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Gene panel NGS of gliomas for prognostic evaluation and up-front diagnostics

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Abstract (250 words)

AIMS We aimed to reclassify a population-based cohort of 529 adult glioma patients to evaluate the prognostic impact of the 2016 World Health Organization (WHO) central nervous system tumour classification. Moreover, we evaluated the feasibility of gene panel next generation sequencing (NGS) in daily diagnostics of 225 prospective glioma patients.

METHODS The retrospective cohort was reclassified according to WHO 2016 criteria by immunohistochemistry for IDH-R132H, fluorescence *in situ* hybridization for 1p/19q codeletion and gene panel NGS. All tumours of the prospective cohort were subjected to NGS analysis up-front.

RESULTS The entire population-based cohort was successfully reclassified according to WHO 2016 criteria. NGS results were obtained for 98% of the prospective patients. Survival analyses in the population-based cohort confirmed three major prognostic subgroups, i.e. isocitrate dehydrogenase (IDH)-mutant and 1p/19q-codeleted oligodendrogliomas, IDH-mutant astrocytomas and IDH-wildtype glioblastomas. The distinction between WHO grade II and III was prognostic in patients with IDH-mutant astrocytoma. The survival of patients with IDH-wildtype diffuse astrocytomas carrying *TERT* promoter mutation and/or *EGFR* amplification overlapped with the poor survival of IDH-wildtype glioblastoma patients.

CONCLUSIONS Gene panel NGS proved feasible in daily diagnostics. In addition, our study confirms the prognostic role of glioma classification according to WHO 2016 in a large population-based cohort. Molecular features of glioblastoma in an IDH-wildtype diffuse glioma were linked to poor survival corresponding to IDH-wildtype glioblastoma patients. The distinction between WHO grade II and III retained prognostic significance in patients with IDH-mutant diffuse astrocytic gliomas.

List of Abbreviations

+7/-10 - combined whole chromosomal imbalances on chromosome 7 and 10
ATRX - alpha-thalassemia/mental retardation X-linked syndrome
cIMPACT-NOW - The Consortium to Inform Molecular and Practical Approaches to CNS Tumour Taxonomy
CNS – central nervous system
CNV – copy number variation
FFPE – formalin-fixed paraffin-embedded
FISH –fluorescence *in situ* hybridization
IDH – isocitrate dehydrogenase
IHC – immunohistochemistry
NGS – next-generation sequencing
OS – overall survival
P53 - tumour protein p53
WHO – World Health Organization

Introduction

Since the introduction of integrated "histomolecular" classification into central nervous system (CNS) tumour diagnostics in 2016 [1], the diagnosis of adult diffuse gliomas has been based on the combination of histopathologic appearance and three key defining molecular alterations, namely mutations in the isocitrate dehydrogenase genes 1 and 2 (*IDH1* and *IDH2*), whole-arm codeletion of chromosomal arms 1p and 19q, as well as the histone 3 K27M missense mutation. With these novel and more precise diagnoses, the glioma research field faces difficulties of reinterpreting results of prior prognostic studies in which tumour diagnoses were only based on histological classification. Large population-based survival analyses of patients with gliomas stratified according to WHO 2016 integrated diagnoses to further support the prognostic consequences of the WHO 2016 classification are still missing. Moreover, the role of conventional histological grading within the WHO 2016 defined glioma entities, in particular in IDH-mutant astrocytomas, is still unclear and a matter of ongoing debate [2].

New clinically relevant molecular markers continue to emerge and targeted next-generation sequencing (NGS) has become a promising approach in daily diagnostics allowing screening for genetic alterations in several diagnostic, prognostic, and predictive genes in a single analysis with a short turn-around time and at reasonable costs. Several studies have been published on NGS panels used for detection of genetic alterations in brain tumours [1, 3-13], but most studies are based on retrospective analyses. Moreover, most neuropathologic laboratories still use single gene analyses for routine detection of diagnostically important molecular biomarkers. The composition of the investigated NGS panels vary from small customized 20-130 gene panels [1, 3-5, 7-9] designed to cover the most common alterations in gliomas, to large commercially available comprehensive cancer gene panels including more than 250 genes [6, 10-12]. These studies reported NGS panels as an accurate and sensitive technique for detection of defining molecular alterations matching established methods such as immunohistochemistry (IHC) and fluorescence *in situ* hybridization (FISH).

Recently, 20 of the most common genes harbouring molecular alterations in diffuse gliomas have been selected for a customized targeted glioma-tailored gene panel (glioma panel) by Zacher and colleagues, covering mutations in *ATRX, BRAF,CDKN2A, CDKN2B, CDKN2C, CIC, EGFR, FUBP1, H3F3A IDH1, IDH2, NF1, NF2, NRAS, PIK3CA, PIK3R1, PTEN, RB1, TERT* and *TP53* [14]. This glioma panel was designed for diagnostic use in a clinico-pathological setting where quick cancer diagnostics is required and since mid-2016, it has been implemented in our institution and used up-front on all brain tumours in daily diagnostics.

The aims of this study thus were twofold: (1) To use IHC, FISH and the 20-gene glioma NGS panel to reclassify the tumours of a well-annotated population-based cohort including 529 adult glioma patients in order to obtain more precise insights in the prognostic impact of the WHO 2016 CNS tumour classification; (2) To evaluate the use of the 20-gene glioma panel NGS in up-front daily diagnostics incorporating some of the novel diagnostic mutations by performing prospective analyses of 225 glioma patients. The genetic alterations detected in both studies were combined and mutational frequencies and profiles in the distinct entities of diffuse gliomas were explored.

Materials and Methods

Patient population and glioma specimens

The retrospective population-based cohort comprised archived human glioma tissue (formalin-fixed paraffin-embedded (FFPE)) of 529 adult patients (age 18 years and over) with diffuse gliomas from two regions in Denmark: Southern Denmark and Central Denmark (Table 1). All tumours were originally classified according to the 2007 WHO CNS tumour classification [15] by neuropathologists from the Dept. of Pathology, Odense University Hospital, and the Dept. of Pathology, Aarhus University Hospital. Histologic grading was performed according to the WHO 2007 criteria which have been retained in the WHO 2016 classification [1]. All glioma tissue samples were obtained in routine clinical practice when the patients underwent initial surgery at the Dept. of Neurosurgery, Odense University Hospital between 1991-2014 or at the Dept. of Neurosurgery, Aarhus University Hospital between 2005-2009.

The prospective cohort comprised 225 adult patients with diffuse gliomas diagnosed according to WHO 2016 criteria as part of routine clinical practice when the patients underwent initial surgery at the Dept. of Neurosurgery, Odense University Hospital between February 2016 and August 2018 (Supporting Information Table S1). The patients from both cohorts had not received any treatment, except glucocorticoids, prior to initial surgery.

Molecular testing using immunohistochemistry (IHC), fluorescent *in situ* hybridization (FISH), DNA methylation profiling and glioma panel next generation sequencing (NGS)

An algorithm including IHC, FISH and targeted NGS results was used to reclassify the 529 diffuse gliomas in the retrospective cohort according to the WHO 2016 classification (Supporting Information Fig. S1). All 225 diffuse gliomas in the prospective cohort underwent targeted NGS sequencing, and results were used in the integrated diagnostic work-up together with results from IHC for IDH1-R132H, nuclear expression of alpha-thalassemia/mental retardation X-linked syndrome (ATRX) and tumour protein (p53) (Supporting Information Fig. S2). IHC staining as well as detection of 1p/19q-codeletion by FISH were performed as previously reported [16]. Twenty-six of the glioma specimens were submitted for DNA methylation profiling using Illumina Infinium Methylation EPIC BeadChip array analysis (Illumina, San Diego, USA) as previously described [17].

To investigate the prognostic impact of recent cIMPACT-NOW recommendations for diffuse astrocytic gliomas in the retrospective cohort, IDH-wildtype were stratified into tumours with or without molecular features of glioblastomas, based on identification of *TERT* promoter mutation and/or *EGFR* amplification according to the cIMPACT-NOW update 3 recommendation [18]. Diffuse astrocytic gliomas, IDH-mutant were stratified with or without *CDKN2A/B* homozygous deletion according to the cIMPACT-NOW update 5 recommendation [19].

