

Molecular differences in *IDH* wildtype glioblastoma according to *MGMT* promoter methylation

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Abstract

Background. O⁶-methylguanine-DNA-methyltransferase (*MGMT*) promoter methylation status is a predictive biomarker in glioblastoma. We investigated whether this marker furthermore defines a molecularly distinct tumor subtype with clinically different outcome.

Methods. We analyzed copy number variation (CNV) and methylation profiles of 1095 primary and 92 progressive isocitrate dehydrogenase wildtype glioblastomas, including paired samples from 49 patients. DNA mutation data from 182 glioblastoma samples of The Cancer Genome Atlas (TCGA) and RNA expression from 107 TCGA and 55 Chinese Glioma Genome Atlas samples were analyzed.

Results. Among untreated glioblastomas, *MGMT* promoter methylated (*mMGMT*) and unmethylated (*uMGMT*) tumors did not show different CNV or specific gene mutations, but a higher mutation count in *mMGMT* tumors. We identified 3 methylation clusters. Cluster 1 showed the highest average methylation and was enriched for *mMGMT* tumors. Seventeen genes including gastrulation brain homeobox 2 (*GBX2*) were found to be hypermethylated and

downregulated on the mRNA level in *mMGMT* tumors. In progressive glioblastomas, platelet derived growth factor receptor alpha (*PDGFRA*) and *GLI2* amplifications were enriched in *mMGMT* tumors. Methylated *MGMT* tumors gain *PDGFRA* amplification of *PDGFRA*, whereas *uMGMT* tumors with amplified *PDGFRA* frequently lose this amplification upon progression. Glioblastoma patients surviving <6 months and with *mMGMT* harbored less frequent epidermal growth factor receptor (*EGFR*) amplifications, more frequent *TP53* mutations, and a higher tumor necrosis factor–nuclear factor-kappaB (TNF-NFκB) pathway activation compared with patients surviving >12 months.

Conclusions. *MGMT* promoter methylation status does not define a molecularly distinct glioblastoma subpopulation among untreated tumors. Progressive *mMGMT* glioblastomas and *mMGMT* tumors of patients with short survival tend to have more unfavorable molecular profiles.

Key words

glioblastoma biomarker | NFκB | O⁶-methylguanine-DNA-methyltransferase (*MGMT*) | *PDGFRA* | *TERT*

Importance of the study

This study compares more than 1200 glioblastomas from 3 sources for differences in methylation, CNV, mutation, and RNA expression according to their *MGMT* promoter methylation status. There was an uneven distribution of *MGMT* promoter methylation in 3 defined methylation clusters, despite otherwise similar molecular profiles. The study is of major relevance for upcoming clinical trials that will stratify

and include patients with newly diagnosed glioblastoma according to their *MGMT* promoter methylation status and frequently withhold temozolomide in the experimental study arms. Differential analysis of primary and progressive tumors revealed differences upon progression, including activation of the TNF-NFκB pathway and CNV changes in *mMGMT* tumors that are distinct from *uMGMT* tumors.

O⁶-methylguanine-DNA-methyltransferase (*MGMT*) promoter methylation status has been consistently identified and used as a predictive biomarker for response to alkylating chemotherapy in patients with glioblastoma.^{1–3} *MGMT* is a protein that repairs damage induced by alkylating chemotherapies, including temozolomide. Methylated *MGMT* (*mMGMT*) promoter leads to reduced *MGMT* expression.⁴ Especially patients with wildtype isocitrate dehydrogenase 1/2 genes (*IDH1/2*) and an unmethylated *MGMT* (*uMGMT*) promoter show minimal response to chemotherapy, with a median survival of just over one year when treated with radiation and chemo after maximal safe resection.⁵

The concept of trials with replacement of temozolomide in the experimental arm in favor of a combination of an experimental systemic therapy with radiotherapy has been successfully established.^{6–9} Preclinical data do not suggest an impact of *MGMT* on radiotherapy, but other treatments have not been systematically evaluated.¹⁰ The German Neuro-Oncology Society NOA-04 trial provided a clinical basis for *MGMT* promoter methylation status being predictive also in *IDH* wildtype anaplastic gliomas,^{11,12} as did NOA-08 for elderly patients with glioblastoma.¹³

In the present study, we aimed to evaluate the molecular differences between glioblastomas with a methylated or unmethylated *MGMT* promoter in different datasets.

Methods

Glioblastoma Patient Cohorts

As of August 2, 2016, we screened the Heidelberg 450k methylation array database. We identified 1028 glioblastoma samples at diagnosis and 66 glioblastoma samples at tumor relapse (see Supplementary Methods) with *IDH* wildtype. *MGMT* promoter methylation status was determined by the algorithm of Bady et al.¹⁴ Samples were classified as “unsure” and excluded from the analysis if the confidence interval of the prediction included the cut-off value of 0.358. Patients provided informed consent concerning the use of their tissue samples for research purposes. The study was approved by the local ethics committee (#206/2005). Of the 1028 primary tumors, 143 with clinical trial grade follow-up data and treatment according to the present standard of care were accessible for survival analysis.

Illumina 450k Array Platform Analysis

The Illumina Infinium HumanMethylation450 (450k) array was used to obtain the DNA methylation status at 482421 cytosine-phosphate-guanine (CpG) sites at the Genomics

and Proteomics Core Facility of the German Cancer Research Center (DKFZ) (see Supplementary Methods).¹⁵

Copy Number Variation Analysis

Copy numbers of single genes were assessed from 450k array data (details in the Supplement).

The Cancer Genome Atlas Data Analysis

Data from The Cancer Genome Atlas (TCGA) of 261 glioblastoma specimens at diagnosis were downloaded from the cBio portal (www.cbioportal.org)^{16,17} and from Firebrowse (Broad institute, <http://firebrowse.org>)¹⁸ on September 12, 2016 (Supplementary Table S1). Only tumor samples with *IDH1/2* wildtype and defined *MGMT* promoter methylation status were used for analysis. Clinical data, copy number variations (CNVs), and mutation and expression subtype according to the classification of Verhaak et al¹⁹ were downloaded from the cBio portal. Methylation cluster types of Ceccarelli et al²⁰ and Sturm et al²¹ were obtained from the original paper. RNA sequencing (RNAseq) V3 normalized data were downloaded from Firebrowse and available for 107 samples meeting the above-mentioned criteria. Data from 450k array methylation were downloaded from the data portal of TCGA (<https://portal.gdc.cancer.gov/>) and available for 67 of the above-mentioned tumor samples.

Chinese Glioma Genome Atlas Data Analysis

Messenger RNA microarray data from the Chinese Glioma Genome Atlas (CGGA) of diffuse gliomas²² including clinical information were downloaded from the CGGA website (<http://www.cgga.org.cn>) on October 25, 2016. Tumors with *IDH* mutations were excluded. For analysis of short and long surviving patients, only samples of patients with survival times <6 months and >12 months of altogether 55 patients were used (Supplementary Table S1).

