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ORIGINAL ARTICLE

Novel roles of VAT1 expression in the immunosuppressive action of diffuse gliomas

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Abstract

Standard treatment regimen of gliomas has almost reached a bottleneck in terms of survival benefit. Immunotherapy has been explored and applied in glioma treatment. Immunosuppression, as a hallmark of glioma, could be alleviated by inhibiting certain abnormally expressed biomarkers. Here, transcriptome data of 325 whole grade gliomas were collected from the CGGA database. The TCGA RNA sequencing database was used for validation. Western blot was used to verify the expression level of VAT1 on cellular level. The results showed that the expression of VAT1 was positively correlated with the grades of glioma as classified by WHO. A higher expression level of VAT1 was observed in the mesenchymal subtype of gliomas. The area under the curve suggested that the expression level of VAT1 might be a potential prognostic marker of mesenchymal subtype. In survival analysis, we found that patients with high VAT1 expression level tended to have shorter overall survival, which indicated the prognostic value of VAT1 expression. The results of gene ontology analysis showed that most biological processes of VAT1-related genes were involved in immune and inflammatory responses. The results of GSEA analysis showed a negative correlation between VAT1 expression and immune cells. We also identified that the expression of immune checkpoints increased with VAT1 expression. Therefore, the high expression level of VAT1 in patients with glioma was a potential indicator of a lower survival rate for patients with gliomas. Remarkably, VAT1 contributed to glioma-induced immunosuppression and might be a novel target in glioma immunotherapy.

Keywords VAT1 · Glioma · Immunosuppression · Immune checkpoint · Prognosis

Abbreviations

CGGA	Chinese Glioma Genome Atlas
DA	Dopamine
GBM	Glioblastoma
GO	Gene ontology
GSEA	Gene set enrichment analysis
GSVA	Gene set variation analysis
IDH	Isocitrate dehydrogenase
Mfn	Mitofusin
NK	Natural Killer
OS	Overall survival
siRNA	Small interfering RNA
TCGA	The Cancer Genome Atlas

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TMZ Temozolomide
VAT1 Vesicle amino transport protein 1

Highlights

1. Immunosuppression could be alleviated by inhibiting certain abnormally expressed biomarkers.
2. Biological processes of VAT1-related genes were functionally involved in the immune response.
3. VAT1 overexpression was a potential indicator of poor survival prognosis.

Introduction

Gliomas are the most common supratentorial brain tumors in adults; their recurrence is inevitable and the mortality rate is high. They are characterized by sustained proliferation, enhanced invasiveness and migration ability, and genetic abnormalities, patients suffering from malignant glioma have poor prognosis despite undergoing the standard treatment regimen [1]. According to the WHO 2016 classification of central nervous system tumors, gliomas are graded into five types based on their cell sources and biomolecular features [2]. For patients with newly diagnosed diffuse glioma, standard treatments should be performed, which includes gross-total resection followed by radiotherapy with concomitant and adjuvant temozolomide (TMZ) chemotherapy, as referred to in the European Association for Neuro-Oncology updated recommendations, published in 2017 [3]. In most patients, the disease caused by malignant gliomas follows an unusually aggressive clinical course, with rapid deterioration of the patients' health and high fatality. Especially for glioblastoma (GBM), the average overall survival is merely 14.6 months, and less than 5% of patients survive beyond five years following diagnosis [4]. For recurrent or secondary GBMs, the median overall survival duration is in the range of 6–9 months despite repeated surgery, radiotherapy, or TMZ chemotherapy [5]. Therefore, a novel therapeutic strategy is urgently needed to overcome the limitations of current treatments and improve the clinical outcome.

As the hallmark of GBM, local immunosuppression has been widely investigated, and recent studies have elucidated the limitations and advantages of immunotherapy. The mechanisms of this phenomenon have been identified in glioma, including a paucity of tumor-infiltrated lymphocytes, high expression level of immune checkpoints, modulation of the local tumor microenvironment by regulatory T cells secreting immunosuppressive cytokines, etc. [6, 7]. Notably, blocking of inhibitory immune-checkpoint proteins such as PD-1, CTLA-4, TIM-3, etc., has emerged as a major potential immunotherapy strategy [8]. Several clinical trials investigating the safety and efficiency of anti-immune checkpoint antibodies have been carried out [9]. The curative effect was satisfactory in general, however, the resistance to the antibodies due to the individual heterogeneity or genetic features of immune checkpoint expression status was inevitable and limited the progress of the immunotherapy. Hence, it is worth exploring potential targets to overcome the resistance to immunotherapy and enhance the efficiency of the treatment.

Vesicle amino transport protein 1 (VAT1) is a 41 kDa integral membrane protein. The majority of VAT1 localizes in the cytoplasm and also resides in the out membrane of mitochondria. VAT1 plays an important role in regulating the storage and secretion of neurotransmitters in the nerve cell terminal [10]. Research into VAT1 expression and its role in diseases has been carried out in the past few years. VAT1 is regarded as a pathogenic factor and even an oncogene in a wide range of tumor types [11, 12]. However, studies focusing on gliomas are rare. Mertsh et al. revealed that the overexpressed VAT1 in GBM was associated with tumor migration, but the exact mechanism and clinical significance of VAT1 remained unknown [13]. In our previous study, we identified that high expression of VAT1 is an indicator of poor prognosis and predicts malignancy in GBM [14].

To further explore the mechanism of VAT1-promoted tumor development and malignant progression, we performed an integrative investigation of VAT1 in glioma using transcriptome data. We found that the differentially expressed genes related to VAT1 were remarkably enriched in immunological processes. Here, we investigated the molecular and clinical characteristics of VAT1 in gliomas. We hypothesized that VAT1 may contribute to glioma-induced immunosuppression and be a suitable target for glioma immunotherapy.

