Original Article

Variants in the Promoter Region of *HLA-DQA1* were Associated with Idiopathic Membranous Nephropathy in a Chinese Han Population

Xiao-Song Qin[1], Jian-Hua Liu[1], Guan-Ting Lyu[2], Meng-Le Peng[3], Fu-Ning Yang[4], Dong-Chun Qin[3], Yong-Zhe Li[4], Yong Liu[1]

[1]Department of Medical Laboratory, Shengjing Hospital of China Medical University, Shenyang, Liaoning 110011, China
[2]Department of Blood Transfusion, Tangdu Hospital, The Fourth Military Medical University, Xi'an, Shaanxi 710038, China
[3]Department of Medical Laboratory, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan 450052, China
[4]Department of Rheumatology and Clinical Immunology, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Key Laboratory of Rheumatology and Clinical Immunology, Ministry of Education, Beijing 100005, China

Abstract

Background: Idiopathic membranous nephropathy (IMN) is an autoimmune disease and the leading cause of adult nephritic syndrome. HLA-DQA1 had been identified to be associated with IMN in Europeans and the result was replicated in Chinese Han population. In this study, six single nucleotide polymorphisms (SNPs) in the promoter of *HLA-DQA1* and other two SNPs with IgA nephropathy were included for the association analysis.

Methods: The SNPs were genotyped in 509 patients and 601 controls by the MassArray iPLEX. The quantification of anti-phospholipase A2 receptor (PLA2R) antibodies in sera of IMN patients was performed by anti-PLA2R ELISA (IgG) kit.

Results: After analysis, four SNPs were significantly associated with IMN, with rs2187668 and rs28383345 as the top two signals ($P = 8.42\times10^{-5}$ and 2.48×10^{-5}, respectively). Even under dominant model, the two SNPs were still significantly associated with IMN ($P = 3.50\times10^{-3}$ for rs28383345 and $P = 6.55\times10^{-5}$ for rs2187668). After conditional study with rs2187668, rs28383345 was the only variant significantly correlated with IMN after Bonferroni correction ($P = 0.016$). The minor alleles of the two SNPs were also mutually exclusive in our cohort. This indicated that the two SNPs were independently associated with IMN in Chinese Han population. Levels of anti-PLA2R autoantibodies were correlated with the genotypes of the two SNPs, but not significantly ($P>0.05$).

Conclusions: Our results revealed that a novel independent variant in the promoter of *HLA-DQA1* was associated with IMN in Chinese Han population. The locus possessed regulatory role according to the data of RegulomeDB. The exact role of the SNPs on the expression of HLA-DQA1 needs further investigation.

Key words: Autoimmune Disease; HLA-DQA1; Idiopathic Membranous Nephropathy; Promoter; Chinese

Introduction

Idiopathic membranous nephropathy (IMN) is considered as an autoimmune disease, which is the leading cause of adult nephritic syndrome.[1,2] The incidence of IMN was increased significantly from 6.48% to 22.79% during 1997–2011; especially in 2009–2011, IMN has become the second common primary glomerular diseases after IgA nephropathy (IgAN) in Chinese.[3] In recent years, phospholipase A2 receptor 1 (PLA2R1) and thrombospondin type-1 domain-containing 7A were identified as the two major antigens of IMN in adults.[4] Immune deposits containing antibodies and their targeted antigens were formed on the outer layer of the glomerular basement membrane, which would evoke the complement activation cascade resulting in podocyte injury and urinary protein loss.[5] Although IMN is not a typical hereditary disease, some researches had confirmed the importance

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Address for correspondence: Dr. Xiao-Song Qin, Department of Medical Laboratory, Shengjing Hospital of China Medical University, 36# Sanhao Street, Heping District, Shenyang, Liaoning 110004, China
E-Mail: qinxs@sj-hospital.org

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of genetic factors in its pathogenesis.[6-8] Especially, a genome-wide association study (GWAS) for Caucasians identified HLA-DQA1 and PLA2R1 as the two most closely associated signals with IMN.[9]

HLA-DQA1 is located on chromosome 6p21, belonging to the group of major histocompatibility complex (MHC) Class II genes. A major function of MHC Class II molecule is to present antigens derived from extracellular proteins for recognition by T-cells.[10] Since 1970s, genes in the *HLA* locus have been implicated to be associated with IMN.[11,12] *HLA-DR2*, *DR3*, *B8*, and *B18* were reported to be associated with IMN in Japanese and Caucasians.[11,13,14] To date, most studies about association between HLA variants and risk of IMN have been performed in Westerners; only a few reports for Chinese Han population are available.[15,16] In later studies, merely, the proxy single nucleotide polymorphism (SNP) rs2187668 of *HLA-DQA1* was detected in different cohorts of IMN patients.

