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ASSOCIATION OF RS35006907 POLYMORPHISM WITH RISK OF DILATED CARDIOMYOPATHY IN HAN CHINESE POPULATION

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ABSTRACT

Background: Several investigations have demonstrated the association of *MTSS1* with left ventricular (LV) structure and function. A recently published study has even revealed that rs35006907 was associated with both *MTSS1* expression and the risk of dilated cardiomyopathy (DCM).

Objective: Our study intended to investigate the relationship between rs35006907 and the risk of DCM in the Han Chinese population.

Methods: A total of 529 DCM and 600 healthy controls were recruited. We conducted genotyping for rs35006907 in all participants. Gene association studies were performed to assess the association between rs35006907 and the risk of DCM. A series of functional assays including western blot, realtime PCR and firefly luciferase reporter gene assays were conducted to illuminate the underlying mechanism.

Results: We found that rs35006907-A allele was significantly associated with reduced risk of DCM in additive (p= 0.004; OR=0.78; 95% CI=0.66–0.93) and recessive models (p= 0.0005; OR=0.56; 95%CI=0.41-0.78) when compared with the rs35006907-C allele. There were significant differences in the left ventricular end-diastolic diameter (LVEDD) and left ventricular ejection fraction (LVEF) between rs35006907-CC/AC and AA genotypes. Furthermore, the variant rs35006907-A allele presented lower reporter gene activity, reduced mRNA and protein expression levels when compared with the C allele.

Conclusions: Our findings demonstrated that rs35006907-C allele increased the risk of DCM in Han Chinese population. Besides, rs35006907-C displayed higher reporter gene activity and increased *MTSS1* expression in human samples.

Keywords: Dilated cardiomyopathy, risk, *metastasis suppressor 1 protein*, genetic polymorphism, rs35006907.

INTRODUCTION

Characterized by ventricular dilatation and diminished contraction, dilated cardiomyopathy (DCM) is the leading cause of chronic heart failure with high mortality worldwide [1]. The prevalence of DCM ranges from 1/2500 to 1/250 people and the cause of DCM is multifactorial, among which hereditary factors play an important role [2, 3]. More than 100 DCM-related genes have been reported, but only a handful of these have been definitively linked to human disease, including genes encoding cytoskeletal, sarcomere and nuclear envelope proteins [4, 5]. Approximately 40% of DCM cases could be explained by rare variants in cardiomyopathy-related genes, which represents the current research priority [6]. Recently, the role of common variants in DCM has been widely investigated, which could explain a portion of DCM cases without the known DCM gene variants [3, 7, 8].

MTSS1, located on human chromosome 8q24.13, is highly expressed in testis, esophagus, spleen and peripheral blood [9]. Consistent with its tissue distribution, *MTSS1* was identified involved in many cancers, including bladder uroepithelium cell carcinoma [10], acute myeloid leukemia [11], gastric cancer [12], colorectal cancer [13] and esophageal cancer [14], among which *MTSS1* always acts as a cancer suppressor. Accumulating evidence have indicated that *MTSS1* was a scaffold protein and could regulate

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actin dynamics by interacting with many partners. An actin monomer-binding site was identified to reside in the WH2 domain of *MTSS1* [15], which confirmed the association of *MTSS1* with cytoskeletal dynamics. Genetic variants in *MTSS1* might lead to structural changes of the heart and eventually DCM by destroying the cytoskeletal dynamics. Recently, a Genome-Wide Association Study (GWAS) conducted by Nay et al. has revealed that rs200712209 and rs34866937 in *MTSS1* were associated with left ventricular end-systolic volume (LVESV) and left ventricular ejection fraction (LVEF), respectively [16]. Charlotte et al. have also demonstrated the association between *MTSS1* and LV systolic function using robust rank aggregation [17]. Importantly, *MTSS1* knockout mice displayed reduced LV end-diastolic dimension and LV end-systolic dimension, as well as trends towards increased LV fractional shortening, when compared with wild-type mice. In the end, rs35006907 was demonstrated as the causal variant that links the expression level of *MTSS1* to LV systolic function [18]. Considering the importance of *MTSS1* in the heart, we attempt to investigate the association between rs35006907 and DCM among a small population of the Han Chinese and elucidate the underlying mechanisms.

