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Transcriptional upregulation of multiple earthworm chitinase genes following bacterial challenge suggests their implications in innate immunity

Beom Jun Park[1] · Yoo Bin Yoon[1] · Dong Ho Lee[2] · Chuog Shin[3] · Louis Juakali[4] · Sung-Jin Cho[5] · Soon Cheol Park[1]

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Abstract

Background Chitinase is a multi-functional enzyme that catalyzes the hydrolysis of β-1,4-linkages between N-acetylglucosamines (GlcNAc) in chitin. Recent studies imply that earthworm chitinase is implicated in self-defense immunity against chitin-containing pathogens. However, a direct relationship of earthworm chitinase with innate immunity has not yet been established.

Objective In this study, earthworm (*Eisenia andrei*) chitinase expression was examined following bacterial challenge by *Bacillus subtilis.*

Methods RNA sequencing (RNA-seq) and real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) were used to quantitatively evaluate mRNA expression changes in response to bacterial stimulation.

Results Multiple chitinase-related mRNAs were found to be upregulated, among which *EaChi3*, *EaChi4*, and *EaChi2* were upregulated by approximately eightfold, eightfold, and 2.5-fold, respectively. This strongly suggested that earthworm chitinases may act as inducible humoral effectors in earthworm innate immunity. The primary structures of all three chitinases contained an N-terminal glycol_18 domain with two chitin-binding and chitin-catalyzing domains, and a C-terminal proline, glycine, serine, threonine (PGST)-rich domain. In addition, EaChi2 had a chitin-binding peritrophin-A domain at the end of the C-terminus with 5 cysteine residues possibly contributing two intradomain disulfide bonds. Multiple sequence alignment of the catalytic domain centers of glycol_18 domain displayed highly conserved chitin-binding and chitin-catalyzing domains in which three essential amino acid residues (D, D, E) for catalyzing activity are well conserved except EaChi4. The critical glutamic acid (E) residue was substituted for glutamine (Q) in EaChi4 indicating that it is devoid of catalytic activity.

Conclusions To our knowledge, this is the first report providing direct evidence that multiple earthworm chitinases are bacteria-responsive, strongly suggesting that earthworm chitinases are inducible humoral effectors in earthworm innate immunity. In addition, our results possibly suggest that earthworm EaChi4 may function as a pattern recognition molecule modulating the downstream immune pathway.

Keywords Earthworm · Multiple forms of chitinase · Bacterial challenge · RNA sequencing · mRNA upregulation · Innate immune effector

Beom Jun Park, Yoo Bin Yoon and Dong Ho Lee have contributed equally to this work.

✉ Sung-Jin Cho
sjchobio@chungbuk.ac.kr

✉ Soon Cheol Park
scpark@cau.ac.kr

[1] Department of Life Sciences, Chung-Ang University, Seoul 06974, Republic of Korea

[2] Da Vinci College of General Education, Chung-Ang University, Seoul 06974, Republic of Korea

[3] Department of Biological Science and Technology, College of Science and Technology, Yonsei University, Wonju 26493, Republic of Korea

[4] Department EGRA, University of Kisangani, Kisangani, Democratic Republic of the Congo

[5] Department of Biological Sciences and Biotechnology, Chungbuk National University, Chungbuk 28644, Republic of Korea

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Introduction

Chitinase (EC 3.2.1.14) catalyzes the hydrolysis of β-1,4-linkages between N-acetylglucosamines (GlcNAc) in chitin, which is one of the most abundant polysaccharides in nature next to cellulose and starch (Garcia-Fraga et al. 2014). This enzyme has widely differing functions and is involved in digestion, molting processes, defense/immunity and growth/development (Arakane and Muthukrishnan 2010). Earthworms are surrounded by microorganisms in their natural environment, including chitinous organisms such as fungi, protozoa, and algae, which are their major sources of nutrients and/or pathogens (Edwards et al. 1988). It is commonly believed that chitinase supports digestive functions for the hydrolysis of dietary materials, and is a host defense factor against chitin-containing pathogens (Kim et al. 2016).

