MOLECULAR PROFILING OF GLIOMAS

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Molecular Profiling of Gliomas

Moleculaire Classificatie van Gliomen

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INTRODUCTION: MOLECULAR SUBTYPES OF GLIOMAS

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Tumors of the central nervous system part II (book). Edited by Dr. M.A. Hayat, Springer, 2011.

Gliomas are the most common type of primary brain tumors in adults with an incidence rate of 5.27 per 100.000 patients every year [1-2]. In 1926, Bailey and Cushing suggested a classification model based on distinct histological morphologies [3], which forms the basis of the currently used WHO classification [1]. Two major subtypes are recognized: Astrocytic (A) and oligodendrocytic (OD) tumors, the latter including pure OD tumors and mixed oligoastrocytic (MOA) tumors. Astrocytic tumors are further separated into grades I (pilocytic astrocytomas [PA]), II (low grade), III (anaplastic), and IV (glioblastoma [GBM]). Oligodendrocytic tumors are further separated into grades II (low grade) and III (anaplastic). Patient survival, time to tumor progression, and response to therapy are all associated with subtype and grade of the tumor [1]. This classification model, combined with the patients' prognostic features (e.g. age and Karnofsky Performance Score [KPS]), guides treatment decisions.

Differences between histological subtypes are very subtle, and classifying gliomas is subject to a large interobserver variability [4-7]. Clearly, this variability can result in misdiagnosis of gliomas, for example by assigning a prognostically favorable lower-grade glioma into a poor prognostic glioma (e.g. "false-positive GBM"), and in assigning a prognostically less favorable higher-grade glioma into a good prognostic glioma (e.g. "false-negative GBM"). Since treatment protocols often dependon the diagnosed histological subtype, accuracy in diagnosis is very important for patients in order to get optimal treatment [6]. Therefore, more accurate methods to diagnose gliomas are urgently required.

The molecular characteristics of gliomas have been studied extensively over the last years, in order to provide more objective and accurate methods of identifying distinct molecular tumor subgroups, and to identify specific molecular tumor markers that can help diagnosis. In the future, these molecular features may also be used to develop personalized targeted therapy.

SINGLE MOLECULAR MARKERS IN GLIOMAS

LOH 1p19q

Loss of heterozygosity (LOH) of 1p19q is a chromosomal aberration that is strongly associated with classical ODs [8-10]. Determination of the 1p19 status in gliomas is clinically relevant because of two important features. First, gliomas with LOH of 1p19q generally grow more slowly than most other gliomas and therefore have a better prognosis. Second, the presence of this mutation is predictive for response to treatment with alkylating agents such as PCV (procarbazine, lomustine, and vincristine) [11-12]. However, many glial tumors benefit from alkylating agents at some level. Nevertheless, 1p19q status is a strong prognostic factor, and the presence or absence of 1p and 19q is currently tested for in patients with a tumor containing oligodendroglial features.

IDH1

Recently, somatic mutations in the gene encoding isocitrate dehydrogenase 1 (*IDH1*) have been identified in gliomas [13]. *IDH1* mutations occur mainly in lower grade gliomas and in secondary GBMs and are therefore thought to be early events in glioma genesis [14]. Interestingly, glioma-specific mutations in *IDH1* always affect the amino acid arginine in position 132 of the amino acid sequence, which belongs to an evolutionary highly conserved region located at the binding site for isocitrate [13,15]. The most frequent mutation occurring in this region is the R132H

mutation (>90%), but other variants also have been found (R132S, R132C, R132G, and R132L) [15-16]. Interestingly, non-R132H mutations segregate in distinct histological and molecular subtypes of glioma [14,16]. Histologically, non-R132H mutations occur sporadically in classic oligodendrogliomas and at significantly higher frequency in other grade II and III gliomas. Genetically, non-p.R132H mutations occur in tumors with *TP53* mutation, are virtually absent in tumors with LOH on 1p and 19q and accumulate in distinct (gene-expression profiling based) intrinsic molecular subtypes [16-17].

Importantly, *IDH1* mutations are associated with improved prognosis [13,18]. Therefore *IDH1* mutation status is likely to be used as a prognostic molecular marker in the near future. Additionally, two studies have examined whether *IDH1* mutation status can predict response to treatment in gliomas. In a group of patients with dedifferentiated low-grade astrocytomas progressive after radiotherapy response to TMZ did not differ between *IDH1* mutant and wild-type tumors [19]. In patients with anaplastic oligodendroglial tumors treated with radiotherapy alone or radiotherapy with adjuvant PCV, *IDH1* mutations reported had no predictive value for response [20].

MGMT

The O^6-methylguanine-DNA methyltransferase (*MGMT*) gene encodes for the nuclear repair enzyme alkyltransferase, which removes alkylating adducts from the O^6 position of thymine. By doing this, the enzyme is involved in maintaining the integrity of the DNA. More specifically, the product of the *MGMT* gene protects the cells from being damaged by alkylating and methylating agents (e.g. BCNU [*N,N*[prime]-bis(2-chloroethyl)-*N*-nitrosourea], procarbazine and TMZ).

In gliomas, the CpG islands located in the promotor of the *MGMT* gene are frequently methylated, causing "epigenetic" silencing of this gene. Theoretically, this methylation would result in a greater susceptibility for alkylating and methylating agents. In daily practice, the meaning and implications of the *MGMT* status are more difficult to interpret. Several studies showed that the epigenetic silencing of the *MGMT* gene is of clinical importance, because its association with increased survival and better response to combined chemo-irradiation in GBMs [21-22]. It was shown that the effect of the *MGMT* silencing was especially present when TMZ was used as chemotherapy [22]. Other studies showed that the time of administering the TMZ seemed to be crucial. One study showed that a positive effect of the methylated *MGMT* gene on survival was only seen if TMZ was administered at the time of radiation, while other studies showed positive effects when administering TMZ before or after irradiation [23-25]. Interestingly, a recent study showed that *MGMT* promotor methylation in GBMs was a predictor for better response to radiotherapy, and a prognostic marker even in patients not receiving adjuvant alkylating chemotherapy [26]. This study suggests that *MGMT* might be a general favorable prognostic factor in GBMs, instead of being a predictive marker for response to alkylating chemotherapy.

Another issue that makes it difficult to interpret the role of *MGMT*, is that there are several different approaches in the assays for measuring the *MGMT* promotor methylation. These different approaches cause variable results, which are difficult to compare. *MGMT* activity can be measured using immunohistochemistry, but also using Q-pcr, or promotor methylation assays. Also, differences in outcome arise using assays on frozen tissue as well as on paraffin samples, as well as by possible interobserver-variability. In conclusion, *MGMT* status is thought to be an important biomarker in gliomas.

GENOME WIDE MOLECULAR MARKERS IN GLIOMAS

Several techniques have been developed to perform genome wide analysis of the tumors' epigenome, genome or transcriptome. Whilst markers for glioma have been identified on all levels [27], this review will discuss the molecular markers identified by the tumor's transcriptome, that are currently used (or show promise to aid) in clinical decision making. Gene expression profiling involves the measurement of the activity (the expression) of thousands of genes at once. In general, gene expression profiling is performed using microarrays; chips that containthe complementary sequence to thousands of target mRNA sequences. mRNA isolated from tumor samples is processed and labeled and subsequently hybridized to the microarray. The signal extracted from the microarray is a measure of gene expression levels, which is visualized using fluorescence.

Expression profiling can be used to identify molecular subtypes of tumors roughly by two methods: Supervised and unsupervised. Supervised clustering uses external information to separate tumors into predefined subgroups (e.g. responders vs. non-responders; long vs. short-survivors), and then specifically screens for genes that are differentially expressed between these groups. In contrast, unsupervised clustering does not use external information and thus classifies tumors based on homologies in gene expression profiles.

One of the first large studies that used supervised clustering (on survival) has identified three large subtypes of glioma with distinct prognosis. Subtypes were named according to the signature of the genes they predominantly expressed: Proneural, Mesenchymal and Proliferative subtypes [28]. These subtypes have also been identified in GBM using unsupervised methods [29]. A different supervised study identified genes associated with response to treatment [30]. Most often however, supervised clustering has been used to define gene expression signatures based on histological subtypes [31].

Thusfar, only threegroups have performed unsupervised analysis to define "intrinsic" molecular subgroups of gliomas [17,29,32]. In all cases, the unsupervised clusters identified more subtypes of gliomas than histology. The molecular clusters correlate better with survival than histology [17,31-32]. Therefore, molecular clustering provides an objective and more accurate method to classify gliomas, and may even be used to predict patients' prognosis. The molecular clusters all contained a wide variety of histological subtypes. The fact that different histological subtypes were assigned to the same molecular cluster means that these phenotypically different tumors have a similar genetic composition. Indeed, two independent studies have demonstrated that genetic changes segregate into distinct molecular subtypes indicating that causal genetic change drives a distinct pattern of gene expression [16-17,29].

For example, gliomas of different histological subtypes with LOH of 1p19q are accumulating within one distinct molecular profile, regardless of their histological appearance, showing significant longer survival times than other molecular subgroups [17]. These findings imply that 1p19q status should be determined in all histological subtypes of gliomas, instead of testing this mutation in oligodendroglial-like tumors only.

In the future, the specific genetic features of molecular glioma subgroups can be used to improve diagnosis, to give a more accurate prognosis, as well as todevelop personalized therapies. It is likely that each molecular glioma subgroup will benefit from its own specific treatment based on the specific underlying molecular pathways and markers. Novel randomized controlled trials should take these molecular clusters into account when comparing different therapy regimens in gliomas.

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INTRINSIC GENE EXPRESSION PROFILES OF GLIOMAS ARE A BETTER PREDICTOR OF SURVIVAL THAN HISTOLOGY

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ABSTRACT

Gliomas are the most common primary brain tumors with heterogeneous morphology and variable prognosis. Treatment decisions in patients rely mainly on histologic classification and clinical parameters. However, differences between histologic subclasses and grades are subtle, and classifying gliomas is subject to a large interobserver variability. To improve current classification standards, we have performed gene expression profiling on a large cohort of glioma samples of all histologic subtypes and grades. We identified seven distinct molecular subgroups that correlate with survival. These include two favorable prognostic subgroups (median survival, >4.7 years), two with intermediate prognosis (median survival, 1–4 years), two with poor prognosis (median survival, <1 year), and one control group. The intrinsic molecular subtypes of glioma are different from histologic subgroups and correlate better to patient survival. The prognostic value of molecular subgroups was validated on five independent sample cohorts (The Cancer Genome Atlas, Repository for Molecular Brain Neoplasia Data, GSE12907, GSE4271, and Li and colleagues). The power of intrinsic subtyping is shown by its ability to identify a subset of prognostically favorable tumors within an external data set that contains only histologically confirmed glioblastomas (GBM). Specific genetic changes (epidermal growth factor receptor amplification, *IDH1* mutation, and 1p/19q loss of heterozygosity) segregate in distinct molecular subgroups. We identified a subgroup with molecular features associated with secondary GBM, suggesting that different genetic changes drive gene expression profiles. Finally, we assessed response to treatment in molecular subgroups. Our data provide compelling evidence that expression profiling is a more accurate and objective method to classify gliomas than histologic classification. Molecular classification therefore may aid diagnosis and can guide clinical decision making.

INTRODUCTION

Gliomas are the most common type of primary brain tumor in adults (1, 2). Despite advances in therapy, the prognosis for most glioma patients remains dismal. Based on their histologic appearance, gliomas can be divided into two major subtypes according to the 2007 WHO classification (1): astrocytic tumors, including pilocytic astrocytomas (PA), astrocytomas, and glioblastomas (GBM), and oligodendroglial (OD) tumors, including pure OD tumors and mixed oligoastrocytic (MOA) tumors. Tumors are further divided into grades I (PA), II (low grade), III (anaplastic), and IV (GBM) depending on the presence of anaplasticfeatures (1). Patient survival, time to tumor progression, and response to therapy are all associated with subtype and grade of the tumor (1). In glioma patients, the histologic classification of tumors, often combined with perceived clinical prognostic features, guides treatment decisions. However, histologic classification of gliomas is troublesome and subject to interobserver variation (3).

Expression profiling provides an objective method to classify tumors (4, 5). Thus far, previous studies have shown that expression profiling correlates better with prognosis than histology (6) and may even be used to predict patients' prognosis (7–11). However, these studies have used external information (histology or clinical parameters) to build molecular classifiers. Furthermore, many studies were performed on a more restricted number of histologic diagnoses and/or tumor grades, contained incomplete clinical annotation, or included a relatively small number of patients (6–9, 12–19). Although these studies show that expression profiling can predict outcome based on supervised analysis, thus far only one study has identified intrinsic (unsupervised) subtypes of glioma and correlated them with patients' prognosis (20). However, no study has compared the prognostic and predictive value of molecular classification methods with that of histologic subtyping in glioma.

In this study, we therefore performed expression profiling on a large cohort of clinically annotated glioma samples of all histologic subtypes and grades. We provide strong evidence that the intrinsic molecular subtypes of gliomas correlate better with survival than histologic diagnosis. Furthermore, our data indicate that certain molecular subgroups clearly benefit from treatment. Our results were validated on several large independent external data sets. Molecular classification therefore may aid diagnosis and may be used to guide clinical decision making.

MATERIALS AND METHODS

Patients and tumor samples

Glioma samples were collected from the Erasmus University Medical Center tumor archive (n = 276) from patients (1989–2005), including seven repeat samples. Samples were collected immediately after surgical resection, snap frozen, and stored at −80°C. Use of patient material was approved by the Institutional Review Board. Medical history is stated in Supplementary Table S1. Survival time was defined as the period from date of surgery to date of death. If unavailable, date of last follow-up was used. Repeat samples were not included in survival analysis. All samples were visually inspected at the time of this study on their extent of tumor (J.M.K.). All histologic diagnoses were made on formalin fixed, paraffin-embedded H&E

sections and were reviewed (J.M.K.) blinded to the original diagnosis according to the 2007 WHO classification (1). GBMs were defined as secondary when symptoms occurred more than 1.5 y before histologic diagnosis or following relapse of a lower-grade glioma. Eight additional control samples (normal adult brain) were obtained from the Erasmus University Medical Center (n = 4) and the Dutch Brain Bank (n = 3) or purchased (n = 1; Qiagen).

Nucleic acid isolation, cDNA synthesis, and array hybridization

Total RNA and genomic DNA were isolated from 20 to 40 cryostat sections of 40-μm thickness using Trizol (Invitrogen) according to the manufacturer's instructions (14) and further purified on RNeasy mini columns (Qiagen). RNA quality was assessed on a Bioanalyzer (Agilent). One to two micrograms of high-quality RNA [i.e., RNA integrity number >6.5 (21)] was used for our experiments. Double-stranded cDNA synthesis and labeled cRNA synthesis were performed according to the Affymetrix Eukaryotic One-cycle cDNA synthesis protocol. Affymetrix HU133 Plus 2.0 microarrays were hybridized overnight with 10 μg of biotin-labeled cRNA. Genechips with a glyceraldehyde-3-phosphate dehydrogenase 5′/3′ ratio >4, present calls <30%, unsuccessful RT controls, or a background >200 were excluded. Robustness of sample processing was assessed using eight biological replicates and three technical replicates. Replicates were not included in any analysis. Sample labeling and array hybridization on 250K NspI arrays was performed using high-quality genomic DNA according to the Genechip Mapping 500K Assay Manual (n = 40). Sample labeling and array hybridization on single-nucleotide polymorphism (SNP) 6.0 arrays were performed using Trizol-extracted, Repli-G (Qiagen)–amplified genomic DNA by AROS Applied Biotechnology AS according to standard Affymetrix protocols (n = 15).

Genetic aberrations

Loss of heterozygosity (LOH) of 1p19q was determined by microsatellite analysis or inferred from genotyping arrays. Microsatellites were amplified by PCR and analyzed as described (14). Allelic losses were statistically determined as described (22). Mutations in exon 4 of *IDH1* were determined by direct sequencing (Supplementary Table S2). Apart from experiments on 250K NspI arrays, all experiments used Trizol extracted, Repli-G–amplified genomic DNA as starting material.

Amplification of *EGFR* was determined by semiquantitative PCR as described (14).

Unsupervised clustering analysis

Expression levels of 17,527 genes were extracted from Affymetrix HU133 Plus 2.0 arrays using updated array annotation (23). Next, hierarchical ordered partitioning and collapsing hybrid (HOPACH) clustering was used to identify molecular subgroups in gliomas on 5,000 genes with highest variance (24). Nonparametric bootstrapping was used to estimate the probability that each sample belongs to a cluster (i.e., fuzzy clustering) and thus determine cluster stability. Samples were assigned to a cluster when at least 50% of bootstraps allocated the sample to that specific cluster.

The Cancer Genome Atlas (TCGA) and GSE4271 data sets used HU133A microarrays; thus, the actual probes used to define a probe set may differ between this and our data set. Cluster

validation was performed by representing each of the molecular clusters in our data set by its centroid and classifying external samples to their nearest centroid. Samples belonging to the original data set that were not assigned to a cluster were similarly assigned to the nearest centroid. Robustness of external validation was estimated with the in-group proportion (IGP) cluster quality measure (25). To validate the groups, the IGP scores are compared with a null distribution of IGPs. A P value for each IGP was calculated based on permutation tests. Both HOPACH and IGP were available as R packages.

Gene set enrichment analysis

Gene set enrichment analysis (GSEA) was done for each cluster versus the remaining samples against the MSigDB gene ontology gene sets (26, 27). Gene sets that are over expressed or under expressed in at least one cluster with P value of <0.01 and false discovery rate (FDR) q value of <0.05 were selected. Enrichment scores were calculated by the negative and positive logarithm (base 10) of the FDR q values for the over expressed and under expressed gene sets, respectively, such that over expressed gene sets are scored on a positive scale and under expressed gene sets on a negative scale.

Data analysis

Statistical processing of data was performed using Excel, Access, Stata 10.0, and Prism 5.02 (GraphPad). The significance of prognostic factors was determined with a multivariate analysis using Cox regression. Differences between Kaplan-Meier survival curves were calculated by the log-rank (Mantel-Cox) test. Comparisons between mean survivals of different groups were assessed by unpaired t tests, and comparisons between frequencies by the Fisher's exact test.

RESULTS

Patient characteristics

A total of 276 glioma samples of following histology were included in this study: 8 astrocytomas grade 1 (PAs), 13 astrocytomas grade 2 (AII), 16 astrocytomas grade 3 (AIII), 159 astrocytomas grade 4 (GBM; 106 primary and 53 secondary), 28 MOAs (3 grade 2 and 25 grade 3), and 52 ODs (8 grade 2 and 44 grade 3). Male-to-female ratio was 2.1:1, median age at diagnosis was 50.2 years (range, 11.7–81.2), and mean Karnofsky Performance Score (KPS) was 78.9. Molecular clustering is an independent prognostic factor (Table 1) and remains independent when the analysis is performed on GBMs only (Supplementary Table S3). Age at time of diagnosis and KPS are well-documented prognostic factors in glioma (28–31). However, age at diagnosis does not remain an independent factor when taking molecular markers into account, as they mostly occur in tumor types associated with lower age at onset (*IDH1*, 1p19q). Due to the long period of inclusion, patients did not receive uniform treatment. One hundred and seventy-four (63%) patients were treated with radiotherapy and 24 (8.5%) with combined chemoradiation therapy, and a "wait and see" policy was applied to 11 (4%) patients until disease progression. Sixty-eight (24.5%) patients only received supportive treatment after diagnosis because of poor performance status, high age at diagnosis, rapid disease progression, or refusal of any other treatment by the patient. Detailed patient characteristics are listed in Supplementary Table S1.

Table 1. Multivariate analysis. Multivariate analysis (Cox regression analysis) on all samples included in the study.

	Haz. Ratio	Std. Err.	z	P>|z|	[95% Conf. Interval]	
Histological diagnosis	0,6737357	0,6098470	-4,36	0,000	0,5642105	0,8045220
Histological grade	0,8869178	0,1263290	-0,84	0,400	0,6708751	1,1725330
Age	1,0349960	0,0056765	6,27	0,000	1,0239300	1,0461820
Sex	0,7155355	0,0976483	-2,45	0,014	0,5476066	0,9349614
KPS	0,9867135	0,0025791	-5,12	0,000	0,9816714	0,9917815
Extent of surgery	0,7946357	0,0726748	-2,51	0,012	0,6642323	0,9506402
Radiotherapy	0,7811229	0,0617660	-3,12	0,002	0,6689783	0,9120669
Chemotherapy	0,8947309	0,1903937	-0,52	0,601	0,5896056	1,3577610
Molecular cluster	1,3198160	0,0543694	6,74	0,000	1,2174420	1,4307980

Molecular clusters differ from histologic subgroups

Principle components analysis based on the 5,000 most variable genes in the data set highlights the relative difference and similarity between samples (Supplementary Fig. S1). Similar clustering results were obtained using all genes, half of the genes, or 1,000 genes. We then identified molecular subgroups in our data set based on similarities in gene expression levels between samples using the HOPACH algorithm. Twenty-four distinct molecular clusters were identified. Nonparametric bootstrapping confirmed that most samples indeed belong to a defined cluster (fuzzy clustering). Only 17 samples were assigned to a cluster different from the original after bootstrapping, indicating the high stability of the clusters (see Materials and Methods). More specifically, all of these samples had very sparse cluster memberships, meaning that for most of these samples the remaining cluster membership was to one or very few clusters. Clustering of samples and bootstrapping results are shown in Supplementary Fig. S2 and Supplementary Table S4.

We then focused our analysis on the five largest clusters containing >10 samples each (clusters 9, 17, 18, 22, and 23) and two smaller subgroups that are molecularly (Supplementary Fig. S1) and histologically distinct: a control sample cluster 0 (n = 8; a merge of clusters 0, 1, and 3) and cluster 16 that contains PAs (n = 6; a merge of clusters 14, 15, and 16) and 3 recurrences of PAs. All samples were then reassigned to one of these seven clusters. After reassignment, clusters 0, 9, 16, 17, 18, 22, and 23 contained 23, 44, 10, 38, 64, 26, and 79 samples, respectively.

All clusters contain a wide variety of histologic diagnosis and malignancy grades. However, distinct histologic subtypes segregate into different molecular clusters. For example, clusters 18, 22, and 23 predominantly contain GBMs; cluster 9 contains most of the ODs (grade 2 and 3); and six of eight PAs cluster in group 16. Cluster 17 is the most histologically diverse cluster. The histologic composition of all molecular subgroups is shown in Fig. 1A.

Because control brain samples are markedly different in gene expression profile from the large majority of glioma samples (Supplementary Fig. S1), we were surprised that cluster 0 contained 15 glioma samples in addition to the 8 control brain samples. We hypothesized that the tumor samples in cluster 0 might contain a large amount of nonneoplastic tissue. Indeed, histologic reexamination of all samples by a blinded experienced neuropathologist (J.M.K.) confirmed that all 15 samples that were reassigned to cluster 0, but none of the other samples, contained a substantial (>50%) amount of nonneoplastic brain tissue. Because of the high amount of nonneoplastic tissue in the samples in this cluster, we were unable to extract a clear glioma-derived expression profile and, therefore, did not involve cluster 0 in the survival analysis.

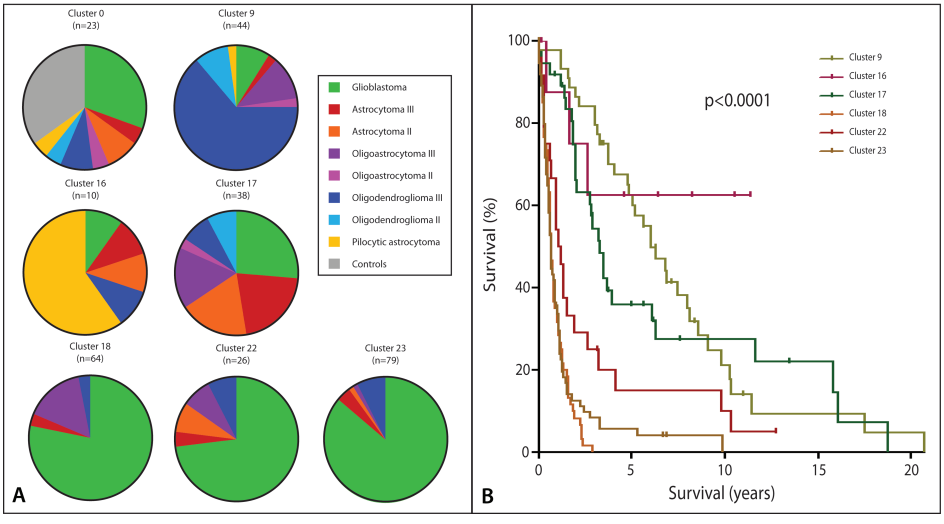

Figure 1. Molecular subgroups are distinct from histological subgroups and correlate with survival. A: Pie charts show the composition of individual molecular subgroups by their histological subtypes. Each molecular subgroup is composed of a variety of different histological subtypes, although distinct histological subtypes predominate distinct molecular subgroups. B: Kaplan Meijer survival analysis of molecular clusters. Intrinsic gene expression profiles identify two molecular clusters with poor prognosis (18 and 23), two clusters that have intermediate prognosis (17 and 22), and two clusters with relatively favorable prognosis (9 and 16).

Molecular clusters correlate with survival

Kaplan-Meier survival analysis highlighted that the molecular clusters differ significantly with respect to their survival ($P < 0.0001$). As illustrated in Fig. 1B, molecular clusters are separated in two favorable prognostic subgroups (9 and 16; median survival of 6.06 and >4.7 years, respectively), two subgroups with intermediate prognosis (17 and 22; median survival of 1.1 and 3.32 years, respectively), and two subgroups with poor prognosis (18 and 23; median survival of 0.68 and 0.67 years, respectively). Cluster 16 has a median survival of >4.7 years, as 6 of 10 patients were still alive at the time of last follow-up. Clinical characteristics of the molecular subgroups are summarized in Table 2. In repeat samples, cluster assignment either remained identical or changed to a molecular subgroup with poorer prognosis (Supplementary Table S5).

Table 2. Clinical characteristics of the molecular subgroups. Median age at diagnosis, median survival, and mean KPS per molecular cluster. Median survival of cluster 16 could not be defined since 6/10 patients were still alive at time of last follow-up.

	Cl 9	Cl 16	Cl 17	Cl 18	Cl 22	Cl 23
Median Age at Diagnosis (yrs)	48,50	36,50	38,30	58,00	46,00	54,70
Median Survival (yrs)	6,06	Undefined	3,32	0,68	1,12	0,67
Mean Karnofsky Performance score	82,9	70,9	89,2	77,0	79,2	79,7

Cox regression analysis showed that molecular clustering is an independent significant prognostic variable in survival (P < 0.012; Table 1). Other factors in survival are age at diagnosis, KPS, and sex (28–31). These results are illustrated by Supplementary Fig. S3 and show that additional prognostic factors can help estimate prognosis.

Molecular clusters correlate better with patient survival than histologic subgroups

Because histology also correlates with patient survival, we compared the accuracy of survival prediction by the two classification methods. To this end, we separated samples of the same histologic diagnosis by their molecular profile. Conversely, samples of the same molecular subgroup were further separated by their histologic appearance. The median survival of all GBMs used in our study was 0.73 years (range, 0.02–9.8). However, GBMs in the three poorest prognostic molecular subgroups (18, 22, and 23) have a significantly shorter median survival compared with GBM sin the three most favorable molecular clusters (9, 16, and 17; 0.70 versus 2.05 years; P = 0.0024; Fig. 2A). Conversely, within the poor prognostic subgroups (18, 22, and 23), no significant difference was observed in median survival between samples with poorest histologic diagnosis (GBM) versus those with a histologically more favorable prognosis (all

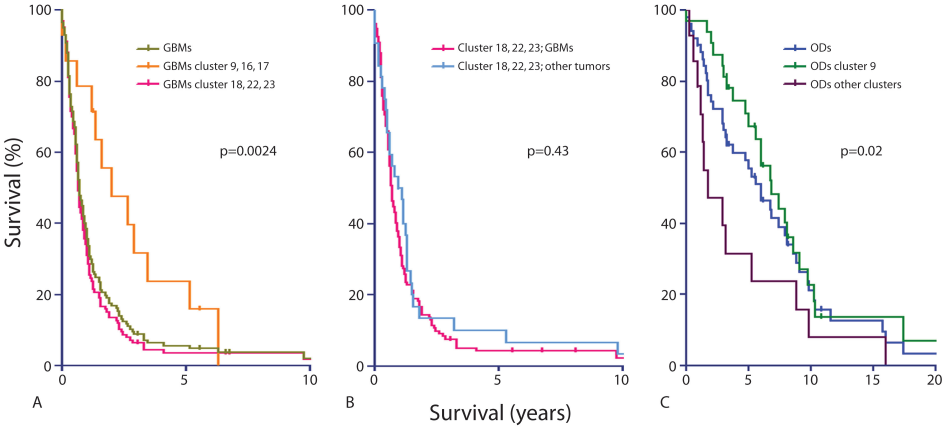

Figure 2. Molecular subgroups correlate better with survival than histological subgroups. Comparison between molecular and histological classification: A: Samples of the same histological diagnosis (GBM) were separated by their molecular profile into those present in poorest prognostic molecular subgroups (18, 22 and 23) and those present in relatively favorable prognostic molecular subgroups (9, 16 and 17). The median survival of all GBMs was 0.73 years, but patients with a GBM in a poor molecular subgroup perform worse than patients with a GBM in a relatively favorable subgroup (0.70 vs. 2.1 years, P<0.01). B Samples present in poor molecular subgroups (median survival 0.82 years) cannot be further separated by their histological appearance into those of poor (GBMs) and less poor (all other tumors) prognostic subgroups (median survival 0.71 and 1.04 years). C: Similar to A, but using ODIIs and ODIIIs as histological subgroup (median survival 6.04 years) further separated by their poor and relatively favorable molecular subgroup (median survival 6.87 and 1.78 years, P<0.023). These results demonstrate that molecular clustering has additional prognostic value to histological diagnosis whereas histological diagnosis does not have additional prognostic value to molecular subgroups.

non-GBM; Fig. 2B). Similar to GBMs, ODs in the favorable molecular cluster 9 have a significantly better prognosis than the ODs in the poor prognostic subgroups (P = 0.02; Fig. 2C). Our data show that unsupervised molecular clustering has additional prognostic value to histologic diagnosis, whereas histologic diagnosis does not add prognostic value to molecular subgroups. The intrinsic molecular subtypes of glioma therefore predict survival more accurately than histology in our data set.

