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Angiogenin Upregulation Independently Predicts Unfavorable Overall Survival in Proneural Subtype of Glioblastoma

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Abstract

Objective: Angiogenin is a small protein that exerts potent stimulating effects on angiogenesis. In this study, we aimed to examine the expression of angiogenin in different subtypes of glioblastoma and estimated its independent prognostic value. **Methods:** The genomic and survival data from The Cancer Genome Atlas-glioblastoma were extracted for a secondary study. Results The expression of angiogenin was upregulated in glioblastoma tissues and varied significantly in different subtypes. Although the proneural subtype had the lowest angiogenin expression, high angiogenin expression was associated with significantly worse overall survival. However, this association was not observed in other subtypes. By performing univariate and multivariate analysis using Cox regression model, we observed that high angiogenin expression was an independent indicator of shorter overall survival in proneural glioblastoma (hazard ratio: 1.669, 95% confidence interval: 1.033-2.696, $P = .036$), after adjustment of age, gender, isocitrate dehydrogenase 1 mutation, temozolomide chemotherapy and radiation therapy. In addition, we also observed a correlation between elevated angiogenin expression and the hypomethylated status of its DNA. The hypermethylation group had significantly better overall survival. **Conclusions:** Angiogenin upregulation might serve as a biomarker for unfavorable overall survival in the proneural subtype of glioblastoma.

Keywords

ANG, overall survival, proneural subtype, glioblastoma

Abbreviations

ANG, Angiogenin; CI, confidence interval; GBM, glioblastoma; HR, hazard ratio; IDH1, isocitrate dehydrogenase 1; MGMT, O(6)-methylguanine-DNA methyltransferase; OS, overall survival; ROS, receiver–operating characteristic; TCGA, The Cancer Genome Atlas

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Introduction

Glioblastoma (GBM) is a group of heterogeneous diseases, among which 4 subtypes (proneural, neural, classical, and mesenchymal) were identified by gene expression pattern established by The Cancer Genome Atlas (TCGA) researchers.[1] Proneural subtype has a higher frequency of *PDGFRA* or isocitrate dehydrogenase 1 (*IDH1*) mutations. Classical subtypes show an abnormally high level of *EGFR* amplification and homozygous deletions of *CDKN2A*.[1] Mesenchymal tumors usually contain hemizygous deletions of NF1.[1] In comparison, no unique mutations have yet been observed in neural cases.[1,2] Each subtype varied significantly in survival length and treatment response. Among the 4 subtypes, the proneural cases have the best survival.[1,2] Although several strong prognostic indicators have been identified in GBM, such as isocitrate dehydrogenase (*IDH*) mutations and O(6)-methylguanine-DNA methyltransferase (*MGMT*) promoter methylation,[3] the use of

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these markers in clinical practices still has some limitations due to the heterogeneous properties of GBM.

Angiogenin (encoded by *ANG* gene) is a 14-kD ribonuclease that exerts potent stimulating effects on angiogenesis during neovascularization process. It shares 33% sequence identity to the pancreatic RNAse A sequence and is also called RNase5.[4] Angiogenin interacts with actin of both endothelial and smooth muscle cell surface and forms protein complex that initiates the proteolytic cascades via elevating the expression of proteases and plasmin.[5,6] This process leads to the degradation of the basement membrane and extracellular matrix, which enables the endothelial cells to penetrate and migrate into the perivascular tissue.[7] Therefore, it does not only play an essential role in normal physiological processes but also involves in the pathological development of tumors.

Previous studies found that *ANG* expression was elevated in numerous tumors, such as colorectal,[8] breast,[9] cervical,[10] ovarian,[11] stomach,[12] liver,[13] pancreatic[14] tumors and gliomas.[15] Nuclear translocation of *ANG* is essential to its angiogenic activity.[16] However, not all tumors had nuclear expression of *ANG*.[17] One previous study demonstrated that the glioblastoma (GBM) cells had nuclear expression of *ANG*.[17] Inhibition of its nuclear translocation suppresses its ribonucleolytic activity and 45S ribosomal RNA synthesis.[17] Another study observed that *ANG* promotes GBM cell proliferation via activating the nuclear factor κB signaling pathway and inhibiting the expression of its binding partner Four and a half LIM domains protein 3.[18] These findings confirmed that *ANG* has functional roles in GBM cells.

