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Association between Thrombophilic Gene Polymorphisms and Recurrent Pregnancy Loss in Iranian Women

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ABSTRACT

***Background*:** Recurrent pregnancy loss (RPL) is a common problem among couples, and acquired thrombophilia is the well-known etiology of RPL. The aim of this study was to establish the association between inherited thrombophilic gene polymorphisms and RPL.

***Methods*:** This case-control study was conducted on 50 women with unexplained RPL and 50 parous women with no history of miscarriage (age range: 17–48 years). The data were collected during 2013–2015 in Sarem Hospital, Tehran, Iran. Genotyping was performed using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) for glycoprotein IIIa (PLA1/PLA2), KDR (Q472H), and β-fibrinogen (-455G/A); tetra-primer amplification refractory mutation system (ARMS)-PCR for glycoprotein Ia (807c/t) and vascular endothelial growth factor (VEGF) (2578c/a), and ins/del PCR for angiotensin I-converting enzyme (ACE) (intron 16 I/D). The association between the frequency of the genotypes and RPL was determined by Chi-square and Fisher's exact tests.

***Results*:** The results of the present study revealed a significant relationship between glycoprotein Ia (807C/T), VEGF (2578C/A), and ACE (intron 16 I/D) polymorphisms and RPL (P=0.00, 0.02, and 0.00, respectively). In contrast, no relationship was observed between β-fibrinogen (-455G/A), KDR (Q472H), and glycoprotein IIIa (PLA1/PLA2) polymorphisms and increased risk of RPL (P>0.05).

Conclusion: This study demonstrated that glycoprotein Ia (807C/T), VEGF (2578C/A), and ACE (intron 16 I/D) polymorphisms may be a risk factor for the women with a history of RPL.

Keywords: Polymorphism, Recurrent pregnancy loss, Thrombophilia

Introduction

Recurrent pregnancy loss (RPL) is described as failed pregnancy before 20 weeks of gestation, which globally affects 1-3% of women (1, 2). The main causes of RPL have been recognized, but approximately 50% of them have unknown etiology (3).

Thrombophilic risk factors have been considered as an etiology for RPL. However, other factors including uterine anomalies, abnormalities in chromosomes, endocrinological disorders, and environmental factors can be the main reasons for RPL, as well (4-6). Thrombus formation depends on platelet aggregation, fibrinolytic inhibitors, coagulation factors, and thrombophilic factors (7). Many studies have been performed to discover the role of thrombophilic gene mutations in unexplained miscarriage (8- 10). Mutations in thrombophilic genes could lead to blood clots in small vessels in placenta, decreased oxygen delivery to the fetus, and fetal loss (11). GPIa/IIa is a collagen receptor on the platelet membrane. The 807C/T polymorphism of glycoprotein Ia has been identified in the GPIa gene, which is related to numbers of receptors on the platelet membrane (12, 13). Glycoprotein IIIa (GPIIIa) is the main receptor on the surface of platelets. The PLA1/PLA2 polymorphism is recognized in the *GPIIIa* gene. The PLA2 allele is associated with aggregation and activation of platelets (14). Vascular endothelial growth factor (VEGF) is attached to specific receptors on the vascular endothelial cells. The kinase insert domain

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receptor (KDR) is a receptor of VEGF. The VEGF and KDR genes are essential to embryonic angiogenesis (15). Structure of thrombosis may result in β-fibrinogen polymorphisms (7). The β-fibrinogen (-455G/A) polymorphism could enhance fibrinogen levels during gestation (16). The angiotensin I-converting enzyme (ACE) hydrolyzes angiotensin I and transforms it into angiotensin II. In addition, ACE plays a key role in the alignment of homeostasis (17).

The aim of this study was to analyze six variants of the thrombophilic gene that may be the risk factors for RPL. The gene polymorphisms included GPIa (807C/T), GPIIIa (PLA1/PLA2), ACE (intron 16 I/D), FGB (-455G/A), VEGF (2578C/A), and KDR (Q472H).