Since the fundamental knowledge as to the presence (Tracey 1951) and origin (Parle 1963) of the earthworm chitinase was reported in the mid-twentieth century, little information has been accumulated on its molecular level properties, preventing us from further understanding its biological significances in terms of earthworm nutrition and self-defense. Recently, two GH18 family chitinase genes from two sibling earthworm species (*Eisenia andrei* and *Eisenia fetida*) were found to exhibit 99% amino acid sequence similarity, with *EaChi* from *E. andrei* being mainly expressed in the gut epithelium and epidermis, suggesting that its prime functions are related to digestion and self-defense immunity against chitin-containing pathogens (Kim et al. 2016; Ueda et al. 2017). However, the direct relationship of earthworm chitinase with innate immunity has not yet been identified.

In this study, RNA sequencing (RNA-seq) and real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) were used to quantitatively evaluate mRNA expression changes in response to bacterial stimulation. To our knowledge, this is the first report providing direct evidence that multiple earthworm chitinases are bacteria-responsive, strongly suggesting that earthworm chitinases are inducible humoral effectors in earthworm innate immunity.

Materials and methods

Animals

Sexually mature earthworms *Eisenia andrei* with well-developed clitella were obtained from a commercial source (Seoul, Korea) and reared as described previously (Park et al. 2017). Before use, earthworms were placed on moistened filter paper in Petri dishes with earthworm saline (Storey 1989) for 48 h to purge the gut contents and avoid sample contamination.

Microbial challenge and sample collection

After purging the gut contents for 24 h, 50 µL phosphate buffered saline with or without gram-positive *Bacillus subtilis* (1×10^4 colony forming units, CFU) was administered via parenteral injection into the post-clitellum. Earthworms were then severed to obtain the middle ~ 15–20 segments of the post-clitellum for RNA isolation.

Primary sequence and phylogenetic analyses

Amino acid sequences of the GH18 family were retrieved from the GenPept database via protein Basic Local Alignment Search Tool (BLASTP) and UniProt* (http://www.uniprot.org/). Amino acid sequence alignment and phylogenetic analysis were performed using MEGA X via the neighbor joining method (https://www.megasoftware.net/) using bootstrap analysis with 1000 replicates. The phylogenetic tree was built with the GH18 family of metazoan animal models using Lophotrochozoan chitinase orthologs: *E. andrei* (A0A0N9MGM8*), *Pecten maximus* (XP_033739583), *Lottia gigantea* (XP_009063567), *Helobdella robusta* (XP_009018499), *Lingula anatina* (XP_013402309), *Capitella teleta* (ELT90300) *Mizuhopecten yessoensis* (XP_021340846) and *Crassostrea gigas* (CAI96027); Deuterostomia chitinase orthologs: *Homo sapiens* (Q13231* and P36222*), *Mus musculus* (Q9D7Q1* and Q61362*), *Gallus gallus* (F1NMM2*), and *Xenopus laevis* (Q6GP67*); with outgroups: *Cg*-Clp from *Crassostrea gigas* (Q1RQ17* and Q1RQ22*), and di-N-acetylchitobiase from *Homo sapiens* (Q01459*), and *Mus musculus* (Q8R242*). Putative disulfide bonds were predicted through the DiANNA 1.1 Web server at http://clavius.bc.edu/~clotelab/DiANNA/. Putative signal peptide sequence was predicted through the SignalP-5.0 server at http://www.cbs.dtu.dk/services/SignalP/.

RNA-seq and annotation

The previously described procedure by (Kim et al. 2020) was followed. Briefly, RNA samples were prepared according to (Bhambri et al. 2018) at 6 h post-injection with *B. subtilis* and sequenced using the Illumina HiSeq 2500 system at Theragen Etex Bio Institute (Suwon, Korea). The Illumina reads that did not meet the minimum quality score (30 per base) across the whole read including unknown nucleotides ('Ns') were removed using Trim Galore! version 0.6.2. De novo transcriptome assembly was generated

using Trinity version 2.8.5 (Haas et al. 2013). The transcripts were annotated using BLAST (standalone blastx version 2.8.1) searches against the non-redundant protein database of the National Center for Biotechnology Information (NCBI). Paired-end reads were mapped to our assembled transcriptomes using Bowtie 2 version 2.3.5 and the fragments per kilobase of transcript per million fragments mapped (FPKM) were calculated using Cufflinks version 2.2.1 (Trapnell et al. 2010).

