Differential expression of genes associated with low-level vancomycin resistance in *Staphylococcus aureus*

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ABSTRACT.

Heterogeneous vancomycin-intermediate *Staphylococcus aureus* (hVISA) and vancomycin-intermediate *S. aureus* (VISA) have been associated with treatment failure, and hVISA cannot be detected by routine disc diffusion test. Currently, there is no specific genetic marker for detection of these strains. The aim of the current study was to investigate the differential expression of six genes (viz., *isaA, atl, rplN, pbp4, walK, walR*) in VISA compared to those of hVISA and vancomycin-susceptible *S. aureus* (VSSA) isolates. The expression of six genes of 69 MRSA isolates (26 VSSAs, 28 hVISAs, and15 VISAs) was studied by using real-time PCR. The mean respective relative expression (i.e., the relative quantification (RQ) value) for five (*isaA, atl, pbp4, walK, walR*) vs. four (*isaA, atl, walK, walR*) genes in the VISA group was significantly higher than those in the VSSA and hVISA groups. In contrast, the respective mean RQ value for all genes in the hVISA and VSSA groups showed no significant difference. The mean RQ value for *isaA* in the VISA group showed the highest increased level compared with those in the hVISA (5.3-fold) and VSSA (9.1-fold) groups. The expression of six genes in VISA and hVISA compared to

VSSA strains were analysed and found that *isaA* was up-regulated in VISA compared to hVISA and VSSA. The variation in gene expression levels reflects the diversity in genetic alterations of hVISA and VISA, involving the development of reduced vancomycin-susceptibility among hVISA and VISA.

Keywords: Antimicrobial susceptibility; *Staphylococcus aureus*; Vancomycin resistance; Relative gene expression; Heterogeneous vancomycin-intermediate *S. aureus*

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) is an important cause of serious bacterial infections. The widely used of vancomycin for many decades leading to the immerging of MRSA with intermediate resistance to vancomycin (VISA) in 1997 (Hiramatsu et al., 1997). Though such strains are designated as 'intermediately resistant', they are practically resistant to the concentrations of vancomycin used in clinical therapy (Fridkin et al., 2003). VISA strains are frequently generated from MRSA strains which are heterogeneously resistant to vancomycin or hetero-VISA (hVISA). The hVISA isolates contain sub-populations that exhibit reduced susceptibility to vancomycin while their vancomycin minimum inhibitory concentrations (MICs) are within the susceptible range (<2 µg/mL) (Hiramatsu, 2001). These organisms are clinically important because they can persist in hospital environments and become VISA during vancomycin therapy in patients infected with hVISA (Liu, 2003). Now-a-days, hVISA cannot be detected by routine susceptibility test, thus several screening methods have been proposed (Satola et al., 2011). A confirmatory test of population analysis profile with area under the curve (PAP-AUC) is still needed albeit it is not appropriate for routine service because it is laborious and time-consuming (Wootton et al., 2001; Howden et al., 2010). The resistance mechanism of the hVISA and VISA phenotypes is related to the functional alteration of several metabolic genes especially those involving the alteration of cell wall biosynthesis and thickening (Cui et al., 2003). The specific genes contributing to low-level vancomycin-resistance have not yet been elucidated. Cumulative mutations in diverse genetic pathways have been found when vancomycin-susceptible strains were transformed to hVISA and VISA (Mwangi et al., 2007; Howden et al., 2008; Cui et al., 2009). Several point mutations in the regulatory system modulating cell wall metabolisms were reported (i.e., the *walRK*, *graSR* and *agr* quorum sensing system) (Mwangi et al., 2007; Howden et al., 2008; Cui et al., 2009). Proteomic analysis of hVISA demonstrated that there is an up-regulation of *isaA* gene compared to vancomycin-susceptible *S. aureus* (VSSA) (Stapleton et al., 2007; Matsuo et al., 2011; Chen et al., 2013). In the current study, the respective quantitative expression of 5 genes (*isaA, atl, walK, walR, pbp4*) related to bacterial cell wall metabolism and one related to peptide formation (*rplN*) were determined among hVISA, VISA and VSSA isolates. This study could provide a broad view of the expression level of these selected genes among *S. aureus* strains with different resistance to vancomycin and might be useful in categorization of hVISA, VISA and VSSA.