In this study, using genomic and survival data from the TCGA-GBM, we explored the expression of *ANG* in different subtypes of GBM and estimated its independent prognostic value in each subtype.

Materials and Methods

In Silico *Analysis Using Data From TCGA-GBM*

The data in TCGA-GBM were acquired with the access provided by the UCSC Xena Browser (https://xenabrowser.net/heatmap/). Only the primary tumor cases without histological neoadjuvant treatment were included in this study. Gene expression was quantified by Affymetrix Human Genome U133 Array Strip (AffyU133a). The following variables were extracted from the database for subsequent analysis: age at initial pathologic diagnosis, gender, *IDH1* mutation status, gene expression subtypes, karnofsky performance score, overall survival (OS) status and time, temozolomide chemotherapy status, radiation therapy status, *ANG* expression, and *ANG* DNA methylation (quantified by Infinium HumanMethylation27 BeadChip, which includes cg22723026 and cg19211827 in *ANG* DNA). O(6)-methylguanine-DNA methyltransferase promoter methylation status was determined by the mean methylation status of the MGMT-STP2 model,[19] which includes 2 CpG sites (cg12434587 and cg12981137).

Statistical Analysis

Statistical analyses were conducted using SPSS 25.0 software (SPSS, Chicago, Illinois) and GraphPad Prism 7.04 (GraphPad Inc, La Jolla, California). One-way analysis of variance with Bonferroni post hoc tests and Welch t test (unequal variances t test) were performed to assess the statistical differences. Characteristics of patients (categorical variables) in the 2 groups were analyzed by χ^2 test with 2-sided Fisher exact test. Kaplan-Meier OS curves were generated using GraphPad Prism 7.04. Receiver–operating characteristic (ROC) analysis for death detection was performed to identify the best cutoff (Youden index) for *ANG* expression to separate the patients. Log-rank testing was used to assess the differences between the curves. The independent prognostic value of *ANG* expression in the proneural subtype was assessed using the univariate and multivariate Cox regression models. $P < .05$ was considered statistically significant.

Results

ANG *Expression Was Upregulated in GBM Tissues and Varied Significantly in Different Subtypes*

By extracting the microarray data of gene expression in TCGA-GBM, we studied the expression of *ANG* in GBM tissues and the adjacent normal tissues. Results showed that *ANG* expression was generally upregulated in GBM tissues (Figure 1A and B). Besides, its expression varied significantly among the 4 subtypes of GBM. Mesenchymal subtype and proneural subtype had the highest and lowest *ANG* expression, respectively (Figure 1C and D).

High ANG *Expression Was Associated With Significantly Shorter OS in Proneural Subtype But Not Other Subtypes of GBM*

Then, we generated Kaplan-Meier survival curves to investigate the association between *ANG* expression and OS in each subtype of GBM. By using the best cutoff model, we found that high *ANG* expression was associated with significantly shorter OS in proneural subtype ($P = .002$; Figure 2D) but not other subtypes of GBM (Figure 2A-C).

Expression of ANG *in Proneural Subtype Might Be Related to its DNA Methylation Status*

The *IDH1* mutation does not only serve as a powerful prognostic marker but also influences the expression of a series of genes via indirectly influencing the activity of DNA demethylases.[20] In this study, we examined the correlation between *IDH1* mutation and *ANG* expression in proneural subtype.