Messenger RNA Data and Ingenuity Pathway Analysis

RNAseq data from TCGA samples were downloaded from firebrowse.org as described above. We only used samples with available RNAseq data and *IDH* wildtype as well as determined *MGMT* promoter methylation status ($n = 107$) (details in the Supplement).

Gene Set Enrichment Analysis

GSEA was performed with RNAseq data from TCGA samples and microarray data from CGGA samples downloaded as described above from firebrowse.org and cgga.org.cn (see Supplement).

Cluster Analysis

Consensus clustering using k-means was performed with the 10000 most variable methylation positions of the indicated dataset. Clusters were calculated with the

R extension package “ConsensusClusterPlus”²³ version 1.38.0. Euclidean distance was used for distance measure. The maximum number of evaluated clusters was $k = 8$.

Statistical Analysis and Graphics

Univariate survival analyses were performed using the Kaplan–Meier estimator and the log-rank test using Sigmaplot 12.5 software (Systat Software). Fisher’s exact test was used for significance testing for experiments with a 2×2 matrix. Values of $P < 0.05$ were considered significant and asterisked. Multiple testing was corrected using the Benjamini–Hochberg procedure. Circos plots were generated using R version 3.3.2 with the extension package “OmicCircos”²⁴ Heatmaps and all further graphics were generated using the extension packages “gplots” and “ggplot2.”

Results

Differences in the Molecular Profile of *mMGMT* and *uMGMT* Tumors at Diagnosis

There were 435/1028 (42%) *mMGMT* and 593 (58%) *uMGMT* samples. Patients with *mMGMT* promoter in the Heidelberg cohort had a better outcome ($P = 0.001$; Fig. 1A). In the cohort from TCGA, survival analysis of patients who received chemotherapy showed a better outcome in the *mMGMT* tumor subgroup, but no difference in patients not receiving chemotherapy ($P = 0.008$, $P = 0.001$, and $P = 0.94$, respectively; Fig. 1A, B).

No significant differences in cyclin dependent kinase 4 (*CDK4*), mouse double minute human homolog (*MDM2*), cyclin dependent kinase inhibitor 2A (*CDKN2A*), and platelet derived growth factor alpha (*PDGFRA*) were found when stratifying for *MGMT* promoter methylation status (Supplementary Figure S1A).

There were no differences between *mMGMT* and *uMGMT* tumors in the general DNA copy number profile (Fig. 1C). The percentage of copy number altered genomes between *mMGMT* and *uMGMT* tumors did not differ in the dataset from TCGA (Fig. 1D).

In TCGA,²⁵ a higher overall number of mutations was reported for *mMGMT* tumors. The absolute number of mutations was higher in *mMGMT* tumors in the current TCGA cohort (46 vs 38, $P < 0.001$; Fig. 1E). None of the mutation frequencies of any single gene differed significantly between *mMGMT* and *uMGMT* tumors, but there was a trend toward a higher frequency of phosphatase and tensin homolog (*PTEN*) mutations in the *mMGMT* group (31.4% vs 18.5%, $P = 0.071$). Telomerase reverse transcriptase (*TERT*) promoter mutations and *BRAF* mutations were analyzed in a large dataset by Arita et al.²⁶ After exclusion of *IDH* mutant and lower-grade tumors, no differences in *TERT* promoter mutations and *BRAF* mutation frequencies were found according to *MGMT* promoter methylation (*TERT* 57.9% vs 57.8% and *BRAF* 2.8% vs 2.5%, for *mMGMT* and *uMGMT*, respectively).

After excluding CpGs in proximity to the *MGMT* gene, we found 2024 differentially methylated positions (DMPs) with an adjusted P -value ≤ 0.001 and 438 differentially methylated

