Molecular classification of anaplastic oligodendroglioma using next-generation sequencing: a report of the prospective randomized EORTC Brain Tumor Group 26951 phase III trial

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Background. Histopathological diagnosis of diffuse gliomas is subject to interobserver variation and correlates modestly with major prognostic and predictive molecular abnormalities. We investigated a series of patients with locally diagnosed anaplastic oligodendroglial tumors included in the EORTC phase III trial 26951 on procarbazine/lomustine/vincristine (PCV) chemotherapy to explore the diagnostic, prognostic, and predictive value of targeted next-generation sequencing (NGS) in diffuse glioma and to assess the prognostic impact of *FUBP1* and *CIC* mutations.

Methods. Mostly formalin-fixed paraffin-embedded samples were tested with targeted NGS for mutations in *ATRX*, *TP53*, *IDH1*, *IDH2*, *CIC*, *FUBP1*, *PI3KC*, *TERT*, *EGFR*, *H3F3A*, *BRAF*, *PTEN*, and *NOTCH* and for copy number alterations of chromosomes 1p, 19q, 10q, and 7. *TERT* mutations were also assessed, with PCR.

Results. Material was available from 139 cases, in 6 of which results were uninformative. One hundred twenty-six tumors could be classified: 20 as type II (*IDH* mutation [mut], “astrocytoma”), 49 as type I (1p/19q codeletion, “oligodendroglioma”), 55 as type III (7+/10q- or *TERT*mut and 1p/19q intact, “glioblastoma”), and 2 as childhood glioblastoma (*H3F3A*mut), leaving 7 unclassified (total 91% classified). Molecular classification was of clear prognostic significance and correlated better with outcome than did classical histopathology. In 1p/19q codeleted tumors, outcome was not affected by *CIC* and *FUBP1* mutations. *MGMT* promoter methylation remained the most predictive factor for survival benefit of PCV chemotherapy.

Conclusion. Targeted NGS allows a clinically relevant classification of diffuse glioma into groups with very different outcomes. The diagnosis of diffuse glioma should be primarily based on a molecular classification, with the histopathological grade added to it. Future discussion should primarily aim at establishing the minimum requirements for molecular classification of diffuse glioma.

Keywords: 1p/19q, methylation, next generation sequencing, oligodendroglioma, PCV.

The diagnosis of grades II and III diffuse glioma is currently made by histopathological criteria according to classification by the World Health Organization (WHO). A diagnostic

classification of brain tumors should be prognostic and preferably also predictive for outcome and should show sufficient specificity and sensitivity. In recent years, however, the

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histopathological classification of diffuse glioma has become the subject of criticism. It is clear that the histopathological diagnosis of diffuse glioma suffers from a considerable interobserver variability (in particular in grades II and III glioma), and tumors with a similar microscopic appearance may have rather different clinical outcomes.¹ This is illustrated by rather different prognoses of patients with anaplastic oligoastrocytoma in various trials, low concordance rates in the diagnosis of “classical for anaplastic oligodendroglioma,” and ongoing discussions about whether after the removal of anaplastic oligoastrocytoma with necrosis from grade III glioblastoma with an oligodendroglial component merits a separate diagnosis.^{2–8} Recent papers have in fact questioned the existence both of true mixed oligoastrocytoma and of glioblastoma with oligodendroglial morphology.^{9–12}

Many of these critical reports are generated by studies on molecular analysis of diffuse glioma, which have revealed a major prognostic role but also a predictive value for outcome to chemotherapy of certain molecular lesions in diffuse glioma.^{13–16} In particular the presence or absence of mutations in *IDH*, *TERT*, *ATRX* and copy number alterations of 1p, 19q, 7, and 10q appear to be candidates for use for a molecular classification of diffuse glioma.^{17–21} This has resulted in propositions of a primarily molecular classification of diffuse glioma. First studies suggested that molecular profiling has more prognostic significance than the diagnosis of tumor lineage by classical histology, and virtually all studies on this topic conclude a rather modest relation between molecularly defined subclasses of diffuse glioma and histology.^{20–22} Moreover, with the recent technical advances allowing routine assessment of the genetic makeup of glioma, this appears now possible in the routine care of brain tumor patients. It remains, however, unclear whether this should be used instead of a classical morphological diagnosis or in addition to it, and what criteria should be used to make certain molecular classification.

