Histone deacetylase 3 expression correlates with vasculogenic mimicry through the phosphoinositide3-kinase/ERK–MMP–laminin5γ2 signaling pathway

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Vasculogenic mimicry was first reported by Maniotis et al.1 in 1999. It refers to the process by which highly aggressive tumor cells independently mimic endothelial cells to form vessel-like structures that aid in supplying enough nutrients to rapidly growing tumors. These VM structures have previously been observed in a number of cancers, such as breast, ovarian, lung, and renal cancers and Ewing sarcoma.2,3 Furthermore, our previous study indicated that VM exists in gliomas and is a significant prognostic factor for patient survival.4

Before VM was reported, researchers attempted to use anti-angiogenesis as an alternative to frequently ineffective surgical resections and chemoradiotherapies, owing to the fact that glioma growth depends on abundant blood perfusion.2,5 Research that involves targeting endothelial cells has been the therapeutic target for gliomas.6,7 However, recent studies have found that anti-angiogenic therapy alone may not be effective; or worse, it may elicit greater malignancy.6,7 As a supplementary theory of angiogenesis, VM may account for the failure of antivascular therapy.8,9 Mounting evidence has focused on VM and its close correlation with poor prognosis.2,3,10,11 Many recent studies have shown that some genes and corresponding proteins are involved in VM, including VE-cadherin,12,13 EphA2,14–17 MMPs17–20 and Ln5γ2 (LAMC2).8–11 The AKT and ERK signaling pathways also play key roles in VM; whereas, in VM, the two signaling pathways did not act upstream or downstream from each other. Taken together, our findings showed that HDAC3 contributed to VM in gliomas, possibly through the PI3K/ERK–MMPs–laminin5γ2 signaling pathway, which could potentially be a novel therapeutic target for gliomas.
inhibitors also inhibited AKT and ERK signaling pathways.\(^{30,31}\) However, the relationship between HDAC3 and VM in glioma is currently unknown. To expand our knowledge regarding VM and the biological function of HDAC3, the current study was designed in an attempt to identify the contribution of HDAC3 to VM, thereby providing novel therapeutic strategies for gliomas.

Materials and Methods

**Tissue specimens.** Tissue collection and analysis in this study were approved by the Research Ethics Committee of Southern Medical University (Guangzhou, China). Glioma tissues were obtained from the Department of Pathology, Zhujiang Hospital at Southern Medical University between 2010 and 2013. All tissues were randomly collected from patients who did not undergo any therapy before undergoing surgery. Tumor sections were reviewed by two neuropathologists to verify the diagnosis of glioma according to the 2007 WHO classification of tumors of the central nervous system.

**Cell culture.** The human U87MG (Laboratory Animal Center, Sun Yat-sen University, GuangZhou, China) glioma cell lines were cultivated in high glucose DMEM (HyClone, Logan, UT, USA) supplemented with 10% FBS (HyClone) in 5% CO\textsubscript{2} at 37°C.

**Immunohistochemical and CD34-PAS histochemical double staining.** For immunohistochemical staining, tumor tissue sections (5 mm) were prepared and deparaffinized in xylene, hydrated by standard procedures described in our previous study.\(^{32}\) To determine the expression of HDAC3, slides were incubated with a rabbit mAb against HDAC3. Five visual fields of each tissue section were selected randomly under a microscope (Leica, Newcastle, UK) at 400× magnification. The number of stained cells and the total number of cells were counted in the five visual fields, and the ratio between the stained and total cells was calculated. The following definitions were used for the stained cell ratio: −/+, <10% negative or weakly positive for expression of HDAC3; ++, 20–50% strongly positive for expression of HDAC3; and ++++, >50% very strongly positive for expression of HDAC3. The −/+ rating was considered as low expression of HDAC3; ++ and ++++ were both considered as high expression of HDAC3.