Materials and methods

Samples and data collection

Transcriptome data of 325 patients diagnosed with diffuse glioma (WHO grade II-IV) were obtained from The Chinese Glioma Genome Atlas (CGGA, http://www.cgga.org.cn) [15, 16]. The follow-up information was complete. Overall survival (OS) was calculated from the date of diagnosis to that of death or last follow-up. The mRNA sequencing database from The Cancer Genome Atlas (TCGA) was downloaded from the publicly available database (https://tcgadata.nci.nih.gov/tcga/tcgaDownload.jsp). All patients included in the present study signed the informed consent paper. This

study was approved by the Beijing Tiantan Hospital, Beijing, China.

Detection of glioma biomarkers

As a valuable marker of glioma, isocitrate dehydrogenase (IDH) mutation has recently been shown to be associated with a lower infiltration rate of immune cells [17]. In the CGGA database, IDH1 mutations were detected by pyrosequencing as described in our previous study [18]. In the TCGA database, information on IDH mutation status, downloaded from the TCGA website, was derived from whole exon sequencing or pyrosequencing.

Gene set variation analysis (GSVA)

The signatures of different immune cells were mainly sourced from a previously published study [19]. The dopamine-related gene sets were obtained from the Molecular Signature Database (MSigDB) (http://www.broad.mit.edu/gsea/msigdb/). GSVA analysis was applied to detect the correlation between VAT1 expression and those gene sets [20].

Gene set enrichment analysis (GSEA)

Enrichment analysis of VAT1-related pathways was performed using GSEA software (http://software.broadinstitute.org/gsea/index.jsp). The gene sets of immune checkpoints-related pathways were acquired from the Molecular Signature Database (MSigDB) (http://www.broad.mit.edu/gsea/msigdb/).

Cell transfection

Human glioma cell lines LN229, U251, U87, and H4 were purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. These cell lines were cultured using DMEM (Gibco) with 10% FBS (Gibco) and maintained at 37 °C under 5% CO_2 conditions. Human stem cell lines T2-4 were isolated from human glioma samples in our laboratory according to the protocol used in previous articles [21]. This cell line was cultured using DMEM-F12 (Gibco) supplemented with 2% B27 (GIBCO), 20 ng/ml basic fibroblast growth factor (PeproTech), and 20 ng/ml epidermal growth factor (EGF, PeproTech), and maintained at 37 °C and 5% CO2. The VAT1 small interfering RNA (siRNA) sequences were as follows: siRNA-1, 5′-GGGAGAAGUUGGGAAGCUACG-3′ and siRNA-2, 5′- GCUUUGGAGGCUACGACAAGG-3′ (Guangzhou RiboBio). The siRNA and negative control (NC) (50 nM) were transfected in the cells when the cell density was 30–50% using the riboFECT CP Transfection kit (C10511-1). After 48 h in the incubator, the total proteins were extracted using RIPA buffer (Cell Signaling Technology).

Western blot analysis

In this study, the antibodies used were as followed: anti-VAT1 polyclonal antibody (1:1000; 22,016–1-AP; Biotechnology); anti-CD11B polyclonal antibody (1:1000; 20,991–1-AP; Biotechnology); anti-CD86 polyclonal antibody (1:1000; 26,903–1-AP; Biotechnology); anti-LAG-3 polyclonal antibody (1:1000; 16,616–1-AP; Biotechnology); anti-IDO1 polyclonal antibody (1:1000; 13,268–1-AP; Biotechnology), and anti-actin (1:5000; CW0098M; CWBIO). The secondary antibodies were goat anti-rabbit and goat anti-mouse (1:5,000; ZB-2301 and ZB-2305; OriGene Technologies). Gray scanning analysis was used to compare the protein bands. The protein concentration was measured by Coomassie brilliant blue (APPLYGEN A1011). SDS-PAGE loading buffer (Beijing Solarbio Science & Technology) was added to the proteins and the solution was heated at 100˚C for 15 min. The protein was separated by 10% SDS-PAGE and transferred to a polyvinylidene fluoride membrane (Millipore). The membrane was then blocked with 5% milk (BD Biosciences) on a shaker at room temperature for 1 h. The antibodies were mixed into the milk according to the manufacturer's specifications, and this milk was added to the membrane and kept overnight at 4˚C. After washing the membrane three times for 10 min, the secondary antibodies were added, and the membrane was kept at room temperature for 1 h. The results were detected using an Enhanced Chemiluminescence Western Blotting Detection system (Bio-Rad Laboratories).

Immunohistochemistry

Immunohistochemistry was performed to detect VAT1, CD1, CD3, and CD68 protein expression according to the protocol described in a previous article [22]. CD1, CD3 and CD8 were used as the marker of dendritic cell, T cell and macrophage, respectively. Anti-VAT1/CD1/CD3/CD68 antibodies were purchased from Proteintech and were used at the dilutions specified in the protocol. Each section was individually reviewed and scored by two independent neuropathologists.

Statistical analysis

R (version 3.5.0, http://www.r-project.org) was the main software used for statistical analysis and visualization in this study. Several R packages were used to generate figures, such as ggplot2, pROC, corrgram, and pheatmap. The Pearson correlation analysis was applied for comparison of VAT1 expression level and checkpoint expression

status. The Kaplan–Meier analysis was performed to show the prognostic value of VAT1. The univariate log-rank test and the Cox's proportional hazard model were used in the univariate and the multivariate analyses, respectively. A p value less than 0.05 was regarded as statistically significant in this study.

Results

VAT1 expression was upregulated in GBM

To characterize the expression pattern of VAT1 in gliomas, we examined VAT1 expression level in different grades of glioma as classified by the WHO. The results demonstrated that VAT1 expression increased with the increasing malignancy of glioma (Fig. 1a, b). Compared with low-grade gliomas, the expression level of VAT1 was significantly higher in patients with anaplastic gliomas and GBMs. The expression status of VAT1 in different TCGA subtypes showed a similar trend in CGGA and TCGA databases (Fig. 1c, d). VAT1 expression was the highest in mesenchymal subtype of glioma and the lowest in neural subtype. Receiver operating characteristic (ROC) curves for VAT1 expression and mesenchymal subtype of whole grades glioma were produced. Notably, the area under the curve (AUC) was up to 78.9% and 79.5% in the CGGA and TCGA, respectively (Fig. 1e, f). The above results indicated that VAT1 was strikingly and specifically expressed in high-grade gliomas as well as in mesenchymal subtype. Thus, VAT1 may exert an influence on the development of malignancy in patients with glioma, and high expression of VAT1 may be a potential prognostic marker for mesenchymal molecular subtype of gliomas.