MATERIALS AND METHODS

Study population

In this study, a total of 529 idiopathic DCM patients and 600 healthy controls were recruited between March 2014 and June 2017 from the Cardiology Division of Panzhihua central hospital in Sichuan. The diagnostic criteria of DCM refers to the modified version of standardized diagnostic criteria for DCM [19]. Patients with a family history of DCM, cardiac valve disease, coronary heart disease, hypertension, tachyarrhythmia, congenital heart disease, pericardial disease, acute viral myocarditis, heavy alcohol intake, skeletal myopathies, systemic diseases of a putative autoimmune origin, diabetes, and nutrition disorders were excluded from our study. Participants of healthy controls are free of cardiac disease, cardiac dysfunction, and a family history of DCM. Echocardiography was conducted for all participants to assess their heart function. 32 human heart samples used in this study were obtained between April 2015 and July 2017 from patients who received heart transplants at Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology. Our study was in accordance with the principles of the Helsinki Declaration and approved by the Review Board of Tongji College of Medicine and Panzhihua central hospital. All patients have signed the informed consent. Detailed about clinical characteristics of patients are listed in Supplemental Table 1

Genotyping

Genomic DNA extraction from peripheral leucocytes were finished using a DNA isolation kit in accordance with the protocol (TIANGEN, Beijing, China) and quantified by a NanoDrop 2000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). The final concentration of the samples ranged from 10 to 30 ng/mL. Probe and primer for rs35006907 genotyping were purchased from ThermoFisher (Assay ID: C____449430_10). The variant rs35006907 was genotyped using a TaqMan assay on the TaqMan 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA) with the following condition: 10 min at 95 °C (enzyme activation) followed by 45 cycles at 95 °C for 15 s and 60 °C for 1 min (annealing/extension).

Plasmids construction, cell culture, transient transfection, and luciferase activity assays

To investigate the effect of rs35006907 on transcription activity of *MTSS1*, we constructed the reporter plasmids using PGL3-promoter. Related primer and restriction enzyme cutting sites are shown below: Forward primer with KpnI 5' GGTACCGCACAATTTGCCAATGAGTTCAAAG 3', reverse primer with MluI 5' ACGCGTGGGAGAGGTTTACCAGCAGAAG 3'. AC16 and HEK293T were used for the luciferase reporter assay. Details about cell culture and transient transfection procedures have been described previously [20]. Cells were harvested 36 to 48 hours after transfection using the Passive Lysis Buffer (SIRIUS, Pforzheim, Germany). The data of luciferase expression levels were adjusted with reference to Renilla luciferase activity and relative to the average values of wild-type variant. Six independent experiments were conducted for each reporter to avoid potential errors.

Western blotting for *MTSS1*

A total of 20ug of protein extracts for each sample were denatured in sample buffer (SDS polyacrylamide) containing β-mercaptoethanol and electrophoretically resolved by 10% SDS-polyacrylamide gels, followed by transferring to polyvinylidene difluoride membranes. Non-specific binding sites were blocked with 5% non-fat milk for 2 h at room temperature. Subsequently, the membranes were incubated with primary antibodies (anti-*MTSS1* and GAPDH antibodies were purchased from ABclonal, article number: A11697 and AC002) overnight at 4 °C, followed by incubation with a peroxidase-conjugated secondary antibody. Bands were visualized by enhanced chemiluminescence reagents (Pierce Chemical, Rockford, IL) and quantified by densitometry.

Realtime fluorescence quantitative PCR

To determine the effect of rs35006907 on *MTSS1* expression, we collected 126 samples of peripheral blood

lymphocytes from participants undergoing coronary angiography. Total RNA was extracted from the peripheral blood lymphocytes using the RNeasy Mini Kit (Qiagen) and DNase-treated with DNA-free reagent (Promega), and then converted to cDNA in 20ul of reverse transcriptase reaction with random hexamers (Roche Diagnostics) and Moloney murine leukemia virus reverse transcriptase (Promega). The mRNA levels of *MTSS1* and GAPDH were measured using realtime fluorescence quantitative PCR with each sample in triplicate. Relevant primer sequences and detailed characteristics of individuals are shown on Supplemental Table 2 and 3, respectively. Expression of *MTSS1* relative to GAPDH was compared among individuals with different genotype.

