doi:10.1093/jmcb/mjx056
Published online January 24, 2018

Article

EZH2-, *CHD4*-, and *IDH*-linked epigenetic perturbation and its association with survival in glioma patients

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Edited by Luonan Chen

Glioma is a complex disease with limited treatment options. Recent advances have identified isocitrate dehydrogenase (*IDH*) mutations in up to 80% lower grade gliomas (LGG) and in 76% secondary glioblastomas (GBM). *IDH* mutations are also seen in 10%–20% of acute myeloid leukemia (AML). In AML, it was determined that mutations of *IDH* and other genes involving epigenetic regulations are early events, emerging in the pre-leukemic stem cells (pre-LSCs) stage, whereas mutations in genes propagating oncogenic signal are late events in leukemia. *IDH* mutations are also early events in glioma, occurring before *TP53* mutation, 1p/19q deletion, etc. Despite these advances in glioma research, studies into other molecular alterations have lagged considerably. In this study, we analyzed currently available databases. We identified *EZH2*, *KMT2C*, and *CHD4* as important genes in glioma in addition to the known gene *IDH1/2*. We also showed that genomic alterations of *PIK3CA*, *CDKN2A*, *CDK4*, *FIP1L1*, or *FUBP1* collaborate with *IDH* mutations to negatively affect patients' survival in LGG. In LGG patients with *TP53* mutations or *IDH1/2* mutations, additional genomic alterations of *EZH2*, *KMC2C*, and *CHD4* individually or in combination were associated with a markedly decreased disease-free survival than patients without such alterations. Alterations of EZH2, KMT2C, and CHD4 at genetic level or protein level could perturb epigenetic program, leading to malignant transformation in glioma. By reviewing current literature on both AML and glioma and performing bioinformatics analysis on available datasets, we developed a hypothetical model on the tumorigenesis from premalignant stem cells to glioma.

Keywords: glioma, epigenetics, bioinformatics, prognosis, gene mutation

Introduction

Molecular mutations are frequent in both acute myeloid leukemia (AML) and lower grade glioma (LGG). The convenience of separating individual cells of AML combined with marker-based fluorescence-activated cell sorting (FACS) purification of hematopoietic stem cells (HSCs, Lin−CD34+CD38−CD99−TIM3−) and leukemia cells (CD99+TIM3+) from blood samples of individual patients has allowed the establishment of cell clones from single HSCs where tracking of molecular mutations in clonal evolution over time is possible (Corces-Zimmerman et al., 2014). This technological advance has enabled the identification of sequential molecular mutations during clonal evolution and the concept of pre-leukemic stem cell (LSC). It was found that gene mutations occur in a sequential pattern in AML where some mutations occur early while others are late events (Corces-Zimmerman et al., 2014).

Pre-LSCs have the potential to give rise to multiple normal hematopoietic lineages and are yet able to develop into full-blown

Received August 30, 2017. Revised November 12, 2017. Accepted December 18, 2017.

leukemia (Sato et al., 2016). On the other hand, the concept of LSCs was demonstrated over 20 years ago (Lapidot et al., 1994). LSCs do not have the potential to produce normal hematopoietic lineages, but they develop into leukemia over time (Sato et al., 2016). The transformation from pre-LSC to LSC occurs as oncogenic mutations accumulate in pre-LSCs. Major molecular mutations identified in AML to date include mutations in *IDH1*, *IDH2*, ten eleven translocation methylcytosine dioxygenase 2 (*TET2*), DNA (cytosine-5-)methyltransferase 3 alpha (*DNMT3A*), structural maintenance of chromosome 1A (*SMC1A*), Wilms tumor 1 (*WT1*), additional sex combs like transcriptional regulator 1 (*ASXL1*), runt-related transcription factor 1 (*RUNX1*), nucleophosmin 1 (*NPM1*), FMS-related tyrosine kinase 3 (*FLT3*), *KRAS/NRAS*, etc. (Busque et al., 2012; TCGA, 2013a; Zhang et al., 2013; Corces-Zimmerman et al., 2014). Among these, mutations in *IDH1*, *IDH2*, *WT1*, *DNMT3A*, *TET2*, and *ASXL1* are well characterized as early, pre-leukemic events, whereas mutations in *FLT3* and *KRAS/NRAS* are thought to be late events, occurring in the leukemic stage (Table 1). Mutation in *NPM1* is considered to occur more often in the later leukemic stage than in the pre-leukemic stage (Corces-Zimmerman et al., 2014). Mutations in both *IDH1/2* and *TET2* result in the DNA hypermethylation phenotypes and they are in the same *IDH/WT1/TET2* axis in AML pathogenesis, as WT1 is a binding partner of TET2 (Cimmino et al., 2011). Thus, genes mutated in pre-LSCs share common function and perform epigenetic modification by regulating DNA or histone methylations or histone acetylations or the reversal of methylations and acetylations.

Wild-type IDH1/2 protein is an enzyme which catalyzes the conversion of isocitrate into alpha-ketoglutarate (α-KG) via decarboxylation in the presence of NADP+. As the result of this reaction, NADPH is produced. Another enzyme named Tet methylcytosine dioxygenase 2 (TET2) is then able to hydroxylate 5-methylcytosine (5 mc), resulting in the production of 5-hydroxymethylcytosine (5 hmc). This process is α-KG dependent, leading to DNA demethylation (Cimmino et al., 2011). Mutant IDH proteins use isocitrate and NADPH to produce R(–)-2-hydroxyglutarate (2-HG) and NADP+. The product 2-HG is a 'oncometabolite' (Dang et al., 2009; Xu et al., 2011; Koivunen et al., 2012; Losman et al., 2013; Pusch et al., 2014). The oncometabolite 2-HG can inhibit the active DNA demethylating functions of TET family proteins with the consequences of impaired DNA demethylation (Xu et al., 2011; Liu et al., 2016). It was revealed that there was strong association between *IDH* mutation and DNA hypermethylation in glioma (Li et al., 2014). *IDH* mutations occur as a single amino acid missense mutations at R132 of *IDH1* or R172 of *IDH2*. The functional consequences of mutation at R132 of *IDH1* are analogous to that of mutation at R172 of *IDH2* in glioma, although *IDH1* mutation is more common than *IDH2* mutation in both AML and glioma (Cohen et al., 2013; Wang et al., 2016). *IDH* mutation rate is about 80% in LGG and secondary glioblastomas (GBM) and 5%–7% in primary GBM (Balss et al., 2008; Sanson et al., 2009). Genetic analysis of sequential biopsy samples from the same glioma patient indicated that *IDH1* mutation was an early event, occurring well ahead of *TP53* mutations or 1p/19q deletion (Watanabe et al., 2009). This result is remarkably similar to that of experiments analyzing clonal evolution in the same patient in AML (Corces-Zimmerman et al., 2014). Based on extensive studies in AML and the clonal evolution model where all the defined gene alterations in pre-LSCs change genomic dynamics by epigenetic perturbation, we speculate that gliomas arising from *IDH* mutations may evolve in a similar fashion.

Another alteration which frequently occurs in pre-LSC is *DNMT3A* mutation. Mutations in *DNMT3A* have been found in 22% of all AML (Ley et al., 2010; Yan et al., 2011). Phenotypic changes from *DNMT3A* mutation include DNA hypomethylation or loss of DNA methylation (Yan et al., 2011; Jeong et al., 2014). The consequences of hypermethylation or hypomethylation in genomic DNA resulted from mutations of *IDH1/2*, *TET2* or *DNMT3A* are the disturbance of methylation homeostasis in the stem cell compartment, leading to deregulation of stem cell self-renewal and differentiation and resulting in the development of cancer (Gereige & Mikkola., 2009). In addition to DNA methylation, other epigenetic modifications such as histone methylation

Table 1 Genes with frequent alterations in AML and glioma and their effects on stem cell function.