MATERIALS AND METHODS

Ethics approval

This study was approved by the Ethics Committee of Khon Kaen University (HE552272).

Bacterial strains

A total of 69 MRSA isolates collected from patients at Srinagarind Hospital between 2002 and 2011 were studied. All isolates were identified by Gram staining and biochemical testing (catalase, coagulase, DNase, and mannitol fermentation) and confirmed by detection of either *femA* or *nuc* gene using PCR (Berger-Bachi et al., 1989). Methicillin-resistance was identified by detection of the *mecA* gene using PCR (Kondo et al., 2007). The strains were determined for Staphylococcal Cassette Chromosome (SCC) *mec* types and *agr* types according to previous reports (Lina et al., 2003; Kondo et al., 2007). Genomic DNA used for all amplifications was extracted

using achromopeptidase (Wako Chemicals, USA) (Shittu et al., 2004). Each isolate was classified as VSSA, hVISA and VISA by determining the minimum inhibitory concentration (MIC) of vancomycin by using an agar dilution method, together with one point analysis, a screening method for hVISA and VISA, and then confirmed by PAP-AUC (Wootton et al., 2001) using Graph Pad Prism 5.0.1 (GraphPad Software Inc., San Diego, USA) for determining the AUC. The PAP-AUC ratios of the test isolate and that of the reference hVISA isolate (Mu3) were calculated. The isolate which had PAP-AUC ratio of 0.90 –1.30 and > 1.30 was interpreted as hVISA and VISA respectively. All isolates were kept at -20°C in skimmed milk containing 20% glycerol until used. *S. aureus* ATCC29213, *S. aureus* ATCC700698 (Mu3), and *S. aureus* ATCC700699 (Mu50) were used as the control VSSA, hVISA and VISA strains, respectively.

Antimicrobial susceptibility testing

The MICs to vancomycin for all of the isolates was determined by an agar dilution method according to the CLSI standard procedure (Clinical and Laboratory Standards Institute, 2010).

RNA extraction and cDNA synthesis

An aliquot of overnight culture was diluted 1:50 in Trypic soy broth (Oxoid, UK) and grown to the exponential phase, which was determined by measuring the OD600 at 0.4 (The Ultrospec™ 1100 pro UV/visible spectrophotometer, GE Healthcare). The bacterial cells were harvested and lysed by incubation with 10 µLof 10 U achromopeptidase at 55°C for 10 min. After incubation, the total bacterial RNA was extracted from the other bacterial components using TRIzol® Reagent (Invitrogen, Australia). The bacterial RNA was re-suspended in 30 µL RNase-free water (Invitrogen). The contaminated bacterial DNA was further eliminated using the Turbo DNA-free Kit (Invitrogen) as per the manufacturer's instructions.

The cDNA was synthesized using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystem). Ten microliters of total RNA was reverse transcribed according to the manufacturer's instructions in a total volume of 20 µL. All cDNA samples were stored at -20°C until used.

Quantitative Real-Time PCR

Relative gene quantifications were performed using a QuanStudioTM 6 Flex Real-Time PCR System (Applied Biosystems). Primers were designed from the corresponding gene sequences derived from GenBank sequences (http://www.ncbi.nlm.nih.gov) using Beacon Designer 8.12 software. The multiple sequence alignment was done using Florence Corpet (http://multalin.toulouse.inra.fr/multalin/). The OligoAnalyzer 3.1 (http://sg.idtdna.com/calc/analyzer) was subsequently used to analyze for potential hairpin-loops, primer-dimers, and hetero-dimers. Gene specificity of all primers was confirmed using BLAST (http://ncbi.nlm.nih.gov/BLAST). All sequences of the primers used in the current study are shown in Table 1.

