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Relationship Between Polymorphism of Thrombin-Activatable Fibrinolysis Inhibitor Gene +1040C/T and a Cohort of Chinese Women With Recurrent Spontaneous Abortion

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Abstract

The balance between coagulation and fibrinolysis is essential for a successful pregnancy. This study aimed to explore the genetic variant of +1040C/T in the coding region of thrombin-activatable fibrinolysis inhibitor (TAFI) gene in women with recurrent spontaneous abortion (RSA) and in unrelated healthy controls and to investigate the possible association between TAFI +1040C/T polymorphism and RSA. Peripheral blood samples were collected from 137 Chinese patients with RSA and 103 unrelated healthy Chinese controls. The TAFI +1040C/T polymorphism was analyzed using SNaPshot SNP typing after DNA extraction. The frequency of the C allele was lower in RSA patients compared with the controls (0.78 vs 0.84). A subanalysis of the TAFI +1040C/T polymorphism in the 2 populations of RSA women (groups 2RSA and >2RSA) showed that the +1040CT genotype was significantly higher and the +1040CC genotype was significantly lower than from that found in controls. The allele +1040C was associated with a reduced risk of RSA in both group 2RSA (OR = 0.418, 95%CI, 0.255-0.685) and group >2RSA (OR = 0.473, 95%CI, 0.274-0.819) compared with controls. Our data indicate a protective role for TAFI +1040C allele against RSA, and may be associated with the genetic susceptibility of RSA.

Keywords

thrombin-activatable fibrinolysis inhibitor, +1040C/T polymorphism, recurrent spontaneous abortion, Chinese, SNaPshot

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Introduction

Recurrent spontaneous abortion (RSA) is defined as 2 or more consecutive spontaneous abortions in the same couple.[1] RSA is a common reproductive health problem that affects approximately 1%-5% of women of reproductive age.[2] However, the etiology of RSA is complex, including genetic abnormalities, structural abnormalities, infections, maternal endocrine abnormalities, maternal immune dysfunction, antiphospholipid syndrome, thrombophilic disorders, and other unexplained causes,[3] with the pathogenesis of 40%-50% of cases of RSA remaining unexplained.[4]

Successful pregnancies require a balance between coagulation and fibrinolysis. Precise regulation of the coagulation–fibrinolysis system under normal physiological hypercoagulability plays an important role in embryo implantation and trophoblastic invasion, and abnormal coagulation mechanisms may be a risk factor in women with RSA.[5] Thrombin-activatable fibrinolysis inhibitor (TAFI), also known as procarboxypeptidase U, plasma procarboxypeptidase B, or procarboxypeptidase R, is a procarboxypeptidase that is mainly synthesized by the liver and

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exists as an inactive zymogen in the blood circulation. TAFI can be activated by thrombin, thrombin–thrombin regulatory protein, and plasmin, and regulates early trophoblast cell invasion. It can also inhibit the activation of plasminogen by degrading fibrin, exerting anti-fibrinolytic effects, and regulating the balance between the coagulation and fibrinolysis systems, and thus plays an important role in thrombotic diseases.[6-8]

High TAFI levels might enhance the development of thromboses and lead to pregnancy loss due to thrombophilia.[9] Maternal levels of TAFI have been reported to increase during pregnancy and return to normal soon after delivery.[10] Furthermore, TAFI levels were negatively correlated with the risk of RSA, and high levels of TAFI reduced the risk of RSA.[11,12] Previous studies indicated that inter-individual differences in plasma TAFI antigen (TAFI-Ag) levels were determined by genetic factors.[13] Several single-nucleotide polymorphisms (SNPs) have been identified in the TAFI gene,[14] including a C/T polymorphism at +1040 in the coding region, resulting in the conversion of a threonine (ACU) to isoleucine (AUU) at position 325 (Thr325Ile). The Ile-325 variant has 60% higher antifibrinolytic activity than Thr-325 variant, and prolongs the half-life of activated TAFI.[15] Brouwers et al[16] found that TAFI-Ag plasma levels were significantly correlated with the +1040C/T SNP in the coding region of the TAFI gene (TAFI+1040C/T): the highest levels of TAFI-Ag were associated with the wild-type C/C genotype and the lowest levels with the homozygous mutant T/T genotype. In addition, carriers of the T/T genotype had a 1.23-fold increased risk of fetal loss compared with C/C carriers and a 1.34-fold increased risk compared with C/T carriers.[17] Although the frequency of the T allele was higher among Egyptian patients with RSA and the frequency of the C allele was higher in controls, the difference was not significant, suggesting that TAFI +1040C/T was not a molecular predictive factor for RSA.[18,19] In addition, the TAFI +1040C/T polymorphism was not related to RSA in Chinese Han women.[20]

