Research Paper

Correlation between genetic polymorphism of angiopoietin-2 gene and clinical aspects of rheumatoid arthritis

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Abstract

The Angiopoietin-2 (Ang2) gene encodes angiogenic factor, and the polymorphisms of Ang2 gene predict risk of various human diseases. We want to investigate whether the single nucleotide polymorphisms (SNPs) of the Ang2 gene can predict the risk of rheumatoid arthritis (RA). Between 2016 and 2018, we recruited 335 RA patients and 700 control participants. Comparative genotyping for SNPs rs2442598, rs734701, rs1823375 and rs12674822 was performed. We found that when compared with the subjects with the A/A genotype of SNP rs2442598, the subjects with the T/T genotype were 1.78 times likely to develop RA. The subjects with C/C genotype of SNP rs734701 were 0.53 times likely to develop RA than the subjects with TT genotype, suggesting the protective effect. The subjects with G/G genotype of SNP rs1823375 were 1.77 times likely to develop RA than the subjects with C/C genotype. The subjects with A/C and C/C genotype of SNP rs11137037 were 1.65 and 2.04 times likely to develop RA than the subjects with A/A genotype. The subjects with G/T and T/T genotype of SNP rs12674822 were 2.42 and 2.25 times likely to develop RA than the subjects with G/G genotype. The T allele over rs734701 can lead to higher serum erythrocyte sedimentation rate level (p = 0.006). The A allele over rs11137037 was associated with longer duration between disease onset and blood sampling (p = 0.003). Our study suggested that Ang2 might be a diagnostic marker and therapeutic target for RA therapy. Therapeutic agents that directly or indirectly modulate the activity of Ang2 may be the promising modalities for RA treatment.

Key words: Angiopoietin-2; single nucleotide polymorphisms; rheumatoid arthritis

Introduction

Rheumatoid arthritis (RA) is manifested by marked hypertrophy, hypervascularity of the synovial tissues and consequent joint destruction, plaguing around 1% of the global population [1, 2]. Despite the recent advent of biological agents enabling some RA patients to achieve disease remission with minimal symptoms, a marked proportion of patients remain treatment-refractory and suffer from progressive joint destruction, functional deterioration or even premature mortality [3-5]. The fact that genetic factors account for about 60% of the overall susceptibility to RA highlights the importance of research into genetic aberrations of this disease [3, 6-8]. Investigations into RA genetics could facilitate risk prediction for individual patients and facilitate personalized regimen.

Single nucleotide polymorphisms (SNPs) denote the single nucleotide variations occurring at specific sites in the genome with substantial frequency within the population [1, 9, 10]. Genotyping SNPs and comparing the frequency of SNPs among subgroups (e.g., controls and patients) are frequently utilized to examine the risk and prognosis of human, including RA [6, 10, 11].

The process of angiogenesis is pivotal in the pathogenesis of RA. The proliferation of the synovial lining of joints and the subsequent invasion by the pannus of underlying cartilage and bone necessitate an increase in the vascular supply to the synovium in RA [12-14]. Angiogenesis is also essential in facilitating the invasion of inflammatory cells and increase in local pain receptors that contribute to structural damage and pain. The angiogenic process is further modulated by the complex interplays between various mediators such as growth factors, notably vascular endothelial growth factor (VEGF) and the angiopoietin 2 (Ang2) [15-18].

The angiopoietin family mediates the process of angiogenesis and has two main members. Angiopoietin-1 is critical for vascular maturation, adhesion, migration, and survival. Ang2 promotes cell death and disrupts vascularization in its singular form but enhances angiogenesis in conjunction with VEGF [19]. The VEGF/Ang2-induced angiogenesis modulates RA-associated angiogenic processes [16]. The genetic polymorphisms of Ang2 harbor prognostic values for various human disease, including retinopathy, lung diseases and secondary lymphedema after breast cancer surgery [17, 20, 21]. Despite the known impact of Ang2 on RA pathogenesis and the recognized prognostic value of Ang2 SNPs for human disease, little is known about the association between Ang2 SNPs and the risk of RA. In this study, we tried to determine the predictive capacity of Ang2 SNPs as candidate biomarkers for susceptibility to RA.

