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Identification of subsets of *IDH*-mutant glioblastomas with distinct epigenetic and copy number alterations and stratified clinical risks

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Abstract

Background. *IDH*-mutant glioblastoma is classified by the 2016 CNS WHO as a group with good prognosis. However, the actual number of cases examined in the literature is relatively small. We hypothesize that *IDH*-mutant glioblastoma is not a uniform group and should be further stratified.

Methods. We conducted methylation profiles and estimated copy number variations of 57 *IDH*-mutant glioblastomas.

Results. Our results showed that 59.6% and 40.4% of tumors belonged to glioma-CpG island methylator phenotype (G-CIMP)-high and G-CIMP-low methylation subgroups, respectively. G-CIMP-low subgroup was associated with significantly worse overall survival (OS) as compared to G-CIMP-high ($P = .005$). *CDKN2A* deletion (42.1%) was the most common gene copy number variation, and was significantly associated with G-CIMP-low subgroup ($P = .004$). Other frequent copy number changes included mesenchymal–epithelial transition (*MET*) (5.3%), *CCND2* (19.3%), *PDGFRA* (14.0%), *CDK4* (12.3%), and *EGFR* (12.3%) amplification. Both *CDKN2A* deletion ($P = .036$) and *MET* amplification ($P < .001$) were associated with poor OS in *IDH*-mutant glioblastomas. Combined epigenetic signature and gene copy number variations separated *IDH*-mutant glioblastomas into Group 1 (G-CIMP-high), Group 2 (G-CIMP-low without *CDKN2A* nor *MET* alteration), and Group 3 (G-CIMP-low with *CDKN2A* and/or *MET* alteration). Survival analysis revealed Groups 1 and 2 exhibited a favorable OS (median survival: 619 d [20.6 mo] and 655 d [21.8 mo], respectively). Group 3 exhibited a significant shorter OS (median survival: 252 d [8.4 mo]). Multivariable analysis confirmed the independent prognostic significance of our Groups.

Conclusions. *IDH*-mutant glioblastomas should be stratified for risk with combined epigenetic signature and *CDKN2A/MET* status and some cases have poor outcome.

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Key points

1. Not all *IDH*-mutant glioblastomas have good prognosis.
2. Combined DNA methylation subgroups and *CDKN2A/*mesenchymal–epithelial transition (*MET*) status identified a subset of *IDH*-mutant glioblastomas with poor outcome.
3. Glioma-CpG island methylator phenotype-low, *CDKN2A* deletion, and *MET* amplification are negative prognostic markers in *IDH*-mutant glioblastomas.

Importance of the Study

The WHO 2016 Classification of Tumors of the Central Nervous System has classified glioblastoma into *IDH*-wildtype and *IDH*-mutant, the latter being described to have a better prognosis and to be more often found in secondary glioblastoma. However, only a small number of cases were actually examined in the literature. We hypothesize that *IDH*-mutant glioblastoma is not a uniform group and should be stratified further for risk to provide more precise prognostication. By profiling DNA methylation of 57 *IDH*-mutant glioblastomas and by mining the epigenetic data for copy number variations, we identified a subset of glioma-CpG island methylator phenotype-low, *IDH*-mutant glioblastomas carrying *CDKN2A* or mesenchymal–epithelial transition alteration and these tumors have poor survivals in spite of their being *IDH* mutant.

The WHO 2016 Classification of Tumors of the Central Nervous System (CNS) has classified glioblastoma into *IDH*-wildtype and *IDH*-mutant, with the latter being described to have a better prognosis and to be more often found in the secondary glioblastoma.[1,2] *IDH*-mutant glioblastoma shows different genetic, epigenetic, and clinical features compared to *IDH*-wildtype counterpart.[3,4] Recurrent mutations in *IDH* genes in glioblastomas were first described in 2008.[5] Afterwards, Yan et al. showed that 11/13 (84.6%) of secondary glioblastomas carried *IDH* mutations whereas such alterations were only observed in 6/123 (4.9%) of primary glioblastomas.[6] Yan et al. also showed that some cases of *IDH*-mutant glioblastomas harbored 1p19q codeletion and *CDKN2A* deletion. Overall, 17 cases of *IDH*-mutant glioblastomas were actually genetically examined in that study. The TCGA database focused on primary glioblastoma and only contained 35 patients diagnosed with *IDH*-mutant glioblastoma.[7] Global mRNA expression analysis revealed that *IDH*-mutant glioblastomas were enriched for the proneural subtypes.[7] Overall, the number of *IDH*-mutant glioblastomas having been evaluated with follow-up data was small at the time of WHO 2016. A very recent paper examined 97 *IDH*-mutant glioblastomas and showed that *CDKN2A* deletion was associated with a poor prognosis.[8] Taken together, these data suggest that *IDH*-mutant glioblastoma is a heterogeneous group that can be further stratified.

At the epigenetic level, researchers including our team have shown that gliomas overall can be divided by the status of glioma-CpG island methylator phenotype (G-CIMP) into G-CIMP positive and G-CIMP negative.[9] G-CIMP positive tumors display extensive DNA hypermethylation at specific loci and are associated with *IDH* mutation.[9] G-CIMP positive gliomas have an improved survival over G-CIMP negative gliomas. Recently, we further showed by unsupervised clustering analysis of methylation profiling that *IDH*-mutant gliomas overall could separate into three methylation subgroups, the Codel, G-CIMP-high, and G-CIMP-low subgroups.[10] However, the number of *IDH*-mutant glioblastoma cases, in contrast to low-grade gliomas, was only 35 in this study, and 7 of 35 cases had available DNA methylation data spanning approximately 450,000 CpG sites. Therefore, the importance of the different DNA methylation subgroups among *IDH*-mutant glioblastomas is not well characterized.