To identify the VM structures, CD34/PAS histochemical double staining was carried out. After immunohistochemical staining for CD34, slides were stained following the PAS staining procedures before lightly counterstaining with hematoxylin. The sections were lightly stained with eosin after these procedures. Detailed information of antibodies used in this study is listed in Table 1.

For diagnosis of VM, sections were scanning under microscope carefully. CD34/PAS vascular-like structures containing red blood cells formed by glioma cells were identified as positive for VM: VM channels were lined with tumor cells with nuclei stained dark blue by hematoxylin. The channels were rich in ECM that can be highlighted pink or pink-purple by PAS, whereas the luminal surfaces of channels could be highlighted brown (negative for CD34 reaction). In hollows, red blood cells stained by eosin red or grey-red can be observed.

**Vasculogenic mimicry channel density.** The median number of VM channels was counted under a microscope. Tumor sections were observed under 200× magnification to first identify the accumulation of VM channels. Next, we chose the areas that contained the most VM channels to determine the median number of VM channels observed per field at 400× magnification.

**Tube formation assay.** Tube formation was observed by 3-D culture, as described in our previous study.\(^{33}\) Briefly, 24-well culture plates were coated with Matrigel Basement Membrane Matrix (0.5 mL/well) (BD Biosciences, Franklin Lakes, NJ, USA), and then allowed to polymerize at 37°C for 60 min. Cells (2.5 × 10\textsuperscript{5} cells/mL) were seeded onto the surface of Matrigel (1 mL/well) and then incubated without serum for 6 h. To investigate the effect of SAHA (Sigma, St. Louis, MO, US), cells were treated with SAHA at indicated concentrations (2 and 4 µM) for 12 h before being seeded onto the Matrigel.\(^{34,35}\) Cultures were photographed by microscope after 6 h. Formation of VM was quantified by the total length of tubes (complete structures) and the number of intersections (complete structures) per field in five randomly chosen fields using image analysis software (Image-Pro Plus, Washington, DC, USA).

**Western blot analyses.** Cell lysates were harvested with the Total Protein Extraction Kit (KeyGEN, Nanjing, China). Protein concentration was determined by the BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Equal quantities (20 µg) of protein were separated electrophoretically on 10% SDS–polyacrylamide gel and transferred onto PVDF membranes (Millipore, Billerica, MA, USA). The blots were incubated with appropriate primary antibodies, followed by incubation with secondary antibodies. The blots were detected using Pierce ECL plus Western Blotting Substrate (Waltham, MA, USA). A monoclonal GADPH antibody was used for protein loading analyses. Detailed information of the antibodies used in this study is listed in Table 1.

**Quantitative real-time PCR.** For the glioma tissue samples, total RNA was extracted by QiaGen RNeasy FFPE Kit
For cells, total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA). RNA integrity was checked by gel electrophoresis. Reverse transcription was carried out with random primers and Reverse Transcriptase M-MLV (RNase H-; Takara, Dalian, China). The mRNA expression level was determined by SYBR Premix Ex Taq (Tli RNaseH Plus; Takara) and ABI ViiA7 Detection System (Applied Biosystems, Foster, CA, USA); the sequences of target gene-specific primers are listed in Table 2. All reactions were carried out with the following program: 30 s at 95°C, followed by 44 cycles of 95°C for 3 s and 60°C for 34 s. For the internal control for normalization, 18S mRNA was used. The relative expression of transcripts was analyzed using the 2^-ΔΔCt method.

