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Integrated DNA methylation and copy-number profiling identify three clinically and biologically relevant groups of anaplastic glioma — Source link

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Abstract: The outcome of patients with anaplastic gliomas varies considerably. Whether a molecular classification of anaplastic gliomas based on large-scale genomic or epigenomic analyses is superior to histopathology for reflecting distinct biological groups, predicting outcomes and guiding therapy decisions has yet to be determined. Epigenome-wide DNA methylation analysis, using a platform which also allows the detection of copy-number aberrations, was performed in a cohort of 228 patients with anaplastic gliomas (astrocytomas, oligoastrocytomas, and oligodendrogliomas), including 115 patients of the NOA-04 trial. We further compared these tumors with a group of 55 glioblastomas. Unsupervised clustering of DNA methylation patterns revealed two main groups correlated with IDH status: CpG island methylator phenotype (CIMP) positive (77.5 %) or negative (22.5 %). CIMP(pos) (IDH mutant) tumors showed a further separation based on copy-number status of chromosome arms 1p and 19q. CIMP(neg) (IDH wild type) tumors showed hallmark copy-number alterations of glioblastomas, and clustered together with CIMP(neg) glioblastomas without forming separate groups based on WHO grade. Notably, there was no molecular evidence for a distinct biological entity representing anaplastic oligoastrocytoma. Tumor classification based on CIMP and 1p/19q status was significantly associated with survival, allowing a better prediction of outcome than the current histopathological classification: patients with CIMP(pos) tumors with 1p/19q codeletion (CIMP-codel) had the best prognosis, followed by patients with CIMP(pos) tumors but intact 1p/19q status (CIMP-non-codel). Patients with CIMP(neg) anaplastic gliomas (GBM-like) had the worst prognosis. Collectively, our data suggest that anaplastic gliomas can be grouped by IDH and 1p/19q status into three molecular groups that show clear links to underlying biology and a significant association with clinical outcome in a prospective trial cohort.

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Integrated DNA methylation and copy-number profiling identifies three clinically and biologically relevant groups of anaplastic glioma

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Key words: Anaplastic glioma, IDH, G-CIMP, 1p/19q, 450k

ABSTRACT

The outcome of patients with anaplastic gliomas varies considerably. Whether a molecular classification of anaplastic gliomas based on large scale genomic or epigenomic analyses is superior to histopathology for reflecting distinct biological groups, predicting outcomes and guiding therapy decisions has yet to be determined.

Epigenome-wide DNA methylation analysis, using a platform which also allows the detection of copy-number aberrations, was performed in a cohort of 228 patients with anaplastic gliomas (astrocytomas, oligoastrocytomas and oligodendrogliomas), including 115 patients of the NOA-04 trial. We further compared these tumors with a group of 55 glioblastomas.

Unsupervised clustering of DNA methylation patterns revealed two main groups correlated with *IDH* status: CpG island methylator phenotype (CIMP) positive (77.5%) or negative (22.5%). $CIMP^{pos}$ (*IDH* mutant) tumors showed a further separation based on copy-number status of chromosome arms 1p and 19q. $CIMP^{neg}$ (*IDH* wild type) tumors showed hallmark copy-number alterations of glioblastomas, and clustered together with $CIMP^{neg}$ glioblastomas without forming separate groups based on WHO grade. Notably, there was no molecular evidence for a distinct biological entity representing anaplastic oligoastrocytoma. Tumor classification based on CIMP and 1p/19q status was significantly associated with survival, allowing a better prediction of outcome than the current histopathological classification: Patients with $CIMP^{pos}$ tumors with 1p/19q codeletion (CIMP-codel) had the best prognosis, followed by patients with $CIMP^{pos}$ tumors but intact 1p/19q status (CIMP-non-codel). Patients with $CIMP^{neg}$ anaplastic gliomas (GBM-like) had the worst prognosis.

Collectively, our data suggest that anaplastic gliomas can be grouped by *IDH* and 1p/19q status into three molecular groups that show clear links to underlying biology and a significant association with clinical outcome in a prospective trial cohort.

INTRODUCTION

Anaplastic gliomas of World Health Organization (WHO) grade III comprise a common primary CNS tumor in adults. They are histologically subdivided into anaplastic astrocytomas, oligodendrogliomas and mixed oligoastrocytomas according to morphological criteria [16]. While this histological classification has prognostic value [28], it is prone to high interobserver variation, especially for anaplastic oligoastrocytomas, but also for the differentiation between anaplastic glioma and glioblastoma (WHO grade IV) [13]. In recent years, large-scale genomic and epigenomic studies have greatly increased our insight into the biology of malignant gliomas, identifying key alterations and molecular groups [4, 8, 23, 26] which may complement the WHO classification. The discovery of a prognostically favorable point mutation in *isocitrate dehydrogenase 1* (*IDH1*) codon 132 [19], resulting in a neomorphic enzymatic capacity to produce 2-hydroxyglutrate from α-ketoglutarate, has considerably changed the understanding of glioma biology [27]. *IDH1* (and less frequently *IDH2*) mutations are rare (~5%) in primary glioblastomas, but are found in the majority of secondary glioblastomas as well as diffuse (WHO grade II) and anaplastic gliomas, especially in oligodendrogliomas [34]. Patients with *IDH* mutant glioblastoma have a better prognosis than *IDH* wild type anaplastic astrocytoma patients [9]. The *IDH* mutation causes epigenetic remodeling [25], resulting in a CpG island methylator phenotype (CIMP) [18]. Indeed, gliomas across histological groups with an *IDH* mutation carry similar epigenetic profiles [6]. This and other studies [14] suggest that *IDH* mutant gliomas form a biologically distinct molecular group from their wild type counterparts. This notion is supported by the finding that methylation of the promoter of *O^6-methylguanine DNA-methyltransferase* (*MGMT*) is merely prognostic in *IDH* mutant anaplastic gliomas, whereas it is predictive for response to alkylating chemotherapy in *IDH* wild type gliomas [29, 31].

Besides *IDH*, it has been known for some time that patients with tumors harboring 1p/19q codeletion have a better prognosis [22]. When two phase III trials reported their long-term

follow-up, both demonstrating an overall survival benefit from combined treatment with radiotherapy and procarbazine, CCNU and vincristine (PCV) chemotherapy mainly in patients with 1p/19q codeleted tumors, the 1p/19q codeletion gained predictive properties [2, 5]. Mutations of the homolog of the Drosophila gene capicua (*CIC*) on chromosome 19q and *far-upstream element binding protein 1* (*FUBP1*) on 1p have been identified as potential mechanisms involved in the biology of 1p/19q codeleted gliomas [3]. Gene expression clustering plus *IDH1* and 1p/19q status revealed molecular groups with prognostic value among oligodendroglial tumors [7].

Mutually exclusive mutations affecting *telomerase reverse transcriptase* (*TERT*) and *alpha-thalassemia/mental retardation syndrome X-linked* (*ATRX*) have been detected in malignant gliomas [11, 21]. Two point mutations in the promoter of *TERT* (C228 and C250) resulting in higher *TERT* mRNA expression were discovered with high frequency in oligodendrogliomas (usually co-occurring with 1p/19q codeletion) and primary glioblastomas. Alternative lengthening of telomeres (ALT) is another, telomerase-independent mechanism of telomere maintenance. *ATRX* mutations, usually leading to reduced or absent ATRX protein [15], have been linked to ALT [10]. Consequently, loss of ATRX expression predominantly occurs in astrocytomas and mixed oligoastrocytomas without 1p/19q codeletion (i.e. *TERT* wild type tumors) and seems to identify a prognostically more favorable molecular group among anaplastic astrocytoma patients [32].

Genome-wide sequencing data in 284 glioblastoma samples from the TCGA consortium showed that roughly 50% harbored at least one, usually mutually exclusive somatic mutation in genes functionally linked to chromatin organization, such as *IDH1/2*, *SETD2*, *EZH2*, the *HDAC* family or *ATRX* [4]. Analysis of genome-wide DNA methylation patterns revealed distinct molecular groups of glioblastoma, associated with defined hotspot mutations (*IDH* and *H3F3A*, respectively) or common genomic aberrations like *EGFR* amplifications [24].

In contrast to glioblastomas, anaplastic gliomas have been analyzed less comprehensively [7, 17]. Here, the feasibility and prognostic value of a molecular classification of anaplastic gliomas based on epigenetic and copy-number analysis is investigated.

MATERIALS & METHODS

Patients and tumor samples

Primary tumor samples of anaplastic gliomas for DNA methylation analysis (n = 228) were collected at the Heidelberg University Hospital (Heidelberg, Germany, n = 113) and from the NOA-04 trial (n = 115) [28].

DNA methylation profiling

For genome-wide assessment of DNA methylation patterns, tumor samples were subjected to microarray analyses at the Genomics and Proteomics Core Facility of the German Cancer Research Center (DKFZ) using the Infinium HumanMethylation450 BeadChip (Illumina, San Diego, CA).

The following filtering criteria were applied: Removal of probes targeting the X and Y chromosomes (n = 11,551), removal of probes containing a single-nucleotide polymorphism (dbSNP132 Common) within five base pairs of and including the targeted CpG site (n = 24,536), and probes not mapping uniquely to the human reference genome (hg19) allowing for one mismatch (n = 9,993). In total, 438,370 probes were kept for analysis.

Statistical analysis of DNA methylation

For unsupervised hierarchical clustering we selected the 10,000 most variable methylated probes that showed highest median absolute deviation (MAD) across beta values. Pairwise similarity between cases was calculated using euclidean distance and hierarchical clustering was performed using Ward's linkage method. To cluster CpG probes we applied hierarchical clustering using 1-centered Pearson correlation as similarity measure and average linkage as hierarchical clustering method.

To predict *MGMT* methylation using methylation (M-values) of CpG probes "cg12434587" and "cg12981137" , we applied the logistic regression model MGMT-STP27 [1].

Detection of *TERT* promoter and ATRX aberrations

Data for *TERT* promoter hotspot mutations and for loss of ATRX expression (using immunohistochemistry (IHC)) in this patient cohort have been reported before [12, 32].

Data repository

Data are accessible at GEO (http://www.ncbi.nlm.nih.gov/geo/), accession number GSE58218. Additional methylation data of 55 adult glioblastoma samples used for comparison with anaplastic gliomas and six non-neoplastic brain tissue samples used as reference to generate copy-number variation plots are publicly available at GEO under the accession number GSE36278.

Statistical analysis of clinical and molecular data

Kaplan-Meier estimator, log rank test and multivariate Cox regression analyses were used to assess survival differences. Comparisons of binary and categorical patient characteristics between molecular groups were performed by the use of a two-sided Fisher's exact test. Continuous variables were compared using ANOVA and post-hoc pairwise comparisons with Holm's multiple testing procedure. Analyses were carried out using Stata IC version 12.1 (StataCorp LP, College Station, TX, USA) and R version 3.0.2 [20].

