

RESEARCH ARTICLE

Prognostic Value of ALDH1A3 Promoter Methylation in Glioblastoma: A Single Center Experience in Western China

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Abstract

Background: Aberrations in gene methylation patterns play important roles in gliomagenesis. However, whether the ALDH1A3 promoter methylation is related to prognoses of primary glioblastomas (GBMs) in Western China remains unclear. **Materials and Methods:** Methylation levels of ALDH1A3 CpG island in 36 GBMs were identified by pyrophosphate sequencing, while ALDH1A3 expression was assessed with matched paraffin section immunohistochemistry. Survival curves were analysed by Kaplan-Meier. **Results:** The hypermethylation status of ALDH1A3 promoter predicted a better prognosis accompanied with low expression of ALDH1A3 protein. **Conclusions:** Our results indicate ALDH1A3 promoter methylation correlates with prognosis in primary GBMs.

Keywords: ALDH1A3 - methylation - glioma - prognosis

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Introduction

Glioblastoma (GBM) is the most common and catastrophic malignant tumor, accounts for about 60% of primary intracranial tumor. Despite multimodal comprehensive treatment, including surgery and radiation with concomitant/adjuvant temozolomide (TMZ), the 5-year survival rate of glioma patients is less than 10% (Sukhdeo et al., 2011; Aran et al., 2013). Nowadays, IDH1 mutation and MGMT promoter methylation have been identified as molecular markers with clinical significance and prognosis value across various studies.

DNA hypermethylation induced silencing of tumor suppressor and DNA repair genes is a frequent phenomenon in variety of cancers (Rivera et al., 2013). Although epigenetic alterations that lead to aberrant gene expression in malignant glioma have been identified, with the advent of high throughput microarray technology makes it possible to gain a comprehensive insight into the molecular basis of glioma. Using this technology, genome-wide DNA methylation patterns and tumor-cell specific molecular targets can be identified. Zhang W et al research show genome-wide DNA methylation profiling identifies ALDH1A3 promoter methylation as a prognostic predictor in primary glioblastoma (Zhang et al., 2013). ALDH1A3 gene mutation has been thought to be an early event and a distinct diagnostic and prognostic marker for glioma patients. Until now, there have been few reports about the role of ALDH1A3 in predicting glioma prognosis. Thus, in the present study, we would explore the relationship between ALDH1A3 promoter methylation and ALDH1A3

protein expression as well as GBM patients prognosis by Meier-Kaplan and cox regression analysis.

Materials and Methods

Patients and samples

All 36 patients random selected in the present study underwent surgical resection between January 2009 and December 2012 and subsequently received adjuvant radiotherapy and/or temozolomide chemotherapy. Tumor tissue samples were obtained by surgical resection before the treatment with radiation and chemotherapy. Respected specimens were storage in -80°C refrigerator until DNA extraction. This study was approved by the Ethics Committee of Kunming Medical University, and written informed consent was obtained from all patients. Primary GBM pathological diagnosis was defined by same two neuropathologists.

DNA extraction

All of the tumor samples were immediately snap-frozen in liquid nitrogen after surgery. A hematoxylin and eosin-stained frozen section was prepared for assessment of the percentage of tumor cells before RNA extraction. Only samples with greater than 85% tumor cells were selected. Genomic DNA was isolated from frozen tumor tissues using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's protocol. DNA concentration and quality were measured using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Houston, TX).

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Pyrophosphate sequencing analysis of ALDH1A3

Pyrosequencing was supported by Genetech (Shanghai, China) and performed using the PyroMark Q96 ID System