These studies suggest that the +1040 SNP in the coding region of the TAFI gene in relation to RSA might differ among ethnic groups. Based on the limited and inconsistent published data for the polymorphism of the TAFI +1040C/T in RSA, including in Chinese women, we aimed to analyze this polymorphism in Chinese patients with and without RSA, to explore the relationship between TAFI +1040C/T and RSA.

Materials and Methods

Study Population

A total of 137 unrelated RSA patients with a history of 2 or more spontaneous abortions were enrolled as a case group and 103 healthy women with no history of spontaneous abortion or complicated pregnancy were recruited as a control group in this case control study. All patients and controls were clinic outpatients from the Department of Obstetrics and Gynecology at Affiliated Huadu Hospital, Southern Medical University (People's Hospital of Huadu District) from January 2018 to December 2019. They were all women in the reproductive period who had been physically healthy and had no family history of genetic diseases. All participants were Han Chinese, and all had at least 1 live birth and complete clinical data. Any women with chromosomal abnormalities, anatomical anomalies of the uterus, abnormal endocrine or autoimmune diseases, diabetes mellitus, thyroid dysfunction, and infections were excluded from the study. Any candidate with abnormal levels of protein C, protein S and antithrombin III, as well as carrying the known thrombophilic gene mutations, such as coagulation factor V [G1691A Leiden], factor II prothrombin [G20210 A] and methylene tetrahydrofolate reductase [MTHFR C677 T], was also excluded from our study. Ethical approval for the study was obtained from the Ethics Committee of Affiliated Huadu Hospital, Southern Medical University (People's Hospital of Huadu District). Written informed consent was obtained from all participants prior to the study.

SNaPshot SNP Typing

DNA was extracted from peripheral blood leukocytes using a standard phenol/chloroform method. DNA quantity and quality were evaluated using a nucleic acid quantifier (NanoDrop 2000 spectrophotometer) and 2% agarose gel electrophoresis. TAFI +1040C/T gene polymorphism was determined by SNaPshot SNP typing. Polymerase chain reaction (PCR) was performed using a 2720 Thermal Cycler (Applied Biosystems,). Forward (5′-ACCTCTAAGCCTTTGAGATGTA-3′) and reverse (5′-GCTTCTGTTCCTAATTATTACAA-3′) primers were designed for a TAFI fragment that included the +1040C/T SNP. Shrimp alkaline phosphatase (SAP) and exonuclease I (Exo I) were used to remove the primers and unexhausted dNTPs from 15 μl of PCR product, which was purified using 5 U SAP and 2 U Exo I for 1 h at 37°C, followed by 15 min at 75°C to inactivate SAP and Exo I. SNaPshot PCR was performed using the SNaPshot PCR primer (5′-TGACACCAC-GACCTAACCTCAGA-3′) and SNaPshot Multiplex Kit with a 2720 Thermal Cycler. A 10 μl reaction volume (containing 5 μl reaction mix, 1 μl SNaPshot PCR primer, 3 μl purified PCR products, and 1 μl dH_2O) was used for single base extension on a 2720 Thermal Cycler. The thermocycling conditions were as follows: 25 cycles of denaturation for 10 s at 96°C, annealing for 5 s at 50°C, and extension for 30 s at 60°C, maintained at 4°C until the next analysis. Electrophoresis sample preparation was conducted in a 10 μl reaction mixture (containing 0.5 μl SNaPshot PCR products, 9.25 μl Hi-Di formamide, and 0.25 μl GeneScan LIZ-120ABI), followed by 5 min at 95°C for denaturation and rapid cooling for 4 min. The Matrix Standard Set DS-02(E5) [dR110, dR6G, dTAMRA, dROX, LIZ] were used for spectral calibration. A 200 ul reaction volume (containing 25 ul Matrix Standard Set DS-02 and 175 ul Hi-Di formamide) was used for spectral calibration, followed by 5 min at 95°C for denaturation and rapid cooling for 5 min. Spectrum calibration was performed on the 3730XL Genetic Analyzer. After spectrum calibration, the prepared

