**EVA1B to Evaluate the Tumor Immune Microenvironment and Clinical Prognosis in Glioma**

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**Background:** Previous research indicated that the tumor cells and microenvironment interactions are critical for the immunotherapeutic response. However, predicting the clinical response to immunotherapy remains a dilemma for clinicians. Hence, this study aimed to investigate the associations between EVA1B expression and prognosis and tumor-infiltrating immune cells in glioma.

**Methods:** Firstly, we detected the EVA1B expression in glioma tissues through biological databases. The chi-squared test, Kaplan-Meier, and univariate and multivariate Cox regression analyses were used to analyze the clinical significance of EVA1B expression. The correlation between EVA1B expression and levels of tumor-infiltrating immune cells in glioma tissues was investigated. Receiver operating characteristic (ROC) analysis was performed to compare the predictive power between EVA1B and other commonly immune-related markers.

**Results:** In the CGGA cohort of 325 glioma patients, we found that EVA1B was upregulated in glioma, and increased with tumor grade. High EVA1B expression was prominently associated with unfavorable clinicopathological features, and poorer survival of patients, which were further confirmed by TCGA (n=609) and GEO (n=74) cohorts. Furthermore, multivariate analysis indicated that EVA1B is an independent prognostic biomarker for glioma. Importantly, EVA1B overexpression was associated with a higher infiltration level of CD4\(^+\) T cells, CD8\(^+\) T cells, B cells, macrophages, and neutrophils in glioma. ROC curves showed that, compared with PD-L1, CTLA-4, and Siglec15, EVA1B presented a higher area under the curve (AUC) value (AUC=0.824) for predicting high immune infiltration levels in glioma.

**Conclusions:** We found that EVA1B was upregulated and could act as a poor prognostic biomarker in glioma. Importantly, EVA1B overexpression was associated with the immune infiltration levels of immune cells including B cells, CD4\(^+\) T cells, CD8\(^+\) T cells, macrophages, and neutrophils, and strongly with the overall immune infiltration levels of glioma. These findings suggested that EVA1B might be a potential biomarker for evaluating prognosis and immune infiltration in glioma.

**Keywords:** EVA1B, prognosis, glioma, biomarker, microenvironment
INTRODUCTION

Glioma is one of the brain malignancies with the highest prevalence, and the incidence of glioma is on the increase worldwide (1, 2). Despite the variety of modern therapies against glioblastoma, it is still a deadly disease with an extremely poor prognosis. The median overall survival (OS) of glioblastoma (GBM) patients is less than two years (3). Most patients have a few months of survival if they are left untreated (4). Therefore, it is of great significance to indicate how to develop further effective therapeutic strategies to improve the OS of glioma patients.

In recent years, immunotherapy has increasingly attracted scholars’ attention in the field of tumor therapy worldwide (5). Since January 2015, cancer immunotherapy has made significant advances (6). The programmed cell death protein 1 (PD-1) blockade is effective in the treatment of Hodgkin’s lymphoma (HL) and raises hopes that it may change the treatment pattern of the disease (7). Immunotherapy, such as cytotoxic T lymphocyte associated antigen 4 (CTLA4), programmed death-1 (PD-1), and programmed death ligand-1 (PD-L1) inhibitors, showed promising antitumor effects in malignant melanoma (8). However, current immunotherapies, such as anti-CTLA4, anti-PD-1, and anti-PD-L1, showed poor clinical efficacy in GBM (9). The reason for the ineffectiveness of the treatment is very complicated, which may be related to the lack of biomarkers for guiding individualized immune targets (9). Increasing studies have also found that tumor-infiltrating lymphocytes, such as tumor associated macrophages (TAMs) and tumor-infiltrating neutrophils (TINs), affect the prognosis and efficacy of chemotherapy and immunotherapy (10). Thus, there is an urgent need for the elucidation of the immunophenotypes of tumor-immune interaction and identification of novel immune-related biomarkers and therapeutic targets in glioma.

