Kafkas Univ Vet Fak Derg
24 (6): 853-858, 2018
DOI: 10.9775/kvfd.2018.20082

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://submit.vetdergikafkas.org

Research Article

Preliminary Study on Association of *EDNRB* Gene with Heterochromia Iridis in Cats *(Felis catus)* [1]

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[1] *The research funding was received from the Development and Promotion of Science and Technology Talents Project (DPST) and the Center of Excellence in Veterinary Bioscience, Chiang Mai University, Chiang Mai, Thailand*

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Article Code: KVFD-2018-20082 Received: 06.05.2018 Accepted: 05.09.2018 Published Online: 11.09.2018

How to Cite This Article

Chomdej S, Leelawattanakul P, Buddhachat K, Pradit W, Siengdee P, Phongroop K, Nganvongpanit K: Preliminary study on association of *EDNRB* gene with heterochromia iridis in cats *(Felis catus)*. *Kafkas Univ Vet Fak Derg,* 24 (6): 853-858, 2018. DOI: 10.9775/kvfd.2018.20082

Abstract

This study conducted an investigation on three exons of the endothelin receptor type B *(EDNRB)* gene of Thai odd-eyed cats to find out the association between the variations in the gene and heterochromia iridis. DNA sequencing analysis was performed on 11 odd-eyed cats compared to 11 normal-eyed cats. Seven variations were found across the studied region (XM_003980457.2: c.610A>G, c.820+40C>T, c.821-14C>T, c.916A>G, c.1025+36G>T, c.1025+69A>T, and c.1025+138C>T) with two of them (c.610A>G and c.916A>G) causing amino acid changes (P.Asn128Ser and P.Val230Ala). There was no statistical association between the variations near the three exons of *EDNRB* and feline heterochromia iridis (chi-square test, P-value >0.05).

Keywords: *EDNRB, Heterochromia iridis, Odd-eyed cat, SNPs, Sequencing*

Kedilerde *(Felis catus)* EDNRB Geni ile Heterokromia İridis Arasında İlişkiye Dair Ön Çalışma

Öz

Bu çalışmada Tayland tek-göz kedilerinde endotelin reseptör tip B *(EDNRB)* geninin üç ekzonunda gen varyasyonu ile heterokromia iridis arasında bir ilişki olup olmadığı araştırıldı. On bir tek-göz kedide DNA sekans analizi yapıldı ve 11 normal gözlü kedi ile karşılaştırıldı. Çalışılan bölge itibarıyla yedi varyasyon (XM_003980457.2: c.610A>G, c.820+40C>T, c.821-14C>T, c.916A>G, c.1025+36G>T, c.1025+69A>T ve c.1025+138C>T) tespit edildi ve bunların ikisi (c.610A>G ve c.916A>G) amino asit değişimine (P.Asn128Ser ve P.Val230Ala) neden olmaktaydı. Çalışmada, üç *EDNRB* ekzonu yakınındaki varyasyonlar ile kedi heterokromia iridis arasında istatistiksel bir ilişki tespit edilmedi (ki kare testi, P-değeri >0.05).

Anahtar sözcükler: *EDNRB, Heterokromia iridis, Tek-göz kedi, SNP, Sekanslama*

INTRODUCTION

Heterochromia iridis is a condition of difference in iris coloration, which occurs in animals. There are three types of heterochromia: complete heterochromia (completely different iris colors of the two eyes), sectoral heterochromia (difference in color in some parts of the iris), and central heterochromia (rings of spikes which have lighter or different color from the rest of the iris, radiating from the pupil). This difference in coloration is either acquired

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(by accident, disease, or some drugs) or congenital [1]. Congenital heterochromia iridis found in mammals is usually associated with mosaicism and chimerism as well as an involvement with white skin/fur and complete heterochromia in which the individual has one iris blue in color and the other iris having some other color. Moreover, there are several reports that abnormality or dysfunction of some genes can cause heterochromia iridis through Waardenburg syndrome (WS) and skin/deafness disease. Examples of such genes include endothelin receptor type B (*EDNRB*), microphthalmia-associated transcription factor (*MITF*), pair-box 3 (*PAX 3*), and sex determining region Y-box 10 (*SOX10*) [2-5].