Category by functions	Genes involved		Corresponding proteins	Effects on stem cell function
	AML	Glioma		
Epigenetic modifiers and stem cell regulators	*ASXL1*		Additional sex combs like 1	HSCs pool maintenance
	DNMT3A		DNA methyltransferase 3A	Important in self-renewal
	IDH1/2	*IDH1/2*	Isocitrate dehydrogenase 1 and 2	Role in differentiation
	TET2		Ten eleven translocation methylCytosine dioxygenase 2	Role in differentiation
	EZH2	*EZH2*	Enhancer of zeste homolog 2	Stem cell maintenance
Signal propagators or proliferation activators	*FLT3*		FMS-related tyrosine K 3	Role in stem cell survival
	K/N-Ras		Rat Sarcoma Viral Homolog	Role in differentiation, etc.
	c-KIT		KIT proto-oncogene RTK	
	CEBPa		CCAAT/EBPα	Maintain quiescent state
		EGFR	EGF receptor	Role in proliferation
Tumor suppressors		*PI3KCA*	Phosphatidylinositol 3-k	Role in self-renewal
	TP53	*TP53*	Tumor protein p53	Role in genome stability
	WT1	*WT1*	Wilms tumor 1	Role in cell growth
	CDKN2A/B	*CDKN2A/B*	CDK inhibitor 2A/B	Role in proliferation
		PTEN	Phosphatase & tensin homolog	Maintain quiescent state
Histone chaperones	*NPM1*		Nucleophosmin	Role in genome stability

CDK, cyclin-dependent kinase; EBP, enhancer-binding protein; EGF, epidermal growth factor.

or acetylation are also important in stem cell function (Zhang and Zhang, 2017). ASXL1 protein is such an epigenetic modifier of gene transcription through histone regulation. Mutation in *ASXL1* is also found in the pre-leukemic phase of AML. Normal function of *ASXL1* is crucial in maintaining the hematopoietic stem cell pool and loss-of-function mutation in *ASXL1* gene impairs hematopoietic stem cell development (Abdel-Wahab et al., 2013).

In contrast to the extensive studies at genetic level in AML, little is known about other genes with alterations in glioma development besides *IDH1/2*. Since almost all the genomic alterations found in pre-LSC occur in genes coding for factors with a role in epigenetic modulation, we are interested in whether changes of these factors or genes are also present in glioma and whether there are other driver genes whose mutations or alterations have an impact on patients' survival among the *IDH1/2*-mutated glioma patients.

Results

Identification of EZH2, KMT2C, and CHD4 as potential epigenetic modulators involved in LGG with an impact on patient survival

In order to investigate whether genes mutated, deleted, or amplified in pre-LSC are also altered in a similar fashion in glioma, we inquired all the genes reported in the literature with a role in AML development and progression plus additional epigenetic modifiers collected from public databases and additional genes with high-mutation rate and high frequency of copy number alterations in glioma. Among the subset of selected epigenetic modifiers, we checked the frequencies of gene mutations and copy number alterations (CNA) of 171 genes whose protein products are considered epigenetic modifiers in all 283 complete LGG tumors in the dataset. Of 171 genes (Supplementary Table S2), 168 are from dbEM (a database of epigenetic modifiers, website: crdd.osdd.net/raghava/dbem/index.php) (Nanda et al., 2016). The other three genes are from previous reports in the literature and include *ASH2L*, *RIOX1*, and *KMT5A*.

In our analysis of LGG database, following *IDH1* (77% mutation rate or 80% alteration rate), *TP53* is the second most frequently altered gene with a frequency of 53%, followed by *ATRX* (51%), *CIC* (29%), *PIK3CA* (18%), *PTEN* (17%), *NOTCH1* (17%), *EGFR* (16%), and *CDKN2A* (15%), *KDM5A* (15%), *FUBP1* (14%), *CHD4* (14%), *ARID1A* (13%), *NF1* (13%), *MYC* (13%), *EXT1* (13%), *NRAS* (12%), *KMT2C* (11%), *EZH2* (10%), *SMARCA4* (10%), *HDAC4* (9%), *KRAS* (9%), *TFPT* (7%), and *DOT1L* (6%) (Figure 1A and B). Among these altered genes, nine are epigenetic modifier genes which include *ARID1A*, *CHD4*, *DOT1L*, *EZH2*, *HDAC4*, *KDM5A*, *KMT2C*, *SMARCA4*, and *TFPT*. Frequently mutated genes in pre-LSC of AML such as *DNMT3A*, *TET2*, and *ASXL1* had a low alteration frequency (<5%) in glioma. After extensive analysis, only three genes *CHD4*, *EZH2,* and *KMT2C* in the epigenetic modifier group, when altered in tumors, showed a significant reduction in disease-free survival in the selected patient group. The other six genes did not have significant effects on either overall survival (OS) or disease-free survival (DFS) in the selected LGG patient group. Presentation of results from the analyses focused on these three genes which included *CHD4*, *EZH2*, and *KMT2C* thereafter. Most of *CHD4*, *EZH2*, and *KMT2C* alterations are gene amplification which is different from many other genes in query such as *IDH1/2*, *DNMT3A*, *ASXL1*, and *TET2*, where mutations are more frequently seen (Figure 1A and B). Alteration of *CHD4*, *EZH2* and *KMT2C* gene occurs less frequently in primary GBM (2%, 4%, and 5%, respectively, among 291 all complete GBM tumors; TCGA, 2013b). *TP53* mutation (35%) has a lower frequency in GBM while *CDKN2A* (59%; TCGA, 2013b) has a much higher frequency of alterations (deletions) in GBM (data not shown). We next studied their impact on survival. Consistent with previous reports (Yan et al., 2009), both OS and DFS were significantly better among all 283 complete LGG patients with *IDH1/2* mutations alone (data not shown) or *IDH1/2* mutations plus *TP53* mutations (Figure 2, upper panel). Patients with both *EZH2* alterations (gene amplification or mRNA upregulation) and *TP53* mutations had no survival advantages compared to unaltered group ($P > 0.1$) (Figure 2, lower panel). Similarly, patients with both *KMT2C* alterations and *TP53* mutations had no significant difference in survival from unaltered group (Logrank test P-value > 0.1). The same observation was true to patients with both *CHD4* alterations and *TP53* mutation (data not shown). We then speculated that effects of epigenetic modifier alterations on tumor initiation and patients' survival could manifest when another important gene was mutated or deleted.

Since over 50% of LGG tumors had *TP53* mutations, a subgroup of 146 patients with *TP53* mutations were selected from the total of 283 all complete LGG. In these 146 cases, patients with and without alterations of epigenetic modifiers *EZH2*, *KMT2C*, or *CHD4* were compared for their OS and DFS. We hypothesized that changes in epigenetic modifiers *EZH2*, *KMT2C*, and *CHD4* act in the premalignant stem cell stage of glioma by epigenetic perturbation to contribute to disease recurrence, and thus decreasing disease-free survival (van Rhenen et al., 2005; Gentles et al., 2010; Wang et al., 2017). We tested this hypothesis by investigating the effect of epigenetic modifier gene alterations in the context of *TP53* mutations, an important driver gene for tumor progression. Our results clearly showed that alteration of either *EZH2* or *KMT2C* in the presence of *TP53* mutations decreased DFS significantly compared to *TP53* mutations only group (Figure 3). The presence of both *EZH2* and *KMT2C* alterations further decreased DFS within the *TP53*-mutated group. The presence of both *IDH* mutation and *CHD4* alteration in combination with *TP53* mutations reduced the DFS within *IDH*-mutated group compared to *IDH1/2* mutation only group (Figure 3), although the presence of *CHD4* alteration did not change either OS or DFS among *TP53*-mutated subgroup ($P > 0.5$, data not shown). Alteration of *EZH2*, *KMT2C*, and *CHD4* epigenetic modifiers contributed to disease recurrence, resulting in shortened disease-free survival time without decreasing overall survival significantly. This is by far the strongest evidence implying that epigenetic perturbation by genomic alteration of *EZH2*, *KMT2C*, or *CHD4* is associated with increased disease recurrence rate. Other genes such as Capicua transcriptional repressor or *CIC* gene, had 20% mutation rate in LGG (Figure 1B). *CIC* gene mutations were reported to contribute to

