ORIGINAL RESEARCH

Association Between Genetic Polymorphisms of lncRNA *NEAT1* and Pulmonary Tuberculosis Risk, Clinical Manifestations in a Chinese Population

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Background: Recent studies have shown that abnormal expression of lncRNA NEAT1 is associated with the progression of pulmonary tuberculosis (PTB). The aim of our study was to analyze the relationship between single nucleotide polymorphisms (SNPs) of *NEAT1* gene and susceptibility to PTB.

Methods: Four SNPs (rs2239895, rs3741384, rs3825071, rs512715) in *NEAT1* gene were genotyped in 479 patients with PTB and 476 controls by improved multiple ligase detection reaction (iMLDR) in a Chinese population.

Results: We found no significant differences in allele and genotype frequencies of *NEAT1* gene rs2239895, rs3741384, rs3825071, rs512715 between PTB patients and controls (all $P > 0.05$). There was no statistically significant association between genotype frequency distribution of dominant model, as well as recessive model, and genetic susceptibility to PTB patients (all $P > 0.05$). The TT genotype, T allele frequencies of rs3825071 were significantly increased in sputum smear-positive PTB patients when compared to sputum smear-negative PTB patients ($P = 0.010$, $P = 0.003$, respectively). Haplotype analysis shown that *NEAT1* haplotype frequency was not associated with PTB susceptibility.

Conclusion: *NEAT1* gene polymorphisms were not associated with the risk of PTB in Chinese population, and rs3825071 polymorphism might be related to sputum smear-positive in PTB patients.

Keywords: long non-coding RNA, NEAT1, pulmonary tuberculosis, single nucleotide polymorphisms

Introduction

Tuberculosis (TB) is a common, chronic infectious disease caused by *Mycobacterium tuberculosis* (MTB), and a serious public health problem around the world, which significantly affect human health.[1] According to the latest World Health Organization Global Tuberculosis report, there is approximately 9.9 million new incident TB patients globally in 2021, with an increasing threat.[2] The pathogenesis of TB is complex, and the occurrence of TB is relatively slow.[3] In addition, the patients normally have sputum smear negative, and no special clinical manifestations besides cough, fever in the early stage. Therefore, it is very important to research the risk factors leading to increased susceptibility to TB and to provide scientific basis for developing disease control strategies. Studies had shown that about a third of the population was infected with MTB, of which 10% would develop TB, while the remaining 90% might never develop symptomatic disease.[4] This suggested that the occurrence and progression of this disease was influenced by a variety of factors, such as genetic variation, nutritional status and environmental factors.[5] The genetic variation of *HLA, killer immunoglobulin-like receptor, Toll-like receptor, vitamin D receptor* might play an important role in the susceptibility to pulmonary TB (PTB).[6–9]

Long noncoding RNAs (lncRNAs) referred to a class of non-coding RNA transcripts with a length of more than 200 nucleotides, which played a key role in many biological processes including transcriptional, posttranslational, and epigenetic regulation of gene expression.[10] There was sufficient evidence that lncRNA was also involved in the regulation of host immune response to invasive pathogens, and abnormal expression of lncRNA was associated with a variety of infectious diseases.[11] Several studies had been reported on lncRNA effected on acquired and innate immunity responses against MTB, and the differential expression of several lncRNA was also observed in PTB patients.[12,13] In addition, the single nucleotide polymorphisms (SNP) in lncRNA genes have been shown to influence the susceptibility and clinical presentation of PTB.[14–16] The data of Zhao et al indicated that lncRNA AC079767.4 polymorphism had the potential to serve as a novel biomarker for PTB diagnosis.[14]

LncRNA NEAT1 was an important inflammatory regulator that not only promoted the activation of inflammasome, but also facilitated the expression of inflammatory cytokines by regulating the expression of miR-17-5p/TLR4 in macrophages.[17,18] It had been reported that the up-regulation of NEAT1 played an anti-TB role in MTB-infected patients.[19] Our recent study suggested that NEAT1 expression level was abnormally increased in patients with PTB compared to normal controls.[20] In addition, previous studies demonstrated that the genetic variations in *NEAT1* were significantly associated with risk of multiple diseases, including lung cancer,[21] lung squamous cell carcinoma,[22] oral squamous cell carcinoma,[23] etc. However, no study explored the association between *NEAT1* gene variation and susceptibility to PTB. Therefore, we performed this study to investigate the association between *NEAT1* gene polymorphism and PTB susceptibility, as well as some clinical manifestations in a Chinese population.

