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Associations of the polymorphisms in long non-coding RNA *H19* with hepatocellular carcinoma risk in a Southern Chinese population

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ABSTRACT

Background: Overexpression of *H19* long non-coding RNA (lncRNA) has been observed in hepatocellular carcinoma (HCC), however, the role of *H19* polymorphisms in the development of HCC was still unclear. Therefore, in this study, we aimed to explore whether the *H19* polymorphisms were related to the susceptibility of HCC.

Materials and Methods: A case-control study of 625 cases and 621 controls was conducted to investigate genetic associations of three potentially functional variants in *H19* (rs2839702, rs2067051 and rs2075745) with HCC risk in a Southern Chinese population.

Results: After adjustment for age, gender, smoking status, drinking status, HBsAg status, and family history of cancers, three *H19* polymorphisms were not associated with HCC risk under any genetic models. The odds ratio (OR) per risk allele for HCC was 1.03 (95% confidence interval [95% CI] = 0.94–1.13) for rs2839702, 1.05 (95% CI = 0.96–1.16) for rs2067051, and 1.04 (95% CI = 0.95–1.14) for rs2075745, respectively. No significant associations were also observed in the stratified analysis, haplotype analysis and combined effect analysis.

Conclusions: Our study indicated that no association of polymorphisms (rs2839702, rs2067051 and rs2075745) in long non-coding RNA *H19* with HCC. Further larger population-based case-control study and a global view to the genetic component of *H19* would be required to identify the causal genetic polymorphisms for HCC.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common primary malignancy and the third leading cause of cancer-related death [1]. Although the treatment of HCC has made great advance, the long-term survival still remains quite low, with the 5 year survival rate of 22% [2]. Thus, elucidation of the etiological factors for the development of HCC would be of great help to develop the effectively preventative and therapeutic approaches for HCC. It is well established that the occurrence and progression of HCC is a complicated process, in which multiple genetic and environmental factors are involved [3]. Among the risk factors, genetic basis could predispose to the HCC development and explain why only a fraction of individuals actually develop HCC when exposed to the same risk environment. In spite of great effort for the research of hepatocellular carcinogenesis, the underlying

molecular mechanisms contributing to HCC need to be further elucidated.

Recently, high throughput transcriptome analysis has revealed that up to 98% of human genome would transcribe into non-coding RNA. Among them, the long non-coding RNAs (lncRNAs), more than 200 bp in length [4], are broadly concerned for their pivotal role in the regulation of gene expression. Recent studies have identified the participant of lncRNAs in a wide range of biological processes, including proliferation, cell cycle, apoptosis, differentiation and invasion [5–7], and that emphasized the prominent effect of lncRNAs in cancer incidence and progression.

H19, located on chromosome 11p15.5, has attracted much interest in research of cancer etiology. The *H19* is a paternally imprinted oncofetal gene and does not encode for a protein, but instead codes for a capped, spliced and polyadenylated 2.7 kb RNA [8, 9]. Emerging evidence demonstrated the relationship between an aberrant expression of *H19* and HCC. For example, the study by Matouk et al. [10] suggested that HCC tumors transfected with *H19* siRNA exhibited significant inhibition of tumor growth, and in certain cases, there was complete inhibition of tumor formation. There was a 82% decrease of mean tumor weights and mean tumor volumes in the two transfected cell lines. Thus, *H19* exerts a tumor suppressor role in hepatocellular carcinogensis.

Multiple lines of evidence revealed that the potentially functional consequences of the genetic polymorphisms in lncRNAs affecting their structure and molecular function. The polymorphisms in the lncRNA *H19* have been investigated in breast, bladder, gastric, lung, colorectal and ovarian cancer [11–16]. However, no studies to date have reported on the association between *H19* polymorphisms and the risk of HCC. Therefore, we used bioinformatics tools to screen out three potentially functional polymorphisms of *H19* (rs2839702, rs2067051 and rs2075745). And then we carried out a hospital-based case-control study of 625 patients with HCC and 621 cancer-free controls in a Southern Chinese population to explore the role of three polymorphisms in susceptibility to HCC and the effect of three polymorphisms on HCC progression.