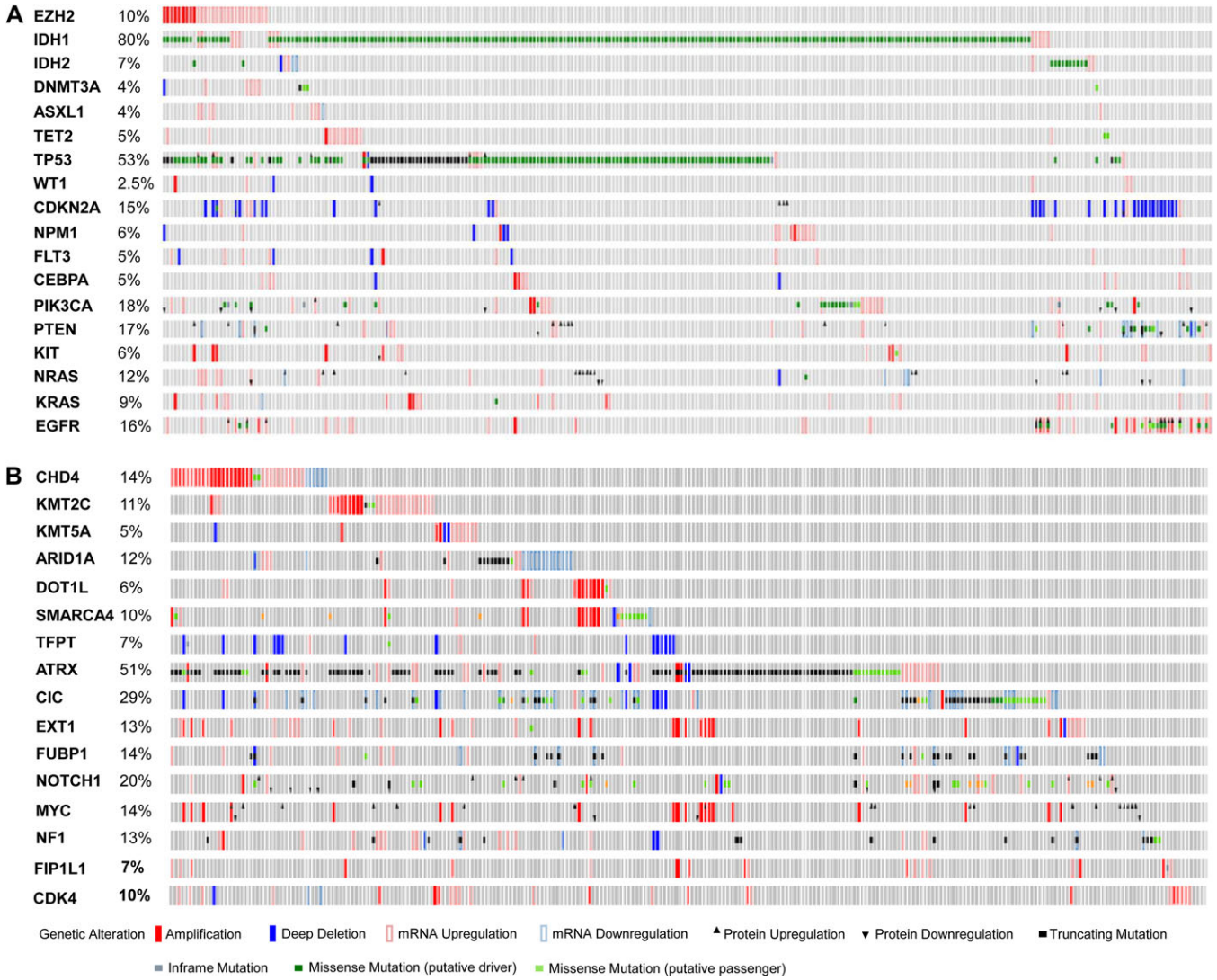

Figure 1 OncoPrint of genes related to AML and glioma. OncoPrint of 18 genes related to AML and glioma (**A**) and 16 genes related to glioma (**B**). Database used: Brain Lower Grade Glioma (TCGA, provisional) 530 samples. All complete tumors (283) were selected for analysis. Alterations include mutations, putative copy number alterations, mRNA expression data, protein expression *Z*-scores (RPPA).

oligodendroglioma (Bettegowda et al., 2011). We selected a total of 56 cases with CIC mutations from 283 all complete LGG. Alterations of *EZH2* markedly reduced the OS of patients compared to those patients without *EZH2* alterations within the selected *CIC*-mutated group (Logrank test $P = 0.00437$). In the meantime, alterations of *CHD4* significantly decreased the DFS within the same group (Logrank test $P = 0.0417$). However, accurate interpretation of the results is limited due to a small sample size.

IDH mutations collaborate with PIK3CA, CDKN2A, CDK4, FIP1L1, or FUBP1 gene alterations to negatively affect survival in a subset of LGG patients

Mutations, deletions, gene amplifications, up- or down-regulations of mRNA and proteins are common events in glioma (Figure 1A and B). Genomic changes in tumor suppressor genes and oncogenes play important roles in tumor initiation, disease progression, and prognosis. *IDH1* mutation occurred with a similar high frequency in all LGG subgroups (Figure 4). In contrast, *IDH2* mutation occurred more often in anaplastic olioastrocytoma (11%) than in oligodendroglioma (7.4%). Its mutation was present in only 1.2% oligoastrocytoma and 3% astrocytoma. *IDH2* gene was deleted in about 3% astrocytoma. No *IDH2* mutations were detected in anaplastic astrocytoma (Figure 4). The combined mutation rate of *IDH1* and *IDH2* is 81% in LGG. Interestingly, mutations of *IDH1* and *IDH2* are mutually exclusive in LGG, which is consistent with previous report in glioma (Yan et al., 2009) and the findings in AML (Papaemmanuil et al., 2016; Figure 1A), but their mutations share the same consequences that are the generation of 'oncometabolite' 2-HG and changed DNA methylation status, as both *IDH1* and *IDH2* are epigenetic modulators (Dang et al., 2009; Xu et al., 2011; Losman et al., 2013; Feinberg et al., 2016). LGG