In total, we performed gene panel NGS on 345 diffuse gliomas, including 120 gliomas of the retrospective cohort and 225 gliomas of the prospective cohort using the Ion AmpliSeq CNS Next Generation Sequencing Panel v1 (CNSv1-NGS) as a glioma-targeted custom-designed gene panel [14]. Library preparation for gene panel sequencing was carried out according to the manufacturer's protocol and has been published elsewhere [14]. NGS data were analysed for sequence variants using Ion Reporter (v5.4 through v5.10). BAM alignment files were visualized using Golden Helix GenomeBrowse 2.1.0 (Golden Helix, Bozeman, MT, USA). The following filter steps were used: (1) UCSC Common SNPs filter, (2) removal of intronic variants except for splice sites, (3) read depth greater or equal 40, (4) minimum allele

frequency of 10%, and (5) at least 10% reads from each strand. Filtered variants were individually evaluated using available public databases to identify pathogenic alterations.

Copy number variation (CNV) of *EGFR* were identified by manual interpretation of sequencing data as focal high-level amplification of the gene. Single-amplicon sample coverage relative to bi-allelic controls of specific *EGFR* exons was furthermore used to systematically define a lower cut off to distinguish low-level amplification from gain of chromosome 7. CNV of *CDKN2A/B/C* deletions and homozygous deletions of *CDKN2A/B* were identified using Ion Reporter™ 5.12 Software CNV analysis (Life Technologies). The software estimates CNVs based on an algorithm (Hidden Markov Model) build on a baseline of 49 tonsil control samples and 6 glioma tumour samples with no known CNVs in any region covered by the 20-gene glioma panel. The algorithm uses normalized read coverage across amplicons to predict the copy number or ploidy states.

Test of inter-laboratory variation and robustness of the glioma panel

NGS on 14 selected samples was performed both at the Dept. of Pathology Odense and Dept. of Pathology, Vejle Hospital, Denmark to test inter-laboratory variation and robustness of the glioma panel. NGS was also performed on 4 selected samples for validation purposes at the Institute of Neuropathology, Heinrich Heine University, Düsseldorf [14].

Statistical analyses

Unsupervised hierarchical clustering was performed by constructing two binary matrices applied to the NGS data obtained from the 225 prospective samples and the other on the 120 retrospective samples in which columns are patients and rows are the 23 selected mutations and/or chromosomal abnormalities. Each cell in these matrices was scored 0 or 1, based on the absence or presence of the particular aberration defined above. Next, the relationships between samples within the two matrices were calculated using the Simple matching coefficient of Sokal & Michener [20]followed by dendrogram construction using average clustering. The binary matrices and dendrograms were visualized by two heatmaps. All calculations were performed using the open source R-environment (R version 3.5.1, (http://cran.r-project.org/). The R-package *ade4* [21-23] and *ComplexHeatmap* [24] were used for calculating the Simple matching coefficient of Sokal & Michener and for heatmap visualization, respectively.

Survival analyses were carried out for the population-based retrospective patient cohort. Relevant clinical characteristics of this cohort stratified according to the WHO 2007 or WHO 2016 classification systems are listed in Tables 1 and 2, respectively, which provide information on patient age, clinical performance status, initial postsurgical treatment and patient survival. Overall survival (OS) was defined as time from primary surgery until death from any cause or date of censoring in November 2018. Kaplan-Meier survival curves were used for exploring differences in overall survival between WHO diagnoses using the 2007 and 2016 WHO CNS tumour classification and the recent cIMPACT-NOW recommendations for the different glioma subgroups. Log–rank tests were used for univariate comparisons, and Cox proportional hazards model were used to evaluate hazards ratios (HRs) to determine the prognostic impact of the two WHO classifications. Application of multivariable regression analysis was not performed due to insufficient numbers of patients per variable per group as well as establishment of therapeutic heterogeneous groups as a result of the WHO 2016

reclassification (Tables 1 and 2). Analyses related to patient characteristics and overall survival were carried out using STATAIC 15 (StataCorp LP) and Prism (GraphPad Software Inc., San Diego, CA, USA). Sensitivity, specificity and the concordance rates between IHC and NGS results for IDH1-R132H, ATRX and TP53 were calculated using standard statistical methods. Differences in mutational frequencies were investigated using Fisher's exact test. Significance was defined at p-values < 0.05.

Results

Reclassification of the retrospective cohort according to the 2016 WHO CNS tumour classification

The combined analysis of IHC, FISH and NGS data resulted in successful reclassification of all 529 retrospectively investigated diffuse gliomas according to the WHO 2016 classification of CNS tumours [1] (Fig. 1A) (Supporting Information, Table S2). Patient characteristics after reclassification are summarized in Table 2. The reclassification resulted in a marked decrease in the number of patients with WHO grade III oligodendroglial tumours (from 63 to 37 patients), an increase in the number of patients with glioblastomas (from 327 to 383 patients) and a reclassification of all oligoastrocytomas. Only smaller changes in patient numbers after reclassification were seen for WHO grade II and III astrocytic gliomas (from 113 to 107 patients) (Fig. 1B). Of note, at revision according to 2016 WHO classification, 19 anaplastic astrocytomas and one anaplastic oligodendroglioma were reclassified as IDH-wildtype glioblastomas based on morphological identification of focal necrosis and/or glomeruloid vascular proliferation and lack of IDH mutation and 1p/19q-codeletion.

The stratification of WHO grade II/III IDH-wildtype astrocytic gliomas according to cIMPACT-NOW recommendation 3 resulted in about half (17/39) of the tumours in the retrospective cohort being categorized as diffuse astrocytic gliomas, IDH-wildtype with molecular features of glioblastoma, WHO grade IV. Further, stratification of WHO grade II/III IDH-mutant astrocytic gliomas according to cIMPACT-NOW recommendation 5 identified *CDKN2A/B* homozygous deletions in 17% (2/12) of the tumours in the retrospective cohort.

Concordance between IHC (IDH1-R132H, ATRX, p53) and NGS results

Correlative analyses between immunohistochemical findings and NGS results were based on the combined retro- and prospective glioma cases that were subjected to NGS. In total, IHC data were available for IDH1-R132H, ATRX and p53 for 345, 299 and 296 of the 345 sequenced gliomas, respectively. IHC analysis of IDH1-R132H was well correlated with targeted NGS analysis showing high sensitivity (95%) and specificity (100%) for this mutation (Supporting information Table S3). Forty-two WHO grade II or III gliomas were positive for IDH1-R132H by IHC analysis and all harboured an IDH1-R132H mutation by sequencing. Three-hundred and one gliomas were negative for IDH1-R132H by IHC and for 2 gliomas staining results were inconclusive. NGS of these 303 gliomas identified 23 additional *IDH1* or *IDH2* mutations (*IDH1*; R132H: n=4, R132G: n=4, R132C: n=4, R132S: n=2, R132L: n=1, *IDH2;* R172K: n=3, R172S: n=2, R172M: n=1, R172W: n=1, R140W: n=1). Nuclear expression of ATRX staining as demonstrated by IHC was strongly correlated with identification of no ATRX mutation (sensitivity 98%), but loss of expression was less tightly correlated with identification of ATRX mutations (specificity 76%) (Supporting Information Table S4). Both low sensitivity and specificity was found when comparing IHC and NGS results for p53 (sensitivity 75%, specificity 63%) (Supporting Information Table S5).

Hierarchical cluster analysis and identification of distinct mutational frequencies of molecular subgroups of diffuse gliomas.

Separate unsupervised hierarchical cluster analyses were performed on the NGS data (including 1p/19q-codeletion data from FISH/850k) obtained from both cohorts (Fig. 2 and 3). In both analyses, three separate molecular subgroups dominated by distinct mutational patterns were identified: (1) IDH-mutant astrocytic gliomas with frequent mutations in *TP53* and *ATRX* (Fig. 2, 3 and 4A, green bars); (2) IDH-mutant and 1p/19q-codeleted oligodendrogliomas with frequent mutations in *TERT* promoter, *CIC* and *FUBP1* (Fig. 2, 3 and 4A, yellow and orange bars) and (3) IDH-wildtype astrocytic gliomas/glioblastomas with frequent mutations in *TERT*, *PTEN, NF1*, *TP53* and *EGFR* with increasing mutational frequencies concomitantly to increasing WHO grade (Fig. 2, 3 and 4A, blue bars).

