Downregulation of TBC1 Domain Family Member 24 (BC1D24) Inhibits Breast Carcinoma Growth via IGF1R/PI3K/AKT Pathway

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Background: TBC1 domain family member 24 (TBC1D24) pathogenic mutations affect its binding to ARF6 and then result in severe impairment of neuronal development. However, there are no reports about the expression and function of TBC1D24 in cancer. The aim of the present study was to evaluate the effect of proliferation, migration, and invasion after silencing TBC1D24 expression in breast cancer MCF-7 cells, and to elucidate the potential mechanism of TBC1D24 in breast cancer.

Material/Methods: The expression of TBC1D24 in breast cancer tissues and the adjacent non-tumor tissues was determined by S-P immunohistochemistry. The malignant behavior, including proliferation, migration, and invasion ability, was determined after silencing TBC1D24 in breast cancer MCF-7 cells. The expression of IGF1R was determined after silencing TBC1D24. The expression of TBC1D24 and IGF1R was determined after transfecting miR-30a mimics or inhibitors. The expression of TBC1D24 on MCF-7 cells growth in vivo was evaluated by a tumor xenograft study.

Results: TBC1D24 expression was elevated and was associated with poor outcome in breast carcinoma. TBC1D24 high expression was significantly correlated with unfavorable OS and RFS for breast cancer patients (p<0.05). Silencing TBC1D24 inhibited the proliferation, migration, and invasion ability of MCF-7 cells. TBC1D24 high expression was significantly correlated with unfavorable OS and RFS for breast cancer patients (p<0.05). Silencing TBC1D24 inhibited the proliferation, migration, and invasion ability of MCF-7 cells. TBC1D24 and IGF1R expression were decreased when transfected with miR-30a mimics. However, TBC1D24 and IGF1R expression were increased when transfected with miR-30a inhibitors (p<0.05). Knockdown of TBC1D24 inhibited the expression of IGF1R, PI3K, and p-AKT (p<0.05). Knockdown of TBC1D24 abolished tumorigenicity of MCF-7 cells. The average volume and weight of tumors was lower after silencing TBC1D24 expression (P<0.05).

Conclusions: Silencing TBC1D24 inhibited MCF-7 cells growth in vitro and in vivo. TBC1D24 promoted breast carcinoma growth through the IGF1R/PI3K/AKT pathway. TBC1D24 is a potential therapeutic target for breast cancer.

MeSH Keywords: Brain Neoplasms • Cell Migration Assays • MicroRNAs

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Background

Breast cancer is a heterogeneous disease that has many clinical and genetic forms. Many attempts have been used to categorize this heterogeneous disease and now molecular classification into 5 groups can be accomplished by gene expression profiling [1,2]. Among these molecular subtypes, basal-like breast cancer existed a more aggressive clinical course than other subtypes. It is commonly regarded as triple-negative breast cancer, which is defined as lacking estrogen receptor, progesterone receptor and human epidermal growth factor receptor 2. Because of highly heterogeneous and many molecular subtypes, triple-negative breast cancer is difficult to treat [3–8].

The TBC1D24 gene encodes a protein of 553 amino acids that includes a TBC domain shared by Rab GTPase-activating proteins (Rab-GAPs) and a TLDc domain [9]. The TBC1D24 protein is mainly expressed in the brain. This protein interacts with the ADP ribosylation factor 6 (ARF6), which is a small GTP-binding protein implicated in membrane exchange between the endocytic compartments and the plasma membrane [9,10]. Previous studies reported that TBC1D24 pathogenic mutations affect its binding to ARF6 and then results in severe impairment of neuronal development [9,11,12]. However, there are no reports about the expression and function of TBC1D24 in cancer. ARF6 can promote breast cancer cell metastasis through activation of EGF-induced E-cadherin internalization [13]. Many studies have observed ARF6 regulation of malignant behaviors of breast cancer [14,15]. TBC1D24 binds ARF6 and promotes ARF6 activation [9,16]. Based on previous studies, we hypothesized TBC1D24 plays a role in breast cancer.

In this study, we report that TBC1D24 functions as a ceRNA to regulate the expression of IGF1R through competition for miR-30a in breast cancer cells. The present work provides the first evidence for a positive TBC1D24/IGF1R correlation and crosstalk between miR-30a, TBC1D24 and IGF1R, which provides new insights for the treatment of breast carcinoma.