In the mid-1990s, two large phase III trials were started investigating the efficacy of (neo)adjuvant procarbazine/lomustine/vincristine (PCV) in anaplastic oligodendroglioma and anaplastic oligoastrocytoma (European Organisation for Research and Treatment of Cancer [EORTC] study 26951 and Radiation Therapy Oncology Group [RTOG] study 9402).^{13,14} Patients were enrolled in the EORTC study based on the local diagnosis of an anaplastic oligodendroglial tumor, but previous molecular analysis of this study using fluorescence in situ hybridization (FISH) probes targeting chromosomes 1p, 19q, 7, and 10q and *EGFR* showed that 20%–30% of patients had in fact a molecular background more consistent with glioblastoma.⁵ Next-generation sequencing (NGS) allows the simultaneous assessment of mutations in a multitude of genes but also of copy number alterations.²³ We used targeted NGS to investigate a molecular classification scheme based on a limited number of molecular alterations within EORTC 26951. This cohort was used because it represents a clinically well-characterized set of patients, locally diagnosed with anaplastic oligodendroglial tumors, with extensive pathology review data available and data on long-term benefit from PCV chemotherapy. This cohort allows therefore a thorough comparison between classical histopathology and a molecular classification. We also explored the prognostic relevance of recently discovered *CIC* and *FUBP1* mutations in 1p/19q codeleted tumors,

as the impact of these mutations on outcome is unclear, and reexamined predictive factors for benefit to adjuvant PCV chemotherapy.^{24,25}

Material and Methods

EORTC 26951 was a phase III study testing the benefit of adjuvant PCV chemotherapy after radiotherapy in anaplastic oligodendroglial tumors, which accrued patients between 1995 and 2002. Details of this study have been published elsewhere.¹⁴ Patients were eligible for this study if they had been diagnosed by the local pathologist with an anaplastic oligodendroglioma or with a mixed anaplastic oligoastrocytoma with at least 25% oligodendroglial elements according to the WHO 1993 classification for brain tumors, with at least 3 of 5 anaplastic characteristics (high cellularity, mitosis, nuclear abnormalities, endothelial proliferation, and necrosis). Central pathology review was conducted (J.M.K.) after inclusion into the trial and confirmation of an anaplastic oligodendroglial tumor in 70% of patients.⁵ All centers had to obtain approval of the study design from their local ethical boards before study activation according to national and institutional regulations, and patients provided written informed consent.

DNA Extraction

Manual microdissection was performed to enrich for neoplastic cells from formalin-fixed paraffin-embedded glioma tissues. The percentage of neoplastic cells was estimated by our local pathologist (J.M.K.). DNA was extracted as previously described.^{26,27} Part of the DNA samples were isolated from frozen sections as previously described.^{28,29}

Primer Panel Design for Targeted (NGS) Analysis

A custom primer panel was designed using the Ion AmpliSeq Designer 2.0. The panel encompasses 468 amplicons covering the genes for *ATRX* (overall coverage in design 91%), *CIC* 94 (94%), *EGFR* (100%), *FUBP1* (95%), *NOTCH1* (96%), *PTEN* (87%), and *TP53* (97%), as well as hot spot mutation regions in *BRAF* (codon 600), *H3F3A* (exon 2), *IDH1* (codon 132), *IDH2* (codons 140 and 172), and *PIK3CA* (exon 9). In addition, primer panels for analysis of polymorphic single nucleotide polymorphisms (SNPs) were added for detection of large genomic aberrations or loss of heterozygosity (LOH) of chromosomes 1p, 7p, 7q, 10q, and 19q and of *EGFR* amplification (*EGFRamp*). Further details on the panel and the assessed chromosomal regions are presented in Supplementary Table S1. Diagnostic use of SNP-based LOH analysis will be described in detail elsewhere (Dubbink et al manuscript in preparation).