Table 1. List of primers for real-time quantitative reverse transcriptase – PCR

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')	Location[a]
isaA	GCAGTATTACCATTGCTTA	GCACAATCAAGTAACTCA	2648126-2648827
atl	ACACCGTTTAATGATGAAA	GTTGGGTAAGTAAAGCATA	1026342-1030088
rplN	GTGCTAATGATACGATTTTCA	TGACGGTTCATACATCAA	2303125-2303493
pbp4	CGGCATTACTAGAATTAGATA	CTACCTGAGTTGAGTAATAC	690688-691983
walK	GTGACTGAACAACAACAA	TGGTGCTAGTTCTTCATC	25100-26926
walR	CCAGACATCGTATTATTAGATA	GCAGTAAGCATTATAATTGG	24386-25087
16S rRNA	GCCTAATACATGCAAGTC	CCAGTCTTATAGGTAGGTTA	

[a] Sequences are derived from GenBank accession number BA000018.3 for the *pbp4*, *atl*, *rplN*, *isaA*, and *16S rRNA* genes and CP002114.2 for the *walk* and *walR* genes. The location refers to the position of each amplicon located within its corresponding sequence.

Real-time PCR reaction was performed using Power SYBR® Green PCR Master Mix (Applied Biosystem). The total volume of each reaction was 20 µL, containing 10 µL of Power SYBR® Green PCR Master Mix, 2 µL of cDNA template, and 0.2 µM final concentration of each primer. The cycle conditions were: 10 min at 95°C; followed by 45 cycles of 20 s at 95°C; 20 s at 53°C; and 30 s at 72°C and a final cycle of 15 s at 95°C; 1 min at 60°C; and 15 s at 95°C. The expression experiment was done in triplicate. The relative quantification (RQ) of gene expression was calculated based on the comparative Ct method (Livak and Schmittgen, 2001) using QunastudioÔTM Real-Time PCR Software version 10.2 (Applied Biosystem®). In brief, the mean of the replicated Ct values of each tested genes were normalized by subtraction with those of their *16S rRNA* gene acted as internal control gene. These normalized values were then subtracted to those normalized values of the respective gene of the control VSSA strain (i.e., *S. aureus* ATCC29213) and displayed as the RQ value.

Statistical analysis

All data are presented as means ± their respective standard error of the mean (SEM). Statistical analyses were performed using One-Way ANOVA followed by an least significant difference (LSD) post-hoc test using SPSS version 19.0. A p value of <0.05 was considered statistically significant.

RESULTS

Characteristics of the bacterial isolates

All 69 MRSA isolates were found to carry the *femA* and *mecA* genes. The respective PAP-AUC ratio ranges for the VSSA, hVISA and VISA groups were 0.42-0.87, 0.9-1.27, and 1.35-3.91. These represented 26 VSSA, 28 hVISA and 15 VISA isolates. The respective ranges for vancomycin MICs were between 1-2, 1-3, and 4->16 µg/mL (Table 2). The genotypes of the isolates were SCC*mec* III *agr* I for 49 isolates, SCC*mec* II *agr* II for 15 isolates, SCC*mec* II *agr* I for 3 isolates and SCC*mec* III a*gr* II for 2 isolates.

Table 2. Phenotypic characteristics of the VSSA, hVISA, and VISA isolates

Isolates (n)	PAP-AUC ratio range (mean±SEM)	Vancomycin MIC range ($MIC_{50/90}$) µg/ml
VSSA (26)	0.42-0.87 (0.61 ± 0.03)	1-2 (1/2)
hVISA (28)	0.9-1.27 (0.99 ± 0.02)	1-3 (1/2)
VISA (15)	1.35-3.91 (2.26 ± 0.30)	4->16 (7/15)

Relative quantification of the 6 genes in the VISA, hVISA, and VSSA groups

The respective expression of the six genes (i.e. *isaA, atl, walK, walR, pbp4, and rplN*) among the VSSA, hVISA and VISA isolates was determined by using real-time RT-PCR and displayed as RQ value. The mean±SEM of RQ values of all six genes and the fold-increase in the mean RQ values among *S. aureus* groups were shown in Table 3. The mean RQ values for all of the tested genes in the VISA groups were significantly higher than those of the VSSA and hVISA groups except for *rplN* in both the VSSA and hVISA groups and *pbp4* in the hVISA group. The respective mean RQ values of *isaA* and *atl* expression in the VISA group were 9.1- and 5.6-fold of that in the VSSA and 5.3- and 3.1-fold of that in the hVISA. The respective mean RQ values of *walK, walR,* and *pbp4* in the VISA group was 2- to 3-fold of those in the VSSA and hVISA groups. However, no significant difference was observed in the mean RQ value of *rplN* among the three groups and that of *pbp4* in the VISA and hVISA groups. Upon comparing the mean RQ values for all six genes of hVISA with those of VSSA, no significant difference was found. Figure 1 presents the relative gene expression as the RQ value of the six genes among the three bacterial

groups. In addition, it should be noted that no correlation of the expression levels of the studied genes with neither MIC nor SCC*mec* type among the strains in three groups (data were not shown).

