Molecular stratification of gliomas using next-generation sequencing: abridged secondary publication

AKY Chan, DTM Chan, JYT Chan, H Chen, NYF Chung, QJQ Huang, JSH Kwan, KKW Li, WC Li, EM Liu, XZ Liu, HHF Loong, TM Malta, Y Mao, HK Ng *, H Noushmehr, MFM Poon, WS Poon, ZF Shi, WW Wang, ZY Zhang

K E Y   M E S S A G E S

1. Adult diffuse gliomas are heterogeneous groups of tumours.
2. A combination of single-gene biomarkers can be used to stratify adult gliomas into molecular groups. Our proposed molecular grading system is superior to the histological grading system in terms of prognostication for adult gliomas.
3. IDH-wildtype, TERTp-wildtype adult glioblastomas clustered into different methylation classes, and CDKN2A/B deletion is an independent prognostic factor in this subset of gliomas.
4. Methylation classes, MGMTp methylation, TERTp mutation, and MMR mutation are prognostic biomarkers for IDH-wildtype, H3-wildtype glioblastomas in adolescents and young adults. These tumours are similar to the above IDH-wildtype, TERTp-wildtype glioblastomas in that they do not frequently exhibit the diagnostic molecular criteria for regular glioblastomas.

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AKY Chan, DTM Chan, JYT Chan, H Chen, NYF Chung, QJQ Huang, JSH Kwan, KKW Li, WC Li, EM Liu, XZ Liu, HHF Loong, TM Malta, Y Mao, HK Ng, H Noushmehr, MFM Poon, WS Poon, ZF Shi, WW Wang, ZY Zhang

Department of Anatomical and Cellular Pathology, The Chinese University of Hong Kong, Hong Kong SAR, China

* Principal applicant and corresponding author: hkng@cuhk.edu.hk

Introduction

Diffuse gliomas are the most common primary brain tumours in adults. According to the World Health Organization (WHO), diffuse gliomas can be classified into grades II to IV (glioblastoma). However, this grading system has considerable interobserver variability and weak reproducibility. It does not fully reflect the variable clinical course and biological diversity, even within a single grade group. Molecular studies have identified multiple biomarkers with diagnostic and prognostic implications, including IDH1/2 mutation, TERT promoter (TERTp) mutation, BRAF mutation, 1p19q codeletion, epidermal growth factor receptor (EGFR) amplification, and chromosome 10q deletion.

We developed a molecular grading system for diffuse gliomas in adults using histological data from 1275 patients in the Hospital Authority.[1] Adult gliomas can be stratified into six molecular grades with distinct clinical outcomes using a combination of single-gene molecular biomarkers. We demonstrated that molecular grading is superior to conventional histological grading for prognostication. We then used next-generation sequencing (NGS) and methylation profiling to delineate the molecular landscapes of 72 IDH-wildtype, TERTp-wildtype adult glioblastomas[2] and 50 IDH-wildtype, H3-wildtype glioblastomas in adolescents and young adults.[3] These glioblastomas are heterogeneous groups of tumours. We also identified molecular biomarkers associated with clinical outcomes; they do not frequently display the molecular diagnostic features of regular IDH-wildtype glioblastomas.

Methods

Crude cell lysate was prepared from formalin-fixed paraffin-embedded sections. DNA from the lysate was amplified using forward and reverse primers. Locus-specific probes were denatured, incubated overnight, washed, and visualised. At least 100 non-overlapping signals were counted and analysed in each case. Amplification was considered present when clusters appeared or when >5% of tumour cells displayed a target/reference signal ratio >2. Deletion was considered present when >25% of counted nuclei exhibited one target signal and two reference signals.

In-house glioma-specific target panels were constructed for targeted sequencing. DNA quality and quantity were assessed. Samples that passed quality control underwent library preparation. DNA libraries were further assessed for quality and quantity before sequencing. Paired-end reads were aligned to the human reference genome. Variants were identified and annotated.

RNA was extracted, and libraries were generated to detect fusion transcripts of 15 cancer-related genes. Paired-end reads were aligned to the human genome assembly GRCh37 (hg19); fusion genes were identified. DNA was subjected to bisulphite modification and hybridisation. Background correction, global dye-bias normalisation, and calculation of DNA methylation levels were then performed.

