

The Variant rs145204276 of GAS5 is Associated with the Development and Prognosis of Gastric Cancer

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ABSTRACT

Background & Aims: Down-regulation of the growth arrest specific transcript 5 (GAS5) (long non-coding RNA) is associated with cell proliferation of gastric cancer (GC) and a poor prognosis. We aimed to investigate whether the variant rs145204276 of GAS5 is associated with the prognosis of GC in the Chinese population, and to unveil the regulatory mechanism underlying the GAS5 expression in GC tissues.

Method: 1,253 GC patients and 1,354 healthy controls were included. The frequency of the genotype del/del and the allele del of rs145204276 were compared between the patients and the controls and between different subgroups of patients classified by clinicopathological variables. The overall survival rate was analyzed according to the Kaplan-Meier method using the log-rank test.

Results: The frequency of genotype del/del was significantly lower in patients than in the controls (7.0% vs. 9.1%, $p = 0.001$). Kaplan-Meier analysis showed that genotype del/del was significantly associated with a higher survival rate ($p = 0.01$). Patients with late tumor stage were found to have a significantly lower rate of genotype del/del than those with an early tumor stage (4.9% vs. 8.8%, $p = 0.01$). Patients with UICC III and IV were found to have a significantly lower rate of genotype del/del than those with UICC I and II (5.3% vs. 8.1%, $p = 0.02$).

Conclusion: The variant rs145204276 of GAS5 is associated with the development and prognosis of GC. The allele del of rs145204276 is associated with a remarkably lower incidence of cancer progression and metastasis.

Key words: Gastric cancer – GAS5 – Polymorphism – Prognosis.

Abbreviations: GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; GAS5: growth arrest specific transcript 5; GC: gastric cancer; HWE: Hardy-Weinberg equilibrium; lncRNA: long non-coding RNA; OR: odds ratio RT-PCR: Real-time PCR;

INTRODUCTION

Gastric cancer (GC) is one of the most common malignant diseases in the world [1]. To date, the precise mechanisms underlying the development of GC remain obscure, although a variety of factors involved in the progression of GC have been reported [2, 3]. As documented in previous studies, neither the histologic subtype nor the clinical classification can be used to effectively predict the prognosis of GC [4, 5]. Therefore, identification of novel

biomarkers involved in the development of GC is of great importance for the prognosis and the treatment.

Instead of the traditional concepts that only protein-coding genes are implicated in biological processes, long non-coding RNA (lncRNA) now provides a novel insight into the etiological research of a variety of cancers [6, 7]. Numerous lncRNAs have been found differentially expressed in the tumor tissues, which subsequently influenced tumor growth or metastasis [8-12]. Among these lncRNAs, the growth arrest specific transcript 5 (GAS5) was found to act as a tumor-suppressor in different types of cancer [13-16]. Down-regulation of GAS5 was also involved in the cell proliferation in GC and associated with a poor prognosis [17, 18].

It is noteworthy that few studies have specifically investigated the regulatory mechanism of GAS5 expression in GC. In earlier studies [19, 20], genetic variants in the promoter region of lncRNAs have been reported to regulate the

expression level through methylation. The variant rs145204276 is 5-bp indel polymorphism shown as „-/AGGCA”. Located in the promoter region of GAS5, rs145204276 was reported to regulate the expression of GAS5 and subsequently significantly add to the risk of hepatocellular carcinoma [20]. In the study of Zheng et al. [16], rs145204276 was significantly associated with the susceptibility for colorectal cancer. Based on these findings, we speculated that polymorphisms in the promoter region of GAS5 could be associated with the susceptibility for GC. In this study, our purposes were to investigate whether the genetic variant rs145204276 is associated with the prognosis of GC in the Chinese population, and to unveil the regulatory mechanism underlying the GAS5 expression in GC tissues.

METHODS

Participants

The current study was approved by the local institutional review board (Huai'an 1st People's Hospital No. HPH20170132). All the subjects signed the written informed consent. A number of 1,253 GC patients treated in our clinic centers between March 2006 and December 2017 were enrolled in this study. The inclusion criteria were: 1. gastric adenocarcinoma confirmed by two senior pathologists, and 2. no history of previous treatment for GC. A number of 1,354 healthy controls were recruited through the free examination program of the local community. Gastric cancer was excluded in all the controls by upper gastrointestinal endoscopy. All the subjects included in our study were inhabitants along the Yangtze River. The demographic and pathological information of the patients were collected from the medical records, including age, gender, tumor size, histologic differentiation, TNM stages, lymphatic metastasis stage, and distant metastasis. Specifically, UICC staging system (7th edition) was applied to determine the TNM stages [21]. The Lauren's classification was used to evaluate the histological subtype [22].