The stratification of WHO grade II/III astrocytic gliomas in accordance to the cIMPACT-NOW recommendations mutational profiles of diffuse astrocytic gliomas, IDH-wildtype, with molecular features of glioblastomas (*TERT* promoter mutation and/or *EGFR* amplification), WHO grade IV, harboured higher, but not significantly different frequencies of mutations in *PTEN* (p=0.14) and *EGFR* (p=0.35), and lower, but also not significantly different frequencies of mutations in *TP53* (p=0.33), *NF1* (p=0.18) and *ATRX* (p=0.15) when compared with IDH-wildtype diffuse astrocytic gliomas without these molecular features (Fig.4B, blue and red). Diffuse astrocytic gliomas, IDH-mutant, with *CDKN2A/B* homozygous deletion showed higher, but not significantly different frequencies of mutations in *TP53* (p=0.99) and *NF1* (p=0.27) and lower, but also not significantly different frequencies of mutations in *ATRX* (p=0.3).

Prognostic impact of integrated diagnostics

Comparison of OS data from patients with WHO grade II diffuse astrocytomas before (WHO 2007) and after reclassification (WHO 2016), showed that patients with IDH-wildtype diffuse astrocytomas (WHO 2016) had significantly shorter OS than patients with diffuse astrocytoma diagnosed according to WHO 2007 (HR 2.74; 95% CI: 1.36-4.49; p=0.003, Fig. 5A and Table 3) as well as patients with IDH-mutant diffuse astrocytomas (HR 3.68; 95% CI: 1.94-6.97; p=0.000, Fig. 5A and Table 4). OS of patients with IDH-mutant diffuse astrocytomas (WHO 2016) showed a trend towards longer OS when compared to patients with diffuse astrocytomas according to WHO 2007, but this difference was not statistically significant (Fig. 5A and Table 3). Patients with IDH-mutant anaplastic astrocytomas (WHO 2016) showed significantly longer OS compared to patients diagnosed with anaplastic astrocytomas (WHO 2007) (HR 0.44; 95% CI: 0.26-0.75; p=0.002, Fig. 5B and Table 3). In contrast, patients with IDH-wildtype anaplastic astrocytomas (WHO 2016) showed a trend towards shorter OS when compared to patients with anaplastic astrocytomas classified according to WHO 2007, although this difference did not reach statistical significance (Fig. 5B and Table 3). Similar associations were found in the WHO grade IV glioblastoma group (Fig. 5C and Table 3). Comparison of OS of patients with WHO grade II versus III oligodendroglial tumours (WHO 2007) showed shorter survival of patients with anaplastic tumours of WHO grade III (Fig. 4D). For patients with IDH-mutant and 1p/19q-codeleted oligodendroglial tumours, this survival difference was smaller, but a trend towards shorter OS remained for patients with WHO grade III anaplastic oligodendroglioma (HR 2.17; CI: 95% 0.88-5.31; p=0.091). For the oligodendroglial tumours, patients with IDH-mutant and 1p/19q-codeleted anaplastic oligodendrogliomas of WHO grade III showed significantly longer OS

when compared to patients with anaplastic oligodendrogliomas classified according WHO 2007 (HR 0.44; 95% CI: 0.21-0.92; p=0.029, Fig. 5D and Table 3).

An overall comparison (Fig. 5E and Table 4) of WHO 2016 diagnoses confirmed a clear prognostic value of IDH mutation in the different glioma subgroups. Importantly, we also found that WHO grading of astrocytic gliomas had a significant effect on OS, including patients with IDH-mutant astrocytomas (WHO grade II vs. III: HR 2.90; 95% CI: 1.61-5.20; p<0.001, WHO grade II vs. IV: HR 4.75; CI: 95% 2.30-9.77; p<0.001) and patients with IDH-wildtype astrocytomas (WHO grade II vs. III: HR 2.67; CI: 95% 1.33-5.38; p=0.006, WHO grade II vs. IV: HR 2.51; CI: 95% 1.48-4.27; p=0.001). Comparing patients with astrocytomas across IDH mutation status and WHO grade showed a more favourable outcome of patients with WHO grade IV IDH-mutant glioblastoma in comparison to patients with WHO grade III IDH-wildtype anaplastic astrocytomas (HR 0.39; CI: 95% 0.18-0.84; p=0.015), but no statistical difference in outcome between WHO grade IV IDH-mutant glioblastoma patients and WHO grade II IDH-wildtype diffuse astrocytoma patients, due to contamination of the IDH-wildtype groups by prognostically unfavourable tumours with molecular features of glioblastoma. Finally, we investigated the prognostic role of novel molecular alterations in WHO grade II/III diffuse astrocytic gliomas. Patients with IDH-wildtype astrocytomas with molecular features of glioblastomas, WHO grade IV [18] (n=17) and IDH-wildtype glioblastomas WHO grade IV (n=370) showed no survival difference (HR; 0.85 CI: 95% 0.51-1.44; p=0.55) (Fig. 5F), whereas comparison of IDH-mutant astrocytomas with (n=2) and without (n=10) *CDKN2A/B* deletions indicated (only twelve tumours included, low statistical power) prognostically unfavourable outcome in patients carrying the molecular alteration (Supporting Information, Fig.S3).

Feasibility, robustness and detection of potential actionable therapeutic targets with the glioma panel in daily routine diagnostics

Integrated histomolecular diagnoses were established for all 225 gliomas prospectively investigated in the daily diagnostic setting. The patient demographics and results of important findings are summarized in Table S6. The feasibility of the glioma panel in a daily clinico-pathological setting was assessed by the number of samples found with reduced quality parameters compared to the number of total sequenced samples in the prospective cohort. Tissue samples to be characterized molecularly included all types from a routine diagnostic setting with varying tissue quality and amounts. We found that 2.2% (5/225) of the samples had reduced quality parameters based on low RNase_P and DNA concentrations. Retrospectively, these samples were evaluated and only one sample was found with abundant necrosis that could explain the reduced quality parameters. Despite reduced quality parameters, we were able to detect mutations in all five gliomas.

Potentially actionable molecular targets, defined as targetable alterations by FDA-approved drugs, were detected in 67% (110/163) of the glioblastomas in the prospective cohort. Some cases had more than one targeted with the genetic alterations being considered as targetable when there is either an approved or investigational therapy available (Supporting Information Table S7). Additionally, the robustness of the glioma panel was evaluated across two external laboratories with an overall good inter-laboratory reproducibility (Supporting Information Table S8).

Discussion

The molecular reclassification of a large population-based cohort of glioma patients revealed considerable changes in the glioma diagnoses between the WHO classifications of 2007 and 2016, with the main shifts being in the number of WHO grade III oligodendroglial tumours and WHO grade IV glioblastomas, and to a lesser extent shifts in the numbers of WHO grade II and III astrocytomas. For example, only 11 of 43 tumours histologically classified as anaplastic oligodendrogliomas were molecularly confirmed to be IDH-mutant and 1p/19q-codeleted anaplastic oligodendrogliomas. The majority of the remaining WHO 2007 anaplastic oligodendrogliomas were reclassified as either IDH-wildtype or IDH-mutant astrocytic gliomas/glioblastomas according to WHO 2016, which of course bears major consequences on post-surgical therapy and prognosis [25]. Recent studies by Iuchi *et al.* [26], Orhirjav *et al.* [27] and Brito *et al.* [28] also reported oligodendroglial tumours and WHO grade II/III astrocytomas as the main targets of reclassification, while the French nationwide POLA cohort (cohort of high-grade glioma with an oligodendroglial component) [29] showed most frequent classification changes among astrocytomas and glioblastomas, but less common re-classification of oligodendroglial tumours. The differences between these studies are largely related to the distribution of glioma subgroups in the individual cohorts and variable stringency in the histological criteria used for oligodendroglioma classification [30]. Our results thus reflect the main effects of the WHO 2016 classification in a population-based cohort of diffuse glioma patients.