On the basis of the literature, we hypothesize that *IDH*-mutant glioblastoma is a not a uniform group and should be further stratified for more precise prognostication and bedside management. In this study, we examined the genome-wide methylation profiles of 57 *IDH*-mutant glioblastomas and determined gene copy number variations (CNVs) from DNA methylation array. We were able to integrate epigenetic signature and CNVs into a stratification scheme for prognostication.

Materials and Methods

Samples

Formalin-fixed, paraffin-embedded (FFPE) tissues were obtained from the archives of the Pathology departments at Prince of Wales Hospital (Hong Kong) and Hua Shan Hospital (Shanghai, China). Local ethical approvals were obtained from The Joint Chinese University of Hong Kong–New Territories East Cluster Clinical Research Ethics Committee and Ethics Committees of Hua Shan Hospital, Shanghai. The cohort contains 57 samples recruited from 2008 to 2017. All patients were ≥ 18 years

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at the time of diagnosis. Histological diagnoses were reviewed by three pathologists (H.K.N., H.C., A.K.C.). Tumor location was determined by neuroradiological examination and intraoperative information. Data on patient demographics and therapeutic treatment were obtained from institutional paper and electronic records. Most of the patients who had undergone adjuvant chemotherapy had temozolomide (TMZ), and a few patients received nimustine (ACNU) as adjuvant chemotherapy. Survival data were ascertained from follow-up visits to clinics or by direct contact with patients or close relatives via telephone.

IDH1/2 and *TERT* promoter mutation analysis

IDH (*IDH1* and *IDH2*) and *TERT* promoter mutations were detected by direct sequencing as described[11] and cases with *IDH1*- or *IDH2*-mutation were included in this study. All mutations were confirmed by independent PCR amplification and sequencing analyses.

Illumina Infinium MethylationEPIC BeadChip Array

FFPE sections were sent to Macrogen, Shenzhen, China (Shenzhen Millennium Spirit Technology Co, Ltd), where the DNA was extracted and subjected to DNA methylation profiling by EPIC Illumina Infinium Human Array (850,000 CpG sites) following manufacture's protocol (Illumina). The raw data of methylation array can be found at http://www.cuhk.edu.hk/med/acp/acp/staff/hkng.html.

Identification of Methylation Subgroups

Background correction, global dye-bias normalization, and calculation of DNA methylation level are parts of Illumina 850k array preprocessing, and were done according to the previous publications.[10,12] Epigenomic subtypes described previously were predicted in this cohort using machine learning algorithm.[10,12]

Determination of Copy Number Variations With EPIC Illumina DNA Methylation Array

Probe-level signal intensities obtained from the IDAT files were first subjected to background correction and dye-bias normalization (shifting of the 5% percentile of negative control probe intensities to 0, and scaling of the mean of normalization control probe intensities to 10,000).[13,14] Probes were excluded if they targeted the sex chromosomes, contained single-nucleotide polymorphisms, or mapped to multiple locations in the human genome. One hundred nineteen control samples from the study by Capper et al. (GSE109381) were used for normalization.[15] As the control samples were profiled through the 450k array platform, probes present in the EPIC array but not in the 450K array were also removed. CNV analysis was then performed from the methylation data using the "*conumee*" package in R, as previously described.[16,17]

Statistical Analysis

Statistical analysis was performed on IBM SPSS software v22 and R software. Overall survival (OS) was defined as the period of time between surgery and death or the last follow-up. Student's *t*-test was used to compare mean age between two populations. Chi-squared or Fisher's test was used to determine relationships between molecular alterations and clinical parameters. Survival curves were evaluated by the Kaplan–Meier method, and survival difference between different groups was determined by the log-rank test. Multivariable analysis was performed by Cox proportional hazards model. $P < .05$ (two-sided) was considered statistically significant.

Results

Samples and Clinical Features

A summary of clinical features of the cohort in this study is shown in Table 1 and Fig. 1. The mean and median ages of this cohort were 39.8 and 38 years old, respectively. Consistent with the literature, patients with *IDH*-mutant glioblastoma were younger at diagnosis compared to those with *IDH*-wildtype glioblastoma.[6] Male to female ratio was 1:0.73. Primary glioblastoma, which developed de novo without previous clinical or histologic evidence of a low-grade glioma, was found in 33/57 (57.9%) of our cohort, and secondary glioblastoma arising from a previous histologically confirmed low-grade lesion accounted for 24/57 (42.1%) of our samples. Histological review of the pre-existing Grade II or Grade III astrocytoma was available in 10 of these cases in our own laboratories. For the rest, such documentation is available in the medical records but histological review was not possible as the patients were treated in other hospitals. Most of the patients in this study cohort (46/57; 80.7%) had total resection (Table 1). Chemotherapy alone and radiotherapy alone were given to 6 (10.5%) and 1 (1.8%) patients, respectively (Table 1). A total of 35 (61.4%) patients were treated with both chemotherapy and radiotherapy. 43 and 52 cases had follow-up data for progression-free survival (PFS) and OS, respectively. The average and median follow-up periods were 22.9 and 13.9 months, respectively (range 1.0–85.4 mo). Univariate survival analysis was then performed in the cohort according to the clinical variables. The results revealed that age at diagnosis, gender, tumor location, operation, chemotherapy, and radiotherapy were not associated with clinical outcomes (Supplementary Table 1).