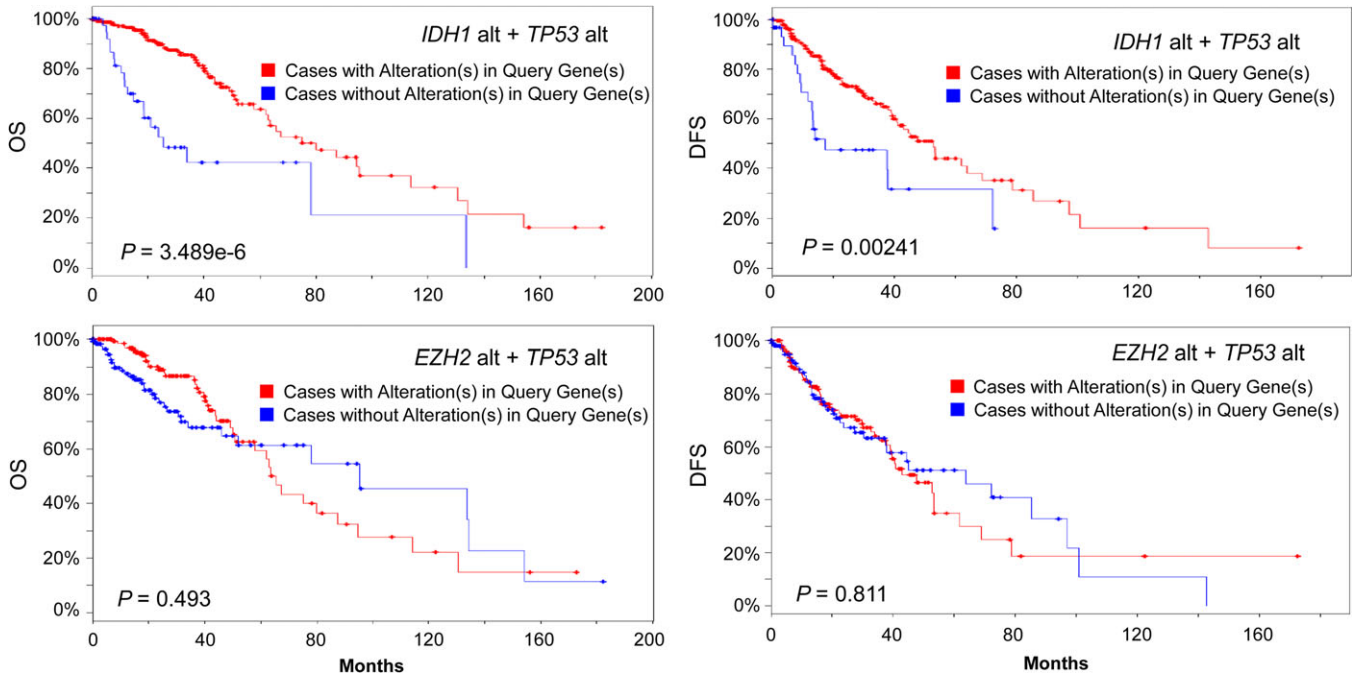

Figure 2 OS and DFS in cases with alterations in *IDH1* and *TP53* (top panel) and in *EZH2* and *TP53* (lower panel) versus no alteration. Database used: Brain Lower Grade Glioma (TCGA, provisional) 530 samples. Alt, alteration. Blue line: cases without alterations in query genes (n = 45, 35, 125, 112 clockwise). Red line: cases with alterations in query genes (n = 236, 224, 156, 147 clockwise). For *EZH2* alt plus *TP53* alt, $P > 0.05$, not significant compared to unaltered group. X-axis: months survival (left panels) or months disease-free (right panels). *P*-value is from Logrank test.

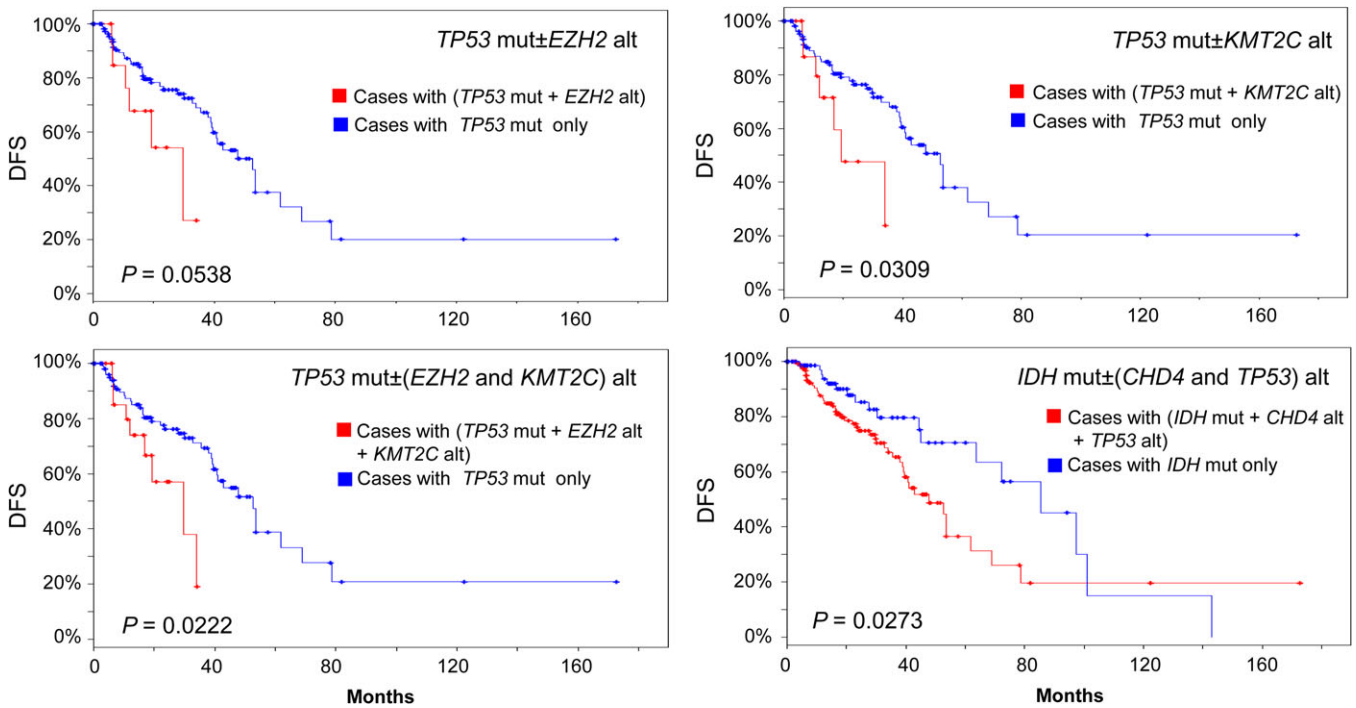

Figure 3 DFS in the *TP53*-mutated (n = 146) or *IDH*-mutated (n = 231) LGG group. Blue line: patients with *TP53* mutation (*TP53* mut–) or *IDH* mutation (*IDH* mut–) only (n = 121, 119, 81, 112 clockwise). Red line: patients with *TP53* mut or *IDH* mut plus other gene alteration (*TP53* mut+ or *IDH* mut+, n = 17, 19, 137, 26 clockwise). Mut, mutated. Alt, alteration. *P*-value is from Logrank test.

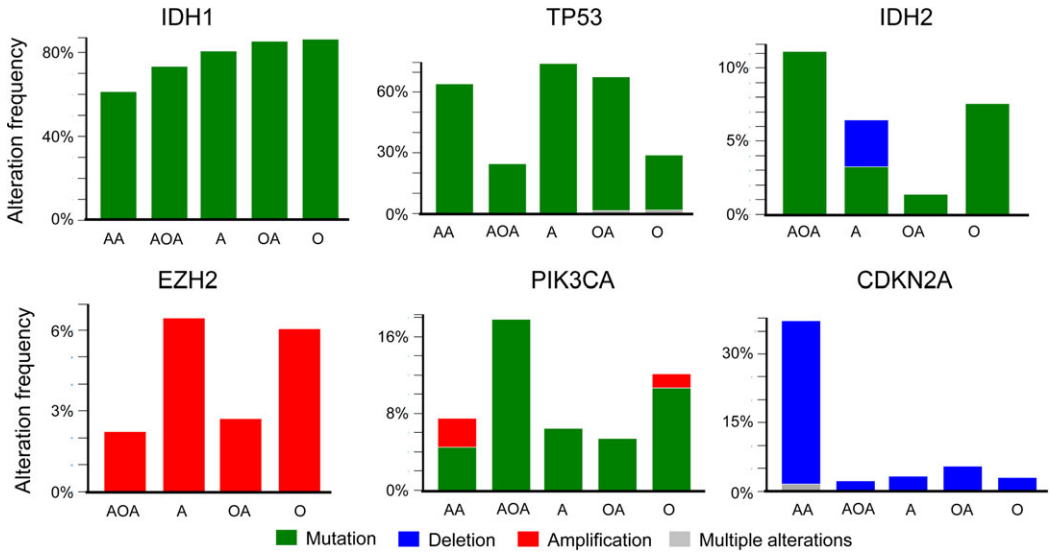

Figure 4 Alterations (mutation in green, deletion in blue, amplification in red, and multi-alterations in gray) of genes in glioma subtypes, from left: anaplastic astrocytoma (AA), anaplastic oligoastrocytoma (AOA), astrocytoma (A), oligoastrocytoma (OA), oligodendroglioma (O). Database used: Brain Lower Grade Glioma (TCGA, provisional) 530 samples.

patients have a better OS and DFS when *IDH1/2* mutations are present (Cohen et al., 2013).

