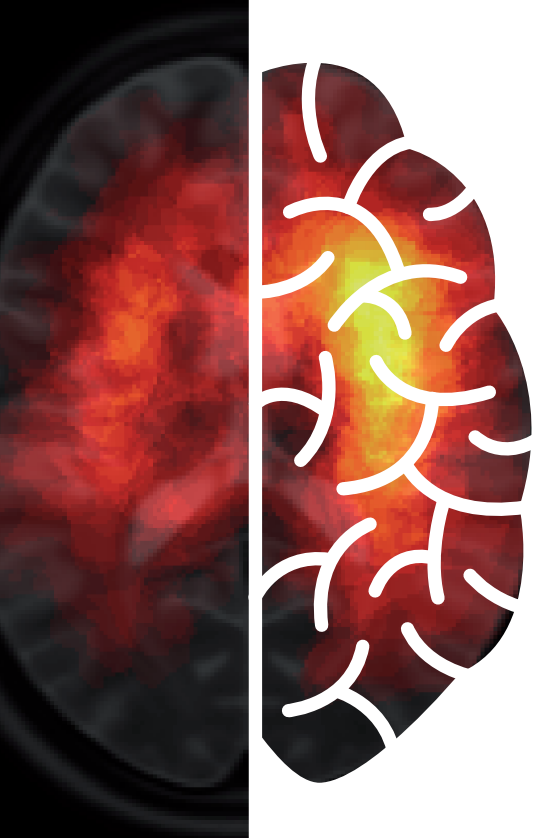

Prognostication and **Surgical Management** of **Diffuse Gliomas** in the Era of **Molecular Diagnostics**

MAARTEN M.J. WIJNENGA

Prognostication and Surgical Management of Diffuse Gliomas in the Era of Molecular Diagnostics

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Prognostication and Surgical Management of Diffuse Gliomas in the Era of Molecular Diagnostics

Prognosestelling en chirurgische behandeling van diffuse gliomen in een tijdperk van moleculaire diagnostiek

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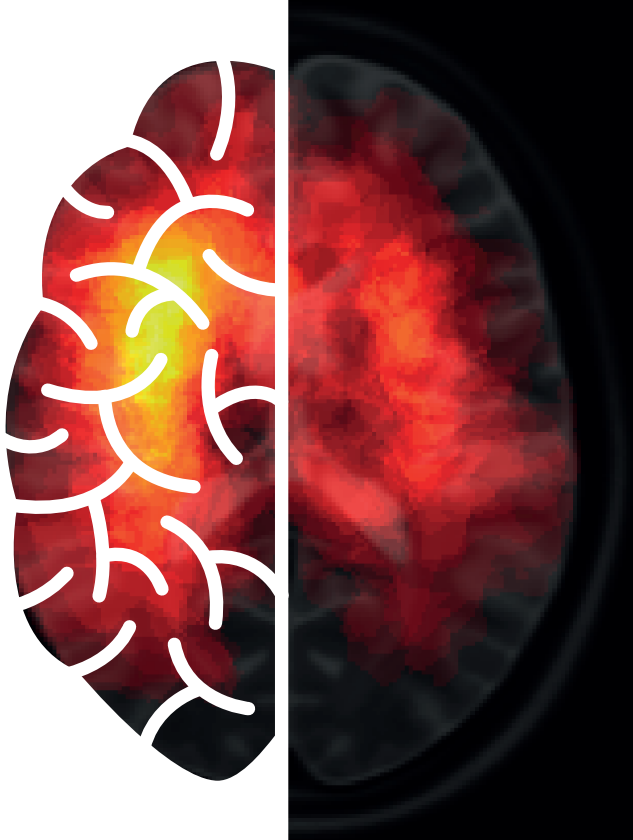

Chapter 1

General Introduction

GENERAL INTRODUCTION

Tumors that originate in the human brain are called primary brain tumors. Distinct subtypes are recognized by the World Health Organization (WHO), as distinct types of brain tissue or anatomic location can give rise to specific tumors. One of them is called glioma, named so as it is hypothesized that this type of tumor arises from glial cells (supporting tissue of the brain). Although it is the most common type of primary malignant brain tumors in human, it is a rare disease with an incidence rate of approximately 6 per 100.000 persons annually in Europe and the United States.[1, 2] Extrapolated to the Dutch situation, this means approximately 1000 persons per year in the Netherlands are newly diagnosed with a glioma.

CLASSIFICATION AND PROGNOSIS OF DIFFUSE GLIOMAS

Diffuse gliomas have a variable prognosis with overall survival rates ranging from only several months to more than 20 years, depending on the subtype.[3, 4] It is clear that very aggressive tumors with an overall survival of only a few months need a different treatment strategy than more indolent tumors with an overall survival of multiple years. Therefore, classifying gliomas into different subtypes that reflect their clinical behavior, prognosis and/or response to treatment is essential.

Gliomas are classified according to the WHO classification of tumors of the central nervous system and traditionally this was based on histological features.[5] However, differences between histological subtypes on microscopic level can be very subtle, and therefore this classification was subject to substantial interobserver variability.[6-8] This potentially results in suboptimal treatment of some patients which is undesirable. The WHO classification scheme was updated in 2016 following many observations that showed better discrimination of clinically relevant subclasses of glioma by classifying on the molecular background of brain tumors.[5] The updated WHO classification now consists of both histologic and molecular features and this has led to marked improvement of objectivity and prognostic significance. Cornerstone of the WHO 2016 classification is testing for presence of mutations in isocitrate dehydrogenase gene 1 or 2 (*IDH1/2*) and presence of a combined deletion (co-deletion) of chromosomal arms 1p and 19q. Based on just these two markers, three subtypes of diffuse lower grade glioma can be recognized; 1) Oligodendroglioma, *IDH1/2* mutant and 1p/19q co-deleted (*IDH1/2* mutation in combination with presence of a co-deletion of the entire 1p and 19q chromosomal arms); 2) Astrocytoma, *IDH1/2* mutated (*IDH1/2* mutation without 1p19q co-deletion); and 3) Astrocytoma, *IDH1/2* wildtype. The highest grade of glioma, glioblastoma, is separated in *IDH1/2* mutated and *IDH1/2*

wildtype (most common form).[5, 9] Molecular aberrations described in *IDH* wildtype glioblastoma are generally equal to the aberrations described in *IDH* wildtype astrocytomas and the outcome is similarly poor (median survival approximately 15 months). Hence, low-grade and anaplastic *IDH* wildtype astrocytomas are often considered as misdiagnosed glioblastoma. Oligodendrogliomas and *IDH* mutated astrocytomas have a much better prognosis with a median overall survival of 12-14 years and 3-8 years respectively. Next to *IDH* gene mutations and 1p19q co-deletion, there are many other frequently reported genetic changes in glioma that are not used for classification criteria, but which can support the diagnosis. For example, *TP53* and *ATRX* mutations are frequently reported in *IDH* mutated astrocytoma. These two mutations are mutually exclusive with 1p/19q co-deletions in glioma. *CIC* and *FUBP1* mutations are frequently reported in *IDH* mutant 1p19q co-deleted oligodendroglioma, but almost never in *IDH* mutated or wildtype astrocytoma. *TERT* promotor mutations are present in almost all *IDH* mutant 1p19q co-deleted oligodendrogliomas and are frequently reported in *IDH* wildtype astrocytoma and glioblastoma, but in principle not in *IDH* mutated astrocytoma.[9-12] Also, mutations or amplifications of the *EGFR* gene are frequently reported, mostly in *IDH* wildtype glioblastoma. Observation of this aberration can support diagnosis, but is not related to prognosis. For a detailed description of the WHO 2016 classification scheme, see Figure 1.

Apart from classification of diffuse gliomas into histomolecular subgroups, diffuse gliomas are also graded (grade II, III, or IV) to further stratify the aggressiveness of the tumors. This is currently still based on the presence of the following histopathological features: nuclear atypia, mitotic activity, microvascular proliferation, and necrosis.[13] Unfortunately, grading of glioma is subject to interobserver variability as scoring of these histological criteria may be difficult due to tumor heterogeneity, small sample volumes, and different interobserver judgement. Therefore, although the updated classification outflanks the previous version for prognosis estimation, there is still variation in prognosis of patients within the major glioma groups. Further improvement and refinement of the classification would be very welcome, especially with markers that reflect aggressiveness/grade within the current WHO subgroups, but so far no molecular markers have been identified that aid in objective grading. **Chapter 2, 3, and 4** of this thesis focus on the efforts to further refine the WHO classification and are described briefly in the last paragraph of this chapter.

GLIOMA TREATMENT

Diffuse gliomas have an infiltrative growth pattern and are often located in or near eloquent areas of the brain (i.e. the sensory cortex, motor cortex, basal ganglia, and

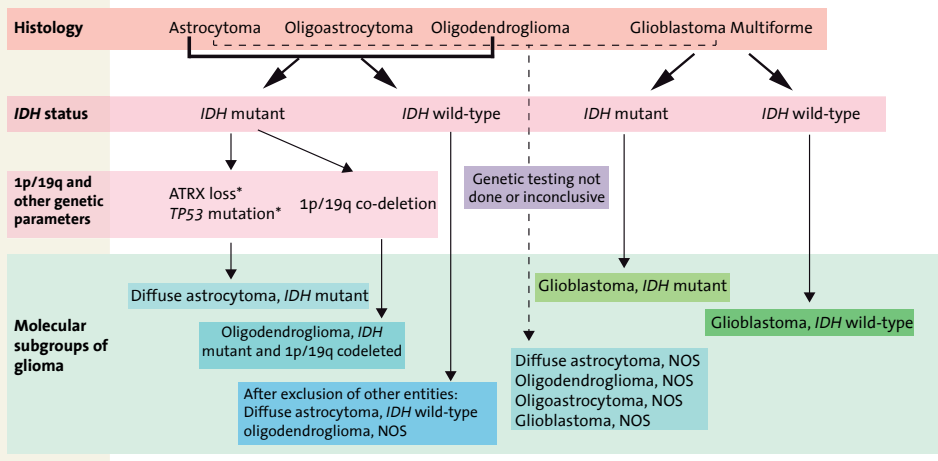

Figure 1. 2016 WHO classification scheme of diffuse glioma. Figure adapted (with permission) from Louis et al.[5]

language/speech area).[4] Therefore it is impossible to fully resect a glioma. As our knowledge on the molecular background of glioma improves, much research nowadays focusses on targeting glioma specific mutations and developing glioma specific immunotherapies. So far this has not led to new standard therapies in daily clinical setting. Therefore, the common available modalities for glioma treatment still are (a combination of) surgical resection, chemotherapy, and radiotherapy.[3, 4, 14-18] How to best employ these different treatment modalities remains a matter of controversy. In individual patients the combination, timing, and sequence is often decided based upon the perception of prognostic factors within a specific patient, such as the clinical condition, location and size of the tumor, and the integrated WHO 2016 diagnosis which is assessed following surgery. The intent of surgery is threefold; to provide tissue for diagnostic purposes (histology and molecular testing), to remove as much tumor as possible to relieve symptoms and to improve survival. Whether that latter objective is actually realistic in low grade glioma has been a topic of debate for years. In the past a so called wait-and-scan approach was the common strategy to treat a lesion suspected for low-grade glioma.[19, 20] This strategy consists of monitoring tumor behavior over time with regular interval MRI scans, with the intention to start active treatment once significant growth of the lesion, clinical deterioration or malignant transformation (signs of contrast enhancement on brain imaging) has occurred. The rationale behind this was the incurable nature of these tumors, the low growth rates and the fact that patients usually present with minor symptoms, such as controllable seizures. Furthermore, the fear for inducing neurological deficits by a neurosurgical procedure withheld many neurosurgeons from aggressive surgical treatment. Performing early surgery on these lesions was therefore generally seen as inappropriate,

as surgery comes with these risks and is not curative. This consensus on treatment of low-grade glioma patients gradually changed in the past decade towards a standard of care where clinicians aim for aggressive resections as early as possible when this is safely possible. This was due to the growing evidence that early and extensive resections are associated with a better clinical outcome (longer overall survival) and the improvement of surgical techniques that allow more safe and extensive resections.[21-26] However, all studies investigating the role of surgery for low grade glioma are retrospective, and are therefore exposed to certain indication and selection bias. Nonetheless, as a prospective study to answer this question is generally considered not feasible for various reasons, retrospective evidence for early and extensive resections is the best option and over time early resection has become part of the international guidelines on glioma treatment. Nevertheless, the timing and extent of resection remain topics of debate in the field. **In chapter 5 and 6** we focus on this still timely topic.

SCOPE OF THIS THESIS

This thesis mainly focusses on lower grade diffuse gliomas (grade II and III). Although the objectivity and prognostic value of glioma classification have improved with the updated WHO classification, further refinement in order to achieve more efficient treatment strategies is mandatory. In **chapter 2** we analyze the publically available whole exome sequencing data of *The Cancer Genome Atlas* (TCGA) of both low and high grade glioma, to find additional prognostic markers within WHO recognized glioma subgroups. In **chapter 3** we report the prognostic relevance of additional mutations and copy number alterations in *IDH* mutated grade II glioma, using a targeted next generation sequencing panel that is also used in routine diagnostic setting. In **chapter 4** we report on a relatively large group of *IDH*-wildtype gliomas, and show this is in fact a molecular and clinical heterogeneous group of tumors. As mentioned above, the role of surgery for lower grade gliomas has been controversial in the past. Consensus in the field shifted from a wait-and-scan approach to early and aggressive resection during the last decade. As the WHO classification of gliomas has been completely revised and is now predominantly based on molecular criteria, the impact of extent of resection needed to be re-evaluated in molecularly defined low grade glioma which we describe in **chapter 5.** In **chapter 6**, we focus on the timing of surgery and the impact on outcome in presumed low-grade glioma, but with a set-up wherein we tried to minimize the above mentioned indication and selection bias as much as possible. In **chapter 7**, we provide insight in the location distribution of specific WHO molecular subgroups of glioma in the human brain. Finally, **chapter 8** discusses the main findings of chapters 2 to 7 and puts this in perspective with recent literature and opinions in the field.

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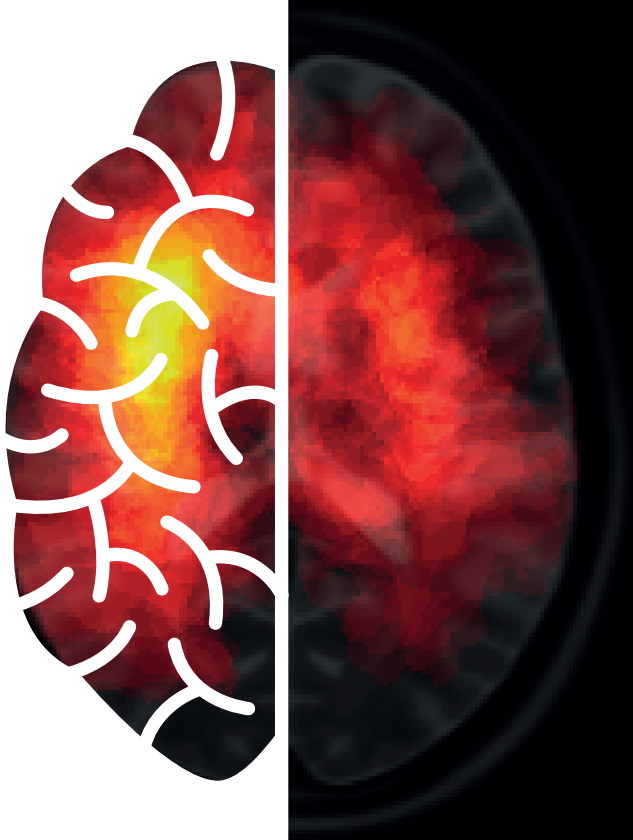

Chapter 2

PI3 kinase mutations and mutational load as poor prognostic markers in diffuse glioma patients.

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ABSTRACT

Recent advances in molecular diagnostics allow diffuse gliomas to be classified based on their genetic changes into distinct prognostic subtypes. However, a systematic analysis of all molecular markers has thus far not been performed; most classification schemes use a predefined and select set of genes/molecular markers. Here, we have analyzed the TCGA dataset (combined GBM and LGG datasets) to identify all prognostic genetic markers in diffuse gliomas in order to generate a comprehensive classification scheme. Of the molecular markers investigated (all genes mutated at a population frequency >1.7% and frequent chromosomal imbalances) in the entire glioma dataset, 57 were significantly associated with overall survival. Of these, *IDH1* or *IDH2* mutations are associated with lowest hazard ratio, which confirms *IDH* as the most important prognostic marker in diffuse gliomas. Subsequent subgroup analysis largely confirms many of the currently used molecular classification schemes for diffuse gliomas (*ATRX* or *TP53* mutations, 1p19q codeletion). Our analysis also identified *PI3*-kinase mutations as markers of poor prognosis in *IDH*-mutated +ATRX/TP53 mutated diffuse gliomas, median survival 3.7 *v.* 6.3 years (P=0.02, Hazard rate (HR) 2.93, 95% confidence interval (CI) 1.16 – 7.38). *PI3*-kinase mutations were also prognostic in two independent datasets. In our analysis, no additional molecular markers were identified that further refine the molecular classification of diffuse gliomas. Interestingly, these molecular classifiers do not fully explain the variability in survival observed for diffuse glioma patients. We demonstrate that tumor grade remains an important prognostic factor for overall survival in diffuse gliomas, even within molecular glioma subtypes. Tumor grade was correlated with the mutational load (the number of non-silent mutations) of the tumor: grade II diffuse gliomas harbor fewer genetic changes than grade III or IV, even within defined molecular subtypes (e.g. ATRX mutated diffuse gliomas). The increase in mutational load may partially explain the increased aggressiveness of higher grade diffuse gliomas when a subset of the affected genes actively contributes to gliomagenesis and/or progression.

INTRODUCTION

Gliomas are the most common primary malignant brain tumors in adults.[1,2] Diffuse gliomas are classified into different subtypes according to their histological features into astrocytomas, oligodendrogliomas and mixed oligoastrocytomas.[3] These subtypes are further divided into various tumor grades (grade II-IV) depending on the number of malignant features present in the tumor (nuclear atypia, mitoses, endothelial proliferation and necrosis). The WHO classification, in combination with clinical parameters such as age and Karnofsky Performance Status (KPS), guides treatment decisions and provides prognostic information for patients and clinicians.

Unravelling the causal genetic changes of diffuse gliomas has been the focus of extensive research in the past decade[4-6] and it is now possible to classify diffuse gliomas based on their molecular characteristics.[7-11] For example, *IDH1* mutations are frequent events in all grade II and III gliomas and in secondary glioblastomas (sGBM, glioblastomas that progress from lower grade gliomas) whereas primary GBMs (pGBM) are usually *IDHwt* and frequently have genetic changes involving the *EGFR* locus, *PTEN* deletions and *TERT* promoter mutations.[4,6,12] In addition, *CIC, FUBP1, TERT* promoter mutations and *1p/19q* codeletion are observed more frequently in oligodendrogliomas than in astrocytic tumors[13-15] whereas *ATRX* and *TP53* mutations are seen more frequently in grade II/III astrocytic tumors.[16-18] The importance of this molecular information is widely acknowledged and guidelines have been made to incorporate them in the WHO classification of gliomas.[19]

Although the genetic changes are used to classify diffuse gliomas into distinct prognostic subtypes[9,10,16,20-23], a systematic analysis of all available molecular prognostic markers has thus far not been performed. In fact, most classification schemes use only a few high frequent genes or molecular markers. It is therefore possible that additional and/or stronger prognostic markers are present that can improve the molecular classification of diffuse gliomas. Furthermore, while the prognostic molecular markers may refine (or even replace) the histological classification of diffuse gliomas, there are thus far no genetic changes that can discriminate between grade II and III tumors. This is remarkable as tumor grade is a strong prognostic marker in diffuse gliomas[3] (although some reports found little prognostic value for tumor grade within defined glioma subtypes).[24,25]

In this study we therefore have analyzed the publicly available TCGA dataset in order to identify additional prognostic molecular markers in diffuse gliomas. Since diffuse gliomas can be classified solely based on molecular markers[9,20], we also evaluated whether tumor grade remains relevant after the molecular classification and/or whether there are genetic markers that can distinguish between tumor grades in diffuse gliomas. Our analysis confirms many of the currently used molecular classifica-

tion schemes for diffuse gliomas: gliomas are first separated based on *IDH*-mutation status and a further stratification is based on *ATRX/TP53* mutation status or 1p19q codeletion. We show that *PI3*-kinase mutations are associated with poor prognosis in molecular astrocytomas (i.e. diffuse gliomas that are *IDH*-mutated and 1p19q intact (or *ATRX*/*TP53* mutated)) and that no other marker investigated in this study appears to further refine this molecular/prognostic classification of diffuse gliomas. Our analysis also shows that, for most driver mutations investigated here (*IDH1/2, ATRX, TP53*), tumor grade remains a prognostic factor in diffuse gliomas with identical driver mutations. This indicates that *IDH*-mutated glioblastomas behave significantly more aggressive than *IDH*-mutated grade III gliomas. Although no single molecular marker was associated with tumor grade, we find that tumor grade is correlated with the overall mutational load: grade II gliomas harbor fewer genetic changes than grade III or IV, even within defined molecular subtypes (e.g. *ATRX* mutated gliomas). The increased mutational load may partially explain the increased aggressiveness of higher grade gliomas when a subset of the affected genes actively contributes to gliomagenesis and/or progression.

METHODS

For this study, we have used publicly available data from the TCGA, both lower grade glioma and glioblastoma datasets. Data include mutation status, copy number variations and clinical data, only cases with complete data were included in current analysis (n=542). All data analyses were based on overall survival (OS). Survival data for patients that are listed as <30 days were omitted from the survival analysis; the cause of death for such patients may not be tumor-related (but e.g. related to complications occurring after surgery). *EGFR* amplification status and *CDKN2A* deletions data were downloaded from the cbioportal site.[26] Although such data could be extracted from the copynumber data (see below), we used cBioportal data to ensure identical thresholds were used to define amplification and allelic loss. All mutation data were filtered for those that result in a change in the primary amino acid sequence. We focused on all genes that are mutated in more than ten samples of the entire study population. We also included the copy number alterations 1p19q codeletion (loss of heterozygosity (LOH) of the 1p and 19q chromosome arms) and trisomy of chromosome 7 and LOH of chromosome 10 (alt 7/10). Combined, we analyzed 128 genetic alterations in 542 samples.

Genome wide SNP 6 Copynumber data was downloaded from the TCGA data portal. This data gives a value per chromosomal region (segment) where values deviating from 0 likely correspond to regions with chromosomal losses (<0) or gains (>0). From

the segment values, we calculated the average an entire chromosome/chromosomal arm and defined 1p19q codeletion as averages over both arms -0.3 or less. When values were discordant between 1p and 19q or values were between 0 and -0.3 (which can occur in tumors with a high content of non-neoplastic tissue), we determined 1p19q codeletion based on visualization of the copynumber plot. This visualization was performed blinded to the patient outcome. Alt 7/10 was determined by a value of 0.3 or higher for chromosome 7 *and* a value of -0.3 or lower for chromosome 10. When values were either discordant between chromosomes 7 and 10, or were between 0 and 0.3 for chromosome 7 and/or between 0 and -0.3 for chromosome 10, we determined alt 7/10 based on visualization of the copynumber plot (blinded to patient outcome). Because *IDH1* and *IDH2* mutations are mutually exclusive and play an identical role in tumor pathogenesis, we have combined mutation data into an additional single *IDH*-mutations variable. Similarly, we combined *EGFR*-mutations and *EGFR* gene amplifications into a single additional *EGFR*-alteration variable. As *PIK3CA* and *PIK3R1* are highly related (and mutually exclusive) genes within the same PI3-kinase pathway, we also combined mutation data into an additional single *PI3*-kinase mutations variable.

To validate the prognostic value of identified genes, we performed survival analysis on two additional datasets containing mutation and survival data.[6,17] Hazard ratios (HR) and survival differences were calculated using a cox proportional hazard model in R (survival CRAN package), unless specifically indicated otherwise. Differences in mutation frequencies were calculated using an ANOVA (3 groups) or T-Test (2 groups). Bonferroni correction was done by using a P value cut-off of 0.0004 (0.05 divided by the total number of calculations (128 genes and copy number changes)). Chi square tests were performed using an online calculator (www.quantpsy.org/chisq/chisq.htm), Graphpad Prism (version 5.00) was used to perform log-rank tests.

Because a large number of genes were tested to determine association with survival, we corrected for multiple testing by estimating the false positive rate. This was done by an in-silico analysis in which a set of 100 genes were randomly mutated across 542 samples (at a population frequency between 2.5-10%) and we then calculated how many of those were associated with survival using the Cox proportional hazards method. These false positive estimations were made using three different population mutation frequencies (2.5%, 5% and 10%) and were done 50 times for each population mutation frequency. In such analysis, we identified between 1-12 genes that were significantly associated with outcome. For all calculations, $P<0.05$ was considered statistically significant.

RESULTS

Prognostic classification of diffuse gliomas

We analyzed the combined GBM and LGG (low grade glioma) datasets from the TCGA (n=542 samples) and identified 128 genes that are mutated (non-silent mutations only) in ten or more samples, consistent with a population frequency >1.7% (i.e. 10/542= 1.8%). Of these, 57 genes were significantly associated with survival and the list included the well-known favorable prognostic markers *IDH1/2*, 1p19q codeletion, *CIC, FUBP1* and *NOTCH1*. Poor prognostic markers included genetic changes in the *EGFR* locus, *PTEN*-mutations and alt 7/10 (supplementary table 1). *IDH1 or IDH2*-mutations (collectively referred to in our analysis as *IDH*-mutations unless specifically stated) were associated with the lowest HR (0.10 95% confidence interval (CI): 0.07-0.14, P<0.0001). Because our aim was to generate a prognostic classification scheme for diffuse gliomas based on molecular aberrations, the gene with lowest HR (i.e. *IDH*-mutations) provided our first molecular prognostic separator for diffuse gliomas.

Genes associated with prognosis in IDH-wt gliomas

We then screened for prognostic markers separately within *IDH*- wildtype (wt) and *IDH*-mutated gliomas. Within the subset of *IDH*-wt gliomas, we identified 4 genes that, when mutated, were significantly associated with prognosis (supplementary table 2). However, a relatively large number of tests were performed to identify these genes. To correct for multiple testing, we performed similar analysis on a set of 100 genes that were randomly mutated across the TCGA dataset at a population mutation frequency of 2.5%, 5% and 10%. In such analysis, we identified between 1-12 genes that were significantly associated with outcome. Identification of 4/128 genes associated with survival in *IDH* wt gliomas is therefore within the range of the false positive frequency (1-12%). By analogy, after Bonferroni correction only one gene (*SLC6A3*) remained significant.

As independent validation is warranted, we screened two additional datasets to confirm the prognostic value of these four genes in *IDH*-wt tumors.[6,17] Clinical and mutation data are listed in supplementary tables 3 and 4. In a dataset of anaplastic astrocytomas, mutations in two of these four genes (*PKHD1* and *MUC16*) were identified and in a set of GBMs, mutations in three genes (*MUC16*, *F5* and *PKHD1*) were identified. Unfortunately, the mutation frequency of individual genes was too low to allow for a statistical comparison, and a combined analysis of mutated genes does not show a difference between wt and mutated samples within one dataset. However, when combining survival of both datasets, mutations in any of these genes is associated with poor prognosis (median survival of 0.88 *v.* 1.33 years for mutated and wt samples respectively, P=0.018 HR 3.81, 95% CI 1.26-11.5). However, because numbers are small,

caution should be taken when interpreting these data as it remains possible that the four prognostic genes identified in *IDH*-wt tumors were false positive candidates and do not represent true prognostic genes.

IDH-wt diffuse gliomas are often further subdivided into those with trisomy on chromosome 7 combined with LOH of chromosome 10 (alt 7/10) and those without (7/10 wt). It should be noted that, in the TCGA dataset, alt 7/10 does not confer any prognostic information in *IDH*-wt diffuse gliomas (supplementary table 2). On the gene expression levels alt 7/10 GBMs correlate with "classical" GBMs (or those assigned to IGS-18); 7/10 wt tumors associate with other molecular subtypes (mesenchymal/neural/proneural or IGS-22/IGS-23) (27, 28). We have therefore screened for prognostic molecular features within the *IDH*-wt, alt 7/10 ('*molecular classical*', n=214) and within the *IDH*-wt, 7/10 wt ('*molecular mesenchymal*', n=86) diffuse gliomas. Within *molecular classical* gliomas, 10 genes were significantly correlated with survival (supplementary table 5) and 11 genes within the *molecular mesenchymal* gliomas (supplementary table 6). It is interesting to note that *TP53* mutations are associated with a more favorable prognosis in the *molecular classical* gliomas and *PIK3CA* (or combined *PIK3CA* and *PIK3R1*) mutations with poor prognosis in the *molecular mesenchymal* gliomas. Unfortunately, we were unable to validate these results due to an absence of copy number data in the two validation datasets.

It should be noted that pilocytic astrocytomas (PAs, brain tumors with favorable prognosis) may be present among the *IDH*-wt tumors. However, detailed analysis shows that only one of the samples included in this study harbored a genetic profile consistent with PA (TCGA-HT-7691; a diploid genome apart from a tandem duplication on chromosome 7q34 involving the *BRAF* locus), and the survival data for this patient is 0.1 months (patient still alive). Omitting this patient from the analysis will therefore not impact the survival data as presented.

PI3 kinase pathway mutations are associated with poor survival in molecular astrocytomas

Within *IDH*-mutated diffuse gliomas, we identified 12/128 genes associated with poor survival (Supplementary table 7). Mutations in three and two genes of these were also identified in validation datasets of anaplastic astrocytomas and GBMs respectively.[6,17] In both datasets, there were too few samples to allow comparison. The absence of a true validation set indicates that caution should be taken as it is possible that the twelve prognostic genes identified in *IDH*-mutant tumors were false positive candidates and do not represent true prognostic genes.

IDH-mutated diffuse gliomas are often further subdivided into *molecular astrocytomas* (i.e. those with mutations in *ATRX* and/or *TP53*) and *molecular oligodendrogliomas* (i.e. those with 1p19q codeletion).[16,23] It should be noted that these genetic changes

by themselves did not reach statistical significance in *IDH*-mutated tumors of the TCGA. This is likely due to the large number of patients alive at time of analysis (205 patients alive out of the 243 *IDH*-mutant glioma patients). We therefore separated IDH-mutated samples into those with *TP53* or *ATRX* mutations (n= 151) and those with 1p19q codeletion (n=74). Seventeen samples had neither genetic change and five samples had both.

Within *molecular oligodendrogliomas* we identified 1 out of 128 genes associated with survival (Supplementary table 8). Unfortunately, there are no external datasets to validate this finding.

Within *molecular astrocytomas*, we identified 8 genes associated with survival (Supplementary table 9). *PIK3CA* was one of the genes identified. Interestingly, a similar trend was observed in a highly related gene, *PIK3R1,* HR 2.45 P=0.075 95% CI 0.91 – 6.56. As *PIK3CA* and *PIK3R1* are highly related (and mutually exclusive) genes within the same PI3-kinase pathway, we combined mutation data into an additional single *PI3*-kinase mutations variable. The median survival in molecular astrocytomas with *PI3*-kinase mutations was 3.7 years *v.* 6.3 years for *PI3*-kinase wt molecular astrocytomas (P=0.02, HR 2.93, 95% CI 1.16 – 7.38, figure 1a). Individual *PI3*-kinase mutations are listed in supplementary table 10. *PIK3CA* mutations are missense mutations or in-frame deletions and often affect the known hotspots of the protein (E542, E545 or the C-terminal domain, see [29]). *PIK3R1* mutations are more heterogeneous (in-frame deletions, nonsense, frame-shifts, splice site or missense) not confined to specific hotspots.

To validate the prognostic value of identified genes, we screened an anaplastic astrocytomas dataset and determined survival within defined molecular subtypes of diffuse glioma.[17] Within the *IDH*-mutated and *TP53* or *ATRX* mutated tumors, mutations in four genes out of the 15 identified in the TCGA dataset (*PIK3R1, PKHD1, NEB1,* and *NOTCH2*) were identified. Of these, tumors with *PIK3R1* mutations (n=4) had poorer prognosis than *PIK3R1* wt tumors (n=20), median survival 2.4 and 5.4 years respectively (supplementary figure 1a). We next downloaded mutation data of a cohort of GBMs.[6] Also in this dataset, we observed a similar poor prognostic trend for *PIK3R1* mutations in *IDH*-mutated and *TP53* or *ATRX* mutated GBMs: Tumors with *PIK3R1* mutations (n=2) had poorer prognosis than *PIK3R1* wt tumors (n=2), median survival 1.4 and 5.5 years respectively (supplementary figure 1b). Although significance was not reached in either of these datasets (perhaps due to the small sample size), a pure molecular classification allows combining both datasets. When this is performed, a median survival of 1.9 *v.* 5.4 years was observed for *PIK3R1* mut and *PIK3R1* wt tumors respectively, HR 17.0, 95% CI (2.40-121), P=0.0046 (figure 1). The fact that *PI3*-kinase mutations showed similar trends in prognosis in three independent datasets, strongly suggests they are prognostic markers for molecular astrocytomas.

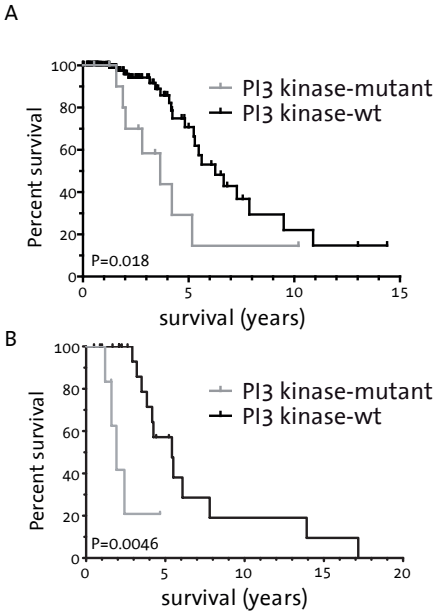

Figure 1. PI3-kinase mutations are prognostic in molecular astrocytomas (those with *ATRX* and/or *TP53* mutations). A: Data from TCGA samples (test cohort). Histology and grade of samples presented are listed in supplementary table 9; B: Data from two validation cohorts (combined) from astrocytomas (17) and glioblastomas (6). In both figures, only samples with an *IDH* mutation and *TP53* or *ATRX* were selected. In these molecular astrocytomas, PI3 kinase mutations are prognostic for overall survival. P values indicated are calculated using the Log-rank test.

Tumor grade remains prognostic in molecular diffuse glioma subtypes and is associated with mutational load of the tumor

Apart from the pure molecular analysis described above, several clinical and histological parameters are also associated with survival. For example, tumor grade is inversely correlated with patient survival within the defined histological subtypes of diffuse glioma[3]; a correlation that was also present in the TCGA dataset. For example, there were 42 grade II and 31 grade III oligoastrocytomas, of which the grade II tumors had a significantly better prognosis than the grade III tumors (median survival was 5.3 vs 6.3 years, P=0.024, HR 0.26, 95% CI (0.08 – 0.84)). A similar trend was observed for astrocytomas (table 1, Supplementary figure 2).

Table 1. Tumor grade is inversely correlated with patient survival within histological subtypes of diffuse glioma

	Grade II survival (y)	Grade III survival (y)	HR	95% CI	P
Astrocytoma	5.2	3.7	0.27	0.06 - 1.16	0.078
Oligodendroglioma	7.9	5.2	0.49	0.2 - 1.2	0.12
Oligoastrocytoma	5.3	6.3	0.26	0.08 - 0.84	0.024

Survival: median overall survival in years. HR calculated using Cox univariate analysis. HR was calculated grade II vs grade III.

As detailed above, an alternative method for histological classification is to classify gliomas based on their genetic aberrations. Within defined molecular subtypes (i.e. all tumors that harbor mutations in one of the lineage specific genes *IDH, CIC, FUBP1, ATRX, TP53, PTEN, EGFR,* 1p19q codeletion or alt 7/10, frequency listed in table 2) tumor grade often remained inversely correlated with survival (Supplementary figure 3, table 3). For example, there were 151 *IDH + ATRX/TP53*-mutated gliomas in the TCGA diffuse glioma datasets of which 73 were of grade II, 65 of grade III and 13 of grade IV (GBM) and median survival was 7.3, 5.2 and 2.8 years (P=0.0024). Similar trends were observed for most other single molecular changes (i.e. selecting samples only on one genetic change, regardless of other molecular changes present). Importantly, tumor grade was a prognostic factor for each of the molecular subtypes identified above: i) *IDH*-wt gliomas; ii) *IDH and TP53 and/or ATRX*-mutated gliomas and; iii) *IDH* and 1p19q codeleted gliomas (figure 2)

Table 2. Frequency of genetic changes listed per histological subtype and grade.

	Low grade	Molecular Oligodendroglioma		Molecular Astrocytoma		Molecular Glioblastoma				
	IDH1/ IDH2	CIC/ FUBP1	LOH 1p19q	ATRX	TP53	EGFR alterations	PTEN	alt 7/10	NF1	N
OD II	95	48	51	28	28	0	2	3	2	65
OD III	82	49	60	18	27	7	2	7	7	45
A II	83	0	0	67	73	0	0	0	0	30
A III	62	1	1	41	65	26	13	26	15	68
OA II	95	14	21	69	74	0	0	2	5	42
OA III	74	10	13	48	58	16	6	16	3	31
GBM	5	0	0	5	30	55	31	71	10	261
N	228	64	74	131	222	170	93	214	44	542

The numbers in the table are percentages of the number of samples mutated (i.e. population frequencies) except the columns listed as N where numbers represent absolute numbers

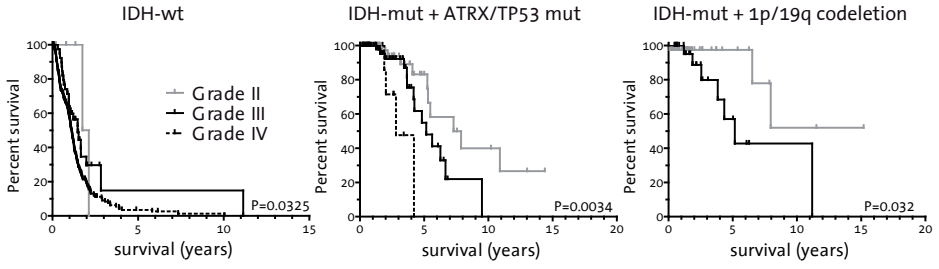

Figure 2. Survival in prognostic molecular subtypes of diffuse glioma stratified by tumor grade. Different subtypes are indicated above each graph. As can be seen, within defined molecular subtypes, tumor grade remains a prognostic factor. Number of samples (grade II, III and IV) for each graph: 10, 42 and 148 (IDH-wt); 73, 69 and 13 (IDH-mut, ATRX/TP53 mut); 41, 32 and 0 (IDH-mut, 1p19q codeleted)

Because tumor grade was associated with patient survival, we further analyzed the TCGA dataset to identify the molecular correlates of tumor grade. When screening for mutations that occur at different frequencies between grade II and III diffuse gliomas, the only genes identified were the lineage specific genetic changes (*IDH*, *CIC*, 1p19q co-deletion, *ATRX*, *EGFR*, and alt 7/10). These genes are listed in table 2 and such a higher rate (where the frequency of mutations in grade II > grade III) has been observed in other studies (although other studies did not find such a difference).[14,30,31] Perhaps the most striking difference between tumors of different grade however was the total number of genetic changes (the mutational load). For example, the average number of non-silent (i.e. those that result in a change in the primary protein sequence) genetic changes in grade II astrocytomas was 18.8 ± 13.1 (n=30), in grade III astrocytomas it was 36.8 ± 47.6 (n=68), P = 0.0050 (table 4). This increase in 'mutational load' was also observed within molecular subtypes of diffuse glioma and is listed in table 5. For example, the mutational load of *ATRX* mutated gliomas increased from 21.6 ± 10.3 and 26.0 ± 11.2 to 65.4 ± 40.1 mutations per sample (P<0.0001) for grade II, III and IV gliomas respectively.

Table 3. Tumor grade is inversely correlated with survival within molecular subtypes of diffuse glioma

	Grade II		Grade III		Grade IV			
Genes	OS (y)	n	OS (y)	n	OS (y)	n	P	P II vs III
IDH+CIC/FUBP1/LOH 1p19q	"not reached"	47	5.2	34		1	0.040	0.04
IDH+ATRX/TP53	7.3	73	5.2	70	2.8	13	0.0029	0.069
EGFR/PTEN/ alt 7/10	1.9	3	1.5	32	1.2	211	0.13	0.5
NF1	2.1	3	1.9	14	1	27	0.034	NA

IDH+CIC/FUBP1/LOH 1p19q refers to mutations in IDH plus any of the subsequent genes, similar for IDH+ATRX/TP53. Statistical tests were performed using a Chi-square test. OS refers to median overall survival in years. Frequency comparisons were done between grade II, III and IV. Exceptions were made for genes with too few/no data in one of the grades (e.g. there are no grade IV tumors with 1p19q codeletion). Therefore, the P value for NF1 is based on comparison between grade III and IV and the P value for IDH+CIC/FUBP1/LOH 1p19q is based on a comparison between grade II and III.