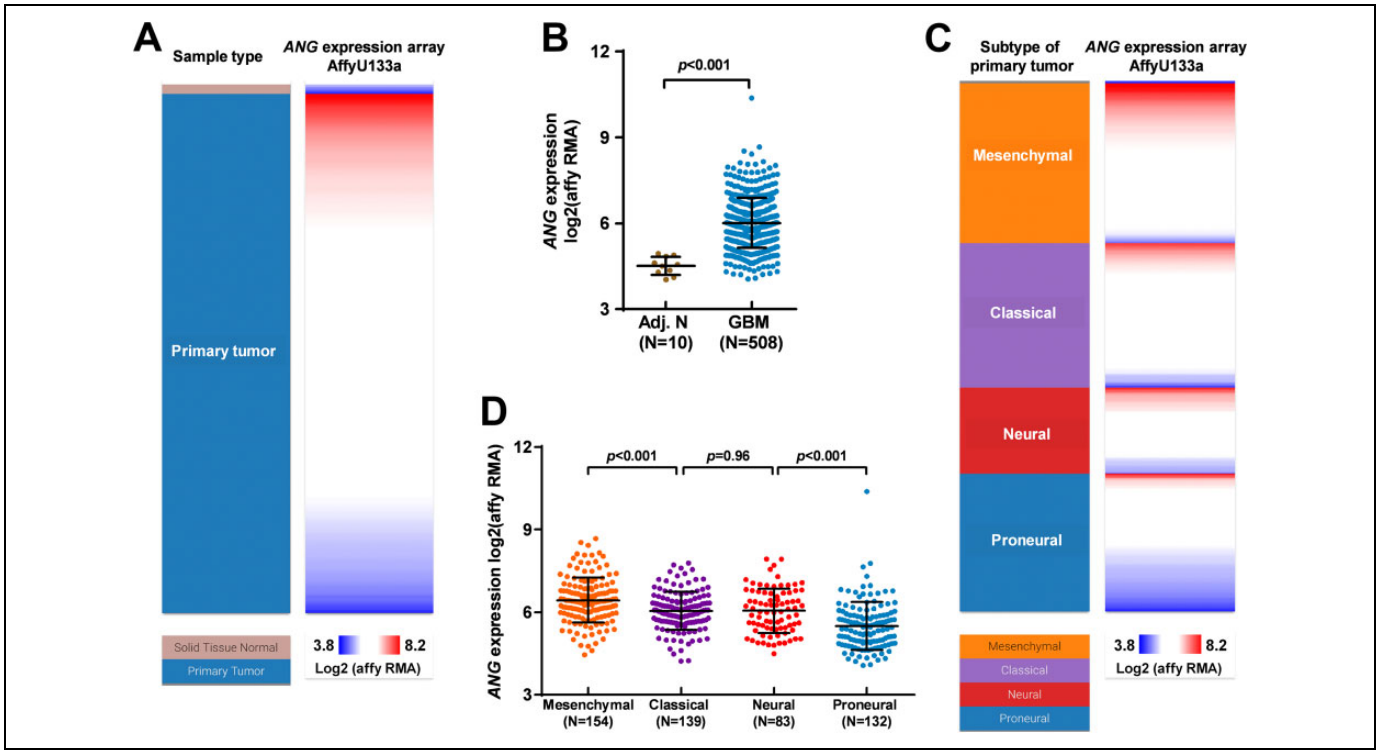

Figure 1. Angiogenin (*ANG*) expression was upregulated in glioblastoma (GBM) tissues and varied significantly in different subtypes. A-D, Heatmap (A and C) and plots chart (B and D) showing the expression of *ANG* in GBM tissues and in the adjacent normal tissues (A-B) and among the 4 subtypes of GBM (C-D). Data were extracted from The Cancer Genome Atlas (TCGA)-GBM.