Real-time quantitative reverse transcription PCR (qRT-PCR)

Total RNA was isolated using TRIzol (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. mRNA was selected from total RNA using biotinylated oligo (dT) primers (Promega, USA) and reverse-transcribed into cDNA using SuperScript II First-Strand Synthesis System for RT-PCR (Invitrogen, USA). qRT-PCR was performed using WizPure qPCR Master (SYBR) (Wizbiosolutions, Korea) on an Applied Biosystems StepOne Plus real-time PCR System with the following specific primer pairs: *Eachi2* (forward), 5′–AGGGTGTGCTATGTGACCAA–3′ and (reverse), 5′–CTGTGCCAACTACAGCGAAG–3′; *Eachi3* (forward), 5′–ATAGCAGTTGGTGGATGGAA–3′ and (reverse), 5′–GATGTCAACTCCGTCGAAAC–3′; *Eachi4* (forward), 5′–ACAGGTCGGCTTTCATCAAC–3′ and (reverse), 5′–AGGACTTCCTCTCCATGCTG–3′; *EanGAPDH* (forward), 5′–GATGGTCCAAGCAACAAGGA–3′ and (reverse), 5′–GATACGTTGGGAGTGGGAAC–3′. Relative mRNA quantification was conducted using the comparative $2-\Delta\Delta$Ct method (Livak and Schmittgen 2001). Due to the significant changes of *actin* and *rps20* expression in response to bacterial injection, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal control.

Results and discussion

RNA-seq showed that multiple chitinase-related mRNAs were upregulated following *B. subtilis* challenge. The three genes that most highly expressed encoded chitinases *EaChi3*, *EaChi4*, and *EaChi2* which were significantly upregulated by approximately 8-fold, 8-fold, and 2.5-fold, respectively, estimated by $\log_2$ and FPKM values (Table 1). Upregulation by RNA-seq was validated by qRT-PCR although there were differences in the degree of upregulation (Supplemental Fig. 2). Phylogenetic analysis showed that each of the earthworm chitinases clustered with those of the Clitellata and are grouped into the Lophotrochozoa clade, distinct from Deuterostomian chitinases, with a high bootstrap value of 97% (Fig. 1).

The *EaChi2* and *EaChi4* open reading frames were 1,656 bp and 1,293 bp, respectively which corresponded to polypeptides of 551 amino acids (59.6 kDa) and 430 amino acids (45.6 kDa), respectively, excluding signal peptides sequence (Supplemental Fig. 1). All three chitinases had an N-terminal glycol_18 domain with two conserved chitin-binding and chitin-catalyzing domains, and a C-terminal proline, glycine, serine, threonine (PGST)-rich domain. In addition, EaChi2 appears to possess a chitin-binding peritrophin-A domain at the end of the C-terminus (Fig. 2). Previous and present reports regarding earthworm chitinases clearly indicate that some earthworm chitinases have a chitin-binding peritrophin-A domain with five to six conserved cysteine residues (Kim et al. 2016). Although the peritrophin-A domain was initially thought to be exclusive to a range of arthropod and nematode chitinases (Tellam et al. 1999), an atypical chitinase gene (*Cg-Chit*) comprising a peritrophin-A type chitin-binding domain with a six-cysteine motif was identified from a bivalve mollusk (*C. gigas*) (Badariotti et al. 2011). This domain likely binds the solid polysaccharide chitin substrate (Arakane et al. 2003) to allow the catalytic part of the enzyme to cleave glycosidic bonds in a random endo-type of cleavage mechanism (Arakane and Muthukrishnan 2010). The cysteine residues in the EaChi2 domain probably form two intradomain disulfide bonds (Fig. 2) and provide an architectural framework upon which a variety of structures may be built which results in proteolytic resistance (Tellam et al. 1999).