Targeted Next-Generation Sequencing

Targeted NGS was performed by semiconductor sequencing with the Ion Torrent Personal Genome Machine (PGM) with supplier's materials and protocols (Life Technologies) as previously described.³⁰ Shortly, genomic DNA input varied between 2.5 and 10 ng, depending on the amount of tissue or DNA available. Library and template preparations were performed consecutively with the AmpliSeq Library Kit 2.0-384 LV and the

Ion PGM Template OT2 200 kit. Templates were sequenced using the Ion PGM Sequencing 200 Kit v2 on an Ion 318v2 chip. Sequence information was analyzed with Variant Caller v3.6 (Life Technologies) and variants were annotated in a local Galaxy pipeline using ANNOVAR.^{31–33}

Details on NGS performance of the different samples are available as shown in Supplementary Tables S2–S4. In cases with *EGFRamp*, which had a negative effect on NGS performance and reliability of the assay outcome (Supplementary Table S3), NGS analysis was also performed with the same panel, but without targeting *EGFR* amplicons (Supplementary Table S4).

TERT Promoter Analysis

TERT promoter analysis was originally part of the custom primer panel described above, but due to the high G/C content, the Ion AmpliSeq Designer 2.0 was not able to design good primers for that region. Therefore, SNaPshot analysis was subsequently performed for analysis of 3 mutational hot spots: C228, C242, and C250 (chr5:1,295,228C>T; chr5:1,295,242–243CC>TT; chr5:1,295,250 C>T, respectively; hg19) in the *TERT* promoter as described previously.³⁴

Classification

Based on several recent studies,^{17,18,20–22,24} for the molecular classification the following criteria were used:

- Type I, “oligodendroglioma”: presence of 1p/19q codeletion (entire 1p and 19q arm)
- Type II, “astrocytoma”: *IDH1* or *IDH2* mutated, no 1p/19q codeletion
- Type III, “glioblastoma”: *TERT* mutation without 1p/19q codeletion, or 10q loss and either *EGFRamp* or chromosome (Chr) 7 imbalance

Strictly speaking, SNP-based LOH analysis does not allow discrimination of LOH or trisomy. Therefore, we have labeled copy number changes on Chr 7 as “imbalance.” To allow comparison with the histological classification, the central review histological diagnosis conducted at the time of the study was recoded in a similar way. Anaplastic and low-grade oligodendroglioma were coded as oligodendroglioma; mixed anaplastic oligoastrocytoma with necrosis was grouped with glioblastoma; and the other mixed oligoastrocytic tumors were coded with the oligodendroglioma. Grade II and anaplastic astrocytoma found at central review were coded as astrocytoma.

MGMT Status and Cytosine–Phosphate–Guanine Island Methylated Phenotype

As part of previous projects, 78 samples (59%) were investigated for epigenetic changes using the Illumina HM450 Beadchip methylation platform.²⁹ This allowed assessment of the cytosine–phosphate–guanine island methylated phenotype (CIMP). Using an algorithm for the methylation of 2 probes within the O⁶-DNA methylguanine-methyltransferase (MGMT) promoter region, MGMT promoter methylation can also be calculated (MGMT-STP27) from the results of this analysis.³⁵ In

previous analysis of tumor samples from EORTC study 26951, assessment of MGMT promoter methylation using MGMT-STP27 provided the most powerful prediction of survival benefit from adjuvant PCV chemotherapy.

Survival

Survival was measured from the date of randomization, and the follow-up of the most recent clinical update of the study was used.¹⁴ For all analysis of prognostic variables, patients treated with radiotherapy (RT) + PCV and RT alone were lumped. In a separate predictive factor analysis, impact of the key variables on benefit from adjuvant PCV was explored.