One of the major benefits of applying the NGS panel sequencing in routine glioma diagnostics concerns detection of a spectrum of genetic alterations with prioritized, diagnostic impact including rare mutations that cannot be investigated by standard IHC panels. For example, non-canonical *IDH1* or *IDH2* mutations are not detectable by IHC with the antibody against IDH1-R132H [31]. In our series of WHO grade II and III gliomas, an additional 8% (23/303) of *IDH1* or *IDH2* mutations were detected by NGS and this led to a change in diagnosis in up to 8% of the cases. Additional important findings provided by NGS were *TERT* promoter mutation, *EGFR* amplification and *CDKN2A/B* homozygous deletion, which have become diagnostically relevant in WHO grade II/III astrocytic gliomas. In our series of WHO grade II and III gliomas, 30% (22/59) of IDH-wildtype gliomas carried a *TERT* mutation and/or *EGFR* amplification and 20% of IDH-mutant gliomas showed *CDKN2A/B* homozygous deletion. This results in change of diagnosis according to cIMPACT-NOW recommendations. *H3F3A* K27M and *BRAF* V600E were also detected by gene panel NGS in our study. In the retrospective cohort, NGS revealed one *H3F3A* K27M mutation and one *BRAF* V600E, which upon review led to reclassification of two anaplastic astrocytomas as a diffuse midline glioma, H3-K27M-mutant and an anaplastic pleomorphic xanthoastrocytoma, respectively. Even though both *H3F3A* K27M and *BRAF* V600E mutations now can be reliably detected by immunohistochemistry [32, 33], targeted NGS facilitate identification of a broad spectrum of mutations up-front, which is useful in the differential diagnosis of diffuse gliomas.

As a result of our setup using both conventional testing with IHC analysis (IDH-R132H, p53, ATRX) and glioma gene panel NGS, we were able to compare results from both assays. In line with previous studies [1, 3, 6, 26] we found that IDH1 R132H IHC analysis was highly concordant with results of NGS, thus further confirming the reliability of this antibody in the diagnostic setting (Supporting information, Table 3). IHC and mutational status of *ATRX* also was found to be well-correlated with a 95% concordance rate, but loss of ATRX expression was not consistent with *ATRX* mutation in 19% of the gliomas. These findings are similar to results found in other studies and have been related to technical

limitations or complex mutational and expressional patterns [14, 34] that need to be further investigated. In contrast, assessment of p53 expression by IHC and detection of *TP53* mutations were not well correlated (concordance rate of 67%), consistent with the wide range of concordance rates shown previously in gliomas [35]. The poor correlation may be explained by imperfect cut-off criteria, rare truncating mutations without nuclear p53 accumulation, and non-tumour cells showing p53 expression [31]. IHC analysis results of p53 should therefore be interpreted with caution in the differential diagnostics.

The mutational profiles identified for each molecular subgroup of gliomas in this study as well as the frequencies of mutations for each subgroup were highly comparable with results obtained in large-scale sequencing studies like The Cancer Genome Atlas (TCGA) project [36, 37] as well as targeted NGS panel studies [1, 3, 5, 7-9], thereby supporting that targeted NGS is a robust technology with high sensitivity and inter-laboratory reproducibility.

Unfortunately, it emerged that the amplicon-based glioma panel NGS was not able to detect mutational frequencies for the challenging *TERT* promoter at high sensitivity on all analysed tumour samples due to low read depth around the two mutational hot spots ("C228T" and "C250T"). As a consequence for example, in IDH-mutant and 1p/19q-codeleted oligodendroglial tumours, we thus observed a frequency of *TERT* promoter mutations of 61% in WHO grade II tumours and 70% in WHO grade III tumours, whereas the reported frequency in studies using deep sequencing and frozen tissue is > 95% [36, 38]. Only few targeted NGS panels include the *TERT* promoter and different authors report challenges to some degree with the detection of mutations in the *TERT* promoter [5, 7, 9, 14]. Challenges related to low or variable read depth from amplicons in GC-rich regions (e.g. the *TERT* promoter) as well as large amplicons (e.g. the coverage for *RB1* was 90%) are known limitations of the IonTorrent AmpliSeq panels [39]. Capture-based sequencing is an alternative and commonly used method, shown to be superior to amplicon-based sequencing by providing higher uniformity of coverage depth and higher sensitivity for variant calling [40]. Novel molecular methods, such as droplet digital PCR and qPCR-based allele specific assays are promising new techniques for fast and sensitive *TERT* promoter mutation detection [41, 42], which indeed is needed in molecular classification of diffuse gliomas.

A major aim of this study was to molecular reclassify a large retrospective cohort of diffuse glioma patients to get further insights into the prognostic associations of integrated diagnostics of adult gliomas according to the 2016 WHO classification. Comparison of survival data from patients with gliomas classified according to WHO 2007 versus WHO 2016 diagnoses revealed that addition of molecular biomarkers significantly alters OS associated with several entities.

Diffuse astrocytomas (WHO grade II) in our retrospective cohort were roughly divided into 70% IDH-mutant and 30% IDH-wildtype tumours consistent with prior studies [43, 44]. Patients with IDH-wildtype diffuse astrocytomas showed significantly shorter OS compared to patients with IDH-mutant diffuse astrocytomas. Similarly, patients with IDH-mutant anaplastic astrocytomas showed a significantly longer OS when compared to patients with the respective IDH-wildtype entity. Taken together, these findings support previous studies that the majority of IDH-wildtype diffuse and anaplastic astrocytomas in adults in fact resemble histologically underdiagnosed IDH-wildtype glioblastomas [45]. The cIMPACT-NOW consortium recently published a third update introducing *EGFR* amplification, *TERT* promoter mutation, and/or combined whole chromosomal imbalances on 7 and 10 (+7/-10) as minimal molecular diagnostic criteria for a new tumour

category termed diffuse astrocytic glioma, IDH-wildtype, with molecular features of glioblastoma, WHO grade IV [18]. In line with this new recommendation, and results published very recently by Tesileanu *et al.* [46], we found that OS of patients with IDH-wildtype diffuse or anaplastic astrocytomas carrying *TERT* promoter mutation and/or *EGFR* amplification completely overlapped with OS of patients with IDH-wildtype glioblastomas. This finding would argue in favour of considering the traditional histological features of malignancy, i.e., necrosis and/or microvascular proliferation, and/or the presence of molecular alterations as defined by cIMPACT-NOW [18] in a revised definition of glioblastoma, IDH-wildtype, WHO grade IV.

As expected, patients with IDH-mutant and 1p/19q-codeleted anaplastic oligodendroglioma showed significantly longer OS when compared to patients with only histologically defined anaplastic oligodendrogliomas, which were found to be significantly contaminated by IDH-wildtype glioblastomas. In patients with WHO grade II oligodendrogliomas, OS was only slightly better for the group with IDH-mutant and 1p/19q-codeleted oligodendrogliomas, which to a larger extent overlapped with the WHO 2007 classified oligodendroglioma WHO grade II group.

Taken together, the comparative survival analyses obtained in this study indicate that part of the results from prognostic association studies prior to WHO 2016 may still have value, but that prognostic results based on cohorts classified only by histology according to prior WHO criteria may not be comparable anymore, in particular for patients with diffuse gliomas of WHO grade II or III.

In agreement with recent molecular reclassification studies, we showed a distinct prognostic segregation of glioma subtypes with the 2016 WHO classification as well as a prognostic significance of IDH mutation over histological tumour grade [26-29, 47]. Interestingly, however, was the significant prognostic difference found in OS between WHO grade II and III IDH-mutant astrocytoma patients. Previous retrospective studies [48, 49] have shown only modest effect of histological grade on OS in IDH-mutant astrocytomas. However, Shirahata *et al.* [50] reported a significant difference in OS between WHO grade II and III IDH-mutant astrocytomas, although the difference decreased after further molecular stratification based on *CDKN2A* homozygous deletion. Because there were only a few retrospective IDH-mutant astrocytomas with sequencing data (n=12), we were not able to investigate whether the difference in OS in our series were caused by uneven distribution of *CDKN2A/B* homozygous deletion. Similarly, and in line with our data, Cimino *et al.* [51] and Yang *et al.* [52] reported on a prognostic role of WHO grade in IDH-mutant astrocytoma patients.