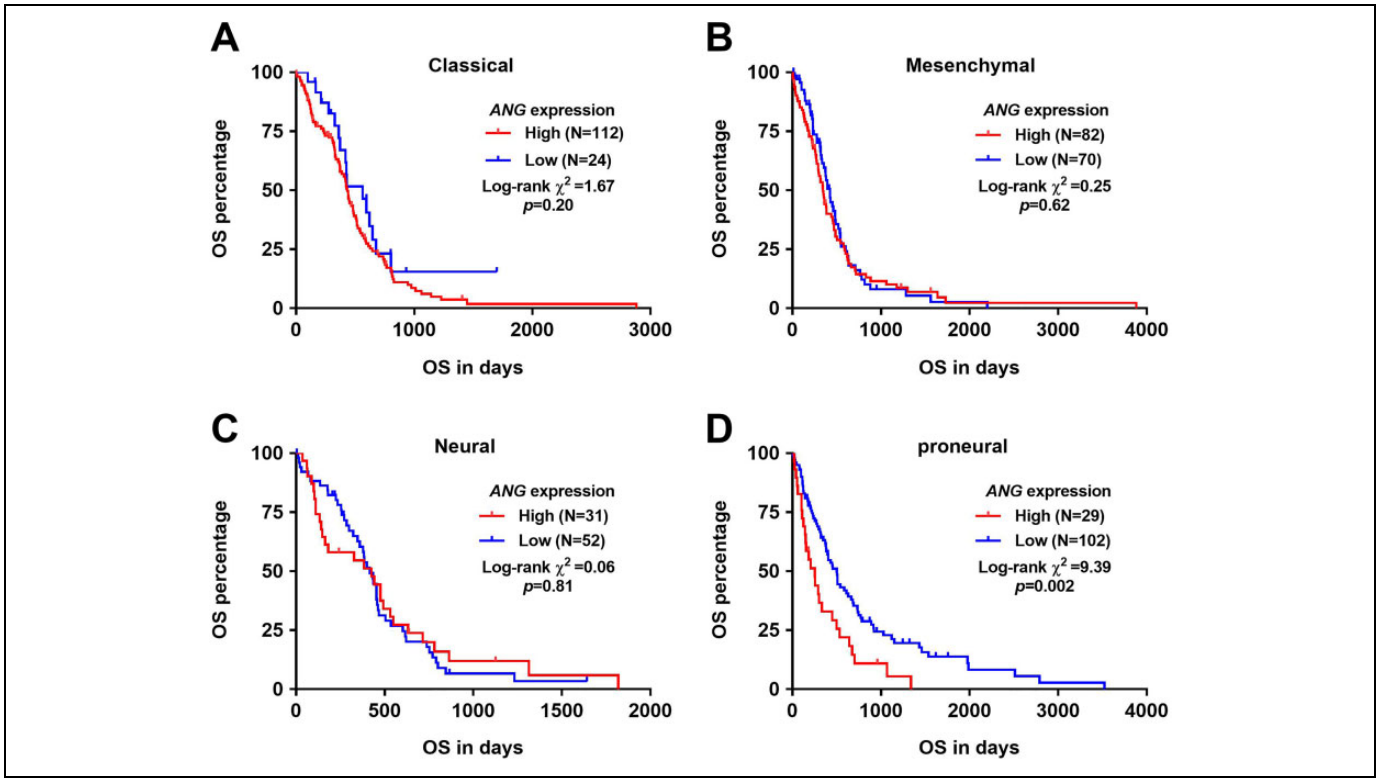

Figure 2. High angiogenin (*ANG*) expression was associated with significantly shorter overall survival (OS) in proneural subtype but not other subtypes of GBM. A-D, Kaplan-Meier curves of OS in each subtype of glioblastoma (GBM): classical (A), mesenchymal (B), neural (C), and proneural (D). The Youden Index of *ANG* expression in the receiver–operating characteristic (ROC) analysis for death detection was applied as the cutoff to separate the patients.