Since the majority of LGG patients have *IDH1/2* mutations, we speculated that further stratification of *IDH1/2*-mutated group may reveal new subsets who may have other driver oncogene mutations or gene amplifications and who may be vulnerable to therapeutic interventions. Thus, we examined the effect of *IDH1/2* mutations in combination with other genomic changes on survival in *IDH1/2*-mutated LGG patient group (231 out of 283 all complete tumors). We tested the effects of 28 other genes whose mutations or copy number alterations (CNA) are relatively common in LGG or GBM or both in the selected 231 patients. These genes include *TP53, ATRX, CIC, NOTCH1, PIK3CA, EGFR, FUBP1, NF1, CDKN2A, PTEN, MYC, EXT1, RAD21, PTK2, AGO2, CDK4, PDGFRA, KIT, MLLT3, CHIC2, MDM4, FIP1L1, MDM2, DDIT3, PIK3R1, SPTA1, FLG, and PCLO*. We found that genetic alterations of individual gene *PIK3CA* or *CDKN2A*, *CDK4* or *FIP1L1* significantly decreased OS of LGG patients among the selected group (Logrank test $P < 0.05$; Figure 5). In the meantime, the presence of *FUBP1* genetic alterations (mainly mutations for *FUBP1* gene) also significantly reduced DFS among the same patient group (Figure 6). Gene alterations of *PIK3CA* and *FIP1L1* had a marginal reduction on DFS (Logrank test $P = 0.0567$ and $P = 0.0723$, respectively; Figure 6, data not shown for *FIP1L1*). The rest of the genes tested did not significantly affect the OS or DFS of the selected patients (data not shown).

EZH2 and CHD4 reduce disease-free survival of patients with other collaborating genetic abnormalities in IDH-mutated LGG group

We next examined the effect of altered *EZH2* or *CHD4* with other collaborating genetic abnormalities on disease-free survival in *IDH*-mutated LGG patients by analyzing the cases with *IDH1/2* mutations ($n = 231$). Interestingly, alterations of either *EZH2* or *CHD4* or *KMT2C* brought down DFS from marginal to a significant level in patients with *PIK3CA* or *FIP1L1* alteration (Logrank test P-value < 0.05). In addition, *CDK4* alteration by itself did not change the DFS significantly in *IDH*-mutated LGG patients compared with those without *CDK4* altertion (Figure 7, Logrank test P-value $= 0.107$), but addition of either *EZH2* or *CHD4* significantly decreased DFS in *CDK4*-altered and *IDH*-mutated patient group, indicating a role of *EZH2*- or *CHD4*-mediated epigenetic purterbation in LGG recurrence (Figure 7). Of note, *CHD4* or *EZH2* alone did not cause significant reduction in DFS without *CDK4* alteration in *IDH*-mutated LGG patients (Logrank test P-value $= 0.0859$ and 0.0558, respectively). Furthermore, *CHD4* alteration collaborated with *RAD21* to decrease DFS in *IDH*-mutated LGG patients compared to those without *CHD4* and *RAD21* alterations (Figure 7, lower right panel). *RAD21* alone was unable to reduce DFS significantly in *IDH*-mutated LGG patients (Logrank test P-value $= 0.203$).

Co-occurrence, mutual exclusivity, and their interaction of altered genes in LGG

Next, we performed analyses to explore whether alterations of these epigenetic modifier genes had the tendency to appear concurrently with each other or with other genes in LGG. The analyses covered 18 genes including epigenetic modifiers *ARID1A, CHD4, DOT1L, EZH2, HDAC4, KDM5A, KMT2C, SMARCA4, TFPT*, and glioma-related genes *ATRX, CDKN2A, CIC, EGFR, FUBP1, MYC, NOTCH1, PIK3CA, TP53*. Results show that *EZH2* and *KMT2C* have a strong tendency to co-occur in LGG ($P < 0.001$). *EZH2* also tends to co-occur with *CDKN2A* deletion, *TP53* mutation, or *EGFR* alterations, respectively. *KMT2C* tends to co-occur significantly with *ATRX*. *CHD4* and *KDM5A* have a strong tendency to co-occur in LGG ($P < 0.001$). Alteration of *CHD4* also tends to

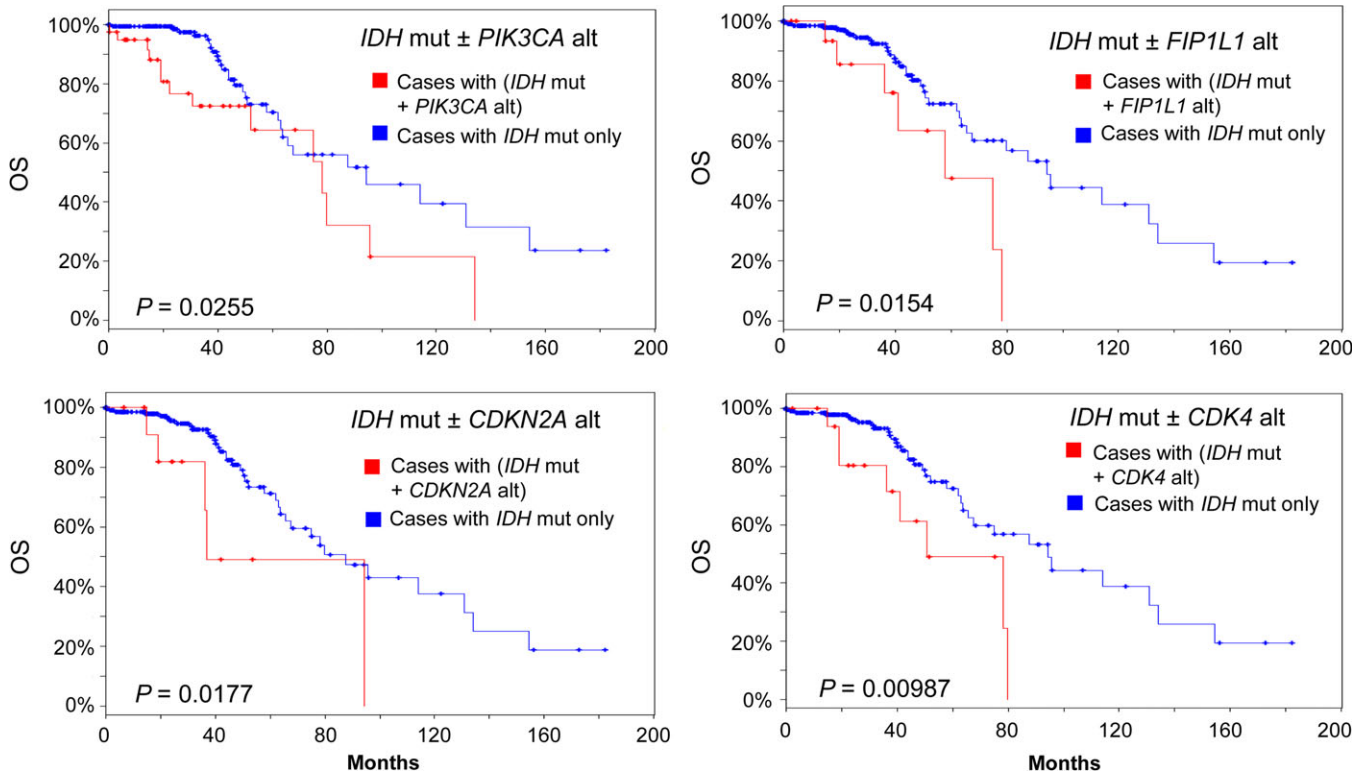

Figure 5 OS within the *IDH*-mutated LGG group. Sample size n = 231. Blue line: patients with *IDH* mutation only (*IDH* mut–, n = 183, 211, 219, 211 clockwise). Red line: patients with *IDH* mut plus other gene alteration (*IDH* mut+). Mut, mutated. Alt, alteration. Alterations of *PIK3CA* (n = 46), *FIP1L1* (n = 18), *CDKN2A* (n = 10), or *CDK4* (n = 18) decreased OS in patients with *IDH* mut. *P*-value is from Logrank test.