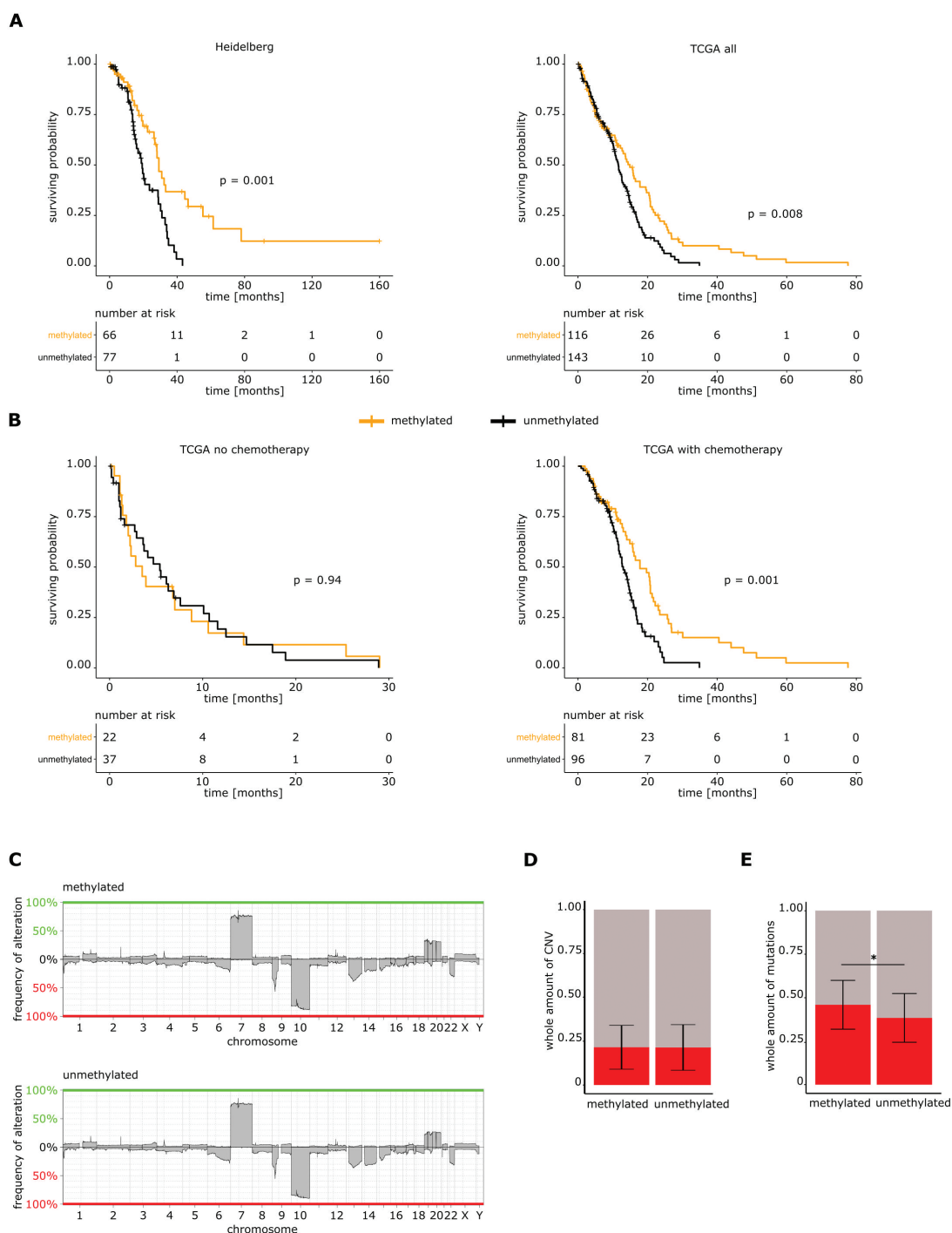


Fig. 1 Copy number variations in primary glioblastoma according to *MGMT* promoter methylation. (A) Left panel: overall survival of 143 glioblastoma patients with *IDH1* wildtype tumors according to *MGMT* promoter methylation. Right panel: survival analysis of patients with tumors from the dataset from TCGA regardless of the treatment. (B) Left panel: survival analysis of tumors of the TCGA glioblastoma database not receiving chemotherapy according to *MGMT* promoter methylation. Right panel: survival analysis of the dataset from TCGA treated with chemotherapy. (C) Graphical illustration of the percentage of CNV in the *mMGMT* and *uMGMT* groups. (D) Relative fractions of DNA copy number variations detected in the database from TCGA according to *MGMT* promoter methylation. Shown are the means \pm standard deviation. (E) Average fraction of mutations detected in the dataset from TCGA according to *MGMT* promoter methylation. Shown are the means \pm standard deviation.

regions (DMRs) between *mMGMT* and *uMGMT* tumors in our 450k array datasets from TCGA (Fig. 3A). Filtering by including only CpGs within the promoter region of a gene identified 814 DMPs in 419 different genes (Supplementary Table S2). All but 3 of these positions were hypermethylated in *mMGMT* tumors. GSEA of differentially methylated positions and regions revealed that the top molecular functions of genes in hypermethylated regions in *mMGMT* tumors involve transcription regulation and DNA binding (Supplementary Table S3). Applying a more stringent gene selection, we included genes with at least 3 differentially methylated CpGs in one DMR. Ingenuity Pathway Analysis (IPA) of the 87 genes matching these criteria found the highest scoring network associated with the functions *cell death and survival* (Supplementary Figure S1B).

Gastrulation brain homeobox 2 (*GBX2*), a transcription factor known to stimulate proliferation of prostate cancer cells,²⁷ was hypermethylated at 5 CpGs in the transcription start site of the methylation dataset (adjusted

P -values = 3.7×10^{-6} to 6.5×10^{-7} ; Supplementary Figure S2A) in the *mMGMT* group and furthermore more than 2-fold downregulated on the RNA level in the *mMGMT* group in the dataset from TCGA, suggesting transcriptional repression through promoter methylation in *mMGMT* tumors (Fig. 2). Direct comparison in 40 samples from TCGA with methylation and RNAseq data revealed relevant *GBX2* expression only in samples with low methylation of *GBX2*, and these tumors were predominantly *uMGMT* (Supplementary Figure S2B). In addition to *GBX2*, 16 genes were hypermethylated at a minimum of 3 CpGs in the combined methylation analysis and downregulated in the RNAseq dataset of TCGA (Fig. 2, Supplementary Table S2).

Consensus clustering using k-means of all samples revealed 3 different subgroups (Fig. 3A–D). Methylated *MGMT* tumors were overrepresented in cluster 1 (44% vs 31%, $P = 0.0001$) and underrepresented in cluster 3 (28% vs 40%, $P = 0.0001$; Fig. 3E). Comparison of tumors from TCGA included in the analyses by Ceccarelli et al²⁰ and

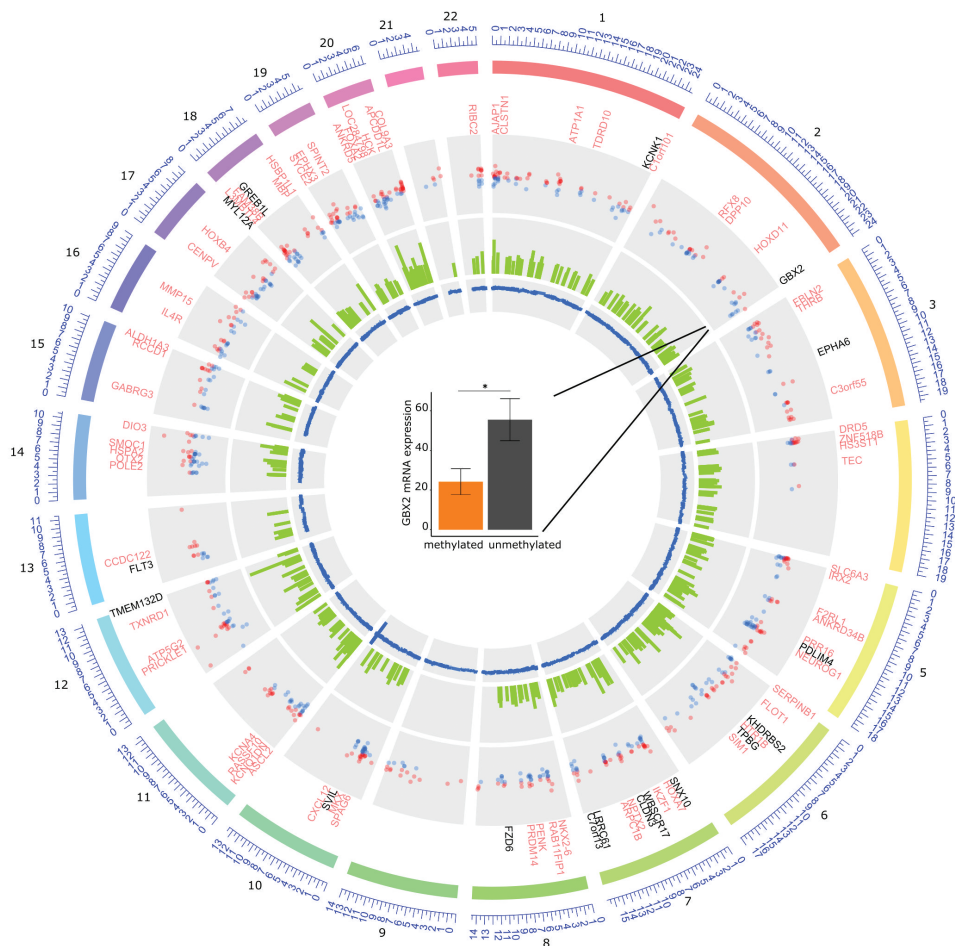


Fig. 2 Differential methylation in primary glioblastoma according to *MGMT* promoter methylation. From outside to inside of the circle: First circle: hypermethylated genes in *mMGMT* tumors according to their chromosome position. Genes that were additionally downregulated in the RNAseq TCGA dataset are labeled in black, others in light red. Second circle: all DMPs within a promoter region of a gene. Third circle: area of each DMR between *mMGMT* and *uMGMT* glioblastomas. Fourth circle: all DMPs, including *MGMT* related on chromosome 10. Center: *GBX2* mRNA expression in the dataset from TCGA in *mMGMT* and *uMGMT* glioblastomas.

