Role of the \textit{UGT2B17} deletion in exemestane pharmacogenetics

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Exemestane (EXE) is an aromatase inhibitor used for the prevention and treatment of breast cancer. The major metabolic pathway for EXE is reduction to form the active 17β-dihydro-EXE (17β-DHE) and subsequent glucuronidation to 17β-hydroxy-EXE-17-O-β-D-glucuronide (17β-DHE-Gluc) by UGT2B17. The aim of the present study was to determine the effects of UGT2B17 copy number variation on the levels of urinary and plasma 17β-DHE-Gluc and 17β-DHE in patients taking EXE. Ninety-six post-menopausal Caucasian breast cancer patients with ER+ breast tumors taking 25 mg EXE daily were recruited into this study. UGT2B17 copy number was determined by a real-time PCR copy number variant assay and the levels of EXE, 17β-DHE and 17β-DHE-Gluc were quantified by UPLC/MS in patients’ urine and plasma. A 39-fold decrease (\(P < 0.0001\)) in the levels of creatinine-adjusted urinary 17β-DHE-Gluc was observed among UGT2B17 (\(*2/*2\)) subjects vs subjects with the UGT2B17 (\(*1/*1\)) genotype. The plasma levels of 17β-DHE-Gluc was decreased 29-fold (\(P < 0.0001\)) in subjects with the UGT2B17 (\(*2/*2\)) genotype vs subjects with UGT2B17 (\(*1/*1\)) genotype. The levels of plasma EXE-adjusted 17β-DHE was 28% higher (\(P = 0.04\)) in subjects with the UGT2B17 (\(*2/*2\)) genotype vs subjects with the UGT2B17 (\(*1/*1\)) genotype. These data indicate that UGT2B17 is the major enzyme responsible for 17β-DHE-Gluc formation \textit{in vivo} and that the UGT2B17 copy number variant may play a role in inter-individual variability in 17β-DHE levels \textit{in vivo}.

\textbf{INTRODUCTION}

Breast cancer is the most frequently diagnosed cancer (excluding cancers of the skin) and second leading cause of cancer death in women, with a lifetime risk for breast cancer of 12.4\%\textsuperscript{1}. An estimated 246,660 new cases of invasive breast cancer are expected to be diagnosed among women in the USA in 2016\textsuperscript{2}. Approximately 75\% of all breast cancers express estrogen receptors (ERs)\textsuperscript{3}. Treatment for early-stage ER+ breast cancer in post-menopausal women has primarily been focused on elimination of growth stimulation caused by estrogens. Aromatase inhibitors (AIs) act to abolish aromatase activity, blocking estrone/estradiol biosynthesis and preventing the growth stimulation that estrogens induce\textsuperscript{4,4}. Clinical trials have shown that the use of AIs increases disease-free survival and decreases the occurrence of contralateral breast cancer as compared to the use of selective estrogen receptor modulators like tamoxifen alone\textsuperscript{5,7,10}. Although AIs represent an improvement in breast cancer treatment, considerable inter-individual variability exists in patient response and in the manifestation of toxicity to these drugs\textsuperscript{11–14}. The causes of this variability have not been elucidated.

Exemestane (EXE), a steroidal substrate analog, is a third generation AI that acts through a mechanism of suicide inhibition to inhibit the target aromatase enzyme, and is widely used for the adjuvant treatment and prevention of breast cancer in post-menopausal women as well as in the metastatic setting\textsuperscript{15–18}. After absorption, EXE is distributed extensively into tissues with 90\% bound to plasma proteins\textsuperscript{19}. It is known that EXE is extensively metabolized by reduction of the 17-keto group by aldo–keto reductases and cytochrome P450s to form an active metabolite, 17β-dihydroexemestane (17β-DHE), and subsequent addition of glucuronic acid (Gluc) to 17β-DHE to form 17β-DHE-glucuronide (17β-DHE-Gluc)\textsuperscript{20–26}. EXE is excreted roughly equally in the urine and feces, with the unchanged drug in urine < 1\% of the dose\textsuperscript{19}.