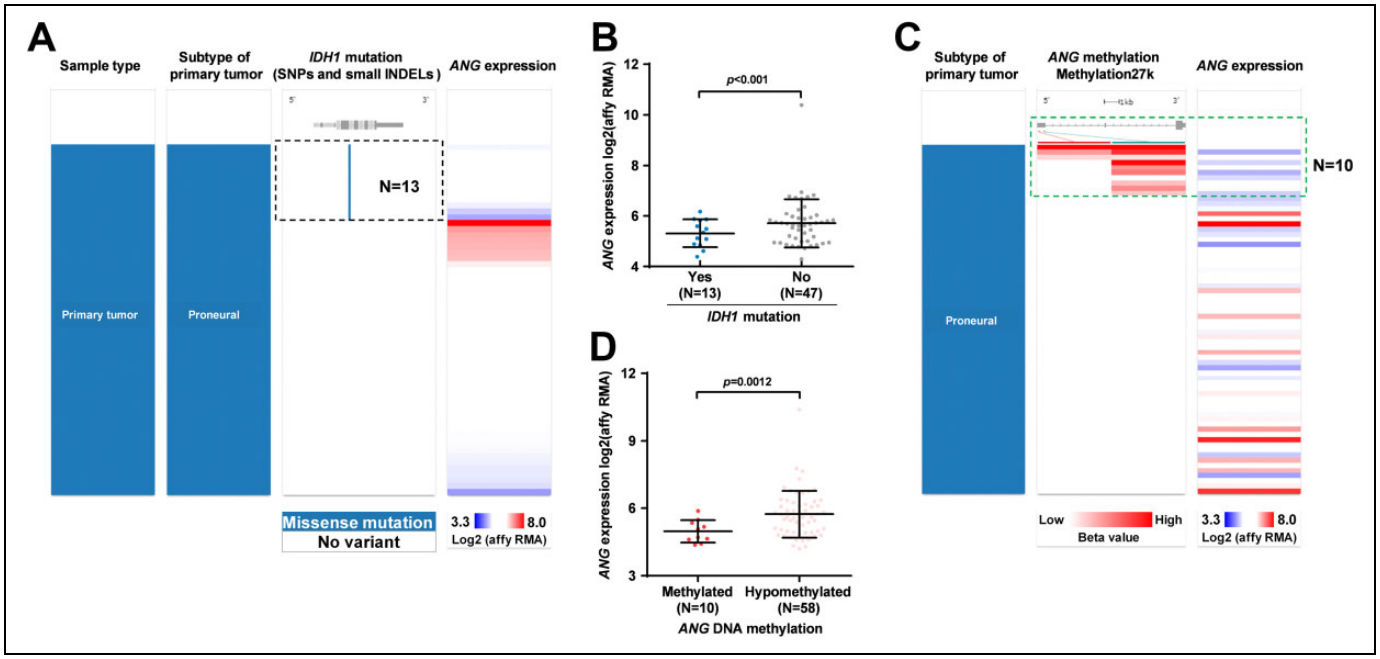

Figure 3. Angiogenin (*ANG*) expression in proneural subtype was related to its DNA methylation status. A-D, Heatmap (A and C) and plots chart (B and D) showing the correlation between isocitrate dehydrogenase 1 (*IDH1*) mutation and *ANG* expression (A-B) and between *ANG* methylation and its expression (C-D) in proneural subtype. Data were extracted from The Cancer Genome Atlas (TCGA)-glioblastoma (GBM).

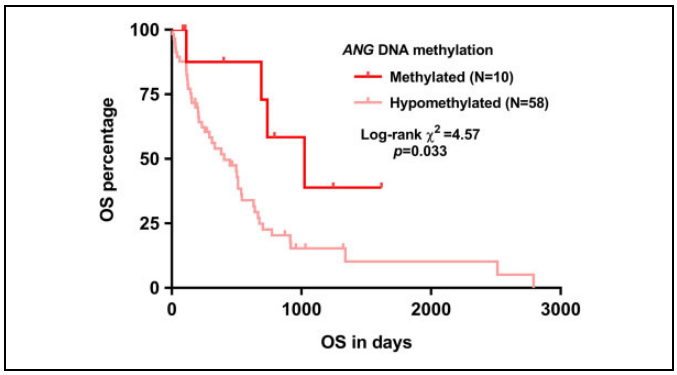

Figure 4. Kaplan-Meier overall survival (OS) curves of patients with proneural glioblastoma (GBM) with angiogenin (*ANG*) DNA methylation measured.