In this study, we detected SNPs in the promoter region of *HLA-DQA1* in a relative large Chinese Han population to evaluate whether polymorphisms were associated with the susceptibility to IMN. After curating the clinical information, we found that about 2.95% (15/509) IMNs were coupled with mild mesangial proliferative IgAN. Therefore, two other SNPs of the HLA region were also included in our analysis.

Except the previously reported variant rs2187668, the SNPs rs28383345, rs36173887, and rs1794275 were also significantly associated with IMN [Table 1]. Conditional analysis indicated that rs2187668 and rs28383345 were independently associated with the susceptibility of IMN. The minor alleles of these SNPs were correlated with levels of anti-PLA2R in sera, but not significantly. Through this study, a novel variant highly linked with IMN was identified in our cohort of Chinese Han population. The exact functional impact of this SNP on *HLA-DQA1* still needs further study.

Methods

Ethical approval

This study was approved by the Ethics Committee of Shengjing Hospital of China Medical University. All methods were carried out in accordance with the approved protocol guidelines of the Shengjing Hospital. Written informed consents were obtained from all participates in this study.

Study population

A total of 509 patients were enrolled from five hospitals (Shengjing Hospital of China Medical University, Peking Union Medical College Hospital, First Affiliated Hospital of Zhengzhou University, Second Hospital of Jilin University, and Yan'an People's Hospital) in different geographical areas of China between December 2014 and April 2016. The diagnosis of IMN was confirmed by renal biopsy. Cases of secondary membranous nephropathy were removed, which included membranous nephropathies induced by other autoimmune diseases, diabetes, infections of hepatitis B virus, malaria, and other pathogens or malignancies. The age- and gender-matched healthy individuals ($n = 601$) were recruited from Peking Union Medical College Hospital. All participants were genetically unrelated Han Chinese, determined by self-report. The clinical features of participants are listed in Table 2.

Selection of single nucleotide polymorphisms

The 5 kb region (chr6:32601004–32606003) around the promoter of *HLA-DQA1* was analyzed using RegulomeDB version 1.1[17] to identify DNA features and regulatory elements. Only SNPs with a score <3 were selected for subsequent screening. Next, SNPs with an MAF >1% were selected based on the genotyping data of Chinese Han population from 1000 Genome Projects. Two variants associated with IgAN were also included for our analysis to verify whether there existed a common pathway shared by IMN and IgAN. Totally, eight SNPs were selected for the association study. Among them, SNP rs2187668 has been identified to be significantly associated with IMN in the previous GWAS research.[9]

DNA extraction and genotyping

Genomic DNAs of the participants were extracted from the peripheral blood using TIANamp Blood DNA Kit (Tiangen Biotech, Beijing, China) according to the manufacturer's instructions.

SNP genotyping was performed using the MassArray system (Sequenom, San Diego, CA, USA). The data were analyzed by the MassArray Typer software version 4.0

Table 1: Association of *HLA-DQA1* variants with IMN in Chinese Han population

Genes	SNPs	Locations	Risk alleles	Minor allele frequency		*P*	*OR* (95% *CI*)	RegulomDB score	Reported diseases
				Case	Control				
HLA-A	rs2523946	Chr6:29941943	C	0.463	0.444	0.429	1.079 (0.894–1.303)	1f	IgAN
HLA-DQA1	rs72848263	Chr6:32602065	C	/	/	/	/	2c	
HLA-DQA1	rs114929610	Chr6:32602075	A	0.045	0.069	0.041	0.641 (0.417–0.985)	2b	
HLA-DQA1	rs36173887	Chr6:32602086	G	0.010	0.028	0.008	0.352 (0.156–0.792)	2b	
HLA-DQA1	rs115222936	Chr6:32605039	T	/	/	/	/	2a	
HLA-DQA1	rs28383345	Chr6:32605234	A	0.042	0.096	2.48×10^{-5}	0.418 (0.276–0.634)	3a	
HLA-DQA1	rs2187668	Chr6:32605884	T	0.102	0.055	8.42×10^{-5}	1.956 (1.393–2.746)	1f	IMN
HLA-DQB1	rs1794275	Chr6:32671248	A	0.093	0.131	0.013	0.684 (0.506–0.923)	/	IgAN