Procedures of genotyping

Genomic DNA was extracted from peripheral blood for each subject using a genomic DNA purification kit (Qiagen, Tokyo, Japan). The SNP rs145204276 of GAS5 was genotyped using TaqMan SNP Genotyping Assay, and the results were interpreted by Roche LightCycler 480 Real-time PCR System (Roche Diagnostics GmbH, Mannheim, Germany). For the quality control, 200 samples were randomly selected to replicate the results of genotyping, and the concordance rate was 100%.

Real-time PCR analysis

The tumor tissues and the adjacent normal tissue were consecutively collected from 154 patients with GC during surgery and stored at -80°C immediately. All the patients signed the informed consent for expression analysis. Total RNA was extracted from the tissue samples with a commercial kit (CWBio. Co. Ltd, Beijing, China). Real-time PCR (RT-PCR) was performed on Roche LightCycler 480 system (Roche Diagnostics GmbH, Mannheim, Germany) to quantify the relative expression of GAS5 with Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used as the internal control. The PCR primers were as follows:

GAS5 sense, 5'-CTTCTGGGCTCAAGTGATCCT-3' and reverse, 5'-TTGTGCCATGAGACTCCATCAG-3'; GAPDH sense, 5'-GTCAACGGATTTGGTCTGTATT-3' and reverse, 5'-AGTCTTCTGGGTGGCAGTGAT-3'. The relative expression of GAS5 was calculated and normalized using the $\Delta\Delta\text{Ct}$ method.

DNA methylation analysis

Quantitative bisulfite pyrosequencing kit (EpigenDx Inc. Worcester, MA, USA) was used to detect promoter methylation level of GAS5 for the 154 patients included in the expression analysis. A total of approximate 100 ng DNA was used for bisulfite conversion using the EpiTect Bisulfite Kit (QIAGEN, Tokyo, Japan), which was subsequently amplified using PyroMark PCR Kit (QIAGEN, Tokyo, Japan). The methylation status of the 7th CpG site in the promoter of GAS5 was analyzed using QCpG software (Qiagen Pyrosequencing) as described previously [20]. Each pyrosequencing assay was performed for at least three times. DNA methylation analysis was subsequently conducted using the Sequenom EpiTYPER system (Sequenom Inc., San Diego, California USA).

Statistical analysis

The Hardy-Weinberg equilibrium (HWE) test was performed for both patients and healthy controls by a goodness-of-fit chi-square test. For the inter-group comparison between the cases and the controls or between the subgroups of the patients stratified according to clinical features the Student's *t* test and the chi-square test were used to analyze the continuous data and the categorical data, respectively. Odds ratios (ORs) and 95% confidence intervals (95% CIs) were calculated to estimate the association between rs145204276 and the risk of development, progression and metastasis of GC. The Kruskal-Wallis test was used to compare the gene expression and the percentage of methylation among different genotypes of rs145204276. Overall survival rate was analyzed according to the Kaplan-Meier method using log-rank test. Patients were classified into the event group and the event-free group according to the 5-year survival. The intergroup comparison of GAS5 expression/methylation was investigated by the Student's *t* test. The relationship between the genotype of rs145204276 and the survival data was investigated by the chi-square test according to different tumor stages separately. A $p < 0.05$ was used as statistical significance. SPSS version 19.0 (SPSS Inc., Chicago, USA) was used for the data analysis.