Survival analysis of VAT1 expression status for gliomas

To detect the influence of VAT1 expression on the survival of patients with glioma, we applied the Kaplan–Meier method to conduct survival analysis. In whole grades, patients with high expression levels of VAT1 showed shorter OS in both the CGGA ($p < 0.001$; Fig. 2a) and TCGA data ($p < 0.001$; Fig. 2b). The survival curve of high-grade gliomas also indicated worse prognosis associated with overexpressed VAT1 (Fig. 2c, d). Univariate analysis showed that age, tumor grade, VAT1, IDH mutation, 1p19q codeletion, and MGMT promoter methylation were significantly associated with OS. In the multivariate analysis, tumor grade, VAT1, 1p19q codeletion, and MGMT promoter methylation remained significant in predicting the OS (Table 1). The results suggested that the expression of VAT1 could be a prognostic marker for glioma, and that a high expression level is an indicator of a poor clinical outcome.

VAT1-related biological processes

To identify the biological processes related to VAT1, we performed gene ontology (GO) analysis in CGGA (Fig. 3a) and TCGA (Fig. 3b). Differentially expressed genes related to VAT1 were used for online analysis (DAVID, https://david.ncifcrf.gov/). According to the heatmap, we found that genes positively correlated with VAT1 expression were mainly involved in immune responses and the inflammatory response, and some other functions including cell adhesion, angiogenesis, cell−cell signaling, etc., while the negatively correlated genes were more involved in physiological processes, such as chemical synaptic transmission, neurotransmitter secretion, etc. We also noted that VAT1 was highly enriched in IDH wild-type gliomas in both CGGA and TCGA.

Tumor-specific T cells can specifically target and kill invading tumor cells by identifying antigenic peptides presented by human antigen class I molecules on the surface of cancer cells [23]. To further explore the correlation between VAT1 expression and immune cells, we performed GSVA analysis on both databases. The signatures of diverse immune cells were obtained from a previous study [19]. In CGGA, the results demonstrated that natural killer (NK) cells with CD56 bright, B cell, effector memory T cells, CD8 + T cells, follicular helper T cells, and central memory T cells presented an obvious negative correlation with VAT1 (Fig. 3c). In TCGA, a similar result was observed (Fig. 3d). These results indicated that VAT1 may play a role in tumor immune escape and immunosuppression of gliomas. These results were further validated by the analysis of tumor tissues in in vitro immunohistochemistry experiments. The results showed that T cell infiltration was positively correlated with VAT1 expression (Fig. 3e).

Given that dopamine (DA) has been reported to be an important molecule, bridging the nervous and immune systems [24], we performed a GSVA analysis between VAT1 and DA pathways-related genes. The results demonstrated that VAT1 expression was negatively correlated with the release of DA, while DA binding related genes were increased with VAT1 expression (Supplementary Fig. 1a, b). The potential mechanism of DA-induced immune response is elaborated upon in the discussion section.

The co-inhibitory function of VAT1 and other immune checkpoints in tumor-induced immune response

Immune checkpoints have been shown to be upregulated in various tumors. Overexpression of immune checkpoints is