Materials and Methods

Study Design and Subjects

A total of 955 subjects were included in this case-control study, including 479 patients with PTB and 476 healthy controls. All PTB patients were selected from Anhui Chest Hospital, and diagnosed by experienced respiratory physician based on the following criteria: clinical symptoms, chest radiograms, sputum and/or bronchoalveolar lavage fluid MTB culture, microscopic examination of Acid-fast bacilli (AFB), and efficacy of anti-tuberculosis treatment. The PTB patients with HIV infection, hepatitis, cancers, pregnancy, and other respiratory diseases were excluded from the study. All healthy individuals without a previous history of TB, cancer, and HIV were enrolled from health examine center in the same area as the controls. Moreover, healthy controls must also be asymptomatic, sputum smear and culture negative, and chest radiographs normal.

The present study was approved by the Ethics Committee of Anhui Medical University (20200250), and informed consent was acquired from each subject. Then, we collected the demographic data of all subjects, as well as the clinical data of PTB patients including fever, drug resistance, drug-induced liver injury (DILI), etc. from the medical record system of Anhui Chest Hospital. Meanwhile, about 5 mL early morning fasting blood samples from all subjects were collected for DNA extraction.

SNP Selection and Genotyping

At present, the study on *NEAT1* gene polymorphism and PTB had not been reported, therefore, we tried to identify the Tag SNP sites in *NEAT1* gene. Firstly, we collected the genotype data of *NEAT1* in the Han Chinese people in Beijing by using Ensembl Genome Browser 85 and CHBS_1000G. Then, the pairwise option of the HaploView 4.0 software (Cambridge, MA, USA) was used to select the tag SNPs of *NEAT1*. The tag SNPs, which located in potentially functional regions such as promoters, exons, untranslated regions (UTRs), were preferred for genotyping in this study. In addition, we also searched the previous studies on the association of *NEAT1* gene polymorphisms with human diseases.[22–24] Finally, we selected four SNPs of *NEAT1* gene, including rs2239895, rs3741384, rs3825071, rs512715. These SNPs all met the following two requirements: r^2 threshold > 0.8, minor allele frequency (MAF) ≥ 0.05 in CHB.

The Flexi Gene-DNA Kit (Qiagen, Valencia, CA) was used to extract genomic DNA from each peripheral blood sample according to the manufacturer's instructions, and the improved multiple ligase detection reaction (IMLDR)

technique was used for genotyping, with the technical support from Genesky Biotechnologies (Inc., Shanghai).[25] The detailed experimental steps were as follows: (1) 1μL DNA sample was extracted, and the quality of the sample was checked and the concentration was estimated by 1% agarose electrophoresis. Then the DNA sample was diluted to 5–10 ng/μL according to the estimated concentration. (2) Multiplex PCR reaction was carried out with 20μL reaction system included 1x HotStarTaq buffer, 3.0 mMMg2+, 0.3mM dNTP, 1U HotStarTaq polymerase (Qiagen Inc.), 1μL sample DNA and 1μL multiple PCR primers. (3) Purification of multiple PCR products: 5U SAP enzyme and 2U Exonuclease I enzyme were added to 20 μL PCR product, 37°C warm bath for 1h, then 75°C inactivated for 15min. (4) Ligating reaction system: 1 μL 10x ligating buffer, 0.25 μL high temperature ligase, 0.4 μL 5' ligating primer mixture (1μM), 0.4 μL primer 3' ligating primer mixture (2 μM), 2 μL purifed multiple PCR products, 6 μL ddH2O mixing. (5) The 0.5 μL diluted product was mixed with 0.5 μL Liz500 SIZE STANDARD, 9 μL Hi-Di, denatured at 95°C for 5 min, then placed on the ABI3730XL sequencer. (6) The raw data collected on the ABI3730XL sequencer are analyzed by GeneMapper 4.1 (AppliedBiosystems, USA). The subjects with 100% genotyping success rate for all SNPs were included for final analysis. Those subjects with 100% genotype success for four SNPs were eventually included in our subsequent analysis.