RESULTS

Demographic data

Baseline characteristics and clinical features of the subjects are summarized in Table I. There was no significant difference between the cases and the controls in terms of age, gender, alcohol status and smoking status. The mean age was 50.9 ± 16.8 years for the patients and 50.5 ± 17.3 years for the controls, respectively. According to the Lauren classification, 636 patients (50.8%) had intestinal type, 471 (37.6%) had diffuse type, 107 (8.5%) had mixed type and 39 (3.1%) had intermediate type carcinomas. Regarding the tumor stage and the other tumor characteristics see Table I. Patients with UICC

Table I. Baseline characteristics of the subjects

	Patients (n = 1253)	Controls (n = 1354)	p
Age (years)			
> 50	615 (49.1%)	642 (47.4%)	0.45
≤ 50	638 (50.9%)	712 (52.6%)	
Mean ± SD	50.9 ± 16.8	50.5 ± 17.3	0.64
Gender			0.39
Male	654 (52.2%)	683 (50.4%)	
Female	599 (47.8%)	671 (49.6%)	
Smoking status			0.41
Smokers	395 (31.5%)	416 (30.7%)	
Non-Smokers	958 (68.5%)	938 (69.3%)	
Alcohol status			0.37
drinkers	326 (26.0%)	332 (24.5%)	
Non-drinkers	927 (74.0%)	1022 (75.5%)	
Tumor size (cm)			N/A
> 5	610 (48.7%)	-	
≤ 5	643 (51.3%)	-	
Lauren's classification			
Diffuse	471 (37.6%)	-	
Intestinal	636 (50.8%)	-	
Mixed	107 (8.5%)	-	
Intermediate	39 (3.1%)	-	
UICC Stages			N/A
I	395 (31.5%)	-	
II	383 (30.6%)	-	
III	323 (25.8%)	-	
IV	152 (12.1%)	-	
Lymphatic metastasis stage			N/A
N0	446 (35.6%)	-	
N1	378 (30.2%)	-	
N2	310 (24.7%)	-	
N3	119 (9.5%)	-	
Tumor Stage			
T1	303 (24.2%)		
T2	382 (30.5%)		
T3	427 (34.1%)		
T4	141 (11.2%)		
Distant metastasis			N/A
Yes	138 (11.0%)	-	
No	1115 (89.0%)	-	

N/A: not applicable.

III and IV received neoadjuvant chemotherapy after surgery. Of 152 patients with UICC IV, 58 received palliative surgery.

Association of rs145204276 with susceptibility of GC

Table II summarizes the frequency of genotype and allele of rs145204276 in cases and controls. HWE test indicated that there was no selection bias regarding the genotype frequency in cases or controls ($p > 0.05$). The frequency of genotype del/del in the patients was significantly lower than in the controls (7.0%

vs. 9.1%, $p = 0.001$). The allele del was significantly associated with a decreased risk of GC (26.3% vs. 31.0%, $p < 0.001$) with an OR of 0.79 (95% CI = 0.71 - 0.89). After classifying the patients into male and female, we found that the allele del of rs145204276 was significantly associated with a decreased risk of GC in both subgroups (26.2% vs. 31.3%, $p = 0.005$ for male; 26.4% vs. 30.7%, $p = 0.01$ for female), with ORs of 0.80 for male (95% CI = 0.69 - 0.93) and 0.81 for female (95% CI = 0.68 - 0.97), respectively.

Association between rs145204276 and prognosis of GC

Clinical follow-up data of 432 patients was available for the analysis of the 5-year survival rate. The mean overall survival was 27.8 months. As shown in Fig. 1, Kaplan-Meier analysis showed that the genotype del/del of rs145204276 was significantly associated with a higher survival rate ($p < 0.001$). Table III summarizes the survival data according to the different tumor stages. Patients with genotype del/del were found to have remarkably higher survival rates than those with genotype ins/ins in different tumor stages ($p = 0.03$ for stage T1/T2; $p = 0.01$ for stage T3/T4). To determine the relationship between rs145204276 and the progression and metastasis of GC, we compared the frequency of rs145204276 between different subgroups of patients classified by the tumor stage, UICC stage, lymph node metastasis and distant metastasis respectively. As shown in Table IV, patients with the late tumor stage were found to have a significantly lower rate of genotype del/del than those with early tumor stage (4.9% vs. 8.8%, $p = 0.01$). Patients with UICC III and IV were found to have a significantly lower rate of genotype del/del than those with UICC I and II (5.3% vs. 8.1%, $p = 0.02$). Moreover, patients with the allele del were less likely to develop lymph node metastasis (23.3% vs. 27.9%, $p = 0.02$), with an OR of 0.78 (95% CI = 0.66 - 0.95). Comparably, allele del was also significantly associated with a decreased risk of distant metastasis of GC (21.0% vs. 26.9%, $p = 0.005$) with an OR of 0.72 (95% CI = 0.53 - 0.98).