Table 3. Comparison of the mean of relative expression of six genes expressed as RQ value in the VISA, hVISA and VSSA groups

Gene	RQ value (mean±SEM)			Fold increase (*p*-value)*		
	VSSA (n=26)	hVISA (n=28)	VISA (n=15)	VISA vs hVISA	VISA vs VSSA	hVISA vs VSSA
isaA	0.875 ± 0.123	1.492 ± 0.500	7.956 ± 3.044	**5.3 (0.001)**	**9.1 (< 0.001)**	1.7 (0.692)
atl	0.407 ± 0.117	0.743 ± 0.322	2.281 ± 0.769	**3.1 (0.009)**	**5.6 (0.002)**	1.8 (0.494)
rplN	0.478 ± 0.096	1.077 ± 0.291	1.030 ± 0.233	0.9 (0.890)	2.2 (0.130)	2.3 (0.052)
walK	1.994 ± 0.318	3.491 ± 0.988	7.057 ± 2.097	**2.0 (0.033)**	**3.5 (0.003)**	1.8 (0.286)
walR	4.665 ± 0.912	6.756 ± 1.183	13.567 ± 3.765	**2.0 (0.013)**	**2.9 (0.002)**	1.5 (0.360)
pbp4	1.566 ± 0.308	3.004 ± 0.703	5.616 ± 2.922	1.9 (0.165)	**3.6 (0.035)**	1.9 (0.367)