Statistical Analysis

Statistical analysis of our study was performed using SPSS 23.0 software (SPSS Inc., IL, USA). Hardy-Weinberg equilibrium (HWE) of all SNPs genotype distributions among controls was evaluated by Chi-square test. Calculated genotypic and allelic frequencies for all SNPs in PTB patients and controls were compared with Chi-square test, and odds ratios (OR), 95% confidence intervals (CI) were analyzed through logistic regression analyses. In addition, two genetic models (dominant model, recessive model) were also analyzed. The Chi-square test was used to assess the possible association between *NEAT1* gene polymorphisms and the patients' clinical features. Haplotype analysis with *NEAT1* polymorphisms was also carried out by SHEsis software.[26] $P < 0.05$ was considered as statistical significance. The Bonferroni correction was used for multiple testing.

Results

Relationship Between *NEAT1* Gene SNPs and PTB Risk

The mean age of 479 PTB patients, including 313 males and 166 females, was 45.07±17.74 years. The average age of 476 controls, including 208 males and 268 females, was 43.36±13.84 years (Table 1). The common clinical characteristics of PTB patients were also summarized in Table 1. The allele, genotype frequencies of *NEAT1* gene rs2239895, rs3741384, rs3825071, rs512715 in PTB patients and controls were shown in Table 2, and the controls were in Hardy-Weinberg's equilibrium (all $P > 0.05$).

Table 1 The Demographic and Clinical Characteristics of PTB Patients and Healthy Controls

Characteristics	PTB Patients	Healthy Controls
Demographic characteristics		
Age (years)	45.07±17.74	43.36±13.84
Sex (male/female)	313/166	208/268
Clinical characteristics[a]		
Fever (+/-)	85/389	NA
Drug resistance (+/-)	74/402	NA
DILI (+/-)	70/408	NA
Pulmonary infection (+/-)	104/374	NA
Hypoproteinemia (+/-)	34/444	NA
Leukopenia (+/-)	32/446	NA
Sputum smear (+/-)	301/129	NA

Note: [a]Part of the study subjects of data missing.
Abbreviations: PTB, pulmonary tuberculosis; DILI, drug-induced liver injury; NA, not applicable; +/−, with/without.

Table 2 Genotypes and Alleles Frequencies of *NEAT1* Gene in PTB Patients and Healthy Controls

SNP	Analyze Model	PTB (N = 479) n (%)	Control (N = 476) n (%)	P value	OR (95% CI)
rs2239895	Genotype				
	CC	13(2.71)	7(1.47)	0.214	0.554(0.219, 1.407)
	GC	120(25.05)	133(27.94)	0.370	1.141(0.855, 1.524)
	GG	346(72.23)	336(70.59)	Reference	
	Allele				
	C	146(15.24)	147(15.44)	0.903	0.992(0.768, 1.263)
	G	812(84.76)	805(84.56)	Reference	
	Dominant model				
	GG	346(72.23)	336(70.59)	0.574	1.041(0.903, 1.201)
	GC+CC	133(27.77)	140(29.41)	Reference	
	Recessive model				
	CC	13(2.71)	7(1.47)	0.180	0.698(0.383, 1.272)
	GC+GG	466(97.29)	469(98.53)	Reference	
rs3741384	Genotype				
	AA	12(2.51)	6(1.26)	0.193	0.518(0.192, 1.395)
	GA	117(24.43)	132(27.73)	0.294	1.168(0.874, 1.562)
	GG	350(73.06)	338(71.01)	Reference	
	Allele				
	A	141(14.72)	144(15.13)	0.802	0.984(0.867, 1.117)
	G	817(85.28)	808(84.87)	Reference	
	Dominant model				
	GG	350(73.06)	338(71.01)	0.478	1.053(0.912, 1.216)
	GA+AA	129(26.93)	138(28.99)	Reference	
	Recessive model				
	AA	12(2.51)	6(1.26)	0.157	0.665(0.345, 1.281)
	GA+GG	467(97.49)	470(98.74)	Reference	
rs3825071	Genotype				
	TT	12(2.51)	11(2.31)	0.821	0.909(0.395, 2.088)
	CT	129(26.93)	124(26.05)	0.743	0.953(0.714, 1.272)
	CC	338(70.56)	341(71.64)	Reference	
	Allele				
	T	153(15.97)	146(15.34)	0.703	0.976(0.861, 1.107)
	C	805(84.03)	806(84.67)	Reference	
	Dominant model				
	CC	338(70.56)	341(71.64)	0.714	1.027(0.891, 1.183)
	CT+TT	141(29.44)	135(28.36)	Reference	
	Recessive model				
	TT	12(2.51)	11(2.31)	0.845	0.959(0.623, 1.476)
	CT+CC	467(97.49)	465(97.69)	Reference	
rs512715	Genotype				
	CC	35(7.31)	27(5.67)	0.399	0.796(0.468, 1.353)
	GC	181(37.79)	194(40.76)	0.460	1.105(0.847, 1.442)
	GG	263(54.91)	255(53.57)	Reference	
	Allele				
	C	251(26.20)	248(26.05)	0.941	0.998(0.946, 1.053)
	G	707(73.80)	704(74.95)	Reference	
	Dominant model				
	GG	263(54.91)	255(53.57)	0.679	1.025(0.912, 1.152)
	GC+CC	216(45.09)	221(46.43)	Reference	
	Recessive model				
	CC	35(7.31)	27(5.67)	0.305	0.983(0.950, 1.016)
	GC+GG	444(92.69)	449(94.33)	Reference	