Methylation status and expression of GAS5

Methylation percentage in the 7th CpG site was significantly different among patients with different genotypes (Fig. 2). Patients with genotype del/del had a remarkably higher percentage of methylation than patients with genotype ins/ins (39.1% ± 19.4% vs. 21.6% ± 6.2%, $p < 0.001$).

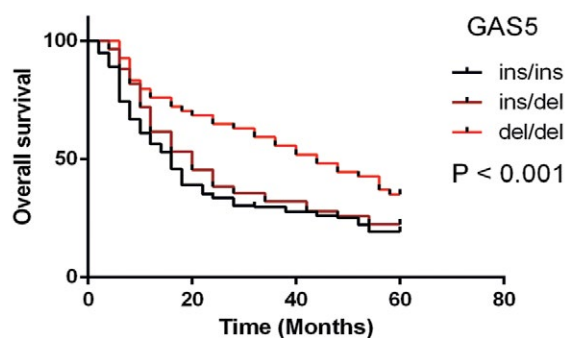


Fig. 1. The overall survival curves of patients with genotype del/del ($n = 53$), genotype del/ins ($n = 142$) and genotype ins/ins ($n = 237$). Log-rank tests showed that patients with genotype del/del had a significantly higher survival rate ($p < 0.001$).

Table II. Comparison of the genotype and allele frequency of rs145204276 in patients and controls

	Genotype			p	Allele		p	Odds ratio (95% CI)
	del/del	del/ins	ins/ins		del	ins		
All subjects								
Patients (n = 1253)	88 (7.0%)	483 (38.6%)	682 (54.4%)	0.001	659 (26.3%)	1847 (73.7%)	< 0.001	0.79 (0.71-0.89)
Controls (n = 1354)	123 (9.1%)	593 (43.8%)	638 (47.1%)		839 (31.0%)	1869 (69.0%)		
Male subjects								
Patients (n = 654)	47 (7.2%)	249 (38.1%)	358 (54.7%)	0.02	343 (26.2%)	956 (73.8%)	0.005	0.80 (0.69-0.93)
Controls (n = 683)	65 (9.5%)	297 (43.5%)	321 (47.0%)		427 (31.3%)	939 (68.7%)		
Female subjects								
Patients (n = 599)	41 (6.8%)	234 (39.1%)	324 (54.1%)	0.04	316 (26.4%)	882 (73.6%)	0.01	0.81 (0.68-0.97)
Controls (n = 671)	58 (8.6%)	296 (44.1%)	317 (47.2%)		412 (30.7%)	930 (69.3%)		

Table III. The relationship between the genotype of rs145204276 and survival data according to different tumor stages

	Genotype			p
	del/del	del/ins	ins/ins	
Overall survival for stage T1/T2	93.8%	90.4%	77.4%	0.03
Event (n = 22)	1	5	16	
Event-free (n = 117)	15	47	55	
Overall survival for stage T3/T4	39.1%	31.2%	24.7%	0.01
Event (n = 210)	14	77	119	
Event-free (n = 83)	9	35	39	

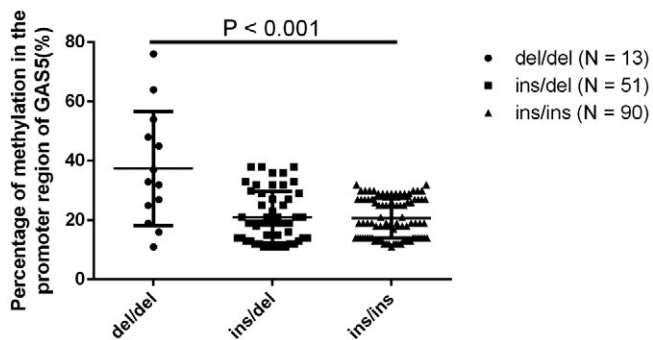
**Fig. 2.** Comparison of the percentage of methylation among different genotypes of rs145204276. Patients with genotype del/del had obviously higher percentage of methylation as compared with those with genotype ins/ins ($39.1\% \pm 19.4\%$ vs. $21.6\% \pm 6.2\%$, $p < 0.001$).

