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Research Paper

Relationship of common variants in Interleukin 33 gene with susceptibility and prognosis of osteosarcoma in Han Chinese population

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Abstract

Osteosarcoma (OS) is one of the most common malignant bone tumors. Many previous studies have indicated that OS is a complex disease and that its development may be affected by multiple genetic factors, which may contribute to its carcinogenesis. The aim of the present study was to evaluate the relationship of *IL-33* with susceptibility and prognosis of OS in Han Chinese individuals. A total of 1,605 study subjects including 507 OS patients and 1,098 controls were recruited. Eighteen SNPs mapped to *IL-33* were selected for genotyping. Genetic associations between selected SNPs and OS disease status were evaluated. Survival analyses, including Kaplan-Meier analysis and Cox model fitting for significant SNPs, were performed. The functional consequences of significant SNPs were analyzed using a publicly available database. SNP rs1048274 was identified to be significantly associated with OS disease status (OR=0.75, $P=1.53\times10^{-4}$). Compared to the GA and GG groups, OS patients with the AA genotype of rs1048274 had better survival rate. The hazard ratio of SNP rs1048274 (AA group compared to GG+GA group) was 0.35 (95% confidence interval of 0.25-0.5) following adjustment for several clinical variables. In conclusion, our results suggested that *IL-33* may play a key role in the etiology of OS, indicating *IL-33* as a potential genetic risk factor of the development and prognosis of OS.

Key words: Osteosarcoma susceptibility; *IL-33*; Common variants; Prognosis; Case-control studies

Introduction

Osteosarcoma (OS) is one of the most common malignant bone tumors and occurs predominantly in children and adolescents [1]. Because of its highly malignant and invasive nature, the metastasis of OS often occurs early in patients and, thus, the prognosis is poor [2]. Although some patients benefit from surgery and chemotherapy, the 5-year survival rate of OS is still lower than 50% [3]. Therefore, it is an urgent problem to clarify the pathology and the molecular mechanism of OS. Previous research have indicated that the process of OS development is complicated and may be influenced by multiple factors [4-5]. Only a portion of people who are exposed to the same environmental factors will develop OS, suggesting that genetic factors may contribute to the carcinogenesis of OS. Therefore, it is necessary to find the genes responsible for susceptibility to OS.

Interleukin-33 (IL-33) was found to be predominantly expressed in the nuclei of stromal cells and activated myeloid cells. Previous studies have confirmed that IL-33 plays an important regulatory role in many physiological and pathological conditions, such as tissue repair, autoimmune diseases and cancer, and it is also considered to be a

damage-associated molecular pattern (DAMP) molecule [6]. IL-33 is a cytokine member of the IL-1 family, which includes IL-18 and IL-1 [7]. Previous studies have shown that high levels of both IL-1α and IL-18 have a positive effect on suppressing the metastasis of OS [8-9]. Additionally, it has been found that the expression of IL-33 in tumor cells can enhance immunogenicity and also promote type 1 antitumor immune responses via CD8+ T cells and NK cells [10-11]. Studies have shown that many cancers can cause an increase in serum IL-33 levels, such as gastric cancer, hepatocellular carcinoma, and lung cancer [12-15]. Moreover, IL-33 is an inhibitor of bone reabsorption that blocks osteoclastic activity in *in vitro* experiments [16]; *in vivo*, researchers have also shown that IL-33 can have anti-osteoclastogenic effects, reducing osteoclast formation and activity by inducing their apoptosis in mice [17]. Taken together, these studies suggest that IL-33 may be important to the occurrence and development of OS.

A recent genome-wide association study (GWAS) involving 632 OS patients, including 523 European patients and 109 from Brazilian patients, produced highly significant association of GLDC/IL33 locus at 9p24.1 with the risk of OS. Using publicly available data, Koster et al. found that the OS susceptible single nucleotide polymorphism (SNP) in GLDC/IL33 locus associated with low expression of IL-33, which was also significantly correlated with poor survival in OS patients [18]. This result suggested that IL-33 might play some roles in the pathogenesis of OS. However, to date, only one report correlating IL-33 with OS is available. Importantly, the study only evaluated OS patients of European and Brazilian descent. Given that different ethnic populations may exhibit genetic heterogeneity of OS, there is a need to confirm these findings with additional samples from different populations. Therefore, in the present study, we aimed to determine whether IL-33 was associated with susceptibility to OS in Han Chinese population.