Molecular analysis

We next evaluated the frequencies of known molecular markers in gliomas within the subgroups. LOH of 1p19q was determined in 149 of 276 (54%) samples. Virtually all samples in cluster 9 have LOH on 1p (85%) or on 19q (85%; regardless of histology), with 82% of samples showing combined loss. 1p/19q LOH was observed at significantly lower frequencies in samples not associated with cluster 9, with 11% showing combined loss (P < 0.0001, Fisher's exact test). Epidermal growth factor receptor (*EGFR*) status was determined in 151 (55%) samples (unbiased toward histologic diagnosis). *EGFR* amplification was predominantly observed in clusters 18 and 23 (71%; 27%); no amplification was seen in clusters 9, 17, and 22. Sequencing of *IDH1* exon 4 was successfully evaluated in 226 (82%) cases. Mutations in the highly conserved Arg132 were predominantly identified in clusters 9 (69%) and 17 (70%). Clusters 16, 18, 22, and 23 contained 13%, 16%, 45%, and 22% R132 mutations, respectively. A complete overview of genetic changes is stated in Fig. 3A and Supplementary Table S6. Figure 3B shows the clustering of the GSEA enrichment scores using average linkage hierarchical clustering using cosine similarity. Our data show that each molecular subgroup has a distinct pattern of genetic changes.

Cluster validation

Five independent external data sets were used to validate our clustering results: The Cancer Genome Atlas data set (n = 236; ref. 12), the Repository for Molecular Brain Neoplasia Data 9 data set [REMBRANDT; n = 296; National Cancer Institute (2005), assessed 2008 December; ref. 32), a data set containing 21 PAs (GSE12907; ref. 33), data set GSE4271 (8), and a data set froma recent study (20). In all cases, the molecular clustering results correlate with the composition of the data set. For example, the TCGA data set only consists of GBMs, which is reflected by the predominance of clusters 18, 22, and 23. The REMBRANDT data set has a more diverse histologic makeup (ODs, MOAs, astrocytomas, and GBMs), which is reflected by the recurrence of all molecular clusters (Fig. 4A). Finally, GEO data set GSE12907 contains 21 PAs, which is reflected by 20 of 21 samples being grouped in cluster 16. When comparing the survival of molecular clusters in both the TCGA and REMBRANDT data sets, the relative differences between molecular subgroups are virtually identical to those identified inour data set (Fig. 4B). For example, clusters 9 and 17 have significantly longer median survival in the REMBRANDT data set compared with clusters 18, 22, and 23 (P < 0.0001). Eighteen of the GBM samples in the TCGA data set were assigned to the relatively more favorable molecular cluster 17. These patients indeed had significantly longer survival (799 days) compared with those of clusters 18, 22, and 23 (420 days; P = 0.0002). Only one GBM sample of the TCGA data set was assigned to cluster 9. This patient had the longest survival in the entire data set (9.7 years). The identification of prognostically favorable samples in a data set that contains only GBMs highlights the prognostic power of intrinsic expression profiles. Analysis of genetic changes in the TCGA data set showed that both

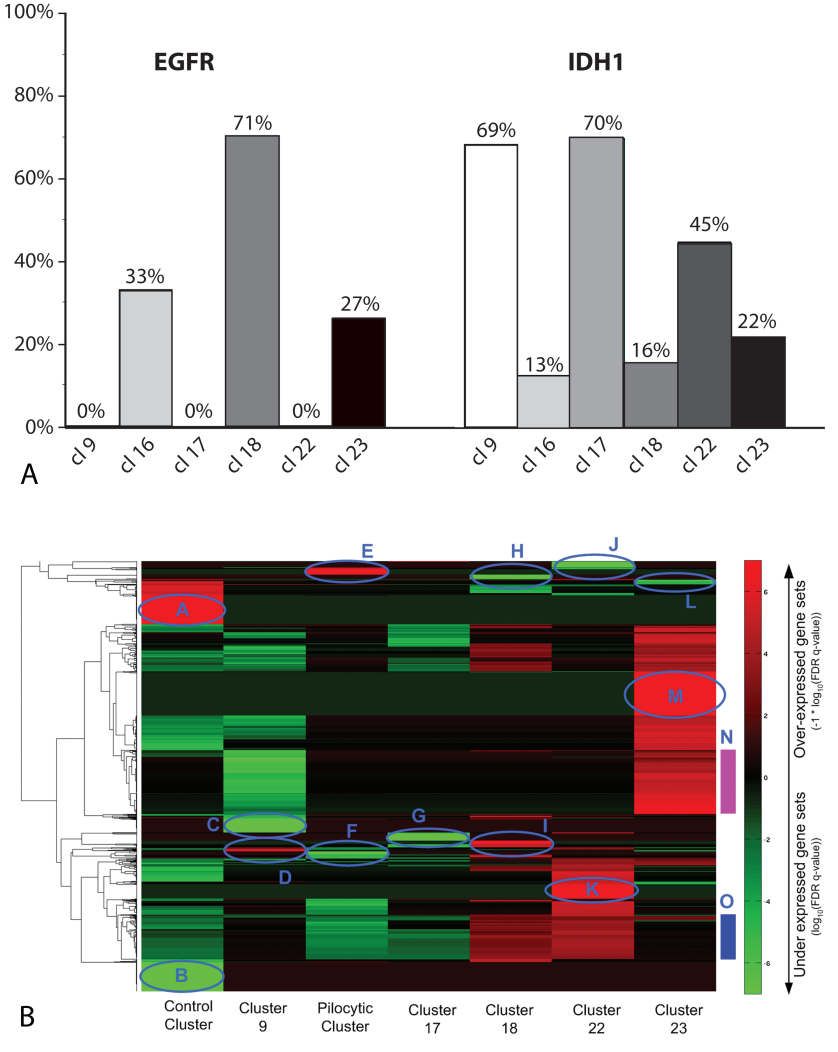

Figure 3. Genetic and pathway differences between molecular subgroups. A: distinct genetic changes are associated with distinct molecular clusters. Amplification of *EGFR* is predominantly observed in clusters 18 and 23, whereas mutations in *IDH1* are more prevalent in clusters 9 and 17. These data strongly suggest that distinct molecular subtypes have different underlying causal genetic changes. B: clustering of GSEA scores. A to O correspond to gene set clusters that are differentially expressed in at least one subtype. These functional categories were investigated by extracting overlapping genes in >10% of all gene sets in a particular cluster. Functional categories: A, cyclic AMP binding, neurogenesis, GnRH signaling, long-term potentiation; B, protein transport and regulation; C, no significant functional categories; D, ribosome, metabolic processes, histone and chromatin modification, RNA transcription; E, fatty acid metabolism; F, oxidative phosphorylation, transport; G, no significant functional categories; H, G-protein coupled receptor, neuropeptide binding; I, RNA polymerase and transcription; J, amino acid transport; K, ribosome, mitochondrion; L, cell-cellsignaling, nervous system development, ion channel activity; M, immune response; N, Janus-activatedkinase–signal transducer and activator of transcription signaling, response to stress and wounding, apoptosis, immune response; O, cell cycle, mitosis, response to DNA damage.

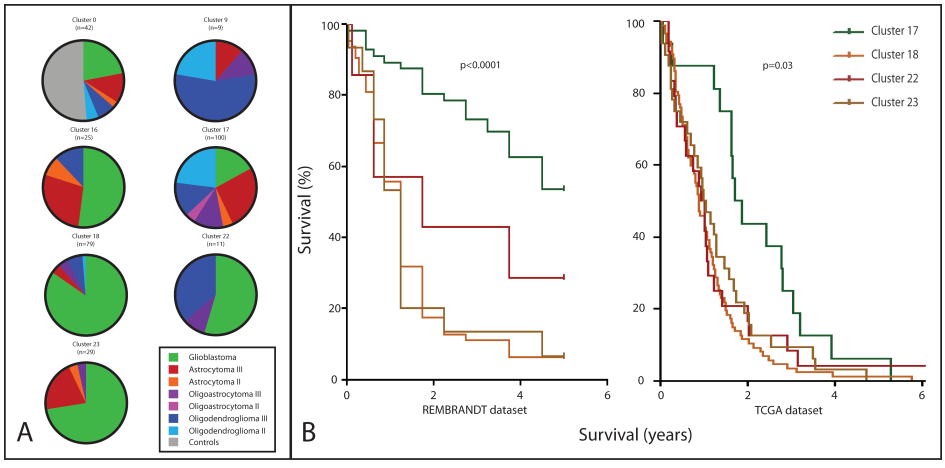

Figure 4. Molecular subgroups can be confirmed in external data sets. A: composition of individual molecular subgroups by their histologic subtype. Each molecular subgroup is composed of a variety of different histologic subtypes in the REMBRANDT data set. Similar to our set, histologic subtypes predominate distinct molecular subgroups. B: Kaplan-Meier survival analysis of molecular clusters in the REMBRANDT data set (left) and the TCGA data set (right). Both sets show similar trends in survival compared with our data. Distinct genetic changes are associated with distinct molecular clusters.

EGFR amplification and *IDH1* mutation frequencies have virtually identical distribution across the different subgroups (Supplementary Fig. S4; refs. 12, 34). Distributions of other frequently mutated genes (*NF1* and *P53*) are shown in Supplementary Fig. S5.

Furthermore, the single patient assigned to cluster 9 in the TCGA data set indeed showed LOH on 1p19q (34), as did one of the samples from the REMBRANDT data set assigned to cluster 9. SNP chip data were not available for other samples of the REMBRANDT data set assigned to cluster 9. In summary, the intrinsic glioma subgroups identified in our study can be validated in external data sets with respect to histologic, molecular, and clinical features.

We overlaid our clustering results onto GSE4271 (8), which again resulted in similar survival between molecular subgroups (Supplementary Fig. S6). The three different signatures identified by Phillips and colleagues (proneural, proliferative, and mesenchymal; see also ref. 35) segregated into specific molecular subgroups. For example, the proneural signature was predominantly found in clusters 16 and 17, whereas the proliferative and the mesenchymal signatures were mostly found in clusters 18, 22, and 23 (Supplementary Fig. S7A). Conversely, these results are confirmed when imposing these signatures onto our data set (Supplementary Fig. S7B). However, one of our best prognostic groups (cluster 9) is classified as poor prognostic group (proliferative) by Phillips and colleagues. This shows that, at least for some molecular subtypes, our clustering method predicts prognosis more specifically. Li and colleagues (20) also identified intrinsic molecular subtypes of gliomas by expression profiling. These subgroups can be confirmed in our data set, with similar survival to reported (Supplementary Fig. S8A). However, samples in the poor prognostic subgroup identified by Li and colleagues (G-groups) can be further separated based on our molecular classification into a poor prognostic

group (n = 132) and a more favorable prognostic group (n = 18) with significantly better survival (P = 0.03; Supplementary Fig. S8B). Similarly, samples that cluster in good prognostic subgroups of Li and colleagues (OA and OB group) can be separated by our profiles into a poor prognostic group (n = 13) and a favorable prognostic group (n = 67; P = 0.009; Supplementary Fig. S8C). The reverse analysis is shown in Supplementary Fig. S9. GSEA shows that some of the pathways identified by Li and colleagues show differential distribution in our subgroups (Supplementary Fig. S10). This indicates that the different clustering methods show some overlap.

Molecular clusters and treatment response

Finally, we examined whether there are differences in treatment response between molecular subgroups. The efficacy of treatment in molecular subgroups could be assessed in our sample cohort as patients were treated heterogeneously. A clear effect of radiotherapy was observed in clusters 18 and 23 (P < 0.001 and P = 0.01, respectively). In other clusters, the effects were either less pronounced or contained too few samples to reach statistical significance. However, to eliminate potential effects of sample bias, we used an external data set (GSE7696; ref. 16) that consisted of 80 GBM patients from a randomized controlled trial in which patients were treated with radiotherapy versus combined chemoradiation. We identified the following molecular clusters in this data set: clusters 0 (n = 4), 16 (n = 3), 17 (n = 10), 18 (n = 52), 22 (n = 4), and 23 (n = 11; Supplementary Fig. S11A). Results showed that both clusters 18 and 23 seem to benefit from combined chemoradiation therapy (Supplementary Fig. S11B) compared with radiotherapy only. Other clusters contain too few samples to assess effect of treatment.

DISCUSSION

In this study, we have examined whether expression profiling can serve as a more objective method to classify gliomas than histology. Our data show that expression profiling identifies molecular subgroups that are distinct from histologic subgroups and that these molecular subgroups correlate better with patient survival. In addition, our data indicate that distinct molecular subgroups benefit from treatment. As a confirmatory step, the molecular subgroups and their prognostic values were validated on five independent sample cohorts. Finally, specific genetic changes (*EGFR* amplification, *IDH1* mutation, and 1p19q LOH) segregate in distinct molecular subgroups.

At present, the treatment pathway for glioma patients has been optimized for the different histologic subtypes. However, classification based on histologic appearance has a high degree of interobserver variability (3). The intrinsic subtypes of glioma identified in our study therefore provide a robust and objective alternative to histologic classification. The power of intrinsic subtyping was shown by its ability to identify a subset of prognostically favorable tumors within an external data set that contains only histologically confirmed GBMs (TCGA).

Distinct genetic changes segregate into different intrinsic molecular clusters. For example, amplification of *EGFR* is predominantly observed in clusters 18 and 23, whereas mutations in *IDH1* are significantly more prevalent in clusters 9 and 17. All but four samples in cluster 9 have LOH on 1p19q regardless of histologic subtype. This observation is confirmed in external data sets. Interestingly, the molecular changes and clinical features associated with secondary GBMs (high mutation rate of *IDH1*, absent *EGFR* amplification, and lower age at time of diagnosis) are

reflected in a distinct molecular subgroup (cluster 22) that indeed is enriched in the number of secondary GBMs. Differences in genetic changes between molecular subtypes can indicate that novel targeted agents may only be effective in distinct molecular subtypes (Supplementary Fig. S12).

Cluster 0 contains all nonneoplastic tissue samples in our study (n = 8), in the REMBRANDT data set (n = 21), and in GSE7696 (n = 4). However, cluster 0 also contains several glioma samples in our dataset (n = 15), in the TCGA data set (n = 3), and in the REMBRANDT data set (n = 20). In our data set, samples that are associated with cluster 0 have significant amounts of nonneoplastic tissue (>50%). Similarly, two of three samples of the TCGA data set contained >90% nonneoplastic tissue (data on other sample not available) and have virtually no genetic aberrations. The samples of the REMBRANDT data set that associate with cluster 0 might therefore also contain substantial amounts of non tumor tissue.

Our data indicate that both clusters 18 and 23 benefit from chemoradiation. This benefit seems to be reflected by an increase in overall survival between our data set, in which few samples received chemoradiation, and the TCGA, in which most samples received chemoradiation (0.68 versus 0.89 years and 0.67 versus 1.02 years in clusters 18 and 23, respectively). However, in the TCGA data set, cluster 22 does not show improved survival (1.1 versus 0.98 years), and it is possible that current treatment standards do not affect this specific molecular subgroup. Unfortunately, too few samples from this chemoradiotherapy (16) were assigned to cluster 22 to assess treatment efficacy.

Another study also identified intrinsic molecular subtypes of gliomas by expression profiling (20). Although these molecular clusters also correlate with patient survival, a comparison with histologic diagnosis and molecular markers was not attempted. In addition, several histologic subtypes were not included (control tissue, MOAs, and PAs) in building molecular glioma classifiers. Our data show that our molecular clustering has additional prognostic value both to histologic diagnosis (20) and to alternative clustering methods (8, 20). There are several limitations using unsupervised hierarchical clustering for subclassification based on mRNA expression profiles. For example, tumor types that are not included in present study (e.g., brain metastasis) and rare tumor types with insufficient sample size to form a separate molecular cluster will be incorrectly classified. Histologic examination to detect such histologies therefore remains required. To some extent, molecular cluster definition also depends on the algorithms used (both for clustering and data extraction) so that individual samples may switch between molecular subgroups.

In conclusion, our data indicate that the intrinsic subtypes identified improve on histologic classification of gliomas and are an accurate predictor of prognosis. Molecular classification can contribute to diagnosis and may form a rationale for clinical decision making and novel targeted therapies.

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2

GENE EXPRESSION PROFILES OF GLIOMAS IN FORMALIN-FIXED PARAFFIN-EMBEDDED MATERIAL

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ABSTRACT

Background: We have recently demonstrated that expression profiling is a more accurate and objective method to classify gliomas than histology. Similar to most expression profiling studies, our experiments were performed using fresh frozen (FF) glioma samples whereas most archival samples are fixed in formalin and embedded in paraffin (FFPE). Identification of the same, expression-based intrinsic subtypes in FFPE stored samples would enable validation of the prognostic value of these subtypes on these archival samples. In this study, we have therefore determined whether the intrinsic subtypes identified using FF material can be reproduced in FFPE stored samples.

Methods: We have performed expression profiling on 55 paired FF-FFPE glioma samples using HU133 plus 2.0 arrays (FF) and Exon 1.0 ST arrays (FFPE). The median time in paraffin of the FFPE samples was 14.1 years (range 6.6 - 26.4 years).

Results: In general, the correlation between FF and FFPE expression in a single sample was poor. We then selected the most variable probe sets per gene (n=17,583), and of these, the 5,000 most variable probe sets on FFPE expression profiles. This unsupervised selection resulted in a better concordance (R^2= 0.54) between expression of FF and FFPE samples. Importantly, this probe set selection resulted in a correct assignment of 87% of FFPE samples into one of seven intrinsic subtypes identified using FF samples. Assignment to the same molecular cluster as the paired FF tissue was not correlated to time in paraffin.

Conclusion: We are the first to examine a large cohort of paired FF and FFPE samples. We show that expression data from FFPE material can be used to assign samples to intrinsic molecular subtypes identified using FF material. This assignment allows the use archival material, including material derived from large- randomized clinical trials, to determine the predictive and/or prognostic value of "intrinsic glioma subtypes" on Exon arrays. This would enable clinicians to provide patients with an objective and accurate diagnosis and prognosis, and a personalized treatment strategy.

INTRODUCTION

Classification of tumor subtypes influences treatment decisions for many types of cancer. Accuracy in classifying cancer subtypes is therefore necessary to provide patients with correct diagnosis, prognosis and an optimal treatment strategy. Since histological classification is often difficult in poorly differentiated tumors, this classification method urgently needs improvement. Gene expression profiling of cancer offers an accurate and objective method for classifying cancer subtypes (Sorlie et al., 2001; Valk et al., 2004). For example, in gliomas, the most common primary brain tumor in adults, gene expression profiling has identified distinct intrinsic subtypes of gliomas (Freije et al., 2004; Gravendeel et al., 2009; Li et al., 2009; Louis et al., 2007; Madhavan et al., 2009; Nutt et al., 2003; Phillips et al., 2006; Shirahata et al., 2007; Shirahata et al., 2009; Verhaak et al., 2010). We have performed unsupervised gene expression profiling in a cohort of 276 gliomas of all histological subtypes (Gravendeel et al., 2009). In this largest single-institution study conducted to date, we identified seven molecular glioma clusters. The molecular clusters were a significantly better predictor of survival than histology, and were characterized by specific genetic changes. Data were validated and confirmed on six large external datasets. When validated in prospective studies these molecular clusters could contribute to clinical decision making. However, this study was conducted using RNA isolated from fresh frozen (FF) tissue. Unfortunately, FF tissue is scarce; most of the tissue archives with matched clinical outcome data are fixed in formalin and embedded in paraffin (FFPE). RNA isolated from FFPE material is often degraded and chemically modified as a result of the archiving method (Farragher et al., 2008; Masuda et al., 1999). However, new techniques have shown promising results in genome wide expression profiling of RNA isolated from FFPE (Hall et al., 2011; Hoshida et al., 2008; Linton et al., 2009; Linton et al., 2008; Mittempergher et al., 2011).

Techniques used to study gene expression with FFPE material thus far have mostly been limited to single gene analysis with RT-qPCR. Such techniques have demonstrated to be clinical relevant on a limited set of "classifier" genes (Colman et al., 2010; Ma et al., 2006). Other multiplex assays (DASL, Quantigene, Nanostring, Fluidigm) have also shown promising results using distinct classifier genes (Canales et al., 2006; Geiss et al., 2008; Hall et al., 2011; Hoshida et al., 2008; Linton et al., 2009; Linton et al., 2008; Mittempergher et al., 2011; Spurgeon et al., 2008). Although whole genome approaches for degraded RNA samples have improved over the last few years, the performance of current techniques to detect more subtle differences between cancer subtypes remains to be confirmed.

In this study we therefore have performed expression analysis using a large cohort paired FF-FFPE glioma tissue using Exon 1.0 ST "exon" arrays (Affymetrix). Expression profiling using such a cohort has thus far not been performed. Most expression profiling studies using FF tissues have been performed on HU133 –type arrays (A, A+B or the +2.0 version), whereas best results using FFPE samples have been obtained using the Exon 1.0 ST arrays. In this study we therefore compare expression of FF samples on HU133 plus 2.0 arrays with FFPE samples on Exon 1.0 ST arrays. Previous studies have demonstrated an good overall correlation of HU133 Plus 2.0 with Exon 1.0 ST arrays (Okoniewski et al., 2007) (supplementary figure 1). We show that expression data from FFPE glioma material is concordant with expression data from matched FF tissue, and can be used for molecular profiling in gliomas. Furthermore, this molecular profiling is able to identify the subtle differences between the molecular glioma subtypes.

MATERIALS AND METHODS

Patient samples

We selected 55 paired FF-FFPE samples from the Erasmus University Medical center glioma tumor archive. The FF and the FFPE samples were taken simultaneously from the tumor as parallel biopsies. All samples were visually inspected at the time of this study for tumor content by the neuropathologist (J.M.K.) so that samples containing at least 80% of tumor tissue were selected. The FF samples selected were used in a previous study in which seven molecular clusters were identified (Gravendeel et al., 2009). The selection contained ~10 samples from each molecular cluster. FF expression profiling results were reported previously (Gravendeel et al., 2009). The RNA from the FF tissue was extracted and hybridized in 2008. The RNA from the FFPE tissue was extracted and hybridized in 2010. Clinical and molecular data from the glioma samples included were reported previously (Gravendeel et al., 2009). The use of patient material was approved of by the Institutional Review Board of the ErasmusMC, Rotterdam, the Netherlands (nr MEC 221.520/2002/262; date of approval July 22, 2003, and MEC-2005-057, date of approval February 14, 2005). For this use, patients gave written informed consent according to institutional and national guidelines. The fixation method of tissue in the Erasmus MC did not change over the last 25 years.

RNA from FFPE extraction

Five sections of 10 µm thick were cut from each tissue block. The High Pure RNA Paraffin Kit (Roche Applied Science, Mannheim, Germany) was used to isolate the RNA from the paraffin. After isolation the RNA was purified by ethanol precipitation (supplementary table 1). The quantity and integrity of the RNA was measured using a Nanodrop, and an Agilent 2100 BioAnalyzer RNA 6000 Nano Assay (Agilent Technologies). After total RNA isolation and purification, samples were diluted to 50 ng/µl and stored at -80°C until use.

qPCR

We randomly selected eleven samples that were assigned to two molecular clusters based on the fresh frozen expression data (*Cluster 9* (n=5) and *Cluster 18* (n=6)) (Gravendeel et al., 2009). Four genes (two up-regulated, two down-regulated) that discriminate between the two subtypes were examined for differential gene expression (*EMP3*, *SLC2A10*, *SUSD5*, and *CSMD3*). These genes were identified using a t-test in combination with fold change. *ACTB*, *GAPDH* were used as control. All reactions were performed in duplicate. Primers and conditions are described in supplementary table 2.

Arrays

A total of 150 ng per sample of the extracted RNA (FFPE) was used for the Exon 1.0 ST arrays (Affymetrix). Sample labeling and array hybridization were performed by AROS Applied Biotechnology AS (Arhus, Denmark) according to standard Affymetrix protocols in combination with Nugen WT-Ovation technology (FFPE V2 and Exon modules) (San Carlos, CA, USA) (n=55). Expression arrays (HU 133 plus 2.0 (Affymetrix) using FF material was reported previously (Gravendeel et al., 2009).

Quantile normalized RMA expression levels of 22,011 genes and 287,329 exons were extracted from Affymetrix Exon 1.0 ST arrays using Expression Console (Affymetrix). ClusterRepro (an R package) was used to assign a sample to a defined molecular subtype (Kapp & Tibshirani, 2007).

Statistics

Differences between Kaplan-Meier survival curves were calculated by the log-rank (Mantel-Cox) test. Differences in age and RIN-scores of the tissue blocks were calculated using a t-test and a Mann-Whitney test. Significance of correlation coefficients was calculated using the p-value calculator for correlation coefficients (www.danielsoper.com/statcalc3).

RESULTS

Sample characteristics

At total of 55 FFPE samples was included in the study. These samples included 29 glioblastomas (GBMs), 5 astrocytomas grade III (A III), 5 As grade II, 4 mixed oligoastrocytoma grade III (OA II), 2 OAs grade II, 8 oligodendrogliomas grade III (OD III), and 2 pilocytic astrocytoma (PA). The median time in paraffin of the FFPE samples was 14.1 years (range 6.6 - 26.4 years). The median RIN score of the RNA was 2.4 (range 1.1-2.7). Sample characteristics are listed in table 1.

Table 1. Patient and sample characteristics. GBM = Glioblastoma; A III = Astrocytoma grade III; A II = Astrocytoma grade II; PA = Pilocytic Astrocytoma; OA III = Oligoastrocytoma grade III; OA II = Oligoastrocytoma grade II; OD III = Oligodendroglioma grade III; OD II; Oligodendroglioma grade II.

