Original Article
Immunostaining of IDH-1^{R132H} and ATRX proteins in the classification of adult glioblastomas

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Abstract: Classification of gliomas based on genetic alternations was of great significance in understanding tumor behavior and treating individually. Immunohistochemistry (IHC) offered an easy, cheap and efficient way to detect mutations, compared to direct sequencing. Herein, we characterized the mutations of IDH-1 and ATRX detected by IHC, in a cohort of 217 glioblastomas. IDH-1$^{R132H\ mutations}$ was more common in younger patients (42.31 ± 1.56 *vs.* 53.45 ± 1.02, *P*=0.000) and secondary glioblastomas [12.8% (23/180) *vs.* 75.7% (28/37), *P*=0.000]. ATRX loss was closely correlated with a younger age (44.59 ± 1.94 *vs.* 52.51 ± 1.00, *P*=0.000) and secondary glioblastomas [51.4% (19/37) *vs.* 15.0% (27/180), *P*=0.000]. IDH-1^{R132H} mutations were significantly overlapped with ATRX loss [56.9% (29/51) *vs.* 10.2% (17/166), *P*=0.000]. Moreover, the median overall survival significantly differed among IDH-1$^{R132H-mut}$-ATRX^{neg} (28.70 months, 95% CI 12.44-44.96), IDH-1$^{R132H-mut}$-ATRX^{pos} (20.87 months, 95% CI 9.57-32.17), IDH-1$^{R132H-wt}$-ATRX^{neg} (14.27 months, 95% CI 10.01-18.53) and IDH-1$^{R132H-wt}$-ATRX^{pos} (12.20 months, 95% CI 10.33-14.07). Also patients in the group of IDH-1$^{R132H-mut}$-ATRX^{neg} or IDH-1$^{R132H-mut}$-ATRX^{pos} were both significantly younger than those had IDH-1$^{R132H-wt}$-ATRX^{pos} subtypes (*P*<0.05). In conclusion, immunostaining of IDH R132H and ATRX proteins help better classify glioblastomas. We recommend IHC as a necessary tool to diagnose glioblastomas, when direct sequencing was not performed.

Keywords: Gliomas, IDH-1$^{R132H\ mutation}$, prognosis, ATRX loss, immunohistochemistry

Introduction

Glioblastomas accounts for 15.1% in central nervous system (CNS) tumors, with a median survival of 1.5 years approximately [1, 2]. Glioblastomas are further classified as primary and secondary (pGBM & sGBM), and the later originates from lower-grade gliomas [3, 4]. Alternatively, glioblastomas are divided into classical, mesenchymal, neural and proneural subtypes, according to gene expression. Interestingly, there is a varied proportion of primary and secondary glioblastomas in the molecular subtypes [5-7]. Because mutations of IDH1/2 (isocitrate dehydrogenase), TP53, and ATRX (alpha thalassemia/mental retardation syndrome X-linked) are more frequent in secondary GBMs than in pGBM [4, 8-13]. Recently, the WHO 2016 classification of CNS tumors is revised with the inclusion of IDH status and 1p19q co-deletion [14].

Most importantly, molecular classification helps us better understand tumorgenesis, clini-

cal behavior, and treat individually. However, gene testing is expensive and complicated, and is unlikely to be performed globally especially in developing countries. IHC offered a robust, cheap and efficient to detect mutations, especially in IDH-1^{R132H} and ATRX [9, 15-18]. Moreover, it is well observed that mutations of IDH and ATRX could subclassify gliomas [9, 19-22]. In this study, we investigate the status of IDH and ATRX with the method of IHC, in a single institute. We hope this IHC method could provide an efficient classification for glioblastomas.

