MyD116 reverse, 5’-CGGACTGTGGAAAGATG-3’) were used to amplify a 284 bp product that was specific for the γ34.5-MyD116 chimaera. The samples were initially denatured for 5 min at 94°C; followed by 35 cycles of denaturation at 94°C for 30 sec, re-naturation at 52°C for 30 sec and extension at 72°C for 20 sec; with a final extension at 72°C for 10 min. pVec91 or pRB4871 plasmids and PBS were used as the positive and blank controls, respectively. The PCR products were separated by size in 1% agarose gels.

Western blotting. A total of 5x10⁴ Vero cells were mock treated or treated with viruses at an MOI (multiplicity of infection) of 2 for 24 h, and then cells were lysed using a Whole Cell Lysis kit (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). A total of 30 µg protein (quantified using the BCA protein quantification kit; Nanjing KeyGen Biotech Co., Ltd.) was separated by 15% SDS-PAGE and transferred to a 0.22-µm thick nitrocellulose membrane (EMD Millipore, Billerica, MA, USA). Following blocking with 5% non-fat dry milk in TBS-Tween-20 (TBS-T, 0.1% Tween) for 1 h at room temperature, the membrane was incubated overnight at 4°C with primary antibodies against ICP34.5 (amino terminus) (diluted 1:500, rabbit polyclonal; cat no. HX5191; TSZ Biosciences, San Francisco, CA, USA; http://www.tsz-biological.com/) or β-actin (diluted 1:1,000; rabbit monoclonal; cat no. 4970; Cell Signaling Technology, Inc., Danvers, MA, USA). The membrane was then washed and blotted with a horseradish peroxidase (HRP)-linked anti-rabbit secondary antibody (diluted 1:6,000; cat no. 7074; Cell Signaling Technology, USA) for 1 h at room temperature. The Protein antibody complexes were visualized with the chemiluminescent HRP substrate (EMD Millipore) using a chemiluminescence imaging system (Tanon-5200; Tanon, Guangzhou, China).

Virus replication assay. Cells were seeded in 12-well plates at 1x10⁵ cells per well and infected at 70-80% confluence with GD116 or GD-empty at a MOI of 0.1 (Vero, MCF-7, SK-BR-3 and MDA-MB-468) or 0.3 (MDA-MB-231). The inoculum was...
removed after 2 h and replaced with DMEM supplemented with 1% heat-inactivated FBS (iFBS). The cells and the supernatant were harvested at the indicated times (24 and 48 h) post-infection, processed with three freeze/thaw cycles, and virus titres were determined by plaque assays on Vero cells. In brief, Vero cells were seeded in 12-well plates at 1x10^4 cells/well and infected with 500 µl virus dilute (10^3/ml, 10^4/ml, 10^5/ml or 10^6/ml) at 100% confluence. Then, the infected Vero cells were cultured at 37°C in 5% CO_2 for an additional 48 h prior to subsequent X-gal staining for plaque counting. The X-gal staining was performed using the in situ β-galactosidase staining kit (Beyotime Institute of Biotechnology, Haimen, China) according to the manufacturer’s protocol. The virus titres were calculated using the following equation: Virus titres=plaque numbers/0.5 x dilution ratio. Experiments were repeated at least three times.

**Cell susceptibility assay.** Cells were seeded in 48-well plates at 8,000 cells per well. After 16 h, the cells were infected with either GD116 or GD-empty at MOIs of 0.01 and 0.1 (MCF-7, SK-BR-3 and MDA-MB-468) or at MOIs of 0.1 and 0.3 (MDA-MB-231). Cells were incubated for up to 5 days in DMEM medium supplemented with 1% iFBS at 37°C. The cell viability was assessed daily with an MTT assay and expressed as a percentage of the mock-infected control. Formazan crystals were dissolved using 400 µl DMSO and the visualization wavelength was 490 nm. Meanwhile, infection was monitored using β-galactosidase activity via X-gal staining at the indicated times (1, 2, 3, 4 and 5 days post-infection). Experiments were repeated at least three times for each condition in quadruplicate.

**Statistical analysis.** Student's t-test was used for statistical analyses. P<0.05 were considered to be statistically significant. Data are presented as the mean ± standard deviation. SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA) was used for all of the statistical analyses.