Materials and Methods

Patients and blood samples

We collected 335 blood specimens from the patients who had been diagnosed with RA at Dongyang People's Hospital as the RA group from 2016 to 2018. For the control group, 700 health participants without RA history or cancers were enrolled. All of the participants provided written informed consent, and this study was approved by the Ethics Committee of Dongyang People's Hospital Ethics Committee and Institutional Review Board (2016-YB002). Clinical and pathological characteristics of all patients were determined based on medical records. A standardized questionnaire and electronic medical record system were used to acquire detailed clinical data on age, sex and disease duration, as well as concurrent treatment with methotrexate, prednisolone, and tumor necrosis factor-α (TNF-α) inhibitors. At baseline, serum samples were collected from all RA patients and analyzed for the level of anti-citrullinated protein antibodies (ACPAs), rheumatoid factor (RF), erythrocyte sedimentation rate (ESR), and C-reactive protein (CRP). Samples were ACPA-positive if anti-CCP2 titers were ≥17 IU/mL and RF-positive if IgM RF titers were ≥30 IU/mL. Whole blood samples (3 mL) were collected from all study participants and stored at −80 °C for subsequent DNA extraction.

Selection of *Ang-2* polymorphisms

Five *Ang-2* SNPs were selected from the intron of *Ang-2*; all SNPs had minor allele frequencies of greater than 5%. Most *Ang-2* SNPs were known to be associated with lung injury or secondary lymphedema after breast cancer surgeries [21, 22].

Genomic DNA extraction

Genomic DNA was extracted from peripheral blood leukocytes using a QIAamp DNA blood kit (Qiagen, CA, USA) according to the manufacturer's instructions. Extracted DNA was stored at -20°C and prepared for genotyping by polymerase chain reaction (PCR).

Genotyping by real-time PCR

Total genomic DNA was isolated from whole blood specimens using QIAamp DNA blood mini kits (Qiagen, Valencia, CA), following the manufacturer's instructions. DNA was dissolved in TE buffer (10 mM Tris pH 7.8, 1 mM EDTA) and stored at −20°C until quantitative PCR analysis. Five *Ang-2* SNP probes were purchased from Thermo Fisher Scientific Inc. (USA), and assessment of allelic discrimination for *Ang-2* SNPs was conducted using a QuantStudio™ 5 Real-Time PCR system (Applied Biosystems, CA, USA), according to the manufacturer's instructions. Data were further analyzed with QuantStudio™ Design & Analysis Software (Applied Biosystems), and compiled statistics with clinical data [6]. Genotyping PCR was carried out in a total volume of 10 μL, containing 20–70 ng genomic DNA, 1 U Taqman Genotyping Master Mix (Applied Biosystems, Foster City, CA, USA), and 0.25 μL probes. The sequence of four Ang2 SNP probes were described as follows: rs2442598, TATGTGTGCGA GGACAGTGTGTGTT[A/T]ATTTTGTCCTCTTCTTG ATGGTTGA; rs734701, TGTGATATTGTGGAAAG ACCTGGTA[T/C]TCAAGTAATTTGTTATTCTATT

CTC; rs1823375, GTGACTTCTCTTAGGGAGCACACTT[C/G]CCTTCACCTGCCCTGACCACATGGA; rs11137037, CCCACCATCCCCCATTGCATGCCCT[A/C]AGCAAAGATACTCGTTTTGTGTTTC; rs12674822, GCAATCACTTGTCTGGCCCAACCCT[G/T]TATATTATTTGAGGCCCAGAAAAGG. The protocol included an initial denaturation step at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min [23, 24].

Statistical analysis

Differences between the two groups were considered significant if *p* values were less than 0.05. Hardy-Weinberg equilibrium (HWE) was assessed using chi-square goodness-of-fit tests for biallelic markers. Since the data was independent and normal distribution, Fisher's exact test was used to compare differences in demographic characteristics between healthy controls and patients with RA. The odds ratios (ORs) and 95% confidence intervals (CIs) for associations between genotype frequencies and the risk of RA or clinical and pathological characteristics were estimated by multiple logistic regression models, after controlling for other covariates. All data were analyzed using Statistical Analytic System software (v. 9.1, 2005; SAS Institute, Cary, NC, USA).

