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Molecular classification of diffuse cerebral WHO grade II/III gliomas using genome- and transcriptome-wide profiling improves stratification of prognostically distinct patient groups

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Abstract Cerebral gliomas of World Health Organization (WHO) grade II and III represent a major challenge in terms of histological classification and clinical management. Here, we asked whether large-scale genomic and transcriptomic profiling improves the definition of prognostically distinct entities. We performed microarray-based genome- and transcriptome-wide analyses of primary tumor samples from a prospective German Glioma Network cohort of 137 patients with cerebral gliomas, including 61 WHO grade II and 76 WHO grade III tumors. Integrative bioinformatic analyses were employed to define molecular subgroups, which were then related to histology, molecular biomarkers, including isocitrate dehydrogenase 1 or 2 (*IDH1/2*) mutation, 1p/19q co-deletion and telomerase reverse transcriptase (*TERT*) promoter mutations, and patient outcome. Genomic profiling identified five distinct glioma groups, including three *IDH1/2* mutant and two *IDH1/2* wild-type groups. Expression profiling revealed evidence for eight transcriptionally different groups (five *IDH1/2* mutant, three *IDH1/2* wild type), which were only partially linked to the genomic groups. Correlation of DNA-based molecular stratification with clinical outcome allowed to define three major prognostic groups with characteristic genomic aberrations. The best prognosis was found in patients with *IDH1/2* mutant and 1p/19q co-deleted tumors. Patients with *IDH1/2* wild-type gliomas

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For the German Glioma Network.

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and glioblastoma-like genomic alterations, including gain on chromosome arm 7q (+7q), loss on chromosome arm 10q (−10q), *TERT* promoter mutation and oncogene amplification, displayed the worst outcome. Intermediate survival was seen in patients with *IDH1/2* mutant, but 1p/19q intact, mostly astrocytic gliomas, and in patients with *IDH1/2* wild-type gliomas lacking the +7q/−10q genotype and *TERT* promoter mutation. This molecular subgrouping stratified patients into prognostically distinct groups better than histological classification. Addition of gene expression data to this genomic classifier did not further improve prognostic stratification. In summary, DNA-based molecular profiling of WHO grade II and III gliomas distinguishes biologically distinct tumor groups and provides prognostically relevant information beyond histological classification as well as *IDH1/2* mutation and 1p/19q co-deletion status.

Abbreviations

AII	Diffuse astrocytoma WHO grade II
AAIII	Anaplastic astrocytoma WHO grade III
AOIII	Anaplastic oligodendroglioma WHO grade III
AOAIII	Anaplastic oligoastrocytoma WHO grade III
ATRX	ATP-dependent X-linked helicase
BRAF	v-Raf murine sarcoma viral oncogene homolog B1
CDK	Cyclin-dependent kinase
CGH	Comparative genomic hybridization
CIC	*Drosophila* homolog of capicua
EGFR	Epidermal growth factor receptor
GB IV	Glioblastoma WHO grade IV
gCIMP	Glioma CpG island methylator phenotype
GGN	German Glioma Network
GO	Gene ontology
IDH	Isocitrate dehydrogenase
KPS	Karnofsky performance score
MAPK	Mitogen-activated protein kinase
MDM	Murine double minute
MGMT	O^6-methylguanine DNA methyltransferase
OII	Oligodendroglioma WHO grade II
OAII	Oligoastrocytoma WHO grade II
OS	Overall survival
PDGFRA	Platelet-derived growth factor receptor-α
PFS	Progression-free survival
PTEN	Phosphatase and tensin homolog on chromosome 10
SOM	Self-organizing map
TERT	Telomerase reverse transcriptase
TMZ	Temozolomide
TP53	Tumor protein p53
WHO	World Health Organization

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Introduction

The World Health Organization (WHO) classification of tumors of the central nervous system [22] separates cerebral gliomas into distinct disease entities based on histological criteria. In addition to tumor typing, a malignancy grade ranging from WHO grade I to IV is allocated to each tumor considering morphological features of anaplasia, such as mitotic activity, microvascular proliferation and necrosis. This serves the overall goal of providing clinicians with information on the assumed natural disease course and strongly impacts clinical decision making today. In clinical practice, histopathological classification is greatly aided by immunohistochemical markers, and, more recently, by an increasing set of molecular markers, including *isocitrate dehydrogenase 1* or *2* (*IDH1/2*) mutation, co-deletion of chromosome arms 1p and 19q, O^6-methylguanine DNA methyltransferase (*MGMT*) promoter methylation, and others [35].

Combined classification approaches have resulted in a satisfactory segregation of WHO grade I pilocytic astrocytoma as a distinct entity characterized by a benign clinical

course and almost universally driven by mitogen-activated protein kinase (MAPK) pathway activation, most commonly caused by fusion of the v-raf murine sarcoma viral oncogene homolog B1 (*BRAF*) proto-oncogene to other genes or activating *BRAF* point mutations [16]. On the other end of the glial tumor spectrum, primary glioblastoma has been delineated as a distinct entity of highly malignant tumors characterized by the absence of *IDH1/2* mutation, gains on chromosome 7 and losses on chromosome arm 9p and chromosome 10, frequent mutations in the phosphatase and tensin homolog on chromosome 10 (*PTEN*) gene and the human telomerase (*TERT*) promoter, as well as activation of receptor tyrosine kinase pathways, in particular the epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor pathways [17, 25].

In contrast, the histologically defined groups of astrocytic, oligodendroglial and oligoastrocytic (mixed) gliomas of WHO grades II and III remain a major challenge in various ways: (1) there is poor interobserver agreement when diagnoses and grading are made by histological criteria alone [30], in particular concerning the classification of oligoastrocytomas [10] (2) the clinical course is highly variable, and (3) the clinical management remains poorly standardized [1, 35]. Current molecular marker-based approaches explore the validity of a three-tiered approach delineating (1) *IDH1/2*-mutant tumors with 1p/19q co-deletion which often carry mutations in the *Drosophila* homolog of capicua (*CIC*) gene as well as the *TERT* promoter and predominantly include oligodendroglial tumors, (2) *IDH1/2* mutant tumors without 1p/19q co-deletion which often carry tumor protein p53 (*TP53*) as well as ATP-dependent X-linked helicase (*ATRX*) gene mutations and predominantly include astrocytic tumors, and (3) *IDH1/2* wild-type tumors that remain poorly characterized in terms of other molecular alterations [15, 17, 38]. In addition, recent data from exome sequencing of anaplastic astrocytomas suggested a distinct mutation profile from primary glioblastomas, including frequent mutations in Notch pathway genes [18].

In the present study, we sought to determine whether a molecular profiling approach for genomic copy number and mRNA expression changes might help to overcome some of the limitations of the current histology-based diagnostic approaches to these tumors and to provide better stratification for future clinical trials. Therefore, we performed high-resolution array-based comparative genomic hybridization (array-CGH) analyses and Affymetrix gene chip expression profiling on 137 cerebral gliomas of WHO grade II or III from patients of the German Glioma Network (GGN) with prospectively collected clinical follow-up data. We found that molecular profiling distinguishes distinct subtypes among these gliomas that carry prognostically relevant information beyond histological classification, grading as well as *IDH1/2* mutation and 1p/19q co-deletion.

Patients and methods

Patients and tumors

The GGN is a prospective, non-interventional cohort study involving eight clinical centers at University Hospitals in Germany (www.gliomnetzwerk.de), and was supported by the German Cancer Aid from 2004 to 2012. All patients gave written informed consent for participation in the GGN and its translational research projects. For this study, we analyzed tumors of 137 patients with WHO grade II or III gliomas by array-CGH analysis, Affymetrix chip-based gene expression profiling and candidate gene analyses (Table 1). All tumors were subjected to central pathology review (T.P.) and classified according to the WHO classification of tumors of the central nervous system [22]. For comparison of molecular profiles and prognostic outcome determined for the poor prognosis group of patients in this study (group V, see below), we additionally used published molecular and clinical data from two previous GGN studies on glioblastoma [26, 34].