Material and Methods

The tissue microarray slides, including invasive ductal carcinoma and cancer adjacent normal breast tissue (n=110; BC081116a), were purchased from the US BioMax Inc cancer tissue bank collection (US BioMax Inc., MD, USA).

Immunohistochemistry

All slides were deparaffinized in xylene, dehydrated in a graded series of ethanol, subjected to antigen retrieval in sodium chloride citrate buffer (00526; Sigma) for 30 min in an antigen retrieval box, and washed in phosphate-buffered saline (PBS). PBS was used for all subsequent washes and for antiserum dilution. Tissue sections were quenched sequentially in 3% hydrogen peroxide and blocked with PBS containing 10% goat serum (sigma) for 1 h at 37°C. Next, we added primary antibody to the slides at 37°C for 2 h, which included rabbit polyclonal to TBC1D24 (1: 100; ab101933; Abcam). Irrelevant primary antibodies and omission of primary antibody were used for negative controls. After several washes (3×3 min) to remove excess antibody, the slides were incubated with diluted (1: 300) anti-rabbit biotinylated antibodies (Beijing Biosynthesis Biotechnology Co., LTD, China) for 1 h. All the slides were washed in PBS and were incubated in avidin biotin peroxidase complex (ABC, Beijing Zhongshan Golden Bridge Biotechnology Co. Ltd., Beijing, China) diluted 1: 300 in PBS for 30 min in humidified chambers at 37°C. DAB (Beijing Biosynthesis Biotechnology Co., LTD, China) was used as a chromogen. Hematoxylin was used as a nuclear counter stain. We scored the intensity of TBC1D24-positive cells as follows: 0, 1, 2, or 3, from negative, weak, moderate, and strong intensity, respectively. The percentage of TBC1D24-positive cells was scored as follows: 0 for no cytoplasm expression, 1 for 1–25% positive tumor cytoplasm, 2 for 26–50% positive tumor cytoplasm, 3 for 51–75% positive tumor cytoplasm, and 4 for 76–100% positive tumor cytoplasm [17]. The final TBC1D24 staining score was obtained by multiplying the intensity and percentage scores, and was defined as follows: staining score less than 6 were considered low expression, while staining scores of 7 or more were considered high expression. All values are showed as the mean ± standard error (mean ±SEM).

Cell culture, reagents, and transfection procedure

All cells were cultured in Rosewall Park Memorial Institute (RPMI) 1640 medium. The medium was supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin in an atmosphere with 5% carbon dioxide at 37°C. The siRNA sequences were obtained from GenePharma Co., Ltd. (Shanghai, China). The siRNA sequences were as following: TBC1D24-1: 5’-GGUUCUGGAGCAAAAGACAAAAG-3’; TBC1D24-2: 5’-GUGCUCUACUCUGAGUGAAG-3’; and NC (negative control) siRNA: 5’-UUCUGAAAGGUGUCAGUTT-3’. The lentiviral vectors expressing shRNA targeting TBC1D24 (called LV3-shTBC1D24-1 and LV3-shTBC1D24-2) were purchased from GenePharma Co., Ltd. (Shanghai, China). The miR-30a mimics and inhibitors were obtained from GenePharma Co., Ltd. (Shanghai, China). Breast cancer cells were infected with LV3-shTBC1D24-1, LV3-shTBC1D24-2 or LV3-NC. After 48 h, the purinomycin was added at 5 ug/ml.

Total RNA isolation and RT-qPCR

Total RNA was extracted from the cancer cells using the RNA pure High-purity Total RNA Rapid Extraction Kit (BioTeke, Shanghai, China). Total RNA was reverse transcribed into cDNA using a Maxima First Strand cDNA Synthesis Kit (Thermo Scientific). RT-qPCR was performed using the iQ SYBR Green Supermix (Bio-Rad) and an iCycler (Bio-Rad) according to the manufacturer’s instructions. The primers were shown in Table 1.
RP1201, China) following the manufacturer’s protocol. Real-time (RT) and quantitative polymerase chain reaction (qPCR) kits (Bio-Rad) were used to determine the mRNA expression levels. The RT and qPCR reactions were conducted as previously described [18]. Relative mRNA expression was calculated using the comparative cycle threshold (CT) (2-ΔΔCT) method. GAPDH was used as an endogenous control to normalize the data.