Note: After Bonferroni correction, *P* value was still not statistically significant.

Regarding the allele frequency of *NEAT1* gene rs2239895, rs3741384, rs3825071, rs512715 between PTB patients and controls, we noted no significant association (all $P > 0.05$). Similarly, no significant difference was existed in genotype frequency of all SNPs among PTB patients and controls (all $P > 0.05$). We also constructed dominant model and recessive model to further investigate the differences in the frequencies of different genotypes between PTB patients and the control group, and no significant differences were found (all $P > 0.05$).

Association of *NEAT1* Gene SNPs with Several Clinical Features in PTB Patients

Considering that the clinical manifestations and complications of PTB might affect the disease progression of patients, we determined to explore the potential association between *NEAT1* gene polymorphism and the common clinical characteristics of PTB patients in a case-only study (Table 3). We found that the TT genotype, T allele frequencies of rs3825071 were significantly increased in sputum smear-positive PTB patients when compared to sputum smear-negative PTB patients ($P < 0.0167$, $P = 0.003$, respectively). The rs512715 CC genotype frequency appeared to be increased in PTB patients with hypoproteinemia, while the significant association disappeared after multiple testing by Bonferroni correction ($P > 0.0167$). Similarly, no statistically significant association was found between rs512715 gene variation and pulmonary infection in PTB patients after Bonferroni correction ($P > 0.0167$).

Nonetheless, there was no significant difference between rs2239895, rs3741384 polymorphism and these clinical characteristics of PTB patients (all $P > 0:05$).

Haplotype Analysis

We used SHEsis software to detect four major haplotypes of *NEAT1* gene, including CACC, GGCC, GGCG, GGTG, and analyzed the association between the frequency of these haplotypes and susceptibility to PTB. The results suggested that the association between these haplotype frequencies and susceptibility to PTB was not statistically significant (Table 4).

Discussion

Abnormal expression and gene variation of multiple lncRNA had been proved to be closely related to the occurrence and development of tumors, autoimmune diseases, etc.[27,28] At the same time, the regulatory roles of lncRNA in PTB had also attracted widespread attention. Yi et al used microarray to detect the expression profile of lncRNA in CD4+T cells, and found that compared with healthy control group, there was abnormal expression of lncRNA in peripheral blood CD4+T cells in patients with active and latent TB.[29] According to another recent study, the T-cell inhibitory factor CD244 might control the CD8+T cell immune response during TB infection through inducing the lncRNA CD244 expression.[30] One recent study shown that lncRNA NEAT1, which known as a novel regulatory molecule, had two subtypes including NEAT1_1(3.7 kb) and NEAT1_2 (23 kb), and was actively involved in immune regulation.[31] Zhang et al found that NEAT1 was increased in T cells infected by HIV-1, whereas NEAT1 knockout stimulated the replication of HIV-1, and this was achieved through promoting the transport of HIV-1 mRNA from the nucleus to the cytoplasm.[32] In addition, the results by Huang et al demonstrated that the NEAT1 expression level was higher during MTB infection, and it might be related to the outcome of TB.[19] Similarly, our recent study also confirmed that the NEAT1 expression level in peripheral blood mononuclear cells of PTB patients was abnormally elevated.[20] These results suggested that NEAT1 might be involved in the development of PTB, however, no study analyzed the association between *NEAT1* gene polymorphism and PTB susceptibility. The present study was the first to explore the association between *NEAT1* gene rs2239895, rs3741384, rs3825071, rs512715 and the risk of PTB. Our results demonstrated that there was no significant association between these SNPs and PTB.