In humans, the EVA1 family includes three main groups: EVA1A, EVA1B, and EVA1C. Previous studies have revealed that EVA1A, an endoplasmic reticulum-related protein were involved in regulating autophagy and apoptosis-related mechanisms in many cancers (11, 12). Additionally, Bang-Yi Lin et al. found that EVA1A promotes papillary thyroid cancer progression and epithelial-mesenchymal transition via upregulating immune-related signaling pathways (11). Hippo signaling pathway. MST1/2 is the core kinases of the classical Hippo signaling pathway in mammals (13), which also plays a key role in the immune microenvironment by regulating the adhesion and trafficking of immune cells (14). A paralogous gene of the EVA1A gene is EVA1B, which both belong to the EVA1 family. EVA1B has high sequence similarity with the EVA1A gene. We also found that the EVA1B protein has the same domain as the EVA1A protein using the GenesLikeMe database. However, what role EVA1B plays in tumors has not yet been reported, especially its correlation with immune infiltration.

Consequently, this study was conducted to comprehensively identify EVA1B expression levels in glioma, and its correlation with malignant features and the prognosis of glioma patients. Moreover, we also investigated the correlation of EVA1B with infiltration levels of different immune cells and further assessed the predictive performance of EVA1B for high infiltration levels of glioma.

MATERIALS AND METHODS

Gene Expression Profiles

The mRNA expression profiles of EVA1B were obtained from the GEO (https://www.ncbi.nlm.nih.gov/geo/) and TCGA database (http://cancergenome.nih.gov/). Moreover, a pan-cancer analysis of EVA1B was conducted using the Oncomine (http://www.oncomine.org) and the online tool TIMER (https://cistrome.shinyapps.io/timer/). Additionally, the localization of EVA1B protein was analyzed by immunofluorescence images from the Human Protein Atlas (HPA) database (https://www.proteinatlas.org/).

Patients

The included glioma patients were retrospectively collected from the CGGA database (http://cgga.org.cn/, Dataset ID: mRNAseq_325) and acted as a CGGA cohort. A total of 325 glioma patients were analyzed, and general clinical data and RNA-sequencing data were retrieved from the CGGA database. Among them, the mean age of included patients was 42.94 years (age range: 8-79 years). The details of patients’ characteristics are summarized in Supplementary Table 1. Ethical approval was obtained through the Ethics Committee of Lishui People’s Hospital, written informed consent was waived by the ethics committee because all data comes from public databases.

Validation of External Datasets (TCGA and GEO Databases)

To validate the prognostic value of EVA1B in glioma, two independent datasets from TCGA and GEO databases were used to validate the TCGA (n=609) and GEO (n=74) cohorts. In the TCGA cohort, 609 glioma patients (mean age=47.3 years, range: 14-89 years) were included. The details of patients’ characteristics are summarized in Supplementary Table 1. In the GEO cohort, 74 glioma patients (age range, 18-82) were included, and 32 (43.2%) of them were male and 42 (56.8%) were female. The correlation between EVA1B expression and prognosis of patients was analyzed by the comprehensive platform PrognoScan website (http://www.prognoscan.org/).

Biological Functions of EVA1B Participation in Glioma

First of all, the top 500 genes most co-expressed with EVA1B were extracted from the LinkedOmics database (http://www.linkedomics.org) (15). Function annotations were conducted to identify the biological processes potentially involved, cellular components, molecular function, and signaling pathway using DAVID 6.8 (https://david.ncifcrf.gov/) (16). To identify the...
potential interacting proteins, a functional protein network was built by GeneMANIA software (http://www.genemania.org/) (17).

**Systematic Analysis of Immune Infiltration**

To comprehensively investigate the associations between EVA1B and immune cells and immune molecules, we used the TIMER and EPIC algorithm to estimate the abundance of six immune infiltrates including B cells, CD4+ T cells, CD8+ T cells, Neutrophils, Macrophages, and Dendritic cells (DCs) (18).

**Statistical Analysis**

In this study, statistical analysis was performed using SPSS (Version 23.0; IBM), GraphPad (version 6.0), and R software (version 3.6.1). Chi-square test was used to calculate categorical variables. For continuous variables, when continuous variables follow the normal distribution, an independent Student’s t-test was conducted. Otherwise, a Mann-Whitney test was conducted. Spearman rank tests were also performed for correlation analysis. Kaplan-Meier analysis was performed to compare the OS of patients. Univariate and multivariate analyses were used to explore the prognostic factor for glioma patients. A time-dependent ROC curve was performed to evaluate prediction power. All P-value <0.05 was considered statistically significant (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001).