Classification of *IDH*-mutant glioblastomas based on genome-wide DNA Methylation Profiling

IDH-mutant glioblastomas (N = 57) were analyzed by Illumina MethylationEPIC (850k) arrays. We applied Random Forest (machine learning algorithm) with a two-step process and divided our 57 samples into one of the two *IDH*-mutant methylation-based gliomas subgroups (G-CIMP-high and G-CIMP-low) according to the previous publication.[10] The results revealed that the majority of the

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Table 1. Clinical characteristics of G-CIMP-high and G-CIMP-low glioblastomas

	All tumors (*N* = 57)	G-CIMP-high (*N* = 34)	G-CIMP-low (*N* = 23)	*P* value
Age				
mean/median/range	39.8/38/21–68	38.9/36/24–64	40.9/40/21–68	.508
Gender				
Male	33	16	17	.058
Female	24	18	6	
Tumor location				
Frontal	36	25	11	.207
Temporal	15	7	8	
Occipital	2	1	1	
Non-hemisphere	4	1	3	
Primary or secondary GBM				
Primary	33	24	9	**.029**
Secondary	24	10	14	
Operation				
Total	46	28	18	.592
Non-total	7	3	4	
Not available	4	3	1	
Adjuvant therapy				
No therapy	9	4	5	.441
Chemotherapy only	6	2	4	
Radiotherapy only	1	1	0	
Chemotherapy and radiotherapy	35	22	13	
Not available	6	5	1	

G-CIMP, glioma-CpG island methylator phenotype.

samples belonged to G-CIMP-high (34/57; 59.6%), and that G-CIMP-low was present in 23/57 (40.4%) of our cohort (Fig. 1 and Table 1). The prevalence of these glioma subtypes is consistent with previous findings.[10]

We then investigated the association between methylation subgroups and clinical parameters. We found G-CIMP-high tumors were markedly associated with primary glioblastomas (P = .029; Table 1). Methylation-based subgroups were not associated with other clinical parameters including age, gender, tumor location, operation, and adjuvant therapy (Table 1).

In agreement with a previous report[10], G-CIMP-low tumors exhibited a significantly shorter OS compared to G-CIMP-high tumors (median: 407 d [13.6 mo] vs 619 d [20.6 mo], P = .005; Fig. 2A). Methylation-based subgroups were not associated with PFS in our cohort (Fig. 2B).

TERT Promoter Mutation in *IDH*-Mutant Glioblastomas

By Sanger sequencing, *TERT* promoter mutation was identified in 3/57 (5.3%) of *IDH*-mutant glioblastomas. Out of these three samples, two cases had the C250T mutation, and one case had the C228T mutation (Fig. 1). *TERT* promoter mutation appeared in both primary (N = 2) and secondary (N = 1) glioblastomas. An association between *TERT* promoter mutation and clinical parameters (age, gender, and tumor location) was not formed. All *TERT* promoter mutations were found in G-CIMP-low tumors (Supplementary Table 2). *TERT* promoter mutation was not associated with PFS or OS.

Clinical Significance of Gene CNVs in *IDH*-Mutant Glioblastomas

CNVs have been recognized as a useful prognostic tool in glioblastomas.[18] Therefore, we derived copy number status from EPIC 850k array data according to previous publications.[14,15] We then looked at genes with established relevance in gliomas for amplification or deletion.[14] These included *CCND1, CCND2, CDK4, CDK6, CDKN2A, EGFR, MDM4, MET, MYC, MYCN, NF1, NF2, PDGFRA, PPM1D, PTEN, RB1*, and *SMARCB1*. We used the cutoff established in Shirahata et al. study to determine amplification and deletion.[14] We found *CDKN2A* deletion in 24/57 (42.1%) *IDH*-mutant glioblastomas, and this was the most common alteration among the gene list (Fig. 1, Supplementary Table 3).

We then evaluated the association between CNVs and methylation subgroups. We found that *CDKN2A* deletion was markedly associated with the G-CIMP-low subgroup

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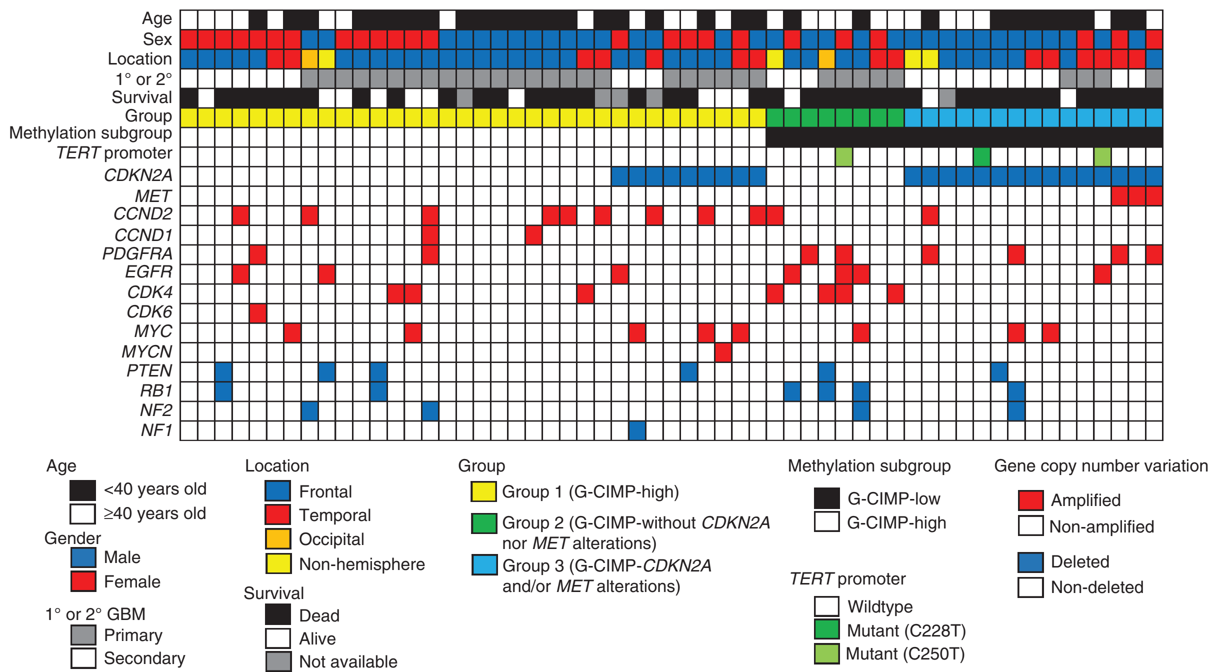

Fig. 1. Summary of molecular and clinical features of 57 *IDH*-mutant glioblastomas. Each column represents a single case.

