

ORIGINAL ARTICLE

Increased soluble HLA-DRB1 in B-cell acute lymphoblastic leukaemia

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Abstract

Soluble HLA (sHLA) are potential tumour markers released in order to counter immune surveillance. sHLA-class II is less known especially in acute lymphoblastic leukaemia (ALL). This study aimed to investigate soluble, surface and allelic expression of HLA Class II (sHLA-DR) in B-cell ALL patients and compare with soluble expression in normal individuals. A sandwich enzyme-linked immunosorbent assay (ELISA) was developed to measure soluble HLA-DRB1 in plasma. Flow cytometric analysis was performed to determine median fluorescence intensity in HLA-DR surface expression. HLA-DNA typing by polymerase chain reaction, sequence specific oligonucleotides, PCR-SSO was performed to determine HLA-DRB1 type in ALL samples. Results showed sHLA-DRB1 (mean±SEM) was significantly increased ($p=0.001$) in plasma of ALL patients ($0.260\pm 0.057 \mu\text{g/mL}$; $n=30$) compared to healthy controls ($0.051\pm 0.007 \mu\text{g/mL}$; $n=31$) of Malay ethnicity. However, these levels did not correlate with percentage or median fluorescence intensity of HLA-DR expressed on leukemia blasts (CD19+CD34+/-CD45^{lo}HLA-DR+) or in the normal B cell population (CD19+CD34-CD45^{hi}HLA-DR+) of patients. No significant difference was observed in gender (male/female) or age (paediatric/adult). Only a trend in reduced sHLA was observed in patients carrying HLA-DR04. These results have to be validated with a larger number of samples.

Keywords: Human leukocyte antigen (HLA), acute lymphoblastic leukaemia (ALL), soluble HLA.

INTRODUCTION

The major histocompatibility complex (MHC) molecules are known as the most polymorphic genetic system in humans with excess of 220 genes or loci and classified into Class I (HLA-A, -B and -C) and Class II (HLA-DP, -DQ and -DR). Many of these alleles are associated with various inflammatory diseases. In immunity, these two main classes present antigens to T cells which lead to final elimination of the offending pathogen.

Biochemical analysis of HLA class I (sHLA-I) products in serum and cell supernatants by Western Blot revealed heterogeneity in the molecular masses, which is most likely the result of multiple mechanisms of production including shedding from the cell membrane (size of 44-46

kDa), proteolytic cleavage of membrane bound molecules (35-37 kDa) and/or alternative splice variants (39-41 kDa).¹

Further analysis of plasma of multiple-myeloma and leukaemia patients, normal controls and cultured cancer cells identified thousands of sHLA peptides including cancer-related peptides present in the sHLA peptidomes of the cancer patients.² More precisely, HLA-I was increased in non-Hodgkin's lymphoma and Hodgkin's diseases³ AML and MDS⁴ and pancreatic cancer.⁵ Elevated serum sHLA-E levels were observed in melanoma cases supporting this as a marker for cancer.⁶ Another well studied sHLA, HLA-G (of HLA Class-I) was reported to be significantly increased in ALL and AML cases.^{7,8} Increased levels of sHLA-G were also

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observed in CLL, non-Hodgkin lymphoma (B-NHL and T-NHL).^{9,10} sHLA-G molecules, an inhibitory ligand for natural killer (NK) cells, could be a factor in tumoural escape from immune surveillance during acute leukaemia.⁷

Less is known about the biochemistry of soluble HLA class II molecules, but some studies have reported the molecular mass of sHLA-II molecules as 60 kDa, while another identified both a 28 kDa and a 43 kDa fragment.^{1,11,12} Early studies using ¹²⁵I labelled antibodies showed increased levels of circulating HLA-DR antigen in ALL but not AML or other cancers.^{13,14}

sHLA-II antigen are elevated in immune related disorders such as multiple sclerosis where levels were raised in paired serum, saliva and cerebrospinal fluid (CSF) of patients.¹⁵⁻¹⁷ Soluble HLA-DR was also increased in sepsis patients¹⁸ as well as in chronic hepatitis C.¹⁹ High levels of sHLA-I were also described in pre-transplantation of kidney and pancreas recipients.²⁰ sHLA levels in liver, heart and kidney recipients varied with time post-operation and increased in episodes of infection or organ rejection²¹ suggestive of its potential as a marker in transplant management.