The UDP glucuronosyltransferase (UGT) superfamily of enzymes catalyzes the glucuronidation of various compounds, including endogenous compounds as well as xenobiotics. Previous \textit{in vitro} study have strongly implicated UGT2B17 as the major enzyme responsible for the glucuronidation of 17β-DHE.\textsuperscript{23} A polymorphic whole-gene deletion of the UGT2B17 gene has been identified with an allelic prevalence of ~ 30\% in Caucasians\textsuperscript{27–30} and this copy number variant (CNV) was associated with decreased formation of 17β-DHE-Gluc in human liver microsomes (HLMs) \textit{in vitro}\textsuperscript{24}.

The goal of the present study was to examine the effect of the UGT2B17 CNV on 17β-DHE-Gluc formation and 17β-DHE levels \textit{in vivo}, by examining the levels of EXE, 17β-DHE, and 17β-DHE-Gluc in 96 post-menopausal women with breast cancer taking EXE.

\textbf{MATERIALS AND METHODS}

Chemicals and materials

EXE and creatinine were purchased from Sigma-Aldrich (St Louis, MO, USA). 17β-hydroxy (OH)-EXE-17-O-β-D-Glucuronide (17β-DHE-Gluc), 17β-OH-EXE-D3-17-O-β-D-Glucuronide (D3-17β-DHE-Gluc), 17β-OH-EXE-D3 (D3-17β-DHE), EXE-19-D3 (D3-EXE), and D3-
creatinine were purchased from Toronto Research Chemicals (North York, ON, Canada). 17β-DHE was synthesized as previously described to a purity of > 99%.25 Ammonium formate was obtained from Sigma-Aldrich while ammonium acetate and formic acid were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Acetonitrile was purchased from Merck (Kenilworth, NJ, USA). Solvent and buffer modifiers for liquid chromatography-mass spectrometry (LC/MS) analysis including acetonitrile, ammonium formate, ammonium acetate and formic acid were all LC/MS grade. Milli-Q water was used for making any solutions when needed. PureLink Genomic DNA Kits were purchased from Thermo Fisher Scientific, and the TaqMan Copy Number Reference Assay (RNase P, Human) and the UGT2B17 TaqMan Copy Number Assay were purchased from Life Technologies (Carlsbad, CA, USA).

Subjects and samples

Ninety-six post-menopausal Caucasian breast cancer patients (age range: 35–89 years) with ER+ breast tumors taking 25 mg EXE daily (orally) and one healthy volunteer not taking EXE (used as a control) were recruited from the breast oncology clinic at the Penn State Hershey Cancer Institute into this study. All recruited subjects provided blood (10 cc) and urine (up to 50 ml). Patients were excluded from the study if they had been given EXE concurrently with adjuvant chemotherapy or if they were taking other adjuvant endocrine therapies, or were on chronic corticosteroid or megestrol acetate therapies. Approval was obtained from the Institutional Review Board at Penn State University with informed consent obtained from all subjects and with all specimens being de-identified. Specimens were obtained 4–6 h after last pill ingestion by a trained nurse coordinator after patients had been taking EXE for at least 28 days. Pretreatment medical histories including a comprehensive list of current medications and results of physical and laboratory examinations were also collected for each subject. Blood was separated by differential centrifugation and buffy coat was used to extract genomic DNA. Aliquotted urine samples and plasma fractions of blood samples were stored at −80 °C until analysis.

Sample preparation

Genomic DNA was purified from blood samples using PureLink Genomic DNA Kits. DNA quantity and purity were determined photometrically at 260 and 280 nm using the Thermo Scientific Nanodrop 2000 spectrophotometer (Waltham, MA, USA).

For EXE metabolite analysis, a 50-μl aliquot of each urine sample was first spiked with 10 μl of a mixture of deuterium-labeled internal standards in methanol, including D2-EXE (0.17 μM), D2-17β-DHE (1.7 μM) and D4-17β-DHE-Gluc (1.1 μM). Ninety μl of methanol was then added to extract EXE and its metabolites. After vortexing and subsequent centrifugation at 16 100 g for 10 min at 4 °C, an aliquot of 50 μl of supernatant was transferred to a sample vial for analysis by ultra-pressure liquid chromatography (UPLC)/mass spectrometry (MS).