Results

We stratified 1275 cases of adult gliomas into six molecular grades based on IDH mutations, 1p19q codeletion, TERT promoter mutation, BRAF mutation, EGFR amplification, 10q loss, and H3.1 and H3.3 mutations.[1] The clinical features of molecular grading were classified into six groups: (1) IDH mutant and 1p19q codeleted; (2) IDH mutant, 1p19q non-codeleted, and TERT mutant; (3) IDH mutant, 1p19q non-codeleted, and TERT wildtype; (4) IDH wildtype and BRAF mutant; (5) IDH wildtype, BRAF wildtype, and without evidence of TERTp mutation, EGFR amplification, 10q loss, or H3 mutation; and (6) IDH wildtype and positive for TERTp mutation, EGFR amplification, 10q loss, or H3 mutations (Table).

Of all cases, 1028 were classified based on sufficient molecular data. Molecular grades were strongly associated with prognosis across the entire cohort (P<0.001, Fig 1). Multivariable analysis showed that molecular groups had independent prognostic value across the cohort after adjustments for age, sex, histological grade, tumour resection, radiotherapy, and chemotherapy.

Molecular grading was superior to conventional histological grading for prognostication. For example, WHO grade III (molecular group 1) tumours had a longer overall survival (OS) than WHO grade II (molecular group 6) tumours (P<0.001), and WHO grade IV (molecular group 4) tumours had a longer OS than WHO grade III (molecular group 6) tumours (P=0.004).

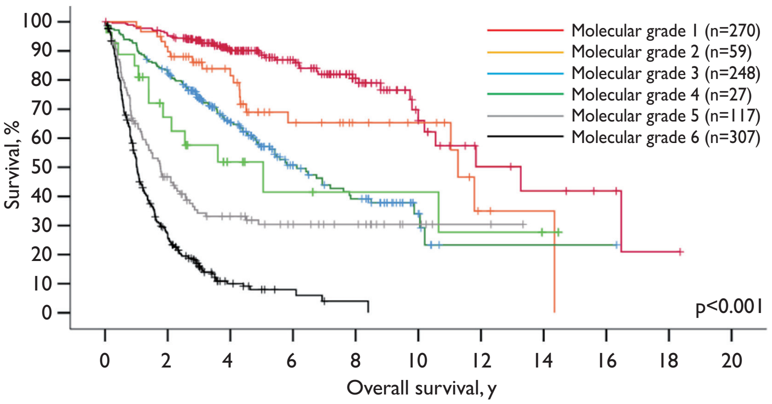
We investigated 72 IDH-wildtype, TERTp-wildtype adult glioblastomas.[2] Global methylation profiling revealed three methylation subtypes—classic-like, mesenchymal-like, and LGm6-GBM—in IDH-wildtype, TERTp-wildtype adult glioblastomas. The LGm6-GBM subtype was the most common (57%). There was, however, no survival difference among epigenetic subtypes.

EGFR amplification, TERTp mutation, and combined chromosome 7 gain and chromosome 10 loss (+7/-10) are common molecular features in regular glioblastomas. However, in this cohort, only 34.7% of samples demonstrated EGFR amplification. None showed TERTp mutation or chromosome +7/-10. Additionally, cyclin-dependent kinase (CDK) 4 amplification was detected in nearly 60% of samples, and platelet-derived growth factor receptor A (PDGFRA) amplification was observed in 38.9% of the cohort.

DNA-targeted sequencing was performed in 67 cases. Overall, 4.15±6.187 mutations were detected per sample, with values expressed as mean±standard deviation. Mismatch repair (MMR) gene mutations (MSH2, MSH6, MLH1, and PMS2) were mainly found in LGm6-GBM tumours. As expected, cases exhibiting MMR mutations showed a higher mutation load than those lacking MMR mutations (9.00±11.832 vs 3.49±4.794 mutations

TABLE. Multivariate analysis of clinical and molecular features of IDH-wildtype, TERTp-wildtype glioblastomas.