This study aimed to investigate the levels of soluble, membrane bound and identify HLA Class II (sHLA-DR) allele in acute lymphoblastic leukaemia (ALL) patients, as well as soluble HLA Class II in normal individuals and determine for associations.

MATERIALS AND METHODS

Patients and controls

Thirty bone marrow and peripheral blood samples from untreated pre-B ALL patients of Malay ethnicity were obtained from the Haematology Diagnostic Laboratory, Hospital Kuala Lumpur between June 2011 and February 2013. Patients were matched with 31 normal controls by ethnicity and gender from staff and students from the Faculty of Medicine and Health Sciences, UPM and the Institute for Medical Research. Peripheral blood samples were collected after informed consent. All procedures on human subjects were in accordance with the Helsinki Declaration of 1975, as revised in 2008.

Patients were diagnosed as pre-B ALL according to the 2008 WHO classification.^{22,23} Diagnosis of B-cell ALL patients were obtained from reports made by haematologists, Haematology Diagnostic Laboratory, Hospital Kuala Lumpur which functions as one of

referral centres for leukaemia cases. Diagnosis was based on morphology criteria, presence of $\geq 20\%$ lymphoid blasts, cytochemistry staining positive for Periodic Acid-Schiff (PAS) and immunophenotyping positive panels based on Table 1. The majority of plasmas from pre-B ALL samples were collected from bone marrow (90%) samples while the rest were from peripheral blood (10%). All patients were *de novo* cases, at diagnosis, except for two; one a CML in blast crisis and the other in relapse.

Plasma and bone marrow mononuclear cells (BMMCs) or peripheral blood mononuclear cells (PBMCs) from pre-B ALL patients and normal controls were isolated after centrifugation at 1100g (2600 rpm) with Ficoll-paque PLUS (GE Healthcare Life Sciences, Sweden) and cryopreserved in freezing media (50% RPMI, 40% FBS and 10% DMSO).

Sandwich enzyme-linked immunosorbant assay (ELISA)

Sandwich ELISA techniques for soluble HLA-DRB1 detection were developed in the laboratory using 2.0 $\mu\text{g}/\text{mL}$ unlabelled rabbit HLA-DRB1 polyclonal antibodies (Novus Biologicals, USA, Cat No: H00003123-D01P) in carbonate coating buffer, pH 9.5. Diluted coating antibodies were transferred into high-binding 96-well ELISA microplates (Greiner-Bio, Germany, Cat No: 655081), which were then sealed before being incubated 4°C overnight. Non-specific binding sites were then blocked by adding 1.0% milk, and the plates were sealed and incubated at room temperature for 1 hour. Two-fold diluted plasmas of pre-B ALL patients or normal controls were added into each well and the plates were sealed and incubated for 2 hours at room temperature. One $\mu\text{g}/\text{mL}$ of biotin-labelled mouse monoclonal anti-HLA-DR (clone L243, BD Biosciences, San Jose, USA, Cat No: 347361) in blocking buffer was added and incubated at room temperature for another hour in the dark. Avidin-horse-peroxidase (Av-HRP) conjugate (BioLegend, USA, Cat No: 405103) diluted in blocking buffer (1:500) was subsequently added, incubated at room temperature for 30 minutes in the dark, followed by TMB substrate (BioLegend, San Diego, CA, USA, Cat No: 421101) with incubation at room temperature for 15 minutes in the dark for colour development. Stop solution (1M Phosphoric acid, H_3PO_4) was used to inhibit the colour reaction. Absorbance (450 nm) was then read with an ELISA microplate reader (Dynex Technologies, USA). In between each step, washing was done

TABLE 1: Characteristics of Pre-B ALL patients (n=30) and healthy controls (n=31) of Malay ethnicity