We have used glioma gene panel NGS up-front in daily diagnostics since mid-2016 where the panel has been part of the routine diagnostics workflow at our institution. Successful prospective NGS analyses were obtained for 98% of the gliomas in the prospective cohort. Since the panel was implemented in 2016, our practice and workflows have been stepwise optimized to shorten the turn-around time. Initially, we had a glioma NGS panel run once per week, later on twice per week and now in our current setup, we run the glioma panel daily together with other cancer panels (lung, colon) providing a sufficient sample load to fill up the chips used in each run. With this setup, our turn-around time from time of arrival of the sample until the final integrated pathology report is at best 7 working days (Supporting Information, Fig. S4). The procedure comprises different steps and delays can occur due logistic challenges between the steps. For a few

cases, where the NGS must be repeated, due to e.g. poor tissue quality, the turn-around time can increase up to around 2 weeks.

Panel sequencing is a relatively expensive diagnostic method in comparison with conventional methods like IHC. The costs of a single IHC section with a mutation-specific antibody are approximately 20 € with personnel time included and since we usually perform around 6 immunostains per tumour, the overall costs per patient sample are close to 120 €. Overall costs per patient sample using the 20-gene glioma panel are close to 600 € (Supporting Information Table S9). Even though the costs and turn-around times for IHC are only a fraction of those of panel sequencing, we consider panel sequencing as cost-effective, especially if the approach is focussed on those gliomas patients not suspected to have a glioblastoma and being 55 years or older. When used up-front, the information on the broad spectrum of mutations in multiple genes is available in time and therefore NGS panels can be a more cost-effective way to reach an accurate molecular diagnosis as costs of additional testing are reduced. Further, the costs of treatment are significantly higher than the costs of NGS testing. Allocation of patients to the right treatment early on avoid unnecessary treatment costs and lead to more efficient use of healthcare resources. These economic aspects must be taken in to consideration, when justifying the use of NGS panels.

From a 2020 diagnostic perspective, the glioma panel we have been using too date has some limitations. Detection of 1p and 19q loss was not reliable due to limitations in the NGS panel design. Detection of CNVs in addition to 1p/19q-codeletion, such as +7/-10 and *EGFR* amplification, has become of increasing importance especially after these biomarkers have been included in a recent recommendation by cIMPACT-NOW [18]. To improve identification of CNVs in general, incorporation of highly polymorphic SNPs (single-nucleotide polymorphisms) across relevant chromosomes in the panel would significantly improve the assessment of combined losses on 1p and 19q, combined +7/-10 as well as deletions of *CDKN2A/B,* with the latter becoming diagnostically relevant for prognostic assessment of IDH-mutant astrocytic gliomas [50]. The principle of SNP incorporation into a targeted gene panel enables a single test for routine brain tumour diagnostics in a molecular pathology laboratory and offers an attractive alternative to methods such as FISH and immunohistochemistry [53].

In conclusion, we report that glioma gene panel NGS is a robust approach for detection of diagnostically relevant genetic alterations in gliomas in the daily routine diagnostic setting. Using NGS in combination with IHC and FISH for reclassification allowed for the comparison of prognostic associations in glioma subgroups defined by histological criteria according to the WHO 2007 classification or by integrated histomolecular criteria according to the WHO 2016 classification and recent cIMPACT-NOW updates. For the classification of IDH-wildtype diffuse astrocytic gliomas, we provide further support for a role of *TERT* promoter mutation and *EGFR* amplification as molecular markers for aggressive tumours corresponding prognostically to IDH-wildtype glioblastoma. We additionally found that distinction of WHO grade II and III astrocytic tumours retained prognostic significance in the group of IDH-mutant astrocytoma patients. The future value of NGS panels in daily brain tumour diagnostics will require continued adjustment to novel diagnostic criteria so that the need of complementary single gene-based methods can be reduced.

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Ethical approval

The study was approved by the Regional Committee on Health Research Ethics for Southern Denmark (Project-ID S-20150148) as well as the Danish Data Protection Agency (file number: 16/11065). The use of tissue was not prohibited by any patient according to the Danish Tissue Application Register.

Data Sharing

All data underlying the findings reported in the manuscript have been submitted as part of the article.

Disclosure

The authors declare no conflicts of interest.

Conflict of Interest

The authors declare that they have no conflict of interest. Prof. Guido Reifenberger, member of the NAN editorial committee, has not been involved in the review process of this manuscript.

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777

778 **Figure legends**

779

780 Figure 1

781 Results of molecular reclassification of 529 adult diffuse gliomas from the retrospective cohort according to the WHO
782 2016 CNS tumour classification. A) The diagram shows the diagnostic change between histological diagnosis (WHO
783 2007, left side) and integrated histomolecular diagnosis (WHO 2016, right side). B) Summary of the diagnostic changes
784 in the glioma groups.

785

786 Figure 2

787 Unsupervised hierarchical cluster analysis performed on NGS data from 120 gliomas from the retrospective cohort.
788 1p/19q-codeletion status (FISH/850k) is included in the analysis. The analysis revealed three separate molecular
789 subgroups in line with the WHO 2016 Classification of CNS tumours; IDH-mutant astrocytic tumours, IDH-mutant and
790 1p/19q-codeleted oligodendroglial tumours and IDH-wildtype astrocytic tumours. Presence and absence of a mutation
791 were coloured black and grey, respectively. The WHO 2007 and WHO 2016 diagnoses are shown and coloured in the
792 column annotation above the heatmaps indicating the shift between the former histology-based classification and the
793 current integrated histomolecular classification. Army green bar indicate failed detection of *TERT* in some samples due

to low coverage. * indicate *EGFR* mutations, ** indicate high copy number *EGFR* amplification, *** indicate *EGFR* deletion variant, **** indicate homozygous deletion of *CDKN2A/B*.

Figure 3

Unsupervised hierarchical cluster analysis performed on NGS data from 225 gliomas from the prospective cohort. 1p/19q-codeletion status (FISH/850k) is included in the analysis. The analysis revealed three separate molecular subgroups in line with the WHO 2016 Classification of CNS tumours; IDH-mutant astrocytic tumours, IDH-mutant and 1p/19q-codeleted oligodendroglial tumours and IDH-wildtype astrocytic tumours. Presence and absence of a mutation were coloured black and grey, respectively. The WHO 2016 diagnoses are shown and coloured in the column annotation above the heatmaps. Army green bar indicate failed detection of *TERT* in some samples due to low coverage. * indicate *EGFR* mutations, ** indicate high copy number *EGFR* amplification, *** indicate *EGFR* deletion variant, **** indicate homozygous deletion of *CDKN2A/B*.

Figure 4

A summary of genetic alterations identified in 345 gliomas analysed (combined retro- and prospective data) with the glioma panel.

A) Frequencies of gene mutations and CNVs across the glioma entities of the 2016 WHO classification. The different entities have distinct mutational profiles.

B) Frequencies of gene mutations and CNVs found in WHO grade II/III astrocytic gliomas. These molecular alterations are of diagnostic importance as described in the cIMPACT-NOW recommendation 3 (*TERT* promoter mutation, *EGFR* amplification, combined whole chromosome 7 gain and whole chromosome 10 loss) and the cIMPACT-NOW 5 recommendation (*CDKN2A/B* homozygous deletion).

* indicate *EGFR* mutations, ** indicate high copy number *EGFR* amplification, *** indicate *EGFR* deletion variant, **** indicate homozygous deletion of *CDKN2A/B*.

Figure 5

Kaplan-Meier overall survival curves showing the association between glioma subtypes classified in accordance to the current 2016 CNS tumour classification and the former WHO 2007 CNS tumour classification. Log-rank p-values are coloured, indicating comparison of astrocytic tumours: green: IDH-mutant, WHO 2O16 vs. WHO 2O07, blue: IDH-wildtype, WHO 2016 vs. WHO 2007, grey: IDH-mutant, WHO 2016 vs. IDH-wildtype, WHO 2016. For the oligodendroglial tumours yellow p-value indicate comparison between WHO grade II tumours (WHO 2016 vs. WHO 2007) and orange p-value indicate comparison between WHO grade III tumours (WHO 2016 vs. WHO 2007). E) Overall comparison of WHO 2016 diagnoses. F) Comparison of diffuse astrocytic gliomas, IDH-wildtype, with molecular features of glioblastomas, WHO grade IV and IDH-wildtype glioblastomas, WHO grade IV.