RESULTS

Characteristics of the study subjects

The characteristics of 625 HCC cases and 621 controls are summarized in Table 1. The distributions of gender and age between cases and controls were similar ($P > 0.05$). As expected, more HBsAg positive status proportion was observed among HCC cases compared with controls (76.21% and 31.72%, respectively). Significant associations were also seen between smoking, drinking, tumor family history and HCC (all $P < 0.01$). The proportions of TNM stages of I/II and III/IV were 13.76% and 70.72% in HCC cases, respectively. Furthermore, the proportions of metastasis and non-metastasis were 13.60% and 81.92%, respectively. Besides, the proportions of cancer embolus and non-cancer embolus were 10.24% and 84.80%, respectively.

Association analysis between the polymorphisms in *H19* and HCC incidence risk

For all the three polymorphisms, genotype frequencies in controls were in Hardy-Weinberg equilibrium (Supplementary Table 1). The distributions of genotypes and alleles in cases and controls were presented in Table 2. No statistically significant genetic association with HCC was found for all the polymorphisms (rs2839702, rs2067051 and rs2075745) under any genetic models. The odds ratio (OR) of per risk allele for HCC was 1.03 (95% confidence interval [95% CI] = 0.94–1.13) for rs2839702, 1.05 (95% CI = 0.96–1.16) for rs2067051, and 1.04 (95% CI = 0.95–1.14) for rs2075745. Moreover, further stratified analysis also showed no association between the polymorphisms and HCC risk under the recessive model for the maximum effect of rs2067051 (Table 3). No associations were also seen in the rest two polymorphisms under the recessive model (Supplementary Table 2). Additionally, the interaction analysis between gene and environment was performed to explore their role on HCC susceptibility. However, no significant interaction was observed between the rs2067051 and environmental factors (Table 3). Meanwhile, there were no significant interaction effect for rs2075745 and rs2839702 (Supplementary Table 2).

To investigate the combined genetic effect, haplotype analysis was conducted for the rs2839702, rs2067051, and rs2075745. Three haplotypes were constructed, of which, the haplotype ACA was the highest frequency (Table 4). However, no significant association of the haplotype was found with the HCC risk. We also compared the carrying numbers of risk alleles between cases and controls, but did not find any statistically significant result (Table 5).

The association analysis between the polymorphisms and clinicopathological characteristics of HCC

There were also not any statistically significant associations between the polymorphisms and the clinicopathological characteristics of HCC, including the TNM stage, metastasis and cancer embolus (Table 6).

DISCUSSION

In the present study, we conducted a hospital-based case-control study in a Southern Chinese population

Table 1: Characteristics of the patients with hepatocellular carcinoma and controls

Variables	Controls (%) *N* = 621	Cases (%) *N* = 625	*P*[*]
Mean age, years (SD)	58.5 (12.1)	58.8 (11.9)	0.608
Gender			0.707
Male	535 (86.15)	543 (86.88)	
Female	86 (13.85)	82 (13.12)	
HBsAg status			< 0.001
Positive	197 (31.72)	477 (76.21)	
Negative	424 (68.28)	148 (23.79)	
Smoking status			< 0.001
Yes	352 (56.68)	425 (68.00)	
No	269 (43.32)	200 (32.00)	
Drinking status			< 0.001
Yes	279 (44.93)	351 (56.16)	
No	342 (55.07)	274 (43.84)	
Family history[a]			< 0.001
Yes	86 (13.85)	168 (26.88)	
No	535 (86.15)	457 (73.12)	
TNM stage			
I+II		86 (13.76)	
III+IV		442 (70.72)	
NA		97 (15.52)	
Distant metastasis[b]			
Yes		85 (13.60)	
No		512 (81.92)	
NA		28 (4.48)	
Cancer embolus[b]			
Yes		64 (10.24)	
No		530 (84.80)	
NA		31 (4.96)	

Abbreviations: NA, Not available; SD, Standard deviation.

[*]The *P* values were calculated by using the student's *t* test or χ^2 test.

[a]The subjects have a family history of malignancy in the first-degree relatives.

[b]Distant metastasis and cancer embolus were determined at first diagnosis of hepatocellular carcinoma.

to explore the potential association between the *H19* polymorphisms and HCC risk. Our results demonstrated that rs2839702, rs2067051 and rs2075745 in *H19* were not associated with the risk of HCC incidence or progression among Chinese population, and negative results were also observed in all of the subgroups stratified by gender, HBsAg status, smoking status, drinking status and family history.