**RESULTS**

**Expression of EVA1B in Various Tumor Types**

Firstly, we compared the differential mRNA level of EVA1B between the normal brain tissue and glioma tissues from the GEO and TCGA database. Compared with normal tissues, EVA1B mRNA expression was remarkably upregulated in gliomas based on GSE50161 (normal: 13, glioma: 49, Figure 1A) and GSE4290 (normal: 23, WHOII: 45, WHOIII: 31, WHOIV: 77, Figure 1B), and the EVA1B expression level increased with tumor grade. Similar results were obtained by analyzing TCGA sequencing data of glioma tissues (normal: 5, GBM: 156, Figure 1C). To gain insight into the subcellular localization of EVA1B protein, we obtained the immunofluorescence images of EVA1B in the U251 cell line from the HPA database, which contains high-resolution, multi-color immunofluorescence images of cells that detail the subcellular distribution pattern of proteins. Immunofluorescence analysis showed that EVA1B protein mainly localized to the cytoplasm and membrane (Figure 1D). In total, the EVA1B expression levels in glioma were significantly overexpressed and were increased with tumor grade.

We also explored the EVA1B expression level in pan-cancer. As expected, compared with normal tissue, the EVA1B expression was also upregulated in breast cancer, colorectal cancer, lymphoma, melanoma, and sarcoma based on the

![FIGURE 1](image_url)

**FIGURE 1** | The expression of EVA1B is highly expressed in glioma compared to the normal brain tissues. (A–C) The EVA1B expression is significantly regulated in glioma in the GSE50161, GSE4290, and TCGA, and increases with tumor grade. (D) The EVA1B protein is predominantly localized in the cytoplasm and cellular membrane of U251 (images obtained from the Human Protein Atlas).
Oncomine database (Supplementary Figure 1A). To confirm these findings, we further analyzed the TCGA data of tumors and obtained similar results (Supplementary Figure 2B).

The Association Between EVA1B Expression and the Clinicopathological Features of Glioma Patients

In the above, we reported that EVA1B expression was positively related to tumor grade. To further explore EVA1B overexpression and malignant behavior of glioma, 325 patients with glioma were obtained from the CGGA database. First, we compared the differential mRNA level of EVA1B in different subgroups stratified by WHO grade, IDH mutation, 1p/19q codeletion, age, recurrence, and sex. As shown in Figures 2A–F, EVA1B expression was significant different between subgroups stratified by WHO grade (P<0.0001, P<0.0001, respectively, Figure 2A), IDH mutation (P<0.0001, Figure 2B), 1p/19q codeletion (P<0.0001, Figure 3C), age (P<0.0001, Figure 3D), but not in subgroups stratified by recurrence (P=0.053, Figure 2E), and sex (P=0.929, Figure 2F).

Second, we analyzed its correlation with MKI67 (Ki-67 proliferation index) and VIM expression level (Vimentin invasion index) and found that EVA1B was weakly correlated with Ki-67 (r=0.320, P<0.001, Figure 2G), but strongly

![FIGURE 2](image_url)

**FIGURE 2** | The association between EVA1B expression and clinicopathological features in glioma cohort (CGGA cohort, n = 325). (A–F) The EVA1B expression was significantly associated with WHO grade, IDH wildtype, 1p/19q non-codeletion, and age, but not with recurrence of glioma and sex. (G, H) EVA1B expression was weakly associated with proliferation marker (Ki-67 expression), and strongly associated with invasion marker (Vimentin expression). (****P < 0.0001).
correlated with Vimentin expression ($r=0.690$, $P<0.001$, Figure 2H). This result was also verified by the TCGA cohort of 609 glioma patients (Supplementary Figures 2A, B).

Third, according to the median value of EVA1B expression, all patients were divided into low or high EVA1B groups. Chi-square test results showed that overexpressed EVA1B was significantly associated with age ($P<0.001$), WHO grade ($P<0.001$), histopathology ($P<0.001$), IDH status ($P<0.001$), and 1p/19q status ($P<0.001$) (Table 1, Supplementary Table 2). These results suggested that EVA1B overexpression was related to the malignant clinicopathological features of glioma patients.

