Int J Clin Exp Med 2015;8(11):21343-21350
www.ijcem.com /ISSN:1940-5901/IJCEM0015544

Original Article

A tagging *ALOX5AP* polymorphism and risk of ischemic stroke in a northeastern Chinese Han population

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Received September 3, 2015; Accepted November 1, 2015; Epub November 15, 2015; Published November 30, 2015

Abstract: Objective: 5-lipoxygenase-activating protein gene (*ALOX5AP*) has been recognized as a susceptibility gene for stroke. In this work, we explored the association of 6 *ALOX5AP* SNPs with cerebral infarction (CI) in a northeastern Chinese Han population, using a case-control design. Methods: A group of patients with cerebral infarction were randomly chosen as case group in northeastern Chinese Han population. Another comparative group of individuals without stroke were chosen as the control group. By utilizing TaqMan probe based real-time fluorescent PCR and DNA sequencing method, this study focused on 6 SNPs of *ALOX5AP* gene and analyzed the association with the hereditary susceptibility of cerebral infarction. Results: We found that, the rs9579646 G allele frequency was significantly associated with higher ischemic cerebral infarction. There was no significant difference of rs9551963, rs9315050, rs4769874, rs10507391 and rs4147064 genotype frequencies between the case and control group. Haplotype-based association analysis of the block involving rs9579646 and rs10507391 revealed that the increased risk of stroke was significantly associated with haplotype GT and GA. Conclusion: These results suggested that the genetic variants in *ALOX5AP* might be related to the risk of stroke in northeastern Chinese Han population. The SNP rs9579646 may be a diagnostic index of cerebral infarction.

Keywords: 5-lipoxygenase activating protein (*ALOX5AP*), cerebral infarction, single nucleotide polymorphisms (SNPs), stroke, TaqMan-PCR

Introduction

Ischemic cerebral vascular diseases (ICVDs) are common diseases of the nervous system, and the leading causes of disability and death in developed countries [1]. ICVDs have become one of the three major causes of human death currently. Complex environmental factors and genetic background play an important role in the occurrence and development of ICVDs.

A number of different pathogenic events may lead to ICVDs, including atherosclerosis, small vessel disease, cardiac arrhythmias, and hypercoagulation [2]. Atherothrombosis is considered to be the main cause [3]. Previous studies had established age, sex, obesity, smoking, hypertension, diabetes, and dyslipidemia as reliable stroke risk predictors [4]. Recent studies found that type 2 diabetes mellitus (T2DM) is a major independent risk factor for cardiovascular disease, including stroke [5]. A meta-analysis reported patients with T2DM had 2.27 times higher risk for ischemic stroke [5]. Given the rising prevalence of T2DM due to the epidemic of obesity, the number of patients suffering cerebral diseases due to T2DM is expected to further increase [6]. However, the molecular association between diabetes and ICVDs are unrevealed.

In addition, these conventional risk factors do not fully account for the overall risk of stroke. Evidences from twins, family, and animal studies have consistently suggested a genetic contribution to the risk of ischemic stroke [7]. Although the detailed genetic mechanism of ICVDs remains unclear, studies on gene polymorphism in diabetes or cerebral infarction have reported several potential pathogenic factors. The De Code study highlighted the implication of single nucleotide polymorphisms (SNPs)

Table 1. Evaluation and clinical characteristics of the cases and controls

Background parameters	Cases (n = 456)	Controls (n = 452)	*P*
Sex (Males/Females)	297/159	301/151	0.693
Age (years)	59.7 ± 11.5	53.7 ± 8.2	< 0.001***
Diabetes (%)	165 (36.1%)	21 (4.6%)	< 0.001***
TC (mmol/L)	5.29 ± 1.28	4.98 ± 0.81	< 0.001***
TG (mmol/L)	2.16 ± 0.63	1.17 ± 0.76	< 0.001***
HDL-C (mmol/L)	1.14 ± 0.25	1.35 ± 0.33	< 0.001***
LDL-C (mmol/L)	3.15 ± 0.39	2.91 ± 0.31	< 0.001***

TC, total cholesterol; TG, triglycerides; HDL, high-density lipoprotein cholesterol; LDL, low density lipoprotein. Data are presented as mean ± standard deviation. ***$P < 0.001$.

