RESEARCH ARTICLE

IDH mutant lower grade (WHO Grades II/III) astrocytomas can be stratified for risk by CDKN2A, CDK4 and PDGFRA copy number alterations

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Abstract

In the 2016, WHO classification of tumors of the central nervous system, isocitrate dehydrogenase (IDH) mutation is a main classifier for lower grade astrocytomas and IDH-mutated astrocytomas is now regarded as a single group with longer survival. However, the molecular and clinical heterogeneity among IDH mutant lower grade (WHO Grades II/III) astrocytomas have only rarely been investigated. In this study, we recruited 160 IDH mutant lower grade (WHO Grades II/III) astrocytomas, and examined PDGFRA amplification, CDKN2A deletion and CDK4 amplification by FISH analysis, TERT promoter mutation by Sanger sequencing and ATRX loss and p53 expression by immunohistochemistry. We identified PDGFRA amplification, CDKN2A homozygous deletion and CDK4 amplification in 18.8%, 15.0% and 18.1% of our cohort respectively, and these alterations occurred in a mutually exclusive fashion. PDGFRA amplification was associated with shorter PFS (P = 0.0003) and OS (P < 0.0001). In tumors without PDGFRA amplification, CDKN2A homozygous deletion or CDK4 amplification was associated with a shorter OS (P = 0.035). Tumors were divided into three risk groups based on the presence of molecular alterations: high risk (PDGFRA amplification), intermediate risk (CDKN2A deletion or CDK4 amplification) and low risk (neither CDKN2A deletion and CDK4 amplification nor PDGFRA amplification). These three risk groups were significantly different in overall survival with mean survivals of 40.5, 62.9 and 71.5 months. The high-risk group also demonstrated a shorter PFS compared to intermediate- (P = 0.036) and low-risk (P < 0.0001) groups. One limitation of this study is the relatively short follow-up period, a common confounding factor for studies on low-grade tumors. Our data illustrate that IDH mutant lower grade astrocytomas is not a homogeneous group and should be molecularly stratified for risk.

INTRODUCTION

The 2016 WHO classification of tumors of the central nervous system has for the first time incorporated molecular features in the classification of diffuse gliomas (18). Astrocytomas are classified as isocitrate dehydrogenase (IDH) mutant or IDH wild type based on IDH genotype and they lack 1p19q codeletion. Approximately 70%–80% of WHO Grades II and III astrocytomas possess IDH mutations and the rest are IDH wild type (28). Diffuse astrocytomas with IDH mutations are regarded as the better prognostic group by the WHO classification based on available literature (1, 3, 21, 38). Determination of the IDH status has become a standard practice in the diagnosis of lower grade astrocytomas.

However, recent studies have shown that just the IDH status alone is inadequate for stratification of risk for gliomas. For instance, IDH wild type lower grade gliomas is not a single group with uniform dismal prognosis as originally thought and the poor prognostic groups among IDH wild type diffuse astrocytomas were found to be those carrying TERT promoter mutations, EGFR amplification and concomitant gain of chromosome 7 and loss of chromosome 10 (+7/−10) (1, 3, 36). However, such molecular and clinical diversity have not been extensively examined in IDH mutant astrocytomas and whether IDH mutant lower grade (WHO Grades II/III) astrocytomas is a group with uniformly longer survival has not been vigorously evaluated.

There have been a few papers in which IDH mutant astrocytomas have been included as one of the cohorts of the studies (2, 4, 5, 24, 28). However, molecular heterogeneity within the group was not demonstrated in majority of the reports. And in Reuss *et al* cohort, histological grade lost its significance within the IDH mutant astrocytomas (25).

Reis *et al* was the first group to show that CDKN2A deletion was associated with a shorter overall survival in Grade II and Grade III astrocytomas (24). They demonstrated that CDKN2A loss in IDH/TP53 mutated tumors was strongly associated with worse overall survival. Shirahata *et al* examined methylation profiles of 211 IDH mutant astrocytic tumors and identified CDKN2A deletion as a molecular biomarker to stratify astrocytic tumors (28). They showed that none of the Grade II astrocytomas had CDKN2A deletion. Grade III astrocytic gliomas harboring CDKN2A deletion had a poorer survival compared to Grade IV glioblastomas without the deletion (28). Aoki *et al* investigated the genetic alterations in diffuse low-grade gliomas, and they found that PIK3R1 mutations and altered RB pathway genes (RB1, CDKN2A and CDK4) were independent predictors of poor survival in 109 IDH mutant astrocytomas (2).

Based on these studies, we speculated that not all IDH mutant lower grade (WHO Grades II/III) astrocytomas behave uniformly well and can be stratified for risk using molecular markers. In this study, we recruited a large series of 160 IDH mutant lower grade (WHO Grades II/III) astrocytomas, and studied them for PDGFRA amplification, CDKN2A homozygous deletion and CDK4 amplification by FISH analysis, TERT promoter mutation by Sanger sequencing and ATRX loss and p53 expression by immunohistochemistry. Our data illustrate that IDH mutant lower grade (WHO Grades II/III) astrocytomas is not a homogeneous group, some do not have longer survival and as a group, should be molecularly stratified for risk.

MATERIALS AND METHODS

Patients and tissue samples

A total of 160 consecutive and unselected IDH mutant lower grade (WHO Grades II/III) astrocytomas were collected between years 2010 and 2018, and they were diagnosed at the Prince of Wales Hospital, Hong Kong, Hua Shan Hospital, Shanghai and the First Affiliated Hospital of Zhengzhou University, Zhengzhou. All patients were aged 16 or above at the time of diagnosis. Histological review for a diagnosis of astrocytomas was performed by two pathologists (HKN and HC). Histological features were unequivocally astrocytic and grading was based on WHO 2016 and Grade III astrocytomas were diagnosed based on mitosis, hypercellularity and nuclear atypia, as per our previous publication (1). The clinicopathological features of the cohort are summarized in Table 1.

Data on patient demographics and therapeutic treatment were obtained from paper and electronic records of the institutions. Survival data were ascertained from records of follow-up visits in clinics, or by contacting with patients or close relatives by telephone. This study was approved by The Joint Chinese University of Hong Kong—New Territories East Cluster Clinical Research Ethics Committee, Ethics Committees of Huashan Hospital, Shanghai and First Affiliated Hospital of Zhengzhou University, Zhengzhou.

1p19q codeletion

1p19q codeletion was examined by FISH analysis as described (6). A sample was considered 1p or 19q deleted when >25% of counted nuclei exhibited one target signal and two reference signals and ratio of target to reference signals was <0.8 (13, 23). In this study, we only included astrocytomas that were 1p19q intact and carried IDH1/2 mutation (described below).

DNA extraction from formalin-fixed paraffin-embedded (FFPE) tissues

DNA was extracted from FFPE tissues for mutational analysis. In brief, representative areas with tumor content of at least 80% on FFPE sections were marked. Slides were then dewaxed by xylene and rehydrated in a series of descending grades of alcohol. Tissues within marked areas were scrapped and treated with proteinase K in 10 mM Tris HCl buffer (pH 8.5) at 55°C for 12 h followed by 98°C for 10 minutes. The cell lysate was centrifuged, and supernatant was collected and used in subsequent polymerase chain reaction (PCR) reaction.