Db no	Survival (yrs)	Status	PA diagnosis	Age FFPE material (yrs)	RIN score	correlation (r^2) FF and FFPE	P-value correlation FF and FFPE
8	9.82	dead	OD III	15.3	1.90	0.24	<0.001
40	10.28	dead	OD III	18.9	2.20	0.29	<0.001
55	17.49	dead	OD III	23.9	2.30	0.30	<0.001
63	3.28	dead	GBM	9.9	2.40	0.12	<0.001
77	1.30	dead	GBM	12.2	2.60	0.03	0.02
92	1.26	dead	GBM	11.3	1.10	0.20	<0.001
98	0.98	dead	GBM	12.1	2.30	0.13	<0.001
99	0.86	dead	GBM	13.3	N/A	0.03	0.024
104	0.21	dead	GBM	10.4	N/A	0.15	0.024
105	1.03	dead	GBM	16.0	N/A	0.08	<0.001
112	0.59	dead	GBM	16.0	N/A	0.10	<0.001
119	0.18	dead	GBM	13.1	2.30	0.15	<0.001
123	2.05	dead	GBM	13.9	2.50	0.15	<0.001
130	6.31	dead	GBM	15.1	1.40	0.08	<0.001
134	1.48	dead	A II	13.9	N/A	0.06	<0.001
143	4.79	dead	A II	13.4	2.70	0.10	<0.001
174	0.28	dead	GBM	19.3	N/A	0.05	<0.001
183	0.12	dead	GBM	12.3	N/A	0.14	<0.001
194	0.19	dead	A III	20.8	1.90	0.13	<0.001

Db no	Survival (yrs)	Status	PA diagnosis	Age FFPE material (yrs)	RIN score	correlation (r^2) FF and FFPE	P-value correlation FF and FFPE
198	6.27	dead	A III	21.8	2.40	0.11	<0.001
199	1.11	dead	GBM	20.2	2.10	0.14	<0.001
206	0.29	dead	GBM	11.7	2.50	0.03	0.028
209	1.96	dead	OA III	11.0	2.50	0.09	<0.001
227	3.10	lost to follow up	GBM	10.9	N/A	0.01	p=0.22
253	0.62	dead	GBM	9.3	2.20	0.32	<0.001
256	0.27	dead	GBM	14.1	2.40	0.15	<0.001
257	3.30	dead	OD III	17.9	2.50	0.23	<0.001
258	2.26	dead	GBM	18.4	2.50	0.12	<0.001
259	5.02	dead	OD III	13.5	2.40	0.02	0.087
286	5.56	lost to follow up	GBM	24.6	2.30	0.13	<0.001
291	0.63	dead	OA III	20.3	2.10	0.24	<0.001
293	2.99	dead	OD III	22.7	2.50	0.10	<0.001
315	0.61	dead	GBM	15.4	N/A	0.14	<0.001
336	1.19	dead	A II	11.9	2.40	0.15	<0.001
353	9.79	dead	GBM	26.0	2.10	0.10	<0.001
380	1.61	dead	GBM	7.5	2.20	0.04	p=0.002
387	3.32	dead	GBM	17.0	N/A	0.12	<0.001
393	0.06	dead	GBM	14.2	2.10	0.17	<0.001
416	15.82	dead	OD III	26.4	2.50	0.14	<0.001
420	1.32	dead	A II	17.4	N/A	0.06	<0.001
441	3.76	dead	OA II	18.9	2.50	0.02	p=0.06
445	1.18	dead	OA III	18.9	2.20	0.25	<0.001
446	0.45	dead	A III	8.9	1.60	0.04	<0.001
467	0.05	dead	A III	10.9	2.50	0.01	p=0.15
473	1.20	dead	A III	22.7	2.50	0.20	<0.001
515	0.35	dead	GBM	7.4	2.50	0.00	0.45
536	6.39	alive	OA II	6.6	2.40	0.13	<0.001
565	2.79	dead	GBM	14.2	1.90	0.23	<0.001
566	0.48	dead	GBM	13.8	N/A	0.18	<0.001
568	16.31	alive	OA III	16.5	2.60	0.20	<0.001
619	0.48	dead	OD III	11.9	2.50	0.28	<0.001
628	7.52	dead	A II	10.9	N/A	0.21	<0.001
629	2.22	dead	GBM	10.8	2.50	0.08	<0.001
711	14.18	alive	PA	14.3	1.30	0.10	<0.001
712	0.19	lost to follow up	PA	13.9	2.60	0.17	<0.001

qPCR

We first aimed to determine whether differences identified using expression profiling on snap frozen tissue could be found on RNA isolated from FFPE samples. For this initial test, we selected for samples that were assigned to two distinct molecular clusters based on the fresh frozen expression data (*Cluster 9* (n=5) and *Cluster 18* (n=6)) (Gravendeel et al., 2009).

Cluster 9 shows a favorable prognosis compared to the other clusters, and is specific for loss of heterozygosity (LOH) of 1p and 19q, as well as a high frequency of *IDH1* mutations. *Cluster 18* has poor prognosis and is characterized by *EGFR* amplifications and *CDKN2A* deletions. The RT-qPCR results showed that both direction and fold change of all four genes in all samples could be recapitulated on RNA isolated from FFPE samples. Overall correlation was relatively strong r^2 =0.61 (p<0.001). Correlations (r^2) and p values for individual genes *EMP3*, *SUSD5*, *CSMD3* and *SLC2A10* were 0.34 (p=0.024), 0.840 (p<0.001), 0.849 (p<0.001) and 0.255 (p=0.047) respectively. These results demonstrate that differences in gene expression are retained in RNA isolated from FFPE samples (figure 1).

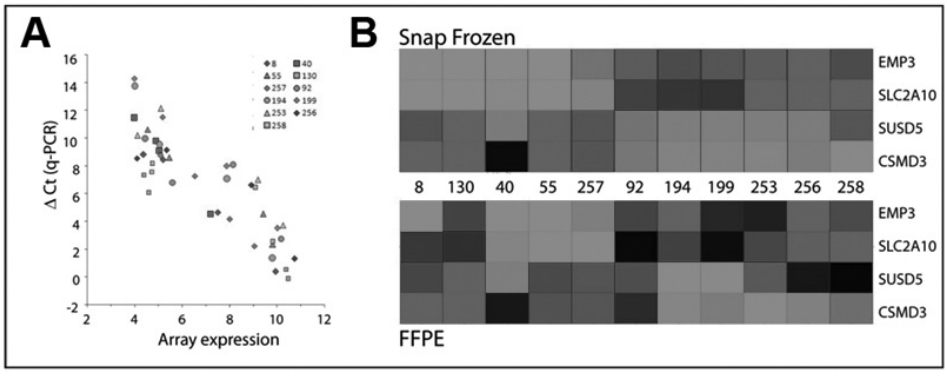

Figure 1. Correlation of the expression of the RNA (FFPE) and the RNA (FF) of eleven matched FF-FFPE samples. The four genes (*EMP3*, *SLC2A10*, *SUSD5*, *CSMD3*) chosen are the most discriminating genes between two very distinct molecular subtypes. A) The correlation between the ΔCt of the qPCR results (FFPE material) and the expression data of the HU133plus 2.0 arrays (FF tissue). A high ΔCt value is indicative for a low expression value. The correlation plot shows a good correlation between the FF expression and the FFPE expression. B) This correlation view shows the correlation of the expression of the FF tissue (HU133 plus 2.0 array) and the expression of the FFPE tissue (qPCR). The green color represents high expression, red represents low expression. The molecular clusters can be identified using the RNA isolated from FFPE.

Exon array expression data & Molecular clustering analysis

After the hybridization of the exon 1.0 ST arrays, we compared the Robust Multichip Average (RMA) normalized expression data of the exon arrays (FFPE) with exon 1.0 ST arrays that were analyzed in earlier studies (FF tissue) (French et al., 2007; Schutte et al., 2008). The exon arrays with FF tissue showed expression of more probe sets, as well as higher expression levels than the exon arrays with FFPE material (supplementary figure 2). On Hu133 plus 2.0 arrays (using FF samples), 9261±117 (52.9%) probe sets are expressed at RMA levels > 6.5. On exon arrays, 59618 ±20337 (20.7%) probe sets are detected at p < 0.01, using DABG values. It should be noted that significantly more probe sets are detected on exon arrays using fresh frozen tissue (141493±12924 [49.2%], see also supplementary figure 1. In addition, the distribution of the RMA expression histograms of the FFPE glioma tissue is shifted compared to the expression histograms of exon arrays with FF tissue (supplementary figure 3).

Correlation expression FF vs. FFPE

Exon arrays contain one or more probe sets per exon for each gene (287,329 core probe sets for 22,011 genes), whereas only one data point per gene is generated on HU133plus2 arrays (17,583 genes, when using the alternative .cdf based on entrezgene) (Dai et al., 2005). We therefore first selected the probe sets on exon arrays that likely contain most of the biological information. Because genes that discriminate between molecular subtypes are by definition differentially expressed, and thus show a relatively high variance in expression, we selected the probe set with highest variance per gene (n=17,583 probe sets [6.2% of all "core" probe sets]) (log2 normalized data). Selecting the most variable probe set on exon arrays does not always identify those with highest correlation to expression on HU133plus2.0 arrays. However, selection based on variance approaches both datasets independently and avoids potential circular arguments. All further analysis was therefore done using the selection of exon-array probe sets based on variance as starting data set.

In our previous study we performed molecular clustering with FF tissue based on the 5,000 most variable genes (FF: 5,000). The overlap between the most variable probe sets in FF tissue and the most variable probe sets in FFPE tissue (FF: 5,000/ FFPE: 17,853) consisted of 4,620 matching probe sets (figure 2A).

The set of 17,853 probe sets assumes that all genes have at least one informative probe set per gene. It is however possible all probe sets that belong to the same gene perform

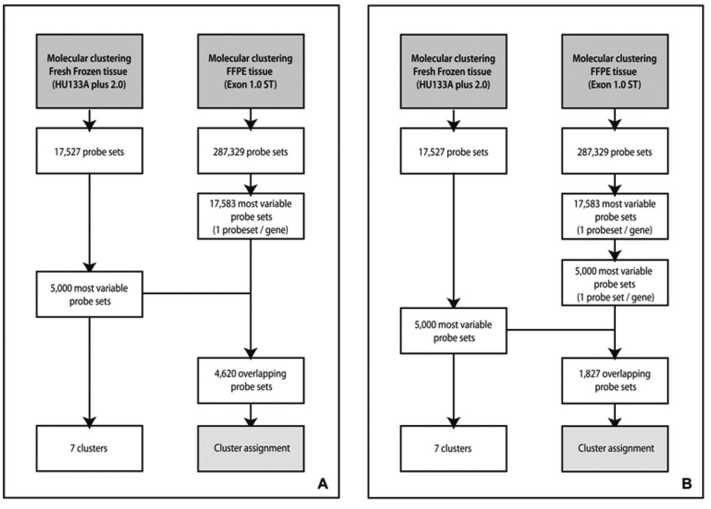

Figure 2. Flow charts of the selection of probe sets containing the most informative gene expression data. A) We first selected the probe set with highest variance per gene (n=17,583 probe sets; FFPE: 17,583). In our previous study we performed molecular clustering with FF tissue based on the 5,000 most variable genes (FF: 5,000). The overlap between the most variable probe sets in FF tissue and the most variable probe sets in FFPE tissue (FF: 5,000/ FFPE: 17,853) consisted of 4,620 matching probe sets. Based on these 4,620 probe sets, samples were assigned to one of the seven molecular clusters using ClusterRepro. B) When using exon arrays, it is possible that no informative probe sets are available for a single gene, and such probe sets may be filtered out by selecting not only the most variable probe set per gene, but, of these, the most variable 5,000 probe sets (FF: 5,000/ FFPE:5,000). By using this filter, there are 1,827 overlapping probe sets. ClusterRepro was used to assign the samples to one of the seven molecular clusters based upon these overlapping probe sets.

poorly. We therefore also performed a further selection of the 17,583 exon array probe sets, by selecting the 5000 most variable probe sets of these 17,583 (1.74% of the total number of "core" probe sets, figure 2B). Using the 5,000 most variable probe sets for both FF and FFPE material showed an overlap of 1,827 matching probe sets (FF: 5,000/ FFPE:5,000, 1,827 overlapping probe sets) (figure 2B).

We first compared the gene expression data (FF) of the genes used in the qPCR analysis (*EMP3*, *SUSD5*, *CSMD3* and *SLC2A10*) with the expression data of the FFPE material. For this analysis we used the expression data of the same 11 samples that were also used in the qPCR analysis. Figure 3 shows the correlation of the qPCR genes (*EMP3*, *CSMD3* and *SLC2A10*) between the expression of the FF RNA and the FFPE RNA. These results show a weak correlation between the FF and FFPE expressions for both *EMP3* and *SLC2A10* (r^2=0.32; p=0.028; and r^2= 0.21; p=0.067), and a good correlation for *CSMD3* (r^2= 0.97; p<0.001). There was no data available of SUSD5 since there were no probe sets of this gene on exon 1.0 ST arrays. These results highlight that exon arrays can show a concordance in gene expression compared to FF tissue, but that a selection of the biologically most informative probe sets is required.

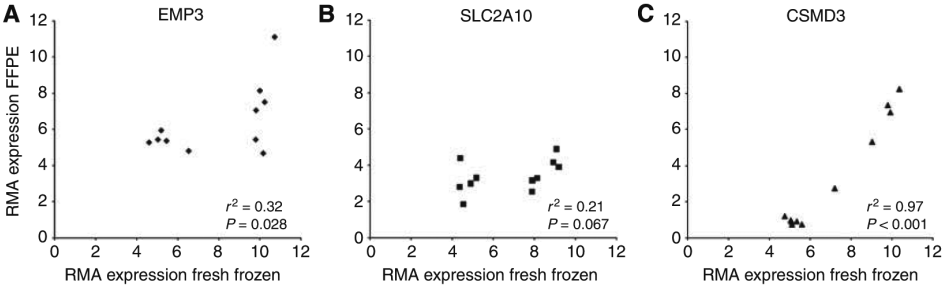

Figure 3. Correlation of the qPCR genes (*EMP3*, *CSMD3* and *SLC2A10*) between the expression of the FF RNA and the FFPE RNA. We compared the gene expression data (FF; HU133 plus 2.0) of the four genes used in the qPCR analysis (*EMP3*, *SUSD5*, *CSMD3* and *SLC2A10*) with the expression data of the FFPE material (Exon 1.0 ST). We used the expression data of the same 11 samples that were also used in the qPCR analysis. Panel 3A and panel 3B show a weak correlation for both *EMP3* (r^2= 0.32) *SLC2A10* (r^2= 0.21). A good correlation is seen for *CSMD3* (r^2= 0.97) (panel 3C). There was no data available of *SUSD5* since there were no probe sets of this gene on exon 1.0 ST arrays.

We next compared the normalized expression data of the 5,000 most variable genes as used in our previous study, with the expression of the most variable exons (FF: 5,000/ FFPE: 17,583, 4,620 matching probe sets) (Gravendeel et al., 2009). In general, the strength of correlation between FF and FFPE expression in a single sample (sample 8) was weak (r^2= 0.24, p<0.001). It should be noted that part of the between FF and FFPE sample variability is biological: The snap frozen and FFPE tissues are not taken from exactly the same location within a tumor. For this analysis we compared differential gene expression between samples of cluster 9 and 18 (separately for FF and FFPE). The correlation (r^2) in differential gene expression between FF and FFPE was 0.38.

We did the same analysis for the most variable 5,000 probe sets (FF: 5,000/ FFPE: 5,000, 1,827 overlapping probe sets). Differential gene expression between samples of *Cluster 9* and *Cluster 18* then showed a relatively strong correlation between FF on HU133 plus 2.0 and FFPE on HuEx 1.0 ST arrays (R^2 = 0.54; p<0.001 (figure 4). Our results demonstrate that differential gene expression between samples observed using RNA isolated from FF tissue is at least partially retained on RNA isolated from FFPE samples.

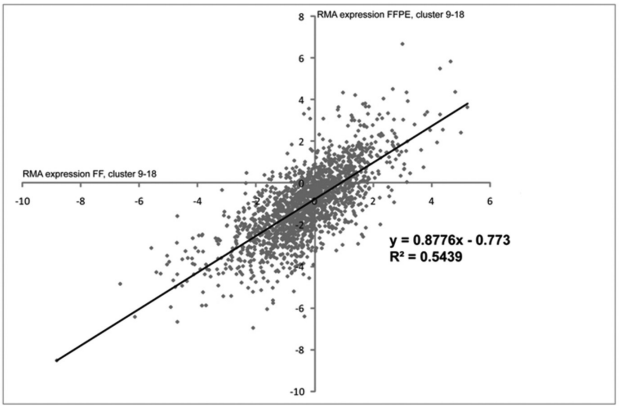

Figure 4. Differential gene expression between samples of Cluster 9 and Cluster 18. The differential gene expression between samples of two molecularly very distinct clusters (Cluster 9 and 18) showed a relatively good correlation between 18 FF samples on HU133 plus 2.0 and 18 FFPE samples on HuEx 1.0 ST arrays (R^2= 0.54). For this analysis we used the 5,000 most variable probe sets of the exon expression data and subtracted the median expression of Cluster 18 from the median expression of Cluster 9.

Expression data of fresh frozen samples is performed HU133 Plus 2.0 arrays, a platform that is different from the platform used for FFPE samples (HuEx 1.0 st arrays). We have therefore compared expression of all probes (287,329) between FF and FFPE on exon arrays of eight matched samples (8, 40, 130, 206, 257, 259, 275, 293). In general, correlation between FF and FFPE samples on the same platform was reasonable (r^2 = 0.315 ± 0.093 range 0.210-0.450, P<0.001). This correlation was much better than the overall correlation (also using 287,329 probe sets) between FF on HU133 Plus 2.0 arrays and FFPE on HuEx 1.0 st arrays (0.034±0.023). The better correlation between FF and FFPE samples on the same platform therefore indicates that differential gene expression is better retained when using the same platform.

Cluster assignment

Recently, we described the identification of seven molecular glioma subtypes based on gene expression profiling, which are a better predictor of survival than histology (Gravendeel et al., 2009). Our final assessment to determine the suitability of RNA isolated from FFPE samples was to confirm sample assignment to individual molecular subtypes. Clustering results are represented in table 2. Overall, assignment to the correct cluster (e.g. assignment to the same molecular cluster as the FF tissue in the previous study) was seen in 76% (n=42) of the

Table 2. Cluster assignment of samples. FF = fresh frozen; FFPE = fixed in formalin and embedded in paraffin.

Db no	Clustering RNA (FF)	Clustering RNA FFPE 17,853 most variable exons	Clustering RNA FFPE 5,000 most variable exons
8	9	9	9
40	9	9	9
55	9	9	9
63	0	17	0
77	0	23	23
92	18	18	18
98	22	22	22
99	23	17	0
104	23	23	23
105	23	23	23
112	23	23	23
119	22	22	22
123	17	17	17
130	9	9	9
134	17	17	17
143	0	0	0
174	23	23	23
183	23	23	23
194	18	18	18
198	17	17	17
199	18	18	18
206	22	0	0
209	17	17	17
227	22	17	17
253	18	18	18
256	18	18	18
257	9	9	9
258	18	23	23
259	9	23	23
286	17	17	17
291	18	18	18
293	0	9	9
315	17	17	17
336	22	9	0
353	22	22	22
380	9	23	23
387	22	22	17
393	23	23	23
416	17	17	17
420	22	0	0
441	9	0	0
445	18	18	18
446	16	23	23
467	22	22	22

Db no	Clustering RNA (FF)	Clustering RNA FFPE 17,853 most variable exons	Clustering RNA FFPE 5,000 most variable exons
473	9	9	9
515	23	23	23
536	17	17	17
565	23	23	23
566	23	23	23
568	17	17	17
619	18	18	18
628	0	0	0
629	23	23	23
711	16	16	0
712	16	16	16

samples (FF: 5,000/ FFPE: 17,583). However, part of the variability between FF and FFPE samples is biological and may represent tumor heterogeneity. This heterogeneity is specifically notable for assignment to *Cluster 0*, as assignment to this cluster depends on the relative amount of non-neoplastic tissue present. Indeed, the overlap between FF and FFPE cluster assignment when excluding samples that are assigned to *Cluster 0* is 86% (n=42/49). A total of 13 samples were assigned to a different molecular cluster, 7 without *Cluster 0*.

Similar performance in cluster assignment was observed when using the 5,000 most variable exon probe sets (FF: 5,000/ FFPE: 5,000) (figure 2B). Assignment to the identical cluster was seen in 75% (n=41) of the samples, 87% without *Cluster 0* (n=41/47).

Assignment to the same molecular cluster as the paired FF tissue did not have a significant correlation with the time in paraffin. The "wrongly" assigned blocks even showed a slightly shorter median time in paraffin than the "correctly" assigned samples (11.9 years vs. 14.3 years; p=0.07). The average RIN score of the incorrectly assigned samples was 2.18 ±0.40 and was not significantly different from the RIN scores of the correctly assigned samples 2.24±0.34, p=0.71).

The high degree of overlap between FF and FFPE sample assignment (both FF: 5,000/ FFPE: 17,583 and FF: 5,000/ FFPE: 5,000) is reflected in a highly similar patient survival curves (figure 5). However, FFPE survival curves also include three samples that originally were assigned to *Cluster 0*.

DISCUSSION

In this study, we describe a method that allows analysis of gene expression profiling of FFPE cancer tissue using HuEx 1.0 ST arrays. Our data show that expression data of RNA isolated from FFPE and FF tissues are comparable. However, a selection on the most informative probe sets (based on highest variance for each probe set/gene and highest variance between genes) is required. RIN score and the age of the FFPE tissue blocks do not influence the gene expression results. The average RIN score and the average time in paraffin of the incorrectly assigned samples were not significantly different from the RIN scores and time in paraffin of the correctly

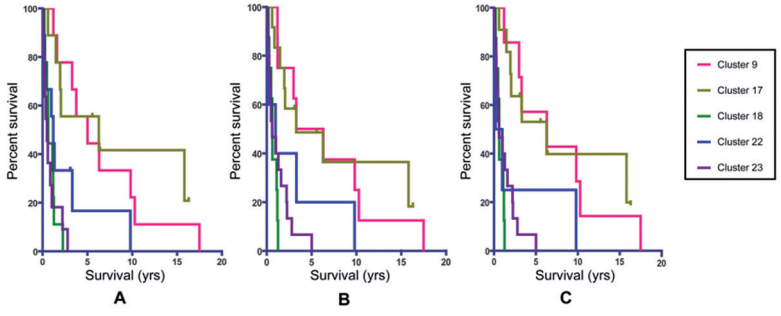

Figure 5. Kaplan-Meier survival curves of the seven molecular clusters identified in FF and FFPE material show a high resemblance. A) This panel shows the survival of the seven molecular clusters identified, using FF material of the fifty-five patients included in this study (HU133 plus 2.0 arrays). B) Panel B shows the survival curves of the molecular clusters to which the FFPE tissue was assigned based on the 17,852 most variable probe sets from the Exon 1.0 ST arrays. C) Panel C shows the survival curves of the molecular clusters to which the FFPE tissue was assigned by filtering the exon data on the 5,000 most variable probe sets.

assigned samples. The probe sets identified in this study can be used in other profiling studies that lack paired FF samples.

Differential gene expression between samples is well retained (figure 4). This is also illustrated by the identical assignment to intrinsic molecular subtypes for both FF and FFPE glioma tissue in up to 87% of the samples (table 2). It should be noted that FF and FFPE tissues are resected from different parts of the tumor. Therefore, tumor heterogeneity may also contribute to the differential assignment between FF and FFPE samples.

Previous studies demonstrated that FFPE samples can be used for gene expression profiling either using the Affymetrix (Exon 1.0 and HU133 plus 2.0) or Illumina (DASL) platforms (Hall et al., 2011; Hoshida et al., 2008; Linton et al., 2009; Linton et al., 2008; Mittempergher et al., 2011). However, these studies had limited sample size, lacked controlled experiments with paired FF-FFPE sample analysis, or were used to differentiate between very distinct cancers. Our study is the first to use a large cohort of paired FF-FFPE glioma samples for expression profiling with exon 1.0 ST arrays. We show that degraded RNA that is up to 25 years old, is suitable to identify subtle differences between subtypes within one specific cancer.

Other genome wide techniques are also available that can perform expression profiling on FFPE samples, including the DASL platform (Illumina). The platform chosen for this study was based on reports from literature (Linton et al., 2009; Linton et al., 2008), and it is beyond the scope of this manuscript to compare performance of both platforms. Although it is possible other platforms perform better on FFPE samples, our study demonstrates that sufficient information is stored in FFPE samples so that it can be used for expression profiling using exon 1.0 ST arrays. Our method allows molecular classification of archived clinical trial samples to evaluate the predictive and prognostic values of the molecular glioma clusters. Furthermore, it allows assignment of FFPE material of newly diagnosed patients to molecular clusters. Such assignment would allow clinicians to improve patients' diagnosis and would contribute to treatment decisions.

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3

SEGREGATION OF NON-P.R132H MUTATIONS IN *IDH1* IN DISTINCT MOLECULAR SUBTYPES OF GLIOMA

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ABSTRACT

Mutations in the gene encoding the isocitrate dehydrogenase 1 gene (*IDH1*) occur at a high frequency (up to 80%) in many different subtypes of glioma. In this study, we have screened for *IDH1* mutations in a cohort of 496 gliomas. *IDH1* mutations were most frequently observed in low grade gliomas with c.395G>A (p.R132H) representing >90% of all *IDH1* mutations. Interestingly, non-p.R132H mutations segregate in distinct histological and molecular subtypes of glioma. Histologically, they occur sporadically in classic oligodendrogliomas and at significantly higher frequency in other grade II and III gliomas. Genetically, non-p.R132H mutations occur in tumors with *TP53* mutation, are virtually absent in tumors with loss of heterozygosity on 1p and 19q and accumulate in distinct (gene-expression profiling based) intrinsic molecular subtypes. The *IDH1* mutation type does not affect patient survival. Our results were validated on an independent sample cohort, indicating that the *IDH1* mutation spectrum may aid glioma subtype classification. Functional differences between p.R132H and non-p.R132H mutated *IDH1* may explain the segregation in distinct glioma subtypes.

INTRODUCTION

Approximately 200.000 people worldwide are diagnosed with primary brain cancer each year (1). The most common type of primary brain tumor (~40%) are gliomas. Based on their histological appearance, gliomas can be divided into three distinct types of tumors according to the standard WHO classification (2): Astrocytic tumors (75%), pure oligodendroglial tumors (ODs) and mixed oligoastrocytic tumors (MOAs) (25%). Tumors are further classified into grades II, III (anaplastic) and IV (Glioblastomas, GBMs) depending on the number of malignant features present. Despite advances in neurosurgery, chemotherapy and radiotherapy the prognosis for most glioma patients remains dismal (3).

Recently, a genetic screen identified somatic mutations in the isocitrate dehydrogenase 1 gene (*IDH1* [OMIM 147700],) in glioblastomas (4). *IDH1* catalyzes the oxidative decarboxylation of isocitrate to α-ketoglutarate and uses NADP(+) as the electron acceptor (5-6). Additional analysis showed that *IDH1* mutations occur at a very high frequency (up to 80%) in all grade II and III gliomas (astrocytic, oligodendrocytic and oligoastrocytic) and secondary GBMs (4, 7-13). *IDH1* mutations do not occur at significant frequencies in other tumor types, with the notable exception of acute myeloid leukemia (AML) (12, 14-15). The reported mutations in *IDH1* all result in a reduced enzymatic activity towards its native substrate, isocitrate (9, 12, 16).

Mutations in the *IDH1* gene are heterozygous and virtually always affect only a single residue (arginine 132) which is replaced by a histidine in approximately 90% of tumors (c.395G>A resulting in p.R132H (7, 9-10, 12). However, non-p.R132H mutations in the *IDH1* gene (e.g. p.R132C) have been reported to accumulate at higher frequencies in histological subtypes of glioma (17), in astrocytomas of Li-Fraumeni patients (18) and in patients with AML (15). Distinct mutations within the same gene but affecting the same codon therefore appear to segregate in distinct tumor subtypes.

In this study, we demonstrate that non-p.R132H mutations specifically accumulate in distinct histological subtypes of glioma. Because histological classification of gliomas is subject to significant interobserver variability (19), we also used molecular markers (*TP53* [OMIM 191170], 1p/19q LOH and intrinsic molecular subtypes) to identify the glioma subtypes. Our results demonstrate that non-p.R132H mutations indeed segregate in distinct histological, genetic and molecular subtypes of gliomas

MATERIALS AND METHODS

Samples

Glioma samples were collected from five hospitals in the Netherlands (Erasmus MC, Rotterdam; UMCU, Utrecht; NCI and VUMC, Amsterdam; RUNMC, Nijmegen) from patients, operated from 1989-2009. Use of patient material was approved by the Institutional Review Board. All samples are listed in the supplementary data table 1. Histological diagnoses were made on formalin-fixed, paraffin-embedded Haematoxylin & Eosin sections and were reviewed by the neuropathologist (J.M.K.). Genomic DNA from snap frozen or formalin fixed, paraffin embedded tissue samples was isolated as described (20)2009; (21). Of the 496 glioma samples reported in this study, 247 were derived from a series of gliomas of all histologies (set A) (20), 54 from a series of low

grade astrocytomas (set B) (21), 24 were derived from samples operated in Rotterdam that were included in EORTC 26951 (set C) (22) and 171 novel glioma samples (set D). The *IDH1* status of 297 samples was described previously (20-21). *IDH1* (NM_005896.2, GI:28178824) mutational status of the remaining 199 samples was determined as described (14), see also supplementary data table 2.

1p/19q status was determined in 317 samples. 1p19q LOH was determined by fluorescent in-situ hybridization (FISH) (23), inferred from genotyping arrays or by microsatellite analysis. Microsatellites were amplified by PCR on 20 nanogram genomic DNA, using a fluorescently labeled forward primer and a reversed primer. Allelic losses were statistically determined as described and scored by two independent researchers (24). *TP53* (NM_000546.4, GI:187830767) status was determined in 169 samples. Primers and cycling conditions to determine the *TP53* mutation status are stated in the supplementary data table 2.

