Gene Variations of Sixth Complement Component Affecting Tacrolimus Metabolism in Patients with Liver Transplantation for Hepatocellular Carcinoma

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Abstract

Background: Orthotopic liver transplantation (OLT) improves the prognosis of patients with hepatocellular carcinoma (HCC). Moreover, the complement system is a powerful immune effector that can affect liver function and process of liver cirrhosis. However, studies correlating the complement system with tacrolimus metabolism after OLT are scarce. In this study, the role of single nucleotide polymorphisms (SNPs) associated with the sixth complement component (C6) in tacrolimus metabolism was investigated during the early stages of liver transplantation.

Methods: The study enrolled 135 adult patients treated with OLT for HCC between August 2011 and October 2013. Ten SNPs in C6 gene and rs776746 in cytochrome P450 3A5 (CYP3A5) gene were investigated. The tacrolimus levels were monitored daily during 4 weeks after transplantation.

Results: Both donor and recipient CYP3A5 rs776746 allele A were correlated with decreased concentration/dose (C/D) ratios. Recipient C6 rs9200 allele G and donor C6 rs10052999 homozygotes were correlated with lower C/D ratios. Recipient CYP3A5 rs776746 allele A (yielded median tacrolimus C/D ratios of 225.90 at week 1 and 123.61 at week 2), C6 rs9200 allele G (exhibited median tacrolimus C/D ratios of 211.31 at week 1, 110.23 at week 2, and 99.88 at week 3), and donor CYP3A5 rs776746 allele A (exhibited median C/D ratios of 210.82 at week 1, 77.49 at week 3, and 85.60 at week 4) and C6 rs10052999 homozygote (exhibited median C/D ratios of 167.59 at week 2, 157.99 at week 3, and 155.36 at week 4) were associated with rapid tacrolimus metabolism. With increasing number of these alleles, patients were found to have lower tacrolimus C/D ratios at various time points during the 4 weeks after transplantation. In multiple linear regression analysis, recipient C6 rs9200 group (AA vs. GG/GA) was found to be related to tacrolimus metabolism at weeks 1, 2, and 3 (P = 0.005, P = 0.045, and P = 0.033, respectively), whereas donor C6 rs10052999 group (CC/TT vs. TC) was demonstrated to be correlated with tacrolimus metabolism only at week 4 (P = 0.001).

Conclusions: Recipient C6 gene rs9200 polymorphism and donor C6 gene rs10052999 polymorphism are new genetic loci that affect tacrolimus metabolism in patients with HCC after OLT.

Key words: C6; Cytochrome P450 3A5; Hepatocellular Carcinoma; Liver Transplantation; Single Nucleotide Polymorphism; Tacrolimus Metabolism

Introduction

Hepatocellular carcinoma (HCC) ranks fifth among all the malignancies and third among cancer deaths worldwide. Orthotopic liver transplantation (OLT) increases survival in HCC patients. However, immune suppressants are required in appropriate amounts to prevent transplant rejection. Tacrolimus, which is the first-line immunosuppressant after organ transplantation, reduces the incidence of rejection and improves graft and recipient survival. Merely, tacrolimus has a narrow therapeutic interval, with varying pharmacokinetics. Standard doses of tacrolimus administered postoperatively are not effective in all patients.
patients equally. Therefore, tacrolimus treatment requires monitoring for efficacy and safety. Nonetheless, it is a challenge to tailor individualized interventions during early postoperative period. Previous studies suggested that some gene polymorphisms are associated with drug metabolism. Pharmacogenomics focuses on the relationship between host genetics and drug metabolism. Most previous studies focused on pharmacogenomics regardless of pathogenesis. Patients with HCC were treated with different therapeutic regimens, which are associated with complications and poor prognosis. Therefore, the identification of markers to facilitate individualized tacrolimus therapy, especially in patients with HCC undergoing transplantation, is imperative.

Tacrolimus is mainly metabolized by cytochrome P450 (CYP) 3A isoenzymes. A cytochrome P450 3A5 (CYP3A5) gene variation involving an A-to-G transition at position rs776746 within intron 3 was significantly associated with CYP3A5 expression. Tacrolimus is poorly metabolized in CYP3A5 rs776746 GG carriers. However, the effects of CYP3A5 rs776746 do not explain the total individual differences in tacrolimus metabolism. Therefore, additional markers can play a role in individual variation.