The Prognostic Value of EVA1B Expression in Glioma Patients
To assess whether EVA1B could act as a prognosis marker for glioma patients, Kaplan-Meier survival analysis was conducted to compare the OS of patients between high and low EVA1B expression groups. Overall, the Kaplan-Meier curve showed that patients from the high EVA1B expression group had a remarkably poorer OS (HR=0.38, 95%CI=0.30-0.49, $P<0.0001$, Figure 3A). Furthermore, stratified survival analysis was performed to assess the prognostic significance of EVA1B in different subgroups of glioma patients stratified by age, WHO grade, IDH status, and 1p/19q status. The results of stratified survival analysis were consistent with the results shown in Figure 3A (Figures 3B–I). Next, the univariate and multivariate analysis results revealed that EVA1B expression was an independent prognostic biomarker for glioma patients (HR= 1.93, 95% CI=1.42-2.62, $P<0.001$, Table 2).

A time-dependent ROC curve was also used to assess the predictive power of EVA1B in predicting 1-, 3- and 5-years OS, and the AUC for 1-, 3- and 5-year survival rate of glioma patients.
results of the TCGA validation cohort are consistent with the results of the CGGA cohort in WHO grade (P<0.0001, 
Supplementary Figure 3A), IDH status (P<0.0001, 
Supplementary Figure 3B), and 1p/19q status groups (P<0.0001, 
Supplementary Figure 3C). Similarly, Supplementary Table 3 
revealed the comparison of clinicopathological features between the 
high and low EVA1B groups, which yielded similar results.

Next, to validate the prognostic value of EVA1B for glioma 
patients, Kaplan-Meier analysis was used to compare the OS 
between Low-EVA1B and high-EVA1B groups and showed that 
patients with high EVA1B expression had a remarkably worse OS 
than patients with low-EVA1B expression (P<0.0001, 
Supplementary Figure 3D). Likewise, all patients were further 
stratified by WHO grade and IDH mutation. Kaplan-Meier 
curves showed that patients with low EVA1B were significantly 
correlated with a much better OS than patients with high EVA1B 
in the LGG subgroup (P<0.05, Supplementary Figure 3E), HGG 
subgroup (P<0.0001, Supplementary Figure 3F), and IDH-
wildtype subgroup (P=0.0092, Supplementary Figure 3H). Although 
there was no statistical difference between patients 
with low and high EVA1B in the IDH-mutation subgroup 
(P>0.05, Supplementary Figure 3G), the median survival of 
patients with low-EVA1B expression was better than patients 
with high-EVA1B expression (105.1 months and 75.1 
months, respectively).

We also verified the prognostic value of EVA1B for glioma 
patients by the GEO dataset (GSE4412-GPL96, n=74). The details 
of GSE4412-GPL96 were summarized in Supplementary Figure 
4A. Likewise, the 74 glioma patients were divided into low-EVA1B 
and high-EVA1B groups and showed that 
EVA1B overexpression was an unfavorable prognostic indicator for glioma patients.

**Gene Function Annotation and Pathway Analysis**

After determining the prognostic value of EVA1B in glioma, we 
next explored what kind of biological function EVA1B protein
might be associated with. GO and KEGG enrichment analyses were performed, and the top twenty GO terms and signaling pathways are shown in Figures 5A, B. EVA1B gene was associated with many biological processes such as focal adhesion, regulation of actin cytoskeleton, cell proliferation, and tumor necrosis factor. Of note, EVA1B was also associated with NF-kappa B signaling, apoptotic signaling pathway, and TNF signaling pathway. These three signaling pathways are known to be associated with immune and inflammation processes.

To understand the potential interaction proteins of EVA1B, a visual interaction network was generated by GeneMANIA software (Figure 5C). The GeneMANIA results covered 20 EVA1B-interacting proteins, and the proteins are tightly interconnected around BIRC7, NRPI, NAAADL1, CRYBA4, ADAMTS20, CRYBB3, DSPP, CCDC71L, CTDSP1, and LTBP4.