EDNRB is a gene that synthesizes the receptor protein located on the cell surface called endothelin receptor type B (OMIM: 131244) which interacts with the endothelin protein. The mechanism regulates some critical biological processes involving the stimulation of cell growth and division of some structure or molecules such as blood vessels and hormones [6]. This receptor has an important role during fetal development, which involves determining cell differentiation for melanocytes and regulating melanin which is also involved in the normal function of inner ear [7]. Some studies have reported that defects in this gene can cause heterochromia iridis in humans. There was a case in which mutation of *EDNRB* had caused Waardenburg's syndrome type IV in Chinese population and caused blue/brown complete heterochromia iridis as well as deafness and white patches of hair (NM_000115.3: c.-121G>T; c.-26G>A; c.552T>C; c.831A>G) [3]. There also had a report of heterochromia iridis that have been observing in a Brazilian family that had a defect in the *EDNRB* gene [8]. Moreover, homozygous mutation in the same region of *EDNRB* has been reported that can cause heterochromia and white hair , which are symptoms of the ABCD syndrome (WS type IV), without hearing impairment in a family's children[9]. These suggested that the dysfunction of *EDNRB* may relate to heterochromia iridis or odd-eye trait in other animals as it does in human.

Heterochromia iridis can be found in cats as well, usually as blue-yellow or blue-green irises with mostly with white coat. These cats are called odd-eyed cats, of which Turkish Van, Turkish Angora, Sphynx, Persian, Oriental Shorthair, Japanese Bobtail, and Khao Manee are the examples [10]. These cats have several conditions similar to those in other animals affected by dysfunction of genes that mentioned above, particularly the Waardenburg syndrome in humans and cattle [2,3,5,11]. Thus, the study of these genes should facilitate cat breeding for improving chances of the heterochromia trait together with decreasing or preventing chances of hearing impairment that could come with the trait. The objective was to investigate the association between feline *EDNRB* gene and heterochromia iridis in Thai domestic cats.

MATERIAL and METHODS

Collection of Samples

Phenotypic data and blood samples were obtained from 11 cats having normal eye color (male=8, female=3) and 11 white, heterochromia cats (male=5, female=6) *(Fig. 1)*. Blood samples from cats were collected in blood collecting tubes coated with EDTA as anticoagulant. Among the cats used in this study, there were three kittens born in the same liter in which two of them had heterochromia iridis while the other did not *(Fig. 2)*. The Ethics Committee of the Faculty of Veterinary Medicine, Chiang Mai University, Thailand, approved this study in 2016 (S35/2559).

DNA Extraction, Polymerase Chain Reaction (PCR), and Sequencing

Each blood sample (200 µL) was used for obtaining the genomic DNA. DNA extraction method was performed by phenol-chloroform extraction, which adapted from Taş (1990) [12]. The quality and quantity of the DNA were verified by 1% agarose gel electrophoresis and UV absorbance at 260 and 280 nm.

Three exons of feline *EDNRB* (exon 1, 2, and 3) were amplified by polymerase chain reaction (PCR) using two pairs of primers designed from feline genome (Abyssinian

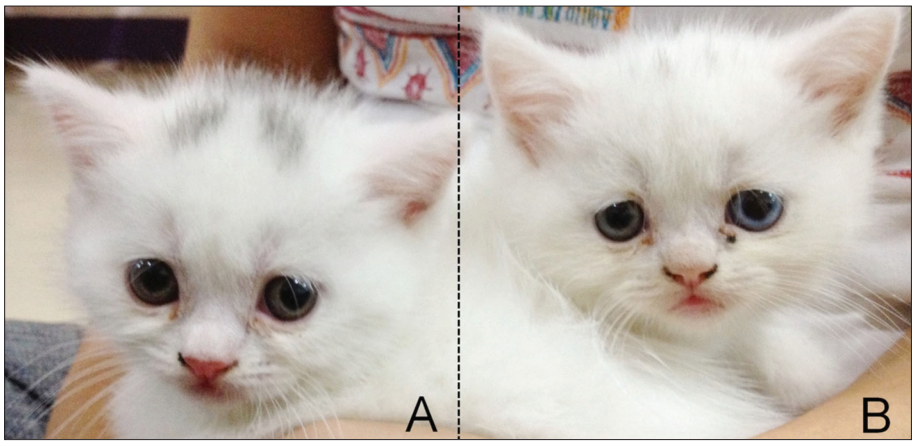

Fig 1. Representative of cats with normal eye color (A) and cats with heterochromia eyes (B)