**Results**

**Cloning of the γ34.5-MyD116 chimaera into the shuttle plasmid.** The genomic structure of the mutants is depicted in Fig. 1A. The pRB4871 plasmid was cleaved using the BamHI and Stul restriction endonucleases, and then a 1.51 kb fragment containing the γ34.5-MyD116 chimaera was purified from an agarose gel and directionally cloned into the pVec91 vector to generate pVec91-116. To identify the recombinant plasmid pVec91-116, pRB4871 and pVec91-116 were digested with BamHI and Stul, and the products were separated on a 1% agarose gel. As shown in the map of double restriction endonuclease digestions (Fig. 1B), the desired 1.51 kb bands on an agarose gel confirmed the successful construction of the recombinant pVec91-116 vector.

**Construction of recombinant oHSVs with or without the γ34.5-MyD116 chimaera.** The generation of two novel oHSV-1 mutants was performed by a previously described two-step replacement procedure (22). In the first step, the shuttle plasmid pVec91-116 or pVec91-empty were integrated into pG47A-BAC using Cre recombinase in a tube and an integrated BAC clone (pG47A-BAC-Vec91-116 or pG47A-BAC-empty) was isolated in E. coli by selection with chloramphenicol and kanamycin. The integrated plasmids were collected and the DNA structures of the plasmids were confirmed by gel analyses following Hind III restriction endonuclease digestion (Fig. 1C).

In the second step, the integrated plasmid pG47A-BAC-Vec91-116 or pG47A-BAC-empty and the FLP expression plasmid were co-transfected into Vero cells, and the BAC backbone and lambda stuffer sequences were excised by FLP recombinase. As a result, the novel oHSV-1 mutants were generated and were designated GD116 and GD-empty.
To verify the insertion of the target sequences into the novel mutants, specific PCR analyses were conducted using pVec91 and pRB4871 plasmids as the positive controls. The PCR products were checked by electrophoresis on 1% agarose gels. GD116 and GD-empty produced the desired 113 bp band on an agarose gel when used as templates to amplify the CMV sequence (Fig. 1D). However, only GD116 generated the expected size band of 284 bp when \( \gamma_{34.5}-\text{MyD116} \)-specific PCR products were detected (Fig. 1D). To determine the expression of the \( \gamma_{34.5}-\text{MyD116} \) protein, Vero cells were treated with PBS, GD-empty or GD116. Western blot analysis revealed that the expression of \( \gamma_{34.5}-\text{MyD116} \) was shown only in the GD116-treated group (Fig. 1E). In addition, the two mutants could form blue plaques on Vero cells by X-gal staining 48 h after infection, which confirmed the insertion and expression of the LacZ gene (data not shown).

**Replication of GD116 and GD-empty in vitro.** Viral replication is one of the key determinants of oHSV-1 efficacy against tumour cells. The replication of the two mutants was compared in Vero cells and a panel of human breast cancer cell lines at 24 and 48 h after infection (Figs. 2A-E). Compared with GD-empty, GD116 had a significantly increased viral yield in breast cancer MCF-7 and MDA-MB-231 cell lines at 24 and 48 h (Fig. 2B and E). Additionally, the viral yields of GD116 in MDA-MB-468 cells were greater than that of GD-empty at 48 h (Fig. 2D). However, the insertion of \( \gamma_{34.5}-\text{MyD116} \) did not significantly increase the replication rate of oHSV-1 in SK-BR-3 cells (Fig. 2C), and even reduced the viral replication in Vero cells (Fig. 2A).

**In vitro cytotoxicity of GD116 and GD-empty in breast cancer cell lines.** Next, the oncolytic activity of these two mutants was examined in human breast cancer cell lines. The breast cancer cells MCF-7, SK-BR-3 and MDA-MB-468 were infected with GD116 or GD-empty at MOIs of 0.01 and 0.1, whereas MDA-MB-231 cells were infected with these two mutants at MOIs of 0.1 and 0.3. In addition, cells infected with the virus could express LacZ and thus were stained blue by X-gal; staining of MCF-7 and SK-BR-3 cells with X-gal was presented in Fig. 3A and B. Similar to what was observed with virus replication, GD116 was more effective at inhibiting the growth of MCF-7 cells at MOIs of 0.01 and 0.1 (Fig. 3C). On the fifth day after infection with GD116 at MOIs of 0.01 and 0.1 (Fig. 3C), the killing activity of GD-empty at the different MOIs was only 24.3 and 37.8%, respectively (Fig. 4D). Aside from that effect, no significant difference in cytotoxicity was observed between the two mutants at the other indicated times (Fig. 3D).