	Pre-B ALL patients (n=30)	% (No)	Normal controls (n=31)
Gender			
Male	63.3 (19)		45.2 (14)
Female	36.7 (11)		54.8 (17)
Age			
Adult (>15 years)	53.3 (16)		100 (31)
Children (≤ 15 years)	46.7 (14)		
Source			
Bone marrow	90.0 (27)		
Peripheral blood	10.0 (3)		100 (31)
Stages/condition			
<i>De novo</i> /at diagnosis	90.0 (27)		
CML blast crisis	6.7 (2)		
Relapse	3.3 (1)		
Basis of diagnosis			
Morphology	Lymphoid blasts (>20%)		
Cytochemistry	Periodic Acid-Schiff + and Myeloperoxidase-		
Immunophenotyping	CD19+, CD10+, CD20+, CD22+, CD79a+, CD34+/-, HLA-DR+		

Abbreviation: pre-B ALL- precursor B Acute Lymphoblastic Leukaemia, CML – Chronic Myeloid Leukaemia, CD - Cluster of differentiation, + : expressed, - : not expressed.

by adding PBS/Tween (0.05% Tween 20) for 5 times.

The standard curve was prepared using soluble HLA-DRB1 protein purchased from Pure Protein LLC. (Texas USA, Lot No: PZP051). The amino acid sequences of this protein were verified from IMGT/HLA database available online. All experiments were conducted in duplicates and protocols of sandwich ELISA were based on procedures by BioLegend® (San Diego, CA, USA). Soluble HLA-DRB1 protein (1 µg/mL) was used as positive control while 1X PBS was used as negative control. Standard curves with R² value >0.99 were obtained providing good confidence in correlating two values. The concentration of sHLA was extrapolated from the absorbance value using this curve.

HLA-DNA typing by PCR-SSO

HLA-DNA typing for the B-cell ALL samples was determined using polymerase chain reaction, sequence-specific oligonucleotides, PCR-SSO (LIFECODES HLA-DRB Typing Kit, Cat No: 628710-50, Gen-Probe, Inc. USA) by Luminex™ xMAP technology. Results were analysed with the Quick Type Life Match 2.6.1 software

provided by the manufacturer. DNA extraction was performed by the salting out method,²⁴ while the purity and concentration of the DNA were estimated by NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, USA).

Flow cytometric analysis

Flow cytometric analysis was performed on BD FACSCanto™ flow cytometer (Becton Dickinson, Mountain View, CA, USA) for 27 of pre-B ALL patients (90.0%). Blast cells from whole blood were stained with monoclonal antibodies against CD45, CD34, CD19, and HLA-DR bound to the different fluorochromes allophycocyanin (APC), fluorescein isothiocyanate (FITC), phycoerythrin (PE), and perinidin chlorophyll protein–cyanine 5 (PerCP-CY5). Cells were gated using forward and side scatter and analysed with FACSDiva Version 6.1.3. for HLA-DR expression on CD19+ leukaemic blasts and normal lymphocytes.

Statistical analysis

Results were analysed with statistical software, SPSS 20.0 using test of normality, Kolmogorov-Smirnov and non-parametric Mann-Whitney test to compare two groups and Pearson

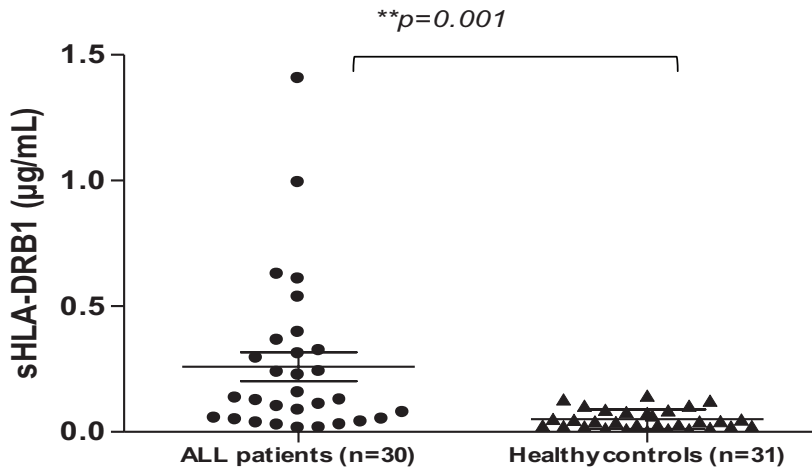


FIG.1: Scatter plot of soluble HLA-DRB1 levels in ALL patients and normal controls. Plasma levels were determined using sandwich ELISA method with plates coated with HLA-DRB1 polyclonal antibodies. Standard curve was set up with soluble recombinant HLA-DRB1 proteins. **Indicates statistical significance.

correlation test. Results were considered significant when p value <0.05.