Table 4. Tumor grade is correlated with mutational load within histological subtypes of diffuse glioma.

	Grade II	Grade III	Grade IV	P
Oligodendroglioma	21.8 ± 10.3(65)	28.1 ± 13.5(45)		0.011
Astrocytoma	18.8 ± 13.1(30)	36.8 ± 47.6(68)		0.0050
Oligoastrocytoma	20 ± 9(42)	29.3 ± 14.3(31)		0.0025
GBM			57.3 ± 19.9 (261)	

Values are listed as the average number of non-silent mutations +/- SD (number of tumors analyzed. P values were calculated using an anova.

Table 5. Tumor grade is correlated with mutational load within molecular subtypes of diffuse glioma.

	Grade II	Grade III	Grade IV	P	P II v. III
Overall	20.6 ± 10.6 (137)	32.4 ± 34.3 (144)	57.3 ± 19.9 (261)	< 0.0001	
IDH1/IDH2	21.1 ± 10.1 (127)	26.7 ± 12.1 (102)	52 ± 22.1 (13)	< 0.0001	0.00023
CIC/FUBP1	21.9 ± 10.3 (37)	28 ± 10.7 (26)		0.030	
LOH 1p19q	21.7 ± 10.1 (42)	28.2 ± 10.2 (32)		0.0081	
ATRX	21.6 ± 10.3 (67)	26 ± 11.2 (51)	65.4 ± 40.1 (14)	< 0.0001	0.034
TP53	21.4 ± 10.2 (71)	33 ± 46.1 (74)	60.5 ± 23 (78)	< 0.0001	0.038
EGFR		41.9 ± 12.7 (15)	60.3 ± 16.6 (69)	< 0.0001	
PTEN		42.8 ± 10.8 (12)	62.7 ± 21.3 (80)	< 0.0001	
alt 7/10	24 ± 10.6 (3)	43.5 ± 10.1 (26)	59.6 ± 16.7 (185)	< 0.0001	
NF1	12 ± 7.2 (3)	57.6 ± 101.6 (14)	56.6 ± 15.5 (27)	0.97	

Values are listed as the average number of non-silent mutations +/- SD (number of tumors analyzed). Alt 7/10: Trisomy chromosome 7 and LOH of chromosome 10. P values were calculated using an anova. P II *v.* III indicates significance of grade II *v.* grade III tumors based on a T-test. Total number of cases analysed = 542.

The mutational load is associated with patient age

Because age is a well-known prognostic factor in diffuse glioma patients, we included age in the analysis. Similar to previously reported, grade II tumors occur in patients that were younger than those with grade III or grade IV tumors, 39.6 ± 12.5 (n=137), 45.6 ± 13.5 (n=144) and 61.3 ± 13.0 (n=261) years respectively (average ± standard deviation (SD), P<0.0001 for any comparison, ANOVA).[1,32,33] As patient age and tumor grade were correlated, and tumor grade was correlated to the mutational load, it is not surprising that age was also correlated with the mutational load of the tumor (figure 3). This correlation was observed not only in the entire dataset but also within histologically and molecularly defined subtypes (table 5 and 6). Indeed, when analyzing the type of mutations that occur in the TCGA dataset, a large proportion (2962/9281, 32%) of all mutations were C>T transitions in the sequence xCG (where x represents any nucleotide). Only 4/96 possible combinations would lead to this specific mutation, and this type of signature has been identified as an age related mutation signature [34].

Univariate analysis confirmed that histology (oligoastrocytoma vs. oligodendroglioma: P=0.41 HR 1.33 95% CI 0.68-2.61; astrocytoma vs. oligodendroglioma: P=0.0029 HR 2.52 95% CI 1.37-4.63; GBM vs oligodendroglioma: P=<0.0001 HR 10.6 95% CI 6.47-17.3), tumor grade (grade III vs. II: P= 0.0001, HR 3.14 95%CI 1.76-5.60; grade IV vs. II: P<0.0001, HR 14.4 95%CI 8.48-24.5), the number of mutations (P<0.00001, HR 4.52, 95%CI 3.42 – 5.97) and patient age (P<0.00001, HR 5.51, 95%CI 4.03 – 7.54) were associated with patient overall survival. In a multivariate analysis, the number of mutations remained a significant prognostic factor when including histology and tumor grade in the analysis. However, when the multivariate analysis also included

patient age, the number of mutations was no longer a significant prognostic marker (table 7). Similar results were obtained when performing multivariate analysis within defined molecular subtypes (mutations in *IDH*, *CIC* or *FUBP1*, *TP53*, *EGFR*, *PTEN*, *NF1* or trisomy of Chr7 combined with LOH of Chr 10 or 1p19q codeletion), data not shown. Therefore, patient age appears to be stronger associated with patient survival than mutational load.

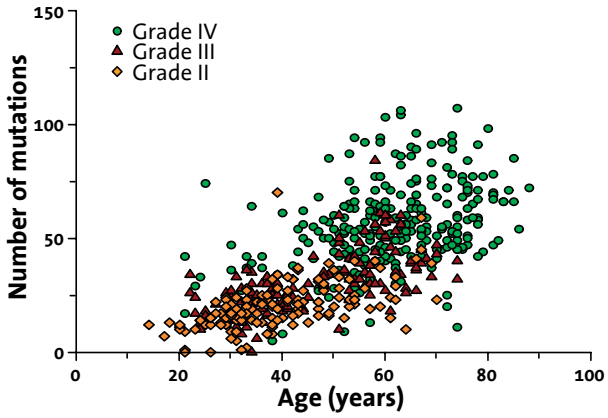

Figure 3. Correlation between patient age and mutational load in diffuse gliomas. The number of non-silent genetic changes increases with patient age. This increase is irrespective of histological subtype (not shown) or tumor grade

Table 6. Tumor grade is correlated with patient age within molecular subtypes of diffuse glioma.

	Grade II	Grade III	Grade IV	P	P II v. III
Overall	39.6 ± 12.5 (137)	45.6 ± 13.5 (144)	61.3 ± 13 (261)	< 0.0001	
IDH1/IDH2	39.6 ± 12.3 (127)	42 ± 12.1 (102)	39.6 ± 15.7 (13)	0.32	0.14
CIC/FUBP1	42.3 ± 13.4 (37)	48 ± 10.8 (26)		0.065	0.065
LOH 1p19q	42 ± 12.4 (42)	49.4 ± 11.8 (32)		0.012	0.012
ATRX	37.4 ± 11.9 (67)	38.1 ± 11.3 (51)	41.6 ± 17.2 (14)	0.30	0.74
TP53	37.2 ± 11.8 (71)	39.9 ± 11.7 (74)	59.2 ± 15.5 (78)	< 0.0001	0.18
EGFR		61.7 ± 7.5 (15)	61.2 ± 11.7 (69)	0.84	
PTEN		56.8 ± 10.5 (12)	62.8 ± 11.9 (80)	0.092	
alt 7/10	49.7 ± 8.3 (3)	59.4 ± 6.8 (26)	62.8 ± 10.8 (185)	0.015	
NF1	51 ± 18.4 (3)	43.7 ± 12.7 (14)	64.4 ± 13.2 (27)	0.00050	

Values are listed as the mean patient age +/- standard deviation (number of tumors analysed). P values were calculated using an anova. P II *v.* III indicates significance of grade II *v.* grade III tumors based on a T-test. Total number of cases analysed = 542.

DISCUSSION

In this study, we have aimed to identify genetic changes associated with patient prognosis within defined histological and molecular subtypes of diffuse glioma by analyzing the TCGA glioma datasets. Our analysis shows that diffuse gliomas are first

Table 7. Multivariate Cox analysis of prognostic markers for overall survival in diffuse glioma patients.

	HR	P value	95% CI
Histology	1.00		
Oligoastrocytoma vs. oligodendroglioma	1.53	0.22	0.78 - 3.02
Astrocytoma vs. oligodendroglioma	2.19	0.015	1.17 - 4.11
Grade	1.00		
III vs. II	2.46	0.0040	1.33 - 4.54
IV vs. II	6.41	< 0.0001	2.11-4.57
Age	1.00		
> 50 vs. ≤50	3.10	< 0.0001	2.11 - 4.57
Mutational load	1.00		
> 40 vs. ≤ 40	0.69	0.066	0.47 - 1.03

A total of 542 samples were analyzed for this table. HR: Hazard Rate; CI: Confidence interval. Grade levels were 2, 3 and 4. Three histology levels were used (oligodendroglioma, oligoastrocytoma and astrocytoma), GBMs were categorized as astrocytomas.

classified based on their *IDH*-mutation status. Further stratification into molecular oligodendrogliomas and molecular astrocytomas involves determining the *ATRX* and/or *TP53* mutation status or determining 1p19q codeletion (these changes are mutually exclusive). Within molecular astrocytomas, mutations in *PI3* kinase genes *PIK3CA* and *PIK3R1* are likely to be associated with poor prognosis. Additional prognostic factors include tumor grade and patient age, both of which are correlated to the mutational load of the tumor. A scheme for the prognostic classification is proposed in figure 4.

A novel prognostic marker identified by current analysis are *PI3* kinase mutations. Such mutations are frequently observed in various cancer types including diffuse gliomas.[29,35] They act as lipid kinase downstream of various receptor tyrosine kinases, ultimately resulting in activation of signaling cascades involved in cell growth and proliferation, survival and migration.[36] It has been speculated that, as *PI3* kinase mutations are frequently observed in diffuse gliomas, specific inhibitors may provide clinical benefit for *PI3* kinase mutated diffuse glioma patients.[37] Here we show that PI3 kinase mutations also act as prognostic markers for molecular astrocytoma patients, providing the first evidence to demonstrate they are associated with poor outcome within a defined glioma subtype.

Our analysis also shows that grade is associated with mutational load of the tumor. This is an interesting observation as the mutational load may provide a biological explanation for tumor grade. Even if only a subset of the affected genes contributes to gliomagenesis and/or progression, an increase in mutational load would increase tumor aggressiveness. Indeed, several studies on genes mutated at a low population frequency ('low frequency genes') have demonstrated that they can contribute to tumor formation or progression.[38-43] In a larger study, we have shown that many (but not

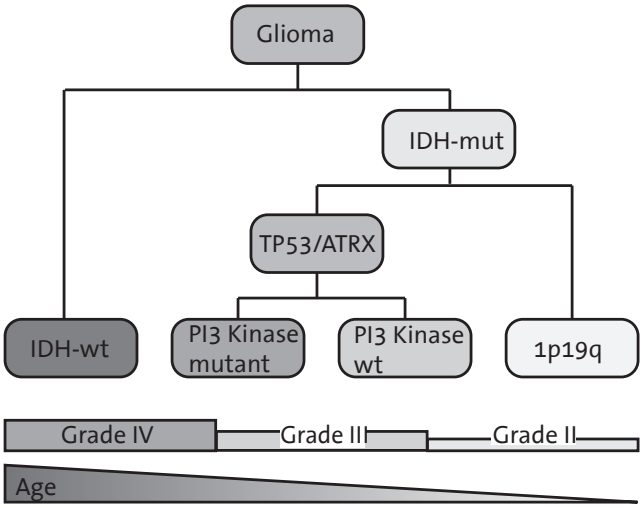

Figure 4. Proposed scheme for the prognostic classification of diffuse gliomas. Diffuse gliomas are first stratified based on their *IDH*-mutation status. Further classification is based on the *ATRX* and/or *TP53* mutation status or determining 1p19q codeletion (these changes are mutually exclusive). Within the *ATRX* and/or *TP53* mutated samples, mutations in *PI3* kinase genes *PIK3CA* and *PIK3R1* are associated with poor prognosis. It should be noted that there are genetic changes that associate with each molecular subtype (like *EGFR* amplification with *IDH*-wt tumors). They are however, not important for prognostic classification and may occur in several molecular subtypes. For example, PI3K mutations occur in all molecular subtypes but are only prognostic in *IDH*-mutated, *TP53/ATRX* mutated diffuse gliomas) it merely says the additional markers are irrelevant in this study for prognostic classification. Additional prognostic factors include tumor grade and patient age, both of which are correlated to the mutational load of the tumor and are listed below the classification scheme. These additional markers are often correlated to the mutational profile of the tumors: Patients with *IDH*-wt tumors are often older and most are diagnosed as grade IV. ATRX/TP53 indicates mutation of either/both genes; 1p19q indicates codeletion of these chromosomal arms.

all) mutations in low frequency genes affect their functional property.[44] In addition, mouse experiments have demonstrated that the age of the cells in which a glioma is generated largely determines their survival and not the age of the mouse into which the tumor is transplanted. These data argue for an intrinsic (age-related) property of the tumor initiating cell, perhaps mutational load.[45] Interestingly however, in a multivariate analysis, the mutational load is no longer a significant prognostic marker when patient age is included. The mutational load therefore cannot fully explain the increased aggressiveness of tumors of higher grade.

Our analysis also indicates that each malignancy grade is associated with a different prognosis within molecularly similar tumors. These results appear to be in contrast with a recent publication that failed to identify differences in survival between grade II and III *IDH*-mutant astrocytic tumors.[24] Similarly, a second paper found only a modest impact of tumor grade in *IDH*-mutated grade II and III gliomas.[25] However, our analysis

included all tumor grades (II-IV) whereas those studies focused only on grade II and III. In addition, our analysis did not preselect for a specific histological subtype.

It is often reported that *IDH1* mutated GBMs have a better prognosis than IDH1-wt gliomas.[6,12] The analysis presented here (using TCGA data) also shows that *IDH1* mutated grade IV tumors have a poorer prognosis than IDH1-mutated lower grade gliomas, which has also been observed in other studies. For example, *IDH1* mutated GBMs have a survival in the range of 24-30 months whereas IDH1 mutated grade III astrocytic tumours, median survival is significantly longer surpassing 50-60 months[7,12] and similarly, *IDH1*-wt GBMs have median survival of 11-15 months whereas *IDH1*-wt grade III astrocytic tumours have a median survival in the range of 21 months.[12] Here we show that the correlation between grade and prognosis is also true for other molecularly similar tumors. These data therefore argue for inclusion of tumor grade as prognostic factor when molecularly classifying diffuse gliomas and indicate that molecularly similar tumors of different grade should not be treated identical.

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SUPPLEMENTARY MATERIAL

Supplementary table 1. Genetic changes associated with survival in the entire TCGA (GBM and LGG) datasets

Gene	Hazard Ratio	lower	upper	p.value
IDH1.or.IDH2	0,103	0,071	0,15	0
IDH2	0,113	0,016	0,807	0,009
IDH1	0,123	0,085	0,179	0
ARID1A	0,17	0,042	0,686	0,005
1p19q codeletion	0,181	0,099	0,334	0
CIC	0,195	0,092	0,415	0
PCDHAC2	0,204	0,051	0,821	0,013
SMARCA4	0,206	0,051	0,831	0,014
ATRX	0,252	0,167	0,381	0
ZBTB20	0,261	0,065	1,051	0,042
LOC283788	0,354	0,113	1,107	0,062
FUBP1	0,364	0,15	0,884	0,02
NOTCH1	0,365	0,172	0,776	0,006
MUC2	0,379	0,094	1,524	0,155
AFF2	0,409	0,13	1,281	0,113
RP1	0,444	0,11	1,785	0,24
ESPNP	0,453	0,185	1,105	0,074
TP53	0,481	0,363	0,637	0
ATRX.or.TP53	0,494	0,374	0,653	0
UBC	0,499	0,124	2,009	0,318
PCDHGC5	0,506	0,209	1,229	0,125
MYH8	0,513	0,164	1,603	0,242
C3	0,537	0,2	1,445	0,211
RYR1	0,619	0,23	1,666	0,338
TPTE	0,631	0,235	1,698	0,358
IL32	0,653	0,209	2,044	0,461
HLA.J	0,656	0,243	1,769	0,401
MLL2	0,683	0,219	2,136	0,51
STK19	0,692	0,172	2,79	0,603
LOC100233156	0,695	0,327	1,477	0,341
FAM47C	0,699	0,26	1,879	0,475
NEB	0,701	0,311	1,579	0,389
KIF2B	0,704	0,262	1,894	0,485
MYH4	0,746	0,306	1,815	0,517
ABCA13	0,764	0,189	3,089	0,705
PCDH19	0,771	0,191	3,11	0,714
NBPF1	0,779	0,345	1,755	0,545
RPL13AP20	0,78	0,29	2,098	0,622
TUBBP5	0,785	0,292	2,111	0,63
HSD17B7P2	0,821	0,459	1,47	0,507
CSMD3	0,832	0,343	2,019	0,683
CHD9	0,834	0,343	2,024	0,687
HSPG2	0,843	0,347	2,046	0,705
HRNR	0,873	0,43	1,773	0,708
FRG1B	0,875	0,509	1,504	0,629
CHEK2	0,875	0,432	1,773	0,711
MUC5B	0,898	0,461	1,752	0,753
ZNF845	0,902	0,372	2,191	0,82
SCN10A	0,907	0,337	2,44	0,847
DNAH3	0,912	0,291	2,857	0,875
MUC4	0,937	0,511	1,717	0,832
CACNA1S	0,948	0,39	2,303	0,907
TCF12	0,949	0,353	2,552	0,917
FRAS1	0,953	0,486	1,87	0,888
NOTCH2	0,981	0,435	2,209	0,963
FAT2	1,006	0,495	2,045	0,987
LAMA3	1,026	0,381	2,76	0,959
GOLGA8DP	1,037	0,494	2,173	0,924
BCOR	1,039	0,532	2,027	0,912
ABCB1	1,052	0,467	2,371	0,902
GRIN2A	1,057	0,469	2,38	0,893
MYH2	1,066	0,502	2,264	0,868
MYO3A	1,073	0,399	2,887	0,889
DSG3	1,083	0,446	2,632	0,86
LAMA5	1,089	0,404	2,931	0,867
UGT2B10	1,103	0,49	2,483	0,812
LRP2	1,105	0,567	2,155	0,769
KDR	1,109	0,522	2,357	0,788
LRP1	1,119	0,497	2,521	0,786
ZNF292	1,132	0,532	2,407	0,748
CACNA1E	1,133	0,534	2,407	0,744
C15orf2	1,146	0,565	2,326	0,705
DSP	1,149	0,473	2,794	0,759
PIK3R1	1,193	0,782	1,822	0,413
DOCK5	1,194	0,589	2,421	0,622
THSD7B	1,202	0,616	2,343	0,589

Gene	Hazard Ratio	lower	upper	p.value
ZNF814	1,209	0,536	2,729	0,647
MST1P9	1,235	0,546	2,792	0,612
NBPF10	1,25	0,892	1,753	0,194
DNAH9	1,256	0,592	2,668	0,552
WASH3P	1,272	0,674	2,401	0,456
HEATR7B2	1,286	0,701	2,359	0,415
FBN2	1,294	0,481	3,483	0,609
PRDM9	1,296	0,61	2,753	0,499
DNAH5	1,309	0,731	2,344	0,363
SYNE1	1,313	0,696	2,478	0,399
DNAH11	1,315	0,649	2,665	0,445
FLG2	1,337	0,593	3,016	0,483
HMCN1	1,351	0,771	2,365	0,291
CDH18	1,377	0,511	3,707	0,525
LZTR1	1,425	0,529	3,84	0,481
OBSCN	1,426	0,814	2,497	0,213
COL1A2	1,43	0,705	2,901	0,319
TRPV6	1,44	0,591	3,506	0,419
RIMS2	1,441	0,71	2,927	0,309
MYH13	1,459	0,648	3,287	0,359
SDK1	1,476	0,728	2,992	0,278
PI3K	1,486	1,085	2,033	0,013
KRTAP4.11	1,489	0,734	3,019	0,267
USH2A	1,494	0,791	2,821	0,213
EPPK1	1,51	0,669	3,406	0,318
FLG	1,512	0,982	2,33	0,059
RYR2	1,519	0,986	2,338	0,056
KRTAP4.9	1,532	0,915	2,563	0,096
RELN	1,537	0,757	3,12	0,23
CNTNAP2	1,546	0,82	2,916	0,175
POTEC	1,553	0,796	3,031	0,193
NLRP5	1,557	0,768	3,158	0,216
ZNF844	1,557	0,577	4,2	0,378
PIK3CA	1,577	1,051	2,365	0,026
MACF1	1,583	0,745	3,364	0,228
TTN	1,61	1,178	2,201	0,003
RB1	1,615	0,954	2,733	0,072
CXorf22	1,635	0,606	4,416	0,327
MXRA5	1,637	0,808	3,317	0,167
DNAH8	1,659	0,85	3,235	0,133
TCHH	1,674	0,912	3,072	0,093
FCGBP	1,688	0,833	3,421	0,142

Gene	Hazard Ratio	lower	upper	p.value
MUC17	1,693	1,079	2,655	0,02
NF1	1,718	1,154	2,555	0,007
KRTAP4.7	1,727	1,025	2,909	0,032
AHNAK2	1,763	1,005	3,092	0,045
CHD8	1,773	0,729	4,314	0,2
PCLO	1,799	1,11	2,917	0,016
GPR98	1,844	1,004	3,388	0,045
MLL3	1,849	0,947	3,611	0,068
COL6A3	1,856	1,008	3,417	0,044
LRP1B	1,863	0,875	3,966	0,101
DCAF12L2	1,871	0,829	4,224	0,125
GABRA6	1,873	0,961	3,652	0,061
ACACB	1,886	0,887	4,011	0,094
SDHAP2	1,951	0,962	3,955	0,059
F5	1,96	1,005	3,825	0,044
ANO2	1,97	0,874	4,437	0,095
SPAG17	1,991	0,981	4,04	0,052
KEL	1,997	1,088	3,668	0,023
ABCC9	1,999	0,884	4,521	0,09
DDX11L2	2,004	0,889	4,519	0,087
APOB	2,069	1,203	3,559	0,007
MUC16	2,086	1,459	2,982	0
DMD	2,086	1,026	4,238	0,038
PKHD1	2,108	1,245	3,571	0,005
ANK2	2,131	1,05	4,326	0,032
DNAH2	2,193	1,219	3,943	0,007
RYR3	2,212	1,284	3,812	0,003
PDGFRA	2,241	1,184	4,243	0,011
SLC6A3	2,262	0,839	6,1	0,097
FBN3	2,321	1,141	4,723	0,017
DRD5	2,326	1,142	4,738	0,017
ADAMTS16	2,34	1,035	5,291	0,035
SPTA1	2,422	1,523	3,85	0
HCN1	2,426	1,282	4,593	0,005
PTEN	2,517	1,852	3,419	0
WBSCR17	2,522	1,186	5,364	0,013
STAG2	2,547	1,305	4,97	0,004
tri7loh10	2,61	1,928	3,534	0
PLEKHG4B	2,71	1,389	5,287	0,002
AHNAK	2,755	1,295	5,858	0,006
SEMA3C	2,883	1,351	6,152	0,004
TAF1L	3,127	1,464	6,678	0,002

Gene	Hazard Ratio	lower	upper	p.value
SLIT3	3,238	1,595	6,577	0,001
EGFR	3,299	2,517	4,324	0
LAMA1	3,346	1,644	6,807	0
PCDH11X	3,55	1,745	7,223	0
SCN9A	3,769	1,927	7,37	0

Supplementary table 2. Genetic changes associated with survival in IDH-wt gliomas

Gene	Hazard Ratio	lower	upper	p.value
RP1	0,214	0,053	0,867	0,018
NOTCH1	0,297	0,041	2,176	0,206
PCDHAC2	0,302	0,042	2,155	0,205
MUC2	0,312	0,077	1,257	0,083
ZBTB20	0,361	0,05	2,579	0,289
TPTE	0,43	0,159	1,164	0,087
NOTCH2	0,447	0,143	1,399	0,155
HLA.J	0,453	0,063	3,237	0,418
ARID1A	0,459	0,064	3,283	0,427
DNAH3	0,464	0,148	1,454	0,177
KIF2B	0,474	0,151	1,486	0,19
CACNA1E	0,475	0,21	1,077	0,068
DSG3	0,486	0,18	1,309	0,145
SCN10A	0,494	0,158	1,548	0,217
CHD9	0,522	0,214	1,272	0,145
ABCA13	0,531	0,131	2,143	0,366
GRIN2A	0,538	0,238	1,218	0,131
RPL13AP20	0,564	0,209	1,521	0,251
MYH8	0,565	0,181	1,77	0,321
DOCK5	0,595	0,292	1,212	0,148
TCF12	0,625	0,087	4,466	0,636
DNAH9	0,632	0,278	1,436	0,269
CDH18	0,656	0,243	1,768	0,401
ATRX	0,659	0,242	1,793	0,411
HSD17B7P2	0,662	0,347	1,264	0,208
ZNF292	0,664	0,243	1,813	0,421
CACNA1S	0,671	0,249	1,809	0,427
TUBBP5	0,677	0,251	1,826	0,438
RIMS2	0,687	0,338	1,398	0,298
LAMA5	0,696	0,258	1,874	0,47
DSP	0,709	0,292	1,727	0,447
ZNF814	0,715	0,317	1,614	0,418
KDR	0,725	0,321	1,639	0,438
MYH4	0,735	0,235	2,302	0,595
RB1	0,75	0,443	1,271	0,284
ESPNP	0,761	0,272	2,128	0,602
NLRP5	0,762	0,375	1,549	0,451
HSPG2	0,764	0,314	1,861	0,552
AFF2	0,766	0,245	2,4	0,647
C3	0,777	0,248	2,438	0,665
FRAS1	0,778	0,379	1,597	0,493
HRNR	0,782	0,346	1,766	0,553
ABCB1	0,804	0,356	1,813	0,598
MST1P9	0,806	0,35	1,854	0,611
MUC5B	0,813	0,4	1,653	0,566
GOLGA8DP	0,833	0,415	1,672	0,605
CSMD3	0,843	0,312	2,274	0,735
MLL2	0,85	0,271	2,663	0,78
LZTR1	0,861	0,319	2,323	0,768
TCHH	0,861	0,441	1,684	0,662
OBSCN	0,872	0,492	1,546	0,64
TP53	0,877	0,627	1,227	0,442
UGT2B10	0,884	0,363	2,15	0,785
FBN2	0,888	0,283	2,78	0,838
HMCN1	0,892	0,506	1,574	0,693
THSD7B	0,898	0,44	1,832	0,767
RYR2	0,9	0,566	1,433	0,657
TRPV6	0,91	0,374	2,216	0,836
SYNE1	0,912	0,466	1,784	0,788
PCDH19	0,914	0,226	3,708	0,9
ATRX.or.TP53	0,919	0,662	1,276	0,614
NEB	0,926	0,23	3,735	0,914
LOC100233156	0,927	0,296	2,904	0,896
PRDM9	0,928	0,435	1,979	0,847
LRP2	0,93	0,476	1,817	0,832
RYR1	0,934	0,346	2,524	0,894
FLG	0,945	0,594	1,503	0,811
MUC4	0,946	0,484	1,847	0,87
CNTNAP2	0,952	0,469	1,935	0,893
CXorf22	0,953	0,353	2,572	0,924

Gene	Hazard Ratio	lower	upper	p.value
FLG2	0,958	0,424	2,164	0,917
COL6A3	0,966	0,524	1,78	0,912
CHEK2	0,98	0,46	2,091	0,959
NBPF1	0,985	0,365	2,656	0,976
CIC	1	1	1	1
FUBP1	1	1	1	1
IDH1	1	1	1	1
IDH1.or.IDH2	1	1	1	1
IDH2	1	1	1	1
FAT2	1,005	0,472	2,142	0,989
HEATR7B2	1,01	0,516	1,975	0,977
ZNF844	1,016	0,377	2,739	0,975
MYH2	1,027	0,482	2,187	0,945
RELN	1,027	0,481	2,194	0,945
NF1	1,029	0,675	1,568	0,894
FRG1B	1,037	0,547	1,966	0,91
SPAG17	1,038	0,511	2,11	0,918
PCDHGC5	1,041	0,386	2,807	0,936
PTEN	1,046	0,769	1,422	0,776
MACF1	1,049	0,431	2,552	0,917
tri7loh10	1,071	0,791	1,451	0,657
AHNAK2	1,082	0,588	1,992	0,799
WASH3P	1,086	0,556	2,121	0,81
SDK1	1,091	0,538	2,216	0,809
TTN	1,105	0,792	1,541	0,556
ABCC9	1,106	0,489	2,501	0,808
EGFR	1,151	0,865	1,532	0,335
DNAH2	1,152	0,625	2,122	0,651
GABRA6	1,153	0,567	2,345	0,694
RYR3	1,16	0,673	2	0,594
GPR98	1,172	0,618	2,22	0,627
ANK2	1,198	0,562	2,552	0,639
KRTAP4.9	1,199	0,729	1,971	0,472
PCLO	1,2	0,738	1,952	0,461
SPTA1	1,213	0,763	1,928	0,414
DMD	1,223	0,597	2,507	0,581
FAM47C	1,229	0,456	3,312	0,684
KEL	1,236	0,652	2,342	0,516
DCAF12L2	1,24	0,549	2,798	0,604
MUC17	1,24	0,77	1,997	0,375
STK19	1,251	0,31	5,054	0,752
USH2A	1,251	0,638	2,451	0,513

Gene	Hazard Ratio	lower	upper	p.value
COL1A2	1,257	0,618	2,555	0,527
FBN3	1,26	0,62	2,561	0,523
LAMA3	1,279	0,408	4,009	0,672
NBPF10	1,29	0,941	1,769	0,11
PDGFRA	1,302	0,688	2,465	0,416
MYO3A	1,316	0,487	3,551	0,587
PIK3R1	1,317	0,799	2,172	0,278
FCGBP	1,322	0,65	2,688	0,439
UBC	1,324	0,328	5,344	0,692
KRTAP4.7	1,372	0,835	2,257	0,205
PLEKHG4B	1,376	0,644	2,94	0,408
PI3K	1,385	0,974	1,969	0,068
HCN1	1,391	0,734	2,636	0,309
PIK3CA	1,397	0,907	2,151	0,127
DRD5	1,403	0,688	2,86	0,349
DNAH5	1,425	0,773	2,626	0,253
MYH13	1,426	0,631	3,226	0,391
LRP1B	1,433	0,671	3,062	0,35
DNAH11	1,491	0,732	3,037	0,268
LRP1	1,514	0,619	3,702	0,36
ACACB	1,545	0,684	3,491	0,291
ADAMTS16	1,557	0,638	3,8	0,327
MUC16	1,559	1,08	2,25	0,017
EPPK1	1,568	0,693	3,548	0,276
PKHD1	1,58	0,895	2,788	0,111
MXRA5	1,585	0,743	3,383	0,23
SDHAP2	1,654	0,801	3,412	0,169
MLL3	1,658	0,815	3,374	0,158
APOB	1,692	0,963	2,976	0,065
C15orf2	1,739	0,714	4,236	0,217
POTEC	1,745	0,816	3,731	0,146
DNAH8	1,748	0,77	3,971	0,176
CHD8	1,794	0,733	4,391	0,194
PCDH11X	1,815	0,892	3,692	0,095
KRTAP4.11	1,842	0,864	3,927	0,108
SLIT3	1,885	0,927	3,836	0,075
LAMA1	1,886	0,927	3,835	0,075
SMARCA4	1,906	0,266	13,664	0,514
SCN9A	1,92	0,981	3,754	0,052
STAG2	2,051	1,046	4,022	0,033
TAF1L	2,123	0,936	4,815	0,065
ZNF845	2,181	0,806	5,902	0,115

Gene	Hazard Ratio	lower	upper	p.value
F5	2,355	1,151	4,82	0,016
BCOR	2,395	1,12	5,12	0,02
IL32	2,535	0,624	10,302	0,178
WBSCR17	2,662	1,175	6,031	0,015
SEMA3C	2,683	1,252	5,748	0,008
AHNAK	2,804	1,236	6,362	0,01

Gene	Hazard Ratio	lower	upper	p.value
DDX11L2	3,835	1,69	8,703	0,001
SLC6A3	4,313	1,356	13,714	0,007
ANO2	5,38	2,346	12,337	0
1p19q codeletion	NA	NA	NA	NA
LOC283788	NA	NA	NA	NA

Supplementary table 3. Genetic changes associated with survival in IDH-mutant gliomas

Gene	Hazard Ratio	lower	upper	p.value
tri7loh10	1	1	1	1
IDH1	2,178	0,296	16,038	0,433
TP53	1,615	0,776	3,362	0,196
ATRX	1,226	0,626	2,402	0,552
TTN	1,554	0,601	4,022	0,359
PTEN	1	1	1	1
MUC16	0,534	0,073	3,921	0,531
PIK3CA	2,871	0,838	9,838	0,079
CIC	0,698	0,304	1,599	0,392
NBPF10	1,266	0,488	3,281	0,627
NF1	2,73	0,818	9,108	0,089
FRG1B	2,744	0,933	8,067	0,056
FLG	2,174	0,657	7,191	0,192
PIK3R1	2,038	0,885	4,693	0,088
RYR2	2,382	0,713	7,955	0,146
MUC17	1,567	0,37	6,639	0,539
PCLO	NA	NA	NA	NA
HSD17B7P2	0,818	0,194	3,449	0,784
MUC4	1,224	0,291	5,152	0,782
NOTCH1	1,315	0,545	3,177	0,541
RB1	1	1	1	1
SPTA1	NA	NA	NA	NA
FUBP1	1,594	0,612	4,148	0,335
HMCN1	NA	NA	NA	NA
AHNAK2	9,585	2,147	42,782	0
LRP2	NA	NA	NA	NA
OBSCN	NA	NA	NA	NA
DNAH5	0,501	0,068	3,69	0,489
APOB	0,848	0,114	6,308	0,872
PKHD1	6,123	1,377	27,223	0,007
WASH3P	0,625	0,085	4,628	0,643
PCDHGC5	0,496	0,068	3,639	0,482

Gene	Hazard Ratio	lower	upper	p.value
RYR3	NA	NA	NA	NA
TCHH	5,623	1,305	24,227	0,009
USH2A	2,308	0,3	17,742	0,408
COL6A3	NA	NA	NA	NA
FAT2	0,547	0,073	4,091	0,551
GPR98	2,833	0,378	21,212	0,289
KEL	2,113	0,282	15,802	0,456
CHEK2	0,908	0,122	6,768	0,925
FRAS1	0,556	0,074	4,163	0,562
PCDHAC2	0,445	0,061	3,273	0,414
SYNE1	0,883	0,118	6,582	0,903
DNAH8	5,496	1,596	18,933	0,002
KRTAP4.11	2,76	0,359	21,23	0,309
MUC5B	0,589	0,079	4,358	0,6
RELN	2,76	0,369	20,653	0,302
COL1A2	NA	NA	NA	NA
DNAH11	NA	NA	NA	NA
DOCK5	NA	NA	NA	NA
HEATR7B2	1,666	0,395	7,024	0,482
MYH2	NA	NA	NA	NA
PDGFRA	NA	NA	NA	NA
ZNF814	NA	NA	NA	NA
ARID1A	0,332	0,045	2,442	0,255
BCOR	0,98	0,232	4,148	0,978
CNTNAP2	2,758	0,646	11,766	0,153
EPPK1	NA	NA	NA	NA
ESPNP	0,339	0,046	2,494	0,265
FLG2	NA	NA	NA	NA
HRNR	0,785	0,186	3,309	0,74
LOC100233156	1,516	0,528	4,353	0,436
MST1P9	NA	NA	NA	NA
POTEC	1,462	0,346	6,183	0,604

Gene	Hazard Ratio	lower	upper	p.value
DMD	NA	NA	NA	NA
DNAH2	NA	NA	NA	NA
FBN3	NA	NA	NA	NA
LOC283788	2,178	0,646	7,342	0,198
LRP1	1,774	0,237	13,284	0,572
MLL3	0,91	0,122	6,795	0,927
MXRA5	2,051	0,275	15,272	0,474
NBPF1	1,002	0,237	4,244	0,998
NEB	2,695	0,934	7,776	0,056
SDHAP2	NA	NA	NA	NA
STK19	NA	NA	NA	NA
TPTE	NA	NA	NA	NA
UBC	NA	NA	NA	NA
ZBTB20	0,646	0,087	4,784	0,666
CACNA1E	24,548	2,527	238,439	0
DNAH9	7,372	0,94	57,836	0,026
DSP	NA	NA	NA	NA
FCGBP	NA	NA	NA	NA
GABRA6	2,113	0,282	15,802	0,456
HCN1	NA	NA	NA	NA
HSPG2	NA	NA	NA	NA
KRTAP4.7	NA	NA	NA	NA
KRTAP4.9	NA	NA	NA	NA
MYH8	NA	NA	NA	NA
PRDM9	NA	NA	NA	NA
SMARCA4	0,304	0,041	2,238	0,216
SPAG17	NA	NA	NA	NA
STAG2	NA	NA	NA	NA
THSD7B	1,118	0,151	8,266	0,913
ABCB1	NA	NA	NA	NA
C15orf2	1,699	0,514	5,621	0,379
CACNA1S	2,713	0,359	20,49	0,314
CHD9	NA	NA	NA	NA
CSMD3	1,185	0,16	8,767	0,868
F5	2,309	0,31	17,219	0,401
KDR	2,809	0,365	21,633	0,3
MACF1	5,804	1,344	25,055	0,008
MYH13	NA	NA	NA	NA
MYH4	1,084	0,254	4,628	0,914
NLRP5	NA	NA	NA	NA
PCDH19	NA	NA	NA	NA
RIMS2	1	1	1	1

Gene	Hazard Ratio	lower	upper	p.value
RPL13AP20	NA	NA	NA	NA
SDK1	NA	NA	NA	NA
TUBBP5	NA	NA	NA	NA
ABCC9	NA	NA	NA	NA
ACACB	4,427	0,57	34,409	0,12
AFF2	NA	NA	NA	NA
C3	0,573	0,078	4,219	0,58
CDH18	1	1	1	1
CHD8	NA	NA	NA	NA
DCAF12L2	NA	NA	NA	NA
DNAH3	1	1	1	1
DRD5	NA	NA	NA	NA
FAM47C	NA	NA	NA	NA
FBN2	6,702	0,868	51,725	0,035
GRIN2A	NA	NA	NA	NA
IDH2	0,459	0,062	3,382	0,433
KIF2B	1,28	0,173	9,478	0,808
LAMA1	NA	NA	NA	NA
LAMA3	2,625	0,349	19,751	0,33
LRP1B	NA	NA	NA	NA
LZTR1	NA	NA	NA	NA
MLL2	NA	NA	NA	NA
MUC2	NA	NA	NA	NA
NOTCH2	5,799	1,719	19,567	0,001
PCDH11X	1	1	1	1
RP1	NA	NA	NA	NA
SEMA3C	NA	NA	NA	NA
TCF12	5,085	1,507	17,159	0,004
UGT2B10	1,467	0,198	10,878	0,706
WBSCR17	5,72	0,735	44,514	0,059
ABCA13	NA	NA	NA	NA
ADAMTS16	16,369	1,967	136,219	0
AHNAK	5,606	0,734	42,816	0,061
ANK2	8,049	1,037	62,448	0,017
ANO2	NA	NA	NA	NA
CXorf22	NA	NA	NA	NA
DDX11L2	NA	NA	NA	NA
DSG3	9,026	1,154	70,577	0,011
GOLGA8DP	NA	NA	NA	NA
HLA.J	1,547	0,465	5,14	0,473
IL32	1,408	0,186	10,632	0,739
LAMA5	NA	NA	NA	NA

Gene	Hazard Ratio	lower	upper	p.value
MYO3A	NA	NA	NA	NA
PLEKHG4B	28,862	5,812	143,325	0
RYR1	NA	NA	NA	NA
SCN10A	3,379	0,449	25,412	0,209
SCN9A	1	1	1	1
SLC6A3	6,361	0,816	49,596	0,043
SLIT3	NA	NA	NA	NA
TAF1L	36,377	3,783	349,822	0
TRPV6	NA	NA	NA	NA

Gene	Hazard Ratio	lower	upper	p.value
ZNF292	4,513	1,338	15,228	0,008
ZNF844	NA	NA	NA	NA
ZNF845	1,369	0,183	10,225	0,759
PI3K	2,746	1,308	5,767	0,005
IDH1.or.IDH2	1	1	1	1
ATRX.or.TP53	1,601	0,769	3,334	0,204
1p19q codeletion	0,689	0,338	1,406	0,303
EGFR	NA	NA	NA	NA

Supplementary table 4. Genetic changes associated with survival in IDH- mutated and 1p19q codeleted gliomas