For analysis of plasma, 10 μl of each plasma sample was first mixed with 10 μl of a mixture of deuterium-labeled internal standards as described above. Eighty microliters of methanol was then added to precipitate proteins. After vortexing and subsequent centrifugation at 16 100 g for 10 min at 4 °C, an aliquot of 50 μl of supernatant was transferred to a sample vial for analysis by UPLC/MS.

UPLC/MS conditions

For the simultaneous analysis of EXE, 17β-DHE and 17β-DHE-Gluc in urine and plasma, samples prepared as described above were analyzed using a UPLC/MS system (Waters), consisting of an Acquity UPLC pump, an Acquity sample manager-FTN, an ACQUITY UPLC BEH column C18 (2.1 × 100 mm, 1.7 μm particle size), and a XEVO G2-S QTOF mass spectrometer. UPLC was performed at a flow rate of 0.4 ml/min with solvent A (5 mM ammonium formate and 0.01% formic acid in water) and solvent B (100% acetonitrile) using the following conditions for both urine and plasma specimens: 1 min at 35% solvent B, a linear gradient to 52% solvent B for 0.5 min, 2.5 min at 52% solvent B, a liner gradient to 100% solvent B for 0.5 min, followed by re-equilibrium with 35% solvent B for 1.5 min. The injection volume of each prepared urine and plasma sample was 2 μl. The column temperature was 35 °C. The retention times were 2.96 min for EXE, 2.66 min for 17β-DHE and 0.99 min for 17β-DHE-Gluc (Supplementary Figure 1). Corresponding peaks from the urine or plasma matched well with the internal standards. No EXE, 17β-DHE or 17β-DHE-Gluc peaks were observed in the urine or plasma specimens from the subject not treated with EXE.

The Waters XEVO G2-S QTOF MS was equipped with an electrospray ionization (ESI) probe operated in the positive-ion mode, with capillary voltage at 0.6 kV. Nitrogen was used as both the cone and desolvation gases with flow rates maintained at 50 and 800 l h−1, respectively. Ultra-pure argon was used as the collision gas with a flow rate of 0.11 l h−1. The source and desolvation gas temperatures were 120 and 500 °C, respectively, with the mass spectrometer was operated in the MS/MS mode. The dwell time for each ion was 100 ms. The ion-related parameters for the 6 transitions monitored are listed in Table 1. The detection limits at which the signal to noise ratio was ⩾ 3:1 for EXE, DHE and DHE-Gluc were 1.0, 0.6 and 0.5 nM, respectively, in plasma, and 0.3, 1.0 and 5.0 nM, respectively, in urine. The limit of quantification at which the signal to noise ratio was ⩾ 10:1 for EXE, DHE and DHE-Gluc were 3.1, 1.8 and 5.0 nM, respectively, in plasma, and 0.9, 3.0 and 15.0 nM, respectively, in urine.

Standard curves were constructed by plotting the ratio of analyte peak area to peak area of the corresponding internal standard (described above) versus concentration of analyte standard. The concentrations of stock standards were 1000 ppm, which were 3, 4, 8, and 16 μM in methanol for EXE, 17β-DHE and 17β-DHE-Gluc, respectively. A serial dilution of standards at concentrations ranging from 0.4 nM to 3.4 μM, 0.4 nM to 3.4 μM and 0.25 nM to 2.1 μM were used to establish standard curves for EXE, 17β-DHE and 17β-DHE-Gluc, respectively. Analyte concentrations were determined by measuring the peak area ratios of analyte to internal standard and then calculating analyte concentration from the appropriate standard curve using Waters’ TargetLynx software (Milford, MA, USA).

Table 1. MS/MS transitions and ion optic parameters for EXE and EXE metabolites

| ES+MS/MS transition (m/z) | Cone voltage (V) | Collision energy (eV) |
|--------------------------|------------------|-----------------------|
| 17β-DHE-Gluc 475.23 > 281.19 | 20 | 15 |
| D2-17β-DHE-Gluc 478.25 > 284.21 | 20 | 15 |
| 17β-DHE 299.20 > 299.20 | 25 | 10 |
| D2-17β-DHE 302.22 > 302.22 | 25 | 10 |
| EXE 297.19 > 297.19 | 25 | 10 |
| D2-EXE 300.20 > 300.20 | 25 | 10 |

Abbreviations: EXE, exemestane; 17β-DHE, 17β-dihydro-EXE; 17β-DHE-Gluc, 17β-hydroxy-EXE-17-O-β-D-glucuronide.
Determination of urinary creatinine