Nucleic acid extraction, mutation and promoter methylation analyses

DNA and RNA were extracted by ultracentrifugation from deep-frozen tumor samples [14]. Tumor cell content was histologically estimated as 80 % or more in most samples except for individual cases with lower tumor cell content but still detectable copy number aberrations by array-CGH analysis. High quality of extracted DNA was assured by spectrophotometric analysis and agarose gel electrophoresis. RNA quality was determined on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Only samples showing a RNA integrity number of 7 or more were used for microarray analyses. Mutational analyses of the *IDH1*, *IDH2*, *TERT*, *BRAF* and *H3F3A* genes were carried out by Sanger sequencing or pyrosequencing [9, 12, 19]. The *MGMT* promoter methylation status was determined by methylation-specific PCR [8].

Array-based comparative genomic hybridization (array-CGH)

Array-CGH was performed using genomic DNA microarrays with 10,000 large insert clones allowing for an average resolution of better than 0.5 Mb. Array assembly, probe labeling, array hybridization and scanning were carried out essentially as reported elsewhere [43]. To identify amplification events, genomic profiles were generated for each tumor and amplifications scored if $\log_2$ test/reference ratios were ≥ 1. All other analyses of array-CGH data were done using aCGHPipeline [20].

Table 1 Patient characteristics

	$N = 137$
Age (years)	
Median	41
Range	21–80
Gender	
Male	84 (61.3 %)
Female	53 (38.7 %)
KPS	
Median	90
Range	60–100
80–100	114 (94.2 %)
70 or less	7 (5.8 %)
Unknown	16
Histological diagnosis	
Diffuse astrocytoma (A2)	45 (32.8 %)
Oligoastrocytoma (OA2)	13 (9.5 %)
Oligodendroglioma (O2)	3 (2.2 %)
Anaplastic astrocytoma (AA3)	42 (30.7 %)
Anaplastic oligoastrocytoma (AOA3)	31 (22.6 %)
Anaplastic oligodendroglioma (AO3)	3 (2.2 %)
IDH1/2 status	
Mutant	115 (83.9 %)
Wild-type	22 (16.1 %)
1p/19q status	
1p/19q co-deleted	37 (27.0 %)
1p/19q non-co-deleted	100 (73.0 %)
MGMT promoter status	
Unmethylated	25 (18.4 %)
Weakly methylated	13 (9.6 %)
Strong methylated	98 (72.1 %)
Unknown	1
TERT promoter status	
Mutant	45 (33.8 %)
Wild-type	88 (66.2 %)
Unknown	4

Affymetrix gene chip analyses

Gene expression profiles were determined with Affymetrix Gene Chip® Human Genome U133 Plus 2.0 arrays (Affymetrix, Santa Clara, CA). Sample preparation was done with 2.5 μg total tumor RNA using the One Cycle Target Labelling and Controls kit (Affymetrix) [26]. Hybridization and scanning of the chips were performed at the Center for Biological and Medical Research at Heinrich Heine University Düsseldorf.

Statistical analysis

Array-CGH data were evaluated as reported before using aCGHPipeline [21]. Gene expression data were analyzed

after reduction to metagenes using self-organizing map (SOM) machine learning [41]. As a result, each tumor tissue is characterized by the expression values of 1600 metagenes that are visualized as expression landscape by color coding. Metagene-based clustering using either hierarchical or maximum spanning graph-partitioning methods, visualization and downstream analysis of expression data were performed with the program OpoSOM after hook calibration of the raw data, quantile normalization and centralization in log10 scale [13, 26]. In addition, pairwise testing between groups of samples was carried out by regularized *t* testing as implemented in OpoSOM [42]. Samples were classified separately based either on array-CGH (five groups I–V) or on mRNA expression (eight groups 1–8) data as described in the Results section. In addition, both classifications were combined by taking the intersections of groups I and 7 and of groups V and 1 to obtain the "integrated molecular groups" A and C, respectively. Samples not matching criteria for either group A or C were assigned to group B.

Progression-free survival (PFS) was calculated from the day of first surgery until tumor progression, death, or end of follow-up. Overall survival (OS) was calculated from the day of first surgery until death or end of follow-up. Log-rank test was used to analyze survival data. When more than two groups were compared, we tested for the equality of groups regarding PFS or OS and present global *p* values indicating that at least two groups were different. Cox regression models were built to assess the association of clinical parameters and molecular groups defined by array-CGH and expression profiling with OS. The statistic deviance (minus twice the logarithm of the maximized likelihood) was used to measure the model fit. All statistical analyses were performed with the IBM SPSS Statistics (version 20.0.0) software.

Results

Patient characteristics and outcome

Table 1 summarizes clinical findings and molecular genetic tumor characteristics. Individual patient data are provided in Supplementary Table 1. Clinical data, molecular marker characteristics, and therapeutic measures over the course of disease until the last follow-up, broken down by histology, are summarized in Supplementary Table 2. The median follow-up was 71.1 months; 92 (67.2 %) patients have experienced a PFS event, 47 (34.3 %) patients have died. Histological grading was not associated with PFS, but there was a trend towards longer OS with WHO grade II as compared to WHO grade III tumors (Supplementary Fig. 1a). Astrocytic gliomas were associated with inferior

PFS, but not OS, although there were few OS events (Supplementary Fig. 1b, Supplementary Table 2). WHO grade was prognostic by trend also within histological entities (Supplementary Figs. 1c, d). *IDH1/2* mutation and 1p/19q co-deletion were both associated with prolonged PFS and overall survival (OS) (Supplementary Table 3, Supplementary Figs. 1e, f). Among *IDH1/2* mutant tumors, 1p/19q co-deletion was associated with improved outcome (Supplementary Fig. 1g). *TERT* promoter mutation was linked to outcome neither in the entire cohort of 137 patients (Supplementary Fig. 1h) nor in the subgroup of 112 patients with *IDH1/2* mutant tumors (Supplementary Fig. 1i). The lack of significance for the latter comparison likely reflects the low number of events. In oligodendroglial and oligoastrocytic gliomas, *TERT* promoter mutation was more common in cases with than without 1p/19q co-deletion (27/30 versus 2/19, $p < 0.001$). In patients with 1p/19q non-deleted anaplastic gliomas of WHO grade III ($n = 51$), *TERT* promoter mutation was associated with less favorable outcome (median OS 2.4 versus 8.8 years, $p = 0.048$) (Supplementary Fig. 1k). *MGMT* promoter methylation was associated with prolonged OS (Supplementary Table 3, Supplementary Fig. 1l).

Genomic copy number profiling

Unsupervised clustering of the array-CGH data separated the 137 gliomas into three major clusters characterized by (a) an oligodendroglial genomic profile with 1p/19q co-deletion, (b) an intermediate group consisting mostly of *IDH1/2* mutant tumors without 1p/19q co-deletion, and (c) a glioblastoma-like genomic profile with partial or complete gains on chromosome 7, losses on 9p, partial or complete losses on 10, and frequent gene amplifications (Fig. 1a). Based on these findings and the well-known differences in tumor biology and prognosis between *IDH1/2* mutant and wild-type gliomas, we performed supervised analyses of the array-CGH data stratified by *IDH1/2* status. This analysis revealed three distinct tumor groups among the *IDH1/2* mutant tumors defined by 1p/19q co-deletion (group I), chromosome arm 7q gain associated with a usually limited number of copy number changes on other chromosomes (group II), or gains and losses affecting multiple chromosomes (group III). *IDH1/2* wild-type tumors were divided into one group with relatively few genomic changes variably affecting different chromosomes and *TERT* promoter mutation restricted to a single case (group IV), and another group with primary glioblastoma-like copy number changes, typically including combined gains on chromosome 7 and losses on chromosome 10 affecting at least the long arms of both chromosomes (+7q/−10q; group V) (Fig. 1b, Supplementary Table 4). Additional analyses for *BRAF*-V600E and *H3F3A*-K27 or -G34 mutations in 8 of 9 and 7 of 9 group

IV tumors did not reveal any mutation (data not shown). Tumors in group V often carried *TERT* promoter mutations (9/13; 75 %) as well as gene amplifications (11/13; 85 %), including *TERT* amplification in one tumor without *TERT* promoter mutation. Unsupervised analyses of array-CGH data from group V tumors together with array-CGH data of primary *IDH1/2* wild-type glioblastomas reported elsewhere [26] revealed that group V tumors did not form a distinct cluster, but were distributed among the glioblastoma cases, indicating that group V tumors carry genomic imbalances typical of glioblastoma (Supplementary Fig. 2a).