RESULTS

Unsupervised analysis identifies two main clusters of anaplastic gliomas

Unsupervised clustering methods using genome-wide DNA methylation patterns generated from 228 anaplastic gliomas revealed two main clusters, which were closely associated with *IDH* mutation status (**Figure 1**): In the first cluster, consisting of 177 (77.5%) samples, 156/160 samples with available mutation status harbored an *IDH* mutation (94%, *IDH* status was unavailable for 17 samples). Only 1 out of 35 assessable samples (3%) in the second cluster (in total 51 tumors, 22.5%) carried an *IDH* mutation (*IDH* status was unavailable for 16 patients). In analogy to similar DNA methylation patterns observed in glioblastoma, we thus refer to the first (hypermethylated) cluster as $CIMP^{pos}$ and to the second cluster as $CIMP^{neg}$ (**Supplementary Figure 1**). Patients with $CIMP^{pos}$ tumors had significantly longer overall survival than patients with a $CIMP^{neg}$ tumor (log rank $p < 0.001$; **Supplementary Figure 2**). Anaplastic astrocytomas were more frequently $CIMP^{neg}$ (34/93, 36.5%) than oligodendrogliomas (10/71, 14%) or oligoastrocytomas (7/64, 11%; Fisher's exact test $p < 0.001$, **Table 1**).

The effect of *IDH* mutation on the methylome is so pronounced that identification of further molecular groups beyond $CIMP^{pos}$ and $CIMP^{neg}$ is challenging when assessing all samples together. We therefore performed an additional analyses using copy-number and methylation data in order to investigate potential substructures within these two clusters, and also included previously published glioblastomas with known CIMP status [24].

$CIMP^{pos}$ tumors are subtyped by 1p/19q status

Unsupervised hierarchical clustering of the 177 $CIMP^{pos}$ anaplastic gliomas and 14 $CIMP^{pos}$ / *IDH* mutant glioblastomas showed a clear separation of samples into two main clusters (**Figure 2**). Copy-number analysis revealed that this separation is based on 1p/19q status, as 79/82 samples (96%) in one cluster had 1p/19q codeletion as opposed to 8/109 (7%) in the

other cluster (Fisher's exact test, $p < 0.001$). As expected from the distribution of 1p/19q codeletion in anaplastic gliomas, the group with 1p/19q codeletion was significantly enriched for oligodendrogliomas and oligoastrocytomas, as was the group with intact 1p/19q status for astrocytomas (**Table 1**). We therefore termed these molecular groups CIMP-codel and CIMP-non-codel, respectively. There was no further subtyping based on histology, e.g. a split between astrocytomas and oligoastrocytomas in the CIMP-non-codel cluster. None of the 14 $CIMP^{pos}$ glioblastomas displayed a 1p/19q codeletion and all clustered with the CIMP-non-codel tumors without forming a separate molecular group. Furthermore, characteristic glioblastoma copy-number alterations (*EGFR* amplification, gain of chromosome 7 and loss of chromosome 10) were evenly rare in both $CIMP^{pos}$ molecular groups. Homozygous *CDKN2A* deletion occurred in 13/109 CIMP-non-codel tumors (two glioblastomas, 11 anaplastic gliomas) and in no CIMP-codel tumor. However, the low numbers precluded further statistical analysis of survival differences in the above mentioned smaller groups.

The biological differences between CIMP-non-codel and CIMP-codel tumors are further evidenced by the distribution of *TERT* promoter mutations and ATRX expression in the two molecular groups: *TERT* promoter mutations (C228 and C250) were detected in 57/65 CIMP-codel tumors (88%), but only in 7/80 in the CIMP-non-codel group (9%, notably, 6 of these had 1p/19q codeletion). Conversely, loss of ATRX expression determined by immunohistochemistry was common in the CIMP-non-codel group, where 32/40 tumors (80%) had lost ATRX expression as opposed to the CIMP-codel group, where only 1/34 tumors with available ATRX status showed this feature. Of note, no tumor (of 86 samples being analyzed both for *TERT* and ATRX status) displayed both, an ATRX loss and a *TERT* promoter mutation, consistent with these two pathways of telomere lengthening being mutually exclusive.

Interestingly, of the eight tumors with 1p/19q codeletion that were found in the CIMP-non-codel group by cluster analysis, seven clustered tightly together and had *TERT* mutations.

These tumors stably clustered in the CIMP-non-codel group even when using different similarity measures and linkage methods for the hierarchical clustering, indicating that these tumors, even while carrying 1p/19q codeletion, have a different methylation profile than the majority of the 1p/19q codeleted tumors. By histology, 6/7 of these tumors were diagnosed as anaplastic oligodendroglioma.

Assessment of *MGMT* promoter methylation using the MGMT-STP27 algorithm [1] revealed a high frequency of *MGMT* promoter methylation in CIMP^{pos} tumors: Of 191 tumors, only nine tumors (5%) were predicted to have an unmethylated *MGMT* promoter. These nine samples (eight anaplastic gliomas, one glioblastoma) all belonged to the CIMP-non-codel group, while all 82 tumors in the CIMP-codel group were scored as *MGMT* methylated. Importantly though, as the MGMT-STP27 was trained on glioblastoma DNA samples from fresh frozen tissue its ability to correctly predict the MGMT status of DNA samples coming from FFPE material might be limited (see **Supplementary Discussion**).

CIMP^{neg} anaplastic gliomas share copy-number aberrations and methylation profiles with *IDH* wild type glioblastomas

As with the CIMP^{pos} tumors, we grouped the 51 CIMP^{neg} anaplastic gliomas with 41 glioblastomas with known copy-number and methylation profiles from the study of Sturm *et al.* [24]. Unsupervised clustering did not separate anaplastic gliomas and glioblastomas into distinct clusters, with tumors of both WHO grades largely mixing (**Figure 3**). Subsets of CIMP^{neg} anaplastic gliomas clustered together with glioblastomas of each of the mesenchymal, RTK I and RTK II groups, respectively. The seeming interposition of the RTK I samples in between RTK II samples is most likely caused by different normal cell amounts and/or hybridization quality and not a real biological difference between the two RTK II clusters. This biological similarity of CIMP^{neg} anaplastic gliomas and glioblastomas based on methylation data is also reflected in the copy-number profiles: hallmark glioblastoma

alterations were found at similar frequency in anaplastic gliomas and glioblastomas (Fisher's exact test, $p > 0.1$ for each comparison; **Figure 3**). Furthermore, copy-number aberrations showed typical patterns, for example with *EGFR* amplifications and *CDKN2A* deletions occurring mostly in RTK II glioblastomas as well as the anaplastic gliomas clustering with them. *MGMT* promoter methylation was also found at similar frequency in both WHO grades: 23/51 anaplastic gliomas (45%) and 18/41 glioblastomas (44%) were predicted to have a methylated *MGMT* promoter according to the *MGMT*-STP27 algorithm. In $CIMP^{neg}$ samples from NOA-04 (n = 24), *MGMT* status clearly was associated with differences in progression-free survival in the chemotherapy arm, while no such obvious effect was seen in the radiotherapy arm (**Supplementary Figure 3**). However, the small number of samples in each subgroup precluded further statistical analysis.

Integrated molecular classification is significantly associated with survival

Taken together, these data suggest that anaplastic gliomas can be robustly divided into three main molecular groups based on DNA methylation and copy-number data and independent of histology: $CIMP^{neg}$ tumors, which molecularly resemble glioblastomas, CIMP-non-codel tumors with intact 1p/19q and CIMP-codel tumors with 1p/19q codeletion (**Figure 5**).

Our series of anaplastic gliomas contained 115 samples from the biomarker cohort of the NOA-04 trial, which investigated the optimal sequence of radio- and chemotherapy in newly-diagnosed anaplastic gliomas [28]. Baseline patient characteristics of these 115 samples closely resembled those of the entire NOA-04 trial cohort (**Supplementary Table 1**), except for an enrichment of patients with tumor resection as opposed to biopsy due to the tissue requirements for DNA methylation analyses. As observed in the entire NOA-04 cohort, both treatment sequences performed very similar (log rank test, $p = 0.94$).

Of the 115 samples, 24 were $CIMP^{neg}$, 48 CIMP-non-codel and 43 CIMP-codel. Time to treatment failure (TTF), the primary endpoint of NOA-04, showed a clear separation of

patients based on molecular classification (**Figure 4a**): CIMP-codel tumors had the best prognosis (median TTF not reached [95% CI 1840 days – not reached]), followed by CIMP-non-codel tumors (median TTF 1691 days [95% CI 793 – 2315 days]), while $CIMP^{neg}$ tumors had the shortest median TTF (496 days [95% CI 326 – 1071 days]). This was also true for overall survival (**Figure 4b**). As opposed to reference histology (**Figure 4c,d**), this led to a clearer separation of prognostic subgroups. Consequently, in a multivariate Cox regression model including molecular classification, reference histology and age, only molecular classification and age, but not reference histology, were significantly associated with outcome (**Table 2**). To account for potential collinearity between molecular classification and reference histology, we analyzed the prediction error curves for Cox models based on reference histology or molecular classification only or including both. This indicated again a better prediction of survival through the molecular classification as opposed to reference histology and only a small improvement of prediction when including both reference histology and molecular classification (**Supplementary Figure 4**).

DISCUSSION

Using DNA methylation profiling and copy-number analysis, we identified three principal molecular groups of anaplastic gliomas in a cohort of 228 samples: CIMP^{neg} tumors, which resemble glioblastomas, CIMP-codel tumors with 1p/19q codeletion as a hallmark genomic alteration and CIMP-non-codel tumors, which lack this copy-number aberration (key characteristics are summarized in **Table 1**); the classification scheme is shown in **Figure 5**. Importantly, this classification not only reflects molecular differences between these groups, but also better translates into clinically relevant survival differences when compared with the WHO classification as evidenced in the biomarker cohort of the NOA-04 trial (**Figure 4, Table 2**).

The majority of oligoastrocytomas and oligodendrogliomas [34] belonged to the CIMP^{pos} cluster. Based on their epigenome-wide DNA methylation profile, tumors belonging to the CIMP^{pos} cluster can be further subdivided by 1p/19q status (also the first split to occur after CIMP positive *vs.* negative when looking at all samples; **Figure 1**) as suggested before for a smaller (n = 46) series of purely oligodendroglial tumors [17]. We termed these clusters CIMP-codel (mainly 1p/19q codeleted with oligodendroglial histology) and CIMP-non-codel (mainly 1p/19q intact and astrocytic). Notably, the 14 CIMP^{pos} glioblastomas included in this analysis all clustered well within the CIMP-non-codel molecular group. On the other hand, CIMP^{neg} (and hence *IDH* wild type) anaplastic gliomas were molecularly indistinguishable from CIMP^{neg} glioblastomas: Hallmark glioblastoma copy-number aberrations were found in comparable frequency in both tumor grades. Consequently, unsupervised clustering did not sort WHO grade III and IV CIMP^{neg} tumors into separate clusters. In contrast, these copy-number aberrations are rare in CIMP^{pos} tumors (of WHO grade III or IV). Thus, anaplastic gliomas and glioblastomas with a given CIMP status are more similar to each other than CIMP^{pos} *vs.* CIMP^{neg} tumors of a certain histology, strongly supporting the notion that *IDH* mutant and *IDH* wild type tumors are in fact distinct entities [14].