(*P* = .004; Supplementary Table 4). Examining the association between CNVs and clinical parameters revealed that *CDKN2A* deletion was enriched in secondary glioblastoma (*P* = .034; Supplementary Table 5). Other frequent copy number changes included *CCND2* amplification (11/57; 19.3%), *PDGFRA* amplification (8/57; 14.0%), *MYC* amplification (8/57; 14.0%), *CDK4* amplification (7/57; 12.3%), and *EGFR* amplification (7/57; 12.3%). Mesenchymal–epithelial transition (*MET*) amplification was identified in 3/57 (5.3%) of our cohort. The prevalence of gene alterations is summarized in Supplementary Table 3. We used fluorescence in situ hybridization (FISH) analysis to validate some findings of CNVs. *CDKN2A* deletion was confirmed in 7 *CDKN2A*-deleted cases with sufficient tissues. Similarly, by FISH analysis, *EGFR* amplification was confirmed in five *EGFR*-amplified samples with sufficient tissues.

We then investigated if these CNVs were associated with clinical parameters including age, gender, operation, chemotherapy, and radiotherapy. We found *CCND2* amplification was significantly associated with younger age (mean ± SD, 33.91 ± 7.45 versus 41.15±11.00; *P* = .044). *NF2* loss displayed a trend toward younger age (*P* = .091). No other association between gene CNV and clinical features was detected.

Log-rank test revealed that *CDKN2A* deletion was significantly associated with shorter OS (*P* = .036) (Fig. 3A). Yet it had no impact on PFS (Fig. 3B). We then separated the tumors according to methylation subgroups. We found *CDKN2A* deletion was associated with shorter OS (*P* = .035; Supplementary Figure 1A) and PFS (*P* = .040; Supplementary Figure 1B) in G-CIMP-low tumors. The significance was lost in G-CIMP-high tumors (Supplementary Figures 1C–D). We also found *MET* amplification was markedly associated with shorter OS (*P* < .001; Fig. 3C), but was not associated with PFS due to insufficient cases with PFS data (only two *MET*-amplified cases with PFS data). Clinical significance was not detected for other gene CNVs (Supplementary Table 3). The results indicated that *CDKN2A* deletion and *MET* amplification are prognostic markers in *IDH*-mutant glioblastomas.

We then asked if combined *CDKN2A* and *MET* status could improve prognostication. We separated the cohort into (1) *CDKN2A* deletion + *MET* amplification; (2) *CDKN2A* deletion; and (3) Neither *CDKN2A* deletion nor *MET* amplification. Survival analysis revealed that *CDKN2A* deletion + *MET* amplification predicted poor survival (*P* < .001; Fig. 3D). Pair-wise comparison indicated that *CDKN2A*-deleted + *MET*-amplified tumors had a shorter OS compared to *CDKN2A*-deleted tumors (*P* = .008; Fig. 3D). Survival analysis for PFS was not conducted given that only two cases of *CDKN2A* deletion + *MET* amplification had the PFS data.

Stratification of *IDH*-Mutant Glioblastomas With DNA Methylation Subgroup and *CDKN2A*/*MET* Status

We then investigated the prognostic values of combined DNA methylation subgroups and CNVs in our cohort. Given that DNA methylation subgroup, *CDKN2A* deletion, and *MET* amplification all showed prognostic value on their own, we used these three factors in the analysis. The cohort was separated into three molecular groups: Group 1 (G-CIMP-high), Group 2 (G-CIMP-low without *CDKN2A* nor *MET* alteration), and Group 3 (G-CIMP-low with *CDKN2A* and/or *MET* alterations). A log-rank test demonstrated that groups based on combined methylation subgroups and

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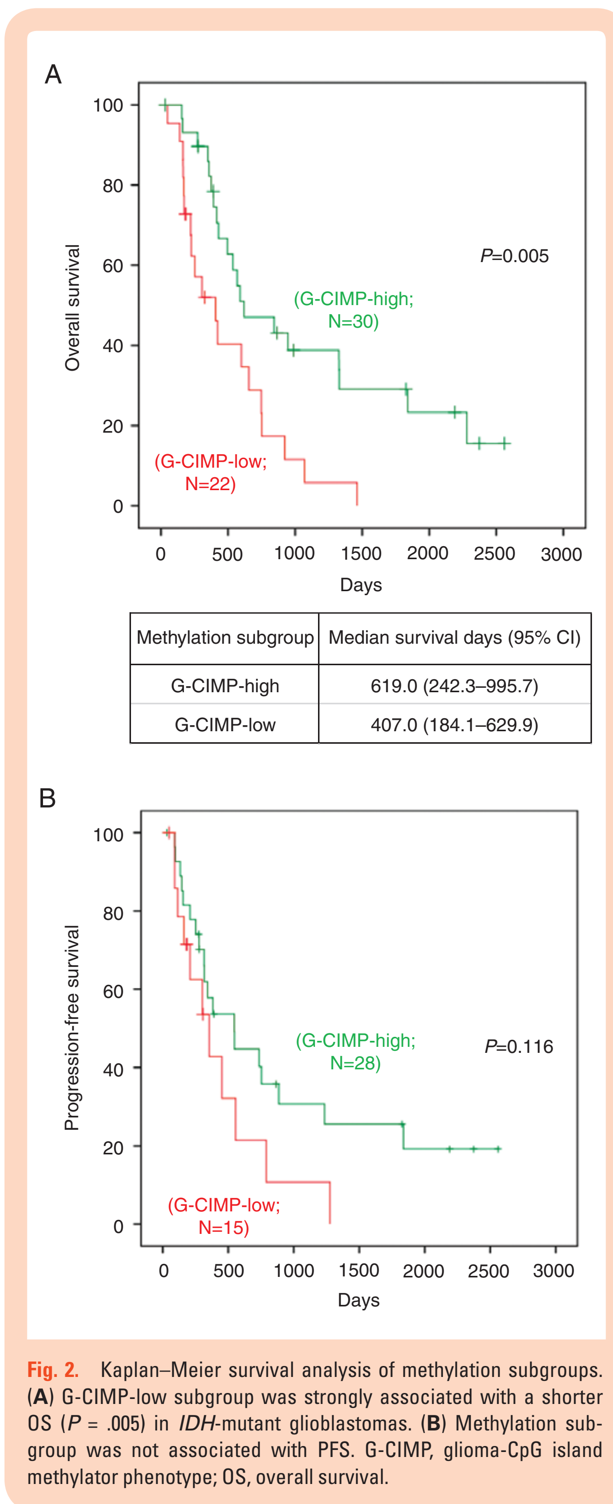