Variables	Overall survival		Progression-free survival	
	Hazard ratio (95% confidence interval)	P value	Hazard ratio (95% confidence interval)	P value
Age	1.011 (0.990-1.032)	0.303	0.999 (0.977-1.021)	0.931
Sex				
Male	1	0.697	1.281 (0.698-2.353)	0.424
Female	1.127 (0.617-2.059)			
Location				
Hemisphere	1	0.114	0.974 (0.383-2.477)	0.956
Non-hemisphere	0.447 (0.165-1.214)			
Operation				
Gross total resection	1	0.047	1	0.003
Non-total resection	1.824 (1.007-3.302)		2.598 (1.369-4.931)	
Chemotherapy				
Yes	1	0.003	1	0.003
No	6.644 (1.901-23.225)		6.711 (1.917-23.494)	
Radiotherapy				
Yes	1	0.688	1	0.147
No	1.220 (0.462-3.223)		2.095 (0.771-5.692)	
MGMTp				
Unmethylated	1	0.096	1	0.745
Methylated	0.609 (0.340-1.092)		0.908 (0.509-1.622)	
Epidermal growth factor receptor amplification				
No	1	0.456	1	0.627
Yes	1.269 (0.679-2.372)		1.177 (0.610-2.270)	
Platelet-derived growth factor receptor A amplification				
No	1	0.866	1	0.884
Yes	0.947 (0.506-1.775)		0.952 (0.489-1.851)	
CDKN2A/B homozygous deletion				
No	1	0.023	1	0.020
Yes	2.068 (1.107-3.864)		1.996 (1.115-3.572)	



Molecular grade	Median overall survival, y	Hazard ratio (95% confidence interval)	P value
1	13.3	1	<0.001
2	11.3	2.06 (1.21-3.51)	0.008
3	6.1	3.57 (2.51-5.08)	<0.001
4	5.1	4.26 (2.28-7.95)	<0.001
5	1.8	7.7 (5.29-11.2)	<0.001
6	1.0	18.1 (13-25.3)	<0.001

FIG 1. Overall survival curves of adult gliomas according to molecular grading (n=1028).

per sample, P=0.017). ATRX mutation was detected in four (6%) of 67 cases. ATRX-mutated cases had a higher mutation load than those without (22.25±13.022 vs 3.00±3.172 mutations per sample, P<0.001). A possible explanation is a link between ATRX deficiency and impaired non-homologous chromosomal end joining, which renders cells more sensitive to DNA-damaging agents. No prognostic implication of single-gene mutations was detected throughout the cohort.

Cyclin-dependent kinase inhibitor 2A/B (CDKN2A/B) homozygous deletion was associated with shorter OS (P=0.031) and a trend towards worse progression-free survival (P=0.088). Multivariate analysis revealed that CDKN2A/B deletion was an independent prognostic factor (Table).

Global methylation profiling revealed that 50 tumours clustered into the following subgroups: (1) glioblastoma, IDH wildtype, subclass midline (GBM_midline); (2) glioblastoma, IDH wildtype, subclass RTK I/II/III/mesenchymal (GBM_RTK/mesenchymal); (3) anaplastic pleomorphic xanthoastrocytoma (PXA); and (4) low-grade glioma (LGG) classes, including LGG_GG (ganglioglioma), LGG_MYB (MYB/MYBL1), and LGG_SEGA (subependymal giant cell astrocytoma).[3]

LGG-clustered tumours had significantly longer OS than those clustered with GBM_midline and GBM_RTK/mesenchymal methylation classes (Fig 2). PXA-clustered tumours also showed longer OS than GBM_midline-clustered tumours. The survival difference among methylation groups remained significant in multivariate analysis (P=0.040).

Combined chromosome 7 gain and chromosome 10 loss (+7/-10), TERTp mutation, and EGFR amplification are common in regular glioblastomas. However, only one case showed combined +7/-10, whereas TERTp mutation and EGFR amplification were detected in 20.4% and 14.0% of samples, respectively. The most frequent gene copy number variations and mutations in these glioblastomas were CDKN2A/B deletions (>60%) and TP53 mutation (40.8%), respectively.

Targeted NGS was successfully performed in 49 cases. The most prevalent mutation identified was TP53 (40.8%), followed by KMT2C (38.8%), BRAF (28.6%), NF1 (28.6%), ATRX (24.5%), and SETD2 (20.4%). BRAF mutation was associated with younger age (P=0.034) and was a favourable prognostic factor within the PXA-clustered group (P=0.018). SETD2 mutation was associated with shorter OS in the entire cohort (P<0.001). Additionally, 10 (20.4%) of 49 cases carried either the C228T or C250T hotspot mutation of TERTp, and tumours with TERTp mutation had significantly worse OS (P<0.001).