Recently, accumulating evidences suggested that *H19* was up-regulated in a variety of cancer types, including esophageal cancer [18], bladder cancer [19], colorectal cancer [20] and breast cancer [21]. Previous studies have indicated that *H19* involves in the complex biological process of oncogenesis [22–24]. Liang et al. reported that *H19* functioned as a competing endogenous RNA (ceRNA) for miR-138 and miR-200 and antagonized their functions, leading to the de-repression of their endogenous targets Vimentin, ZEB1, and ZEB2, all of which were core marker genes to promote epithelial-mesenchymal transition (EMT) in colorectal cancer [25]. Furthermore, *H19* has the potential to produce the "91H RNA", which regulates insulin like growth factor

Table 2: The association between the polymorphisms of *H19* and HCC susceptibility in the case-control study

Genotypes	Controls (%) *N* = 621	Cases (%) *N* = 625	χ^2	*P*	Crude OR (95% CI)	Adjusted OR (95% CI)*
rs2839702[a]						
AA	256 (41.29)	251 (40.48)			1.00	1.00
AC	290 (46.77)	286 (46.13)	0.002	0.962	1.01 (0.79–1.28)	1.04 (0.79–1.36)
CC	74 (11.94)	83 (13.39)	0.540	0.463	1.14 (0.80–1.64)	1.16 (0.77–1.74)
AC+CC	364 (58.71)	369 (59.52)	0.083	0.773	1.03 (0.82–1.30)	1.06 (0.82–1.37)
AA+AC	546 (88.06)	537 (86.61)			1.00	1.00
CC	74 (11.94)	83 (13.39)	0.591	0.442	1.14 (0.82–1.59)	1.13 (0.77–1.67)
A	802 (64.68)	788 (63.55)			1.00	1.00
C	438 (35.32)	452 (36.45)	0.344	0.558	1.05 (0.89–1.24)	1.03 (0.94–1.13)
rs2067051[b]						
CC	289 (46.61)	274 (44.26)			1.00	1.00
CT	274 (44.19)	278 (44.91)	0.320	0.572	1.07 (0.85–1.35)	1.10 (0.84–1.44)
TT	57 (9.20)	67 (10.82)	1.163	0.281	1.24 (0.84–1.83)	1.26 (0.80–1.97)
CT+TT	331 (53.39)	345 (55.74)	0.689	0.407	1.10 (0.88–1.38)	1.13 (0.87–1.46)
CC+CT	563 (90.81)	552 (89.18)			1.00	1.00
TT	57 (9.19)	67 (10.82)	0.914	0.339	1.20 (0.83–1.74)	1.20 (0.78–1.84)
C	852 (68.71)	826 (66.72)			1.00	1.00
T	388 (31.29)	412 (33.28)	1.121	0.290	1.10 (0.93–1.30)	1.05 (0.96–1.16)
rs2075745[c]						
AA	268 (43.30)	256 (41.09)			1.00	1.00
AT	281 (45.40)	288 (46.23)	0.338	0.561	1.07 (0.85–1.36)	1.11 (0.84–1.45)
TT	70 (11.30)	79 (12.68)	0.803	0.370	1.18 (0.82–1.70)	1.15 (0.76–1.75)
AT+TT	351 (56.70)	367 (58.91)	0.619	0.432	1.09 (0.87–1.37)	1.12 (0.86–1.45)
AA+AT	549 (88.69)	544 (87.32)			1.00	1.00
TT	70 (11.31)	79 (12.68)	0.554	0.457	1.14 (0.81–1.60)	1.09 (0.74–1.62)
A	817 (65.99)	800 (64.21)			1.00	1.00
T	421 (34.01)	446 (35.79)	0.874	0.350	1.08 (0.91–1.28)	1.04 (0.95–1.14)

Abbreviations: HCC, hepatocellular carcinoma; OR, odds ratio; CI, confidence interval; SNPs, single nucleotide polymorphisms.

*Adjusted ORs and 95% CIs were calculated by the logistic regression model after adjusting for age, gender, smoking status, drinking status, HBsAg status and family history.

[a]Five and one individuals were not successfully genotyped in cases and controls, respectively.

[b]Six and one individuals were not successfully genotyped in cases and controls, respectively.