**Western blot analysis**

Expressions of TBC1D24, IGF1R, PI3K, and p-AKT and GAPDH protein were analyzed by Western blot as described previously [19–23]. The TBC1D24 (ab101933), IGF1R (ab39675), p-AKT (ab38449), and GAPDH (ab8245) antibody were purchased from Abcam, Inc. The antibody was dilution at 1: 1000.

**CCK-8 and EdU assay**

The ability of cell proliferation was determined by the CCK-8 assay according to the manufacturer’s instructions. Cells were seeded into 96-well plates, and then cultured for an additional 24 h. After treatment with OT, 10 μl of the kit reagent was added and then incubated for another 2 h. The O.D. value was read at 450 nm to obtain the final results [24,25]. Cell proliferation was analyzed using the Cell-Light EdU imaging detecting kit (Ruibo Biotechnology, Guangzhou, China) according to the manufacturer’s instructions [26–28].

**Wound healing migration assay and cell invasion assays**

Wound healing assay was performed as described previously [18]. MCF-7 cells were infected with LV3-shTBC1D24-1, LV3-shTBC1D24-2, or LV3-NC. After 48 h the purinomycin was added at 5 μg/ml. Then, the cells were cultured in 6-well plates to about 90% confluence. The medium was replaced with serum-free medium. After the wounding, the distance between 2 wounds was measured at 0 and 72 h. Transwell invasion assays were performed as described previously [18]. The upper side was coated using Matrigel basement membrane matrix (BD Biosciences, Bedford, MA) for 2 h at 37°C. The MCF-7 cells were added into the top chamber, and then incubated for 48 h. We used 4% paraformaldehyde to fix the invasive cells, after which they were stained in 0.5% crystal violet (Beyotime) and counted.

**In vivo animal experiment**

All animal experimental protocols were approved by the Committee on the Use and Care of Animals (Jiamusi University, China). MCF-7 cells were infected with LV3-NC or LV3-shTBC1D24-1. The 6-week-old BALB/c nude mice were grouped into an LV3-NC-infected group (n = 5) and an LV3-shTBC1D24-1-infected group (n = 5). The infected MCF-7 cells were injected subcutaneously into the left armpit. Twenty-eight days later, the weight and volume of tumors were calculated.

**Statistical analysis**

All statistical analyses were performed using SPSS software, version 19.0 (Chicago, IL). Each experiment was performed in triplicate. Student’s t-test or analysis of variance (ANOVA) was used for statistical analysis. The differences were considered significant when P<0.05.

**Results**

**TBC1D24 expression was elevated and was associated with poor outcome in breast carcinoma**

The expression of TBC1D24 in breast cancer has not been previously reported. We firstly analyzed the expression pattern in breast cancer. The Oncomine database is a cancer microarray database and web-based data-mining platform aimed at facilitating discovery from genome-wide expression analyses. Differential expression analyses comparing most major types of cancer with respective normal tissues, as well as a variety of cancer subtypes and clinical-based and pathology-based analyses, are available for exploration [29]. Using Oncomine analysis, we investigated levels of TBC1D24 genes in breast cancers. Three independent data sets (Finak Breast, Gluck Breast, and Zhao Breast) showed that the mRNA level of TBC1D24 in invasive breast carcinoma stroma, invasive breast carcinoma, and invasive ductal breast carcinoma was higher than in normal breast tissues (fold changes were 4.683, 1.366, and 2.329, respectively) (p=4.45E-8, 2.20E-4 and 8.91E-5, respectively) (Figure 1A–1C). Then, we analyzed the expression of TBC1D24 in other breast cancer histological types (data derived from TCGA Breast). The expression level of TBC1D24 in invasive breast carcinoma stroma, invasive breast carcinoma, and invasive ductal breast carcinoma was higher than in normal breast tissues (fold changes were 1.937, 1.216, and 2.078, respectively) (p=0.003, 0.016, and 0.009, respectively) (Figure 1D–1I).