The complement system affects liver function and process of liver cirrhosis and eliminates virus-infected and cancer cells as well. The sixth component of the complement system (C6) is involved in the formation of a membrane attack complex (MAC) with other components (C5–C9) following activation of the complement cascade. The MAC was reported to mediate transplant rejection, C6 is an acute phase protein, which was studied in a rat heart transplantation model. Interestingly, we demonstrated that patients with C6 rs9200 GA presented with HCC recurrence following OLT. However, to the best of our knowledge, no research has yet found the association between C6 gene polymorphisms and tacrolimus pharmacokinetics. Therefore, we initially hypothesized that C6 gene polymorphisms might regulate MAC function and mediate tacrolimus metabolism. This study was to investigate the association between tacrolimus metabolism and C6 single nucleotide polymorphisms (SNPs) in a large cohort of patients undergoing OLT for HCC and to evaluate the possible role of tacrolimus as an immunotherapeutic intervention tailored to individual patients during early postoperative period.

**Methods**

**Ethical approval**

The study was conducted in accordance with the *Declaration of Helsinki* and was approved by the Institutional Review Board of Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine. Informed written consent was obtained from all patients before their enrollment in this study.

**Patients**

The adult patients treated with OLT for HCC between August 2011 and October 2013 at the Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine were enrolled in the study. Patients with etiologically different liver disease (e.g., hepatic failure and autoimmune hepatitis) were excluded from the study. In addition, patients who were not treated with tacrolimus-based immunosuppressive regimens were excluded from the study. All the patients underwent immunosuppressive therapy using tacrolimus (Prograf, Astellas Pharma, Japan) administered orally twice daily, starting with a preliminary dose of 0.06 mg·kg⁻¹·d⁻¹. The dose was modified during the 1st month posttransplantation based on the target blood levels (7–10 ng/ml).

**Determination of tacrolimus concentration**

Tacrolimus levels were assayed using apron-TRAC TM II Tacrolimus ELISA kit (Diasorin, USA) and microparticle enzyme immunoassays (ELx 800NB analyzer, BioTek, USA). The concentration/dose (C/D) ratio was determined by dividing the trough concentration by the weight-adjusted daily dose (mg·kg⁻¹·d⁻¹). C/D ratios were determined at 4 weeks posttransplantation. The mean C/D ratios at weeks 1–4 were used to monitor weekly changes in tacrolimus metabolism.

**Genomic DNA isolation and genotype determination**

Recipient (ethylenediaminetetraacetic acid-anticoagulated whole blood) and donor (fresh-frozen liver tissue) genotypes were determined separately. Both the recipient and the donor DNA were determined using the AllPrep DNA/RNA Mini kit (Qiagen, Venlo, The Netherlands) according to the manufacturer’s instructions. Genotype variations were determined using a Sequenom MassARRAY SNP genotyping platform (Sequenom, San Diego, CA, USA). Quality control was performed to exclude individual SNPs or samples with genotype efficiency call rates <95% and SNP assays with poor-quality spectra/cluster plots, using LDR-PCR sequencing technology for further verification.

**Statistical analysis**

Hardy-Weinberg equilibrium and allele frequency were analyzed using SHEsis online version (http://analysis.bio-x.cn/myAnalysis.php). The patients’ age and weight and tacrolimus C/D ratios are expressed as median (interquartile range). Quantitative variables among the groups were compared using nonparametric tests. Mann-Whitney U-test was used to compare differences between two groups, and Kruskal-Wallis test was used to analyze the differences among several groups. All the analyses were two sided and performed using SPSS software version 17.0 (SPSS Inc., Chicago, IL, USA). A P < 0.05 was considered statistically significant.