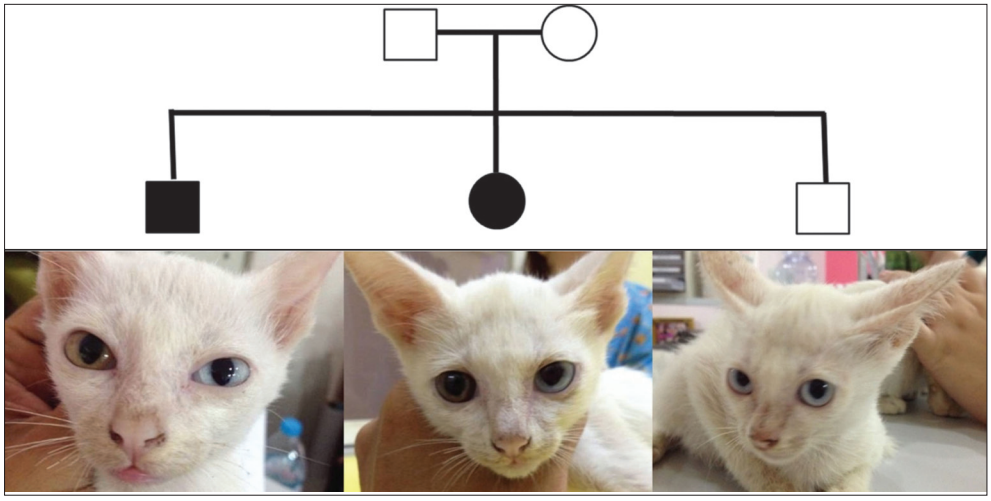

Fig 2. Three kittens born in the same litter: two of them had heterochromia iridis, while the other did not

Table 1. *Primers used in this study*

Region	Primer Sequence (5′→3′)	Product Size (bp)
EDNRB exon 1	Fw: TCCTAACTAGGCACCCTCCC	595
	Rw: CAGTCTTTCTTCCCTGCGGT	
EDNRB exon 2 and 3	Fw: TGGCAGTCCTTATGGAGGAGA	801
	Rw: AGGGCACCGTGTGAAAATCT	

cat, GenBank: GCF_000181335.2) by using BLAST PRIMER (http://blast.ncbi.nlm.nih.gov) *(Table 1)*. The location of studied region was chosen based on the orthologous loci of human *EDNRB* gene (GenBank: NM_000115.3), reported by Jiang et al.[3]. The PCR reaction contained 20 ng genomic DNA; 1X reaction buffer (RBC Bioscience, Taiwan); 0.25 mM dNTP (Vivantis Technologies, Malaysia); 0.25 µM primers (BioDesign, Thailand); 1 U *Taq* DNA polymerase (RBC Bioscience, Taiwan); and deionized distilled water with a total volume of 25 µL. The PCR was performed in Major Cycler, CYCLER-25 thermal cycler (Major Science, USA), with the following cycling profile: 95°C for 5 min; 45 cycles of 95°C for 30 sec, 62°C for 30 sec, and 72°C for 30 sec; and a final extension at 72°C for 10 min. The PCR products were detected by gel electrophoresis using 2% agarose gel as medium and GelRed (Biotium, USA) as visualizer. The amplified products were then scanned for variations by Sanger sequencing (1st Base, Malaysia). The secondary structures of the EDNRB polypeptide with/without mutations were predicted by using the SPIDER3 program (http://sparks-lab.org/server/SPIDER3).

Analysis of Association

The sequencing data were compared between the normal eye group and the heterochromia iridis group using *Felis catus* genome database version 8.0 on GenBank as the reference (ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/181/335/GCA_000181335.3_Felis_catus_8.0). The range of locus that was searched for variation/mutation included 200 bp of intron regions adjacent to exon. The collected data were analyzed for correlation by using chi-square test.