RESULTS

Significantly higher plasma soluble HLA-DRB1 in B-cell ALL patients

Figure 1 displays sHLA-DRB1 concentrations in plasma of ALL patients and normal subjects measured by sandwich ELISA. Mean values of plasma sHLA-DRB1 levels were 0.259 ± 0.057 µg/mL (mean ± SEM) for ALL patients (n=30) and 0.051 ± 0.007 µg/mL (mean ± SEM) for normal controls (n=31). When data disregarded samples from childhood B-cell ALL (paediatric), age ≤15 years, levels of sHLA-DRB1 were still significantly higher (p=0.034) with mean sHLA-

DRB1 increased to 0.293 ± 0.110 µg/mL (mean ± SEM; n=16; age range: 17—67 years old).

A comparison between gender and age showed no significant difference between these subtypes (Figure 2). Mean values of plasma sHLA-DRB1 for male ALL was 0.221 ± 0.076 µg/mL (mean ± SEM; n=19; age range between 1—58 years old) while for female ALL it was 0.317 ± 0.088 µg/mL (mean ± SEM; n=11; age range between 1—67 years old). Mean values of plasma sHLA-DRB1 in adult pre-B ALL group was 0.293 ± 0.110 µg/mL (mean ± SEM, n=16; age range between 17—67 years old), while for children (paediatric) ALL group it was 0.226 ± 0.037 µg/mL (mean ± SEM; n=14; age range between 1—14 years old).

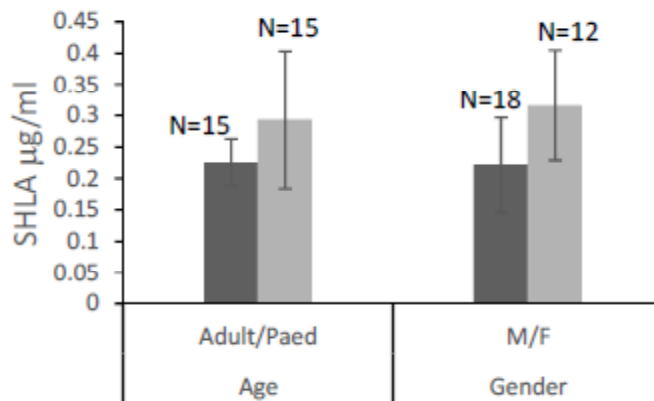


FIG. 2: Comparison of soluble HLA-DRB1 levels (sHLA) by age and gender. No significant difference was observed.

No significant correlation between sHLA levels and HLA-DR expression on leukaemia blasts and normal B cells in B-cell ALL samples

Surface expression of HLA-DR was determined by flow cytometric analysis on bone marrow and peripheral blood samples of B-cell ALL on both leukaemic blasts and normal B lymphocytes (Figure 3). Median fluorescence intensity (MFI) was also obtained using available software. More than 90.0% of patients were positive for HLA-DR expression on cells with mean percentage of 93.5%.

As expected, a significant inverse correlation was observed between percentage of leukaemic blasts and normal lymphocytes ($R=-0.696$, $p=0.000$, $N=27$) in the samples showing as leukaemic blasts increases, percentage of lymphocytes will decrease. Interestingly, a positive correlation was observed between percentage of normal lymphocytes and MFI of normal B cells ($R=0.452$, $p=0.018$, $N=27$). This however, was not observed for percentage of leukaemic blasts and MFI of leukaemic B cells ($R=-0.004$, $p=0.986$, $N=27$).

No significant correlation was observed between sHLA levels and MFI of leukaemic blasts or normal B lymphocytes in B-cell ALL cases. Neither did it correlate with percentages of these cells.

No relationship between sHLA-DRB1 levels and HLA-DRB1 alleles in B-cell ALL

HLA-DRB1 alleles were also determined for B-cell ALL patients using PCR-SSO based kit. Only HLA-DRB1 allele identified in more than 2 cases were included in the analysis. Figure 4 shows mean plasma sHLA-DRB1 levels in patients with a specific HLA-DRB1 allele compared to mean values on patient of other alleles. Total number of cases analysed was 30 ($n=30$). Lowest levels were observed for those expressing HLA-DRB1*04 (0.0872 $\mu\text{g}/\text{mL}$) while the highest levels were observed in patients positive for DRB1*16 (0.3762 $\mu\text{g}/\text{mL}$). None of the groups showed significant difference except a decreased trend for those with HLA-DRB1*04.