Gene	Hazard Ratio	lower	upper	p.value
tri7loh10	1	1	1	1
IDH1	1,509	0,186	12,27	0,699
TP53	2,545	0,307	21,094	0,37
ATRX	NA	NA	NA	NA
TTN	2,641	0,317	22,013	0,351
PTEN	1	1	1	1
MUC16	NA	NA	NA	NA
PIK3CA	2,158	0,399	11,659	0,36
CIC	0,934	0,269	3,237	0,914
NBPF10	4,012	0,797	20,197	0,069
NF1	3,689	0,734	18,542	0,09
FRG1B	2,008	0,229	17,604	0,521
FLG	5,03	0,578	43,766	0,104
PIK3R1	0,99	0,208	4,703	0,99
RYR2	NA	NA	NA	NA
MUC17	NA	NA	NA	NA
PCLO	NA	NA	NA	NA
HSD17B7P2	0,953	0,117	7,783	0,964
MUC4	1,294	0,156	10,724	0,811
NOTCH1	1,869	0,56	6,235	0,302
RB1	1	1	1	1
SPTA1	NA	NA	NA	NA
FUBP1	2,579	0,738	9,019	0,124
HMCN1	NA	NA	NA	NA
AHNAK2	NA	NA	NA	NA
LRP2	NA	NA	NA	NA
OBSCN	NA	NA	NA	NA
DNAH5	NA	NA	NA	NA

Gene	Hazard Ratio	lower	upper	p.value
APOB	23,766	2,11	267,696	0
PKHD1	1	1	1	1
WASH3P	NA	NA	NA	NA
PCDHGC5	NA	NA	NA	NA
RYR3	NA	NA	NA	NA
TCHH	9,637	1,812	51,261	0,001
USH2A	NA	NA	NA	NA
COL6A3	1	1	1	1
FAT2	NA	NA	NA	NA
GPR98	3,907	0,459	33,27	0,179
KEL	NA	NA	NA	NA
CHEK2	NA	NA	NA	NA
FRAS1	0,849	0,098	7,365	0,882
PCDHAC2	NA	NA	NA	NA
SYNE1	1,216	0,143	10,314	0,858
DNAH8	18,856	1,858	191,407	0,001
KRTAP4.11	NA	NA	NA	NA
MUC5B	NA	NA	NA	NA
RELN	4,657	0,528	41,107	0,128
COL1A2	1	1	1	1
DNAH11	NA	NA	NA	NA
DOCK5	NA	NA	NA	NA
HEATR7B2	NA	NA	NA	NA
MYH2	NA	NA	NA	NA
PDGFRA	NA	NA	NA	NA
ZNF814	1	1	1	1
ARID1A	NA	NA	NA	NA
BCOR	1,203	0,147	9,841	0,863

Gene	Hazard Ratio	lower	upper	p.value
CNTNAP2	6,795	0,742	62,256	0,05
EPPK1	NA	NA	NA	NA
ESPNP	1,648	0,196	13,827	0,642
FLG2	NA	NA	NA	NA
HRNR	1,269	0,154	10,473	0,825
LOC100233156	2,071	0,521	8,231	0,291
MST1P9	NA	NA	NA	NA
POTEC	1,286	0,15	11,066	0,818
DMD	1	1	1	1
DNAH2	NA	NA	NA	NA
FBN3	1	1	1	1
LOC283788	3,852	0,448	33,122	0,186
LRP1	NA	NA	NA	NA
MLL3	1,367	0,156	11,981	0,777
MXRA5	4,387	0,504	38,216	0,144
NBPF1	NA	NA	NA	NA
NEB	1,669	0,202	13,802	0,631
SDHAP2	NA	NA	NA	NA
STK19	NA	NA	NA	NA
TPTE	NA	NA	NA	NA
UBC	NA	NA	NA	NA
ZBTB20	NA	NA	NA	NA
CACNA1E	NA	NA	NA	NA
DNAH9	NA	NA	NA	NA
DSP	1	1	1	1
FCGBP	1	1	1	1
GABRA6	1	1	1	1
HCN1	1	1	1	1
HSPG2	NA	NA	NA	NA
KRTAP4.7	NA	NA	NA	NA
KRTAP4.9	NA	NA	NA	NA
MYH8	NA	NA	NA	NA
PRDM9	1	1	1	1
SMARCA4	1,456	0,178	11,909	0,725
SPAG17	1	1	1	1
STAG2	1	1	1	1
THSD7B	NA	NA	NA	NA
ABCB1	NA	NA	NA	NA
C15orf2	1,344	0,161	11,223	0,784
CACNA1S	NA	NA	NA	NA
CHD9	1	1	1	1
CSMD3	NA	NA	NA	NA

Gene	Hazard Ratio	lower	upper	p.value
F5	NA	NA	NA	NA
KDR	NA	NA	NA	NA
MACF1	1	1	1	1
MYH13	1	1	1	1
MYH4	NA	NA	NA	NA
NLRP5	NA	NA	NA	NA
PCDH19	NA	NA	NA	NA
RIMS2	1	1	1	1
RPL13AP20	NA	NA	NA	NA
SDK1	1	1	1	1
TUBBP5	NA	NA	NA	NA
ABCC9	NA	NA	NA	NA
ACACB	NA	NA	NA	NA
AFF2	NA	NA	NA	NA
C3	NA	NA	NA	NA
CDH18	1	1	1	1
CHD8	NA	NA	NA	NA
DCAF12L2	NA	NA	NA	NA
DNAH3	1	1	1	1
DRD5	1	1	1	1
FAM47C	NA	NA	NA	NA
FBN2	1	1	1	1
GRIN2A	NA	NA	NA	NA
IDH2	0,663	0,082	5,39	0,699
KIF2B	1	1	1	1
LAMA1	NA	NA	NA	NA
LAMA3	4,464	0,518	38,48	0,136
LRP1B	NA	NA	NA	NA
LZTR1	NA	NA	NA	NA
MLL2	NA	NA	NA	NA
MUC2	NA	NA	NA	NA
NOTCH2	NA	NA	NA	NA
PCDH11X	1	1	1	1
RP1	NA	NA	NA	NA
SEMA3C	1	1	1	1
TCF12	5,759	0,642	51,658	0,076
UGT2B10	NA	NA	NA	NA
WBSCR17	1	1	1	1
ABCA13	1	1	1	1
ADAMTS16	1	1	1	1
AHNAK	NA	NA	NA	NA
ANK2	1	1	1	1

Gene	Hazard Ratio	lower	upper	p.value
ANO2	1	1	1	1
CXorf22	NA	NA	NA	NA
DDX11L2	NA	NA	NA	NA
DSG3	1	1	1	1
GOLGA8DP	1	1	1	1
HLA.J	6,748	0,698	65,226	0,056
IL32	2,663	0,291	24,417	0,368
LAMA5	1	1	1	1
MYO3A	NA	NA	NA	NA
PLEKHG4B	41,661	5,691	304,965	0
RYR1	NA	NA	NA	NA
SCN10A	1	1	1	1
SCN9A	1	1	1	1
SLC6A3	23,766	2,11	267,696	0
SLIT3	1	1	1	1
TAF1L	NA	NA	NA	NA
TRPV6	NA	NA	NA	NA
ZNF292	6,156	1,19	31,854	0,013
ZNF844	NA	NA	NA	NA
ZNF845	NA	NA	NA	NA
PI3K	1,676	0,485	5,794	0,409
IDH1.or.IDH2	1	1	1	1
ATRX.or.TP53	2,123	0,259	17,435	0,473
1p19q codeletion	1	1	1	1
EGFR	NA	NA	NA	NA

Supplementary table 5. Genetic changes associated with survival in IDH-and TP53/ATRX mutated gliomas

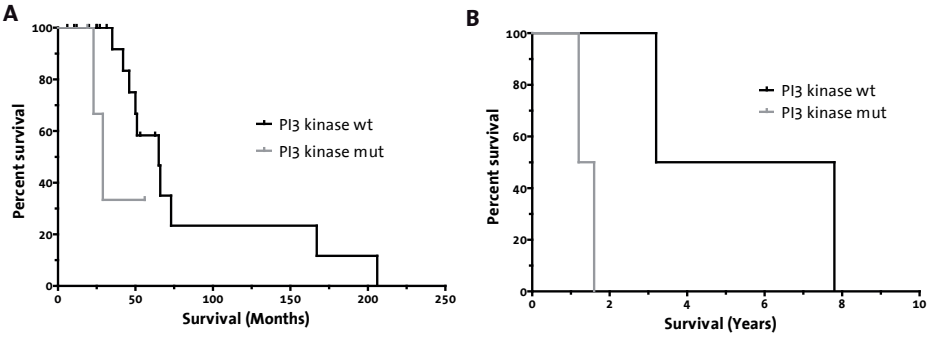
Gene	Hazard Ratio	lower	upper	p.value
tri7loh10	1	1	1	1
IDH1	NA	NA	NA	NA
TP53	NA	NA	NA	NA
ATRX	0,471	0,149	1,484	0,188
TTN	1,16	0,395	3,412	0,787
PTEN	1	1	1	1
MUC16	0,473	0,064	3,515	0,454
PIK3CA	64,622	4,026	1037,338	0
CIC	NA	NA	NA	NA
NBPF10	0,842	0,284	2,492	0,756
NF1	3,958	0,507	30,883	0,156
FRG1B	2,641	0,763	9,144	0,111
FLG	1,475	0,342	6,368	0,6
PIK3R1	4,938	1,813	13,453	0,001
RYR2	1,192	0,274	5,193	0,814
MUC17	1,253	0,289	5,437	0,763
PCLO	NA	NA	NA	NA
HSD17B7P2	0,705	0,094	5,279	0,732
MUC4	1,864	0,239	14,518	0,546
NOTCH1	2,649	0,602	11,65	0,18
RB1	1	1	1	1
SPTA1	1	1	1	1
FUBP1	1,957	0,256	14,993	0,51
HMCN1	NA	NA	NA	NA
AHNAK2	4,748	0,596	37,843	0,104
LRP2	NA	NA	NA	NA
OBSCN	NA	NA	NA	NA
DNAH5	0,568	0,075	4,317	0,58
APOB	NA	NA	NA	NA
PKHD1	6,106	1,291	28,887	0,009
WASH3P	0,763	0,1	5,801	0,793
PCDHGC5	0,551	0,074	4,122	0,556
RYR3	NA	NA	NA	NA
TCHH	NA	NA	NA	NA
USH2A	2,32	0,289	18,627	0,415
COL6A3	1	0	Inf	1
FAT2	0,461	0,06	3,561	0,448
GPR98	1	1	1	1
KEL	1,698	0,222	12,986	0,606
CHEK2	1,223	0,159	9,384	0,846
FRAS1	NA	NA	NA	NA
PCDHAC2	0,376	0,05	2,847	0,326
SYNE1	NA	NA	NA	NA
DNAH8	3,818	0,84	17,354	0,062
KRTAP4.11	4,049	0,503	32,623	0,155
MUC5B	1,993	0,262	15,185	0,497

Gene	Hazard Ratio	lower	upper	p.value
RELN	NA	NA	NA	NA
COL1A2	NA	NA	NA	NA
DNAH11	NA	NA	NA	NA
DOCK5	NA	NA	NA	NA
HEATR7B2	1,585	0,367	6,84	0,534
MYH2	NA	NA	NA	NA
PDGFRA	1	0	Inf	1
ZNF814	1	0	Inf	1
ARID1A	0,94	0,123	7,182	0,952
BCOR	0,97	0,127	7,411	0,977
CNTNAP2	1,483	0,194	11,318	0,702
EPPK1	NA	NA	NA	NA
ESPNP	NA	NA	NA	NA
FLG2	NA	NA	NA	NA
HRNR	0,493	0,063	3,838	0,491
LOC100233156	1,733	0,393	7,638	0,462
MST1P9	NA	NA	NA	NA
POTEC	2,996	0,388	23,107	0,269
DMD	NA	NA	NA	NA
DNAH2	1	1	1	1
FBN3	NA	NA	NA	NA
LOC283788	1,626	0,369	7,172	0,517
LRP1	1,654	0,215	12,72	0,625
MLL3	1	0	Inf	1
MXRA5	NA	NA	NA	NA
NBPF1	0,784	0,181	3,395	0,744
NEB	3,493	0,976	12,498	0,041
SDHAP2	NA	NA	NA	NA
STK19	NA	NA	NA	NA
TPTE	NA	NA	NA	NA
UBC	NA	NA	NA	NA
ZBTB20	0,618	0,081	4,742	0,641
CACNA1E	5,05E+31	0	Inf	0
DNAH9	9,3	1,118	77,365	0,012
DSP	NA	NA	NA	NA
FCGBP	NA	NA	NA	NA
GABRA6	1,698	0,222	12,986	0,606
HCN1	NA	NA	NA	NA
HSPG2	NA	NA	NA	NA
KRTAP4.7	1	1	1	1
KRTAP4.9	1	1	1	1
MYH8	NA	NA	NA	NA

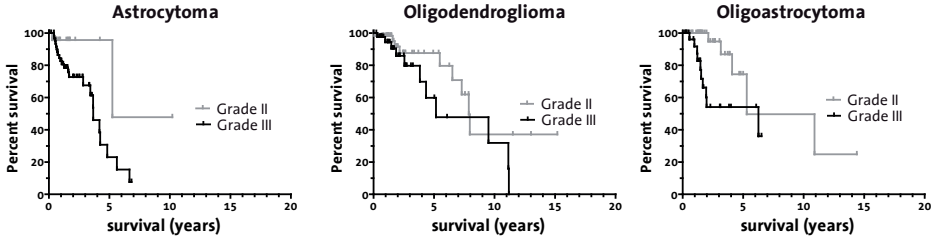
Gene	Hazard Ratio	lower	upper	p.value
PRDM9	NA	NA	NA	NA
SMARCA4	NA	NA	NA	NA
SPAG17	NA	NA	NA	NA
STAG2	NA	NA	NA	NA
THSD7B	0,974	0,129	7,328	0,979
ABCB1	NA	NA	NA	NA
C15orf2	2,837	0,648	12,428	0,148
CACNA1S	2,539	0,327	19,686	0,355
CHD9	NA	NA	NA	NA
CSMD3	3,091	0,391	24,441	0,26
F5	2,253	0,295	17,211	0,421
KDR	4,885	0,596	40,023	0,102
MACF1	5,548	1,239	24,838	0,012
MYH13	NA	NA	NA	NA
MYH4	0,828	0,189	3,627	0,801
NLRP5	1	1	1	1
PCDH19	NA	NA	NA	NA
RIMS2	1	1	1	1
RPL13AP20	NA	NA	NA	NA
SDK1	NA	NA	NA	NA
TUBBP5	NA	NA	NA	NA
ABCC9	NA	NA	NA	NA
ACACB	4,488	0,549	36,709	0,125
AFF2	NA	NA	NA	NA
C3	0,812	0,108	6,116	0,839
CDH18	1	1	1	1
CHD8	NA	NA	NA	NA
DCAF12L2	NA	NA	NA	NA
DNAH3	1	1	1	1
DRD5	NA	NA	NA	NA
FAM47C	NA	NA	NA	NA
FBN2	7,112	0,873	57,97	0,032
GRIN2A	1	1	1	1
IDH2	NA	NA	NA	NA
KIF2B	0,974	0,129	7,328	0,979
LAMA1	1	0	Inf	1
LAMA3	NA	NA	NA	NA
LRP1B	NA	NA	NA	NA
LZTR1	1	1	1	1
MLL2	NA	NA	NA	NA
MUC2	NA	NA	NA	NA
NOTCH2	5,477	1,554	19,302	0,003

Gene	Hazard Ratio	lower	upper	p.value
PCDH11X	1	1	1	1
RP1	1	0	Inf	1
SEMA3C	NA	NA	NA	NA
TCF12	4,102	0,925	18,189	0,044
UGT2B10	12,56	1,395	113,113	0,004
WBSCR17	5,843	0,712	47,917	0,062
ABCA13	NA	NA	NA	NA
ADAMTS16	19,903	2,066	191,789	0
AHNAK	5,385	0,678	42,741	0,074
ANK2	7,791	0,957	63,422	0,023
ANO2	NA	NA	NA	NA
CXorf22	NA	NA	NA	NA
DDX11L2	NA	NA	NA	NA
DSG3	7,791	0,957	63,422	0,023
GOLGA8DP	NA	NA	NA	NA
HLA.J	0,753	0,172	3,299	0,705
IL32	NA	NA	NA	NA
LAMA5	NA	NA	NA	NA

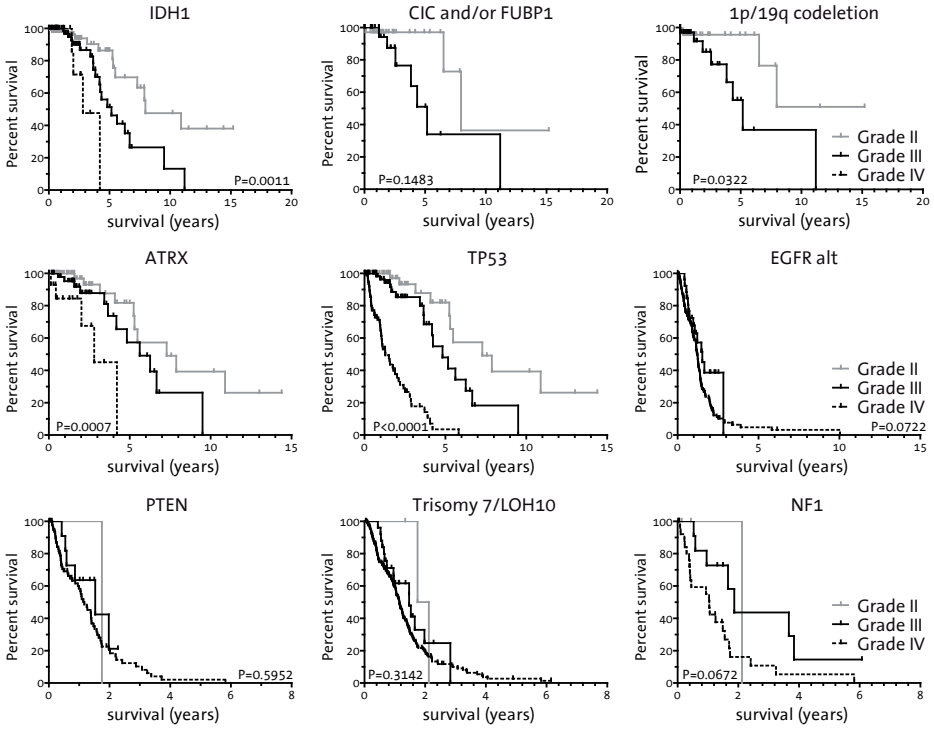
Gene	Hazard Ratio	lower	upper	p.value
MYO3A	NA	NA	NA	NA
PLEKHG4B	NA	NA	NA	NA
RYR1	NA	NA	NA	NA
SCN10A	2,996	0,388	23,107	0,269
SCN9A	1	1	1	1
SLC6A3	NA	NA	NA	NA
SLIT3	NA	NA	NA	NA
TAF1L	NA	NA	NA	NA
TRPV6	NA	NA	NA	NA
ZNF292	4,578	0,582	36,005	0,112
ZNF844	NA	NA	NA	NA
ZNF845	1,594	0,209	12,157	0,65
PI3K	6,246	2,358	16,549	0
IDH1.or.IDH2	1	1	1	1
ATRX.or.TP53	1	1	1	1
1p19q codeletion	1,957	0,256	14,993	0,51
EGFR	NA	NA	NA	NA



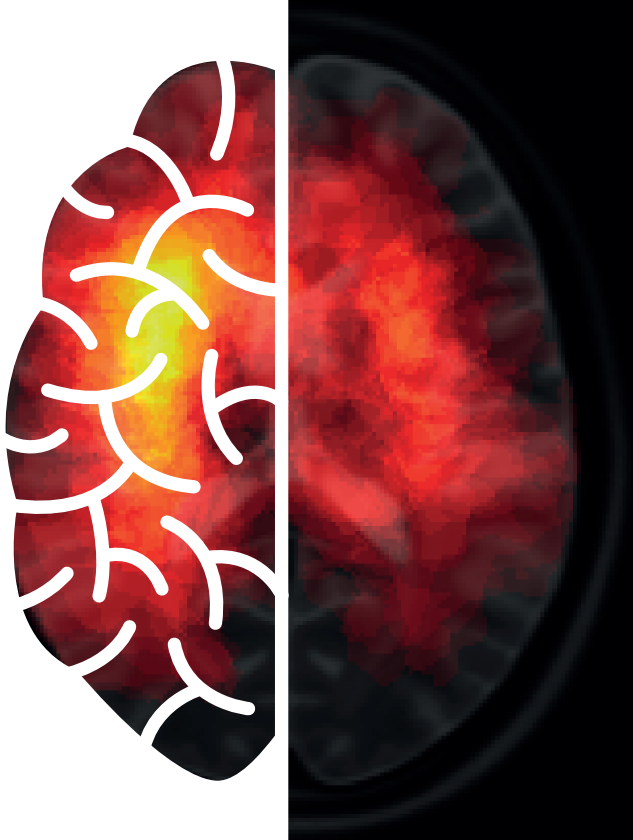
Supplementary figure 1. *PI3*-kinase mutations are prognostic for survival in independent datasets. Validation showing that *PI3*-kinase mutations are prognostic for survival in a dataset of astrocytomas (A) and glioblastomas (B, n=4) after selecting for tumors for *IDH1* and *TP53/ATRX* mutations (1, 2). *PI3*-kinase mutations were found in 4/24 and 2/4 samples in the astrocytoma and glioblastoma datasets.



Supplementary figure 2. Survival in histological subtypes of glioma stratified by tumor grade of samples included in the TCGA dataset. Median overall survival (OS) for astrocytomas was 5.2 and 3.7 years for grade II (n=30) and grade III (n=68). Median OS for oligodendrogliomas was 7.9 and 5.2 years for grade II (n=65) and grade III (n=45). Median OS for oligoastrocytomas was 5.3 and 6.3 years for grade II (n=42) and grade III (n=31), all P values stated in the figures are calculated using a Log-rank test. A Gehan-Breslow-Wilcoxon test, which gives more weight to early events, was also performed using Graphpad Prism software and yields P values of 0.19, 0.28 and 0.0013 for astrocytomas, oligodendrogliomas and oligoastrocytomas respectively.



Supplementary figure 3. Overall survival in distinct molecular subtypes of glioma stratified by tumor grade of samples included in the TCGA dataset. As can be seen, within most defined molecular subtypes, tumor grade remains a prognostic factor. P values were calculated using a log rank test. Number of samples (grade II, III and IV) for each graph: 119, 96 and 13 (IDH1); 37, 26 and 1 (CIC and/or FUBP1); 42, 32 and 0 (1p/19q codeletion); 67, 50 and 14 (ATRX); 71, 73 and 78 (TP53); 0, 26 and 144 (EGFR); 1, 12 and 80 (PTEN); 3, 26 and 185 (trisomy 7/LOH10); 3, 14 and 27 (NF1). Most cases with CIC/FUBP1, 1p/19q codeletion, ATRX or TP53, also have an IDH mutation



Chapter 3

Prognostic relevance of mutations and copy number alterations assessed with targeted next generation sequencing in IDH mutant grade II glioma

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ABSTRACT

Background

At current prognostication of low grade glioma remains suboptimal and might be improved with additional markers. These may guide treatment decisions, in particular on early adjuvant therapy versus wait and see after surgery.

Methods

We used a targeted Next-Generation Sequencing panel to assess mutational and copy number status of selected genes and chromosomes in a consecutive series of adult grade II supratentorial glioma, and assessed the impact of molecular markers of interest on overall survival.

Results

207 *IDH* mutated grade II glioma samples were analyzed with a median follow-up of 6.9 years. Loss of region 9p21.3 did not show a correlation with outcome in *IDH* mutated 1p/19q-codeleted oligodendroglioma or *IDH* mutated astrocytoma. We found a significant shorter overall survival with univariable analysis in *IDH* mutated astrocytoma patients with polysomy of chromosome 7 (Log rank P = 0.044) and in *IDH* mutated 1p/19q-codeleted oligodendroglioma patients with a *PTEN* mutation (Log rank P = 0.033). We could not validate these findings in multivariate analysis or in the TCGA dataset.

Conclusions

Loss of 9p21.3 is not associated with outcome in a molecularly defined cohort of grade II glioma and therefore it remains unclear if loss of 9p21.3 can be used as additional marker of anaplasia or to guide treatment decisions. Trisomy of chromosome 7 in *IDH* mutated astrocytoma and *PTEN* mutations in *IDH* mutated oligodendroglioma are potential markers of poor prognosis, but require confirmation in larger series.

INTRODUCTION

In 2016 the World Health Organization Classification of Tumors of the Central Nervous System (WHO) was updated, resulting in a major change in the classification of diffuse gliomas.[1] The 2016 WHO classification provides an integrated diagnosis of glioma by combining histopathological with genotypic features. Moreover, in the case of discrepancy between genotypic and histopathological features, the genotypic features are leading in classifying a glioma subtype. As a result the 2016 CNS WHO markedly improved the objectivity of classification and prognosis estimation as compared to the previous version.[1-3]

Three clinically relevant subgroups of diffuse low-grade (grade II) gliomas are identified and recognized by the WHO 2016 classification based on two molecular markers: 1) Oligodendroglioma, *IDH* mutant and 1p/19q-codeleted (*IDH1*/2 mutation in combination with presence of a co-deletion of the entire 1p and 19q chromosomal arms), 2) diffuse astrocytoma, *IDH* mutant; (*IDH1/2* mutation without 1p19q co-deletion), and 3) diffuse astrocytoma, *IDH1/2* wildtype. Other frequently reported genetic changes in glioma are *CIC*, *FUBP1*, *TP53*, *ATRX*, *TERT* promoter mutations and copy number changes of chromosome 7, 9, and 10. [1, 2, 4, 5] Although the 2016 CNS WHO update is robust and provides a more accurate prognosis estimation, there is still variation in outcome within the different entities and the grading of glioma is still depending on histological features. Prognostication might be further improved if additional molecular markers can be identified that correlate with prognosis. These markers may facilitate treatment decisions, in particular for early radiotherapy and adjuvant chemotherapy versus a wait and watch policy after surgery. This is in particular relevant, as the currently used criteria (age of 40, less than gross total resection) are quite arbitrary, and in some patients that are 40 years or older with a less than gross total resection, a wait and watch period of years is possible.[6-8] Thus, molecular factors showing a clinically significant relation with outcome would be highly welcome.

Several studies have tried to further stratify molecularly defined glioma, most of them in anaplastic glioma. For example, some studies showed that loss of chromosome 9p or specifically the 9p21.3 region is associated with a worse prognosis in various subtypes of grade III and IV glioma.[9-11] This could however not be validated in a recently published large cohort of grade II and III glioma.[12] In the present study we aimed to identify molecular prognostic markers for grade II *IDH* mutated astrocytoma (in particular loss of 10q, trisomy of chromosome 7, *PTEN* mutations) and in grade II *IDH* mutated 1p19q-codeleted oligodendroglioma (in particular loss of 9p21.3, *CIC*, *FUBP1*, and *PTEN* mutations). To our knowledge there are no published studies that investigated prognostic impact of these markers specifically in molecularly defined grade II glioma. Therefore, in a well-defined cohort of histologically proven supraten-

torial adult grade II glioma we used a targeted Next-Generation Sequencing panel for molecular classification and evaluated the prognostic value of glioma specific molecular markers.

METHODS

Patient selection

For this study we used a cohort of patients from a project on extent of resection in grade II glioma.[13] Adult patients (age ≥ 18 years) with histopathologically confirmed supratentorial grade II glioma were included. Tissue samples were collected in two Dutch hospitals (Erasmus MC Cancer Institute, Rotterdam; and Elisabeth-TweeSteden Hospital, Tilburg). Histopathological diagnosis and low grade was confirmed by a dedicated neuropathologist (J.M.K.). Time-window of patient inclusion was 2003-2016. For clinical factors, age, KPS, type of surgery, and treatment after surgery were collected. This study was approved by the medical ethics committee of Erasmus MC.

DNA extraction

DNA was isolated from formalin-fixed-paraffin-embedded (FFPE) tissue blocks. Tissue areas with high percentage of neoplastic cells (preferably >70%, but at least 50%) were manually macrodissected from 10µm sections. Macrodissected tissue was digested using Proteinase K incubation at 56°C overnight in presence of 5% Chelex 100 resin (Bio-Rad). After overnight incubation, proteinase K was inactivated at 90°C for 10 minutes. Next, dissolved DNA was separated from Chelex resin and cell debris by centrifugation at 20g for 5 minutes. DNA concentration was measured using the Qubit 3.0 Fluorometer according to manufacturer's protocol (Life Technologies).

Next-Generation-Sequencing

We used Next-Generation Sequencing to assess mutational and copy number status of selected genes and chromosomes. The primer panel consisted of primers for glioma specific genes of interest (hot spot regions, or whole gene) and primers for highly polymorphic single nucleotide polymorphisms to detect large genomic alterations in chromosomes of interest. Chromosomal imbalances and loss of heterozygosity (LOH) were estimated as described previously.[3] An overview of the targeted hotspots/whole genes and chromosomes is shown in Supplementary Table 1. Sequencing was performed with the Ion Torrent Personal Genome Machine or Ion S5 (Life Technologies). *TERT* promoter mutational status (C228T & C250T mutation) was assessed in a separate assay as described before (SNaPshot, Life Technologies).[3]

Survival

All patients were followed until death or censored at date of last follow-up. Overall survival (OS) was measured from the date of diagnostic scan until date of death or censorship. Date of death was provided by patient records or the Municipal Personal Records Database. The database was developed and maintained at Erasmus MC, and locked on March 21st 2018.

Statistical analysis

All analyses were performed using R (3.3.2) and RStudio (1.0.44). Overall survival was measured as time between date of diagnostic scan and date of death or censorship. Overall survival is shown in Kaplan-Meier plots (ggplot2 package in R). Univariable analyses were performed using the Log-rank test and multivariable analyses with Cox proportional-hazards models. Categorical data were analyzed with Pearson's chi-square test or Fisher's exact test when assumptions of the chi-square test were violated (as indicated in the respective tables). Kruskal-Wallis test was used for continuous data. All calculations were two-sided tests, with a p-value <0.05 considered as statistically significant.

RESULTS

We identified 246 patients with a pathologically confirmed grade II glioma with available FFPE material. Of these, 2 patients were excluded due to insufficient DNA yield and no remaining tissue for DNA isolation, and 14 were excluded from analysis due to sequencing failure (very low coverage and/or uniformity for most amplicons after two attempts). At the time of analysis, 53 patients were reported dead, 15 of the 95 IDH mutated 1p/19q-codeleted oligodendroglioma patients and 38 of the 112 IDH mutated astrocytoma patients.

Molecular classification

As we were interested in additional markers for *IDH* mutated grade II glioma, 23 patient samples that were classified as *IDH* wildtype were excluded from further analyses. 207 patients were included in final analyses with a median follow-up of 6.9 years (range 0.4-21.7 years). Clinical characteristics are shown in Table 1. An oncoprint plot with all mutations and copy number alterations for the 207 patients is shown in figure 1. Mutation frequencies per molecular subgroup are shown in table 2. Median overall survival per CNS WHO 2016 molecular subgroup was consistent with literature (Supplementary Figure 1).

Table 1. Patient characteristics

	Oligodendroglioma		Astrocytoma IDHmt		
Characteristics	N	%	N	%	P
Patients (n)	95		112		
Sex					0.149
Male	49	51,6	71	63.4	
Female	46	48,4	41	36.6	
Age					<0.0001
Median (IQR)	45	(37-52)	37	(29 - 45)	
Type of 1st surgery					0.002†
Awake craniotomy	51	53,7	54	48.2	
Normal resection	23	24,2	49	43.8	
Open biopsy	7	7,4	2	1.8	
Stereotactic biopsy	14	14,7	7	6.2	
Preoperative KPS					0.064
Median (IQR)	100	(100-100)	100	(90 - 100)	
Histopathological diagnosis					
Grade II Astrocytoma	8	8,4	87	77.7	
Grade II Oligodendroglioma	77	81,1	9	8.0	
Grade II Oligo-astrocytoma	10	10,5	16	14.3	
Treatment after 1st surgery					<0.0001
Wait & Scan	52	54,7	52	46.4	
Chemotherapy	24	25,3	5	4.5	
Radiotherapy	16	16,8	42	37.5	
Chemoradiation	3	3,2	13	11.6	
Follow-up (years)					
Median (range)	7.3	(0.8-20.4)	5.7	(0.3 - 15)	

† Fisher's exact test

IDH mutated astrocytoma

107 of the 112 *IDH* mutated astrocytomas could be reliably evaluated for imbalance of chromosome 7: 13 samples showed imbalance compatible with trisomy of the entire chromosome 7. In univariable analysis trisomy of chromosome 7 was significantly associated with shorter overall survival (Log rank P = 0.044). However, this survival difference lost significance when correcting for age and KPS (HR 2.22; 95% CI 0.95-5.20; P =0.066). Only 2 samples showed loss of entire chromosomal arm 10q, both with a relatively poor overall survival of less than 8 years (figure 2). Loss of 9p21.3 (n = 18) was not associated with outcome. Out of the 18 patients with loss of 9p21.3, 13 showed loss of the entire 9p chromosomal arm, which was also not associated with outcome. A *PTEN* mutation was detected in 3 patients (all three no loss of 10q) and did not show an impact on outcome (figure 2).

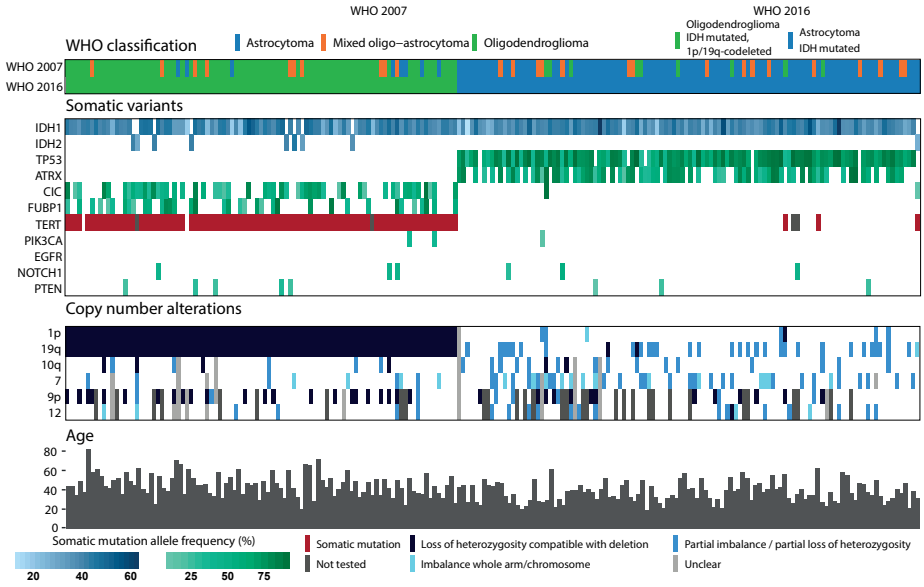

Figure 1. Oncoprint plot with overview of somatic alterations per patient. At the top of the figure the WHO 2007 and WHO 2016 classification are shown. In the middle part all somatic variants and copy number alterations are shown. Patients are separated based on the WHO 2016 classification. *IDH* mutated 1p/19q-codeleted patients are depicted in the left part of the figure and *IDH* mutated astrocytoma patients on the right part of the figure. The bottom part of the figure shows the clinical characteristic age per patient.

Table 2. Frequencies of gene mutations per WHO subgroup

	IDH mutated astrocytoma		IDH mutated 1p/19q-codeleted oligodendroglioma	
Gene	N	%	N	%
IDH1	111	99,1	87	91,6
IDH2	1	0,9	8	8,4
TP53	105	93,8	0	0
ATRX	75	67	0	0
CIC	2	1,8	52	54,7
FUBP1	0	0	36	37,9
TERT	3	2,7	91	95,8
PIK3CA	1	0,9	2	2,1
EGFR	0	0	0	0
NOTCH1	3	2,7	3	3,2
PTEN	3	2,7	5	5,3

IDH mutated 1p/19q-codeleted oligodendroglioma

In *IDH* mutated 1p/19q-codeleted oligodendroglioma, both *CIC* and *FUBP1* mutations were frequent events, but neither was associated with prognosis (figure 3). In 27 out of the 77 patients that could be reliably evaluated for copy number changes on chromosome 9p, loss of 9p21.3 was found. Of these, 23 showed loss of entire chromosomal arm 9p. No homozygous deletions or mutations of *CDKN2A* were detected. Loss of 9p21.3 did not have significant impact on overall survival (Log rank P=0.12) (figure 3). Additional analysis for impact of loss of entire 9p did not show different results. Trisomy of chromosome 7 and loss of chromosomal arm 10q were present in a few samples, both without impact on overall survival (figure 3). Five patients were *PTEN* mutated and showed a significantly shorter OS in univariable analysis (Log rank P = 0.033). This survival difference was not significant anymore when correcting for age and KPS (HR 3.73; 95% CI 0.78-17.76; P =0.097).

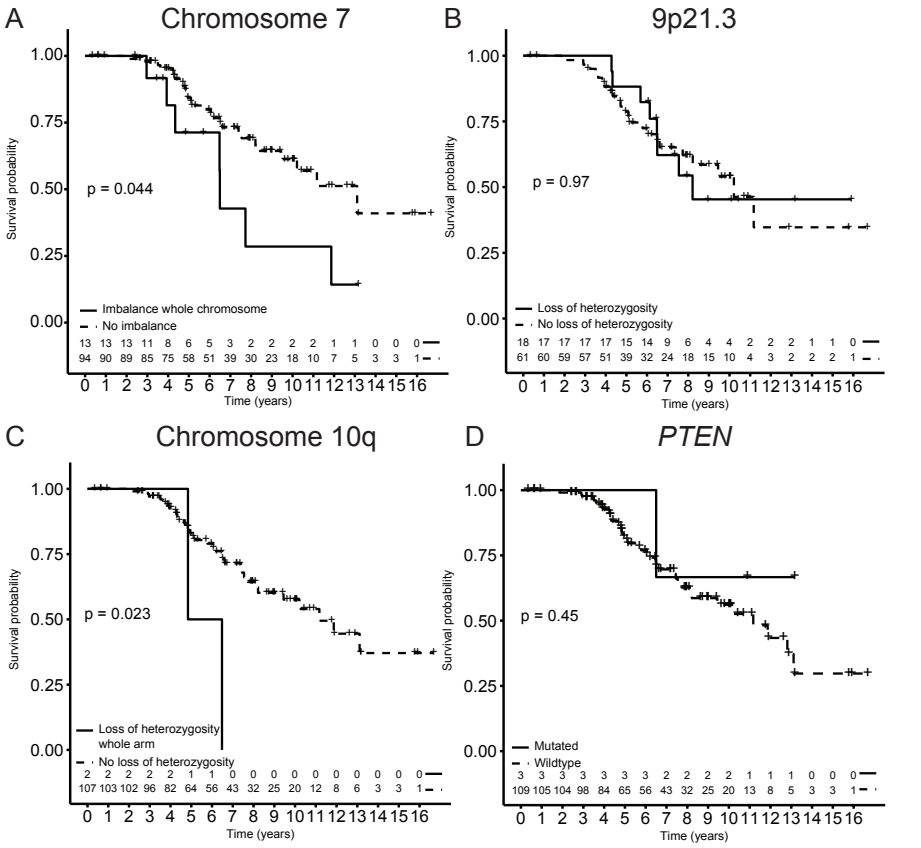

Figure 2. Kaplan-Meier plots with overall survival of IDH mutated astrocytoma patients stratified for presence of (A) Imbalance pattern consistent with trisomy of chromosome 7, (B) loss of 9p21.3 region, (C) loss of chromosomal arm 10q, (D) and presence of a PTEN mutation.