**High level of TBC1D24 expression was associated with cancer progression**

We next detected the location of TBC1D24 protein in breast cancer tissues. TBC1D24 was mainly localized on the plasma membrane and cytoplasm (Figure 2A). The TBC1D24 expression was shown by high staining in breast carcinoma (Figure 2A). The TBC1D24 in glandular cells was shown by medium staining. However, the TBC1D24 in medium showed staining in...
glandular cells and was not detected in adipocytes (Figure 2B). The TBC1D24 expression was significantly increased in breast carcinoma samples compared with adjacent normal breast tissue (p<0.05; Figure 1A, 1B and Table 1). The Mann-Whitney U test was used to determine the associations between TBC1D24 expression and clinicopathologic variables of 100 breast cancer tissues (Table 1). The relationship between TBC1D24 expression and age was not significant (p>0.05; Table 1). TBC1D24 expression was significantly increased in cancer samples with advanced stages (stage III/IV) as compared with those in the normal breast tissues.

**Figure 1.** (A–I) TBC1D24 expression was elevated and associated with poor outcome in breast carcinoma. The mRNA expression of TBC1D24 in breast cancer was analyzed. Data derived from Oncomine database. The mRNA expression of TBC1D24 in breast cancer was increased compared to normal breast tissues.

**Figure 2.** TBC1D24 expression was detected by immunohistochemistry (IHC). TBC1D24 was located in cytoplasm and membranes (A). The expression of TBC1D24 in breast cancer tissues (A) was increased compared to normal breast tissues (B). Original magnification, 200×.
early stages of disease (stage I/II) (p<0.05). The staining intensity of TBC1D24 was significantly associated with the tumor grade (grades 2–3 versus 1, p<0.05).

**TBC1D24 regulates cellular proliferation, migration, and invasion**

We determined the mRNA level of TBC1D24 in 2 cell lines by real-time quantitative PCR (qPCR). We found that TBC1D24 expression was upregulated in the MCF-7 cell line compared with ZR-75-30 cell lines (Figure 3A). Therefore, the MCF-7 cell line was chosen for the silencing of TBC1D24 to determine its function. The expression of TBC1D24 was reduced in LV3-shTBC1D24-1- and LV3-shTBC1D24-2-infected MCF-7 cells compared with LV3-NC-infected MCF-7 cells (Figure 3B). Our results indicate that cell proliferation, migration, and invasion in MCF-7 cells infected with LV3-shTBC1D24-1 and LV3-shTBC1D24-2 were decreased compared to MCF-7 cells infected with LV3-NC (P <0.05; Figure 2C–2I).

**TBC1D24 is a ceRNA of IGF1R through sponging miR-30a**

Bioinformatics analyses (Starbase) showed that TBC1D24 is a putative ceRNA of IGF1R. We observed that IGF1R mRNA and protein expression were significantly reduced in MCF-7 cells infected with LV3-shTBC1D24-1 and LV3-shTBC1D24-2 relative to MCF-7 cells infected with LV3-NC (Figure 4A; p<0.05). Interestingly, bioinformatics analyses showed that TBC1D24 and IGF1R are predictive targets of miR-30a (TargetScan). We explored whether the regulation of TBC1D24 on IGF1R is miR-30a-dependent. Bioinformatics analyses (Starbase) showed that the miR-30a-specific binding site was located in the 3’ UTR of TBC1D24 and IGF1R mRNAs (Figure 4B). To investigate whether miR-30a could directly target the 3’ UTR of TBC1D24 and IGF1R, the vector was constructed. Our results showed that miR-30a significantly inhibited luciferase activity after the TBC1D24 and IGF1R 3’ UTRs were inserted downstream of the luciferase cDNA in the reporter vector (pMIR-TBC1D24 3UTR, pMIR-IGF1R 3UTR). In contrast, no suppression of luciferase activity was detected in MCF-7 cells transfected with the control vector, including in mutant TBC1D24 and IGF1R 3’ UTRs (MIR-TBC1D24 and IGF1R 3UTRm) when miR-30a expression was increased (Figure 4B; p<0.05). We also observed that miR-30a mimics reduced the expression of TBC1D24 and IGF1R (Figure 4C; p<0.05). However, miR-30a inhibitor upregulated the expression of TBC1D24 and IGF1R (Figure 4D; P<0.05).

