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MiR-135b-5p affected malignant behaviors of ovarian cancer cells by targeting KDM5B

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Abstract. – OBJECTIVE: The aim of this study was to investigate the potential effect of microRNA-135b-5p (miR-135b-5p) on the development of ovarian cancer (OC) and to explore the relevant mechanism.

PATIENTS AND METHODS: The expression of miR-135b-5p in OC tissues and cells was detected by quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR). MicroRNA online prediction websites were used to screen the potential targets of miR-135b-5p. Subsequently, luciferase reporter gene assay and Western blot (WB) were performed further confirmation. In addition, the effects of miR-135b-5p on cell function were analyzed by relevant experiments *in vitro*.

RESULTS: MiR-135b-5p was lowly expressed both OC tissues and cell lines. Combined with online prediction software, luciferase reporter gene assay and WB, KDM5B was predicted and verified as a downstream target gene of miR-135b-5p. Down-regulating the expression of KDM5B by over-expressing miR-135b-5p could effectively control the proliferation and apoptosis of OC cells. Cell proliferation was significantly reduced, while cell apoptosis was promoted after miR-135b-5p transfection in OC cells.

CONCLUSIONS: By targeting KDM5B, miR-135b-5p exerted an excellent anti-cancer effect in OC cells. Our findings indicated that miR-135b-5p/KDM5B might become a feasible and new target of OC treatment.

Key words:
MicroRNA-135b-5p (miR-135b-5p), Ovarian cancer (OC), KDM5B, Proliferation, Apoptosis.

Introduction

Ovarian cancer (OC) is the fifth most common cause of cancer death among females. Currently, the morbidity rate of OC is second only to that of cervical cancer and endometrial cancer among female reproductive tract malignant tumors. The latent invasion of OC makes it difficult to be detected in early stage. Moreover, there are few effective screening tools and early detection methods for OC. Recent studies have demonstrated that the five-year survival rate of OC is still low, which seriously threatens women's health.

MicroRNAs (miRNAs) are a class of small single-stranded endogenous and conserved non-coding RNAs (ncRNAs) with 19-25 nucleotide length. In 1993, miRNAs were initially found in Caenorhabditis elegans by Lee et al[2]. MiRNAs are characterized by high sequence conservation, time-ordered expression, and tissue specificity. In the early 21st century, numerous researches have indicated that multiple miRNAs exist in different organisms and they play important roles in the metabolism of cells. Typical miRNAs can interact with specific messenger RNAs (mRNAs) by complementing base pairing, eventually influencing target gene translation or stability. Furthermore, miRNAs have been verified to affecting vital activities of cancer cells[3,4]. Changes in the expression patterns of miRNAs have been detected in many diseases[5-8], including malignant tumors[9-11].

MicroRNA-135b-5p (miR-135b-5p) is an important component of the miRNA regulatory network, wich exerts critical regulatory effects on many diseases[12-14]. However, the effects of miR-135b-5p on OC development has not been fully elucidated yet. Therefore, the aim of this study was to discover the role and possible mechanism of miR-135b-5p in OC.

Patients and Methods

OC Cases and Cell Lines

A total of 83 paired OC tissues and para-normal ovarian tissues were collected in Gansu

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Provincial Hospital from March 2016 to October 2018. Chemotherapy and radiotherapy were forbidden before the operation. Collected tissue samples were immediately preserved in liquid nitrogen after surgical resection. This investigation was approved by the Ethics Committee of Gansu Provincial Hospital. Signed written informed consents were obtained from all participants before the study.

Human ovarian serous papillary cystadenocarcinoma cell line (SKOV3) and normal human ovarian surface epithelial cell line (HOSEpiC) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). All cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; HyClone, South Logan, UT, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin in a cell incubator at 37°C with 5% CO_2.