RESULTS

Compared to reference data from brown coat Abyssinian cat genome (GenBank: GCF_000181335.2), analysis of the DNA sequences on exon 1, exon 3, and adjacent introns of *EDNRB* showed that there were two variations with amino acid changes found on odd-eyed cats and five variations found on both normal-eyed and odd-eyed cats *(Fig. 3)*. They were c.610A>G, c.916A>G, c.820+40C>T, c.821-14C>T, c.1025+36G>T, c.1025+69A>T, and c.1025+138C>T, respectively (GenBank: XM_003980457.2). Various allele frequencies were calculated, and they are shown in *Table 2*. On c.610, a heterozygous mutation (A/G) was found on one of the odd-eyed cats. The substituted base G changed amino acid from asparagine to serine (P.Asn128Ser). On c.916, one of the odd-eyed cats was found to have base transition from A to G, which caused amino acid to change from valine to alanine (P.Val230Ala), while there was no variation in the normal-eyed cats, which made allele frequency to be 1.0A (and 0.91A/0.9G for heterochromia cats). On c.820+40, the base substitution between C and T (reference allele=T) was found to be sporadic among normal-eyed cats and odd-eyed cats, and heterozygous genotype (C/T) was found in three heterochromia cats. On c.821-14, heterozygous genotype C/T was found in one normal cat and homozygous genotype T/T was found in one heterochromia cat, while the majority of genotype was C/C. On c.1025+36, however, the majority

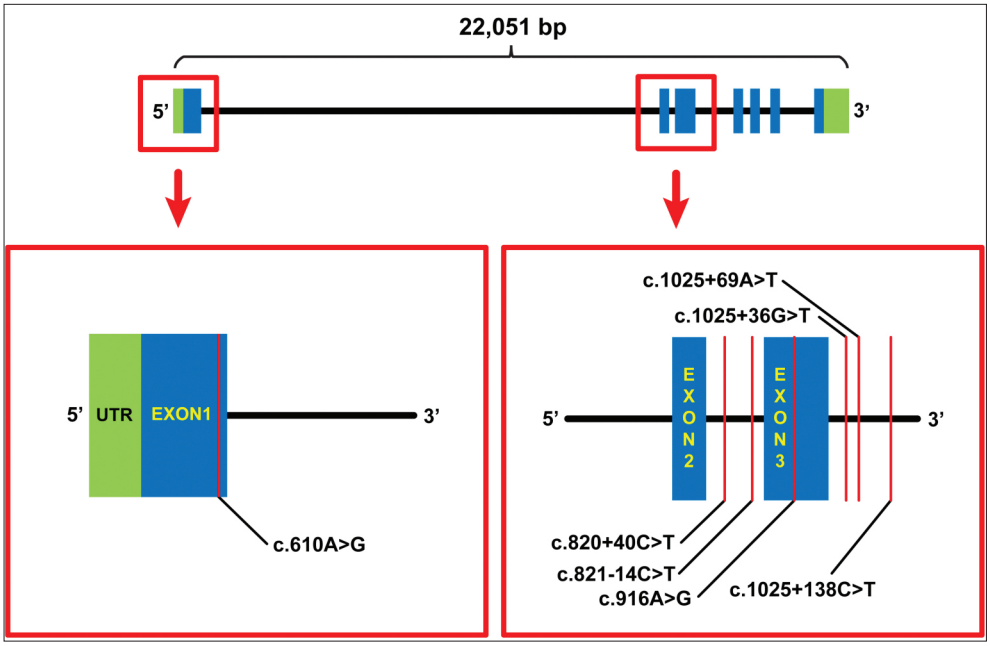

Fig 3. The seven variations found on exon 1, 3, and adjacent intron of the *EDNRB* gene (Feline *EDNRB*, GenBank: XM_003980457.2)

Table 2. *Allele frequencies and Chi-square results*

Locus	Allele Frequency				Chi-square* (*P*-value)	Association (*P*≤0.05)
	Normal-eyed Cats		Odd-eyed Cats			
	Allele 1	Allele 2	Allele 3	Allele 4		
c.610	1.00A	0.00G	0.95A	0.05G	0.311748	no
c.820+40	0.55C	0.45T	0.59C	0.41T	0.760858	no
c.821-14	0.95C	0.05T	0.91C	0.09T	0.549773	no
c.916	1.00A	0.00G	0.91A	0.09G	0.147759	no
c.1025+36	0.73G	0.27T	0.82G	0.18T	0.471847	no
c.1025+69	0.91T	0.09A	0.91T	0.09A	1.00	no
c.1025+138	0.91C	0.91T	0.91C	0.91T	1.00	no