Statistical analysis

Comparison between frequencies of different groups was assessed using the Fisher exact test. The significance of *IDH1* mutation type was assessed by univariate and multivariate analysis using Cox Regression. Differences between Kaplan-Meyer survival curves were calculated by the Log-rank (Mantel-Cox) test. Survival time was defined as the period from date of surgery to date of death. If date of death was not available, date of last follow-up was used.

RESULTS AND DISCUSSION

Dataset

We have screened for mutations in *IDH1* in a large cohort of 496 glioma samples. In this dataset, a total of 246 mutations in the *IDH1* gene were identified (49.6%). The *IDH1* mutation frequency for all grades II and III gliomas was high and ranged from 49% in anaplastic oligoastrocytic tumors to 79% in low grade oligodendrogliomas (table 1). The frequency of genetic changes in our sample cohort is similar to reported by others (7, 12-13, 17, 25). The frequency of *IDH1* mutations in grade II gliomas (76%, n=110/144) was higher than in grade III tumors (58%,

Table 1. The frequency of *IDH1* (NM_005896.2, GI:28178824) mutations, LOH on 1p and 19q and mutations in *TP53* (NM_000564.4, 187830767) in different histological. ODII/ODIII: grade II/III oligodendroglioma, OAII/OAIII: grade II/III oligoastrocytoma, AII/AIII: grade II/III astrocytoma, GBM: glioblastoma. nd: not determined.

	n	p.R132H	p.R132X	*IDH1* wt	1p/19 LOH	no loss	nd	TP53 mut	TP53 wt	nd
ODII	43	34	0	9	21	9	13	0	6	37
ODIII	106	63	1	42	57	31	18	7	18	81
OAII	28	21	1	6	6	10	12	1	3	24
OAIII	39	15	4	20	3	23	13	8	2	29
AII	73	48	6	19	2	53	18	29	18	26
AIII	32	15	4	13	1	18	13	12	6	14
GBM	175	31	3	141	10	73	92	27	32	116
total	496	227	19	250	100	217		84	85	

n=102/177, P<0.001) (see also (10)). More specifically, low grade oligodendrogliomas (79%, n=34/43) and oligoastrocytomas (79%, n=22/28) have a higher *IDH1* mutation frequency then their corresponding grade III tumors (60%, n=64/106 and 49%, n=19/39 respectively P<0.05). A similar difference in frequency can also be extracted from at least two external sample cohorts (11, 17).

The observation of a higher mutation incidence in lower grade tumors has thusfar been described for only a few genes (see e.g. (26)). This differential frequency indicates that low grade tumors can accumulate distinct genetic changes and thus represent a different disease entity. Moreover, such differential frequency indicates that the *IDH1* mutation status can, at least to some extent, serve as a marker for low grade gliomas (see also (27-28)).

Non-p.R132H mutations in *IDH1* segregate in distinct glioma subtypes

The majority of mutations (92.3%) in the *IDH1* gene were heterozygous c.395G>A missense mutations that result in an arginine to histidine substitution on position 132 (p.R132H). However, 19/246 mutations (7.7%) were non-p.R132H mutations: Single nucleotide changes that result in different amino acid substitutions albeit on the same position. Similar to reported, c.394C>T (p.R132C) was the second most common mutation type in our sample cohort and was identified in nine gliomas. Other non-p.R132H mutations were p.R132S (n=5), p.R132G (n=3), p.R132L (n=1) and p.R132P (n=1).

When focusing on non-p.R132H *IDH1* mutations in our dataset, we observed that in classical oligodendrogliomas (combined grades II and III), only one out of the total of 98 *IDH1* mutations (1.0%) was non-p.R132H (figure 1). A significantly larger proportion of non-p.R132H mutations was present in oligoastrocytic tumors (5/41, P<0.01) and in astrocytic tumors (10/73, P<0.001). Significance remained within the different tumor grades (figure 1). These results indicate that, although non-p.R132H mutations are rare, they are not uniformly distributed across the different histological subtypes of glioma.

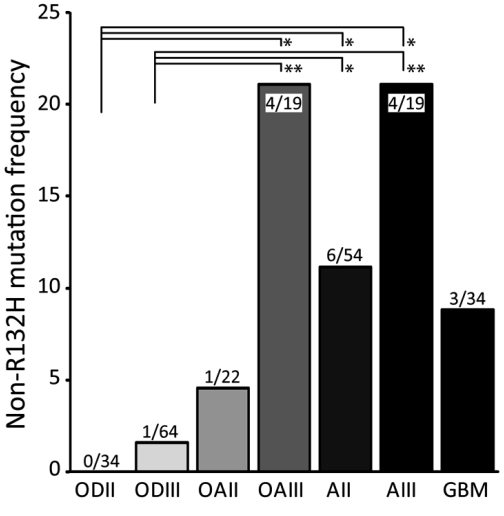

Figure 1. Distribution of non-p.R132H mutations in *IDH1* in different histological subtypes of glioma. Plotted is the relative frequency of non-p.R132H mutations in *IDH1* mutated histological subtypes of glioma. Absolute numbers for each subtype is stated in/above individual bars. For example, *IDH1* mutations are observed in 54 grade II astrocytomas. Of these, six are non-p.R132H. As can be seen, non-p.R132H mutations are sporadically observed in oligodendrogliomas and more frequent in oligoastrocytomas and astrocytomas. *: P<0.05, **: P<0.01, Fishers' exact test.

We aimed to confirm the differential distribution of non-p.R132H mutations using objective molecular markers. We first screened for combined LOH on 1p and 19q; chromosomal losses that are frequently observed in oligodendrogliomas and relatively rare in astrocytic tumors (29-30)). These losses are caused by an unbalanced translocation between chromosomes 1 and 19 [t(1;19)(q10,p10)] (31-32). In our entire dataset, 1p/19q LOH was determined in 317 (63.9%) samples. Of the tumors with combined 1p/19q LOH (n=100), a mutation in the *IDH1* gene was detected in 75 samples (75%). In these 75 samples, only one non-p.R132H mutation was identified (1.3%, figure 2). Conversely, in tumors that have retained 1p and/or 19q (n=217), the proportion of non-p.R132H mutations was significantly higher, 13/106 (12.3%, P<0.01). Non-p.R132H mutations therefore segregate in tumors that do not show combined 1p/19q LOH.

We then correlated the type of *IDH1* mutation to the *TP53* mutation status. *TP53* mutations are frequently observed in astrocytomas and secondary glioblastomas but relatively rare in oligodendrogliomas and primary glioblastomas (29, 33). In our entire dataset, *TP53* mutation status was determined in 169 samples (34.1 %). Of the tumors with a mutation in *TP53* (n=84), a mutation in the *IDH1* gene was detected in 65 samples (77.4%). Non-p.R132H mutations were identified in 12 of these 65 samples (17.9%, figure 2). Conversely, in tumors with wt *TP53* (n=85), the proportion of non-p.R132H mutations was significantly lower (3/54) (5.6%, P<0.05). Non-p.R132H mutations therefore accumulate in tumors with a mutation in the *TP53* gene.

Finally, we determined whether non-p.R132H mutations segregate in intrinsic, gene expression based-, molecular subtypes of glioma. In a recent study, we identified seven intrinsic glioma subtypes based on gene expression profiles of 276 gliomas of all histological subtypes and grades (20). As reported, *IDH1* mutations were most frequently observed in clusters 9 and 17 (27/40 and 22/32 respectively). When focusing on the non-p.R132H mutations (n=8) in this cohort, they were relatively rare in cluster 9 (n=1/27, 3.7%) and more frequent in cluster 17 (5/22, 22.7%, P=0.05). Other non-R132 samples were identified in clusters that have relatively few *IDH1*

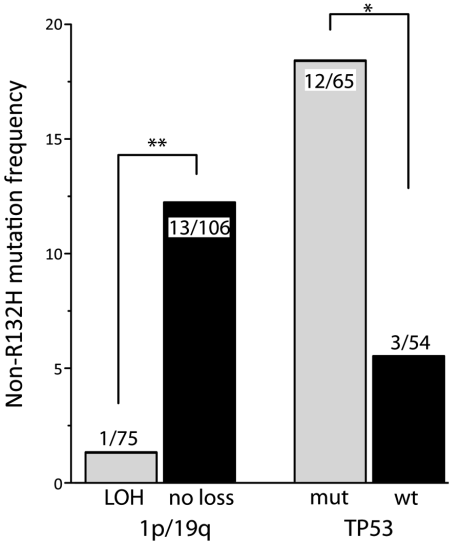

Figure 2. The frequency of non-p.R132H mutations in *IDH1* is correlated to distinct genetic changes in gliomas. Non-p.R132H mutations are more prevalent in gliomas that do not show combined LOH on 1p and 19q (left) and in gliomas with a *TP53* mutation (right). Absolute numbers for non-p.R132H mutations/total number of *IDH1* mutations are stated in/above the individual bars. *: P<0.05, **: P<0.01, Fishers' exact test.

mutations; one in cluster 0 and one in cluster 18 (20). Non-p.R132H mutations therefore appear not to be uniformly distributed across intrinsic molecular subtypes of glioma.

Validation of non-p.R132H segregation

The segregation of non-p.R132H mutations into distinct molecular subtypes of gliomas was validated on a recently described independent cohort of glioma samples (25). In this study, a total of 119 mutations in *IDH1* were identified across various histological subtypes, nine of which were non-p.R132H. Non-p.R132H mutations are clearly more prevalent in *IDH1* mutated astrocytomas (7/45) compared to *IDH1* mutated oligodendrogliomas (0/35, $P<0.05$). Furthermore, a significantly higher proportion of non-p.R132H mutations was observed in tumors with both *IDH1* and *TP53* mutation (8/65) compared to tumors with wt *TP53* (1/52, $P<0.05$). Finally, all non-p.R132H mutations were identified in tumors that have retained 1p and 19q (9/74 vs. 0/45, $P<0.05$). In summary, non-p.R132H mutations are more prevalent in *IDH1* mutated astrocytomas, gliomas with *TP53* mutation and gliomas that have retained 1p and 19q in this external dataset. These data therefore confirm our hypothesis that non-p.R132H mutations in *IDH1* are more prevalent in distinct subtypes of gliomas.

IDH1 mutation and patient survival

In gliomas, the presence of an *IDH1* mutation has been associated with prolonged survival within gliomas of similar malignancy grade (4, 10). In our dataset, the *IDH1* mutation status was also positively correlated to prolonged survival in the entire dataset (3.3 vs. 0.84 years, $P<0.0001$) and in glioblastomas (2.0 vs. 0.72 years, $P<0.001$). Survival of our sample cohort was similar to population based studies (see e.g. (34) and www.cbtrus.org). To determine whether the *IDH1* mutation type affects patient prognosis, we performed survival analysis on p.R132H vs. non-p.R132H mutated samples. Univariate analysis indicates that the type of *IDH1* mutation does not affect patient survival ($P=0.74$). The *IDH1* mutation type also does not affect patient survival within histological and/or molecular subtypes of glioma. For example, in the molecular glioma subtype that has a relatively large proportion of non-p.R132H mutations (Cluster 17) the median survival between p.R132H (n=17) and non-p.R132H (n=5) mutated samples is similar (3.2 and 3.7 years respectively). It should be noted that the low numbers of non-p.R132H mutations within defined subtypes may obscure relatively small differences. However, multivariate analysis (in which known prognostic factors tumor type, grade and age at diagnosis were included), also indicates that the type of *IDH1* mutation does not affect patient survival ($P=0.44$). Therefore, the *IDH1* mutation type does not largely affect patient survival within our sample cohort.

Non-p.R132H mutations in *IDH1* have been reported to accumulate at higher frequencies in distinct histological subtypes of gliomas (17) and other tumor types (15, 18, 35). However, histological diagnosis of gliomas is subject to significant interobserver variability (19). This study therefore provides evidence for the preferential accumulation of distinct types of *IDH1* mutations not only in histological subtypes of glioma but also using molecular markers such as *TP53*, 1p/19q LOH and intrinsic molecular subtypes. We demonstrate that non-p.R132H mutations are relatively frequent in astrocytic tumors, tumors with mutation in *TP53* and gliomas that do not show combined LOH on 1p and 19q. Although our results indicate that non-p.R132H mutations occur preferentially in tumors with distinct molecular features,

it remains to be investigated whether these changes are causal for- or associated with- this increased frequency. The sequence in mutation events may also influence the type of *IDH1* mutation acquired.

There are two possible explanations for the differential distribution of non-p.R132H mutations in glioma subtypes. First, it is possible that (epi) genetic differences between tumor types (e.g. differences in DNA repair due to e.g. *MGMT* [OMIM 156569] promoter methylation) result in the preferential accumulation of distinct mutations (see e.g. (36)). For example, *MGMT* promoter methylation is associated with transition type mutations in *TP53* in colorectal cancer and in gliomas (37-38). Indeed, a higher percentage of oligodendroglial tumors show *MGMT* promoter hypermethylation compared to astrocytic tumors (39-41). Differences in the *MGMT* promoter methylation status between glioma subtypes may therefore explain the differential distribution of non-p.R132H mutations in *IDH1*. However, the transition/transversion ratio in *IDH1* (i.e. p.R132H, C/p.R132S, G, L, P) between GBMs, astrocytic tumors (AII/III), oligoastrocytic tumors (OAII/III) or oligodendrocytic tumors (ODII/III) is similar (2/32, 5/68, 2/39 and 1/97 respectively). We also failed to observe a difference in transition/transversion ratio in the *TP53* gene in the respective histological subgroups (14/12, 26/13, 8/1 and 5/2). The transition/transversion ratio in the *TP53* gene between R132H and non-R132H mutated samples is also similar (39/15 and 8/3 respectively). Any possible difference in DNA repair between tumor types therefore is not reflected by a difference in the type of mutations acquired.

A second tentative possibility is that different mutations have different functional properties. For example, non-p.R132H mutations may show some residual activity towards isocitrate either in *cis* (activity of the mutated enzyme itself) or in *trans* (by influencing the activity of the remaining wt enzyme (16)). Alternatively, a recent study described that mutated *IDH1* is able to convert α-ketoglutarate into 2-hydroxyglutarate (42). Interestingly, different *IDH1* mutation types showed a differential activity towards α-ketoglutarate. Whether this differential activity can explain the differential distribution of specific types of *IDH1* mutations requires further detailed analysis.

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Supplementary Information accompanies the paper on the Human Mutation website (http://onlinelibrary.wiley.com)

4

A HYPERMETHYLATED PHENOTYPE IN ANAPLASTIC OLIGODENDROGLIAL BRAIN TUMORS IS A BETTER PREDICTOR OF SURVIVAL THAN *MGMT* METHYLATION IN THE EORTC 26951 STUDY

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ABSTRACT

Purpose: The *MGMT* promoter methylation status has been suggested to be predictive for outcome totemozolomide chemotherapy in patients with glioblastoma (GBM). Subsequent studies indicated that*MGMT* promoter methylation is a prognostic marker even in patients treated with radiotherapy alone, bothin GBMs and in grade III gliomas.

Experimental Design: To help determine the molecular mechanism behind this prognostic effect, we have conducted genome-wide methylation profiling and determined the *MGMT* promoter methylationstatus, 1p19q LOH, *IDH1* mutation status, and expression profile on a series of oligodendroglial tumors[anaplastic oligodendrogliomas (AOD) and anaplastic oligoastrocytomas (AOA)] within EORTC study26951. The series was expanded with tumors of the same histology and treatment from our own archive.

Results: Methylation profiling identified two main subgroups of oligodendroglial brain tumors of whichsurvival in the CpG island hypermethylation phenotype (CIMP +) subgroup was markedly better than the survival of the unmethylated (CIMP-) subgroup (5.62 vs. 1.24 years; P <0.0001). CIMP status correlated with survival, *MGMT* promoter methylation, 1p19q LOH, and *IDH1* mutation status. CIMP status stronglyincreases the predictive accuracy of survival in a model including known clinical prognostic factors such asage and performance score. We validated our results on an independent data set from the Cancer GenomeAtlas (TCGA).

Conclusion: The strong association between CIMP status and *MGMT* promoter methylation suggests that the *MGMT* promoter methylation status is part of a more general, prognostically favorable genome-wide methylation profile. Methylation profiling therefore may help identify AODs and AOAs with improved prognosis.

INTRODUCTION

Glioblastomas (GBM) are the most common and aggressive type of glial brain tumor (1, 2). GBMs in which the *MGMT* promoter is methylated show an improved survival when treated with temozolomide, a finding that has resulted in *MGMT* promoter methylation to serve as a predictive biomarker in GBMs (3, 4). The predictive effect of *MGMT* promoter methylation can be explained by a reduced ability to respond to DNA damage by alkylating agents for methylated tumors.

Two large randomized controlled trials that focused on patients with grade III glioma unexpectedly also revealed a prognostic effect of *MGMT* promoter methylation in patients treated with radiotherapy only (5, 6). Similarly, a retrospective survey in GBM suggests that *MGMT* promoter methylation also has prognostic significance in patients with GBM treated with radiotherapy only (7). The current understanding of the function of the *MGMT* protein does not explain the observed prognostic effect of *MGMT* promoter methylation in patients treated with radiotherapy only.

Methylation in cancer often occurs in the promoter regions of tumor suppressor genes (8, 9). Inactivation of gene expression by promoter methylation thus contributes to tumor formation as the second "hit" in tumor suppressor genes [Knudson 2-hit hypothesis (10)]. Several groups have therefore conducted genome-wide methylation profiling in GBMs (11) and astrocytomas (12). Importantly, whole-genome methylation profiling on GBMs can identify a subset of tumors that have a more favorable prognosis (11, 13). These tumors show an overall increase in DNA methylation at CpG sites (CIMP: CpG island methylator phenotype; ref. 13). Later studies showed that histologic subtype was associated with methylation class and *IDH1* mutation, and that GBMs generally show less overall CpG methylation than lower grade gliomas (14, 15). Identification of CpG methylation sites in gliomas may therefore identify genes involved in the initiation and/or progression of gliomas [see e.g., (16, 17)]. Moreover, an association between CIMP and *MGMT* promoter methylation may also provide an explanation for the prognostic effect of *MGMT* promoter methylation.

In this study, we conducted methylation profiling on a set of 68 anaplastic oligodendrogliomas (AOD) and anaplastic oligoastrocytomas (AOA) and correlated the results to molecular (*MGMT* methylation, *IDH1* mutation, LOH on 1p and 19q, and gene expression profiles) and clinical [overall survival (OS)] parameters. Fifty of these tumors were treated as part of the EORTC 26951 clinical trial of which the primary objective was to see if the addition of adjuvant PCV chemotherapy immediately after radiotherapy (59.4 Gy) would improve OS in patients with AOD or AOA (18, 19). Our results indicate that methylation profiling identified 2 main subgroups of anaplastic oligodendroglial brain tumors that highly correlate with survival, *MGMT* promoter methylation, 1p19q LOH, and *IDH1* mutation status. The strong association between CIMP status and *MGMT* promoter methylation suggests that the *MGMT* promoter methylation status in anaplastic glioma is part of a more general, prognostically favorable genome-wide methylation profile.

MATERIALS AND METHODS

Samples

Glioma samples were collected from EORTC study 26951 (n = 50) or the Erasmus MC tumor archive (n = 18). One additional control sample (normal adult brain) was obtained from the Dutch Brain Bank. Samples were collected immediately after surgical resection, snap frozen, and stored at -80°C. Clinical data are summarized in Supplementary Table S1. The number of samples chosen was based on the availability of 50 fresh frozen samples from the EORTC study 26951. Additional samples with similar clinical and treatment characteristics were included to increase power of the study whilst maintaining the predominance of EORTC 26951 samples. Part of the molecular data of the EORTC 26951 samples was previously reported (5). Survival time was defined as the period from date of surgery to date of death. Patients were censored at the date of last follow-up. Patients were eligible for EORTC study 26951 if they had been diagnosed by the local pathologist with AOD or AOA with at least 25% oligodendroglial elements according to the 1994 edition of the WHO classification of brain tumors; had at least 3 of 5 anaplastic characteristics (high cellularity, mitoses, nuclear abnormalities, endothelial proliferation, or necrosis); were between 16 and 70 years of age; had an Eastern Cooperative Oncology Group (ECOG) performance status of 0 to 2; and had not undergone prior chemotherapy or RT to the skull. Patients provided written informed consent according to national and local regulations for the clinical study and correlative tissue studies. For details of the EORTC study 26951 see reference (18). Additional samples from the Erasmus MC archive were selected on the basis of similar inclusion criteria as the EORTCstudy 26951: Histologic diagnosis AOA or AOD, age 16 to 70, and patients having undergone similar treatment as the patients in EORTC 26951 (radiotherapy with or without chemotherapy) and with similar clinical characteristics. For research on these samples approval has been obtained from the Erasmus MC institutional review board. Sample collection time for EORTC 26951 samples is from 1998 to 2002. Sample collection time for non-EORTC 26951 samples is from 1989 to 2004. Mean follow-up time for all samples is 96.4 months. Survival data are collected till death or, for censored patients, either until lost to follow-up or to date.

Nucleic acid isolation, cDNA synthesis, and array hybridization

Genomic DNA was isolated from 5 to 40 cryostat sections of 40 mm thickness using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions. Quality was assessed on agarose gel electrophoresis, in which a dominant high molecular weight DNA species should be present. For methylation profiling, 1 mg of genomic DNA was subjected to bisulphite modification using the EZ DNA methylation kit (Zymo research company). Bisulphite-converted DNA was then hybridized to Illumina Infinium Human Methylation27 arrays (Illumina) by Service XS (Leiden) according to standard Illumina protocols. Infinium Human Methylation27 arrays interrogate 27,578 Cp Gsites across 14,476 genes. Robustness of sample processing was assessed using 2 biological replicates, and resulted in replicates clustering very tightly to each other. These replicates were not included in any analysis. Data are available on request.

Unsupervised clustering analysis

Clustering was conducted using the hierarchical ordered partitioning and collapsing hybrid (HOPACH) algorithm (20). This clustering was used to identify molecular subgroups in gliomas

on 2,000 CpG sites with highest variance. Virtually identical clusters were identified using all or the 500, 1,000, 5,000, or 10,000 CpG sites with highest variance.

Nonparametric bootstrapping was used to estimate the probability that each sample belongs to a cluster (i.e., fuzzy clustering) and thus determine cluster stability. Samples were assigned to a cluster when at least 50% of bootstraps allocated the sample to that specific cluster.

Cluster validation was conducted by representing each of the molecular clusters in our data set by its centroid and classifying external samples to their nearest centroid. Samples belonging to the original data set that were not assigned to one of the 2 large clusters were similarly assigned to the nearest centroid. Robustness of external validation was estimated with the in-group proportion cluster quality measure (21).

Molecular analysis

The *IDH1* mutation status was determined by direct sequencing as described (22, 23). 1p19q LOH was determined previously by FISH (19). Probes used were D1S32 (for 1p36), pUC1.77 (for centromere 1), equivalent amounts of bacterial artificial chromosome (BAC) RPCI 11-959O6, 11-957I1, and 11-153P24 (for 19p), and BAC 426G3 (for 19q). Expression profiles were determined previously (20). The *MGMT* methylation status was determined on the snap-frozen DNA used for methylation profiling, using the methylation-specific multiplex ligation–dependent probe amplification (MS-MLPA) assay (ME011, MRC Holland) essentially as described (24). This assay uses a methylation-sensitive restriction enzyme (HhaI) and contains 3 probes within the *MGMT* promoter/gene. PCR products were separated by capillary gel electrophoresis (ABI Prism 3130 x l; Applied Biosystems) and quantified by use of the GeneMarker Software version 1.7 (SoftGenetics).

The MS-MLPA results were normalized by dividing the peak height for each *MGMT* probe signal by the mean peak height for 8 control fragments within the same sample. The fraction of methylated *MGMT* promoter DNA was calculated by normalized values of each probe of HhaI-digested DNA divided by normalized values of corresponding undigested DNAs (24). A sample was considered to have a methylated *MGMT* promoter when the average fraction of the 3 *MGMT* probes was more than 0.25.

Statistical analysis

Spearman correlation coefficient was used to assess the strength of the association between parameters. The Fisher exact test was used in the comparisons of patient and disease characteristics between subgroups. The Kaplan–Meier technique was used to assess OS and the log-rank test was computed to compare the survival of subgroups. The Cox regression was used for multivariate OS analysis including age, performance status, *IDH1* mutation status, 1p19q LOH, *MGMT* methylation status, and CIMP. The forward stepwise method was used to select variables with independent prognostic value.

The models' predictive accuracy was assessed by the Harrel c-index. The c-index is the probability that for 2 patients chosen at random, the patient who had the event first had a higher probability of having an event according to the model. C-index equal to 0.50 represents agreement by chance; c-index = 1.0 represents perfect discrimination. In the absence of an independent data set, the bootstrap technique was used to obtain a c-index corrected for

optimism, that is, the model overfitting the data. No adjustment for multiple testing was conducted in this exploratory analysis. The power of multivariate analyses was limited and P values were presented to point out what the main effects or differences are, but not to provide definitive conclusions. The descriptive comparisons of the c-index among different multivariate models allow assessing the relative contribution of CIMP to the classification of patients for their survival. SAS version 9.2 (SAS Institute Inc.) was used for all statistical analyses except the computation of the c-index which was conducted with R software.

RESULTS

Samples

Methylation profiling was conducted on a total of 68 gliomas and 1 control brain. Two biological replicates (i.e., experiments carried out in parallel on independently isolated DNA) were also included. Patients and tumor characteristics are summarized in Table 1, and detailed in Supplementary Table S1. All glioma samples were diagnosed either with AOD or AOA by an expert neuropathologist (J.M. Kros). Fifty of the glioma samples were treated as part of the EORTC study 26951 (18), the remaining 18 samples were treated within the Erasmus Medical Centre. Sixteen of these patients received treatment paradigms similar to that used in the EORTC study 26951 (14 radiation therapy and 2 chemoradiation). One patient received PCV chemotherapy only. One patient refused further treatment after surgical resection; this sample was omitted from any analysis involving clinical parameters.

Our cohort of samples both from EORTC 26951 and Erasmus Medical Centre did not differ from the entire EORTC 26951 cohort (368 patients) with respect to age, performance status, sex, diagnosis (AOD or AOA), *IDH1* mutation, 1p19q LOH, and OS (Supplementary Table S2). *MGMT* promoter methylation was slightly less frequently observed (63.5% vs. 78.7%) in the present series than the entire EORTC data set.

Methylation profiling

A frequency distribution of the percentage of CpG methylation across our sample cohort shows an approximately bimodal distribution: predominantly unmethylated CpG sites (by far the most frequently observed) and predominantly methylated CpG sites (Supplementary Fig. S1). Few CpG sites show an intermediate percentage of methylation (between 10% and 75%). Unsupervised hierarchical clustering (HOPACH) using the 2,000 most variably methylated CpG loci identified 14 distinct molecular methylation subtypes (list available on request). Virtually identical subgroups were identified using all CpG loci, or the most variable 500, 1,000, 5,000, or 10,000 CpG loci (data not shown). Nonparametric bootstrapping (Fuzzy clustering) confirmed that samples indeed belonged to a defined methylation cluster.

We then focused our analysis on the 2 largest clusters that contain more than 10 samples and reassigned all samples to one of these 2 clusters. Cluster stability was shown as only 2 samples (samples 29 and 54) were assigned to a cluster that was different from the original cluster. The 2 large subgroups are defined by either predominantly methylated or unmethylated CpG islands (Fig. 1). Tumors with a high frequency of CpG island methylation are further referred to as CIMP+ tumors.

CIMP status was strongly correlated to other molecular markers such as *IDH1* mutation (rho = 0.75; P < 0.001), 1p19q LOH (rho = 0.54; P < 0.001), and *MGMT* promoter methylation (rho = 0.61; P < 0.001; see also Supplementary Table S3). CIMP status was not correlated to clinical parameters such as age (rho = -0.06; P = 0.65), sex (rho = 0.23; P = 0.064), performance status (rho = 0.0008; P = 0.99), or histologic diagnosis (rho = - 0.01; P = 0.91).

Univariate analysis indicates that CIMP status [P < 0.0001; HR = 0.23 (0.12, 0.43)] is a strong prognostic factor for OS (Fig. 2). Other prognostic factors include *IDH1* mutation status [P < 0.0001; HR = 0.30 (0.16, 0.57)], 1p19q LOH [P = 0.004; HR = 0.34 (0.16, 0.74)], *MGMT* promoter methylation [P = 0.016; 0.49 (0.27, 0.89)], and performance status [P = 0.032; HR = 1.55 (1.04, 2.33)]. In this series, age (P = 0.199) and histopathologic diagnosis (P = 0.67) are not correlated to OS. When stratified into the different treatment arms, CIMP+ tumors showed an increase in OS in RT-PCV–treated tumors compared with RT only (7.53 vs. 4.33 years, see also Supplementary Fig. S2). This increase in OS was, however, not significant (P = 0.45). No difference in OS can be observed in CIMP- tumors (1.0 vs. 1.21 years). Numbers are, however, too low to draw firm conclusions.