Methods

Sample collection

This case-control study was conducted on 50 women with at least two live births and 50 women with a history of at least two consecutive miscarriages, who visited Women Reproductive Health Research Center, Sarem Hospital, Tehran, Iran, during 2015–2016. This study was carried out at the Biology Research Center of Islamic Azad University, Zanjan, Iran. The inclusion criteria for the case group were women within the age group of 17-48 with unexplained abortions. In addition, the participants were examined for chromosomal disorders, vaginal infection, hormonal imbalance, and uterine malformation. Written informed consents were obtained from all the participants. The study was approved by the Ethics Committee of the clinic (reference No.: ZUM.REC.1395.139).

DNA extraction and polymorphism determination

Genomic DNA was extracted from ethylene-diamine tetraacetic acid (EDTA) blood samples using CinnaPure DNA extraction kit (cat. no. PR881612) and following the manufacturer's instructions. The forward and reverse primer pairs were designed for GPIa (807C/T), GPIIIa (PLA1/PLA2), ACE (intron 16 I/D), FGB (-455G/A), VEGF (2578C/A), and KDR (Q472H) by Gene Runner software after obtaining gene sequences from Gene Bank (http://www.ncbi.nlm.nih.gov; Table 1). Genotyping was performed using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) for GP IIIa (PLA1/PLA2), KDR (Q472H), and FGB (-455G/A); tetra-primer amplification refractory mutation system (ARMS)-PCR for glycoprotein Ia (807C/T) and VEGF (2578C/A); ins/del PCR for ACE (intron 16 I/D). Amplification was carried out by using PCR model of Gradient Thermal Cycler (Eppendorf, Germany). PCR products were analyzed by 2% agarose gel. Digestion of PCR products was manifested by 3% agarose gel. The gels were stained with ethidium bromide to visualize fluorescent bands while using UV in the gel documentation system, except for β-fibrinogen (-455G/A) bands that were stained with SYBER Green.

Primer mixes and PCR assay components:

The tetra-primer ARMS-PCR was used for the identification of glycoprotein Ia (807C/T) and VEGF (2578C/A), PCR-RFLP for glycoprotein IIIa (PLA1/PLA2), β-fibrinogen (-455G/A), and KDR (Q472H), and ins/del PCR for ACE (intron 16 I/D) in amounts of 20 µL that contained 1X PCR Master

Table 1. Primer pairs used for the detection of GPIa, GPIIIa, VEGF, KDR, β-fibrinogen, and ACE gene polymorphisms

Gene	Polymorphism	Primer sequences 5'-3'	PCR product size (bp)	Detection methods
Glycoprotein Ia	807 C/T	FO:GTC TCC TCT GTTG AAG GTG GGG TTA ATT RO: CCA GCT GCC TTCT CAA AGT ATT CAA GAC FI:ATA TGG TGG GGAC CTC ACA AAC ACA GTT RI: AAA ACT TAC CTT GCA TAT TGA ATT GCTACG	350 174 234	Tetra-ARMS-PCR
Glycoprotein IIIa	(PLA1/PLA2)	F: CTTAGCTATTGGGAAGTGGTA R: ACTGACTTGAGTGACCTGGGA	Normal allele: 256 Mutant allele: 154, 102	PCR-RFLP MspI
VEGF	2578 C/A	FO:GCCAGCCCTTTTCCTCATAAGGGCCTTA RO: ACATCTTCCCTAAGTGCTCCCAAAGGCC FI: GCCAGCTGTAGGCCAGACCCTGGTAA RI:CCAGTCAGTCTGATTATCCACCCAGACCG	406 205 256	Tetra-ARMS-PCR
KDR	Q472H	F: CCTCCTGTATCCTGAATGAATCT R: TGGTACTGCTAAAAGTCAATGGT	Normal allele: 493 Mutant allele: 283, 212	PCR-RFLP ALU1
β-fibrinogen	455G/A	F: CCTAACTTCCCATCATTTTGTCCAATTAA R: TGTCGTTGACACCTTGGGACTTAACTAG	Normal allele: 265,97 Mutant allele: 362	PCR-RFLP HindIII
ACE	intron 16 I/D	F: CTGGAGAGCCACTCCCATCCTTTCT R: GACGTGGCCATCACATTCGTCAGAT	490 = Insertion 190= Deletion	Ins/del PCR

VEGF: vascular endothelial growth factor
KDR: kinase insert domain receptor
ACE: angiotensin I-converting enzyme

