

Original Article

Circulating Inc-ITSN1-2 expression presents a high value in diagnosis of rheumatoid arthritis and correlates with disease activity

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Received January 16, 2016; Accepted February 22, 2017; Epub October 1, 2017; Published October 15, 2017

Abstract: This study was aimed to investigate the correlation of Inc-ITSN1-2, Inc-APOC3-2 and Inc-AL355149.1 expressions in plasma by qPCR with rheumatoid arthritis (RA) risk and disease activity. 30 RA patients and 30 health controls (HC) were enrolled in this study. Plasma sample were collected from RA patients before any treatment carried out and HCs. Top 3 RA related long non-coding RNAs (lncRNAs) (Inc-ITSN1-2, Inc-APOC3-2 and Inc-AL355149.1) were selected by a computational framework prediction. The expression of Inc-ITSN1-2, Inc-APOC3-2 and Inc-AL355149.1 were determined by qPCR method. Age (P=0.350) and gender (P=0.542) were similar between RA patients and HCs. Inc-ITSN1-2 level was extremely increased in RA patients compared with HCs (P<0.001), while both Inc-APOC3-2 and Inc-AL355149.1 expressions were numerically higher in RA patients but with no statistical significance (P=0.152 and P=0.139 respectively). Receiver Operating Characteristic (ROC) curves were performed and we found Inc-ITSN1-2 disclosed a great diagnostic value for RA with area under curve (AUC) 0.898, 95% CI 0.813-0.983, and sensitivity was 90.0% and specificity was 80.0% respectively at the best cut-off point. In addition, plasma Inc-ITSN1-2 level was illuminated to be positively associated with erythrocyte sedimentation rate (ESR) (P=0.049), C-reactive protein (CRP) (P<0.001) and disease activity score in 28 joints (DAS28) (P=0.007). Circulating Inc-ITSN1-2 expression was observed to be a novel and convincing biomarker for RA diagnosis as well as disease management.

Keywords: Long non-coding RNA (lncRNA), Inc-ITSN1-2, plasma, rheumatoid arthritis

Introduction

Rheumatoid arthritis (RA), as one of the most common inflammatory disease, affects more than 1% of the general population worldwide, which characterized by inflammation, synovitis and damage of articular cartilage as well as bone, and if not treated well 40%-70% patients would eventually progress to disability [1, 2]. Despite of joint involved, patients with longer disease duration could have various extra-articular manifestations such as interstitial lung disease and cardiovascular disease [3, 4]. These increasemuch disease and social burden in RA patients which pinnacles early diagnosis and sufficient treatment in RA management [5].

Long non-coding RNA (lncRNA), as an important part of non-coding RNA (ncRNA), consists of longer than 200 nucleotides, which involved in lots of important biological phenomena includ-

ing imprinting genomic loci, shaping chromosome conformation, regulating enzymatic activity and so on [6]. Accumulating evidences have revealed that lncRNA is implicated in numerous diseases, such as cancers, diabetes as well as inflammatory diseases [7-9].

However, less researches on the role of lncRNA in RA were reported, especially the circulating lncRNAs. This study was aimed to predict potential lncRNAs associated with RA development through computational framework, and subsequently validate the correlation of top 3 related candidate lncRNAs in plasma by qPCR with RA risk and disease activity.

Patients and methods

Participants

30 RA patients from July 2016 to August 2016 in Guanghua Hospital were recruited in this

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Table 1. Primers of candidate lncRNAs and U6

Gene name	Primer sequence (5' to 3')	Amplicon size
Lnc-ITSN1-2-F	GCCTTTGGACACTTCTTCGGAA	127 bp
Lnc-ITSN1-2-R	GGAGTGCATCCATCAGCTGAA	
Lnc-APOC3-2-F	AGAACTGAAGCGTGGCAAGAT	233 bp
Lnc-APOC3-2-R	TCCAGTCATCACATTGTACACCT	
Lnc-AL355149.1-F	TTCCTCCGTTCCGTCTATTCT	221 bp
Lnc-AL355149.1-R	ATGATGATGCTACTCCTCCAAG	
U6-F	CTCGCTTCGGCAGCAC	94 bp
U6-R	AACGCTTCACGAATTTGCGT	