Urine samples were first diluted 1000-fold with water. A 10 μl aliquot of each diluted urine sample was then spiked with 10 μl of internal standard (0.1 ppm D3-creatinine) before injecting 1 μl onto an ACQUITY UPLC HSS T3 column (2.1 x 100 mm, 1.8 μm particle size). UPLC was performed at a flow rate of 0.4 ml min⁻¹ using the following conditions: 100% solvent A (5 mM ammonium acetate) for 1 min, a linear gradient to 100% solvent B (100% acetonitrile) for 2 min, 100% B for 1 min, followed by re-equilibration for 1 min with 100% solvent A. The mass spectrometer was operated in positive MS scan mode with cone voltage at 20 V, other MS operation condition were the same as described above for EXE and its metabolites. Standards at concentrations ranging from 0.002 to 2 pmol were used to establish standard curves for quantification of urinary creatinine levels.

Genotyping of UGT2B17

The UGT2B17 CNV was determined by real-time PCR using the UGT2B17 TaqMan Copy Number Assay and the TaqMan Copy Number Reference Assay (RNase P, human). All real-time PCR analyses were performed in the Washington State University- Spokane Genomics Core Facility using a Bio-Rad CFX384 real-time PCR machine (Hercules, CA, USA).

Statistical analysis

Statistical analyses were performed using Prism (version 6, Graph Pad Software, San Diego, CA, USA). The Pearson correlation test was used to test the association between the levels of EXE and its metabolites in both urine and plasma. The Student’s t-test (or the Mann–Whitney rank test when data were not normally distributed) and the trend test were used to compare levels of EXE, 17β-DHE and 17β-DHE-Gluc in patients stratified by UGT2B17 copy number genotypes. Hardy–Weinberg equilibrium was used to test the genotype distribution.

RESULTS

EXE and its two major metabolites, 17β-DHE and 17β-DHE-Gluc, were separated by UPLC-MS as described in the Materials and methods section. The levels of EXE and its two major metabolites, 17β-DHE and 17β-DHE-Gluc, were determined simultaneously in individual urine and plasma specimens from 96 subjects taking EXE, and also in one control subject who did not take EXE. At least one of these metabolites were detected in all 96 plasma specimens and in 92 of the urine specimens (EXE and its metabolites were not detected in 4 urine specimens; these specimens were not incorporated into the genotype:phenotype analysis described below). Among the subjects taking EXE, the mean concentrations of EXE, 17β-DHE and 17β-DHE-Gluc were 23.6 and 46 nmol, respectively, in plasma (n = 96 specimens), and 161.9 and 1048 nmol, respectively, in urine (92 specimens; Table 2).

UGT2B17 genotypes were obtained for all 96 EXE-treated patients tested. In addition to the 35 subjects (37%) who exhibited the UGT2B17 (+1/+1) genotype (two wild-type UGT2B17 alleles), one subject (1.0%) exhibited three copies of the UGT2B17 gene, suggesting that this individual contained one wild-type UGT2B17 allele and one allele (termed the *3 allele) that contained two CNV genotypes versus creatinine-adjusted 17β-DHE-Gluc concentrations in 96 plasma and 92 urine specimens from EXE-treated subjects

**Table 2.** Summary of EXE, 17β-DHE and 17β-DHE-Gluc concentrations in 96 plasma and 92 urine specimens from EXE-treated subjects

|                         | Plasma (nmol/mg) | Urine (nmol/mg) | Urine, creatinine-adjusted (nmol/mg) |
|-------------------------|------------------|-----------------|-------------------------------------|
|                         | Mean (95% CI)    | Range           | Mean (95% CI)                       | Range                       | Mean (95% CI)                       | Range                       |
| EXE                     | 23 (17–29)       | 0–157           | 161 (101–221)                      | 0.24–1709                   | 0.41 (0.24–0.57)                   | 0.001–4.0                    |
| 17β-DHE                 | 6.0 (5.1–6.9)    | 0.03–30         | 9.1 (6.1–12)                      | 0–86                       | 0.022 (0.014–0.029)                | 0–0.21                      |
| 17β-DHE-Gluc           | 46 (31–60)       | 0–461           | 1048 (525–1572)                  | 0.48–22562                  | 1.8 (1.0–2.7)                      | 0.001–34                     |

Abbreviations: CI, confidence interval; EXE, exemestane; 17β-DHE, 17β-dihydro-EXE; 17β-DHE-Gluc, 17β-hydroxy-EXE-17-O-β-D-glucuronide.