Mix (CinnaGen PCR Master Mix, Iran), 200 ng of DNA template, and 10 pmol of each forward and reverse primer (Gen Fanavaran, Iran). PCR amplifications were performed as follows: the first denaturation cycle of DNA at 94°C for 5 min followed by 35 cycles, each consisting of 45 s denaturation at 94°C, 1 min annealing at 55.8°C for glycoprotein Ia (807c/T), 63°C for VEGF (2578C/A), 56.1°C for glycoprotein IIIa (PLA1/PLA2), 55°C for β-fibrinogen (-455G/A), 51°C for KDR (Q472H), 60.5°C for ACE (intron 16 I/D), and 45 s extension at 72°C, the final extension cycle at 72°C for 5 min. PCR products (10 µL) for glycoprotein IIIa (PLA1/PLA2), β-fibrinogen (-455G/A), and KDR (Q472H) were digested with 1 unit of the MspI , HindIII, and Alu1 restriction enzymes, respectively.

Statistical analysis

Chi-square test was used to calculate the expected level of genotypes' frequencies. Odds ratio calculator (online software) was employed for statistical analysis. Statistical analysis was performed by SPSS, version 16. Estimation of the relative risk was performed by logistic regression with 95% confidence interval. P-values less than 0.05 were considered statistically significant.

Results

The participants were aged 17 to 48 years old; the control group had 2-8 children, and the case group had history of 2 to 11 spontaneous miscarriages. The mean body mass index (BMI) and age were 45.2±0.1 kg/m^2 and 33.7±6.4 years, respectively. Furthermore, the mean age at the onset of puberty was 13.7±1.4 years. Menstrual disorders existed in nine (18%) patients. The subjects were tested for sexually transmitted diseases, and one (2%) patient was shown to have genital herpes. Moreover, five (10%) patients had hyperthyroidism and two (4%) patients had hypothyroidism. Urinary tract infection was observed in two (4%) patients. Stillbirth and curettage surgery were experienced by 2 (4%) and 15 (30%) cases, respectively. The control group was normal from these aspects.

Genotyping results

Glycoprotein Ia (807C/T): The results of tetra-primer ARMS-PCR for amplification of this polymorphism are shown in Figure 1A. The frequencies of the mutant genotype in the case and control groups were 34% and 16%, respectively. Statistical analysis showed that genotype frequencies were significantly different between RPL patients and controls (OR=3.791; 95% CI 1.4547-3.2454; P=0.00; Table 2).

VEGF (2578C/A): The results of tetra-primer ARMS-PCR are presented in Figure 1B for amplification of VEGF (2578C/A) gene. The mutant homozygous and heterozygous polymorphisms of VEGF (2578C/A) were 46% and 26% in the case group and 20% and 56% in the control group, respectively. The genotype frequencies were significantly different between the RPL patients and controls (OR=1.5589; 95% CI: 1.0498-2.3151; P=0.02; Table 2).

Glycoprotein IIIa (PLA1/PLA2): The frequencies of mutant homozygous polymorphisms were 6% and 2% in the case and control groups, respectively (Table 2). The difference between the case and control groups was not statistically significant (OR=1.8571; 95% CI: 0.9401-3.6686; P=0.07). Results of PCR-RFLP have been exhibited in Figure 1C.

KDR (Q472H): The frequency of mutant homozygous polymorphism of KDR (Q472H) was 30% in the case group, while the frequency of mutant homozygous polymorphism was 42% in the control group (Table 2). There was no significant difference between the case and control groups in this regard (OR=1.04; 95% CI: 0.6949-1.5688; P =0.83). Results of PCR-RFLP have

Table 2. The frequencies of the six thrombophilic gene mutations in the case and control groups

Polymorphism	Case			Control			P-value	OR	95%CI
	Mutant homozygote %	Heterozygote %	Normal homozygote %	Mutant homozygote %	Heterozygote %	Normal homozygote %			
Glycoprotein Ia (807C/T)	34	42	24	16	40	44	0.0002	3.791	1.4547 - 3.2454
VEGF (2578C/A)	46	26	28	20	56	24	0.0277	1.5589	1.0498 -2.3151
Glycoprotein IIIa (PLA1/PLA2)	6	14	80	2	10	88	0.0747	1.8571	0.9401 - 3.6686
β-fibrinogen (-455G/A)	4	34	62	6	40	54	0.2390	0.7566	0.4755 - 1.2037
KDR (Q472H)	30	68	2	42	42	16	0.8355	1.0441	0.6949 -1.5688
ACE (intron 16 I/D)	72	26	2	52	44	4	0.0070	1.9910	1.2070 - 3.2842