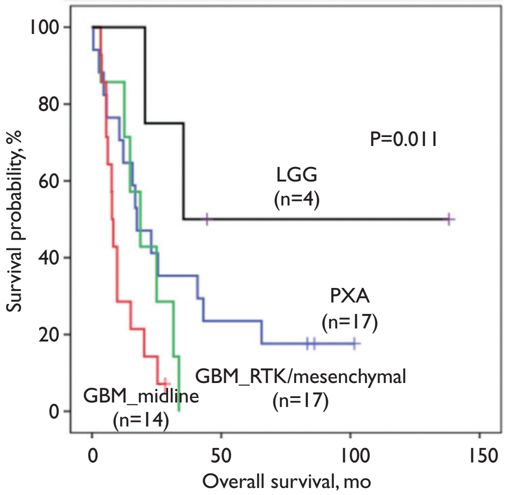
MGMTp methylation was found in 20% of cases and was associated with better OS for the entire cohort (P=0.032), even after adjustment for confounders (P=0.025). Tumours with mutations in any MMR genes (MLH1, MSH2, MSH6, and PMS2) were associated with worse OS (P=0.046); this association remained significant after adjustment for confounders (P=0.002). Tumours with TERTp mutation had worse OS (P<0.001), although significance was lost in multivariate analysis.

Discussion

Molecular criteria were first introduced in the 2016 WHO classification of central nervous system tumours. Many centres now use a combination of histological features, WHO grades, and molecular characteristics for pathological diagnosis. There remains a need to develop a simple molecular grading algorithm for gliomas based on single-gene testing.

Adult gliomas can be categorised into six molecular subgroups based on the biomarkers IDH1/2, TERTp, BRAF, H3.1, H3.3, and chromosome 10q status.[1] The prognostic power of these molecular groups is superior to that of histological grading alone. These molecular biomarkers can be readily assessed in routine pathology laboratories. Accordingly, we established a simple molecular grading system for clinical practice to refine prognostic stratification in adult gliomas.

IDH-wildtype, TERTp-wildtype adult glioblastomas can be divided into three methylation subtypes.[2] CDKN2A/B deletion was identified as



	P value			
Methylation classes	PXA	GBM_RTK/ mesenchymal	GBM_ midline	LGG-like
PXA	-	0.345	0.030	0.219
GBM_RTK/mesenchymal	0.345	-	0.125	0.023
GBM midline	0.030	0.125	-	0.011
LGG-like	0.219	0.023	0.011	-

FIG 2. Kaplan-Meier survival curves for methylation classes of IDH-wildtype, H3-wildtype glioblastomas in adolescents and young adults: low-grade glioma (LGG) clustered tumours show the best survival, followed by anaplastic pleomorphic xanthoastrocytoma (PXA), GBM_RTK/mesenchymal, and GBM_midline tumours.

an independent prognostic factor in this genotype-specific subset of glioblastomas. Only one-third of these glioblastomas exhibited the molecular diagnostic features of regular IDH-wildtype glioblastomas. Tumour grading should be assessed cautiously when evaluating TERTp-wildtype gliomas. PDGFRA alterations should be incorporated into the molecular diagnostic criteria for IDH-wildtype, TERTp-wildtype adult glioblastomas.

We previously characterised 50 IDH-wildtype, H3-wildtype glioblastomas in adolescents and young adults using global methylation profiling and NGS.[3] These tumours clustered into four methylation subgroups associated with different clinical outcomes. They did not commonly exhibit the diagnostic molecular criteria of regular glioblastomas, a finding similar to that observed in IDH-wildtype, TERTp-wildtype glioblastomas. Our findings illustrate the heterogeneity among these glioblastomas; careful consideration of patient age is warranted when diagnosing IDH-wildtype, H3-wildtype gliomas in adolescents and young adults who lack the molecular features of adult IDH-wildtype glioblastomas.

Conclusion

We developed a molecular-based grading system for adult gliomas, which has become part of the molecular diagnostic service for CNS tumours at our hospitals. We implemented NGS using our customised panel for selected glioma patients. Our work has advanced the practice of personalised medicine.

Funding

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Disclosure

The results of this research have been previously published in:

1. Chan AK, Shi ZF, Li KK, et al. Combinations of single-gene biomarkers can precisely stratify 1,028 adult gliomas for prognostication. Front Oncol 2022;12:839302.
2. Liu EM, Shi ZF, Li KK, et al. Molecular landscape of IDH-wildtype, TERT-wildtype adult glioblastomas. Brain Pathol 2022;32:e13107.
3. Shi ZF, Li KK, Huang QJ, et al. Molecular landscape of IDH-wild-type, H3-wild-type glioblastomas of adolescents and young adults. Neuropathol Appl Neurobiol 2022;48:e12802.

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