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A Comparison Between Full-COLD PCR/HRM and PCR Sequencing for Detection of Mutations in Exon 9 of *PIK3CA* in Breast Cancer Patients

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Abstract

One of the most common somatic mutations in breast cancer is found in *PIK3CA* with a prevalence rate of 18–45%. Different variants of this gene are considered as resistance markers for treatment with HER2-targeted medicines. Conventional molecular methods such as Sanger sequencing are not able to detect mutations with low abundance in a mixture of wild-type DNA, especially in the early stages of cancer development. In this study, two methods of co-amplification at lower denaturation temperature PCR (COLD-PCR) and high-resolution melting (HRM) were combined for detection of mutations in exon 9 of *PIK3CA*; DNA, therefore, was extracted from MCF-7 and BT-474 as mutant and wild-type cell lines respectively. Thereafter, serial dilutions of extracted DNA were used to determine sensitivity of full-COLD PCR/HRM in comparison with conventional PCR sequencing as the gold standard method. Cell line experiments resulted in almost 30 fold increase in sensitivity by use of full-COLD PCR/HRM. In addition, 40 patients with primary breast cancer were investigated with the mentioned methods. As a result of this part of study, four mutations were detected by conventional PCR sequencing including E542K and E545K mutations in three and one samples respectively. Whereas, full-COLD PCR/HRM was able to detect one E542K mutation more than gold standard method which caused the percentage of sensitivity to get improved by 2.5% (10 to 12.5%). Our results clearly demonstrated that full-COLD PCR/HRM could detect lower levels of mutations in wild-type background as a sensitive method with simple and cost-effective procedure; therefore, it can prospectively be used in screening of patients with early-stage breast cancers.

Keywords Full-COLD PCR/HRM · Sanger sequencing · *PIK3CA* · Breast cancer

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Introduction

Breast cancer is one of the most common malignancies in the world, and also a leading cause of death among women [1]. In last decades, the incidence of breast cancer has been increased; thus, early diagnosis and screening should be considered more necessary [2]. It is estimated that about 5–10% of breast cancer cases are hereditary caused by familial transmission of mutations such as *BRCA1* and *BRCA2*. The remaining percentage (90–95%) are non-hereditary forms of breast cancer due to somatic mutations [3]. Coming from valuable studies, detection of somatic mutations is essential for individualized genotype-driven patient management [4, 5].

The most somatic mutations in patients with breast cancer occur in *PIK3CA* gene (*OMIM* no. 171834) with frequency of 18–45% [6]. *PIK3CA* is an oncogene encoding the catalytic subunit of phosphatidylinositol-3-kinases (PI3K) [7]. PI3K signaling pathway plays a key role in cell signaling, proliferation, differentiation, and cell survival; mutations in *PIK3CA*, thus, are involved in tumorigenic processes and can activate the PI3K/AKT/mTOR signaling pathway [8]. In addition to high frequency of *PIK3CA* mutations in breast cancer incidence, these mutations are associated with resistance to HER2-targeted medicines like trastuzumab; consequently, identification of drug resistance can improve effectiveness of therapeutic protocols [9–11]. According to the COSMIC (Catalogue of Somatic Mutations in Cancer) database, the most important mutations in this gene are located in exons 9 and 20. The variants of p.E542K c.1624G>A and p.E545K c.1633G>A as two hotspot mutations are located in exon 9 of *PIK3CA* [12].

Amplification by PCR (polymerase chain reaction) followed by Sanger sequencing has been selected as the gold standard method for mutation detection, while it can detect variants with frequencies above 10–20% [13]. Given the inadequate sensitivity of PCR sequencing for low-abundance mutations in dominate context of wild-type DNA, it is necessary to selectively enrich the mutant DNA [14, 15]. A fast and cost-effective screening method for mutation detection, high-resolution melting (HRM) is based on PCR product denaturation in the presence of a saturating fluorescent dye [16]. Amplicon size and composition, type of mutation, PCR quality, and the analytical equipment are determining factors for sensitivity of HRM. According to published studies, general sensitivity of HRM, as a non-enrichment screening method, is about 5–10% mutant alleles among wild-type DNA [12]. Low-abundance mutations barely can be determined by performing sequencing stages without using an enrichment method. Also most often, tumors have heterogeneity and mosaicism of mutations which increases the importance of precise enrichment method [17–19]. Amplification refractory mutation system (ARMS), as an enrichment method, detects only known variants [20]. Peptide nucleic acids (PNAs) and locked nucleic acids (LNAs) improve amplification of mutations through binding to wild-type alleles and preventing them to amplify [21, 22]. Despite the fact that PNA and LNA have the ability to enrich minority alleles presenting among wild-type DNA, their optimization is almost time and cost consuming [23].