Multivariate analyses

Two models were fit with different variables grouping atentry and models were compared by their c-index. Final models of the predictive accuracy for patient survival are shown in Supplementary Table S4. Details of the full analysis are stated in Supplementary Table S5. Our first model included all biological factors (*IDH1* mutation,1p19q LOH, and *MGMT* methylation) and histology. In this model, *IDH1* mutation status (P = 0.0011) and 1p19q LOH (P = 0.06) were the most influential factors (c-index = 0.70). When CIMP was included in this model, CIMP status was the only factor selected (P < 0.0001; c-index = 0.71) with comparable predictive accuracy to the model with *IDH1* mutation status and 1p19q LOH (0.71 vs. 0.70).

Our second model included all biological factors (*IDH1* mutation, 1p19q LOH, and *MGMT* methylation), clinical factors (age and performance status), and histology. In this model, performance status (P = 0.006), age (P = 0.002), *IDH1* mutation status (P < 0.0001), and 1p19q LOH (P = 0.06) were selected (c-index = 0.74). When CIMP was included in this model, CIMP status was not selected for.

Integrated analysis with expression profiling

We next integrated our methylation data with gene expression profiles to determine the effect of methylation on gene expression. Expression and methylation data were available for 27 samples included in this study and were integrated on the basis of "gene symbol" as indicated by the respective annotation files (20). Results are shown in Fig. 3. In general, few genes (994 of 2,965, 33.5%) that are highly methylated (>75% methylation) are expressed (RMA levels > 6.5). In contrast, genes that are unmethylated (<10% methylation) show a much wider range in gene expression levels, though most (7,476 of 9,422, 79.3%) are expressed at RMA levels exceeding 6.5. The absence of methylation of genes therefore appears to be permissive for gene expression. An unmethylated gene is not necessarily expressed at high levels as a multitude of additional molecular mechanisms are involved in gene expression.

We have recently conducted expression profiling on a large cohort of gliomas and identified 7 "intrinsic molecular subtypes" that correlate better with survival than histology (20). We determined whether these "intrinsic molecular subtypes" correlate with the tumors'

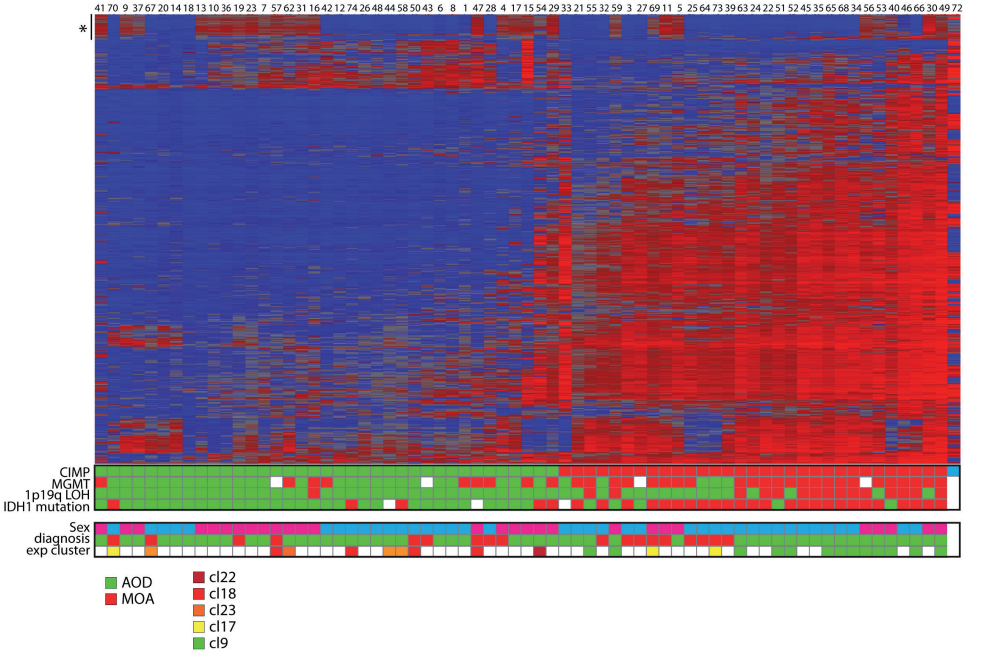

Figure 1. Unsupervised clustering based on the 2000 most variably methylated CpG loci identifies two subgroups of gliomas. Subgroups can be separated based on predominantly unmethylated or methylated CpG loci. Sample numbers are shown above the graph. CIMP status as determined by HOPACH clustering (red= CIMP+, green= CIMP-), *MGMT* methylation status (red= *MGMT* methylated, green= unmethylated, white= not determined), 1p19qLOH (red= LOH, green = retention of either or both arms) and *IDH1* mutation status (red= mutated, green= wildtype) is show below the figure. A second bar below the figure indicates sex (blue= males, pink= females), diagnosis (red= anaplastic oligoastrocytoma, green= anaplastic oligodendroglioma) and molecular cluster based on gene expression profiling (darkred= cl22; red= cl18; orange= cl23; yellow= cl17 and green= cl9). * denotes genes associated with gender (see supplementary figure 3).

CIMP status. In our study, all but one CIMP+ tumors are in the prognostically favorable molecular clusters 9 (characterized by a high incidence of 1p19q LOH and *IDH1* mutation) and 17 (characterized by a high incidence of *IDH1* and *TP53* mutations). Conversely, tumors within the prognostically unfavorable molecular clusters 18 (characterized by a high frequency of *EGFR* amplification) and 23 (no clear molecular marker identified) are all CIMP- ($P < 0.0001$, the Fisher exact test). Only one sample was available of cluster 22 (secondary tumors and high frequency of *IDH1* mutations), which clustered among the CIMP- tumors. Prognostically favorable tumors are therefore identified both by methylation profiling (CIMP+) and expression profiling (clusters 9 and 17). However, methylation profiling does identify fewer prognostically relevant molecular subgroups than expression profiling (20, 25, 26).

Cluster validation

We next aimed to determine whether our CIMP+ and CIMP- methylation clusters can also be identified in anexternal data set. We therefore validated our methylation clusters on the

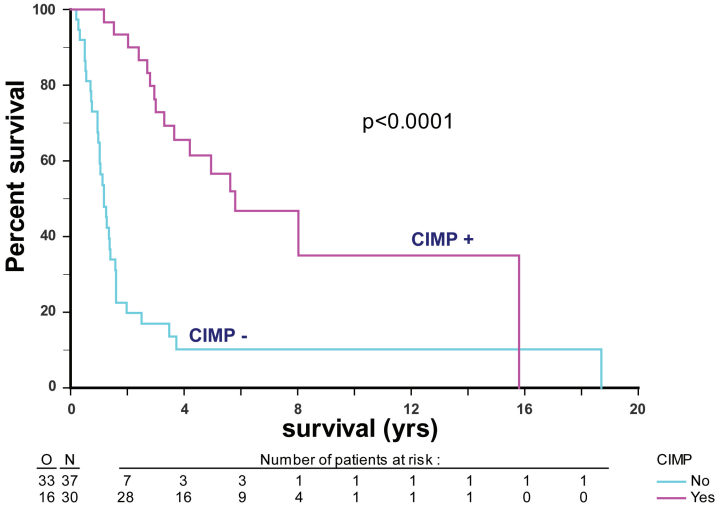

Figure 2. Kaplan Meyer survival curves based on CIMP status. Gliomas that are CIMP+ have a significantly better survival than CIMP- gliomas.

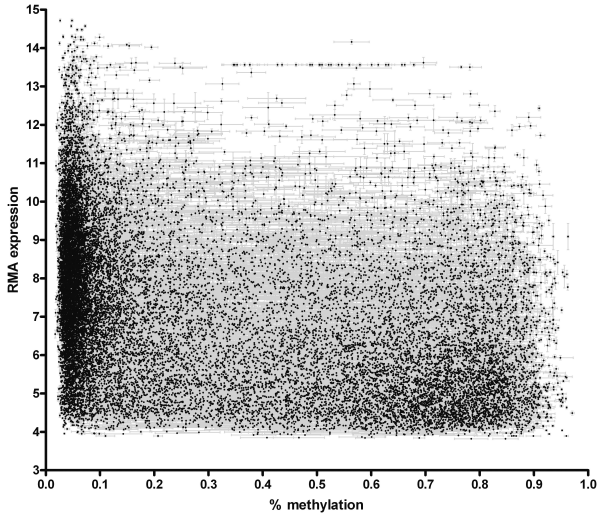

Figure 3. Integrated analysis of CpG methylation and expression. As can be seen, most genes that are methylated are expressed at RMA levels <6.5 whereas genes that are unmethylated (<10% methylation) show a much wider range in gene expression levels. Values are +/- SE both for expression and methylation.

methylation data from the Cancer Genome Atlas (TCGA) external data set (assessed December 2010) containing GBMs only (13). The "clusterRepro" R-package (see methods) was used to classify eachTCGAsample to one of the 2 clusters (CIMP+ or CIMP-) identified in the present study. Of the 269 samples included in this study, 245 were assigned to the CIMP- cluster, the

remaining 24 samples were assigned to the CIMP+ cluster. Survival data were available for 190 of these samples of which 176 wereassigned to the CIMP- cluster and 14 to the CIMP+ cluster. Similar to our data set, the CIMP+ tumors had a significantly better survival than the CIMP- tumors (Fig. 4; P = 0.0001).

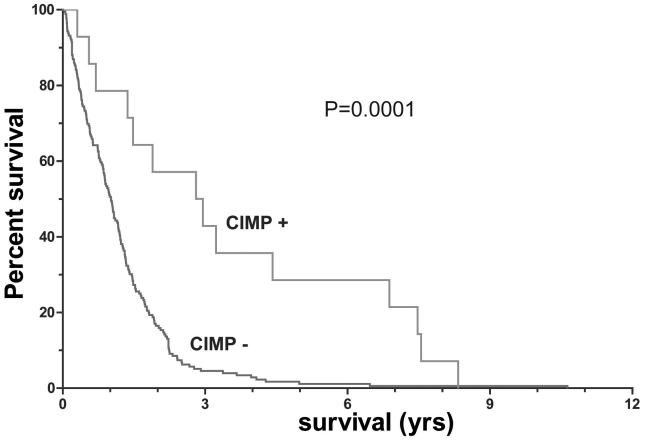

Figure 4. Validation of the prognostic value of methylation subgroups on an external dataset. Of the 269 samples included the TCGA study, 245 were assigned to the CIMP- cluster, the remaining 24 samples were assigned to the CIMP+ cluster. Survival data was available for 190 of these samples of which 176 were assigned to the CIMP- cluster and 14 to the CIMP+ cluster. Similar to our dataset, CIMP+ tumors had a significantly better survival compared to the CIMP- tumors

In summary, our CIMP+ and CIMP- methylation clusters can be identified in an external data set and, similar to our data set, these methylation clusters are prognostically relevant.

These results therefore validate our molecular clustering approach.

CIMP statuswas originally defined in gliomas by the TCGA (13). We have therefore also assigned our data set according to the TCGA-defined CIMP status based on 1,503 CpG sites as reported (13). When the 68 samples of current study are assigned to one of these clusters, the CIMP status remained identical in 64 of 68 samples (Supplementary Table S1) with highly concordant survival (Supplementary Fig. S3).

We next used samples of the TCGA to validate the association between CIMP status and "intrinsic molecular subtype." For 217 samples of the TCGA data set, paired expression and methylation data are available. Each sample was assigned to a defined gene expression–based intrinsic subtype as described previously (20). When integrating the molecular expression clusters with methylation, all but one (8 of 9) samples within the CIMP+ subgroup were found in the prognostically favorable cluster 17 (*IDH1* and *TP53*) and all but one 172 of 173 CIMP- tumors are within prognostically unfavorable tumors (clusters 18 and 23). Both CIMP+ (9 of 35) and CIMP- (26 of 35) tumors are found in cluster 22, which has unfavorable prognosis but contains secondary GBMs and a high percentage of tumors with *IDH1* mutation. This observation largely confirms our hypothesis that prognostically favorable/unfavorable tumors can be identified both by methylation and expression profiling.

DISCUSSION

In this study, we have identified 2 prognostically different molecular subgroups of anaplastic oligodendroglioma based on methylation profiling. These subgroups can be separated on overall CpG island methylation: CIMP+ tumors generally have a more favorable outcome than CIMP- tumors. CIMP was the only factor selected when all biological factors were included. Predictive accuracy was slightly higher in the model with CIMP alone. CIMP status was not selected in a model including all biological factors (*IDH1* mutation, 1p19q LOH, and *MGMT* methylation), clinical factors (age and performance status), and histology. The prognostic value of CIMP status could be validated on an external data set from the TCGA.

Our results indicate that CIMP status is a strong predictor of survival in AODs and AOAs. Other prognostic markers in gliomas include 1p19q LOH (27), *IDH1* mutation status (6, 28, 29), and *MGMT* methylation status (3, 6, 28) or whole-genome approaches such as gene expression profiling (20, 25, 26, 30) and, similar to reported in this study, methylation profiling (13–15). These prognostic markers may help classify gliomas as histologic classification is troublesome and subject to interobserver variation (31– 34).

CIMP was first identified in colorectal cancer in 1999 (35). In this study, colorectal cancers could be distinguished on the basis of low or high levels of tumor-specific methylation, the latter being referred to as the CpG island methylator phenotype (CIMP). CIMP has been identified in several other cancer types including gastric, lung, liver, ovarian, and leukemias (36). Although CIMP+ gliomas are associated with a more favorable prognosis, a high methylation index is associated with a reduced progression free or OS in many other cancer subtypes, including bladder cancer, esophageal adenocarcinoma, neuroblastoma, ovarian cancers, and leukemia [see (36) and references therein]. Furthermore, in colorectal cancer, CIMP is associated with *KRAS* and *BRAF* mutations and negatively associated with *TP53* mutations and chromosomal instability (37–39). In gliomas, CIMP status appears to be positively correlated with *TP53* mutations: astrocytic grade II and III tumors often are CIMP+, and these histologic subtypes have a high percentage of *TP53* mutations (13, 40). Two recent papers have shown a more causal effect of *IDH1* mutation on chromatin remodeling and CIMP status (41, 42). In thepresent study, however, not all *IDH1* mutated tumors are CIMP+ (Fig. 1), which indicates that *IDH1* mutation status is not the only determinant for CIMP status.

CIMP has been associated with increased (protein) expression of DNA methyl transferase [*DNMT*; e.g., see ref. 43]. As a result, many genes involved in tumor initiation and or progression become methylated in CIMP+ tumors (44). Reversal of CIMP status may thus provide a potential method in the treatment of CIMP+ gliomas. Aberrant methylation in tumors may be reversed by treatment of *DNMT1* inhibitors such as decitabine (9, 44). However, in our expression profiling data, the prognostically favorable tumors do not show an increase in *DNMT1*, 3A, or 3B (data not shown). It therefore remains to be determined whether *DNMT1* inhibition is an effective treatment for gliomas.

In summary, our data using mainly samples that were included in the large, prospective, randomized phase III EOTRC study 26951 show the association of CIMP status and prognosis in anaplastic oligodendroglial tumors, and their close association with 1p19q co-deletion, *MGMT* promoter association, and *IDH1* mutations. The strong association between CIMP status and *MGMT* promoter methylation suggests that the *MGMT* promoter methylation status is part of a more general, prognostically favorable genome-wide methylation profile. This

finding appears to explain the observations of the improved outcome in patients with *MGMT* promoter methylated tumors treated with radiotherapy only. Thus, in anaplastic glioma, *MGMT* promoter methylation may well be an epiphenomenon of genome-wide methylation. Methylation profiling may thus be helpful for identification of patients with AOD or AOAs with improved prognosis.

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5

A MOLECULAR SUBTYPE OF GLIOMA CONSISTS OF TWO DISTINCT TUMOR ENTITIES WITH MARKEDLY DIFFERENT CLINICAL OUTCOMES

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Submitted.

ABSTRACT

Gene expression profiling is a powerful approach to identify molecular subtypes in cancer. It is generally believed that each "intrinsic subtype" represents a distinct tumor entity that requires its own unique treatment paradigm. Here, we demonstrate that a specific intrinsic subtype of glioma consists of two different tumor types with remarkably different outcomes. Tumors of this subtype can be separated by their genomic, but not by their epigenomic, aberrations: Patients that survive have a near diploid tumor genome apart from a tandem duplication on 7q34. In contrast, most non-survivors are near tetraploid and a near tetraploid primary cell culture from one of these tumors has been established. All tumors are wt for *IDH1, TP53* and *BRAF*. Eventhough pathology is also able to separate the two subtypes, classification based on histology is difficult as similar features are observed in both. Indeed, our screen for genomic aberrations has led us to identify at least one patient with highly favorable prognosis despite poor histological characteristics. The different outcomes (tetraploidy and long term survival) are confirmed in the TCGA and REMBRANDT external datasets. In summary, intrinsic tumor subtypes often mark molecularly similar tumors that have similar genetic changes and therefore may be clinically relevant. However, our study demonstrates that at least one intrinsic subtype is highly heterogeneous and thus that care should be taken when using intrinsic subtypes to screen for targeted therapies.

INTRODUCTION

Unsupervised analysis of gene expression profiling in cancer is used to identify different molecular subtypes that sometimes are hard to separate by their histological characteristics [1-3]. Often, these "intrinsic subtypes" share similar genetic aberrations, and therefore form a basis to screen for targeted therapies [3, 4].

In a previous study we performed expression profiling on a cohort of 276 gliomas of all histological subtypesin which we identified seven distinct subtypes of glioma [5]. In general, the intrinsic molecular classification was a better predictor of survival than histology and genetic changes segregate in the different subtypes [5, 6]. However, in one distinct molecular subtype (*Cluster 16*) pilocytic astrocytomas (PAs) clustered among gliomas of higher grades and with much poorer prognosis. This seems peculiar since PAs show a distinct biological behaviour with much longer survival times (5-year survival rate of 96% after surgical resection) than higher grade gliomas [7, 8]. Indeed, all PA patients are still alive to date, while most patients diagnosed with other glioma subtypes died within a few years. Validation with external datasets (GSE12907) confirmed that PAs are virtually always assigned to this specific molecular cluster. However, the TCGA (The Cancer Genome Atlas [9]) and REMBRANDT (the Repository for Molecular Brain Neoplasia Data [10]) datasets also contained a small subset of higher grade gliomas that are also assigned to this subtype.

The aim of present study was to perform a detailed analysis this specific intrinsic subtype of glioma. Review of the pathological reports indicates that these tumors share similar characteristics; pilocytic features are recognized by the pathologist in many tumors of this intrinsic subtype, regardless of clinical outcome. However, tumors of this intrinsic subtype can be separated into two clinically relevant subtypes by their genomic aberrations. Such separation could not be made by genome-wide methylation profiling. Our results have implications for individual patients that have been diagnosed with a histologically malignant glioma subtype, but who can be identified as a long-term survivor using these molecular profiling techniques. Our data also indicate that tumors of the same intrinsic subtype can harbor different tumor subtypes and thus that intrinsic subtyping should be treated with caution when using it to screen for targeted therapies.

MATERIALS AND METHODS

Patients and samples

Fresh frozen (FF) tumor samples from patients were collected from the Erasmus MC tumor archive from patients operated from 1989-2005. Samples were collected immediately after surgical resection, snap frozen, and stored at -80°C. Four matched formalin-fixed paraffin-embedded (FFPE) tissue blocks were also included for Fluorescence In-Situ Hybridization (FISH) analysis and immunohistochemistry. Use of patient material was approved by the Institutional Review Board. The medical history of all patients in our dataset was available as described [5]. Samples were assigned to molecular *Cluster 16* using the cluster-repro package as described [11].

Medical history from REMBRANDT(National Cancer Institute (2005), http://rembrandt.nci.nih.gov, Assessed December 2008) and the TCGA samples were obtained via the respective repositories [5, 9, 10].

Molecular *Cluster 16* is a relatively small intrinsic subtype of gliomas. It originally contains 6 PAs, 1 astrocytoma (A) grade II, 1 A grade III, 1 oligodendroglioma (OD) grade III, and 1 GBM. In this study we also included one sample that was assigned to *Cluster 16* (sample 17) derived a previous study [12] and one sample that was recently profiled (EGT 16). For survival analysis we also included four samples (242, 300, 628 and 631) that were originally assigned to *Cluster 0* (a cluster containing all control samples), but are assigned to *Cluster 16* when performing cluster-repro without *Cluster 0*. This was done because lower grade samples are diffuse and often contain a significant proportion of control brain tissue. Omitting *Cluster 0* therefore forces all tumor samples to cluster into its nearest subtype. It should be noted differences in survival time between PAs and non-PAs remain similar without these samples. This way, a total of 16 samples are assigned to *Cluster 16*. Patient characteristics are described in table 1.

Table 1. Patients characteristics

Db No	Histological diagnosis	age at diagnosis	Survival	Status
451	astrocytoma grade II	24	9,5	alive
710	pilocytic astrocytoma	34	14,3	alive
711	pilocytic astrocytoma	37	14,4	alive
224	pilocytic astrocytoma	16	11,2	alive
300	pilocytic astrocytoma	22	6,5	alive
308	pilocytic astrocytoma	12	20,2	alive
709	pilocytic astrocytoma	18	17,2	alive
712	pilocytic astrocytoma	32	13,9	alive
362	glioblastoma	64	2,7	dead
351	oligodendroglioma grade III	79	1,6	dead
EGT 16	glioblastoma	68	1,3	dead
446	astrocytoma grade III	50	0,4	dead
17	mixed oligoastrocytoma grade III	47	1,0	dead
242	glioblastoma	65	0,4	dead
628	astrocytoma grade II	50	7,2	dead
631	glioblastoma	55	0,0	dead

Microarray data & SNP chip data

DNA and RNA were extracted from fresh frozen material using the Qiagen Allprep DNA/RNA mini Kit. Sample labeling and array hybridization on single-nucleotide polymorphism (SNP) 6.0 arrays using the extracted DNA was performed by AROS Applied Biotechnology AS (Aarhus, Denmark) according to standard Affymetrix protocols. SNP 6.0 analysis was performed using Affymetrix Genotyping Console software. SNP 250k Nspl (Affymetrix) data of 21 samples that were included in previous studies were collected for analysis [5, 13].

Methylation profiling was performed as described [14]. In brief, 1 μg of genomic DNA was subjected to bisulphite modification using the EZ DNA methylation kit (Zymo research company,

Orange, CA) and hybridized to IlluminaInfinium Human Methylation 27 arrays (Illumina, San Diego, CA). Arrays were hybridized by Service XS (Leiden, the Netherlands) according to standard Illumina protocols. Snap frozen DNA was available from eight *Cluster 16* samples.

A primary cell culture of a surgically resected tumor sample (EGT16) was established and propagated as described [15].

Molecular analysis

Detection of *BRAF* V600E mutations was performed by PCR analysis using primers as described [16]. *TP53* mutation status was determined using the primers and conditions as describedand by immunohistochemistry [12]. To perform immunohistochemistry for *TP53*, sections of 10 µm thick were cut from each of the four FFPE blocks. *TP53* immunohistochemistry was performed as described [17]. FISH analysis was performed using probes for the centromeres of chromosome 1, 2 and 7, and for *EGFR* (Kreatech Diagnostics) and performed as described [18].

Statistical analysis

Differences between Kaplan-Meier survival curves were calculated by the log-rank (Mantel-Cox) test.

RESULTS

Cluster 16 samples contain pilocytic features

Molecular *Cluster 16* is a relatively small intrinsic subtype of gliomas. Despite its small size a dramatic difference in survival between patients was observed: All seven patients with a PA were all still alive at the time of writing this article (survival 6.5-20.2 years), while 8/9 patients with non-PAs had died within 0.5-7.2 years (table 1). The only patient with a non-PA (an astrocytoma grade II) who remains alive has a survival time of 9.5 years (figure 1). The average age at diagnosis of *Cluster 16* patients is 42 years. However, patients diagnosed with a PA are much younger (24 years, consistent with the age distribution of this tumor subtype) than patients with any other diagnosis in this cluster (56 years, P= 0.0012). Interestingly, the only patient not diagnosed with a PA that remains alive to date was diagnosed as a young adult (24 years).

To further investigate this molecular subtype, we performed a detailed analysis of the original pathology reports. We observed that several of the non-PAs contained pilocytic features. The word "pilocytic" was in found in the pathology report in 3/8 of the non-PAs. One glioblastoma (GBM) was a recurrence from a PA diagnosed two years earlier, another tumor is described as a "reasonably characteristic image of a pilocytic astrocytoma" and the third as "astrocytoma with focal pilocytic features". Pilocytic features were specific for tumors in *Cluster 16*; there were no pilocytic features identified in any of 60 samples of the other molecular subtypes. Tumors of *Cluster 16* therefore are not only molecularly similar, many also share similar histological features.

Genotyping separates survivors from non-survivors

We performed genotyping to analyze the molecular differences between tumors of this cluster. Three of five of the examined *survivors* showed an amplification of chromosome 7q34, consistent with a tandem duplication which results in the *KIAA1549-BRAF* fusion gene (figure 2) [19]. It should

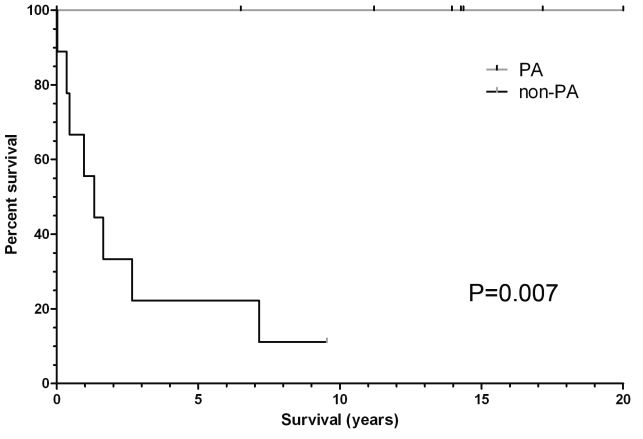

Figure 1. Samples of a single molecular subtype show dramatic differences in overall survival when separated by histological diagnosis. Within a defined molecular subtype of glioma (Cluster 16), a large difference in survival between patients diagnosed with a PA or other diagnosis is observed. All patients with a PA were all still alive at the time of writing this article (survival 6.5-20.2 years) whereas 8/9 patients with non-PAs had died within 0.5-7.2 years.

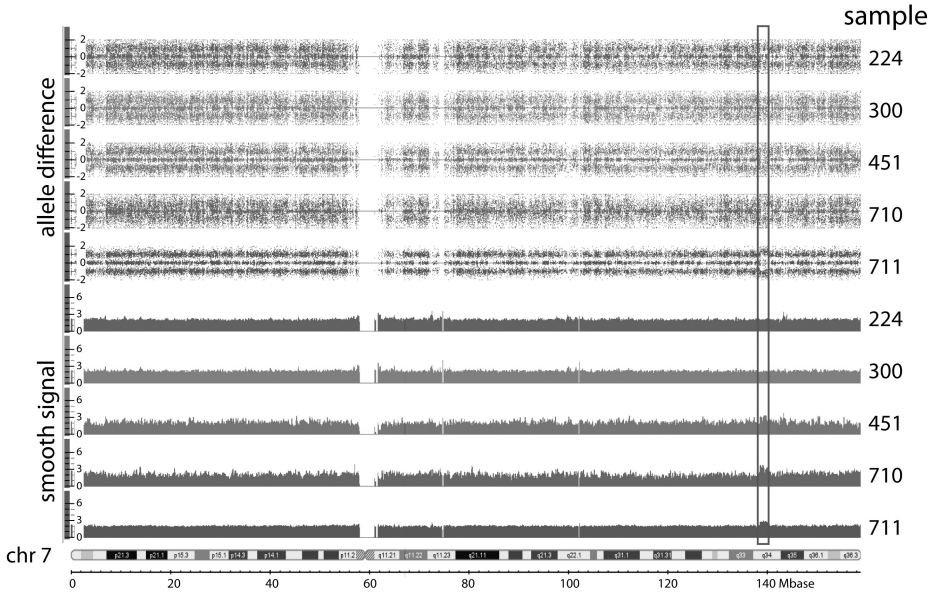

Figure 2. Amplification of chromosome 7q34 in surviving patients. SNP 6.0 data of five survivors show an amplification of chromosome 7q34 in three. Amplification on 7q34 is associated with an increase in the smooth signal plots (bottom traces) and, on the allele difference plots (top traces) the appearance of a fourth 'band' (each band representing either AAA, AAB, ABB and BBB alleles) concomitant with a disappearance of a band on the '0' line (the AB allele in a diploid situation). This amplification is consistent with a recurrent tandem duplication identified in PAs that results in a *KIAA1549-BRAF* fusion gene. This amplification is also reflected by the allele difference which is also aberrant for this exact location.

be noted that *KIAA1549-BRAF* fusion proteins are less frequent in adult than in child PAs [20]. This amplification is common for PAs and was not seen in any of the non-survivors. Apart from this single genetic change, no other change was identified in any of the survivors.