X-gal staining of MDA-MB-468 and MDA-MB-231 cells was presented in Fig. 4A and B. As shown in Fig. 4C, GD116 had a similar cytotoxic effect to GD-empty in MDA-MB-468 cells. However, the insertion of \( \gamma_{34.5}-\text{MyD116} \) chimaera could significantly increase the oncolytic activity of GD116 on MDA-MB-231 cells (Fig. 4D). On day 5 after infection, 35.0 and 50.2% of MDA-MB-231 cells were killed by GD116 at MOIs of 0.1 and 0.3, respectively; however, the killing activity of GD-empty at the different MOIs was only 24.3 and 37.8%, respectively (Fig. 4D). In vitro, the spread of oHSV-1 was associated with cytotoxicity; therefore, X-gal staining visually showed the difference in oncolytic activity between GD116 and GD-empty in different breast cancer cell lines (Figs. 3A and B, and 4A and B).
Figure 3. Cytotoxicity of GD116 and GD-empty in MCF-7 and SK-BR-3 cells in vitro. Monolayers of cells were mock-infected or infected with viruses at multiplicities of infection of 0.01 and 0.1 and further incubated at 37°C for 5 days. (A and B) X-gal staining of MCF-7 and SK-BR-3 cells. Infected cells expressing LacZ were stained positive (blue) with X-gal. Original magnification, x100. (C and D) Cell viability was assessed using an MTT assay from days 1 to 5 following infection. The relative cell viability was normalised to that of the mock-infected control. Statistical comparisons (independent t-test) were between two viruses at the indicated times. *P<0.05 and **P<0.01.

|       | Control | GD116 (MOI=0.01) | GD116 (MOI=0.1) | GD-empty (MOI=0.01) | GD-empty (MOI=0.1) |
|-------|---------|-------------------|-----------------|---------------------|-------------------|
| Day 1 |         |                   |                 |                     |                   |
| Day 2 |         |                   |                 |                     |                   |
| Day 3 |         |                   |                 |                     |                   |
| Day 4 |         |                   |                 |                     |                   |
| Day 5 |         |                   |                 |                     |                   |

Figure 4. Cytotoxicity of GD116 and GD-empty against MDA-MB-468 and MDA-MB-231 cells in vitro. Monolayers of cells were mock-infected or infected with viruses at multiplicities of infection of 0.01 and 0.1 (MDA-MB-468) or 0.1 and 0.3 (MDA-MB-231) and further incubated at 37°C for 5 days. (A and B) X-gal staining of MDA-MB-468 and MDA-MB-231 cells. Infected cells expressing LacZ were stained positive (blue) with X-gal. Original magnification, x100. (C and D) Cell viability was assessed by an MTT assay from days 1-5 following infection. The relative cell viability was normalised to that of the mock-infected control. Statistical comparisons (independent t-test) were made between two viruses at the indicated times. *P<0.05 and **P<0.01.

|       | Control | GD116 (MOI=0.1) | GD116 (MOI=0.3) | GD-empty (MOI=0.1) | GD-empty (MOI=0.3) |
|-------|---------|-----------------|-----------------|---------------------|-------------------|
| Day 1 |         |                 |                 |                     |                   |
| Day 2 |         |                 |                 |                     |                   |
| Day 3 |         |                 |                 |                     |                   |
| Day 4 |         |                 |                 |                     |                   |
| Day 5 |         |                 |                 |                     |                   |

|       | Control | GD116 (MOI=0.1) | GD-empty (MOI=0.1) | GD-empty (MOI=0.3) |
|-------|---------|-----------------|--------------------|-------------------|
| Day 1 |         |                 |                    |                   |
| Day 2 |         |                 |                    |                   |
| Day 3 |         |                 |                    |                   |
| Day 4 |         |                 |                    |                   |
| Day 5 |         |                 |                    |                   |
Discussion