Table 1. Comparison of Main Characteristics of the 2 Groups of Subjects.[a]

		RSA cases (n = 137)	Controls (n = 103)	*P* value
Age	Mean ± SD	29.95 ± 3.847	29.26 ± 3.786	0.143
	Range	22-41	21-40	
Number of pregnancies	Mean ± SD	3.91 ± 1.333	2.83 ± 0.845	<0.001
	Range	3-13	2-5	
Number of births	Mean ± SD	1.20 ± 0.467	1.75 ± 0.776	<0.001
	Range	1-4	1-5	
Numbers of pregnancy losses	Mean ± SD	2.71 ± 1.208	0	<0.001
	Range	2-11	NA	

Abbreviations: SD, standard deviation; NA, not applicable.
[a]*P* were determined using the Mann-Whitney U test.

Table 2. Comparison of Main Characteristics of Ages: 22-30 and 31-41 in RSA Cases.[a]

		Ages: 22-30 (n = 78)	Ages: 31-41 (n = 59)	*P* value
Age	Mean ± SD	27.27 ± 2.237	33.49 ± 2.374	<0.001
	Range	22-30	31-41	
Number of pregnancies	Mean ± SD	3.79 ± 1.188	4.05 ± 1.502	0.132
	Range	3-8	3-13	
Number of births	Mean ± SD	1.17 ± 0.495	1.24 ± 0.429	0.118
	Range	1-4	1-2	
Numbers of pregnancy losses	Mean ± SD	2.63 ± 1.046	2.81 ± 1.395	0.398
	Range	2-7	2-11	

Abbreviation: SD, standard deviation.
[a]*P* were determined using the Mann-Whitney U test.

samples were separated by capillary electrophoresis using an ABI 3730XL Genetic Analyzer. Data were analyzed using GeneMapper software v4.0.

Statistical Analysis

Measured data were presented as mean ± standard deviation and compared using *t*-tests between patients and controls, and ages 22-30 and 31-41. Deviations of genotype distributions from Hardy-Weinberg equilibrium were assessed by χ^2 tests for patients and controls. The χ^2 tests were used to compare the TAFI+1040C/T allele and genotype frequencies. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated for the association analysis. A *P*-value <0.05 was considered statistically significant. All statistical analyses were performed using SPSS (version 20.0; SPSS Inc., Chicago, IL, USA).

Results

As shown in Table 1, the 137 women with RSA included in this study had experienced at least 2 spontaneous abortions. Although the ranges of age were relatively large, the SD is low in both groups. There was no significant difference in age distribution between the 2 groups ($P = 0.143$). However, the numbers of pregnancies, births, and pregnancy losses differed significantly between the RSA patients and healthy controls ($P < 0.001$).

According to ages and numbers of RSA, the 137 women with RSA were then stratified into age subgroups (22-30, n = 78; 31-41, n = 59) and numbers of pregnancy losses subgroups (2RSA: women with 2 pregnancy losses, n = 81; >2RSA: women with 3 or more pregnancy losses, n = 56). Comparison of main characteristics in the age subgroups of women (22-30 and 31-41) was shown in Table 2. Among the 137 RSA patients, there was no significant difference between ages subgroups (22-30 and 31-41) and the numbers of pregnancies, births, and pregnancy losses ($P > 0.05$, Table 2).

The TAFI+1040C/T polymorphism genotype distributions in patients with RSA and controls were in Hardy-Weinberg equilibrium ($P = 0.500$ and $P = 0.398$, respectively), indicating that the study population was representative (Table 3). We independently examined the distributions of the TAFI+1040C/T SNP in RSA patients and normal individuals, but found no significant difference in the genotype and frequencies between the RSA cases and the controls ($P > 0.05$) (Table 3). The frequency of the C allele was lower among RSA cases (0.78 vs 0.84) and was associated with a reduced risk of RSA (OR = 0.690; 95%CI, 0.434-1.099), but the difference was not significant ($P = 0.073$).