Figure 2a shows the frequency plots of genomic imbalances in each of the five genomic groups defined by array-CGH patterns within the subsets of *IDH1/2* mutant and wild-type tumors, as well as an independent cohort of *IDH1/2* wild-type primary glioblastomas reported previously [26]. Group V tumors showed a virtually identical genomic aberration pattern as the primary glioblastomas. High-level copy number gains indicative of gene amplification occurred at similar frequencies in both groups, most commonly affecting epidermal growth factor receptor (*EGFR*) [group V 8/13 (62 %) versus primary glioblastoma 26/54 (48 %)], murine double minute (*MDM) 4* [group V 3/13 (23 %) versus glioblastoma 6/54 (11 %)], cyclin-dependent kinase (*CDK) 4* [group V 2/13 (15 %) versus glioblastoma 9/54 (17 %)], *MDM2* [group V 1/13 (8 %) versus glioblastoma 5/54 (9 %)], and platelet-derived growth factor receptor-α (*PDGFRA)* [group V 0/13 (0 %) versus glioblastoma 4/54 (7 %)] (each comparison: $p > 0.05$, Fisher's exact test). In contrast, amplification of *MDM4*, *EGFR*, and *CDK4* was less common in genomic groups I–IV when compared to group V (Supplementary Table 5), and tumors with amplification events were less frequent in groups I–IV versus group V [group I: 1/37 (3 %); group II: 10/31 (32 %); group III: 6/47 (13 %); group IV: 4/9 (44 %); group V: 11/13 (85 %) (groups I–III versus group V: $p < 0.005$, group IV versus group V: $p > 0.05$, Fisher's exact test)].

Figure 2b illustrates the association of the array-CGH groups with PFS and OS. Patients with *IDH1/2* mutant and 1p/19q co-deleted (group I) tumors had the best outcome while patients with *IDH1/2* wild-type and +7q/−10q (group V) tumors showed the worst prognosis. However, compared with a cohort of 270 *IDH1/2* wild-type primary glioblastoma from our previous GGN study [34], group V patients had a longer median OS (2.4 years versus 1.0 years, $p = 0.008$). Patients in groups II–IV demonstrated similar survival, with outcomes being intermediate between group I and group V patients.

mRNA expression profiling

We analyzed mRNA expression using the SOM method and identified six major clusters of highly correlated tumor
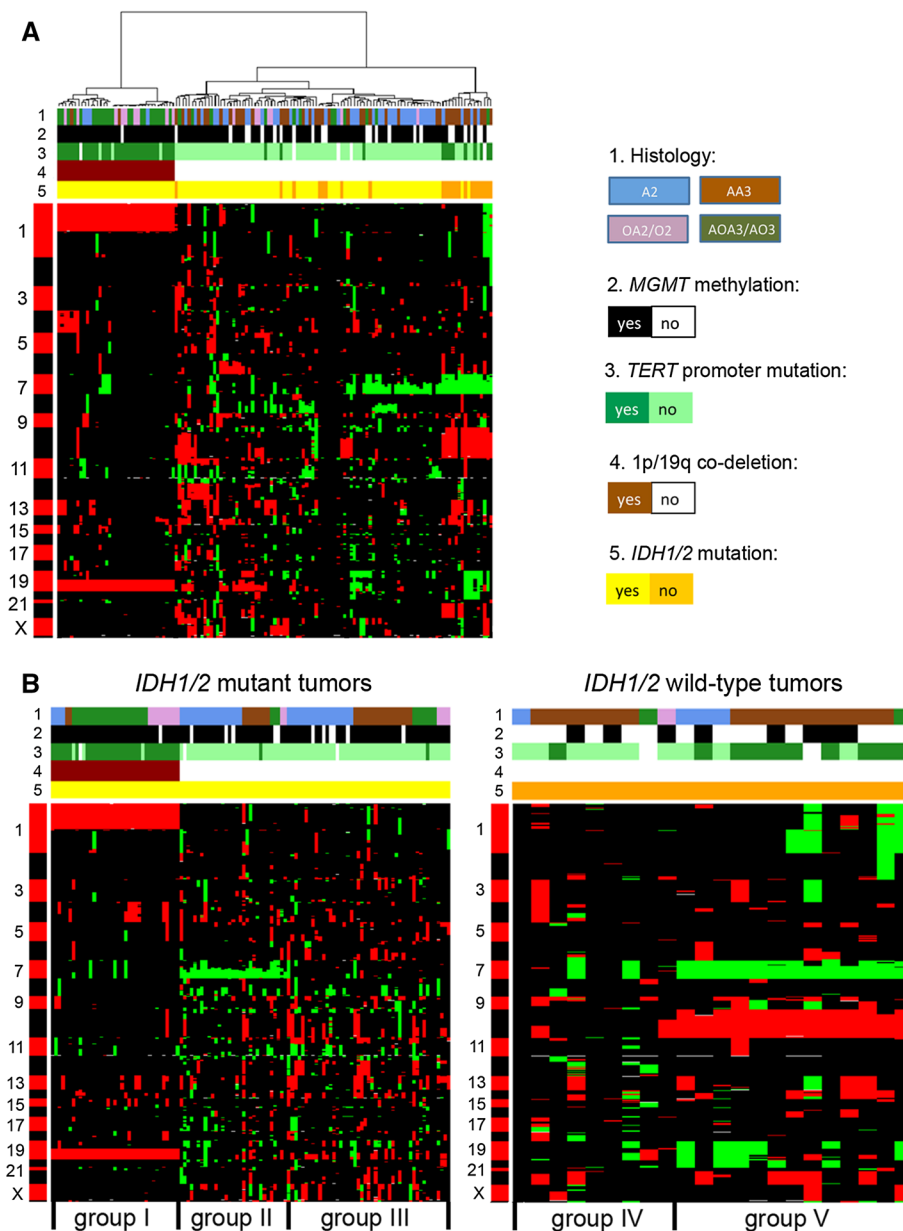
Fig. 1 *Results of array-CGH analyses.* **a** Unsupervised analysis of genomic profiles in 137 WHO grade II and III gliomas distinguishes three major clusters, one with all 1p/19q co-deleted *IDH1/2* mutant tumors (*left*), one mostly including *IDH1/2* mutant tumors without 1p/19q co-deletion (*middle*), and one mostly including *IDH1/2* wild-type tumors with +7q/-10q (*right*). A heat map indicating genomic losses in *red* and genomic gains in *green* is shown. The individual chromosomes are indicated on the left side of the heat map. The color bars on top of each heat map refer to the histological classification (1), as well as the status of *MGMT* promoter methylation (2), *TERT* promoter mutation (3), 1p/19q co-deletion (4) and *IDH1/2* mutation (5) in each tumor. **b** Supervised analyses of array-CGH data stratified by *IDH1/2* status revealed three distinct molecular groups among the *IDH1/2*-mutant gliomas (groups I–III), with group I including all 1p/19q co-deleted tumors, while group II and III tumors lacked 1p/19q co-deletion but demonstrated either gains of 7 or 7q (group II) or multiple on other chromosomes (group III). *IDH1/2* wild-type gliomas comprised two distinct tumor groups: one characterized by relatively few genomic aberrations affecting diverse chromosomes (group IV) while the other (group V) was characterized by a glioblastoma-like genomic pattern with gain of 7 or 7q and loss of 10 or 10q as marker lesions

samples, two of which closely overlapped with genomic groups I and V (Supplementary Figure 3). The individual expression clusters visible in the correlation heatmap were characterized by (1) a classical glioblastoma-like profile associated with *IDH1/2* wild-type status and anaplastic astrocytic histology (corresponding to genomic group V except for one sample without +7q/−10q status), (2) a heterogeneous glioblastoma-like profile associated with both