In our cohort, median overall survival was longer in CIMP^{neg} anaplastic gliomas than in glioblastomas (**Supplementary Figure 5**). However, the cohort for this comparison is very heterogeneous with respect to treatment and clinical follow-up, including prognostically favorable NOA-04 study patients. Longer overall survival for *IDH* wild type anaplastic astrocytomas compared to glioblastomas has been reported in another retrospective series [9]. However, in elderly patients with newly diagnosed WHO grade III or IV astrocytomas, which are almost always *IDH* wild type [33], there was no survival difference [30]. Since most anaplastic gliomas eventually progress to secondary glioblastomas at recurrence, a survival difference may just reflect that CIMP^{neg} anaplastic gliomas are diagnosed in an earlier stage of tumor development compared to CIMP^{neg} glioblastoma. Another explanation may be yet to be defined groups in the CIMP^{neg} anaplastic gliomas which (i) share a glioblastoma-like clinical course or show (ii) a markedly longer survival. For the time being, our findings suggest that WHO grading retains a prognostic role within the group of CIMP^{neg} high-grade gliomas.

Histological classification of anaplastic gliomas, in particular of mixed oligoastrocytomas, has considerable interobserver variability [13]. Molecularly, oligoastrocytomas do not form a separate biological entity, as they are evenly distributed between the CIMP-codel and CIMP-non-codel clusters (24 and 33 cases, respectively), while pure oligodendrogliomas and astrocytomas were predominantly sorted in the CIMP-codel or CIMP-non-codel cluster, respectively (**Table 1**). Since there is no further subtyping based on histology in either CIMP^{pos} cluster, e.g. a split between astrocytomas and oligoastrocytomas in the CIMP-non-codel cluster, there seems to be no clear biological basis for the diagnosis of an oligoastrocytoma. Mean age at diagnosis significantly differed between all three groups (**Table 1**, $p < 0.0001$, ANOVA).

With a stringent central histology, NOA-04 demonstrated a clear survival benefit for patients with oligodendrogliomas and oligoastrocytomas compared to astrocytomas, which was also retained in our cohort of 115 NOA-04 patients (**Supplementary Table 2**). This cohort is

therefore well suited to compare the clinical significance of this molecular classification of anaplastic gliomas. In a multivariate Cox regression model, histological assessment did not significantly correlate with survival anymore once molecular subtyping was added as a covariate, pointing to a diagnostic superiority of molecular subtyping.

In the long-term follow-up analysis of two large randomized phase III trials, 1p/19q codeletion was concordantly shown to predict benefit from radio-chemotherapy [2, 5]. However, in both trials several patients with 1p/19q codeletion did not benefit from this intensified treatment. In our classification, while 1p/19 status is clearly the defining feature for the two molecular groups, 8 out of 95 tumors in the CIMP-non-codel group carried 1p/19q codeletion (and *TERT* mutation). Whether these tumors represent a distinct group is currently unclear. An influence of other factors on the DNA methylation profile, such as infiltrating normal (reactive) astrocytes can also not be excluded. Based on this observation, however, it seems possible that these might be the rare 1p/19q codeleted tumors not benefitting from intensified treatment. This hypothesis could be tested in either RTOG-9402 or EORTC-26951. Furthermore, both trials were designed to include only oligodendroglial tumors, but no astrocytomas, although reference histology revealed a relevant proportion of grade II gliomas, anaplastic astrocytoma and even glioblastoma [13]. While 1p/19q codeletion occurs only infrequently in astrocytomas (three out of 93 anaplastic astrocytomas belonged to the CIMP-codel group), our observation that anaplastic astrocytoma with 1p/19q codeletion molecularly resemble codeleted oligodendroglial tumors supports the notion that this subset should also be treated with combined radio-chemotherapy at initial diagnosis.

In summary, we demonstrate a classification of anaplastic gliomas based on (epi)genome-wide DNA methylation and copy-number profiles, where tumors can be divided into $CIMP^{pos}$ tumors with or without 1p/19q loss, and $CIMP^{neg}$ tumors which closely resemble glioblastomas (and may in fact be true glioblastomas biologically, but diagnosed at an earlier stage of tumor development, for example). This information, together with *MGMT* status, can

be readily obtained from one platform (450k DNA methylation array), suggesting a possible simplification of routine diagnostic testing for this set of tumors. This biological classification is also significantly associated with outcome: patients with CIMP-codel tumors have the best prognosis, followed by those with CIMP-non-codel tumors and lastly $CIMP^{neg}$ tumors. Defining such groups will allow for a clearer understanding of their pathophysiological drivers, enabling a more rational search for therapeutic targets. The presented molecular classification has the potential to greatly complement the standard histological WHO classification by providing a biologically meaningful and clinically relevant stratification algorithm, and warrants further examination in future clinical trials.

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FIGURE LEGENDS

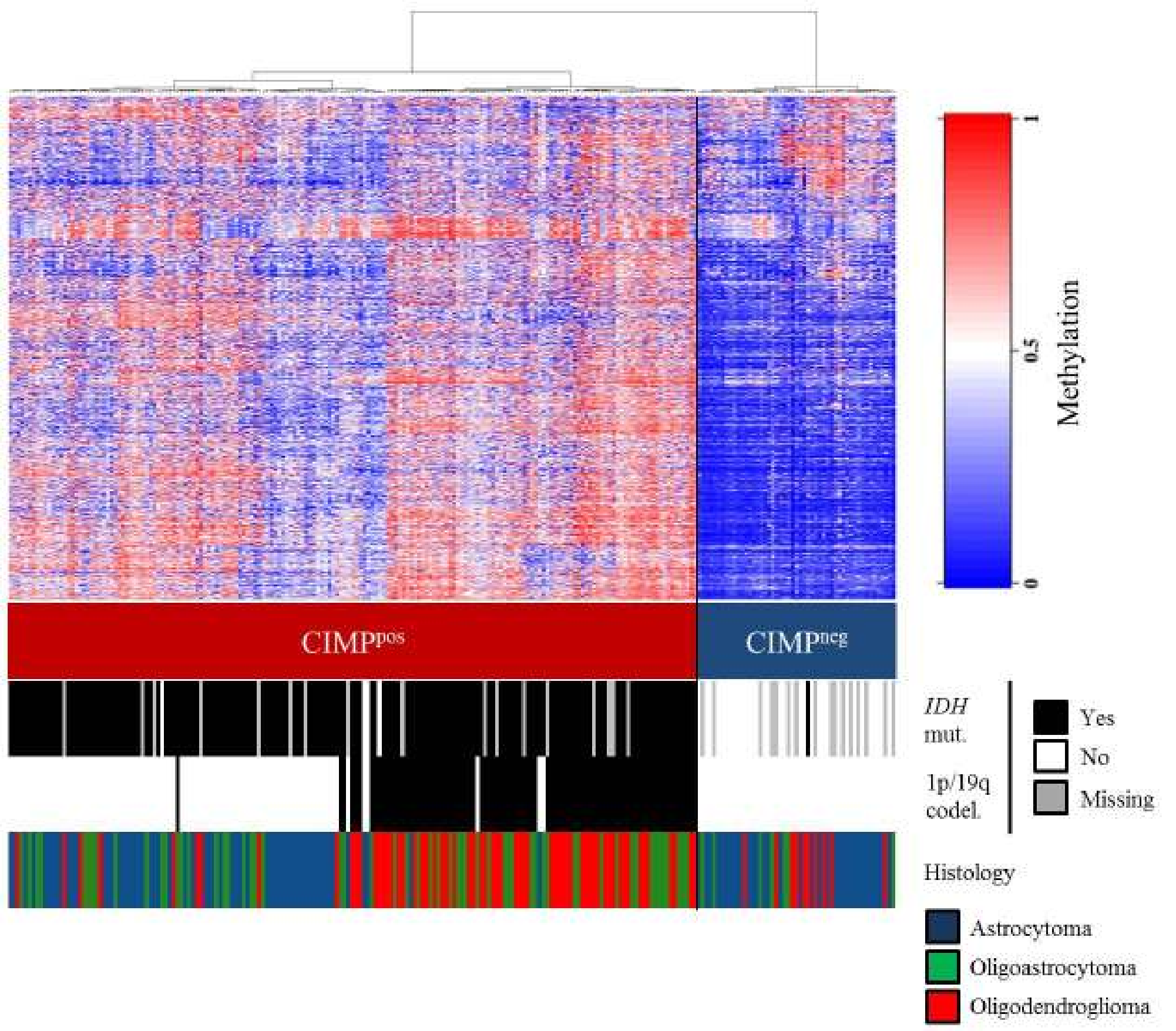
Figure 1. DNA **methylation profiling separates anaplastic gliomas into two main clusters based on *IDH* status.** Heatmap of DNA methylation levels in 228 anaplastic gliomas. Each row represents a probe and each column a sample. The level of DNA methylation (beta-value) is represented with a color scale as depicted. For each sample, *IDH* mutation and 1p/19q copy-number status as well as histology are indicated.