Table 2. Characteristics of participants

Characteristics	RA patients (n=30)	Health controls (n=30)	P Value
Age (years)	50.35±11.21	47.82±9.54	0.350
Gender-Female (n/%)	24 (80%)	22 (73.3%)	0.542
RF positive (n/%)	25 (83.3%)	-	-
Anti-CCP (n/%)	26 (86.7%)	-	-
ESR (mm/h)	25.54 (12.28-40.37)	-	-
CRP (mg/l)	18.7 (7.99-33.26)	-	-
DAS28	4.85±1.12	-	-
HAQ	1.83±1.35	-	-

Data was presented as mean values ± standard deviation, median and 25th-75th or counts (percentage). Significance of the comparison was determined by the Student test or Chi-square test. P Value <0.05 was considered significant. RF, rheumatoid factor; CCP, cyclic citrullinated peptide; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; DAS28, disease activity score in 28 joints; HAQ, health assessment questionnaire.

study. All patients were diagnosed according to 1987 American College of Rheumatology (ACR) classification of RA with age above 18 years, and no treatments were performed before entering this study. Patients with the following conditions were excluded: history of severe infection, malignant tumor or joint operations; abnormal hepatic and renal function; infection or tuberculosis (TB). 30 health controls (HC) were also enrolled during the same period with age and gender matched. This research was approved by the Ethics Committee of Guanghua Hospital. All participants provided written informed consents.

Disease assessments

In order to explore the association of studied lncRNAs with disease severity, several disease indexes were determined. Rheumatoid factor (RF) and anti-cyclic citrullinated peptide (CCP) antibody were determined in all RA patients. Inflammatory index including erythrocyte sedi-

mentation rate (ESR) and C-reactive protein (CRP) was measured in RA patients as well. In addition, disease activity score in 28 joints (DAS28) and health assessment questionnaire (HAQ) score were assessed at the time blood sample were obtained from RA patients.

Prediction of RA related lncRNAs

A computational framework to predict lncRNAs associated with RA was analyzed by combining human lncRNA expression profiles, gene expression profiles, and human disease-associated gene data as described in previous study [10]. And Top 3 related lncRNA (lnc-ITSN1-2, lnc-APOC3-2 and lnc-AL355149.1) were selected as candidates to be validated in this study.

Samples

Blood samples were collected in RA patients before any treatment initiation and HCs into sodium citrate tubes. The whole blood was stand for 3 h at -4°C before

centrifuging at 1,500 g for 10 min at room temperature. The resultant plasma was then collected and total RNA was subsequently extracted from plasma using TRIzol Reagent (TaKaRa, Japan).

qPCR analysis

Total RNA was reversely transcribed using PrimerScript Real-time reagent kit (TaKaRa, Japan) according to the manufacturer's instructions. Quantitative analysis of studied lncRNAs expression was performed using SYBR Premix Ex TaqTM II (TaKaRa, Japan) with the primers presented in **Table 1**. Expression levels of candidate lncRNAs were calculated utilizing the 2^{-ΔΔt} method with U6 as the internal reference.

Statistics

Statistical analysis was performed using SPSS V19.0 (SPSS, USA). Data was presented as mean values ± standard deviation, median and

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Table 3. Top 3 RA-related lncRNAs by computational framework prediction

TCONS_00029004	Gene_id	Transcript_id	NONCODE_id	Disease	P-value	Corrected P-value (Bonferroni)	FDR	Chr
TCONS_00000848	lnc-ITSN1-2	lnc-ITSN1-2:6	NONHSAT081856	Arthritis, Rheumatoid	1.56351E-13	8.05206E-10	2.92988E-09	chr21
TCONS_00019155	lnc-AL355149.1-1	lnc-AL355149.1-1:2	NONHSAT001134	Arthritis, Rheumatoid	2.37216E-10	1.22166E-06	3.1873E-06	chr1
TCONS_00029004	lnc-APOC3-2	lnc-APOC3-2:1	NONHSAT024352	Arthritis, Rheumatoid	1.2405E-09	6.38857E-06	2.20968E-05	chr11

FDR, false discovery rate.