IDH1/2 wild-type and mutant status and with anaplastic astrocytic and oligoastrocytic histology, (3) a proneural glioblastoma-like profile associated with *IDH1/2* mutation and astrocytic histology, (4) an expression profile unrelated to known glioblastoma signatures and associated with *IDH1/2* mutation and predominantly diffuse astrocytoma WHO grade II histology, (5) a proneural glioblastoma-like profile associated with *IDH1/2* mutation, 1p/19q co-deletion and oligodendroglial histology (corresponding to genomic group I), and (6) a normal brain-like profile associated with both *IDH1/2* mutant and wild-type tumors of predominantly astrocytic histology, but also including a fraction of mixed oligoastrocytic tumors (Supplementary Fig. 3). The first expression cluster (1) consisted predominantly of *IDH1/2* wild-type gliomas whereas the vast majority of tumors in the other five expression clusters were *IDH1/2* mutant. Therefore, we additionally performed supervised analysis of metagene expression data stratified according to *IDH1/2* mutation status, which separated *IDH1/2* wild-type tumors into three major expression groups (groups 1–3). The *IDH1/2* mutant tumors were subdivided into five major expression clusters (groups 4–8) (Fig. 3a). Metagene expression data of *IDH1/2* wild-type gliomas resembled those of primary glioblastomas, while expression profiles in *IDH1/2* mutant gliomas were shared with those of *IDH1/2* mutant glioblastomas (Supplementary Fig. 2B). Gene set enrichment analysis revealed that the top enriched sets in the lists of up-regulated genes were linked to the classical (group 1) and proneural (groups 5 and 7) glioblastoma expression signatures [33], immune response processes and increased transcriptional activity (groups 2 and 4), normal brain expression signature (groups 3 and 8), astrocytic expression signatures (group 5), oxygen transport with high expression levels of hemoglobin and cytoglobin (group 6) and concerted transcriptional and mitochondrial activities associated with oligodendroglial tumor histology (group 7).

Clinical and histological correlates of the expression-based classification are summarized in Supplementary Table 6 and Fig. 3b. Analysis of patient outcome stratified according to the distinct expression groups revealed the best outcome for patients whose tumors carried a proneural glioblastoma-like profile, frequently associated with 1p/19q co-deletion (corresponding to genomic group I and expression group 7), but also for expression groups with brain-like signatures independent of *IDH1/2* mutation status (expression groups 3 and 8) and expression group 5 (*IDH1/2* mutant), the worst outcome for patients whose tumors displayed a classical glioblastoma profile associated with +7q/−10q genomic profile (corresponding to genomic group V and expression group 1) and expression group 2 of the *IDH1/2* wild-type tumors without the +7q/−10q genomic profile and without a brain-like expression signature. Patients whose tumors were classified in the remaining two expression clusters of the *IDH1/2* mutant tumors (group 4 and 6) had intermediate prognoses. Expression group 1 tumors had a similarly poor PFS but longer OS when compared to *IDH1/2* wild-type glioblastoma (Supplementary Table 6, Fig. 3b).

Integration of genomic and expression profiling results

Next, we explored the relationships between the five genomic groups identified by array-CGH analyses and the eight clusters obtained by gene expression profiling. Genomic group I (*IDH1/2* mutant, −1p/−19q) consisted mostly of expression group 7 tumors while genomic group V (*IDH1/2* wild-type, +7q/−10q) tumors closely overlapped with expression group 1 tumors (Fig. 3b, Supplementary Table 7). Genomic groups II–IV could not be matched to individual expression groups (Supplementary Table 7). Among the *IDH1/2* wild-type tumors, most genomic group V tumors showed classical glioblastoma-like expression profiles (expression group 1), while most genomic group IV tumors showed other expression profiles related to immune response processes and increased transcriptional activity (expression group 2) or a normal brain expression signature (expression group 3). The *IDH1/2* mutant genomic groups II and III both demonstrated mixed expression profiles without obvious enrichment of a certain profile in one of these groups (Fig. 4a, Supplementary Table 7).

Taken together, genomic and transcriptomic data were compatible with three molecularly distinct tumor groups that were primarily defined by genomic features and strongly linked to clinical outcome in patients with WHO grade II and III gliomas: a prognostically favorable group with *IDH1/2* mutation and 1p/19q co-deletion as the characteristic molecular marker, corresponding to genomic group I and including all expression group 7 tumors, a prognostically intermediate group mostly containing *IDH1/2* mutant tumors without 1p/19q co-deletion (genomic groups II–III) and the small group of *IDH1/2* wild-type tumors without combined gains on 7 and losses on 10 (genomic group IV), and a prognostically unfavorable group of exclusively *IDH1/2* wild-type tumors with glioblastoma-like genotype characterized by +7q/−10q, frequent gene amplification and *TERT* promoter mutation (genomic group V), including mostly expression group 1 tumors (Supplementary Table 8, Fig. 4b). Median PFS and OS differed significantly between the groups [group I versus groups II–IV: PFS 5.9 years versus 3.7 years ($p = 0.046$), OS not reached versus 9.0 years ($p = 0.028$); groups II–IV versus group V: PFS 3.7 years versus 1.5 years ($p = 0.001$), OS 9.0 years versus 2.4 years ($p < 0.001$)]. Median PFS (1.5 years) and OS (2.4 years) of the group V patients appeared to be better
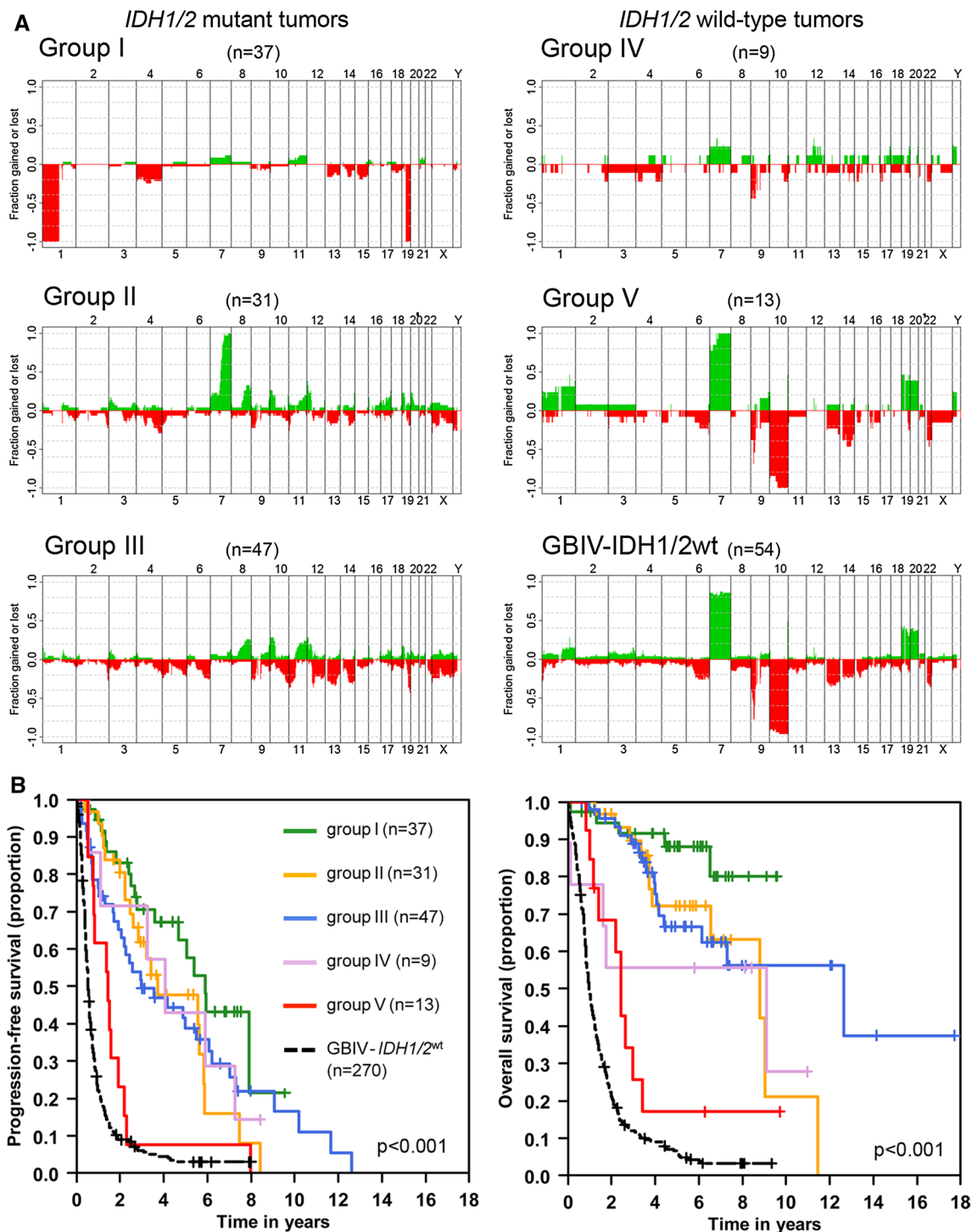
than PFS and OS of unselected glioblastoma patients, as indicated by comparison to survival data from a previous study on glioblastoma patients (PFS 0.5 years, $p = 0.026$; OS 1.0 years, $p = 0.008$) [34]. We also evaluated the prognostic role of WHO grading within these three tumor groups, which revealed that histological grading according to WHO criteria may provide some prognostic information within groups I and II–IV, although the differences in these