All of the examined *non-survivors* showed many genetic changes including *EGFR* amplification and deletion of the *CDKN2A* locus, but no amplification of 7q34 (supplementary figure 1). In addition, 3/4 of the *non-survivors* in this molecular cluster also showed near tetraploidy of virtually all chromosomes. Tetraploidy can be extracted from genotyping by analyzing the copy number plots with allele frequency plots: a loss of copy number is associated with an increase in the number of alleles. Tetraploidy was confirmed in the non-survivors by FISH analysis; all survivors were diploid (except for the tandem duplication on 7q34). Tetraploidy was not observed in samples that were assigned to other molecular clusters (SNP 6.0 and SNP 250k Nspl data), which indicates that tetraploidy is specific for tumors in *Cluster 16*. All tumors of *Cluster 16* were wild type (wt) for *BRAF*, *IDH1* and *TP53*. No LOH on 1p19q was detected. Molecular data are shown in supplementary figure 1 and summarized in supplementary table 1.

Methylation profiling

Methylation profiling was performed on eight samples to further analyze molecular differences within this intrinsic subtype. In general, the methylation profiles of all *Cluster 16* samples differs from those that have been reported, see e.g. [14, 21, 22]. Unsupervised analysis based on the 1503 most variable CpG sites in gliomas [22]-does not discriminate survivors (samples 224, 308, 709, 711 and 712) from the non-survivors (samples 17, 362 and 628, (figure 3). Recently, a genome-wide hypermethylation phenotype (CIMP+) was identified in gliomas that was associated with a more favorable prognosis [14, 21, 22]. When assigning the methylation profiles of *Cluster 16* samples, 2 and 6 samples are assigned to the CIMP+ and CIMP- subtypes respectively. Interestingly, 2/3 of the non-survivors are assigned to the prognostically favorable CIMP+ subtype whereas all of the survivors are assigned to the prognostically poor CIMP- subtype.

Validation on external datasets

As reported, two external datasets (REMBRANDT and the TCGA) without PAs also contained a minority of samples (1% and 2% respectively) in the that were assigned to *Cluster 16* [5]. These samples were 2 oligodendrogliomas (OD) grade II, and 1 GBM in the REMBRANDT dataset and five GBM samples (2%) in the TCGA dataset.

For this study, we obtained the original pathological reports of samples assigned to *Cluster 16* in the REMBRANDT dataset. We first noticed that, similar to our dataset all patients were diagnosed at relatively young age (18, 23, and 30 years). This is much younger than the median age of onset for grade II and III gliomas (CBTRUS statistical report, www.cbtrus.org) and molecular subtypes of glioma [5, 23]. Interestingly, the two patients diagnosed with an OD remain alive to date (survival of 9.8 and 12.8 years respectively) whereas the GBM sample (survival 0.65 years) was a recurrent tumor of a cerebellar astrocytoma (which often is indicative of a pilocytic astrocytoma) that was diagnosed two years earlier. Unfortunately, no tissue was available to genetically analyze these tumors for the presence of a *KIAA1549-BRAF* duplication or tetraploidy. Nevertheless, the remarkable long survival of the two OD patients confirm that our molecular profiling is able to identify patients who have a significant better prognosis than other higher grade gliomas, regardless of their histology.

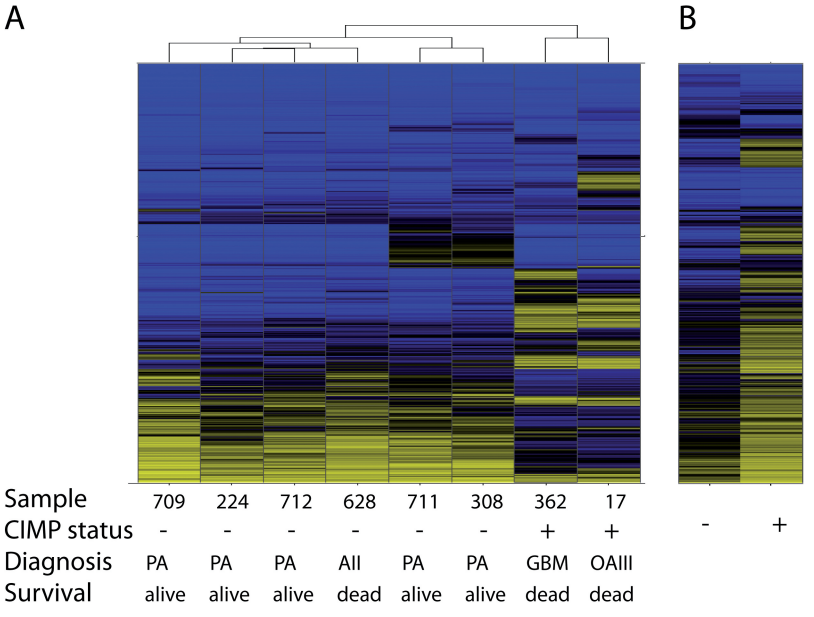

Figure 3. Methylation profiling of Cluster 16 samples. A) Unsupervised analysis based on the 1503 most variable CpG sites in gliomas identifies two major subtypes and does not discriminate survivors from the non-survivors. In contrast to the more favorable prognosis associated with CIMP+ tumors, most 'survivors' are classified as CIMP-. Two samples with a prognostically poor tumor are classified as CIMP+. B) The median methylation % of CIMP- and CIMP+ samples are included for comparison 14.

In the TCGA dataset, SNP 6.0 array data was available of 4/5 samples. All samples showed an amplification of EFGR and had a near tetraploid profile. Detailed analysis of SNP arrays from other clusters indicates that tetraploidy was not seen in TCGA glioma samples that were assigned to other molecular clusters. Patient survival was poor (0.5-1.7 years; survival available of four). These data confirm our observation that tumors with poor prognosis assigned to *Cluster 16* are tetraploid. The patient characteristics of the external datasets are listed in table 2.

Table 2. Patients characteristics external data sets

no	Histological diagnosis	age at diagnosis	Survival	Status	dataset
HF-0897	oligodendroglioma grade II	30	12,78	alive	Rembrandt
HF-1345	oligodendroglioma grade III	18	9,77	alive	Rembrandt
HF-1357	glioblastoma	23	0,65	dead	Rembrandt
TCGA-06-0132	glioblastoma	49,66	0,81	dead	TCGA
TCGA-06-0142	glioblastoma	N/A	N/A	dead	TCGA
TCGA-02-0060	glioblastoma	66,17	0,50	dead	TCGA
TCGA-06-0179	glioblastoma	64,25	1,69	dead	TCGA
TCGA-02-0002	glioblastoma	55,50	0,56	dead	TCGA

Establishment of a polyploid cell line

One of the tetraploid samples was propagated *in vitro* under normal serum conditions. Genotypic analysis of passage 5 indicates that the genomic changes observed in the primary tumor are retained in the primary cell line. The cell line does contain several additional genetic changes that are not present in the primary tumor. In general, the cell line showed a tendency towards chromosomal loss and thus towards triploidy. Genetic changes include loss of a one allele on chromosome 4, 5, 11, 13, 15, 20 (all tumor tetraploid, cell line triploid) and 9q, 10, 14q11-14q23.2, 17, 18, 19, 21 (all tumor triploid, cell line uniparentaldisomy). Cell line showed an additional homozygous deletion on 6q26. An example of the similarity and differences between the tumor and its matched primary cell line is shown in figure 4.

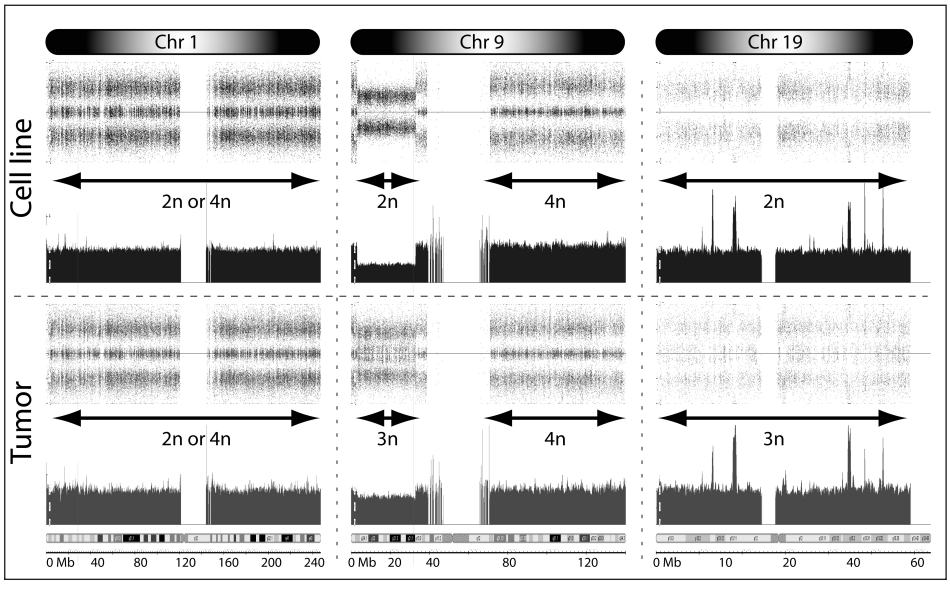

Figure 4. Establishment of a polyploidy cell line. One of the tetraploid samples of Cluster 16 was propagated *in vitro* under normal serum conditions. Examples of three different chromosomes demonstrates that the genomic changes observed in the primary tumor are retained in the primary cell line. Note that the high copy amplicons on chromosome 19 are retained in tumor and cell line. On Chromosome 1, neither the allele difference plots nor the smooth signal can discriminate between a diploid or a tetraploid genome.

DISCUSSION

In this study we demonstrate that a specific intrinsic subtype of glioma is not a homogenous tumor entity but consists of two different subtypes with remarkably different outcomes. Although expression profiling (nor methylation profiling) cannot separate the two tumor subtypes, they can be separated based on their histology (PA vs non-PA). However, many tumors within this intrinsic glioma subtype share the same histological characteristics (i.e. pilocytic features) and a tumor within *Cluster 16* was diagnosed as an ODII but has the genetic

aberration of PAs and remains alive to date without signs of tumor progression. Both the identification of long-term survivors and tetraploidy in non-survivors could be validated on independent datasets.

Gene expression profiling is a powerful technique that is used to identify "intrinsic" subtypes of tumors. Because these subtypes are molecularly similar, they form a basis to screen for targeted therapies. In fact, virtually all manuscripts that have identified intrinsic subtypes of tumors, argue they are relevant for targeted therapies, see e.g. [4, 5, 24-26]. Indeed, intrinsic subtypes of glioma are clinically and biologically relevant: They predict survival better than histological diagnosis and distinct genetic changes segregate into defined intrinsic subtypes. Nevertheless, our study clearly demonstrates that not all intrinsic subtypes are homogeneous. Intrinsic subtyping of tumors therefore does not always identify a single defined subtype, and care should be taken when using intrinsic subtypes to screen for targeted therapies.

It has been reported that the histological distinction between PAs and low grade astrocytomas is difficult [27, 28]. In should be noted however, that very few tumors within *Cluster 16* are diagnosed as low grade astrocytoma. In addition, *IDH1* mutations are frequent (>70%) in low grade astrocytomas, but are noticeably absent in all *Cluster 16* tumors [5, 29].

Relatively few studies have thusfarreported on tetraploidyin gliomas [30, 31]. Mechanstically, tetraploidy in GBM U87 cells is associated with the expression of the dominant-negative PTN which leads to a less aggressive phenotype [32]. A different study stated that the formation of a slower growing tetraploid cell population and astrocytic differentiation can be induced by high doses of specific alkylphosphocholines in rat glioma cells [33].

Tetraploidy has been observed in several other cancers (colon cancer, Barrett's esophagus, breast cancer and cervical cancer) [34-38]. A recent study demonstrated that tetraploidization occurs in a subset of human cancers with loss of function of the *TP53* pathway as an early step [39]. It also showed that persistent telomere damage can lead to tetraploidization and that telomere-driven tetraploidyis *TP53* dependent. However, *TP53* mutations were not observed in any of the samples in this study. Alternative mechanisms for tetraploidy include an excess of centrosomes, which generate aneuploid cells by producing multipolar spindles, or by misregulation or mutation for genes whose products mediate mitotic progression [40-42]. Which mechanism leads to tetraploidy in *Cluster 16* gliomas remains to be determined.

It remains to be determined why tetraploid gliomas share molecular features with PAs. A possible explanation may lie in the relatively few (when compared to other malignant glioma subtypes) genetic changes observed in tetraploidgliomas: Fewer genetic changes may result in a less aberrant gene expression pattern. Nevertheless, tetraploid tumors are a distinct subtype of glioma, and are likely to require a specific treatment paradigm. One interesting treatment pathway worth exploring is a therapy that can specifically target tetraploid cell lines as earlier described for aneuploid celllines [43].

In summary, we demonstrate that intrinsic subtyping of tumors does not always identify a single defined subtype. Our findings have implications when using intrinsic subtypes to screen for targeted therapies. Finally, we recommend all gliomas with pilocytic features and/or assigned to molecular *Cluster 16*, regardless of histological diagnosis, to be further molecularly analyzed for the 7q34 tandem duplication and/or tetraploidy using SNP arrays and/or FISH analysis.

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6

INTRINSIC MOLECULAR SUBTYPES OF GLIOMA ARE PROGNOSTIC AND PREDICTIVE FOR RESPONSE TO PCV CHEMOTHERAPY IN ANAPLASTIC OLIGODENDROGLIOMAS. A REPORT FROM EORTC STUDY 26951

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Submitted.

ABSTRACT

Background: Intrinsic gliomasubtypes (IGS) are molecularly similar tumors that can be identified based on unsupervised gene-expression analysis. Here, we have evaluated the clinical relevance of these subtypes within EORTC26951, a randomized phase III clinical trial investigating adjuvant procarbazine, CCNU (lomustine) and vincristine (PCV) chemotherapy in anaplastic oligodendroglial tumors. Our study is the first to include gene-expression profiles of formalin-fixed and paraffin-embedded (FFPE) clinical trial samples.

Methods: Gene-expression profiling was performed in 140 samples: 47 fresh frozen and 93 FFPE, on HU133_Plus_2.0 and HuEx_1.0_st arrays (Affymetrix), respectively.

Results: All previously identified six intrinsic glioma subtypes are present in EORTC26951. This confirms that different molecular subtypes are present within a well-defined histological subtype. Intrinsic subtypes are highly prognostic for overall- (OS) and progression-free survival (PFS). They are prognostic for PFS independent of clinical (age, performance, tumor location), molecular (1p19qLOH, *IDH1* mutation, *MGMT* methylation) and histological parameters. Combining known molecular (1p19LOH, *IDH1*) prognostic parameters with intrinsic subtypes significantly improves outcome prediction (Proportion of Explained Variation 30% *v* 23%). Specific genetic changes (*IDH1*, 1p19qLOH and *EGFR* amplification) segregate into different subtypes. We identified one subtype, IGS-9 (characterized by a high percentage of 1p19qLOH and *IDH1* mutations), that especially benefits from PCV chemotherapy. Median OS in this subtype was 5.5 years after radiotherapy (RT) alone *v* 12.8 years after RT/PCV; *P*=0.0349; HR 2.181, 95% CI [1.057, 4.50].

Conclusion: Intrinsic subtypes are highly prognostic in EORTC26951 and improve outcome prediction when combined with other prognostic factors. Tumors assigned to IGS-9 benefit from adjuvant PCV.

INTRODUCTION

Unsupervised analysis of gene expression profiling identifies subgroups of tumors that are molecularly similar. This approach has been used to identify distinct 'intrinsic' subtypes of cancer and provides an objective method to classify tumors [1-4]. In gliomas, the identified intrinsic subtypes reportedly correlate better with patient prognosis than histology [5-9]. Intrinsic glioma subtypes are not only similar on the RNA level, specific genetic changes also segregate in distinct intrinsic subtypes [8,10]. It is therefore likely that each molecular subtype will require its own treatment paradigm.

True validation of the prognostic relevance of the intrinsic glioma subtypes requires analysis on homogeneously and prospectively treated patients, ideally within a randomized clinical trial. One of the main problems in analysing clinical trial samples is that they are often of poor quality, most are formalin-fixed and embedded in paraffin (FFPE) [11]. However, recent technological advances have enabled high-throughput analysis of FFPE material, including expression arrays [12-14]. In a large cohort of paired FF-FFPE samples, we have recently demonstrated differences in mRNA expression are retained in FFPE samples. Importantly, the assignment to one of six intrinsic glioma subtypes (IGS) was identical between the FF-FFPE matched samples in 87% of cases [15] and the intrinsic subtypes remain highly prognostic for survival. These results demonstrate that FFPE material can be used for expression profiling.

Not all glioma patients respond similarly to treatment. For example, glioblastomas with a methylated O6-methylguanine-methyltransferase (*MGMT)* promoter respond better to treatment with temozolomide [16,17]. In oligodendrogliomas, uncontrolled trials indicated that loss of heterozygosity on 1p/19q is predictive for response to procarbazine, CCNU (lomustine) and vincristine (PCV) chemotherapy [18,19]. Selection of patients that benefit most from particular therapeutic regimens helps to improve treatment efficacy and to potentially avoid toxicity in patients that are unlikely to benefit from that therapy anyway.

EORTC26951 is a randomized phase III clinical trial investigating whether the addition of PCV chemotherapy to RT would improve OS and PFS in patients with anaplastic oligodendroglioma (AOD) or anaplastic mixed oligoastrocytoma (AOA). This trial showed that the addition of 6 cycles PCV after 59.4 Gy RT increases OS and PFS in these tumors (van den Bent et al., submitted). However, some patients appeared to benefit more from the addition of PCV treatment than others [20]. Here, we have evaluated the clinical relevance of intrinsic subtypes within EORTC26951. Our study is the first to use gene expression profiling on FFPE clinical trial samples. Our data validate that intrinsic subtypes of glioma are prognostic for survival. Intrinsic subtypes significantly improve outcome prediction when combined with other known prognostic parameters (1p19LOH, *IDH1* mutation). Our data also indicate that patients with tumors assigned to a specific intrinsic subtype benefit from PCV treatment.

MATERIALS AND METHODS

Patient samples

Patients were considered eligible in the EORTC26951, if they had been diagnosed by the local pathologist with an AOD or a AOA according to the WHO classification of 1993, with at least 25% oligodendroglial elements; with at least three of five anaplastic characteristics (high cellularity,

mitoses, nuclear abnormalities, endothelial proliferation or necrosis); if they were between the 16 and 70 years of age; if they had an Eastern Cooperative Oncology Group (ECOG) status between the 0 and 2; if they had not undergone prior chemotherapy or RT to the skull; if they had no diseases interfering with follow-up; if they had adequate bone marrow, renal, and hepatic function and if written informed consent was obtained from all patients. All centers had to get approval of the study design from their local ethics committee prior to study activation according to local and national regulations [20]. Study accrual was between August 13, 1995, and March 3, 2002. The consolidated standards on reporting trials (Consort) flow diagram has been reported previously [20], and is shown in supplementary figure 1.

RNA isolation and array hybridization

Total RNA extraction, purification and quantification from FF and FFPE material was reported previously [8,15]. 150 ngRNA from FF and FFPE tissueswas used for expression profiling. FF samples (n=47) were profiled as described on HU133plus 2.0 arrays (Affymetrix, High Wycombe, UK) [8]; FFPE samples (n=93) were profiled using HuEx_1.0_st arrays (Affymetrix) in combination with Nugen Ovation kits (Nugen, San Carlos, Ca) as reported [13,15].

Molecular assessments

Analysis of loss of heterozygosity on 1p/19q, *EGFR* amplification, *IDH1* mutations and *MGMT* promoter methylation on EORTC26951 samples was described previously [20-22], see also paper by van den Bent et al. (submitted).

Statistical analysis

Samples were assigned to one of the six 'intrinsic' molecular subtypes of glioma using ClusterRepro (an R package) as described previously, omitting control cluster 0[8,23]. In our previous manuscripts, these intrinsic subtypes were designated 'cluster' followed by the cluster number (0, 9, 16, 17, 18, 22 or 23). For clarity, we here annotate these 'clusters' as 'intrinsic glioma subtype' (IGS) followed by the same cluster number (e.g. IGS-9).

Differences between the Kaplan-Meier survival curves were calculated by the Log-rank (Mantel-Cox) test using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego, CA. Comparisons between frequencies were calculated by the Fisher's exact test. In this exploratory analysis$P<0.05$ was considered to indicate significant differences. The significance of prognostic factors was determined with a multivariate analysis by Cox regression analysis using Stata version 12.0 (Statacorp, College Station, TX). The importance of prognostic factors was compared in Cox and logistic regression using SAS macros (www.akh-wien.ac.at/imc/biometrie/relimp).

RESULTS

Patients and samples.

A total of 140 samples out of 368 patients within the EORTC26951 trial were available for the current study. Of these, 47 were FF and 93 FFPE. Seventy-three patients had been assigned to the RT plus PCV chemotherapy arm and 67 patients to the RT only arm. Our cohort of samples from the EORTC26951 study did not differ from the entire EORTC26951cohort (368 patients) with

respect to age, sex, performance status, diagnosis, tumor location, *IDH1* mutation, 1p19qLOH, *EGFR* amplification, *MGMT* promoter methylation, OS and PFS (*Table 1*). However, OS within

Table 1. Comparison of baseline characteristics

		Baseline characteristics			
		Included in the cluster analysis			
		No	Yes	Total	
		N=228 (%)	N=140 (%)	N=368 (%)	P-value
Age	**Median** **Range**	49.5 19.4 - 68.7	49.4 18.6 - 68.7	49.5 (18.6 - 68.7)	0.57
	Obs (O)	228	140	368	
Sex	**Male**	123 (53.9)	89 (63.6)	212 (57.6)	0.08
	Female	105 (46.1)	51 (36.4)	156 (42.4)	
Performance status	**0.**	81 (35.5)	53 (37.9)	134 (36.4)	0.75
	1.0	107 (46.9)	64 (45.7)	171 (46.5)	
	2.0	36 (15.8)	22 (15.7)	58 (15.8)	
Histological diagnoses	**AOD**	173 (75.9)	93 (66.4)	266 (72.3)	0.04
	AOA>25% O	53 (23.2)	47 (33.6)	100 (27.2)	
Central diagnosis	**AOD**	100 (43.9)	76 (54.3)	176 (47.8)	0.02
	AOA	46 (20.2)	36 (25.7)	82 (22.3)	
	LGG	28 (12.3)	11 (7.9)	39 (10.6)	
	HGG	26 (11.4)	13 (9.3)	39 (10.6)	
	Other	10 (4.4)	0 (0.0)	10 (2.7)	
Tumor location	**Elsewhere**	119 (52.2)	71 (50.7)	190 (51.6)	0.83
	Frontal	109 (47.8)	69 (49.3)	178 (48.4)	
***IDH1* mutation**	**Normal**	40 (17.5)	57 (40.7)	97 (26.4)	0.37
	Mutated	39 (17.1)	42 (30.0)	81 (22.0)	
1p19q LOH	**Non codeleted**	141 (61.8)	95 (67.9)	236 (64.1)	0.51
	Codeleted	44 (19.3)	36 (25.7)	80 (21.7)	
***EGFR* mutation**	**Normal**	111 (48.7)	82 (58.6)	193 (52.4)	0.76
	Amplified	32 (14.0)	26 (18.6)	58 (15.8)	
***MGMT* promoter methylation**	**Unmethylated**	23 (10.1)	24 (17.1)	47 (12.8)	0.61
	Methylated	60 (26.3)	76 (54.3)	136 (37.0)	
PFS	**Obs (O); HR (95% CI)**	181; 1.00	117; 1.16 (0.92, 1.46)		0.21
	Median (95% CI) (months)	19.65 (15.38, 33.35)	14.52 (9.63, 21.13)		
	% at 2 Year(s) (95% CI)	48.02 (41.39, 54.34)	39.29 (31.20, 47.26)		
OS	**Obs (O); HR (95% CI)**	168; 1.00	113; 1.25 (0.99, 1.59)		0.06
	Median (95% CI) (months)	43.89 (30.03, 61.17)	26.87 (18.69, 38.31)		
	% at 2 Year(s) (95% CI)	63.44 (56.81, 69.33)	51.43 (42.87, 59.33)		

Abbreviations: Obs: observation events; AOD: anaplastic oligodendroglioma; AOA: anaplastic oligoastrocytoma; LGG: low grade gliomas; HGG: high grade gliomas; HR: hazard ratio; CI: confidence interval; PFS: progression-free survival; OS: overall survival. *P*<0.01 is considered statistically significant. The missing values are not included in the tableand are the remaining percentages.

the RT-only treatment arm of included patients, was worse compared to OS in patients not included (OS: 1.59 *v* 3.70, *P*=0.009; HR: 1.59, 95% CI [1.121, 2.251], PFS: 0.77 *v* 1.43 years, *P*=0.012; HR: 1.548, 95% CI [1.103, 2.174], even when corrected for known clinical (age, extent of resection, sex and performance), molecular (1p19qLOH) and histological review diagnosis. There were no such differences in the RT-PCV arm. This effect is likely due to difference in patient selection in the hospitals but not related to tissue sampling. Of note, at the time of randomization patients were stratified per center. Of the included samples, there were no differences in clinical, molecular and histological characteristics between the two treatment arms (*Supplementary Table 1*).

Intrinsic subtypes are prognostic for OS and PFS.

Expression profiling was performed on a total of 140 samples of patients treated within EORTC26951. All expression profiles were then assigned to one of six predefined intrinsic subtypes. These molecularly-similar 'intrinsic subtypes' were identified previously and are based on unsupervised gene expression analysis [8]. All six intrinsic glioma subtypes were identified which shows that patients with different molecular subtypes were enrolled within this trial under the same histopathological diagnosis. After assignment IGS-9, IGS-16, IGS-17, IGS-18, IGS-22 and IGS-23 contained 50, 2, 26, 27, 8 and 27 patients respectively. These subtypes were highly prognostic for both OS and PFS. The median OS for IGS-9, IGS-17, IGS-18 and IGS-23 is 8.5, 2.8, 1.2 and 1.0 years respectively; and the median PFS 5.7, 1.8, 0.5 and 0.5 years *(Figure 1)*. IGS-16 and IGS-22 contained too few samples to draw conclusions. The subtype-specific differences in survival were highly similar to previously reported differences using FF archival samples and confirms the prognostic power of intrinsic subtyping [8].

In a multivariate analysis, intrinsic subtypes are a significant prognostic factor that is independent from clinical (age, sex, performance status and type of surgery), molecular (1p19qLOH) and histological (local diagnosis or review diagnosis) parameters *(Table 2)*. When *IDH1* mutation status is included in this analysis, intrinsic subtyping remains an independent

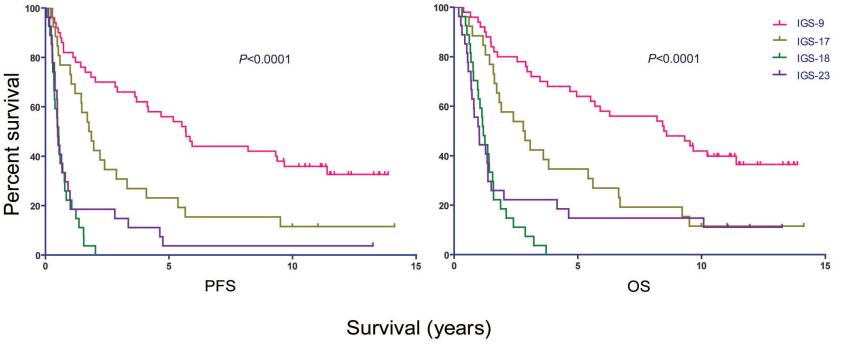

Figure 1. Kaplan-Meier survival curves of the four intrinsic glioma subtypes for OS and PFS. Overall survival (A) and progression free survival (B) of the four major intrinsic glioma subtypes to which the patients from EORTC26951 trial were assigned. The four intrinsic glioma subtypes were highly prognostic for both OS and PFS as patient prognosis is different for each intrinsic subtype. Only two and eight samples were assigned to IGS-16 and IGS-22 respectively (not shown).