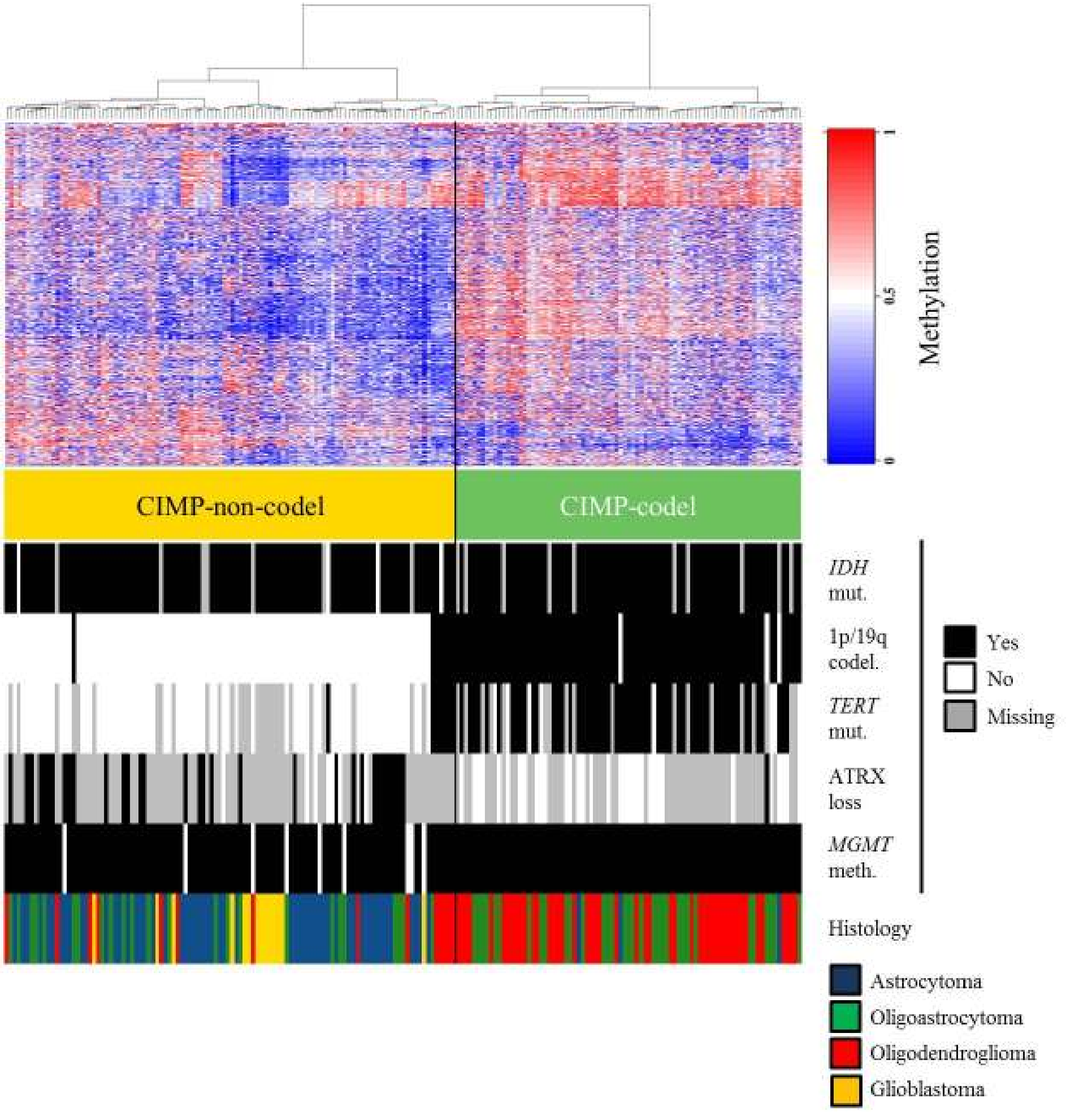
Figure 2. CIMP^{pos} anaplastic gliomas form two separate molecular subypes closely associated with 1p/19q status. DNA methylation heatmap showing the result of an unsupervised clustering of 177 CIMP^{pos} anaplastic gliomas and 14 CIMP^{pos} glioblastomas. Below, *IDH,* 1p/19q*, TERT,* ATRX and *MGMT* promoter methylation status and histology are given.

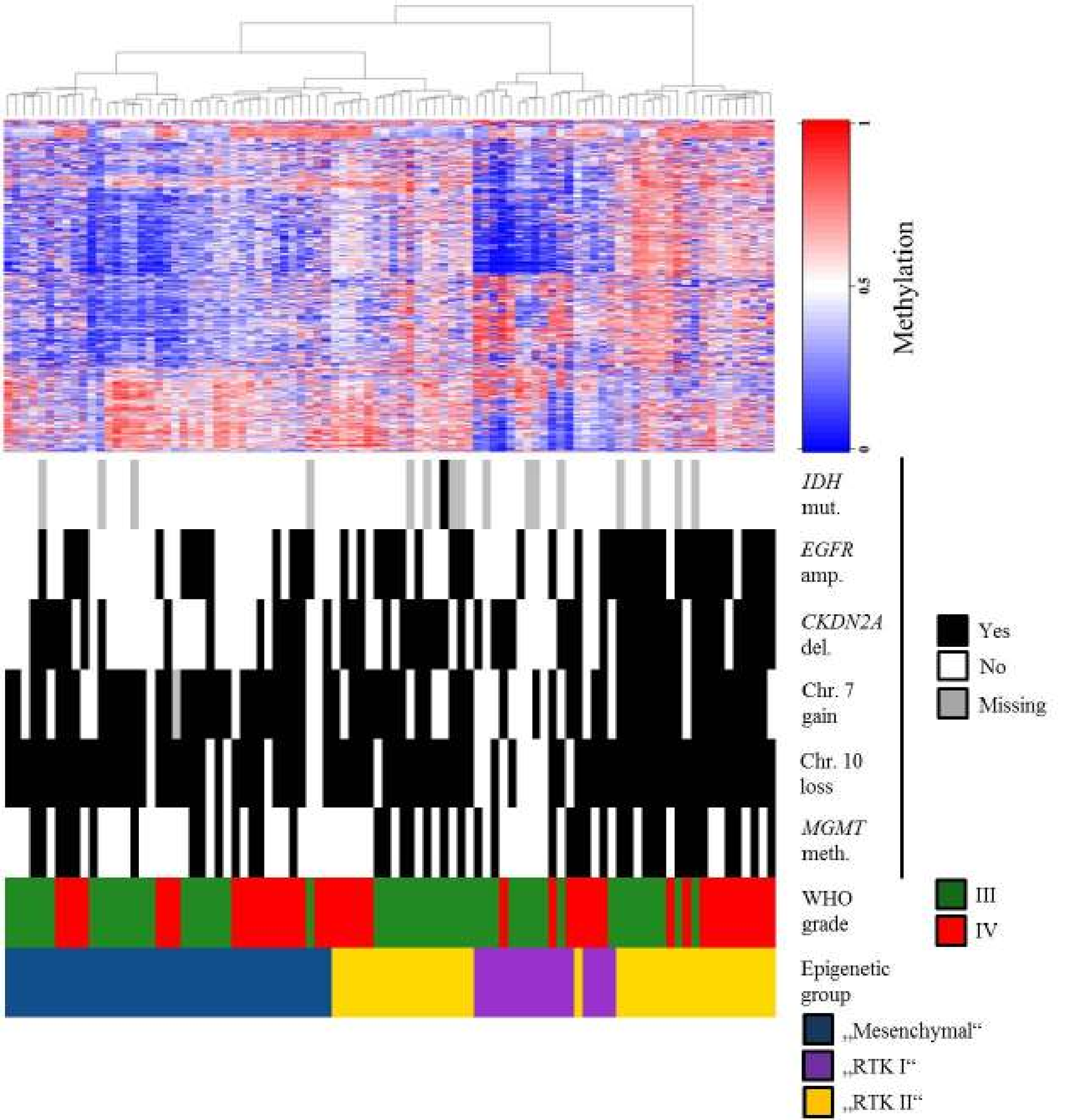
Figure 3. Unsupervised clustering analysis of DNA methylation and copy-number profiles of CIMP^{neg} anaplastic gliomas (n = 51) and CIMP^{neg} glioblastomas (n = 41) reveals a close biological similarity. For each sample, *IDH* mutation status, copy-number aberrations, *MGMT* promoter methylation status and WHO grade are given. Additionally, cluster membership within the six glioblastoma clusters defined by Sturm et al. are given.

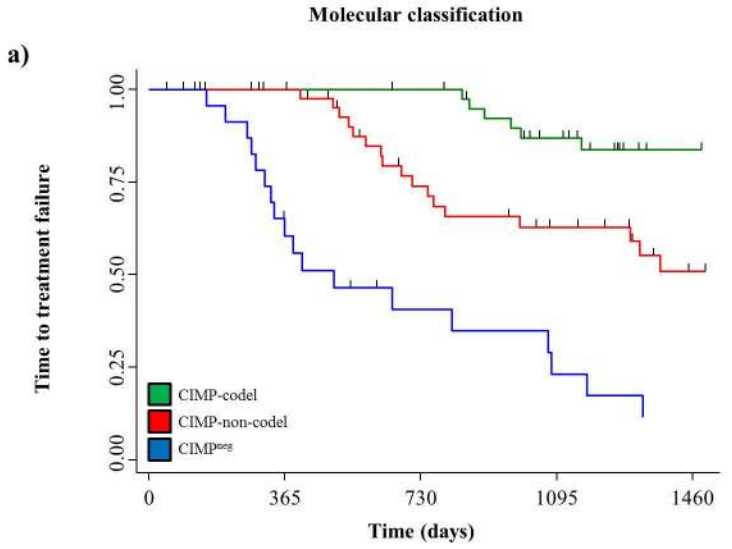
Figure 4. Molecular classification of anaplastic gliomas is a better prognosticator than the WHO classification. Kaplan-Meier plots for time to treatment failure (a, c) and overall survival (b, d) for the 115 NOA-04 trial samples according to molecular classification (a, b) or reference histology (c, d) are shown.

Figure 5. Summary of the proposed molecular classification. CNVs, copy-number variations.


1
0.5
0
Methylation
CIMPpos
CIMPneg
IDH mut.
1p/19q codel.
Yes
No
Missing
Histology
Astrocytoma
Oligoastrocytoma
Oligodendroglioma

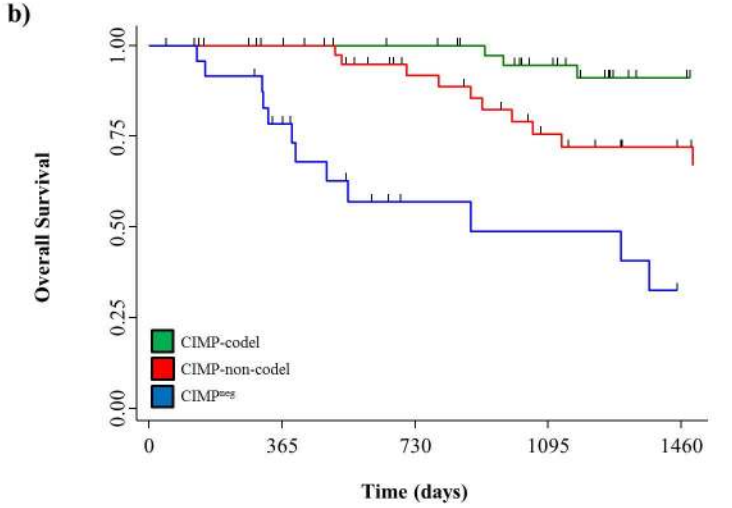

Methylation
1
0.5
0
CIMP-non-codel
CIMP-codel
IDH mut.
1p/19q codel.
TERT mut.
ATRX loss
MGMT meth.
Yes
No
Missing
Histology
Astrocytoma
Oligoastrocytoma
Oligodendroglioma
Glioblastoma


1
0.5
0
Methylation
IDH mut.
EGFR amp.
CKDN2A del.
Chr. 7 gain
Chr. 10 loss
MGMT meth.
Yes
No
Missing
WHO grade
III
IV
Epigenetic group
„Mesenchymal“
„RTK I“
„RTK II“