Score supporting data - 1f: eQTL + TF binding/DNase peak; 2a: TF binding + matched TF motif + matched DNase Footprint + DNase peak; 2b: TF binding + any motif + DNase Footprint + DNase peak; 2c: TF binding + matched TF motif + DNase peak; 3a: TF binding + any motif + DNase peak; IgAN: IgA nephropathy; IMN: Idiopathic membranous nephropathy; *CI*: Confidence interval; *OR*: Odds ratio; SNP: Single nucleotide polymorphism; /: Not available.

Table 2: Demographic and clinical features of individuals

Items	Case	Control
Gender, male/female (*n*)	337/172	403/198*
Age (years), mean ± SD (range)	48.1 ± 14.1 (14–84)	48.6 ± 14.0 (20–89)*
Serum creatinine (μmol/L)	80.27 ± 66.16	/
Urea nitrogen (mg/dl)	6.41 ± 4.34	/
Serum albumin (g/L)	28.01 ± 8.66	/
Proteinuria (g/d)	4.83 ± 4.65	/
eGFR (ml·min^{-1}·1.73 m^{-2})	97.54 ± 24.72	/
Total cholesterol (mmol/L)	6.17 ± 3.02	/
Total triglyceride (mmol/L)	3.72 ± 7.43	/
Anti-PLA2R positive, *n* (%)		
Not treated before assay	122 (68.92)	/
Treated before assay	70 (22.51)	/
Renal pathology, *n* (%)		
Atypical	28 (7.05)	/
Stage 1	65 (16.37)	/
Stage 1–2	51 (12.85)	/
Stage 2	222 (55.92)	/
Stage 2–3	18 (4.53)	/
Stage 3	12 (3.02)	/
Stage 3–4	1 (0.25)	/

*$P > 0.05$ between the case and control. /: Not available; SD: Standard deviation; eGFR: Estimating glomerular filtration rate; PLA2R: Phospholipase A2 receptor 1.

(Sequenom, San Diego, CA, USA). Assay Designer version 4.0 was used to design primers for multiplexed PCR and single-base extension reactions. Briefly, about 10 ng of genomic DNA was used for genotyping by MassArray iPLEX system at Beijing DNALead Co. Ltd., according to the manufacturer's instructions. The reactions were desalted and transferred to a 384-element SpectroCHIP array. Allele identification was obtained using MALDI-TOF mass spectrometry. The mass spectrograms and genotype data were resolved by the MassArray Typer software version 4.0 (Sequenom).

Detection of anti-phospholipase A2 receptor autoantibodies

The quantification of anti-PLA2R antibodies in sera of IMN patients was performed by anti-PLA2R ELISA (IgG) kit (Euroimmun, Luebeck, Germany) according to the manufacturer's instructions. The cutoff value for positive result was set to 20 U/ml for anti-PLA2R antibodies referred by the manufacturer's handbook.

Statistical analysis

The distributions of age, items for routine test, and levels for anti-PLA2R were analyzed through descriptive statistics of SPSS version 13.0 (SPSS Inc., USA). Values for mean and standard deviation were presented.

The statistical analysis was primarily carried out as previously reported.[18,19] A PLINK tool set (Broad Institute, MA, USA) was used to conduct the association analysis.[20] The Chi-square test was applied to compare allele frequencies between case and control groups under dominant and recessive models. The sex and age of samples were used as covariates. The odds ratio (*OR*) and 95% confidence interval were also calculated. SNPs with a $P < 0.05$ were taken as significantly associated with IMN. Conditional logistic regression analyses were also undertaken by including the most significant SNP as a covariate in calculation.