*df=1

of allele was G instead of T compared to reference data. Moreover, it was found that two odd-eyed cats had heterozygous G/T genotype on this locus, while there was no heterozygous genotype in normal-eyed cats. The variations found on c.1025+69 and c.1025+138 were same, with allele frequencies of 0.09A/0.91T for both normal-eyed cats and heterochromia cats, and 0.91C/0.09T for both normal-eyed cats and heterochromia cats, respectively. In addition, there were two samples of odd-eyed cats, which had heterozygous genotypes on both loci (A/T on c.1025+69 and C/T on c.1025+138, respectively). When analyzed for association using chi-square test, it was found that there was no association between those seven variant loci and heterochromia iridis in cats *(Table 2)*. Furthermore, the predicted secondary structures of three possible mutated amino acid sequences (EDNRB polypeptides with Asn128Ser: NS, Val230Ala: VA, and Asn128Ser-Val230Ala: MT) were slightly different from those in wild type: WT *(Fig. 4)*.

DISCUSSION

Since the heterochromia iridis involves with the embryonic development of neural crest, a transient, multipotent, migratory cell population unique to vertebrates that gives rise to a diverse cell lineage such as craniofacial cartilage and bone, smooth muscle cells, peripheral and enteric neurons, glial cells and melanocytes [13], a crucial factor in the development of skin/fur and eye color. The cascade of gene expressions in neural crest cells depends on the stage of neurulation as well. Several genes that link to heterochromia iridis were previously reported such as *EDNRB*, *PAX3*, *SOX10*, and *MITF* [2,3]. Paired box gene 3 (*PAX3*) is a gene that encoding one of the transcription factors called neural plate border specifier, which mediates influence of induction signals (Wnts, bone morphogenic proteins: BMPs, and fibroblast growth factors: FGFs expressions) and regulates a group of genes called neural crest specifiers that turns embryo cells into neural crest