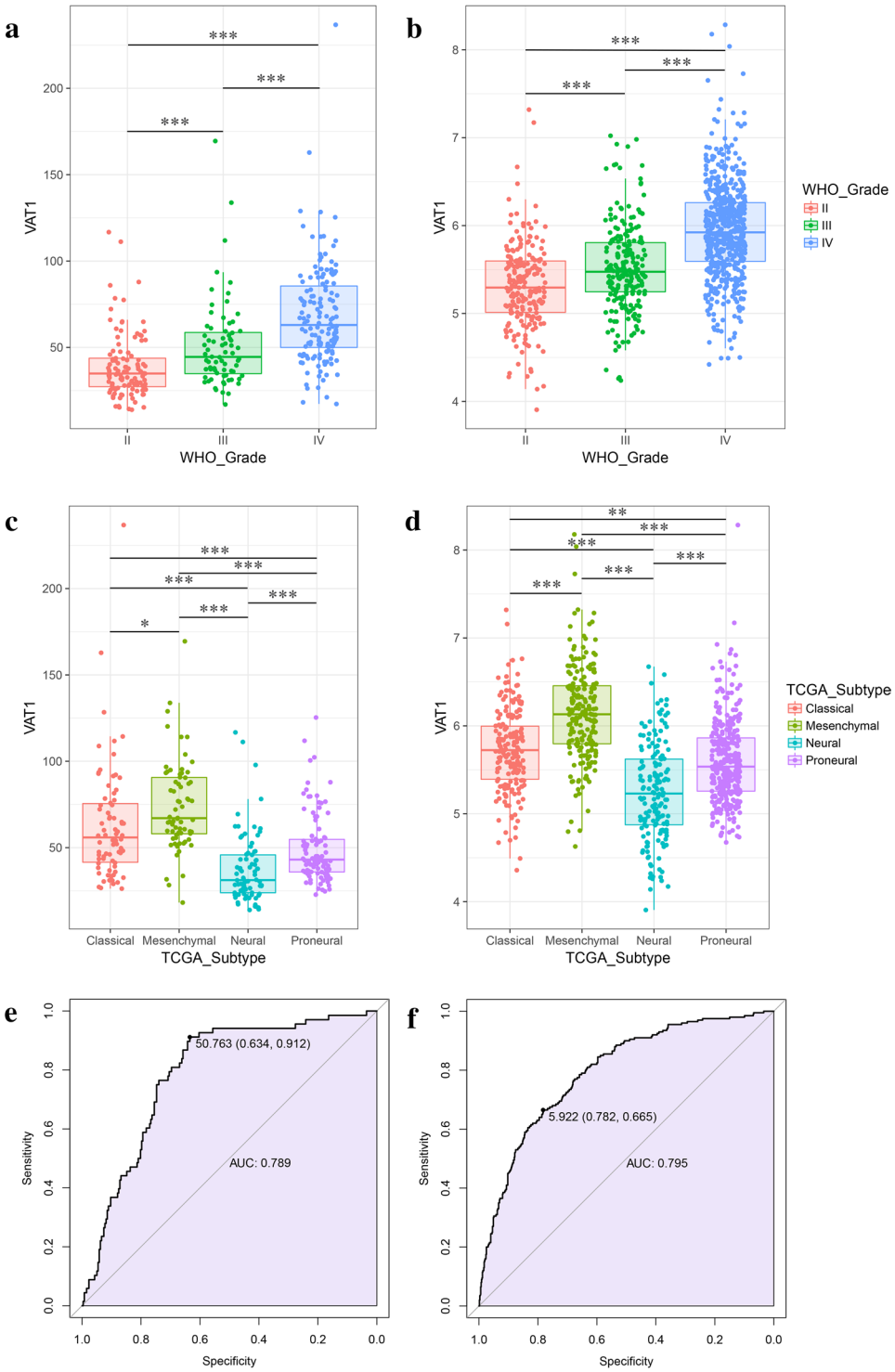

Fig. 1 The expression status of VAT1 in gliomas. **a**, **b** VAT1 expression status in different grades of gliomas taken from the CGGA (left) and TCGA (right) databases. **c**, **d** VAT1 expression status in diverse subtypes of glioma from the CGGA and TCGA databases. **e**, **f** ROC curve showing the predictive value of VAT1 expression for mesenchymal subtype of glioma in the CGGA and TCGA databases. * $p<0.05$; ** $p<0.01$; *** $p<0.001$

related to immune escape as well as immune cell exhaustion. The results of correlation analysis showed that some immune checkpoints were positively correlated with VAT1 expression in CGGA, including HAVCR2 ($r=0.322$, $p<0.001$), PDCD1LG2 ($r=0.293$, $p<0.001$), PDCD1 ($r=0.169$, $p=0.002$), LAG3 ($r=0.211$, $p<0.001$), ITGAM ($r=0.224$, $p<0.001$), CTLA-4 ($r=0.162$, $p<0.001$), and IDO1 ($r=0.246$, $p<0.001$) (Fig. 4a). In TCGA, we found that VAT1 expression was significantly correlated with CD274 ($r=0.160$, $p<0.001$), ITGAM ($r=0.320$, $p<0.001$), PDCD1 ($r=0.211$, $p<0.001$), PDCD1LG2 ($r=0.257$, $p<0.001$), and HAVCR2 ($r=0.372$, $p<0.001$) (Fig. 4b). The subsequent analysis showed the correlation between each marker in CGGA and TCGA, respectively (Fig. 4c,

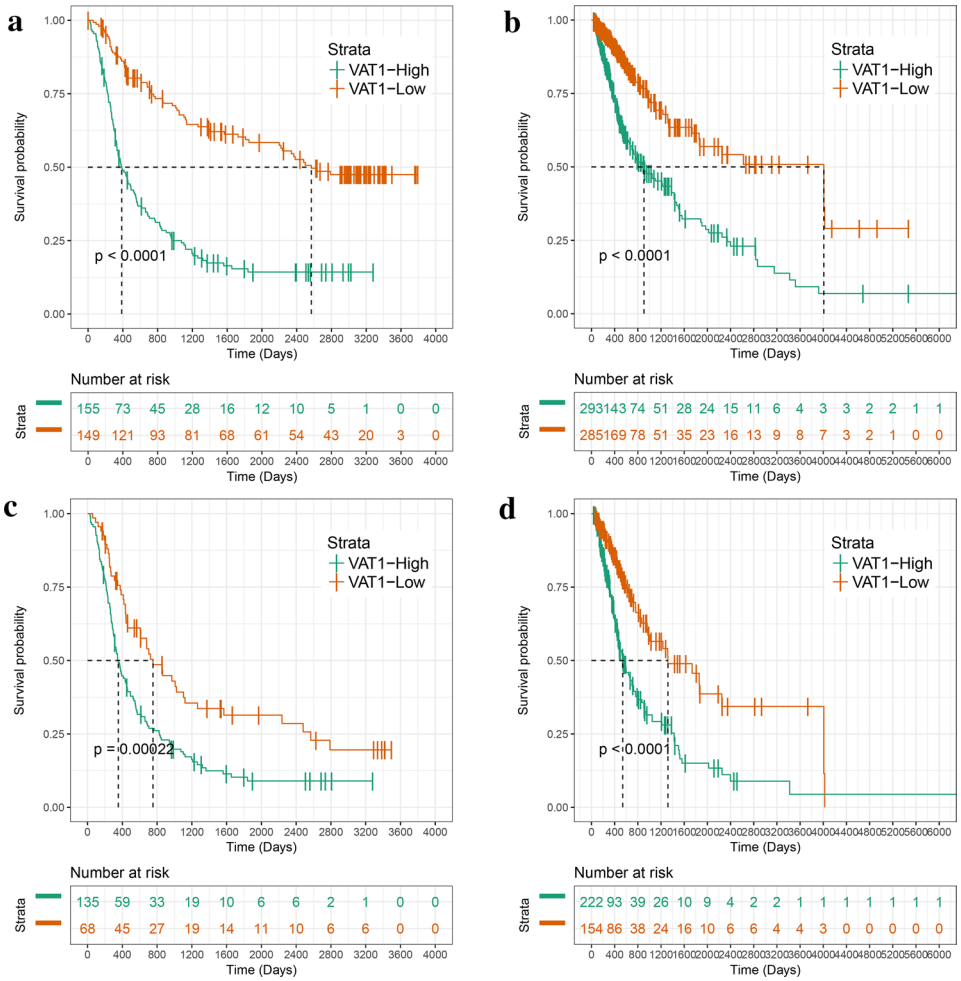

Fig. 2 The influence of VAT1 expression on prognosis for patients with glioma. **a**, **b** The Kaplan–Meier survival curve showing the overall survival of patients with glioma of all grades with different levels of VAT1 expression from the CGGA (left) and TCGA (right) databases. **c**, **d** The Kaplan–Meier survival curves showing the overall survival of patients with high-grade glioma (WHO grade III and IV) with different VAT1 expression levels from the CGGA and TCGA databases

d). These results further confirmed that VAT1 expression showed a positive correlation with immune checkpoint upregulation. Therefore, there might be a synergistic effect between VAT1 and immune checkpoint members in tumor-induced immune responses.