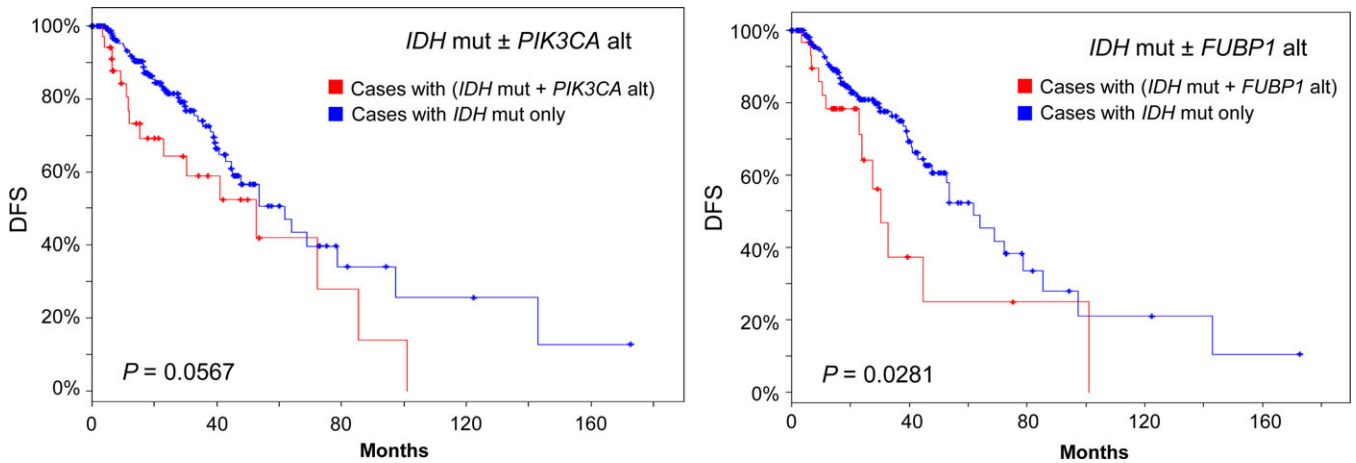

Figure 6 DFS in the *IDH*-mutated LGG group. Sample size n = 231. Blue line: patients with *IDH* mutation only (*IDH* mut–, n = 177 and 185 from left to right). Red line: patients with *IDH* mutation plus other gene alteration (*IDH* mut+). Mut, mutated. Alt, alteration. Alterations of *FUBP1* (n = 33) reduced DFS significantly. *PIK3CA* alt (n = 41) marginally decreased DFS. *P*-value is from Logrank test.

appear with that of *TFPT*, *TP53*, *ATRX*, *MYC*, respectively (P < 0.05). *CHD4* has a significant tendency for mutual exclusivity with *EGFR* (Supplementary Table S1). In addition, *EZH2* amplification tends to occur concurrently with mutated *DNMT3A*, or *ASXL1* (Figure 1A). Besides co-occurrence and mutual exclusivity, alterations of genes could be different across subtypes of LGG. Among 12 genes (*IDH1/2*, *EZH2*, *TP53*, *CDKN2A*, *PIK3CA*, *KMT2C*, *CHD4*, *CDK4*, *FUBP1*, *FIP1L1*, and *CIC*) analyzed (Figure 4 and Supplementary Figure S1), no *EZH2* mutations were found in LGG of this dataset. However, *EZH2* alteration manifested in the form of gene amplification was found in 6.4% astrocytoma and 6% oligodendroglioma patients. Low frequency of *EZH2* gene

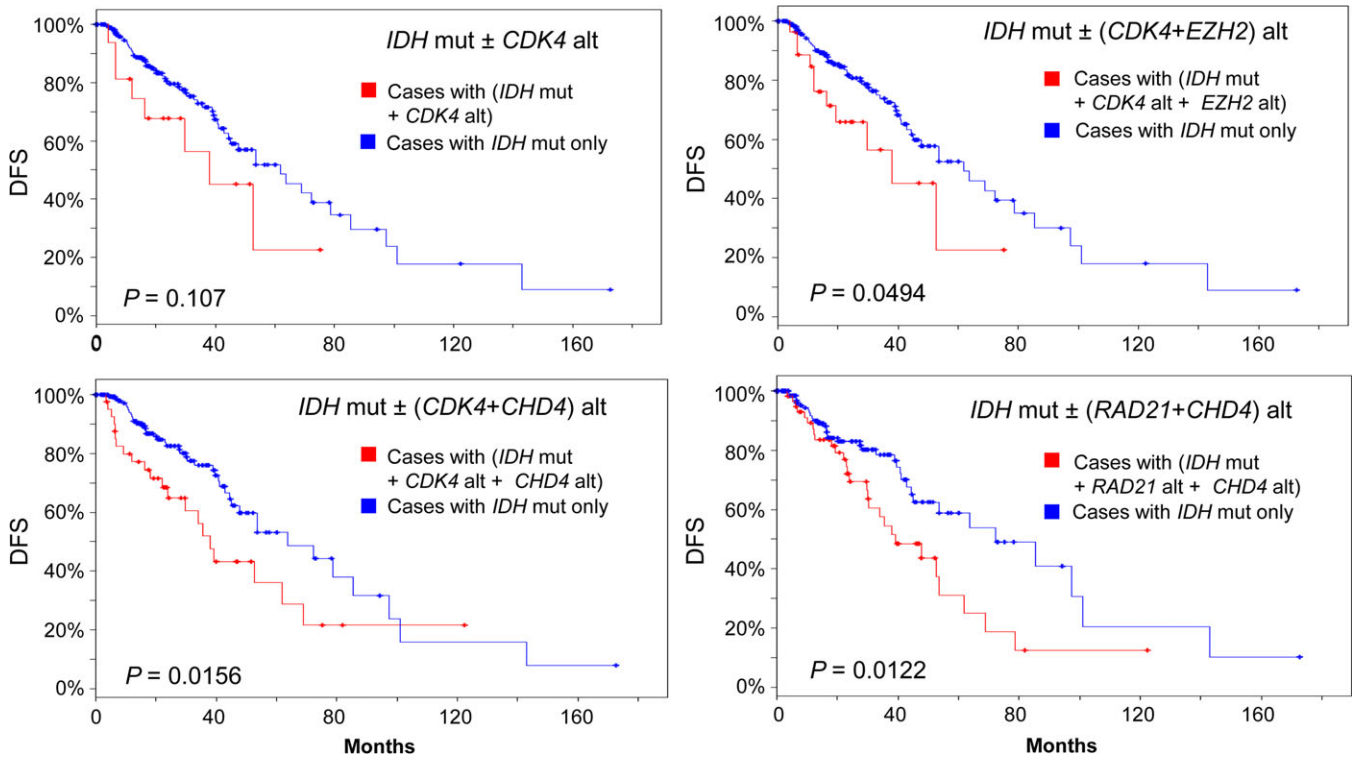

Figure 7 DFS in the *IDH*-mutated (n = 231) LGG group. Blue line: patients with *IDH* mutation only (*IDH* mut−, n = 201, 187, 175, 156 clockwise). Red line: patients with *IDH* mutation plus alteration of other genes (*IDH* mut+). *CDK4* alt, n = 17; (*CDK4*+*EZH2*) alt, n = 31; (*CDK4*+*CHD4*) alt, n = 43; (*RAD21*+*CHD4*) alt, n = 62. Alt, alteration. Mut, mutated.

amplification existed in anaplastic oligoastrocytoma (2.2%) and oligoastrocytoma subtypes (2.5%). Interestingly, no *EZH2* gene amplification was recorded in anaplastic astrocytoma (Figure 4). *TP53* mutations occurred across all subtypes of LGG with some variations in frequency. The majority (99%) of *CDKN2A* alterations were in the form of deletion. All subtypes of glioma had *PIK3CA* gene mutations with a varied frequency. Anaplastic astrocytoma and oligodendroglioma also had *PIK3CA* amplification at 2% or 1%, respectively (Figure 4). *KMT2C* amplification was seen across five subtypes. Low frequency of *KMT2C* mutations (1.5%) was also seen in anaplastic astrocytoma, oligoastrocytoma, and oligodendroglioma (Supplementary Figure S1). *CHD4* gene amplification was seen across all five subtypes, but was more frequent in anaplastic astrocytoma (10%), astrocytoma (13%), and oligoastrocytoma (9%). A few mutations of *CHD4* were seen in anaplastic astrocytoma and oligodendroglioma (Supplementary Figure S1).