Co-amplification at lower denaturation temperature PCR (COLD-PCR) is a novel PCR-based method in which low levels of somatic mutations can be selectively enriched within a wild-type DNA background regardless of the type or position of mutations [24, 25]. COLD-PCR is an accurate method which uses a critical denaturation temperature (Tc) that both known and unknown mutations can simultaneously be enriched with it. The optimized Tc is the temperature in that only WT-mutant heteroduplexes, caused by point mutations, or Tm (melting temperature) reducing mutations (G: C>A: T or G: C>T: A) can be optionally denatured. This optimized Tc must be precisely determined in temperatures lower than wild-type amplicon Tm; subsequently, this stage results in enrichment of mutations in amplification region [26–28].

In this study, sensitivity of COLD PCR/HRM, as a combination of both methods, was evaluated for detection of mutations in exon 9 of *PIK3CA*. Then, it was compared with limit of detection (LOD) of the conventional PCR sequencing in cell line DNA and also in DNA samples from primary breast cancer tissues.

Material and Methods

Cell Lines and DNA Extraction

MCF-7 and BT-474 breast cancer cell lines were purchased from IBRC (Iran Biological and Genetic Resource Center, Tehran, Iran). BT-474 as a wild-type cell line for *PIK3CA* mutations and MCF-7 cell line harboring E545K mutation in exon 9 of *PIK3CA*, as a representative for G>A mutations, were used in this study. Genomic DNA was extracted from cell lines using QIAamp DNA Mini Kit (Qiagen, Milan, Italy) and examined by direct sequencing in order to confirm the mutation. Extracted DNA from MCF-7 cell lines was serially diluted into non-mutated DNA from BT-474 cell lines. Twelve serial dilutions by different percentages of mutant DNA were prepared including 0%, 0.1%, 0.2%, 0.4%, 0.8%, 1.5%, 3.1%, 6.2%, 12.5%, 25%, 50%, and 100%.

Clinicopathological Data for Tissue Specimens

Tissue samples were collected from 40 Iranian women in primary stage of breast cancer. This study is based on local ethical rules and informed consent was obtained from each patient. Clinicopathological characteristics, estrogen receptor (ER), progesterone receptor (PR), and Her2 were evaluated by immunohistochemistry (IHC) for all the patients. The stage of tumor was assessed according to TNM classification, and the tumors were graded based on the Bloom–Richardson grading system [29]. Total DNA was extracted from snap-frozen tumor tissues in liquid nitrogen, using Qiagen DNeasy Blood & Tissue kit (Qiagen, Germany).

Determination of Critical Denaturation Temperature

After amplification of wild-type DNA by conventional PCR, HRM analysis was performed (ramping at 0.2 °C/s from 70 to 85 °C) and Tm of wild-type amplicon was measured. For determination of optimal Tc, several series of full-COLD PCR were performed to check different temperatures (lower than WT amplicon Tm 76.9 °C) decreasing from 76.5 to 74 °C. After the selection of 75.5 °C as the best Tc for heteroduplexes denaturation, full-COLD PCR procedure was performed.

PCR and COLD PCR/HRM

After optimization, the ideal process sequentially included 10 cycles of conventional PCR in which all amplicons were replicated equally, and then 35 cycles of full-COLD PCR which allowed enrichment of amplicons with G>A mutations. PCR and full-COLD PCR were performed, using primers that were designed for exon 9 in a way that amplification of chromosome 22 pseudogene be avoided. Primer sequences included 5′-TGAC AAAGAACAGCTCAAAGCA-3′ and 5′-AGCACTTACCTGTGACTCCA-3 for forward and reverse respectively which leaded to a 96-bp amplicon. For each reaction, 50 ng of DNA,