Cell Transfection

SKOV3 cells in the logarithmic phase were first seeded into 6-well culture plates at a density of 2×10^5/well. Up to 50-70% of fusio[n] transfection was performed according to structions of Lipofectamine™ 2000 (Invi[tro]n, Carlsbad, CA, USA). Three groups were e[sta]lished in this study, including miR-NC gr[oup] (negative control), miR-135b-5p [gro]up (SKOV cells transfected with miR-13[5b-5p] ics), and KDM5P group (SKOV3 c[ells] trans[fec]ted with miR-135b-5p mimics and [pc]DM5P

Quantitative Reve[rse] Transcription-Po[lym]erase C[hain] Reaction (qRT[-PCR]) Analysis

Cells in di[ffer]ups were c[oll]ected at 24 h after tra[nsf]ection[.] RNA in cells was extracted [us]ing TRIzol [reag]ent (Invitrogen, Carlsbad, A, USA). After [det]ermination of optical [den]sity (O[D]), electrophoresis and reverse tran[scr]iption [were] carried out. Subsequently, reve[rse] tion-p[oly]erase chain reaction (RT-PC[R] perfo[rme]d, with complementary ribos[omal] d (cDNA) as the template. U6 used internal reference. Relative exp[res]ion of mR[NA] was calculated by the $2^{-\Delta\Delta Ct}$ m[eth]od. sequences used in this study [we]re as [fol]ows: miR-135b-5p, F: 5'-GGCAGGGTAACCGTCCTTTCTC-3', R: 5'-CGCATATGTGTCGGACAGGA-3'; U6: F: 5'-CTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'.

Luciferase Reporter Gene Assay

The potential targets of miR-135b-5[p] were searched in TargetScan, miRDB, and websites. KDM5B, also known [as] ARID1[B] was screened as the target gene [of] miR-135b-5p. The binding sequence of mi[R-135b-]5p at the 3'-end of KDM5B was m[ut]ated [by] point mutation kit. When cell d[ensi]y reach[ed] i[n] 24-well plates, they we[re] ansfected with type KDM5B 3'UTR [pm]L3-K[DM5]B-WT) and mutant-type KDM[5B] UTR [pm]L3-KDM5B-MT), respectivel[y] te[d] th pla[smid] NC and over-expre[ssed] miR-[135b-]5p pla[smid]. After transfection [for] 48 h, fluor[escence] ivity was determined [acc]ding to the in[struc]tions of the Dual-Lu[ciferase] ter assay kit (Promega, Madison, WI, USA).

Western Blot (WB) Analysis

[To]tal protein was first extracted in tissues and s. Protein c[once]ntration was determined by bicinchonini[c a]cid (BCA) method (Pierce, R[ockford], IL, [USA]). Subsequently, extracted protein [were] separated by electrophoresis (25 μg [to]protein in each well) and transferred [to] membranes. After sealing with 5% skim milk [ov]ernight, the membranes were incubated [wi]th p[rim]ary antibodies for 2 h and corresponding secondary antibody for 2 h. Then, the membranes were washed again. Immunoreactive bands were developed *via* enhanced chemiluminescence (ECL) using a gel imaging analysis system. The ratio of the measured value of target proteins and β-actin was calculated as the relative expression.

Cell Proliferation

Transfected cells in different groups were gathered, digested, dispersed, and counted. The cells were inoculated into 96-well plates (100 μL/well) after the concentration was adjusted to 1×10^5/mL. Methyl thiazolyl tetrazolium (MTT; Sigma-Aldrich, St. Louis, MO, USA) was added to each well at different time points (24 h and 72 h), respectively, followed by inoculation for 4 h in the dark. OD value at 490 nm was determined.

Colony Formation Assay

Cells in the logarithmic phase were collected to prepare the single-cell suspension. Then, the cells were inoculated into 6-well culture plates (3 duplicates in each group), with about 1×10^3 cells per well. The culture plates were shaken slightly to achieve uniform distribution of cells. Subsequently, the cells were continuously cultured in

the incubator until the observation of visible colonies. After discarding the culture medium, the cells were washed with phosphate-buffered saline (PBS) for 3 times, fixed with 2 mL of methyl alcohol for 15 min, and stained with 2 mL of crystal violet dye for 15 min. After air drying, formed colonies in each well were photographed. Finally, the number of colonies was counted.