**RNA-mediated interference.** The siRNAs were purchased from GenePharma (Shanghai, China). Target sequences of siRNAs were 5'-CCGCCAGACAAUCUUGAATT-3' (HDAC3-1), 5'-CGGUGUCCUCCACAAAUATT-3' (HDAC3-2), 5'-GCAGGUGUUUAGAAGUGUAUTT-3' (LAMC2), 5'-CCGACAUCAUGUACUUCUUTT-3' (MMP-14), and 5'-CGGUGUCUCCACAAAUATT-3' (negative control). For siRNA, U87MG cells were plated onto 6-well dishes at a concentration of 2.5–5 × 10^5 cells/well and cultured for 24 h. Then, 50 pmol siRNA was transfected into 70% confluent U87MG cells for 24–48 h using Lipofectamine RNAiMAX Reagent (Life Technologies, Carlsbad, CA, USA). Cells were then lysed for Western blot, and the isolated RNA was subjected to reverse transcription. Meanwhile, we used a positive control (GAPDH siRNA) and fluorescein-labeled (FAM-) negative control to ensure the reliability of the method and transfection efficiency.

**Statistics.** All experiments were carried out at least in triplicate. The data analysis was carried out with spss version 13.0 (SPSS Inc., Chicago, IL, USA). All P-values were two-sided, and P < 0.05 was considered statistically significant.

**Results**

**Relationship between VM and clinicopathological data in glioma tissues.** Twenty-six specimens (25.49%) with VM structures were identified out of 102 glioma specimens by CD34-
### Table 3. Relationship between vasculogenic mimicry (VM), histone deacetylase 3 (HDAC3), and clinicopathological data of patients with glioma

| Parameters | Cases | VM Positive | VM Negative | \( \chi^2 \) | \( P \)-value | HDAC3 High | HDAC3 Low | \( \chi^2 \) | \( P \)-value |
|---|---|---|---|---|---|---|---|---|---|
| Gender | | | | | | | | | |
| Male | 61 | 16 | 45 | 0.044† | 0.834 | 17 | 30 | 14 | 1.677† | 0.432 |
| Female | 41 | 10 | 31 | | | 7 | 23 | 11 | | |
| Age, years | | | | | | | | | |
| <40 | 45 | 11 | 34 | 0.982† | 0.610 | 10 | 23 | 12 | 0.425† | 0.990 |
| \( \geq 40 \) to <60 | 32 | 10 | 22 | | | 10 | 16 | 6 | | |
| \( \geq 60 \) | 25 | 5 | 20 | | | 4 | 14 | 7 | | |
| Tumor size, cm | | | | | | | | | |
| <5 | 53 | 14 | 39 | 0.824† | 0.943 | 15 | 24 | 14 | 2.549† | 0.280 |
| \( \geq 5 \) | 49 | 12 | 37 | | | 9 | 29 | 11 | | |
| Grade, WHO | | | | | | | | | |
| I | 6 | 0 | 6 | 7.502† | 0.048* | 0 | 2 | 4 | 33.390† | 0.000* |
| II | 40 | 6 | 34 | | | 7 | 19 | 14 | | |
| III | 23 | 7 | 16 | | | 6 | 13 | 4 | | |
| IV | 33 | 13 | 20 | | | 11 | 19 | 3 | | |
| KPS | | | | | | | | | |
| <65 | 45 | 12 | 33 | 0.059† | 0.810 | 6 | 16 | 23 | 3.018† | 0.221 |
| \( \geq 65 \) | 57 | 14 | 43 | | | 13 | 24 | 20 | | |
| HDAC3 | | | | | | | | | |
| Low \(-/-\) | 43 | 6 | 37 | 6.315† | 0.043* | – | – | – | | |
| High ++ | 40 | 12 | 28 | | | – | – | – | | |
| High +++ | 19 | 8 | 11 | | | – | – | – | | |
| VM | | | | | | | | | |
| Positive | 23 | – | – | | | 8 | 12 | 6 | 6.203† | 0.045* |
| Negative | 76 | – | – | | | 11 | 28 | 37 | | |

*Statistical analyses were carried out using the \( \chi^2 \)-test (asymptotic significance, two-sided). †Fisher’s exact test (two-sided); *\( P < 0.05 \) was considered significant; \(-/-\), <10% negative or weakly positive HDAC3 expression; ++, 20–50% strongly positive HDAC3 expression; ++++, >50% very strongly positive HDAC3 expression; KPS, preoperative Karnofsky performance scores.