**Results**

**Genotype and allele distributions**

Initially, 135 adult patients treated with OLT for HCC between August 2011 and October 2013 were enrolled in the study. Fifty-five patients with etiologically different liver disease and nine patients who were not treated with tacrolimus-based immunosuppressive regimens were excluded from the study. A total of 71 patients were...
Table 1: Genotype frequencies of CYP3A5 rs776746, C6 rs9200, and rs10052999

| Genotypes   | Frequency of donors, n (%) | Frequency of recipients, n (%) |
|-------------|----------------------------|-------------------------------|
| CYP3A5 rs776746 |                           |                               |
| GG          | 33 (46.5)                  | 36 (50.7)                     |
| GA+AA       | 38 (53.5)                  | 35 (49.3)                     |
| C6 rs9200   |                            |                               |
| AA          | 36 (50.7)                  | 40 (56.3)                     |
| GA+GG       | 35 (49.3)                  | 31 (43.7)                     |
| C6 rs10052999 |                          |                               |
| TC          | 30 (42.3)                  | 31 (43.7)                     |
| CC+TT       | 41 (57.7)                  | 40 (56.3)                     |

Table 2: Effects of recipient CYP3A5 rs776746, C6 rs9200, and rs10052999 polymorphisms on tacrolimus concentration/dose ratios

| Genotypes   | C/D ratio | U   | P  | C/D ratio | U   | P  | C/D ratio | U   | P  | C/D ratio | U   | P  |
|-------------|-----------|-----|----|-----------|-----|----|-----------|-----|----|-----------|-----|----|
| CYP3A5 rs776746 |           |     |    |           |     |    |           |     |    |           |     |    |
| GG (n = 36)   | 322.15 (305.21) | 153.92 (129.19) | 6.816 | 0.009 | 3.868 | 0.049 | 1.376 | 0.241 |
| GA + AA (n = 35) | 225.90 (216.77) | 123.61 (98.59) | 5.059 | 0.025 | 4.903 | 0.027 | 0.716 | 0.397 |
| C6 rs9200    | 292.93 (301.27) | 153.16 (115.80) | 276.97 ± 267.84 | 0.001 | 0.982 | 114.27 (101.99) | 0.011 | 0.917 | 119.62 (105.41) | 0.077 | 0.782 |
| C6 rs10052999 | 211.31 (249.07) | 110.23 (98.42) | 247.25 ± 227.44 | 0.001 | 0.982 | 119.04 (172.01) | 0.011 | 0.917 | 124.37 (143.02) | 0.077 | 0.782 |

The data are shown as median (IQR). C/D: Concentration/dose; IQR: Interquartile range.
allele G carriers and non-G carriers (P > 0.05). Therefore, donor CYP3A5 rs776746 allele A and C6 rs10052999 homozygote represented statistically significant markers of rapid tacrolimus metabolism.

In addition, none of the eight C6 SNP genotypes including rs3805712 (Pmin = 0.053, donor at week 3, AG vs. AA/AG), rs3805715 (Pmin = 0.059, recipient at week 4, AA vs. GG/AG), rs3805716 (Pmin = 0.068, donor at week 4, AA vs. AT/TT), rs6865420 (Pmin = 0.478, donor at week 3, CA vs. CC/AA), rs7443562 (Pmin = 0.089, donor at week 2, AA vs. GG/GA), rs121917779 (all genotypes were AA), rs138105385 (all genotypes were CC), and rs150358068 (all genotypes were CC) was associated with tacrolimus metabolism.

**Combined polymorphisms and tacrolimus concentration/dose ratios**

CYP3A5 rs776746 genotypes (GA and AA) and C6 rs9200 genotypes (GA and AA) of recipient and donor CYP3A5 rs776746 genotypes (GA and AA) and C6 rs10052999 genotypes (CC and TT) were associated with rapid tacrolimus metabolism. All the genotypes were further analyzed to determine the role of SNPs in tacrolimus metabolism. We divided the patients into three groups based on those genotypes: patients carried zero or one related genotypes were defined as poor metabolizers, named Group 1; patients carried two or three related genotypes were defined as intermediate metabolizers, named Group 2, whereas patients carried four related genotypes were defined as junior extensive metabolizers, named Group 3. As shown in Figure 1, in Kruskal-Wallis test, with increasing number of genotypes associated with rapid metabolism, patients exhibited lower tacrolimus C/D ratios at all the time points during the first 4-week posttransplantation (H = 26.842, P < 0.001 for week 1; H = 20.875, P < 0.001 for week 2; H = 18.214, P < 0.001 for week 3; and H = 21.200, P < 0.001 for week 4, respectively).