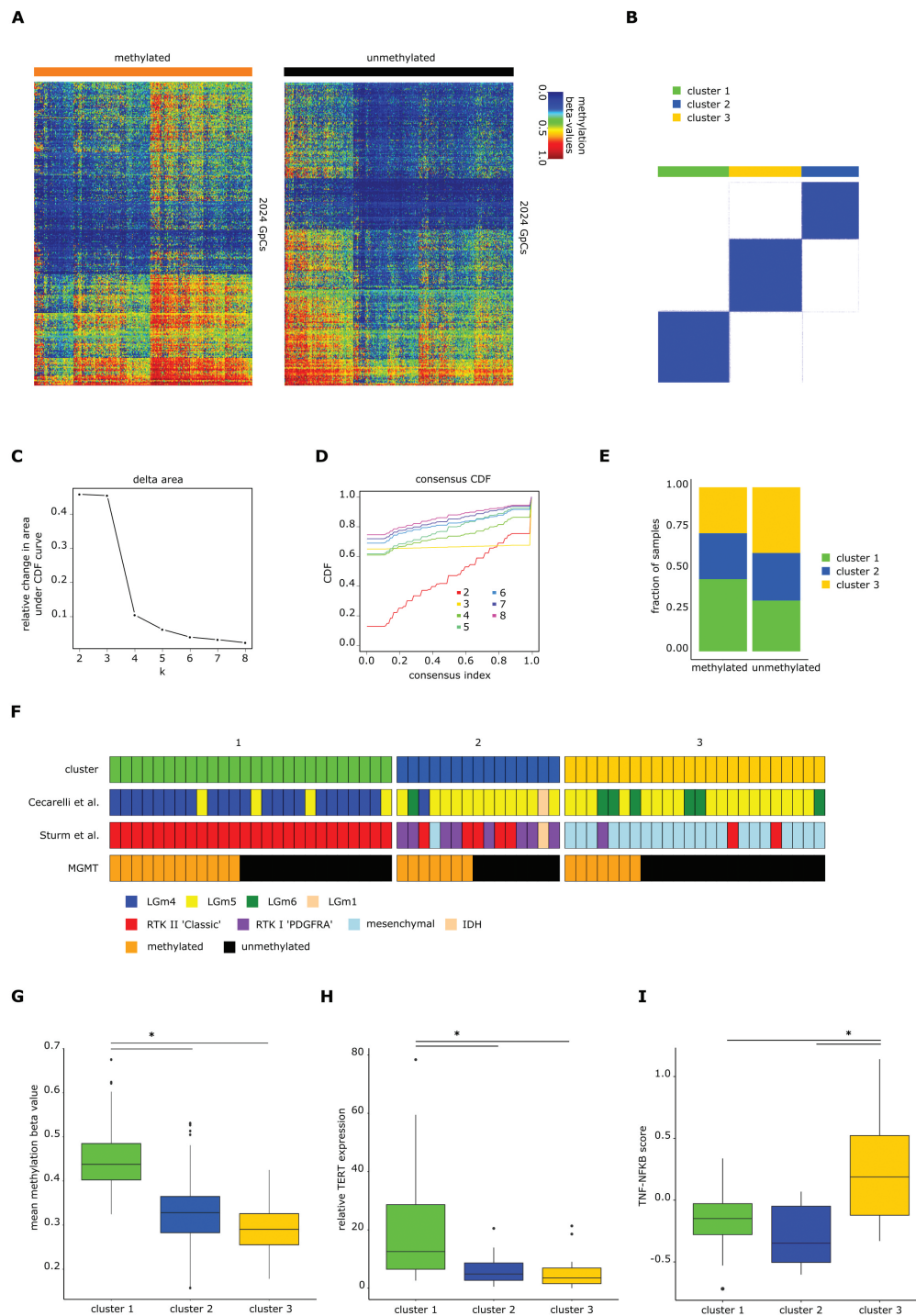


Fig. 3 IDH wildtype glioblastoma cluster into 3 different clusters. (A) Heatmaps of 2024 positions that are differentially methylated between *mMGMT* and *uMGMT* tumors in the dataset of primary tumors, excluding *MGMT* related and intergenomic positions. (B) Consensus clustering using k-means of all samples of the primary tumor dataset. Shown is the result for $k=3$. (C) Plot showing the relative change in area under the cumulative distribution function (CDF) curve in the k-means unsupervised clustering of primary glioblastoma. (D) CDF plot with examples from $k=2$ to $k=8$. Three clusters were chosen as the optimal number. (E) Distribution of *mMGMT* and *uMGMT* primary tumors into the clusters 1, 2, and 3. (F) Comparison of the cluster assignments with the clusters from Ceccarelli et al.²⁰ and Sturm et al.²¹ for 65 glioblastoma samples of the database from TCGA. (G) Mean methylation beta-values of the 2024 differentially methylated CpGs, (H) relative TERT expression, and (I) TNF-NFκB score according to methylation clusters. The lower and the upper hinges correspond to the first and third quartiles. The upper and lower ends of the whiskers correspond to the 1.5× interquartile range from the hinge.

Sturm et al²¹ revealed that cluster 1 contains mainly the “LGm4” tumors of Ceccarelli et al²⁰ and the “RTKII classic” tumors of the Sturm et al classification²¹ (Fig. 3F). Tumor samples in cluster 1 showed higher mean methylation beta-values of differentially methylated CpGs between *mMGMT* and *uMGMT* tumors than tumors in clusters 2 and 3 ($P = 2 \times 10^{-92}$ and $P = 2 \times 10^{-186}$; Fig. 3G). A hotspot of DMRs on chromosome 6p21-6p22 is described in the Supplement. Age at diagnosis was similar between the 3 clusters (cluster 1: 65.5 y, cluster 2: 62.7 y, cluster 3: 61.7 y; P -values: cluster 1 vs 2: 0.09, cluster 1 vs 3: 0.05, cluster 2 vs 3: 0.65). The survival difference between patients with *mMGMT* and *uMGMT* tumors was largest in patients with tumors of cluster 1 ($P < 0.001$ for cluster 1; Supplementary Figure S4A), and no significant survival difference according to *MGMT* promoter methylation was found for clusters 2 and 3. Chemotherapy was given more often in patients of cluster 1, but survival curves were similar when including only patients who received chemotherapy (Supplementary Figure S4B). Overall, patients of cluster 2 showed worse survival (Supplementary Figure S4C). Of note, Nguyen et al²⁸ suggested a survival benefit of patients with *mMGMT* only in *TERT* promoter mutated tumors. As *TERT* promoter mutation increases *TERT* expression, we analyzed the tumors in the different clusters with available RNAseq data. Tumors of cluster 1 showed considerably higher *TERT* expression levels than tumors of clusters 2 and 3 (P -values: cluster 1 vs 2: 0.021, cluster 1 vs 3: 0.010; Fig. 3H). Conversely, the tumor necrosis factor (TNF)-nuclear factor-kappaB (NF κ B) score was highly enriched in tumors of cluster 3 (P -values: cluster 1 vs 3: 0.007, cluster 2 vs 3: 0.002; Fig. 3I).