and haplotypes in the 5-lipoxygenase-activating protein gene (*ALOX5AP* or *FLAP*) in ischemic stroke in the Icelandic population through genome-wide linkage scan [8, 9]. *ALOX5AP* encodes arachidonate 5-lipoxygenase-activating protein, which plays a key role in the biosynthesis of pro-inflammatory leukotriene B mediators, providing a potential link between inflammation and cardiovascular disease. The SNPs of *ALOX5AP* have been a research focus in studies of ICVD populations. In 2012, we investigated the association of the SNPs of *ALOX5AP* with stroke in the Han population in northeastern China. The rs9579646 GG genotype of *ALOX5AP* was found to be associated with a marginally decreased risk for stroke [OR (95% CI) = 1.73 (1.01-2.97)], compared with the AA genotype [10]. Since the number of patients with ischemic stroke was not large enough, we investigated 6 SNPs across *ALOX5AP* sequence in a larger number of stroke patients and matched controls from northeastern China, as part of our ongoing hospital-based case-control study.

Subjects and methods

Subjects

A total of 456 patients with unrelated ischemic cerebral infarction of the northeastern Chinese Han population were included in this study and were taken as the case group. All cases were diagnosed using the Fourth edition of Cerebrovascular Disease Diagnostic Standards [11]. These patients have lived in the northeastern provinces of China for more than 3 generations, with no genetic relationship. The patients were enrolled in the department of neurology of the Fourth Affiliated Hospital of Harbin Medical University (Harbin, Heilongjiang, China) between January 2013 and February 2014 and include in the case group. Among the cases, 165 patients were diagnosed with type 2 diabetic mellitus according to the 1997 American Diabetes Association criteria [12]. Patients complicated with hypertension, blood diseases, tumor, thyroid diseases, cerebral hemorrhage, cerebral embolism, and cerebral infarction induced by infection and severe liver and kidney diseases were excluded in this study.

We also recruited unrelated individuals as controls from the Health Medical Center of the Fourth Affiliated Hospital of Harbin Medical University. The control group composed of 452 individuals who were not related to the case group and had no history of cerebral infarction or transient ischemic attack. The first and second degree relatives of the controls had no cerebrovascular diseases or hypertension. All blood samples were collected following informed written consent of the participating individuals and the study protocol was approved by the institutional ethics committee.

All experiments were performed in compliance with the relevant laws and institutional guidelines. Informed consent was obtained from all the subjects.

Genotyping

Peripheral venous blood was withdrawn from each subject for genotyping of *ALOX5AP*. Genomic DNA was extracted using standard phenol-chloroform protocols. The genotyping was based on TaqMan® SNP Genotyping Assays (Applied Biosystems). The primers and probes were synthesized by Gene Tech (Shanghai) and Applied Biosystems (ABI). For PCR, the reaction mixture included the following: 20 ng genome DNA (2.25 μL), 2.5 μL TaqMan Universal PCR Master Mix (2×), and 0.25 μL SNP Genotyping Assay stock (10×). The reaction volume a total of 5 μL each well was loaded into 384-well plates for PCR. Amplification cycle was as follows: pre-denaturation at 95°C for 10 min, denaturation at 92°C for 15 s, annealing and extension at 60°C for 1 min. The total reaction had 60 cycles and was carried out in an ABI 9700 PCR instrument.

Table 2. Hardy-Weinberg equilibrium test of SNPs in *ALOX5AP* among the cases and controls

#	SNP	Genotype	Cases		χ^2	*P*	Controls		χ^2	*P*
			n	%			n	%		
1	rs9579646	A/A	60	13.2	1.61	0.234	87	19.2	0.001	1
		A/G	228	50			223	49.3		
		G/G	168	36.8			142	31.4		
2	rs10507391	T/T	198	43.4	0	1	189	41.8	0.006	1
		A/T	205	45			206	45.6		
		A/A	53	11.6			57	12.6		
3	rs4147064	C/C	220	48.2	0.277	0.657	215	47.6	0.006	1
		C/T	190	41.7			194	42.9		
		T/T	46	10.1			43	9.5		
4	rs4769874	G/G	429	94.1	0.424	0.315	433	95.8	0.208	1
		A/G	27	5.9			19	4.2		
		A/A	0	0			0	0		
5	rs9551963	A/A	215	47.1	0.768	0.435	206	45.6	0.002	1
		A/C	202	44.3			198	43.8		
		C/C	39	8.6			48	10.6		
6	rs9315050	A/A	427	93.6	0.492	0.359	432	95.6	0.231	1
		A/G	29	6.4			20	4.4		
		G/G	0	0			0	0		