IDH1/2 and TERT promoter (TERTp) mutation analyses

Sanger sequencing was conducted to examine hotspot mutations at promoter region (termed C228T and C250T) of TERT, codon 132 of IDH1 and codon 172 of IDH2 as described previously (6, 40). In brief, PCR was performed in a 20 μL contained 0.5 μL cell lysate, 0.5 μM of forward and reverse primers, 1× KAPA2G Robust HotStart ReadyMix (Sigma) or 1× KAPA HiFi HotStart ReadyMix (Sigma). Amplification was conducted under the conditions of 95°C for 3 minutes, followed by 45 cycles of 95°C for 15 s, 60°C/66°C for 15 s and 72°C for 30 s on Veriti® 96-well Thermal Cycler (Applied Biosystems). PCR products were cleaned by spin column-based nucleic acid purification kit

Table 1. Clinical and molecular characteristics.

		TERT promoter		ATRX expression		P53 expression		PDGFRA amplification		CDKN2A deletion		CDK4 amplification	
	All tumors (n = 160)	wt (n = 131)	mut (n = 26)	Positive (n = 39)	Negative (n = 109)	Negative (n = 78)	Positive (n = 78)	Negative (n = 130)	Positive (n = 30)	Negative (n = 136)	Positive (n = 24)	negative (n = 131)	Positive (n = 29)
Age (mean/median)	40.5/40.0	40.1/39.0	42.8/45.0	39.0/38.0	40.5/40.0	40.5/39.5	40.9/41.0	39.5/39.0	44.8/44.5	40.6/40.5	39.9/39.0	41.2/41.0	37.6/39.0
Gender													
Male	86 (53.8%)	66 (78.6%)	18 (21.4%)	24 (30.8%)	54 (69.2%)	40 (48.2%)	43 (51.8%)	70 (81.4%)	16 (18.6%)	73 (84.9%)	13 (15.1%)	70 (81.4%)	16 (18.6%)
Female	74 (46.3%)	65 (89.0%)	8 (11.0%)	15 (21.4%)	55 (78.6%)	38 (52.1%)	35 (47.9%)	60 (81.1%)	14 (18.9%)	63 (85.1%)	11 (14.9%)	61 (82.4%)	13 (17.6%)
Histological grade													
Diffuse astrocytoma (Grade II)	106 (66.3%)	88 (85.4%)	15 (14.6%)	27 (26.7%)	74 (73.3%)	46 (44.7%)	57 (55.3%)	93 (87.7%)	13 (12.3%)	93 (87.7%)	13 (12.3%)	87 (82.1%)	19 (17.9%)
Anaplastic astrocytoma (Grade III)	54 (33.8%)	43 (79.6%)	11 (20.4%)	12 (25.5%)	35 (74.5%)	32 (60.4%)	21 (39.6%)	37 (68.5%)	17 (31.5%)	43 (79.6%)	11 (20.4%)	44 (81.5%)	10 (18.5%)
Location													
Cerebellum	1 (0.6%)	1 (100.0%)	0 (0.0%)	0 (0.0%)	1 (100.0%)	1 (100.0%)	0 (0.0%)	1 (100.0%)	0 (0.0%)	0 (0.0%)	1 (100.0%)	1 (100.0%)	0 (0.0%)
Midline	17 (10.6%)	14 (82.4%)	3 (17.6%)	3 (20.0%)	12 (80.0%)	10 (58.8%)	7 (41.2%)	15 (88.2%)	2 (11.8%)	13 (76.5%)	4 (23.5%)	13 (76.5%)	4 (23.5%)
Hemisphere	141 (88.1%)	116 (84.1%)	22 (15.9%)	35 (26.7%)	96 (73.3%)	66 (48.2%)	71 (51.8%)	113 (80.1%)	28 (19.9%)	123 (87.2%)	18 (12.8%)	116 (82.3%)	25 (17.7%)
Not available	1 (0.6%)	0 (0.0%)	1 (100.0%)	1 (100.0%)	0 (0.0%)	1 (100.0%)	0 (0.0%)	1 (100.0%)	0 (0.0%)	0 (0.0%)	1 (100.0%)	1 (100.0%)	0 (0.0%)
Extent of resection													
Total resection	126 (78.8%)	103 (83.7%)	20 (16.3%)	32 (27.6%)	84 (72.4%)	59 (48.4%)	63 (51.6%)	101 (80.2%)	25 (19.8%)	111 (88.1%)	15 (11.9%)	102 (81.0%)	24 (19.0%)
Non-total resection	33 (20.6%)	28 (84.8%)	5 (15.2%)	6 (19.4%)	25 (80.6%)	18 (54.5%)	15 (45.5%)	28 (84.8%)	5 (15.2%)	25 (75.8%)	8 (24.2%)	28 (84.8%)	5 (15.2%)
Not available	1 (0.6%)	0 (0.0%)	1 (100.0%)	1 (100.0%)	0 (0.0%)	1 (100.0%)	0 (0.0%)	1 (100.0%)	0 (0.0%)	0 (0.0%)	1 (100.0%)	1 (100.0%)	0 (0.0%)
Adjuvant therapy													
No adjuvant therapy	15 (9.4%)	12 (80.0%)	3 (20.0%)	5 (35.7%)	9 (64.3%)	10 (66.7%)	5 (33.3%)	10 (66.7%)	5 (33.3%)	14 (93.3%)	1 (6.7%)	11 (73.3%)	4 (26.7%)
Chemotherapy only	7 (4.4%)	6 (85.7%)	1 (14.3%)	1 (14.3%)	6 (85.7%)	3 (42.9%)	4 (57.1%)	7 (100.0%)	0 (0.0%)	6 (85.7%)	1 (14.3%)	7 (100.0%)	0 (0.0%)
Radiotherapy only	7 (4.4%)	7 (100.0%)	0 (0.0%)	3 (42.9%)	4 (57.1%)	2 (28.6%)	5 (71.4%)	5 (71.4%)	2 (28.6%)	7 (100.0%)	0 (0.0%)	6 (85.7%)	1 (14.3%)
Chemo-radiotherapy	129 (80.6%)	105 (83.3%)	21 (16.7%)	29 (24.6%)	89 (75.4%)	61 (48.8%)	64 (51.2%)	107 (82.9%)	22 (17.1%)	108 (83.7%)	21 (16.3%)	105 (81.4%)	24 (18.6%)
Not available	2 (1.3%)	1 (50.0%)	1 (50.0%)	1 (50.0%)	1 (50.0%)	2 (100.0%)	0 (0.0%)	1 (50.0%)	1 (50.0%)	1 (50.0%)	1 (50.0%)	2 (100.0%)	0 (0.0%)

Abbreviations: mut = mutant; n = number of cases; wt = wild type.

(iNtRON Biotechnology), and sequenced with BigDye Terminator Cycle Sequencing kit v1.1 (Life Technologies). The products were resolved in 3130xl Genetic Analysis. Primer sequences are listed in Table S1.

Immunohistochemical analysis of ATRX and p53

Immunohistochemistry (IHC) was performed to detect ATRX loss and p53 expression. FFPE sections were de-waxed in xylene and rehydrated in graded ethanol. Sections were then treated with citrate buffer (pH 6.0) in microwave for antigen retrieval. Immunohistochemical staining was done in BenchMark ULTRA automated tissue staining systems (Ventana Medical Systems). The primary antibodies were anti-ATRX (Sigma HPA001906, 1:400) and anti-p53 (Dako DO-7, 1:100).