groups were not significant presumably because of small samples size (group I, WHO grade II versus III, $p = 0.102$ for OS; group II-IV, WHO grade II versus III, $p = 0.103$ for OS) (Fig. 4c). Age at diagnosis was a prognostic factor associated with OS in both groups (group I, ≤40 years versus >40 years, $p = 0.076$; group II–IV, ≤40 years versus >40 years, $p < 0.001$) (Fig. 4d). In group V, WHO grading and age could not be evaluated since these patients

◄ **Fig. 2** Genomic imbalance patterns according to genomic group and relationship to patient outcome. **a** Frequency plots of genomic imbalances detected in each of the five genomic groups and, for reference, a previous series of *IDH1/2* wild-type primary glioblastomas [26]. Results are stratified according to the *IDH1/2* mutation status (*left side IDH1/2* mutant tumor groups; *right side IDH1/2* wild-type tumor groups). Gains of genomic material are indicated by *green bars* while losses are indicated by *red bars*. The *bar* length indicates the percentage of tumors in each group showing gains or losses at the respective chromosomal location. The individual chromosomes are indicated on the bottom and top of each frequency plot, with markers on each chromosomes being sorted from pter on the left to qter on the right. **b** Progression-free survival (*left*) and overall survival (*right*) of patients stratified according to the five genomic groups defined by array-CGH analysis. For reference, survival of a previously published GGN cohort of 270 patients with *IDH1/2* wild-type primary glioblastoma WHO grade IV (GB IV) is shown [34]. Note that patients with group I tumors (*IDH1/2* mutant, 1p/19q co-deleted) had the best survival, while patients with group V tumors (*IDH1/2* wild-type, +7q/-10q) showed the worst survival. However, median overall survival of group V patients was still longer when compared to primary glioblastoma patients. Patients with group II-IV tumors showed similar outcomes, with survival curves running between those of group I and group V patients

were generally older than 40 years and except for three patients had WHO grade III tumors.

We also evaluated the possibility that complementation of the three DNA aberration-based prognostic groups by integrating information on mRNA expression groups might further improve prognostic stratification. Using this approach, we more strictly defined the best prognostic group ("integrated molecular group A", 26 patients) as *IDH1/2* mutant, 1p/19q deleted and group 7 expression profile tumors while the worst prognostic group was exclusively composed of *IDH1/2* wild-type, +7q/−10q and group 1 expression profile tumors ("integrated molecular group C", 10 patients). The remaining tumors not falling into either group A or group C were compiled in one intermediate prognosis group ("integrated molecular group B", 101 patients) (Supplementary Fig. 4a, Supplementary Table 8). This integrated molecular stratification again provided three prognostically distinct groups of patients (Supplementary Fig. 4b), however, it added no improvement of the three-tiered genomic stratification (Fig. 4a). Moreover, the group of patients with intermediate prognosis ("group B") increased to 101 patients and included patients with molecularly heterogeneous tumors lacking a characteristic marker profile, i.e., consisted of *IDH1/2* mutant and *IDH1/2* wild-type as well as 1p/19q co-deleted and 1p/19q intact tumors.

#### Molecular correlates of glioma type and WHO grade

The genomic aberration profiles determined by array-CGH analysis differed substantially between oligodendroglial and astrocytic as well as 1p/19q co-deleted and 1p/19q intact tumors (Supplementary Figs. 5a, b), with *IDH1/2* mutant and 1p/19q co-deleted tumors showing significantly fewer genomic alterations per tumor than *IDH1/2* mutant tumors without 1p/19q co-deletion (Supplementary Fig. 5c). Comparison of expression profiles in the 68 *IDH1/2* mutant tumors classified as astrocytic gliomas (AII, AAIII) and the 47 *IDH1/2* mutant tumors containing an oligodendroglial component (OII, OAII, AOIII, AOAIII) revealed a set of differentially expressed genes (Fig. 5a; Supplementary Table 9). Similar but even more pronounced expression differences were detected when the 37 *IDH1/2* mutant 1p/19q co-deleted tumors were compared with the 78 *IDH1/2* mutant but 1p/19q intact tumors (Fig. 5b; Supplementary Table 10). In general, oligodendroglial tumors and 1p/19q co-deleted tumors demonstrated reduced expression of a subset of genes located on 1p or 19q as well as genes related to 'immune response' and 'inflammatory response' but showed increased expression of gene signatures related to 'normal brain', synaptic transmission' and 'brain development' (Supplementary Table 11). As expected [15, 21, 38], *ATRX* was among the top genes with lower expression in astrocytic versus oligodendroglial tumors as well as 1p/19q intact versus 1p/19q co-deleted tumors (Supplementary Tables 9, 10).

Transcriptomic profiles also differed between WHO grade II and WHO grade III gliomas (Fig. 5c, Supplementary Table 12). Genes expressed at higher levels in WHO grade II gliomas were related to normal brain function, including the gene ontology (GO) terms 'normal brain' and 'synaptic transmission', while genes with higher expression in WHO grade III gliomas were related to cell division and transcriptional activity, including the GO terms 'nucleus', 'mitotic cell cycle', 'DNA repair', 'DNA replication' and 'nucleic acid binding' (Supplementary Table 11). The overall patterns of genomic aberrations were similar in WHO grade III gliomas when compared to WHO grade II gliomas. Moreover, the number of genomic aberrations detected per tumor by array-CGH did not significantly differ between WHO grade II and III gliomas, except for a trend towards more aberrations per tumor in 1p/19q co-deleted WHO grade III versus WHO grade II gliomas. Array-CGH analysis did not detect any dominant progression-associated genomic aberration according to WHO grade (Supplementary Fig. 5).