Methylation subgroup	Median survival days (95% CI)
G-CIMP-high	619.0 (242.3–995.7)
G-CIMP-low	407.0 (184.1–629.9)

Fig. 2. Kaplan–Meier survival analysis of methylation subgroups. (**A**) G-CIMP-low subgroup was strongly associated with a shorter OS (*P* = .005) in *IDH*-mutant glioblastomas. (**B**) Methylation subgroup was not associated with PFS. G-CIMP, glioma-CpG island methylator phenotype; OS, overall survival.

CDKN2A/*MET* status differed significantly with respect to their OS and PFS (*P* = .001 and *P* = .044, respectively; Fig. 4A and B). As illustrated in Fig. 4A, Groups 1 and 2 exhibited prolonged survival with a median survival of 619 (20.6 mo), and 655 (21.8 mo) days, respectively. Group 3 exhibited a poor outcome with a median survival of 252 days (8.4 mo). Although both Groups 2 and 3 were of G-CIMP-low tumors, pair-wise comparison indicated that Group 3 patients performed significantly worse than Group 2 patients (*P* = .035; Fig. 4A), suggesting that *CDKN2A/MET* status would further stratify G-CIMP-low patients. As shown in Fig. 4B, groups based on combined methylation subgroups and *CDKN2A/MET* status also correlated with PFS (*P* = .044). Group 3 tumors showed a shorter PFS compared to Groups 1 and 2. Similar to the OS, *CDKN2A/MET* status predicted a shorter PFS among G-CIMP-low tumors (Groups 2 and 3; *P* = .040; Fig. 4B), highlighting the values of *CDKN2A/MET* status in stratification of G-CIMP-low tumors.

Multivariable analysis was conducted to examine the independent prognostic value of the combined methylation subgroups and *CDKN2A/MET* status by adjusting for age, gender, operation, radiotherapy, chemotherapy, and clinical diagnosis (Table 2). Although there was a significant association between the combined methylation subgroups and clinical diagnosis (*P* = .016; Supplementary Table 6), interaction between these two factors was not significant in the multivariable analysis. Multivariable analysis showed that combined methylation subgroups and *CDKN2A/MET* status was an independent prognostic factor in *IDH*-mutant glioblastomas (Table 2).

Discussion

Even with intensive treatment, the prognosis of glioblastoma is poor with a median OS of less than 15 months.[19,20] However, a minority of glioblastoma patients survives longer than 2–3 years.[21,22] The WHO 2016 classification of CNS tumors has defined many entities by both histology and molecular features, and majority of glioblastomas are classified as *IDH*-wildtype or *IDH*-mutant.[2] *IDH*-wildtype glioblastoma accounts for over 90% of primary glioblastoma and has been well studied.[23–26] *IDH*-mutant glioblastoma constitutes a small proportion of primary glioblastoma and around 60%–70% of secondary glioblastoma,[1,27,28] Yan et al. showed that the median OS of *IDH*-mutant glioblastomas was about two times longer than that of *IDH*-wildtype glioblastomas[6]; however, the number of *IDH*-mutant glioblastomas with follow-up data in that study was small (N = 14) and similarly only 35 *IDH*-mutant glioblastomas are currently listed in TCGA database among which only 7 had available DNA methylation data for 450,000 CpG sites.

Genome-wide DNA methylation analysis revealed that *IDH*-mutant glioblastomas (both primary and secondary) formed a group distinct from other *IDH*-mutant gliomas (Grades II-III).[8] Shirahata et al. demonstrated that *IDH*-mutant astrocytic tumors (Grades II-IV) is not uniform in terms of histological and genetic parameters. It was suggested that the 2016 CNS WHO grading of *IDH*-mutant astrocytic tumors is not as prognostically useful as needed for this group and a novel grading algorithm correlated better to prognosis for *IDH*-mutant astrocytic tumors overall.[14] We too speculate that *IDH*-mutant glioblastoma is a heterogeneous group characterized by tumors with differing in methylation signature, copy number changes, and clinical outcomes. We also speculate that not all *IDH*-mutant glioblastomas have good prognosis and it is necessary to