Cell Apoptosis

Cells were first inoculated into 6-well culture plates (containing the cover glass treated with polylysine). 48 h after transfection, the cells were fixed with 4% paraformaldehyde for 25 min. Then, they were stained in accordance with the instructions of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) and developed *via* diaminobenzidine (DAB; Solarbio, Beijing, China). Finally, counterstaining, dehydration, permeabilization, mounting, and observation were performed.

Statistical Analysis

Prism 6.02 software (La Jolla, CA, USA) was used for all statistical analysis. Statistical analysis was performed with Student's *t*-test or *F*-test. All *p*-values were two-sided, and $p<0.05$ was considered significant.

Results

MiR-135b-5p Expression Was Significantly Reduced in Both Tissues and Cells

The expression level of miR-135b-5p in both OC tissues and cells was detected by qRT-PCR. The results demonstrated that miR-135b-5p was lowly expressed in both OC tissues and cells compared to normal controls (Figure 1).

The Relationship Between KDM5B With MiR-135b-5p

Target genes of miR-135b-5p were predicted using online prediction software. KDM5B contained a matching-sites with miR-135b-5p on positions 1494-1500 (Figure 2A), indicating that KDM5B might be the potential target of miR-135b-5p.

Transfection efficiency was confirmed by qRT-PCR. Thanks to this, we confirmed the effects of miR-135b mimics transfection up-regulating the expression of miR-135b-5p in cells (Figure 2B), which was a prerequisite for *in vitro* experiments.

Luciferase reporter gene assay indicated that miR-135b-5p could significantly inhibit the activity of luciferase in cells transfected with pGL3-KDM5B-WT. However, it had no effect on luciferase activity in cells transfected with pGL3-KDM5B-MT (Figure 2C). The above findings prove the negative regulation of miR-135b-5p on KDM5B in OC cells.

MiR-135b-5p Decreased the Expression Level of KDM5B

Previous studies[3] have shown that the regulatory mechanism of miRNAs is to regulate the expression of target proteins at the post-transcriptional level. Therefore, we validated our pre-results by WB experiment. We found that the protein expression of KDM5B was significantly inhibited by up-regulating the expression level of miR-135b-5p in SKOV3 cells (Figure 3A).

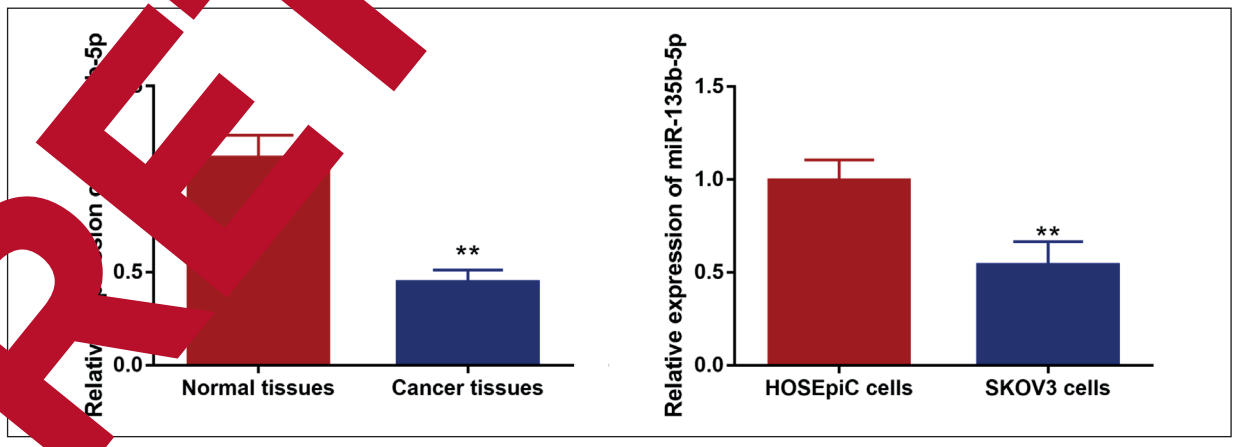

Figure 1. The expressions of miR-135b-5p in OC tissues and cell lines. (**$p< 0.01$).