**Table 3: Effects of donor CYP3A5 rs776746, C6 rs9200, and rs10052999 polymorphisms on tacrolimus concentration/dose ratios**

| Genotypes          | Week 1 |          | P     | Week 2 |          | P     |
|--------------------|--------|----------|-------|--------|----------|-------|
|                    | C/D ratio | U      |       | C/D ratio | U      |       |
| CYP3A5 rs776746    |         |        |       |         |        |       |
| GG (n = 33)        | 360.74 (364.92) | 12.202 | <0.001 | 144.22 (135.36) | 5.585 | 0.018 |
| GA+AA (n = 38)     | 210.82 (205.46) |        |       | 111.06 (109.80) |        |       |
| C6 rs9200          |         | 2.100   | 0.147 |         | 1.719   | 0.190 |
| AA (n = 36)        | 286.09 (250.90) |        |       | 137.84 (142.61) |        |       |
| GA+GG (n = 35)     | 227.19 (312.24) |        |       | 120.86 (111.06) |        |       |
| C6 rs10052999      |         | 3.398   | 0.065 |         | 6.388   | 0.011 |
| CC+TT (n = 41)     | 226.55 (237.69) |        |       | 107.39 (87.00) |        |       |
| TC (n = 30)        | 317.84 (318.74) |        |       | 167.59 (153.47) |        |       |
| CYP3A5 rs776746    |         |        |       |         |        |       |
| GG (n = 33)        | 148.31 (91.33) | 13.104 | <0.001 | 143.96 (174.98) | 11.645 | 0.001 |
| GA+AA (n = 38)     | 77.49 (90.17) |        |       | 85.60 (70.91) |        |       |
| C6 rs9200          |         | 0.460   | 0.497 |         | 0.331   | 0.565 |
| AA (n = 36)        | 121.02 (99.98) |        |       | 129.35 (140.12) |        |       |
| GA+GG (n = 35)     | 104.66 (120.89) |        |       | 120.60 (99.78) |        |       |
| C6 rs10052999      |         | 9.086   | 0.003 |         | 11.856  | 0.001 |
| CC+TT (n = 41)     | 86.21 (78.04) |        |       | 90.15 (66.34) |        |       |
| TC (n = 30)        | 157.99 (113.41) |        |       | 155.36 (194.37) |        |       |

The data are shown as median (IQR). C/D: Concentration/dose; IQR: Interquartile range.

**Figure 1:** Combined effect of four related genotypes on tacrolimus concentration/dose ratios in various groups during the first 4-week posttransplantation (n = 20 in Group 1, n = 46 in Group 2, and n = 5 in Group 3).

**Metabolism factors associated with tacrolimus concentration/dose ratios in the multiple linear regression analysis**

To have a better understanding about the associations of these genotypes with tacrolimus metabolism during early...
postoperative period, stepwise multiple linear regression analysis was performed. To avoid unnecessary errors from patient characteristics, age and gender were also considered in the statistical analysis. Notably, final regression models were established to predict the C/D ratios of tacrolimus during the first 4-week posttransplantation [Table 4], recipient C6 rs9200 group (AA vs. GG/GA) was found to be related to tacrolimus metabolism at weeks 1, 2, and 3 (P < 0.05), whereas donor C6 rs10052999 group (CC/TT vs. TC) was demonstrated to be correlated with tacrolimus metabolism only at week 4.

**DISCUSSION**

Drug resistance is a potentially fatal challenge post-OLT for HCC. Pharmacogenomics provides effective methods to investigate the mechanisms underlying interactive gene networks. An understanding of these interactions may contribute to the development of individualized drug therapy. The risk of early organ rejection is the highest immediately posttransplantation. Tacrolimus concentrations during the 1st week after transplantation determine the extent of rejection. Therefore, the identification of key predictors of tacrolimus concentrations in the early stage after liver transplantation is clinically valuable. Pharmacogenetic dose algorithms for tacrolimus based on genotypes and clinical variables may facilitate the development of stable therapeutic doses. In the current study, researchers investigated the associations of CYP3A5, interleukin (IL)-6, IL-10, and IL-18 genetic polymorphisms with tacrolimus metabolism. Based on our findings, we propose the existence of a gene interaction network that affects tacrolimus pharmacogenomics in patients with HCC after OLT.