Association Between EVA1B Expression Levels and Tumor-Immune Microenvironment in Glioma

We further explored the correlation between EVA1B expression and the infiltration levels of different immune cells in a cohort of 609 glioma patients. We obtained the immune infiltration status of glioma patients by the TIMER algorithm. As shown in Figures 6A–G, EVA1B expression was shown to be weakly correlated with B cells (r=0.37, P<0.0001), CD4⁺ T cells (r=0.39, P<0.0001), moderately with macrophages (r=0.50, P<0.0001), and also strongly with CD8⁺ T cells (r=0.71, P<0.0001). However, EVA1B was very weakly or not correlated with DCs (r=0.17, P<0.0001). Importantly, there was a strong correlation between EVA1B expression and the overall immune-infiltration levels in glioma (r=0.64, P<0.0001). These findings suggested that EVA1B might be associated with the regulation of immune infiltration of glioma. Next, to verify the correlation between EVA1B and immune infiltration levels, we also calculated the infiltration levels of different immune cells by another commonly EPIC algorithm. As expected, we obtained similar results (Supplementary Figure 5). In addition, Figure 6H shows the correlation between EVA1B expression and the various immune marker genes.

Finally, to assess the predictive performance of EVA1B for the high infiltration levels of immune cells in glioma, ROC curves were conducted to compare the AUC value between EVA1B, PD-L1, CTLA-4, and Siglec15. The results revealed that, compared with other markers, EVA1B presented a higher predictive power (AUC=0.824, 95%CI=0.79-0.86, P<0.0001, Supplementary Figure 6).

DISCUSSION

In this study, we found, for the first time, that EVA1B expression was highly upregulated in glioma tissues compared with normal brain tissues, and increased with tumor grade. The pan-cancer analysis also revealed that EVA1B was broadly and highly expressed in multiple kinds of tumors. In glioma, we observed that EVA1B overexpression was associated with age, WHO grade, histopathology, IDH wildtype, 1p/19q non-codeletion, and marker of invasion, which suggested that EVA1B overexpression may be involved in the malignant behavior of glioma. What is more, multivariate Cox regression analysis showed that EVA1B overexpression is an independent poor factor for the prognosis of glioma patients, which was also confirmed in two other independent cohorts.
enrichment analysis (DAVID) also indicated that EVA1B overexpression was associated with tumor proliferation, focal adhesion, regulation of actin cytoskeleton, anti-apoptotic effect, and the inflammatory response, which was in agreement with the above findings. These results implied that EVA1B overexpression may have played a key role in the malignant phenotypes of gliomas, and is a potentially unfavorable prognostic biomarker for glioma patients. However, the role EVA1B plays in the tumor-immune microenvironment is not reported.

The EVA1 family protein has three members, EVA1A, EVA1B, and EVA1C. To date, functional studies of EVA1B protein in glioma are still not reported. Lan Wang et al. first reported that EVA1A is a novel transmembrane protein (19), and a highly homologous protein of EVA1B. Recently, Bang-Yi Lin et al. further found that, compared with the normal tissues, the EVA1A was overexpressed in thyroid cancer tissues, and overexpression of EVA1A significantly promoted cell proliferation, metastasis, and apoptosis through the Hippo signaling pathway (20). In this study, we observed that EVA1B is associated with many biological processes such as focal adhesion, cell proliferation, and tumor necrosis factor. Focal adhesion mediates cell migration, survival, proliferation, and apoptosis, which are regulated by tyrosine kinase and small G-protein (21). Focal adhesion proteins are integrin-rich microdomains, which transmit mechanical signals from the ECM to activate signaling pathways inside the cell and structurally link the cytoskeleton to the ECM (22). This result is consistent with the
strong correlation between \textit{EVA1B} and vimentin in glioma. Additionally, \textit{EVA1B} was also associated with NF-kappa B signaling, the apoptotic signaling pathway, and TNF signaling pathway. These three signaling pathways were known to be associated with immune and inflammation processes.