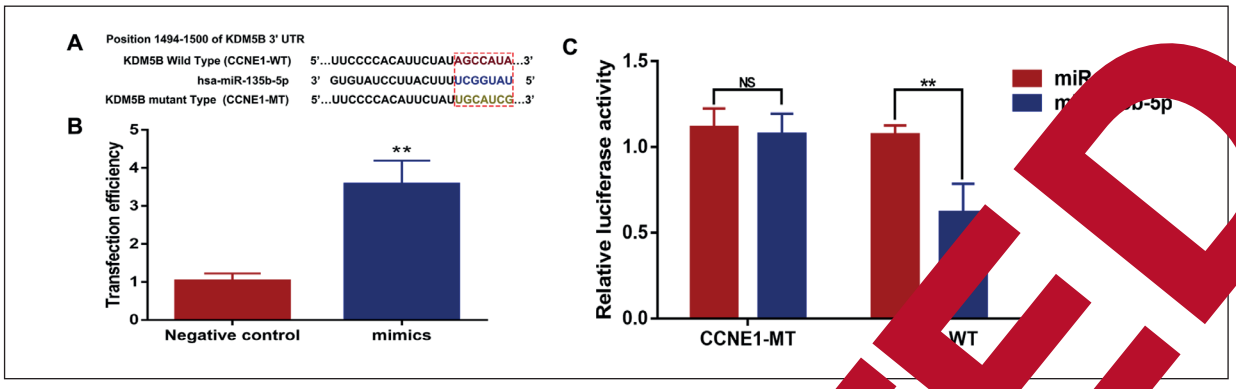

Figure 2. KDM5B was a direct and functional target of miR-135b-5p. **A,** Diagra putative mi ng sites of KDM5B. **B,** Transfection efficiency was determined by qRT-PCR. **C,** Relative a of luciferase re (**p<0.01).

MiR-135b-5p Suppressed Proliferation of OC Cells

MTT assay results showed that over-expression of miR-135b-5p could significantly suppress the proliferation capacity of SKOV3 cells. However, when we restored KDM5B to the level of control grou proliferation y of cells was also re his indicated th miR-135b-5p affect- he proliferative capacity by regulating the ression of K 5B in SKOV3 cells (Figure Moreover, t umber of formed colonies in ells trans d with miR-135b-5p mimics