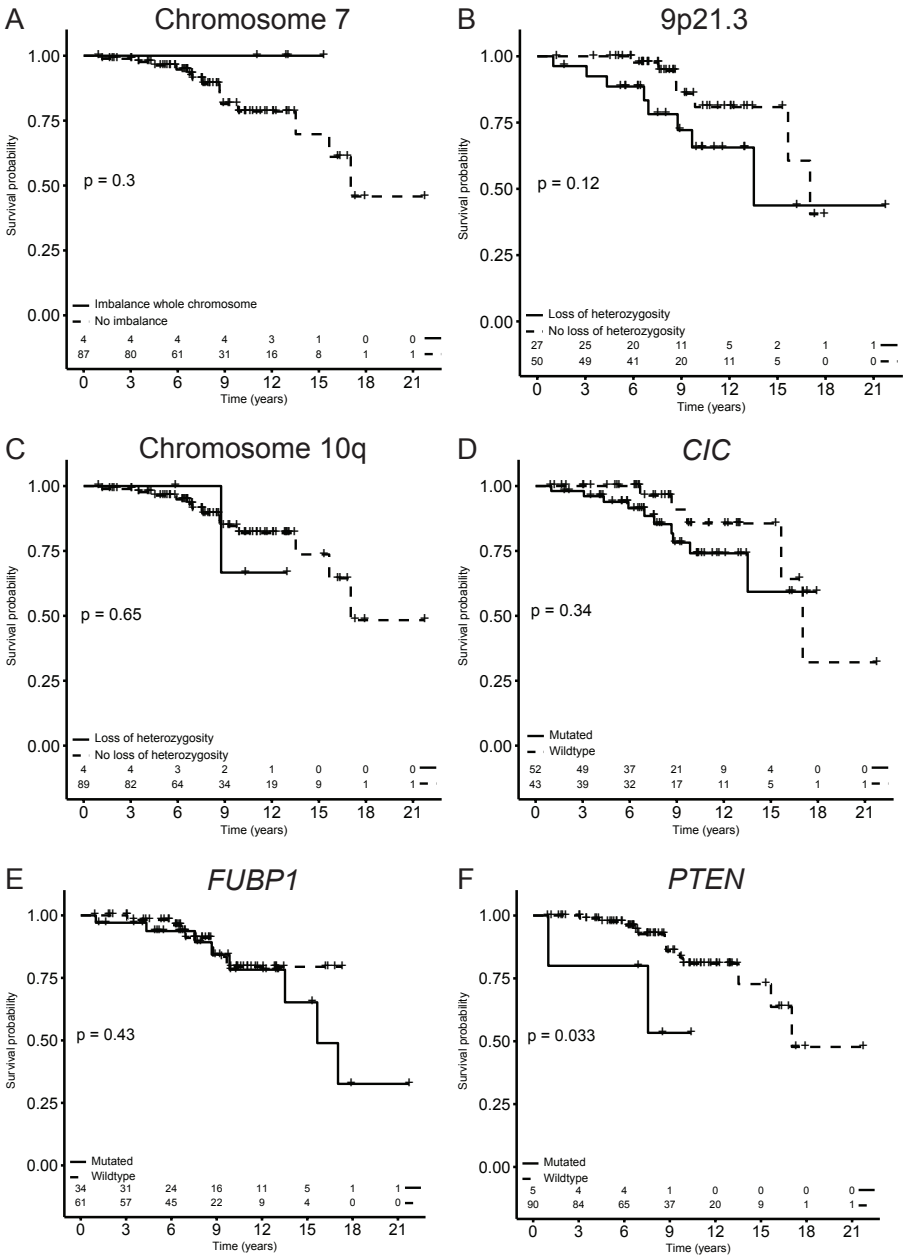

Figure 3. Kaplan-Meier plots with overall survival of *IDH* mutated 1p/19q-codeleted oligodendroglioma patients stratified for presence of (A) Imbalance pattern consistent with trisomy of chromosome 7, (B) loss of 9p21.3 region, (C) loss of chromosomal arm 10q, (D) presence of *CIC* mutation, (E) presence of *FUBP1* mutation, (F) and presence of a *PTEN* mutation.

Exploratory analyses

On further exploratory analyses, we found no other molecular markers that significantly impact on overall survival in molecularly defined LGG with the targeted sequencing panel we used.

Validation of findings in the TCGA

We aimed to validate our findings in the publically available dataset *The Cancer Genome Atlas* (TCGA). In the TCGA dataset, 9 out of 72 grade II *IDH* mutated astrocytoma showed trisomy of chromosome 7, but this was not significantly associated with a difference in overall survival in univariate analysis (Log rank P = 0.3). We also analyzed the TCGA dataset for presence of loss of 10q in grade II *IDH* mutated astrocytoma. Only one sample showed loss of 10q, with a poor survival (1.75 years). Among the 47 grade II *IDH* mutated 1p/19q-codeleted oligodendroglioma samples in the TCGA dataset, there were no samples with a *PTEN* mutation, confirming the rarity of this event.

DISCUSSION

In this study we aimed to investigate the prognostic impact of additional molecular markers in grade II *IDH* mutated astrocytoma and grade II *IDH* mutated 1p/19q-codeleted oligodendroglioma. We used a targeted NGS panel to investigate genes that are frequently mutated in glioma and to investigate regions that are frequently reported to have copy number changes.

Loss of chromosome 9p or in particular the 9p21.3 region is a frequently reported copy number variation in all glioma subtypes.[2, 9-11] Several studies investigated the impact of loss of 9p/9p21.3 on prognosis. A meta-analysis published in 2015 pooled the data of 13 different studies that were published between 2002 and 2013.[10] Although most individual studies in this meta-analysis did not show a survival difference, the pooled data showed that loss of 9p was significantly associated with a poorer prognosis. However, most studies in this analysis did not take into account confounding factors such as *IDH1/2* mutational status, and a subgroup analysis of types of gliomas showed that impact of loss of 9p on overall survival was particularly present in the glioblastoma subtype. A recent study specifically focused on the impact of 9p loss in grade II and III glioma and found that loss of 9p is an independent prognostic factor in IDH mutated glioma, however, the effect was most clear in *IDH* mutated astrocytomas.[11] In opposite, Alentorn et al. found loss of 9p to be a poor prognostic marker in anaplastic oligodendroglioma.[9] A later study by Aoki et al. did not confirm this finding however.[12] Our study is the first that specifically focusses on the impact of loss of 9p21.3 and entire 9p in histologically defined grade II glioma. We could not confirm impact of

loss of 9p21.3 region or entire 9p on prognosis neither in IDH mutated astrocytoma nor in *IDH* mutated 1p/19q-codeleted oligodendroglioma. However, a trend towards shorter overall survival in *IDH* mutated 1p/19q-codeleted oligodendroglioma with loss of 9p21.3 is visible, and longer follow-up and larger sample size is necessary for final conclusions. Exact comparison of our data with previous literature is difficult, because of different selection criteria in the different cohorts. Therefore, it is yet unclear if in grade II *IDH* mutated glioma loss of 9p21.3 region can be used as marker of anaplasia or to guide more aggressive treatment strategies.

Trisomy of chromosome 7 is also frequently reported in glioma.[2, 14, 15] In combination with loss of 10q it is considered an early event in glioblastoma *IDH* wildtype and is correlated with dismal prognosis in grade II and III *IDH* wildtype glioma.[2, 15-17] Trisomy of chromosome 7 is also described in lower grade glioma, though less frequently. The impact of trisomy of 7 on overall survival in *IDH* mutated low grade glioma is not clear. To our knowledge no large series are published. Wessels et al. reported that polysomy of 7 was associated with a poorer prognosis in grade II astrocytoma, but this report antedates the discovery of the role IDH mutations in glioma.[14] In our cohort we found that trisomy of chromosome 7 might be a marker of poor prognosis in *IDH* mutated astrocytoma. However, this could not be validated in multivariate analysis or in the TCGA data, and this observation requires validation in a larger independent cohort to define the clinical value.

CIC and *FUBP1* mutations are frequently mutated in *IDH* mutated 1p/19q-codeleted oligodendroglioma and the prognostic impact has been investigated in several series. One study by Gleize et al reported that inactivating *CIC* mutations in *IDH* mutated glioma correlate with poorer outcome. In other cohorts this effect was not observed.[3, 18-20] In our cohort we also did not find a correlation between *CIC* nor *FUBP1* mutation and prognosis.

Our study has several limitations. The retrospective nature comes with the risk of a selection bias. We tried to avoid selection bias by analyzing a consecutive cohort of all grade II gliomas undergoing surgery within a specified period. Also due to the retrospective nature, treatment was heterogeneous. Furthermore, we may have missed smaller region copy number alterations and other mutations in regions not covered by our NGS panel. We used a diagnostically validated[21] and targeted NGS panel that consists of highly polymorphic SNPs that cover whole chromosomes of interest with roughly 1 SNP per 3MB. Large scale copy number variations of whole chromosomes or large parts of chromosomes can therefore be reliably detected, but small region or subclonal copy number variations are potentially missed. However, the aim of this study was to validate the impact of large region copy number variations which were described before and that are considered to be early events, and these can be reliably detected with the panel used.[21] Also, it is known that lengthy follow-up in studies on

low-grade glioma is necessary for definitive conclusions, so longer of follow up of this dataset is necessary.

In conclusion, in univariable analysis we found a significant shorter overall survival in *IDH* mutated astrocytoma patients with trisomy of chromosome 7, and in *IDH* mutated 1p/19q-codeleted oligodendroglioma patients with a *PTEN* mutation. However, we could not confirm these findings in multivariate analysis or in the TCGA validation set and therefore these findings require validation in other larger series. We could not confirm the impact on OS of LOH of 9p21.3 (the CDKN2A region) which is frequently reported as a progression marker particularly in higher grade glioma. However, we need lengthy follow-up for definitive conclusions. Also, other strategies should be pursued to identify prognostic relevant molecular markers within these IDH mutant glioma subgroups, like methylation patterns and total number of chromosomal aberrations.

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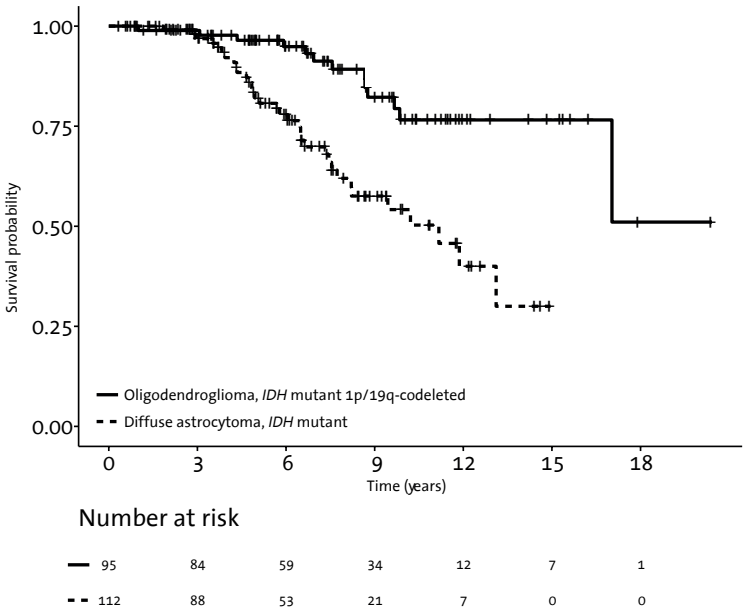
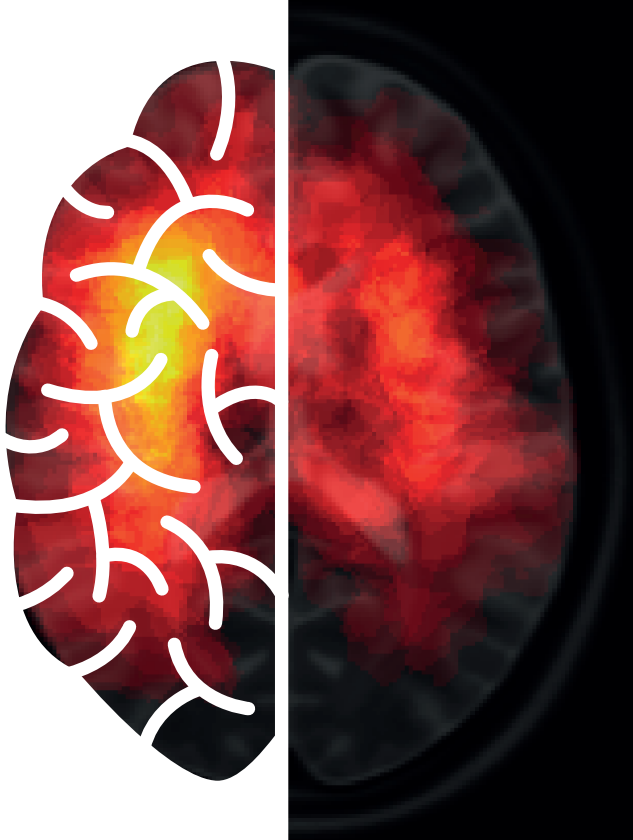
SUPPLEMENTARY FILES

Supplementary table 1. Overview of targeted genes and chromosomes

Whole Gene	Hotspots	SNPs	SNaPshot
TP53	*EGFR* (exon 3+15)	chr1	*TERT* promoter
FUBP1	*H3F3A* (exon 2)	chr10 (including *PTEN*)	
PTEN	*IDH2* (exon 4)	chr12 (including *MDM2*)	
CIC	*IDH1* (exon 4)	chr19	
CDKN2A	*PIK3CA* (exon 10+21)	chr7 (including *EGFR* and *MET*)	
NOTCH1	*BRAF* (exon 11+15)	chr9 (including *CDKN2A*)	
ATRX		chrY	



Supplementary figure 1. Kaplan-Meier plot with overall survival stratified by WHO 2016 molecular subgroup. IDH mutated 1p/19q-codeleted patients have significantly longer overall survival (median OS not reached) compared to IDH mutated astrocytoma patients (median OS 10.2 years) (Log rank test: P = 0.0001).



Chapter 4

Molecular and clinical heterogeneity of adult diffuse lower-grade IDH wildtype gliomas: assessment of TERT promoter mutation and chromosome 7 and 10 copy number status allows superior prognostic stratification

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Acta Neuropathol. 2017 Dec;134(6):957-959.

With the 2016 revision of the World Health Organization classification of tumors of the central nervous system (WHO 2016) testing for the presence of mutations in isocitrate dehydrogenase 1 and 2 (*IDH*) and chromosome 1p/19q status is the cornerstone of glioma classification.[5] Approximately 80% of diffuse lower-grade (grade II & III) gliomas (DLGG) are *IDH* mutated and have a relatively favorable prognosis compared to their *IDH* wildtype (*IDHwt*) counterparts.[2] The prognosis of *IDHwt* DLGG is almost similar to primary glioblastoma and genetic aberrations that are seen in primary glioblastoma are also reported in *IDHwt* DLGG: the combination of trisomy of whole chromosome 7 and loss of chromosomal arm 10q (+7/-10q), and telomerase reverse transcriptase gene promoter (*TERTp*) mutations.[2, 6, 7] However, +7/-10q or *TERTp* mutations are not part of the WHO 2016 criteria and not all *IDHwt* DLGG have these specific molecular aberrations.[2, 5] Although clinical trials have not been performed, in view of their poor prognosis aggressive treatment regimens for *IDHwt* DLGG has been suggested. However, as this is not a well-defined separate entity, the question remains whether *IDHwt* DLGG classified according to current WHO classification qualifies as a single entity, with sufficient information to estimate prognosis adequately and therefore guide treatment, or if the assessment of additional markers is necessary and if so which. A recent study by Aibaidula et al. showed that *IDHwt* DLGG are prognostically heterogeneous and that markers like *TERTp*, *EGFR* amplification and *H3F3A* mutation could be of additional value.[1] The prognostic role of +7/-10q and its relationship with *TERTp* mutations were not reported however. In this study we report on the impact of additional molecular markers, including +7/-10q and *TERTp*, on overall survival in adult *IDHwt* DLGG.

In our institute targeted Next-Generation Sequencing is part of routine diagnostics for DLGG. We assess copy number changes of chromosome 1, 7 (including *EGFR* amplification), 9p, 10, 12, 19, and mutational status of genes *IDH1/2*, *TP53, ATRX, CIC, FUBP1, EGFR, PIK3CA, CDKN2A, PTEN, H3F3A, BRAF, NOTCH1, TERT*p. In our routine diagnostics we use gain of whole chromosome 7 and loss of whole chromosomal arm 10q as criterion for +7/-10q status. Our sequencing protocol has been described previously.[3, 4] Between January 2003 and January 2017 we sequenced a total of 639 tumors as part of daily diagnostic routine (samples since 2013) and as part of a project on extent of resection in DLGG (samples since 2003).[8] Of these, 510 tumors were histologically classified as DLGG and on sequencing 74 were *IDHwt*. We collected Karnofsky Performance Status (KPS) at diagnosis, age, gender and overall survival which was defined as time between date of diagnostic imaging and date of death. Patients that were alive at the time of analysis were censored.

Further stratification of these *IDHwt* DLGG showed a molecularly heterogeneous group of tumors. Only 52,7% of patients (n=39) showed a +7/-10q phenotype that is presumed to be a molecular characteristic of glioblastoma. Of these, all but one also

Table 1. Patient characteristics

	All patients	IDH 7+/10-	Only *TERTp*	*BRAF*	*H3F3A*	Unclassified
	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)
Characteristic						
Number of patients	74	39	14	3	3	15
Gender						
Male	49 (66.2%)	24 (61.5%)	11 (78.6%)	3 (100.0%)	2 (66.7%)	9 (60.0%)
Female	25 (33.8%)	15 (38.5%)	3 (21.4%)	0 (0.0%)	1 (33.3%)	6 (40.0%)
KPS at diagnosis						
100	29 (39.2%)	14 (35.9%)	1 (7.1%)	3 (100.0%)	2 (66.7%)	9 (60.0%)
90	21 (28.4%)	12 (30.8%)	4 (28.6%)	0 (0.0%)	1 (33.3%)	4 (26.7%)
80	19 (25.7%)	10 (25.6%)	7 (50.0%)	0 (0.0%)	0 (0.0%)	2 (13.3%)
70	3 (4.1%)	2 (5.1%)	1 (7.1%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
60	2 (3.7%)	1 (2.6%)	1 (7.1%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Age at diagnosis						
median (IQR)	56 (47-63)	57 (52-64)	60 (51-68)	47 (32-49)	25 (24-32)	52 (42-62)
age <40	11 (14.9%)	2 (5.1%)	1 (7.1%)	1 (33.3%)	3 (100.0%)	4 (26.7%)
age 40 - 60	34 (45.9%)	21 (53.8%)	6 (42.9%)	2 (66.7%)	0 (0.0%)	5 (33.3%)
age >60	29 (39.2%)	16 (41.0%)	7 (50.0%)	0 (0.0%)	0 (0.0%)	6 (40.0%)

had a *TERTp* mutation. In contrast, 18.9% of patients (n=14) were *TERTp* mutated, but showed no +7/-10q pattern. 4.1% (n=3) were classified as *BRAF* mutated glioma, and 4.1% (n=3) as *H3F3A* mutated glioma. Age at diagnosis of *BRAF* and *H3F3A* mutant patients was generally younger compared to the other groups (table 1). A substantial part of samples (20.3%; n=15) could not be further stratified (either no known classifying variants or no variants found at all). The clinical importance of this heterogeneity becomes clear by overall survival analysis(Figure 1). The few *BRAF* mutant patients (median overall survival not reached) and the unclassified patients had better outcome compared to +7/-10q, *TERTp* mutated only and *H3F3A* mutated patients. The latter three all have a very poor prognosis. The longer overall survival of unclassified patients is remarkable. An explanation might be that these unclassified *IDHwt* DLGG belong to a specific, not yet identified, molecular subset with better prognosis. Additional immunohistochemistry with *IDH1* R132H antibody was negative in 12 unclassified patients. For the 3 other unclassified patients there was no available tissue anymore for immunohistochemistry. However, in each of these 3 samples we found somatic variants or copy number alterations suggestive of tumor, although not classifying. In these samples there was sufficient coverage of both *IDH1* and *IDH2*. Therefore, it is less likely that these tumors are actually true low-grade gliomas of oligodendroglial or astrocytic lineage wherein the corresponding molecular aberrations escaped detection due to technical limitations or unexpected low tumor cell percentage. Another

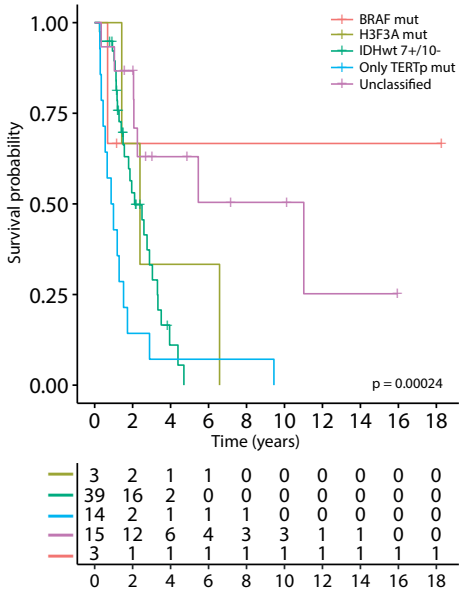

Figure 1. Overall survival of all 74 patients stratified by molecular subgroup.

interesting observation is the survival difference between the *IDH*wt +7/-10q (also *TERTp* mutated, except for one patient) patients and the patients with only a *TERTp* mutation; the patients with only a *TERTp* mutation have a significant shorter overall survival (Log-Rank test: p = 0.024). To confirm our findings we analyzed the 56 *IDHwt* DLGG in the publically available Cancer Genome Atlas (TCGA) and found that 20 samples showed a +7/-10q phenotype (all but two also *TERTp* mutated) and 18 samples were only *TERTp* mutated. Unfortunately the small sample size and limited follow-up do not allow to adequately give conclusions about prognostic differences between these groups. Therefore our findings require confirmation in an independent dataset to determine whether the *TERTp* mutated subgroup without +7/-10q is a biological distinct group.

In conclusion, we showed that adult *IDHwt* DLGG is a molecularly heterogeneous group of tumors with a widespread variation in prognosis. Consequently, assessment of only *IDH* and 1p19q status is not accurate enough to label *IDHwt* DLGG as prognostically poor and therefore to guide treatment decisions of these tumors. According to our results at least assessment of *TERTp* mutational status or +7/-10q status is necessary in *IDHwt* glioma to confirm whether a patient with DLGG has poor survival. However, when validated in independent series, assessment of both +7/-10q status and *TERTp* status seems preferable since *TERTp* mutation without +7/-10q shows shorter overall survival in our dataset. When *TERTp* is wildtype or +7/-10q is not present, further testing for other markers (at least *H3F3A* and *BRAF*) is necessary to adequately inform patients about prognosis and to decide on treatment.

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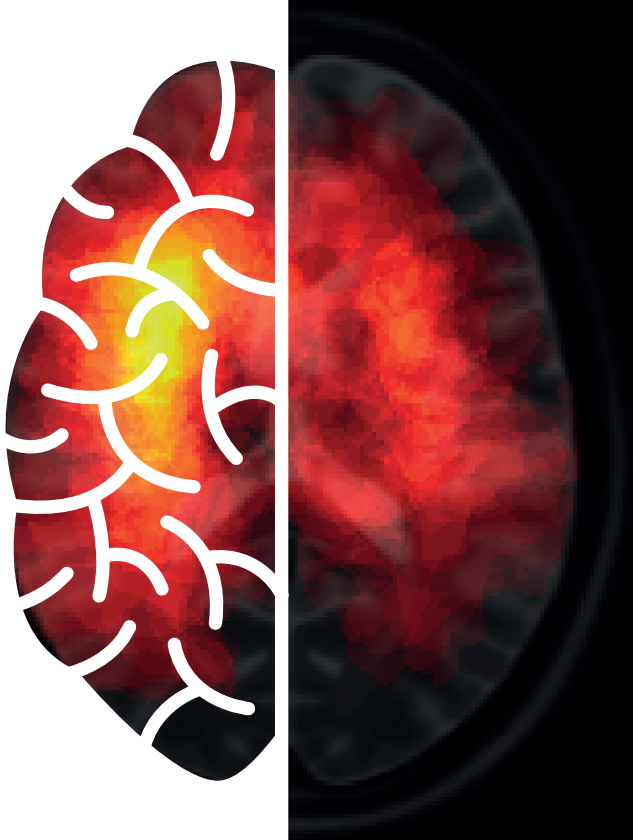

Chapter 5

The impact of surgery in molecularly defined low-grade glioma: an integrated clinical, radiological and molecular analysis.

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ABSTRACT

Background

Extensive resections in low-grade glioma are associated with improved overall survival. However, WHO classification of gliomas has been completely revised and is now predominantly based on molecular criteria. This requires re-evaluation of the impact of surgery in molecularly defined low-grade glioma subtypes.

Methods

We included 228 adults who underwent surgery since 2003 for a supratentorial low-grade glioma. Pre-and postoperative tumor volumes were assessed with semi-automatic software on T2-weighted images. Targeted Next-Generation Sequencing was used to classify samples according to current WHO classification. Impact of postoperative volume on overall survival, corrected for molecular profile, was assessed using a Cox proportional-hazards model.

Results

Median follow-up was 5.79 years. In 39 (17.1%) of histopathologically classified glioma the subtype was revised after molecular analysis. Complete resection was achieved in 35 patients (15.4%), and in 54 patients (23.7%) only small residue (0.1-5.0 cm^3) remained. In multivariable analysis, postoperative volume was associated with overall survival with a HR of 1.01 (95% CI 1.002-1.02; p=0.016) per cm^3 increase in volume. The impact of postoperative volume was particularly strong in IDH mutated astrocytoma patients, where even very small postoperative volumes (0.1-5.0 cm) already negatively affected overall survival.

Conclusions

Our data provides the necessary re-evaluation of the impact of surgery in molecularly defined low-grade glioma and supports maximal resection as first-line treatment for molecularly defined low-grade glioma.

INTRODUCTION

Low-grade (WHO grade II) gliomas (LGG) are primary brain tumors that grow relatively slow but with a diffuse infiltrative pattern, which makes them impossible to fully eradicate. Recurrences always occur, and despite all advances in the field of oncology, the treatment modalities for LGG remain to be surgery, radiotherapy, and chemotherapy.[1,2] The optimum use of these regimens has been controversial for many years, though the use of chemo-and radiotherapy for LGG has been studied in several randomized controlled clinical trials.[3-7] The role of surgery has never been investigated in a randomized manner, and it is unlikely this ever will be due to ethical reasons in view of the assumptions about equipoise and the duration of such a trial. However, more extensive surgery was associated with an increased overall survival (OS) in several retrospective series.[8-13] Unfortunately, interpretation of these studies is not straightforward, due to probable selection bias. For example, the resectability of a brain tumor and the decision to operate depends on its location, size, and delineation: small and superficially located tumors in non-eloquent areas are more likely to be extensively resected. However, location, eloquency and size are major prognostic factors by themselves.[14] In addition, previous studies were based on the histopathological diagnosis of gliomas which is subject to a large degree of interobserver variability, particularly for LGG.[15] Nowadays, gliomas can also be classified based on a limited set of molecular markers (isocitrate dehydrogenase 1/2 (*IDH1/2)* gene mutation, chromosome 1p and 19q co-deletion), and this classification outperforms the prognostic value of the previous histopathological classification of gliomas. [16,17] This molecular approach to glioma diagnosis is now incorporated in the 2016 WHO Classification of tumors of the Central Nervous System (WHO 2016).[15,18,19] Since some mutations preferentially manifest in distinct brain regions, the impact of extent of resection in the different molecular subtypes remains to be determined, as well as to what extent previous studies are confounded by this major prognostic factor. [20,21] To address this issue, we examined the impact of surgery on OS in molecularly defined LGG in a large retrospective cohort.

METHODS

Patient selection and clinical data acquisition

We studied a cohort of adult patients with a supratentorial LGG (age ≥ 18 years) who underwent a resection or biopsy in one of two neurosurgical centers (Erasmus MC Cancer Institute, Rotterdam, The Netherlands (Erasmus MC); Elisabeth-TweeSteden Hospital, Tilburg, The Netherlands (ETZ)), with tumor material (formalin-fixed-paraffin-embedded (FFPE) tissue) available for tissue analysis and pre- and post-operative MR

scans (T2-weighted and/or T2-weighted Fluid Attenuated Inversion Recovery (FLAIR); either 2D or 3D) available for radiological review. We included only histopathologically confirmed LGG (grade II) diagnosed by a dedicated neuropathologist (J.M.K.). As the extent of resection can be improved with the advent of awake craniotomy for tumors in eloquent areas, the time-window of patient inclusion was based on the year of introduction of awake craniotomies in the respective institutes (2003-2016 for Erasmus MC and 2008-2016 for ETZ).[22] Patients that underwent their first resection or biopsy in this period were included, even when radiological diagnosis was made before this time window. Patients only undergoing a biopsy were also included, since those samples potentially reflect a distinct subset of patients with tumors that are more difficult to resect and/or with a distinct molecular profile. We excluded patients with WHO grade II histology who had a radiological appearance (extensive tumor enhancement) suggestive of a high-grade lesion. Clinical data were collected from patient records. All included patients were followed until death or censored at the date of last follow-up. Date of death was provided by patient records or the Municipal Personal Records Database. OS was defined as time between date of diagnostic scan and death or censorship. Progression free survival (PFS) was defined as time between date of first surgical intervention and date of first clinical or radiological progression as indicated by the treating clinician. The database was developed and maintained at Erasmus MC, and locked on January 30th 2017. This study was approved by the medical ethics committee of Erasmus MC.

Volumetric analysis and acquisition of MRI characteristics

Pre-and postoperative tumor volumes were assessed in a semi-automatic fashion using the SmartBrush tool in Brainlab Elements (version 2.1.0.15; see supplemental figure 1A-C). A 3D-volume-of-interest can be created by first manually segmenting the tumor on one MRI slice of a chosen plane. Then, a second manual segmentation is carried out on one perpendicular slice. Next, the software calculates the full 3D-volume-of-interest, which can be easily manually adjusted where necessary. If available, the T2-weighted FLAIR sequence (3D where possible) was used for pre-and postoperative volumetric assessment, otherwise the T2-weighted (T2w) sequence was used. All T2w and T2w-FLAIR signal abnormalities were included in the segmentations. Within single patients the same MRI sequence was used for pre-and postoperative analysis. Preferentially, we used the postoperative scan ≥ 3 months to minimize overestimation of postoperative volume due to postoperative edema or ischemia. Proportion of resection was calculated as (preoperative volume – postoperative volume) / preoperative volume. Localization of tumor in/near eloquent area was assessed using the criteria of Chang et al.[14]

Mutation analysis, copy number analysis and molecular classification

We used a targeted Next-Generation Sequencing panel to assess mutational and copy number status as described in detail elsewhere, using an Ion Torrent Personal Genome Machine or Ion S5XL (Life Technologies).[16,23] DNA was isolated from selected tissue areas composed of a high percentage of neoplastic cells on 10µm FFPE sections using Proteinase K digestion in presence of 5% Chelex 100 resin (Bio-Rad). TERT promoter mutations (C228T & C250T) were assessed in separate assays (SnaPshot) as described.[16]

The following criteria for molecular classification were used (consistent with the current WHO 2016 classification for brain tumors):

- Oligodendroglioma: *IDH1* or *IDH2* mutated and loss of heterozygosity consistent with co-deletion of the entire 1p and 19q chromosomal arms.
- Astrocytoma IDH mutated (IDHmt): *IDH1* or *IDH2* mutated.
- Glioblastoma-like (GBM-like): *IDH1* or *IDH2* wildtype and; *TERT* promoter mutation without 1p19q co-deletion, or loss of heterozygosity of chromosome 10q and imbalance of chromosome 7, or *EGFR* amplified (in the WHO 2016 classification described as astrocytoma IDHwt)[18]

Statistical analysis

All primary analyses were carried out according to a predetermined analysis protocol; all other analyses are indicated as post-hoc. Categorical data were analyzed with Pearson's chi-square test or Fisher's exact test when assumptions of the chi-square test were violated (as indicated in the respective tables). Kruskal-Wallis test was used for continuous data. We used postoperative tumor volume as primary measure of extent of resection. The same calculations were made using resection percentage as alternative measure, which can be found in the supplementary material. Multiple linear regression models were used to explore correlations of molecular subtype with postoperative tumor volume and resection percentage. To meet the assumption of normal distribution of residual errors in multiple linear regression, postoperative volume was log10 transformed, and resection percentage was arcsin square root transformed. To investigate impact of surgery, univariable and multivariable analyses of overall survival were performed using a Cox proportional-hazards model. In this model pre- and postoperative tumor volumes were treated as continuous variable, and postoperative tumor volume was log transformed to prevent inappropriate weighting of results by very large preoperative tumor volumes. The assumption of proportional hazards was tested based on the scaled Schoenfeld residuals. All calculations were two-sided tests, with a p-value <0.05 considered as statistically significant. All analyses were performed using R (3.3.2) and RStudio (1.0.44) using ggplot2 and CRAN survival packages.[24,25]

RESULTS

Our initial cohort included 246 patients with confirmed LGG, and with MR scans and FFPE material available. Of these, 18 patients were excluded from analyses; two due to insufficient amount of DNA for sequencing, 14 due to sequencing failure, and two due to a preoperative radiological appearance suggestive of glioblastoma (both stereotactic biopsy samples, molecularly classified as GBM-like). 228 patients were included in final analyses. Median follow-up was 5.79 years (range 0.3 – 20.4). Clinical characteristics are shown in table 1. When comparing histopathological classification with molecular subtype, there was a change of diagnosis in 39 patients (17.1%, mixed oligo-astrocytomas not included); all mixed oligo-astrocytomas were reclassified according to the WHO 2016 update. There were no significant differences in clinical characteristics between oligodendroglioma and astrocytoma IDHmt, except for age and type of first surgery: oligodendroglioma patients were significantly older (median age 45 vs. 37 years; p<0.0001) and type of first surgery was more often a biopsy (p=0.006). We observed however several statistically significant different characteristics in the GBM-like group. Patients in this group were significantly older (median age 61 years), had a different presentation (only 11 (47.8%) patients presenting with epileptic seizures, vs. 77 (82.8%) and 82 (73.2%) in oligodendroglioma and astrocytoma IDHmt respectively), and a different tumor localization (more often situated in eloquent areas, N=16; 69.6%). Interestingly, the majority of GBM-like patients (N=19; 82.6%) underwent a biopsy, compared to only a small fraction in the oligodendroglioma and astrocytoma IDHmt group (N=20 (21.5%) and N=9 (8%) respectively; p<0.0001). This is also reflected in postoperative tumor volume: although preoperative tumor volume did not differ between the molecular groups, the postoperative tumor volume was significantly higher in the GBM-like tumors compared to oligodendroglioma and astrocytoma IDHmt (median volume 30.0 cm^3; 8.0 cm^3; 8.9 cm^3 respectively, p=0.005). Postoperative tumor volume did not differ between oligodendroglioma and astrocytoma IDHmt (p=0.553). An overview of salvage treatments is shown in supplementary table 3 and an overview of surgical outcome is shown in supplementary table 4.

Factors influencing postoperative tumor volume

We used multiple linear regression to explore which factors influence the amount of postoperative tumor volume. Results are shown in table 2. Factors such as increasing age, higher preoperative tumor volume (see also supplementary figure 2) and insular localization were significantly correlated with a higher postoperative tumor volume (p=0.002; p<0.0001; p<0.0001 respectively). Tumors located in eloquent areas were also significantly correlated with a higher postoperative volume (p<0.0001). Interestingly, corrected for the factors mentioned above, the molecular subtype of the tumor

Table 1. Clinical characteristics

	All patients		Oligodendroglioma		Astrocytoma IDHmt			GBM-like		
Characteristics	N	%	N	%	N	%	P*	N	%	P**
Patients (n)	228		93		112			23		
Sex							0.119			0.078
Male	136	59.6 %	48	51.6 %	71	63.4 %		17	73.9 %	
Female	92	40.4 %	45	48.4 %	41	36.6 %		6	26.1 %	
Age							<0.0001			<0.0001
Median (IQR)	42	(34 - 51)	45	(37 - 52)	37	(29 - 45)		61	(52 - 65)	
< 40	99	43.4 %	32	34.4 %	67	59.8 %		0	0.0 %	
40-60	104	45.6 %	50	53.8 %	43	38.4 %		11	47.8 %	
> 60	25	11.0 %	11	11.8 %	2	1.8 %		12	52.2 %	
Presenting symptom							0.239†			<0.0001†
Epilepsy	170	74.6 %	77	82.8 %	82	73.2 %		11	47.8 %	
Incidental	24	10.5 %	8	8.6 %	15	13.4 %		1	4.3 %	
Headache	8	3.5 %	1	1.1 %	6	5.4 %		1	4.3 %	
Miscellaneous neurologic complaints	26	11.4 %	7	7.5 %	9	8.0 %		10	43.5 %	
Watch-and-wait before first surgery? Yes	66	29.1 %	35	38.0 %	28	25.0 %	0.064	3	13.0 %	0.025
Type of 1st surgery							0.006†			<0.0001†
Awake craniotomy	105	46.1 %	50	53.8 %	54	48.2 %		1	4.3 %	
Normal resection	75	32.9 %	23	24.7 %	49	43.8 %		3	13.0 %	
Open biopsy	15	6.6 %	7	7.5 %	2	1.8 %		6	26.1 %	
Stereotactic biopsy	33	14.5 %	13	14.0 %	7	6.2 %		13	56.5 %	
Preoperative KPS							0.055			<0.0001
Median (IQR)	100	(90 - 100)	100	(100 - 100)	100	(90 - 100)		90	(85 - 95)	
100	148	64.9 %	71	76.3 %	71	63.4 %		6	26.1 %	

Table 1. Clinical characteristics (continued)

	All patients		Oligodendroglioma		Astrocytoma IDHmt			GBM-like		
Characteristics	N	%	N	%	N	%	P*	N	%	P**
90	62	27.2 %	17	18.3 %	34	30.4 %		11	47.8 %	
<=80	18	7.9 %	5	5.4 %	7	6.2 %		6	26.1 %	
Histopathological diagnosis										
Grade II Astrocytoma	112	49.1 %	7	7.5 %	87	77.7 %		18	78.3 %	
Grade II Oligodendroglioma	86	37.7 %	76	81.7 %	9	8.0 %		1	4.3 %	
Grade II Oligo-astrocytoma	30	13.2 %	10	10.8 %	16	14.3 %		4	17.4 %	
Molecular diagnosis										
Oligodendroglioma	93	40.8 %	93	100 %	0	0.0 %		0	0.0 %	
Astrocytoma IDHmt	112	49.1 %	0	0.0 %	112	100.0 %		0	0.0 %	
GBM-like	23	10.1 %	0	0.0 %	0	0.0 %		23	100.0 %	
Tumor location							0.299†			<0.0001†
Frontal	121	53.1 %	62	66.7 %	58	51.8 %		1	4.3 %	
Parietal	19	8.3 %	8	8.6 %	10	8.9 %		1	4.3 %	
Temporal	37	16.2 %	9	9.7 %	19	17.0 %		9	39.1 %	
Occipital	8	3.5 %	3	3.2 %	4	3.6 %		1	4.3 %	
Insula	28	12.3 %	9	9.7 %	19	17.0 %		0	0.0 %	
Basal Ganglia	4	1.8 %	0	0.0 %	0	0.0 %		4	17.4 %	
Gliomatosis Cerebri	11	4.8 %	2	2.2 %	2	1.8 %		7	30.4 %	
Eloquent Area: yes	90	39.5 %	35	37.6 %	39	34.8 %	0.786	16	69.6 %	0.007
Side of lesion										
Right	98	43.0 %	38	40.9 %	52	46.4 %		8	34.8 %	
Left	118	51.8 %	50	53.8 %	58	51.8 %		10	43.5 %	
Bilateral	12	5.3 %	5	5.4 %	2	1.8 %		5	21.7 %	

Table 1. Clinical characteristics (continued)

	All patients		Oligodendroglioma		Astrocytoma IDHmt			GBM-like		
Characteristics	N	%	N	%	N	%	P*	N	%	P**
Pre-operative tumor volume, cm3							0.785			0.891
<25.0	66	28.9 %	26	28.0 %	32	28.6 %		8	34.8 %	
25.1-50.0	54	23.7 %	24	25.8 %	24	21.4 %		6	26.1 %	
50.1-100.0	67	29.4 %	24	25.8 %	39	34.8 %		4	17.4 %	
100.1-250.0	38	16.7 %	17	18.3 %	16	14.3 %		5	21.7 %	
250.1-351.0	3	1.3 %	2	2.2 %	1	0.9 %		0	0.0 %	
Median (range)	47.3	(3.01 - 350.5)	46.1	(4.29 - 350.5)	50.95	(3.01 - 302.8)		33	(9.05 - 213.1)	
Postoperative tumor volume, cm3							0.553			0.005
0.0	35	15.4 %	15	16.1 %	19	17.0 %		1	4.3 %	
0.1-5.0	54	23.7 %	27	29.0 %	26	23.2 %		1	4.3 %	
5.1-15.0	41	18.0 %	12	12.9 %	23	20.5 %		6	26.1 %	
> 15.0	98	43.0 %	39	41.9 %	44	39.3 %		15	65.2 %	
Median (range)	10.95	(0 - 263.6)	8.02	(0 - 263.6)	8.9	(0 - 232.7)		30	(0 - 213.1)	
Extent of tumor resection (%)							0.166			<0.0001
0-40	67	29.4 %	28	30.1 %	20	17.9 %		19	82.6 %	
41-89	90	39.5 %	35	37.6 %	53	47.3 %		2	8.7 %	
90-94	22	9.6 %	10	10.8 %	11	9.8 %		1	4.3 %	
95-99	14	6.1 %	5	5.4 %	9	8.0 %		0	0.0 %	
100	35	15.4 %	15	16.1 %	19	17.0 %		1	4.3 %	
Median (range)	76.1	(0 - 100)	75.4	(0 - 100)	82.5	(0 - 100)		0	(0 - 100)	
Treatment after 1st surgery							<0.0001			<0.0001
Wait & Scan	104	45.6 %	51	54.8 %	52	46.4 %		1	4.3 %	
Chemotherapy	35	15.4 %	24	25.8 %	5	4.5 %		6	26.1 %	

Table 1. Clinical characteristics (continued)

	All patients		Oligodendroglioma		Astrocytoma IDHmt			GBM-like		
Characteristics	N	%	N	%	N	%	P*	N	%	P**
Radiotherapy	71	31.1 %	15	16.1 %	42	37.5 %		14	60.9 %	
Chemoradiation	18	7.9 %	3	3.2 %	13	11.6 %		2	8.7 %	
Follow-up (years)										
Median (range)	5.8	(0.3 - 20.4)	7.3	(0.8 - 20.4)	5.7	(0.3 - 15)		2.1	(0.3 - 4.7)	

* Comparison between Oligodendroglioma and Astrocytoma IDHmt

** Three group comparison (Oligodendroglioma vs. Astrocytoma IDHmt vs. GBM-like)

† Fisher's exact test

did not correlate with postoperative tumor volume. A similar model but using resection percentage instead of postoperative tumor volume is shown in supplementary table 1.