Table 3. Association of *ALOX5AP* SNP alleles with ischemic cerebral infraction

#	SNP	Allele	Cases		Controls		χ^2	*P*	OR (95% CI)
			n	%	n	%			
1	rs9579646	A	348	38.2	397	43.9	Ref		
		G	564	61.8	507	56.1	5.986	0.014	1.27 (1.05-1.53)
2	rs10507391	T	601	65.9	584	64.6	Ref		
		A	311	34.1	320	35.4	0.282	0.595	0.94 (0.78-1.15)
3	rs4147064	C	630	69.1	624	69	Ref		
		T	282	30.9	280	31	0	1	1 (0.82-1.22)
4	rs4769874	G	885	97	885	97.9	Ref		
		A	27	3	19	2.1	1.031	0.31	1.42 (0.78-2.57)
5	rs9551963	A	632	69.3	610	67.5	Ref		
		C	280	30.7	294	32.5	0.614	0.433	0.92 (0.75-1.12)
6	rs9315050	A	883	96.8	884	97.8	Ref		
		G	29	3.2	20	2.2	1.271	0.26	1.45 (0.81-2.59)

OR, odds ratio; CI, confidence interval.

Statistical analysis

The data in this work were analyzed with Excel 2003. Continuous variables were compared between groups with the Student's *t* test by SPSS 21. Chi-square test or Fisher's exact test, as appropriate, was used to compare categorical variables between groups by the R Project (http://www.R-project.org). Odds ratios (ORs) and their 95% confidence intervals (CIs) were also calculated using the R Project. Hardy-Weinberg equilibrium tests to evaluate the genotype distributions were performed with the R package "genetics" (version 1.3.8.1, http://CRAN.R-project.org/package = genetics). A *P* value of less than 0.05 was considered to be statistically significant. The D' (D-Prime) measurement was used to determine linkage disequilibrium (LD) with the software Haploview 4.2.

Results

Clinical characteristics of cases and controls

In this work, we collected the clinical data and blood samples of 456 patients with unrelated ischemic cerebral infarction in total, which are classified as the case group. 452 individuals without ischemic cerebral infarction were also included in this work as the control group. The clinical characteristics of cases and controls are presented in **Table 1**. Briefly, the mean age was 59.7 ± 11.5 years for the cases and 53.7 ± 8.2 years for the controls (mean ± standard deviation, ***$P < 0.001$). As diabetic and ICVD patients, cases had a higher triglyceride level (TG, ***$P < 0.001$) and a lower level of high density lipoprotein-cholesterol (HDL-C, ***$P < 0.001$). The differences also exist in total cholesterol (TC, ***$P < 0.001$) and low density lipoprotein-cholesterol (LDL-C, ***$P < 0.001$) between two groups.

Analysis of SNP distribution between cases and controls

The SNP IDs and allele frequencies are listed in **Table 2**. Hardy-Weinberg equilibrium tests were used to evaluate the genotype distributions of

Table 4. Genotype distributions of *ALOX5AP* SNPs in the case and control groups

#	SNP	Genotype	Cases		Controls		χ^2	*P*	OR (95% CI)
			n	%	n	%			
1	rs9579646	AA	60	13.2	87	19.2	Ref		
		AG	228	50	223	49.3	3.83	0.050	1.48 (1.02-2.16)
		GG	168	36.8	142	31.4	6.613	0.010	1.72 (1.15-2.55)
2	rs10507391	TT	198	43.4	189	41.8	Ref		
		AT	205	45	206	45.6	0.085	0.77	0.95 (0.72-1.25)
		AA	53	11.6	57	12.6	0.197	0.657	0.89 (0.58-1.36)
3	rs4147064	CC	220	48.2	215	47.6	Ref		
		CT	190	41.7	194	42.9	0.059	0.808	0.96 (0.73-1.26)
		TT	46	10.1	43	9.5	0.006	0.941	1.05 (0.66-1.65)
4	rs4769874	GG	429	94.1	433	95.8	Ref		
		AG	27	5.9	19	4.2	1.058	0.304	1.43 (0.79-2.62)
		AA	0	0	0	0	-	-	-
5	rs9551963	AA	215	47.1	206	45.6	Ref		
		AC	202	44.3	198	43.8	0.009	0.926	0.98 (0.74-1.29)
		CC	39	8.6	48	10.6	0.888	0.346	0.78 (0.49-1.24)
6	rs9315050	AA	427	93.6	432	95.6	Ref		
		AG	29	6.4	20	4.4	1.307	0.253	1.47 (0.82-2.63)
		GG	0	0	0	0	-	-	-