**TBC1D24 regulated IGF1R/PI3K/AKT pathway**

Previous studies revealed that the IGF1R/PI3K/Akt pathway is involved in the development of breast cancer via the PI3K/Akt pathway [30,31]. Based on the above results, we hypothesized that TBC1D24 regulates the IGF1R/PI3K/AKT pathway. PI3K and p-AKT protein levels were decreased in MCF-7 cells when silencing TBC1D4. These data indicate that TBC1D4 acts as a ceRNA of IGF1R by sponging miR-30a, which activates the IGF1R/PI3K/AKT signaling axis in breast cancer (Figure 5).

**Downregulation of TBC1D24 abolished tumorigenicity of MCF-7 cells**

To investigate the role of TBC1D24 in vivo, an animal model was constructed. MCF-7 cells infected with LV3-NC and LV3-shTBC1D24-1- and LV3-shTBC1D24-2-infected MCF-7 cells were used in a nude mouse xenograft model. The data showed that TBC1D24 expression was significantly lower in LV3-shTBC1D24-1- and LV3-shTBC1D24-2-infected MCF-7 cells compared with LV3-NC-infected MCF-7 cells (Figure 5A–5D; p<0.05). These results indicated that TBC1D24 expression was significantly associated with breast cancer progression.

### Table 1. Association of TBC1D24 expression with clinicopathological characteristics in 100 patients of breast cancer.

| Characteristics          | No. of patients (n=110) | TBC1D24 expression | P value |  |
|--------------------------|------------------------|--------------------|---------|
|                         |                        | Low no. (%)        | High no. (%) |  |
| Age (years)              |                        |                    |         |
| <50                      | 58                     | 21 (36.20%)        | 37 (63.80%) | >0.05 |
| ≥50                      | 52                     | 28 (53.85%)        | 24 (46.15%) |         |
| Normal ovarian           | 10                     | 8 (80.00%)         | 2 (20.00%)  | <0.05 |
| Cancer tissues           | 100                    | 41 (41.00%)        | 59 (59.00%) |         |
| FIGO stage               |                        |                    |         |
| I/II                     | 56                     | 31 (55.36%)        | 25 (44.64%) | <0.05 |
| III/IV                   | 44                     | 10 (22.73%)        | 34 (72.27%) |         |
| Grade                    |                        |                    |         |
| 1                        | 43                     | 33 (76.74%)        | 10 (23.26%) |         |
| 2                        | 32                     | 5 (15.63%)         | 27 (84.37%) |         |
| 3                        | 25                     | 3 (12.00%)         | 22 (88.00%) |         |

Grade 2–3 versus 1 <0.05

[30,31]
LV3-shTBC1D24-1 formed tumors in all nude mice. The average volume of tumors was decreased in the LV3-shTBC1D24-1 group compared to that in the LV3-NC group (p<0.05) (Figure 6A, 6B). The average weight of tumors in the LV3-shTBC1D24-1 group was decreased compared to that of the LV3-NC group (Figure 6C). The expression of IGF1R, PI3K, and p-AKT in tumors derived from the LV3-shTBC1D24-1-infected group was decreased compared to that in the LV3-NC group (Figure 6D).
In this study, we noted an increased expression of TBC1D24 in breast cancer tissues compared with the adjacent normal breast tissue. The up-regulation of TBC1D24 in breast cancer was correlated with histological grades and tumor stage. Our data also show that the silencing of TBC1D24 expression in breast cancer cells inhibited the proliferation, migration, and invasion of breast cancer. These results indicate that the down-regulation of TBC1D24 reduced an aggressive behavior. Based on these findings, TBC1D24 can serve as a biomarker as well as a potential therapeutic target for breast cancer. Our data also demonstrate that TBC1D24 regulates IGF1R in a miR-30a-dependent manner.