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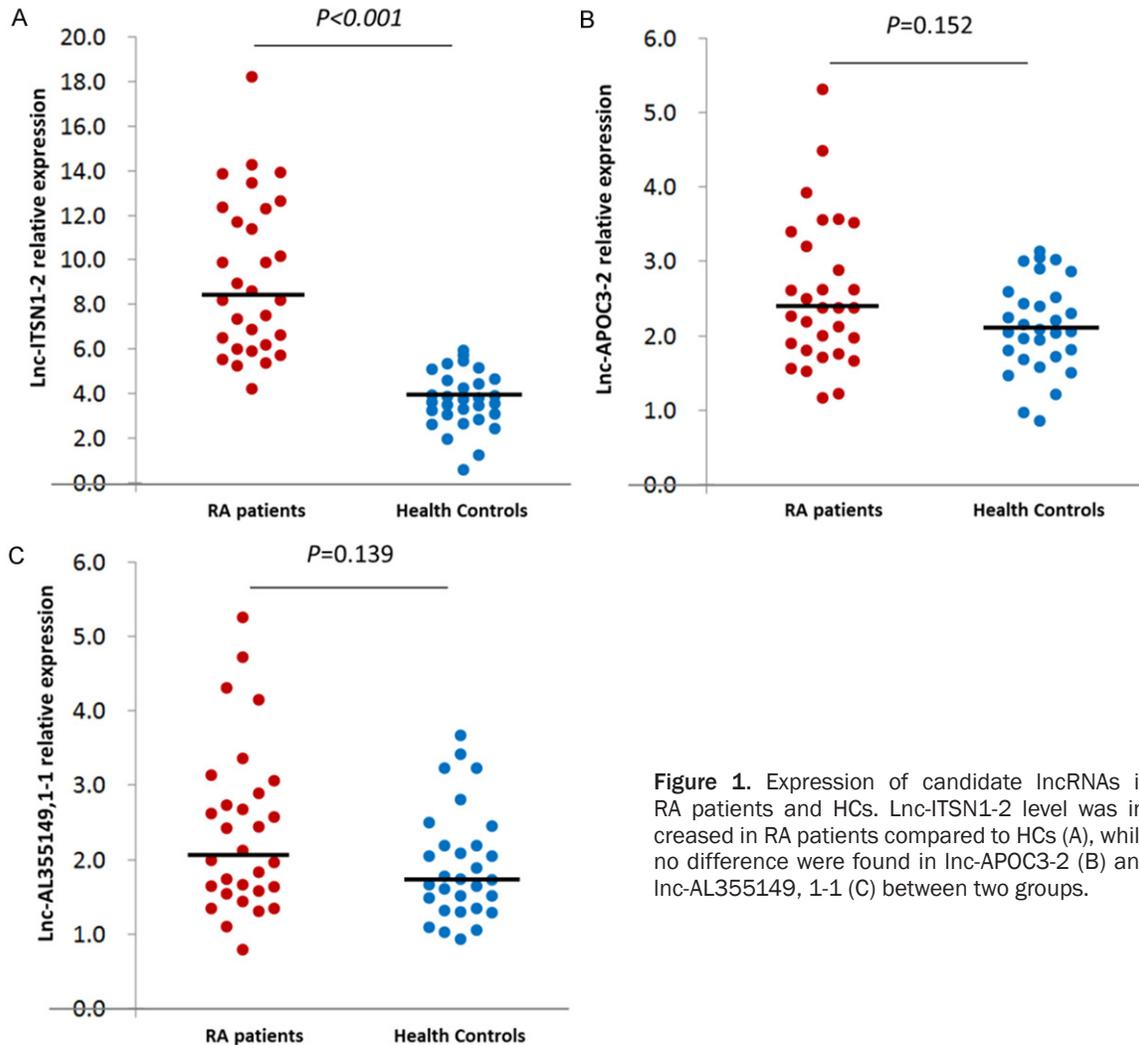


Figure 1. Expression of candidate lncRNAs in RA patients and HCs. lnc-ITSN1-2 level was increased in RA patients compared to HCs (A), while no difference were found in lnc-APOC3-2 (B) and lnc-AL355149, 1-1 (C) between two groups.