ATRX loss was defined by a lack of nuclear staining for ATRX by IHC in >10% of tumor cells (37). Endothelial cells and infiltrating inflammatory cells served as internal positive controls. Cases were classified as failed/non-informative when internal control cells were not immunopositive (n = 12 cases). In p53 staining, a tumor was considered as positive when >10% of tumor nuclei showed strong nuclear staining or the slides were completely immune negative (31, 32).

Fluorescence in situ hybridization (FISH) analysis

CDKN2A homozygous deletion, CDK4 amplification and PDGFRA amplification were evaluated by FISH as described (40). Vysis LSI CDKN2A SpectrumOrange/CEP 9 SpectrumGreen Probes (Vysis) were employed to investigate CDKN2A deletion. The Zytolight SPEC CDK4/CEN 12 Dual Color Probe (ZytoVision) was applied in CDK4 amplification detection. The target probe for CDK4 was labeled in green and the reference probe was labeled in red. Probes of PDGFRA (CTD-2054G11 and RP11-231C18) were generated from bacterial artificial chromosome clones using nick translation with the presence of Spectrum Orange deoxyuridine triphosphate (dUTP). The reference probe for PDGFRA was labeled with Spectrum Green dUTP. In brief, 4-μm-thick FFPE sections were de-waxed by xylene, heated in 1 M sodium thiocyanate for 10 minutes at 80°C and digested with pepsin at 37°C for 20 to 30 minutes. Sections were then rinsed in molecular biology grade water to stop the digestion and dehydrated by heating. Locus-specific probe was then denatured at 80°C for 10 minutes followed by incubation on slides for 16 h at 37°C. Next day, sections were washed twice in 1.5M Urea/2X saline sodium citrate at 50°C for 10 minutes, and stained with Vectashield mounting medium containing 4′,6-diamidino-2-phenylindole (Vector Laboratories). Fluorescent signals were visualized under a fluorescent microscope (Carl Zeiss). At least 100 non-overlapping signals were counted and analyzed in each case.

CDKN2A homozygous deletion was considered when >20% of tumor cells showed loss of two signals, in the presence of two reference signals (14). Polyploid cells were not common in this study (<5% of tumor cells), and they were excluded from our scoring. CDK4 and PDGFRA amplification were defined as over 10% of tumor cells showing over 12 target signals or over 40% of tumor cells showing 6 or 12 target signals (22). Representative photos showing tumors positive for CDKN2A homozygous deletion, CDK4 amplification and PDGFRA amplification are shown in Figure S1.

Statistical analysis

Statistical analysis was performing using IBM SPSS software v20 (IBM Corporation, NY, USA). Chi-squared test or Fisher's exact test was used to examine the correlation between molecular markers and clinical parameters. Progression-free survival (PFS) was defined as the time between tumor diagnosis to recurrence or progression as evidenced by radiological imaging. Overall survival (OS) was defined as the time between diagnosis to death or last follow-up. Survival curves were performed by Kaplan–Meier method. Log-rank test was done to compare survival distribution between groups. In univariate and multivariate analyses, Cox's proportional hazards regression model was applied. All hazard ratios were reported with 95% CIs. *P*-value of <0.05 (two sided) was considered statistically significant.

RESULTS

Clinical characteristics of IDH mutant lower grade (WHO Grades II/III) astrocytomas

All tumors in this series were morphologically astrocytomas and were 1p19q non-deleted by FISH and IDH mutant by sequencing. The mean and median ages of our 160 tumors were 40.5 and 40.0 years old, respectively. 106 cases were Grade II diffuse astrocytomas and 54 cases were Grade III anaplastic astrocytomas. Male to female ratio was 1.16:1. 141 tumors were located in the hemispheres. Tumors involving the ventricular system, brain stem, thalamus, spinal cord, sellar region and pineal region were defined as midline tumors. 17 tumors were located in midline and one was located in cerebellum (Table 1). 126 patients received total resection. Adjuvant therapy data were available in 158 cases. 129 patients were given both chemo- and radiotherapy. Seven patients had chemotherapy alone or radiotherapy alone. 15 patients did not receive any adjuvant therapy.

Follow-up data were available in 155 of 160 (96.9%) patients. For overall survival, 113/155 (72.9%) patients were still alive upon the completion of study and 42/155 (27.1%) were passed away. For progression-free survival, 102/149 (68.5%) patients did not experience a tumor progression at the end of this study. The mean OS was 64.7 months but caution has to be taken in interpretation because majority of the patients were censored. The median OS was not yet reached. The mean OS for Grades II and III were 72.5 and 39.5 months respectively ($P < 0.0001$).

Univariate Cox proportional hazards analyses were performed, and histological grade was highly associated with PFS ($P < 0.001$) and OS ($P < 0.001$). Gender, location, extent of resection, radiotherapy, chemotherapy and chemo-

radiotherapy were not associated with PFS and OS (Table 2).

TERTp mutations in IDH mutant lower grade (WHO Grades II/III) astrocytomas

TERTp mutation was examined in 157 cases with sufficient tissue for analysis, and 26 of them (16.6%) carried the mutation. Majority of mutant cases (20/26; 76.9%) had C228T mutation. TERTp mutation was not associated with age, gender, histological grade, tumor location and extent of resection (Table S2). Co-occurrence of TERTp mutation and PDGFRA amplification was identified in one Grade III tumor as described below. One other tumor exhibited both TERTp mutation and CDKN2A homozygous deletion (below). We did not detect a prognostic significance of TERTp for PFS (P = 0.718; Figure 1A) and OS (P = 0.647; Figure 1B).

ATRX loss in IDH mutant lower grade (WHO Grades II/III) astrocytomas

IHC was employed to detect ATRX loss in our cohort. ATRX loss was found in 109/148 (73.6%) of our cohort. Uninformative result was noted in 12 cases caused by the failure of internal control. We did not detect a difference in age, gender, histological grade, tumor location and extent of resection between ATRX-loss and ATRX-retent tumors (Table S2). ATRX loss and TERTp mutation were mutually exclusive. ATRX loss was not associated with PFS (P = 0.477; Figure 1C) and OS (P = 0.341; Figure 1D).

P53 expression in IDH mutant lower grade (WHO Grades II/III) astrocytomas

P53 accumulation was detected in 78 of 156 (50.0%) of IDH mutant lower grade astrocytomas. P53 immunopositivity was not associated with age, gender, histological grade, tumor location and extent of resection (Table S2). P53 expression was not associated with PFS (P = 0.918; Figure 1E) and OS (P = 0.100; Figure 1F).

PDGFRA amplification is a poor prognostic marker in IDH mutant lower grade (WHO Grades II/III) astrocytomas

PDGFRA amplification was identified in 18.8% (30/160) of tumors. PDGFRA amplification was significantly associated with histological grade (Tables 1 and S2). More than 30% of anaplastic astrocytomas harbored PDGFRA amplification. In contrast, the alteration was identified in only 12.3% of diffuse astrocytomas. Furthermore, PDGFRA-amplified patients were older than non-amplified patients (P = 0.011; Table S2). PDGFRA amplification was not associated with gender, tumor location and extent of resection (Table S2). We identified a 56-year-old man diagnosed with anaplastic astrocytoma carrying TERTp mutation and PDGFRA amplification.