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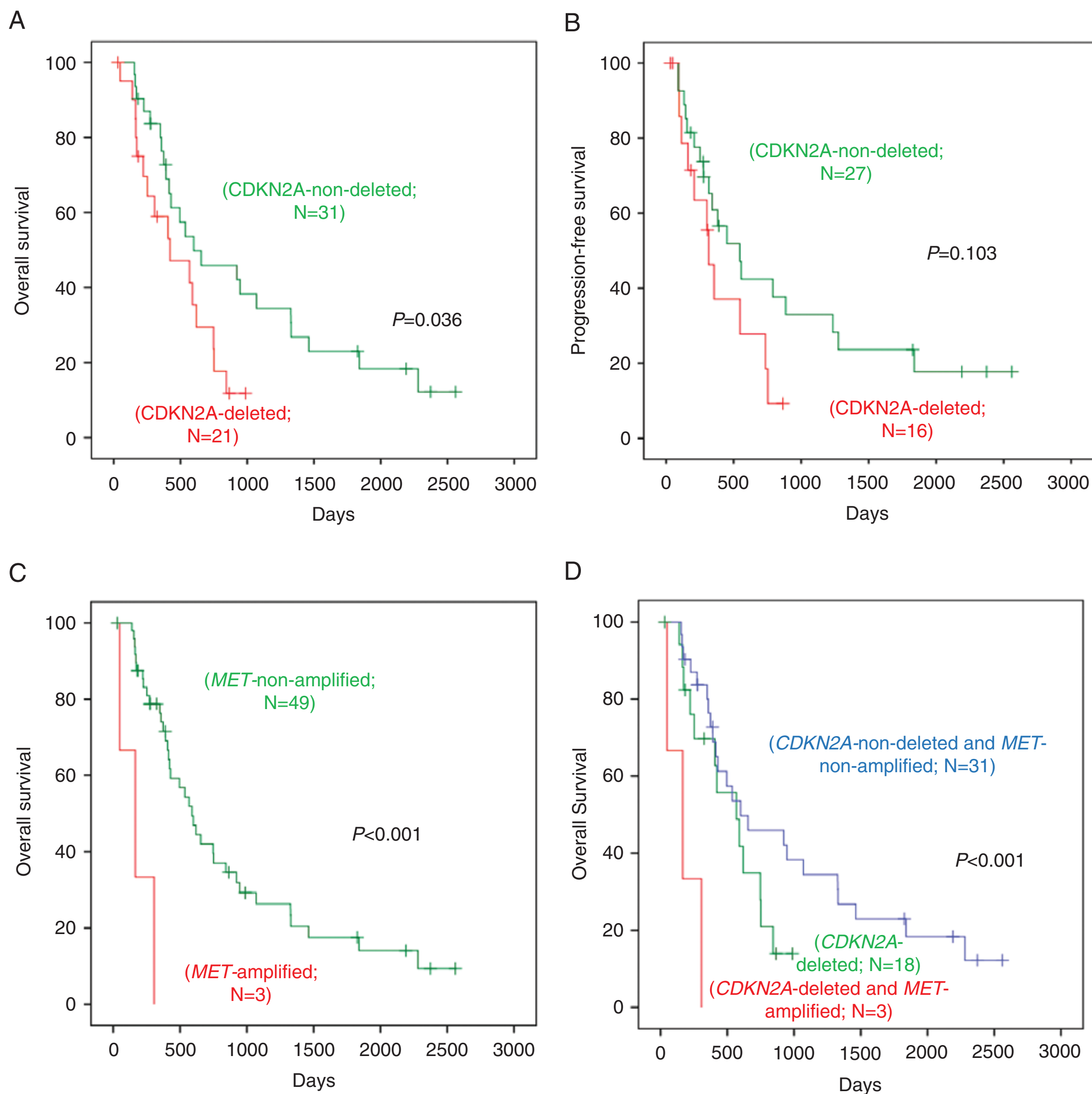

p value	Median survival days (95% CI)	*CDKN2A + MET*	*CDKN2A* only	None
CDKN2A + MET	165.0 (0.0–350.6)			
CDKN2A only	567.0 (261.5–872.5)	**0.008**		
None	599.0 (74.2–1123.8)	**<0.001**	0.122	

Fig. 3. Kaplan–Meier survival analysis of *CDKN2A* deletion and *MET* amplification. *CDKN2A* deletion was significantly associated with a shorter (**A**) OS. *CDKN2A* deletion lost had no impact on (**B**) PFS. (**C**) *MET* amplification was correlated with a shorter OS. (**D**) Combined *CDKN2A* deletion and *MET* amplification was associated with a poor OS in *IDH*-mutant glioblastomas. *MET*, mesenchymal–epithelial transition; OS, overall survival.

provide a better stratification for risk. We showed that a combination of methylation subgroups and copy number changes provided good prognostication of *IDH*-mutant glioblastomas.

CpG island methylator phenotype (CIMP) is defined by genome-wide hypermethylation of CpG islands and later was defined to include other non-CpG islands. CIMP alterations has been shown to lead to an inactivation of

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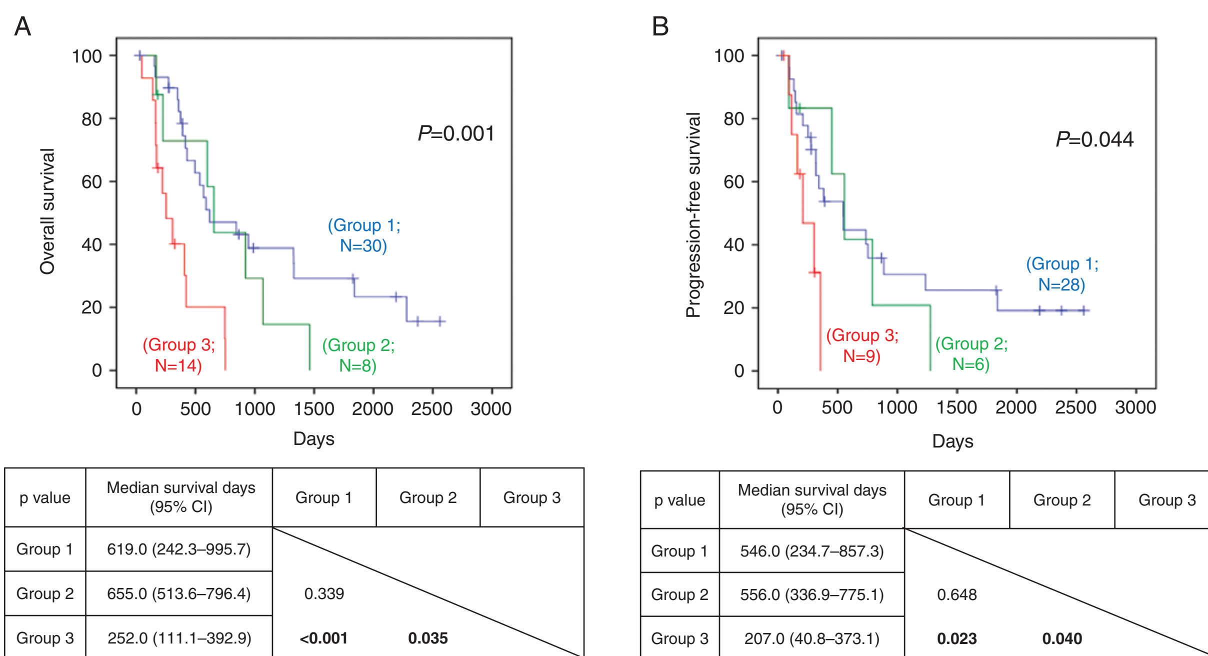