25th-75th or counts (percentage). Significance of the comparison was determined by the Student test, Chi-square test or Wilcoxon rank sum test. Significance of the correlations was determined by Spearman test. Receiver Operating Characteristic (ROC) curve was drawn to evaluate the diagnostic value of candidate lncRNAs for RA. P Value < 0.05 was considered statistically significant.

Results

Participants

30 RA patients with age 50.35 ± 11.21 years and 24 (80%) female were included in the study. There were 25 cases (83.3%) with RF positive and 26 (86.7%) with anti-CCP positive. There were no difference found between RA patients and HCs in age and gender ($P = 0.350$ and $P = 0.542$ respectively). The other detailed

clinical characteristics of RA patients were presented in **Table 2**.

Candidate lncRNAs selection by computational framework prediction

Three top RA related lncRNAs were selected by computational framework prediction as presented as follows: lnc-ITSN1-2 (TCONS_00029004, NONCODE id: NONHSAT081856), lnc-APOC3-2 (TCONS_00019155, NONCODE id: NONHSAT024352) and lnc-AL355149.1-1 (TCONS_00000848, NONCODE id: NONHSAT001134). The other detailed information about the three lncRNAs was listed in **Table 3**.

Expressions of lnc-ITSN1-2, lnc-APOC3-2 and lnc-AL355149.1 in plasma of RA patients and HCs

As presented in **Figure 1**, lnc-ITSN1-2 level was extremely increased in RA patients (8.433

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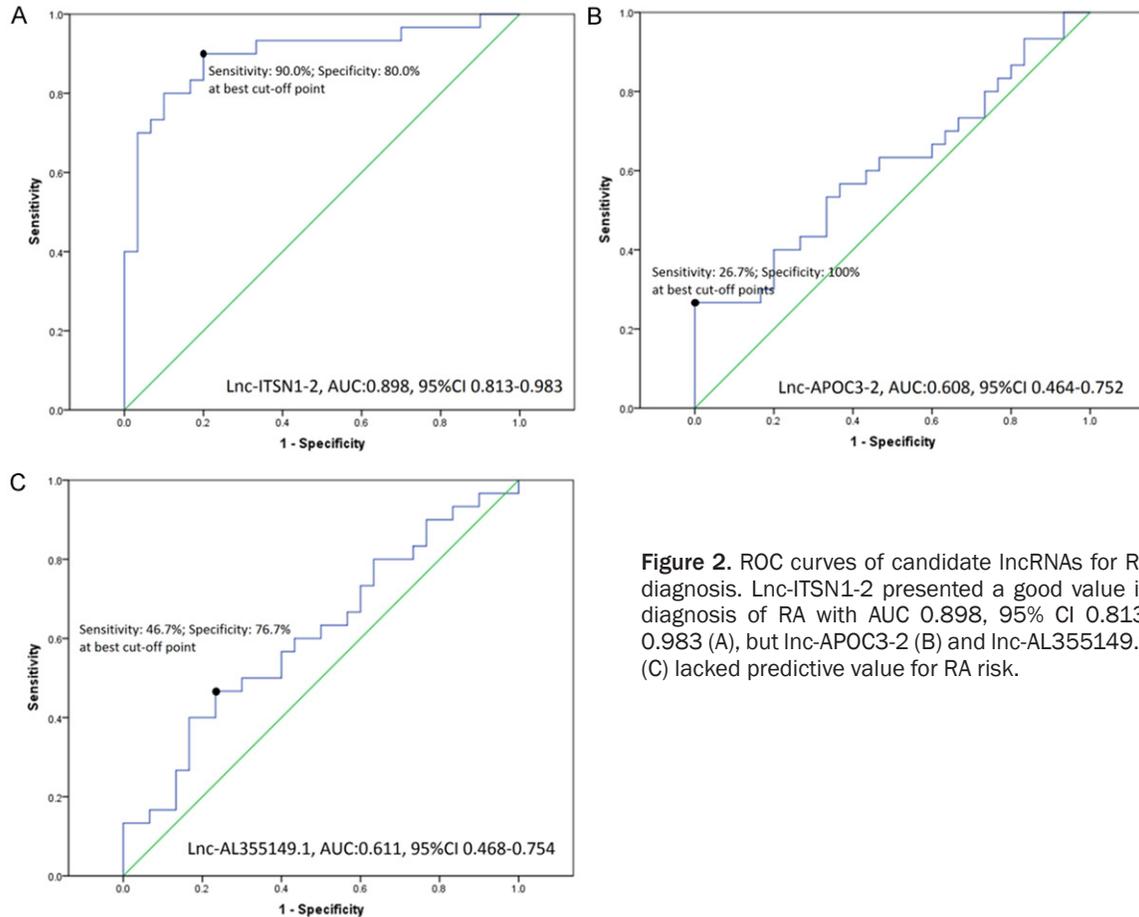