[c]Two and two individuals were not successfully genotyped in cases and controls, respectively.

2 (IGF2) expression [26]. *H19* acts as an oncogene in the above cancers [10], however, is proposed as a tumor suppressor gene in hepatocellular carcinoma [27]. Based on the orthotopic xenograft experiments by Zhang et al. [27], the HCC tissue, with lower *H19* expression showed more regressive and metastatic properties. Moreover, their analysis also indicated that, by altering the epigenetic activation of miR-200 with cooperation of the protein complex hnRNP U/PCAF/RNA Pol II, *H19* could suppress HCC metastasis and cause EMT. Furthermore, c-Myc could induce the expression of the *H19* RNA and bind to the E-boxes near the imprinting control region to facilitate histone acetylation and transcriptional initiation of the *H19* gene in HCC, c-Myc could also down-regulate the expression of IGF2, the reciprocally imprinted gene at the H19/IGF2 locus [28]. Interestingly, the *H19* gene was

Table 3: Stratified analysis of rs2067051 in *H19* by participants' characteristics

Stratified variables	Genotypes	Controls $N = 621$	Cases $N = 625$	Adjusted OR* (95% CI)	P^a	P^b
Gender					0.384	0.518
Males	CC+CT	490	483	1.00		
	TT	45	56	1.31 (0.82–2.10)		
Females	CC+CT	73	69	1.00		
	TT	12	11	0.77 (0.27–2.19)		
HBsAg status					0.343	0.511
Positive	CC+CT	180	419	1.00		
	TT	17	55	1.42 (0.79–2.56)		
Negative	CC+CT	383	133	1.00		
	TT	40	12	0.96 (0.48–1.91)		
Smoking status					0.399	0.306
Yes	CC+CT	321	385	1.00		
	TT	31	37	1.01 (0.57–1.81)		
No	CC+CT	242	167	1.00		
	TT	26	30	1.48 (0.78–2.79)		
Drinking status					0.175	0.132
Yes	CC+CT	254	318	1.00		
	TT	25	29	0.86 (0.46–1.61)		
No	CC+CT	309	234	1.00		
	TT	32	38	1.56 (0.86–2.83)		
Family history					0.974	0.969
Yes	CC+CT	78	146	1.00		
	TT	8	20	1.08 (0.39–3.02)		
No	CC+CT	485	406	1.00		
	TT	49	47	1.20 (0.75–1.93)		

Abbreviations: OR, odds ratio; CI, confidence interval.

*Adjusted ORs and 95% CIs were calculated by the logistic regression model after adjusting for age, gender, smoking status, drinking status, HBsAg status and family history.

[a] *P* values for multiplicative interaction between genetic and environmental factors.

[b] *P* values for additive interaction between genetic and environmental factors.

Table 4: Haplotype analysis of *H19* rs2839702, rs2067051, and rs2075745 in cases and controls

Haplotype*	Cases, $N = 1250$ (%)	Controls, $N = 1242$ (%)	OR (95% CI)	*P*
ACA	788 (63.04)	799 (64.33)	1.00	
CTT	413 (33.04)	386 (31.08)	1.08 (0.92–1.29)	0.348
CCT	29 (2.32)	33 (2.66)	0.89 (0.54–1.48)	0.656
Others	20 (1.60)	24 (1.93)	0.85 (0.46–1.54)	0.583

Abbreviations: OR, odds ratio; CI, confidence interval.

*The alleles were in the order of rs2839702, rs2067051, and rs2075745.

Table 5: Combined effect of the 3 polymorphisms in H19 on HCC

Number of risk alleles*	Cases (%) *N* = 625	Controls (%) *N* = 621	OR (95% CI)	*P*	*P* for trend
0	254 (41.17)	247 (40.10)	1.00		0.409
1–2	36 (5.83)	25 (4.06)	0.74 (0.42–1.23)	0.221	
3–4	258 (41.82)	267 (43.34)	1.06 (0.83–1.36)	0.618	
5–6	69 (11.18)	77 (12.50)	1.15 (0.79–1.66)	0.465	

Abbreviations: OR, odds ratio; CI, confidence interval; SNPs, single nucleotide polymorphisms.
*The risk alleles were C, T, T for rs2839702, rs2067051, and rs2075745, respectively.

reported to induce P-glycoprotein expression and multi-drug resistance 1 (MDR1)-associated drug resistance at least in liver cancer cells through regulating MDR1 promoter methylation [29]. Additionally, recent studies suggested that individual therapy targeting the miRNA and lncRNA in combination may afford more curative effects on HCC, such as *H19* [27]. Despite of extensive evidence, the complex role of *H19* in tumorigenesis is still needed to be further elucidated.