MGMT showed the lowest P -value for downregulation of all recorded genes in the dataset from TCGA ($P = 3.9 \times 10^{-13}$, fold change 0.38 for methylated vs unmethylated). Applying P -value for difference of $P < 0.05$ and a fold change of >2 by RNAseq analysis, there were 175 differentially regulated genes.

Analysis of Paired Tumor Samples

Twenty (48%) of paired tumor samples showed *MGMT* promoter methylation, whereas 29 (52%) did not (Fig. 4A). Only samples with the same *MGMT* promoter methylation status at diagnosis and progression were included for paired analysis. Chemoradiotherapy was received by 42/49 (86%) patients, 3 patients received chemotherapy alone, and 4 received radiotherapy alone (Supplementary Table S4). Three of the 20 (15%) *mMGMT* tumors acquired *PDGFRA* amplification or gain at progression, whereas none of the *uMGMT* tumors did. Instead, 3 progressive *uMGMT* tumors lost their *PDGFRA* amplification (Fig. 4A, B). The *uMGMT* tumor 19 is shown (Fig. 4C upper panel) as an example of an acquired loss of *PDGFRA* and an acquired gain of *CDKN2A* gene dosage at progression that occurred frequently only in *uMGMT* tumors (21% vs 0%, $P = 0.031$; Fig. 4A, B). Loss of the *CDKN2A* deletion in *uMGMT* tumor 16 was accompanied by acquired *CDK4* amplification upon progression (Fig. 4C middle panel). Methylated *MGMT* tumor 15 is an example of loss of *CDKN2A* upon progression paralleled by loss of preexisting *CDK4* and *MDM2* amplifications (Fig. 4C lower panel). No major differences

were observed in the global CNV profiles of paired tumor samples according to *MGMT* promoter methylation status (Supplementary Figure S5A, B).

The Frequency of *PDGFRA*, *CDK4*, and *MDM2* Amplification in Progressive Glioblastomas Differs According to *MGMT* Promoter Methylation

In 85 samples of progressive glioblastoma (including 66 samples from the Heidelberg database, 13 samples from TCGA patients, and 6 samples from Wang et al²⁹), CNV of *CDK4*, *MDM2*, *PDGFRA*, and *CDKN2A* was assessed (Fig. 5A). Of these tumors, 32 (38%) were *MGMT* promoter methylated and 53 (62%) were unmethylated. Amplification or gain of the *PDGFRA* gene was found in 22% of the *mMGMT* tumors and 7.5% of *uMGMT* samples among progressive tumors ($P = 0.092$; Fig. 5B right panel). Furthermore, amplification or gain of *CDK4* and *MDM2* was found more frequently in progressive *uMGMT* tumors compared with *mMGMT* tumors (3.1% vs 18.9%, $P = 0.046$ and 0% vs 13.2%, $P = 0.042$, respectively; Fig. 5B left and middle panels). When compared with newly diagnosed glioblastomas, the frequency of *CDK4* or *MDM2* amplifications was significantly lower in *mMGMT* tumors in the progressive setting but was comparable in *uMGMT* tumors (Supplementary Figure S6). In progressive tumors with 450k array data available, we analyzed a set of 19 frequently altered genes in glioblastoma. Besides the differences in *CDK4*, *MDM2*, and *PDGFRA* amplifications described above, we found a gain or amplification of the *C19MC* microRNA cluster and the *GLI2* gene more often in *mMGMT* compared with *uMGMT* tumors (54% vs 22% and 14% vs 0%, respectively). A higher percentage of myeloblastosis (*MYB*) deletions was detected in *uMGMT* tumors (36% vs 9%; Fig. 5C). The higher rate of *C19MC* amplifications in the *mMGMT* group was accompanied by a trend toward a higher amplification rate of the whole chromosome 19 (Fig. 5D).

Principal component analysis of progressive tumors in the 450k methylation array dataset did not separate tumors according to *MGMT* status (Supplementary Figure S7A). Applying the same clustering algorithm as we did for primary tumors, we identified 3 different methylation groups. Methylated *MGMT* tumors were overrepresented in cluster 1 (57% vs 20%, $P = 0.005$) and underrepresented in cluster 3 (10% vs 43%, $P = 0.009$; Supplementary Figure S7B–E).

We found 346 DMPs and 182 DMRs between progressive *mMGMT* and *uMGMT* tumors (Supplementary Figure S7F). After filtering for positions within the promoter region of a gene, we identified 196 positions in 152 genes that were differentially regulated between both groups. These positions were all hypermethylated in *mMGMT* tumors. GSEA did not show significantly enriched molecular functions in both groups.

Twenty-one of the 152 genes (14%) had at least one DMP in the dataset of primary tumors. Of these, within the promoter region of the myosin light chain (*MYL12A*), which may be involved in DNA damage repair,³⁰ we identified 5 positions that were hypermethylated in progressive *mMGMT* tumors (Supplementary Figure S2C). Three of exactly these 5 positions were already found to be hypermethylated in the dataset of primary tumors.