Another important finding in this study is that \textit{EVA1B} expression has a positive correlation with the abundance of infiltrating B cells, CD4$^+$ T cells, CD8$^+$ T cells, macrophages, and neutrophils in glioma. Furthermore, the immune cells were involved in the tumor immune network (23). For instance, Neeta Somalia et al. found that LV305 could selectively induce \textit{NY-ESO-1} expression of DCs, which further promotes tumor response in patients (24). The abundance of immune cells affects the outcomes of patients in head and neck squamous cell carcinoma (HNSCC) and lung adenocarcinoma (LUAD) (25, 26), which is in agreement with the results of this study. However, the role of immune cells in tumors is very complex. As studies have reported, on the one hand, DCs are professional antigen-presenting cells, which can capture and process antigen to present antigenic peptides on MHC Class I and Class II to activate CD8$^+$ and CD4$^+$ cells respectively (27). On the other hand, DCs could promote breast cancer bone metastasis via increasing Treg cells and reducing CD8$^+$ cytotoxic T cells (28) and play a crucial role in cell proliferation, invasion, and intercellular communication (5, 29). At present, Treg cells are considered to be one of the main reasons for glioma producing an immunosuppressive microenvironment (30). Several studies have suggested that the proportion of Treg cells increases with the malignant degree of glioma (31).

Previous studies have mainly focused on the correlations between new antigens and survival by genomics (32). A comprehensive understanding of the immune infiltration status of
tumor patients is of great significance for patients in choosing individual immunotherapy (33). In recent years, some immune-associated markers, such as PD-L1, tumor mutational burden (TMB), have been reported to become the indicators for immunotherapy response (34, 35). However, the dynamics of the immune microenvironmnet, other checkpoint molecules, infiltrating immune cells, and immune biomarkers may also be important for the response to immunotherapy (36). In recent years, the study of immune checkpoints has made a breakthrough in the field of tumor immunotherapy, and immune checkpoint blockade has also been approved for the treatment of melanoma and lung cancer (37). Although PD-L1 expression could predict the immunotherapy response of some patients, PD-L1 expression is not enough to predict which patients should be treated with immunotherapy (38, 39). Therefore, an accurate precision biomarker is of great importance to individualized immunotherapy. Jing-Hua Pan et al. (40) recently found that LAYN overexpression is closely related to the abundance of immune cell infiltration in gastric and colon cancer, and may be involved in the regulation of tumor-associated macrophages, DCs, T cell exhaustion, and Tregs function. They concluded that overexpression of LAYN can be used as a molecular biomarker of immune infiltration and survival prognosis in patients with gastric or colon cancer. Similarly, we found that EVA1B was significantly correlated with immune checkpoint blocking molecules PD-1, CTLA4, LAG3, and TIM3, which suggested that EVA1B has the potential to act as a predictive biomarker for the effectors of immune checkpoint blockade in glioma. However, whether the same is true for other types of cancer remains to be studied.

There were limitations to the present study. Firstly, this study did not further verify the functional role of EVA1B in the immune microenvironment of glioma. This subject is very novel and worthy of further study. Secondly, although we found that EVA1B was mainly localized to cytoplasm and membrane in immunofluorescence images from HPA, the result should be verified by different cell lines. Thirdly, the present findings may be the basis for further studies to validate the outcomes.

In conclusion, we discovered for the first time that EVA1B was significantly upregulated in glioma, and increased with tumor grade. EVA1B overexpression was remarkably associated with malignant behavior and poorer prognosis in glioma patients. Another important finding is that the expression level of EVA1B correlates with the infiltration levels of immune cells including B cells, CD4+ T cells, CD8+ T cells, macrophages, and neutrophils. Thus, EVA1B is strongly correlated with the overall immune infiltration levels of glioma and could serve as an independent prognosis biomarker for glioma patients.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: CGGA (http://cgga.org.cn/, Dataset ID: mRNAsseq_325) and TCGA (http://tcga.org.cn/, Dataset ID: mRNAsseq_325) database, and requests for further access to datasets can be directed to huayuanlu402@126.com.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of Lishui People’s hospital. The ethics committee waived the requirement of written informed consent for participation.

AUTHOR CONTRIBUTIONS

JL and HW conceived the study. JL and SQ performed data analysis. SQ wrote the manuscript. HW revised the manuscript. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.648416/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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