Results

All of the enrolled participants were identified as Chinese Han ethnicity. The mean age was 56.16 ± 12.31 years old for the RA cohort and 43.60 ± 17.85 years old for the control cohort (*p* < 0.001). The proportion of female subjects was 82.7% in the RA cohort and 51.3% for the control cohort (*p* < 0.001). The interval between the onset of RA and the blood sampling was 71.36 ± 91.45 months. At the time of blood sampling, 39.4% of the RA cohort were receiving TNF-α inhibitors, 49.3% were receiving methotrexate, and 53.4% were receiving prednisolone. The majority of RA patients were rheumatoid factor (RF) positive (84.2%) and anti-citrullinated protein antibody (ACPA) positive (80.9%) (Table 1). To mitigate the possible impact of confounding variables, AORs with 95% CIs were estimated by multiple logistic regression models after controlling for age in each comparison.

The details of polymorphism frequencies in both cohorts are shown in Table 2. All genotypes were in Hardy-Weinberg equilibrium (*p*>0.05). The most frequent genotypes for SNPs rs2442598, rs734701, rs1823375 and rs12674822 in both groups were A/T, T/C, C/C and G/T respectively. The genotypes of highest frequency for rs 11137037 were AC for RA cohort and AA for control cohort.

Table 1. Comparison of demographic characteristics and clinical parameters of 700 healthy controls and 335 patients with RA.

Variable	Controls N=700 (%)	RA Patients N=335 (%)	p value
Age (y)	Mean ± S.D.	Mean ± S.D.	
	43.60 ± 17.85	56.16 ± 12.31	p<0.001
Gender			
Female	359 (51.3)	277 (82.7)	
Male	341 (48.7)	58 (17.3)	p<0.001
RA duration (months)			
		71.36 ± 91.45	
Serum CRP (mg/L)			
		21.39 ± 68.37	
ESR (mm/h)			
		32.65 ± 25.71	
RF status			
Negative		53 (15.8)	
Positive		282 (84.2)	
ACPA status			
Negative		64 (19.1)	
Positive		271 (80.9)	
Anti-TNF drugs use			
Non-users		203 (60.6)	
Current users		132 (39.4)	
Methotrexate use			
Non-users		170 (50.7)	
Current users		165 (49.3)	
Prednisolone use			
Non-users		156 (46.6)	
Current users		179 (53.4)	

The Mann-Whitney U test or Fisher's exact test was used to compare values between controls and patients with RA. RA = rheumatoid arthritis; y = years; S.D. = standard deviation; CRP = C-reactive protein; ESR = erythrocyte sedimentation rate; RF = rheumatoid factor; ACPA = anti-citrullinated protein antibodies; TNF = tumor necrosis factor.

When compared with the subjects with the A/A genotype of SNP rs2442598, the subjects with the T/T genotype were 1.78 times likely to develop RA (AOR 1.78; 95% CI 1.17 to 2,71; *p*<0.05). The subjects with C/C genotype of SNP rs734701 were 0.53 times likely to develop RA (AOR 0.53; 95% CI 0.34 to 0.83; *p*<0.05) than the subjects with T/T genotype. The subjects with G/G genotype of SNP rs1823375 were 1.77 times likely to develop RA (AOR 1.77; 95% CI 1.12 to 2.79; *p*<0.05) than the subjects with C/C genotype. The subjects with A/C and C/C genotype of SNP rs11137037 were 1.65 (AOR 1.65; 95% CI 1.19 to 2.29; *p*<0.05) and 2.04 (AOR 2.04; 95% CI 1.37 to 3.04; *p*<0.05) times likely to develop RA than the subjects with A/A genotype. The subjects with G/T and T/T genotype of SNP rs12674822 were 2.42 (AOR 2.42; 95% CI 1.67 to 3.51; p<0.05) and 2.25 (AOR 2.25; 95% CI 1.48 to 3.42; p<0.05) times likely to develop RA than the subjects with GG genotype.

The respective SNPs were all analyzed for their correlation with the demographic characteristics and clinical parameters. The T allele over the rs12674822 site was associated with 1.36 (AOR 1.36; 95% CI 1.00 to 1.85; *p*<0.05) times the likelihood to require steroid use than the G allele (Table 3). The T allele over rs734701 can lead to higher serum ESR level (*p* = 0.006) (Table 4). The A allele over rs11137037 was

associated with longer duration between disease onset and blood sampling (*p*=0.003) (Table 5).