Materials and methods

Study population

From 2009 to 2015, 217 cases with glioblastoma were retrospectively reviewed in the study. All of them underwent an operation at Sanbo Brain Hospital. Postoperative therapies were adjuvant chemoradiotherapy according to Stupp protocols [2]. Unluckily, only 58 (59.8%)
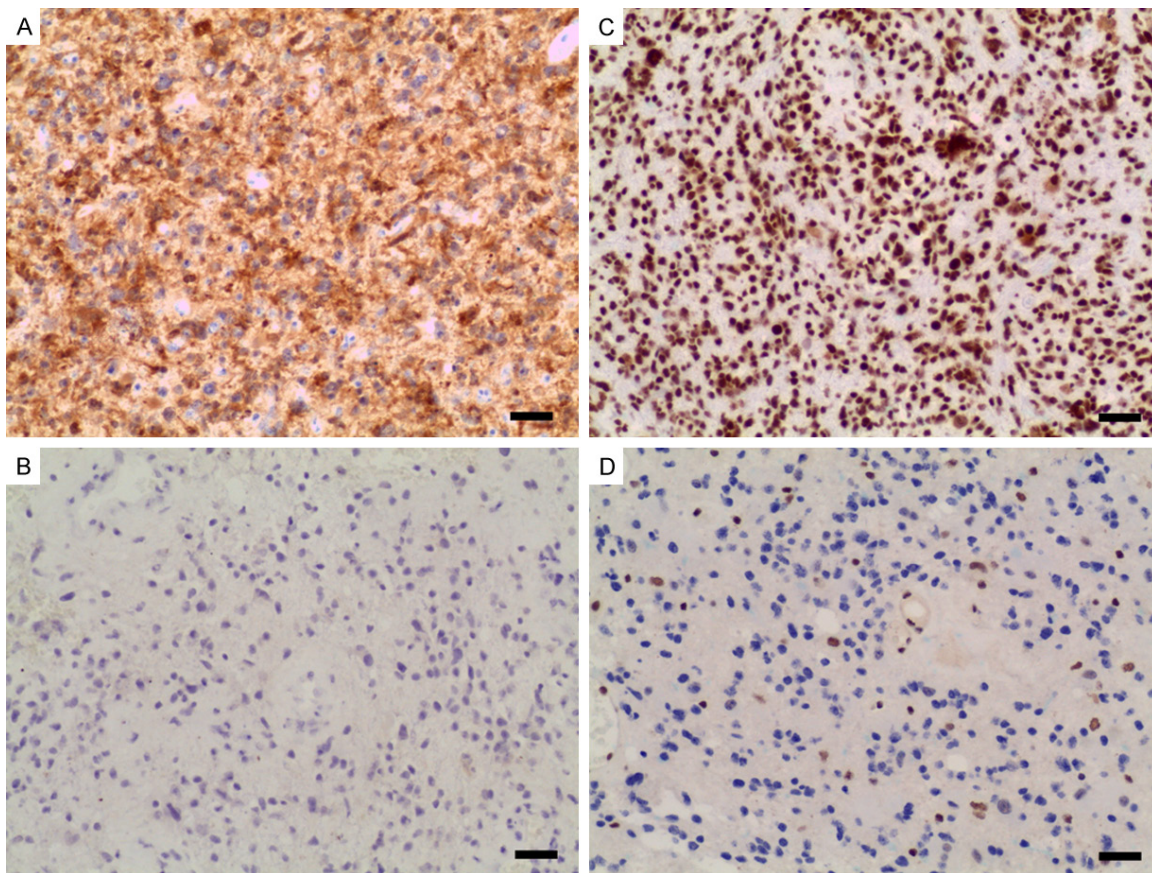
Figure 1. Immunostaining for IDH-1$^{R132H\ mut}$ positive (A), negative (B), ATRX expression (C), loss (D).

patients were administered with full treatment protocols (adjuvant chemoradiotherapy plus concomitant temozolomide). Overall survival (OS) was defined as the period from operation to death or censored. Only 2 cases are missing from the follow-up. The deadline of the follow-up was Feb, 2016. The study was approved by the ethics committee of Sanbo Brain Hospital, and a written informed consent was obtained from all patients.

Immunohistochemical staining and assessment

Immunoperoxidase staining for IDH1^{R132H} and ATRX was performed on formalin-fixed, paraffin-embedded tissue sections as previously reported [13]. Each slide stained for IDH1^{R132H} (Dianova 1:100) and ATRX (1:1000 sigma) was individually reviewed and scored by 2 independent observers. The cutoff values were 10% for IDH1^{R132H} and ATRX. Briefly, the cytoplasm Primary antibodies against IDH1^{R132H} and ATRX were applied overnight at 4°C. After washing

with PBS, the sections were then incubated with poly-HRP Anti-Mouse/Rabbit IgG Detection System (PV-9000 ZSGB-BIO, China) for 30 min at 37°C. Two experienced pathologists (Xueling Qi and Kun Yao) reviewed the sections under microscope (Leica DM3000) independently, blind to clinical outcomes.

Statistical analysis

SPSS 22.0 was used for data analysis. χ^2 test and student test were used for data analysis appropriately. Survival curves were evaluated by the Kaplan-Meier method, and confirmed by log-rank test. Multivariate analysis was performed with Cox regression model. $P<0.05$ was defined as statistically significant.