PAS dual staining (Fig. 1). Here, clinicopathological data of glioma specimens with VM (n = 26) were compared to those without VM (n = 76). The results are summarized in Table 3.

The results showed that the pathological grade (based on WHO standards) of glioma differed significantly between groups with VM and without VM (\( P = 0.048 \)). Vasculogenesis mimicry was detected preferentially in high-grade gliomas: 13 of 33 WHO grade IV (39.39%), seven of 23 WHO grade III (30.43%), and six of 40 WHO grade II (15%). Vasculogenesis mimicry was not detected in any WHO grade I gliomas. No \( +/+ \) expression was detected in any WHO grade I gliomas. No \( \geq 65 \) cases. Moreover, a significant difference in VM numbers was observed when HDAC3 was compared to HDAC3++ or without VM. This difference was statistically significant (\( P = 0.045 \)). There was no significant association between the expression of HDAC3 and gender, age, tumor size, or Karnofsky performance score (Table 3).

The mRNA levels of HDAC3 in 102 glioma tissues were also analyzed by qPCR. Prior to qPCR, samples were divided into two groups based on the presence or absence of VM. The results show that the mRNA levels of HDAC3 in VM-positive gliomas were significantly higher than those in VM-negative gliomas (\( P < 0.001 \); Fig. 2d).

More VM detected in glioma tissues with increased HDAC3 expression. The analysis presented in Table 3 shows that VM was detected in six cases (13.95%) with HDAC3 \(-/-\), 12 cases (30%) with HDAC3++, and eight cases (42.11%) with HDAC3+++ (Fig. 3a). The positive rate of VM showed a significantly sharp increase with increased HDAC3 expression (\( P = 0.043 \)). Higher expression of HDAC3 (HDAC3++ and HDAC3++++) showed a greater VM-positive rate than did HDAC3 \(-/-\) (\( P = 0.022 \)).

Vasculogenesis mimicry channel density showed that the median value of the VM channels was 0.5 ± 0.29 in HDAC3 \(-/-\) samples, 2.07 ± 0.32 in HDAC3++ samples, and 3.38 ± 0.37 in HDAC3+++ samples (Fig. 3b). The highest median value of VM channels was detected in HDAC3+++ cases. Moreover, a significant difference in VM numbers was observed when HDAC3+++ was compared to HDAC3++ or
HDAC3++ combined with HDAC3 $-/-$ groups ($P = 0.024$ and $P = 0.007$, respectively). Furthermore, Figure 3(c) shows a positive correlation between VM and HDAC3 in relation to tumor grade in glioma tissues.

**Downregulation of HDAC3 inhibited VM in U87MG cells.** The U87MG cell line was transfected with HDAC3-siRNAs. The tube formation assay was then carried out after the HDAC3-depleted cells had been evaluated by qPCR and Western blot. Quantitative PCR (Fig. 4a) showed that HDAC3 mRNA transcripts apparently declined in the HDAC3-1 ($P < 0.001$) group and HDAC3-2 group ($P < 0.001$) compared with that in the U87MG group. Additionally, for HDAC3-1 and HDAC3-2 groups, HDAC3 mRNA transcript in the HDAC3-1 group declined the most ($P < 0.001$). Consistent with the qPCR results, the Western blot analysis (Fig. 4b) showed a lower HDAC3 expression in the HDAC3-2 group and, in particular, in the HDAC3-1 group, compared to that in the U87MG group. The tube formation assay results showed that relatively more well-formed tubular structures were found in the HDAC3 group (for number of intersections, $45.71 \pm 0.57$; for total tube length, $7382 \pm 116 \mu m$) (Fig. 4c,d). By contrast, the HDAC3-1 group (for number of intersections, $6.57 \pm 0.57$; for total tube length, $905 \pm 29 \mu m$) and HDAC3-2 group (for number of intersections, $29.71 \pm 0.61$; for total tube length, $4490 \pm 59 \mu m$) did not form tubular networks efficiently (Fig. 4c). Similar results were also observed when we reduced HDAC3 expression by SAHA, an HDAC3 inhibitor (Doc. S1, Fig. S1).