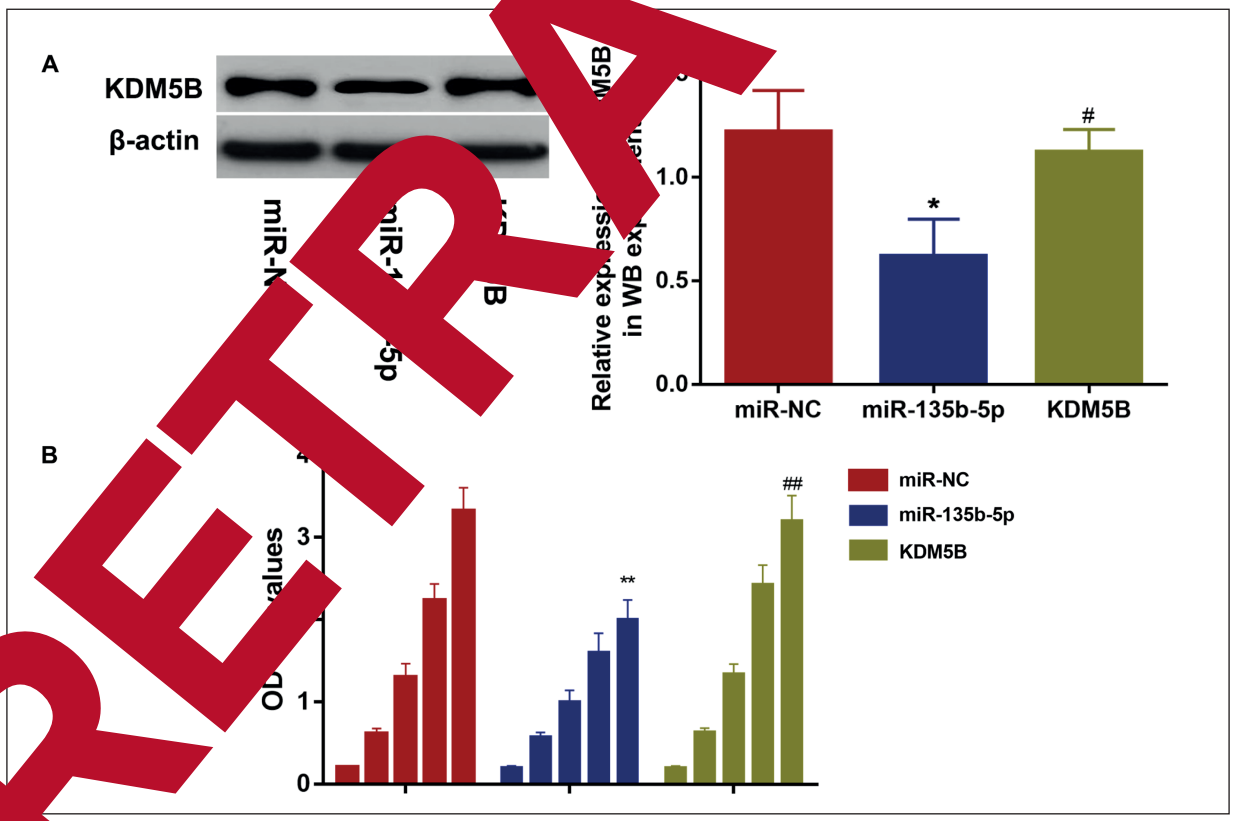

Figure 3. A. MiR-135b-5p decreased the protein expressions of KDM5B in OC cells. B. MiR-135b-5p suppressed the pro ion ability of OC cells. Data were presented as means ± standard deviations. (*p<0.05, **p<0.01 *vs.* miR-NC group; #p<0.05, ##p<0.05 *vs.* miR-135b-5p group).

was significantly more than that of other groups, with bigger sizes (Figure 4A).

MiR-135b-5p Promoted Apoptosis of OC Cells

TUNEL assay indicated a significant number of apoptosis in miR-135b-5p group. Similarly, up-regulation of KDM5B reversed the effects of miR-135b-5p on the apoptosis of SKOV3 cells (Figure 4B).

Discussion

OC is still one of the major diseases affecting the health of women worldwide, with up to 140,000 deaths every year. Currently, how to effectively detect and diagnose OC in early stages and to improve the effectiveness of treatment has become an urgent problem which needs to be solved.

The occurrence and development mechanism of tumors is complicated with various reasons. Uncontrolled proliferation of cells is one of the important features during the progression o- lignant tumors. Exploration of the mecha- imbalance between cell proliferation and a- o- sis can provide a theoretical basis for treatm- With further research and the understanding miRNAs in recent years, mult- research- have proved that miRNAs pl- roles in the proliferation, apoptosis, conduction, and other processes of tumor cells[15,16]. MiRNAs, as -gula- tors, are involved in tumorigenesis an- biological behaviors by regulatin- wnstrea- key genes and signal pathways- iRNAs can serve as new targets and mark- detecting, judging prognosis, and ass-ssing t- -cts of diagnosis and treatment in- [17-20].

In our research, 83- -ed OC tissu- para-cancer normal ti- -s were- -ected. Q- PCR assay was use- -nalyz- expre- -ion of miR-135b-5p. Th- res- that th- xpres- sion of miR-135- -p was- -icantl- -wn-reg- ulated in O- -ssues com- -n that in matched p- -r normal tis- Meanwhile, the expr- -n o- -135b-5p was detected in the cellular level a- The results indicated that th- -ression of m- -5b-5p was evidently lo- -C cells than tha- -f HOSEpiC cells.

-ngle miRNA can regulate over 200 target -es, and abo- -/3 protein-coding genes in -mals are r- -lated by miRNAs. Up till n- any fun- -s of miRNAs remain unclear. The- -o find out more miRNAs and their targ- genes has become a hot spot in recent -ars. To further detect the expression of genes -sitively and effectively, the luciferase -por- gene assay was used in this study to identify the targets of miRNAs.