Tacrolimus is extensively metabolized by the CYP system. CYP3A5 rs776746 allele A noncarriers produce a truncated, nonfunctional CYP3A5 enzyme due to a splicing defect; therefore, these noncarrier patients metabolize tacrolimus more slowly than carriers. In the current study, the CYP3A5 rs776746 genotype in donors altered tacrolimus C/D ratios during the 4-week posttransplantation, which was consistent with earlier studies. In contrast, in this study, the recipient CYP3A5 rs776746 genotype was found to influence tacrolimus levels only at weeks 1, which was consistent with a recent meta-analysis and systematic review. In the final combination analysis, an additional Kruskal-Wallis test including CYP3A5 polymorphisms alone revealed significantly lower tacrolimus C/D ratios in patients with rapid-metabolizing genotypes at 4-week posttransplantation (P < 0.001, P = 0.004, P = 0.007, and P = 0.001, respectively, data not shown). Thus, we hypothesized that CYP3A5 rs776746 polymorphisms play a key role in tacrolimus pharmacokinetics although donor CYP3A5 polymorphisms have a more significant influence. A limited number of clinical markers have been associated with tacrolimus pharmacokinetics. However, these markers or genetic polymorphisms do not account for all the observed variations. Interestingly, in this study, variations in tacrolimus C/D ratios among individual patients were partially correlated with C6 polymorphisms.

Recipients of liver grafts are known to be at increased risk of ischemia-reperfusion injury (IRI), immunological injury, and drug toxicity during the posttransplantation period. The complement system includes a series of complex biochemical and immunological pathways that eliminate foreign components from an individual. Complement mediates inflammation and injury after ischemia. Complement inhibition, therefore, is a potential therapeutic strategy to reducing IRI. Wu et al. reported that C6-deficiency inhibits a late step in complement activation resulting in delayed xenograft rejection. Furthermore, C6-deficiency in rats ameliorates IRI. Activation of the complement system triggers apoptosis and opsonization. Wu et al. reported that C6-deficiency inhibits a late step in complement activation triggering cytokine production and hepatocyte proliferation. Therefore, a potential therapeutic strategy to reducing IRI. Wu et al. reported that C6-deficiency inhibits a late step in complement activation resulting in delayed xenograft rejection. Furthermore, C6-deficiency in rats ameliorates IRI. Activation of the complement system triggers apoptosis and opsonization. Thus, the MAC can induce transplant rejection and also accelerates the restoration of liver function.

The complement system is involved in several cytolytic activities. Surface expression of complement regulatory proteins is reduced in liver allografts during the first 4-week posttransplantation. The risk of early organ rejection is the highest immediately posttransplantation. Tacrolimus concentrations during the 1st week after transplantation determine the extent of rejection. Therefore, the identification of key predictors of tacrolimus concentrations in the early stage after liver transplantation is clinically valuable. Pharmacogenetic dose algorithms for tacrolimus based on genotypes and clinical variables may facilitate the development of stable therapeutic doses. In the current study, researchers investigated the associations of CYP3A5, interleukin (IL)-6, IL-10, and IL-18 genetic polymorphisms with tacrolimus metabolism. Based on our findings, we propose the existence of a gene interaction network that affects tacrolimus pharmacogenomics in patients with HCC after OLT.

**Table 4: Metabolism factors associated with tacrolimus concentration/dose ratios in the multiple linear regression analysis**