Statistical Analysis

SPSS version 13.0 (SPSS, Inc, Chicago, Illinois) for Windows (Microsoft Corp, Redmond, WA) and Prism (GraphPad) were used for the statistical analyses in our study. Normality was assessed using Shapiro–Wilk's test with SPSS version 13.0. Polymorphism was tested for Hardy-Weinberg equilibrium (HWE) among the DCM patients and controls using χ^2 test with SPSS version 13.0. We conducted binary logistic regression to test the association of rs35006907 with DCM in different genetic models (additive, dominant and recessive models) using SPSS version 13.0. The odds ratio (OR) and its 95% confidence intervals (95% CIs) were calculated to evaluate the effect of any differences between alleles or genotypes with SPSS version 13.0. The Mann–Whitney U test was used for comparison between two continuous data with Prism (GraphPad), while categorical data were compared using Pearson's chi-square test. Data are expressed as mean ± SEM of n experiments. $P<0.05$ was considered to be significant.

RESULTS

Clinical characteristics of participants

As shown in Table 1, a total of 529 DCM and 600 healthy controls were matched by age with mean age of 56.6±10.5 and 55.8±9.6, respectively. There were no statistical differences between DCM and the control group in age (p=0.15), gender (p=0.5), SBP (p= 0.054), DBP (p= 0.09) or IVSD (p=0.3) between DCM and control groups. Compared with controls, the LVEDD and LVPWD were significantly higher in DCM group, while the LVEF was clearly lower.

Association between rs35006907 and risk of DCM

The genotype distribution was in HWE in both groups (χ^2 =2.7, p = 0.1 in DCM group; χ^2 =1.8, p = 0.18 in controls). As shown in Table 2, the frequency of A allele among DCM group was remarkably lower compared with controls (38% vs 44%). The results showed that rs35006907-A allele is significantly associated with reduced risk of DCM in additive (p= 0.004; OR=0.78; 95% CI=0.66–0.93) and recessive model (p=0.0005; OR=0.56; 95%CI=0.41-0.78) when compared with rs35006907-C allele.

Table 1. Baseline characteristics of population

Variable	DCM (n=529)	Control (n=600)	P Value
Age	56.6±10.5	55.8±9.6	0.15
Gender (males/females)	175/354	210/390	0.5
SBP (mmHg)	123±28	126±26	0.054
DBP (mmHg)	82±11	81±13	0.09
IVSD (mm), mean±SD	9.7±1.7	9.8±2.6	0.3
LVEDD (mm), mean±SD	65.3±8.4	46.04.2	<0.001
LVPWD (mm), mean±SD	9.6±1.4	9.2±1.6	<0.001
LVEF (%)	35.1±12.5	61.5±7.7	<0.001

SBP, Systolic blood pressure; DBP, Diastolic blood pressure; IVSD, end-diastolic interventricular septal diameter; LVEDD, left ventricular end-diastolic diameter; LVPWD, end-diastolic left ventricular posterior wall diameter; LVEF, left ventricular ejection fraction; SD, standard deviation.

Table 2. Association of rs35006907 with DCM.

SNP	Function	Population	MAF	Genotype			Model	P-Value	OR (95%CI)
				CC	CA	AA	Additive	0.004	0.78 (0.66-0.93)
rs35006907	Enhancer	Control	0.44	194	280	126	Dominant	0.16	0.84 (0.66-1.07)
C>A		DCM	0.38	192	268	69	Recessive	0.0005	0.56 (0.41-0.78)

Odds Ratios (ORs) and 95% confidence intervals (CIs) were obtained by logistic regression.