Histone methylation is one of the most important modifications after the translation of

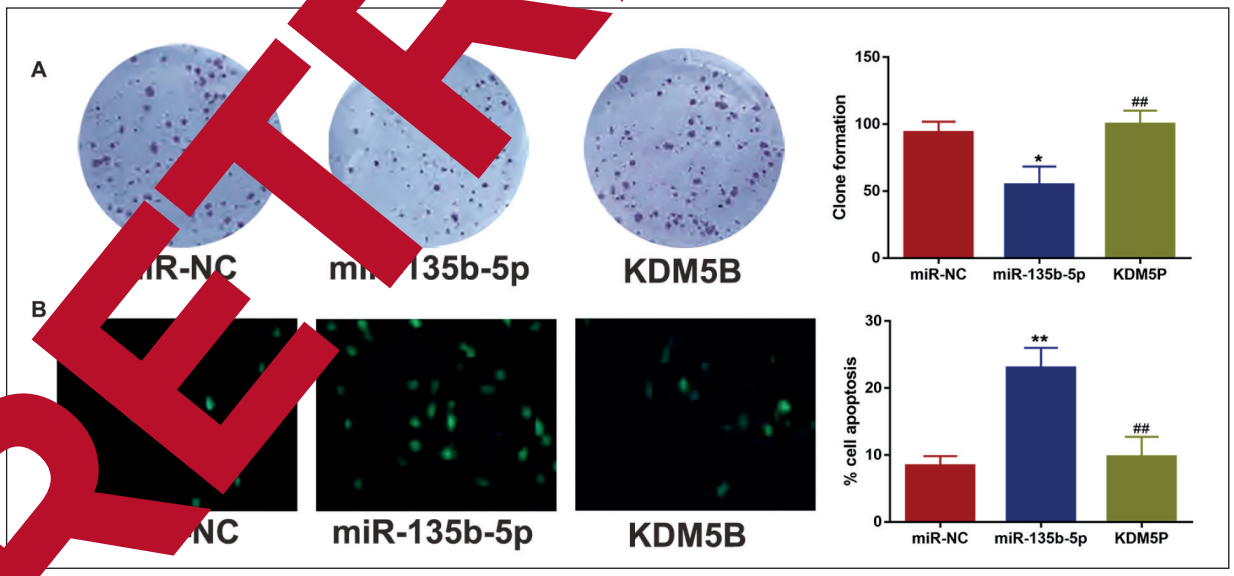

Figure 4. A, Assessment of colony formation (magnification × 40). **B,** Apoptosis level tested by TUNEL staining (mag- -ation × 40). Data were presented as means ± standard deviations. (*p<0.05, **p<0.01 *vs.* miR-NC group; ##p<0.05 *vs.* miR-135b-5p group).

nuclear chromatin. It has many influences on the functions of the cell nucleus, including epigenetic inheritance, transcriptional modification, and maintaining genomic integrity. Changes in the state of histone methylation are involved in many pathological and physiological processes[21,22]. KDM5B is a specific demethylase enzyme of histone H3 at Lys 4 (H3K4). Previous studies[22,23] have shown that it possesses the functions of inhibiting genetic transcription, regulating cell proliferation and embryonic development, etc. Meanwhile, its expression is up-regulated in various malignant tumor tissues, such as hepatoma carcinoma[24], breast cancer[25], and cervical cancer[26]. Up-regulated KDM5B contributes to the proliferation of tumor cells[27,28]. However, some reports[29] have suggested that KDM5B may act as a cancer suppressor gene in some tumors. Wang et al[30] have found that KDM5B expression level is significantly elevated in OC, serving as an important biomarker for poor prognosis and chemotherapy resistance of OC patients.

Combined with online prediction software, luciferase reporter gene assay and WB, the mutual binding relation between miR-135b-5 d KDM5B in OC gradually emerged. As stream target gene of miR-135b-5p, dow u-lating the expression of KDM5B by over-exp ing miR-135b-5p in SKOV3 cells could achi satisfactory results in controllin ll prolifer tion and apoptosis.

Conclu

For the first tim e highli the regulation of miR-135b OC cells. B ting to inhibit the exp KDM5B, mi 135b-5p exerted an e lent ncer effect in OC cells. Our dings indica at miR-135b-5p/KDM5B ght become a new et of OC treatment.

Conflic erest

thors th y have no conflict of interests.

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