Figure 1. Association between the UGT2B17 CNV and urinary creatinine-adjusted 17β-DHE-Gluc, 17β-DHE and EXE levels in EXE-treated subjects. Subjects were stratified by UGT2B17 CNV genotype, with the UGT2B17*1 allele corresponding to the wild-type single-gene copy number and the UGT2B17*2 allele corresponding to the UGT2B17 gene deletion variant. (a) UGT2B17 CNV genotypes versus creatinine-adjusted 17β-DHE-Gluc (nmol/mg creatinine); (b), UGT2B17 CNV genotypes versus creatinine-adjusted 17β-DHE; and (c), UGT2B17 CNV genotypes versus creatinine-adjusted EXE. All values are expressed as the median for 91 subjects.
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Figure 2. Association between the UGT2B17 CNV and plasma 17β-DHE-Gluc, 17β-DHE and EXE levels in EXE-treated subjects. Subjects were stratified by UGT2B17 CNV genotype, with the UGT2B17*1 allele corresponding to the wild-type single-gene copy number and the UGT2B17*2 allele corresponding to the UGT2B17 gene deletion variant. (a) UGT2B17 CNV genotypes versus 17β-DHE-Gluc; (b) UGT2B17 CNV genotypes versus 17β-DHE; and (c) UGT2B17 CNV genotypes versus EXE. All values are expressed as the median for 95 subjects.

DISCUSSION

Previous studies have demonstrated that 17β-DHE is the primary metabolite of EXE and that it exhibits an in vitro anti-aromatase activity similar to that observed for EXE. A major mode of detoxification of 17β-DHE is by glucuronidation, with UGT2B17 catalyzing greater than 95% of total 17β-DHE formation in HLM. In the present study, it was demonstrated that the common UGT2B17 gene deletion polymorphism is associated with large decreases in both urinary and plasma levels of 17β-DHE-Gluc, with the levels of 17β-DHE-Gluc reduced by 39- and 29-fold in urine and plasma, respectively, in subjects exhibiting the UGT2B17 (*2/*2) homozygous deletion genotype vs subjects exhibiting the (*1/*1) genotype. This is consistent with that observed previously in HLM in vitro. It is also consistent with that observed in a recent small pharmacokinetic study of healthy volunteers which demonstrated an ~8-fold difference in the AUC and Cmax of conjugated 17β-DHE between subjects with the UGT2B17 (*1/*1) vs (*2/*2) genotypes.21 Together, these studies are consistent with an important role for UGT2B17 in the glucuronidation of this active EXE metabolite.

The UGT2B17 deletion polymorphism was associated with a 28% increase in plasma 17β-DHE levels in the present study. This contrasts with that observed in a recent study where no difference in 17β-DHE levels were observed in non-β-glucuronidase-treated plasma.31 The difference in plasma 17β-DHE levels vs UGT2B17 genotype observed between studies is likely due to sample size or study design differences. The previous study sampled multiple time points from 14 healthy individuals after a single dose of EXE, while the current study examined samples from single time points from 96 breast cancer patients who daily doses of EXE for at least 4 weeks. In addition, the major toxicity associated with AIs including EXE is joint pain, bone loss, the propensity for bone fractures, and increased risk for osteoporosis. Other toxicities reported for AIs include a variety of gynecological events including vaginal bleeding, vaginal dryness or discharge and hot flashes. Variability in changes of lipid profiles, manifestation of osteoporosis, and time of recurrence, were observed in many patients in several AI clinical trials. EXE-induced toxicities are not universal, with only a fraction of patients reporting any one of them, and the UGT2B17 deletion polymorphism could be contributing to this inter-individual variability. Also, the UGT2B17*2 deletion allele frequency varies between different racial groups, with subjects exhibiting the homozygous (*2/*2) deletion genotype most common in the Asian population (for example, Korean, 67%; Chinese, 77%) as compared to that...
observed in Caucasians (for example, Swedish, 9%)\(^{36,37}\). Therefore, 17β-DHE glucuronidation may not be a major pathway for EXE elimination in Asians and this could potentially affect EXE clinical efficacies between racial groups.