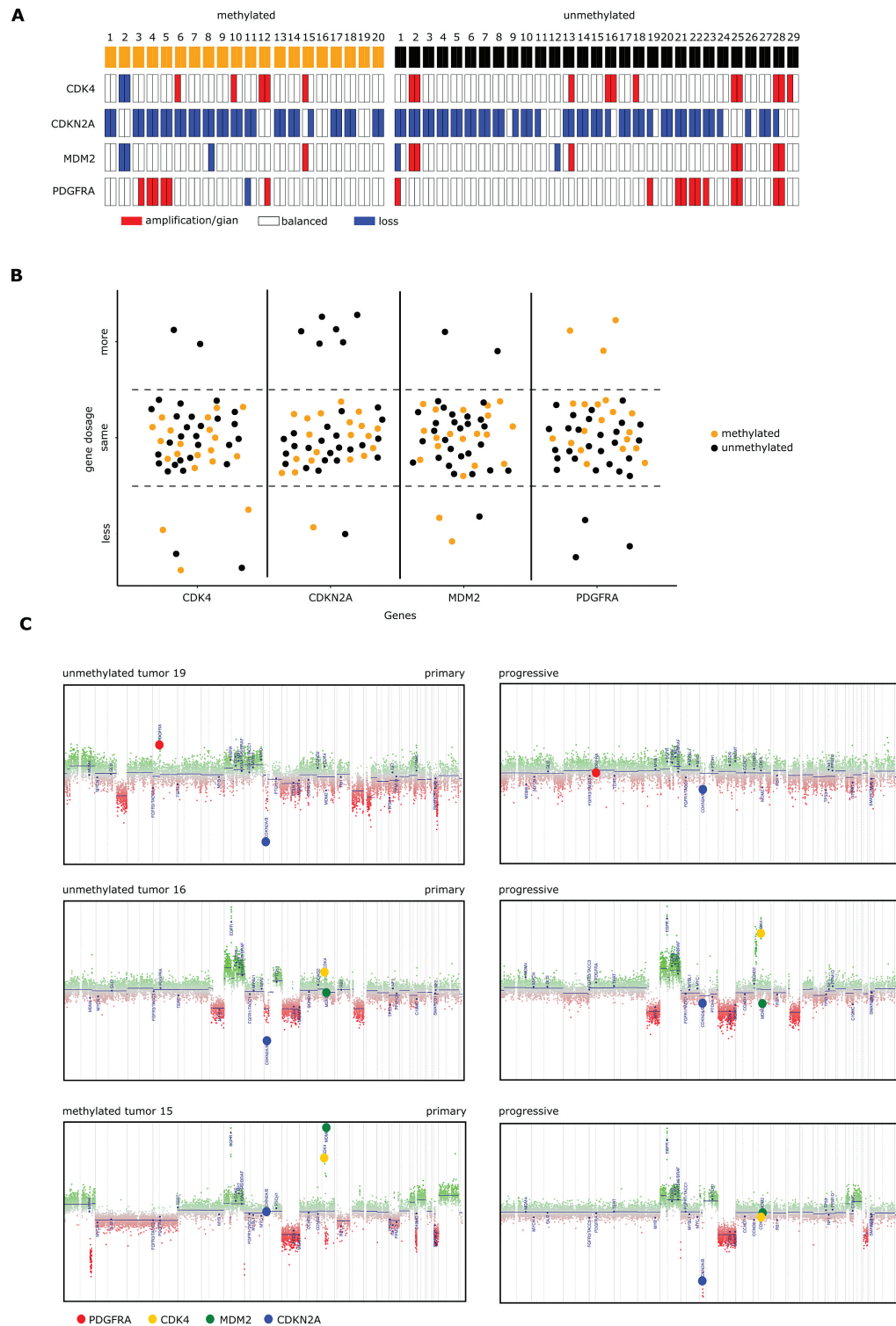


Fig. 4 CNV changes in pairs of primary and progressive glioblastoma. (A) CNV of the genes *CDK4*, *CDKN2A*, *MDM2*, and *PDGFRA* in a set of 49 paired glioblastoma samples at primary diagnosis and progression. *MGMT* promoter methylation is indicated in the first row. (B) Shift in gene dosages of *CDK4*, *CDKN2A*, *MDM2*, and *PDGFRA* in paired samples of primary and progressive glioblastoma according to *MGMT* promoter methylation. (C) Examples of copy number profiles of 3 paired primary and progressive glioblastoma samples. Color code: *PDGFRA* = red, *CDKN2A* = yellow, *CDK4* = blue, *MDM2* = green.

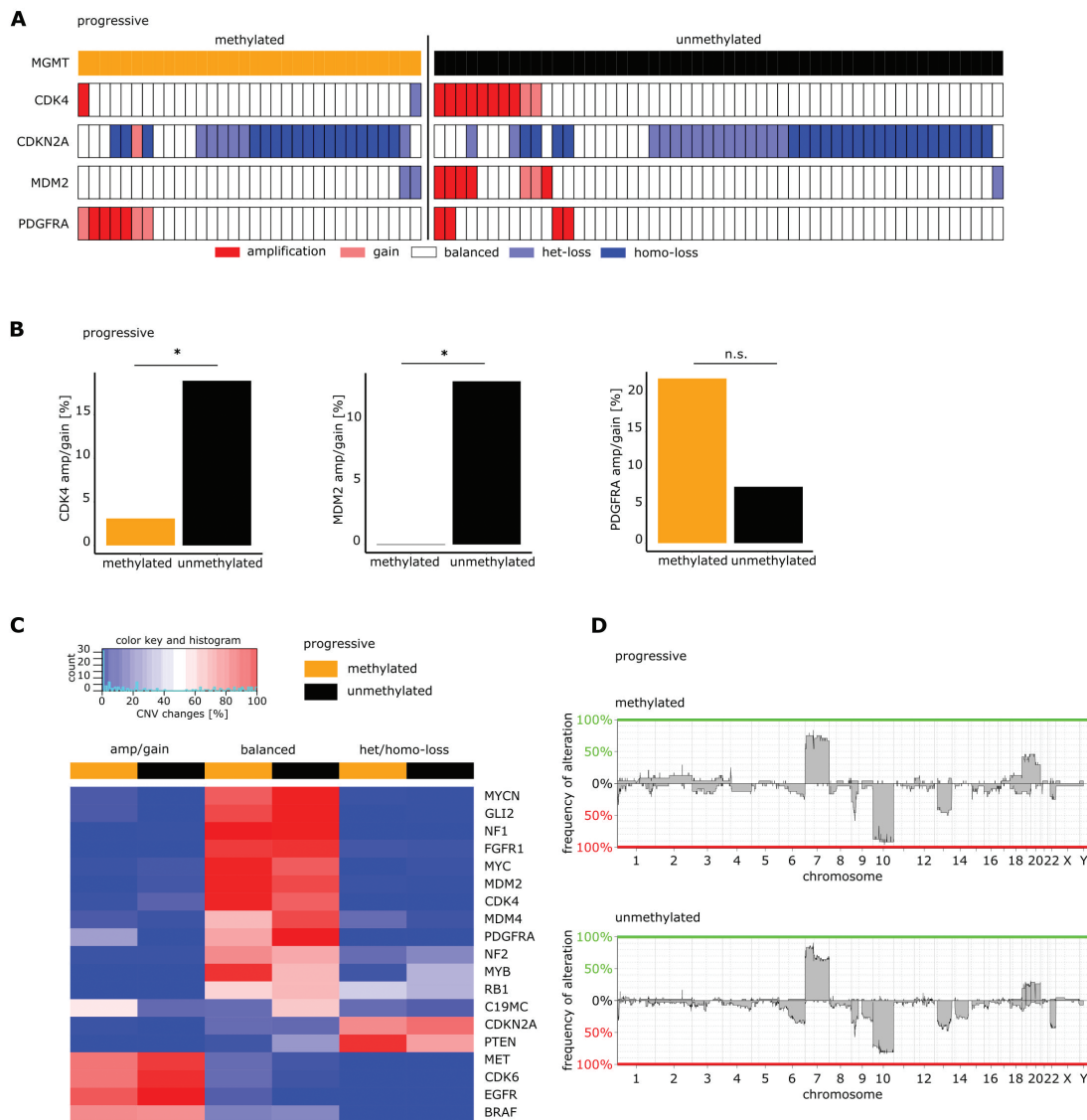


Fig. 5 Molecular differences between *uMGMT* and *mMGMT* progressive glioblastomas. (A) CNV of *CDK4*, *MDM2*, *PDGFRA*, and *CDKN2A* in the cohort of the progressive tumors, grouped according to *MGMT* promoter methylation. (B) Comparison of *CDK4*, *MDM2*, and *PDGFRA* copy number variations between *mMGMT* and *uMGMT* tumors in the progressive situation. (C) Heatmap of CNVs of a set of 19 frequently altered genes in the progressive tumor collective. High frequencies of alterations are shown in red, low frequencies in blue. *MGMT* promoter methylation is indicated in the first row. (D) Copy number variation profiles in *mMGMT* and *uMGMT* tumors in the progressive situation.