Figure 2. ROC curves of candidate lncRNAs for RA diagnosis. lnc-ITSN1-2 presented a good value in diagnosis of RA with AUC 0.898, 95% CI 0.813-0.983 (A), but lnc-APOC3-2 (B) and lnc-AL355149.1 (C) lacked predictive value for RA risk.

(6.186-12.341) compared with HCs (3.999 (3.338-4.896)), $P < 0.001$. Both lnc-APOC3-2 and lnc-AL355149.1 expressions were numerically higher in RA patients than in HCs, but with no statistical significance ($P = 0.152$ and $P = 0.139$ respectively).

Diagnostic value of lnc-ITSN1-2 level for RA

In order to further investigate the prediction of candidate lncRNAs for RA risk, ROC curve was performed (Figure 2). We found lnc-ITSN1-2 disclosed a great diagnostic value for RA with area under curve (AUC) 0.898, 95% CI 0.813-0.983, and sensitivity was 90.0% and specificity was 80.0% respectively at the best cut-off point (defined as the point at which the value was highest by adding sensitivity to specificity). However, lnc-APOC3-2 and lnc-AL355149.1 lacked predictive value for RA risk with AUC 0.608, 95% CI 0.464-0.752 and AUC 0.611, 95% CI 0.468-0.754 respectively.

The correlation of lnc-ITSN1-2, lnc-APOC3-2 and lnc-AL355149.1 level with clinical features in RA patients

Correlation between candidate lncRNAs and clinical features was subsequently determined by Spearman test. As shown in Table 4, plasma lnc-ITSN1-2 level was illuminated to be positively associated with ESR ($P = 0.049$), CRP ($P < 0.001$) and DAS 28 ($P = 0.007$), while no difference was found to be correlated with RF positive ($P = 0.398$), anti-CCP positive ($P = 0.338$) and HAQ ($P = 0.109$). Except for that lnc-AL355149.1 level was positively associated with DAS 28 score ($P = 0.026$), no other correlation of lnc-APOC3-2 and lnc-AL355149.1 with clinical features were found in our study.

Discussion

In the present study, we found plasma lnc-ITSN1-2 was dramatically elevated in RA

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Table 4. Correlation of lnc-ITSN1-2, lnc-APOC3-2 and lnc-AL355149.1 level with clinical features in RA patients

Items (N=30)	Parameters	RF positive	Anti-CCP positive	ESR	CRP	DAS28	HAQ
lnc-ITSN1-2	<i>r</i>	-0.160	-0.181	0.363	0.636	0.480	0.299
	<i>P</i> value	0.398	0.338	0.049	<0.001	0.007	0.109
lnc-APOC3-2	<i>r</i>	-0.191	-0.227	-0.025	0.274	-0.051	0.005
	<i>P</i> value	0.312	0.229	0.894	0.143	0.787	0.978
lnc-AL355149.1	<i>r</i>	-0.202	-0.351	0.174	0.212	0.406	0.194
	<i>P</i> value	0.286	0.057	0.357	0.260	0.026	0.305

Significance of the correlation was determined by Spearman test. *P* Value <0.05 was considered significant. RF, rheumatoid factor; CCP, cyclic citrullinated peptide; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; DAS28, disease activity score in 28 joints; HAQ, health assessment questionnaire.

patients compared with HCs, and it had a good diagnostic value for RA with AUC 0.898. In addition, lnc-ITSN1-2 was positively associated with disease activity (ESR, CRP and DAS 28), while lnc-AL355149.1 was found to be associated with DAS 28 as well.

lncRNAs, which are a diverse class of molecules lack potential of coding protein composed of longer than 200 nucleotides, are characterized by unique regulatory mechanisms, alternative forms of biogenesis, cis-regulatory activities and functional structured RNA domains [6, 11]. Although the deregulation of lncRNAs was reported to be associated with various diseases including cancers, nutrition metabolism and endocrine disorder, cardiovascular disease as well as autoimmune disease [8, 9, 12, 13].