Statistical Analysis

Three molecularly defined glial subtypes were compared for the presence of molecular abnormalities by computing the non-parametric Kruskal–Wallis test. Agreement between measurements (NGS vs FISH) was assessed by kappa coefficient. A coefficient lower than 0.8 was considered poor agreement. The Kaplan–Meier technique and Cox regression models were used for survival analyses. Schemper’s percentage of explained variation (PEV) was computed at 0.^{36,37} A PEV of at least 20% was considered a minimum requirement for a model to provide sufficiently precise individual survival predictions.³⁸ Conditional inference tree (CTREE) was performed as complementary analysis.³⁹ CTREE is a nonparametric decision tree learning technique which produces either classification or regression trees. SAS version 9.2 was used for descriptive and survival analyses. CTREE analyses were performed with the “Party” R package (<http://cran.r-project.org/web/packages/party/index.html>). In this exploratory analysis, a *P*-value of .05 was taken as statistical significance, and no attempt was made to correct for multiple testing. For exploratory purposes, multiple correspondence analysis (MCA) was done to detect underlying structure in the dataset containing multiple binary markers by representing data as points in a lower dimensional space. The R package FactoMineR (<http://cran.r-project.org/web/packages/FactoMineR/index.html>) was used for fitting MCA technique and to generate factorial plans based on the 2 main dimensions.

Results

From 139 of the 368 randomized patients, sufficient material was available for NGS; in 6 patients, results were felt to be of questionable reliability because of low coverage rates, leaving 133 samples for further analyses. There was no major difference between the subgroup included in the analysis and other patients recruited in the trial. Patients in the present analysis less often had good WHO performance status (0 or 1, 63% vs 81%), and fewer patients had a tumor biopsy only (7.5% vs 17%). There was no significant difference in survival outcome between patients included and excluded in the molecular analysis (*P* = .778). From 113 samples formalin-fixed paraffin-embedded tissue was used; for 26 samples DNA from snap frozen samples were available.

The applied NGS LOH analysis was first validated by direct comparison of microsatellite LOH analysis (based on 5 markers

for 1p and 5 markers for 19q) and SNP-based LOH analysis by NGS (29 SNPs for 1p and 16 for 19q) of 48 EORTC 26951 samples (of which 18 were 1p/19q codeleted). Of these, 10 samples showed distinct results with the 2 methods. In 7 cases, SNP-based analysis showed partial or complete 1p or 19q LOH not shown by microsatellite analysis, apparently due to the higher sensitivity of SNP-based analyses. The other 3 discrepancies involving partial losses might reflect the distinct, nonoverlapping, genomic locations and difference in density of the SNP and microsatellite markers and underlying complex chromosomal rearrangements. Overall 1p/19q codeletion status remained the same in all cases. The sensitivity of SNP-based LOH analysis was further assessed by serial dilutions of normal and glioma tissue of the same patient and was found to be higher than microsatellite-based analysis. Validation of *EGFRamp* detection by targeted NGS, based on SNP- and amplicon-coverage analysis, was performed by comparison of NGS with the panel described above and *EGFR* FISH of 50 glioma cases. No discrepancies were observed between methods, showing 23 cases with and 27 without *EGFRamp*.

NGS revealed significantly more copy number alterations of 1p, 19q, 7, and 10q compared with the previous analysis using FISH. Table 1 summarizes the molecular findings in all tumors and the various tumor classes. In 68 patients, mutations of isocitrate dehydrogenase (*IDH*) were identified (66 *IDH1* mutations and 2 *IDH2* mutations); in 50 cases, a 1p/19q codeletion was found. NGS diagnosed more tumors with 1p/19q codeletion (entire arms) compared with the previous analysis with FISH probes (with which 34 of these 133 cases were found codeleted, 4 of which were not confirmed with NGS; kappa score 0.59). The 20 discordant 1p/19q codeleted cases not diagnosed as codeleted by FISH were found to be *TERT* and *IDH* mutated, whereas the 4 not confirmed cases were either *EGFRamp* or *TERT* wild type (wt). Median survival of 1p/19q codeleted tumors assessed with NGS was similar to that of codeleted tumors assessed with FISH (9.4 y vs 10.6 y). *TERT* mutations were found in 100 tumors (75%). In 47 tumors, 10q loss was found in combination with either *EGFRamp* or imbalance of 7; in 2 of these tumors, an *IDH* mutation was found (both without *EGFRamp*). Gain of chromosome 7 (imbalance) could not be established in the *EGFRamp* cases. It was found in 17% of tumors, mainly in glioblastoma.