Methods

Study subjects

A total of 1,605 study subjects including 1,098 healthy controls and 507 OS patients were recruited from 3 clinical centers located in Xi'an (Shaanxi Traditional Chinese Medicine Hospital, Affiliated Hospital of Shaanxi University of Traditional Chinese Medicine and Honghui Hospital of Xi'an Jiaotong University). All patients were diagnosed with OS based on histological and pathological evidence by at least two experienced pathologists. All healthy controls were identified by health screening and recruited from the same hospitals mentioned above. All subjects were Han Chinese individuals from the same geographic region of Xi'an, China. This study has been approved by the Medical Ethics Committee of Xi'an Jiaotong University. Informed consent forms were obtained from all participants. The characteristic information for all study subjects were summarized in Table 1. No differences were identified for age, gender or family history between OS case and control groups.

Table 1. Distributions of selected variables in all subjects

Variables	Subjects (N=1605)		Statistics	*P*-value
	OS (N=507)	Control (N=1098)		
Mean age, years (%)	21.08±6.1	21.12±5.8	*T*=-0.12	0.90
≤20	318 (62.7)	700 (63.8)		
>20	189 (37.3)	398 (36.2)	χ^2=0.12	0.73
Gender (%)				
Male	261 (51.5)	563 (51.3)		
Female	246 (48.5)	535 (48.7)	χ^2= 0.0005	0.98
Cancer family history (%)				
Yes	158 (31.2)	333 (30.3)		
No	349 (68.8)	765 (69.7)	χ^2= 0.08	0.78
Tumor location (%)				
Long tubular bones	408 (80.5)	-		
Axial skeleton	99 (19.5)	-	-	-
Pathological fracture (%)				
Yes	87 (17.2)	-		
No	420 (82.8)	-	-	-
Metastasis (%)				
Yes	112 (22.1)	-		
No	395 (77.9)	-	-	-
Enneking stages (%)				
I	47 (9.3)	-		
II	391 (77.1)	-		
III	69 (13.6)	-	-	-

SNP selection and Genotyping

SNPs in the gene region of the *IL-33* with minor allele frequencies (MAF) ≥ 0.01 based on 1000 genome data were extracted. Then, MAF ≥ 0.01 by pair-wise tagging and $r^2 \geq 0.8$ were used as selection criteria for tag SNPs, and 18 tag SNPs were generated for genotyping. The information regarding the 18 tag SNPs is summarized in Supplemental Table S1. Genomic DNA was isolated from the peripheral blood using a Tiangen DNA extraction kit (Tiangen Biotech Co. Ltd, Beijing, China). Sequenom MassARRAY platform was utilized for SNP genotyping. Typer 4.0 was used for genotyping results processing. We randomly re-performed the analysis on 5% of the samples and found a concordance of 100%. The MAFs of these SNPs in our study subjects ranged from 0.039-0.484, and all of these SNPs were in Hardy-Weinberg equilibrium (HWE) in our controls (Supplemental Table S1). Power analyses were performed using GAS power calculator, and the results were showed in Ssupplemental Figure S1. As seen from this figure, 500 OS cases could achieve statistical power of 78.7% to detect a SNP with relative risk equal to 1.4 at α=0.05 level.

Statistics and bioinformatics analysis

HWE was tested for all SNPs by Haploview v4.2 [19]. Differences of multiple clinical and characteristic variables between cases and controls were examined by Student's *t* and χ^2 tests. Genetic associations between selected SNPs and OS risk were tested using Plink [20]. The linkage disequilibrium (LD) structure was plotted using Haploview v4.2 [19]. Survival analyses, including Kaplan-Meier analysis and Cox model fitting for significant SNPs, were performed using R project (using "survival" and "survminer" packages) [21]. Bonferroni corrections were applied if necessary to address the problem of multiple comparisons. To examine the functional consequences of the significant SNP, we performed bioinformatics analyses using the GTEx [22] and the PolymiRTS Database [23]. The eQTL effects of significant SNPs on gene *IL-33* were investigated using gene expression data from multiple human tissues that were extracted from GTEx. The effects of an SNP on miRNA-mediated gene expression were evaluated using the PolymiRTS Database.