The distribution of TAFI+1040C/T polymorphism was also analyzed in the age subgroups of RSA women, by comparing group 22-30 and group 31-41 to controls and to each other. The distributions of TAFI+1040C/T genotype and allele frequencies in group 22-30 and group 31-41 were not significantly different from that found in control women (both $P > 0.05$, Table 4).

Table 3. Genotype and Allele Frequencies of TAFI 1040C/T in Cases and Controls.

TAFI 1040C/T		Cases	Controls	OR (95%CI)	*P* value
Genotype	T/T	3 (2.19%)	2 (1.94%)	Reference value	NA
	C/C	79 (57.66%)	71 (68.93%)	0.742 (0.120-4.568)	0.555
	C/T	55 (40.15%)	30 (29.13%)	1.222 (0.193-7.724)	0.587
	Total	137	103	NA	NA
HWE	χ^2 value	1.385	1.842	NA	NA
	P value	0.500	0.398	NA	NA
Allele	T	61 (0.22)	34 (0.16)	Reference value	NA
	C	213 (0.78)	172 (0.84)	0.690 (0.434-1.099)	0.073

Abbreviations: OR, odds ratio; CI, confidence interval; HWE, Hardy-Weinberg equilibrium test; NA, not applicable.

Table 4. Comparison of TAFI 1040C/T Genotype and Allele Frequency Distributions Between Controls and RSA Patients According to Ages and Numbers of Pregnancy Losses.

		Genotype frequency [n (%)]			Allele frequency [n(n/N)]	
Group	N	TT	CC	CT	T	C
Age						
22-30	78	2 (2.56)	49 (62.82)	27 (34.62)	31 (0.20)	125 (0.80)
31-41	59	1 (1.69)	30 (50.85)	28 (47.46)	30 (0.25)	88 (0.75)
Controls	103	2 (1.94)	71 (68.93)	30 (29.13)	34 (0.16)	172 (0.84)
χ^2 value			5.684		3.760	
P value			0.224		0.153	
Numbers of pregnancy losses						
2RSA	81	2 (2.47)	31 (38.27)[a]	48 (59.26)[a]	52 (0.32)[a]	110 (0.68)[a]
>2RSA	56	1 (1.79)	24 (42.86)[a]	31 (55.36)[a]	33 (0.29)[a]	79 (0.71)[a]
Controls	103	2 (1.94)	71 (68.93)	30 (29.13)	34 (0.16)	172 (0.84)
χ^2 value			20.159		13.538	
P value			<0.001		0.001	

Abbreviations: N, total number of samples; RSA, recurrent spontaneous abortion; 2RSA, women with 2 pregnancy losses; >2RSA, women with 3 or more pregnancy losses.
[a] $P < 0.05$ vs controls.

Among the RSA patients, 81 (59.12%) had 2 miscarriages, the remaining 56 (40.88%) experiencing 3 or more miscarriages, including 32 (23.36%) who had 3, 17 (12.41%) who had 4, 4 (2.92%) who had 5, 2 (1.46%) who had 7, and 1 woman (0.73%) who had up to 11 miscarriages. Similarly, we also compared the distributions of TAFI+1040C/T polymorphism between group 2RSA and >2RSA subgroups. Compared with the controls, genotype distributions of TAFI +1040C/T in group 2RSA and group >2RSA were significantly different ($P < 0.05$). The frequency of CC genotype in the group 2RSA and group >2RSA group was significantly lower than that in controls, while the CT genotype was significantly higher than that in controls. The frequency of C allele decreased significantly in both group 2RSA and group >2RSA (Table 4).

Genotype and allele frequencies of TAFI 1040C/T were also analyzed independently by comparing group 2RSA and group >2RSA to controls and to each other. The distribution of TAFI+1040C/T allele reached statistical significance with a value of $P < 0.001$. However, the genotype frequencies between the group 2RSA and group >2RSA failed to show significant differences (Table 5). The allele +1040C was associated with a reduced risk of RSA in both group 2RSA (OR = 0.418, 95%CI, 0.255-0.685) and group >2RSA (OR = 0.473, 95%CI, 0.274-0.819) compared with controls.