Table 2. Multivariate Cox's regression prognostic analysis of intrinsic subtyping

Number of observations	126
Number of failures	102

	P-value	HR	95% CI for HR
Intrinsic subtype	0.002	1.072	1.025 - 1.120
Age	0.002	1.031	1.011 - 1.051
Sex	0.650	0.908	0.598 - 1.378
Type of surgery	0.023	0.685	0.495 - 0.949
Performance status	0.018	1.393	1.059 - 1.832
1p19qLOH	0.001	0.338	0.183 - 0.626
Review diagnosis	0.134	0.792	0.584 - 1.074

Abbreviations: HR: hazard ratio (compared to RT only arm); CI: confidence interval. Calculations are based on 126 observations.

prognostic factor for PFS (*P*=0.003) but not OS (*P*=0.052). When *MGMT* promoter methylation is included, intrinsic subtyping remains an independent prognostic factor for both PFS (*P*<0.0001) and OS (*P*=0.008), though the number of patients analyzed becomes relatively small (n=80). Of note, even in the confirmed anaplastic oligodendroglial tumors at central pathology review, each histological subtype was found to contain several intrinsic subtypes. Within the AOD and AOA review diagnosis, intrinsic subtypes remain an independent prognostic factor.

1p19qLOH was significantly more frequently present in tumors assigned to IGS-9 (29/46, 63%), compared to IGS-17 (6/25, 24%, *P*<0.01), 18 (0/26, 0%*P*<0.001) and IGS-23 (0/25, 0% *P*<0.001), (*Figure 2*). *EGFR* amplification was predominantly observed in IGS-18 (14/24, 58%) and, to a lesser extent, in IGS-23 (8/21, 38%). *EGFR* amplification was rarely observed in samples assigned to IGS-9 (3/39, 7.6%, *P*<0.001) and IGS-17 (0/19, 0%, *P*<0.001). *IDH1* mutations were predominantly identified in patientsassigned to IGS-9 (23/33, 70%) and IGS-17 (14/22, 64%) compared to IGS-18 (2/19, 11%, *P*<0.001) and IGS-23 (4/21, 19%, *P*<0.001). *MGMT* promoter methylation was detected at high frequency in samples assigned to IGS-9 (27/28, 96%), and at somewhat lower frequencies in IGS-17 (16/20, 80%), IGS-18 (8/13, 62%, *P*<0.01 compared to IGS-9) and IGS-23 (10/13, 77%). This segregation was highly similar to that reported by us previously using archival samples and demonstrates that each intrinsic subtype has a different set of causal genetic changes [8].

Molecular *(IDH1*, 1p19qLOH) parameters [24] and intrinsic subtypes are both important prognostic factors. To assess the added predictive value of intrinsic subtyping, we combined molecular (*IDH1*, 1p19qLOH) data with intrinsic subtypes and performed an analysis of variance for the percentage of survival explained by the two models. Both molecular (*IDH1,* 1p19qLOH) parameters and intrinsic subtypes have similar ability to explain outcome as measured by the proportion of explained variation (PEV) (*P=0.88*) *(Supplementary Table 2)*. However, the combined model has a larger PEV (30%) compared to 23% for each individual group of factors. A PEV>20% indicates a model providing sufficiently precise survival estimates [25,26]. This does not mean that both models explain the same variability. Combining them significantly improves the power to predict outcome.

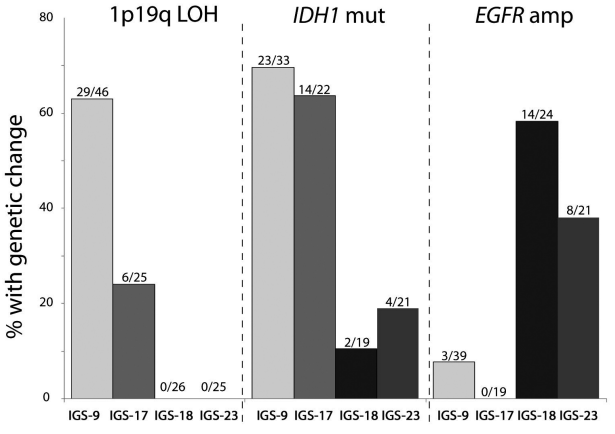

Figure 2. Genetic differences between intrinsic molecular subtypes of gliomas. Specific genetic changes segregate into distinct intrinsic subtypes. 1p19q LOH was predominantly observed in tumors assigned to IGS-9 and to a lesser extent in IGS-17 and not seen in tumors assigned to IGS-18 and IGS-23. *IDH1* mutations were significantly more observed in samples assigned to IGS-9 and IGS-17 compared to IGS-18 and IGS-23. *EGFR* amplification was predominantly identified in IGS-18 and IGS-23, but rarely identified in samples assigned to IGS-9 and IGS-17. Our data strongly suggest that each intrinsic subtype has a different set of causal genetic changes.

Prediction of benefit from adjuvant PCV

We then evaluated whether the benefit from adjuvant PCV chemotherapy is specific to selected intrinsic subtypes. The addition of adjuvant PCV improved OS in samples assigned to IGS-9 (12.8 years in the RT-PCV arm *v* 5.5 years in the RT-only arm, *P*=0.035; HR 2.181, 95% CI[1.057, 4.502]). An improvement was also observed for PFS (12.8 *v* 3.6 years, *P*=0.0018; HR 3.183,95% CI[1.537, 6.590]). Data are shown in *Figure 3* and *Table 3*. PCV treatment also improved PFS, but not OS, in samples assigned to IGS-18(0.78*v*0.39 years *P*=0.028; HR 2.506, 95% CI[1.104, 5.687]). A trend towards an increase in both OS and PFS was observed between the two treatment arms

Table 3. Median Overall and Progresson-free survival per treatment arm.

	Overall survival				Progression-free survival			
	Median survival (years)		Hazard Ratio, [95%CI]	P-value	Median survival (years)		Hazard Ratio, 95% CI	P-value
	RT	RT/PCV			RT	RT/PCV		
IGS-9	5.52	12.77	2.18; [1.067, 4.502]	0.0349	3.62	12.77	3.18; [1.537, 6.590]	0.0018
IGS-17	1.83	5.42	2.21; [0.869, 5.597]	0.0962	1.05	2.21	2.12; [0.847, 5.301]	0.1086
IGS-18	1.13	1.38	1.43; [0.657, 3.123]	0.3669	0.39	0.78	2.51; [1.104, 5.687]	0.0280
IGS-22	1.49	3.19	1.92; [0.413, 8.954]	0.4051	0.88	1.25	3.12; [0.585, 16.600]	0.1829
IGS-23	1.14	0.97	0.89; [0.391, 2.020]	0.7771	0.61	0.49	0.98; [0.444, 2.180]	0.9678

Abbreviations: RT= radiotherapy; RT/PCV= radiotherapy followed by procarbazine, lomustine and vincristine chemotherapy.

in samples assigned to IGS-17 (OS: 5.42 *v* 1.82 years, *P*=0.096; HR 2.205, 95% CI[0.869, 5.597]; PFS: 2.21 *v* 1.05 years, *P*=0.109; HR 2.119, 95% CI[0.847, 5.301]). No difference in PFS or OS was observed between the treatment arms in samples assigned to IGS-23.

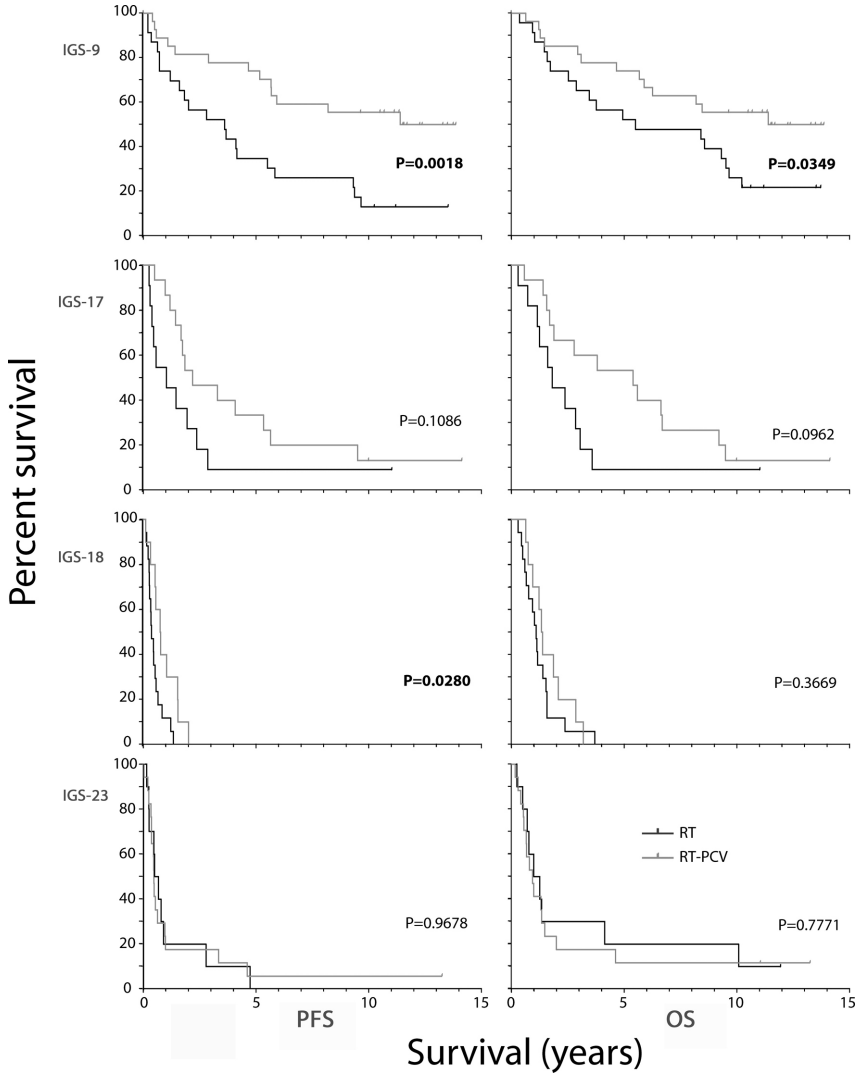

Figure 3. Kaplan-Meier survival curves of the four intrinsic glioma subtypes per treatment arm. (A) shows that adjuvant PCV chemotherapy improved OS and PFS in samples assigned to IGS-9 (B) shows a trend towards an increase in both OS and PFS between the two treatment arms in samples assigned to IGS-17 (C) Adjuvant PCV also improved PFS, but not OS in samples assigned to IGS-18. (D) No difference between the two treatment arms was observed in both OS and PFS in samples assigned to IGS-23. One specific intrinsic glioma subtype (IGS-9) seems to benefit from adjuvant PCV chemotherapy whereas patients with tumors assigned to IGS-23 do not.

DISCUSSION

In this study, we have performed intrinsic subtyping within a prospective clinical trial, EORTC26951. Our data demonstrate that all six intrinsic glioma subtypes are present in EORTC26951, despite the fact that only AODs and AOAs (as diagnosed by the local pathologist) were included in this study. After central pathology review, each histological diagnosis still contains various intrinsic subtypes. Similarly to reported on archival samples, the intrinsic subtypes are an independent prognostic factor both for overall- (OS) and progression-free survival (PFS). Combining known molecular (1p19LOH, *IDH1*) prognostic parameters with intrinsic subtypes significantly improves outcome prediction (PEV 30% *v* 23%). Histological classification of gliomas is troublesome and subject to interobserver variation [27]. In this study, we confirm that expression profiling, compared to the diagnoses made by the local pathologists, is a more accurate and objective method to classify gliomas [6-10,28-32]. Also similar to reported on archival samples, specific genetic changes (*IDH1*, 1p19qLOH and *EGFR* amplification) segregate into different subtypes. Intrinsic subtypes therefore are not only similar in their RNA expression profile, but are also similar on the DNA level in their genetic aberrations. Our data validate the prognostic significance of intrinsic subtypes, and therefore can be used to determine the molecular heterogeneity of samples included in clinical trials.

As said, clinical trial samples are often of poor quality as most are formalin-fixed and embedded in paraffin [11]. However, recent technological advances have indicated that expression profiling is feasible on FFPE material [12,13,15]. Here, we demonstrate for the first time using clinical trial samples that FFPE material can be used for gene expression based, intrinsic subtyping of tumors.

Long term follow up of this trial shows that adjuvant PCV improves OS in AOA and AODs (see accompanying paper). Here, we further demonstrate that not all patients benefit equally from this treatment. Samples assigned to IGS-9 (characterized by a high percentage of 1p19qLOH and *IDH1* mutations) significantly benefit from PCV chemotherapy whereas samples assigned to IGS-23 do not show any improvement in outcome (neither PFS nor OS). This outcome for IGS-9 samples is remarkable as there was a large degree of crossover in the RT-only arm at time of progression [20].

Tumors with 1p19qLOH have been reported to show durable responses to chemotherapy [18,19,33]. Independently, both the EORTC trial 26951 and its North-American counterpart RTOG9402 have shown that the addition of PCV to RT improved OS in 1p19q co-deleted oligodendrogliomas [34] (Cairncross et al, submitted). Our data validate these observations as IGS-9 contains gliomas with the highest percentage 1p19qLOH. Moreover, the median survival time between the RT-PCV and RT-only arm is highly comparable to the median survival observed in RTOG9402 (14.7 in the RT-PCV arm *v* 7.3 years in the RT only arm for the RTOG9402 trial and 12.8 in the RT-PCV arm *v* 5.5 years in the RT only arm for IGS-9 samples in the EORTC26951 trial). It should be noted that not all samples assigned to IGS-9 have 1p19qLOH, and that not all samples with 1p19qLOH are assigned to IGS-9. Differences between the two classification methods should therefore be further examined, preferably on a larger cohort of samples.

Identifying patients that do not benefit from PCV chemotherapy is of equal clinical relevance. In our study, samples assigned to IGS-18 or IGS-23 showed no benefit from PCV chemotherapy in OS (though an increase in PFS was observed for IGS-18). In RTOG9402, OS is not improved by PCV chemotherapy in AODs and AOAs that have retained either 1p and/or 19q.

Combined, these data indicate that patients harboring an AOD or AOA that have retained 1p and/or 19q, assigned to IGS-18 or IGS-23, do not show a benefit in OS from PCV chemotherapy.

Samples assigned to IGS-17 showed a trend towards improved outcome from PCV chemotherapy, both in PFS and OS. IGS-17 contains predominantly tumors that have retained 1p19q but have *IDH1* mutations. A similar trend towards improved outcome was observed in the entire EORTC26951 cohort tumors with retained 1p19q and mutated *IDH1*. Nevertheless, our sample cohort is relatively modest in size (140) and our analysis is post hoc (retrospective testing) which is hypothesis generating. Therefore our data should be validated in an additional independent cohort to firmly establish the predictive effect of these intrinsic molecular subtypes.

Interestingly, in a separate clinical trial (EORTC22981/26981) on glioblastoma, patients with tumors assigned to IGS-18 also failed to show a marked response to the addition of temozolomide to radiotherapy [8,35]. In this study too few samples were assigned to other subtypes to draw firm conclusions. It should be noted that there are some important differences between EORTC26951 and EORTC22981/26981. For example, EORTC22981/26981 examined the efficacy of temozolomide chemotherapy in combination with radiotherapy as opposed to PCV. However, PCV and temozolomide are both alkylating agents with similar mechanism of action. Another important difference is the histological subtype investigated: only glioblastomas (GBM) were included in EORTC22981/26981 whereas EORTC26951 made use of AOD and AOAs. However, intrinsic subtypes have similar molecular and clinical characteristics and are independent of histological diagnosis. We therefore hypothesize that samples assigned to a defined intrinsic subtype will show similar responses to similar chemotherapy regimens regardless of the histological diagnosis.

In summary, our data demonstrate, on clinical trial samples and using FFPE material, that the intrinsic molecular subtypes are highly prognostic for OS and PFS. Our data also indicates that at least one intrinsic subtype of glioma responds favorably to PCV chemotherapy. Intrinsic subtypes are easily determined and therefore this approach provides a novel, straightforward and promising way to improve outcome prediction, also when combined with other prognostic factors.

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DISCUSSION

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DISCUSSION

Molecular profiling

At present, treatment of gliomas is based on the histological typing and grading, with treatment paradigms based on large clinical studies using histological entry criteria. The histological typing and grading based on morphological similarities with similar outcome is however, especially in diffuse glioma, far from perfect with a classification that is subject to inter-observer variation [1-2]. Slowly however, it has become clear that the presence or absence of specific molecular abnormalities contain much more information on the outcome of patients as compared to the classical histological diagnosis. Theoretically, a classification based on molecular abnormalities will be more rational, as tumors will be lumped based on the mechanism of the disease and not just on the phenotype that is the result of both genotype and environmental circumstances. Potentially, this may lead to more specific treatments, especially once the treatment is aimed at specific molecular alterations. Such a personalized medicine approach holds promise for a more rational treatment that may avoid both over and under treatment of tumors. Still, even in the absence of molecularly targeted approaches, classical treatment (e.g. with chemotherapy) may be more effective in certain molecularly defined subgroups.

In this thesis several methods of the molecular profiling of gliomas have been investigated. The studies presented in this thesis demonstrate results of profiling using genome-wide expression analysis, methylation analysis, genotyping and by using additional specific molecular features such as *IDH1* mutation, *EFGR* amplification, LOH of 1p19q, *KIAA1549-BRAF*, as well as FISH (to determine tetraploidy) [3-4].

In chapter 1, we describe an RNA expression profiling study of a large series of snap frozen gliomas. This study was the first large unsupervised analysis which included all histological glioma subtypes, and showed an improved predictive effect on survival of tumor clustering based on expression analysis as compared to classical histology. A fundamental observation in this study was, that tumors with similar histologies were assigned to different clusters, suggesting that tumors with seemingly similar morphologic features may have completely different molecular backgrounds [3]. Similarly, genome-wide methylation analysis of a series of anaplastic oligodendroglial tumors showed that this approach divided the tumors basically in two entities: those tumors that have a CpG Island Hypermethylated Phenotype (CIMP), and those without that phenotype [5]. Moreover, tumors with a CIMP status had a much better prognosis. A similar widespread CpG island methylation phenomenon has been described in other tumors as well (leukemia, colorectal cancers), and was in these diseases also related to outcome.

Still, that any of these techniques in itself also has limitations became clear from one of the RNA expression clusters, which contained both pilocytic astrocytomas (PA) (very favorable prognosis) and glioblastoma (GBM) (poor prognosis). Here, further analysis looking at chromosome copy numbers using SNP arrays showed a clear difference between these groups of tumors. This shows that any single technique may fail to distinguish between relevant glial subtypes, and warns against a too enthusiastic welcome for these novel tools.

In the initial expression analysis of a glioma repository as described in chapter 1 we used snap frozen tissue samples. That has a major drawback however, as in many patients only

formalin-fixed paraffin-embedded (FFPE) samples are available. In order to use the potential value of molecular based diagnostics, assays that can use FFPE to predict outcome to specific treatments are pivotal. We therefore analyzed whether expression analysis using RNA from FFPE material is possible. In chapter 2, we analyzed in a series of 55 tumors the correlation between expression analysis using snap frozen material and FFPE from the same surgery [6]. In these paired samples a good correlation between expression analysis on snap frozen tumor samples and on FFPE samples was observed. This opens a whole new window in glioma research, since this implies FFPE material can be used to correlate treatment responses to the specific molecular subtypes as defined by RNA expression analysis. The possible role of expression analysis on FFPE and its relation to treatment outcome was further investigated on tumor material from patients that were included in multi-centered prospective phase III randomized controlled trial. This analysis using tumor specimens that were at least ten years old suggests that molecular subtypes as determined by RNA expression analysis may indeed respond differently to specific treatment protocols. This is a fascinating result, as the clinical trial was performed on a histologically homogeneous group of tumors, Even within those cases in which the oligodendroglial histology had been confirmed centrally, expression analysis of FFPE revealed major differences which were found to correlate with clinical outcome.

This is obviously just the beginning, and at present not without limitations. Our studies have been performed retrospectively. Before they can be implemented in daily practice, both the prognostic and the predictive value of the molecular clusters must be validated in new prospective and properly controlled studies. In addition, the differences in treatment response between the clusters must be validated in independent datasets treated with similar regimens. Indeed, well defined classical treatments may be more effective in specific molecularly defined subtypes that are based on RNA expression, and a realistic scenario is that for each molecular subtype an optimal treatment paradigm is developed, even in the absence of molecularly targeted treatments. Ideally, specific molecular aberrations within the molecular clusters should be mechanistically linked to prognosis and treatment response. Although as expected, specific genetic aberrations are segregating into the molecular clusters, none of these are that specific and exclusive that it can be used for assignment to a specific molecular cluster.

A relevant clinical question is whether the more comprehensive expression analysis (e.g., RNA expression, methylation profiling) contributes to assessment of specific, single, molecular abnormalities. In our series, analysis shows that the combination of molecular profiling using RNA expression and the determination of specific biomarkers such as *IDH1*, *MGMT* and LOH of 1p19q improves the prognostic and predictive value compared to using these profiling techniques separately. And importantly, the combination of these molecular profiling techniques gives a more accurate prognosis than histological classification. This also implies that expression analysis has added value to the basic of any of the more specific genetic aberrations. It also warns against placing too much confidence into one single technique or marker.

In conclusion, when validated in prospective studies, the molecular glioma profiles can be used to give patients the most accurate information about their diagnosis, prognosis, and treatment possibilities.

Molecular biomarkers

As stated above, within the molecular glioma profiles identified, specific genetic aberrations and gene (co) expression signatures were seen, which supports the hypothesis that these molecularly defined glioma subtypes are fundamentally different. The individual molecular biomarkers investigated were all useful in improving diagnosis, prognosis and/or treatment strategy in comparison to classical histology alone. In this discussion we will further focus on several specific molecular biomarkers that are at present clinically relevant for adult patients with diffuse gliomas or GBMs. More sophisticated assays need to show added clinical value as compared to these markers.

IDH1

In 2008, Parsons et al. identified mutations in a gene encoding isocitrate dehydrogenase (*IDH1*) in a subset (12%) of GBM samples [7]. These mutations result in a reduced enzymatic activity towards the native substrate, isocitrate, and mutant *IDH1*catalyses the formation of 2-hydroxyglutarate (2HG) from α-ketoglutarate. Mutations in a homologous gene, *IDH2*, have also been identified, although they occur sporadically. The occurence of *IDH1* and *IDH2* mutations is mutually exclusive. The *IDH1* mutations were most common in young patients with GBMs that had progressed from low-grade gliomas to secondary GBMs. Subsequently, other studies showed much higher frequencies (60-80%) of the *IDH1* mutation in lower grade gliomas [7-10]. Interestingly, the *IDH1* mutations are detected in only a few tumor types: diffuse gliomas, acute myeloid leukemia (AML), in central and periosteal cartilaginous tumours and in intrahepatic cholangiocarcinoma [11-13]. Sporadic mutations have been described in paragangliomas, pheochromocytomas, prostate carcinomas, and in a colon cancermetastasis [14-16]. In gliomas, a very high prevalence (60-80%) of *IDH1* mutations is found in diffuse low grade (grade II and III) gliomas and secondary gliomas. Since *IDH1* mutations are rare in primary GBMs, *IDH1* mutation status can help to distinguish between primary and secondary GBMs as well as in differentiating anaplastic (grade III) gliomas from GBMs. Also they can be used to distinguish diffuse gliomas from pilocytic astrocytomas (PA), since *IDH1* mutations are virtually absent in the latter.

IDH1 mutations are associated both with the *TP53* mutations and with LOH of 1p19q. Interestingly, *TP53* mutations and LOH of 1p19q are mutually exclusive in glioma, occurring in astrocytomas and oligodendroglial tumors respectively. Therefore, a combination of testing these molecular markers can be used to differentiate between different glioma subtypes.

In this thesis we showed that the *IDH1* mutations are correlated with certain molecular profiles with a relatively good prognosis. The co-existence of *IDH1* and either *TP53* or LOH of 1p19q in tumors was confirmed in our studies, as one expression cluster harbored gliomas with high frequencies of both *IDH1* and LOH of 1p19q, while another other gliomas with both *IDH1* mutation and *TP53* mutation were assigned to another cluster.

There are several different hot spot mutations within the *IDH1* gene. There is a some preponderance of *IDH* mutations for specific molecular glioma subtypes, confirming the genetic differences between the various subgroups [17]. For example, the most common hot spot mutation (p.R132H) was found in gliomas with LOH of 1p19q, while non-p.R132H mutations were seen in gliomas harboring a *TP53* mutation. This supports the hypothesis that the different *IDH1* mutations may cause different changes in different tumor pathways. The current data support the use of *IDH1* and its mutational subtypes as a marker to identify glioma subtypes.

IDH1 is presumed to play a role in the early tumorigenesis, but the exact pathogenetic mechanism of the *IDH1* mutation in gliomas remains unknown until now [18]. Functional analysis of the R132H mutation showed a decreased proliferation *in vitro* as *in vivo* if introduced in a glioblastoma like cell line. A similar analysis of the non-132H mutations could reveal differences in proliferation rate between the mutational subtypes [19]. If there are differences found between the mutational subtypes, this would be an explanation why the mutational *IDH1* subtypes segregate into specific glioma subtypes.

Further studies have shown that mutated *IDH1* inhibits histone demethylases and TET 5-methylcytosine hydroxylases, leading to genome-wide histone and DNA methylation alterations [20]. This finding reveals a relationship between the presence of *IDH1* mutations and a hypermethylated tumor profile, including *MGMT* methylation. This "epigenetic control" function of the *IDH1* mutation could be one of the first important events in the development of gliomas. Inhibition of the epigenetic effects of the *IDH1* mutation, for example by trying to reduce the 2HG production, and/or by administration of mimic α-ketoglutarate could serve as a targeted therapy in *IDH1* mutated tumors.

Several studies have analyzed the prognostic and predictive value of the *IDH1* mutations [10,21-23]. These studies conclude that there is a correlation in grade III tumors with prognosis, but any predictive effects on treatment response has not been shown yet. If a predictive effect can be shown in the future, the question is whether this is an effect of a specific metabolic change caused by *IDH1* mutations, or a secondary effect by inducing (*MGMT)* methylation . As stated earlier, determination of the *IDH1* mutation status can help to distinguish between the different subtypes of gliomas, and when histological morphology and/or other clinical factors give inconclusive information about the diagnosis. For examples, a recent study showed that all patients aged over sixty, diagnosed with an anaplastic astrocytoma lacking an *IDH1* mutation, actually had an outcome that resembled outcome of GBM patients. This raises the question whether patients should be treated accordingly. Analysis of *IDH* status also allowed the distinction between reactive gliosis and tumor [23].

Since *IDH1* mutation status can give us valuable prognostic and diagnostic information in such cases, we recommend the determination of the *IDH1* mutation status to be implemented in clinical practice.

MGMT and methylation profiling

O^6-methylguanine-DNA methyltransferase (*MGMT*) is a DNA repair enzyme that is highly associated with resistance to alkylating and methylating agents (e.g., temozolomide, nitrosourea's). The *MGMT* gene product reverses the alkylation caused by this type of chemotherapy, and thereby reduces the toxic effect. Epigenetic silencing of the *MGMT* gene by methylation of the CpG island located in the promoter region reduces the DNA-repair function of the *MGMT* gene, and thereby induces an improved effect on alkylating chemotherapy. In the late nineties, the potential predictive value of *MGMT* protein levels in glioma patients was first described, and in 2000 the prognostic effect of *MGMT* methylation in BCNU treated GBM patients was shown by Esteller et al. [20]. Five years later, Hegi et al. showed that *MGMT* methylation was predictive for response to additional temozolomide in GBM patients treated with radiotherapy. This confirmed the hypothesis that *MGMT* methylation predicts benefit from alkylating chemotherapies. This is further supported by

data from the German NOA-8 study, that suggest that in elderly glioblastoma patients the choice between RT and temozolomide should primarily be determined by *MGMT* status [24]. To complicate this straight forward assumption, recent studies showed that *MGMT* promoter methylation in anaplastic gliomas is prognostic irrespective of initial treatment with radiotherapy or alkylating chemotherapy [25-29]. This raises the question whether in grade III glioma *MGMT* promoter methylation isn't merely an epiphenomenon of other biomarkers and/or part of a more general genome wide methylated profile. In the study presented in this thesis, we identified two main subgroups of oligodendroglial brain tumors by whole genome methylation profiling [30]. The survival of the patients with a hypermethylated profile (CIMP+) was significantly better than the survival of the unmethylated (CIMP-) subgroup. CIMP+ status was seen almost exclusively in the molecular clusters with a favorable prognosis (also characterized by *1p-19q* co-deletion and *IDH* mutations). As already mentioned in the section about *IDH1*, it is now assumed that *IDH1* mutations induce genome wide methylation. In our study, the CIMP+ status indeed was highly associated with the *IDH1* mutation status, In addition, the strong association between CIMP status and *MGMT* promoter methylation confirms the hypothesis that the *MGMT* promoter methylation status is part of a more general, prognostically favorable genome wide methylation profile. In future experiments, genes that are epigenetically altered in different glioma subtypes should be functionally analyzed to see whether they play a role in tumor formation, or may be a predictor of prognosis, and/or be predictive for response to therapy.