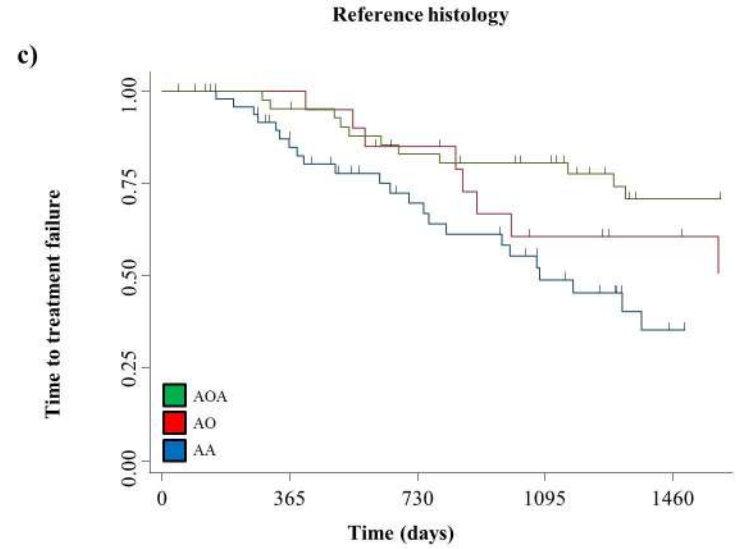
Number at risk

CIMP-O	43	41	33	18
CIMP-A	48	38	22	10
CIMP^{neg}	24	10	6	2



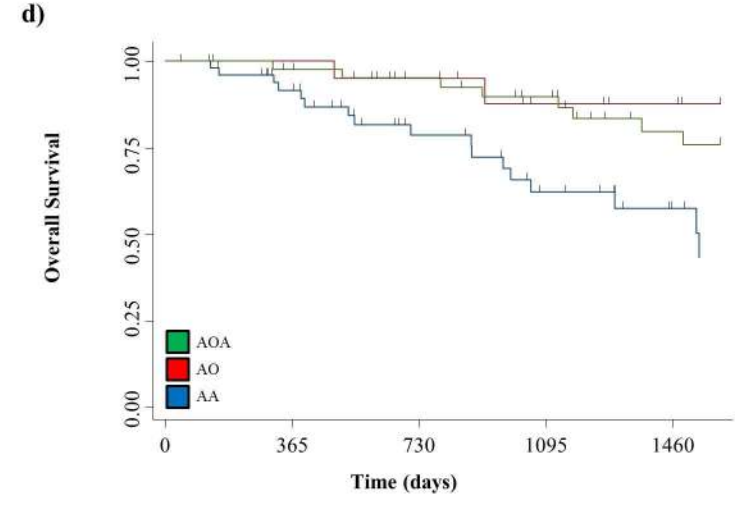
Number at risk

CIMP-O	43	41	35	18
CIMP-A	48	40	24	13
CIMP^{neg}	24	12	6	3



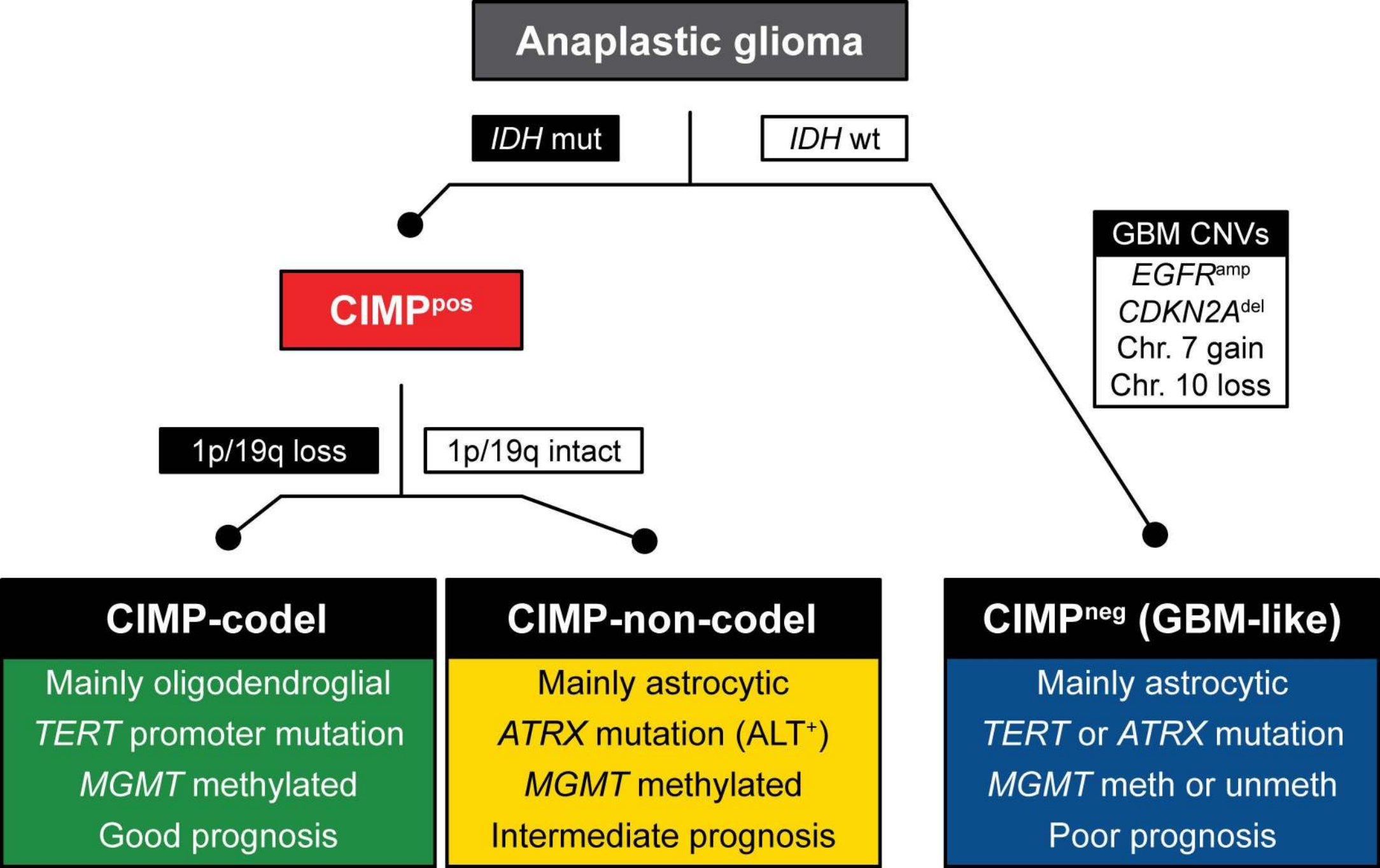
Number at risk

AA	50	32	19	5
AO	20	19	10	6
AOA	45	38	32	19



Number at risk

AA	50	35	20	8
AO	20	19	12	6
AOA	45	39	33	20


Anaplastic glioma
IDH mut
IDH wt
GBM CNVs
EGFRamp
CDKN2Adel
Chr. 7 gain
Chr. 10 loss
CIMPpos
1p/19q loss
1p/19q intact
CIMP-codel
Mainly oligodendroglial
TERT promoter mutation
MGMT methylated
Good prognosis
CIMP-non-codel
Mainly astrocytic
ATRX mutation (ALT+)
MGMT methylated
Intermediate prognosis
CIMPneg (GBM-like)
Mainly astrocytic
TERT or ATRX mutation
MGMT meth or unmeth
Poor prognosis

TABLES

Table 1: Key characteristics of molecular groups

	CIMP-non-codel (1p/19q intact)	**CIMP-codel (1p/19q codeleted)**	**CIMPneg (*IDH* wild type)**
Histology			
Astrocytoma	56 (51%)	3 (4%)	34 (37%)
Oligodendroglioma	15 (14%)	46 (56%)	10 (11%)
Oligoastrocytoma	24 (22%)	33 (40%)	7 (7%)
Glioblastoma	14 (13%)	0	41 (45%)
Mean age (years), (95%CI)	38.7 (36.6 – 40.7)	45.7 (43.4 – 48.1)	49.3 (46.9 – 51.8)
IDH			
Mutated	97 (96%)	73 (100%)	1 (1.5%)
Wild type	4 (4%)	0	75 (98.5%)
Not available	8	9	16
1p/19q			
Codeleted	8 (7%)	79 (96%)	0
Intact	101 (93%)	3 (4%)	92 (100%)
***TERT* promoter**			
Mutated	7 (9%)	57 (88%)	21 (49%)
Wild type	73 (91%)	8 (12%)	22 (51%)
Not available	29	17	49
ATRX			
Lost	32 (80%)	1 (3%)	2 (10%)
Expressed	8 (20%)	33 (97%)	20 (90%)

Not available	69	48	70
MGMT			
Methylated	100 (92%)	82 (100%)	41 (45%)
Unmethylated	9 (8%)	0	51 (55%)
***EGFR* amplification**			
Yes	2 (2%)	1 (1.5%)	45 (59%)
No	107 (98%)	81 (98.5%)	47 (51%)
***CDKN2A* deletion**			
Yes	13 (12%)	0	51 (55.5%)
No	96 (88%)	82 (100%)	41 (44.5%)
Chr. 7 gain			
Yes	3 (2.5%)	2 (2.5%)	67 (73.5%)
No	106 (97.5%)	80 (97.5%)	24 (26.5%)
Chr. 10 loss			
Yes	1 (1%)	0	77 (83%)
No	108 (99%)	82 (100%)	15 (17%)

Table 2: Multivariate Cox PH regression analysis for TTF in the NOA-04 cohort

Variable	HR	95% CI	p
AO *vs.* AA	1.76	0.72 – 4.12	0.211
AA *vs.* AOA	1.85	0.9 – 3.84	0.093
CIMP-non-codel *vs.* CIMP-codel	5.27	2.0 – 13.9	**0.001**
$CIMP^{neg}$ *vs.* CIMP-codel	12.54	5.02 – 31.32	**< 0.001**
Age, per year increase	1.05	1.02 – 1.07	**0.001**

AA, anaplastic astrocytoma; AO, anaplastic oligodendrogliomas; AOA, anaplastic oligoastrocytoma

SUPPLEMENTARY INFORMATION

Integrated DNA methylation and copy-number profiling identifies three clinically and biologically relevant groups of anaplastic glioma

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Supplementary Methods

Patients and tumor samples

The NOA-04 trial (NCT00717210) for patients with newly diagnosed anaplastic gliomas compared the efficacy and safety of initial radiotherapy, followed by chemotherapy (temozolomide or procarbazine, lomustine and vincristine) at progression or occurrence of unacceptable toxicity with the inverse sequence in patients with newly diagnosed anaplastic gliomas. In this trial, both sequences achieved similar results [9]. Median follow-up time was 54 months. All patients consented to exploratory molecular analyses performed with study data and materials. The original phase III trial was approved by the Ethics Committee (EC) at the University of Tuebingen, Germany, and subsequently all local ECs of the participating clinical centers.