VEGF: vascular endothelial growth factor
KDR: kinase insert domain receptor
ACE: angiotensin I-converting enzyme

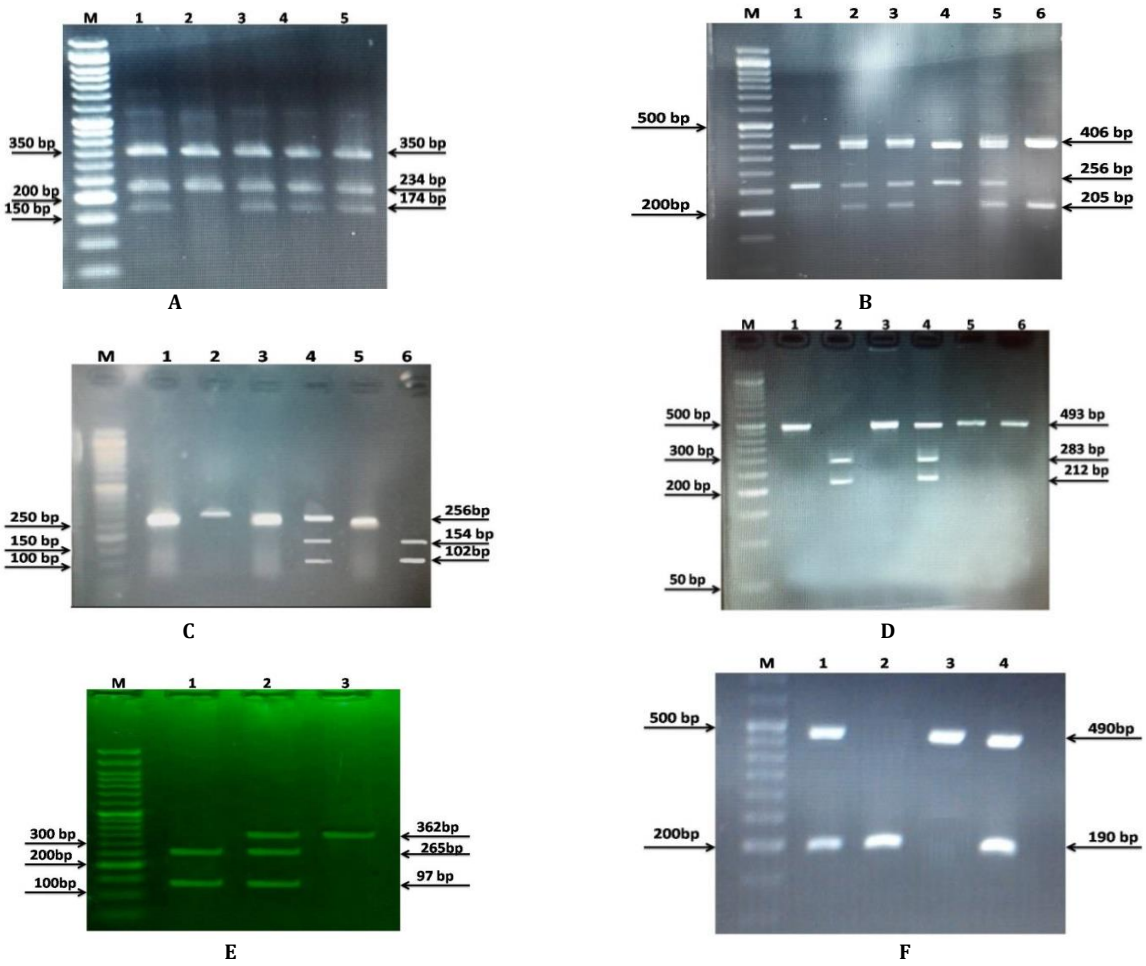

Figure 1. Agarose gel electrophoresis of polymerase chain reaction (PCR) assay for the identification of thrombophilic gene polymorphisms:
A: PCR products of glycoprotein Ia (807C/T) polymorphism: Lane M: 50 bp marker; lanes 1, 3, 4, and 5 : heterozygote (174, 234, and 350 bp); and lane 2: mutant homozygote (234 and 350 bp)
B: PCR products of vascular endothelial growth factor (VEGF) (2578C/A) polymorphism. Lane M: 50 bp marker; lanes 1 and 4: Mutant homozygote (256 and 406 bp); lanes 2, 3, and 5 : heterozygote (205, 256, and 406 bp); and lane 6: normal homozygote (205 and 406 bp)
C: PCR-RFLP analysis of glycoprotein IIIa (PLA1/PLA2) polymorphism. Lane M: 50 bp marker; lanes 1, 3, and 5: PCR product (256 bp) before digestion; lane 2: PCR product after digestion, normal homozygote (256 bp); lane 4: PCR product after digestion, heterozygote (102, 154, and 256 bp); and lane 6: PCR product after digestion, mutant homozygote (154 and 102 bp)
D: PCR-RFLP analysis of kinase insert domain receptor (KDR) (Q472H) polymorphism. Lane M: 50 bp marker; lanes 1, 3, and 5: PCR product (493 bp) before digestion; lane 2: PCR product after digestion, mutant homozygote (212 and 283 bp); lane 4: PCR product after digestion, heterozygote (212, 283, and 493 bp); and lane 6: PCR product after digestion, normal homozygote (493 bp)
E: Three representatives of Hind III restriction site analysis for β-fibrinogen (-455G/A). Lane M: 50 bp marker; lane 1: normal homozygote (97 and 265 bp); lane 2: heterozygote (97, 265, and 362 bp), and lane 3: mutant homozygote (362 bp)
F. PCR products of angiotensin I-converting enzyme (ACE) (intron 16 I/D) polymorphism. Lane M: 50 bp marker; lane 1: heterozygote (190 and 490 bp); lane 2: mutant homozygote (190 bp); lane 3: normal homozygote (490 bp); and lane 4: heterozygote (190 and 490 bp)

been presented in Figure 1D.