Results showed that among the 60 cases with proneural subtype having *IDH1* mutation identified, 13 cases had mutations. This group of patients also had significantly lower *ANG* expression, compared to the group without *IDH1* mutation (Figure 3A-B). Therefore, we hypothesized that *ANG* expression might be related to its DNA methylation status. To test this hypothesis, we examined the correlation between *ANG* expression and the methylation of 2 CpG sites in its DNA. Ten of 68 cases that had at least weak methylation (accumulated methylation $\geq$0.2) of these 2 CpG sites were defined as the methylated group (Figure 3C). Group comparison showed that the methylated group had significantly lower *ANG* expression compared to the hypomethylation group (Figure 3D). By generating Kaplan-Meier survival curves, we also examined the association between *ANG* expression and OS. Results confirmed that the methylated group had significantly longer OS compared to the hypomethylated group (Figure 4).

High ANG *Expression Might Independently Predict Shorter OS in Patients With Proneural GBM*

According to the Youden Index of *ANG* expression in ROC analysis of death, patients with proneural GBM were separated into *ANG* high (n = 29) and low (n = 109) expression group. Their clinicopathological features and OS outcome were given and are compared in Table 1. By performing univariate analysis, we found that older age, male patients, without *IDH1* mutation, without temozolomide chemotherapy, without radiotherapy, and high *ANG* expression were risk factors of unfavorable OS (Table 2). Following multivariate analysis confirmed that high *ANG* expression was an independent indicator of shorter OS in proneural GBM (hazard ratio [HR]: 1.669, 95% confidence interval [CI]: 1.033-2.696, P = .036) after adjustment of other factors (Table 2).

Discussion

Besides the oncogenic effect of the aberrantly expressed ANG, a series of studies also found that its upregulation might be an unfavorable survival marker in some cancers, such as gastric cancer,[12] patients with stage IV melanoma,[21] and non-Hodgkin lymphoma.[22] One previous study found a trend of increased *ANG* concentration in the higher grade of malignancy,[15] suggesting that *ANG* might participate in the malignant transformation of GBM. In this study, we confirmed that *ANG*

Table 1. Comparison of the Clinicopathological Parameters Between High and Low *ANG* Expression Groups in Proneural Subtype.

Parameters	*ANG* Expression		*P* Value
	High, n = 29	Low, n = 102	
Age, Mean ± SEM	63.34 ± 3	52.31 ± 1.692	.002
Gender			
Female	12	40	.83
Male	17	62	
KPS			
≤80	22	67	.29
>80	1	11	
No data	6	24	
IDH1 mutations			
No	11	36	.43
Yes	1	12	
No data	17	54	
MGMT promoter methylation, Mean ± SEM	0.31 ± 0.06	0.30 ± 0.04	.89
Radiation therapy			
No	7	18	.43
Yes	20	77	
No data	2	7	
Temozolomide chemotherapy			
No	12	43	1.00
Yes	15	52	
No data	2	7	
Living status			
Living	2	26	.039
Dead	27	76	

Abbreviations: ANG, Angiogenin; IDH1, isocitrate dehydrogenase 1; KPS, karnofsky performance score; MGMT, O(6)-methylguanine-DNA methyltransferase; SEM, standard error of mean.