All tumors were banked at the time of primary diagnosis between 1994 and 2013 in accordance with research ethics board approval from the respective institutes. Informed consent was obtained from all patients included in this study. All samples were independently reviewed by neuropathologists (T.P. (NOA-04), A.v.D. and A.K.) according to the WHO guidelines. The study additionally included data from 55 glioblastoma samples reported before [8].

Preprocessing of the 450k Infinium methylation data

The raw methylation data collected from different sources was preprocessed together by using the function *preprocessIllumina* of the Bioconductor Software package Minfi [1]. This function is an open source implementation of the normalization procedure developed by Illumina which is also available in Illumina's GenomeStudio Software. In brief, this procedure uses different internal control probes located on the array to perform background subtraction and normalization of the two measured intensity channels.

Unsupervised clustering of methylation data

For unsupervised hierarchical clustering we selected the 10,000 most variable methylated probes that showed highest median absolute deviation (MAD) across beta values. Pairwise similarity between cases was calculated using euclidean distance and hierarchical clustering was performed using Ward's linkage method. To cluster CpG probes we applied hierarchical clustering using 1-centered Pearson correlation as similarity measure and average linkage as hierarchical clustering method. The same procedure was applied to all three data sets. As indicated by the volcano plot in Figure 1 of the Supplementary Material, the CIMP status is the dominating signal that might overlay other structures in the data, i.e., unknown molecular subgroups. Therefore, to select probes not affected by the CIMP signal and reveal such underlying subgroups, unspecific variance filtering and clustering was applied in the same way as described above to the two subgroups $CIMP^{pos}$ and $CIMP^{neg}$.

Detection of copy-number aberrations

Copy-number data were computed from the 450k Infinium methylation array as described previously [8]. Low-resolution copy number variations were detected from the 450k Infinium methylation array in a custom approach using the sum of both methylated and unmethylated signals. Probes found to be highly variant in the six normal cerebellum samples were excluded from the analysis according to the following criteria: Removal of probes not within the 0.05 and 0.85 quantile of median summed values or over the 0.8 quantile of the median absolute deviation. Log-ratios of samples to the median value of control samples were calculated, and sample noisiness was determined as the median absolute deviation of adjacent probes. Probes were then combined by joining 20 adjacent probes, and resulting genomic windows less than 100kb in size were iteratively merged with adjacent windows of smaller size. Windows of more than 5Mb were excluded from analysis, resulting in a total of 8,654

windows throughout the genome. For each window, the median probe value was calculated and shifted to minimize the median absolute deviation from all windows to zero for every sample. Segmentation was performed by applying the circular binary algorithm using the following settings: min.width=10, nperm=32000, alpha=0.001, undo.splits="sdundo", undo.SD=2.2 [7]. The median value of windows contained in each segment was calculated, and classified as homozygous or hemizygous deletion, neutral, gain or high-level amplification by the following arbitrary thresholds: -0.96 , -0.24, 0.12 and 0.72.

The resulting copy-number plots were individually curated for 1p/19q codeletion, *EGFR* amplification, homozygous *CDKN2A* deletion and copy-number changes affecting chromosomes 7 and 10 (examples in **Supplementary Figure 6**).

Supplementary Discussion

Methylation of the *MGMT* promoter is a well-established biomarker predicting benefit from temozolomide in glioblastomas [5], which has recently been prospectively confirmed in the RTOG-0525 trial [4]. In anaplastic gliomas however, the situation is more complex, as the prognostic role of *MGMT* promoter methylation has been shown to be independent of treatment in two phase III trials [3, 9]. A recent analysis suggested an interdependence between *IDH* status and *MGMT* methylation. In *IDH* wild type tumors, *MGMT* is predictive specifically for benefit from alkylating chemotherapy, while it is prognostic in *IDH* mutant tumors [10]. The molecular concordance between $CIMP^{neg}$ (*IDH* wild type) anaplastic gliomas and glioblastomas, supports the similar relevance of *MGMT* in *IDH* wild type anaplastic gliomas and glioblastomas (**Supplementary Figure 3**). In $CIMP^{pos}$ tumors on the other hand, the MGMT-STP27 algorithm, a logistic regression model based on the methylation in two CpGs in the differentially methylated regions 1 and 2 of *MGMT* [2], indicated a methylated *MGMT* promoter in 182 of 191 tumors. Of note however, in our database at the DKFZ which comprises methylation data of more than 4000 brain tumors covering most known entities we observed consistent batch effects between samples coming from FFPE and fresh frozen material for specific CpG probes. Probe cg12434587 which is one of the two probes involved in the MGMT-STP27 model seems to be affected by this batch effect. Our current estimate for the batch effect for probe cg12434587 is a log2 difference around 2 for the methylated fluorescence channel. Such an effect may result in methylation difference of up 0.3 on the beta scale. Therefore, as the MGMT-STP27 was trained on GBM samples from fresh frozen material its ability to correctly predict the MGMT status of samples coming from FFPE material might be limited. On the other hand, this high rate of *MGMT* promoter methylation has been reported before in *IDH* mutant gliomas [6]. Interestingly, the 9 *MGMT* unmethylated tumors all belonged to the CIMP-non-codel

group, an observation which is further corroborated when considering the results of the centrally performed methylation-specific PCR (MSP) in NOA-04: per MSP, 38 / 42 (90%) CIMP-codel tumors, but only 24 / 46 (52%) of CIMP-non-codel tumors had a methylated *MGMT* promoter (Fisher's exact test, $p < 0.001$). Mulholland et al. suggested that the discrepancy between their finding of nearly 100% *MGMT* promoter methylation and the MSP results might be due to low methylation especially in the MSP-forward region, while methylation is higher in surrounding CpGs, still leading to reduced gene expression [6]. Hence, it seems possible that the prognostic effect seen with MSP might rather reflect a separation between CIMP-codel tumors (which are almost always methylated per MSP) and some CIMP-non-codel tumors.

Supplementary Figure 1. Volcano plot for differentially methylated probes (using median difference and Wilcoxon's rank sum test). Probes with a significantly ($p < 0.05$) different median methylation between both groups after false discovery rate correction are shown in red.

Supplementary Figure 2. Overall survival by CIMP status in anaplastic gliomas.

Supplementary Figure 3. Progression-free survival by *MGMT* status and treatment arm (radio- *vs.* chemotherapy) in the $CIMP^{neg}$ NOA-04 samples (n = 24).

Supplementary Figure 4. Prediction error curves for time to treatment failure in Cox regression models including histology only, molecular classification only, or both.

Supplementary Figure 5. Overall survival by WHO grade in $CIMP^{neg}$ tumors.

Supplementary Figure 6. Copy-number plots examples for a case with 1p/19q codeletion (a) and another case with *EGFR* amplification, *CDKN2A* deletion, gain of chromosome 7 and loss of chromosome 10 (b).

Supplementary Table 1. Baseline patient characteristics of the NOA-04 cohort

	NOA-04 Biomarker Cohort		**NOA-04 Trial Cohort**	
	RT (n = 65)	**PCV / TMZ (n = 50)**	**RT (n = 139)**	**PCV / TMZ (n = 135)**
Median age (range), years	42 (23 – 74)	41.5 (25 – 64)	44 (23 – 74)	42 (20 – 77)
Astrocytoma, n	28 (43%)	22 (43%)	70 (50%)	74 (55%)
Oligoastrocytoma, n	25 (38%)	20 (40%)	47 (34%)	44 (32%)
Oligodendroglioma, n	12 (19%)	8 (17%)	22 (16%)	17 (13%)
Resection, n				
Total	31 (48%)	22 (44%)	53 (38%)	47 (35%)
Subtotal	33 (50.5%)	27 (54%)	61 (44%)	57 (42%)
Biopsy	1 (1.5%)	1 (2%)	25 (18%)	31 (23%)
1p/19q co-deletion, n				
Yes	27 (46%)	19 (46%)	41 (43%)	33 (38%)
No	31 (54%)	22 (54%)	54 (57%)	53 (62%)
Information missing	7	9	44	49
***MGMT* promoter, n**				
Methylated	37 (60%)	33 (70%)	59 (57%)	64 (64%)
Unmethylated	24 (40%)	14 (30%)	44 (43%)	35 (36%)
Information missing	4	3	36	36
***IDH* mutation, n**				
Yes	48 (78%)	35 (74%)	65 (66%)	68 (70%)

No	13 (22%)	12 (26%)	33 (34%)	29 (30%)
Information missing	4	3	41	38

RT, radiotherapy; PCV, procarbazine, CCNU & vincristine; TMZ, temozolomide

Supplementary Table 2. Cox regression for TTF by reference histology in NOA-04

Variable	**HR**	**95% CI**	**p**
AO *vs.* AA	0.64	0.29 – 1.38	0.257
AOA *vs.* AA	0.39	0.2 – 0.77	0.006

AA, anaplastic astrocytoma; AO, anaplastic oligodendrogliomas; AOA, anaplastic oligoastrocytoma