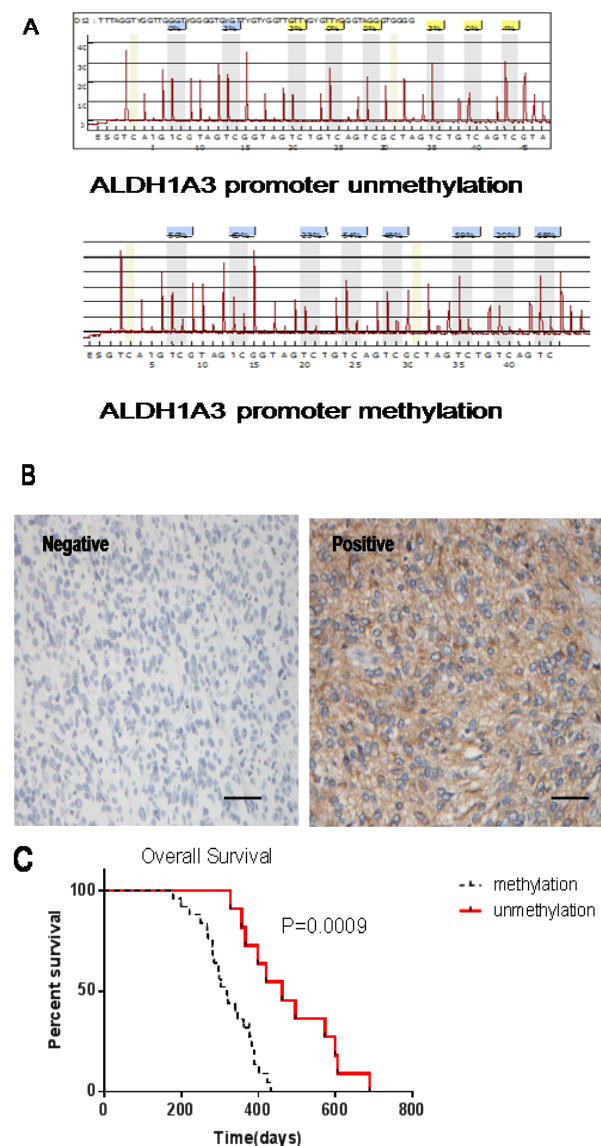


Figure 1. ALDH1A3 Methylation Status, Immunohistochemical Staining and Surviving Analysis.(A) ALDH1A3 promoter methylation status by pyrophosphate sequencing (B) ALDH1A3 differential expression in immunohistochemical staining $\times 200$ (C) ALDH1A3 hypomethylation group having worse prognosis than hypermethylation group by Kaplan-Meier survival curves analysis

Table 1. Relationships between Protein Expression of ALDH1A3, ALDH1A3 Promotor Methylation Status and Clinicopathological Factors

Parameters	n	Methylation status		χ^2	p	ICH		χ^2	p	
		M	U			high	low			
Gender	Male	22	8	14	0.899	0.343	6	16	0.007	0.932
	Female	14	3	11			4	10		
Age	≤ 50	21	7	14	0.183	0.669	5	16	0.613	0.434
	> 50	15	4	11			2	13		
Tumor size	< 4	13	5	8	4.698	0.03	6	7	6.893	0.009
	≥ 4	23	2	21			21	2		
KPS	≤ 70	16	2	14	4.425	0.035	2	14	6.916	0.009
	> 70	20	9	11			10	11		

(QIAGEN) according to the manufacturer’s protocol. Bisulite modification of the DNA was performed using the EpiTect Kit (Qiagen). The primers 50-GGG TTT TGG GAT GGA AG-30 and 50-biotin-ACR TAC CCT ACT CTT AAA TCC AAC-30 were used for PCR amplification and the primer 50-AGG GTT TAG GGG AGA T-30 for Pyrosequencing. The primary GBM samples were considered to be ALDH1A3 promoter-methylated with an average methylation of $> 10\%$.

Immunohistochemistry

Briefly, surgical samples from the above patients were fixed in formalin, routinely processed and paraffin embedded. Four micron-thick sections were prepared, and immunohistochemical staining with streptavidin–biotin immunoperoxidase assay was performed using rabbit monoclonal antibody to ALDH1A3 (Abcam). Negative controls were obtained by substituting primary antibodies with non-immune serum. Sections with fewer than 30% labeled cells indicated low expression of ALDH1A3. Sections with labeling of 30% indicated high expression of ALDH1A3.

Statistical analysis

T-tests were performed using Matlab software. A two-sided χ^2 test was performed using SPSS 13.0. Kaplan–Meier survival curves were obtained, and differences in the overall survival were tested for statistical significance using the log-rank test (GraphPad Prism 6). $p < 0.05$ was considered significant.

Results

ALDH1A3 protein expression in GBM

ALDH1A3 protein expression was primarily identified in the membranes and cytoplasm of cancer cells (Figure B). 10 (10/36) patients were classified as ALDH1A3 positive; 26 (26/36) were negative in the GBM.

ALDH1A3 methylation status in GBM

ALDH1A3 methylation in primary GBM patients are shown in Figure A. ALDH1A3 promoter hypermethylation was detected in 11/36 cases (30.6%), whereas 25/36 (69.4%) samples were unmethylated. Survival analysis showed that primary GBM patients with a hypermethylated ALDH1A3 promoter had a better outcome in overall survival at a statistically significant level (Figure C and Table 2).

Table 2. Univariate and Multivariate Cox Regression Analysis for Determining Disease outcome Based on Methylation Status of ALDH1A3

	Univariate			Multivariate		
	HR	95%CI	p-value	HR	95%CI	p-value
Age	1.019	0.987-1.051	0.252			
Gender	1.22	0.617-2.414	0.567			
KPS	0.969	0.933-1.006	0.099			
Tumor size	5.118	2.217-11.454	0.001	6.691	2.734-16.374	0
Promotor	2.857	1.395-5.855	0.004	3.74	1.670-8.303	0.001

Table 3. ALDH1A3 Promoter Methylation Status and Protein Expression

Protein expression	Methylation	Unmethylation	χ^2	P
Positive	2	20	10.021	0.002
Negative	9	7		

Correlation between ALDH1A3 protein expression and promoter methylation status

In the matched 36 GBM samples, ALDH1A3 protein expression was analyzed via immunohistochemistry. A significant negative correlation was observed between ALDH1A3 promoter methylation status and ALDH1A3 protein expression (Pearson correlation: $R=0.67$, $p<0.01$) (Table 3).