Figure S1

Flow diagram on molecular testing approach used on the retrospective cohort

All gliomas in the retrospective cohort were initially stained by IHC with antibodies against IDH1-R132H and in most cases also ATRX and p53. To identify less common *IDH1* and *IDH2* mutations not detectable by IHC, targeted NGS analysis was performed when the IDH1-R132H staining was negative. Status on *TERT* and *EGFR* amplification was investigated in the remaining WHO grade II and III IDH-wildtype astrocytomas for stratification according to cIMPACT-NOW recommendation 3. All WHO grade II and III IDH-mutant gliomas with retained nuclear ATRX expression were further tested for 1p/19q-codeletion by FISH (or 850k DNA methylation arrays) to refine the classification of gliomas into IDH-mutant astrocytomas or IDH-mutant and 1p/19q-codeleted oligodendrogliomas. IDH-mutant gliomas without 1p/19q-codeletion were tested for *CDKN2A/B* homozygous deletions for stratification according to cIMPACT-NOW recommendation 5. For glioblastomas, IDH status was investigated by IHC and not followed by targeted NGS analysis. NGS was not performed on WHO grade IV gliomas as the majority of included patients with IDH-wildtype glioblastoma (86%, 280/327) were older than 55 years at the time of diagnosis. Thereby, we followed the proposed age cut-off given by the WHO 2016 classification for IDH molecular testing [54].

Figure S2

Flow diagram on molecular diagnostic approach used on the prospective cohort (2016-2018)

All gliomas in the prospective cohort underwent NGS panel sequencing and results were used in the integrated diagnostic work-up together with results from IHC including IDH1-R132H, ATRX, p53 and 1p/19q-codeletion status (FISH or 850k). Stratification according to cIMPACT-NOW recommendation 3 and 5 was done to obtain mutational profiles and not used for final diagnostic classification in the study period.

Figure S3

Kaplan-Meier overall survival curve of WHO grade II/III IDH-mutant astrocytomas with and without *CDKN2A/B* homozygous deletion.

Figure S4

Flow diagram of NGS workflow and the 7-day turn-around time.

Turn-around time for the used NGS setup from time of arrival of the sample until the final integrated pathology report.is 7 working days.

Table 1 Patient characteristics – retrospective cohort

	DA		AA		GBM		OA		AOA		OD		AOD	
	N	%	N	%	N	%	N	%	N	%	N	%	N	%
Patients	60		53		327		11		15		20		43	
Age in years (mean, range, ±SD)	43.6 (18.2-78.5) ±15.45		57.4 (20.35-83.9) ±16.17		62.8 (27.1-89.0) ±11.60		53.6 (27.0-74.8) ±17.20		60.0 (31.9-82.3) ±15,34		46.3 (26.2-75.2) ±11.72		57.3 (25.8-79.1) ±12.13	
Gender														
Male	39	65	33	62.2	201	61.5	10	90.9	6	40	11	55.0	27	62.8
Female	21	35	20	37.8	126	38.5	1	9.1	9	60	9	45.0	16	37.2
Performance status														
0-1	30	88.2	24	68.6	222	70.6	10	90.9	12	80.0	16	88.9	36	83.7
2-4	4	11.8	11	31.4	93	29.4	1	9.1	3	20.0	2	11.1	7	16.3
Unknown	26		18		12		0		0		2		0	
Status														
Alive	15	25	0	0	6	1.8	5	45.4	0	0	10	50.0	7	16.3
Dead	45	75	53	100	321	98.2	6	54.6	15	100	10	50.0	36	83.7
Treatment														
None	35	92.1	5	13.9	35	10.8	9	81.8	3	20	17	85.0	6	14
Stupp protocol	0	0	4	11.1	208	64.1	0	0	2	13.3	0	0	5	11.6
Radiotherapy, 59 Gy/- chemotherapy	2	5.3	21	58.3	23	7.1	2	18.2	9	60	3	15.0	27	62.8
Radiotherapy, 34 Gy +/- chemotherapy	1	2.6	6	16.7	51	15.8	0	0	0	0	0	0	4	9.3
Chemotherapy alone	0	0	0	0	8	2.2	0	0	1	6.7	0	0	1	2.3
Unknown	22		17		2		0		0		0		0	
Survival (median, months)	65.9		13.9		11.97		72.0		17.0		138.8		12.4	

DA = diffuse astrocytoma, WHO grade II, AA = anaplastic astrocytoma, WHO grade III, GBM = glioblastoma, WHO grade IV, OA = oligoastrocytoma, WHO grade II, AOA = anaplastic oligoastrocytoma, WHO grade III, OD = oligodendroglioma WHO grade II, AOD = anaplastic oligodendroglioma, WHO grade III.

Table 2 Patient characteristics after reclassification of the retrospective cohort in accordance to the 2016 WHO CNS classification.

	DA IDH mutant		DA IDH wildtype		AA IDH mutant		AA IDH wildtype		GBM IDH mutant		GBM IDH wildtype		OD IDH mutant		AOD IDH mutant	
	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%
Patients	46		16		22		23		13		370		22		15	
Age in years (mean, range, ±SD)	37.3 (18.2-69.2) ±11.45		57.1 (23.6-78.5) ±14.36		42.5 (20.4-66.2) ±12.74		59.6 (31.8-83.9) ±14.47		50.8 (27.2-79.1) ±17.30		63.4 (25.8-89.0) ±10.83		49.1 (26.2-74.8) ±13.26		56.4 (33.9-72.7) ±13.25	
Gender																
Male	28	60.9	11	68.8	14	63.6	12	52.2	5	38.5	229	61.9	15	68.2	11	73.3
Female	18	39.1	5	31.3	8	36.4	11	47.8	8	61.5	141	38.1	7	31.8	4	26.7
Performance status																
0-1	28	93.3	5	71.4	17	94.4	8	44.4	7	63.6	255	72.2	17	85.0	14	93.3
2-4	2	6.7	2	28.6	1	5.6	10	55.6	4	36.4	98	27.8	3	15.0	1	6.7
Unknown	16		9		4		5		2		17		2		0	
Status																
Alive	16	34.8	1	6.3	2	9.1	0	0.0	1	7.7	6	1.6	11	50.0	6	40.0
Dead	30	65.2	15	93.8	20	90.9	23	100.0	12	92.3	364	98.4	11	50.0	9	60.0
Treatment																
None	30	93.8	6	75	1	5.6	6	33.3	0	0	42	11.6	21	95.5	3	20.0
Stupp protocol	0	0	0	0	2	11.1	0	0	8	72.7	206	56.9	0	0	2	13.3
Radiotherapy, 59 Gy/- chemotherapy	2	6.2	1	12.5	14	77.8	9	50.0	0	0	53	14.7	1	4.5	8	53.3
Radiotherapy, 34 Gy +/- chemotherapy	0	0	1	12.5	0	0	3	16.7	3	27.3	54	14.9	0	0	1	6.7
Chemotherapy alone	0	0	0	0	1	5.6	0	0	0	0	7	1.9	0	0	1	6.7
Unknown	14		8		4		5		2		8		0			
Survival (median, months)	83.7		19.4		37.1		10.0		26.0		11.4		138.8		86.7	

DA IDH wildtype = diffuse astrocytoma, IDH-wildtype, WHO grade II, AA IDH wildtype = anaplastic astrocytoma, IDH-wildtype, WHO grade III, GBM IDH wildtype = glioblastoma, IDH-wildtype, WHO grade IV, DA IDH mutant = diffuse astrocytoma, IDH-mutant, WHO grade II, AA IDH mutant = anaplastic astrocytoma, IDH-mutant, WHO grade III, GBM IDH mutant = glioblastoma, IDH-mutant, WHO grade IV, OD IDH mutant 1p/19q-codeleted = oligodendroglioma, IDH-mutant and 1p/19q-codeleted, WHO grade II, AOD IDH mutant 1p/19q-codeleted = anaplastic oligodendroglioma, IDH-mutant and 1p/19q-codeleted, WHO grade III. Data on the two patients with PXA and DMG are not shown.