Table 2. Multiple linear regression of factors influencing postoperative tumor volume

	Estimate	SE	t	p-value
Intercept	0.307	0.134	2.291	0.023
WHO 2016 classification				
Oligodendroglioma	*	*	*	*
Astrocytoma IDHmt	0.007	0.062	0.107	0.915
GBM-like	0.183	0.127	1.438	0.152
Age	0.008	0.003	3.064	0.002
Preoperative tumor volume	0.008	0.001	14.831	0.000
Eloquency				
Eloquent location	*	*	*	*
Non-eloquent location	-0.320	0.061	-5.230	0.000
Tumor location				
Frontal	*	*	*	*
Parietal	-0.005	0.106	-0.047	0.963
Temporal	0.074	0.082	0.894	0.372
Occipital	0.078	0.151	0.515	0.607
Insula	0.482	0.086	5.577	0.000
Basal ganglia	0.316	0.240	1.319	0.189
Gliomatosis cerebri	0.161	0.157	1.027	0.305

Multiple linear regression model with log10 postoperative tumor volume as dependent variable.
* = Reference variable

Patient outcome per molecular subtype and extent of resection

Projected median OS was 13.1 years at a median follow-up of 5.79 years. At time of analysis 69 patients had died and the remaining 159 patients had a median follow-up of 6.3 years (IQR 3.6 - 9.6 years). There were no surgery related mortalities. GBM-like tumors had a significantly shorter median OS (2.1 years) compared to astrocytoma IDHmt (10.2 years) and oligodendroglioma (not reached) (three group comparison; p = 0.0001, GBM-like vs astrocytoma IDHmt; p = 0.0001, astrocytoma IDHmt vs oligodendroglioma; p =0.0001) (supplementary figure 3). The factors age, Karnofsky Performance Status (KPS), log preoperative volume, resection percentage and eloquency were also significantly correlated with OS in univariable analysis (Table 3, supplementary table 2).

Table 3. Univariable and multivariable Cox regression with postoperative volume as measure of extent of resection

	Univariable			Multivariable		
Variable	HR	95% CI	P-value	HR	95% CI	P-value
Age	1.04	1.02 - 1.06	0.0002	1.01	0.98 - 1.04	0.527
Gender						
Male	*					
Female	0.68	0.41 - 1.12	0.129			
KPS	0.94	0.91 - 0.96	<0.0001	1.01	0.97 - 1.06	0.666
Eloquency						
Yes	*			*		
No	0.49	0.3 - 0.79	0.004	1.59	0.90 - 2.82	0.110
Log preoperative volume	1.66	1.22 - 2.26	0.001	1.70	1.06-2.75	0.029
Postoperative volume	1.01	1 - 1.01	<0.0001	1.01	1.002 - 1.02	0.016
Molecular diagnosis						
Oligodendroglioma	*			*		
Astrocytoma IDHmt	3.77	1.9 - 7.46	0.0001	5.31	2.40 - 11.75	<0.0001
GBM-like	112.9	45.93 - 277.55	<0.0001	218.81	68.75 - 696.38	<0.0001
RTx after surgery						
No	*			*		
Yes	2.45	1.52 - 3.94	0.0002	1.13	0.59 - 2.13	0.716
Chemo after surgery						
No	*			*		
Yes	1.19	0.68 - 2.09	0.545	0.64	0.27 - 1.52	0.315
* = Reference category						

In univariable analysis the amount of postoperative tumor volume (as a continuous variable) was significantly associated with OS with a Hazard Ratio (HR) of 1.01 per 1 cm^3 increase in volume (95% CI 1.01 – 1.01; $p < 0.0001$). A particularly strong effect on OS was seen in patients with no detectable tumor after resection (Figure 1A). Interestingly, any residual volume negatively affects OS, but this effect was most notable in astrocytoma IDHmt (Figure 1B & 1C). In this group, even if residual volume was only 0.1-5.0 cm^3, OS was impaired compared to 0.0 cm^3 residue. No difference in OS was seen between 5.1-15.0 cm^3 and >15.0 cm^3 residue. In oligodendroglioma patients, a trend towards better OS with more extensive resection was observed, though the difference in OS benefit of no detectable tumor versus small residues was less impressive than in astrocytoma IDHmt patients. This analysis was not feasible in GBM-like tumors, due to small sample size. A similar pattern was observed when looking at resection percentages rather than postoperative tumor volume (supplementary figure 4). Progression free survival stratified by postoperative volume is shown in supplementary figure 5,

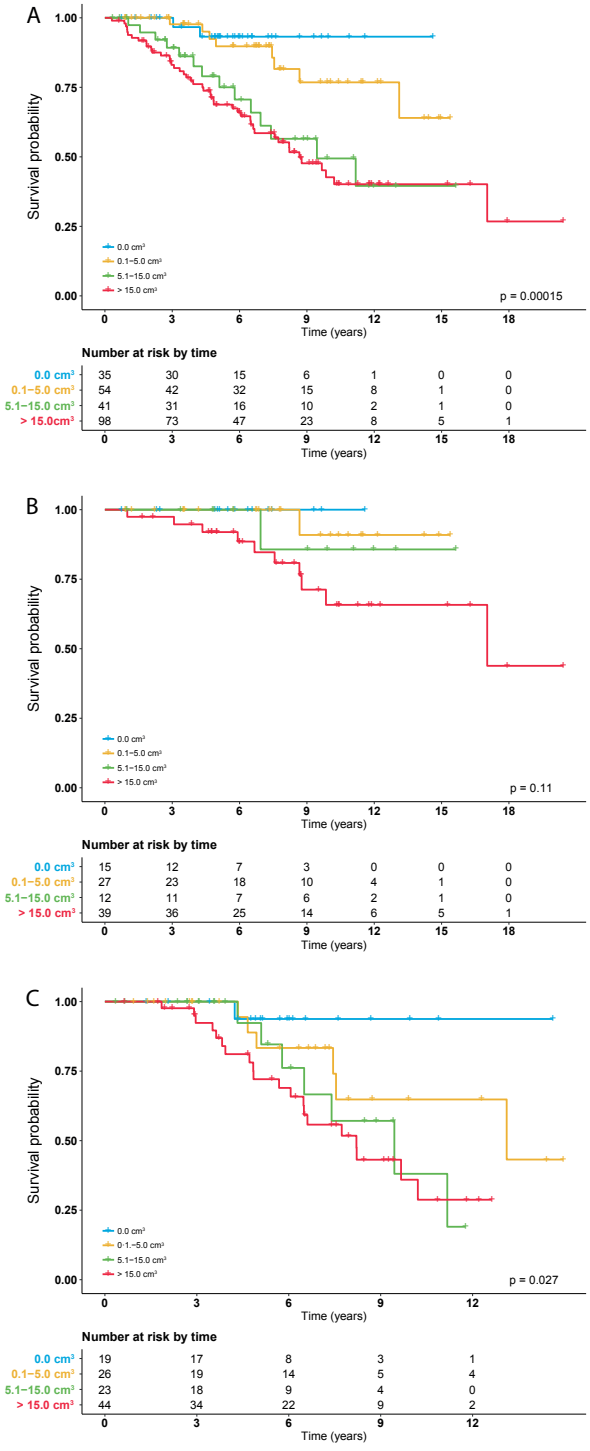


Figure 1. OS stratified by subgroups of postoperative volume for all patients (A), oligodendroglioma patients (B), and astrocytoma IDHmt patients (C).

which shows similar patterns. Postoperative tumor volume remained an independent prognostic factor in a multivariable analysis (HR 1.01; 95% CI 1.002-1.02; p=0.016; per 1 cm^3 increase in volume). Univariable and multivariable Cox-regression with resection percentage as measure of extent of resection is shown in supplementary table 2.

In a post-hoc analysis we dichotomized postoperative volume with different cut-offs to explore at what maximal postoperative volume a resection is still associated with improved OS. In astrocytoma IDHmt, a postoperative volume of up to 25 cm^3 still showed a significant longer OS compared to > 25cm^3. More importantly though, any residual tumor (i.e. greater than 0.0 cm^3) already impacts OS negatively (Figure 2 & supplementary Figure 6). In oligodendroglioma patients such cut-off points for postoperative tumor volume and patient benefit could not be defined (supplementary Figure 7).

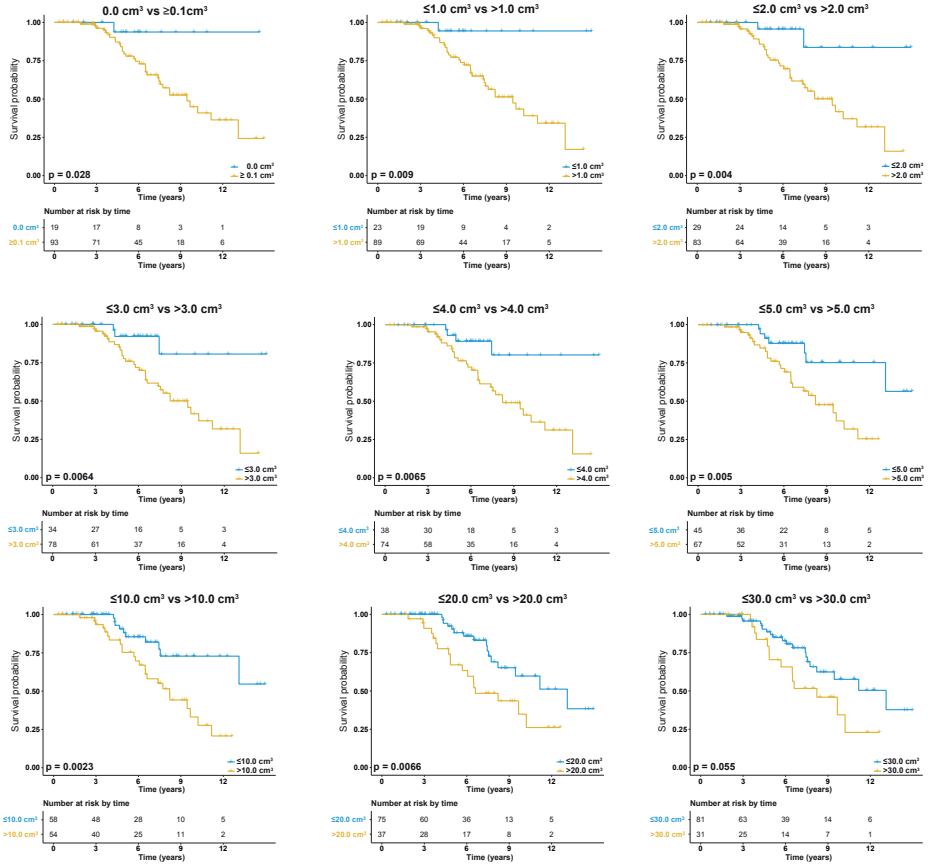


Figure 2. OS in astrocytoma IDHmt stratified by different cut-offs of postoperative tumor volume to explore at what maximal postoperative volume a resection is still beneficial. A postoperative volume of up to 25 cm^3 still shows a significant better OS compared to >25cm^3. However any residual tumor negatively impacts survival. The same figure with more cut-offs is shown in supplementary figure 6.

DISCUSSION

With the incorporation of a molecular classification for glioma, WHO classification of tumors of the central nervous system has been completely revised and molecular markers now overrule the histopathological diagnosis.[18,19] Differences in sensitivity and overall outcome to chemo- and/or radiotherapy according to molecular subgroup have emerged in past years, but the assessment of the role of surgery in the molecularly defined subgroups of low grade glioma was lacking so far. Since the molecular markers in the WHO 2016 update are prognostically very powerful, a re-evaluation of the impact of surgery is important to validate and evaluate current clinical practice.

Several studies have suggested that early and extensive surgery has a positive impact on survival for LGG patients. Unfortunately, most of these studies used qualitative measures for extent of resection or used tumor diameter to calculate tumor volume.[9-13,26,27] These approaches do not allow accurate assessment of extent of resection. A recent report by Duffau et al. incorporated volumetric measurements in a large molecularly defined LGG cohort. The authors showed that resectability was independent of molecular markers, however, survival data were not reported.[28] One report that used a volumetric approach and included survival analysis was the study by Smith et al.[8] These authors showed that a larger extent of resection predicts significant longer OS and that even small postoperative tumor volumes negatively influence OS. Their 2008 study, however, was based on histopathologically diagnosed LGG (WHO 2007) without molecular classification. A recent paper by Wahl et al. reported on the impact of postoperative tumor volumes on OS corrected for molecular subtype. Every 10cm^3 increase in postoperative tumor volume was associated with shorter OS. However, definite conclusions cannot be drawn since this was an exploratory, post-hoc analysis in a selected cohort of 71 patients with only significant residual disease after first surgical treatment.[29]

In this study we show that postoperative volume remains a prognostic factor for OS in molecularly defined LGG. This is an important finding, since it supports the policy of maximal safe resection in all molecular subtypes of LGG. Our data also shows that even very small tumor residues already negatively impact OS in astrocytoma IDHmt tumors. This is exemplified by the clear survival difference between tumor residues as little as 0.1-5.0 cm^3 and 0.0 cm^3, and the absence of a survival difference between 5.1-15.0 cm^3 and >15.0 cm^3 residue. Also, in a post-hoc analysis with dichotomization of postoperative volume we show that up to 25 cm^3 residue is still associated with a significant better OS compared to > 25cm^3. Since this is a post-hoc analysis, we should interpret this specific cut-off value with caution. More importantly though, this analysis shows that any residual tumor (i.e. greater than 0.0 cm^3) already impacts survival negatively in astrocytoma IDHmt. In oligodendroglioma it seems that a small residue

does not have that strong impact on OS as is observed in astrocytoma IDHmt. The absence of a strong relationship between OS and limited amounts of residual tumors might be explained by the more indolent natural course of these tumors and their increased sensitivity to treatment which may have more impact on survival than surgical intervention.[19,30] Although residual tumor probably is located in eloquent areas in the majority of cases, one may argue that a second-look look operation if safely possible might be something to consider in the few astrocytoma IDHmt patients with minor residual tumor located in non-eloquent area. On the other hand, our data also encourages further efforts to implement new methods that aid in safely maximizing extent of resection. Imaging techniques like intraoperative MRI or ultrasound to assess extent of resection during surgery might be valuable in this setting. At current these techniques are not available in most clinics however.

Our data suggest that some of the findings in previous studies may have been impacted by the presence of GBM-like tumors, which were more likely to undergo a biopsy only in this series. This might be explained by the more frequent location of GBM-like tumors in eloquent areas (N=16; 69.6%) and thus a higher frequency of biopsy or limited resections.

Our study has limitations that need to be addressed. The main limitation is the retrospective nature. However, due to ethical and practical reasons a randomized trial on the impact of early and of extent of resection is considered not feasible, and retrospective data with detailed clinical and molecular annotation is the best available data to address this issue.[31] Clearly, the preoperative volume of LGG has a major impact on outcome, and this also influences the postoperative volume. A larger series is necessary to further study this. Due to the retrospective nature of this study the follow-up, postoperative treatment and the used MRI protocol were heterogeneous. We corrected OS for administration of chemo- and/or radiotherapy, but correcting for different timing and sequence of these treatments is not informative due to the large variety of possible combinations. T2w-FLAIR sequences were not available for both pre- and postoperative measure for some patients. When this was the case, T2-weighted images were used. We used the late postoperative scan to minimize overestimation of postoperative tumor volume due to edema or ischemia and to minimize potential differences between sequences.[32] Also, non-standardized MRI follow-up limits the reliability of evaluation of PFS in terms of the importance of the extent of resection. Another important limitation that comes with the retrospective nature of this study is the inclusion based on histology. This has to be taken into account when generalizing results, since histology is only known after surgery.

Also, several recent studies have shown that final conclusions in studies on LGG require lengthy follow-up for definitive conclusions.[33] Further follow-up and more importantly expanding this dataset is very important. This especially concerns the

oligodendroglioma group, since the impact of small tumor residues in this commonly slow-growing tumor might become more clear with longer follow-up. Lastly, confirmation of our findings in an independent dataset is needed before definitive clinical conclusions are drawn.

In conclusion, we validated and added substantial and necessary evidence to current practice of early maximal resection for LGG. Importantly, we show that even very small tumor residues in astrocytoma IDHmt patients already have negative impact on OS.

Table s1. Multiple linear regression of factors influencing resection percentage[a]

	Estimate	SE	t	p-value
Intercept	1.384	0.124	11.177	<0.0001
WHO 2016 classification				
Oligodendroglioma	*	*	*	*
IDH mutated, 1p19q intact	0.049	0.057	0.850	0.396
IDH wildtype, TERT mutated	-0.400	0.117	-3.405	0.001
Age	-0.008	0.002	-3.545	0.001
Preoperative tumor volume	-0.003	0.000	-5.802	<0.0001
Eloquency				
Eloquent location	*	*	*	*
Non-eloquent location	0.256	0.057	4.531	<0.0001
Tumor location				
Frontal	*	*	*	*
Parietal	-0.039	0.098	-0.396	0.693
Temporal	-0.031	0.076	-0.412	0.681
Occipital	-0.120	0.139	-0.862	0.390
Insula	-0.269	0.080	-3.364	0.001
Basal ganglia	-0.363	0.222	-1.637	0.103
Gliomatosis cerebri	-0.260	0.145	-1.794	0.074

[a]Linear regression model with resection percentage as dependent variable. To meet the assumption of normal
distribution of residual errors, resection percentage was arcsin square root transformed.
* = Reference category

Table s2. Univariable and multivariable Cox-regression with resection percentage as measure of extent of resection

	Univariable			Multivariable		
Variable	HR	95% CI	P-value	HR	95% CI	P-value
Age	1.04	1.02 - 1.06	0.0002	1.00	0.98 - 1.03	0.734
Gender						
Male	*					
Female	0.68	0.41 - 1.12	0.129			
KPS	0.94	0.91 - 0.96	<0.0001	0.97	0.93 - 1.01	0.112
Eloquency						
Yes	*			*		
No	0.49	0.3 - 0.79	0.004	1.40	0.77 - 2.54	0.273
Resection percentage	0.16	0.08 - 0.3	<0.0001	0.41	0.16 - 1.05	0.062
Molecular diagnosis						
Oligodendroglioma	*			*		
Astrocytoma IDHmt	3.77	1.9 - 7.46	<0.0001	4.23	2.03 - 8.81	0.0001
GBM-like	112.9	45.93 - 277.55	<0.0001	63.77	22.35 - 181.99	<0.0001
RTx after surgery						
No	*			*		
Yes	2.45	1.52 - 3.94	0.0002	1.26	0.93 - 1.01	0.481
Chemo after surgery						
No	*			*		
Yes	1.19	0.68 - 2.09	0.545	1.19	0.56 - 2.51	0.652

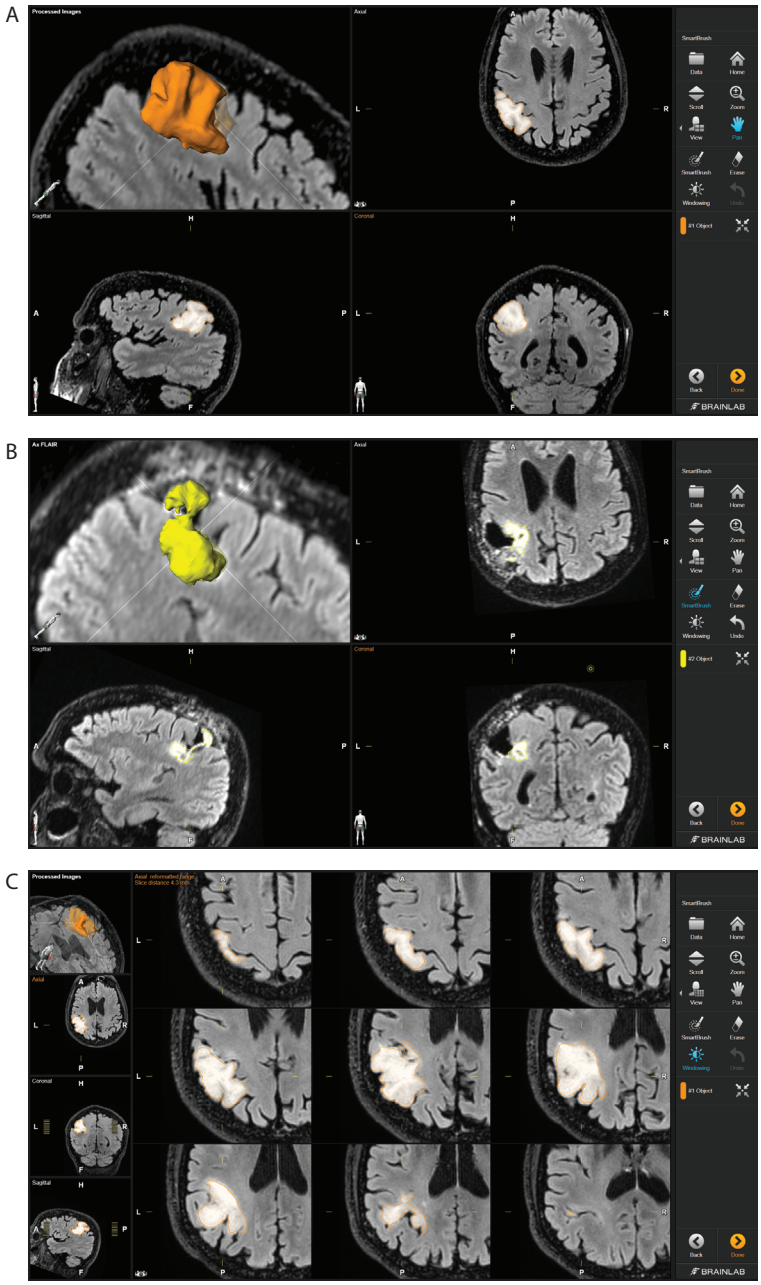
* = Reference category

Table s3. Overview of salvage treatments

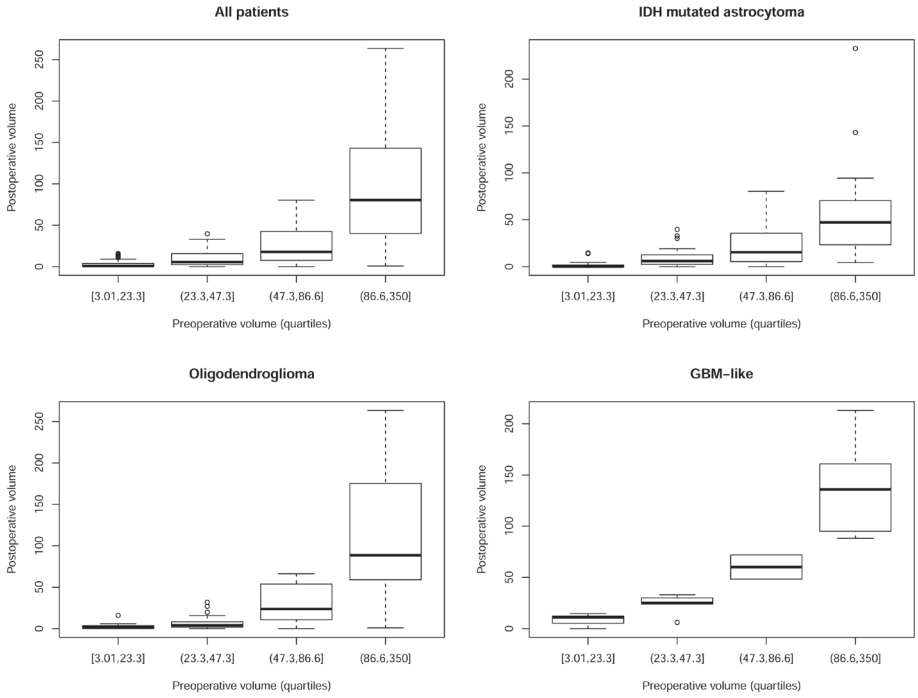
	All patients	Oligodendroglioma	Astrocytoma IDHmt	GBM-like
Number of re-resections				
0	182 (79.82%)	83 (89.25%)	78 (69.64%)	21 (91.30%)
1	43 (18.86%)	10 (10.75%)	31 (27.68%)	2 (8.70%)
2	3 (1.32%)	0 (0.0%)	3 (2.68%)	0 (0.0%)
Treatment after 1st surgery				
Wait & Scan	104 (45.61%)	51 (54.84%)	52 (46.43%)	1 (4.35%)
Chemotherapy	35 (15.35%)	24 (25.81%)	5 (4.46%)	6 (26.09%)
Radiotherapy	71 (31.14%)	15 (16.13%)	42 (37.50%)	14 (60.87%)
Chemoradiation	18 (7.89%)	3 (3.23%)	13 (11.61%)	2 (8.70%)
Ever radiotherapy				
Yes	155 (67.98%)	38 (40.86%)	97 (86.61%)	20 (86.96%)
No	73 (32.02%)	55 (59.14%)	15 (13.39%)	3 (13.04%)
Ever chemotherapy				
Yes	146 (64.04%)	54 (58.06%)	78 (69.64%)	14 (60.87%)
No	82 (35.96%)	39 (41.94%)	34 (30.36%)	9 (39.13%)
No progression during f/u yet				
N (%)	74 (32.46%)	37 (39.78%)	37 (33.04%)	0 (0.0%)

Table s4. Overview of surgical outcome

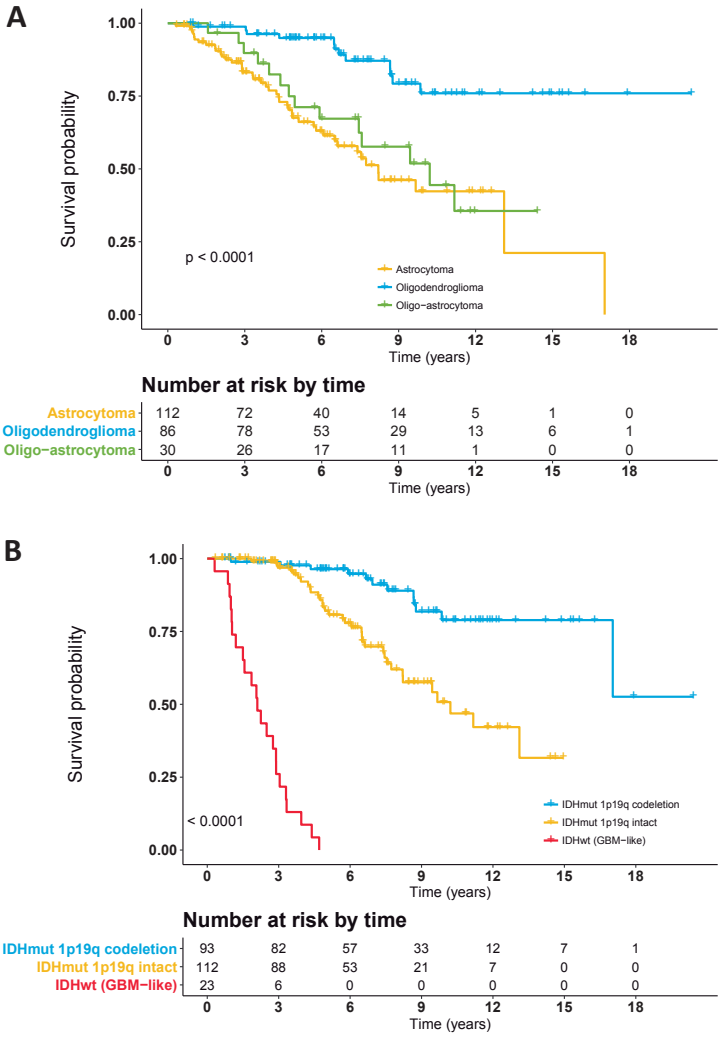
Frequencies of deficits after surgery/biopsy	N (%)
No deficitis	182 (79.8%)
Speech disorder	23 (10.1%)
Palsy	23 (10.1%)
Recovery of deficits	N (%)
Speech disorder	
Full recovery within 3 months	16 (69.6%)
Partial recovery	7 (30.4%)
Permanent deficit	0 (0.0%)
Palsy	
Full recovery within 3 months	11 (47.8%)
Partial recovery	8 (34.8%)
Permanent deficit	4 (17.4%)
Work resumption after surgery	N (%)
Yes	153 (67.1%)
No	17 (7.5%)
Unknown	58 (25.4%)



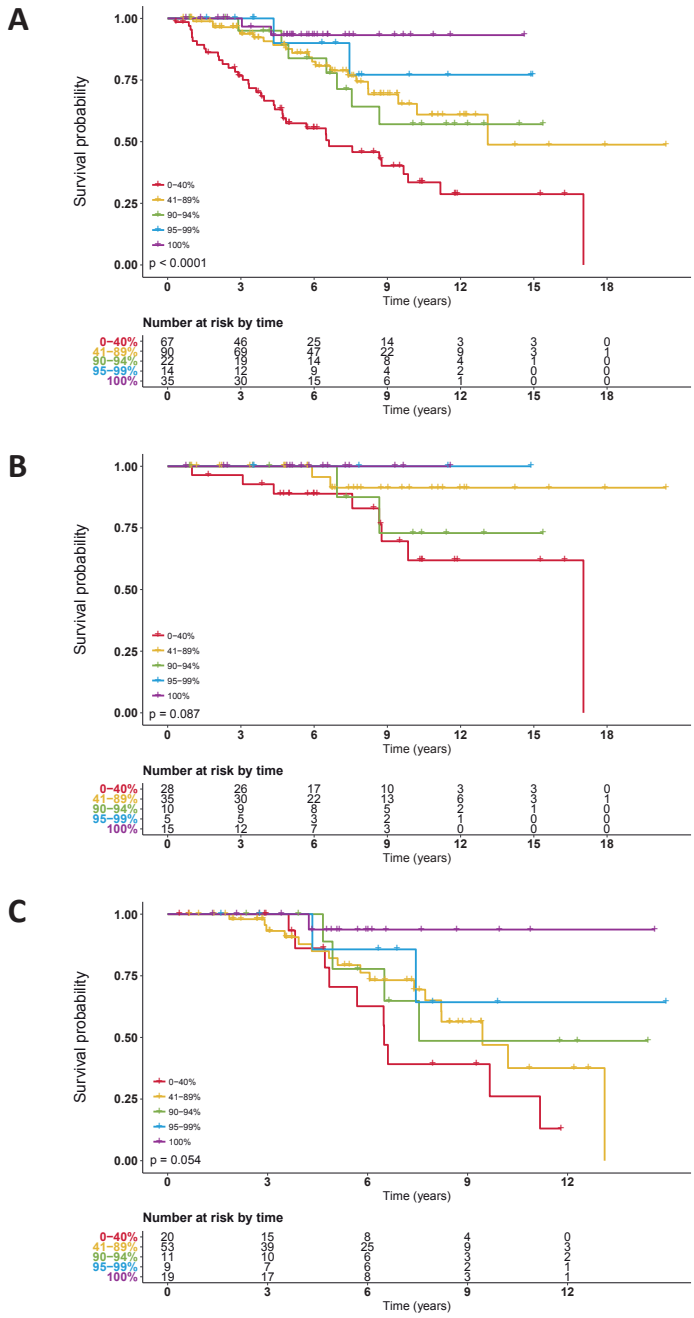
Supplementary figure 1. (A) Assessment of preoperative tumour volume. A 3D volume of interest can be created by first manually segmenting the tumour on one MRI slice of a chosen plane (i.e.the top-right panel of this figure). Then, a second manual segmentation is carried out on one perpendicular slice (i.e. the bottom-right panel). Next, the software automatically calculates the full 3D volume-of-interest (see top-left panel). (B) Assessment of postoperative tumour volume. (C) The volume of interest can be check slice by slice and where necessary, the volume can be easily manually adjusted.



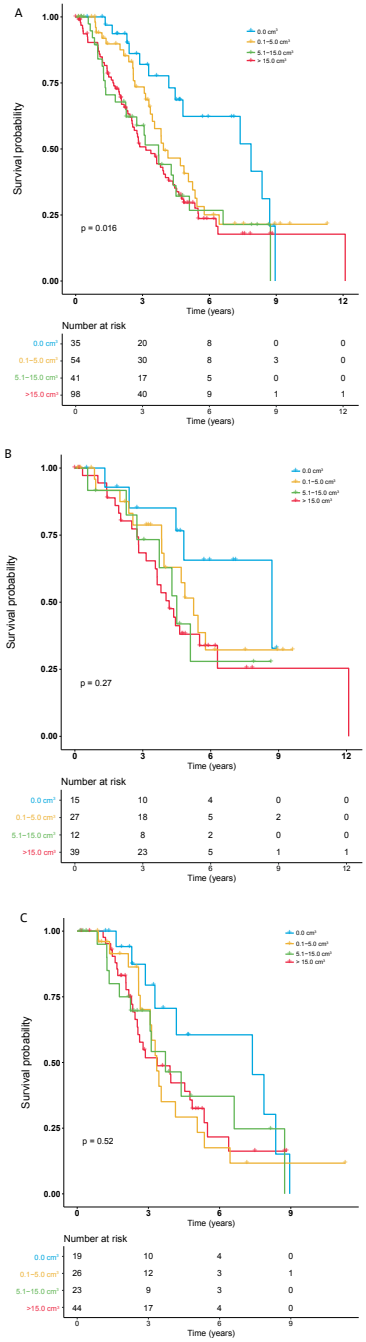
Supplementary figure 2. Quartiles of preoperative tumour volume vs postoperative tumour volume.



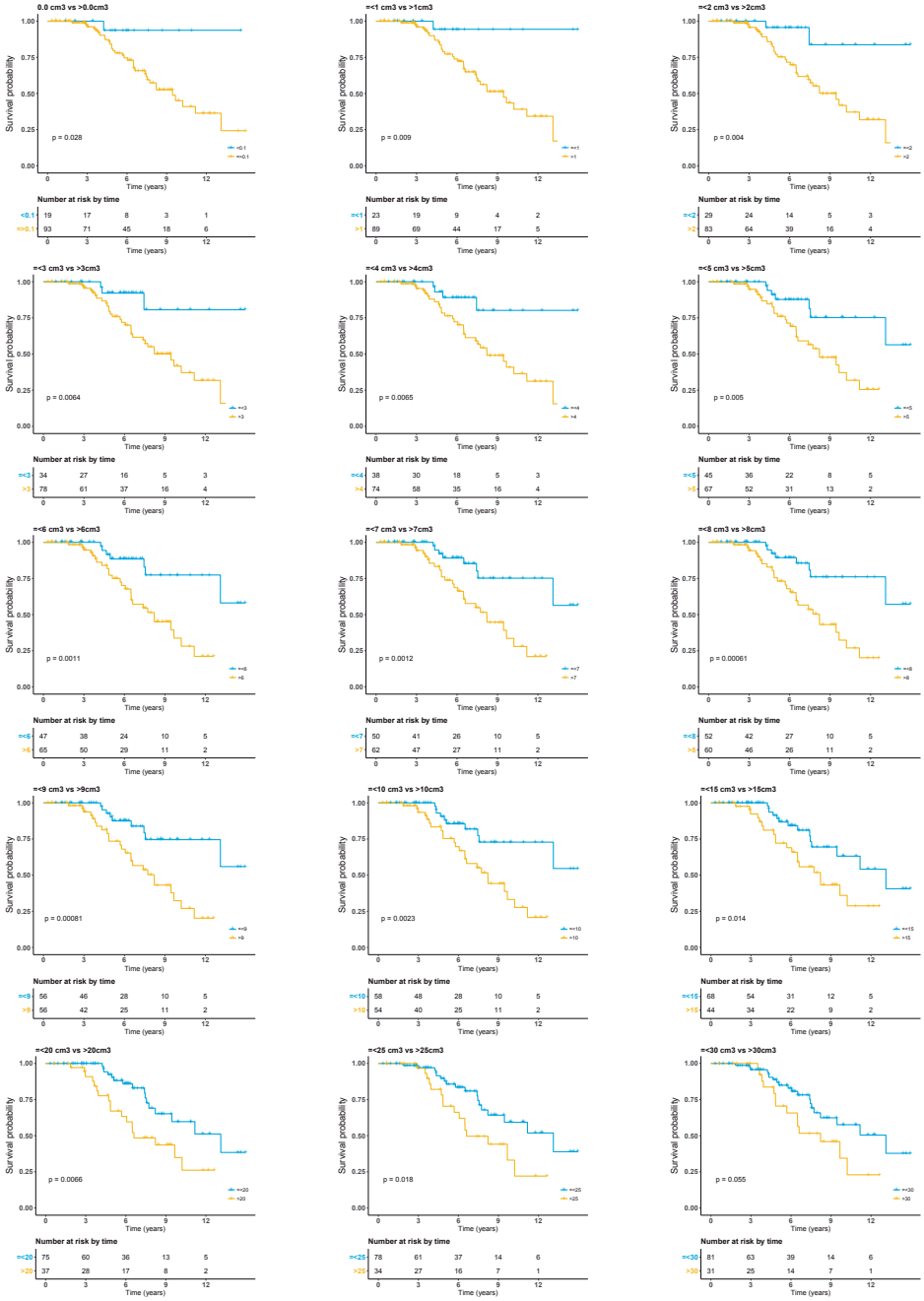
Supplementary figure 3. Overall survival stratified by histopathologic subtype (A) and molecular subtype (B).



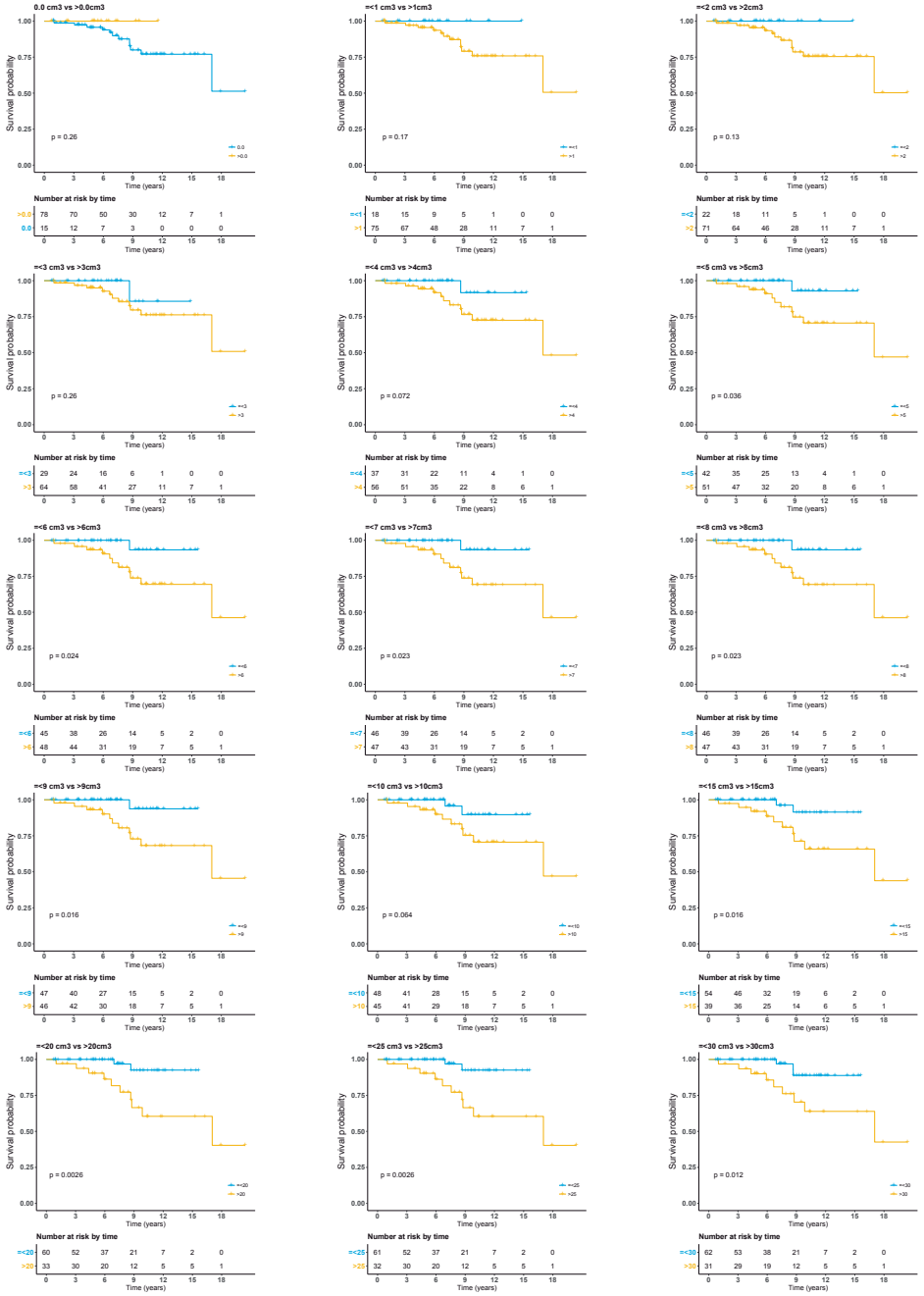
Supplementary figure 4. Overall survival stratified by resection percentage. (A) Overall survival of all patients stratified by resection percentage. (B) Overall survival of oligodendroglioma patients stratified by resection percentage. (C) Overall survival of IDH mutated astrocytoma stratified by resection percentage



Supplementary figure 5. Progression free survival stratified by postoperative tumor volume. (A) Progression free survival of all patients stratified by postoperative tumor volume. (B) Progression free survival of oligodendroglioma patients stratified by postoperative tumor volume. (C) Progression free survival of IDH mutated astrocytoma stratified by postoperative tumor volume.