Discussion

Primary GBM is considered the most fatal brain tumor with low survival rate and rapid progression in adults. It's indicated that deregulation of epigenetic mechanism including DNA methylation changes played an important role in glioma carcinogenesis. Nowadays, only MGMT promoter methylation and IDH1 mutation are validated as stable prognostic factors across various institutes (Aran et al., 2013; Rivera et al., 2013).

ALDH1A3 is a member of Aldehyde dehydrogenase isozymes, locate in 15q26.3, that are thought to play a important role in the detoxification of aldehydes generated by lipid peroxidation and alcohol metabolism (Everts et al., 2007; Corominas-Faja et al., 2013). Recently, the biological role of ALDH1A3 in carcinogenesis has been unveiled gradually. LUO Yu Chun et al study indicated that silencing ALDH1 leads to melanoma cell cycle arrest, apoptosis, decreased cell viability in vitro, sensitizing melanoma cells to chemotherapy and reduced tumorigenesis *in vivo* (LUO et al., 2012). Marcato Paola et al. research revealed that ALDH activity of breast tumor CSCs and cell lines correlates best with expression of isoform, ALDH1A3, not ALDH1A1 by performing shRNA knockdown expression of the various ALDH isoforms and found that only ALDH1A3 knockdown reduced ALDH activity of breast cancer cells. ALDH1A3 expression in breast cancer patient correlates significantly

with tumor grade, metastasis, and cancer stage (Marcato et al., 2013). Saw Yu-Ting et al research indicate that ALDH enzyme expression and activity may be associated with specific cell types in ovarian tumor tissues and the function of the ALDH isozymes in lineage differentiation and pathogenesis may have significant implications for ovarian cancer pathophysiology (Saw et al., 2012).

In 2013, MAO P et al validate ALDH1A3 involved in glycolysis chaos of GBM, knockout the ALDH1A3 expression can obviously decrease the activity of ALDH result in cancer cell resensitve to radioation. Consequently, ALDHs not only may be considered markers of these cells, but also may well play a functional role in terms of selfprotection, differentiation, and/or expansion of stem cell populations. (Mao et al., 2013)

Until now, there have been few reports about the role of ALDH1A3 in predicting new diagnosis GBM prognosis. Chen J et al study suggested that miR-125a/b expression plays a key role in chemoresistance through upregulating ALDH1A3 and Mcl1 gene expression.

Thus, in the present study, we selected 36 primary GBM samples to validate the prognostic value of the promoter methylation of ALDH1A3. we have explored the relationship between ALDH1A3 promoter methylation and ALDH1A3 protein expression as well as GBM patients prognosis by Meier-Kaplan and cox regression analysis. The presence of aldehyde dehydrogenase positive cells in tumor from patients with GBM has been associated with decreased survival (Muzio et al., 2012; Zhou et al., 2013; Selvam et al., 2013; Ohanna et al., 2013; Yang et al., 2013; Zhang et al., 2013; Parajuli et al., 2014). Besides, our data revealed that ALDH1A3 promoter unmethylation indicated a long time of OS ($p=0.0009$) (Table 3) suggesting ALDH1A3 methylation status could be considered an indicator of patients prognosis. In addition, we examined the relationship between protein expression and promoter methylation for ALDH1A3 in the 37 GBM samples used for the independent validation cohort. Correlation analysis showed that the methylation status negatively correlated with ALDH1A3 protein expression ($p=0.002$) (Table 3). These results indicated that ALDH1A3 promoter methylation affects the prognosis of primary GBM patients by regulating its protein expression.

To determine the independent pretreatment clinicopathological factors of tumor, a logistic regression model was used. By multivariate analysis, we found methylation of ALDH1A3 showed significant correlation with good outcome (HR=3.74; 95%CI1.670-8.303, $p=0.001$). It means tumors with methylation of ALDH1A3 promoter are more sensitive to chemotherapy than those with unmethylation. Moreover, the tumor size was also as a predictive factor for tumor response (HR=6.691; 95%CI2.734-16.374, $p=0.001$) (Table 2).

In summary, we have validate ALDH1A3 methylation status is associated with prognosis is primary GBMs. Furthermore, hypermethylation of ALDH1A3 was regarded as a better prognosis fator in primary GBMs. Finally, ALDH1A3 may also serve as a marker of glioma stem cells and should be further investigated.

Taken together, the present study had several

limitations, including the limitations inherent to a retrospective analysis, which may alter the statistical association between ALDH1A3 methylation status and survival outcome. In addition, the patients include in this study did not received identical treatments, which may have been a source of bias in the evaluation of treatment outcome.

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