260 lncRNAs are illustrated to be differentially expressed in the synovium between adjuvant-induced arthritis (AA) and normal rats by lncRNA/mRNA microarray, and six lncRNAs consisting of XR_008357, U75927, MRAK04-6251, XR_006457, DQ266363 and MRAK00-3448 are further validated to be increased in AA rats by qPCR [14]. Furthermore, 135 differentially expressed lncRNAs were observed in fibroblast-like synoviocytes (FLSs) from RA patients compared to normal FLSs, and lnc-ENST00000483588 was up regulated while lnc-ENST00000438399, lnc-uc004afb.1, and lnc-ENST00000452247 was down regulated by qPCR in RA FLSs [9]. These indicate lncRNAs deregulations play important role in RA pathogenesis, however, comprehensive understanding about how lncRNAs function in RA etiology was seldom investigated.

Hotair, as one of the most key imbalanced lncRNA which involved in pathogenesis of various diseases, is found to be notably elevated in blood mononuclear cells and serum exosome of RA patients and leads active macrophage migration, while Hotair level was remarkably reduced in differentiated osteoclasts and synoviocytes with down-regulating MMP-2 as well MMP-3 expressions [15]. Oncofetal H19 RNA is revealed to be increased in synovial tissue of RA patients than controls, and isolated low expression of H19 in FLSs was raised markedly on starvation despite of addition of interleukin-1beta (IL-1beta), tumor necrosis factor-alpha (TNF-alpha) or platelet-derived growth factor [16]. What's more, C5T1 lncRNA which starts in the 3' untranslated region (UTR) of C5 influences mRNA expression of RA candidate gene C5 [17]. These studies provided some but insufficient information about lncRNA functions in RA etiology.

lnc-ITSN1-2, located in chromosome 21, with NONCODE gene ID NONHSAG032726.2 and NONCODE transcript ID NONHSAT081856.2, start from 33976355 to end site 33976982 and length was 451 bp (http://www.noncode.org/show_rna.php?id=NONHSAT081856.2). No previous report has been described about the function of lnc-ITSN1-2 or any biological information. In our study, we found lnc-ITSN1-2 was extremely increased in plasma of RA patients compared with HCs, and was positively correlated with inflammatory indexes (ESR and CRP) and disease activity (DAS 28). ESR and CRP are regarded as critical parameters for RA diagnosis and disease assessments for inflammation, ACR as well as European League

against Rheumatism (EULAR) recommended ESR and CRP as routine examination for RA patients, while DAS 28 is also a gold standard for disease activity assessing score and goal of treating-to-target therapy [18-20]. These indicated Inc-ITSN1-2 could be served as a novel circulating biomarker for RA diagnosis and disease management.

Some limitations existed in this study. Firstly, sample size of this study was relatively small with 30 RA patients and 30 HCs; however, it has been the largest sample for circulating lncRNA determination study in RA patients. Secondly, the patients and HCs were enrolled only in one center, so some confounding factors might exist. Thirdly, we identified Inc-ITSN1-2 deregulation in RA patients and its correlation with clinical features, but how Inc-ITSN1-2 function in the pathogenesis of RA was not investigated, which we would explore in the future study.

In conclusion, circulating Inc-ITSN1-2 expression was observed to be a novel and convincing biomarker for RA diagnosis as well as disease management.

Acknowledgements

This research was supported by Shanghai New Three-year Action Plan-Innovation Connotation Construction of Traditional Chinese Medicine (ZY3-CCCX-3-3021). This research was supported by Psoriasis Key Specialty (20161003) and Project of XELJANZ (tofacitinib CP-690550) in the immunoregulatory effects of rheumatoid arthritis (CNKW2013207).

Disclosure of conflict of interest

None.

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