Table 3 Hazard ratio of overall survival between different glioma subgroups based on WHO 2007 and WHO 2016 classifications.

Reference WHO 2007	DA			AA			GBM			OD			AOD		
	Hazard ratio	95% CI	P	Hazard ratio	95% CI	P	Hazard ratio	95% CI	P	Hazard ratio	95% CI	P	Hazard ratio	95% CI	P
WHO 16															
DA IDH mutant	0.74	(0.47-1.17)	0.199												
DA IDH wildtype	2.47	(1.36-4.49)	0.003												
AA IDH mutant				0.44	(0.26-0.75)	0.002									
AA IDH wildtype				1.59	(0.96-2.64)	0.073									
GBM IDH mutant							0.55	(0.31-0.98)	0.043						
GBM IDH wildtype							1.1	(0.92-1.25)	0.355						
OD IDH mutant, 1p/19q-codel.										0.94	(0.40-2.22)	0.893			
AOD IDH mutant, 1p/19q-codel.													0.44	(0.21-0.92)	0.029

WHO 2007: DA = diffuse astrocytoma, WHO grade II, AA = anaplastic astrocytoma, WHO grade III, GBM = glioblastoma, WHO grade IV, OA = oligoastrocytoma, WHO grade II, AOA = anaplastic oligoastrocytoma, WHO grade III, OD = oligodendroglioma WHO grade II, AOD = anaplastic oligodendroglioma, WHO grade.

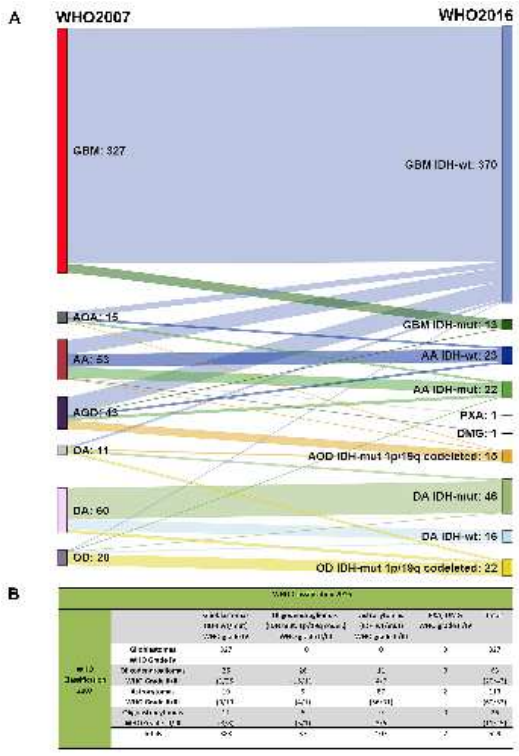
WHO 2016: DA IDH wildtype = diffuse astrocytoma, IDH-wildtype, WHO grade II, AA IDH wildtype = anaplastic astrocytoma, IDH-wildtype, WHO grade III, GBM IDH wildtype = glioblastoma, IDH-wildtype, WHO grade IV, DA IDH mutant = diffuse astrocytoma, IDH-mutant, WHO grade II, AA IDH mutant = anaplastic astrocytoma, IDH-mutant, WHO grade III, GBM IDH mutant = glioblastoma, IDH-mutant, WHO grade IV, OD IDH mutant 1p/19q-codeleted = oligodendroglioma, IDH-mutant and 1p/19q-codeleted, WHO grade II, AOD IDH mutant 1p/19q-codeleted = anaplastic oligodendroglioma, IDH-mutant and 1p/19q-codeleted, WHO grade III.

Table 4 Hazard ratio of overall survival between different glioma subgroups based on WHO 2016 classification.

Reference WHO 2016	DA IDH mutant			DA IDH wildtype			AA IDH mutant			AA IDH wildtype			GBM IDH mutant			OD IDH mutant, 1p/19q-codeleted		
	Hazard ratio	95% CI	P	Hazard ratio	95% CI	P	Hazard ratio	95% CI	P	Hazard ratio	95% CI	P	Hazard ratio	95% CI	P	Hazard ratio	95% CI	P
WHO 16																		
DA IDH mutant	1.00																	
DA IDH wildtype	3.68	(1.94-6.97)	0.000	1.00														
AA IDH mutant	2.90	(1.61-5.20)	< 0.001	0.68	(0.35-1.35)	0.276	1.00											
AA IDH wildtype	18.5	(7.90-43.50)	< 0.001	2.17	(1.08-4.35)	0.028	4.58	2.24-9.33	< 0.001	1.00								
GBM IDH mutant	4.75	(2.30-9.77)	< 0.001	0.93	(0.43-1.99)	0.844	1.58	0.77-3.26	0.215	0.39	0.18-0.84	0.015	1.00					
GBM IDH wildtype	7.10	(4.72-10.70)	< 0.001	1.92	(1.14-3.25)	0.015	2.89	1.82-4.57	< 0.001	0.81	0.53-1.23	0.318	1.93	1.08-3.44	0.026			
OD IDH mutant, 1p/19q-codel.																1.00		
AOD IDH mutant, 1p/19q-codel.																2.17	0.88-5.31	0.091

WHO 2007: DA = diffuse astrocytoma, WHO grade II, AA = anaplastic astrocytoma, WHO grade III, GBM = glioblastoma, WHO grade IV, OA = oligoastrocytoma, WHO grade II, AOA = anaplastic oligoastrocytoma, WHO grade III, OD = oligodendroglioma WHO grade II, AOD = anaplastic oligodendroglioma, WHO grade.

WHO 2016: DA IDH wildtype = diffuse astrocytoma, IDH-wildtype, WHO grade II, AA IDH wildtype = anaplastic astrocytoma, IDH-wildtype, WHO grade III, GBM IDH wildtype = glioblastoma, IDH-wildtype, WHO grade IV, DA IDH mutant = diffuse astrocytoma, IDH-mutant, WHO grade II, AA IDH mutant = anaplastic astrocytoma, IDH-mutant, WHO grade III, GBM IDH mutant = glioblastoma, IDH-mutant, WHO grade IV, OD IDH mutant 1p/19q-codeleted = oligodendroglioma, IDH-mutant and 1p/19q-codeleted, WHO grade II, AOD IDH mutant 1p/19q-codeleted = anaplastic oligodendroglioma, IDH-mutant and 1p/19q-codeleted, WHO grade III.



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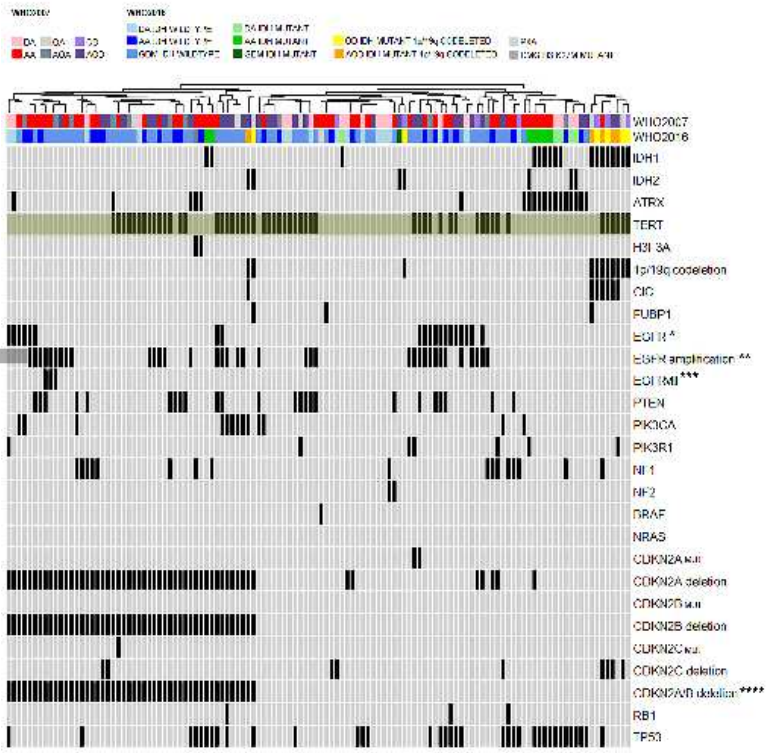