Results

Immunohistochemistry

Immunohistochemistry of IDH-1^{R132H} and ATRX was carried on 217 patients. Positive expres-

Table 1. Patients characteristics

Variables	No	mOS (95% CI) months	HR (95% CI)	P value
Age				
<60	152	15.13 (12.65-17.61)	0.62 (0.45-0.86)	0.005
≥60	65	11.67 (10.02-13.33)		
Gender				
Female	85	14.63 (10.84-18.42)	0.91 (0.65-1.26)	0.908
Male	132	13.50 (11.52-15.48)		
Preoperative KPS				
≤70	99	11.56 (9.23-13.89)	0.75 (0.55-1.04)	0.082
>70	118	16.17 (13.52-18.82)		
Pathology				
pGBM	180	13.50 (11.33-15.67)	1.42 (0.89-2.25)	0.143
sGBM	37	16.90 (7.10-26.70)		
Location				
Frontal	47	15.13 (10.15-20.11)	1.03 (0.94-1.13)	0.550
Temporal	34	17.00 (8.32-25.68)		
Parietal	13	12.00 (10.32-13.68)		
Other site	39	8.53 (6.64-10.42)		
Mixed	84	15.50 (12.65-18.35)		
Size				
≤5 cm	91	13.33 (9.26-17.40)	1.09 (0.79-1.50)	0.585
>5 cm	126	13.83 (10.70-16.96)		
Resection				
GTR	152	13.83 (10.98-16.68)	0.74 (0.52-1.03)	0.076
Non-GTR	65	12.60 (8.44-16.76)		
Chemoradiotherapy				
Incomplete	76	7.73 (5.75-9.71)	0.35 (0.25-0.49)	0.000
Complete	141	16.77 (15.06-18.48)		
IDH-1^{R132H}				
Mutant	51	23.97 (7.85-40.09)	0.48 (0.31-0.73)	0.001
Wild-type	166	12.30 (10.73-13.87)		
ATRX				
Loss	46	16.23 (13.00-19.46)	0.70 (0.46-1.05)	0.082
Expression	171	12.80 (11.18-14.42)		

Table 2. Multivariate analysis of clinicopathological factors

Independent variables	HR	95%		P value
		Lower	Upper	
Age	.746	.527	1.056	.098
Gender	1.227	.871	1.728	.243
KPS≤70	.785	.568	1.086	.144
Classification	.892	.655	1.216	.892
Surgical resection	.841	.591	1.196	.334
Chemoradiotherapy	3.349	2.302	4.871	.000
IDH-1$^{R132H \ mutation}$	.502	.176	.755	.007
ATRX expression	1.018	.642	1.612	.941

sion of IDH-1^{R132H} (**Figure 1A**) was observed in cytoplasm, while ATRX expression (**Figure 1C**) was found in nuclear. Notably, endothelial cells served as positive controls, when ATRX (**Figure 1D**) loss was presented in glioblastomas.

Patient characteristics

There were 180 pGBM and 37 sGBM involved in this study, comprising 85 females and 132 males. The mean age was 50.83 ± 13.50 years, and the median was 52 years. In the univariate analysis, we observed that improved survival was significantly associated with age, complete treatment postoperatively, and IDH-1$^{R132H \ mut}$. (**Table 1**) Moreover, IDH$^{R132H \ mutation}$ was more common in secondary glioblastomas [75.7% (28/37) *vs.* 12.8% (23/180), *P*= 0.000], younger patients (42.31 ± 1.56 *vs.* 53.45 ± 1.02, *P*=0.000) and ATRX loss. Our results were in consistence with the previous studies [12, 13, 23-25]. ATRX loss was significantly associated with younger age (44.59 ± 1.94 *vs.* 52.51 ± 1.00, *P*=0.000) and secondary glioblastomas [51.4% (19/37) *vs.* 15.0% (27/180), *P*=0.000], corresponding well to Ikemura's study [18].