DISCUSSION

The importance of the immune system in prevention of cancer supports the search to understand immunological factors that influence the course of the disease and its potential clinical significance in diagnosis, prognosis and therapeutic cure.

There is currently no soluble cancer marker from serum used to diagnose leukaemia. Diagnosis is performed on cells and utilize expensive equipment. Analysis on soluble

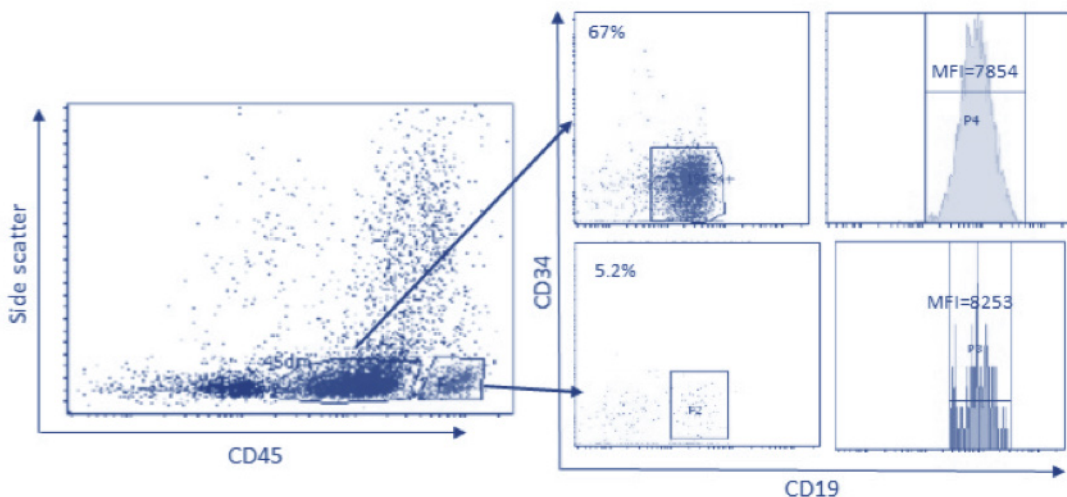


FIG. 3: Flow cytometric analysis of B-cell ALL sample stained for CD45, CD19, CD34 and HLA-DR. Percentages were determined for CD45 dim and low side scatter population which identified leukaemic blasts and CD45 hi and low scatter population which represented normal lymphocytes (left panel). These populations were further analysed for CD19 and CD34 expression (centre panel). Histograms were then used to determine MFI value on CD19+ populations, using statistical software provided (right panel).

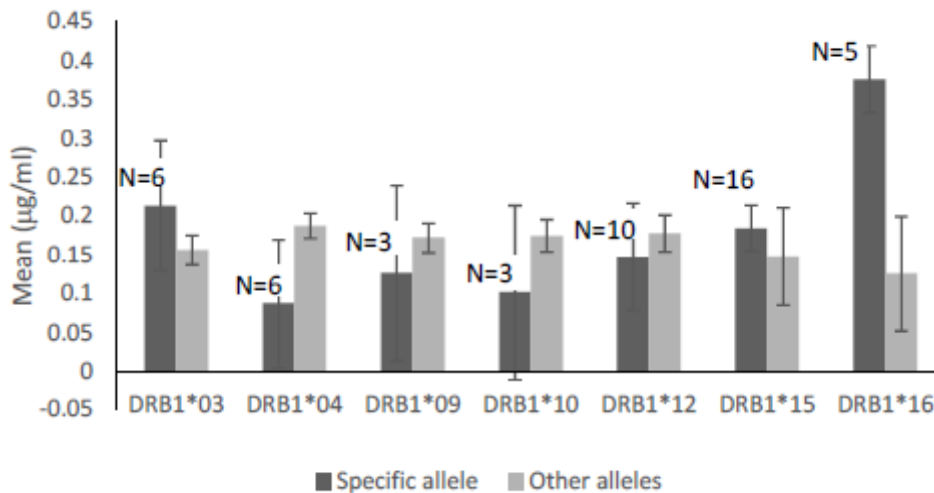


FIG. 4: Mean plasma sHLA-DRB1 levels in B-cell ALL patients of specific HLA-DRB1 allele (n=30).

markers is easier and has the potential to be developed into rapid diagnostic tests available to non-skilled personnel. These tests are also more suitable for multiple sample collection valuable in treatment monitoring.