5 μl of 2× HRM Master Mix (Type-it HRM PCR Kit QIAGEN—Germany), 0.7 μM of each primer, and RNase-free water were used to reach the final volume of 10 μl. PCR was performed with an initial step of 95 °C for 5 min to activate HotStarTaq *plus* DNA polymerase, continued by 10 cycles of 95 °C for 10 s, 64 °C for 30 s, and 72 °C for 10 s. Afterwards, 35 cycles of 95 °C for 10 s, 70 °C for 420 s, 75.5 °C for 3 s as Tc, 64 °C for 30 s, 72 °C for 10 s, and 72 °C for 900 s was performed as the main COLD-PCR step. Then, HRM analysis was done in Corbett Research Rotor Gene-6000 (Qiagen—Germany), ramping at 0.2 °C/s from 70 to 85 °C in the presence of saturating levels of the EvaGreen fluorescent dye as intercalating agent for double-stranded DNA (ds DNA). Q 5plex HRM software was used to generate fluorescent differential curve plots, and also to compare each obtained curve with wild-type.

Sequence Analysis

Two primers including 5′-CATCTGTGAATCCAGAGGGGA-3′ as forward primer and 5′-AGCACTTACCTGTGACTCCA-3′ as reverse primer were designed and synthetized in order to use in dideoxy sequencing (BigDye Terminator). Each PCR master mix included 2 μM of each primer, 1 μl mix dNTP, 1.5 μl MgCl2, 5 μl of 10× PCR buffer, (5 U/μl) AmpliTaq Gold DNA polymerase, 3 μl of DNA sample, and double-distilled water to reach the total volume of 50 μl. PCR condition contained initial denaturation at 95 °C for 5 min and 40 cycles of 95 °C for 30 s, 64 °C for 30 s, and 72 °C for 45 s, and then the final step of 72 °C for 5 min as final extension was performed. After purification of PCR products using QIAquik PCR purification kit (Qiagen—Germany), cycle sequencing was performed by BigDye Terminator Kit (Applied Biosystems—USA) according to expressed method by Sanger F et al. [30]. And fragment sequence reads were analyzed using Applied Biosystems ABI 3730 (Chromas software version 2.33).

Results

Cell Line Experiments

In order to evaluate the sensitivity of full-COLD PCR/HRM theoretically, we prepared serial dilutions of DNA from MCF-7 and BT-474 respectively for mutant and WT DNA of *PIK3CA* exon 9. The ratios of mutant to WT DNA in serial dilutions were 100, 50, 25, 12.5, 6.2, 3.1, 1.5, 0.8, 0.4, 0.2, 0.1, and 0%. After amplification of all samples by conventional PCR and full-COLD PCR, sensitivity was determined using HRM analysis and sequencing. Based on our results, conventional PCR coupled with HRM leads to detection of a minimum of 3.1% mutant DNA (Fig. 1, right graph), which then followed by sequencing that was able to detect 12.5% mutant DNA (Fig. 2, left chromatograms). On the other hand, full-COLD PCR/HRM was able to discover 0.4% of mutated DNA among WT (Fig. 1, left graph); all concentrations, thereafter, were assessed by sequencing for mutation, which leads to detection of 6.2% mutant DNA minimally (Fig. 2, right chromatograms).

Tumor Tissue Experiments

For further examination, full-COLD PCR/HRM was performed to be compared with two common methods of conventional PCR sequencing and HRM/PCR. Therefore, extracted