Table 2. Results of single marker based association analyses

CHR	SNP	POS	A1	F_A	F_U	χ^2	*P*	OR
9	rs1157505	6216240	G	0.265	0.262	0.043	0.84	1.02
9	rs11791561	6216516	C	0.449	0.453	0.054	0.82	0.98
9	rs72614080	6218595	T	0.274	0.279	0.071	0.79	0.98
9	rs1891385	6219845	C	0.223	0.226	0.035	0.85	0.98
9	rs143589217	6220187	A	0.048	0.047	0.031	0.86	1.03
9	rs16924144	6221246	C	0.314	0.317	0.036	0.85	0.98
9	rs183791076	6222545	G	0.052	0.053	0.004	0.95	0.99
9	rs10975495	6223819	A	0.057	0.055	0.086	0.77	1.05
9	rs1418385	6226295	T	0.476	0.472	0.058	0.81	1.02
9	rs16924159	6229417	A	0.298	0.293	0.084	0.77	1.02
9	rs189217453	6230731	T	0.050	0.048	0.092	0.76	1.06
9	rs182211245	6230736	C	0.050	0.051	0.019	0.89	0.98
9	rs117414011	6242939	C	0.071	0.076	0.256	0.61	0.93
9	rs4742170	6242950	C	0.487	0.482	0.068	0.79	1.02
9	rs79981454	6247232	C	0.042	0.038	0.391	0.53	1.13
9	rs76864631	6248408	A	0.248	0.242	0.124	0.73	1.03
9	rs10758751	6252044	C	0.074	0.077	0.064	0.80	0.96
9	**rs1048274**	**6256292**	**A**	**0.414**	**0.486**	**14.340**	**1.53×10^{-4}**	**0.75**

POS: position; A1: tested allele; F_A: allele frequency in cases; F_U: allele frequency in controls. Threshold of *P* value was 0.0028 (0.05/18). Significant results were highlighted in bold.

Results

Single marker based association analyses

SNP rs1048274, which is located at 3' untranslated region of *IL-33*, was identified to be significantly associated with the disease status of OS (Table 2), and its significance was retained after Bonferroni correction (Table 2). Our results showed that the minor allele (A) of this SNP had significant protective effect against OS (OR=0.75, $P=1.53\times10^{-4}$). The LD structure of our 18 selected tag SNPs is shown in Supplemental Figure S2, but there was no LD block which included the associated SNP of rs1048274. Furthermore, we examined the distribution of multiple clinical variables in the OS groups with different genotypes of rs1048274 (Table 3). None of those variables were identified as significantly associated with any of the genotypes of rs1048274 within OS patients.

Table 3. Association between genotypes of rs1048274 and several clinical variables within our OS subjects

	Genotypes of rs1048274 (N=507)			Statistics	*P*
	AA (N=83)	AG (N=254)	GG (N=170)		
Age in years, mean±sd	20.6±5.8	21.4±6.2	20.8±6.0	*F*=0.03	0.87
Gender (%)					
Male (N=261)	43 (16.5)	130 (49.8)	88 (33.7)		
Female (N=246)	40 (16.3)	124 (50.4)	82 (33.3)	χ^2=0.02	0.99
Tumor location (%)					
Long tubular bones (N=408)	62 (15.2)	209 (51.2)	137 (33.6)		
Axial skeleton (N=99)	21 (21.2)	45 (45.5)	33 (33.3)	χ^2=2.29	0.32
Pathological fracture (%)					
Yes (N=87)	13 (14.9)	47 (54.0)	27 (31.1)		
No (N=420)	70 (16.7)	207 (49.3)	143 (34.0)	χ^2=0.65	0.72
Metastasis (%)					
Yes (N=112)	18 (16.1)	55 (49.1)	39 (34.8)		
No (N=395)	65 (16.5)	199 (50.4)	131 (33.1)	χ^2=0.11	0.95
Enneking stages (%)					
I (N=47)	11 (23.4)	23 (48.9)	13 (27.7)		
II (N=391)	58 (14.8)	199 (50.9)	134 (34.3)		
III (N=69)	14 (20.3)	32 (46.4)	23 (33.3)	χ^2=3.43	0.49

