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Prognostic Significance of Zinc Finger E-Box-Binding Homeobox Family in Glioblastoma

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Background: Epithelial-mesenchymal transition (EMT) is an essential progress for tumor cell invasion to both epithelial and non-epithelial cancers, and zinc finger E-box-binding homeobox 1/2 (ZEB1/2) is a well-known promoter of EMT. In glioma cell lines, both ZEB1 and ZEB2 have been demonstrated to facilitate cancer cell proliferation and invasion with experiments *in vitro*. However, the clinical significance of ZEB1 and ZEB2 in glioblastoma (GBM) is still controversial.





Material/Methods: We detected the expression of ZEB1 and ZEB2 in 91 cases of GBM with immunohistochemistry and investigated the correlation between clinicopathological factors and ZEB family expression with Fisher test. By univariate analysis with Kaplan-Meier test, we explored the prognostic significance of ZEB1/2 expression and the clinicopathological factors in GBM. By multivariate analysis with the Cox regression model, we identified the independent prognostic factors in GBM.

Results: The percentages of ZEB1 high expression and ZEB2 high expression were 31.9% (29/91) and 41.9% (36/91), respectively. High expression of ZEB2 was significantly associated with lower survival rate of GBM patients ($P=0.001$). ZEB2, lower KPS score ($P=0.004$), gross total resection ($P<0.001$) and higher Ki67 percentage ($P=0.001$) were notably correlated to worse prognosis of GBM. With multivariate analysis, high expression of ZEB2 was demonstrated to be an independent prognostic factor indicating unfavorable prognosis of GBM ($P=0.001$, HR=3.86, and 95%CI=1.61–9.23).

Conclusions: High expression of ZEB2 is an independent prognostic factor predicting unfavorable prognosis of GBM, indicating that ZEB2 or its downstream proteins may be potential drug targets of GBM therapy.

MeSH Keywords: **Biological Markers • Epithelial-Mesenchymal Transition • Glioblastoma • Prognosis • Zinc Fingers**

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Background

Glioblastoma (GBM) is the most prevalent primary malignant brain tumor, with a median survival time of less than 2 years [1]. High levels of therapy resistance, strong cellular invasiveness, and rapid cell growth demand aggressive multimodal therapies involving resection followed by radio-chemotherapy [2,3]. Although the treatment strategies have been improved remarkably, including surgical equipment or adjuvant therapy, the overall survival rate of GBM is still very poor and new therapies are urgently needed [4–6]. The finding of new biomarkers can help discover new targeted drugs and therapies. For example, the monoclonal antibody against the vascular endothelial growth factor (VEGF) Bevacizumab was demonstrated to improve the survival rate of GBM [4,7]. However, the prognosis of GBM is still very unfavorable compared with many other tumors, requiring the continuous discovery and medical translation of new biomarkers.

Epithelial-mesenchymal transition (EMT) is an important progress of cells losing cell polarity and gaining invasive properties, which is critical for embryonic development and tissue repair [8]. In neoplasms, the process of EMT is significantly associated with cancer progression. Many molecules, including zinc finger E-box-binding homeobox 1/2 (ZEB1/2), N-cadherin, and TWIST or SNAIL, have been demonstrated to promote the EMT process, while E-cadherin can inhibit the EMT process. EMT was also proved to correlate to the tumorigenesis and progression of GBM [9–11]. The ZEB family members, including ZEB1 and ZEB2, are transcriptional inhibitors which can repress transcription of E-cadherin and therefore facilitate the EMT. Though the ZEB signaling pathway was proved to regulate the cell proliferation, migration, invasion, and apoptosis in glioma with experiments *in vitro* [12–14], the clinical significance of ZEB in glioma is still controversial. For example, a previous study reported that the increased ZEB1 expression demonstrated a favorable prognosis of IDH1-mutant lower-grade glioma [15]. On the contrary, another report showed that ZEB1 had no association with the survival of glioma patients [11]. Moreover, the clinical relevance of ZEB family with GBM, the most aggressive glioma, is still unexplored.

In the present study, we detected the expression of ZEB1 and ZEB2 in 91 cases of GBM with immunohistochemistry (IHC) and further investigated the correlation between clinicopathological factors and ZEB family expression. With univariate and multivariate analysis, we explored the prognostic significance of ZEB1 and ZEB2 expression in GBM.