CIC was mutated with a high frequency in anaplastic oligoastrocytoma (44%) and oligodendroglioma (38%) subtypes. Low frequency of gene deletion of *CIC* (<5%) was seen across all subtypes. *FUBP1* was also mutated in anaplastic oligoastrocytoma (24%), oligodendroglioma (13%), and oligoastrocytoma (4%). Only 1.6% anaplastic astrocytoma subtype had *FUBP1* mutation and no mutation or other alterations of this gene were seen in astrocytoma subtype. Common alteration of *FIP1L1* gene was gene amplification seen mainly in two subtypes anaplastic astrocytoma (7.4%) and anaplastic oligoastrocytoma (9%). Low frequency mutation of *FIP1L1* was seen in oligodendroglioma (1.4%). No *FIP1L1* gene alterations were seen in either astrocytoma or oligoastrocytoma subtypes. *CDK4* gene was mostly amplified in anaplastic astrocytoma (12%), with lower gene amplification in oligoastrocytoma (1.2%). No gene amplification or other changes of *CDK4* were detected in anaplastic oligoastrocytoma (Supplementary Figure S1). Although the mutation spectrum and frequency differ across subtypes of LGG, the significance of this difference remains a topic for further investigations.

EZH2, KMT2C, CHD4, and TP53 and their networks in LGG

EZH2 is connected to epigenetic regulators such as *HDAC*s, *DNMT1* in the *EZH2–TP53* network. *KMT2C* is connected with *NCOA2* (nuclear receptor coactivator 2) and others. *CHD4* is connected to other epigenetic modifier *TAF1A, HDAC*, and others such as TATA-box binding protein (*TBP*), a critical molecule with an important role in gene transcription. Changes in *EZH2, KMT2C*, or *CHD4* genomic status by gene amplification mutation, or expression could have profound effects on its neighbors and connection nodes in the network (Supplementary Figure S2). Changes in the network status could dysregulate gene transcription, leading to oncogenic transformation and activation. These networks show the complexity of changes which may happen to other connection nodes or neighbors when *EZH2, KMT2C, CHD4* alterations are combined with *TP53* mutation or *CDKN2A* deletion or others.

Discussion

Gliomas can be categorized into four grades (Grades I–IV) based on World Health Organization (WHO) criteria (Louis et al., 2007). Grade I is generally regarded as benign and curable by surgery. Grades II and III are considered as malignant and invasive. By tradition, Grades II and III are often described as LGG. Grade IV, also called glioblastoma multiforme (GBM), is the most invasive and advanced form. GBM is further divided into primary and secondary GBMs based on clinical presentation at diagnosis. Primary GBMs are often *de novo* advanced gliomas, whereas secondary GBMs often develop from LGG.

In this study, we showed that LGG patients with somatic *IDH1/2* mutations had a worse overall survival when alteration of another gene such as *PIK3CA, CDKN2A, CDK4*, or *FIP1L1* was present. Mutation of *PIK3CA*, or deletion of *CDKN2A*, or gene amplification of *CDK4* will likely increase the kinase activities of cancer cells, contributing to LGG pathogenesis (Peng et al., 2014; Zhang and Yang, 2014). LGG patients with *IDH1/2* mutations and alteration of *FUBP1* (mainly mutation for *FUBP1*) had a significant reduction in disease-free survival as well. Alteration of *PIK3CA* (mainly mutation) or *FIP1L1* (mainly gene amplification) had a marginal reduction in DFS within *IDH1/2*-mutated patient group. Our findings bring out a very important point. Namely, targeted therapy should be considered in *IDH1/2*-mutated LGG patients when *PIK3CA* mutation, *CDKN2A* deletion, *CDK4* gene amplification, *FIP1L1* gene amplification, or *FUBP1* mutation is detected. For example, PIK3CA inhibitors can be used for patients with *PIK3CA* mutations in LGG patients who also have somatic *IDH1/2* mutations. By the same token, CDK inhibitors can be used for patients with either *CDK4* gene amplification or *CDKN2A* gene deletion. To the clinician's advantage, CDK4/6 inhibitors palbociclib (PD0332991) and ribociclib (LEE011) have been approved for the treatment of breast cancer (Zhang and Yang, 2014; Xu et al., 2017). Excitingly, PI3K inhibitors Alpelisib (BYL719) and Sonolisib (PX-866) have been developed and are being tested in clinical trials for the treatment of different types of cancers (Pons-Tostivint et al., 2017). Our results indicate that there is an urgent need to test these pathway inhibitors (PI3K inhibitors, CDK4/6 inhibitors, or other inhibitors) in the treatment of LGG when patients are positive for *IDH1/2* mutations and have *PIK3CA* mutation, *CDK4* gene amplification, *CDKN2A* gene deletion, or other gene alterations.

Many studies have been done to link *IDH* mutations with known oncogenes such as *HIF-1α* (Zhao et al., 2009). However, subsequent studies demonstrated that IDH mutations may not be sufficient to upregulate HIF 1a (Williams et al., 2011; Koivunen et al., 2012). In our analysis, *HIF1A* alteration (mainly mRNA upregulation without mutations) was detected in only 4% of cases with *IDH1/2* mutations. This upregulation of *HIF-1α* mRNA did not change either OS or DFS in *IDH*-mutated patient group (Logrank test *P*-value = 0.507 for OS and 0.739 for DFS). The drawback from our analysis is that data of HIF1-α protein level in these tumors were not available in the database.

We have also demonstrated that *EZH2* is altered in around 10% LGG patients in the form of genomic gene amplification or mRNA upregulation. This alteration frequency is markedly higher than that in GBM (2.2%–4%, data not shown). Alteration of *EZH2* can occur concurrently with *DNMT3A* or *ASXL1* mutations, the two mutations frequently seen in pre-LSC. This co-occurrence implies that they have a coordinated role in epigenetic modulation or perturbation and change in glioma stem cell function. We also showed that *CHD4* and *KMT2C* were altered with similar frequency in glioma as *EZH2*. Similar to *DNMT3A* and *ASXL1* in pre-LSC, alterations of *EZH2*, *KMT2C,* and *CHD4* are likely present early in pre-malignant glial stem cells (GSC). These pre-malignant GSCs accumulate other genetic mutations such as *TP53* mutation, *CDKN2A* deletion, and mutations in *EGFR*, *PIK3CA*, *KRAS*, or *FLT* later in clonal evolution. Indeed, alterations of *EZH2*, *CHD4,* and *KMT2C* decreased DFS in either *TP53*-mutated LGG or *IDH1/2*-mutated LGG together with other genetic events, strongly implying that they acted in the glioma stem cell stage. Our hypothetical model is illustrated in Figure 8 where similar genetic events starting from stem cells lead to a common disease cancer in different organs. When it happened