Multiple sequence alignment of the catalytic domain center of glycol_18 domain with those of other lophotrochozoan chitinases displayed highly conserved chitin-binding and chitin-catalyzing domains in which the characteristic

Table 1 Top 3 upregulated chitinase-related mRNAs following *B. subtilis* challenge in *E. andrei*

Trinity ID	Accession No.	Identifier	Size (bp)	Fold change ($\log_2$)	*P* value/*E* value	Intact FPKM	Challenged FPKM
DN3614_c0_g1_il	MZ615704	*EaChi4*	1293	3.27	< 0.01/0.00	0.48	4.64
DN8211_c0_g1_il	MZ615705	*EaChi3*	1506*	3.1	< 0.01/0.00	2.74	23.59
DN2491_c0_g1_i4	MZ615707	*EaChi2*	1656	1.42	< 0.05/0.00	26.17	69.95

*Partial sequence. Each gene is a putative chitinase matched to *Eisenia fetida* (BAS18737.1)

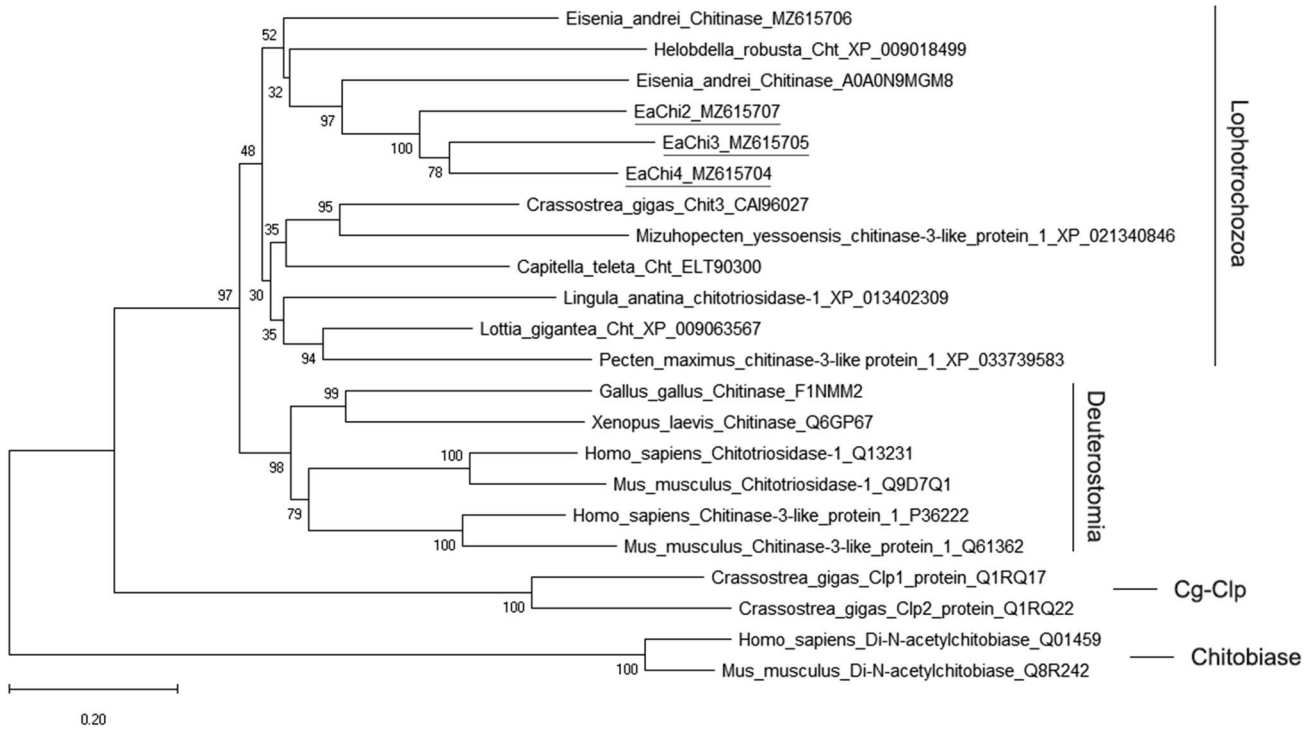

Fig. 1 Phylogenetic analysis of the GH18 chitinase family, constructed using the neighbor joining method based on the amino acid sequences of the GH18 family chitinase. All earthworm chitinases have been clustered with those of the Clitellata and can be grouped into a clade with those of the Lophotrochozoa. The mammalian chitobiases and *Crassostrea gigas* chitinase-like proteins (*Cg*-Clps) are out-grouped. The accession number of each sequence is denoted after the species name. The numbers at the nodes are scores from 1000 bootstrap re-samplings of the data. Amino-acid sequences of GH18 family were retrieved