B-fibrinogen (-455G/A): The distribution of mutant homozygous polymorphism was similar between the case and control groups. Whereas the control group had a higher prevalence of heterozygous polymorphism (OR=0.7566; 95% CI: 0.4755-1.2037; P=0.2390; Table 2). There was no evidence supporting the association of β-fibrinogen (-455G/A) polymorphism with RPL. Results of PCR-RFLP have been shown in Figure 1E.

ACE (intron 16 I/D): The genotype frequencies of the ACE (intron 16 I/D) gene mutations in the case and control groups are presented in Table 2. The frequencies of mutant genotype for the case and control groups were 72% and 52%, respectively, indicating significant differences between the two groups (OR=1.9910; 95% CI: 1.2070-3.2842; P=0.00). Results of ins/del PCR have been displayed in Figure 1F.

Discussion

RPL is known as a multifactorial disease, which is associated with polymorphisms of thrombophilic gene (18). Several studies have investigated the relationship between thrombophilic factors and RPL, which have yielded discrepant results (19-22). Polymorphisms in the GPIa/IIa and GPIIb/IIIa genes can affect platelet aggregation. GPIa (807C/T) polymorphism is correlated with a variable expression of the platelet surface receptor, 807TT and CC genotypes were associated with higher and lower receptor densities, respectively. Differences in the receptor density are directly correlated with the rate of platelet adhesion to collagen under flow conditions (14). Poursadegh Zonouzi et al. (2013) revealed that the glycoprotein Ia (807C/T) polymorphism is associated with RPL and frequency of mutant homozygote was 40.44% in case group (23). However, in our study, glycoprotein Ia (807C/T) polymorphism was a risk factor for RPL, and the frequency of mutant homozygote was 34% in the case group**.** The GPIIb/IIIa is a receptor on the platelet membrane with high frequency. The human platelet alloantigens, PLA1 and PLA2, localized on *GPIIIa* gene are associated with neonatal alloimmune thrombocytopenia (24).