Multivariate analysis of clinical, histological and molecular parameters

To analyze the relative impact of clinical and histological parameters (age, histology, WHO grade), and molecular tumor groups as defined in Fig. 4, Supplementary Fig. 4 and Supplementary Table 8, we performed Cox regression analyses with regard to OS (Table 2). We first assessed the
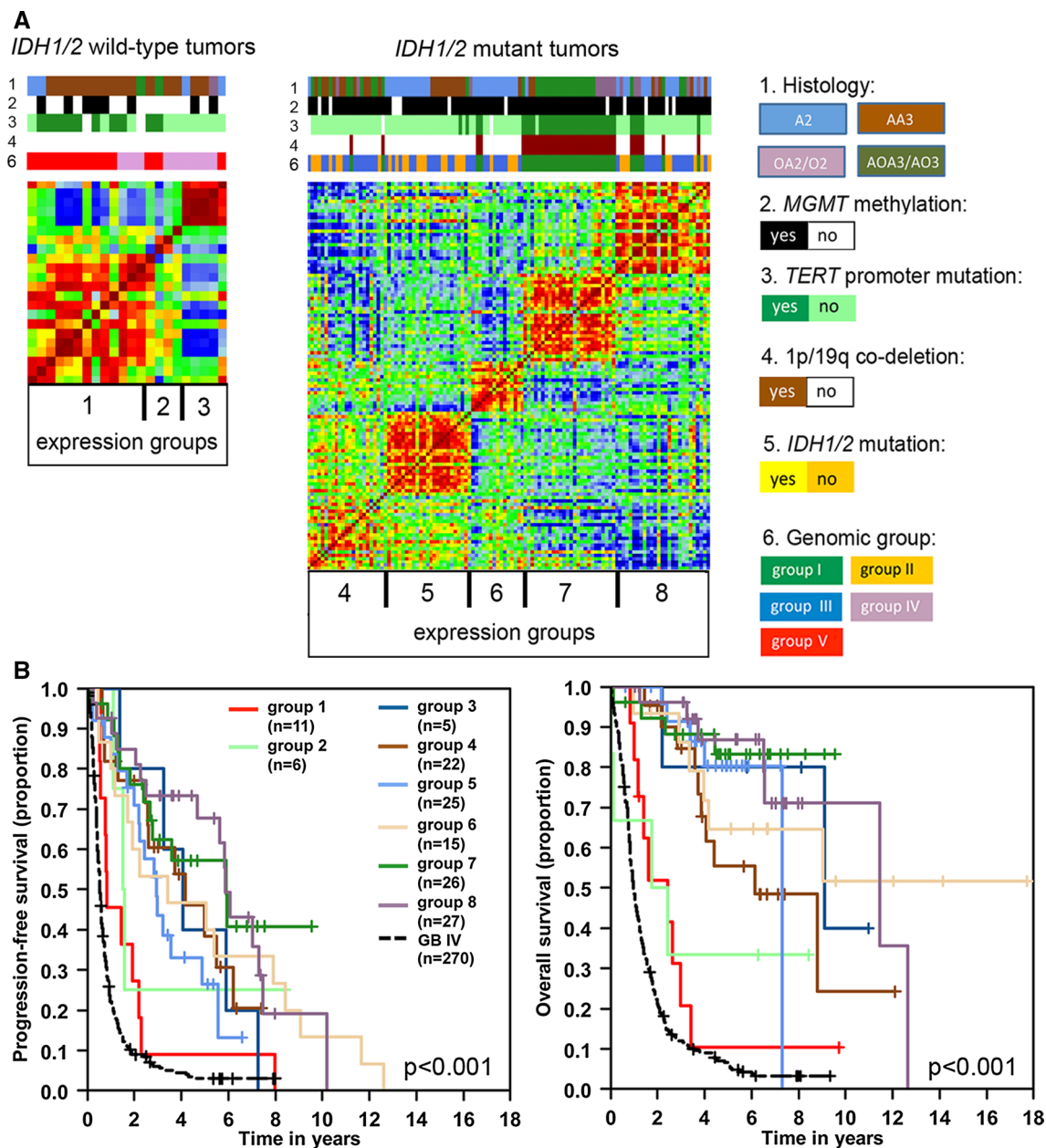
Fig. 3 *Results of mRNA expression profiling.* **a** Pairwise correlation matrices stratified according to *IDH1/2* status (left, wild-type tumors; right, mutant tumors). Note that 3 distinct expression groups (groups 1–3) were delineated in *IDH1/2* wild-type tumors while the *IDH1/2* mutant tumors were stratified in 5 distinct groups (groups 4–8). The color bars on top of each correlation matrix refer to the histological classification (1), as well as the status of *MGMT* promoter methyla-

tion (2), *TERT* promoter mutation (3), 1p/19q co-deletion (4), *IDH1/2* mutation (5), and the genomic group defined by array-CGH analysis for each tumor (6). **b** *PFS and OS in the 8 patient groups defined by expression profiling.* Survival curves of an unselected cohort of 270 *IDH1/2* wild-type glioblastoma patients [34] were added for comparison

genomic classification of groups I, II–IV or V (Fig. 4). In the first model, we only considered the most relevant clinical parameters, histology (astrocytic versus oligodendroglial), WHO grade (grade III versus II) and age (>40 years versus ≤40 years). Compared with the null model (model without any variable), this model reduced the deviance significantly. Higher age had the most significant association

with OS (HR 4.4, $p < 0.001$) followed by histology (HR 2.59, $p = 0.007$). In the second model, we replaced histology and WHO grading by an indicator variable for the three distinct genomic tumor groups I, II–IV or V. The tumor group I with the best outcome was defined as the reference group (Fig. 4b). The highest hazard ratio (HR) of 8.28 ($p < 0.001$) was seen in group V tumors and the effect
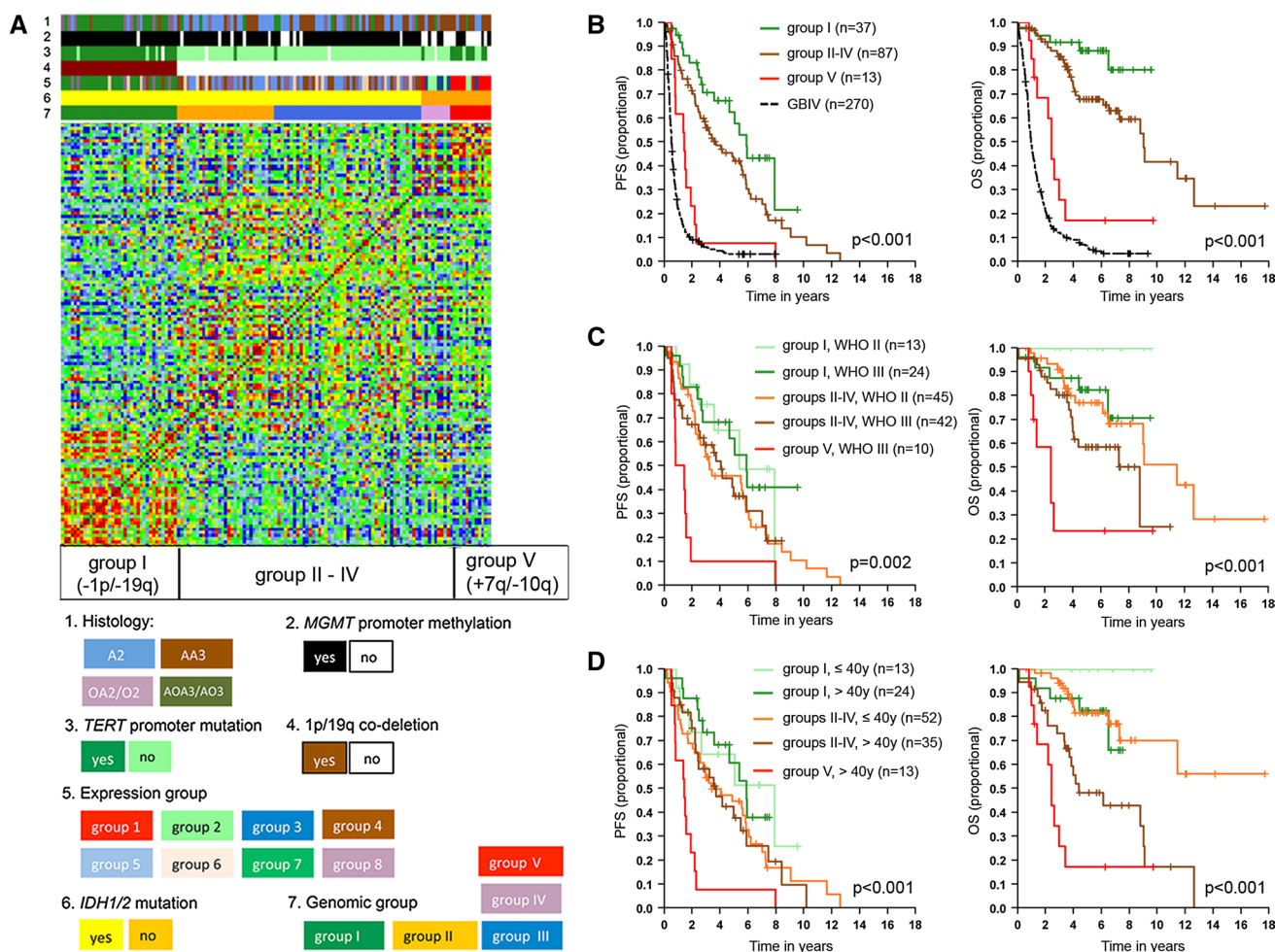
Fig. 4 Molecular classification of WHO grade II and III gliomas based on genomic profiling data. **a** Supervised pairwise correlation matrix of metagene expression data obtained by SOM analysis stratified according to *IDH1/2* status and genomic groups I–V identified by array-CGH analysis. The *color bars* on top refer to the histological classification (*1*), as well as the status of *MGMT* promoter methylation (*2*), *TERT* promoter mutation (*3*), 1p/19q co-deletion (*4*), expression group (*5*) *IDH1/2* mutation (*6*), and genomic group defined by array-CGH analysis (*7*). Note that genomic group I (*IDH1/2* mutant, 1p/19q co-deleted) tumors and group V (*IDH1/2* wild-type, +7q/−10q) tumors show an enrichment for distinct expression profiles, i.e., expression group 7 or expression group 1, further strengthening the hypothesis that these two groups reflect biologically distinct tumor entities. Genomic groups II–IV show expression profiles