p value	Median survival days (95% CI)	Group 1	Group 2	Group 3
Group 1	619.0 (242.3–995.7)			
Group 2	655.0 (513.6–796.4)	0.339		
Group 3	252.0 (111.1–392.9)	**<0.001**	**0.035**	

p value	Median survival days (95% CI)	Group 1	Group 2	Group 3
Group 1	546.0 (234.7–857.3)			
Group 2	556.0 (336.9–775.1)	0.648		
Group 3	207.0 (40.8–373.1)	**0.023**	**0.040**	

Fig. 4. Combined methylation subgroups and *CDKN2A/MET* status in stratification of *IDH*-mutant glioblastomas. Kaplan–Meier survival curves of (**A**) OS and (**B**) PFS according to Groups ($P < .001$). Group 1 (blue line) belongs to tumors of G-CIMP-high. Group 2 (green line) belongs to G-CIMP-low tumors without *CDKN2A* nor *MET* alteration. Group 3 (red line) belongs to G-CIMP-low tumors with *CDKN2A* and/or *MET* alterations. G-CIMP, glioma-CpG island methylator phenotype; *MET*, mesenchymal–epithelial transition; OS, overall survival.

tumor suppressor genes or other tumor-related genes.[29] We previously described CIMP in adult low-grade gliomas and glioblastomas[9] and confirmed the findings in a larger cohort.[7,10] Gliomas can be separated into CIMP positive (CIMP+) and CIMP negative (CIMP–), and the name glioma-CIMP (G-CIMP) was designated for a subgroup of gliomas with CIMP to distinguish from other non-glioma CIMP tumors.[9] Integrative analysis of DNA methylation data and transcriptome profiling revealed G-CIMP+ subgroup was highly enriched for proneural subtype, which is one of the four genetic types described in glioblastomas.[18] G-CIMP+ tumors were associated with younger age and *IDH* mutation compared to the G-CIMP– tumors.

Recently, an integrative analysis of 1122 adult low- and high-grade gliomas revealed that they can be divided into six methylation subgroups that are closely associated with *IDH* mutation status.[10] *IDH*-wildtype tumors could be separated into three methylation subgroups and the same applied to *IDH*-mutant tumors. The three discrete methylation subgroups among the *IDH*-mutant gliomas were Codel, G-CIMP-high and G-CIMP-low. The Codel subgroup was mainly made up of low-grade gliomas with 1p19q codeletion. G-CIMP-high and G-CIMP-low tumors were subgroups of G-CIMP+ and presented with high and low degrees of DNA methylation, respectively. This study also showed that G-CIMP-low gliomas resembled *IDH*-wildtype gliomas and had the worst OS among the three methylation subgroups of *IDH*-mutant gliomas. As >90% of the *IDH*-mutant gliomas in this study were low-grade gliomas,[10] the clinical impact of methylation subgroup in *IDH*-mutant glioblastoma remained unknown. In addition, the clinical significance of gene copy number was not investigated in depth.

In this study, we examined genome-wide DNA methylation profiling of 57 *IDH*-mutant glioblastomas. We showed G-CIMP-high and G-CIMP-low in 59.6% and 40.4% of our cohort, respectively. The prevalence of DNA methylation subgroups in the current study is similar to the reported literature.[10] We demonstrated that DNA methylation subgroups correlated with survival, and G-CIMP-low tumors showed a poorer survival compared to G-CIMP-high tumors, indicating that DNA methylation subgroup is clinically relevant in *IDH*-mutant glioblastomas.

We then uploaded the raw data of methylation array to German Cancer Research Center (DKFZ) classifier (molecularneuropatohlogy.org). Thirty-six cases were classified by the DKFZ classifier as high-grade gliomas. Twenty-one cases were classified as "not defined" ($N = 19$) or "no matching methylation classes with calibrated score" ($N = 2$). The histology of some "not defined" cases was put up in Supplementary Figure 2. It is not clear from the published literature how well *IDH*-mutant glioblastomas are represented in the methylation classifier. *IDH*-mutant glioblastomas may well be under "un-defined" by the classifier and we hope our contribution to the literature and the classifier will help clarify the issue.