Survival analyses

An overall survival plot for all of our OS patients stratified by genotypes of rs1048274 (AA, GA and GG groups) was made, and Kaplan-Meier analysis was conducted (Figure 1). Significant differences of survival curves were obtained (*P*=0.0072) between the three genotype groups. Compared to the GA and GG groups, OS patients with the AA genotype of rs1048274 had better survival rate. Next, we performed Kaplan-Meier analysis stratified by Enneking stages (Supplemental Figure S3-S5) and gender (Supplemental Figure S6 and S7). The patterns of the survival curves for each stratification variable were similar to the overall survival curves. For all survival curves, OS patients with AA genotype had better survival rate, although this statistical difference was not very significant when the curves were stratified by gender (*P*=0.025 for male and *P*=0.13 for female).

A Cox model adjusted with multiple clinical variables was fitted (Figure 2) to the curves. The coding scheme is shown in Supplemental Table S2. Since the survival curves of the GG and GA groups were very similar to each other (Figure 1), we combined the two groups in the Cox model. The adjusted hazard ratio of SNP rs1048274 (AA group compared to GG+GA group) was 0.35 (with 95% confidence interval of 0.25-0.5). The two other variables, gender and Enneking stages, were also significant in our Cox model.

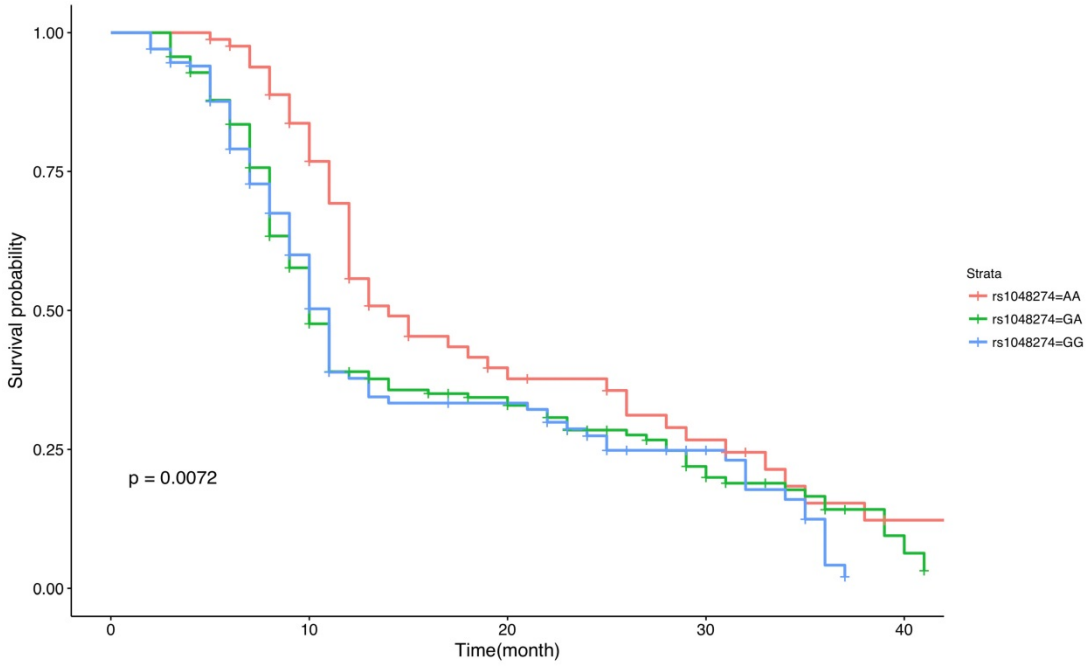

Figure 1. Kaplan-Meier analysis and survival plot for the overall survival of OS patients with different genotypes of s1048274. *P* values for Kaplan-Meier analysis are indicated on the plot.