Material and Methods

Patients and follow-ups

Our study was performed after the approval by the Ethics Committee of Yidu Central Hospital. We collected data on 91 patients who underwent surgical resection of GBM in Yidu Central Hospital from 2006 to 2016. The selection criteria of these patients were: (1) available follow-ups more than 2 months; (2) enough specimens for IHC; and (3) no adjuvant therapy after operation. The final diagnosis as GBM was confirmed by routine pathology. The overall survival time was obtained via follow-up by telephone and confirmed as the time from the operation date to the date of death. Survival time of censored cases was to the date of the last follow-up. The Karnofsky Performance Scale (KPS) was used to evaluate the situation of every patient.

Immunohistochemistry

The expression and location of ZEB family was revealed by detection of IHC. The streptavidin-biotin-immunoperoxidase method was used as described in previous studies in detail [16]. Briefly, boiled citrate buffer at pH 6.0 was used for antigen retrieval in a microwave oven for 10 min after dewaxing. After rinsing with phosphate buffer saline 3 times, 3% hydrogen peroxide was used to incubate the specimens for 10 min to achieve endogenous peroxidase inactivation. Followed by soaking in 5% bovine serum albumin for blockage of unspecific binding, the specimens were incubated in primary antibodies of ZEB1 (1: 200, Sigma-Aldrich, St. Louis, USA), ZEB2 (1: 200, Abcam, Cambridge, UK) or Ki67 (1: 100, DAKO, Denmark) at 4°C overnight. After rinsing with phosphate buffer saline, the corresponding secondary antibodies and streptavidin peroxidase complex reagent were then applied. The final results were visualized by incubation in 3,3'-diaminobenzidine solution.

Evaluation of IHC results

The results of IHC were blindly evaluated by 2 senior pathologists unaware of the clinical data. The score system of ZEB1/ZEB2 IHC results was applied according to a previous study [17]. The IHC scores were defined as: 0, staining in less than 1% of the tumor cells; 1, staining in 1% to 10% of the cells; 2, staining in 10% to 25% of the cells; 3, staining in 25% to 50% of the cells; 4, staining in 50% to 75% of the cells; and 5, staining in more than 75% of tumor cells. The cohort was divided into high-expression or low-expression subgroups according to the cut-off. The cut-off was calculated with receiver operating characteristic (ROC) curve and selected as the point with the highest sum of sensitivity and specificity, referring to a previous study.