Kaplan–Meier survival analysis revealed PDGFRA amplification was associated with shorter PFS (P = 0.0003) and OS (P < 0.0001) (Figure 2A,B). After adjusting for age, histological grade, tumor location and extent of resection in multivariate analysis, PDGFRA amplification remained as an

Table 2. Univariate Cox proportional hazards regression models of clinical characteristics.

	PFS		OS	
Variables	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Age	1.036 (1.006-1.066)	0.018	1.037 (1.007-1.067)	0.015
Gender				
Male	1		1	
Female	1.019 (0.573-1.811)	0.950	1.031 (0.561-1.894)	0.923
Histological grade				
Diffuse astrocytoma (Grade II)	1		1	
Anaplastic astrocytoma (Grade III)	4.187 (2.256-7.771)	<0.0001	5.723 (2.985-10.975)	<0.0001
Location				
Hemisphere	1	0.192	1	0.212
Cerebellum	0.000047*	0.980	0.00013*	0.979
Midline	2.036 (0.945-4.384)	0.069	2.086 (0.921-4.725)	0.078
Extent of resection				
Total resection	1		1	
Non-total resection	1.048 (0.533-2.064)	0.891	1.412 (0.722-2.761)	0.313
Radiotherapy				
Yes	1		1	
No	1.042 (0.411-2.644)	0.931	0.944 (0.336-2.656)	0.914
Chemotherapy				
Yes	1		1	
No	0.706 (0.279-1.785)	0.462	0.810 (0.318-2.064)	0.659
Chemo-radiotherapy				
Yes	1		1	
No	0.685 (0.290-1.613)	0.386	0.653 (0.256-1.662)	0.371

*The hazard ratio did not have significance. Abbreviation: n = number of cases with data available.

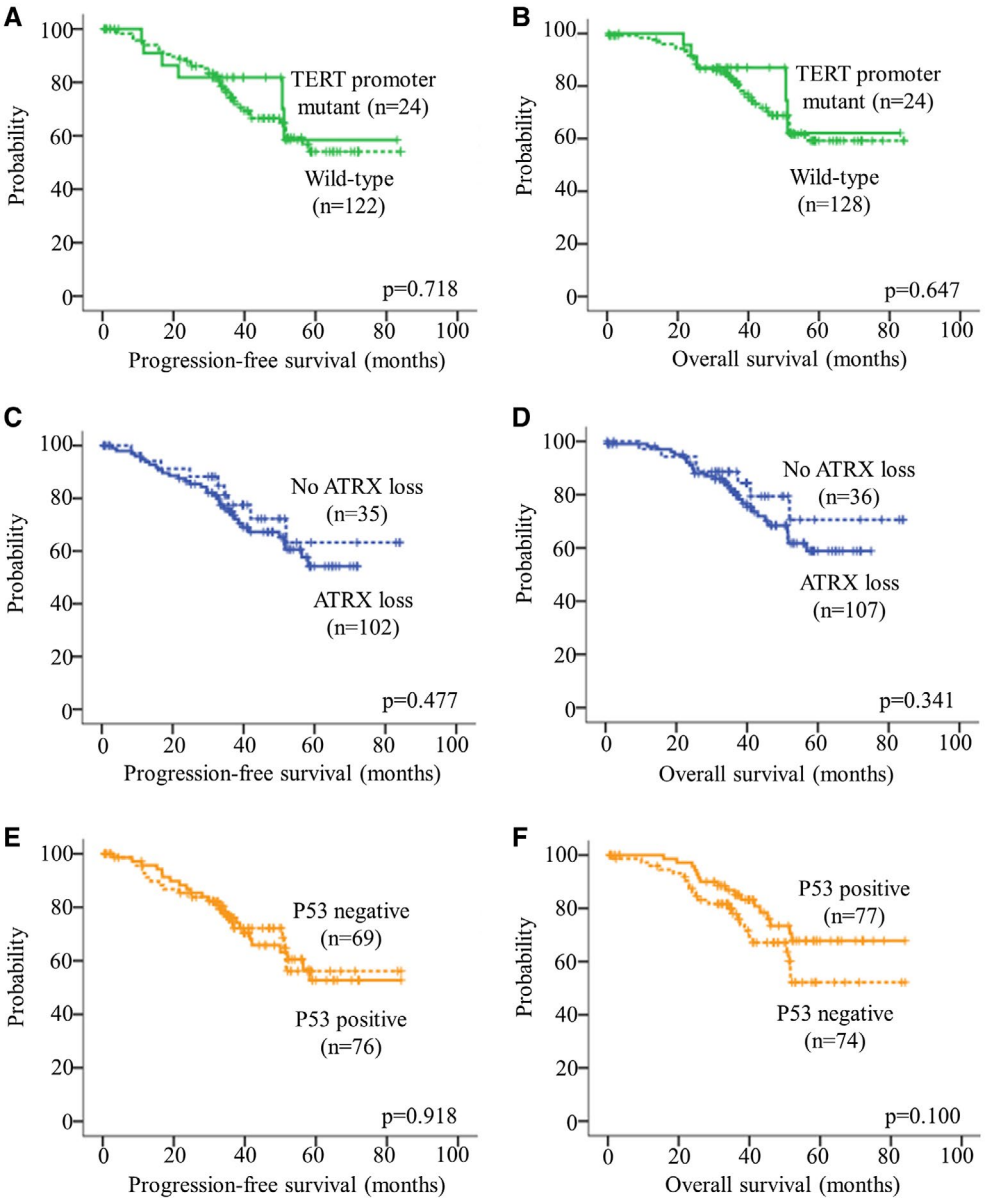


Figure 1. *Kaplan–Meier survival curves of TERTp mutation, ATRX loss and p53 accumulation.* TERTp mutation had no clinical impact on (**A**) PFS and (**B**) OS. ATRX loss was not associated with (**C**) PFS and (**D**) OS. P53 accumulation was not associated with (**E**) PFS and (**F**) OS. The hash marks represent censored patients.

independent factor for PFS [HR 2.567 (95% CI 1.254–5.258); $P = 0.010$] and OS [HR 2.904 (95% CI 1.372–6.149); $P = 0.005$].

CDKN2A homozygous deletion and CDK4 amplification were associated with poor outcomes in a subset of IDH mutant lower grade (WHO Grades II/III) astrocytomas

We then studied CDKN2A homozygous deletion and CDK4 amplification. These genes are involved in the RB signaling pathway and they are frequently altered in astrocytomas (30). CDKN2A homozygous deletion was detectable in 24 of 160 (15.0%) of IDH mutant lower grade (WHO Grades II/III) astrocytomas, including 13 diffuse astrocytomas and 11 anaplastic astrocytomas. CDK4 amplification was identified in 18.1% (29/160) of our cohort. 19 cases were diffuse astrocytomas and 10 cases were anaplastic astrocytomas. Changes in CDKN2A or CDK4 were not associated with sex, age, histological grade, tumor location and extent of resection (Table S2). Alterations in PDGFRA, CDKN2A and CDK4 were mutually exclusive. A single case of an anaplastic astrocytoma from a 33-year-old female carried both TERTp mutation and CDKN2A homozygous deletion.