![Figure 4](image) **Figure 4.** TBC1D24 regulates IGF1R mRNA and protein levels. (A) TBC1D24 mRNA and protein levels were downregulated by infection with LV3-shTBC1D24-1 or LV3-shTBC1D24-2. (B) Putative binding sites targeted by miR-30a were predicted to be located in the 3’ UTR of TBC1D24 and IGF1R mRNA. MCF-7 cells were cotransfected with miR-30a mimics or control RNA (NC) with luciferase reporter plasmids containing either wild-type (pMIR-TBC1D24-3UTR and pMIR-IGF1R-3UTR) or mutant 3’ UTR (pMIR-TBC1D24-3UTRm and pMIR-IGF1R-3UTRm) of TBC1D24 and IGF1R genes. Luciferase expression was measured. The fold changes of the relative luciferase activity in miR-30a mimics with the indicated plasmids transfected cells were normalized to NC with the corresponding indicated plasmid-transfected cells. (C) Breast cancer MCF-7 cells were transfected with miR-30a mimics or control RNA (NC). (D) Breast cancer MCF-7 cells were transfected with miR-30a inhibitor or control RNA (NC). Error bars represent standard error. The symbols * indicate p<0.05.

![Figure 5](image) **Figure 5.** The expression of p-AKT2 and PI3K was determined by western blotting.
Figure 6. TBC1D24 regulated tumorigenesis in nude mice model. (A–C) Mean tumor volume and weight on day 28 after tumor cell injection. LV3-shTBC1D24-1 or LV3-NC infected MCF-7 cells were implanted s.c. into the left armpit. (D) Immunohistochemical analysis of TBC1D24, IGF1R, PI3K, or p-AKT expression was performed on tumor xenografts. Representative images are shown (original magnification ×200). * p<0.05.
a common feature in cells. The ceRNA hypothesis states that IncRNA, mRNA, pseudogene transcripts, and circular RNAs compete to bind the same miRNAs to regulate translational activity or target gene stability, thereby influencing gene expression at the post-transcriptional level [32–34]. In the present study, we first predicted that IGF1R is a ceRNA of TBC1D24 using Starbase. We found that silencing TBC1D24 inhibited the expression of IGF1R. MiR-30a can regulate the expression of TBC1D24 and IGF1R. Luciferase assays confirmed that TBC1D24 and IGF1R are the direct targets of miR-30a. These results indicate that TBC1D24 regulates IGF1R as a ceRNA by competing for miR-30a.

Many reports have shown that miR-30a has low expression levels in bone tumors [35], non-small cell lung cancer [36], breast cancer [37], liver cancer [38], and other malignant tumors. miR-30a is involved in the apoptosis, proliferation, migration, invasion, and other biological functions of different tumor cells. In breast cancer, miR-30a regulates the IGF1R-AKT pathway by targeting IGF1R. Our results also show that IGF1R is a direct target of miR-30a. Our results are consistent with a previous study [39]. Our data provide evidence that TBC1D24 acts as a cancer-promoting gene.

IGF1 and IGF1 receptors (IGF1R) play an important role in the early transformation of mammary cells. IGF1R expression patterns in epithelial cells of normal terminal duct lobular units in benign breast biopsies were associated with an increased risk of subsequent breast cancer [40]. Inhibition of IGF1R activity enhances the response to trastuzumab in HER-2-positive breast cancer cells [41]. Conditional ablation of IGF1R in the mouse mammary epithelium dramatically increased the latency of kras-induced tumors. Treatment of tumor-bearing animals by administration of picropodophyllin, which is a specific Igf1r inhibitor, resulted in a significant decrease in tumor mass of the main forms of basal-like carcinomas [42]. In the present study, we found that silencing TBC1D24 inhibited the proliferation, migration, and invasion of MCF-7 cells. Silencing TBC1D24 also reduced the expression of IGF1R, PI3K, and p-AKT. Our data indicate that TBC1D24 regulates breast cancer malignant behavior through the IGF1R/PI3K/AKT pathway.

Conclusions

In conclusion, the present study found that TBC1D24 is an oncogene in breast cancer, and TBC1D24 promoted malignant behavior through the IGF1R/PI3K/AKT pathway in breast carcinoma. This suggests that TBC1D24 may be a potential therapeutic target in breast carcinoma. There are some limitations to our study. Firstly, we did not insert the TBC1D24 gene into normal breast cells and then show that the parameters were accelerated. Secondly, we found that TBC1D24 regulated the IGF1R/PI3K/AKT pathway in breast carcinoma, but the direct target was not elaborated. In future research, we plan to insert the TBC1D24 gene into normal breast cells and then show that the parameters are accelerated. Also, how TBC1D24 regulates the IGF1R/PI3K/AKT pathway will be further investigated.

Conflict of interest

None.

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Conflict of interest

None.
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