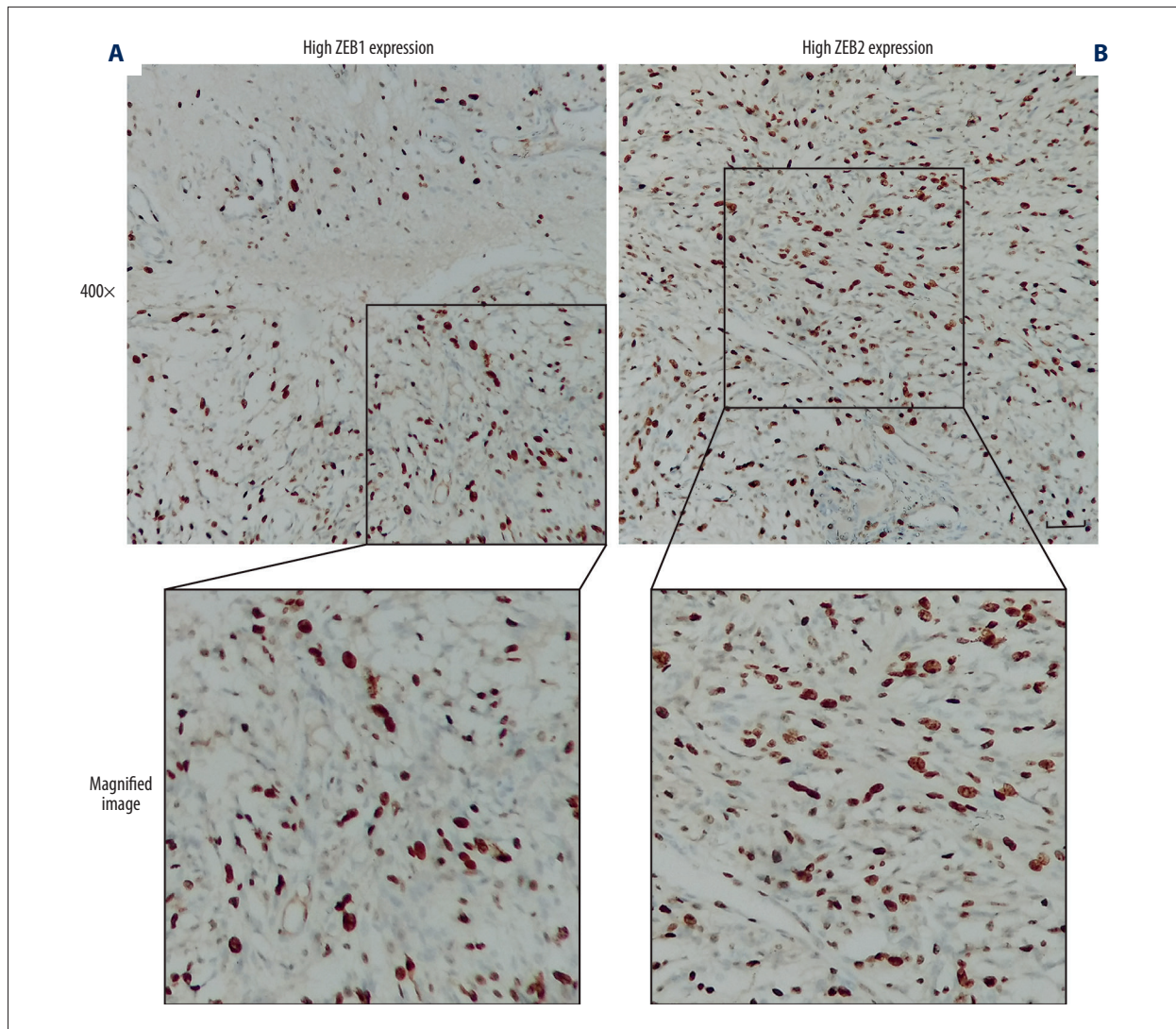


Figure 1. The representative images of ZEB1 and ZEB2 expression. **(A)** Upper panel: Representative image of ZEB1 high expression. **Bottom panel:** magnified image of ZEB1 high expression. **(B)** Upper panel: Representative image of ZEB2 high expression. **Bottom panel:** magnified image of ZEB2 high expression. Scale bar 50 μm .

MVD assessment

Microvascular density (MVD) staining was assessed by the method of Weidner by detecting CD34 with immunohistochemistry. The CD34-positive endothelial cells were labeled as brown and tubular form. The microvasculars were counted under 100 \times magnification of 8 random visual fields and the average number was considered as the MVD number of this sample. The cut-off of MVD was defined by ROC curve and we divided the cohort into a high MVD group and a low MVD group.

Statistical analysis

We used SPSS 22.0 (IBM Corporation, New York, USA) software to analyze all the data, without special instructions. Fisher test

was applied to calculate the correlation between ZEB family expression and clinicopathological features. Survival curves of all the factors were displayed by Kaplan-Meier method and the statistical difference was evaluated by log-rank test. The Cox regression proportional hazards model was used to identify the independent prognostic factors. *P* value less than 0.05 was considered as statistically significant.

Results

Expression of ZEB1 and ZEB2 in GBM

The expression of ZEB family was first detected with IHC. The expression of ZEB1 and ZEB2 was mainly observed in the

Table 1. Correlation between ZEB family and clinicopathological factors.

Parameters	ZEB1		p*	ZEB2		p*
	Low	High		Low	High	
Age						
≤50	31	17	0.503	29	19	0.996
>50	31	12		26	17	
Sex						
Male	36	14	0.498	27	23	0.131
Female	26	15		29	12	
KPS						
<80	24	14	0.495	20	18	0.277
≥80	38	15		35	18	
Extent of resection						
Subtotal resection	23	12	0.818	21	14	0.946
Gross total resection (95%)	39	17		34	22	
Ki67						
<10%	45	15	0.061	36	24	0.905
≥10%	17	14		19	12	
MVD						
Low	35	24	0.375	30	18	0.925
High	22	10		28	15	
ZEB1						
Low				39	23	0.499
High				16	13	
ZEB2						
Low	41	15	0.248			
High	21	14				

* Means calculated by Fisher test.

nucleus (Figure 1), which corresponds to the function of the ZEB family as a transcriptional repressor. According to the criteria described in Materials and Methods, the cohort was divided into the high-expression and low-expression subgroups of ZEB1 and ZEB2 according to their cut-offs. The percentages of ZEB1 high expression and ZEB2 high expression were 31.9% (29/91) and 41.9% (36/91), respectively.