distinct from group 1 and group 5, however, cannot be separated by distinctive expression profiles from each other. Since these groups shared similar patient outcomes (Fig. 2b), we combined them into a single group II–IV for further prognostic correlations. **b** Progression-free (PFS) and overall survival (OS) according to the three major genomic groups I, II–IV or V. A cohort of patients with *IDH1/2* wild-type glioblastoma WHO grade IV (GB IV) published previously [34] is included for comparison. **c** PFS and OS in the three major genomic groups stratified by WHO grade (grade II versus III) in groups I and II–IV. **d** PFS and OS in groups I and II–IV stratified by patient age (≤40 years versus >40 years). Patients in group V were all >40 years old and except for three patients had WHO grade III tumors. Thus, group V was not further stratified according to WHO grade and age

for tumors in the intermediate group II–IV was of relevant dimension (HR 3.81, $p = 0.006$). Relative risk for death was higher for age >40 years (HR 3.99, $p < 0.001$). This model showed a much better model fit than the first model. Adding histology and WHO grade to model 2 resulted only in insignificant improvements of the model fit ($p = 0.318$). The three genomic groups were by far most informative, with age ranking second. Histology and WHO grading provided the lowest independent association with OS (HR 1.36, $p = 0.426$ and HR 1.55, $p = 0.175$), indicating that

much of the information carried in histology and WHO grading has been captured by the genomic information.

When mRNA expression profiling data were introduced, that is integrated molecular profiling (Supplementary Fig. 3) considered instead of genomic profiling only, the prognostic separation into three distinct groups became less clear (Supplementary Table 13). Conversely, when the models were built on *IDH1/2* and 1p/19q status alone, some prognostic information was lost, with the HR for the poor prognosis group decreasing to 6.82, likely as a
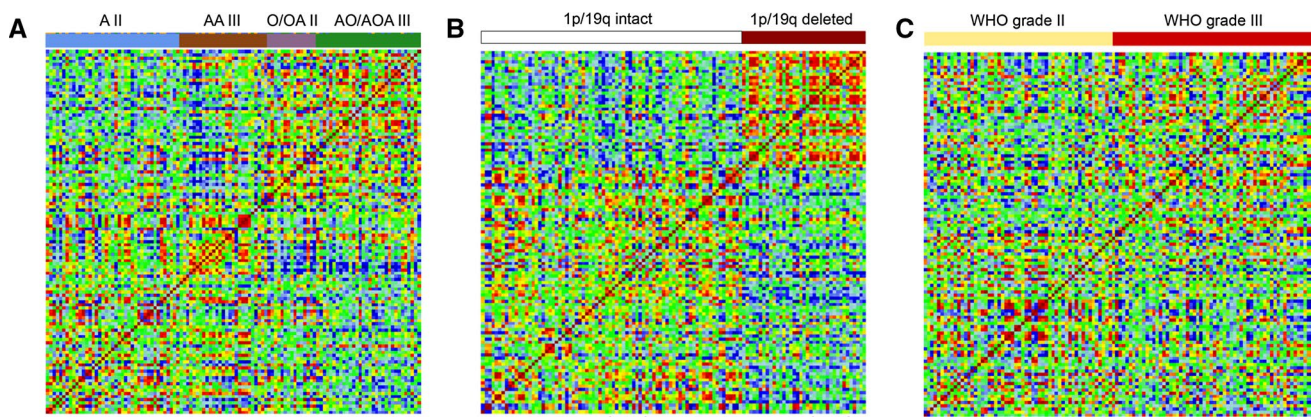
Fig. 5 Expression profiles in 115 *IDH1/2* mutant gliomas according to histological classification (**a**), 1p/19q status (**b**) and WHO grade (**c**). Supervised pairwise correlation matrices based on SOM analysis of mRNA expression data for each group-wise comparison are shown. Note distinct expression profiles between astrocytic and oligodendroglia/oligoastrocytic tumors (**a**) as well as 1p/19q intact and co-deleted gliomas (**b**), with differential gene expression differences being more when tumors were stratified according to 1p/19q status. Comparison of WHO grade II versus WHO grade III gliomas revealed evidence for differential gene expression (**c**), however, the differences being less prominent as compared to those detected between 1p/19q intact versus co-deleted tumors. Lists of differentially expressed genes and associated gene ontology terms for each group-wise comparison are provided as Supplementary Tables 9–12

consequence of including *IDH1/2* wild-type tumors without glioblastoma-like genomic alteration (Supplementary Table 13).

Discussion

Diffusely growing gliomas of WHO grade II and III remain a diagnostic and therapeutic challenge. Outcome seems to be determined more by age and molecular genetic features of the tumors than by the current treatment options of surgery, radiotherapy and alkylating agent chemotherapy. Molecular markers, in particular *IDH1/2* mutation/gCIMP and 1p/19q co-deletion, have been employed to define three major prognostic groups of WHO grade III gliomas [39]. The present study explored whether large-scale genomic or transcriptomic profiling improves prognostic stratification in comparison to histology and established molecular markers like *IDH1/2* mutation and 1p/19q co-deletion in WHO grade II and III gliomas.

Genomic and expression profiling each revealed groups of tumors with distinct genetic imbalance and gene expression patterns (Figs. 1, 2, 3). However, the independently identified genomic and transcriptomic groups only partially overlapped. Prognostic evaluation of molecular subgrouping revealed the worst survival for patients whose tumors showed a glioblastoma-like genomic profile associated with *IDH1/2* wild-type status, frequent gains on 7q and losses on 10q, often a classical glioblastoma-like expression profile, and mostly anaplastic astrocytic or oligoastrocytic histology (Fig. 4). Most tumors in this group additionally demonstrated gene amplifications and *TERT* promoter mutations.

However, despite sharing genomic imbalances and expression signature with primary glioblastomas, patients with these tumors may survive longer than *IDH1/2* wild-type glioblastoma patients (Figs. 2b, 4b), although <50 % received combined radiochemotherapy as upfront treatment (Supplementary Table 8). Our findings thus suggest that the absence of histological features of glioblastoma, that is microvascular proliferation and necrosis, in *IDH1/2* wild-type gliomas with glioblastoma-like genotypes is of prognostic relevance. In line, retrospective analyses of the NOA-04 cohort of anaplastic glioma patients and glioblastoma patients of the GGN similarly indicated a longer survival of patients with *IDH1/2* wild-type anaplastic gliomas compared to patients with *IDH1/2* wild-type glioblastomas [11, 39]. However, this assumption would need confirmation in an independent larger cohort of patients who ideally should have received identical treatment.