Correlation between rs35006907 and clinical characteristics

We attempted to explore the relationship between rs35006907 and clinical characteristics in DCM patients. As shown in Figure 1, patients carrying AA genotype displayed reduced LVEDD and increased LVEF when compared with CC (p=0.0001 for LVEDD and p<0.0001 for LVEF) and AC (p=0.003 for LVEDD and p=0.03 for LVEF) genotype. While no differences were observed in IVSD and LVPWD between different genotypes of rs35006907.

Functional Analysis

Firstly, we attempted to compare the *MTSS1* expression among 3 genotypes of rs35006907 using human heart samples. The results indicated that *MTSS1* mRNA level of CC genotype showed significantly higher than AC genotype (p=0.0028) and AA genotype (p=0.0034) (shown in Figure 2A). The difference between AC and AA genotypes had no statistical significance but showed a trend, probably owing to the limited samples. Furthermore, western blot results also demonstrated that CC genotype displayed increased protein expression when compared with the AC and AA genotypes (shown in Figure 2B).

Subsequently, we investigated the effect of rs35006907 on *MTSS1* expression in lymphocytes including 126 samples (33 CC genotype, 71 AC and 22 AA genotype). As shown in Figure 2C, *MTSS1* mRNA of participants with the CC genotype were significantly higher than AC genotype (p<0.0001) and AA genotype (p<0.0001). Similarly, the difference between the AC and AA genotypes was also statistically significant (p=0.005).