Supplementary References

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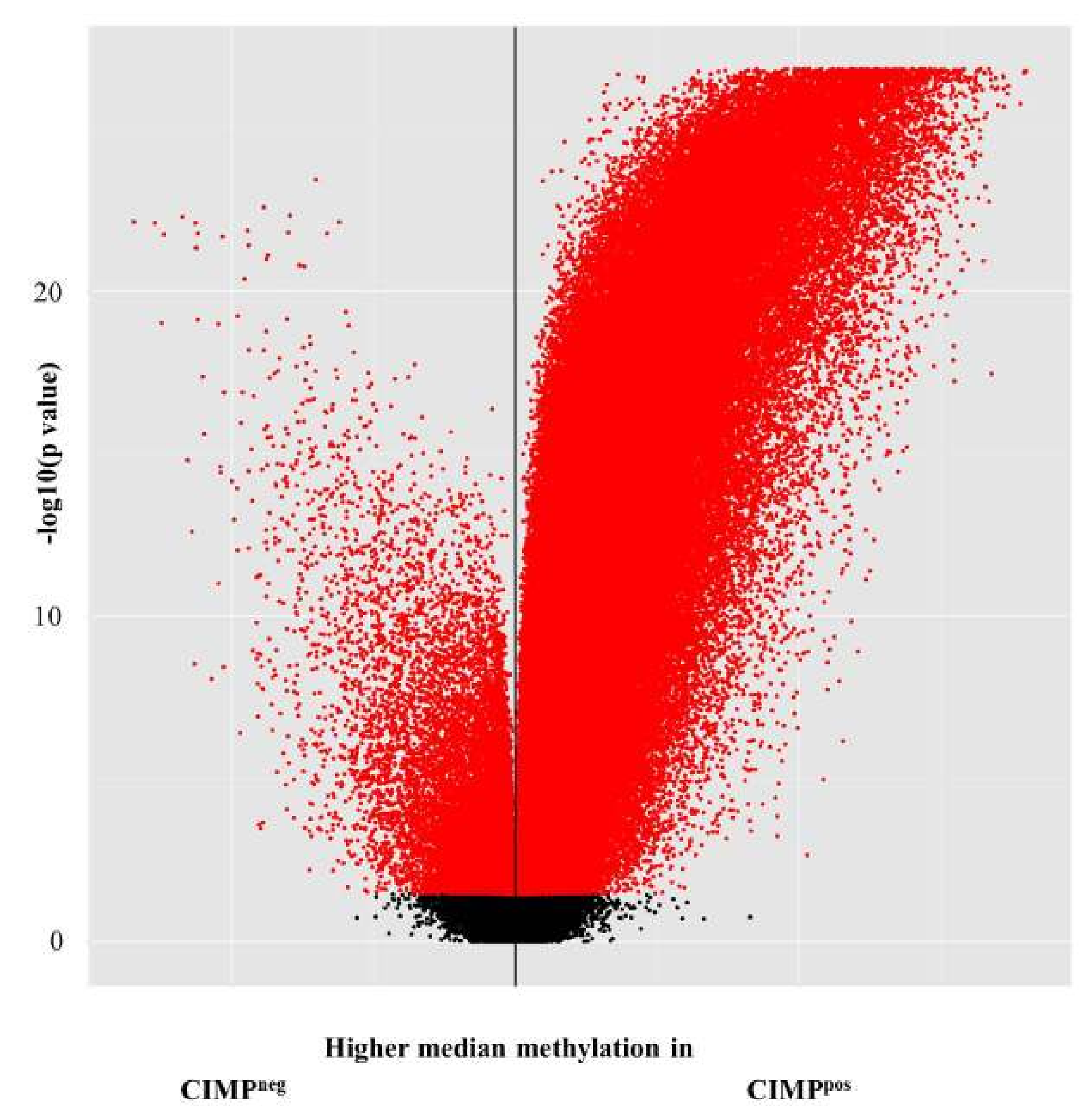
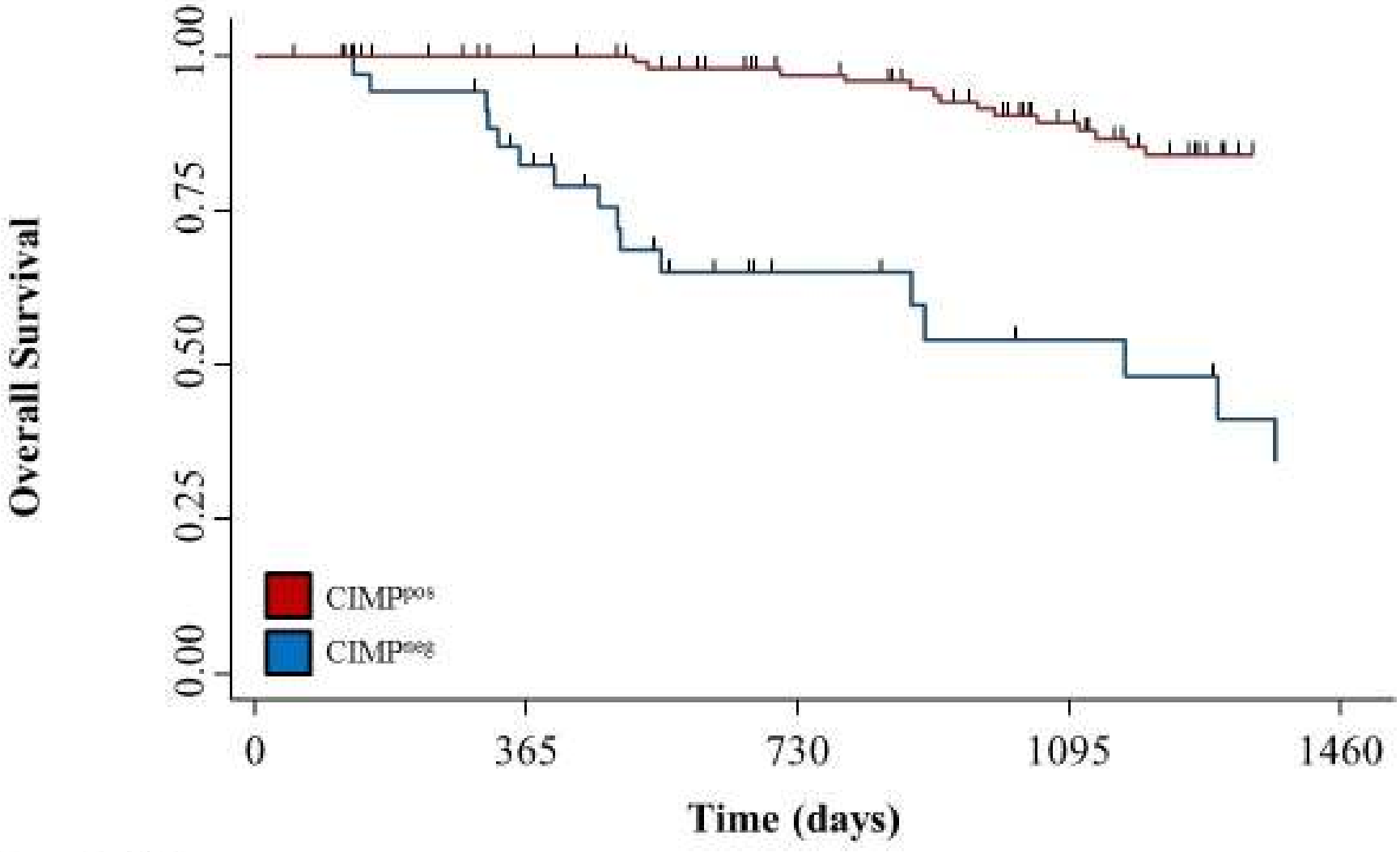
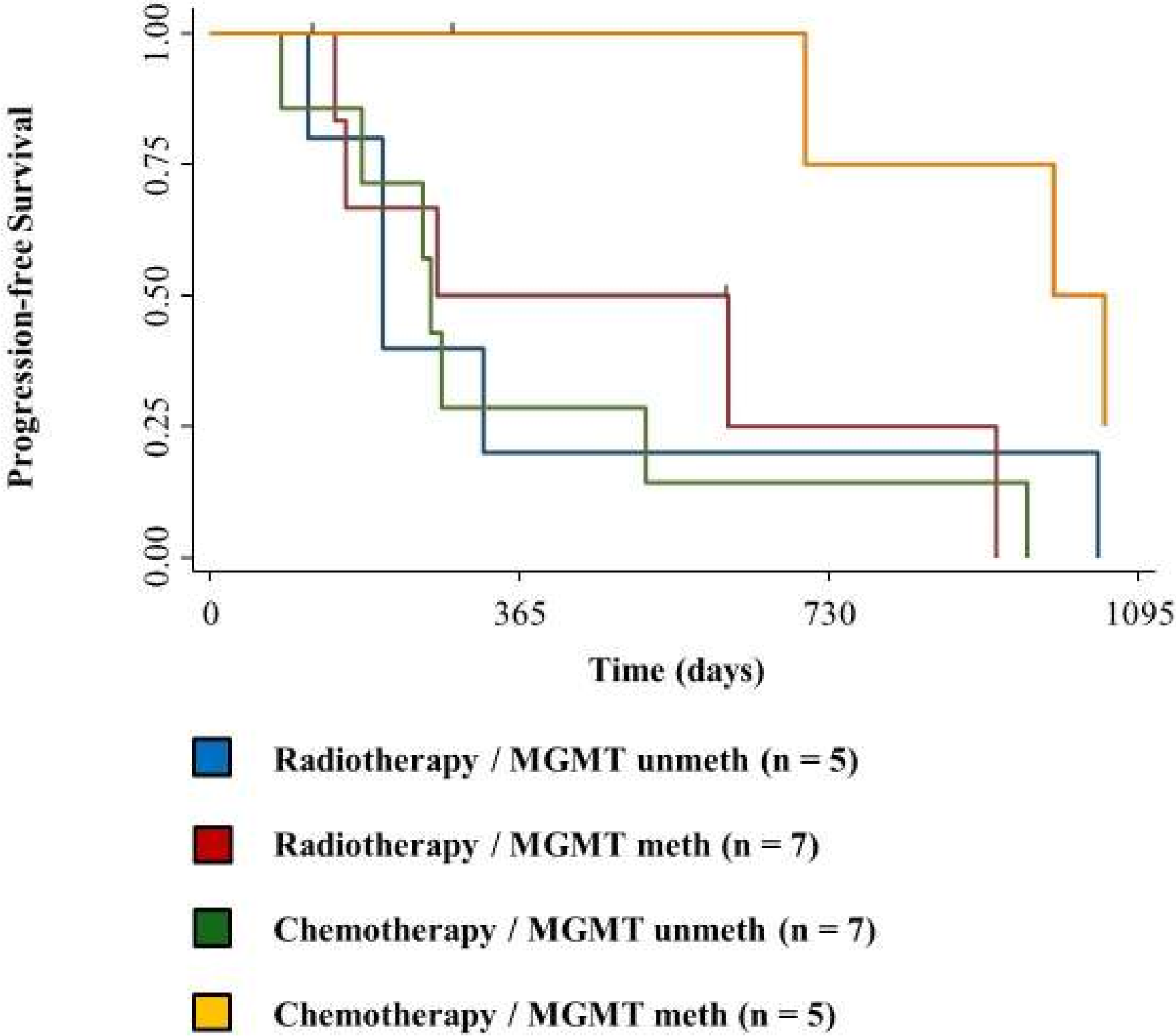
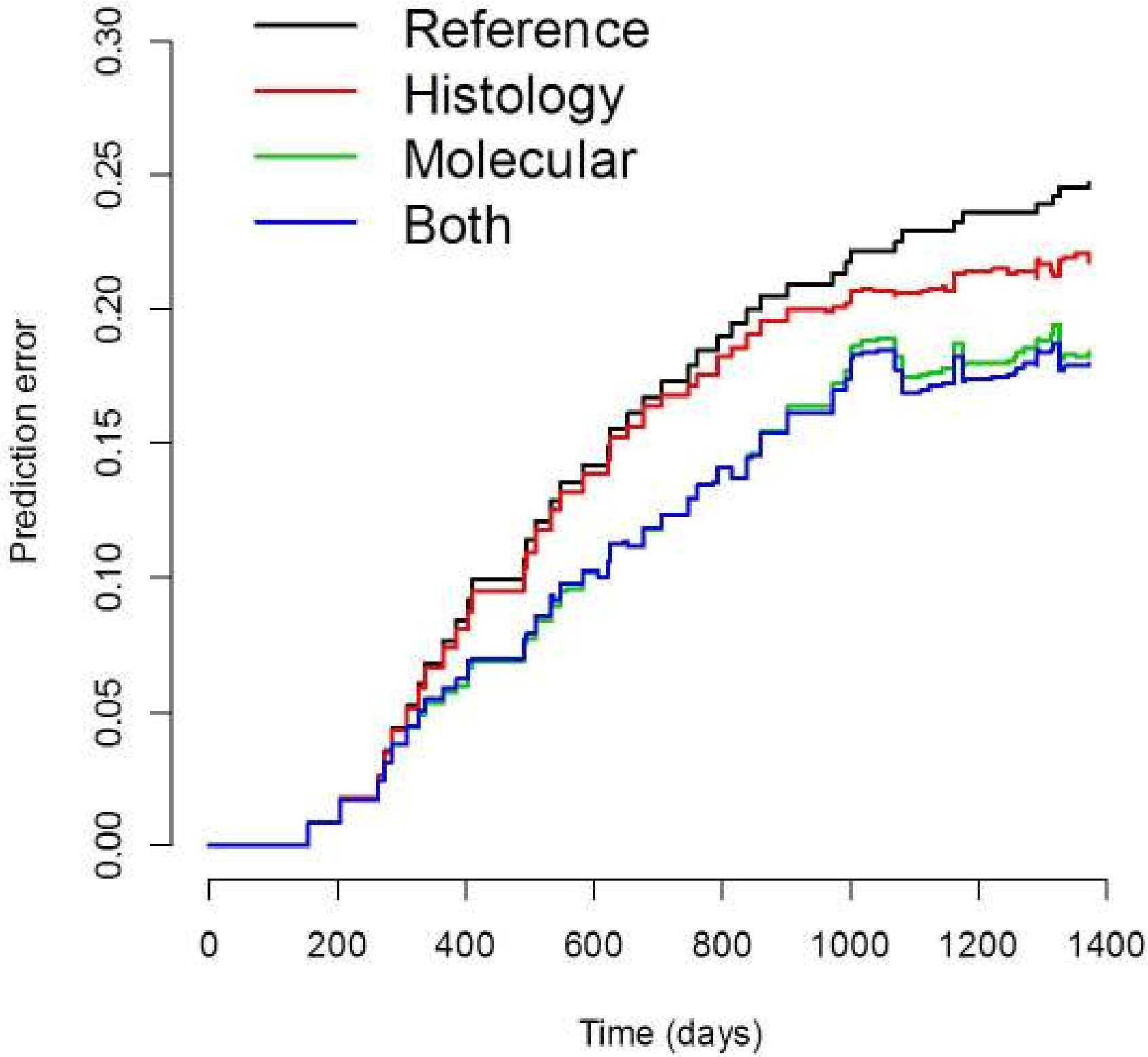