| Factors | Unstandardized coefficients | Standardized coefficients | P | Determination coefficients |
|--------|-----------------------------|---------------------------|---|---------------------------|
| Week 1 | Donor CYP3A5 rs776746 (1 = GG, 2 = GA/AA) | −188.848 | −0.380 | <0.001 | 0.385 |
| | Recipient CYP3A5 rs776746 (1 = GG, 2 = GA/AA) | −171.655 | −0.346 | 0.001 | 0.126 |
| | Recipient C6 rs9200 (1 = AA, 2 = GA/GG) | −142.139 | −0.285 | 0.005 | 0.057 |
| Week 2 | Recipient C6 rs9200 (1 = AA, 2 = GA/GG) | −60.012 | −0.239 | 0.045 | 0.027 |
| | Donor CYP3A5 rs776746 (1 = GG, 2 = GA/AA) | −78.841 | −0.249 | 0.031 | 0.057 |
| | Recipient C6 rs9200 (1 = AA, 2 = GA/GG) | −78.604 | −0.247 | 0.033 | 0.027 |
| Week 3 | Donor C6 rs10052999 (1 = CC/TT, 2 = TC) | 79.145 | 0.383 | 0.001 | 0.223 |
| | Donor CYP3A5 rs77674 (1 = GG, 2 = GA/AA) | −49.929 | −0.243 | 0.027 | 0.126 |
proteins (CRP) prevents cytolysis of cancer cells.\cite{20} As well, cancers such as HCC overexpress CRPs including CD46 and CD59.\cite{21} Increased CD46 expression in HCC represents an early step in disease progression.\cite{22} Interestingly, upregulation of CD46, which is the cellular receptor for adenovirus type 35, may enhance the antitumor efficacy of oncolytic adenoviruses in HCC cells.\cite{23} Tumor cells resist complement-mediated death through different mechanisms.\cite{24,25} Specifically, reduction of C6 and C7 expression levels in tumor tissues have been reported to enhance the capacity of tumors to escape complement-dependent cytotoxicity.\cite{26} Otherwise, the MAC assembly by C6 and other complement components\cite{27} induces cell lysis by generating pores in the cell membrane. Several human neoplasias are associated with genes located on chromosome 5\cite{34-36} and genes for C6 and C7 are closely linked on 5p13.\cite{37} In this study, the recipient C6 rs9200 allele G and donor C6 rs10052999 homozygote were linked with rapid tacrolimus metabolism after OLT in patients with HCC. Hence, C6 may play a decisive role in hepatocyte function in patients with HCC undergoing OLT.

Complement mediates hepatic injury and regeneration, as well as cancer immune surveillance. Antibody and complement play a key role in allograft rejection. Although the underlying mechanisms remain to be elucidated, complement activation products, including C6, play a critical role in inflammatory reactions, allograft rejection, and IRI. The results of this study indicated that both donor and recipient C6 might be closely related to tacrolimus metabolism after OLT. Liver is the primary site of C6 synthesis. In rats, extrahepatic synthesis of C6 has been reported during transplantation of livers from C6-deficient donors to normal recipients.\cite{37-39} Therefore, according to the results of this study, we speculated that extrahepatic sites of C6 biosynthesis play a key role in tacrolimus metabolism immediately after OLT, and C6 biosynthesis in hepatocytes is restored after liver function recovery. Evidence suggested that reduced complement activity resulted from genetic variations in C6.\cite{40} Further, a CAAT/enhancer binding protein site in the C6 promoter has been found to be essential for normal C6 expression.\cite{41} Thus, C6 SNPs can regulate C6 synthesis.

In this study, C6 and CYP3A5 polymorphisms were further analyzed in stepwise multiple linear regression analysis. As shown in Table 4, the determination coefficients were 0.385, 0.057, 0.126, and 0.223 for the C/D ratios, respectively. It indicated that those regression models were bringing a discontent explanatory power, which just showed that amounts of genetic polymorphisms accounting for the C/D ratios were not in the models, and the individual differences of tacrolimus metabolism could be related to others. As such, studies in larger cohorts of patients with some more related genetic polymorphisms are required in the future.

The limitations of this study were as follows: First, the relatively small sample size comprising Chinese Han population precluded generalization of the results to different ethnic populations. Second, the complement system is a complex network, and the relationships between C6 gene polymorphisms, C6 expression, and MAC activity require validation in further studies involving larger liver transplant cohorts of patients with HCC.

In conclusion, patients with OLT for HCC require different therapeutic regimens compared with patients with etiologically distinct liver disease. Specifically, this study found that recipient C6 rs9200 allele G was associated with rapid tacrolimus metabolism during the first 3 weeks after transplantation, and donor C6 rs10052999 homozygote represented a marker for rapid tacrolimus metabolism at weeks 2, 3, and 4 after OLT. Further, this study confirmed the association of CYP3A5 rs776746 SNPs with tacrolimus C/D ratios. The combination of C6 and CYP3A5 polymorphisms exerted a greater effect on tacrolimus metabolism than individual SNPs and developed better equations that described the association between genotype and tacrolimus metabolism. The findings facilitated the design of appropriate and safe therapeutic regimens for the management of patients with OLT for HCC.

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Conflicts of interest
There are no conflicts of interest.

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