Numerous studies have examined the role of this polymorphism in RPL. Ruzzi et al. (2005) and Ivanov et al. (2008) demonstrated that PLA1/PLA2 polymorphism was a risk factor for RPL. According to Ruzzi et al. (2005), PLA2 polymorphism was reported in 44.2% of case group (25, 26), whereas Pihusch et al. (2001) and Jeddi-Tehrani et al. (2011) could not support these findings (11, 27). In this survey, there was no significant difference in the prevalence of glycoprotein IIIa (PLA1/PLA2) between the two groups and the prevalence of mutant homozygote in the patients was 6%. However, the relationship of polymorphisms of glycoprotein Ia (807C/T) and glycoprotein IIIa (PLA1/PLA2) with RPL remains controversial.

Extensive body of evidence suggest that VEGF (2578C/A) and KDR (Q472H) cannot be a risk factor for RPL (15). The most functional polymorphisms are -2578C/A and Q472H in the VEGF and KDR genes, respectively (28, 29).

Previously, El-baz et al. (2014) clarified that the VEGF (2578C/A) mutation is associated with RPL and revealed a significant difference between case and control groups in terms of frequency of homozygous mutant genotype (OR=2.18; 95% CI: 1.86-2.55) (30). Nonetheless, our study showed that the frequency of homozygous mutant genotype among patients was higher than controls. A study by Amirchaghmaghi et al. (2015) exhibited that variation in the expression of VEGF and its receptors (VEGFR1 and VEGFR2/KDR [Q472H]) may considerably impact RPL (31). However, in our study, there was no significant difference in the VEGFR2/KDR (Q472H) genotype between the two groups. The β-fibrinogen (-455G/A) polymorphism can increase the levels of fibrinogen (7). Al-Astal et al. (2014) revealed no relationship between β–fibrinogen -455G/A and RPL, and the prevalence of mutant homozygote was low in patients (3.9%) and controls (4.1%) (7). Further, Torabi et al. (2012) reported that the frequency of β-fibrinogen (-455G/A) polymerphism in women with a history of RPL was significantly higher than in the controls (32). Conversely, we did not find any significant relationship between β-fibrinogen (-455G/A) polymorphism and RPL, but we noted that the presence of mutant homozygote was higher in controls.

ACE is one the main elements of the rennin–angiotensin system effective on homeostasis. Furthermore, the relationship between ACE and I/D polymorphism is proximate, and the D allele is related to the enhancement of ACE (1). Yang et al. (2012) demonstrated that polymorphism of the ACE I/D is significantly correlated with the level of ACE enzyme circulation. The average plasma ACE level in individuals with D/D genotype is approximately two times higher than that of the individuals with I/I genotype. However, those with the I/D genotype have the average level of enzyme circulation. ACE D allele leads to increased expression of plasminogen activator inhibitor-1 (PAI-1), which can increase the risk of thrombotic events and enhance the production of angiotensin II from angiotensin I. On account of the thrombophilic defects in gravidas, the risk for pregnancy-associated thromboembolism and other vascular complications, such as preeclampsia and abortion, will be improved. According to Yang et al. (2012), the prevalence of DD genotype was 30.2% in women with a history of RPL (1). Similarly, our findings showed a significant association between ACE (intron 16 I/D) and RPL in the patient and normal groups. The discrepancy in results may be attributed to differences in sample size, population genetic variation(s), genomic mutations, presence of nucleotide polymorphism in different loci or in the vicinity of the investigated loci, epigenetic alterations, and linkage disequilibrium to other sequence variants

(7). Our study suggested that glycoprotein Ia (807C/T), VEGF (2578C/A), and ACE (intron 16 I/D) polymorphisms may increase the risk of RPL, although there was no significant difference between the patient and normal groups in glycoprotein IIIa (PLA1/PLA2), β-fibrinogen (-455G/A), and KDR (Q472H) polymorphisms. Future studies with larger sample sizes are recommended.

Conclusion

This study supports the role of glycoprotein Ia, VEGF, and ACE genes in RPL. These findings also imply the importance of screening for thrombophilic gene polymorphisms in high-risk women with unexplained miscarriage.

Acknowledgments

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Conflicts of interests

The authors declare they have no conflict of interests.

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