The correlation between ZEB1/2 expression and clinicopathological factors

As well-known stimulators of EMT, overexpression of ZEB1 or ZEB2 can contribute to the progression of cancer by invasion or proliferation. Therefore, we detected the correlation between ZEB1/2 expression and clinicopathological factors to

screen the potential parameters relevant to ZEB overexpression. Fisher test was used to evaluate the correlations between ZEB family and clinicopathological factors including sex, age, KPS score, extent of resection, and MVD and Ki-67 percentage (Table 1). However, no significant association with ZEB1 or ZEB2 expression was observed among all the detected clinicopathological factors. Patients with high ZEB1 expression seemed to have higher Ki-67 percentage ($P=0.061$), but this correlation was not statistically significant.

Prognostic significance of ZEB family expression

The prognostic significance of all the clinicopathological factors were first evaluated with univariate analysis (Table 2). High expression of ZEB2 was significantly associated with lower survival

Table 2. Prognostic value of ZEB family.

Parameters	Univariate analysis		Multivariate analysis		
	1-year survival rate (%)	P*	HR	95%CI	P#
Age					
≤50	53.0				
>50	51.9	0.545			
Gender					
Male	60.6				
Female	46.0	0.345			
KPS					
<80	35.7		1		
≥80	66.8	0.004	0.48	0.22–1.05	0.066
Extent of resection					
Subtotal resection	64.3		1		
Gross total resection (95%)	0	<0.001	4.42	1.92–10.2	<0.001
Ki67					
<10%	64.4		1		
≥10%	38.7	0.001	3.05	1.33–6.99	0.008
MVD					
Low	56.2	0.192			
High	46.7				
ZEB1					
Low	54.3				
High	53.1	0.279			
ZEB2					
Low	63.7		1		
High	25.4	0.001	3.86	1.61–9.23	0.002

* Means calculated by Log-rank test; # means calculated by Cox proportional hazards regression.

time of GBM patients ($P=0.001$). The 1-year survival rates of ZEB2 low expression and high expression were 63.7% and 25.4%, respectively. There was no significant association observed between ZEB1 and the overall survival rate in our study ($P=0.279$). The correlations between the survival rate and ZEB1 or ZEB2 are displayed as survival curves in Figure 2A and 2B with Kaplan-Meier test. Moreover, lower KPS score ($P=0.004$), gross total resection ($P<0.001$), and higher Ki67 percentage ($P=0.001$) were all associated with worse prognosis of GBM patients.

Furthermore, all the factors demonstrated to be of prognostic significant were enrolled into a Cox regression model to identify the independent prognostic factor (Table 2). With the multivariate analysis, ZEB2 high expression was proved to be an independent prognostic factor, indicating unfavorable prognosis of GBM ($P=0.001$, HR=3.86, and 95%CI=1.61-9.23). Additionally, extent of resection and Ki-67 status were both

demonstrated to be independent prognostic factors. Gross total resection and higher Ki67 were risk factors for worse prognosis ($P<0.001$ and $P=0.008$, respectively).

Discussion

E-cadherin is a major cell-cell adhesion molecule located in the adherens junctions and is responsible for intercellular interactions. Loss of E-cadherin is considered as the hallmark of EMT and the central event of tumor metastasis [18]. In epithelial tumors, EMT and EMT-related process is widely acknowledged to play an essential role in progression, dissemination, and therapy resistance [19]. Recently, increasing evidence has identified the oncogenic function of EMT in non-epithelial tumors such as brain tumors, hematopoietic malignancies, and sarcomas [20]. Among these tumors, accumulating evidence