Supplementary figure 6. Overall survival with dichotomisation of postoperative tumour volume in IDH mutated astrocytoma patients.



Supplementary figure 7. Overall survival with dichotomisation of postoperative tumour volume in oligodendroglioma.

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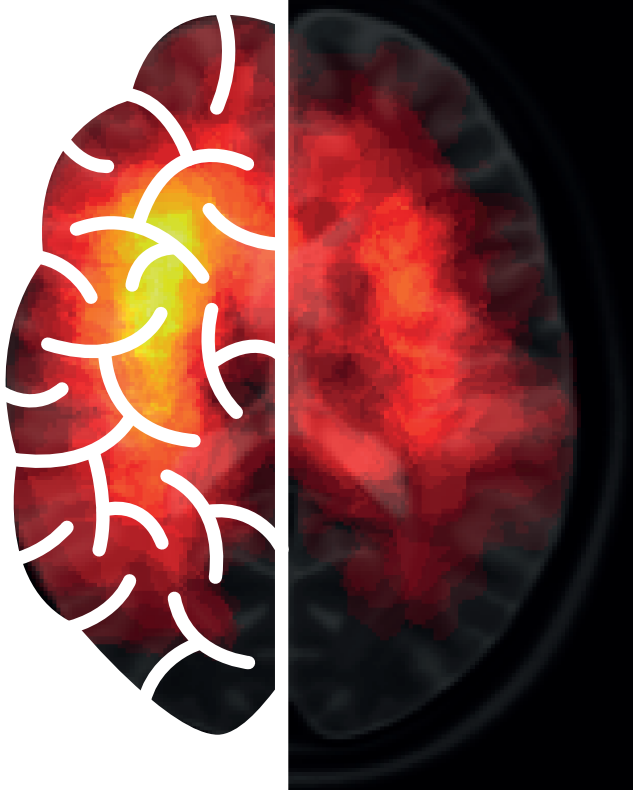

Chapter 6

Does early resection of presumed low-grade glioma improve survival? A clinical perspective

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ABSTRACT

Background

Early resection is standard of care for presumed low-grade gliomas. This is based on studies including only tumors that were post-surgically confirmed as low-grade glioma. Unfortunately this does not represent the clinicians' situation wherein he/she has to deal with a lesion on MRI that is suspect for low-grade glioma (i.e. without prior knowledge on the histological diagnosis). We therefore aimed to determine the optimal initial strategy for patients with a lesion suspect for low-grade glioma, but not histologically proven yet.

Methods

We retrospectively identified 150 patients with a resectable presumed low-grade-glioma and who were otherwise in good clinical condition. In this cohort we compared overall survival between three types of initital treatment strategy: a wait-and-scan approach (n=38), early resection (n=83), or biopsy for histopathological verification (n=29).

Results

In multivariate analysis, no difference was observed in overall survival for early resection compared to wait-and-scan: hazard ratio of 0.92 (95% CI 0.43-2.01; p=0.85). However, biopsy strategy showed a shorter overall survival compared to wait-and-scan: hazard ratio of 2.69 (95% CI 1.19-6.06; p=0.02).

Conclusions

In this cohort we failed to confirm superiority of early resection over a wait-and-scan approach in terms of overall survival, though longer follow-up is required for final conclusion. Biopsy was associated with shorter overall survival.

INTRODUCTION

Diffuse low-grade gliomas (LGGs) are primary brain tumors that, due to their infiltrative nature, cannot be fully eradicated by resection, chemotherapy, radiation, or a combination of these regimens. Most LGGs will gradually evolve into higher-grade gliomas and almost all patients will ultimately die from the disease.[1,2]

The typical LGG patient presents with a first epileptic seizure and a lesion on MRI that is suspect for a LGG (isointense to hypointense and non-enhancing on T1-weighted images; hyperintense on T2-weighted and fluid attenuated inversion recovery (FLAIR) images).[3] Consequently, in combination with clinical parameters, but yet without confirmed histology, physicians have to decide on a treatment strategy. They can opt for a wait-and-scan policy, take a biopsy for histopathological verification, or opt for immediate resection. Treatment strategy is patient dependent, influenced by the clinical condition of the patient, seizure control, size of the tumor and resectability of the tumor.[4] An initial wait-and scan approach is usually followed by resection or biopsy at the time the lesion starts to show growth or enhancement on MRI, or when clinical deterioration occurs. Resection and biopsy can either be followed by a wait-and-scan policy, radiotherapy, chemotherapy, or a combination of the latter two.[4] Both chemotherapy and radiotherapy have been extensively investigated in randomized controlled trials.[5-11] Controlled trials exploring the role and timing of surgery are lacking and, therefore, surgery for LGG has been controversial for many years.

In the past an initial wait-and-scan approach was advocated, since LGGs tend to grow slowly and patients usually present with controllable seizures as the only clinical symptom.[12-14] However, in the last 20 years, general opinion has shifted and early maximal resection is now widely accepted for patients with LGG-like lesions that are eligible for resection. Indeed, multiple retrospective studies showed that a more extensive resection is associated with a marked improvement of overall survival.[15-23] Also, a study in Norway showed that early resection significantly improves overall survival compared to a biopsy with a subsequent watch-and-wait period.[24,25] This growing bulk of evidence, although retrospective, has logically resulted in early maximal resection being standard of care and being incorporated in international guidelines nowadays.[4]

However, we have to bear in mind that these retrospective studies are subject to at least some form of selection and indication bias. Firstly, these studies excluded the non-enhancing presumed LGGs that are diagnosed as a higher grade after early surgery. Secondly, these studies discarded the presumed LGGs that progressed to a higher grade during the wait-and-scan period. Thirdly, these studies included patients with confirmed LGG, but with preoperative enhancement on MRI, which is usually not a presumed low-grade glioma.[26-28] Possibly there is also indication bias present in these studies; the physicians choice for initial treatment is potentially influenced by factors

that also have impact on prognosis itself.[29] In conclusion, the cohorts used in these previous studies are not entirely representative for the daily clinical situation in which physicians are confronted with a LGG-like lesion on MRI without histological confirmation and, consequently, in combination with clinical parameters, have to decide for an initial treatment strategy. Therefore, a study with patient selection based solely on preoperative clinical and imaging characteristics is more clinically relevant and can add significant evidence to support current daily clinical practice. A prospective trial is warranted but is unlikely to be conducted due to the duration of such a study (median survival of ≥ 15 years in oligodendroglioma subtype[13]), ethical considerations raised by physicians who strongly believe in early resection, as well as obtaining patients' consent to randomize between radically different treatment strategies.

In this retrospective study we approached the issue of treatment strategy from a more clinical and preoperative point of view and selected patients with a resectable LGG-like lesion based on diagnostic imaging and not on histopathological confirmation. We included those patients that we retrospectively consider equally eligible for either a wait-and-scan approach, a biopsy for histological verification, or early resection as initial treatment strategy; i.e. patients had to have limited neurological deficits that allowed a wait-and-scan strategy but also a LGG-like lesion that was eligible for extensive resection (estimation of at least 80% volume reduction possible, with use of current available techniques like awake surgery). In this manner we eliminated selection bias by histology and we avoided selection bias on indication as much as possible.

The aim was to determine the optimal initial treatment strategy for a resectable, presumed low-grade glioma by comparing overall survival between wait-and-scan, early resection and a biopsy approach.

METHODS

Patient selection

Three large neurosurgical institutions participated in this cohort study, together serving a population of 6.5 million people in the southwest of the Netherlands. The institutions involved were the Erasmus MC Cancer Institute in Rotterdam (EMC), Elisabeth-TweeSteden Hospital in Tilburg (ETZ), and Medical Centre Haaglanden in The Hague (MCH).

We identified patients with a presumed LGG (LGG-like lesion) that were retrospectively eligible for either initial treatment strategy: i.e. initial wait-and-scan approach, biopsy for histopathological verification, or immediate resection. Well-established prognostically favorable radiological and clinical characteristics were used as inclusion criteria [30-32]. Radiological criteria were: supratentorial location of lesion, no contrast

enhancement, no midline shift, maximal diameter <6 cm, sharply defined borders, and no involvement of corpus callosum, basal ganglia or thalamus. Clinical criteria were: age ≥18 years, Karnofsky Performance Status (KPS) >70, neurologically stable (with only epilepsy or minimal neurological deficits), and no dexamethasone dependency. Patients with active synchronous cancer of other origin were excluded.

To identify glioma patients, the digital archives of patient letters were searched for all neurological and neurosurgical patients registered 1990-2010 in EMC, 1996-2010 in ETZ, and 1992-2010 in MCH. In this first selection, high-grade gliomas were included so as not to exclude patients who progressed to a higher grade during a wait-and-scan period. In this search, of the 1115 glioma patients identified, a diagnostic scan could be retrieved for 498 of them, while the other part mainly originated from the pre digital era and was not available anymore. All diagnostic scans were reviewed to check if they met the criteria for a) the radiological diagnosis of a low-grade glioma and b) for the feasibility of an extensive resection (estimation of at least 80% volume reduction possible with modern surgical techniques like awake surgery) by a single neurosurgeon (AJPEV). Tumor near eloquent location was not an exclusion criterion per se, since the vast majority is eligible for resection with modern surgical techniques. The reviewing neurosurgeon has more than 10 years' experience in awake surgery and was blinded for clinical information such as initial treatment strategy, the histopathological diagnosis and outcome. Of these 498 patients, 348 were excluded: 305 did not meet the radiological criteria, 21 did not meet the clinical criteria, and for 22 the complete medical records were not available. Eventually, 150 patients remained for analysis. An overview of the selection procedure is shown in Figure 1.

Study variables

Baseline characteristics of the patients were collected from the medical records and diagnostic scan; i.e. initial treatment strategy, gender, age, KPS, presenting symptom, tumor location, mean tumor diameter and tumor eloquence; eloquence was graded with the criteria of Chang et al.[32]

Three types of initial treatment strategy were compared: initial wait-and-scan strategy after radiological diagnosis, early resection, and initial biopsy procedure for histological verification. Treatment decisions were based on local, national and international guidelines in each individual center at that time, by an experienced multidisciplinary team. Postoperative characteristics were also collected: first histology and grade, total number of resections, type of surgery (awake vs. general anesthesia), subsequent strategy after early resection, or biopsy and administration of radiotherapy and/or chemotherapy. Because postoperative MRI or CT scans were not available for most operated patients, the extent of resection could not be reliably investigated. In most tumors, molecular markers were not available and therefore not included in the analysis.

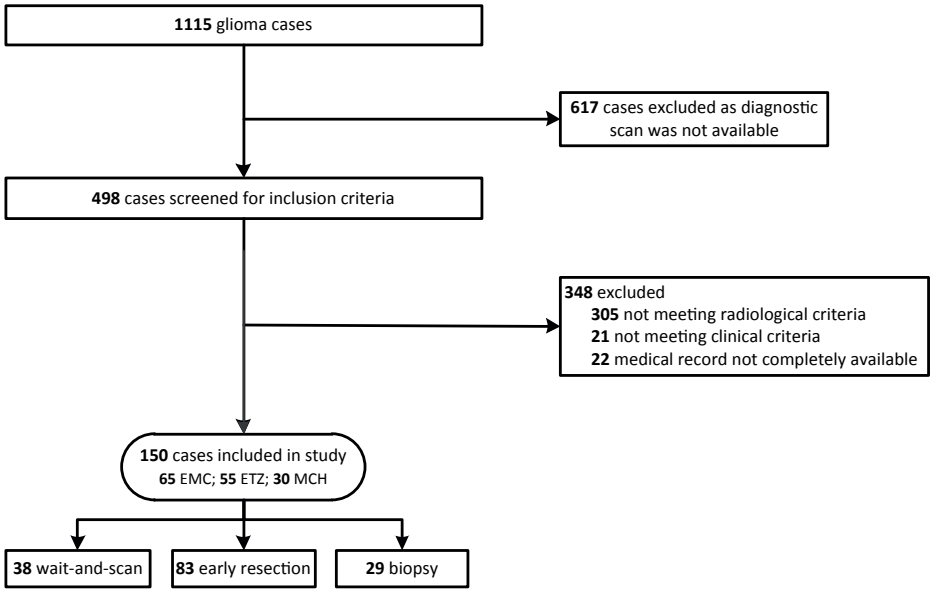

Figure 1. Consort flow diagram of patient inclusion. Of the 1115 glioma patients identified with a search in the digital patient archives, a diagnostic scan could be retrieved for 498 of them. Of these, 305 were excluded as they did not meet radiological criteria, 21 did not meet clinical criteria, and for 22 the complete medical record was not available. A total of 150 cases remained for analysis.

Outcome measure

The primary outcome measure was overall survival (OS), which was defined as the time between the diagnostic scan and death. All included patients were followed until death or censored at the date of last follow-up. Date of death was provided by patient records or the Municipal Personal Records Database.

Statistical analysis

All analyses were performed using R (3.1.3) and RStudio (0.99.486). Categorical data were analyzed with Pearson's chi-square test or Fisher's exact test when assumptions of the chi-square test were violated. Continuous data were analyzed with a Kruskal-Wallis test. Overall survival is shown in Kaplan-Meier plots (ggplot2 package in R). Univariate and multivariate analyses were performed using a Cox proportional hazard model (survival CRAN package in R). All calculations were two-sided tests, with a p-value <0.05 considered as statistically significant.

Ethics and approvals

Need for informed consent was waived by the Medical Ethical Committee of Erasmus MC, Rotterdam.

RESULTS

The medical records and diagnostic scans of 498 identified glioma patients were screened with our selection criteria to select patients with a resectable lesion and relatively favorable prognostic characteristics. A total of 150 patients with a resectable presumed LGG were included (Figure 1). The initial treatment strategy was either an initial wait-and-scan approach (n=38), a biopsy for histopathological verification (n=29), or early resection (n=83). Median follow-up was 7.1 years (25-75% interquartile range: 5.4-9.8 years). Baseline characteristics were equally distributed between treatment groups, except for tumor location in eloquent area (15.8% in wait-and-scan vs. 10.3% in biopsy and 32.5% in early resection; p=0.02) (Table 1).

Median time between diagnostic scan and intervention was 35.4 months in the wait-and-scan group, 0.8 months in the biopsy group, and 2.9 months in the early resection group. In 80% (n=66) of patients in the early resection group surgery was performed within 6 months after the diagnostic scan. Of the remaining 20% (n=17), all received surgery within 1 year, without any sign of tumor growth, enhancement or clinical deterioration at time of surgery. In these latter patients the physicians' initial choice of treatment was an early resection. However, the time between diagnosis and resection was ≥ 6 months, mainly due to practical reasons; either because referral from the diagnosing center to neurosurgical center was delayed, or due to patients' doubts about the treatment strategy. Nevertheless, these 17 patients were not excluded from the analysis as the actual initial choice of treatment was early resection and the intervention took place when there was still no sign of clinical deterioration, tumor growth or contrast enhancement on the control MRI. However to rule out bias, a sensitivity analysis was performed with the exclusion of these patients (see below).

In the group with wait-and-scan as initial treatment strategy, 79% of the patients eventually underwent a resection during follow-up. In these patients, surgery was initiated because of signs of growth or enhancement on follow-up imaging. There were no patients with uncontrolled seizures in the wait-and-scan group, nor was this a reason for surgery during follow-up. In the biopsy group, 51.7% eventually underwent resective surgery. Distribution of the postoperatively obtained tumor characteristics (histology and grade) differed between the groups: the biopsy group consisted of more astrocytomas (75.9% vs. 42.1% in wait-and-scan and 48.2% in early resection; p=0.01) and the wait-and-scan group consisted of more gliomas of higher-grade (24.3% vs. 10.8% in resection and 3.4% in biopsy; p=0.04).

Median OS in the early resection group was not reached and showed no significant difference (p=0.42) from the wait-and-scan group in which the median OS was 11.9 years (95% CI, 9.5-∞) (Figure 2). However, the median OS of 9.1 years (95% CI, 5.8-∞)

Table 1. Patient characteristics at baseline

	Treatment strategy			P
	Wait-and-scan	Early resection	Biopsy	
	(N = 38)	(N = 83)	(N = 29)	
Characteristic	N (%)[a]	N (%)[a]	N (%)[a]	
Gender				0.14
Male	25 (65.8)	40 (48.2)	18 (62.1)	
Female	13 (34.2)	43 (51.8)	11 (37.9)	
Age in years				
Median (IQR[b])	38 (16.3)	39 (14.6)	41 (21.4)	0.14
<40	23 (60.5)	46 (55.4)	13 (44.8)	0.43
KPS at diagnosis				0.28
100	37 (97.4)	77 (92.8)	24 (82.8)	
90	1 (2.6)	5 (6.0)	4 (13.8)	
80	0 (0.0)	1 (1.2)	1 (3.4)	
Presenting symptom				0.56
Epilepsy	35 (92.1)	71 (85.5)	23 (79.3)	
Cognitive disorder	0 (0.0)	1 (1.2)	0 (0.0)	
Hemiparesis	0 (0.0)	1 (1.2)	2 (6.9)	
Speech disorder	0 (0.0)	1 (1.2)	1 (3.4)	
Incidental finding	3 (7.9)	6 (7.2)	3 (10.3)	
Headache	0 (0.0)	3 (3.6)	0 (0.0)	
Tumor location				0.94
Frontal	18 (47.4)	46 (55.4)	13 (44.8)	
Temporal	7 (18.4)	14 (16.9)	6 (20.7)	
Parietal	5 (13.2)	11 (13.3)	4 (13.8)	
Occipital	1 (2.6)	1 (1.2)	0 (0.0)	
Insula	7 (18.4)	11 (13.3)	6 (20.7)	
Eloquent area				0.02
Yes	6 (15.8)	27 (32.5)	3 (10.3)	
No	32 (84.2)	56 (67.5)	26 (89.7)	
Tumor diameter (mm)				
Median (IQR[b])	39.5 (12.0)	41.0 (16.5)	41.0 (10.0)	0.67

[a]Data are numbers (%) unless indicated otherwise. [b]Interquartile range (25-75%)

in the biopsy group was significantly shorter compared to both the wait-and-scan and early resection group (log-rank test; p=0.04 and p=0.001, respectively) (Figure 2).

In the univariate analysis, histology, grade, tumor location and tumor eloquence also had a significant impact on OS (Table 3) and were, therefore, included in the multivariate Cox regression. In this multivariate analysis, the difference in OS remained with a hazard ratio (HR) of 2.53 (95% CI 1.1-6.1; p=0.04) (Table 3) for the biopsy group

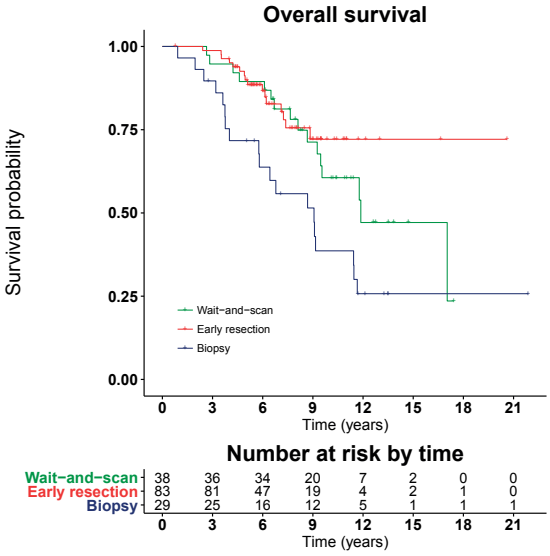

Figure 2. Kaplan-Meier plot showing the overall survival per treatment strategy. The p-value is calculated by the log-rank test including all three treatment groups. In the lower table, data indicate the numbers of patients at risk at the given time.

compared to wait-and-scan, whereas no difference was observed for early resection compared to wait-and-scan (HR 0.92; 95% CI 0.43-2.01; p=0.85).

A sensitivity analysis was also performed excluding those patients in the early resection group that did not undergo a resection within 6 months after diagnosis. No difference in OS was found for early resection compared to wait-and-scan with a HR of 0.70 (95% CI 0.33-1.46; p=0.34) in univariate analysis and 0.83 (95% CI 0.37-1.86; p=0.65) in multivariate analysis (including also histology, grade, and tumor location/eloquence as variables). A significant difference in OS remained for biopsy versus wait-and-scan, with a HR of 2.03 (95% CI 1.03-3.99; p=0.04) in univariate analysis and 2.82 (95% CI 1.24-6.43; p=0.01) in multivariate analysis.

DISCUSSION

Early maximal safe resection is considered standard of care for presumed LGG. Evidence to support this approach is mainly derived from retrospective studies; clear evidence from prospective trials for this early aggressive surgical approach is not available. Arguments in favor of early resection include uncertainty about the radiological diagnosis, the assumption that resection will postpone malignant transformation and will improve overall survival.[20,26] Indeed, several retrospective studies affirm the hypothesis that extensive resection for LGG improves overall survival.[15-23] Concerns that resection in a later stage of the disease could technically be more difficult and induce malignant transformation are not unimaginable. In the light of these concerns and the associa-

Table 2. Tumor and treatment characteristics of the three groups.

	Treatment strategy			P
	Wait-and-scan	Early resection	Biopsy	
	(N = 38)	(N = 83)	(N = 29)	
Characteristics	N (%)[a]	N (%)[a]	N (%)[a]	
Number of resections				<0.001
Zero	8 (21.1)	0 (0.0)	14 (48.3)	
One	25 (65.8)	47 (56.6)	10 (34.5)	
Two	5 (13.2)	34 (40.9)	5 (17.2)	
Three	0 (0.0)	2 (2.4 %)	0 (0.0)	
Procedure of first surgery				0.36
Awake	14 (46.7)	38 (45.8)	4 (26.7)	
General anesthesia	16 (53.3)	45 (54.2)	11 (73.3)	
Subsequent treatment after initial resection or biopsy[b]				<0.001
Wait-and-scan	Not applicable	53 (66.3)	3 (11.5)	
Radiotherapy	Not applicable	26 (32.6)	22 (84.6)	
Other	Not applicable	1 (1.3)	1 (3.8)	
Ever radiotherapy				0.01
Yes	28 (73.7)	57 (68.7)	28 (96.6)	
No	10 (26.3)	26 (31.3)	1 (3.4)	
Ever chemotherapy				0.02
Yes	23 (60.5)	30 (36.1)	16 (55.2)	
No	15 (39.5)	53 (63.9)	13 (44.8)	
First histology				0.01
Astrocytoma	16 (42.1)	40 (48.2)	22 (75.9)	
Oligodendroglioma	12 (31.6)	30 (36.1)	7 (24.1)	
Oligo-astrocytoma	9 (23.7)	13 (15.7)	0 (0.0)	
Not yet known	1 (2.6)	0 (0.0)	0 (0.0)	
Grade				0.04
II	28 (75.7)	74 (89.2)	28 (96.6)	
III	7 (18.9)	9 (10.8)	1 (3.4)	
IV	2 (5.4)	0 (0.0)	0 (0.0)	

[a]Data are shown as numbers (%). [b]Treatment after intervention is shown for the groups in which the initial strategy was immediate resection or biopsy

tion between extent of resection and overall survival, one may argue that the attempt for an extensive resection should be made as early as possible. These concerns that extent of resection can be influenced by timing of surgery in LGG have not been investigated by any study so far. Timing of treatment itself without incorporation of extent of resection has been studied before however. In a study by Jakola et al., a unique

Table 3. Univariate and multivariate analysis of overall survival using the Cox proportional hazards model.

	Univariate		Multivariate	
Variable	HR[a] (95% CI)	P	HR[a] (95% CI)	P
Treatment				
Wait-and-scan	1		1	
Early resection	0.72 (0.36-1.46)	0.37	0.92 (0.43-2.01)	0.85
Biopsy	2.04 (1.05-3.99)	0.04	2.69 (1.19-6.06)	0.02
Eloquency				
Yes	1		1	
No	2.36 (1.00-5.54)	0.05	1.41 (0.57-3.49)	0.46
Histology				
Astrocytoma	1		1	
Oligodendroglioma	0.40 (0.19-0.87)	0.02	0.49 (0.22-1.09)	0.08
Oligo-astrocytoma	1.10 (0.52-2.30)	0.81	1.34 (0.58-3.11)	0.49
Grade				
>II	1		1	
II	0.49 (0.24-0.98)	0.04	0.40 (0.17-0.93)	0.03
Tumor location				
Frontal	1		1	
Temporal	3.40 (1.65-6.88)	<0.001	3.49 (1.66-7.35)	<0.001
Parietal	1.90 (0.88-4.24)	0.10	1.60 (0.72-3.56)	0.25
Occipital	0.00 (0.0-∞)	0.99	0.00 (0.00-∞)	0.99
Insula	2.30 (1.03-4.94	0.04	2.79 (1.21-6.40)	0.02

[a]HR = hazard ratio, ∞=infinite

situation in Norway was studied wherein treatment outcome was compared between two centers: one center favored biopsy with subsequent watchful waiting strategy and the other center early resection. The early resection strategy in one center was clearly associated with a longer overall survival.[24,25] For several years now, after years of controversy, the approach of early maximal resection logically is incorporated in treatment guidelines for LGG. However, despite current guidelines, we have to bear in mind those studies were biased by histopathological diagnosis as inclusion criterion. This selection is actually not representative for the daily clinical setting whereby an initial treatment decision is based on imaging and patient characteristics. Although a prospective trial is the golden standard to clarify this issue, this is generally considered to be infeasible. We therefore tried to confirm the observations and assumptions from earlier studies that early resection prolongs overall survival in a cohort that more closely mimics daily clinical situation.

The strength of the present study is that we selected patients in a way that was not done before. We included patients with a presumed LGG that were equally eligible for all three treatment strategies, by using preoperative characteristics typical for a prognostically favorable LGG, and not histopathological diagnosis. Survival was measured from the date of the first diagnostic scan. We consider this design to result in more clinically relevant conclusions than those of earlier studies, since our selection resulted in more unbiased inclusion and, therefore, a more equitable comparison of strategies compared to previous studies.

We observed no difference in OS between early resection and an initial wait-and-scan approach. This suggests that a wait-and-scan strategy can be safely proposed until evident growth, contrast enhancement or clinical deterioration occurs, and that the timing of surgery does not influence the prognosis. How to interpret this result? Similar findings have been found in other cancers with typically long survival times; early prostatectomy did not increase survival as compared to a watchful waiting policy in a large prospective trial in localized prostate cancer with 10 years follow-up.[33,34] This trial shows that timing of the intervention does not have the impact as expected. Overall survival is not influenced by early intervention as long as the patient is monitored and intervention takes place when necessary. Although our study was not set-up prospectively, the results are comparable. It suggests that in tumors with relatively long overall survival, the relative short timing to treatment intervention is not influencing prognosis. The intrinsic biological behavior of the tumor (molecular markers) has more impact than the timing of treatment. It also implies that potential morbidity of surgery or biopsy can be safely delayed in these patients and lead to higher quality of life until treatment.[35] On the other hand, surgical techniques like awake craniotomy have been shown to be safe and could also decrease seizure frequency and medication intake in patients with LGG. The data are however not mature yet to give final conclusions, but might already argue that a prospective trial is urgently needed to investigate if surgery can be safely delayed in a subset of presumed LGG patients.

In contrast to early resection versus wait-and-scan, this study shows that biopsy as initial strategy has a negative impact on OS. This observation is in line with that of the Norwegian study[24] and suggests that this strategy should be avoided. It is difficult to explain the significantly shorter OS for the biopsy group compared to the wait-and-scan group. In our cohort, we tried to select patients that were equally eligible for all treatment strategies. Nevertheless, we did observe a higher percentage of astrocytomas in the biopsy group, which may partly explain the poorer prognosis. Alternatively, this difference in histology might be caused by sampling error in the biopsy group. Indeed, in a study examining histological diagnosis in paired biopsy and resection samples, an oligodendroglial component was missed in 50% of the biopsy samples.[36] If this is so, our study implies that biopsy is associated with a less favorable outcome. Moreover,

in our multivariate analysis that corrected for histology, a worse prognosis remained for the biopsy group. Nevertheless, it cannot be ruled out that confounding factors that we missed/did not consider might have influenced physicians' decision to choose for a biopsy procedure and, therefore, biased the selection for patients with poorer prognosis for the biopsy. To be on the safe side we think a biopsy should be avoided when possible. A negative effect of the biopsy itself seems unlikely although an acute inflammatory response induced by biopsies is reported to promote metastasis and proliferation in other types of cancer and recently it was shown in a murine model that reactive astrocytes can potentiate glioma aggressiveness after resection.[37-40]

This study has a few limitations. First, this study is retrospective in design. Although our selection criteria aimed to diminish the possible selection and indication bias, which comes with such a design, bias is never ruled out completely. Also, the stringent selection criteria that were used resulted in a relatively small cohort size, but they were used to identify those patients in whom an extensive resection is possible according to current standards. Secondly, a longer follow-up is required before definite conclusions can be drawn, as we have not yet reached the median OS in the early resection group. Longer follow-up time is necessary.

Thirdly, the extent of resection also has an impact on OS.[15-17,20,22,23] Perhaps the most important limitation of our study is that the extent of resection was not measured in our cohort, since this might have influenced survival. Moreover, the awake craniotomy procedure that has emerged in glioma surgery, is reported to increase resection percentage and decrease morbidity compared to general anesthesia.[41] In our three treatment groups, the type of anesthetic procedure at the time of resection was chosen based on the best practice at that time. Of the 128 patients that had any resection during follow-up, 56 (43%) were operated with an awake craniotomy procedure and the use of this procedure was equally distributed between the treatment groups. Given this equal distribution and the fact that we selected patients with a lesion eligible for extensive resection, it is unlikely that the extent of resection plays an explanatory role in our results. It can never be ruled out however. Also, of the patients in the early resection group, 20% underwent the actual intervention within 6-12 months after the diagnostic scan. Nevertheless, as the initial choice of treatment by the physician was early resection and the intervention took place without any sign of clinical deterioration, tumor growth or contrast enhancement on MRI scan, we decided not to exclude these patients from the analysis. The sensitivity analysis performed after exclusion of these patients, failed to show different results.

It should be noted that there were imbalances in the treatment and tumor characteristics that were obtained after initial treatment decision, which should be taken into account when interpreting the data. The wait-and-scan group consisted of more high-grade gliomas; this is, however, to be expected since the histological diagnosis

in the wait-and-scan group was obtained at a median of 35.4 months after the initial imaging diagnosis. Also, there was an imbalance in chemotherapy administration, showing a lower percentage of patients exposed to chemotherapy in the early resection group. This is possibly explained by the fact that a significant part of the patients in the early resection group had a subsequent wait-and-scan approach after initial resection. It is to be expected that also these patients will receive chemotherapy when follow-up is longer. On the other hand, although median follow-up in the early resection group is shorter, this might suggest that patients in the early resection group are more clinically stable than the other treatment groups. If this is so, we would expect the survival curves to further diverge with longer term follow-up.

Recently the WHO classification of tumors of the central nervous system was updated and now incorporates molecular markers. This new classification outflanks classic histopathological classification in terms of prognosis estimation and can also tailor therapy. Impact of surgery possibly differs between molecular subgroups, but this remains to be investigated. The integration of these markers in our study would be very interesting in the light of the new WHO classification. Unfortunately, the status of these markers was not available for the majority of our population.[42,43] However, these molecular markers are only determined after resection and should therefore not play a role in the selection and decision criteria of this study. New techniques are now developed to determine the molecular make up of presumed LGG on preoperative MRI's.[44] This will hopefully lead to optimal treatment strategies of these tumors in the near future, which will also require further analysis of the value of early and of extent of resection in the molecular glioma subtypes.

CONCLUSION

Investigation of three different treatment strategies in a clearly defined set of presumed LGG patients who were candidates for extensive resection could not confirm superiority of early resection over wait-and-scan. In agreement with previous studies, biopsy as first treatment strategy seems to be associated with significantly shorter overall survival. Still, this observation is difficult to explain. However, to be on the safe side, we think avoidance of this strategy should be considered when possible. To our knowledge this is the first study to investigate treatment strategies for presumed LGG with this design based on preoperative imaging characteristics, which is highly representative for daily clinical presentation of this patient group. We need longer term follow-up upon final conclusion, but this data highlights prospective data is of vital importance.

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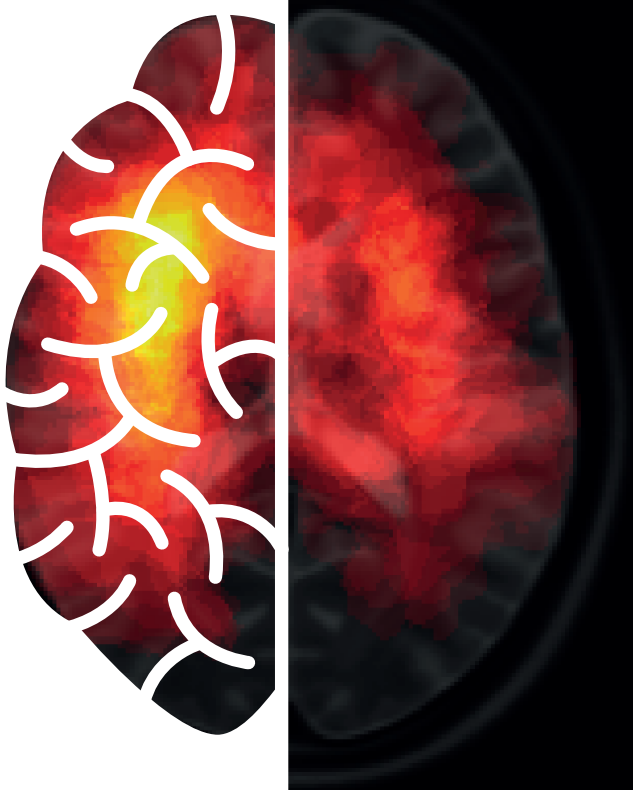

Chapter 7

Differences in spatial distribution between WHO 2016 low-grade glioma molecular subgroups

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Submitted

ABSTRACT

Background

Several studies reported a correlation between anatomic location and genetic background of low-grade gliomas (LGG's). As such, tumor location may contribute to pre-surgical clinical decision-making. Our purpose was to visualize and compare the spatial distribution of different WHO 2016 gliomas, frequently aberrated single genes and DNA copy number alterations within subgroups, and groups of postoperative tumor volume.

Methods

Adult grade II glioma patients (WHO 2016 classified) diagnosed between 2003 and 2016 were included. Tumor volume and location were assessed with semi-automatic software. All volumes of interest were mapped to a standard reference brain. Location heatmaps were created for each WHO 2016 glioma subgroup, frequently aberrated single genes and copy numbers (CNV's), as well as heatmaps according to groups of postoperative tumor volume. Differences between subgroups were determined using voxelwise permutation testing.

Results

110 IDH mutated astrocytoma patients, 92 IDH mutated and 1p19q co-deleted oligodendroglioma patients, and 22 *IDH* wildtype astrocytoma patients were included. We identified small regions in which specific molecular subtypes occurred more frequently. *IDH* mutated LGG's were more frequently located in the frontal lobes and *IDH* wildtype tumors more frequently in the basal ganglia of the right hemisphere. We found no localizations of significant difference for single genes/CNV's in subgroups, except for loss of 9p in oligodendrogliomas with a predilection for the left parietal lobes. More extensive resections in LGG were associated with frontal locations.

Conclusions

WHO low-grade glioma subgroups show differences in spatial distribution. Our data may contribute to pre-surgical clinical decision-making in LGG patients.

INTRODUCTION

Classification of diffuse gliomas is based on histological and molecular criteria according to the 2016 WHO classification of tumors of the central nervous system.[1] Three major subtypes of diffuse low-grade (grade II) glioma (LGG) are recognized based on testing of two molecular markers: mutations of isocitrate dehydrogenase 1 or 2 gene [further abbreviated as *IDH*] and combined deletion of chromosomal arms 1p and 19q. Next to the WHO classification, (eloquent) location, size, presence of contrast enhancement, and delineation of the tumor margins on magnetic resonance (MR) imaging are important prognostic factors.[2–5]

Previous studies suggest that the anatomic location of a glioma is correlated with the molecular background of the tumor.[6–9] If so, tumor location may contribute to pre-surgical clinical decision-making and may provide a non-invasive marker for prediction of molecular subtype. However, current evidence to support this is mainly derived from relatively small series which are often single molecular marker studies. [6–9] For example it was shown that loss of 1p is associated with more frequent location in the frontal lobes and that 1p19q co-deletion is absent in insular tumors.[7,8] In a series of 47 patients Metellus et al. showed that *IDH* wildtype LGG are preferentially located in the insular region.[9] We recently reported on a cohort of resected LGG samples, wherein we found that *IDH* wildtype tumors were more often located in eloquent areas. In that study, just as in many other previous studies on glioma location, we used the cerebral lobes as the location description factor, which is not accurate. A recent study by Tejada Neyra et al. showed a location predilection for *IDH* mutated tumors in the frontal lobes in the rostral extension of the lateral ventricles.[10] Despite these data, no large series have been described that actually visualize anatomic location of WHO 2016 low-grade (grade II) glioma subtypes in a voxel-based manner. As the WHO 2016 criteria are more objective than the previous WHO 2007 criteria and result in a more refined and prognostic classification of tumors, it is possible that the spatial distributions of different WHO 2016 glioma subtypes are more distinct than previously shown for the classical histopathological classification.[11]

Previously we investigated the impact of extent of resection in WHO 2016 classified LGG and assessed tumor volume location and volume in a semi-automatic manner.[12] We used this cohort in the present study to visualize the spatial distribution of different WHO 2016 LGG subtypes, by creating voxel-based probability maps of tumor location for every subtype. Using this cohort also enabled us to visualize spatial distributions of LGG's according to the extent of resection. These maps might be helpful in pre-surgical decision-making.

METHODS

Patient selection

Adult patients (age ≥ 18 years) with histopathologically confirmed supratentorial grade II glioma were included. Patients and samples were previously described in a study investigating the extent of resection in grade II glioma.[12] All patients were treated in a single institute between 2003 and 2016, The Brain Tumor Center at Erasmus MC Cancer Institute in Rotterdam, the Netherlands. The study was approved by the medical ethics committee of Erasmus MC and conducted according to national and European regulations.

Image acquisition and processing

We used the preoperative MR scans that were available from routine clinical setting. MR sequence protocols varied per patient, as patients were diagnosed in several centers without the use of a uniform tumor MR imaging protocol. For inclusion in this study, at least a T2-weighted (T2w) fluid attenuated inversion recovery (FLAIR) or T2w sequence needed to be available. We did not use inclusion or exclusion criteria for voxel size and/or slice thickness. We segmented preoperative and postoperative glioma lesions on MR imaging in a semi-automated fashion with the SmartBrush tool that is incorporated in Brainlab Elements (version 2.1.0.15). With this tool a three-dimensional (3D) volume-of-interest (VOI) can be created by manually segmenting the lesion on two perpendicular slices, from which the software calculates a full 3D VOI, which was manually adjusted where necessary. We used the T2w FLAIR sequence when available (3D where possible), otherwise the conventional T2w sequence was used for segmentation. All tumor related T2w and T2w-FLAIR signal abnormalities were included in the segmentation. The T2w and T2w-FLAIR images of all patients were affinely registered (preserving parallel lines and planes) to the Montreal Neurological Institute (MNI) International Consortium for Brain Mapping (ICBM) 152 non-linear T2w atlas[13,14], and the 3D VOIs were transformed accordingly for further analysis. We registered all MR images using an automated algorithm based on maximization of mutual information,[15] as implemented in the open-source SimpleElastix software (version 9dfa8cb).[16] All registrations were manually checked to assure proper alignment with the atlas, and adjusted where necessary.