expression was significantly upregulated in GBM tissues compared to that in adjacent normal tissues. In addition, we found that *ANG* expression varied significantly in different subtypes of GBM. Therefore, we determined to explore its prognostic value in the subtypes. By generating Kaplan-Meier OS curves in the 4 subtypes of GBM, we found that *ANG* upregulation might only have prognostic value in the proneural subtype. Since *IDH1* mutation and *MGMT* promoter methylation are 2 well-established prognostic indicators in GBM, we included these 2 factors in the univariate and multivariate analyses to assess the independent prognostic value of *ANG* expression. Results showed that high *ANG* expression was an independent indicator of shorter OS in proneural GBM (HR: 1.669, 95%CI: 1.033-2.696, $P = .036$), after adjustment of age, gender, isocitrate dehydrogenase 1(*IDH1*) mutation, temozolomide chemotherapy, and radiation therapy. However, we found that *MGMT* promoter methylation is not associated with OS in proneural subtype of GBM. Although *IDH1* mutation has strong predictive value, they are uncommon in primary GBM.[20,23] This is a major limitation of its prognostic value. Our study found that *ANG* expression showed independent prognostic value, even after adjustment of *IDH1* mutation. Therefore, it might be used as a biomarker to identify the proneural subtype of GBM with better prognosis.

By checking the association between *IDH1* mutation and *ANG* expression, we found that mutation group had significantly decreased *ANG* expression. Mechanistically, the mutations result in the loss of isocitrate dehydrogenase activity and following enhanced production of 2-hydroxyglutarate (2-HG),[24] which inhibit the enzymic activity of DNA

Table 2. Univariate and Multivariate Analysis of OS in Patients With Proneural GBM.

Parameters	Univariate Analysis				Multivariate Analysis			
	P	HR	95% CI (Lower/Upper)		*P*	HR	95% CI (Lower/Upper)	
Age (Continuous)	**<.001**	1.038	1.024	1.053	**.018**	1.020	1.003	1.036
Gender								
Male (N = 79)		1.000						
Female (N = 52)	**.044**	0.657	0.436	0.989	**.001**	0.460	0.287	0.737
KPS								
>80 (N = 12)		1.000						
≤80 (N = 89)	.062	2.105	0.964	4.595				
IDH1 mutations								
Yes (N = 13)		1.000						
No (N = 47)	**.004**	3.930	1.541	10.020	**.042**	2.884	1.039	8.007
MGMT promoter methylation	.767	0.841	0.268	2.641				
Temozolomide chemotherapy								
True (N = 67)		1.000						
False (N = 55)	**.010**	1.718	1.141	2.586	**.963**	1.010	0.649	1.572
Radiation therapy								
True (N = 97)		1.000						
False (N = 25)	**<.001**	4.803	2.895	7.967	**<.001**	5.326	2.920	9.715
ANG expression								
Low (N = 102)		1.000						
High (N = 29)	**.003**	1.989	1.270	3.116	**.036**	1.669	1.033	2.696

Abbreviations: ANG, Angiogenin; CI, confidence interval; GBM, glioblastoma; HR, hazard ratio; IDH1, isocitrate dehydrogenase 1; KPS, karnofsky performance score; MGMT, O(6)-methylguanine-DNA methyltransferase; SEM, standard error of mean.
Bold-face values indicates p<0.05.

demethylases. Therefore, *IDH1* mutation could indirectly increase DNA methylation status of a series of genes. Considering the effect of *IDH1* mutation, we examined whether the DNA methylation status of *ANG* was altered in proneural subtype. As hypothesized, a small proportion of proneural subtype had elevated *ANG* DNA methylation and decreased *ANG* expression. In addition, this group also had significantly better OS. These findings suggest that DNA methylation status might play a role in *ANG* expression. However, since this is an *in silico* study, no molecular studies were performed to validate these findings. In future, it is meaningful to demonstrate that the causative effect of *IDH1* mutation or *ANG* methylation on *ANG* expression.

Conclusion

Angiogenin upregulation might serve as an independent predictor of unfavorable OS in proneural subtype of GBM.

Authors' Note

This is a retrospective study based on online databases. No primary data were collected by any authors in this manuscript.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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