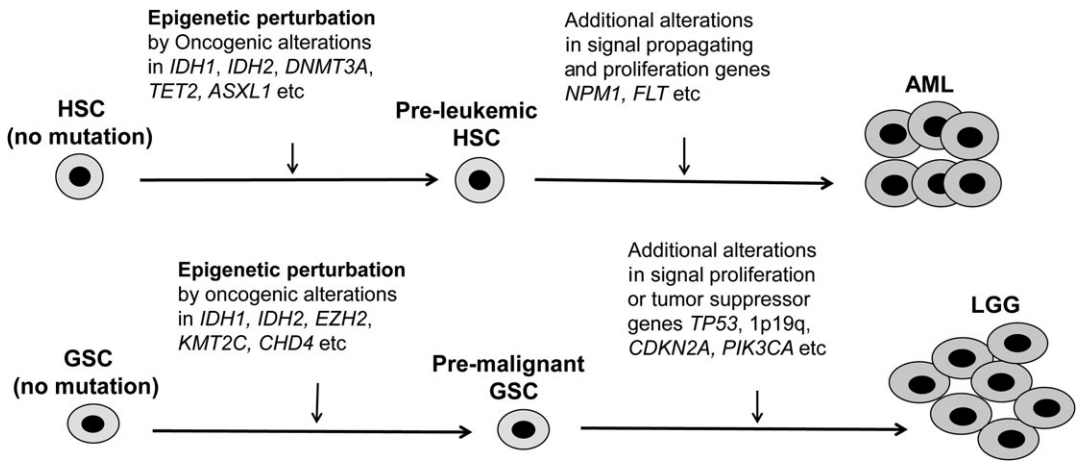

Figure 8 Hypothetical model of malignant transformation of glial stem cells and the development of glioma. HSC, hematopoietic stem cell; AML, acute myeloid leukemia; GSC, glial stem cell; LGG, lower grade glioma.

in HSCs, AML would form. If it occurred in GSCs, then LGG would be the outcome.

EZH2 mRNA upregulation was detected in some AML cases, but its gene amplification has never been documented in AML. *EZH2* is not a major gene in pre-LSC based on current research in AML. However, both gene amplification and mRNA upregulation of *EZH2* were detected in LGG, indicating that this gene likely plays a distinctive role in glioma. The alteration in *EZH2* may lead to epigenetic perturbation of the genome in the stem cell compartment and prime pre-malignant GSCs to malignant transformation along the way when additional genetic changes such as *TP53* mutation, *CDKN2A* deletion, or 1p/19q deletion are acquired. Indeed, it was reported that *EZH2* is crucial for glioma stem cell maintenance (Suva et al., 2009). Currently, EZH2 as therapeutic target in cancer is under investigation (Kim and Roberts, 2016). Genomic alterations of *KMT2C* and *EZH2* in LGG have a strong tendency to emerge at the same time (Supplementary Table S1). KMT2C (also called MLL3) belongs to the mixed lineage leukemia (MLL) family of histone lysine methyltransferases. The enzyme methylates histone at lysine 4 (Lys-4) position which serves as a tag for epigenetic activation of transcription (Fujimoto et al., 2012). Different somatic mutations of *KMT2C* may have different or even opposing effects in cancer (Weirich et al., 2015). The biological effects of *KMT2C* gene amplification in glioma remain an interesting topic to be elucidated.

CHD4 (chromodomain-helicase-DNA-binding protein 4) is part of the histone deacetylase NuRD complex that remodels chromatin through histone deacetylation. CHD4 can recruit DNA methyltransferases to sites of damaged DNA where it methylates DNA de novo. CHD4 plays a role in maintaining DNA hypermethylation-associated transcriptional silencing of tumor suppressor genes (Xia et al., 2017a). CHD4-associated NuRD complex was found to be enriched at nascent DNA in embryonic stem cells, but not in NIH3T3 fibroblasts. Its presence helps maintain protein stability of replication-associated UHRF1 factor (Aranda et al., 2014). CHD4 is important in the maintenance of liver cancer stem cells and is involved in resistance to chemotherapy (Nio et al., 2015). CHD4 was also reported to promote cancer cell proliferation and correlate with a poor prognosis in non-small cell lung cancer patients (Xu et al., 2016). CHD4 interacts with a stem cell transcription factor ZFHX4 to regulate tumor initiation of glioblastoma (Chudnovsky et al., 2014). Somatic CHD4 mutation is not frequent in glioma in our analysis. However, somatic CHD4 mutation is as high as 17% in serous endometrial tumors, implying a possibly critical role in cancer (Gallo et al., 2012). Finally, CHD4 is a coregulator of PAX3–FOXO1 and potential therapeutic target in solid tumor and leukemia as well (Sperlazza et al., 2015; Böhm et al., 2016). *RAD21*, double-strand-break repair protein rad21 homolog, is a component of the cohesion complex and a target gene of Wnt–β-catenin signaling pathway (Xu et al., 2014). RAD21 is also a critical transcriptional regulator of genes and long interspersed retrotransposons. Elevated gene amplification and expression of RAD21 and its mediation of retrotransposon expression were seen in cancer with unstable genome and dysregulated gene expression (Xu et al., 2014). It is not surprising that epigenetic perturbation by CHD4 and activation of RAD21 can result in reduced DFS in *IDH1/2*-mutated LGG patients.

Copy number gain or gene amplification of *EZH2, KMT2C*, and *CHD4* correlates with the upregulation of their mRNA expression (Supplementary Figure S3 for *EZH2* and *KMT2C*; Supplementary Figure S4 for *CHD4*). This is especially apparent for *CHD4*. Therefore, it is reasonable to speculate that copy number gain or gene amplification of *EZH2, KMT2C*, or *CHD4* will lead to increased expression of their respective mRNA and protein. Increased expression of *EZH2, KMT2C,* and *CHD4* leads to the change of their enzymatic activity, causing epigenetic perturbation and malignant transformation. Accumulation of other genetic changes eventually leads to the formation of glioma. Since alteration of *EZH2, KMT2C,* or *CHD4* epigenetic modifier genes increases recurrence rate in *TP53*-mutated glioma patients, targeting these epigenetic modifiers could be the treatment of choice in preventing disease recurrence and increasing disease-free survival in glioma.

Though this study did reveal several genes related to the survival time of the LGG patients, it only employed the well-developed online tools for bioinformatics research (Cerami et al., 2012; Gao et al., 2013). In the future, more advanced data mining techniques (Jiang et al., 2011, 2015; Zhang et al., 2014, 2015a, b, 2016; Jiang, 2015; Kim et al., 2015; Melamed et al., 2015; Tanaka and Ogishima, 2015; Wang et al., 2015; Xia et al., 2017b) will be integrated to continue this research.

Materials and methods

We used four datasets which included the following: (i) Brain Lower Grade Glioma (TCGA, provisional) with 530 samples, for which we selected 283 all complete tumors for analysis; (ii) Glioblastoma (TCGA, 2008) with 206 samples, from which only 91 all complete tumors were chosen for analysis from 206 samples; (iii) Glioblastoma (TCGA, 2013b) with 580 samples, from which 291 all complete tumors were selected for analysis out of 580 samples; (iv) AML (TCGA, 2013a) with 200 samples, in which 166 all complete tumors were used for analysis. For these datasets, chosen genomic profiles included mutations, putative copy number alterations, protein expression by reverse phase protein array (RPPA), mRNA expression by RNAseq (V2 RSEM). Genes entered for query are the following: *EZH2, CHD4, IDH1, IDH2, DNMT3A, ASXL1, TET2, TP53, WT1, CDKN2A, NPM1, FLT3, CEBPA, PIK3CA, PTEN, KIT, NRAS, KRAS, KMT2C,* and *EGFR*. These chosen genes had frequent alterations either in AML or glioma, or both based on available literature. Occasionally, fewer genes or additional genes were entered in query based on search needs. Data retrieval were done via cBioPortal for Cancer Genomics (website: http://www.cbioportal.org). Data generation was made by using the bioinformatics tool at our disposal (Cerami et al., 2012; Gao et al., 2013).

Supplementary material

Supplementary material is available at *Journal of Molecular Cell Biology* online.

Funding

This work was supported by the Natural Science Foundation of China (61372138), the Chinese Recruitment Program of Global Youth Experts, Chongqing Collaborative Innovation Center for Brain Science, and U.S. National Institutes of Health (U01 CA166886-03) (to L.Z.). Y.L. was supported by the start-up fund from the Vivian L. Smith Department of Neurosurgery, McGovern Medical School, the University of Texas Health Science Center at Houston, and Craig H. Neilsen Foundation (338617). J.Z. was supported by the National Institutes of Health (R01HL093195 and R21CA178513).

Conflict of interest: none declared.

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