from the GenPept Database via protein Basic Local Alignment Search Tool (BLASTP) and UniProt* (http://www.uniprot.org/). The phylogenetic tree was built with the GH18 family of metazoan animal models using Lophotrochozoan chitinase orthologs: *Eisenia andrei* (A0A0N9MGM8*), *Pecten maximus* (XP_033739583), *Lottia gigantea* (XP_009063567), *Helobdella robusta* (XP_009018499), *Lingula anatina* (XP_013402309), *Capitella teleta* (ELT90300) *Mizuhopecten yessoensis* (XP_021340846) and *Crassostrea gigas* (CAI96027); Deuterostomia chitinase orthologs: *Homo sapiens* (Q13231* and P36222*), *Mus musculus* (Q9D7Q1* and Q61362*), *Gallus gallus* (F1NMM2*), and *Xenopus laevis* (Q6GP67*); outgroups: *Cg*-Clp orthologs: *Crassostrea gigas* (Q1RQ17* and Q1RQ22*), di-N-acetylchitobiase orthologs: *Homo sapiens* (Q01459*), and *Mus musculus* (Q8R242*)

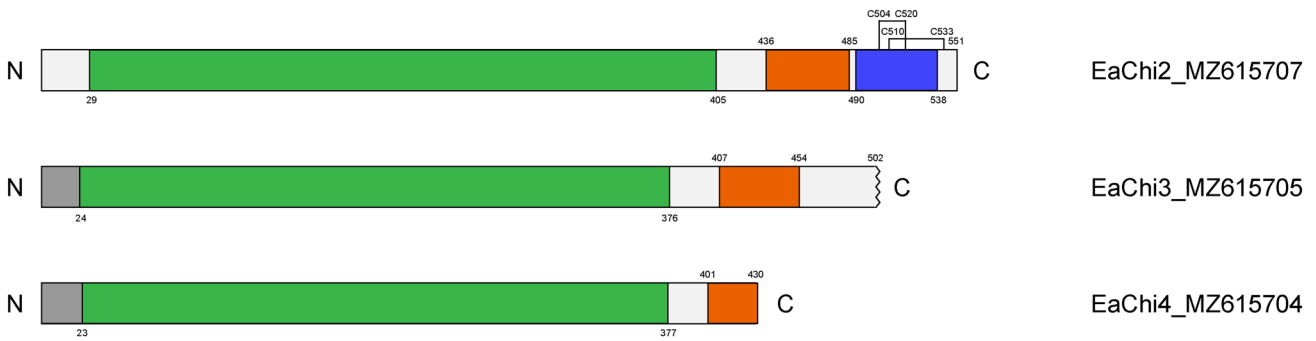

Fig. 2 Primary structures of *E. andrei* chitinases showing the arrangement of the glycol_18 domain, PGST-rich domain and peritrophin-A domain. Signal peptide sequence and protein domains are represented by the colored boxes: grey, signal peptide sequence; green, glycol_18 domain; orange, PGST-rich domain; blue, chitin binding peritrophin-A domain. Predicted disulfide bonds relevant to the peritrophin-A domain are bracketed. The indented line indicates a partial sequence (color figure online)