DNA extraction and Next-Generation-Sequencing

Areas with high tumor content were manually macrodissected from formalin-fixed-paraffin-embedded (FFPE) tissue slides as described previously.[12] We used a targeted Next-Generation Sequencing (NGS) panel to classify samples according to the WHO 2016 criteria, using an Ion Torrent Personal Genome Machine or Ion S5XL (Life Tech-

nologies). The panel assesses mutational status of *IDH1/2, TP53, FUBP1, PTEN, CIC, CDKN2A, NOTCH1, ATRX* (whole gene) and hotspots of *EGFR* (exon 3+15), *H3F3A* (exon 2), *PIK3CA* (exon 10+21), *BRAF* (exon 11+15), and also copy number variations of chromosome 1, 7, 9, 10, 12, and 19. *TERT* promoter mutations (C228T & C250T) were assessed in a separate assay (SnaPshot). Detailed methods were described previously.[12,17]

The following criteria for molecular classification were used:

- Oligodendroglioma: *IDH1* or *IDH2* mutated and loss of heterozygosity consistent with co-deletion of the entire 1p and 19q chromosomal arms.
- *IDH* mutated astrocytoma: *IDH1* or *IDH2* mutated.
- *IDH* wildtype astrocytoma, with molecular features of glioblastoma (according to recent cIMPACT-NOW update 3[18], in further text named as "*IDH* wildtype astrocytoma"): *IDH1* or *IDH2* wildtype and: *TERT* promoter mutation without 1p19q co-deletion, or loss of heterozygosity of chromosome 10q and imbalance of chromosome 7, or *EGFR* amplification.

Statistical analysis

We created tumor location heatmaps for the different WHO subtypes by iterating over all voxels in the MNI atlas and counting the number of tumor occurrences for each group in each voxel. Via this procedure we created location heatmaps for the WHO 2016 molecular subgroups. Additional heatmaps were generated for glioma specific single genes and chromosomes of interest (*CIC, FUBP1*, chromosome 7 and 9p), as well as location heatmaps stratified for extensiveness of resection (four groups of postoperative tumor volumes; 0 cm^3/0.1-5.0cm^3/5.1-15cm^3/>15cm^3).

To test for differences in spatial distribution between WHO 2016 subgroups, we assessed the cluster-wise significance at the voxel-level between distributions, using permutation testing with Threshold Free Cluster Enhancement in the software package "FSL Randomise"[19] (version 5.0.9, using 15000 permutations). This approach corrects p-values for the family wise error in testing the many voxels, considering a corrected P-value of <0.05 as statistically significant. We first compared all *IDH* wildtype LGG's with all *IDH* mutated LGG's. In a subsequent analysis we assessed statistical significance of location differences of *IDH* mutated astrocytoma and oligodendroglioma separately.

RESULTS

Our initial cohort consisted of 246 patients with confirmed LGG, for which FFPE material and MR scans were available. 22 patients were excluded: 16 due to sequencing failure, 2 due to a preoperative contrast enhancement suggestive of glioblastoma, and another 4 due to insufficient image quality. Of the remaining 224 patients, 110 were

IDH mutated astrocytomas, 92 were oligodendrogliomas, and 22 were *IDH* wildtype astrocytomas. Clinical characteristics of this cohort were consistent with LGG patient characteristics known from the literature and are shown in Table 1.

Table 1. Patients characteristics

Characteristics	Total cohort (n=224)		Oligodendroglioma (n=92)		Astrocytoma, IDH mutated (n=110)		Astrocytoma, IDH wildtype (n=22)	
	n	%	n	%	n	%	n	%
Sex								
Male	133	59.4 %	47	51.1 %	70	60.9 %	16	72.7 %
Female	91	40.6 %	45	48.9 %	40	36.4 %	6	27.3 %
Age								
Median	42		45		37		59	
IQR	33 - 51		37 - 52		29 - 45		52 - 63	
< 40	99	44.2 %	32	34.8 %	67	60.9 %	0	0 %
40-60	101	45.1 %	49	53.3 %	41	37.3 %	11	50 %
> 60	24	10.7 %	11	12 %	2	1.8 %	11	50 %
Presenting symptom								
Epilepsy	167	74.6 %	76	82.6 %	81	73.6 %	10	45.5 %
Incidental	23	10.3 %	8	8.7 %	14	12.7 %	1	4.5 %
Headache	8	3.6 %	1	1.1 %	6	5.5 %	1	4.5 %
Miscellaneous neurologic complaints	26	11.6 %	7	7.6 %	9	8.2 %	10	45.5 %
Type of 1st surgery								
Awake craniotomy	103	46 %	49	53.3 %	53	48.2 %	1	4.5 %
Normal resection	74	33 %	23	25 %	48	43.6 %	3	13.6 %
Open biopsy	15	6.7 %	7	7.6 %	2	1.8 %	6	27.3 %
Stereotactic biopsy	32	14.3 %	13	14.1 %	7	6.4 %	12	54.5 %
Preoperative KPS								
Median	100		100		100		90	
IQR	90 - 100		100 - 100		90 - 100		82 - 90	
100	145	64.7 %	70	76.1 %	70	63.6 %	5	22.7 %
90	61	27.2 %	17	18.5 %	33	30 %	11	50 %
<=80	18	8 %	5	5.4 %	7	6.4 %	6	27.3 %
Eloquent Area	90	40.2 %	35	38 %	39	35.5 %	16	72.7 %
Side of lesion								
Right	95	42.4 %	37	40.2 %	51	46.4 %	7	31.8 %
Left	117	52.2 %	50	54.3 %	57	51.8 %	10	45.5 %
Bilateral	12	5.4 %	5	5.4 %	2	1.8 %	5	22.7 %

Table 1. Patients characteristics (continued)

Characteristics	Total cohort (n=224)		Oligodendroglioma (n=92)		Astrocytoma, IDH mutated (n=110)		Astrocytoma, IDH wildtype (n=22)	
	n	%	n	%	n	%	n	%
Pre-operative tumor volume, cm^3								
<25.0	64	28.6 %	26	28.3 %	31	28.2 %	7	31.8 %
25.1-50.0	54	24.1 %	24	26.1 %	24	21.8 %	6	27.3 %
50.1-100.0	65	29 %	23	25 %	38	34.5 %	4	18.2 %
100.1-250.0	38	17 %	17	18.5 %	16	14.5 %	5	22.7 %
250.1-351.0	3	1.3 %	2	2.2 %	1	0.9 %	0	0 %
Median	47.3		45.9		50.95		38.2	
Range	3.01 - 350.5		4.29 - 350.5		3.01 - 302.8		9.05 - 213.1	
Postoperative tumor volume, cm^3								
0.0	33	14.7 %	15	16.3 %	17	15.5 %	1	4.5 %
0.1-5.0	54	24.1 %	27	29.3 %	26	23.6 %	1	4.5 %
51-10.0	21	9.4 %	6	6.5 %	13	11.8 %	2	9.1 %
10.1-50.0	66	29.5 %	19	20.7 %	38	34.5 %	9	40.9 %
50.1-265.0	50	22.3 %	25	27.2 %	16	14.5 %	9	40.9 %
Median	11		7.13		9.26		31.5	
Range	0 - 263.6		0 - 263.6		0 - 232.7		0 - 213.1	
Follow-up (years)								
Median	5.8		7.3		5.7		2.2	
Range	0.3 - 20.4		0.8 - 20.4		0.3 - 15		0.3 - 4.7	

Spatial distribution of WHO 2016 glioma subgroups

Spatial distribution heatmaps for each WHO 2016 grade II glioma subgroup are shown in Figure 1. Upon visual inspection most oligodendrogliomas were located in the frontal lobes and cortex, while *IDH* mutated astrocytomas were more frequently located in the frontotemporal lobes and the insular region. *IDH* wildtype astrocytomas were more frequently located in the basal ganglia and rostral areas of the hemispheres. In this cohort, tumors were slightly more frequently located in the left hemisphere, both for *IDH* mutated astrocytomas and oligodendrogliomas.

Statistical analysis of the spatial distributions indicated that tumors were more frequent in the anterior extensions of the lateral ventricles for *IDH* mutated LGG's (oligodendrogliomas and *IDH* mutated astrocytomas combined) compared to *IDH* wildtype astrocytomas (Figure 2A). With *IDH* wildtype astrocytomas as reference category, our analysis indicated that *IDH* wildtype astrocytomas are more frequently located in the basal ganglia of the right hemisphere (as compared to *IDH* mutated LGG's) (Figure 2B).

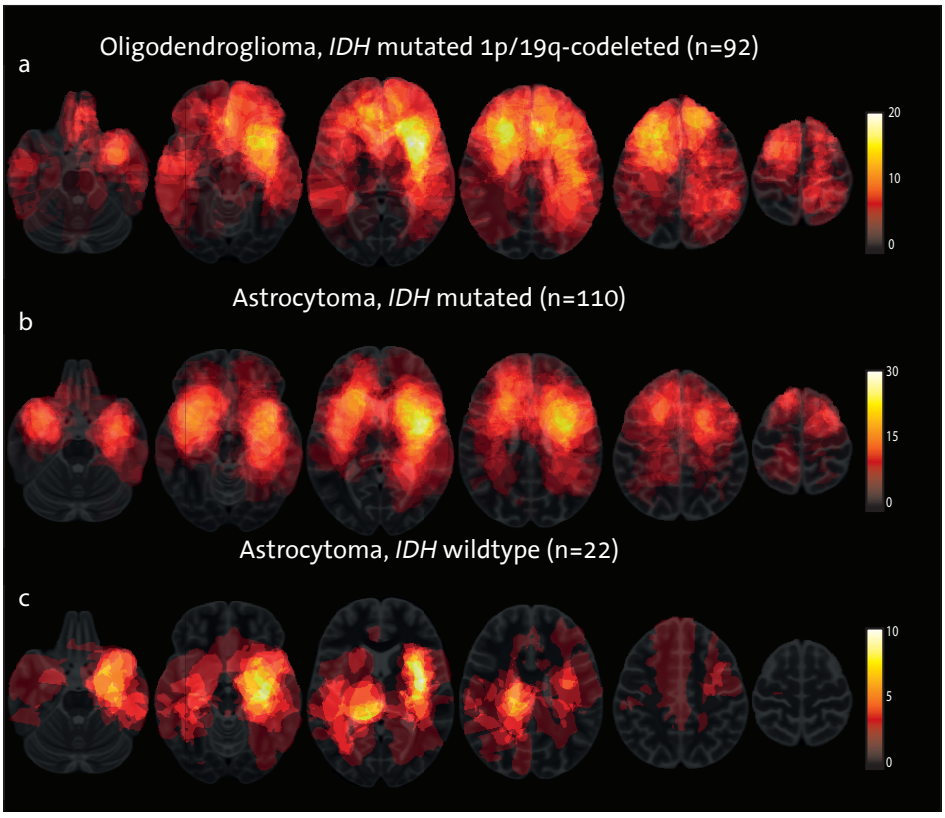


Figure 1. Spatial distribution heatmaps of WHO 2016 glioma subgroups. The color of a voxel corresponds with the number of tumors localized at that location, ranging from red (low number), to white (high number). The color bars on the right indicate the frequencies corresponding with the color per voxel; (a) location distribution of oligodendroglioma shows most are located in the frontal lobes. (b) *IDH* mutated astrocytoma shows a distribution with most tumors located in or near the insular region. (c) *IDH* wildtype astrocytomas are more often located in midline region and basal ganglia.

Direct comparison between *IDH* mutated astrocytomas and oligodendrogliomas showed a small area in the left frontal cortex where oligodendrogliomas occurred more frequently, and a small region in the right temporal lobe where *IDH* mutated astrocytomas (supplementary Figure 1) occurred more frequently. However, in a subsequent 3-group comparison including *IDH* wildtype astrocytomas, there were no locations of significant differences for *IDH* mutated astrocytomas or oligodendrogliomas individually, only for *IDH* wildtype astrocytomas individually.

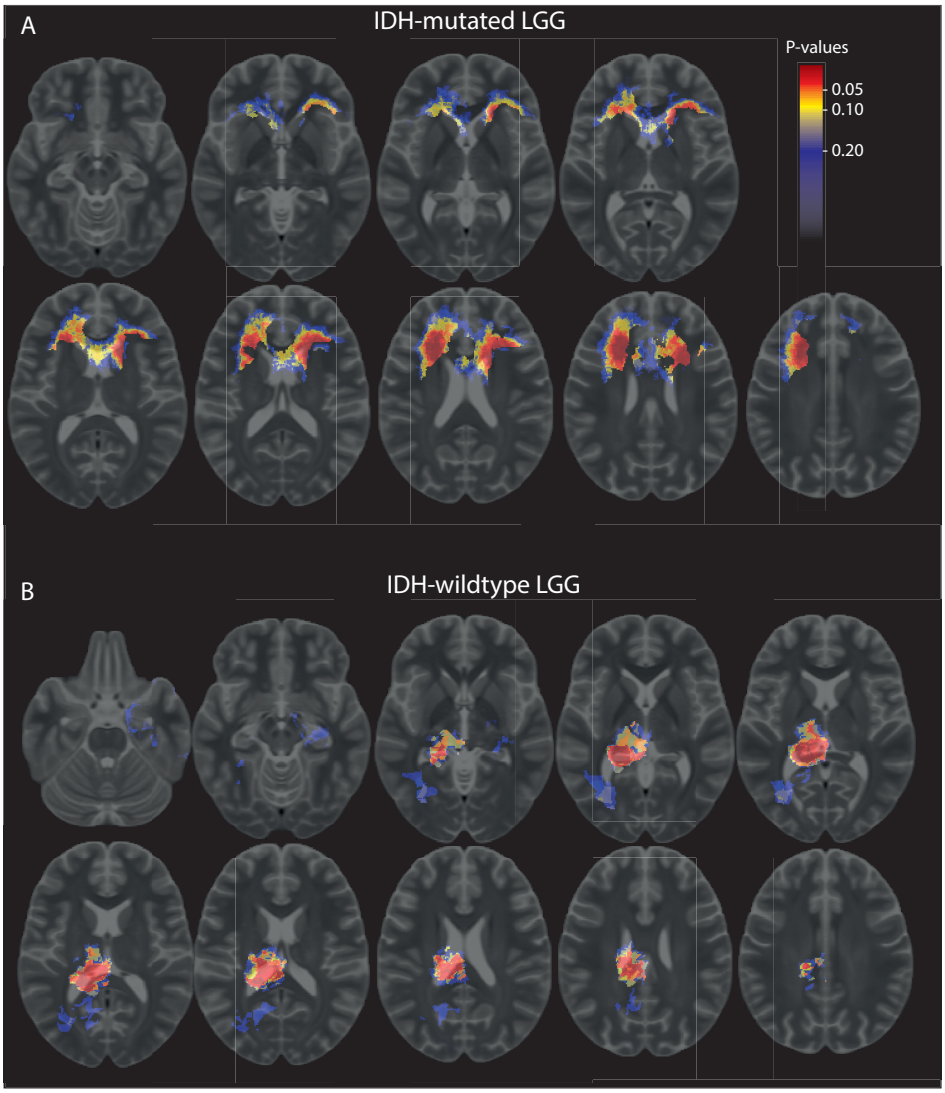


Figure 2. Differences in location distribution between *IDH* wildtype and mutated low-grade gliomas. Voxel-color indicates corrected p-value with color bar for scale; (a) regions more often occupied by *IDH* mutated low-grade gliomas; (b) regions more often occupied by *IDH* wildtype low-grade gliomas.

Exploratory analysis of location predilection of single gene and copy number variations

As an exploratory analysis, we also generated spatial distribution heatmaps of the additional genes and copy number variations we tested with our dedicated NGS panel that were frequently mutated or aberrant. For this, we analyzed *CIC* and *FUBP1* mutations, and loss of chromosomal arm 9p for oligodendroglioma (supplementary

Figure 2). We found no preferential locations for any of those molecular aberrations, except for loss of 9p (compared to oligodendroglioma with intact 9p), which seemed to be more frequently located in the left parietal area (supplementary Figure 3). In *IDH* mutated astrocytoma we created heatmaps of loss of chromosomal arm 9p and imbalance of chromosome 7, and found no preferential brain locations for either of those (supplementary Figure 4).

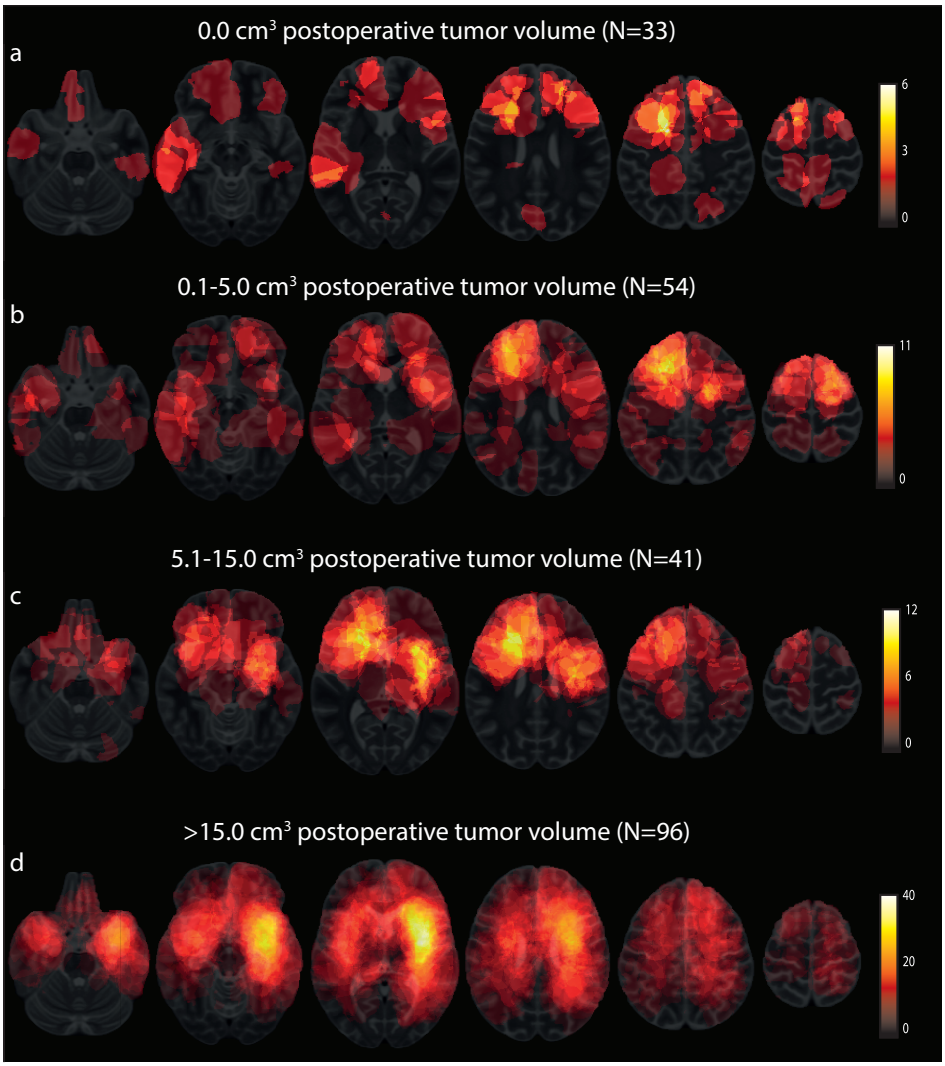


Figure 3. Spatial distribution heatmaps of WHO 2016 grade II glioma stratified according to postoperative tumor volume. The color bars on the right indicate the frequencies corresponding with the color of the voxels; (a) gliomas with a total resection (0.0 cm^3 residue); (b) gliomas with a postoperative tumor volume of 0.1 - 5.0 cm^3; (c) gliomas with a postoperative tumor volume of 5.1-15.0 cm^3; (d) gliomas with a postoperative tumor volume of more than 15.0 cm^3.

Resection probability of low grade glioma

For a previous study on the extent of resection in the same cohort, postoperative tumor volumes were also assessed with the BrainLab Elements SmartBrush Tool. We assigned patients into one of four groups based on the postoperative tumor volume: 0.0 cm^3, 0.1-5.0 cm^3, 5.1-15.0 cm^3, and > 15.0 cm^3 postoperative tumor volume. We generated location distribution heat maps stratified by these four groups, to investigate if there are preferential localizations for gross total resections. Results are shown in Figure 3. All tumors with a total resection (0.0 cm^3 postoperative residue) were located in the frontal lobe. Similarly, the majority of tumors with a low postoperative tumor volume (0.1 - 5.0 cm^3) were located in the frontal lobes. Tumors with a postoperative volume of more than 5.0 cm^3 more frequently occurred in the insular region, temporal lobes, and in or near the primary sensory and motor cortex.

DISCUSSION

In this study we aimed to visualize and compare the spatial distribution of WHO 2016 grade II glioma subgroups. By using advanced image processing analyses, we were able to generate accurate spatial distribution maps, especially compared to previous studies that were primarily based on location description/scores.[7–9,20] Our data indicate there are significant differences in spatial distribution patterns dependent on *IDH* status, with *IDH* mutated LGG's more frequently located in the rostral extensions of the lateral ventricles, and *IDH* wildtype astrocytomas more frequently in the basal ganglia of the right hemisphere. Our data are in line with earlier observations and confirm there is a correlation between molecular background of a glioma and anatomic location.[6–10] On the other hand, our data also indicate an overlap in anatomic location between WHO 2016 subgroups.

Upon visual inspection, a distinct pattern is clearly recognized between groups: most oligodendrogliomas are located in the frontal lobes and cortex, while *IDH* mutated astrocytomas are more frequently located in the frontotemporal and insular region. However, the substantial overlap between *IDH* mutated astrocytomas and oligodendrogliomas can be appreciated as well. This is also indicated by our voxel-cluster based statistical analysis, wherein we find a significant predilection for *IDH* mutated LGG's in the rostral extensions of the anterior lateral ventricles (*IDH* mutated astrocytomas and oligodendrogliomas grouped together), while we could not find regions significantly associated with either *IDH* mutated astrocytomas or oligodendrogliomas when we analyzed them as individual entities. Although oligodendrogliomas and *IDH* mutated astrocytomas differ in clinical behavior (overall survival, sensitivity to chemotherapy) and are recognized as independent entities by the WHO classification, both entities

share the *IDH* mutation. It is suggested that the cell of origin for *IDH* mutated gliomas is localized within the subventricular zone.[21] If oligodendrogliomas and *IDH* mutated astrocytomas share the cell-type of origin this might explain the significant predilection of *IDH* mutated LGG's in the rostral extensions of the anterior lateral ventricles, and the absence of a location difference between *IDH* mutated astrocytomas and oligodendrogliomas.

Compared to *IDH* mutated LGG's, *IDH* wildtype astrocytomas showed a distinct spatial distribution with more lesions located in the midline and basal ganglia. This different spatial distribution is an interesting observation, as it shows that, in the setting of grade II gliomas, *IDH* wildtype astrocytomas have a different anatomical and thus clinical presentation. The spatial distribution explains the high percentage of biopsies in these patients we reported previously, as tumors in these locations are not eligible for safe resections.[12] We also reported previously[12] that these tumors often do not present with epilepsy, in contrast to *IDH* mutated grade II gliomas, and this might also be explained by their preferential, non-cortical location. On the other hand, it has also been postulated that high frequency of epilepsy in *IDH* mutated gliomas is explained by mimicking the activity of glutamate on the NMDA receptor due to high levels of d-2-hydroxyglutarate.[22]

Upon visual inspection in this series, both oligodendrogliomas as well as *IDH* mutated astrocytomas were slightly more frequently located in the left hemisphere, especially in insular location. An explanation might be a selection bias of patient referral due to the fact that our center was one of the first in the Netherlands that performed awake craniotomies, which are performed for tumors located in presumed eloquent regions such as the left insular, frontal, and temporal region.

A current hot topic in glioma research is the development of non-invasive prediction of WHO 2016 classification of tumor diagnosis based on preoperative MR scans. This would be very helpful for pre-surgical decision making. In a previous study for example we showed that minor postoperative tumor residues have more impact on survival in *IDH* mutated astrocytomas than in oligodendrogliomas.[12] Our data shows that anatomic location could contribute to this non-invasive prediction, but requires the combination with other parameters to accurately predict WHO subtype.

We performed an exploratory analysis to assess the spatial distribution of other frequently reported mutations and copy number variations in glioma. Potential differences in spatial distribution might generate hypotheses or clues about the origin of glioma or specific subgroups and aggressiveness of certain glioma subtypes. We found no specific spatial distributions for the tested aberrations however, except for loss of chromosome 9p in the context of oligodendroglioma. Oligodendrogliomas with loss of 9p were significantly more frequently located in the left parietal area. This finding

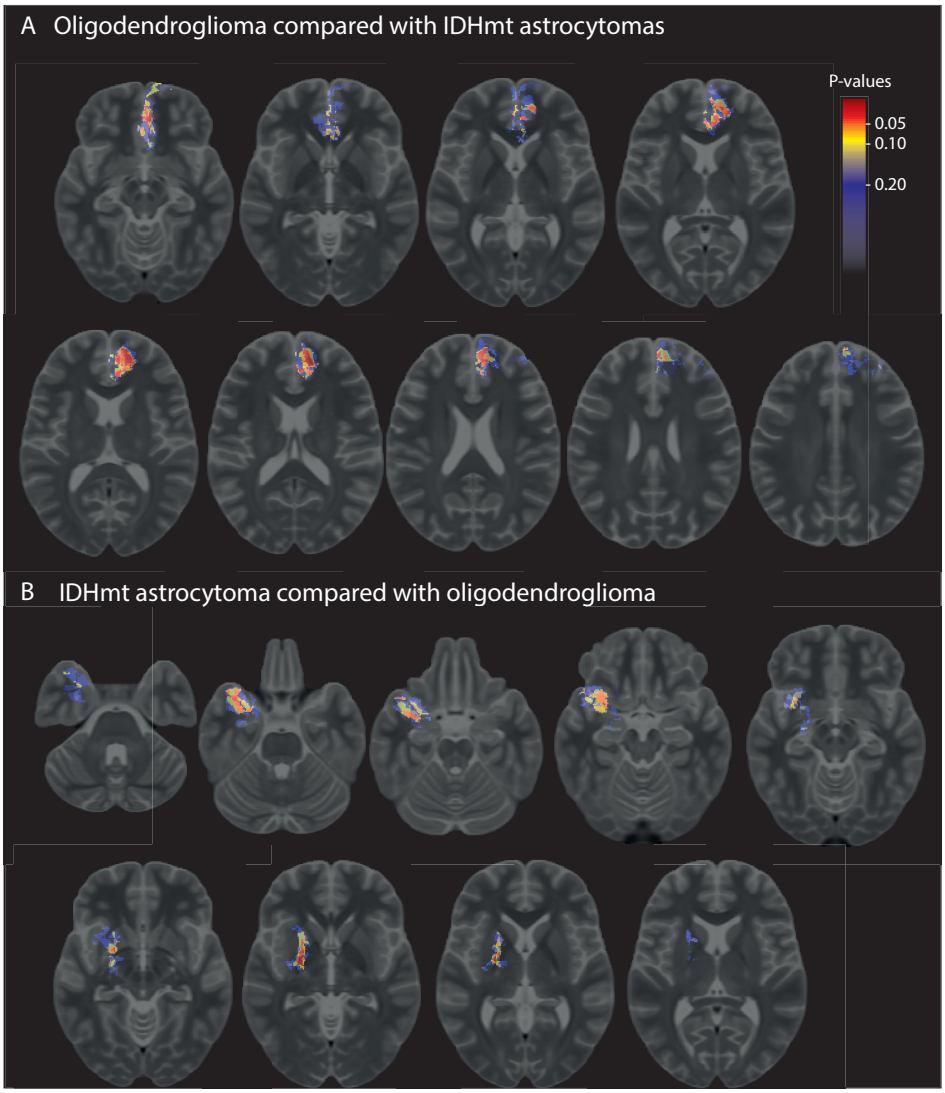
needs to be confirmed in independent series before any assumptions regarding the relevance on the biological level can be made.

As a second aim we visualized the possible correlation between anatomic location of LGG and the extent of their resection. State-of-the-art neurosurgical techniques including awake craniotomies were used in this cohort to achieve resections as extensive as possible in a safe way. Upon visual inspection we found that LGG's with no or very small postoperative tumor residues (more extensive resections) were more frequently located in the frontal lobes, while LGG's with larger postoperative tumor volumes were more frequently located in the insular and temporal regions. This is an expected result, as extent of resection is associated with anatomic location and also with proximity of eloquent areas of the brain.[12] Our heatmaps provide an insightful visualization which might be helpful in surgical planning and in informing patients on resectability of a LGG.

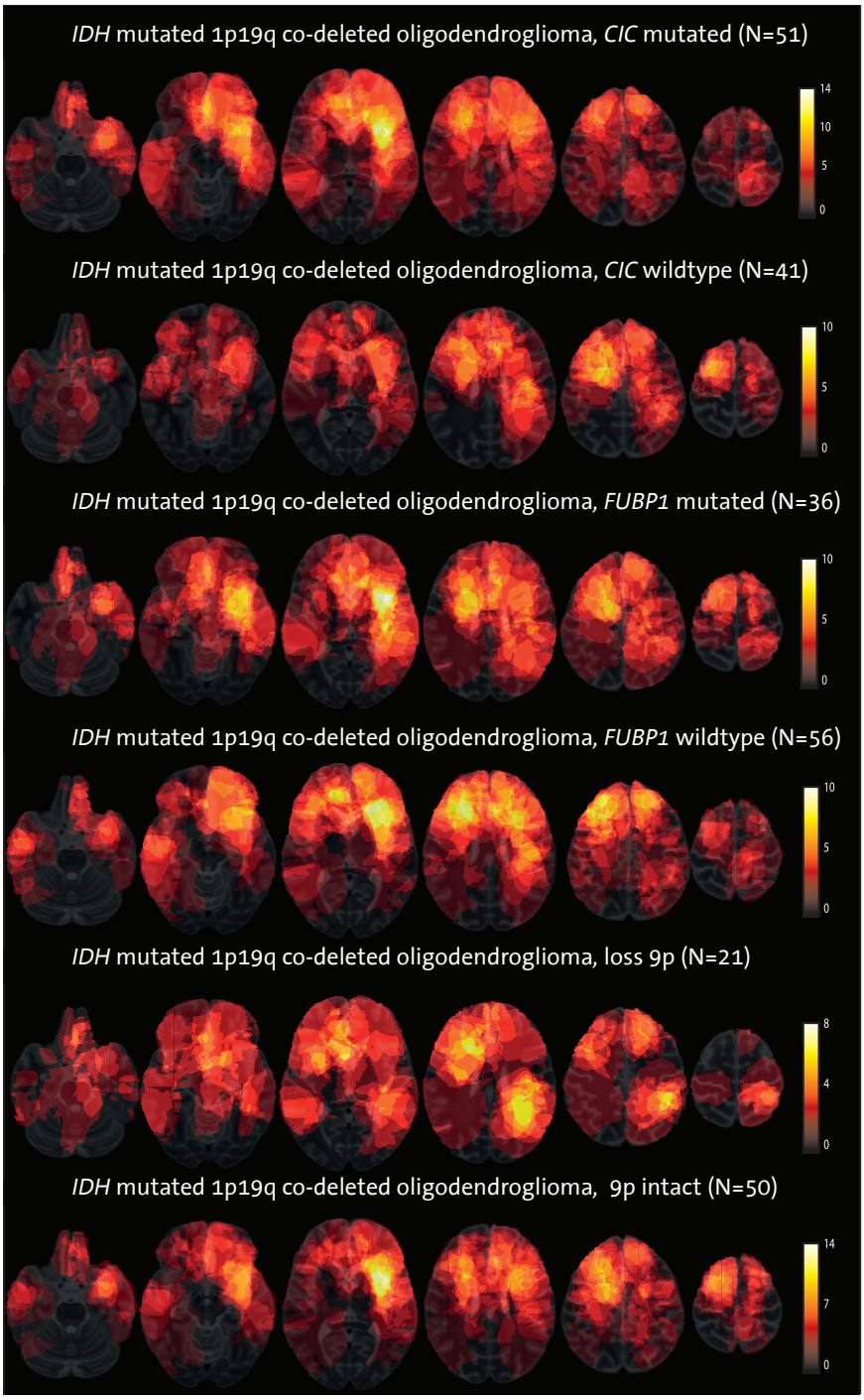
Our study has several contributions compared to previous studies. We used a relatively large and representative consecutive cohort of LGG's, which were all classified with NGS according to the integrated WHO 2016 criteria. To our knowledge this is the first study that visualizes spatial distribution of gliomas classified according to the WHO 2016 classification. Also, we scored location in a voxel-based manner with the use of semi-automated segmentation software, which gives a far more accurate representation of location compared to a manual scoring of location. Some limitations have to be addressed as well. Our study was retrospective in nature, and the MRI protocols were not standardized. Consequently, voxel size and slice thickness were not homogeneous in the cohort. Images with large voxel size and slice thickness pose challenges for accurate segmentation and registration. More subtle differences in location between groups might be missed. However, our aim was not to find subtle differences, but clinically relevant differences between groups, and our cohort with heterogeneous MR protocols is sufficient for this aim (and also reflects the 'real life' situation). More importantly, the mapping of patient MR scans with segmentations to the MNI standard brain can lead to distortion and a slight change of location on the standard brain compared to the original MR scan. This is especially relevant for relatively large lesions with mass effect, for example tumors that compress the ventricles. Because of this our heatmaps erroneously showed some tumors to be located in the ventricles. However, if a registration that perfectly mapped the tumor to its position along the ventricle (instead of mapping it inside the ventricle), the tumor would have be compressed. As such the resulting mapping would misrepresent the actual tumor volume. Therefore, we have chosen to accept the erroneous mappings into the ventricles in favor of these volume effects. To be as accurate as possible, we manually checked all registrations and corrected where necessary. Furthermore, although this is a relatively large consecutive series of molecular defined grade II glioma, a further expansion of

our dataset is needed, as especially the group of *IDH* wildtype astrocytomas is small. We need larger numbers to confirm that the spatial distribution of *IDH* wildtype LGG is indeed different compared to *IDH* mutated LGG, as it is known that glioblastomas are also frequently located in the frontal lobes. However, a recent study showed that in the context of glioblastoma, the frontal cortex is also significantly associated with the presence of *IDH* mutations.[10]

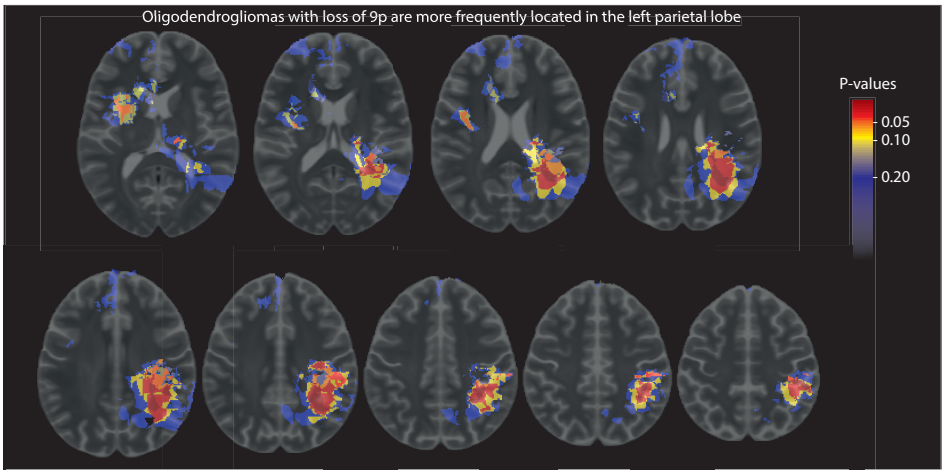
In conclusion, WHO 2016 low-grade glioma molecularly defined subgroups show both differences and similarities in spatial distribution, with *IDH* mutated LGG's significantly more frequently located in the frontal lobes and *IDH* wildtype tumors more frequently in the basal ganglia of the right hemisphere.



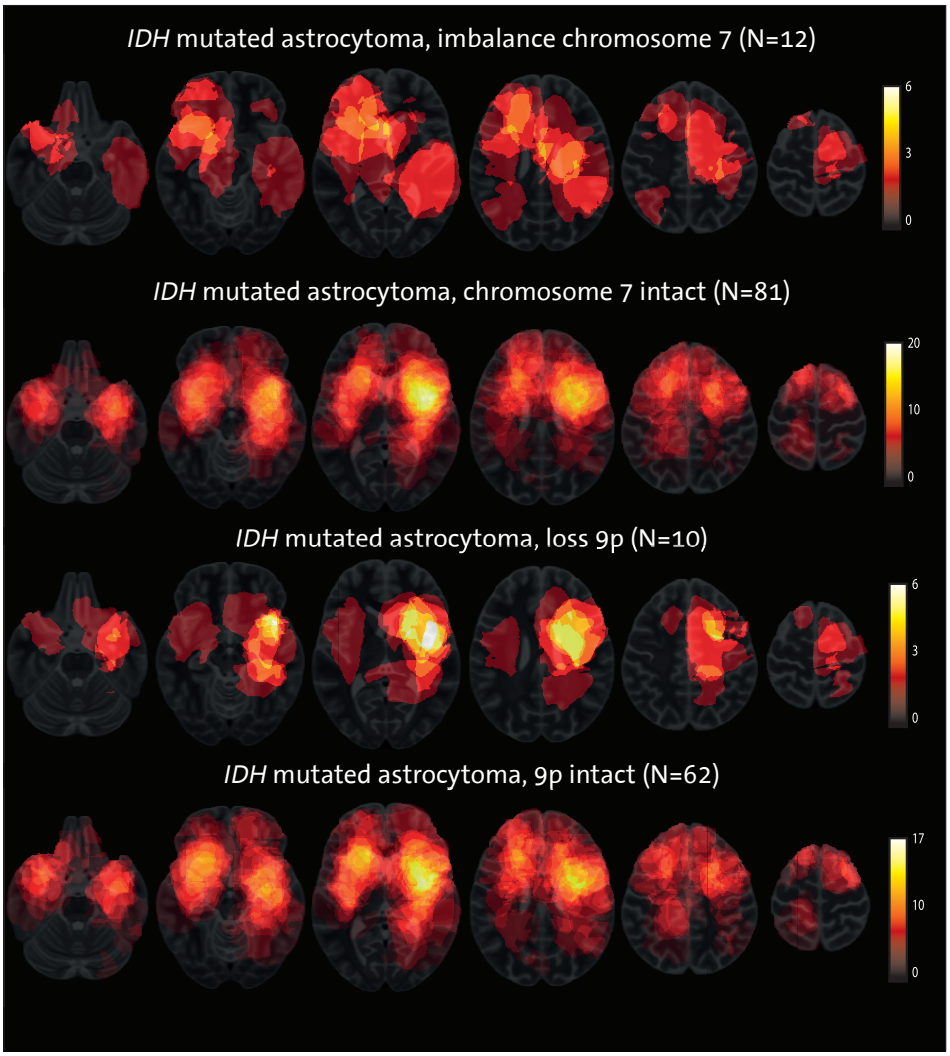
Supplementary figure 1. Results of voxelwise permutation testing with 15000 permutations for statistical differences in location distribution. The color of a voxel inidcates the p-value as indicated in the top-right color bar; (a) heatmap of significantly more frequent occuring localizations for oligodendroglioma compared with IDH mutated astrocytomas (b) heatmap of significantly more frequent occuring localizations for IDH mutated astroctyomas compared with oligodendrogliomas.



Supplementary figure 2. Spatial distribution heatmaps of presence of individual gene or copy number alterations in the context of oligodendroglioma.



Supplementary figure 3. Results of voxelwise permutation testing showing significant more frequently occuring localizations for loss of 9p in the context of oligodendroglioma, compared with 9p intact oligodendroglioma.



Supplementary figure 4. Spatial distribution heatmaps of presence of individual copy number alterations in the context of IDH mutated astrocytoma.