The low number of reports on soluble HLA-DR molecules in the many disorders associated with its increased levels may be due to absence of a commercial kit for this antigen unlike those available for sHLA-G detection, for which publication has been many. Establishment of the ELISA-based method here for leukaemia study offers a step closer to its prospective large scale use to relate to treatment outcome. sHLA has been shown to have prognostication value as sHLA-I was differentially expressed in the different stages of gastric cancers with low levels associated with very advance cancer.²⁵ In melanoma, sHLA-II identifies early stage patients at high risk of disease progression.²⁰ Prognostication in leukaemia continues to be intensively studied.

Healthy normal individuals express approximately 1 µg/mL of sHLA-I. Nevertheless, high variability between individuals is observed which is believed to be influenced by HLA haplotypes. The relationship of sHLA-II to allotypes remains unclear but it was reported that sHLA-DR were highest in DR8 positive and the lowest in DR9 in normal Japanese individuals.^{1,11,26} The impact of haplotype on cancers is still unknown. Though we observed variation in levels of sHLA-DRB1 in patients with different alleles, our results were not conclusive as a larger population is needed.

The increased levels of sHLA-DRB1 in B-cell

leukaemia supported the same observations seen in other cancers. The high release of sHLA in these cancer cells may probably be used to counter immune surveillance.² As described for non-Hodgkin’s lymphoma (NHL) tumour cells, high levels of sHLA-I molecules carrying tumour derived peptide antigens released bind to the TCR of anti-tumour specific T cells, inducing either apoptosis or anergy, and also possibly bind to killer inhibitory receptors (KIR) of NK cells to induce NK cell apoptosis.^{1,3,4}

In fact, sHLA class II was said to have immunoregulatory roles, as specified by *in vitro* studies; through phagocytosis of these sHLA-II by APCs (antigen presenting cells) which is then degraded into peptides and presented to CD4+ T cells in the context of membrane HLA class II antigens. This indirect presentation may lead to either immune tolerance or activation depending on the tolerogenic or stimulating capacity of the HLA-derived peptides presented by HLA class II antigens.²⁷

The mean sHLA-DRB1 level in normal individuals in this study, 0.051 ± 0.007 µg/mL is not comparable to other reports such as soluble class II in serum of healthy individuals which was reported as 0.286 ± 0.163 µg/mL (sHLA-DR),¹⁹ and in the range of 186–362 unit/mL in saliva.¹⁶ This is due to different ELISA methods developed in each laboratory, where different types of antibodies and reagents were used and optimised. Availability of a commercial kit may help reduce this problem. Importantly, sHLA peptidomes analysed in both multiple myeloma and ALL patients revealed high level of similarity in plasmas collected from the peripheral blood

and from the bone marrow.²

Availability of sHLA in plasma or serum might be due to consequence of cell shedding, or probably from active secretion from the cells to the environment.¹¹ To this end, median fluorescence intensity (MFI) for surface expression of HLA-DR was determined in leukemic blasts and normal B cells. Lack of correlation between sHLA-DRB1 with MFI of leukaemic blasts and normal lymphocytes nevertheless, does not exclude the mechanism through membrane shedding. Oddly a higher percentage of normal lymphocytes also saw an increase in MFI of normal B cells in B-cell ALL. This may be that suppression of normal lymphocytes by leukaemia cells may also affect expression of HLA-DR on these cells and possibly affecting normal function of these cells.

In conclusion, an increased level of plasma soluble HLA-DRB1 was observed in pre-B ALL compared to normal individuals of Malay ethnicity. However, a larger number of samples will need to be tested before its potential as a diagnostic marker can be made. It will be interesting to observe if similar findings are indeed found in other ethnic groups.

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The authors declare no conflict of interest.

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