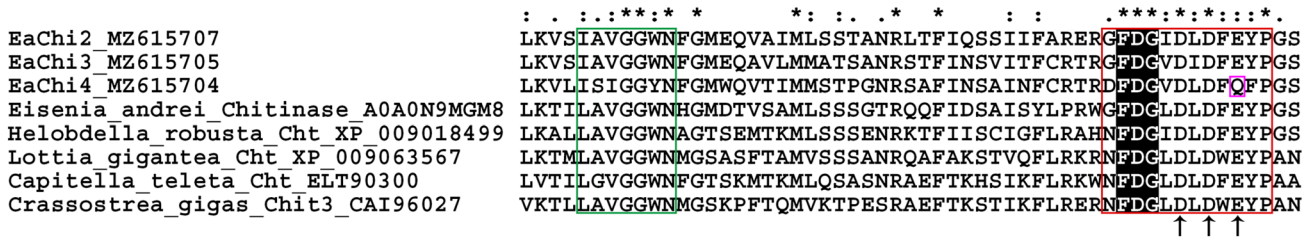

Fig. 3 Multiple sequence alignment of the EaChi catalytic domain center of glycol_18 domain with those of other Lophotrochozoan chitinases. All aligned chitinases show highly conserved chitin-binding (green box) and chitin-catalyzing (red box) domains. The characteristic FDG sequence preceding the catalytic motif is highlighted in black. The positions of essential amino acid residues for their catalyzing activities (D and E) are denoted by black arrows, and a non-conservative substitution is indicated with a magenta box. The accession number of each sequence is denoted after the species name. Conserved residues are indicated with an asterisk (*), while (:) and (.) indicate conservative and semi-conservative substitutions, respectively (color figure online)

FDG sequence and three essential amino acid residues (D, D, E in the D-X-D-X-E stretch) for catalyzing activity are well conserved. The exception was EaChi4 with a substitution of glutamic acid (E144) to glutamine (Q), (Fig. 3). The glutamate residue is critical since it is the likely proton donor required for glycosidic bond cleavage (Lu et al. 2002). This implies that EaChi4 is devoid of catalytic activity. The role of this inactive chitinase in innate immunity is unknown. However, chitinase-like proteins (*Cg*-Clp1 and *Cg*-Clp2) of a mollusk, *C. gigas*, with the same E-Q substitution as EaChi4 were transcriptionally upregulated in hemocytes in response to bacterial lipopolysaccharide challenge (Badariotti et al. 2007a). This strongly suggests that these proteins fulfil an important function as immunity regulators and/or effectors in mollusks. In *Drosophila*, CLPs belonging to the GH18 family retain structural similarity to chitinases but lack enzymatic activity. They are involved in innate immunity as pattern recognition molecules that bind chitin or related carbohydrates on the surface of nematodes and other parasites, and activate immune effector mechanisms (Kucerova et al. 2016; Sutherland et al. 2014). Moreover, coelomic cytolytic factor (CCF) displays significant homology with the catalytic motif of β-1,3-glucanase and β -1,4-glucanase and acts as a pattern recognition molecule in earthworm innate immunity despite lacking glucanase activity (Beschin et al. 1998; Prochazkova et al. 2020). Therefore, it is worthwhile testing whether earthworm EaChi4 functions as a pattern recognition molecule and modulates the downstream immune pathway.

Although the chitinase has widely different functions involved in digestion, molting processes, defense/immunity and growth/development (Kim et al. 2016), accumulating evidence in many invertebrates shows that the chitinase and chitinase-like protein are actively implicated in innate immunity as an immune effector or immune modulator (Liu et al. 2021). In a cnidarian, a chitinase has been reported to exhibit a dual role in pattern formation and immunity, based on its expression pattern in the basal portion of the polyp, the likely origin for most pathogens (Mali et al. 2004). Moreover, in the oyster, another lophotrochozoa evolutionally close to the earthworm, GH18 chitinase could be transcriptionally induced in hemocytes by challenging of bacteria and lipopolysaccharides, indicating that this enzyme could play a significant role as an immunity effector (Badariotti et al. 2007b). Very recently, a crustacean chitinase gene, *PcChitinase 2*, was unequally transcribed in different tissues with showing the major expression in hepatopancreas and its expression was significantly upregulated by the challenge with lipolysaccharide or peptidoglycan. In addition, the knockout of the *PcChitinase 2* gene increased the expression of most Toll-pathway-related immune genes, suggesting that this chitinase 2 may be involved in the innate immune responses by modulating the toll pathway (Liu et al. 2021). In insect, *Glossina morsitans*, fat body-specific chitinase gene may have a role in immune defense against chitin-containing pathogens. Similar defense roles for other chitinases from other insects are likely, but this possibility has not been investigated in detail (Arakane and Muthukrishnan 2010). This study showed that multiple earthworm chitinases are implicated in innate immune responses. However, the receptor-ligand specificity and intracellular signaling pathways implicated in inducing the immune responses remain to be elucidated.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s13258-021-01183-z.

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Declarations

Conflict of interest No potential conflict of interest was reported by the authors.

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