LOH 1p19q

LOH of 1p19q was identified as the specific chromosomal lesion of oligodendroglioma by Reifenberger et al.[31]. Subsequently, Cairncross and Louis noted that this co-deletion predicted response to PCV chemotherapy in recurrent anaplastic oligodendrogliomas [32]. Although initial reports of large randomized phase III trials failed to show that co-deletion of 1p/19q identifies newly diagnosed anaplastic oligodendroglial patients that benefit from adjuvant PCV, it clearly can serve as a prognostic marker in oligodendroglial tumors. However, very recent results of long term follow-up of these studies showed that LOH of 1p19q is predictive for treatment response in case of adjuvant chemotherapy (PCV) [33-34] Thus, the determination of 1p/19q status has now immediate therapeutic consequences.

In this thesis we show that tumors that are assigned to molecular *Cluster 9*, regardless of their histological diagnosis, frequently show LOH of 1p19q and have a relatively good prognosis. Our data show that molecular profiling of gliomas (using RNA expression profiling and the determination of LOH 1p19q) identifies more patients with a relatively good prognosis than when only oligodendroglial tumors as determined by morphology are analyzed for 1p/19q loss. We would therefore propose as standard of care to determine the LOH 1p19q status in all tumors with oligodendroglial features . This identifies a subset of patients with a much better prognosis than their histological diagnosis would predict.

In 2011, inactivating mutations of the *FUBP1* gene (chromosome 1p) and the *CIC* gene (chromosome 19q) were shown to be present in a substantial fraction of ODs with LOH of 1p19q [35]. Functional analysis of these genes is required to provide insights into the pathogenesis of these tumors, and to improve methods currently used for their diagnosis, prognosis, and treatment.

Other molecular markers

KIAA1549-BRAF is an oncogenic fusion gene which was first described in 2008 by Jones et al. [36-37]. The mutation was specifically found in pilocytic astrocytomas. Korshunov et al. showed that combined analysis of *IDH1* mutation status and for the *KIAA1549-BRAF* fusion gene could be used for distinguishing pilocytic astrocytomas from diffuse astrocytomas [38]. *IDH1* was exclusively seen in the diffuse astrocytomas, as the *BRAF* fusion gene was exclusive for pilocytic astrocytomas. In this thesis we showed that tumors with pilocytic features are all assigned to one specific molecular cluster, thereby sharing the same RNA expression profile. However, while sharing the same expression profile, these tumors segregated into two different entities, showing a dramatic difference in survival. All tumors harboring the *BRAF* fusion gene, regardless of their histological diagnosis, were long-term survivors. This illustrates that , a combination of different molecular profiling techniques can help to distinguish good-prognostic tumors from poor-prognostic tumors. In addition, these molecular biomarkers improve on genome wide profiling for this specific molecular cluster. Because of this strong prognostic and diagnostic value, we recommend all gliomas with pilocytic features (pilocytic morphology, located in the fossa posterior, young age at diagnosis) to be analyzed for the presence of the *KIAA1549-BRAF* fusion gene.

EGFR mutation and amplification occurs in a distinct subset of gliomas. They are typically associated with GBMs . This could enable clinicians to refine histologic diagnosis in ambiguous cases. In this thesis, we confirmed that *EGFR* mutations only occurred in molecular clusters with poor prognosis.

The prognostic and predictive relevance of *EGFR* amplification and *EGFRvIII* mutation in GBMs remains controversial. The *EGFR vIII* mutation is the most common mutation of the *EGFR* receptor in GBMs. Because this genetic aberration is not found in normal tissue, it is a potential target for tumor-specific therapy. In 2005, it was suggested that coexpression of *EGFRvIII* by glioblastoma cells and *PTEN* is associated with responsiveness to *EGFR* kinase inhibitors [39]. However, trials using *EGFR* tyrosine kinase inhibitors (erlotinib, imatinib and gefitinib) all had low response results even in subgroups that showed *EGFRvIII* variant and *PTEN* expression [40-42]. Until now, there is not enough evidence to use *EGFR* as a prognostic or predictive marker in clinical practice.

The future of molecular profiling

In the past, classification of gliomas has been based on histopathological findings. In the near future, molecular profiling on various levels will become more and more important in addition to glioma morphology. Pathologists will have the possibility to make a selection of biomarkers which they can use to make the most accurate tumor classification. Biomarkers such as *IDH1*, LOH of 1p19q, *MGMT*, but also more comprehensive assays such as RNA expression profiling and methylation profiling could be useful to establish the most accurate diagnosis, to make a prediction of the prognosis and to determine the best treatment. The role of these markers to tailor treatments on the molecular profile of the tumor requires large prospective randomized trials to assess predictive value of specific markers. Preferably, analysis of these markers should be part of the prospective trial design, as post hoc analysis of identified markers are likely to remain on the level of exploratory subgroup analysis.

In the near future, we expect that next generation sequencing will make a large contribution to the profiling of gliomas. The exact single-base level information will provide more detailed insights in the tumorigenesis, and the specific genetic differences of the molecular glioma subtypes will lead to a further optimization of treatment.

The significance of the mutations found need however to be functionally analyzed *in vitro* and *in vivo*. Only then, these genetic data may also allow developing novel therapies to target vulnerable points embedded within the genetic blueprint of gliomas. This targeted therapy is currently a dream for the future of all cancer treatment. However, it remains a sobering fact that, until now, there are only few therapies available. Resection, radiation and classical chemotherapy have been the most important pillars for glioma treatment for decades, on which all clinical trials have been built. At present molecular assays may allow a more rational choice for of in particular chemotherapy, but we still use old treatment paradigms . The identification of new targets and the development of targeted therapies will take many more years before those can be implemented in daily practice. Which will be the only way to significantly improve the overall outcome of brain tumor patients .

Nowadays, large multi-centered networks such as The Cancer Genome Atlas (TCGA) are collecting large-scale data to provide a basis of complex diseases on a great variety of molecular levels [43]. Especially in glioma, which represent a relatively small group of tumors, these extensive data sets allow researchers to investigate gliomas on a large scale. This will increase the possibilities of the identification new biomarkers and –more importantly- new targets and new treatments. That will be the only way to significantly improve outcome.

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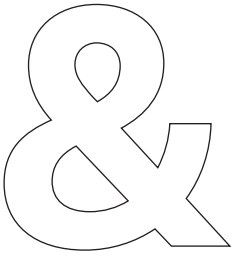
ADDENDUM



SUMMARY

Gliomas are the most common primary brain tumors in adults. Although improvements in treatment possibilities, almost every patient diagnosed with this tumor is facing a fatal disease. Currently, the diagnosis of gliomas is based on the histological morphology. This classification system discriminates between three distinct subtypes: astrocytomas (A), oligodendrogliomas (OD), and mixed oligoastrocytomas (OA). These subtypes are further separated into grades I to IV, with grade IV, the glioblastoma (GBM), being the most aggressive subtype. Prognosis and treatment decisions are strongly related to the histological subtype. Unfortunately, this histological classification is often difficult and subject to a lot of interobserver-variation. This can cause inaccuracy in diagnosis, prognosis and treatment decisions. Our hypothesis in this thesis was that molecular profiling of gliomas would provide a more objective and accurate classification system of gliomas.

In **Chapter 1** we describe the identification of seven intrinsic glioma clusters, which were indentified using whole genome expression profiling. The clustering was unsupervised, and all histological glioma subtypes were included in the study. The results showed that the seven clusters identified were a significant better predictor of prognosis than histology. Two clusters were a predictor of poor prognosis (median survival 0.67 and 0.68 years), two cluster were a predictor of intermediate prognosis (median survival 1.12 and 3.32 years), and two clusters predicted a favorable prognosis compared to the other clusters (median survival 6.06 and >4.7 years). Several distinct genetic aberrations segregate into specific clusters. *IDH1* mutations were specific for clusters with an intermediate prognosis including one cluster that was enriched for secondary GBMs and one of the clusters with good prognosis. LOH of 1p19q was specifically seen in one of the favorable prognostic clusters, and amplification of *EGFR* was seen in the clusters predicting a poor prognosis.

All results were validated and confirmed in three large external datasets, thereby confirming our molecular cluster is a robust clustering method. We concluded that the intrinsic subtypes identified improve on histological classification of gliomas and are an accurate predictor of prognosis. Molecular classification can contribute to diagnosis and may form a rationale for clinical decision making and novel targeted therapies.

To see whether specific clusters show different responses to therapy, we require to analyze material of patients who were included in large randomized phase III trials. Unfortunately, tissue from these trials are most often formalin-fixed paraffin-embedded (FFPE) material. RNA isolated from FFPE material is notorious for being degraded and chemically modified. In **Chapter 2** we analyzed whether it is possible to reproduce our molecular clustering using RNA isolated from FFPE material. We used matched FFPE material of patients that were included in the study described in **Chapter 1**. Affymetrix HuEx 1.0 "Exon" arrays were used as a platform to analyze the expression of RNA isolated from FFPE material. Our molecular clustering showed a reproducibility of ~85%. These results show that molecular profiling can be performed using RNA from FFPE which opens the possibility of using FFPE material from phase III clinical trials.

In low grade gliomas mutations in the *IDH1* gene are frequently seen. The R132H mutation is the most frequent mutation within this gene. Nevertheless, other point mutations do occur on the same position (non-p.R132H mutations e.g. p.R132C). **Chapter 3** describes the segregation of non-p.R132H mutations in *IDH1* in distinct molecular subtypes of glioma. We showed that

non-p.R132H mutations occur in gliomas with *TP53* mutation, are virtually absent in tumors with LOH of 1p and 19q and accumulate in distinct (gene-expression profiling based) intrinsic molecular subtypes. The *IDH1* mutation type does not affect patient survival. Our results were validated on an independent sample cohort, indicating that the *IDH1* mutation spectrum may aid glioma subtype classification. Functional differences between p.R132H and non-p.R132H mutated *IDH1* may explain the segregation in distinct glioma subtypes.

In **Chapter 4** another molecular profiling method is described, based on whole genome methylation profiling. Methylation is the addition of methyl groups to cytosines, which often ultimately results in inactivation of the gene. Methylation can occur in tumor suppressor genes and so may contribute to tumor formation. We performed genome wide methylation profiling on a series of oligodendroglial tumors within EORTC 26951. The series was expanded with tumors of the same histology and treatment from our own archive. Methylation profiling identified two main subgroups of which survival in the hypermethylated phenotype (CIMP+)" subgroup was markedly better than the survival of the unmethylated (CIMP-) subgroup. CIMP status correlated with survival, *MGMT* promoter methylation, 1p19q LOH and *IDH1* mutation status. CIMP status strongly increases the predictive accuracy of survival in a model including known clinical prognostic factors such as age and performance score. We validated our results on an independent dataset from the cancer genome atlas (TCGA). The strong association between CIMP status and *MGMT* promoter methylation suggests that the *MGMT* promoter methylation status is part of a more general, prognostically favorable genome wide methylation profile. Methylation profiling therefore may help identify AODs and AOAs with improved prognosis.

In **Chapter 5** we described the molecular features of one particular cluster, *Cluster 16* that contained all pilocytic astrocytomas (PAs). PAs have a good prognosis after resection of the tumor. However, within this molecular cluster, the PAs cluster together with glioma subtypes of higher grades. Remarkably, although these tumors that shared a similar molecular profile, PAs and non-PAs showed a dramatic difference in survival. The pathology reports showed that all gliomas within this cluster were characterized as having pilocytic features. Genotyping of these tumors resulted in two marked observations. We demonstrated that *Cluster 16* marks tumors with long term survival (without signs of tumor progression), regardless of their histological subtype. Second, *Cluster 16* marks tumors with poor survival that are near tetraploid. We are the first to identify this near tetraploid subtype of gliomas and it is likely that this subtype requires its own treatment paradigm. These two results were confirmed in independent external datasets. Our findings have major consequences for the patients that have been diagnosed with a histologically malignant glioma subtype, but who can be identified as a long-term survivor using these molecular profiling techniques. Based on the results we recommend all gliomas with pilocytic features, or tumors that are assigned to *Cluster 16,* to be molecularly analyzed on the presence of the *KIAA1549-BRAF* fusion gene and/or a tetraploid karyotype. Using this genetic information, the prognosis of the patient can be predicted accurately.

In **Chapter 6** we performed expression profiling on samples of a large European phase 3 controlled trial (EORTC 26951) to evaluate the possible different treatment responses of the molecular subgroups identified in **Chapter 1.** Both fresh frozen and paraffin embedded glioma material was analyzed, using the platforms and clustering methods for FF and FFPE material as described in **Chapter 1** and **Chapter 2** respectively. We found that all six glioma subtypes could be identified within the anaplastic oligodendrogliomas included in the EORTC 26951 trial. All

these molecular subtype were highly prognostic for progression free survival, as for overall survival. Also, the specific subtypes showed different treatment responses. Especially cluster 9 showed to have a beneficial response to adjuvant PCV chemotherapy. With this study, we show that the intrinsic molecular glioma subtypes are both prognostic and predictive.

SAMENVATTING

Gliomen zijn de meest voorkomende primaire hersentumoren bij volwassenen. Ondanks verbeteringen in behandelingsmogelijkheden, hebben vrijwel alle patienten met dit type tumor een ongeneeselijke ziekte. Op dit moment wordt de diagnose van gliomen gebaseerd op de histologische morfologie. Dit classificatie systeem onderscheidt 3 verschillende subtypen: astrocytomen (A), oligodendrogliomen (OD), en gemengde oligoastrocytomen (OA). Deze subtypen worden verder onderscheiden met behulp van gradering (graad I-IV), waarbij graad IV, het glioblastoom (GBM), het meest aggressieve subtype is. De prognose en de behandeling van de patient is sterk gerelateerd aan het het histologische subtype. Helaas is het indelen van gliomen met behulp van de histologische classificatie vaak moeilijk en onderhevig aan veel interobserver variabiliteit. Dit kan een onnauwkeurig beeld geven van de diagnose en de prognose, en kan zorgen voor een suboptimale behandelingsstrategie. Onze hypothese bij het schrijven van dit proefschrift was dat door middel van het maken van moleculaire profielen in gliomen, een objectievere en nauwkeurigere classificatie van gliomen zou kunnen worden gecreëerd.

In **Hoofdstuk 1** beschrijven we zeven intrinsieke groepen (clusters) van gliomen, die werden geïdentificeerd op basis van de expressie van alle genen in het genoom (whole genome expression profiling). De clustering was niet gesuperviseerd, en alle histologische subtypes van gliomen werden geïncludeerd in de studie. De resultaten lieten zien dat de zeven clusters een significant betere voorspelling gaven van de prognose ten opzichte van de histologische classificatie. Twee clusters waren voorspellend voor een slechte prognose (mediane overleving 0.67 en 0.68 jaar), twee clusters waren voorspellend voor een gemiddelde prognose (mediane overleving 1.12 en 3.32 jaar), en 2 clusters voorspelden een relatief goede prognose vergeleken met de andere clusters (6.06 en >4.7 jaar). Eén van deze goede prognostische clusters omvatte vrijwel alle pilocytaire astrocytomen (PA; graad 1) die in de studie waren geïncludeerd. Aan een ander cluster waren alle controle samples en alle tumor samples toegewezen die veel niet-neoplastisch materiaal bevatten. Een aantal genetische afwijkingen waren specifiek voor de verschillende clusters. *IDH1* mutaties werden specifiek gezien in de clusters met een gemiddelde of een goede prognose, waaronder ook een cluster waar veel secundaire glioblastomen in zaten. Verlies van 1p19q werd specifiek gezien in één van de clusters met een goede prognose, en amplificatie van *EGFR* werd gezien in de clusters met een slechte prognose.

Alle resultaten werden gevalideerd en bevestigd in drie grote externe datasets, waardoor werd bevestigd dat onze moleculaire clustering een robuuste manier is om gliomen van elkaar te onderscheiden. We concludeerden dat de geïdentificeerde groepen een verbetering zijn ten opzichte van de histologische classificatie van gliomen en dat zij een nauwkeurige voorspeller zijn van de prognose. Moleculaire classificatie kan bijdragen aan het stellen van een diagnose en zou een rol kunnen spelen bij het maken van klinische beslissingen, en bij het ontwikkelen van nieuwe, op specifieke tumortypes gerichte therapieën.

Om te zien of de specifieke clusters verschillend reageren op behandeling, moeten we tumor materiaal analyseren van patiënten die zijn geïncludeerd in grote gerandomiseerde fase 3 trials. Helaas zijn deze trials bijna allemaal uitgevoerd door het gebruik van tumor materiaal dat is gefixeerd in paraffine (FFPE). RNA dat wordt geïsoleerd uit FFPE materiaal staat er om bekend dat het is afgebroken en chemische veranderingen heeft ondergaan, in tegenstelling tot RNA dat is geïsoleerd uit vers ingevroren tumor materiaal (FF).

In **Hoofdstuk 2** hebben we geprobeerd onze moleculaire clusters te reproduceren door RNA te gebruiken dat is geïsoleerd uit FFPE materiaal. We hebben FFPE materiaal gebruikt van dezelfde patiënten waarvan het FF materiaal is gebruikt in **Hoofdstuk 1**. Affymetrix HuEx 1.0 "Exon" arrays worden gebruikt als platform om de expressie van de het RNA uit het FFPE materiaal te analyseren. De moleculaire clustering kon in ongeveer 85% van de tumor samples gereproduceerd worden. Deze resultaten laten zien dat het maken van moleculaire profielen kan worden uitgevoerd met gebruik van RNA dat is geïsoleerd uit FFPE materiaal, wat de mogelijkheid creëert om FFPE materiaal uit grote fase 3 studies te gebruiken.

In laaggradige gliomen worden zeer vaak mutaties in het *IDH1* gen gezien. De meest voorkomende mutatie in dit gen is de R132H mutatie. Desalniettemin komen er ook andere puntmutaties voor op dezelfde positie (non-p.R132H mutaties, bijvoorbeeld p.R132C). **Hoofdstuk 3** beschrijft het voorkomen van non-p.132H mutaties in het *IDH1* gen in specifieke moleculaire subtypen van gliomen. Wij hebben laten zien dat non-p.R132H mutaties voorkomen in gliomen die ook een *TP53* mutatie hebben, en dat de non-p.132H mutaties vrijwel altijd afwezig zijn in tumoren met verlies van 1p19q. De tumoren met een non-p.132H mutatie groeperen zich in specifieke moleculaire subtypen. Het type *IDH1* mutatie heeft geen invloed op de overlevingsduur van patiënten. Onze resultaten zijn gevalideerd op een onafhankelijke dataset, en laten zien dat het de verschillende *IDH1* mutaties mogelijk kunnen bijdragen aan de classificatie van tumoren in de moleculaire subtypes. Functionele verschillen tussen de p.R132H en de non-p.R132H mutaties zouden kunnen verklaren waarom de verschillende mutaties optreden in verschillende moleculaire clusters.

In **Hoofdstuk 4** wordt een andere moleculaire methode beschreven om profielen van gliomen te maken. Deze methode is gebaseerd op het maken van profielen door naar de methylatie van het genoom te kijken. Methylatie is de toevoeging van methylgroepen aan cytosinen, wat vaak resulteert in deactivatie van een gen. Methylatie kan voorkomen in tumor suppressor genen en kan zo bijdragen aan tumorvorming. Wij hebben methylatie profielen gemaakt van een serie olidodendrogliale tumoren uit de EORTC 26951 studie. Deze serie is uitgebreid met oligodendrogliale tumoren die dezelfde behandeling hadden ondergaan uit ons eigen archief. Door het maken van methylatie profielen werden 2 hoofdgroepen onderscheiden, een groep met veel methylatie (CIMP+), en een groep met weinig methylatie (CIMP-). In de CIMP+ groep was de overleving opmerkelijk langer dan in de CIMP- groep. CIMP status correleerde dus met overleving, maar ook met *MGMT* promotor methylatie, met verlies van 1p19q en met *IDH1* mutaties. De CIMP status verbeterde de nauwkeurigheid van het voorspellen van de overleving van patiënten aanzienlijk, wanneer het werd gebruikt in een model waarin de bekende klinische factoren zoals leeftijd en de *Karnofsky Performance Score* werden meegenomen. We hebben onze resultaten gevalideerd op een onafhankelijke dataset van de Cancer Genome Atlas (TCGA). De sterke associatie tussen de CIMP status en de *MGMT* promotor methylatie suggereert dat de *MGMT* promotor methylatie onderdeel is van een meer algemeen, *genome-wide* methylatie profiel, dat prognostisch beter is. Het maken van glioom profielen door het bepalen van de methylatie status kan bijdragen aan het identificeren van anaplastische oligodendrogliomen en anaplastische oligoastrocytomen met een goede prognose.

In **Hoofdstuk 5** beschrijven we de moleculaire kenmerken van een specifiek cluster, *Cluster 16*, dat alle pilocytaire astrocytomen (PAs) die in de studie voorkwamen bevatte. Patienten met een PA hebben een goede prognose na resectie van de tumor. Opmerkelijk genoeg clusterden

deze PAs samen met gliomen met een hogere histologische graad. Ondanks dat ze hetzelfde moleculaire profiel deelden, lieten de PAs en de niet-PAs een dramatisch verschil in overleving zien. De pathologie verslagen van deze tumoren lieten zien dat alle gliomen in dit specifieke cluster werden gekenmerkt door het hebben van morfologische pilocytaire kenmerken. Het genotyperen van deze tumoren resulteerden in 2 opvallende observaties.

We hebben aangetoond dat *Cluster 16* tumoren bevat met een lange overleving (zonder tekenen van tumor progressie), niet gerelateerd aan het histologische subtype en de graad van de tumoren. Alle tumoren van patiënten met een lange overleving hadden het *KIAA1549-BRAF* fusie gen. Ten tweede bevat *Cluster 16* tumoren met een zeer korte overleving, die allemaal tetraploid zijn. Zo'n tetraploide subgroep is nooit eerder beschreven in gliomen en het is waarschijnlijk dat deze groep een eigen behandelingsstrategie vereist.

Deze resultaten werden bevestigd in onafhankelijke externe datasets.

Onze bevindingen kunen grote consequenties hebben voor patiënten die zijn gediagnosticeerd met een histologisch kwaadaardig glioom subtype, die kunnen worden geïdentificeerd als een *lange-overlever* met behulp van deze moleculaire technieken. Wij adviseren op basis van deze resultaten alle gliomen die pilocytaire kenmerken hebben en/of toegewezen zijn aan *Cluster 16* moleculair te onderzoek op de aanwezigheid van het *KIAA1549-BRAF* fusie gen en/of op de aanwezigheid van een tetraploïd karyotype. Met behulp van deze genetische informatie kan de prognose an patiënten nauwkeuriger worden bepaald.

In **Hoofdstuk 6** hebben we expressie profielen gemaakt van tumor samples die geïncludeerd waren in een grote Europese fase 3 gecontroleerde trial (EORTC 26951), om te zien of de moleculaire clusters die we identificeerden in **Hoofdstuk 1** verschillend reageren op behandeling met radio- en/of chemotherapie. Zowel FF als FFPE materiaal werd geïncludeerd, waarbij er gebruik werd gemaakt van de verschillende platformen en clustering methoden zoals beschreven in **Hoofdstuk 1 en 2**. Alle zes de glioom clusters konden worden geïdentificeerd in de groep tumoren uit de EORTC 26951 studie, waarin alleen anaplastische oligodendrogliale tumoren waren geïncludeerd. Alle moleculaire subtypen hadden een grote prognostische waarde voor zowel progressie-vrije overleving als voor algehele overleving. Tevens reageerden de specifieke subtypes anders op verschillende vormen van behandeling.

LIST OF ABBREVIATIONS

A	Astrocytoma
AA	Anaplastic astrocytoma
AML	Acute myeloid leukemia
AOA	Anaplastic oligoastrocytoma
AOD	Anaplastic oligodendroglioma
BAC	Bacterial artificial chromosome
CBTRUS	Central Brain Tumor Registry of the United States
CCNU	Lomustine
CIMP	CpG island methylator phenotype
DABG	Detection Above BackGround
DASL	cDNA-mediated Annealing, Selection, Extension, and Ligation
DNA	Deoxyribonucleic acid
ECOG	Eastern Cooperative Oncology Group
EGFR	Epidermal growth factor receptor
EORTC	European Organisation for Research and Treatment of Cancer
FDR	False discovery rate
FF	Fresh frozen
FFPE	Formalin-fixed paraffin-embedded
FISH	Fluorescence in situ hybridization
GBM	Glioblastoma
GSEA	Gene set enrichment analysis
HOPACH	Hierarchical ordered partitioning and collapsing hybrid
IDH1	Isocitrate dehydrogenase 1 gene
IDH2	Isocitrate dehydrogenase 2 gene
IGP	In-group proportion
KPS	Karnofsky performance score
LOH	Loss of heterozygosity
MGMT	O-6-methylguanine-DNA methyltransferase
MOA	Mixed oligoastrocytoma
MS-MLPA	methylation-specific multiplex ligation-dependent probe amplification
OA	Oligoastrocytoma
OD	Oligodendroglioma
OS	Overall survival
PA	Pilocytic astrocytoma
PCR	Polymerase chain reaction
PCV	Procarbazine, Lomustine, and Vincristine chemotherapy
PFS	Progression free survival
REMBRANDT	Repository for Molecular Brain Neoplasia Data
RIN score	RNA integrity number score
RMA	Robust Multi-array Average
RNA	Ribonucleic acid
RT	Radiotherapy

SNP	Single-nucleotide polymorphism
TCGA	The Cancer Genome Atlas
TMZ	Temozolomide
WHO	World Health Organization

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&

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LIST OF PUBLICATIONS

Publications included in this thesis:

- Intrinsic molecular subtypes of glioma are prognostic and predictive for response to PCV chemotherapy in anaplastic oligodendrogliomas. A report from EORTC study 26951
  Lale Erdem, **Lonneke A Gravendeel**, Peter AE Sillevis Smitt, Thierry Gorlia, Martin J van den Bent, Pim J French
  Submitted
- A molecular subtype of glioma consists of two distinct tumor entities with markedly different clinical outcomes
  Lonneke A.M. Gravendeel, Nanne K. Kloosterhof, Hein Sleddens, Rutger Balvers, Martine Lamfers, Linda B.C. Bralten, Peter A.E. Sillevis Smitt, Martin J. van den Bent, Pim J. French.
  Submitted
- Gene expression profiles of gliomas in formalin-fixed paraffin-embedded material **Gravendeel LA**, de Rooi JJ, Eilers PH, van den Bent MJ, Sillevis Smitt PA, French PJ.
  Br J Cancer. 2012 Jan 31;106(3):538-45. doi: 10.1038/bjc.2011.547.
- A hypermethylated phenotype in anaplastic oligodendroglial brain tumors is a better predictor of survival than MGMT methylation in the EORTC 26951 study
  Martin J. van den Bent, **Lonneke A. Gravendeel**, Thierry Gorlia, Johan M. Kros, Pieter Wesseling, Johannes L. Teepen, Ahmed Idbaih, Marc Sanson, Peter A.E. Sillevis Smitt and Pim J. French.
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International conferences

- EANO-EORTC. Malignancies of the Central Nervous System. *Budapest, Hungary, 2009.*
- Society of Neuro-Oncology (SNO). *New Orleans, U.S.A, 2009.*
- American Association of Cancer Research (AACR). *Orlando, U.S.A, 2011.*

Presentations

- Neuro-oncologie werkbespreking, 2/year (oral presentation)
- Moleculaire subgroepen bij gliomen. *Landelijke Werkgroep Neuro-Oncologie, UMCG.* (oral presentation)
- JNI Lecture 2009 - Intrinsic Expression Profiles of Gliomas are a better Predictor of Survival than Histology (oral presentation)
- Society of Neuro-Oncology. *New Orleans, U.S.A.* (poster presentation)
- KWF Tumorcelbiologie, *Lunteren* (oral presentation)
- JNI Lecture 2010 - Molecular profiling of gliomas (oral presentation)
- Molecular profiling of gliomas - Referaat afdeling Neurologie, Erasmus MC, Rotterdam. (oral presentation)
- American Association of Cancer Research , *Orlando, U.S.A.* (poster presentation)
- American Association of Cancer Research , *Orlando, U.S.A.* (poster presentation)
- Neuro Tumor Club Meeting 2011, *Orlando, U.S.A.* (oral presentation)