n = Number of isolates

p< 0.05 = Significant difference

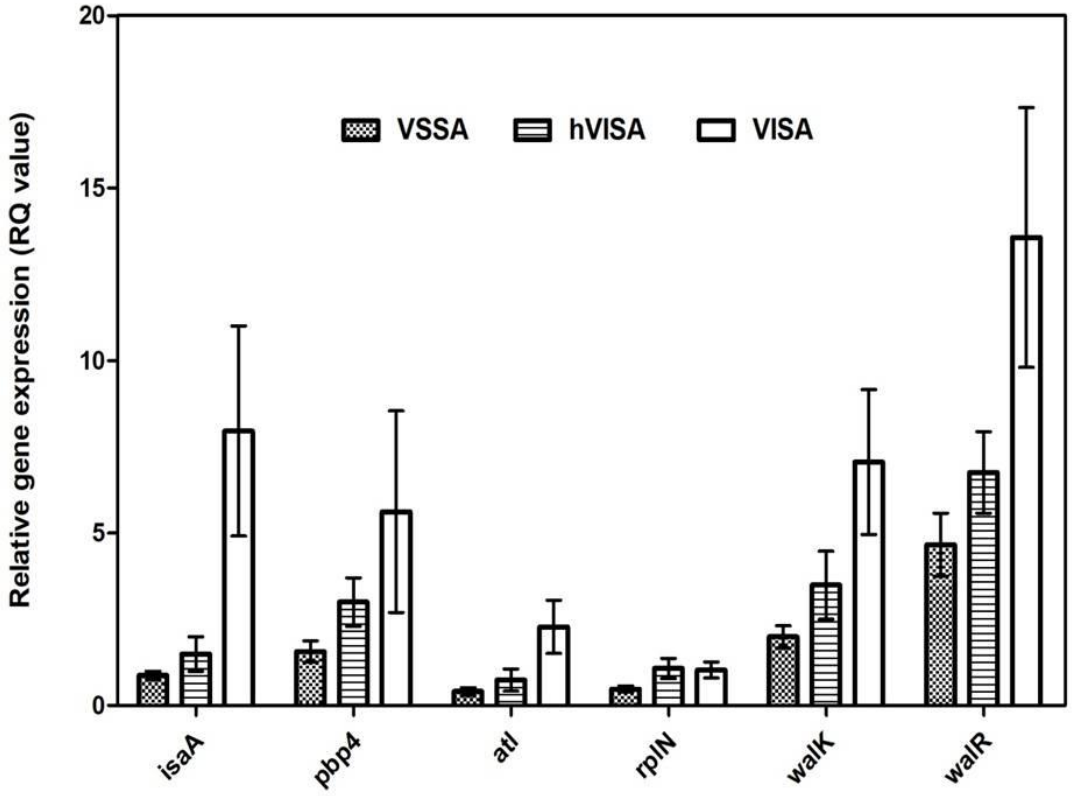

Figure 1. The relative *isaA, pbp4, atl, rplN, walK,* and *walR* gene expression as determined by quantitative real-time PCR and normalized to*16S rRNA* gene expression in the VSSA, hVISA and VISA groups. Data are expressed as RQ values and the mean±SEM values

Using a cut-off value based on an RQ value above the mean + 2 SEMs of the VSSA group, we found more than half of the VISA isolates for all genes fell into this group (except for *pbp4*). Six, 10, and 11 of the 15 VISA isolates (40%, 66.7%, and 73.3%) had RQ values for *pbp4, rplN, and walR* above the cut-off values respectively. Nine VISA isolates (60%) had RQ values for *isaA, atl*, and *walK* above the cut-off values. In contrast, less than half

of the hVISA isolates had RQ values for all the genes above the cut-off values. Based on the cut-off values for the six genes, 13 of 15 (86.7%) VISA and 17 of 26 (60.7%) hVISA isolates had at least one gene with an RQ value above the RQ cut-off value.

DISCUSSION

The definite molecular pathway of hVISA/VISA occurrence from VSSA remains unclear. It has been reported that MRSA strains with low level vancomycin-resistance have undergone metabolic changes; such as, slowed growth rate, increased autolytic activity, and decreased *agr* expression (Lina et al., 2003). In addition, reduced the function of the *agr* operon leads to the reduced RNAIII expression and a reduction of certain virulence gene (s) (i.e.,*spa, hla*), (Sieradzki and Tomasz, 2003; Peleg et al., 2009) which facilitate bacterial evasion of the host immune system (Boisset et al., 2007). Point mutations, moreover, were found on the regulatory genes *walKR* and *rpoB* and other effector genes among the induced and natural VISA strains (Ruef, 2004; Gardete et al., 2012). The present study documented a significant difference in the mean expression of *isaA* and *atl* in VISA which were 5.3- and 3.1-fold of that in hVISA, and 9.1- and 5.6-fold of that in the VSSA. The significant increase in *isaA* expression in the VISA but not significant between hVISA and VSSA strains was partially consistent with a previous report that found an up-regulation of the IsaA protein in hVISA compared with the isogenic VSSA from the same patient (Chen et al., 2013). IsaA is a lytic transglycosylase enzyme which hydrolyzes peptidoglycan allowing cell growth, division, (Stapletonet al., 2007) including septation, while Atl—the most predominant peptidoglycan hydrolase in staphylococci with an amidase domain and a glucosaminidase domain—functions as an autolysin, degrading the peptidoglycan cell wall layer (Vidaillac et al., 2013).

This bifunctional hydrolase plays a key role in (a) bacterial cell wall metabolism, (b) daughter-cell separation, and (c) antibiotic mediated cell lysis (Vollmer et al., 2008). Increasing atl expression possibly reflects an increase in cell wall turnover rates. The increase of *atl* expression in VISA and hVISA strains in the current study is similar to a previous report in which was found an abundant increase of peptidoglycan hydrolase and transglycosylase protein in the cell envelope fraction of a VISA strain compared to the isogenic hVISA and VSSA from the same patient (Ramadurai et al., 1999). Notwithstanding, Utaida et al. (2006) reported a reduction in the whole-cell autolytic activity of the VISA strain Mu50. The discrepancy may be due to the different approach used in their study and/or to the complexity of cell wall synthesis/remodeling autolytic regulation. Such findings, however, imply that VSSA may develop into hVISA and VISA via differentiation through various metabolic pathways. In the current study, *rplN* coding for RplN protein—which functions as a peptidyltransferase forming peptide bonds between adjacent amino acids (Garret and Grisham, 2002)—showed no significant difference in mean *rplN* expression among the three groups. In our previous proteomic study, we found that VISA group had higher RplN expression than the isogenic hVISA and VSSA groups (Sirichoat et al., 2016). The discordance results of *rplN* expression in both studies may be due to the difference of study technique (gene expression vs. protein expression) and the phase of bacteria used in the studies (exponential vs. stationary phase). The histone-like protein (Hup) and RplN are important for optimal survival of bacterial cells in the stationary phase and under stress conditions (Balandina et al., 2002).