After detecting the correlation between VAT1 and immune checkpoints, we investigated the pathway enrichment status related to VAT1 by GSEA analysis. The result showed that VAT1 positively related genes were primarily involved in TIM-3-related signaling pathways (Fig. 5a, b). We also performed GSEA analysis using other checkpoints-related pathways (CTLA-4, PDCD1, LAG3, and PDCD1LG2), and similar results were observed, supporting a close connection between VAT1 and immunity (Supplementary Fig. 2).

VAT1 knockdown decreased the expression level of immune checkpoints

According to the results described above, we identified a correlation between VAT1 expression and immune checkpoints at the transcriptome level. To further verify the relationship, we used glioma cell lines to demonstrate the correlation at the cellular level. The results of western blotting showed that the expression of immune checkpoints decreased after knocking down the expression of VAT1 by transfecting with siRNA1 or siRNA2 (Fig. 6a, Supplementary Fig 3). The bar plots showed that the expression of IDO1 decreased remarkably in the three VAT1-deficient cell lines (Fig. 6b–d). The expression of ITGAM declined in VAT1-deficient LN229 and H4 (Fig. 6b, d), and the expression of CTLA-4 was positively correlated with VAT1 in U251 and H4 (Fig. 6c, d). However, we failed to detect any correlation between VAT1 expression and LAG-3 based on the western blot results.

Considering that the main features of tumor stem cells may closely resemble the original tumor, the experiments were repeated using glioma stem cells. In accordance with our expectation, the expression of immune checkpoints was decreased after knocking down VAT1 expression (Fig. 6e, f).

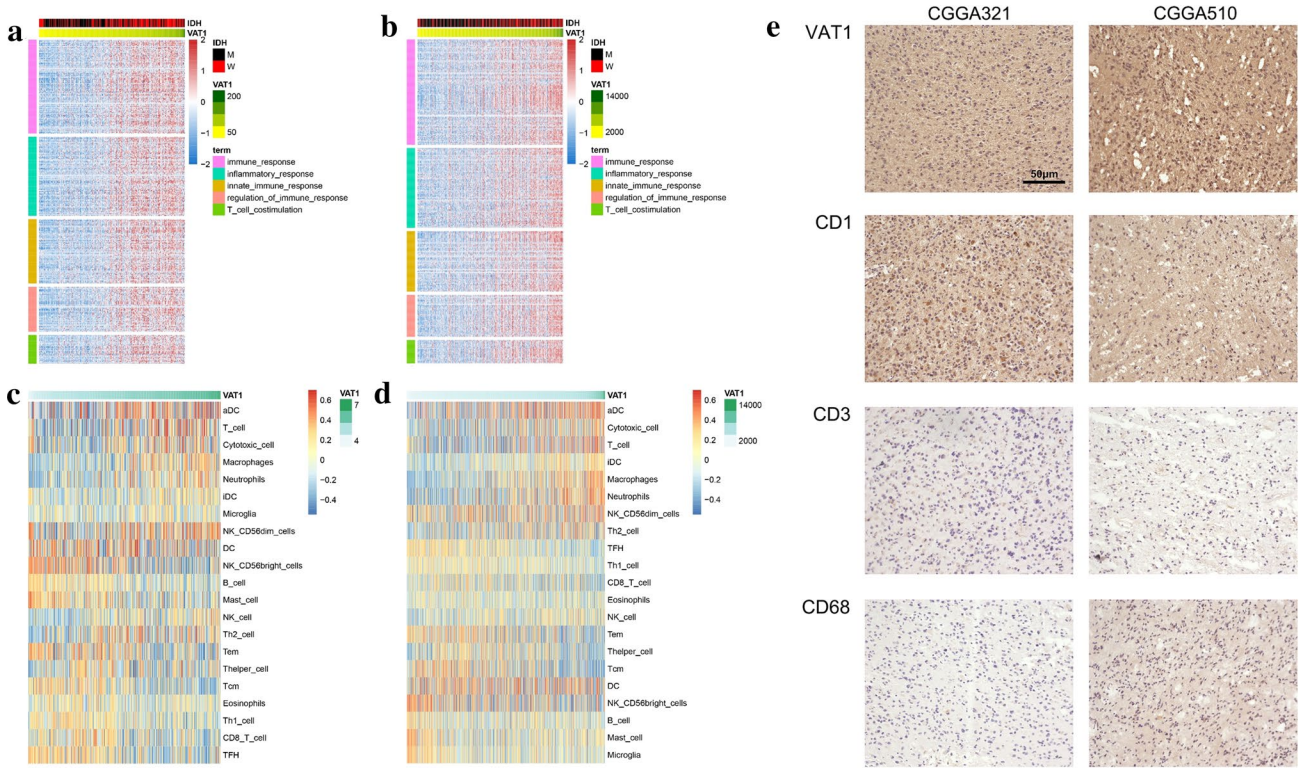

Fig. 3 **a, b** Gene ontology analysis demonstrating the biological processes related to VAT1 expression in data from the CGGA (left) and TCGA (right) databases. **c, d** GSVA analysis on CGGA and TCGA data showing the correlation between VAT1 expression and the expression of immune cell markers. **e** Immunohistochemistry results showing that the expression of dendritic cell, T cell and macrophage markers (CD1, CD3, and CD68) was based on VAT1 expression