While increased plasma 17β-DHE levels were associated with increasing numbers of the UGT2B17 deletion allele, the magnitude of this increase was less than the corresponding decrease in plasma 17β-DHE-Gluc levels (for example, 29-fold decrease for 17β-DHE-Gluc vs 1.3-fold for 17β-DHE, comparing UGT2B17 (*2/*2) subjects with UGT2B17 (*1/*1) subjects). This may be due to other excretion pathways also playing a role in EXE metabolism. Previous studies have shown that there was similar excretion of EXE in the urine vs bile, but it is unknown whether this was studied in subjects with wild-type UGT2B17.\(^{18}\) It is possible that EXE excretion patterns (urine vs bile) also differ between UGT2B17 (1/*1) vs (*2/*2) subjects. It is also possible that other 17β-DHE metabolites accumulate in UGT2B17-deleted subjects, including the inactive 17β-hydroxy-6-hydroxymethylandrost-1,4,6-trien-3-one, and its secondary metabolite, 60β, 17β-diiodohydroxy-6α/β-hydroxyxymethylandrost-1,4-dien-3-one, both of which were implicated as important metabolites of 17β-DHE.\(^{38}\) Studies investigating these possibilities are on-going.

The detection limits observed in the present study were between 0.5 and 1 nm for EXE, 17β-DHE and 17β-DHE-Gluc in plasma, 0.3 and 5 nm in urine. In addition, the quantification limits for EXE, DHE and DHE-Gluc were 3, 1.8 and 1.5 nm, respectively, in plasma, and 0.9, 3 and 15 nm, respectively, in urine. These limits are comparable to those observed in previous studies, where the lower limits of quantification were 0.2 and 0.1 ng ml\(^{-1}\) for EXE and 17β-DHE, respectively, in plasma and the lower limits of detection were 1 ng ml\(^{-1}\) for both in urine.\(^{39,40}\) Of the 96 subjects analyzed in the present study, all exhibited detectable EXE, 17ß-DHE or 17ß-DHE-Gluc in plasma with only 4 exhibiting levels that were below cation limits as performed in the present study for the detection of EXE, 17ß-DHE and 17ß-DHE-Gluc is highly sensitive.

While UGT2B17 is the major enzyme responsible for DHE glucuronidation, many other enzymes are in involved EXE metabolism. Cytosolic keto steroid reductases AKR1Cs and CBR1 are highly active in EXE reduction to DHE in vitro and several common variants in the cytosolic keto steroid reductases were associated with altered enzymatic activity in vitro.\(^{26}\) Cytochrome P450s 4A11 and 1A1/2 were also found to be responsible for formation of DHE, while CYP3A was active in EXE oxidation to form 6-hydroxymethylxestemastane.\(^{46}\) Therefore, in addition to the UGT2B17 deletion polymorphism, functional polymorphisms in these aldo–keto reductases and CYP450s may also play a role in the therapeutic efficacy or toxicity of EXE in vivo.

A limitation of the present study was that the urine and blood specimens were obtained from subjects taking EXE near the \(t_{\text{max}}\). Since previous pharmacokinetic studies demonstrated a \(t_{\text{max}}\) for EXE at 1–2 h post-dose,\(^{31,42}\) it is possible that metabolite concentrations may differ at the 4–6 hour time point that specimens were collected post-pill ingestion in the present study. Another potential limitation was that spot urine collections were performed in the present study. However, while 24-h urine collections are considered to be the most reliable type of urine sample,\(^{43}\) previous studies have also reported that a group average of many individual spot urine samples (as performed in the present study) also provides a good estimate of exposure.\(^{44}\)

In conclusion, the present study demonstrates that UGT2B17 is the major enzyme responsible for 17β-DHE-Gluc formation in vivo. It also showed that UGT2B17 genotypes are associated with altered 17β-DHE levels in the plasma of women taking EXE, and may therefore play a role in variability in EXE-induced toxicity as well as therapeutic efficacy between patients. Clinical trials examining this important possibility will be necessary to examine this directly.

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

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