OHSV-1 vectors that specifically replicate in and kill tumour cells, sparing normal cells, are promising cancer therapeutic agents. In the OHSV-1 genome, \( \gamma34.5 \) is transcribed as a leaky-late gene that encodes ICP34.5, whose expression can be detected as early as 2-3 h post-infection (23). ICP34.5 is involved in multiple aspects of viral pathogenesis, and one of its key roles is to promote neurovirulence. OHSV-1 mutants with deletions in ICP34.5 are unable to replicate in neuronal cells and are not neurovirulent (24). In this regard, ICP34.5 has currently been deleted in all OHSV vectors in clinical trials for treating malignant gliomas, such as 1716 and G207 (18,25). However, at the C-terminus of ICP34.5, a region homologous to murine MyD116 and GADD34 can bridge phosphorylated eukaryotic initiation factor 2\( \alpha \) (eIF2\( \alpha \)) and protein phosphatase-1b (PP1\( \alpha \)) to prevent the accumulation of the former and dephosphorylate it (26,27). As a result, ICP34.5 can counteract PKR-mediated innate immune responses and allow translation to proceed (26,28). Therefore, mutants with \( \gamma34.5 \) gene deletions reveal markedly reduced lethality in the peripheral or intracerebral routes of infection but replicate poorly in certain confluent cell types in vitro (23). Thus, it is necessary to reconstruct novel OHSV-1 mutants that maintain low neurovirulence yet replicate efficiently in cancer cells.

The present study used a BAC-based recombinase system to reconstruct novel OHSV-1 mutants. This recombinase system enabled the rapid generation of recombinant OHSV-1 vectors with the desired transgene inserted in the place of the deleted ICP6 locus. The C-terminus of MyD116/GADD34 was used to substitute that of \( \gamma34.5 \) and to create a novel OHSV-1 mutant, GD116. Compared with GD-empty, the mutation enhanced the replication and increased the cytopathicity of GD116 in breast cancer MCF-7 and MDA-MB-231 cell lines. Although the insertion of the \( \gamma34.5 \)-MyD116 chimaera promoted the replication of the virus in MDA-MB-468 cells, no increased cytotoxic effect was obtained. The insertion of \( \gamma34.5 \)-MyD116 slightly inhibited viral replication in Vero cells, but the viral cytopathicity was not attenuated in cancer cells. Therefore, the murine MyD116/GADD34 gene could be used to substitute the \( \gamma34.5 \) gene to promote the cytopathicity of OHSV-1 without increasing the neurovirulence. However, substitution of the carboxyl-terminus of the \( \gamma34.5 \) gene with the corresponding domain of murine MyD116 did not markedly enhance the virulence of G47A in breast cancer cell lines, and even SK-BR-3 cells were not sensitive to the mutation of G47A.

Recently, a region in the N-terminus of \( \gamma34.5 \) [amino acids (aa) 68 to 87] that interacts with Beclin 1 and inhibits autophagy, which contributes to neurovirulence in a PKR-dependent fashion, has been identified (29,30). Deletion of the Beclin 1-binding domain (BBD) (aa 68-87) resulted in mutants that could induce autophagy in neurons and was attenuated for neurovirulence following intracerebral inoculation (18). In addition, BBD deletion mutants remain able to dephosphorylate eIF2\( \alpha \) and inhibit host protein shutoff, and thus the replication and cytotoxicity are not attenuated. Therefore, promoting the anti-tumour efficacy by expressing BBD-deleted ICP34.5 for OHSV-1 mutants with the deletion of the two copies of the \( \gamma34.5 \) gene, such as G47A may represent a promising strategy for generation of a therapeutic vector for the treatment of breast cancer.

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Availability of data and materials

The datasets generated and analyzed in the present study are included in this published article.

Authors’ contributions

LC, HJ and RL conceived and designed the experiment. LC, HJ and JF performed the experiments. JW, PH and YR collected the data and performed the statistical analyses. LC wrote the manuscript. JW and RL reviewed and revised the manuscript. All authors read and approved the final manuscript.

Ethics and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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