Discussion

RSA is a serious pregnancy complication. The cause of RSA remains unknown in nearly 50% of cases and cures are unsatisfactory, highlighting RSA as a clinically refractory infertility disease. Normal pregnancy may be associated with physiological hypercoagulation, but abnormal coagulation mechanisms may result in placental microthrombi and placental dysfunction, leading to RSA. TAFI is essential for the precise regulation of the local coagulation–fibrinolysis system.[5,18] ElDanasori et al[18] investigated the TAFI function in early pregnancy population and found that in addition to regulating the balance of coagulation–fibrinolysis during pregnancy, the high concentration level of TAFI in the low fibrinolytic state could inhibit the formation of fibrin degradation products, reduce the apoptosis of placental trophoblast cells, and was beneficial to normal pregnancy. RSA can be caused by genetic polymorphisms in the maternal hemostatic system. Arauz et al[21] found that changes in TAFI gene polymorphisms could affect the

Table 5. Genotype and Allele Frequencies of TAFI 1040C/T Between Women With 2 Pregnancy Losses (2RSA), Women With 3 or More Pregnancy Losses (>2RSA), and Controls.

TAFI 1040C/T		2RSA	>2RSA	Controls	OR (95%CI)	*P* value
Genotype	T/T	2 (2.47%)		2 (1.94%)	Reference value	NA
	C/C	31 (38.27%)		71 (68.93%)	0.437 (0.059-3.242)	0.368
	C/T	48 (59.26%)		30 (29.13%)	1.600 (0.214-11.969)	0.510
	Total	81		103	NA	NA
Allele	T	52 (0.32)		34 (0.16)	Reference value	NA
	C	110 (0.68)		172 (0.84)	0.418 (0.255-0.685)	<0.001
Genotype	T/T		1 (1.79%)	2 (1.94%)	Reference value	NA
	C/C		24 (42.86%)	71 (68.93%)	0.676 (0.059-7.793)	0.591
	C/T		31 (55.36%)	30 (29.13%)	2.067 (0.178-24.006)	0.500
	Total		56	103	NA	NA
Allele	T		33 (0.29)	34 (0.16)	Reference value	NA
	C		79 (0.71)	172 (0.84)	0.473 (0.274-0.819)	0.006
Genotype	T/T	2 (2.47%)	1 (1.79%)		Reference value	NA
	C/C	31 (38.27%)	24 (42.86%)		1.548 (0.132-18.104)	0.605
	C/T	48 (59.26)	31 (55.36%)		1.292 (0.112-14.857)	0.664
	Total	81	56		NA	NA
Allele	T	52 (0.32)	33 (0.29)		Reference value	NA
	C	110 (0.68)	79 (0.71)		1.132 (0.671-1.910)	0.372

Abbreviations: OR, odds ratio; CI, confidence interval; NA, not applicable.

coagulation and fibrinolysis status, and the level of TAFI was closely related to gene polymorphisms. TAFI is a plasma protease that can be activated by a variety of substances to inhibit fibrinolysis. Most studies of TAFI have focused on its role in inhibiting fibrinolysis and its participation in regulating inflammatory diseases.[22,23]

TAFI +1040C/T is a commonly studied polymorphic variant for RSA.[14,17-20] Nevertheless, the protective role of the C allele of this polymorphism is controversial. The frequency of the C allele was also related to ethnicity among women with RSA, with incidences of 59.3% in 86 Italian women,[14] 59.33% in 59 Serbian women,[17] 73% in 50 Egyptian women,[18,19] and 15.6% in 426 Chinese women.[20] Although all the frequencies of the C allele mentioned above were lower in RSA patients compared with controls, the differences were not significant.

In addition to the controversial relationship between genetic polymorphisms and RSA, the impact of TAFI-Ag levels on RSA also remains unclear. Both Knol et al[11] and Legnani et al[12] suggested that high TAFI levels reduced the risk of early RSA and prevented early and recurrent early fetal loss; however, TAFI-Ag levels were significantly higher in the RSA group compared with controls in another study,[24] and Eser et al[25] found no change in TAFI levels in women with RSA. Although the exact clinical significance of the TAFI +1040C/T SNP thus remains unclear, existing research results suggest that the functional +1040C/T (Thr325Ile), associated with an threonine to isoleucine substitution at position 325 in TAFI, is not associated with a reduced risk of RSA.