The expression level of NEAT1 could be affected by genetic variation during the development of disease, thus researchers focused on the role of *NEAT1* gene polymorphisms in the risk of many diseases. Wang et al found that *NEAT1* gene rs2239895 polymorphism could upregulate its expression, and was associated with the risk of lung squamous cell carcinoma in a Chinese Northeast population.[22] Similarly, the *NEAT1* gene rs3825071 variant might affect gastric cancer susceptibility by modulated biological effects to improve the NEAT1 expression.[24] Another study indicated that *NEAT1* gene rs3741384 variant might influence the survival of oral squamous cell carcinoma patients, and identified a predictive role of rs3741384 GG in a worse prognosis of this disease.[23] Previous studies had confirmed that

Table 3 The Associations Between *NEAT1* Gene Polymorphisms and Several Clinical Features of PTB Patients

SNP	Allele (M/m)	Clinical Features	Group	Genotype n (%)			P value[a]	Allele n (%)		P value
				MM	**Mm**	**mm**		**M**	**m**	
rs2239895	G/C	Fever	+	59	25	1	0.407	143	27	0.814
			−	283	94	12		660	118	
		Drug resistance	+	56	17	1	0.624	129	19	0.359
			−	287	103	12		677	127	
		DILI	+	53	16	1	0.667	122	18	0.390
			−	292	104	12		688	128	
		Pulmonary infection	+	71	29	4	0.519	171	37	0.254
			−	274	91	9		639	109	
		Hypoproteinemia	+	24	10	0	0.528	58	10	0.893
			−	321	110	13		752	136	
		Leukopenia	+	24	8	0	0.615	56	8	0.523
			−	321	112	13		754	138	
		Sputum smear	+	220	76	5	0.080	516	86	0.452
			−	94	28	7		216	42	
rs3741384	G/A	Fever	+	60	24	1	0.489	144	26	0.831
			−	286	92	11		664	114	
		Drug resistance	+	57	16	1	0.609	130	18	0.324
			−	290	101	11		681	123	
		DILI	+	53	16	1	0.759	122	18	0.494
			−	296	101	11		693	123	
		Pulmonary infection	+	74	26	4	0.600	174	34	0.463
			−	275	91	8		641	107	
		Hypoproteinemia	+	24	10	0	0.519	58	10	0.992
			−	325	107	12		757	131	
		Leukopenia	+	24	8	0	0.643	56	8	0.599
			−	325	109	12		759	133	
		Sputum smear	+	223	73	5	0.081	519	83	0.421
			−	95	27	7		217	41	
rs3825071	C/T	Fever	+	67	17	1	0.148	151	19	0.052
			−	266	112	11		644	134	
		Drug resistance	+	48	24	2	0.520	120	28	0.305
			−	287	105	10		679	125	
		DILI	+	44	23	3	0.252	111	29	0.100
			−	293	106	9		692	124	
		Pulmonary infection	+	76	26	2	0.778	178	30	0.482
			−	261	103	10		625	123	
		Hypoproteinemia	+	28	6	0	0.243	62	6	0.094
			−	309	123	12		741	147	
		Leukopenia	+	24	7	1	0.786	55	9	0.661
			−	313	122	11		748	144	
		Sputum smear	+	196	95	10	0.010	487	115	0.003
			−	103	24	2		230	28	

(Continued)

Table 3 (Continued).