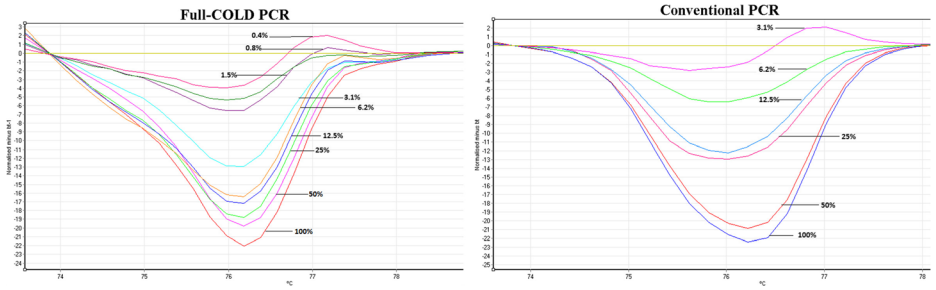

Fig. 1 Comparison between HRM differential graphs in cell line experiments. 10 different serial dilutions (100, 50, 25, 12.5, 6.2, 3.1, 1.5, 0.8, 0.4, 0.2, 0.1, and 0) of mutant DNA in WT (wild-type) DNA as diluent were prepared to compare the LOD (limit of detection) and sensitivity of conventional PCR/HRM and full COLD PCR/HRM in right and left graph respectively for detection of G>A mutations in exon 9 of *PIK3CA*. Results showed the LOD of 3.1% for conventional PCR/HRM and 0.4% for full-COLD PCR/HRM as the minimum concentrations of mutant among WT DNA which could be detected by each method

DNA from 40 samples of breast cancer tumor were screened in order to detect mutations in exon 9 of *PIK3CA*. As the result of PCR sequencing (Fig. 3, bottom chromatograms) and HRM/PCR (Fig. 3, top graph), two variants of E545K and E542K were found in 3/40 (7.5%) and 1/40 (2.5%) of samples respectively. The remaining 36 samples were investigated by full-COLD PCR/HRM to clarify the influence of enrichment in tissue samples, which resulted in clear screening of one E542K variant according to full-COLD PCR/HRM graph (Fig. 4) which then was confirmed by subsequent sequencing.

Discussion

One of the main restrictions of molecular diagnosis in early stages of breast cancer is low-abundance somatic mutations in comparison to wild-type DNA in clinical specimens. Additionally, heterogeneity and mosaicism of mutations within tumors are considered as a limitation of common cancer detection methods. Sanger sequencing as the gold standard method for detection of sequence variations has had inadequate sensitivity of at least 10–20% mutant DNA among WT background [13]. In order to improve the indicated limitations, identification of low-level mutations should be promoted by an efficient enrichment method. COLD-PCR is a novel strategy for enrichment of pre-amplified low-abundance mutant alleles, using critical denaturation temperature (Tc). COLD-PCR that is followed by high-resolution melting (HRM) curve analysis is used to detect mutant alleles as a high-sensitive screening method. HRM, by itself, is considered as a screening method based on binding of a saturating fluorescent dye to dsDNA.

In cell line experiments of this study, using full-COLD PCR/HRM for mutation detection in exon 9 of *PIK3CA* in comparison to using PCR/HRM method interestingly caused almost 7 fold improvement of LOD from 3.1 to 0.4% of mutant DNA in a mixture of WT DNA; afterwards, following full-COLD PCR/sequencing showed about 2 fold improvement of LOD from 12.5 to 6.2% of mutant DNA in WT context in comparison to conventional PCR/sequencing. Overall, full-COLD PCR/HRM by LOD of 0.4% in comparison to conventional PCR/sequencing by LOD of 12.5% showed 30 fold improvement. Additionally, in 40 patients' tumor tissues, 10% (4/40) of samples were determined to have mutations by HRM/PCR, and also Sanger sequencing as well. These results have been subsequently improved to 12.5% (5/40) of samples harboring mutations by use of full-COLD PCR before HRM which showed 2.5% improvement of detection.