Considering that the TAFI+1040C/T polymorphism may be affected by sample size, geographical region, and ethnicity.[14,17-20] In the present study, we detected the +1040C/T SNP in the TAFI gene coding region in 137 Chinese women with RSA and 103 healthy controls using SNaPshot SNP typing to assessed whether TAFI +1040C allele represent a protective factor for RSA. Among the 240 subjects included in the study, although the age ranged from 21 to 41, the average age of the 2 groups was about 29 years old, and the SD was small, indicating that the gap between the subjects of childbearing age included in the study was small. In addition, there was no statistically significant difference in age between the 2 groups ($P = 0.143 > 0.05$), suggesting that they were comparable. All cases and controls had at least 1 successful pregnancy and a history of live births.

Most Chinese obstetrics and gynecology experts consider that 2 consecutive abortions should be considered as a potential problem. Among 137 patients with RSA, the mean number of abortions was (2.71 ± 1.208) (range 2-11), with 56 women (40.88%) experiencing 3 or more miscarriages, including 1 woman who had up to 11 miscarriages. There were significant differences in the number of pregnancies, births and pregnancy losses between the cases and the controls (Table 1). However, among the 137 RSA patients, there was no significant difference between ages (ages: 22-30 and 31-41) and the numbers of pregnancies, births, and pregnancy losses (Table 2).

In this study, we also investigated the association between TAFI polymorphisms and RSA risk, but found no significant differences in the distributions of TAFI genotypes and alleles between patients with and without RSA. Three women (2.19%) with RSA carried the TT genotype, 55 (40.15%) carried the CT genotype, and the other 79 RSA patients (57.66%) carried the CC genotype. The frequency of the C allele was lower among RSA cases than controls (0.78 vs 0.84) and the C/C genotype frequency was also lower among women with RSA (57.66% vs 68.93%), but the differences in allele and genotype distributions were not significant (both $P > 0.05$), in accordance with other studies in different populations.[14,17-20] The overall

prevalence of the CC genotype in the current study population was 62.5%, including 57.66% in the RSA group and 68.93% in the control group.

A subanalysis of the TAFI +1040C/T polymorphism in the 2 populations of RSA women (groups 22-30 and 31-41) showed that the distributions of +1040C/T genotypes and alleles were not significantly different from that found in controls (Table 4). There was no statistically significant difference in the distribution of T and C alleles among the 3 groups (Table 4), indicating that the TAFI +1040C/T polymorphism had no clear relationship with age. In contrast, when women with RSA were stratified according to the number of pregnancy losses (2RSA and >2RSA), the distribution of +1040C/T and +1040C/C genotypes in women with 2 or more pregnancy losses was very similar and was significantly different from that found in controls (Tables 4 and 5). We found that the frequency of +1040C/T genotype was higher in woman both with 2RSA and >2RSA than in woman without RSA (Tables 4 and 5). The same findings was observed also in the case of +1040 T allele frequency. The allele 1040 T was significantly higher in women with 2RSA and >2RSA than in controls, and may act as the risk factor to increase susceptibility to RSA. In addition, we found a significantly higher frequency of +1040C allele in both subgroups (2RSA and >2RSA) than in controls, and the allele +1040C was associated with a decreased risk of RSA in both subgroups (2RSA and >2RSA) compared with controls, suggesting a protective role for this allele (Table 5).

Conclusions

In conclusion, we analyzed and compared the TAFI +1040C/T SNP in Chinese women with and without RSA to assess its protective role for TAFI +1040C allele against RSA. The results suggested that the +104C/C was less common in RSA patients than in healthy controls. A subanalysis of the TAFI +1040C/T polymorphism in the 2 populations of RSA women (groups 2RSA and >2RSA) showed that the +1040CT genotype was significantly higher and the +1040CC genotype was significantly lower than from that found in controls. Our data indicate a protective role for TAFI +1040C allele against RSA, and may be associated with the genetic susceptibility of RSA. Further studies are required to address the molecular mechanisms and clinical diagnoses of RSA.

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Declaration of Conflicting Interests

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