Results

Variants in the promoter region of *HLA-DQA1* were strongly associated with idiopathic membranous nephropathy in Chinese Han population

As for our case group, the cases were predominantly male (male/female = 1.97:1), with a mean age of 48.1 ± 14.1 years. In the group of healthy controls, there were 403 men and 198 women (men/women = 2.03:1), with a mean age of 48.6 ± 14.0 years. No significant differences were observed in sex and age distributions ($P > 0.05$) [Table 2]. In the case group, the positivity for anti- PLA2R autoantibodies was about 68.9% among the first-visits. The frequency dropped to 22.5% for those treated. As for the renal pathology, more than half of the cases (55.92%) were at stage 2 based on biopsy, followed by 16.37% in stage 1, 3.02% in stage 3.

To investigate the promoter region of *HLA-DQA1* with IMN in Chinese Han population, six SNPs were detected in 509 IMN patients and 601 healthy controls. Unexpectedly, two SNPs (rs72848263 and rs15222936) had no polymorphism in our cohort, which were inconsistent with results of the 1000 Genomes Project for Chinese Han population. Among the 103 individuals of Chinese Han population in Beijing, the MAFs for rs72848263 and rs15222936 were 0.238 and 0.039, respectively. As for the remaining four SNPs in the promoter region of *HLA-DQA1*, all of them were significantly associated with IMN based on the criteria with a $P < 0.05$ by Cochran–Armitage Trend Test. Among these SNPs, rs28383345

and rs2187668 were the top two signals associated with IMN ($P = 2.48 \times 10^{-5}$ for rs28383345 and $P = 8.42 \times 10^{-5}$ for rs2187668) [Table 1]. After conditional analysis, rs28383345 was still positively linked with IMN after Bonferroni correction ($P_{Bonf} = 0.016$) [Table 3].

Associations of single nucleotide polymorphisms with idiopathic membranous nephropathy under dominant and recessive models

The associations of these SNPs with IMN were also analyzed under dominant and recessive models. Except the two SNPs without polymorphism, four SNPs of *HLA-DQA1* were significantly associated with IMN under dominant model, still with rs28383345 ($P = 3.50 \times 10^{-3}$) and rs2187668 ($P = 6.55 \times 10^{-5}$) as the top two signals [Table 4]. It was consistent with the results under allelic model [Table 1]. As for the recessive model, rs1794275 was marginally associated with IMN in our cohort ($P = 0.043$). However, since there were no homozygous genotypes of minor alleles for rs36173887, rs28383345, and 2187668 in our cohort, the associations with IMN of these SNPs under recessive models could not be calculated.

IgA nephropathy-associated variant linked marginally with idiopathic membranous nephropathy

Two previously reported IgAN-associated SNPs of HLA in Chinese Han population were also detected in our IMN cohort. Rs2523946 in the *HLA-A* region had negative relationship with the susceptibility of IMN ($P = 0.429$). Only rs1794275 was marginally associated with IMN in the current cohort ($P = 0.013$) [Table 1].

Table 3: Conditional analysis for variants of *HLA-DQA1*

Genes	SNPs	Locations	P_{unadj}	P_{Bonf}
HLA-DQA1	rs2523946	Chr6:29941943	0.379	1
HLA-DQA1	rs72848263	Chr6:32602065	/	/
HLA-DQA1	rs114929610	Chr6:32602075	0.139	1
HLA-DQA1	rs36173887	Chr6:32602086	0.032	0.540
HLA-DQA1	rs115222936	Chr6:32605039	/	/
HLA-DQA1	rs28383345	Chr6:32605234	9.13×10^{-4}	0.016
HLA-DQA1	rs2187668	Chr6:32605884	1	1
HLA-DQA1	rs1794275	Chr6:32671248	0.059	0.832

/: Not available; SNP: Single nucleotide polymorphism.

Most of the selected variants were functional

The functional characters of SNPs were explored by RegulomeDB [Table 1]. Rs2523946 was a reported cis-eQTL for HLA-A in lymphoblastoids and fibroblasts.[21] Rs72848263, rs114929610, rs36173887, rs115222936, and rs28383345 could affect the binding motifs of transcription factors determined by footprinting in RegulomeDB. Interestingly, the previously reported IMN-associated variant, rs2187668, was a cis-eQTL for *HLA-DQA1*, *HLA-DQB1*, *HLA-DRB1*, and *HLA-A* in lymphoblastoid and monocytes.[22] Rs28383345 was located at -2 site of the Open reading frame (ORF) of *HLA-DQA1*, which was the motif of the Kozak sequence influencing translational efficacy.