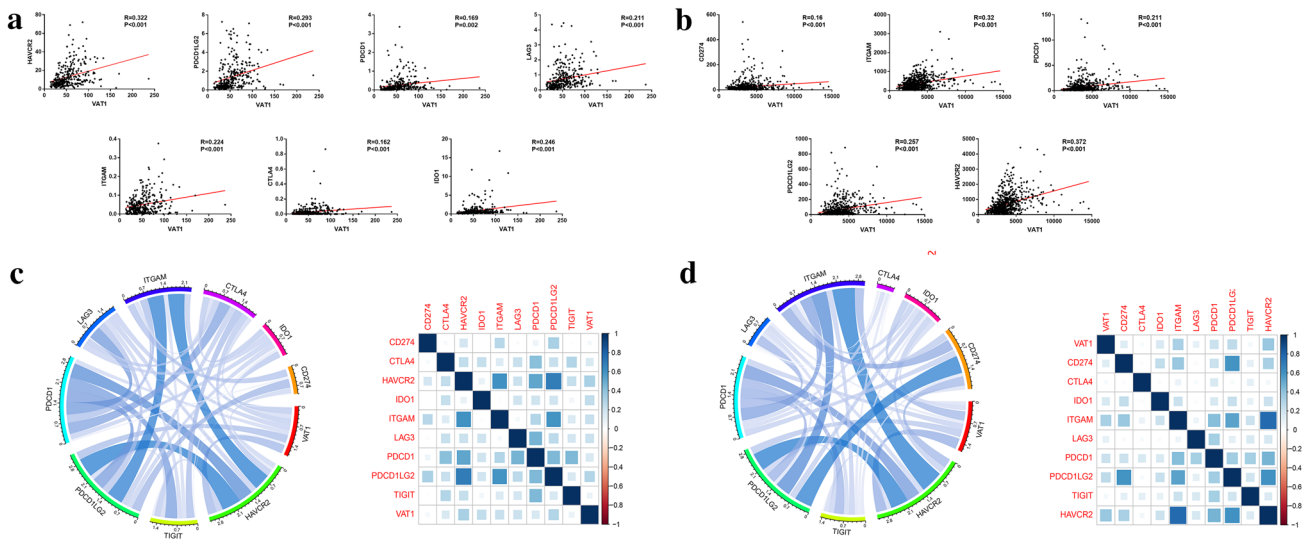

Fig. 4 The correlation between VAT1 expression and the expression of immune checkpoints in data from the CCGA (**a, c**) and TCGA (**b, d**) databases

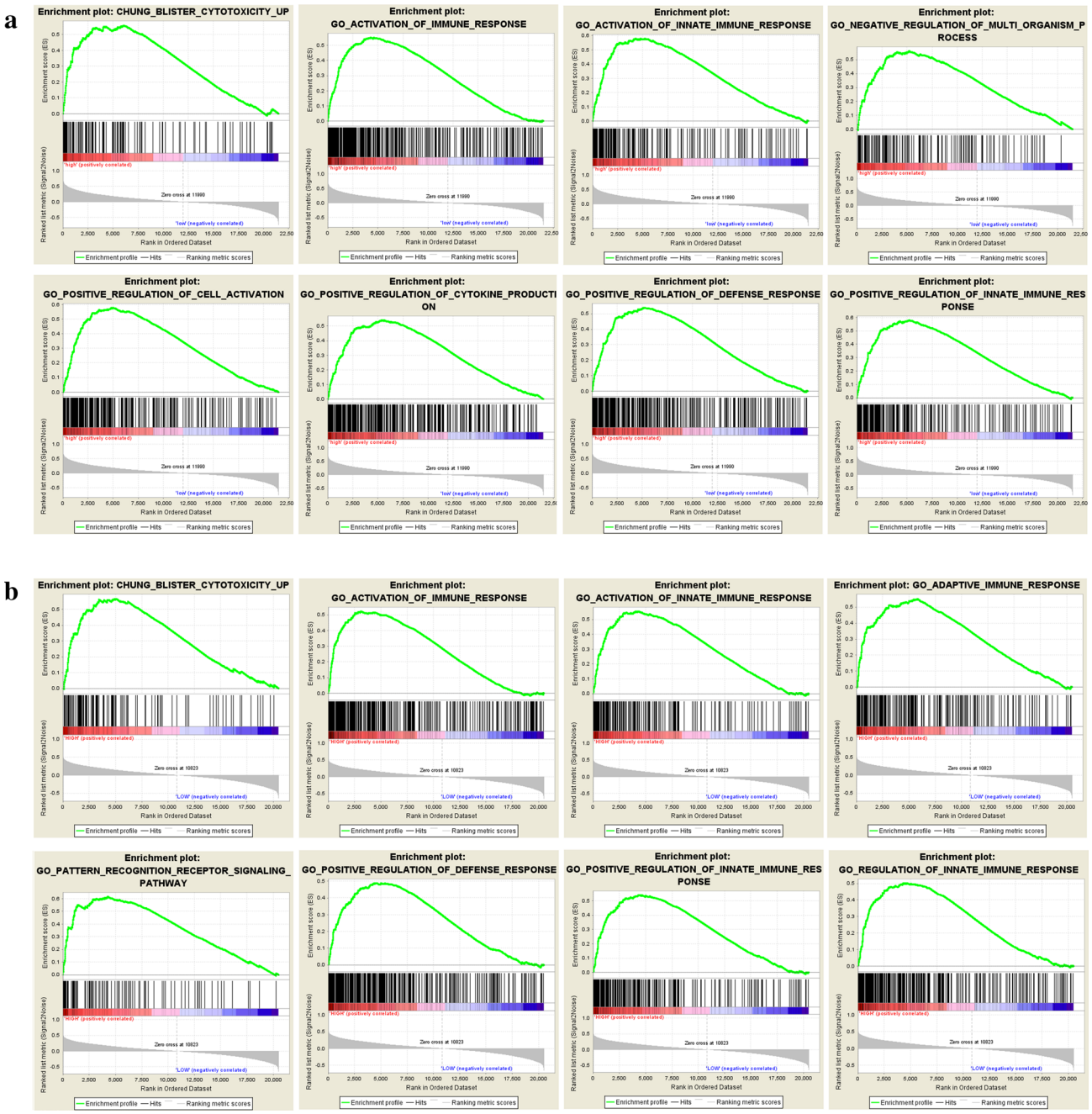

Fig. 5 GSEA analysis of data from the CGGA (**a**) and TCGA (**b**) databases showing the TIM-3-related pathway enrichment status of VAT1 positively associated genes. The NES value is greater than 2 and FDR q value is less than 0.05 in all figures