Table 2. Comparison of the genotype and allele frequencies of the *Ang2* polymorphism in 700 controls and 335 patients with RA.

Variable	Controls N=700 (%)	Patients N=335 (%)	OR (95% CI)	AOR (95% CI)
rs2442598				
AA	205 (29.3)	93 (27.8)	1.00 (reference)	1.00 (reference)
AT	364 (52.0)	151 (45.1)	0.941 (0.671-1.247)	0.970 (0.687-1.369)
TT	131 (18.7)	91 (27.2)	**1.531 (1.065-2.201)***	**1.781 (1.172-2.708)***
AT+TT	495 (70.7)	242 (72.3)	1.078 (0.807-1.439)	1.170 (0.845-1.620)
A allele	774 (55.3)	337 (50.3)	1.00 (reference)	1.00 (reference)
T allele	626 (44.7)	333 (49.7)	**1.222 (1.016-1.469)***	**1.302 (1.059-1.600)***
rs734701				
TT	211 (30.1)	104 (31.0)	1.00 (reference)	1.00 (reference)
TC	321 (45.9)	182 (54.3)	1.150 (0.855-1.548)	1.168 (0.839-1.626)
CC	168 (24.0)	49 (14.6)	**0.592 (0.398-0.879)***	**0.527 (0.337-0.825)***
TC+CC	488 (69.9)	231 (68.9)	0.958 (0.723-1.271)	0.947 (0.691-1.296)
T allele	743 (53.1)	390 (58.2)	1.00 (reference)	1.00 (reference)
C allele	657 (46.9)	280 (41.8)	**0.812 (0.674-0.978)***	**0.784 (0.637-0.965)***
rs1823375				
CC	345 (49.3)	149 (44.5)	1.00 (reference)	1.00 (reference)
CG	289 (41.3)	138 (41.2)	1.106 (0.836-1.462)	1.192 (0.870-1.633)
GG	66 (9.4)	48 (14.3)	**1.684 (1.108-2.559)***	**1.769 (1.121-2.794)***
CG+GG	355 (50.7)	186 (55.5)	1.213 (0.934-1.576)	1.306 (0.975-1.751)
C allele	979 (69.9)	436 (65.1)	1.00 (reference)	1.00 (reference)
G allele	421 (30.1)	234 (34.9)	**1.248 (1.026-1.518)***	**1.314 (1.057-1.634)***
rs11137037				
AA	354 (50.6)	122 (36.4)	1.00 (reference)	1.00 (reference)
AC	240 (34.3)	139 (41.5)	**1.681 (1.253-2.253)***	**1.653 (1.193-2.289)***
CC	106 (15.1)	74 (22.1)	**2.026 (1.412-2.907)***	**2.039 (1.367-3.040)***
AC+CC	346 (49.4)	213 (63.6)	**1.786 (1.367-2.344)***	**1.777 (1.320-2.392)***
A allele	948 (67.7)	383 (57.2)	1.00 (reference)	1.00 (reference)
C allele	452 (32.3)	287 (42.8)	**1.572 (1.300-1.900)***	**1.577 (1.276-1.949)***
rs12674822				
GG	243 (34.7)	62 (18.5)	1.00 (reference)	1.00 (reference)
GT	301 (43.0)	175 (52.2)	**2.279 (1.629-3.187)***	**2.422 (1.674-3.506)***
TT	156 (22.3)	98 (29.3)	**2.462 (1.690-3.587)***	**2.250 (1.481-3.420)***
GT+TT	457 (65.3)	273 (81.5)	**2.341 (1.706-3.213)***	**2.368 (1.670-3.359)***
G allele	787 (56.2)	299 (44.6)	1.00 (reference)	(reference)
T allele	61.3 (43.8)	371 (55.4)	**1.593 (1.324-1.917)***	**1.514 (1.232-1.861)***

The odds ratios (ORs) and with their 95% confidence intervals (CIs) were estimated by logistic regression models. The adjusted odds ratios (AORs) with their 95% confidence intervals (CIs) were estimated by multiple logistic regression models that controlled for age and gender. *RETN* = resistin; RA = rheumatoid arthritis.
* *p* < 0.05 as statistically significant.