Comparison of Patients with Short and Long Survival Revealed Differentially Regulated Pathways

We created groups within the dataset from TCGA containing samples of patients who survived for either less than 6 months (*mMGMT*: $n = 31$, *uMGMT*: $n = 34$) or more than 12 months (*mMGMT*: $n = 43$, *uMGMT*: $n = 44$) after primary diagnosis. Within both the *mMGMT* and *uMGMT* groups, patients with a short survival time less frequently received alkylating chemotherapy (57% vs 88% in the methylated group and 47% vs 86% in the unmethylated group)

and were older at diagnosis (67.9 y vs 56.8 y in the methylated and 65.7 y vs 60.0 y in the unmethylated group; Supplementary Table S5). Amplification of the epidermal growth factor receptor gene (*EGFR*) was less frequent in tumors with a methylated *MGMT* promoter in patients with death within 6 months after initial diagnosis compared with those who lived longer than 12 months or had an unmethylated *MGMT* promoter or both (29% vs 50%, $P = 0.0048$; Fig. 6A). This corresponded to a reduced mRNA expression of *EGFR* in *uMGMT* patients with short survival in 2 independent datasets of the cohort from TCGA derived from RNAseq and microarray data.

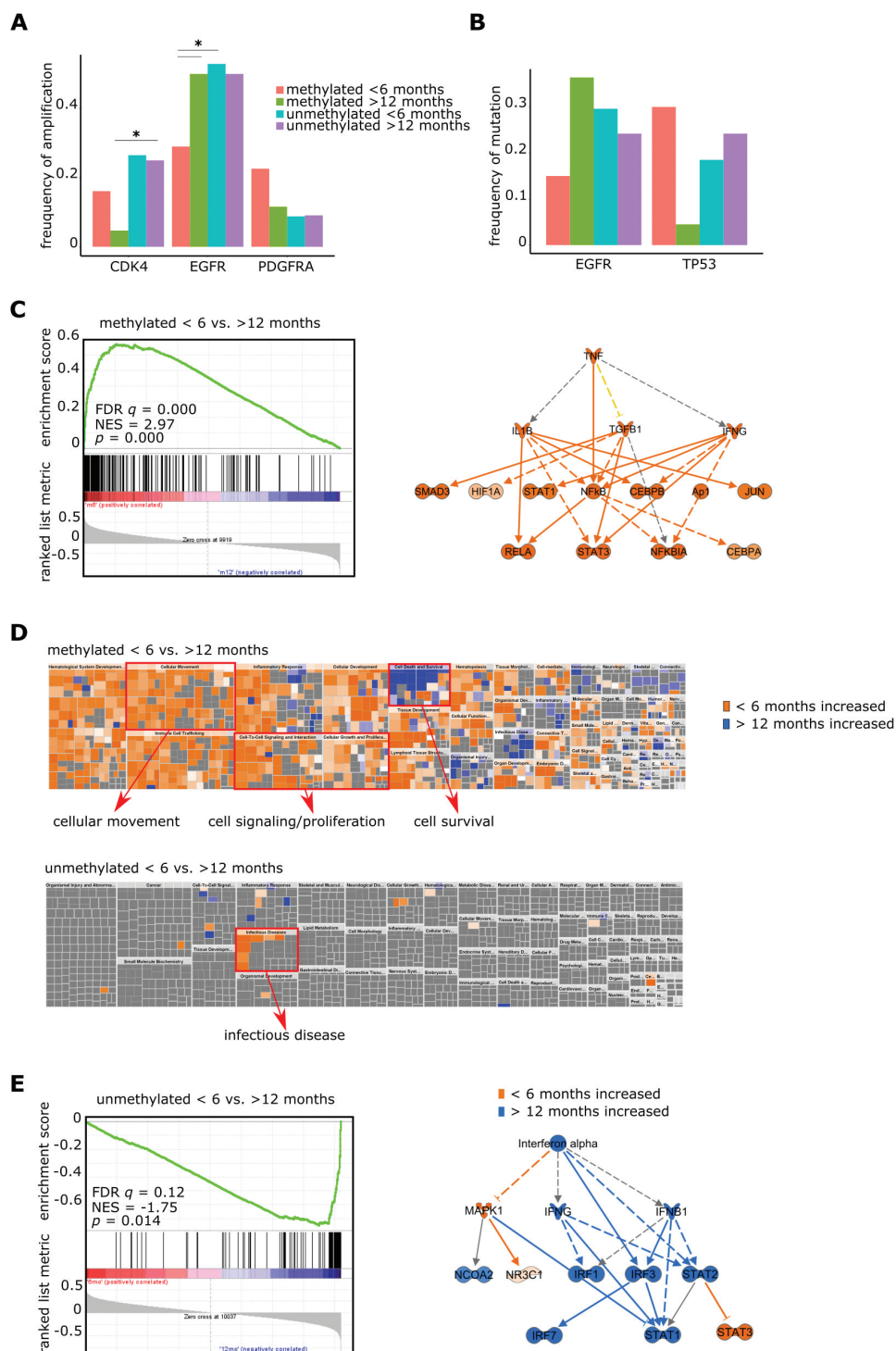


Fig. 6 Comparison between glioblastomas of patients with different survival times (<6 mo vs >12 mo) according to *MGMT* promoter methylation status. (A) Copy number variations of *CDK4*, *EGFR*, and *PDGFRA* according to *MGMT* promoter methylation status and survival groups (<6 mo vs >12 mo) of glioblastoma patients in the cohort of TCGA. (B) Mutations of *EGFR* and *TP53* according to *MGMT* promoter methylation status and survival groups of TCGA glioblastoma patients. (C) GSEA (left) and IPA (right) comparing results obtained for *mMGMT* tumors of patients with survival times <6 months vs >12 months. Prediction for upregulation is indicated in orange. (D) IPA of patients with survival times <6 months vs >12 months. Enriched differentially regulated functions are visualized and the topics cellular movement, cell survival, signaling, and proliferation as well as infectious disease are highlighted in red. (E) GSEA (left) and IPA (right) comparing results obtained for *uMGMT* tumors of patients with survival times <6 months vs >12 months. Prediction for upregulation is indicated in orange, for downregulation in blue.

TP53 mutations were common in *MGMT* promoter unmethylated tumors and in methylated tumors with a short survival but were significantly less common in *mMGMT* tumors and a survival time of more than 1 year compared with the group of patients with short survival of less than 6 months (30% vs 4.5%, $P = 0.041$; Fig. 6B). Like the lower amplification rate of *EGFR*, there was a trend toward a lower *EGFR* mutation frequency in the *mMGMT* patients with short survival.

RNAseq data of tumors of the groups was available for 50 patients. IPA of differentially regulated genes on RNA level (Fig. 6C, Supplementary Table S6) revealed a strong prediction of upregulation of TNF- α and the NF κ B complex in tumors from patients with short survival with *mMGMT* compared with patients with longer survival. To confirm this finding, we conducted a GSEA with a set of genes for signaling of TNF- α through NF κ B provided by the Broad Institute. This gene set was highly enriched in patients with *mMGMT* tumors and short survival (Fig. 6C, Supplementary Table S7). Conclusively, cellular movement and proliferation were enhanced in short surviving *mMGMT* tumors in IPA (Fig. 6D). Further evidence comes from the CGGA cohort, where in *mMGMT* tumors signaling of TNF- α through NF κ B was enriched in a patient cohort with worse prognosis.³¹ Exceptionally high TNF-NF κ B scores were seen in 3/13 (23%) tumors of patients surviving <6 months. Nine of 13 (62%) tumors of the <6 months group and 3/14 (21%) tumors of the >12 months group had scores >0 for NF κ B target genes (Supplementary Figure S9A, B). Additionally, the same pathway was enriched in the dataset of tumors from the collective of TCGA with available microarray data (Supplementary Figure S8A).