Emerging evidence has implied that genetic variants in lncRNAs may modify the risk of multiply tumors [30]. Several molecular epidemiological studies have examined the association between the *H19* polymorphisms and the risks of cancers, including breast, bladder, gastric, lung, colorectal and ovarian cancer. Most reports revealed that *H19* polymorphisms (rs2107425, rs217727 and rs2839698) were associated with cancer risks [11–16, 31, 32]. Based on the Encyclopedia of DNA Elements (ENCODE) DNase I hypersensitive site (DHS) sequencing data set, *H19* polymorphisms (rs2839698 and rs217727) were found in the open chromatin regions that associated with gene regulatory elements, indicating that both of the polymorphisms may affect the binding of transcription factors. ChIP-Seq data from the ENCODE project further demonstrates that rs217727 is located in a region in where may influence the binding of numerous transcription factors. These results show that it is biologically conceivable for the polymorphisms (rs2839698 and rs217727) in *H19* to be potential causal variants that regulate the expression of *H19* and further affect cancer. However, we found rs2839698 was in strong linkage disequilibrium (LD) with rs2839702, rs2067051 and rs2075745 ($r^2 > 0.90$), and the three polymorphisms (rs2839702, rs2067051 and rs2075745) in the role of HCC was far stronger than the above three, according to the bioinformatic we found. Therefore, we aimed to explore whether the H19 polymorphisms were related to the susceptibility of HCC. Nevertheless, in our analysis, no significant association was observed between the *H19* polymorphisms and HCC. One reason for this result may be different pathological role of *H19* polymorphisms in HCC susceptibility as compare to other cancers. The precise mechanism of *H19* polymorphisms action remains unclear, and further investigations are required.

To our knowledge, this is the first investigation of the *H19* polymorphisms in the genetic etiology of HCC. Nevertheless, it should be noted that potential selection bias might occur due to the hospital-based design. Besides, although we had more than 600 pairs of HCC cases and controls, the sample size in current study might not be large enough to detect small effects from low penetrance genes. Additionally, only three common variants were assessed, and this study did not provide a global view to the genetic component of *H19* in development of HCC. And as the research further develops, researchers found that rare mutations may have a stronger causative effect. Therefore, the role of rare mutation on *H19* in HCC should be explored in the future.

In summary, our study indicated that no association of polymorphisms (rs2839702, rs2067051 and rs2075745) in long non-coding RNA *H19* with HCC. Nevertheless, larger population-based case-control studies would be required to confirm the causal genetic polymorphisms in *H19* for HCC.

MATERIALS AND METHODS

Study subjects

This case-control study was approved by the institutional review board of Guangdong Pharmaceutical University, Guangdong, China. A total of 625 HCC patients and 621 cancer-free controls were enrolled, who all have given written informed consent. HCC patients were newly diagnosed from the Shunde First People's Hospital in Guangdong Province from November 2010 to November 2014. The diagnosis of patients was confirmed by pathological examination or α-fetoprotein elevation (> 400 ng/ml) combined with imaging examination (magnetic resonance imaging or computerized tomography). During the same period of case enrollment, Controls were selected from a healthy screening at the same hospital. Controls were cancer free and frequency-matched to cases for residential area, age (± 5 years) and gender. For all subjects, data were collected in standardized interview by trained interviewers using a structured questionnaire, including demographic characteristics, smoking status, drinking status and tumor

Table 6: The associations between the *H19* polymorphisms and clinicopathologic characteristics in patients with HCC (*N* = 625)