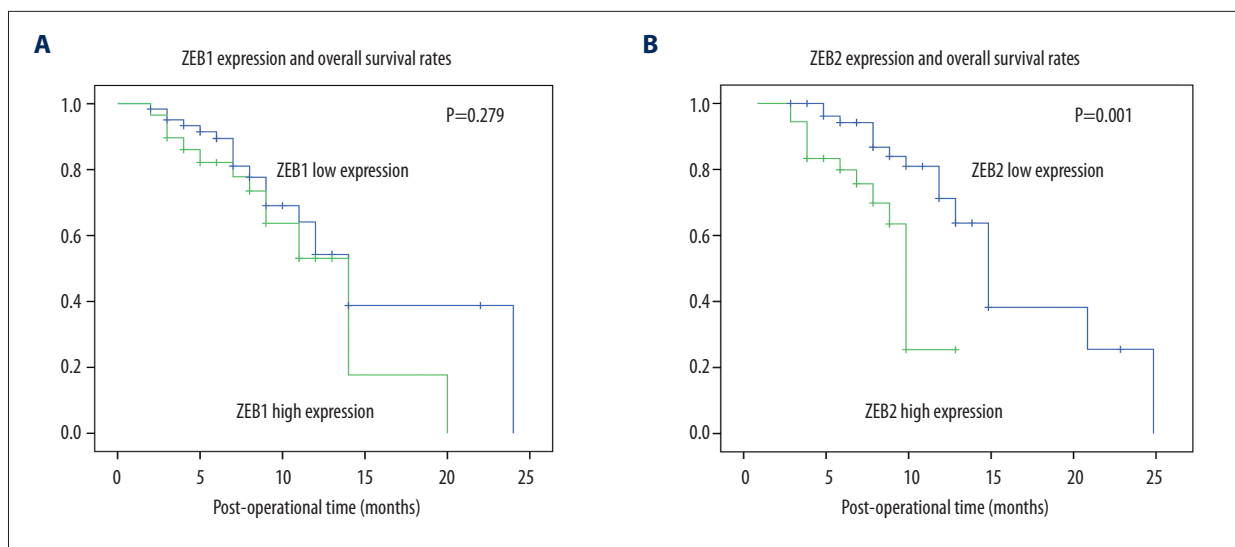


Figure 2. The survival curves of high expression and low expression of ZEB1 or ZEB2. **(A)** The survival curves of high expression and low expression of ZEB1. The survival rates of patients with high or low ZEB1 expression had no statistical significance ($P=0.279$). **(B)** The survival curves of high expression and low expression of ZEB2. Patients with high ZEB2 expression had significantly lower survival rates compared with those with low ZEB2 expression ($P=0.001$).

focused on GBM and demonstrated that EMT could regulate cell stemness, invasion, and therapy resistance in GBM. The mesenchymal state from epithelial or non-epithelial tumor cells has autonomous motility and more aggressive invasion, leading to worse clinical prognosis. EMT features the loss of E-cadherin, leading to loss of intercellular junctions and gain of cell migration ability. The gene encoding E-cadherin is *CDH1*. Some EMT transcription factors regulate E-cadherin expression via suppressing transcription of *CDH1*, including SNAIL/SNAI1, SLUG/SNAI2, E47, and ZEB family.

The oncogenic role of ZEB1 and ZEB2 in certain cancers has been well identified by numerous evidence. Abnormal expression or ectopic function expression of ZEB family has been demonstrated to correlate to progression and prognosis of many cancers, including breast cancer, renal cancer, pancreatic cancer, and ovarian cancer [21–24]. As important inducers of EMT, the oncogenic roles of ZEB1 and ZEB2 in glioma cell lines were also identified in many previous studies, and the suppressing role of ZEB1 and ZEB2 to E-cadherin expression in glioma cell lines is well defined. In a glioma cell line, Songtao et al. demonstrated that ZEB2 suppressed cell proliferation, migration, and invasion and promoted cell apoptosis in glioma cells [13]. Similarly, *in vitro* and *in vivo* experiments [25] also proved that the ZEB1 pathway is involved in initiation, invasion, and chemoresistance of glioblastoma. However, the clinical significance of the ZEB family, including the association with clinicopathological factors and prognosis, is little explored and lacks consensus.

Since the oncogenic function of the ZEB family has been well investigated in glioma cell lines, and ZEB2 expression was

proved to correlate the worse outcome of GBM, anti-ZEB2 therapy may be a promising approach to treat GBM. Unfortunately, there is no available effective inhibitor of ZEB2 in experimental or clinical use. Moreover, although there is an effective inhibitor or monoclonal antibody of ZEB2, the systemic application of ZEB2 inhibitor may be not acceptable based on the key role of the ZEB family in normal development. Therefore, investigating several key effector proteins downstream of ZEB2 is not only essential to elucidate the underlying mechanism, but also to discover a potential drug target for blocking ZEB2 signaling. Several miRNAs were reported to target ZEB1 or ZEB2 and can downregulate their expression in several types of cancers, such as miRNA-429 in cervical cancer and gastric cancer, and miR-200 in non-small cell lung cancer [25–27]. In glioma or GBM cell lines, miR-139-5p or miR-590-3p was also proved to suppress cell migration, invasion, and EMT via targeting ZEB1 or ZEB2 [28,29]. These miRNA findings expand the possibility of the ZEB family as a drug target, but the application of miRNA to treat disease remains in the experimental stage.

Conclusions

In conclusion, we present the results of the first study to investigate the expression of ZEB1 and ZEB2 in 91 cases of GBM with IHC, and demonstrate that ZEB2 high-expression is significantly associated with worse prognosis of GBM. Moreover, ZEB2 high expression is an independent prognostic factor predicting unfavorable GBM prognosis. These results suggest that ZEB2 detection could help stratify high-risk patients and more precisely guide individual treatment.

Conflicts of interest

None.

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