SNP	Allele	Clinical Features	Group	Genotype n (%)			P value[a]	Allele n (%)		P value
	(M/m)			MM	Mm	mm		M	m	
rs512715	G/C	Fever	+	44	34	7	0.790	122	48	0.497
			–	217	144	28		578	200	
		Drug resistance	+	45	26	3	0.374	116	32	0.172
			–	217	153	32		587	217	
		DILI	+	40	25	5	0.926	105	35	0.737
			–	223	155	30		601	215	
		Pulmonary infection	+	56	34	14	0.021	146	62	0.175
			–	207	146	21		560	188	
		Hypoproteinemia	+	22	7	5	0.045	51	17	0.823
			–	241	173	30		655	233	
		Leukopenia	+	19	11	2	0.874	49	15	0.609
			–	244	169	33		657	235	
		Sputum smear	+	170	112	19	0.395	452	150	0.359
			–	70	46	13		186	72	

Notes: Part of the study subjects of data missing. [a]P-value was corrected by Bonferroni correction (0.05/3), $P < 0.0167$ was considered as statistical significance.
Abbreviations: +/-, with/without; M, major alleles; m, minor alleles.

Table 4 Haplotype Analysis of *NEAT1* Gene in PTB Patients and Controls

Haplotype	PTB [n(%)]	Control [n(%)]	P value	OR (95% CI)
rs2239895-rs3741384-rs3825071-rs512715				
CACC	140(14.6)	142(14.9)	0.824	0.972(0.754, 1.252)
GGCC	81(8.5)	74(7.7)	0.566	1.101(0.792, 1.531)
GGCG	580(60.6)	584(61.4)	0.622	0.954(0.789, 1.152)
GGTG	126(13.1)	118(12.4)	0.640	1.066(0.814, 1.397)

Notes: Global χ^2 is 0.623, df=3 (frequency<0.03 in both control and case has been dropped). Fisher's p value is 0.891.

a variety of lncRNA gene variants were associated with the genetic risk of PTB, moreover, the expression of NEAT1 in PTB patients was abnormal.[14,15] To further analyze the role of *NEAT1* gene variation in the pathogenesis of PTB, we explored the role of *NEAT1* gene rs2239895, rs3741384, rs3825071, rs512715 variants in PTB. However, no significant result was found. Contrary to the above studies, our result did not seem to support the involvement of *NEAT1* gene variation in the pathogenesis of PTB. It was worth noting that study regarding the association between *NEAT1* gene variation and PTB was very limited. In addition, this result might be influenced by sample size, experimental methods etc. Thus, relevant studies were very necessary to verify this result.

During the progression of PTB, patients usually accompanied by a variety of complications and clinical manifestations, including pulmonary infection, fever, drug resistance, DILI, hypoproteinemia, which might affect the treatment process of patients. In a previous study, we found that *GC* gene rs7041, rs3733359 variants were significantly associated with pulmonary infection, fever in the PTB patients, respectively.[4] Song et al found a significant association between *lncRNA RP11-37B2.1* rs218916, rs160441 and the occurrence of thrombocytopenia among PTB patients.[15] In this study, we found that rs3825071 variant TT genotype, T allele frequencies were significantly related to sputum smear-positive in PTB patients. Based on these results, we surmised that this SNP might act as a potential predictor of sputum smear status in PTB patients, thus contributing to make more appropriate treatment choices and management measures.

In conclusion, our study suggested that *NEAT1* gene rs2239895, rs3741384, rs3825071, rs512715 polymorphisms might not be related to PTB susceptibility in a Chinese population. While, rs3825071 variant was significantly associated

with sputum smear-positive in PTB patients. It was worth noting that several limitations existed in this study, including sample size, ethnic background, disease activity, course of disease, treatment, etc, which might influence the accuracy of these results of our study. Therefore, in order to further clarify the exact role of *NEAT1* genetic variation in PTB, it was very necessary to carry out replication studies with larger sample sizes in different populations.

Abbreviations

TB, Tuberculosis; lncRNA, Long noncoding RNA; MAF, minor allele frequency; IMLDR, improved multiple ligase detection reaction; HWE, Hardy-Weinberg equilibrium; SNPs, single nucleotide polymorphisms; MTB, Mycobacterium tuberculosis.

Data Sharing Statement

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

Ethics Approval and Consent to Participate

This study was approved by the Ethics Committee of Anhui Medical University (20200250), and the informed consent was obtained from each participant. All methods were carried out in accordance with relevant guidelines and regulations of declaration of Helsinki.

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Disclosure

The authors declare that they have no conflicts of interest in this work.

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