20
10
0
-log10(p value)
Higher median methylation in
CIMPneg
CIMPpos

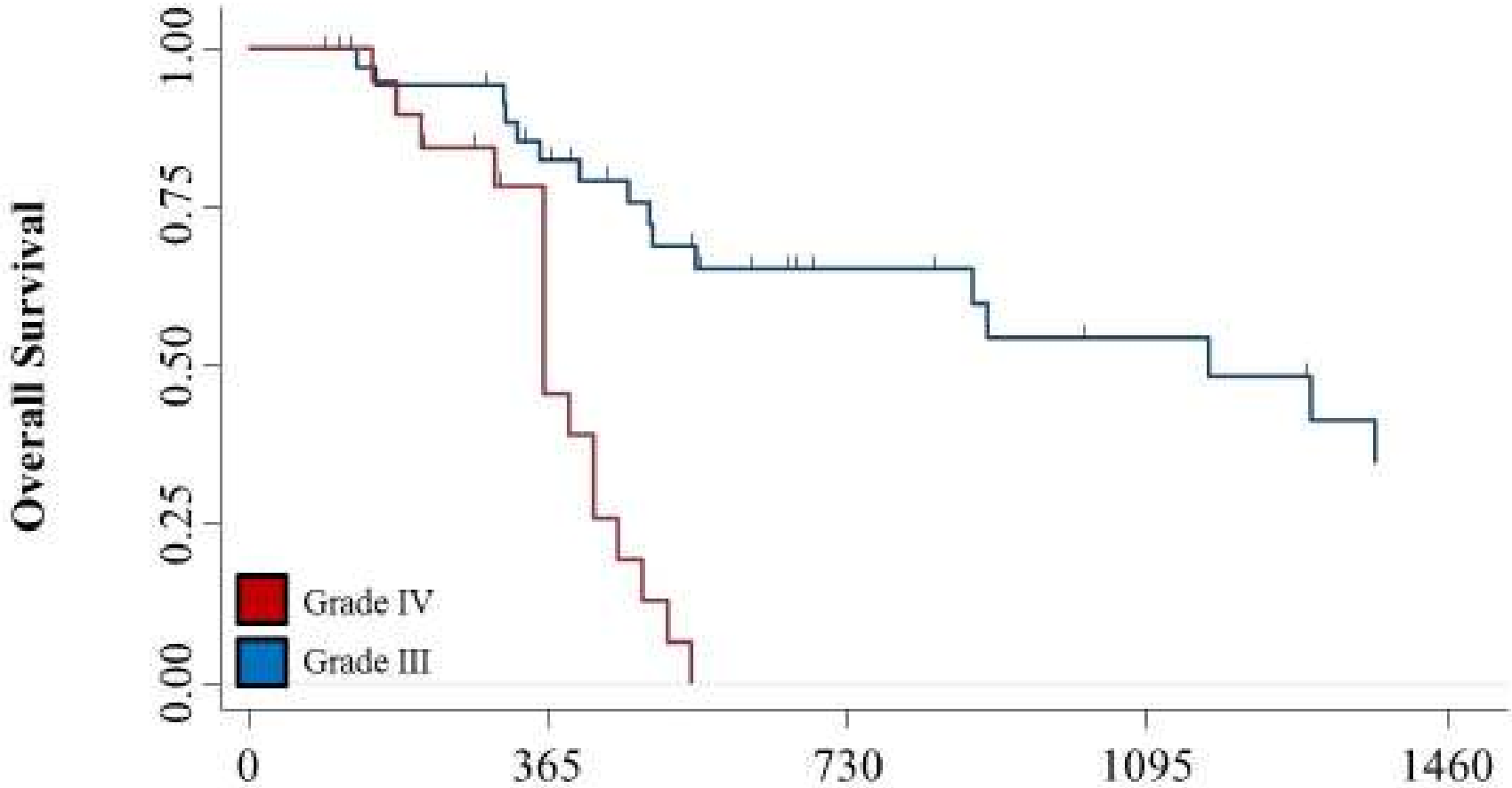


Number at risk

CIMP^{neg}	37	20	10	4
CIMP^{pos}	120	105	80	47

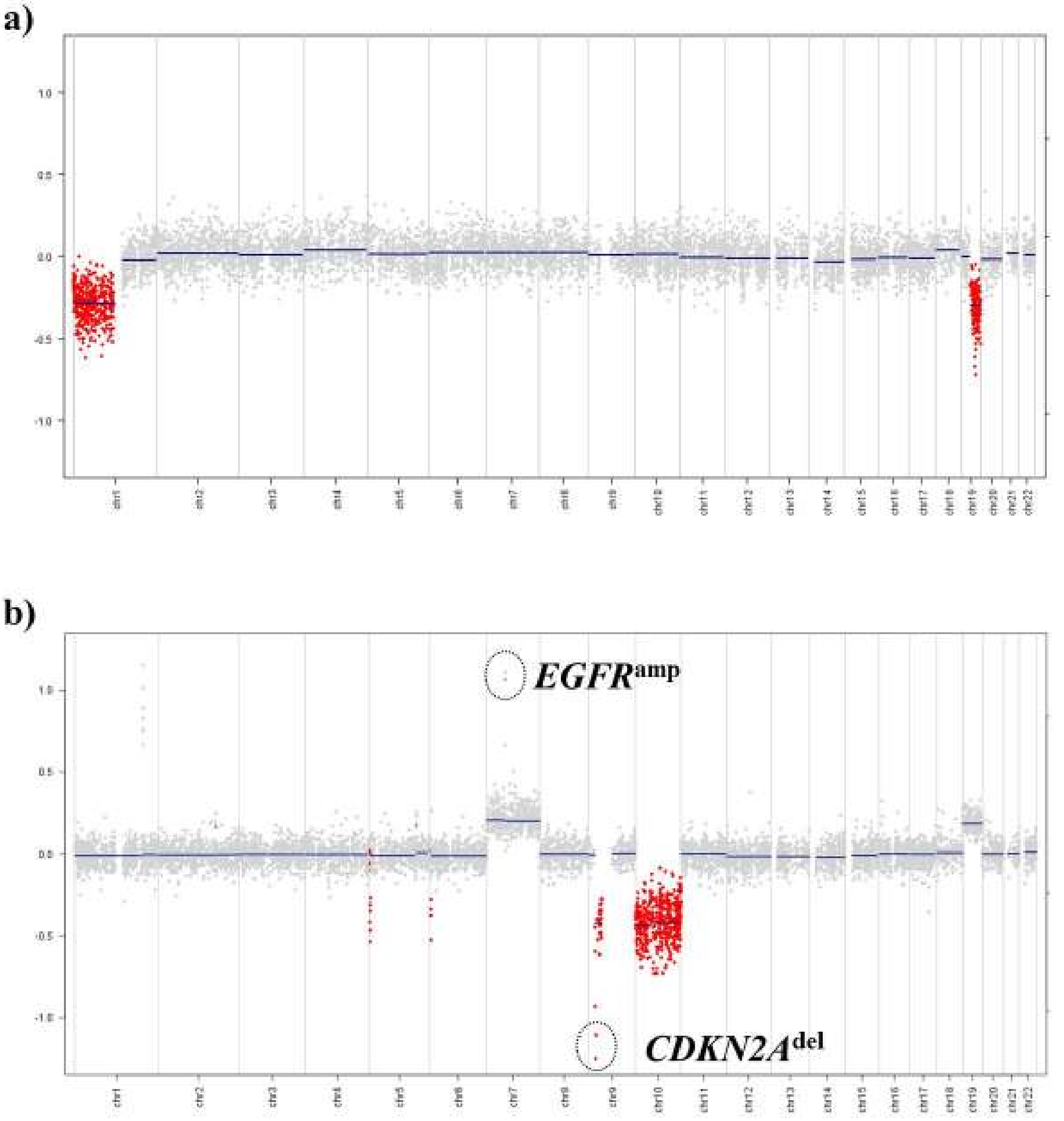

Progression-free Survival
1.00
0.75
0.50
0.25
0.00
0
365
730
1095
Time (days)
Radiotherapy / MGMT unmeth (n = 5)
Radiotherapy / MGMT meth (n = 7)
Chemotherapy / MGMT unmeth (n = 7)
Chemotherapy / MGMT meth (n = 5)


Reference
Histology
Molecular
Both
Prediction error
0.00
0.05
0.10
0.15
0.20
0.25
0.30
0
200
400
600
800
1000
1200
1400
Time (days)



Number at risk

Grade III	37	20	10	4
Grade IV	22	2	0	0


a)
b)
EGFRamp
CDKN2Adel