Prognosis based on molecular subtypes

In the multiple analyses, we only observed complete chemoradiotherapy and IDH-1$^{R132H \ mutations}$ that served as an independent prognostic marker (**Table 2**). The median OS of 23.97 months (95% CI 7.85-40.09) in the IDH-1$^{R132H \ mut}$ group, was longer than the median OS of 12.30 months (95% CI 10.73-13.87) in IDH-1$^{R132H \ wt}$ group (P=0.000, log-rank test).
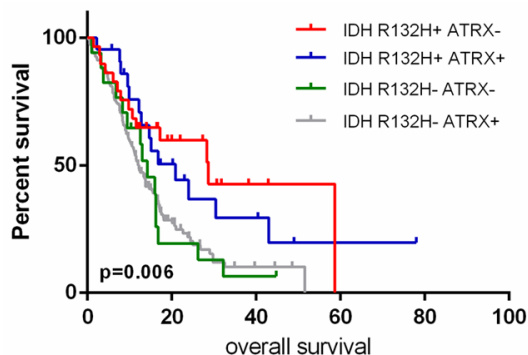
Figure 2. IDH-1$^{R132H\ mutations}$ and ATRX loss in predicting OS in glioblastomas.

ATRX loss was associated with improved OS in the IDH-1$^{R132H\ mut}$ group [median 16.23 months (95% CI 13.00-19.46) vs. 12.80 months (95% CI 11.18-14.42)]; however, the association was not significant (P=0.082, log-rank test).

Next, we subdivided glioblastomas with the inclusion of ATRX status. Patients' age significantly differed among IDH-1$^{R132H-mut}$-ATRX^{neg} (43.31 ± 2.14), IDH-1$^{R132H-mut}$-ATRX^{pos} (41.00 ± 2.28), IDH-1$^{R132H-wt}$-ATRX^{neg} (46.76 ± 3.81) and IDH-1$^{R132H-wt}$-ATRX^{pos} (54.21 ± 1.03). There is significant difference in age between the group of IDH-1$^{R132H-wt}$-ATRX^{pos} and IDH-1$^{R132H-mut}$-ATRX^{neg} (P=0.000) or IDH-1$^{R132H-mut}$-ATRX^{pos} (P=0.000). Moreover, the median overall survival was 28.70 months (95% CI 12.44-44.96) in IDH-1$^{R132H-mut}$-ATRX^{neg}, 20.87 months (95% CI 9.57-32.17) in IDH-1$^{R132H-mut}$-ATRX^{pos}, 14.27 months (95% CI 10.01-18.53) in IDH-1$^{R132H-wt}$-ATRX^{neg} and 12.20 months (95% CI 10.33-14.07) in IDH-1$^{R132H-wt}$-ATRX^{pos} respectively, with a significant difference (P=0.006, **Figure 2**).

Discussion

In the present study, we investigated genetic alternations including IDH mutations and ATRX loss, in a cohort of glioblastomas with IHC. IDH-1$^{R132H\ Mutations}$ and ATRX loss were significantly associated with younger age and secondary glioblastomas. ATRX loss was significantly overlapped with IDH-1$^{R132H\ mut}$, and the later was a predictive marker in this study. Our results corresponded well to the previous data [12, 13, 18, 23-25].

It was well established that IDH mutation and ATRX loss was more frequent in lower grade gliomas (WHO II-III). The incidence of IDH muta-

tions in ATRX-loss gliomas was also higher in lower grade gliomas [9, 10, 19, 20, 24, 26]. Moreover, TERT (telomerase reverse transcriptase) mutations and 1p19q co-deletion was more frequent in lower gliomas [27]. These results suggested a completely different genetic alternation in lower-grade gliomas, compared to the glioblastomas. Interestingly, IDH mutations and ATRX loss were used to subclassify gliomas. Cai et al divided astrocytomas (II-IV) into three types based on IDH mutations and decreased ATRX mRNA. The type of IDH-mut plus ATRX-low had the best clinical outcome [21]. In another study, detection of IDH mutation and ATRX loss by IHC proved is predictive in a cohort of lower-grade gliomas [20]. Similar results were also observed either grade II [28] or grade III gliomas [10]. However, little data was published investigating the IDH mutations and ATRX loss in subdividing glioblastomas. In a cohort of 163 glioblastomas, subtypes based on IDH mutations and ATRX loss were most obvious [25]. The subtype of IDH-1$^{R132H-mut}$-ATRX^{neg} in their study also had best clinical outcome, which matched our results. Additionally, we also found that patients carrying on IDH mutations and ATRX loss were younger than others. These results all indicated that IDH mutations and ATRX loss could subclassify gliomas, not only in lower-grade gliomas, but also in glioblastomas. In conclusion, IHC for detecting the mutations of IDH and ATRX was a reliable, robust and efficient method, and was recommended when classifying glioblastomas.

Acknowledgements

Disclosure of conflict of interest

None.

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