As PDGFRA amplification, belonging to the RTK-PI3K-mTOR pathway, showed a clinical impact on survival, we

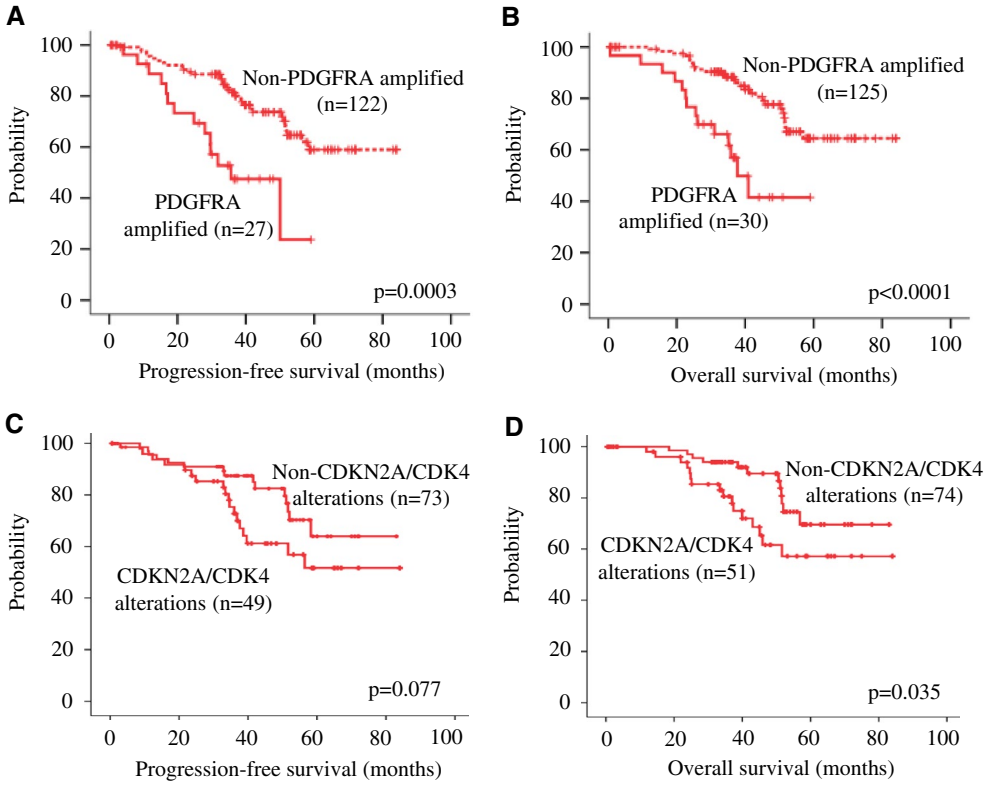


Figure 2. *Kaplan–Meier survival curves of PDGFRA amplification, CDKN2A deletion and CDK4 amplification.* Alteration in PDGFRA (a member in the RTK-PI3K-mTOR pathway) was associated with shorter (**A**) PFS ($P < 0.0001$) and (**B**) OS ($P < 0.0001$). Alteration in CDKN2A or CDK4 (members in the RB pathway) showed a trend toward a poorer (**C**) PFS ($P = 0.077$) and was associated with a shorter (**D**) OS ($P = 0.035$).

questioned if CDKN2A homozygous deletion and CDK4 amplification of the RB pathway is clinically relevant in those case without PDGFRA amplification. The molecular events had been mutually exclusive. We found tumors with CDKN2A homozygous deletion or CDK4 amplification showed a trend toward a poorer PFS ($P = 0.077$; Figure 2C) and a significantly shorter OS ($P = 0.035$; Figure 2D). Upon adjusting for age, histological grade, tumor location and extent of resection, multivariate analysis revealed that alteration in CDKN2A or CDK4 remained an independent factor for OS [HR 2.433 (95% CI 1.110–5.334); $P = 0.026$].

Stratification of IDH mutant lower grade (WHO Grades II/III) astrocytomas into risk groups

We then asked if we could stratify IDH mutant, lower grade (WHO Grades II/III) astrocytomas for risk. Given that PDGFRA of the RTK-PI3K-mTOR pathway and CDKN2A or CDK4 of the RB pathways were associated with clinical outcomes in this study and their alterations remained as independent factors for survivals in multivariate analysis, we included these three molecular markers in the risk stratification scheme. We divided IDH mutant, lower grade (WHO Grades II/III) astrocytomas into three risk groups (low, intermediate and high risk) according to changes in PDGFRA, CDKN2A and CDK4 (RTK-PI3K-mTOR and RB pathways) status (Figure 3). Tumors with PDGFRA amplification (alteration in the RTK-PI3K-mTOR pathway) were assigned into high-risk group (n = 30). Tumors with CDKN2A homozygous deletion or CDK4 amplification (alteration in the RB pathway) were assigned into intermediate-risk group (n = 53). Tumors with no alteration in these genes (two pathways) were classified into low-risk group (n = 77). The high-risk group accounted for nearly 18.8% (30/160) of the cohort. The intermediate- and low-risk groups represented 33.1% (53/160) and 48.1% (77/160) of our cohort respectively. We found a remarkable association between and risk group and histology ($P = 0.001$; Table 3). Nearly 60% of tumors in high-risk group were anaplastic astrocytomas (Grade III). In contrast, low-risk group comprised mainly diffuse astrocytomas (Grade II; 79.2%). Furthermore, risk groups were significantly associated with age ($P = 0.026$), and patients of high-risk group were much older (Table 3). We did not find association between risk groups and other clinical parameters (Table 3). Kaplan–Meier survival analysis indicated that risk groups correlated with PFS ($P < 0.001$; Figure 4A) and OS ($P < 0.0001$; Figure 4B). Compared to patients of intermediate- and low-risk groups, patients of high-risk group had the worst clinical outcomes in term of PFS and OS (Figures 4A,B). Pairwise comparison further indicated that patients of intermediate-risk group had a