Rs2187668 and rs28383345 were correlated, but not significantly, with anti-phospholipase A2 receptor levels

For IMN individuals, anti-PLA2R antibodies were positive in 69.76% of patients among the first visits. After comparing anti-PLA2R level with the genotypes of rs2187668 and rs28383345, the minor alleles of these two SNPs were not significantly associated with the levels of anti-PLA2R ($P = 0.163$ for rs2187668, $P = 0.184$ for rs28383345). However, the minor allele T of rs2187668 had the higher level of anti-PLA2R than that of the major allele C (195.90 ± 62.58 vs. 142.30 ± 22.48). Minor allele A of rs28383345 was with lower level of anti-PLA2R (73.03 ± 19.35 vs. 104.20 ± 13.65) [Figure 1 and Table 5].

Discussion

The variant rs2187668 in *HLA-DQA1* had been reported to be associated with the susceptibility of IMN;[9,15,23] however, the causal SNPs of this gene had not been located. To find out if the variants in the promoter region of *HLA-DQA1* were associated with IMN, four SNPs in the promoter of *HLA-DQA1* and the proxy SNP rs2187668 were genotyped in a cohort of IMN (509 patients and 601 healthy controls).

Except the proxy SNP rs2187668, other four SNPs were also associated significantly with IMN [Table 1]. Even under dominant model, these SNPs were still linked with the susceptibility of the disease [Table 4]. After conditioning analysis with rs2187668, only rs28383345 was significantly

Table 4: Associations of SNPs with IMN in dominant and recessive models

SNPs	Alleles		Dominant model				Recessive model			
	A1	A2	Case	Control	*OR* (95% *CI*)	*P*	Case	Control	*OR* (95% *CI*)	*P*
rs2523946	C	T	239/95	443/188	1.068 (0.797–1.431)	0.661	70/264	117/514	1.165 (0.836–1.622)	0.366
rs72848263	C	T	0/349	0/680	/	/	0/349	0/680	/	/
rs114929610	A	C	22/298	81/579	0.528 (0.323–0.863)	9.77×10^{-3}	7/313	10/650	1.454 (0.548–3.855)	0.449
rs36173887	G	A	6/343	36/643	0.312 (0.130–0.749)	4.59×10^{-3}	1/348	2/677	/	/
rs115222936	T	G	0/346	0/677	/	/	0/346	0/677	/	/
rs28383345	A	G	29/313	96/547	0.528 (0.341–0.818)	3.50×10^{-3}	0/342	27/616	/	/
rs2187668	T	C	71/278	74/602	2.078 (1.456–2.964)	6.55×10^{-5}	0/349	0/676	/	/
rs1794275	A	G	62/287	155/515	0.718 (0.517–0.996)	0.053	3/346	20/650	0.282 (0.083–0.955)	0.043

/: Not available; IMN: Idiopathic membranous nephropathy; *CI*: Confidence interval; *OR*: Odds ratio; SNP: Single nucleotide polymorphism.

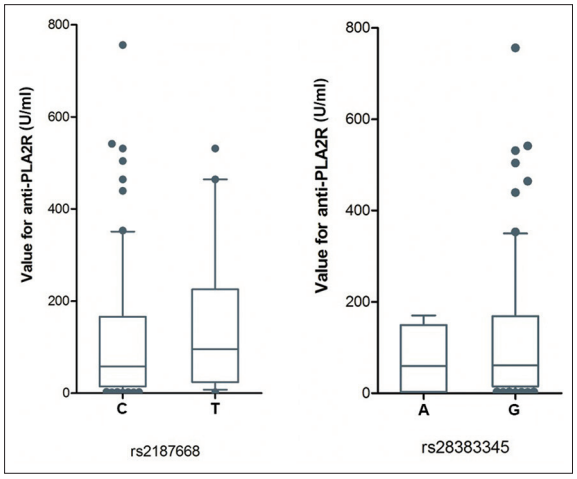

Figure 1: Correlation between genotypes of single nucleotide polymorphisms and levels anti-phospholipase A2 receptor. PLA2R: Phospholipase A2 receptor 1.