PBP4 of *S. aureus* is a carboxypeptidase needed for the secondary cross-linking of peptidoglycan (Henze and Berger-Bachi, 1995). It is a member of the enzyme group for synthesis and modification of cell wall. PBP4 overexpression causes an increase in β-lactam resistance and greater cross-linking of the peptidoglycan (Henze and Berger-Bachi, 1996). In the present study, the VISA and hVISA had a 3.6- and 1.9-fold increase in *pbp4* expression over that of the VSSA. This finding agrees with a previous study that reported cell wall thickening of VISA and hVISA, which may be the result of increasing expression of *pbp4* (Hiramatsu, 2001). This is in agreement with the finding that PBP4 activity was related to peptidoglycan cross-linking (Piepeet al., 2006) which might be involved in cell wall thickening. Sieradzkiet al. (1999) however, found a reduction in *pbp4* activity in the VISA strain from a patient with a persistent infection.

The *walRK*—a two-component regulatory system—is a key regulator of cell wall metabolism in *S. aureus* (Atilinoet al., 2010) and different point mutations in these genes were frequently found among hVISA/VISA strains (Stock et al., 2000). Although the sequences were not determined in the current study, an approximate 2- to 3.5-fold increased expression of the *walRK* genes was found among the VISA/hVISA strains which may have influenced the expression of other genes such as atl (Howden et al., 2011). Cameron et al. (2016) however, found *YycHI* mutations among VISA strains, leading to a reduction in *walRK* activation. It is possible that mutations occurring in different positions within these genes may have an influence on the level of *walRK* expression (Stock et al., 2000). Besides the genes described above, there are a number of genes that have been reported to be associated with vancomycin resistance in *S. aureus* especially the VraSR operon which regulates cell wall stress stimulon was reported to up-regulate in VISA strains (Kuroda et al., 2003). However, this gene was not investigated in the present study.

Using a cut-off value based on the RQ mean+2 SEM of the VSSA group, the simultaneous increase in expression of the 6 genes was found in 1 VISA isolate; of 5 genes was found in 1 VISA; of 4 genes was found in 2 VISA and 3 hVISA; of 3 genes was found in 1 VISA and hVISA each; of 2 genes was found in 1 VISA and 3 hVISA. This finding reflects the diverse gene expression patterns among the VISA/hVISA isolates and suggests that VSSA strains are able to transform to hVISA/VISA strains through various diverse genetic pathways. In addition, mutation in the involved genes and/or their corresponding regulatory region may play a vital role in the development of hVISA/VISA strains (Howden et al., 2008). Further study is needed with larger sample sizes of VISA, hVISA, and VSSA in order to analyze for possible gene expression patterns present among these groups, which might be used for discriminating between the vancomycin-susceptible and non-susceptible *S.aureus* strains and/or lead to a better understanding on the intricate genetic factors involved in the development of vancomycin resistance in *S. aureus*.

CONCLUSION

In conclusion, we analyzed the expression of six genes in VISA and hVISA compared to VSSA strains and found that in particular *isaA* was up-regulated in VISA compared to hVISA and VSSA. Due to the complex and unknown nature of the molecular mechanism for developing VISA and hVISA, further study with more number of all strains are required to find a particular set of diagnostic marker genes to develop as a tool for fast and reliable diagnostics for effective treatment of patients with *S. aureus* infection.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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AUTHORS' CONTRIBUTIONS

A.L. and V.L. designed the experiments and prepared the manuscript; A.S. performed the experiments, analyzes the data and drafts the manuscript, while A.L., A.C., R.T., and S.W. performed the experiments. All authors interpreted the data, reviewed and approved the manuscript.

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