Table 3. Odds ratios (ORs) and 95% confidence intervals (CIs) of the clinical status and genotype frequencies of the *Ang2* rs12674822 polymorphism in 335 patients with RA.

Variable	Genotypic frequencies			
	G allele N=299 (%)	T allele N=371 (%)	OR (95% CI)	AOR (95% CI)
RF status				
Negative	49 (16.4)	57 (15.4)	1.00 (reference)	1.00 (reference)
Positive	250 (83.6)	314 (84.6)	1.080 (0.712-1.637)	1.080 (0.712-1.638)
ACPA status				
Negative	55 (18.4)	73 (19.7)	1.00 (reference)	1.00 (reference)
Positive	244 (81.6)	298 (80.3)	0.920 (0.624-1.357)	0.915 (0.619-1.352)
Anti-TNF drugs use				
Non-users	173 (57.9)	233 (62.8)	1.00 (reference)	1.00 (reference)
Current users	126 (42.1)	138 (37.2)	0.813 (0.596-1.110)	0.814 (0.596-1.111)
Methotrexate use				
Non-users	153 (51.2)	187 (50.4)	1.00 (reference)	1.00 (reference)
Current users	146 (48.8)	184 (49.6)	1.031 (0.760-1.398)	1.026 (0.754-1.396)
Prednisolone use				
Non-users	152 (50.8)	160 (43.1)	1.00 (reference)	1.00 (reference)

Variable	Genotypic frequencies			
	G allele N=299 (%)	T allele N=371 (%)	OR (95% CI)	AOR (95% CI)
Current users	147 (49.2)	211 (56.9)	**1.364 (1.004-1.852)***	**1.362 (1.003-1.850)***

The odds ratios (ORs) and their 95% confidence intervals (CIs) were estimated by logistic regression models. The adjusted odds ratios (AORs) with their 95% CIs were estimated by multiple logistic regression analyses that controlled for gender.
* *p* < 0.05 as statistically significant.
RA = rheumatoid arthritis; RF = rheumatoid factor; ACPA = anti-citrullinated protein antibodies; TNF = tumor necrosis factor.

Table 4. Comparison of the clinical parameters and genotype frequencies of the *Ang2* rs734701 polymorphism in 335 patients with RA.

Parameter	C allele (N=572)	T allele (N=98)	
	Mean ± S.E.M.		p value
RA duration (months)			
	69.88 ± 5.34	80.04 ± 14.07	0.262
Serum CRP (mg/L)			
	21.95 ± 4.32	18.14 ± 3.86	0.566
ESR (mm/h)			
	31.76 ± 1.45	37.88 ± 4.50	**0.006***

Independent sample t test was used to make comparisons between clinical parameters and the C and T alleles of the *Ang2* rs734701 polymorphisms.
**p* ≤ 0.05 was considered to be significant.
RETN = resistin; RA = rheumatoid arthritis; RA = rheumatoid arthritis; S.D. = standard deviation; CRP = C-reactive protein, ESR = erythrocyte sedimentation rate.

Table 5. Comparison of the clinical parameters and genotype frequencies of the *Ang2* rs11137037 polymorphism in 335 patients with RA.

Parameter	A allele (N=522)	C allele (N=148)	
	Mean ± S.E.M.		p value
RA duration (months)			
	75.30 ± 6.04	57.47 ± 7.41	**0.003***
Serum CRP (mg/L)			
	22.12 ± 4.67	18.81 ± 3.81	0.508
ESR (mm/h)			
	32.06 ± 1.57	34.76 ± 3.13	0.378

Independent sample t test was used to make comparisons between clinical parameters and the A and C alleles of the *Ang2* rs11137037 polymorphisms.
**p* ≤ 0.05 was considered to be significant.
RETN = resistin; RA = rheumatoid arthritis; RA = rheumatoid arthritis; S.D. = standard deviation; CRP = C-reactive protein, ESR = erythrocyte sedimentation rate.

Discussion

The RA susceptibility is influenced by genetic factors. Although the advent of biological-based antirheumatic therapies has enabled some patients to achieve very low levels of disease activity, there are still an unignorable number of RA patients who remain treatment-refractory [1, 25, 26]. The unmet need underlines the importance of continuing to investigate the pathogenesis of RA. Genetic studies indicate that specific SNPs are associated with the RA risk [27]. The search for RA-related SNPs seems to be a promising method to understand the pathogenesis of RA and for risk stratification [28].