OR, odds ratio; CI, confidence interval.

Table 5. Stratified analysis of the association between rs9579646 genotypes and ischemic stroke risk

	Genotype	Cases	Controls	χ^2	*P*	OR (95% CI)
Sex						
Male	AA	35	37	Ref		
	AG	138	101	1.517	0.218	1.44 (0.85-2.45)
	GG	124	75	3.547	0.060	1.75 (1.01-3.01)
Female	AA	25	50	Ref		
	AG	90	122	1.558	0.212	1.48 (0.85-2.56)
	GG	44	67	0.516	0.472	1.31 (0.71-2.42)
Age (years)						
≤ 60	AA	29	68	Ref		
	AG	117	175	2.794	0.095	1.57 (0.96-2.57)
	GG	98	111	7.196	0.007	2.07 (1.24-3.46)
> 60	AA	31	19	Ref		
	AG	111	48	0.737	0.391	1.42 (0.73-2.75)
	GG	70	31	0.51	0.475	1.38 (0.68-2.82)
Diabetes						
No	AA	39	81	Ref		
	AG	146	215	2.077	0.150	1.41 (0.91-2.18)
	GG	106	135	3.931	0.047	1.63 (1.03-2.58)
Yes	AA	21	6	Ref		
	AG	82	8	-[a]	0.087	0.35 (0.09-1.35)
	GG	62	7	-[a]	0.181	0.40 (0.10-1.61)

[a]Fisher's exact test was performed.

ALOX5AP. All the allele distributions of *ALOX5AP* SNPs were consistent with the Hardy-Weinberg equilibrium model (**Table 2**, $P > 0.05$). No significant difference of the 6 SNPs was found in allele frequencies among the cases and controls, which suggests a good representation of the population.

The allele and genotype distribution of 6 SNPs of *ALOX5AP* was summarized in **Tables 3** and **4**. The frequencies of rs9579646 SNP allele A and G were significantly different between ischemic stroke patients and the controls (**Table 3**, $*P = 0.014 < 0.05$).

We also calculated the genotype distributions of *ALOX5AP* SNPs in the case and the control groups (**Table 4**). The persons with a genotype of rs9579646 GG had higher risks of developing a cerebral infarction than individuals with other genotype [rs9579646 GG: OR (95% CI) = 1.72 (1.15-2.55), $*P = 0.010 < 0.05$]. The individuals with a genotype of rs9579646 AG were also positively associated with cerebral infarction [OR (95% CI) = 1.48 (1.02-2.16)], with a *P* value of 0.050. No significant difference was found in the other SNP genotypes (**Table 4**).

We performed stratification analysis according to sex, age and diabetes, and found that there was no significant association of rs9579646 with ischemic stroke risk in females (**Table 5**, $P = 0.472$). The frequency of rs9579646 GG had a marginal *P* value between males of the two groups ($P = 0.060$). In individuals under 60, rs9579646 GG was positively associated with cerebral