Molecular Classification

With the above criteria, 49 tumors were classified as type I (oligodendroglioma), 20 as type II (astrocytoma), and 55 as type III (glioblastoma); 9 remained unclassified. Of the 9 unclassified tumors, 2 had an *H3F3A* mutation indicative of a “childhood” glioblastoma (ages 19 and 21).⁴⁰ A total of 126 tumors (91%) were therefore successfully classified with the NGS panel. One tumor diagnosed at central review as an ependymoma had only a *CIC* mutation, 1 had both an *EGFR* and a *TP53* mutation, 2 had minor copy number alterations, and in 3 no abnormalities were identified. One codeleted tumor was classified by the algorithm in the glioblastoma group because it was *IDHwt* and showed imbalance of Chr 7 and 10q LOH (overall survival [OS] in this patient was 1.0 y). One other codeleted tumor was both *IDHwt* and *TERTwt* and showed in addition only a *NOTCH* mutation (OS: alive after 10 y of follow-up).

All other 1p/19q codeleted tumors showed both mutations in both *IDH* and *TERT*, and none showed both LOH of entire 10q and imbalance of the entire Chr 7.

IDH mutations were inversely correlated with 10q LOH, imbalance of 7, and *EGFRamp* (rho between -0.50 and -0.70), and positively correlated with *CIMP* (rho 0.90) and 1p/19q loss. In addition, 1p/19q codeletion was positively correlated with *CIC* and *FUBP1* mutations (rho 0.50–0.70). *TERT* mutations were negatively correlated with mutations in *ATRX* and *TP53* (rho between -0.50 and -0.60).

Table 1 summarizes the molecular findings in each of the subtypes. All but one type I tumor were *TERT* mutated and *IDH* mutated, and virtually all type II tumors (95%) showed *TP53* mutations. *TERT* mutations were identified in 95% of the type III tumors and in 98% of the type I tumors. *CIC* mutations were found in 30 tumors, all but one in type I tumors (prevalence here 59%); similarly all 20 *FUBP1* mutations were found in type I tumors (41% of cases). *ATRX* mutations were found in 65% of type II tumors; in only one tumor (1p/19q codeleted) were both an *ATRX* mutation and a *TERT* mutation found. Twelve *PTEN* mutations were found, all but one in type III tumors. LOH 10q was present in several *IDH* mutated tumors but occurred predominantly in type III tumors (93%).

Survival

Survival was consistent with expectations, with significantly shorter median OS in type III tumors (glioblastoma) (1.1 y) and the longest survival in type I tumors (oligodendroglioma) (9.3 y), but with an interesting plateau of the survival curve of type II tumor (astrocytoma) patients (median: 3.1 y, $P < .001$; Table 2 and Fig. 1A). With the small group of type II tumors, just a trend for better survival was present in type I tumors compared with type II tumors (hazard ratio [HR] = 0.62; 95% CI = 0.31, 1.21); 5-year survival in type II tumors was 47% as opposed to 6% in type III glioblastoma and 71% in type I tumors. The prognostic significance of 1p/19q loss diagnosed with NGS was similar to the significance of the FISH diagnosed cases (HR = 0.28 vs 0.31; both $P < .0001$).