Discussion

Gliomas are the most common primary brain tumor in adults and show highly invasive behavior. As such, they have a high recurrence and mortality rate. The standard treatment regimen has almost reached a bottleneck in the improvement of survival benefits for gliomas. Therefore, novel therapeutic approaches are urgently needed [25]. Although the results are diverse, new technologies and methods are emerging in succession, such as tumor vaccines, oncolytic viral therapies, immune checkpoint inhibitors, chimeric antigen T cell therapy, and tumor-treating field therapy [26–30]. Recently, noteworthy results have shown that some differentially expressed genes or dysfunctional molecules may reveal the tumorigenesis, progression, and recurrence, and they may further serve as potential therapeutic targets. Among them, the use of antibodies of immune checkpoints is regarded as an indisputably important advance in cancer therapy

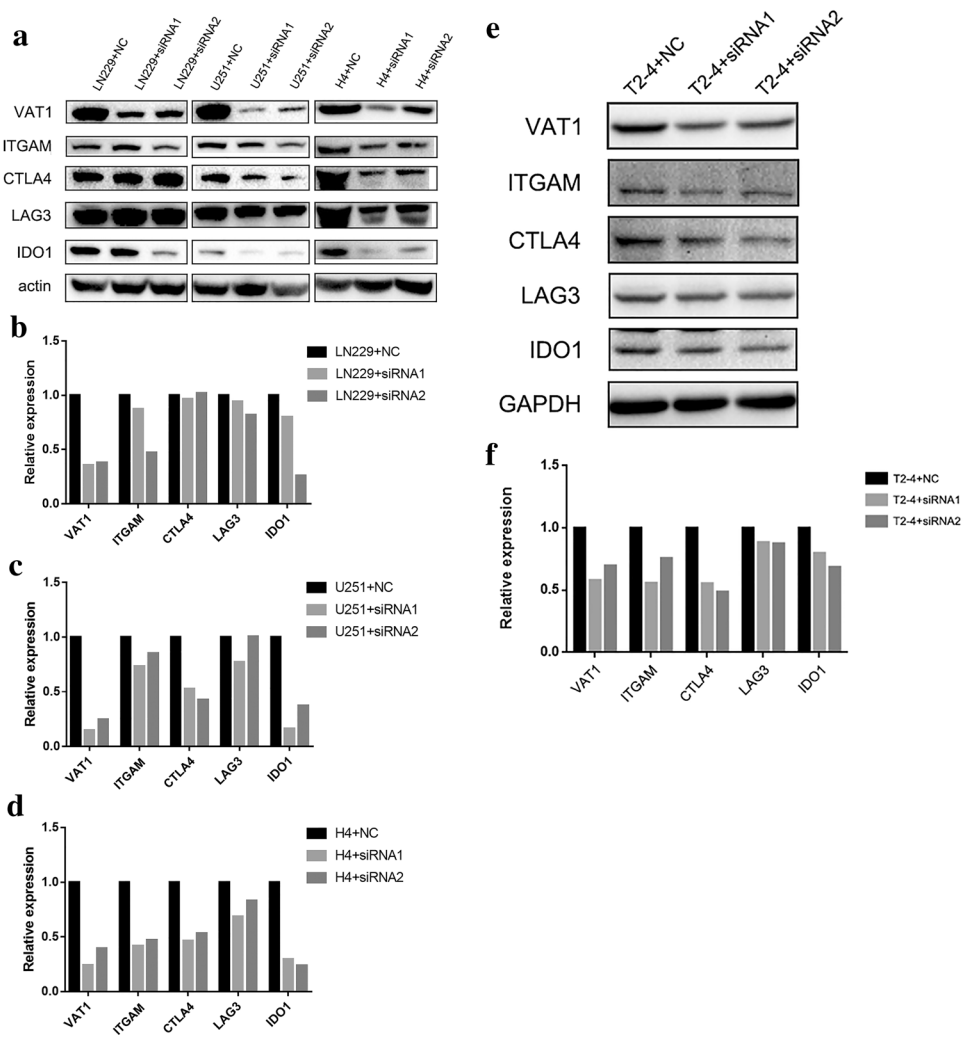

Fig. 6 Western blot analysis results showing the alteration of the expression of immune checkpoints in different VAT1-deficient cell lines (**a**) and in a stem cell line (**e**). Gray scanning analysis was used to compare the protein bands in LN229 (**b**), U251 (**c**), H4 (**d**), and T2-4 (**f**)

Table 1 Univariate and multivariate analyses for overall survival in CGGA database

	Univariate analysis		Multivariate analysis	
	HR (95%CI)	P value	HR (95%CI)	P value
Age	1.035 (1.022–1.049)	**< 0.001**	1.010 (0.997–1.024)	0.139
Gender	0.959 (0.717–1.282)	0.777		
Tumor grade	2.800 (2.307–3.400)	**< 0.001**	1.897 (1.467–2.454)	**< 0.001**
VAT1	1.016 (1.013–1.020)	**< 0.001**	1.008 (1.003–1.013)	**0.002**
IDH mutation	0.347 (0.257–0.469)	**< 0.001**	0.853 (0.551–1.321)	0.477
1p19q codeletion	0.233 (0.146–0.371)	**< 0.001**	0.407 (0.218–0.758)	**0.005**
MGMT promotor methylation	0.576 (0.423–0.784)	**< 0.001**	0.695 (0.498–0.969)	**0.032**
TERT promotor mutation	0.777 (0.551–1.096)	0.151		

* Bold font indicates statistical significance ($p < 0.05$)

considering its effect on improved prognosis [31]. PD-1, PD-L1, CTLA-4, TIM-3, LAG-3, etc., have been reported as immune checkpoints. Blockades of these checkpoints are correlated with survival benefits [32–34]. However, conflicting results limited the development of immunotherapy. The heterogeneous expression of immune checkpoints, resistance to monotherapy, and the complicated regulatory mechanisms of the immune microenvironment of gliomas

may be the probable factors leading to this phenomenon. Therefore, combination or multi-drug treatment may be an appropriate method to overcome this barrier. In this study, we discovered that VAT1 was closely correlated with the immune response by applying RNA sequencing analysis. We also investigated the biological function of VAT1 in gliomas, and fully explored the connection between VAT1 and tumor immunity.