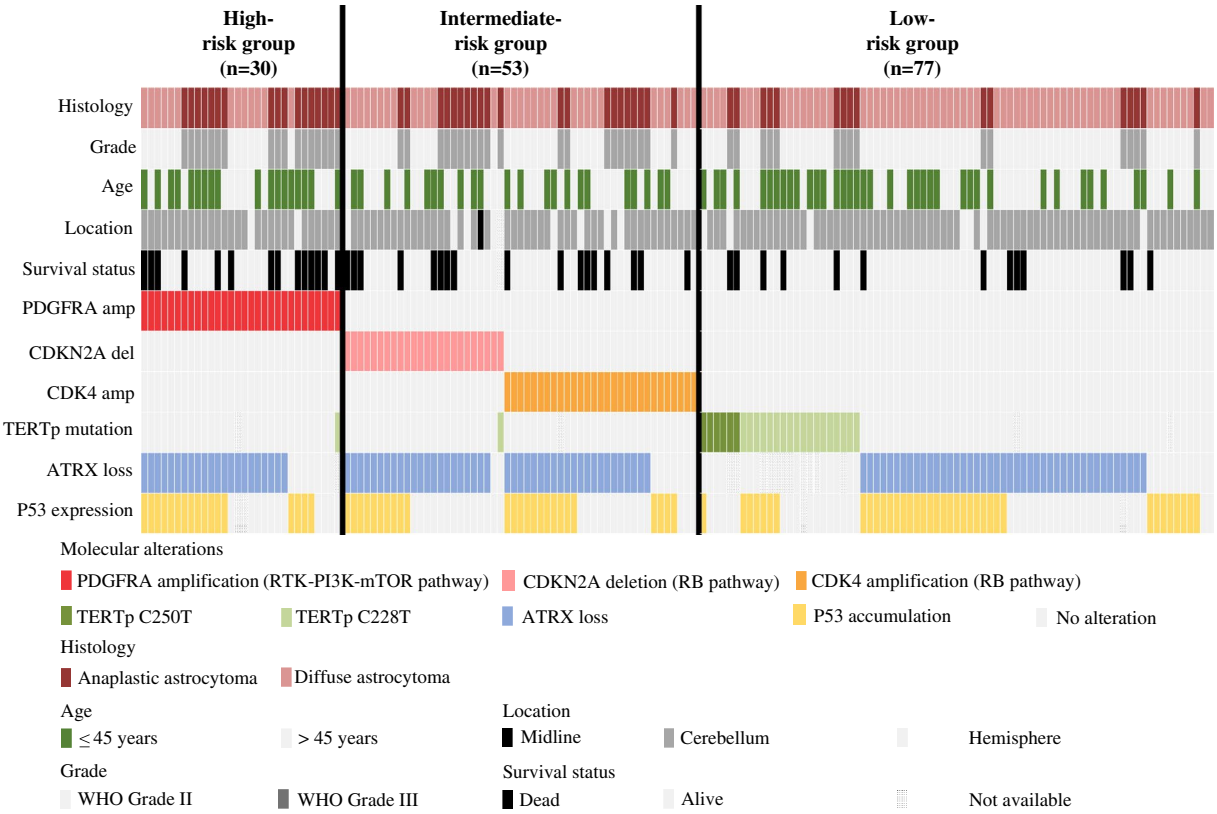


Figure 3. *Summary of clinical data and molecular characteristics of low-, intermediate- and high-risk groups.* IDH mutant lower grade (WHO Grades II/III) astrocytomas were divided into three groups according to PDGFRA, CDKN2A and CDK4 alterations. The high-risk group was assigned to tumors carrying PDGFRA amplification (n = 30). The intermediate-risk group was assigned to tumors showing CDKN2A homozygous deletion or CDK4 amplification (n = 53). The low-risk group was assigned to tumors showing no change in PDGFRA, CDKN2A and CDK4 (n = 78).

significantly shorter OS compared to patients of low-risk group (P = 0.035). Compared to patients in low-risk group, patients in intermediate-risk group tended to have a shorter PFS (P = 0.077). After adjusting covariates including age, histological grade, tumor location and extent of resection in multivariate Cox proportional hazards model, these biomarker-associated risk groups remained as independent prognosticators for PFS (P = 0.009; Table 4) and OS (P = 0.004; Table 4).

DISCUSSION

The updated WHO 2016 classification of tumors of the central nervous system for the first time combined molecular markers and histology to provide an integrated diagnosis (18, 35). IDH mutant lower grade astrocytomas comprise Grade II diffuse astrocytoma, IDH mutant and Grade III anaplastic astrocytoma, IDH mutant. IDH mutant lower grade (WHO Grades II/III) astrocytomas are associated with favorable disease outcome compared to their IDH wild type counterparts (4, 12, 27). IDH mutant lower grade astrocytic tumors are also enriched for p53 mutations (27). IDH mutation impairs histone demethylation and induces major effects on the tumor's methylome and transcriptome (19, 33). In particular, IDH mutation induces G-CIMP (gliomas CpG island methylator phenotype), and downregulates genes known to be involved in glioma initiation and outcome, including CDKN2C and GAP43 (33).

A number of studies have demonstrated that IDH mutation alone is insufficient to stratify astrocytic tumors. Diplas *et al* showed that IDH wild type glioblastomas are molecularly heterogeneous and not all of them are associated with poor outcome (9). IDH wild type glioblastomas bearing the wild type TERTp are associated with prolonged overall survival compared to those carrying mutations in the TERTp (9). Our group has also showed that IDH wild type lower grade gliomas are prognostically heterogeneous and they do not have uniformly poor prognosis (1). IDH wild type lower grade gliomas can be stratified into "molecularly" high grade and "molecularly" low grade based on EGFR, H3F3A and TERTp alterations (1). The cIMPACT-NOW has now recommended that IDH wild type diffuse astrocytic gliomas carrying EGFR amplification, or combined whole chromosome 7 gain and whole chromosome 10 loss (+7/−10), or TERTp mutation have molecular features of a glioblastoma (3). This recommendation highlights the heterogeneity of astrocytic tumors and that IDH mutation alone is not enough to provide accurate information about the natural history

Table 3. Clinical parameters of low-, intermediate- and high-risk groups.

	Low	Intermediate	High	
	(n = 77)	(n = 53)	(n = 30)	*P*-value
Age (mean/median)	40.2/40.0	38.6/39.0	44.8/44.5	0.026
Gender				
Male	41 (53.2%)	29 (54.7%)	16 (53.3%)	0.985
Female	36 (46.8%)	24 (45.3%)	14 (46.7%)	
Histological grade				
Diffuse astrocytoma (Grade II)	61 (79.2%)	32 (60.4%)	13 (43.3%)	0.001
Anaplastic astrocytoma (Grade III)	16 (20.8%)	21 (39.6%)	17 (56.7%)	
Location				
Cerebellum	0 (0.0%)	1 (1.9%)	0 (0.0%)	0.393
Midline	7 (9.1%)	8 (15.1%)	2 (6.7%)	
Hemisphere	70 (90.9%)	43 (81.1%)	28 (93.3%)	
Not available	0 (0.0%)	1 (1.9%)	0 (0.0%)	
Extent of resection				
Total resection	62 (80.5%)	39 (73.6%)	25 (83.3%)	0.622
Non-total resection	15 (19.5%)	13 (24.5%)	5 (16.7%)	
Not available	0 (0.0%)	1 (1.9%)	0 (0.0%)	
Adjuvant therapy				
No adjuvant therapy	5 (6.5%)	5 (9.4%)	5 (16.7%)	0.323
Chemotherapy only	6 (7.8%)	1 (1.9%)	0 (0.0%)	
Radiotherapy only	4 (5.2%)	2 (3.8%)	2 (6.7%)	
Chemo-radiotherapy	62 (80.5%)	44 (83.0%)	22 (73.3%)	
Not available	0 (0.0%)	1 (1.9%)	1 (3.3%)	

Abbreviation: n = number of cases.

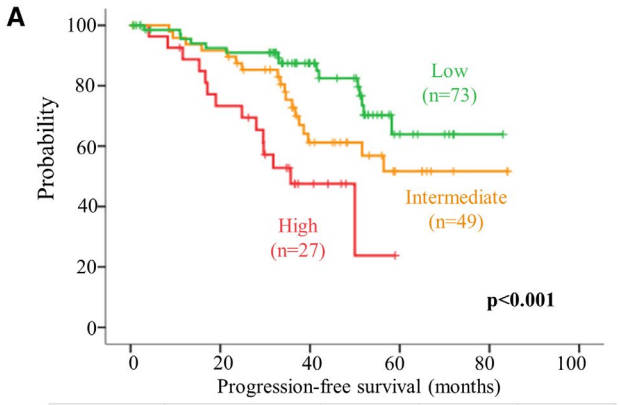
of astrocytic tumors. We therefore postulated that IDH mutant lower grade (WHO Grades II/III) astrocytomas can be stratified for risk, similar to the IDH wild type astrocytomas.