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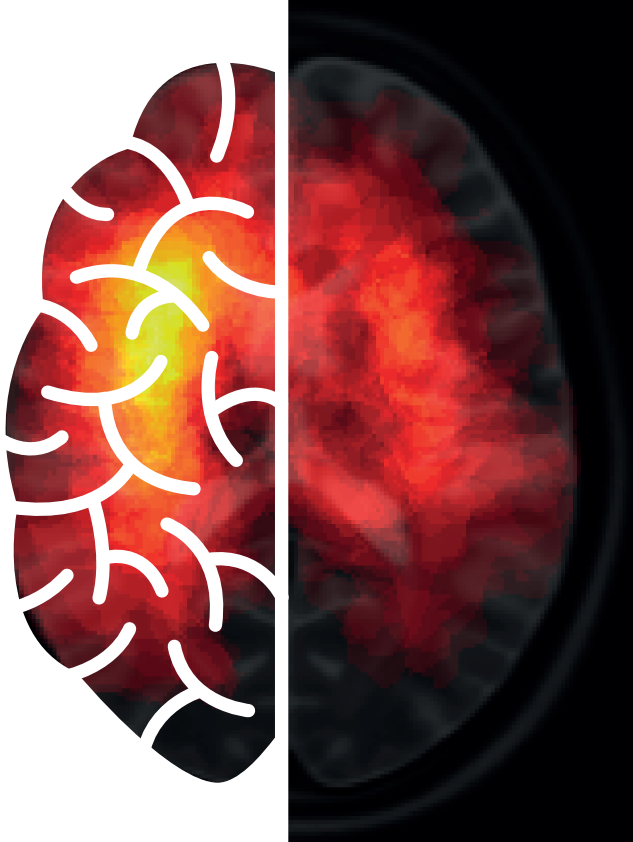

Chapter 8

General Discussion

GENERAL DISCUSSION

In 2016 the World Health Organization (WHO) classification of tumours of the central nervous system was updated.[1] This update marks a historic moment in the glioma field as testing for molecular markers became central in the diagnosis of glioma subtypes. The update altered the composition of diagnostic subtypes, which led to more accurate prognosis estimation.[2] Current treatment strategies are based on several patient factors, of which the WHO subtype is a very important one which determines the treatment intensity. Therefore, due to the changes in diagnostic groups that occurred after the WHO 2016 revision, the clinical management of especially lower-grade gliomas requires reevaluation. Also, the WHO 2016 update does not mark the end of a search for an improved classification, but marks the start of a molecular era in the neuro-oncology field wherein one of the goals is to further refine the molecular classification in order to personalize and further improve clinical management of gliomas. In this thesis we aimed to evaluate surgical management of glioma in the light of the WHO 2016 classification, and to further refine molecular diagnostics.

SURGICAL MANAGEMENT OF (PRESUMED) LOW-GRADE GLIOMAS

Despite the "low-grade" prefix, and the fact that low-grade gliomas never metastasize, it is in principle a fatal disease. Complete eradication (surgical and with systemic therapy) is barely possible due to the infiltrative growth into, and eventually destruction of, vital brain areas, as the skull is a closed and non-compliant compartment.[3, 4] The optimal surgical management of (presumed) low-grade glioma remains a matter of controversy, as surgery comes with a risk of neurological sequelae, especially in low-grade gliomas as these tumors are often located in or near eloquent areas. As no randomized trials exist related to effect of timing and extent of surgery on outcome, surgical treatment strategy varied per treatment center and was mostly depending on local opinions in the past. For a long time, the general consensus on initial treatment for low-grade glioma was a wait and scan policy. With this strategy MR scans (and in the early days CT scans), were performed at regular intervals. Hereby a lesion suspected to be a low-grade glioma was followed over time, with the intention to start active treatment once significant growth, clinical deterioration, or new contrast enhancement (taken as a sign of malignant transformation) were present. This strategy was supported by limited data from some retrospective cohort studies performed in the 1990's that did not find an association between timing of surgery and prognosis.[5-7] In the last two decades the general opinion in the field shifted and nowadays early maximal safe resection is considered standard of care in symptomatic lesions suspected

to be a low-grade glioma. There is a growing bulk of evidence that more extensive resections are associated with a longer overall survival, but the optimal timing of surgery is hardly investigated and most evidence supporting early resections remains retrospective and circumstantial.[8-13] With randomized trails considered not feasible, an influential retrospective study in Norway that was published in 2012 showed that early surgery was associated with a longer overall survival compared to a diagnostic needle biopsy followed by a wait-and-scan approach.[14] In this study the outcome of a unique situation was studied, also labeled as 'postal code randomization'. In this study treatment outcome was compared between two neurosurgical centers in two different regions in Norway with different treatment strategies. One center favored a biopsy followed by a wait-and-scan policy as initial treatment, the other favored early maximal surgical resection; patients were treated in one of these centers depending on their region of residence. The authors concluded that patients treated in the center favoring early resections had a significant longer overall survival.[14] The drawback of this study, and indeed of all studies investigating extent of resection in low grade glioma, is the patient selection that is based on histology obtained during surgery. This is not reflecting the actual clinical situation in which surgery is decided upon preoperative patient characteristics, while the histology is unknown yet. Therefore these retrospective studies are subjected so selection bias due to exclusion of patients with a suspected low-grade gliomas that turn out to be high grade gliomas, and with inclusion of patients with histologically proven low-grade glioma with preoperative enhancement on imaging that was suggestive of a high grade glioma. In our study described in **chapter 6** we tried to address this issue by approaching this clinical issue from a preoperative and more clinically relevant perspective. We retrospectively included patients with a presumed low grade glioma that was eligible for an extensive resection by selection based on imaging and preoperative clinical characteristics and not on histological diagnosis. In this manner we eliminated selection bias by histology and avoided selection bias on indication as much as possible. We investigated three different treatment strategies (wait-and scan, early resection, (needle or open) biopsy) and compared overall survival between groups. We could not confirm superiority of early resection over wait-and-scan (HR 0.92; 95% CI 0.43-2.01; P = 0.85). These data suggest that a wait-and-scan strategy can be safely proposed until some evidence of progression occurs, and that the timing of surgery does not influence the prognosis as long as the patient is monitored and treatment is initiated when necessary. An explanation for this unexpected result could be that the intrinsic biological behavior of the tumor has more impact than the timing of surgery, especially in tumors with a relatively long overall survival. The median time till intervention in the wait-and-scan group was 35.4 months, which is still relatively early in the course of the disease (median overall survival of 11.9 years in the wait-and-scan group). As is showed in **chapter 5** (of which

the results will be discussed later in this chapter), extent of resection is associated with overall survival. We did not have information on extent of resection in the study in **chapter 6**, and that is an important limitation. As low-grade gliomas grow slowly (~4mm per year), it is to be expected that total resections are more achievable when surgery is performed early in the natural course of the disease, especially in larger lesions that are not superficially located. As there is no evidence available that timing of surgery is indeed correlated with extent of resection however, it remains speculative how this might have influenced the survival curves of the cohort of **chapter 5**. There is however a plausible explanation for another observation we made: a worse outcome in the biopsy group, as in **chapter 5** we showed that the percentage of biopsies was significantly higher in patients with *IDH* wildtype low-grade gliomas compared to *IDH* mutated reflecting a difference in location of IDHwt versus IDHmt tumors (which we also show in **chapter 7**). We think it is therefore likely that the shorter overall survival observed in the biopsy group in **chapter 6** is explained by a similar higher proportion of patients with *IDH* wildtype tumors. This does not explain the poor results of the biopsy patients in the Jakola cohort. In 2017 Jakola et al. published an survival update on their 2012 cohort and now added molecular classification as well: after adjustment for molecular markers the positive effect on overall survival with early surgery persisted.[15]

In **chapter 5** we investigated the association between extent of resection and overall survival in low-grade glioma in the light of the WHO 2016 classification. We were the first to report on a relatively large series of molecularly classified low-grade glioma patients that were investigated for extent of resection with a quantitative measure. We could validate current standard of care, as we showed that postoperative tumor volume was inversely correlated with outcome irrespective of molecular subtype. The effect of even small postoperative tumor volumes was particularly strong in *IDH* mutated astrocytomas, as any residual tumor >0 cm^3 already negatively impacted overall survival. This finding argues for second-look surgeries when this is safely possible, when a small residue remains in this subtype. Similar findings were reported for anaplastic glioma, although not in a volumetric manner as in our study. Kawaguchi et al. showed that gross total resection (GTR) was associated with longer overall survival when lumping together all 3 molecular subtypes of anaplastic glioma. However, when looking at molecular subtypes individually, GTR had a positive significant impact on overall survival only in *IDH* mutated astrocytoma, and not in *IDH* wildtype astrocytoma and oligodendroglioma.[16] Another study in 2016 by Wahl et al. reported on clinical outcomes by molecular subtype of a phase II study of adjuvant chemotherapy for low-grade glioma. Although it was not the primary objective of the study, in an exploratory analysis it was found that postoperative tumor volumes were associated with outcome irrespective of molecular subtype.[17]

How to interpret the data form these studies and translate this into treatment guidelines for glioma patients? At first glance the results in **chapter 5 and 6** seem conflicting. However, we have to realize both cohorts are differently selected and try to answer different questions: first, when to resect and second, if one decided to resect how much should be resected. Uniform treatment recommendations that apply to all patients are impossible to give, but one thing is clear: a biopsy should not be part of standard care if a resection is possible. It should only be used in patients wherein a resection is not possible, but with a need for active treatment, to establish an accurate diagnosis. With **chapter 5** we provided the evidence that also for molecularly defined low-grade glioma, lower postoperative tumor volumes are associated with longer overall survival. Therefore a resection should be as extensive as safely possible in all newly diagnosed lower grade glioma patients. That introduces another important element; the question when a lesion is considered eligible for a meaningful extensive resection that is also safely possible (thus with low risk of complications). Resectability is dependent on tumor size, delineation, location, eloquency, and patient condition. Although a total resection is the ultimate aim, this is not possible in most patients, leaving the question what cut-offs for a minimal extent of resection is meaningful in terms of survival benefit. Although the results obtained in **chapter 6** do not show a survival benefit from early surgery, all other presently available data suggests that when a safe and extensive resection is possible, it should be performed early in the course of the disease, once the radiological diagnosis of a presumably low grade glioma is established. We also think this is in patients' best interest as a resection in a later stage will be technically more challenging and may be associated with a less extensive resection. However, when a meaningful resection is considered not possible and a patient has no symptoms other than well controlled seizures, a wait-and-scan period ('active surveillance') can be considered until treatment is necessary. Ideally, in the future we would have prospective data generated from a randomized trial that provides us unbiased evidence on optimal timing of surgery, as well as on the impact of extent of resection, and if so, the minimum extent of resection to aim for. However, it is unlikely that a trial like this will ever be performed and early surgery is now in general accepted as standard of care, despite the lack of prospective data.

MOLECULAR TESTING FOR GLIOMAS

Even though prognosis prediction for patients with glioma improved significantly with the WHO 2016 update, there is still variation in outcome within specific subtypes, and therefore further refinement of this classification would be valuable. Historically, the grade of glioma used to be a strong prognostic marker and grading is still part of the

WHO criteria. Grading is however purely based on microscopic features examined on hematoxylin and eosin stained slides, and currently no molecular markers are available to discriminate outcome within grade II (low-grade) and grade III (anaplastic) gliomas. In **chapter 2** we analyzed the publically available *TCGA* dataset (*The Cancer Genome Atlas*) and found that also for molecular glioma subtypes the histological tumor grade was inversely correlated with patient overall survival. We could not find in this dataset specific single molecular markers that were associated with tumor grade, but we did found that tumor grade was correlated with the mutational load (total number of genetic changes within one sample). This is an interesting observation, as it may partially explain the increased aggressiveness of gliomas with higher tumor grades. However, in the WHO 2016 classification the impact of tumor grade on prognosis seems to be more subtle and not as distinct as in the WHO 2007 classification. Reuss et al performed a study wherein they combined multiple datasets of *IDH* mutated astrocytoma (including the *TCGA* dataset) and found that both low-grade and anaplastic *IDH* mutated astrocytoma present at a similar age and that the difference in overall survival between grade II and III IDHmt astrocytoma is minimal. Partially this may be explained because *IDH* wildtype astrocytomas are now a separate entity and the differences between grades in the WHO 2007 classification were probably predominantly dependent on this subgroup.[18] In **chapter 2** we also showed that within *IDH* mutated astrocytomas, mutations in *PI3* kinase genes *PIK3CA* and *PIK3R1*, are associated with poorer prognosis, and we could confirm this finding in two independent datasets. The prevalence of these mutations in low-grade glioma patients is low however, and does not explain all variation in prognosis within subgroups. Several large efforts have aimed to identify molecular markers that correlate with tumor grade and could replace the current histological grading system. Most studies in the past focused on anaplastic glioma, with different studies showing conflicting results. For example loss of entire chromosomal arm 9p or loss of 9p21.3 region was reported as a marker of poor prognosis, mainly in grade III and grade IV glioma, but results are conflicting.[19-22] It is also unclear which region should be tested for: the entire 9p arm, the 9p21.3 region or the CDKN2A gene only. In **chapter 3** we used a targeted next generation sequencing panel to evaluate the prognostic relevance of frequently reported prognostic glioma markers in a consecutive treated series of grade II glioma. We analyzed 207 *IDH* mutated glioma samples and investigated the impact of loss of 9p21.3 and entire 9p on outcome in both oligodendroglioma and *IDH* mutated astrocytoma. In both groups of our cohort loss of 9p21.3 of entire 9p was not associated with overall survival, although a trend towards shorter overall survival was visible in grade II oligodendrogliomas. Therefore, longer follow-up and expansion of our dataset is necessary for more definitive final conclusions. We did not find homozygous deletions of CDKN2A/B in our dataset, which is in line with a recently published cohort

of grade II,III, and IV *IDH* mutated 1p19q intact tumors. The authors describing that cohort found that CDKN2A/B homozygous deletions and total number of copy number variations were strong predictors of worse outcome in a cohort of grade II,III, and IV *IDH* mutated 1p19q intact tumors. In line with our grade II cohort, they did not find CDKN2A/B homozygous deletions in WHO 2016 grade II gliomas.[23]

In **chapter 4** we showed that *IDH* wildtype low-grade gliomas, in contrast to the current WHO 2016 classification, are not a single entity, but in fact a clinical and molecular heterogeneous group of tumors. We found that *IDH* wildtype gliomas with trisomy of chromosome 7, loss of chromosome 10 (+7/-10q) and *TERTp* mutations have a dismal prognosis almost similar to glioblastoma. We also identified a group of *IDH* wildtype tumors with only a *TERTp* mutation without presence of +7/-10q. These patients even had a worse prognosis than the patients with +7/-10q. More importantly, we found *IDH* wildtype glioma patients without these molecular aberrations, and observed they had a significant better outcome. Our results were confirmed in independent studies.[24-26] This implies that more extensive molecular testing should be performed in *IDH* wildtype gliomas, in order to accurately estimate prognosis and guide treatment decisions. At least assessment of *TERT* promoter status or +7/-10q status is necessary on case of *IDH1/2* wildtype low-grade or anaplastic glioma. When these markers are absent, then further testing for at least *BRAF* and *H3F3A* mutations is necessary. Ideally, however, all makers are included in a single panel for routine diagnostics, to avoid diagnostic delay.

In **chapter 7** we aimed to correlate WHO 2016 molecular subtypes with anatomic location of grade II glioma. Several studies reported a correlation between anatomic location of gliomas and the genetic background of the tumor. [27-29]As such, tumor location might contribute to pre-surgical decision-making and non-invasive prediction of molecular diagnosis, which would be particularly helpful for lesions wherein there is doubt if it is actually a glioma or in patients wherein surgery has too high risk of morbidities but where systemic treatment is eligible. We reported on a large series of WHO 2016 classified tumors of which we created anatomic location heatmaps. Despite relevant overlap between molecular subgroups, we still found unique locations for *IDH* mutated grade II gliomas in the anterior extensions of the lateral ventricles. Interestingly, *IDH* wildtype astrocytomas were predominantly located in the midline and basal ganglia. This explains the relatively high percentage of biopsies in this group (**chapter 5**). Our data shows that location is a potentially important parameter for radiogenomics, but also emphasizes that other parameters are necessary to accurately predict molecular subtype, as there is significant overlap between groups.

In conclusion, we provide several insights that further refine the WHO classification. We suggest testing for mutations in PI3K genes to be added to clinical routine diagnostics, and to further stratify *IDH* mutated astrocytoma. Furthermore, stratifica-

tion of *IDH* wildtype gliomas is essential, as this group of tumors turns out to be very heterogeneous molecularly, and even more important, clinically (strong differences in overall survival), which has direct implications for clinical management. In **Figure 1** we summarize our findings and propose an updated diagnostic scheme. Still, not all variation in clinical outcome is explained by this scheme. To unravel this we need even larger glioma datasets than in *TCGA*, including matched primary and recurrent tumor samples to find markers of progression and of poor outcome. Just as important as sample size, or maybe even more important, these datasets need a more detailed and accurate clinical annotation than is provided in *TCGA*. Including MR imaging with standardized protocols is also important, as this might provide us with imaging correlates of molecular markers and/or prognostic subgroups. This can only be achieved with large international collaborations, as glioma is a rare disease.

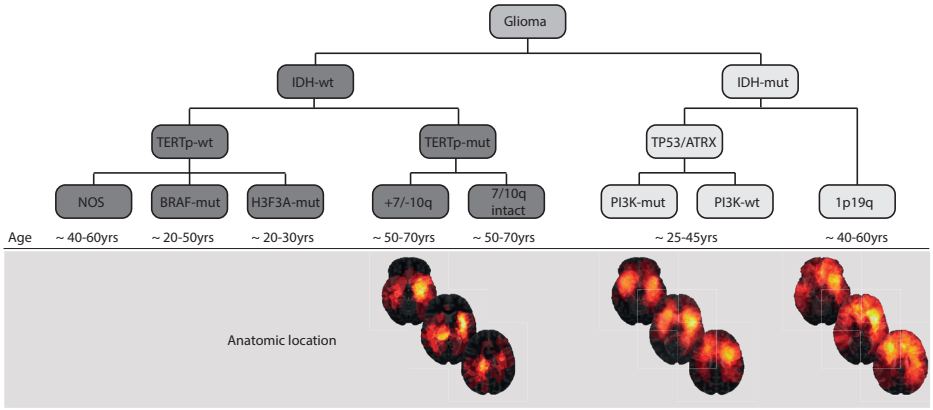

Figure 1. This figure forms a summary of our findings and proposes an updated classification scheme.

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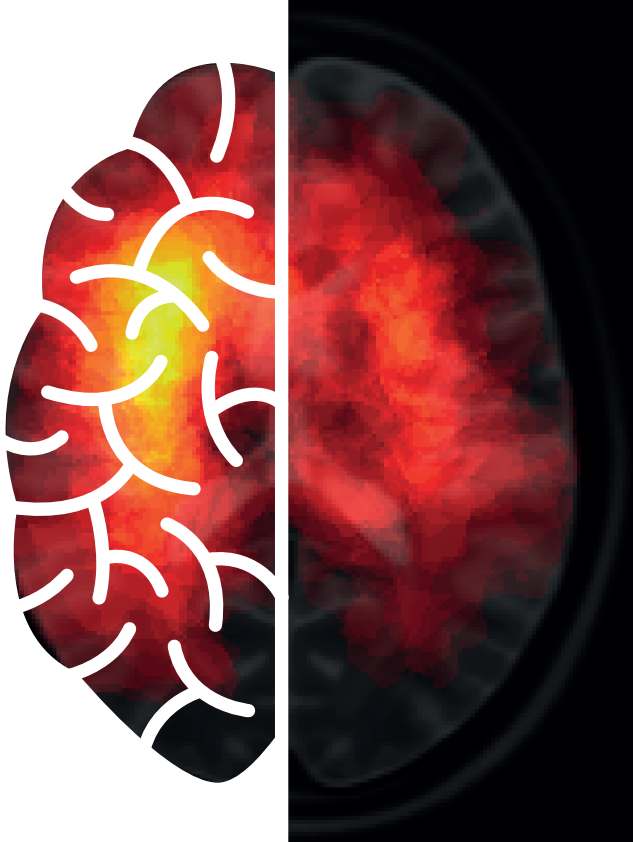


Chapter 9

Summary & Samenvatting

SUMMARY

Gliomas are the most common type of primary malignant brain tumors. Several subtypes of glioma are recognized by the World Health Organization (WHO), based on histological and molecular criteria. The WHO classification of brain tumors was updated in 2016 and in this thesis the results of several studies on classification and clinical management of lower-grade glioma in the context of these updated WHO criteria are described. In **chapter 1**, the general introduction, we provide an overview of the WHO 2016 criteria and current clinical management of low-grade glioma.

In **chapter 2** we analyzed the publically available whole exome sequencing data of the *Cancer Genome Atlas* (TCGA) of both low and high grade glioma, to find additional markers of prognosis within WHO 2016 recognized glioma subgroups. We investigated all genes that were mutated at a population frequency of more than 1.7% and frequent chromosomal imbalances. In the entire dataset 57 genes were significantly correlated with overall survival. *IDH1/2* mutations showed the lowest hazard ratio, confirming *IDH1/2* is the most important prognostic marker in diffuse gliomas. With subsequent subgroup analysis, we found that within *IDH* mutated astrocytomas, mutations in *PI3* kinase genes *PIK3CA* and *PIK3R1*, are associated with poorer prognosis (median OS 3.7 vs. 6.3 years; P=0.02; HR 2.93; 95% CI 1.16-7.38). We also demonstrated that tumor grade remains an independent prognostic factor within molecular subgroups and found that tumor grade is correlated with mutational load.

In **chapter 3** we report on a study wherein we assessed the prognostic relevance of additional mutations and copy number alterations in a consecutive series of *IDH* mutated grade II glioma. In univariate analysis we found a significant shorter overall survival in *IDH* mutated astrocytoma patients with a trisomy of chromosome 7, and in oligodendroglioma patients with a *PTEN* mutation, but could not confirm these findings in multivariate analysis. We could also not confirm the impact of loss of 9p21.3 on overall survival, which is frequently reported as progression marker in high grade glioma. However, we need lengthy follow-up for definitive conclusions.

In **chapter 4** we report on a relatively large group of *IDH*-wildtype low-grade and anaplastic gliomas, which are recognized as a single entity and do not require further stratification according to the current WHO classification. We showed that this is actually a molecular and clinical heterogeneous group of tumors, which requires additional molecular testing beyond *IDH1/2*, in order to accurately estimate prognosis and guide treatment. Only 52.7% of patients showed a phenotype with trisomy of chromosome 7 and loss of 10q, which is a molecular characteristic of glioblastoma. Except one patient, all of these also had a *TERT* promoter mutation. 18.9% of patients had only a *TERT* promoter mutation, and had even a poorer prognosis than patients with the classical molecular characteristics of glioblastoma. More importantly, approximately

20% of patients did not have any of the investigated molecular aberrations, and had a significant better outcome.

In **chapter 5** we describe a study wherein we assessed the impact of extent of surgery on outcome in molecularly defined low grade glioma. As the WHO classification of gliomas has been completely revised and is now predominantly based on molecular criteria, the impact of extent of resection needed to be re-evaluated in molecularly defined low grade glioma. We included 228 adult patients who underwent surgery for a supratentorial low-grade glioma and semi-automatically assessed pre- and post-operative tumor volumes. In multivariable analysis, postoperative tumor volume was inversely correlated with overall survival (HR 1.01 per cm^3 increase in volume; 95% CI 1.002-1.02; *P* = 0.016). Therefore, our data supports maximal resection as first-line treatment for low-grade glioma. The impact of even small tumor residues was particularly strong in *IDH* mutated astrocytomas, where even small postoperative volumes already negatively affect overall survival.

In **chapter 6**, we focused on the timing of surgery and the impact on outcome in presumed low-grade glioma, but with a set-up wherein we tried to indication and selection bias that was present in previous studies. We retrospectively identified a cohort of patients in good clinical condition with a presumed low-grade glioma that was eligible for an extensive resection. We compared outcome between three types of treatment strategies: a wait-and-scan strategy, a biopsy, or early resection. We observed no difference in overall survival for early resection compared to wait-and-scan (HR 0.92; 95% CI 0.43-2.01; p = 0.85). Biopsy as initial treatment strategy was associated with a significant shorter overall survival compared to wait-and-scan (HR 2.69; 95% CI 1.19-6.06; P = 0.02).

In **chapter 7**, we provide insight in the location distribution of specific WHO molecular subgroups of glioma in the human brain. We created heatmaps of anatomic location per WHO molecular subgroup of the patients described in **chapter 5.** We showed there are both differences and similarities in spatial distribution between WHO subgroups. We found a significant predilection for *IDH* mutated low-grade gliomas in the rostral extensions of the anterior lateral ventricles and for *IDH* wildtype astrocytomas in het basal ganglia of the right hemisphere.

Finally, **chapter 8** discusses the main findings of **chapters 2 to 7** and put these results in perspective with recent literature.

SAMENVATTING

Gliomen zijn de meest voorkomende vorm van primaire kwaadaardige hersentumoren. Primair wil zeggen dat deze tumoren ontstaan in het hersenweefsel zelf, een wezenlijk verschil met tumoren die elders in het lichaam ontstaan en soms uitzaaien naar de hersenen. Gliomen ontstaan uit het steunweefsel dat alom aanwezig is in de hersenen, de zogenaamde gliale cellen. Glioom is een overkoepelende term voor de verscheidenheid aan verschillende subtypen die worden onderscheiden door de World Health Organization (WHO) in de WHO classificatie van hersentumoren. De WHO classificatie wordt wereldwijd toegepast voor de indeling van gliomen in verschillende subtypen en deze vormt een leidraad bij het instellen van de juiste behandeling en het informeren van de patiënt over de prognose. De basis van deze classificering was oorspronkelijk de beoordeling van enkele gedefinieerde kenmerken van het tumorweefsel (histologie) onder de microscoop door de patholoog. De afgelopen jaren is er echter steeds meer bekend geworden over de onderliggende DNA mutaties en chromosomale afwijkingen in de tumor, en het is gebleken dat gliomen nauwkeuriger zijn in te delen in subtypen op basis van deze moleculaire kenmerken. De WHO classificatie van hersentumoren werd daarom gereviseerd in 2016, en de subtypering van gliomen vind nu naast alleen histologische criteria, ook plaats op basis van moleculaire criteria. Bij discrepanties tussen de histologische en de moleculaire kenmerken binnen een tumor zijn de moleculaire kenmerken leidend voor het bepalen van het type glioom. De nieuwe criteria hebben in de praktijk geleid tot een verschuiving van diagnostische groepen en dit heeft consequenties voor de dagelijkse praktijk, gezien behandeling en prognose gekoppeld zijn aan deze classificatie. Het is derhalve noodzakelijk om enkele aspecten van bijvoorbeeld de behandeling te her-evalueren in het kader van de nieuwe classificatie. In dit proefschrift worden de resultaten van verschillende studies beschreven naar de prognosestelling en chirurgische behandeling van laaggradige gliomen in de context van deze gereviseerde classificatie. In **hoofdstuk 1**, de algemene inleiding, wordt een overzicht gegeven van de WHO criteria en de huidige behandeling van laaggradige gliomen.

Hoofdstuk 2 beschrijft een analyse van *The Cancer Genome Atlas*: een openbare database met genetische en klinische gegevens van een groot cohort van laag- en hooggradige gliomen. De doelstelling van deze studie was het identificeren van additionele prognostische moleculaire markers binnen de door de WHO 2016 erkende glioom subtypen. We onderzochten frequent voorkomende chromosomale afwijkingen en alle genen met een mutatiefrequentie in de populatie van meer dan 1.7%. In totaal waren 57 genen significant gecorreleerd met overleving. Mutaties in het *IDH1/2* gen waren geassocieerd met de laagste hazard ratio (HR) (relatieve kans op overlijden bij een mutatie in het betreffende gen, ten opzichte van geen mutatie), wat bevestigt dat

een mutatie in het *IDH1/2* gen de belangrijkste prognostische marker is bij diffuse gliomen. Middels een subgroep analyse toonden we aan dat mutaties in *PI3* kinase genen *PIK3CA* en *PIK3R1* bij *IDH* gemuteerde astrocytomen geassocieerd zijn met een slechtere prognose (mediane overleving 3.7 vs. 6.3 jaar; P = 0,02; HR 2,93; 95% CI 1.16-7.38). We toonden ook aan dat tumorgraad (een score van 1-4 die de mate van agressiviteit aangeeft) nog steeds een onafhankelijke prognostische factor is binnen de gedefinieerde moleculaire subgroepen, en dat tumorgraad gecorreleerd is met het aantal DNA mutaties in de tumor.

In **hoofdstuk 3** onderzochten we de prognostische waarde van frequent voorkomende DNA mutaties en chromosomale afwijkingen in een grote groep van *IDH* gemuteerde graad II gliomen. Trisomie van chromosoom 7 in *IDH* gemuteerde astrocytomen en een mutatie in het *PTEN* gen in oligodendrogliomen waren geassocieerd met een slechtere prognose. We konden dit echter niet bevestigen in een analyse gecorrigeerd voor andere prognostische factoren. Ook vonden we geen impact op de prognose van verlies van een deel van chromosoom 9, 9p21.3, wat regelmatig wordt gerapporteerd als marker van progressie in hooggradige gliomen. Langere follow-up is echter nodig voor definitieve conclusies.

In **hoofdstuk 4** beschrijven we een grote groep van *IDH*- wildtype (geen mutatie in het *IDH* gen) laaggradige en anaplastische gliomen en toonden aan dat deze groep tumoren zowel moleculair als klinisch zeer heterogeen is. Slechts 52,7% van de patiënten bleek namelijk een fenotype met trisomie van chromosoom 7 en verlies van chromosom 10q te hebben, wat een moleculair kenmerk is van een glioblastoom (de meest aggresieve vorm van gliomen). Behoudens één patiënt hadden al deze patienten ook een *TERT* promoter mutatie. 18,9% van de patiënten had alleen een *TERT* promoter mutatie en had zelfs een slechtere prognose dan patiënten met de klassieke moleculaire kenmerken van een glioblastoom (trisomie chromosoom 7 en verlies 10q) . Een belangrijke bevinding was dat ongeveer 20% van de patiënten geen van de onderzochte mutaties of chromosomale afwijkingen had, en dit was geassocieerd met een aanzienlijk langere overleving. Uit deze gegevens blijkt dat aanvullende moleculaire diagnostiek naast onderzoek naar het *IDH1/2* gen noodzakelijk is voor een adequate inschatting van prognose en starten van een juiste behandeling.

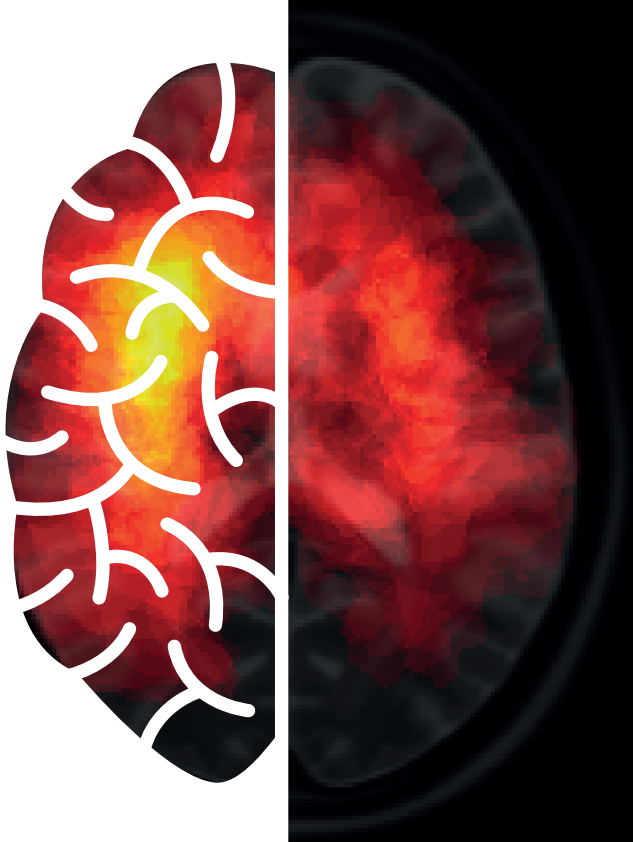
In **hoofdstuk 5** onderzochten we de mogelijke associatie tussen de uitgebreidheid van de operatieve resectie en de prognose bij laaggradige gliomen geclassificeerd volgens de WHO 2016 classificatie. De revisie van de WHO classificatie in 2016 maakte het noodzakelijk om de rol van chirurgie opnieuw te evalueren. We includeerden 228 volwassen patiënten die een operatie hadden ondergaan voor een supratentorieel gelokaliseerd laaggradig glioom. We onderzochten de uitgebreidheid van de resectie door het pre- en postoperatief tumorvolume semi-automatisch te bepalen. In een multivariabele analyse was het postoperatieve tumorvolume omgekeerd evenredig

gecorreleerd met totale overleving (HR 1.01 per cm^3 toename van het postoperatief volume; 95% CI 1.002-1.02; *P* = 0.016). Deze resultaten ondersteunen een maximale resectie als eerstelijnsbehandeling voor laaggradige gliomen. Het effect van postoperatieve tumorrest was met name sterkt in *IDH* gemuteerde astrocytomen, waarin zelfs zeer kleine postoperatieve tumorvolumes al een negatief effect hadden op de overleving.

Hoofdstuk 6 richt zich op de timing van de operatie en het effect op de overleving bij tumoren die op beeldvorming verdacht zijn voor een laaggradig glioom. Middels een unieke studieopzet hebben we geprobeerd de indicatie- en selectiebias te voorkomen die aanwezig is in eerdere studies naar de timing van chirurgie. In een retrospectief cohort van patiënten in goede klinische conditie met een vermoedelijk laaggradig glioom op beeldvorming, en die in aanmerking kwamen voor een uitgebreide operatie, vergeleken we de resultaten tussen drie soorten behandelstrategieën: de zogenaamde wait-and-see benadering, een biopsie of een vroege resectie. We vonden geen verschil in de totale overleving voor vroege resectie in vergelijking met de wait-and-see benadering (HR 0.92; 95% CI 0,43-2.01; p = 0.85). Biopsie als eerste behandelstrategie was geassocieerd met een aanzienlijk kortere totale overleving in vergelijking met wait-and-see (HR 2,69; 95% CI 1.19-6.06; P = 0,02).

In **hoofdstuk 7** geven we inzicht in de anatomische locatie van de door de WHO 2016 erkende subtypen van laaggradige gliomen. Dit door middel van het genereren van heatmaps van de anatomische locatie per WHO 2016 subgroep van de patiënten beschreven in **hoofdstuk 5.** We toonden dat elk subtype een verschillend patroon van anatomische verdeling heeft, maar ook dat er overlap bestaat tussen subtypen. *IDH* gemuteerde laaggradige gliomen komen vaker voor anterieur van de laterale hersenkamers, en *IDH* wildtype laaggradige gliomen zijn significant vaker gelokaliseerd in de basale kernen van de rechter hemisfeer.

Ten slotte bespreken we de belangrijkste bevindingen van **hoofdstukken 2 tot en met 7** en plaatsen deze in perspectief met recente literatuur in **hoofdstuk 8.**



Appendix

LIST OF PUBLICATIONS

Wijnenga MMJ, SR van der Voort, PJ French. S Klein. HJ Dubbink, WNM Dinjens, PN Atmodimedjo, M de Groot, JM Kros, CMF Dirven, AJPE Vincent, M Smits, MJ van den Bent.
Differences in spatial distribution between WHO 2016 low-grade glioma molecular subgroups
Submitted

Wijnenga MMJ, French PJ, Dubbink HJ, Dinjens WNM, Atmodimedjo PN, Kros JM, Fleischeuer R, Dirven CMF, Vincent AJPE, van den Bent MJ. *J Neurooncol. 2018 Apr 16*.
Prognostic relevance of mutations and copy number alterations assessed with targeted next generation sequencing in IDH mutant grade II glioma
Journal of Neuro-Oncology, 2018

Broen MPG, Smits M, **Wijnenga MMJ**, Dubbink HJ, Anten MHME, Schijns OEMG, Beckervordersandforth J, Postma AA, van den Bent MJ.
The T2-FLAIR Mismatch Sign as an Imaging Marker for Non-Enhancing IDH-mutant, 1p/19q-intact Lower Grade Glioma: A Validation Study
Neuro Oncology, 2018

Wijnenga MMJ, French PJ, Dubbink HJ, Dinjens WNM, Atmodimedjo PN, Kros JM, Smits M, Gahrmann R, Rutten GJ, Verheul JB, Fleischeuer R, Dirven CMF, Vincent AJPE, van den Bent MJ.
The impact of surgery in molecularly defined low-grade glioma: an integrated clinical, radiological, and molecular analysis
Neuro Oncology, 2018

Wijnenga MMJ, Dubbink HJ, French PJ, Synhaeve NE, Dinjens WNM, Atmodimedjo PN, Kros JM, Dirven CMF, Vincent AJPE, van den Bent MJ.
Molecular and clinical heterogeneity of adult diffuse low-grade IDH wild-type gliomas: assessment of TERT promoter mutation and chromosome 7 and 10 copy number status allows superior prognostic stratification
Acta Neuropathologica, 2017

Wijnenga MMJ, Mattni T, French PJ, Rutten GJ, Leenstra S, Kloet F, Taphoorn MJB, van den Bent MJ, Dirven CMF, van Veelen ML, Vincent AJPE.
Does early resection of presumed low-grade glioma improve survival? A clinical perspective
Journal of Neuro-Oncology, 2017

Draaisma K, **Wijnenga MMJ**, Weenink B, Gao Y, Smid M, Robe P, van den Bent MJ, French PJ.

PI3 kinase mutations and mutational load as poor prognostic markers in diffuse glioma patients

Acta Neuropathol Communications, 2015

PHD PORTFOLIO

Name PhD student: M.M.J. Wijnenga Erasmus MC Department: Neuro-oncology Research School: Molecular Medicine	PhD period: Feb 2015 –Jan 2019 Promotors: M.J.van den Bent, C Dirven Copromotors: PJ French, A Vincent	
	Year	Workload (ECTS)
Courses		
- Course on basic & translational oncology	2015	1.8 ECTS
- Workshop on microsoft excel 2010: basic & advanced	2015	0.7 ECTS
- Workshop on Microsoft access 2010: basic & advanced	2015	0.7 ECTS
- Course on R	2015	1.4 ECTS
- NGS applications in molecular medicine	2015	0.7 ECTS
- NIHES biostatistical course CC02	2015	5.7 ECTS
- Molmed survival analysis course	2016	0.7 ECTS
- Galaxy for NGS	2016	0.7 ECTS
- Photoshop and illustrator	2017	0.3 ECTS
- Scientific writing	2017	2.0 ECTS
- Research Integrity	2016	0.3 ECTS
Seminars and workshops		
- Landelijke Werkgroep Neuro-Oncologie investigators bijeenkomst (2x)	2015 & 2016	0.5 ECTS
- LWNO wetenschappelijke vergadering (4x)	2015 - 2018	1.2 ECTS
- Neuro-Oncology Retreat (2x)	2015 & 2018	1.5 ECTS
Oral presentations		
- Annual Molmed day elevator pitch	2016, 2018	1 ECTS
- LWNO wetenschappelijke vergadering (3x)	2016-2018	1.5 ECTS
- EANO 2016 Mannheim	2016	0.5 ECTS
- Neuro-Oncology meetings Erasmus MC (1x/year)	2015-2019	1.5 ECTS
- JNI scientific research meetings (2x)	2016,2017	0.5 ECTS
- SNO 2017 San Francisco	2017	0.5 ECTS
- SNO 2018 New Orleans	2018	0.5 ECTS
(Inter)national conferences		
- Annual Molmed Day (5x)	2015-2019	1.5 ECTS
- EORTC-EANO-ESMO Conference 2015 on Trends in Central Nervous System Malignancies Istanbul	2015	0.7 ECTS
- EANO 2016 Mannheim	2016	1.2 ECTS
- SNO 2017 San Francisco	2017	1.2 ECTS
- SNO 2018 New Orleans	2018	1.2 ECTS
Other		
- JNI scientific research meetings (~30x / year). EMC	2015-2018	4 ECTS
- Bi-weekly Neuro-Oncology meetings	2015-2018	2 ECTS
Supervising students		
- Supervising medical student	2015-2016	1 ECTS
- Supervision of Master student	2016-2017	1 ECTS
- Supervision of Master student	2019	1 ECTS

DANKWOORD

Vier jaar geleden kon ik me nauwelijks voorstellen dat er een paar jaar later een proefschrift klaar zou zijn, maar het is er toch echt nu. De tijd is omgevlogen! Uiteraard zijn er een aantal mensen die ik wil bedanken voor hun bijdrage aan de hoofdstukken in dit proefschrift.

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ABOUT THE AUTHOR

Maarten M.J. Wijnenga was born on February 12th, 1989 in Oosterhout NB, the Netherlands. He graduated at the Sint-Oelbert Gymnasium in Oosterhout in 2007 and started his medical education the same year at the Erasmus University in Rotterdam. He obtained his medical degree in February 2014. Subsequently he worked for two months as a resident (ANIOS) at the Neurology department of the Maasstad Hospital in Rotterdam, before returning to the Erasmus Medical Center in Rotterdam as a Neurology resident (ANIOS) in May 2014. In February 2015 he started with his PhD project on low-grade glioma under the supervision of prof.dr. M.J. van den Bent, dr. P.J. French (both department of Neurology), prof.dr. C.M.F. Dirven, and dr. A.J.P.E. Vincent (both department of Neurosurgery). The research in this project led to the current thesis. From April 2019 onwards, Maarten will continue with his residency training (AIOS) in Neurology, under the supervision of dr. J.E.C. Bromberg.