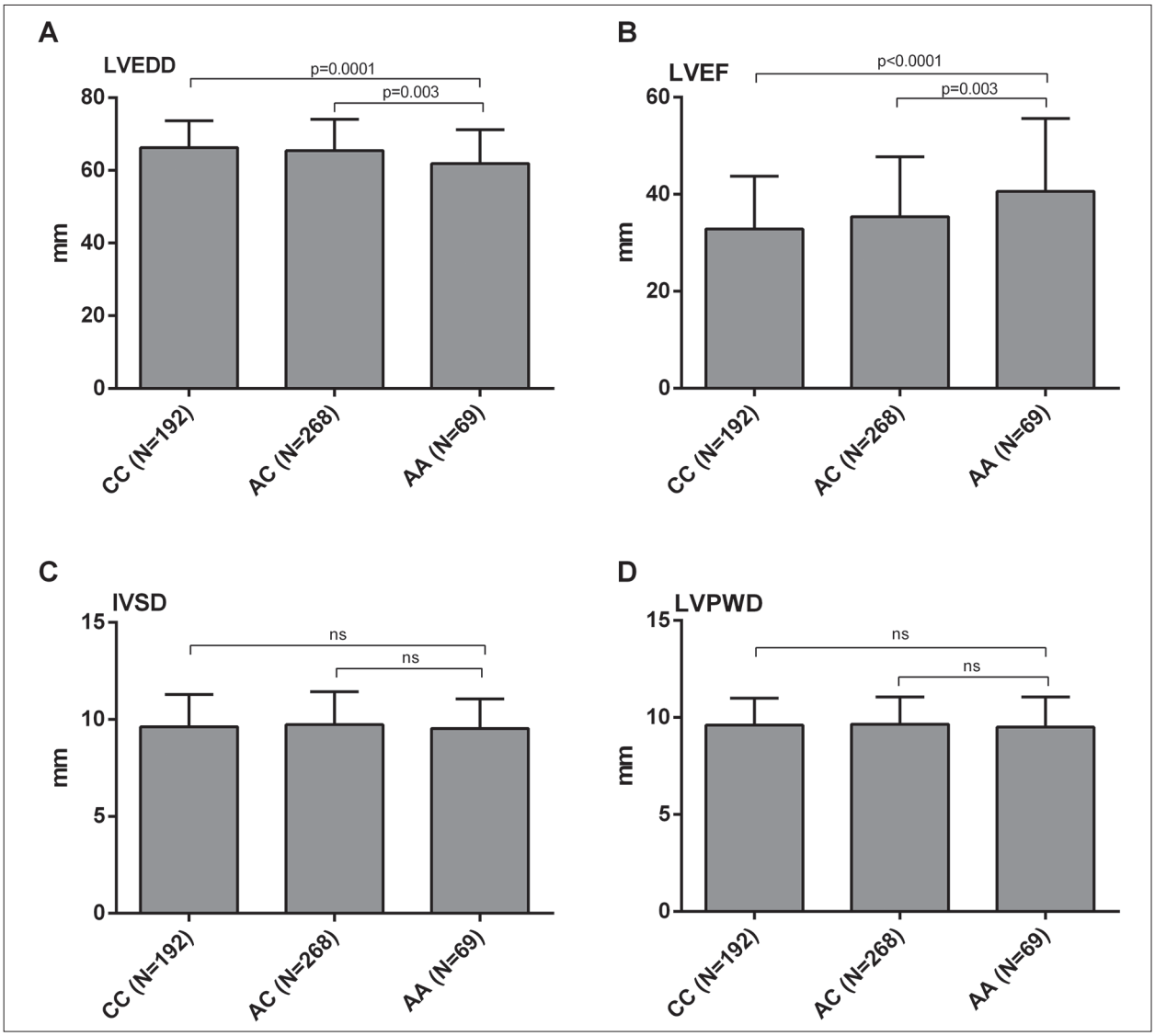

Figure 1. Comparison of clinical characteristics including LVEDD (A), LVEF (B), IVSD (C) and LVPWD (D) among DCM with different genotypes.

Finally, we transfected AC16 and 293T cells with reporter plasmids and found that the reporter gene expression of rs35006907-C allele was significantly increased compared with the rs35006907-A allele (shown in Figure 2D).

DISCUSSION

In this study, we found that rs35006907 was associated with DCM in both additive and recessive models, with the C allele increasing the risk of DCM. While the association showed no statistical significance in the dominant model. Similarly, the C allele of rs35006907 was found to be related to enlarged left ventricle and reduced cardiac function. A series of functional studies demonstrated that the rs35006907-C allele displayed higher reporter gene activity and increased MTSS1 expression when compared with A allele.

DCM is a life-threatening form of heart disease characterized by enlarged ventricle and reduced LVEF. Approximately 30-40% of DCM patients could be attributable to hereditary factors and classified as familial DCM [21]. The majority of DCM-associated genes were identified encoding proteins involved in sarcomere, cytoskeleton, nucleus, the nuclear lamina, and Z-disc [21]. First identified as a metastasis suppressor, *MTSS1* has been demonstrated to be a scaffold protein and could regulate actin dynamic [9, 22]. Pieta et al. have demonstrated that overexpression of mouse *MTSS1* (same as MIM) in NIH 3T3 cells could facilitate the formation of abnormal actin filament structures by interacting with actin monomers [15]. Similarly, the WH2 domain in the C-terminal of *MTSS1* was identified as the actin-monomer binding site [15, 22]. Importantly, many studies have demonstrated the association of *MTSS1* with cardiac structure and function using GWAS [17, 23], further proving the vital role of *MTSS1* in heart as a scaffold protein and that the overexpression of *MTSS1* is harmful. In our study, we first demonstrated higher reporter activity of rs35006907-C allele compared with the A allele. Secondly, the mRNA and protein level of *MTSS1* with rs35006907-C allele were proven significantly higher than the A allele using human heart samples and lymphocytes. These results were in line with those from Michael et al. [18]. Besides, we verified the association of rs35006907 with DCM in the Han Chinese population, which is consistent with the results from Michael et al., which show the that rs35006907-C allele represented increased risk of DCM compared with A allele and *MTSS1* knockout mice displayed better cardiac function when compared with wild-type mouse [18]. Finally, patients carrying the rs35006907-CC or AC genotype displayed