Table 5: Correlation between SNPs and anti-PLA2R levels

SNPs	Alleles	Anti-PLA2R (U/ml), mean ± SD	*P*
rs2187668	T	195.9 ± 62.58	0.163
	C	142.3 ± 22.48	
rs28383345	A	73.03 ± 19.35	0.184
	G	104.2 ± 13.65	

SD: Standard deviation; PLA2R: Phospholipase A2 receptor 1; SNP: Single nucleotide polymorphism.

associated the susceptibility of IMN (P_{Bonf} = 0.01552). Interestingly, the risk allele T of rs2187668 was contributive to the disease (*OR* = 1.956 [1.393–2.746]), the risk allele A of rs28383345 protective against the susceptibility of IMN (*OR* = 0.418 [0.276–0.634]). Besides, in our sample cohort, the risk alleles of these two SNPs were mutually exclusive, indicating that these two SNPs were independently associated with IMN.

Very impressively, after curating the clinical information of our project, about 2.95% (15/509) IMNs were coupled with mild mesangial proliferative IgAN in our disease group. It is inferred that variants associated with IgAN might be also linked with the susceptibility of IMN. In this study, two previously reported IgAN-associated SNPs of the *MHC* gene were discovered to be linked with the pathogenesis of IMN. Rs2523946 in the *HLA-A* had a negative relationship with the susceptibility of IMN (*P* = 0.429). Only rs1794275 in the *HLA-DQB1* was marginally associated with IMN in the current cohort (*P* = 0.013). However, these two variants were strongly associated with IgAN in Chinese ($P = 1.74 \times 10^{-11}$ for rs2523946 and $P = 3.43 \times 10^{-13}$ for rs1794275). This implied that the mechanisms for IgAN might be different from that for IMN at least in the *HLA* although these two diseases shared a common pathway for the pathogenic etiology.

According to eQTL and chromatin modification signatures of RegulomeDB, most of the selected variants possessed regulatory function. Rs2187668 was a cis-eQTL, affecting the expression of *HLA-DQA1*, *HLA-DQB1*, *HLA-DRB1*, and *HLA-A* in lymphoblastoid and monocytes. Rs28383345 was at the -2 site of the ORF of *HLA-DQA1*. This site was at the motif of the well-known Kozak sequence, which could affect the translational efficacy of protein coding genes.[24] As Kozak sequence plays a major role in the initiation of the translation process in eukaryotic cells, the minor allele of rs28383345 might be able to affect the translation of *HLA-DQA1*.

Since anti-PLA2R1 was the most prevalent autoantibodies in IMN, with a prevalence at about 75% in IMN,[25,26] the relationship between variants of *HLA-DQA1* and levels of anti-PLA2R was explored in our cohort. Although with a suggestive trend, the association of two SNPs was not significantly correlated with anti-PLA2R levels (*P* = 0.163 for rs2187668 and *P* = 0.184 for rs28383345) [Table 5]. This might be due to the inadequate number of first-visit nontreated IMN patients with anti-PLA2R detected.

In this study, we assessed, for the first time, the putative association between variants in the regulatory region of *HLA-DQA1* and the pathogenesis of IMN. We applied RegulomeDB to identify variants in regulatory elements of *HLA-DQA1* with a score <3a. The motifs of these categories were verified by footprinting and worthy of further experimental studies. Though we identified novel polymorphisms associated with IMN, there were still existed limitations for this study. First, the signals should be detected in a new cohort with larger samples to verify the relationship between variants and IMN. Second, the interplay between regulatory variants and the missense polymorphisms of *HLA-DQA1* should be studied; it might be highly possible to identify haplotypes more strongly associated with IMN.

From this study, we selected SNPs in the promoter region of *HLA-DQA1* through RegulomeDB and analyzed their association with IMNs in a cohort of Chinese Han individuals. Except the previously reported variant associated with IMN, we also found a novel SNP, rs28383345 in the motif of Kozak sequence, associated with the disease. The functional impact of this SNP on the expression of *HLA-DQA1* is undergoing.

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Conflicts of interest

There are no conflicts of interest.

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