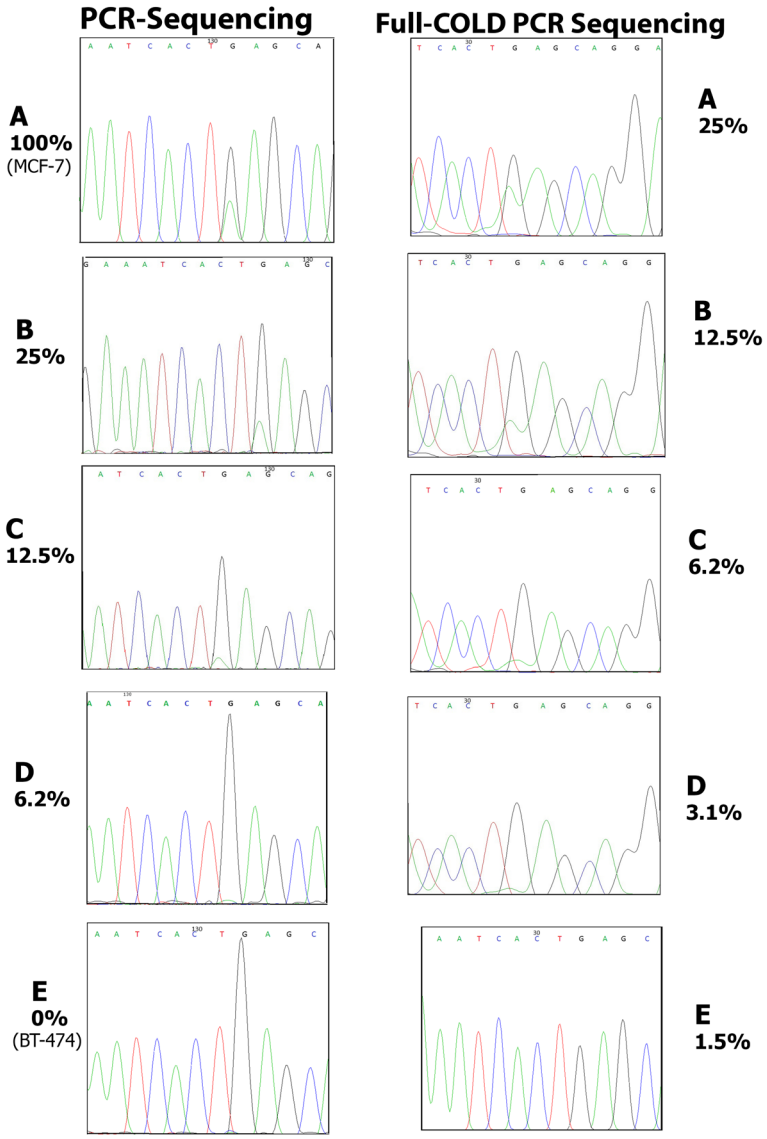

Fig. 2 Comparison between sequencing chromatograms in cell line experiments. 10 different serial dilutions (100, 50, 25, 12.5, 6.2, 3.1, 1.5, 0.8, 0.4, 0.2, 0.1, and 0) of mutant DNA in WT (wild-type) DNA as diluent were prepared to compare the LOD (limit of detection) and sensitivity of conventional PCR sequencing and full COLD-PCR sequencing in left and right chromatograms respectively for detection of G>A mutations in exon 9 of *PIK3CA*. Results showed the LOD of 12.5% for conventional PCR sequencing and 6.2% for full-COLD PCR sequencing as the minimum concentrations of mutant among WT DNA which could be detected by each method

There are different approaches of COLD-PCR which should be opted out for proper enrichment of mutations in different cases. Several studies have applied this method for various genes to enrich different mutations [31]. In a study performed by Boisselier B et al., LOD of 2% was determined for detection of IDH1 mutation through fast-COLD PCR method which was concludes in about 10 fold improvement of sensitivity in comparison with conventional