In this study, we found TERTp mutation in 16.6% of IDH mutant lower grade (WHO Grades II/III) astrocytomas, and the prevalence was similar in both Grades II and III tumors. These tumors were morphologically clearly not oligodendroglial and they were all 1p19q retained (Figure S2). TERTp mutation was not associated with the clinical parameters and outcomes in this study. Similar frequency of TERTp mutation in IDH mutant, 1p19q intact astrocytomas has been reported (16). In a major series by Eckel-Passow *et al*, 13.5% (5/37) of IDH mutant, 1p19q intact astrocytomas showed TERTp mutations, and the mutation was found in both Grades II and III (shown in Figure S4 of the paper) (10). Interestingly, in the TCGA study, among the 65 IDH mutant, 1p19q intact astrocytomas that were examined for TERTp mutation only 2 of them (3.1%) carried the mutation (5). We are uncertain of the reason for discrepancy but it is interesting to note that in our study, Grade II tumors accounted for 2/3 of our cohort, whereas about 2/3 of IDH mutant, 1p19q intact astrocytomas with TERTp data in TCGA study were of Grade III.

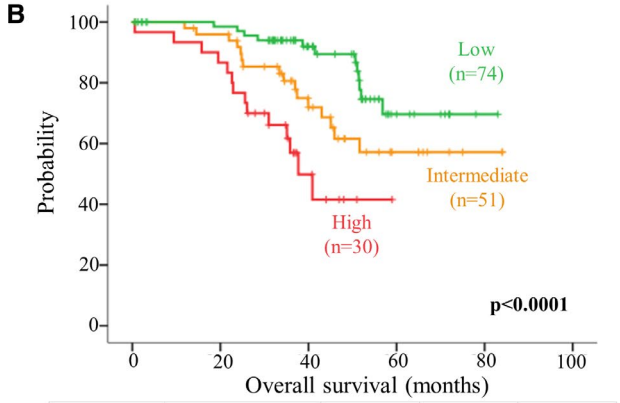
In this cohort, we identified ATRX loss in 73.6% IDH mutant lower grade (WHO Grades II/III) astrocytomas. Previous study by Wiestler and his colleagues also demonstrated that astrocytomas with IDH mutation and intact 1p19q have a similar frequency of ATRX loss (37). However, another study conducted by Reuss *et al* showed that majority of the 1p19q intact, IDH mutant astrocytomas display ATRX loss (95.6%) (26). This discrepancy in prevalence of ATRX loss may be explained by different clinical features between studies. More than 70% of 1p19q intact, IDH mutant astrocytomas in Reuss *et al* study were of Grade III, whereas only one-third of astrocytomas in our study were of Grade III (33.8%; $P < 0.0001$). Moreover, the age of the patients in our study is significantly older compared to that of Reuss *et al* study (mean age 40.5 vs. 37.7 years old; $P = 0.0197$).

Loss of ATRX was not associated with clinical parameters and was not a prognostic factor in our cohort. Wiestler *et al* showed that low-grade glioma patients with ATRX retention and IDH mutations have shorter time to treatment failure than those patients with loss of ATRX and IDH mutation (37). The difference in findings between our study and Wiestler *et al*'s may be explained by the inclusion of tumors of different entities in Wiestler *et al* study. Leeper *et al* also examined the clinical impact of ATRX expression in WHO Grade II gliomas and found that tumors with ATRX retention, IDH mutations and intact 1p19q have similar overall survival compared to tumors with ATRX loss, IDH mutations and intact 1p19q (17).

In this study, we identified p53 accumulation in half of the IDH mutant lower grade (WHO Grades II/III) astrocytomas. P53 protein expression has no impact on clinical outcome in this series. Leeper *et al* examined p53 nuclear staining in WHO Grade II gliomas. Of their IDH mutant astrocytomas without 1p19q codeletion, 60% displayed p53 staining (17). The prevalence of p53 staining in our study is comparable to that in Leeper *et al* study. Analysis of TCGA database revealed the presence of TP53 mutation in 90.2% of IDH mutant lower grade astrocytomas (5).



p value	Mean survival months (95%CI)	Low	Intermediate	High
Low	67.4(60.6-74.2)			
Intermediate	59.7(51.2-68.3)	0.077		
High	37.2(29.7-44.8)	<0.0001	0.036	



p value	Mean survival months (95%CI)	Low	Intermediate	High
Low	71.5(65.8-77.2)			
Intermediate	62.9(54.8-71.1)	0.035		
High	40.5(33.5-47.5)	<0.0001	0.046	

Figure 4. *Risk stratification of IDH mutant lower grade astrocytomas based on molecular markers.* Kaplan–Meier survival curves of molecular-based risk group for (**A**) PFS and (**B**) OS. Green, orange and red lines represent survival curves for low-, intermediate- and high-risk groups respectively. Patients with PDGFRA amplification representing a change in the RTK-PI3K-mTOR pathway were assigned to high-risk group. Patients with CDKN2A deletion or CDK4 amplification representing a change in the RB pathway were grouped into intermediate-risk group. Patients without PDGFRA, CDKN2A and CDK4 aberrations were grouped into low-risk group. These three risk groups had distinct PFS ($P < 0.001$) and OS ($P < 0.001$).

Similar to our findings, TP53 mutation was not associated with PFS and OS in TCGA cohort. The difference in the prevalence of p53 alterations between our study and TCGA database may be explained by the sensitivity of immunohistochemistry. As reported earlier, p53 immunohistochemical detection reached 77.4%–78.8% sensitivity compared to DNA sequencing in detecting TP53 mutation (32). Positive p53 immunohistochemistry correlated well with missense mutations with a sensitivity of 92%; however, only 33% of tumors with truncating mutations would show p53 positivity (11).

A few studies have implicated the use of combination of TERTp, ATRX or p53 with other molecular biomarkers in the classification of gliomas as a whole (5, 10, 17, 39). For instance, IDH, TERTp mutations and 1p19q codeletion can divide lower grade gliomas into five subgroups that are independently associated with prognosis (10). A similar classification system based on IDH and TERTp mutations has been proposed in Grades II and III gliomas (39). A recent study suggested combination of 1p19q codeletion, IDH mutation and ATRX loss can more accurately predict outcome of WHO Grade II gliomas as compared to histology (17). In this study, we showed TERTp mutation, ATRX loss and p53 accumulation have little prognostic value in IDH mutant lower grade astrocytomas.

Overall, a proportion of our cases (19 cases, 11.9%) were ATRX retained and p53 negative. These tumors are morphologically astrocytic and showed fibrillated GFAP positivity and are shown in Figure S2. We appreciate p53 positivity and ATRX retention are important diagnostic criteria for assigning a tumor as astrocytic in the literature. However, in Leeper *et al* 2015s study, 40% of IDH mutant 1p19q retained astrocytic tumors were p53 negative (17) and in Reis *et al* study, 24.6% of IDH mutant "histologically classified as astrocytoma" did not show loss of ATRX (Reis *et al* did not do 1p19q in astrocytomas) (24). Similarly 30% of IDH mutant 1p19q intact astrocytomas in Wiestler *et al* 2013s study showed no ATRX loss, as per data we could retrieve from the papers. We appreciate another major study by Reuss *et al* showed majority (95%) of IDH mutant lower grade astrocytomas showed ATRX loss. Whatever the percentages, our findings confirmed previous studies that the clear majority of IDH mutant lower grade astrocytomas showed p53 positivity and ATRX loss.