The best outcome was detected in patients whose tumors were characterized by *IDH1/2* mutation, 1p/19q co-deletion and oligodendroglial or oligoastrocytic histology. The majority of these tumors demonstrated a proneural glioblastoma-like expression profile, which has previously been associated with favorable outcome and oligodendroglial as opposed to astrocytic tumors [5, 7]. Moreover, the important role of 1p/19q co-deletion in anaplastic glioma patients has been shown in three independent prospective phase III trials [2, 32, 36], with recent long-term follow-up data indicating a predictive role of this biomarker for favorable response to upfront combined radiochemotherapy [3, 31]. Our present data lend further support to the importance of 1p/19q testing for reliable identification of the biologically and clinically distinct group of glioma patients showing

Table 2 Multivariate analysis based on clinical and histological parameters as well as genomic groups determined by array-CGH analysis and IDH1/2 mutation status (groups I–V)

Model	Factor	HR	95 % CI	p value	Model fit deviance	Improvement
1	Astrocytic versus oligodendroglial (ref)	2.59	1.30–5.15	0.007	374.02	Model 1 to null model: p < 0.001
	WHO grade III versus WHO grade II (ref)	1.71	0.92–3.20	0.090		
	Age > 40 versus ≤ 40 (ref)	4.40	2.21–8.79	<0.001		
2	Age > 40 versus ≤ 40 (ref)	3.99	1.95–8.16	<0.001	366.33	Model 2 to null model: p < 0.001
	Groups II + III + IV versus group I (ref)	3.81	1.46–9.93	0.006		
	Group V versus group I (ref)	8.28	2.78–24.71	<0.001		
3	Age > 40 versus ≤ 40 (ref)	4.00	1.94–8.25	<0.001	364.03	Model 3 to 2: p = 0.318
	Groups II + III + IV versus group I (ref)	3.42	1.20–9.80	0.022		
	Group V versus group I (ref)	6.45	1.93–21.60	0.002		
	Astrocytic versus oligodendroglial (ref)	1.36	0.64–2.91	0.426		
	WHO grade III versus WHO grade II (ref)	1.55	0.82–2.93	0.175		

favorable outcome with the current treatment options (genomic group I). Moreover, our data suggest that 1p/19q deletion testing is sufficient to define this particular patient group, i.e., no additional tests for other chromosomal aberrations, gene mutations or expression profiles appear to be required in the diagnostic setting. 450 k methylation bead-array profiling data of the NOA-04 biomarker cohort of anaplastic (WHO grade III) glioma patients independently validate the prognostic distinction of a gCIMP positive and 1p/19q co-deleted subgroup of patients with favorable outcome [39, 40], which essentially corresponds to our *IDH1/2* mutant and 1p/19q co-deleted genomic group I.

The other three genomic groups II–IV defined in this study were associated with similar prognoses, i.e., patients demonstrated intermediate PFS and OS when compared to genomic group I or V patients. Again, this finding fits well to the recent NOA-04 data demonstrating intermediate survival for patients with gCIMP positive, 1p/19q intact and *ATRX* mutant/deficient anaplastic gliomas [40]. However, our intermediate survival group included not only patients with *IDH1/2* mutant astrocytic or oligoastrocytic gliomas without 1p/19q co-deletion, but also a small group of patients with *IDH1/2* wild-type tumors, i.e., genomic group IV. This finding could be clinically important as it suggests that the absence of *IDH1/2* hot spot mutations in diffuse and anaplastic gliomas may not invariably be linked to less favorable outcome when compared to *IDH1/2* mutant tumors without 1p/19q co-deletion. Our data suggest that the association of *IDH1/2* wild-type status with poor survival is mainly restricted to those patients whose tumors additionally carry glioblastoma-like genomic aberrations, in particular gains on 7q combined with losses on 10q (+7q/−10q). This has implications for genetic testing and biomarker-based prognostic stratification, as the sole demonstration of *IDH1/2* wild-type status may not be sufficient to assume a particularly poor outcome. In fact, additional

testing for +7q/−10q would be necessary to identify the *IDH1/2* wild-type WHO grade II and III gliomas associated with particularly poor outcome, and possibly stratify these patients into a distinct treatment regimen. In addition, these group V patients should probably be excluded from future clinical trials on anaplastic glioma patients. Admittedly, it is possible that some of these tumors were initially undergraded simply because of sampling error although none of the diagnoses in this group were made by biopsy alone.

Whether the rare patients with *IDH1/2* mutant gliomas carrying the +7q/−10q genomic profile (6 of 115 patients in our cohort, 5.2 %) should be similarly separated from the bulk of patients with *IDH1/2* mutant gliomas in terms of future trials and clinical management remains to be determined. Recent data indicate that *IDH1/2* mutation, even in the absence of 1p/19q co-deletion, may be linked to more favorable response of anaplastic gliomas to procarbazine, CCNU and vincristine (PCV) chemotherapy when compared to *IDH1/2* wild-type tumors [4]. However, the authors did not further stratify the *IDH1/2* wild-type tumors in that study according to their +7q/−10q status. Conversely, the absence of *IDH1/2* mutations has previously been proposed to be linked to a specific predictive value of *MGMT* promoter methylation for benefit from alkylating agent chemotherapy [37].

While our study indicates an important role for molecular classification of diffuse and anaplastic gliomas, it also supports a role for histological grading according to the WHO criteria, since molecular profiling did not allow for reliable distinction of tumor grades. As stated above, patients with WHO grade III anaplastic gliomas with glioblastoma-like genetic imbalances appear to show better survival than patients with histologically classic WHO grade IV glioblastoma. Moreover, our data suggest that WHO grade tends to be prognostically relevant in patients with 1p/19q co-deleted (group I) tumors as well as in patients with group II–IV

tumors. However, these findings would need to be corroborated on larger and homogeneously treated patient series.

In line with previous studies [24, 27, 29], group-wise comparisons of expression profiling data revealed sets of genes showing differential expression in oligodendroglial versus astrocytic gliomas and/or 1p/19q co-deleted versus 1p/19q intact gliomas. Comparison of the sets of differentially expressed genes identified in our cohort (Supplementary Tables 9, 10) with those reported before [24, 28] revealed considerable overlaps (data not shown). Identified candidate genes included published markers such as *ATRX* [15, 21, 38] and alpha-internexin (*INA*) [6], but also novel candidates (Supplementary Tables 7, 8). The diagnostic utility of these candidate genes as surrogate markers for 1p/19q co-deletion, e.g., by immunohistochemical analysis, remains to be determined. We also detected sets of genes whose expression differed significantly between *IDH1/2* mutant gliomas of WHO grade II or WHO grade III (Supplementary Table 10). It remains to be demonstrated whether any of these candidate genes may serve as a diagnostically useful marker for glioma grading.

In conclusion, our large-scale molecular profiling of WHO grade II and III cerebral gliomas provides important results for improving future classification of these tumors into clinically useful categories based on the integration of histological findings and defined molecular markers as recently proposed by the Haarlem Consensus Meeting [23]. Specifically, our findings support that patients with *IDH1/2* mutant gliomas can be molecularly stratified into two prognostically distinct groups corresponding either to patients with 1p/19q co-deleted oligodendrogliomas or patients with 1p/19q intact astrocytomas. *IDH1/2* mutant oligoastrocytomas can be molecular assigned to either of these groups, thus supporting that oligoastrocytoma does not constitute a molecularly distinct glioma entity [27]. In patients with *IDH1/2* wild-type diffuse and anaplastic gliomas, presence of a +7q/-10q glioblastoma-like genomic signature was linked to poor outcome. However, whether this molecular profile is sufficient to switch the histological diagnosis of a WHO grade II or III glioma to WHO grade IV glioblastoma is currently unclear and will be a point of discussion for the upcoming revision of the WHO classification. In summary, molecular biomarker-based classification of diffuse and anaplastic gliomas may not only improve diagnostic accuracy but will also facilitate clinical decision making, and needs to be considered for patient stratification in future prospective interventional trials.

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