CDKN2A is located on chromosome 9p21, and it encodes for two different proteins, p16INK4a and p14ARF.[30]

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Table 2. Multivariable analysis of *IDH*-mutant glioblastomas

Variables	Hazard ratio (HR) (95% CI)	*P*
Age	1.03 (0.99–1.08)	.135
Gender		
Male	1	
Female	0.79 (0.36–1.76)	.567
Operation		
Non-total resection	1	
Total resection	1.08 (0.25–4.71)	.922
Radiotherapy		
No	1	
Yes	1.12 (0.29–4.32)	.873
Chemotherapy		
No	1	
Yes	0.6 (0.13–2.73)	.507
Clinical diagnosis		
Primary glioblastoma	1	
Secondary glioblastoma	0.32 (0.05–1.99)	.221
Combined methylation subgroup and *CDKN2A/MET* status		
Group 3 (G-CIMP-low with *CDKN2A* and/or *MET* alterations)	1	.009
Group 1 (G-CIMP-high)	0.07 (0.01–0.38)	.002
Group 2 (G-CIMP-low without *CDKN2A* nor *MET* alterations)	0.08 (0.01–0.7)	.022
Combined methylation subgroups and *CDKN2A*/*MET* status by clinical diagnosis interaction		.11

CI, confidence interval; G-CIMP, glioma-CpG island methylator phenotype; *MET*, mesenchymal–epithelial transition.

CDKN2A negatively controls cell cycle, and *CDKN2A* abnormality leads to cellular proliferation and dysregulation of proapoptotic pathways.[31] *CDKN2A* deletion has been described in adult and pediatric low-grade and high-grade gliomas, with frequencies ranging from 20% to 57%.[32,33] Loss of *CDKN2A* is associated with poor outcomes in pediatric and adult low-grade and malignant gliomas.[14,34,35] Recently, Korshunov et al. identified *CDKN2A/B* deletion was associated with shorter survival in *IDH*-mutant glioblastomas.[8] A review of TCGA database revealed 35 *IDH*-mutant glioblastomas with *CDKN2A* deletion status and limited clinical follow-up. *CDKN2A* deletion was found in 14.3% (5/35) of the samples. Survival analysis of the TCGA cases did not reveal a close association between *CDKN2A* deletion and survivals, probably due to a limited number of *CDKN2A* deletion cases. The median OS for deleted and non-deleted samples was 24 and 34 months, respectively.

In this study, we showed that *CDKN2A* deletion is a common event in *IDH*-mutant glioblastomas (42.1%), and it is more often detected in G-CIMP-low tumors (15/23; 65.2%) whereas such alteration is present in about one-quarter of G-CIMP-high tumors. Importantly, *CDKN2A* deletion was a poor prognostic factor for OS in our cohort. *CDKN2A* deletion also exhibited negative clinical impact in a subgroup of tumors with a G-CIMP-low signature (Supplementary Figures 1A and B). Taken together, *CDKN2A* deletion is a poor prognostic marker in *IDH*-mutant glioblastomas, and it can further stratify G-CIMP-low tumors for prognostication.

MET is located on chromosome 7q21–31, and it encodes a receptor for hepatocyte growth factor. Upon binding to its ligands, MET undergoes dimerization and phosphorylation, resulting in recruitment of signal transduction molecules and induction of downstream signaling pathways such as the PI3-K/AKT and RAS/MAPK pathways.[36] MET activation results in cell proliferation, G1/S cell-cycle progression, angiogenesis and resistant to chemotherapy in gliomas.[37,38] In gliomas, *MET* is dysregulated by several mechanisms. *MET* amplification has been described in <10% in glioblastomas.[39,40] Mutation of *MET* leading to a truncated, constitutively active protein has been reported in a small proportion of glioblastomas.[7,41] Recurrent *PTPRZ1-MET* fusion transcript resulting in an increase in migratory activity has also been described in 15% of secondary glioblastomas.[28,42] Overexpression of MET is a frequent event, and the expression is significantly higher in high-grade gliomas compared to the low-grade counterpart.[43,44]

In this study, we showed that *MET* amplification is present in a small proportion of *IDH*-mutant glioblastomas, and can be found in both primary and secondary glioblastomas. Interestingly, all *MET*-amplified tumors belonged to G-CIMP-low subgroup and exhibited *CDKN2A* mutation. In TCGA database where the vast majority of glioblastomas are *IDH*-wildtype and *MET* amplification is found at a low frequency. Yet, none of the *MET*-amplified tumors in TCGA carries *IDH* mutation. Thus, this is the first report of the co-occurrence of *IDH*

mutation, *CDKN2A* deletion, and *MET* amplification in glioblastomas. Our survival analysis revealed that *MET* amplification was associated with a short OS among all *IDH*-mutant glioblastomas ($P < .001$, Fig. 3C) and also among G-CIMP-low, *IDH*-mutant glioblastomas ($P = .017$; data not shown). Furthermore, patients with both *CDKN2A* deletion and *MET* amplification did more poorly compared to patients with only *CDKN2A* deletion or patients without these alterations (Fig. 3D). These data suggest *MET* amplification is a poor prognostic marker, and co-occurrence of *CDKN2A* deletion and *MET* amplification further enhance the aggressiveness of glioblastoma. Thus, *CDKN2A* deletion and *MET* amplification may represent a prognostically unfavorable subset of *IDH*-mutant glioblastomas.

Overall in this study, by integrated methylation signature and gene copy number data, we categorized three molecular subgroups (Groups 1–3) of *IDH*-mutant glioblastomas with different clinical behavior. The prognostic value of such molecular subgroups was also demonstrated in multivariable analysis. In particular, Group 3 (G-CIMP-low with *CDKN2A* and/or *MET* alterations) showed the worst outcomes with a median OS of 252 days (8.4 mo) and a median PFS of 207 days (6.9 mo) so these *IDH*-mutant glioblastomas should not be classified as glioblastomas with good prognosis as they could have been under the current WHO scheme. Our findings suggest that combination of methylation signature and gene CNVs should be used to stratify *IDH*-mutant glioblastomas into prognostic groups, and thus have implications for bedside management.

Supplementary Material

Supplementary material is available at *Neuro-Oncology Advances* online.

Keywords

CDKN2A deletion | DNA methylation profiling | glioblastomas | *IDH* mutation | *MET* amplification.

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