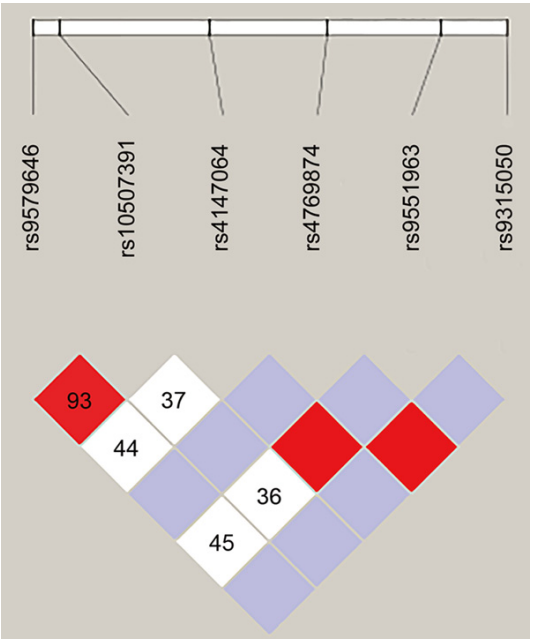

Figure 1. LD structure of the *ALOX5AP* gene. The results of linkage disequilibrium tests were generated by Haploview 4.2 from the genotype of *ALOX5AP* polymorphisms. Linkage was found to be significant between rs9579646 vs. rs10507391, rs4147064 vs. rs9551963, and rs4769874 vs. rs9315050 (D' = 0.933, D' = 1.0 and D' = 1.0 respectively).

infarction [**Table 5**, $^{**}P = 0.007 < 0.01$, OR (95% CI) = 2.07 (1.24-3.46)]. Moreover, individuals with a genotype of rs9579646 GG had higher risks of developing cerebral infarction, who were not diabetics [**Table 5**, $^{*}P = 0.047 < 0.05$, OR (95% CI) = 1.63 (1.03-2.58)]. No significant association of rs9579646 GG with cerebral infarction in patients with diabetes ($P = 0.181$) was found, which may be due to the small sample size of diabetics in the control group (n = 21).

For further analysis, we performed the haplotype distribution tests. Using Haploview 4.2, we found that there were significant linkages between rs9579646 vs. rs10507391, rs4147064 vs. rs9551963, and rs4769874 vs. rs9315050 (**Figure 1**, D' = 0.933, D' = 1.0 and D' = 1.0 respectively). The haplotype distribution in the case and control groups is shown in **Table 6**. Only haplotypes with estimated frequency > 1% were represented. Patients with haplotype rs9579646 & rs10507391 GT or GA had higher risks to have ischemic stroke than haplotype rs9579646 & rs10507391 AA [OR = 1.54 (1.08-2.18), $^{*}P = 0.021 < 0.05$, and OR = 4.07 (1.71-9.72), $^{**}P = 0.002 < 0.01$, respectively].

Discussion

In this work, we selected 6 SNPs of *ALOX5AP* in a northeastern Chinese Han population to investigate their associations with the risk of stroke. The cases and controls were stratified into subgroups according to sex, age and diabetes to evaluate the correlation of *ALOX5AP* SNPs with cerebral infarction in patients. We found that the rs9579646 GG genotype was associated with a significantly increased risk of stroke, compared with the rs9579646 AA genotype. Our findings indicate that the G allele in rs9579646 is markedly increased in cerebral infarction patients in comparison to control subjects of Han ancestry in northeastern China. Furthermore, the rs9579646 GG genotype was also associated with elevated risk of stroke in patients under 60. Together, these findings suggested that these two sequence variants in *ALOX5AP* gene might be linked to the risk of stroke in northeastern Chinese Han population. Patients without diabetes who carried rs9579646 GG genotype were more susceptible to suffer cerebral infarction. We did not find significant association of rs9579646 GG with risk of stroke in diabetic patients, which may be due to the small sample size of diabetics in the control group.

The SNP rs9579646 is an intron variant on chromosome 13 of *Homo sapiens*, and the detailed mechanism of rs9579646 regulating gene function remains unknown. In 2007, Kaushal R et al reported that rs9579646 were found to be significantly associated at both allelic and genotypic level with ischemic stroke among USA whites but not blacks [13], which suggests the associations of rs9579646 with stroke differ among populations. In 2011, Zhang et al showed that the rs9579646 AG genotype was associated with a marginally decreased risk for stroke compared with the AA genotype [14]. The subjects assessed in this work were recruited from Jiangsu province, and from an eastern Chinese Han population. In Zhang's follow-up study, they found rs9579646 AG genotype significantly decreases the risk of stroke in male subjects, but not in female or total subjects [15]. However, in our previous study in 2012, we found that rs9579646 G mutation associated with higher risk of stroke [10], which is inconsistent with Zhang's report. Thus we enlarged the sample size of subjects

Table 6. Comparison of haplotype frequencies of *ALOX5AP* between cases and controls