Figure 3 summarizes the tissue expression of GAS5 in GC patients. Methylation percentage of the 7th CpG site significantly correlated with GAS5 expression in the tumor tissues ($r = 0.383$, $p = 0.01$). Significantly lower expression of GAS5 was observed in the tumor tissues than in the adjacent normal tissues ($p < 0.001$). Moreover, compared with patients with ins/ins, patients with genotype del/del had a remarkably higher expression of GAS5 ($p < 0.001$). The patients with event-free survival were found to have a remarkably higher expression and hypermethylation of GAS5 (Table V).

DISCUSSION

Increasing evidence has shown that lncRNAs could be involved in the cellular processes of human cancer. In earlier studies, numerous lncRNAs have been reported to play a role in the development and progression of GC. Feng et al. [23] reported that lncRNA HOTAIR played a positive role in GC occurrence and development. Yao et al. [24] found that lncRNA CASC15 had a significantly higher level in GC tissues and was associated with the overall survival of patients. As a tumor-suppressive, lncRNA has been involved in a variety of human cancers and the role of GAS5 in the development of GC has also been reported [16, 18]. Aberrant expression of GAS5 was reported to promote GC proliferation and suppress apoptosis via different signaling pathway [16, 18]. Tao et al. [20] reported a functional variant rs145204276 in GAS5, which was significantly associated with the risk of hepatocellular carcinoma. In our study, we confirmed that the allele del of rs145204276 was significantly associated with a decreased risk of GC in both male and female patients. The ORs were 0.80 and 0.81 for male patients and female patients, respectively. Similar to our finding, Zheng et al. [16] observed that the allele del of rs145204276 was significantly associated with a 21% decreased risk of colorectal cancer.

As a suppressor of cancer cells, GAS5 was reported to inhibit cell proliferation and migration and increase cell apoptosis in GC [18]. Sun et al. [18] found that GC patients with low GAS5 expression level tended to have poor disease-free survival. Liu et al. [17] speculated that GAS5 could function as a competing endogenous RNA (ceRNA) and play a role in the pathogenesis of GC. In this study, we further investigated the potential role of rs145204276 in the prognosis of GC. Patients with the allele del of rs145204276 were found to have significantly higher survival rate and lower incidence of progression and metastasis. Collectively, it is probable that rs145204276 may play a protective role in the GC through regulation of GAS5 expression. Further investigations are warranted to determine whether GAS5 can serve as a potential therapeutic target for GC.

Table IV. The relationship between rs145204276 and clinicopathological parameters in the patients with gastric cancer

	Genotype			p	Allele		p	Odds ratio (95% CI)
	del/del	del/ins	ins/ins		del	ins		
Tumor stage				0.01			0.01	0.79 (0.66 - 0.95)
T1/T2 (n = 685)	60 (8.8%)	268 (39.1%)	357 (52.1%)		388 (28.3%)	982 (71.7%)		
T3/T4 (n = 568)	28 (4.9%)	215 (37.9%)	325 (57.2%)		271 (23.9%)	865 (76.1%)		
Lymphatic metastasis stage				0.05			0.02	0.78 (0.66-0.95)
N0/N1 (n = 824)	65 (7.9%)	329 (39.9%)	430 (52.2%)		459 (27.9%)	1189 (72.1%)		
N2/N3 (n = 429)	23 (5.4%)	154 (35.9%)	252 (58.7%)		200 (23.3%)	658 (76.7%)		
UICC Stage				0.02			0.005	0.76 (0.64-0.93)
I/II (n = 778)	63 (8.1%)	312 (40.1%)	403 (51.8%)		438 (28.1%)	1118 (71.9%)		
III/IV (n = 475)	25 (5.3%)	168 (35.6%)	279 (59.1%)		218 (23.1%)	726 (76.9%)		
Distant metastasis				0.04			0.04	0.72 (0.53-0.98)
Yes (n = 138)	9 (6.5%)	40 (29.0%)	89 (64.5%)		58 (21.0%)	218 (78.9%)		
No (n = 1115)	79 (7.1%)	443 (39.7%)	593 (53.2%)		601 (26.9%)	1629 (73.1%)		

Table V. The relationship between the GAS5 expression/methylation and survival data