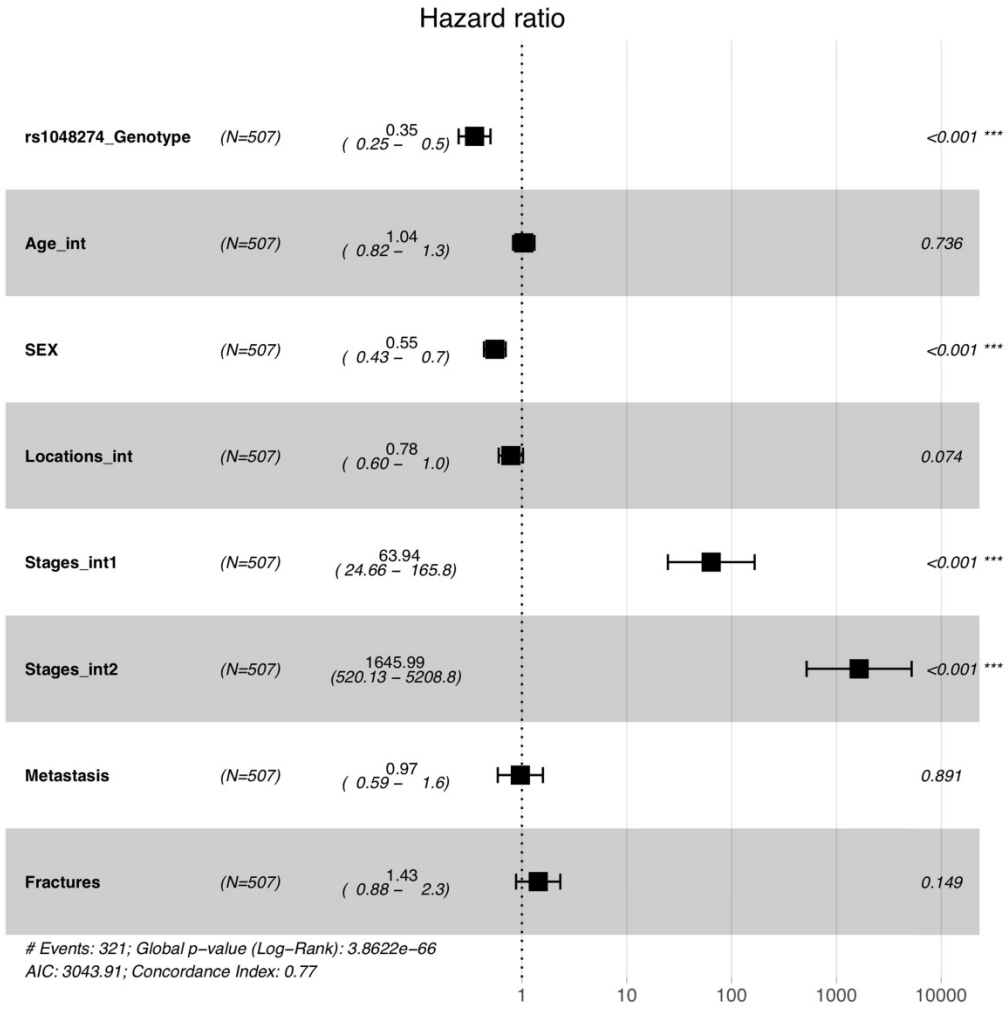

Figure 2. Forest plot for the survival analysis of rs1048274 using a Cox model after adjustment for multiple clinical variables.

Bioinformatics analyses

Gene expression data of *IL-33* from 47 human tissues was extracted. The most significant eQTL finding for rs1048274 was identified in the esophagus (P=0.076 Supplemental Table S3). However, this hit failed to survive after Bonferroni correction. According to the genomic database from the University of California, Santa Cruz (http://genome.ucsc.edu/), rs1048274 acts as a 3' untranslated region (3' UTR) variant, which may affect microRNA binding. We used a free online tool (PolymiRTS Database) to examine the predicted target gain or loss in microRNA binding and found that the A allele of rs1048274 causes the loss of binding of microRNA/SNP target duplexes of two miRNAs (hsa-miR-543 and hsa-miR-570-3p) and *IL-33*. Comparatively, the G allele of rs1048274 maintains a stable microRNA-binding site at the 3' UTR region of *IL-33*.