#	Haplotype	Cases (2n = 912)		Controls (2n = 904)		χ^2	*P*	OR (95% CI)
		n	%	n	%			
HapA	AT	60	6.6	85	9.4	Ref		
	AA	288	31.6	312	34.5	1.799	0.18	1.31 (0.91-1.89)
	GT	541	59.3	499	55.2	5.346	0.021	1.54 (1.08-2.18)
	GA	23	2.5	8	0.9	9.758	0.002	4.07 (1.71-9.72)
HapB	CA	630	69.1	610	67.5	Ref		
	TC	280	30.7	280	31	0.071	0.79	0.97 (0.79-1.18)
HapC	GA	883	96.8	884	97.8	Ref		
	AG	27	3	19	2.1	1.038	0.308	1.42 (0.79-2.58)

HapA: rs9579646 & rs10507391; HapB: rs4147064 & rs9551963; HapC: rs4769874 & rs9315050.

and the results were consistent with our previous reports in 2012. Meanwhile, Merhi M et al reported that the genetic variant rs9579646 G is significantly associated with a increased risk of CAD in 2015 among Lebanese patients [16], which is consistent with our findings. The underlying reason of this difference is unknown, but might be due to different populations, sampling strategies, or phenotype hetero-geneity. The minor allele of rs9579646 among whites was the major allele among blacks (G allele: whites = 0.34, blacks = 0.75) [13]. Merhi M et al considered that the rs9579646 G > A variant may lead to a decreased expression of the *ALOX5AP* gene and its gene product FLAP, diminishing the drastic effects of the leukotriene pathway [16]. There could be linkage disequilibrium (LD) between rs9579646 and different functional SNPs in the *ALOX5AP* gene, which leads to upregulation of the leukotriene pathway in northeastern Chinese Han population and downregulation in eastern Chinese Han population. More work is needed to investigate this.

It is known that cerebral infarction is a group of polygenic diseases, affected by the interaction of genetic and environmental factors. Genetic variation is the molecular basis for human genetic diversity and also the genetic susceptibility of cerebral infarction. In recent years, some ischemic stroke (IS) related genes were found through genome-wide linkage analysis, such as *PED4D* and *ALOX5AP*. *ALOX5AP* was found associated with a greater risk of myocardial infarction in Iceland by a genome-wide scan of 296 multiplex Icelandic families including 713 individuals with myocardial infarction [8]. *ALOX5AP* encodes 5-lipoxygenase-activating protein [17], which is required for the synthesis of leukotrienes [18-20]. Through encoding 5-lipoxygenase-activating protein, *ALOX5AP* plays a role in the initiation and development of inflammation and atherosclerosis. 5-lipoxygenase pathway has been demonstrated elevated in the artery wall of patients with atherosclerosis [21, 22], which induces vascular erosion by inflammatory factors and formation of lipid plaque in the disease area.

It was reported that variants of *ALOX5AP* are involved in the pathogenesis of both myocardial infarction and stroke by increasing leukotriene production and inflammation in the arterial wall [8]. A study of 639 consecutive stroke patients and 736 unrelated population-based controls suggested that sequence variants in the *ALOX5AP* gene are significantly associated with stroke in in a central European population of stroke patients, particularly in males [23]. The *ALOX5AP* SG13S114 variant was reported as an independent risk factor for IS in the Iberian population and is associated with *ALOX5AP* expression levels [9]. Recent work demonstrated that the SNPs of *ALOX5AP* gene have associations with ischemic stroke, particularly in the case atherothrombotic stroke. However, the precise mechanism of *ALOX5AP* variants contributing to the risk of stroke is still unclear. It is possible that the SNP mutation affects the transcriptional process and the gene regulation in other areas such as the promoter region influence the level of the *ALOX5AP* gene transcription regulation. The role of this gene in stroke merits further investigation.

Conclusion

In conclusion, this work suggested that the *ALOX5AP* rs9579646 G polymorphism variant has associations with cerebral infarction risk within a northeastern Chinese population. The relation between *ALOX5AP* SNPs and stroke remains unclear. More studies should be carried out to explore the detailed mechanisms by which *ALOX5AP* polymorphism variants increase susceptibility to cerebral infarction.

Acknowledgements

This study was supported by the Health Department of Heilongjiang Province (Project number: 2012-694).

Disclosure of conflict of interest

None.

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