Genotypes	TNM stage			Distant metastasis			Cancer embolus		
	I + II	III + IV	Adjusted OR* (95% CI)	No	Yes	Adjusted OR* (95% CI)	No	Yes	Adjusted OR* (95% CI)
rs2839702									
AA+AC	69 (15.13)	387 (84.87)	1.00	438 (85.21)	76 (14.79)	1.00	453 (88.65)	58 (11.35)	1.00
CC	16 (23.53)	52 (76.47)	0.59 (0.31-1.09)	69 (88.46)	9 (11.54)	0.76 (0.36-1.63)	73 (93.59)	5 (6.41)	0.55 (0.21–1.42)
rs2067051									
CC+CT	71 (84.52)	393 (89.73)	1.00	448 (88.54)	77 (90.59)	1.00	464 (88.55)	58 (90.63)	1.00
TT	13 (15.48)	45 (10.27)	0.63 (0.32-1.23)	58 (11.46)	8 (9.41)	0.76 (0.34-1.71)	60 (11.45)	6 (9.33)	0.79 (0.32–1.93)
rs2075745									
AA	30 (13.82)	187 (86.18)	1.00	208 (86.67)	32 (13.33)	1.00	210 (88.61)	27 (11.39)	1.00
AT+TT	56 (18.12)	253 (81.88)	0.72 (0.44-1.17)	302 (85.07)	53 (14.93)	1.08 (0.67-1.76)	318 (89.58)	53 (10.42)	0.91 (0.53–1.55)

Abbreviations: OR, odds ratio; CI, 95% confidence interval; SNPs, single nucleotide polymorphisms.
* Adjusted ORs and 95% CIs were calculated by the logistic regression model after adjusting for age, gender, smoking status, drinking status, HBsAg status and family history.

family history. The smokers were defined as individuals who had kept smoking almost every day for more than 1 year till the time of interview. Subjects were considered as drinkers, if they consumed 1–2 alcohol drinks per week for more than 1 year. The clinicopathological parameters of cases were collected from hospital clinical records, including HBsAg status, metastasis status, cancer embolus and TNM stage (according to Union for International Cancer Control-American Joint Committee on Cancer, UICC-AJCC, 2002). 5 ml blood sample was obtained from each subject for detection of HBV and DNA extraction.

Variants selection and genotyping

We used 1000 Genomes Project (http://www.1000genomes.org) and Ensembl database (http://asia.ensembl.org/index.html) to identify all single nucleotide polymorphisms in *H19*, and then SNP info (https://snpinfo.niehs.nih.gov/snpinfo/snpfunc.html), F-SNP software (http://compbio.cs.queensu.ca/F-SNP/) and HaploReg V4.1 tool (http://www.broadinstitute.org/mammals/haploreg/haploreg.php) were used to choose potentially functional SNPs with minor allele frequency > 0.05 in Asian populations (CHB+JPT, based on the 1000GENOMES:pilot_1). A total of three common variants, including rs2839702, rs2067051 and rs2075745, were finally selected in this study based on their potential effects on regulatory motifs (Supplementary Table 1).

Genomic DNA was extracted from peripheral blood leukocytes by TIANamp Genomic DNA Kit. Genotypes were determined by the Sequenom MassARRAY system without knowledge of subjects' status. Five percentage of samples were randomly chosen for duplicately genotyping, with the concordance rate of 100%. The average call rates for the candidate SNPs were over 99%.

Statistical analysis

Differences in the distribution of demographic characteristics and genotypes of SNPs between the cases and controls were evaluated by using the *t* test (for continuous variables) or χ^2 test (for categorical variables). Hardy-Weinberg equilibrium (HWE) for genotypes in controls was tested by the goodness-of-fit χ^2 -test. The associations between SNPs and development of HCC were estimated using the adjusted odds ratio (ORs) and their 95% confidence intervals (CIs), which were calculated in logistic regression models with adjustment for age, gender, HBsAg status, smoking status, drinking status and family history. Multiple genetic models (co-dominant, dominant, recessive and allele models) were assessed in the genetic association analysis. Stratified analysis was conducted by gender, HBsAg status, smoking status, drinking status and family history. Interaction between the polymorphisms and environmental factors were estimated using the multiplicative and additive scales. The *P* values for multiplicative interaction were calculated using the interaction term included in the multivariate logistic regression models, while the *P* values for additive interaction were estimated by binomial regression [17]. Haplotype analysis was conducted using the PHASE 2.1 program. The *P* value of less than 0.05 was considered as statistically significant. All statistical analyses were conducted in the SAS 9.4 software (SAS Institute, Inc.,Cary, NC, USA).

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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