**Influence of HDAC3 on VM-related molecules.** U87MG cells were treated with HDAC3-siRNAs or SAHA, and the expression of HDAC3 and VM-related molecules was determined by qRT-PCR or Western blot analysis. We found that, when HDAC3 was depleted in U87MG cells, the mRNA transcripts (Fig. 5a) of MMP-2 ($P = 0.001$), MMP-14 ($P < 0.001$), and LAMC2 ($P < 0.001$) in the HDAC3-1 group were significantly lower than that in U87MG group; the Western blot results were similar (Fig. 5b). No other molecules showed differences in expression when compared to the U87MG group. Similarly,
the cells subjected to SAHA treatments had lower expression of MMP-2, MMP-14, and LAMC2 compared to that of the U87MG group (Fig. 5c).

Fig. 4. Histone deacetylase 3 (HDAC3) influences vasculogenic mimicry (VM) in U87MG cells. (a) U87MG cells were transfected with siRNAs and HDAC3 mRNA levels were detected by quantitative RT-PCR. Gene expression was normalized to that of the housekeeping gene 18S rRNA. Data are presented as fold induction relative to the expression of the U87MG group and are represented as mean ± SD (*P < 0.05). (b) Western blot and densitometry analysis of HDAC3 protein between groups transfected with siRNA (*P < 0.05). (c) U87MG cells subjected to different treatments were seeded into wells of a 24-well plate coated with Matrigel for 6 h then photographed. Scale bar = 100 μm. (d) Total length of VM tubes and the number of intersections per field were compared between groups in (c) (*P < 0.05 compared with U87MG).

First, U87MG cells were treated with U0126 (ERK inhibitor; 10 μM, 30 min) (Cell Signaling Technology, Boston, MA, USA) or LY294002 (PI3K inhibitor; 2 μM, 60 min; Cell Signaling Technology), the levels of p-ERK or p-AKT showed a significant reduction, respectively (Fig. 6b). We also found that the two inhibitors caused a similar mild reduction in the expression of both MMP-14 and LAMC2 compared to that in U87MG group. Then U87MG cells were treated with LY294002 plus U0126. Results showed that all molecules, except AKT and ERK, detected by Western blot, showed a significant reduction. Interestingly, the expression of MMP-14 and LAMC2 was more significantly reduced in cells treated with LY294002 plus U0126 than those treated with only one inhibitor (LY294002 or U0126) (Fig. 6b).

Discussion
The HDAC3 gene, which has been extensively researched in epigenetics, has been reported to be overexpressed in the
majority of carcinomas, including gliomas, and may be one of the most frequently upregulated genes in cancer.(27,28) However, there were no data supporting the correlation between HDAC3 expression and VM. In this report, we present evidence that HDAC3 has an important facilitative role on VM in gliomas.

We first found that both VM structures and HDAC3 expression have a positive correlation with tumor grades: the higher the tumor grade, the higher the number of VM structures present or HDAC3 expression. These results are consistent with the findings of a previous study.(3,28) Further analysis showed that HDAC3 was upregulated in VM-positive glioma tissues (Table 3, Fig. 2d); furthermore, VM could be frequently detected in glioma tissues with increased HDAC3 expression (Fig. 3a,b). Clearly, all of these results indicate that HDAC3 was closely correlated with VM in glioma tissues. Then we found a significant decrease in VM when HDAC3 expression was altered in U87MG cells (Figs 4,S1), which was consistent with the observations in glioma tissues, indicating that the mechanism underlying the role of HDAC3 in promoting the development of VM in gliomas can be elucidated by cellular level experiments.