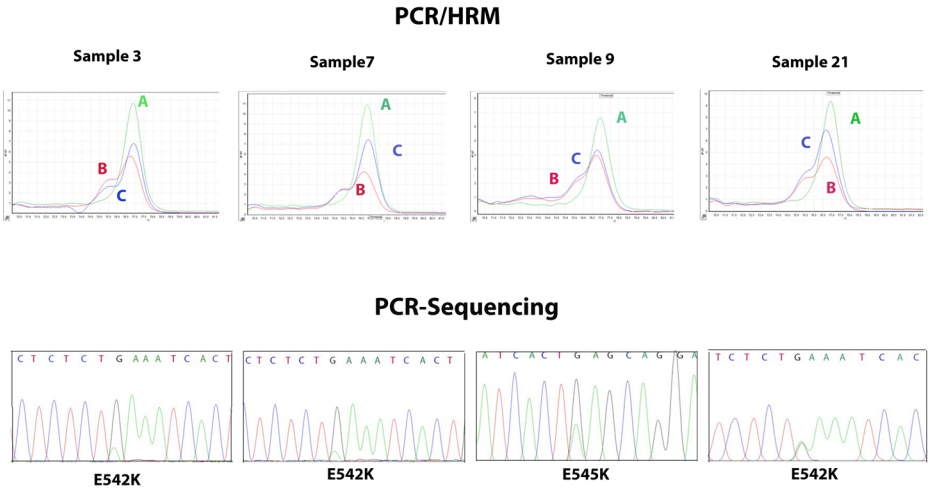

Fig. 3 Results of conventional PCR/HRM and conventional PCR sequencing in tumor tissue experiments. 40 snap-frozen tissues of primary breast cancer patients were investigated to find G>A mutations in exon 9 of *PIK3CA*. Graphs in the upper row are related to positive samples through conventional PCR/HRM in terms of mutation on exon 9 of *PIK3CA*. Chromatograms in the bottom row show the results of conventional PCR sequencing related to the respective upper graph sample. In each graph, melting curves A, B, and C represent negative control (WT DNA), positive control (mutant DNA), and unknown sample respectively. According to results of HRM, 4/40 (10%) of samples were positive for G>A mutation which then was proved and mapped by conventional PCR sequencing. According to results of sequencing, 3 samples were mutated in p.E542K c.1624G>A and 1 sample was mutated in p.E545K c.1633G>A location of exon 9 of PIK3CA

PCR sequencing and HRM/PCR as well, with LOD of about 25%. Additionally, in this study, two consecutive runs of fast-COLD PCR have been used, which alternatively showed LOD of 0.25% of mutant DNA among WT context [32]. This could be interpreted as a significant improvement of 10 and 100 fold in detection in comparison with fast-COLD PCR/HRM in a single run and conventional PCR sequencing respectively. Using "fast" method in study of Boisselier B et al. has been selected in order to raise sensitivity and lower time requirement,

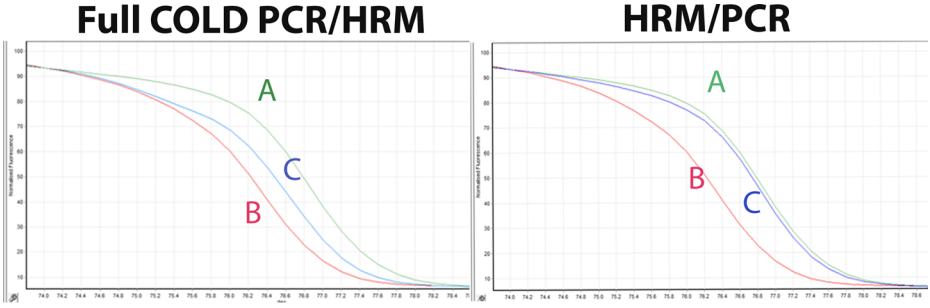

Fig. 4 Comparison between HRM results of sample no. 19 before and after enrichment with full-COLD PCR. 40 snap-frozen tissues of primary breast cancer patients were investigated to find G>A mutations in exon 9 of *PIK3CA*. After exclusion of positive samples discovered through conventional PCR/HRM and conventional PCR sequencing, the 36 remaining samples were enriched and screened by full-COLD PCR/HRM. Results of full-COLD PCR/HRM (left graph) were not concordant with the results of conventional PCR/HRM (right graph) in sample 19 that showed the detection of p.E542K c.1624G>A mutation by use of full-COLD PCR/HRM which was considered as normal by previous conventional PCR/HRM and conventional PCR sequencing method survay on the same sample

whereas it is recommended in this study, the same as Milbury CA et al. study [16], to use "full" method in cases that detection of wider spectrum of mutations has more priority.

A most recent study for detection of mutations on *PIK3CA* gene has revealed LOD of 5–10% by use of HRM/PCR in comparison to 0.5% for PCR-ARMS methodology for detection of "known" mutations [12]. While no other full-COLD PCR enrichment method study on "known and unknown" mutations in breast cancer has been carried out on *PIK3CA* gene so far.

Therefore, our study was the first full-COLD PCR assay which has been performed for screening of mutations in *PIK3CA* gene exon 9. As an enrichment upstream method, full-COLD PCR was done before HRM and sequencing, which finally revealed a better sensitivity in all assays in comparison to conventional PCR followed by HRM and sequencing.

Whereas—as further suggestion to be done in later studies—the sensitivity of this method still could be improved by investigating of wider sample size of tumor tissues and also by assessment of novel modifications in method such as two consecutive runs of full-COLD PCR.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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