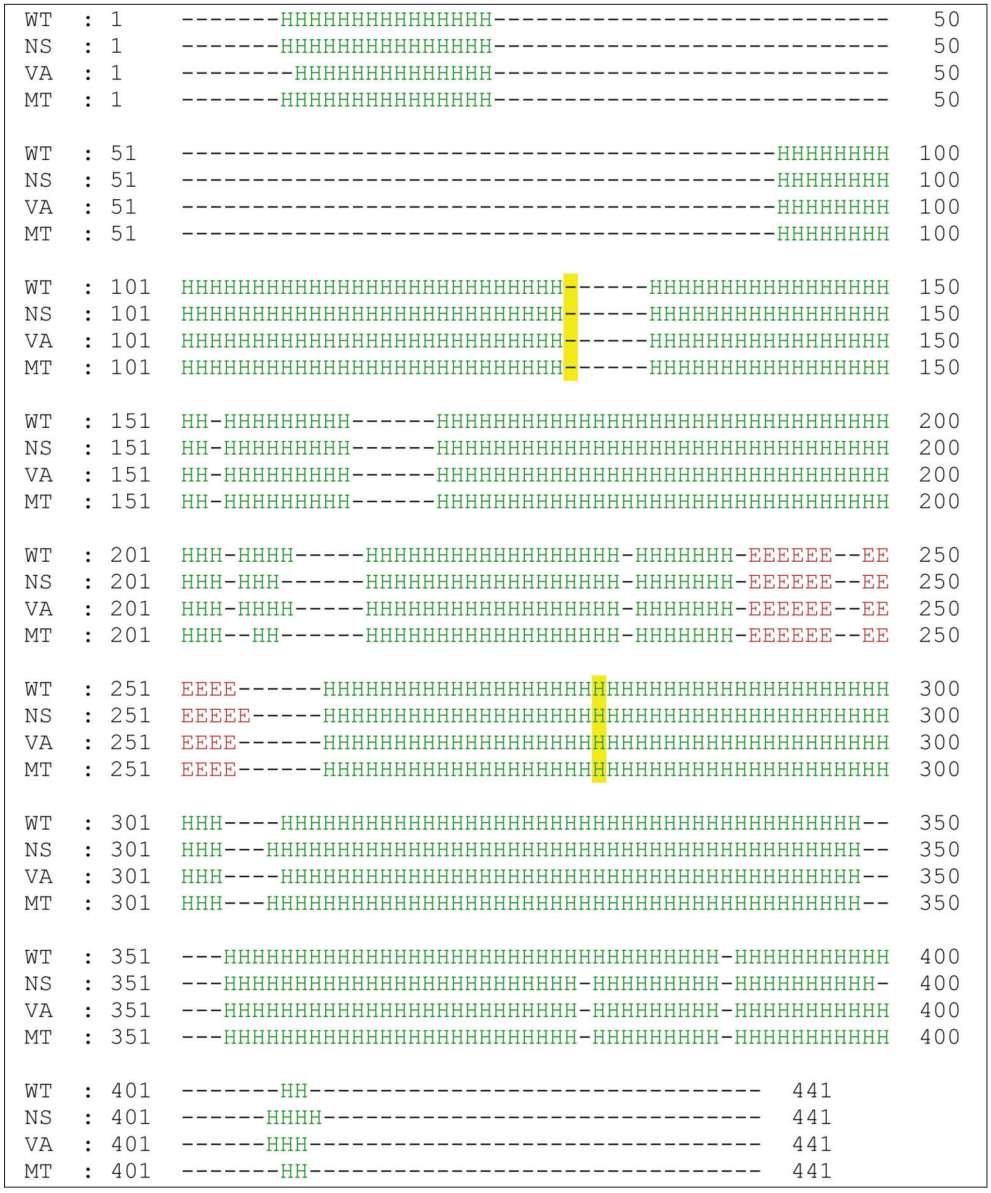

Fig 4. Comparison of the secondary structures of three types of possible mutated EDNRB proteins (NS, VA, and MT) with that of the wild type (WT); H, helix motif; E, strand motif. The yellow highlight indicates the location of the amino acid change (mutation)

cells. SOX10, one of neural crest specifiers, then may turn on most of neural crest effector genes including *MITF*, which is involved in the differentiation of melanocytes, by signaling to *EDNRB*, the receptor protein located on the cell membrane [14]. Hence, as one from many genes involving neural crest development of *EDNRB* gene, two variations (c.610A>G and c.916A>G) found only in heterochromia cats were not statistically related with heterochromia iridis. Moreover, for familial DNA sequence comparison, there was no difference between a white blue-eyed cat and its odd-eyed siblings, suggesting that *EDNRB* may not be the cause of their special eye colors, which is in contrast with other studies [3,8,9,15] that found the mutation at the same location (exon 1 and 3).

Interestingly, the amino acid changes in two exons may be associated with the change in protein properties and conformation due to changes inthe secondary structure of *EDNRB* protein *(Fig. 4)*. Although asparagine and serine are amino acids with uncharged polar side chains, the difference between asparagine and serine is that asparagine has one additional carbon chain with amine group at the end. Valine and alanine are also in the same circumstance: they are amino acids with hydrophobic side chains. Valine is larger than alanine by two methyl groups [16]. With regard to sizes of amino acids, the difference in sizes of the changed amino acids may result in change in protein structure and conformation, which would lead to loss of function of the endothelin receptor protein [17].

In conclusion, the following can be stated: exon 1, 2, and 3 of *EDNRB* were scanned in domestic cats and seven variations were found; two of them were found only in odd-eyed cats and had amino acid changes, but they had no significant relation to heterochromia iridis. Comparison of the DNA sequences between the two odd-eyed cats and their normal-eyed siblings showed no differences in nucleotide sequences as well. The findings of this study

was the first investigation of relationship between three exons of *EDNRB* and heterochromia iridis in odd-eyed cats. To find out genes or DNA regions which involving heterochromia iridis in cats, more samples and candidate genes such *PAX3*, *SOX10*, and *MITF* should be done including with using other novel approaches.

Conflict of Interest

The authors declare that there have no conflict of interests.

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