Compared with low-grade gliomas, high-grade gliomas showed overexpression of VAT1 at the mRNA level, especially in GBMs. The VAT1 expression level was also different among TCGA subtypes, and our results suggested that VAT1 could be a potential prognostic predictor of the mesenchymal subtype of glioma. Based on the results of this study, we postulated that the poor prognosis of the mesenchymal subtype of gliomas might be correlated with the immune escape and immunosuppressive microenvironment of gliomas. Moreover, we investigated the influence of VAT1 on clinical outcome. The survival curves demonstrated that patients with a high expression level of VAT1 were correlated with poor prognosis. The survival time corresponding to 50% survival probability in the VAT1 high expression group was less than half of that in the low expression group. Following GO analysis, the correlation between VAT1 expression and immune processes was observed. With the increase of VAT1 expression level, genes involved in immune response, inflammatory response, and T cell co-stimulation were upregulated strikingly. The subsequent GSVA analysis showed that besides T cells, some other immune cells also presented a negative correlation with VAT1 expression. Given that VAT1 is localized at mitochondrial and cell membranes, we performed a correlation analysis between VAT1 expression and immune checkpoints. We found that VAT1 was positively correlated with those immune checkpoints, which suggested a potential novel treatment target. To further validate the results derived from transcriptome data, we detected the expression level of immune checkpoints in different VAT1 knockdown cell lines. The results demonstrated that the expression of some immune checkpoints was decreased in VAT1-deficient cell lines, which provided validation on cellular level. Given the individual heterogeneity of gliomas and the interaction between biomarkers, combination treatments might be an effective method and could maximize the curative effect. Previous studies have investigated the safety and efficacy of combined anti-PD-1 antibody and anti-CTLA-4 antibody in treating recurrent GBM. However, the combination strategy was not tested beyond phase III clinical trials because of its serious adverse effects [35]. Kim et al. observed that plenty of exhausted tumor-infiltrating T cells presented positive for both PD-1 and TIM-3 in a mouse glioma model, and treatment using both anti-PD-1 and anti-TIM-3 antibodies enabled the glioma to be controlled satisfactorily [9]. The combination of anti-TIGIT (the hallmark of exhausted NK cells) and anti-PD-L1 antibodies could enhance the innate immune response as well as the NK cell-dependent adaptive immunity. Thus, the dual blockade of PD-L1 and TIGIT might be a promising anticancer therapeutic strategy [36]. As stated above, we have reason to believe that VAT1 may be a novel molecular target and the dual blockade of VAT1 and other immune checkpoints may reverse the immunosuppression status induced by tumor. Further experiments are needed to validate the synergistic effect of VAT1 with other immune checkpoints.

To further explore the influence of VAT1 on immune regulation, we examined a large amount of literature and found that VAT1 may also play the role of forming exosome-like vesicles to regulate the immune response. In recent years, the role of extracellular vesicles secreted by tumor cells in modulating the immune response has been given serious attention [37, 38]. VAT1 may facilitate intercellular communication and immunological modulation by storing and transferring monoamines, such as norepinephrine, epinephrine, and DA [10]. DA, as a neuroregulatory and immunoregulatory molecule, aids activation of T cells and promotes the immune response [39, 40]. Therefore, we speculated that the upregulation of VAT1 may lead to a decrease in DA release, and subsequently reduce the activation of T cells.

To highlight another aspect of VAT1, it has been reported that, through possessing ATPase activity and Ca^{2+}-dependent oligomeric-complex-forming activity, VAT1 shows synergy in negatively regulating mitochondrial fusion with mitofusin (Mfn) protein [41–44]. Mitochondria-shaping proteins control the dynamic equilibrium between fusion and fission of mitochondria. The balance is strictly regulated and is required in diverse biological processes, including cell metabolism, death, proliferation, and migration [45]. Pearce et al. found that mitochondrial dynamics could control the fate of T cells through metabolic programming [47]. They identified that mitochondria fusion in memory T cells promoted efficient oxidative phosphorylation, while fission in effector T cells led to imbalanced redox status known as the "Warburg effect." This metabolic insufficiency may contribute to T cell exhaustion. As exhaustion markers identified by previous studies, PD-1, Tim-3, CTLA-4, etc., were found to be immune checkpoint molecules inducing T cell exhaustion. Therefore, we inferred that the high expression level of VAT1-promoted mitochondrial fission by synergizing with Mfn protein. The altered mitochondrial dynamic could unbalance the metabolic processes of T cells, leading to T cell exhaustion. And the upregulated immune checkpoints presenting on T cells further exacerbated the immunosuppression.

In conclusion, we believe that VAT1 is a potential pathogenetic factor promoting tumor development and is closely

linked to tumor-induced immunosuppression. The inhibition of VAT1 may enhance the tumor-induced immune response and provide a promising clinical prognosis for patients with glioma.

Supplementary Fig.1 GSVA analysis of data from the CGGA (A) and TCGA (B) databases showing the correlation between VAT1 expression and the expression of dopamine-related pathways. (PDF 3953 kb) Supplementary Fig.2. GSEA analysis of data from the CGGA database showing the immune checkpoint-related pathway enrichment status of VAT1 positively correlated genes. Enrichment status of CTLA-4-related signaling pathway (A), PDCD1-related pathway (B), LAG3-related pathway (C), and PDCD1LG2-related pathway (D). The NES value is greater than 2 and FDR q value is less than 0.05 in all figures. (PDF 1265 kb) Supplementary Fig.3 (related to Figure 6A) A demonstration of full-length gels and blots in western blot analysis. (PDF 288 kb)

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Compliance with ethical standards

Conflict of interest The authors declare no competing financial interests.

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