Discussion

Previous GWAS have linked IL-33 to several disease states including asthma [24-25] and periodontal microbiota [26]. GWAS using European and Brazilian samples conducted by Koster et al. have shown that *IL-33* might contribute to susceptibility to the risk of OS. However, this evidence was weak because the significant SNP identified by this GWAS was located in the *GLDC* gene and was only linked with *IL-33* based on publicly available data on gene expressions. In addition, study conducted by Koster *et al*. was based on European populations, and the genetic structure of OS related SNPs of *IL33* was still unclear for Chinese population considering the differences in linkage disequilibrium structure between the two ethnic groups. To the best of our knowledge, the present study is the first one to identify the association of *IL33* with the risk of OS in Han Chinese population. Our study replicates and builds on this previous work by using samples with Chinese Han ancestry. In this study, we have identified SNP rs1048274 to be significantly associated with the disease status of OS. The minor allele of this SNP, the A allele, could significantly decrease the risk of OS by 25%. This SNP, located at the 3' untranslated region of *IL-33*, was physically closer to *IL-33* compared to both rs3765555 and rs55933544, which were identified in the previous GWAS. SNP rs1048274 was missed by this previous GWAS. A potential reason might be its difference in the MAF (0.43 for East Asians and 0.32 for Europeans according to the 1000 Genome Project) and the LD structure around it between Chinese and European populations. In addition to its contribution to OS susceptibility, SNP rs1048274 was also associated with prognosis for OS patients. According to our survival analysis, OS patients with the AA genotype could, in general, exhibit significantly better survival rates compared to those with either the GG or GA genotypes. This pattern was not associated with the Enneking stages of OS or gender. Similar to its effect on OS susceptibility, the A allele of SNP rs1048274 has hazard ratio of 0.35 after adjustment for multiple clinical variables.

In the present study, we have identified that the A allele of SNP rs1048274 significantly increases the survival rate of OS patients compared to the G allele. Our bioinformatics analyses have shown that rs1048274's ancestral G allele is a very conserved miRNA binding site which binds miRNA and inhibits gene expression of *IL-33*. The A allele is likely to disrupt this binding site and thus increase its expression. Early studies have shown that IL-33 could promote type I antitumor immune responses through CD8+ T cells and NK cells [10,11]. In other words, the protein product of the gene *IL-33* might be related to the inhibition of tumor cells, and therefore, a higher level of *IL-33* expression might be protective against OS. Although a systematic investigation of the expression levels of *IL-33* is out of the scope of this study, our findings could still provide some clues for the hypothesis stated above. Nevertheless, since the findings from our bioinformatics analyses were functional predictions based on computer algorithms, and no experimental data has been reported, we need to be careful when interpreting these findings.

With the rapid development of high-throughput sequencing technology, genetic association analysis provides an effective method for studying the molecular genetic mechanism of complex diseases, such as schizophrenia, but it is still unknown for us to understand the genetic risk and molecular mechanism of OS [27-36]. Despite our interesting findings on the association between rs1048274 and the susceptibility and prognosis of OS, this study suffered from several limitations. A major issue is that population stratification might be a potential confounder for our association signal. As a candidate gene based study, we did not have enough SNPs to perform some conventional approaches (such as principal component analysis) to address this problem. Nevertheless, we have applied some criteria to restrict the genetic background of our study subjects to minimize their genetic heterogeneity. Thus, due to the subject individuals from the same geographical area, although the potential population stratification cannot be completely excluded, our sampling strategy can effectively avoid significant population stratification to the greatest extent [37-42]. Another limitation is that this study is not a randomized clinical trial, thus selection bias might be present. OS patients and

controls were not randomly selected individuals and our study subjects may not be a representative sample. To the best of our knowledge, the present study is the first one to identify an association signal for rs1048274 and OS susceptibility and its prognosis. Replication studies are needed in the future, especially those based on different ethnic populations.

In summary, we have showed strong evidence for association between SNP rs1048274 of *IL-33* and susceptibility to OS in the Chinese Han population. This SNP was also identified as significantly associated with the prognosis of OS in further analyses of OS patients. This is the first large-scale population based genetic report on polymorphisms of IL-33 using samples with Chinese ancestry. Thus, replication studies based on other populations as well as further investigations of the underlying mechanism responsible for these associations are still needed.

Supplementary Material

Supplementary figures and tables.
http://www.jcancer.org/v10p1138s1.pdf

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Authors' Contributions

Authors Kang C and Zhi L conceived and designed the study. Kang C and Zhao J carried out candidate SNPs selection and statistical analyses. Kang C, Zhao J, Wang Y, and Zhi L conducted subject screening. Zhao J, Yang C, and Chen J contributed to the collection and preparation of control DNA samples. Kang C wrote the paper.

Competing Interests

The authors have declared that no competing interest exists.

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