	Event group (n = 106)	Event-free group (n = 48)	p
Methylation percentage	23.8% ± 11.3%	28.3% ± 13.1%	0.03
Expression level of GAS5	0.0015 ± 0.0011	0.0021 ± 0.0016	0.01

Located in the promoter region of GAS5, rs145204276 was associated with an altered expression of GAS5 in cells as demonstrated by luciferase activity analysis [20]. In previous studies [16, 20], allele del of rs145204276 was observed to be indicative of higher GAS expression in colorectal cancer and hepatocellular carcinoma. Tao et al. [20] speculated that rs145204276 could possibly affect the transcriptional activity of GAS5 and regulate its expression through the methylation of CpG islands in the promoter region. In this study, we also found that GC patients with the allele del of rs145204276 had a remarkably higher expression of GAS5 in the tumor tissue. Moreover, we confirmed that patients with genotype del/del had a remarkably higher methylation percentage in the 7th CpG

site of GAS5 promoter, which was significantly correlated with the GAS5 expression level in GC tissues. Hypermethylation of CpG islands is commonly associated with lower transcriptional activity. Occasionally, the hypermethylation status may result in the shift of transcription start site from one to another, thus leading to active transcription [25]. Interestingly, in the study of Tao et al. [20], hypermethylation of the GAS promoter region was also observed to be correlated with an increased expression of GAS5 in hepatocellular carcinoma. In future studies, the precise mechanism underlying the regulation of rs145204276 on the methylation level of the GAS5 promoter is worthy of further investigation.

The primary limitation of our study is the lack of the follow-up data for some patients. Due to the inherent drawback of a retrospective study, the follow-up period of the patients was relatively short to determine the long-term survival rate of the patients. A longitudinal study could better verify the relationship between the variant rs145204276 and the prognosis of GC. Another limitation lies in that the expression analysis was performed both in patients with and without chemotherapy, which could potentially bias the outcome. In

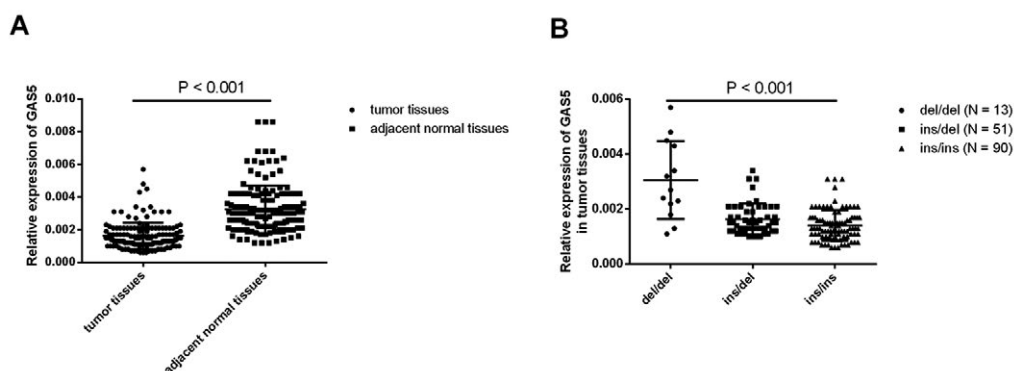


Fig. 3. Comparison of the relative GAS5 expression among different genotypes of rs145204276. (A) Significantly lower expression of GAS5 was observed in the tumor tissues than in the adjacent normal tissues ($p < 0.001$). (B) For tumor tissues, genotype del/del was indicative of a remarkably higher expression of GAS5 than genotype ins/ins ($p < 0.001$).

future studies, the stratification of patients based on the status of chemotherapy can produce a more valid outcome.

CONCLUSIONS

The variant rs145204276 of GAS5 was associated with the development and prognosis of GC. Allele del of rs145204276 was associated with remarkably lower incidence of cancer progression and metastasis. Further investigation on the mechanism underlying the influence of rs145204276 on the methylation status of GAS5 is warranted.

Conflicts of interest. The authors who took part in this study do not have anything to disclose regarding funding or any conflicts of interest with respect to this manuscript.

Authors' contributions: Q.L. and G.M. collected the clinical data. H.G. carried out the basic experiments. S.S. and Y.X. performed the statistical analysis. B.W. conceived of the study and participated in its design. Q.L. drafted the manuscript. All authors read and approved the final manuscript.

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