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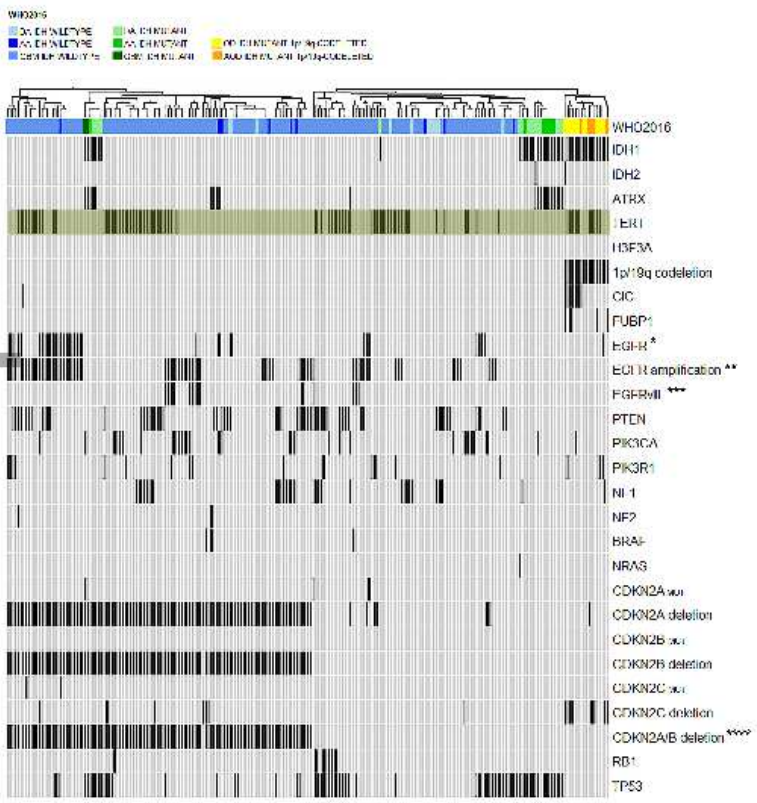

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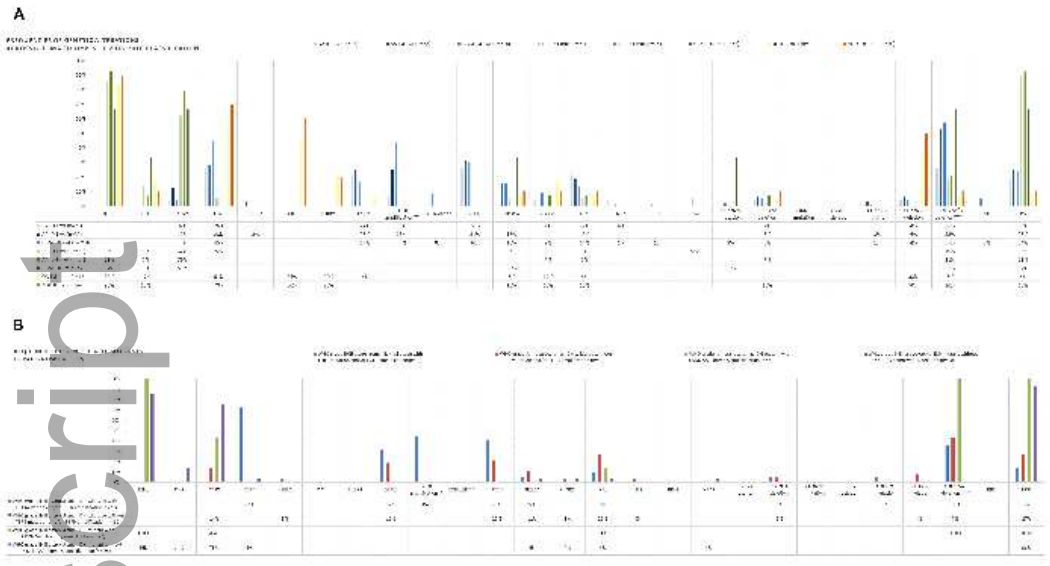

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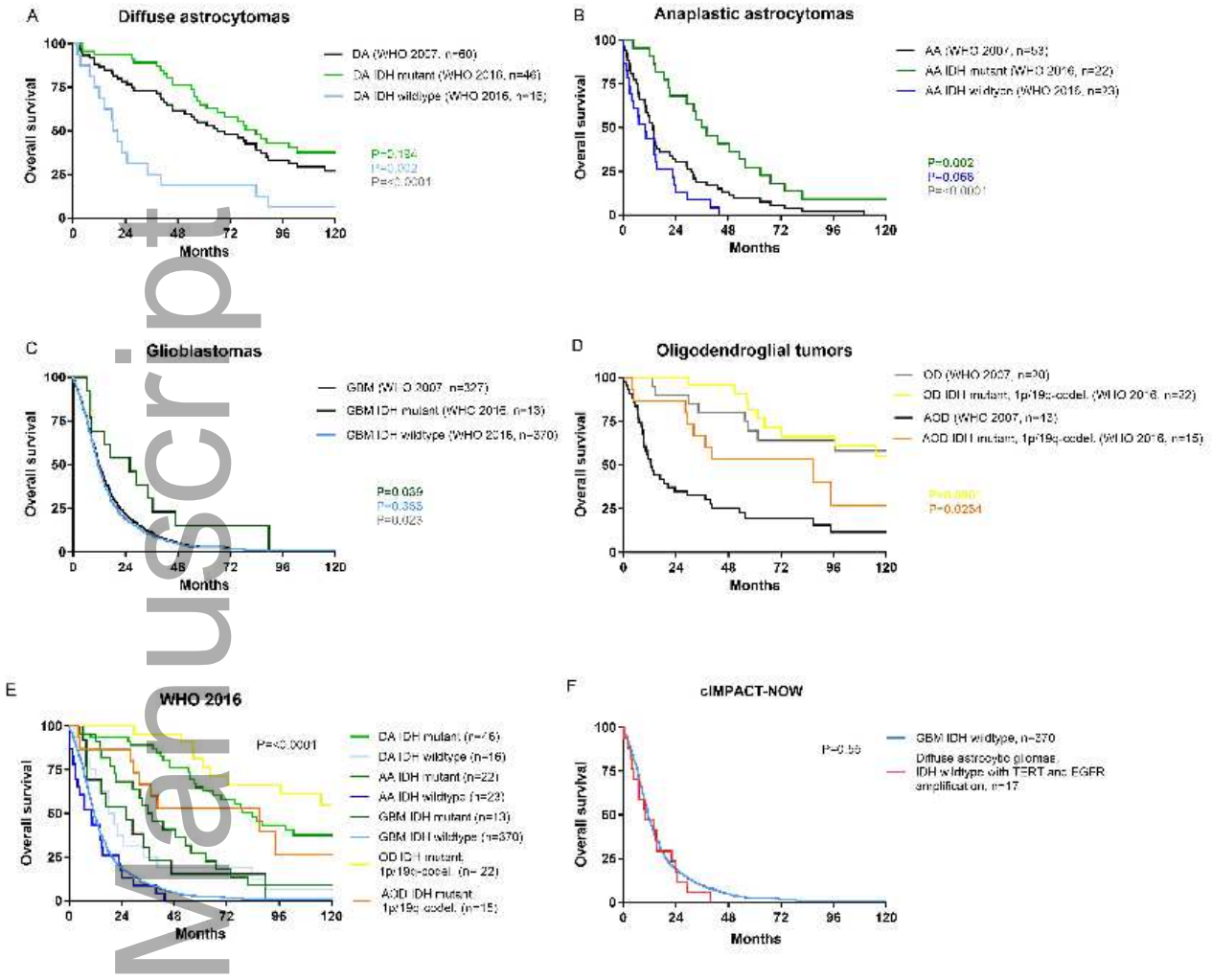

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