Ang2 has been shown to be involved in the pathogenesis of RA. VEGF-induced Ang2 is the main

regulator in the IL-35 suppressed RA angiogenesis [29]. The serum level of Ang2 correlates with disease severity, early onset and cardiovascular disease among RA patients [30]. The bispecific TNF-α-Ang2 molecules showed a dose-dependent reduction in both clinical RA symptoms and histological scores that were significantly better than that achieved by adalimumab alone in mouse RA model [31]. Krausz et al. identified synovial macrophages as primary targets of Ang signaling in RA, and demonstrated that Ang2 promotes the pro-inflammatory activation of human macrophages. The authors thus suggested that targeting Ang2 may be of therapeutic benefit in the treatment of RA [32]. These findings suggest that Ang2 can be enlisted among the factors that dictate the pathogenesis of RA.

The Ang2 SNPs possess prognostic values for various human diseases. The Ma's study proposed Ang2 gene as a susceptibility gene for neovascular age-related macular degeneration and polypoidal choroidal vasculopathy [20]. The rs2442598 polymorphism of Ang2 gene was significantly associated with psoriasis vulgaris [33]. Genetic variants in the Ang2 gene are associated with increased risk of acute respiratory distress syndrome [17].

Despite the evidence inferring a role for Ang2 in the pathogenesis of RA and the prognostic capacity of Ang2 SNPs in various human diseases, few studies have investigated the relationship between Ang2 SNPs and risk of developing RA. Previous studies have been reported the role of Ang1 was involved in RA [34, 35]. However, none of studies explored the correlation between Ang2 gene polymorphism and RA progression. In this study, we sought to determine the prognostic capacity of Ang2 SNPs in predicting RA onset. To the best of our knowledge, our study is the first to identify that the distribution of rs2442598, rs734701, rs1823375, rs11137037 and rs12674822 SNPs is associated with RA development. We examined five *Ang2* SNPs among 700 controls and 335 RA patients. We found that when compared with the subjects with the A/A genotype of SNP rs2442598, the subjects with the T/T genotype were 1.78 times likely to develop RA. The subjects with C/C genotype of SNP rs734701 were 0.53 times likely to develop RA than the subjects with TT genotype, suggesting the protective effect. The subjects with G/G genotype of SNP rs1823375 were 1.77 times likely to develop RA than the subjects with C/C genotype. The subjects with A/C and C/C genotype of SNP rs11137037 were 1.65 and 2.04 times likely to develop RA than the subjects with A/A genotype. The subjects with G/T and T/T genotype of SNP rs12674822 were 2.42 and 2.25 times likely to develop RA than the subjects with

G/G genotype. These findings have not been reported up to now. We also investigated the association of these *Ang2* SNPs with RA treatment regimens and serum inflammatory markers. We found that Ang2 rs11137037 had a high risk in RA patients which correlated with ESR. Although rs734701 had a protective effect, it was associated with clinical ESR of RA patients. These correlations between clinical results and genetic function required to be further explored in the future. On the other hands, our linkage disequilibrium analysis had no significant results between these Ang2 SNPs (Supplement Fig. S1).

A major limitation to this study is that the findings of our study might be mere cross-relationship instead of actual causality. This is a ubiquitous limitation for similar studies and might be partially overcome by the deeper evaluation trying to select and analyze the relationships between all the known SNP elements. In conclusion, our study offers novel insights into Ang2 SNPs in regard to RA susceptibility. We found that the A/A genotype of SNP rs2442598, G/G genotype of SNP rs1823375, A/C and C/C genotype of SNP rs11137037, and GT and TT genotype of SNP rs12674822 were associated with higher risk for RA development, and C/C genotype of SNP rs734701 was associated with decreased RA risk. This is the first study to demonstrate that a correlation exists between Ang2 polymorphisms and RA risk. Ang2 might be a diagnostic marker and therapeutic target for RA therapy. Therapeutic agents that directly or indirectly modulate the activity of Ang2 may be the promising modalities for RA treatment.

Supplementary Material

Supplementary figure.
http://www.medsci.org/v16p0331s1.pdf

Acknowledgments

Competing Interests

The authors have declared that no competing interest exists.

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