Certain molecules, such as VE-cadherin,(12,13) EphA2,(14–17) MMPs,(17–20) and LAMC2(8–11) have been confirmed as VM-related molecules. Of these molecules, we found that MMP-2/14 and LAMC2, but not MMP-9, EphA2, or VE-cadherin, were downregulated in both transfected and inhibitor-treated U87MG cells. These results (Fig. 5) indicated that VM was regulated by HDAC3 probably by way of the MMPs and LAMC2 signaling pathways without the players EphA2, MMP-9, or VE-cadherin.

Previous studies reported that SAHA could inhibit cell proliferation through inhibition of the AKT and ERK signaling pathways, also involved in VM.(31,37–40) In this study, a significant decrease in levels of both phospho-AKT and phospho-ERK were found when we altered HDAC3 levels by siRNA (Fig. 6a), which indicated that AKT and/or ERK signaling pathways may indeed be involved in VM formation regulated by HDAC3. We then used U87MG cells with AKT and ERK inhibitors to further verify AKT and/or ERK involvement and investigate how they worked together, as previous studies had reported that ERK and AKT could interact in various ways.(41,42) Vasculogenic mimicry was evaluated by expression of MMP-14 and LAMC2, as we had confirmed that MMP-14 and LAMC2 were indeed involved in VM (Doc. S1, Fig. S2). Results (Fig. 6b) showed that phospho-AKT decreased, with no change observed in phospho-ERK, when AKT expression was inhibited by LY294002; similarly, phospho-AKT did not
decrease with the inhibition of ERK by U0126, indicating that the ERK or AKT signaling pathways did not act upstream or downstream from each other. However, all molecules except ERK and AKT showed a significant reduction in expression when U0126 and LY294002 were both used and expression of MMP-14 and LAMC2 showed a relatively
higher reduction than when cells were treated with only one inhibitor (LY294002 or U0126). These interesting results (Fig. 6b) clearly indicated that the PI3K and ERK signaling pathways play key roles in VM. More importantly, ERK or AKT signaling pathways did not act upstream or downstream from each other.

Our study provides a novel insight into the mechanisms underlying VM, and may contribute to the development of a novel therapeutic target for gliomas. However, the methods used in our study are mainly in vitro experiments, and all the experimental data were only verified in U87MG cells. Animal experiments are needed to confirm the data obtained from these cellular level experiments. In addition, our laboratory previously reported that TGF-β was required for in vitro VM in U251MG cells, and MMP-14 was correlated with TGF-β-induced VM. However, TGF-β can also regulate the activation of the ERK and PI3K signaling pathways, which need the participation of HDACs, in particular, HDAC3. Thus, we may further investigate whether the HDAC3 pathways stated in this study are involved in the process by which TGF-β regulates VM (Fig. 7). Other studies reported that hypoxia-inducible factor-1α has been shown to induce VM in hepatocellular carcinoma and melanoma, and, interestingly, HDAC3 has been described as a hypoxia-inducible factor-1α-regulated gene. Furthermore, hypoxia enhances HDAC function, for example, HDACs are closely involved in angiogenesis. Hence, it would be of interest to investigate whether the HDAC3 pathway is also involved in processes where hypoxia regulates VM (Fig. 7). All in all, further delineation of the mechanisms of VM regulated by HDAC3 may potentially provide a novel antiglioma therapeutic target.

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Disclosure Statement
The authors have no conflict of interest.

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Histone deacetylase 3 (HDAC3) inhibitor influenced vasculogenic mimicry in U87MG cells.

Supporting Information

Additional supporting information may be found in the online version of this article:

Doc. S1. Histone deacetylase 3 (HDAC3) inhibitor influenced vasculogenic mimicry (VM) in U87MG cells and MMP-14 and Ln5γ2 were involved in VM.

Fig. S1. Histone deacetylase 3 (HDAC3) inhibitor influenced vasculogenic mimicry in U87MG cells.

Fig. S2. Matrix metalloproteinase-14 and laminin5γ2 are involved in vasculogenic mimicry.