Platelet-derived growth factor receptor alpha (PDGFRA) is a member of the receptor tyrosine kinase family and is involved in the RTK-PI3K-mTOR pathway (7, 20). This receptor binds to certain isoforms of platelet-derived growth factor (PDGF) and controls cell proliferation, cell fate specification, migration in the neural stem cell (NSC) compartment and glial development (8, 29). Alterations of PDGFRA, including amplification and overexpression, have been detected in diffuse gliomas and PDGFRA amplification has been identified in 18% of IDH mutant anaplastic astrocytomas (22). Aberrations of PDGFRA together with IDH mutations have been linked to the proneural subtype of glioblastomas (34). PDGFRA amplification is associated with poor PFS and OS in glioblastomas (22). However, the prognostic implication of PDGFRA amplification has not been extensively studied in IDH mutant lower grade (WHO Grades II/III) gliomas. In this study, we showed PDGFRA amplification in 18.8% of our cohort and it is more common in anaplastic astrocytomas. Importantly, PDGFRA amplification is associated with shorter PFS and OS and it is an independent prognostic factor in multivariate analysis. PDGFRA amplification is not prognostically significance in IDH mutant lower grade astrocytomas of TCGA dataset

Table 4. Multivariate Cox proportional hazards regression models of clinico-pathological features and risk groups.

	PFS		OS	
Variables	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Age	1.024 (0.994-1.055)	0.122	1.024 (0.992-1.057)	0.146
Histological grade				
Diffuse astrocytoma (Grade II)	1		1	
Anaplastic astrocytoma (Grade III)	3.079 (1.601-5.918)	0.0007	4.268 (2.145-8.895)	0.00005
Location				
Hemisphere	1	0.135	1	0.145
Cerebellum	0.00004*	0.975	0.00003*	0.984
Midline	2.304 (1.018-5.216)	0.045	2.434 (1.002-5.911)	0.049
Extent of resection				
Total resection	1		1	
Non-total resection	1.009 (0.498-2.044)	0.980	1.291 (0.646-2.582)	0.469
Risk group				
Low-risk group	1	0.009	1	0.004
High-risk group	3.642 (1.587-8.359)	0.002	4.667 (1.890-11.521)	0.0008
Intermediate-risk group	1.988 (0.972-4.065)	0.060	2.378 (1.090-5.189)	0.030

*The hazard ratio did not have significance. Abbreviation: n = number of cases with data available.

and the difference may be caused by the relatively small number of IDH mutant lower grade astrocytomas in the TCGA dataset.

CDKN2A (cyclin-dependent kinase inhibitor 2A) is located at chromosome 9p21.3. The gene encodes two distinct proteins, p16 (or p16INK4a) and p14 (or p14ARF), which are generated through alternative exon usage. Although both p16INK4a and p14ARF are tumor suppressors, they are involved in different pathways. The protein p16INK4a induces G1 cell cycle arrest by inhibiting the phosphorylation of the Rb protein by the cyclin-dependent kinases, CDK4 and CDK6. p14ARF induces a p53-dependent cell cycle arrest by interacting with MDM2 and stabilizing p53. CDKN2A deletion is often detected in pediatric low-grade and high-grade gliomas (15). Reis *et al* showed that CDKN2A deletion is associated with a shorter overall survival in Grades II and III astrocytomas (24). Shirahata *et al* identified CDKN2A deletion as a molecular biomarker to stratify IDH mutant astrocytic tumors. Grade III astrocytic tumors harbored CDKN2A deletion had shorter overall survival compared to non-deleted counterpart and Grade IV glioblastomas without CDKN2A deletion (28). In contrast to Shirahata *et al* study in which no Grade II astrocytic tumor had CDKN2A deletion, we found 12.3% of Grade II IDH mutant astrocytomas harbored CDKN2A deletion. In addition to CDKN2A deletion, we also examined CDK4 amplification in our cohort because CDK4 as well as CDKN2A is a cell cycle regulator in the RB pathway and CDK4 alteration has been implicated in IDH mutant lower grade gliomas (2). We found CDK4 amplification in more than 10% of our samples, a frequency that is concordant with TCGA dataset. We also found after excluding cases with PDGFRA amplification, IDH mutant lower grade (WHO Grades II/III) astrocytomas harboring CDKN2A homozygous deletion or CDK4 amplification had a significantly shorter OS compared to those without the alteration. CDKN2A or CDK4 alteration remained as an independent prognostic factor in multivariate analysis. Both CDKN2A and CDK4 are members of the RB pathway.

As PDGFRA amplification, CDKN2A homozygous deletion and CDK4 amplification appeared in a mutually exclusive fashion in this study, and PDGFRA belongs to the RTK-PI3K-mTOR pathway while the latter two belong to the RB pathway, we hypothesized that these three markers could improve the risk stratification of IDH mutant lower grade astrocytomas. We therefore separated our samples into three groups. Tumors with PDGFRA amplification belonged to high-risk group, and they had the shortest PFS and OS. Patients with CDKN2A homozygous deletion or CDK4 amplification belonged to intermediate-risk group and they showed better prognosis compared to those in the high-risk group. Tumors without PDGFRA amplification, CDK4 amplification and CDKN2A deletion were stratified into low-risk group and they had the best clinical outcome in term of OS. We showed such stratification is independent of histological grade, tumor location and extent of resection.

A limitation of this study is that a significant fraction of patients' survival was censored (72.9%) in this study, a common confounding factor in studies on low-grade cancers, and this has made it difficult to achieve a precise assessment on the effect of molecular alterations on clinical outcomes. Hopefully, future studies with more long-term follow-up with give more insight on this question.

Our data illustrated the potential use of molecular markers to provide a more refined stratification for IDH mutant lower grade astrocytomas. Some IDH mutant astrocytomas, in spite of its IDH genotype, do not have a longer survival and pathologists could convey an inaccurate prognostic implication by merely using a diagnosis of IDH mutant astrocytoma without further molecular grading. We recommend the incorporation of PDGFRA, CDKN2A and CDK4 in the molecular stratification of IDH mutant lower grade astrocytomas. Such stratification likely provides a more precise prognostic information to patients and may influence decisions at the bedside.

The stratification scheme may also have significance toward future classification of lower grade astrocytomas.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web site:

Figure S1. Representative FISH images of IDH mutant lower grade astrocytomas displaying (A) PDGFRA amplification, (B) CDKN2A homozygous deletion and (C) CDK4 amplification.

Figure S2. H&E stained images and immunostaining of GFAP of 19 IDH mutant astrocytomas without ATRX loss or p53 staining. Cases 111 (36yr/F), 148 (39yr/M) and 169 (36yr/F) are high-risk tumors without ATRX loss and p53 staining. All carried wild type TERTp. Cases 99 (33yr/F), 170 (37yr/M), 193 (16yr/F), 201 (24yr/M) and 237 (26yr/M) are intermediate-risk tumors without ATRX loss and p53 staining. Case 99 carried TERTp mutation and the other four cases carried wild type TERTp. Cases 127 (53yr/M), 129 (39yr/M), 137 (46yr/M), 157 (44yr/M), 167 (59yr/F), 184 (45yr/M), 195 (47yr/M), 226 (48yr/M) and 245 (22yr/M) were low-risk tumors that carried TERTp mutation and showed no alteration in both ATRX and p53. Cases 94 (38yr/F) and 138 (35yr/M) were low-risk tumors carrying the wild type TERTp. Both of them showed no alteration in ATRX and p53.

Table S1. Primer sequences.

Table S2. Correlation between clinical features and molecular markers.