Signaling through interferon (INF)- α was the only pathway strongly activated in the group of tumors from patients with longer survival (Fig. 6E, Supplementary Table S6) translating into a decreased function “infectious disease” (Fig. 6D), which has been confirmed by GSEA and CGGA, as response to INF- α was the top enriched in patients with high *MGMT* gene expression and survival times of more than 12 months (Supplementary Figure S8B). Signal transducer and activator of transcription 3 (*STAT3*) was predicted to be upregulated in both *mMGMT* and *uMGMT* tumors of patients with short survival compared with patients with longer survival times (Fig. 6C, E).

Discussion

Most of the *IDH* mutant glioblastomas are *MGMT* promoter methylated.³² In this study, genetic, epigenetic, and transcriptional differences between *MGMT* methylated and unmethylated *IDH* wildtype glioblastomas have been explored.

CNV and mutations do not differ between newly diagnosed *mMGMT* and *uMGMT* tumors. Survival data of patients from the cohort from TCGA who received sole radiotherapy showed no difference in overall survival of *mMGMT* and *uMGMT* patients, making it likely that minor changes in the molecular profile do not affect the clinical course of the disease in the absence of temozolomide.

Methylation analysis with consensus clustering of more than 1000 glioblastoma samples revealed 3 different methylation clusters sharing similarities with published work.^{20,21} The allocation into 3 clusters is relatively stable between different datasets. We found that *mMGMT* tumors were enriched in cluster 1 and *uMGMT* tumors in cluster 3. Cluster 1 shows higher average methylation values, explaining why almost all differentially methylated CpGs between *mMGMT* and *uMGMT* tumors are hypermethylated in *mMGMT* tumors. There are no distinct methylation patterns of *mMGMT* and *uMGMT*, but *MGMT* promoter methylation is unevenly distributed within 3 main methylation clusters, with a higher chance of having a methylated *MGMT* promoter in the cluster with the highest average methylation. The survival advantage of *MGMT* promoter methylation was only present in the more favorable cluster 1, supporting that the prognostic impact of *MGMT* is dependent on the global methylation profile. Tumors of cluster 1 express higher levels of TERT, suggesting that TERT promoter mutations are more frequently in that cluster where survival was strongly associated with *MGMT* promoter methylation. Patients with tumors of clusters 2 and 3 have low TERT expression and do not show a survival benefit with *mMGMT*, confirming *mMGMT* is only a survival advantage in high TERT expressing tumors.²⁸ Furthermore, TERT expression/promoter mutations might be associated with specific epigenetic subgroups with high methylation levels and the enriched high TNF-NF κ B scores in cluster 3 may partly explain the chemoresistance in this cluster. However, these findings should be validated in further studies.

A hypermutated genotype in some *MGMT* promoter methylated tumors upon progression had been published.⁸ We specifically analyzed CNV and found *PDGFRA* predominantly amplified in *MGMT* promoter methylated progressive tumors. In paired samples, we found that *uMGMT* tumors lose *PDGFRA* amplifications upon progression, whereas *mMGMT* tumors tend to gain amplifications of *PDGFRA*. This might also be a resistance mechanism to therapy, especially in *mMGMT* tumors that are more vulnerable to chemotherapy with temozolomide.

The frequency of *CDK4* and *MDM2* amplifications tended to be higher in progressive *MGMT* promoter unmethylated tumors, and in such cases preexisting *CDKN2A* losses may change upon progression to a balanced state, which is consistent with the finding that *CDKN2A* loss and *CDK4* amplification rarely occur in parallel regardless of *MGMT* promoter methylation status.³³ These specific changes cannot be explained by the function of the *MGMT* protein and are likely to be a response of the tumor to different efficacy of the alkylating chemotherapy. However, the CNV differences of *PDGFRA*, *CDK4*, and *MDM2* are based on a limited tumor dataset and should therefore be viewed as preliminary. Robust statistical validation from a larger number of samples will be required.

In our comparison of patient groups with shorter versus longer survival we found differences in specific CNV as well as mutation frequencies and different gene expressions according to *MGMT* promoter methylation. However, a potential bias could be due to the lower number of patients receiving alkylating chemotherapy in both the methylated and unmethylated groups of patients who survived for less

than 6 months compared with the respective groups of patients living longer than 12 months.

The predicted activation of the TNF-NF κ B pathway in the group of short surviving *mMGMT* patients is supported by previous preclinical data showing upregulation of *MGMT* through high NF κ B signaling and therefore increased chemoresistance,³⁴ although this may not be uniformly dependent on the level of promoter inactivity, possibly contributing to the short survival times in patients with otherwise favorable *MGMT* promoter methylated tumors. Of note, it is more likely that glioblastomas of patients with longer survival show a downregulation of TNF-NF κ B signaling, as we also found higher NF κ B signaling in unmethylated tumors not selected for survival compared with all methylated tumors and no difference when comparing tumors from patients with short survival and *MGMT* promoter methylated tumors against all patients with unmethylated glioblastomas.

Likewise, INF signaling through INF- α/β can sensitize *MGMT* promoter unmethylated glioblastomas to temozolomide.^{35,36} There is an upregulation of the INF- α pathway in *uMGMT* tumors and long survival compared with both patients with unmethylated tumors and short survival as well as patients with methylated tumors and long survival, suggesting that patients who survive relatively longer with the unfavorable *uMGMT* might have this survival benefit from increased INF- α signaling and therefore increased chemotherapy sensitivity. This is of particular importance because most of the patients in the group with longer survival despite having tumors with *uMGMT* received temozolomide. The data are supported by a dataset of the CGGA, although caution is required because of the low sample size. The predicted upregulation observed in our study supports the recently proposed therapeutic inhibition of STAT3 in glioblastoma patients,³⁷ at least for patients with glioblastomas lacking *MGMT* promoter methylation.

In conclusion, *MGMT* promoter methylation status serves as a biomarker for temozolomide therapy and does not determine an otherwise molecularly distinct glioblastoma subpopulation in the newly diagnosed setting. The chance to find a molecular target for trial inclusion is not per se worse in the *MGMT* unmethylated population. Further, there is a clear signal from these data to demand a new tissue analysis for every trial at recurrence. Lastly, the data provide evidence for the TNF-NF κ B pathway to be revisited in future trials. Temozolomide might be safely omitted in patients with *MGMT* promoter unmethylated glioblastomas in clinical trials, especially when meaningful molecular markers predicting the response to experimental therapies are found by predictive molecular analyses.

Supplementary Material

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

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