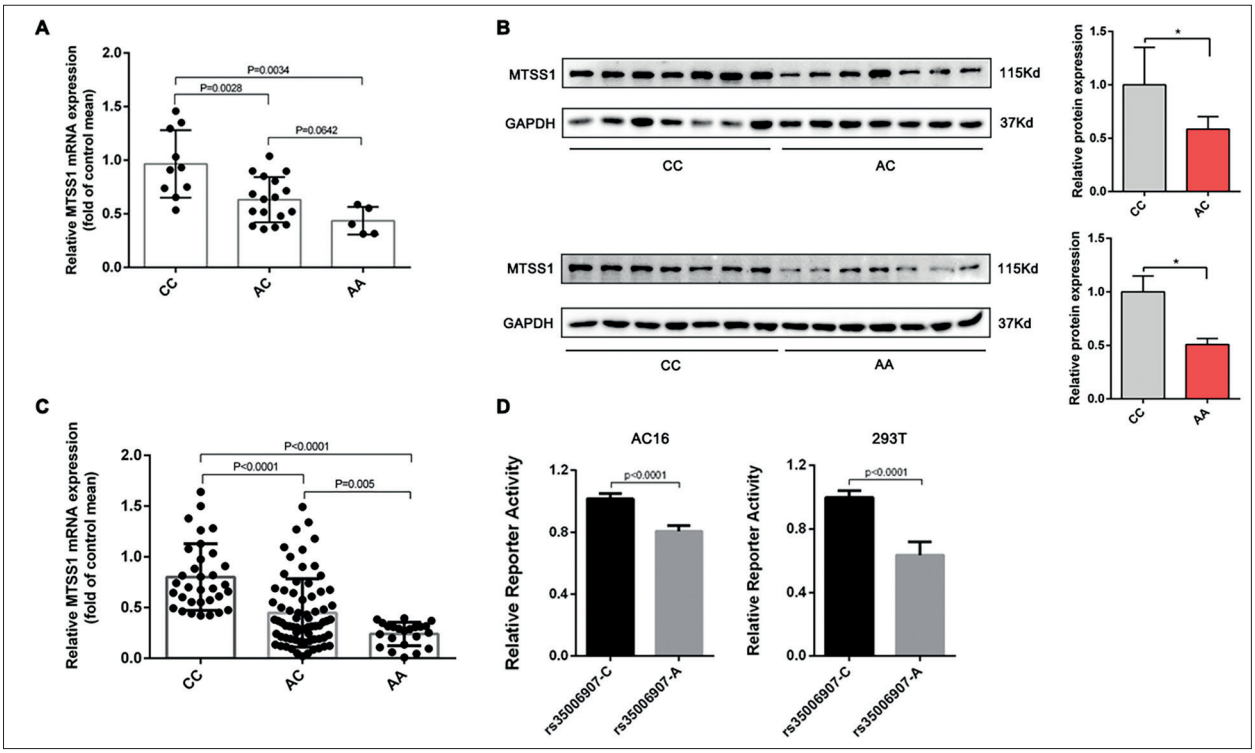

Figure 2. Functional analysis of rs35006907. A, B Expression difference of *MTSS1* mRNA and protein among three genotypes using human heart samples; **C** Comparison of *MTSS1* mRNA in lymphocytes; **D** Firefly luciferase assays using AC16 and 293T for activity comparison of rs35006907-C and A allele.

increase LVEDD and reduced LVEF when compared with the AA genotype. In brief, our results validated the genetic association between *MTSS1* and DCM in the Han Chinese population, which strongly implies the vital role of *MTSS1* in pathological mechanism of DCM. Furthermore, two missense mutations in actin were found related to DCM [24], which implies that *MTSS1* are most probably involved in development of DCM as its regulatory role for actin dynamic. However, the exploration of *MTSS1* in the pathological mechanism of DCM was lacking, needing further investigation.

One limitation of our study is that we only focus on rs35006907. Other genetic loci in strict linkage disequilibrium with rs35006907 could be the causal variants, which needs further exploration. Besides, our DCM population is limited. Although the results were in line with previous reports, additional larger studies would help verify our findings.

In conclusion, our study demonstrated that rs35006907-C allele was associated with increased risk of DCM in Han Chinese population. A series of functional investigations revealed that the rs35006907-C allele represented higher activity and increased *MTSS1* expression. Further understanding of the pathological mechanism and functional roles of *MTSS1* in DCM may promote novel genetic therapeutic interventions involving *MTSS1* in the future.

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Declaration of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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