Methylation Status

Seventy-eight samples (59%) were previously investigated for epigenetic changes by genome-wide methylation profiling; 43 of these were *CIMP*-positive tumors (Table 3).²⁹ Thirty-nine of these tumors were *IDH* mutated, but 4 were *IDHwt*. From 39 type I and type II tumors, both *CIMP* and *MGMT* promoter methylation data were available. All were *CIMP* positive; 2 of the type II tumors, however, did not show evidence of *MGMT* promoter methylation. In 33 type III tumors, methylation data of *MGMT*-STP27 were available; 19 were considered *MGMT* methylated and 32 were *CIMP* negative. Survival in *IDHwt* tumors was not significantly different according to *MGMT* status: median survival was 1.1 years for unmethylated tumors (95% CI = 0.76, 1.39) and 1.4 years for methylated tumors (95% CI = 0.95, 1.98; HR = 0.57; 95% CI = 0.29, 1.1) with respectively 15.8% (95% CI = 3.9, 34.9) and 27.3% (95% CI = 11.1, 46.4) of patients alive at 2 years.

Table 1. Molecular abnormalities present in the 3 molecularly defined glioma subtypes

	No Type (N = 9) N (%)	Type I (N = 49) N (%)	Type II (N = 20) N (%)	Type III (N = 55) N (%)	Total (N = 133) N (%)	P
1p/19q						
No loss	9 (100.0)	0 (0.0)	20 (100.0)	54 (98.2)	83 (62.4)	NA
Loss	0 (0.0)	49 (100.0)	0 (0.0)	1 (1.8)	50 (37.6)	
IDH						
Normal	9 (100.0)	1 (2.0)	0 (0.0)	55 (100.0)	65 (48.9)	NA
Mutated	0 (0.0)	48 (98.0)	20 (100.0)	0 (0.0)	68 (51.1)	
TERT						
Normal	9 (100.0)	1 (2.0)	20 (100.0)	3 (5.5)	33 (24.8)	NA
Mutated	0 (0.0)	48 (98.0)	0 (0.0)	52 (94.5)	100 (75.2)	
Chr 7						
No loss	7 (77.8)	46 (93.9)	16 (80.0)	5 (9.1)	74 (55.6)	NA
Imbalance	2 (22.2)	3 (6.1)	3 (15.0)	15 (27.3)	23 (17.3)	
Missing	0 (0.0)	0 (0.0)	1 (5.0)	35 (63.6)	36 (27.1)	
EGFRamp						
No	9 (100.0)	49 (100.0)	20 (100.0)	20 (36.4)	98 (73.7)	NA
Yes	0 (0.0)	0 (0.0)	0 (0.0)	35 (63.6)	35 (26.3)	
10q						
No loss	7 (77.8)	43 (87.8)	14 (70.0)	4 (7.3)	68 (51.1)	NA
Loss	2 (22.2)	6 (12.2)	6 (30.0)	51 (92.7)	65 (48.9)	
EGFR and Chr 7p						
EGFRwt and Chr 7pNL	7 (77.8)	46 (93.9)	16 (80.0)	5 (9.1)	74 (55.6)	NA
EGFRamp or Chr 7Im	2 (22.2)	3 (6.1)	3 (15.0)	49 (89.1)	57 (42.9)	
Missing	0 (0.0)	0 (0.0)	1 (5.0)	1 (1.8)	2 (1.5)	
ATRX						
Normal	8 (88.9)	48 (98.0)	7 (35.0)	55 (100.0)	118 (88.7)	<.0001
Mutated	1 (11.1)	1 (2.0)	13 (65.0)	0 (0.0)	15 (11.3)	
TP53						
Normal	5 (55.6)	45 (91.8)	1 (5.0)	44 (80.0)	95 (71.4)	<.0001
Mutated	4 (44.4)	4 (8.2)	19 (95.0)	11 (20.0)	38 (28.6)	
CIC						
Normal	8 (88.9)	20 (40.8)	20 (100.0)	55 (100.0)	103 (77.4)	<0.0001
Mutated	1 (11.1)	29 (59.2)	0 (0.0)	0 (0.0)	30 (22.6)	
FUBP1						
Normal	9 (100.0)	29 (59.2)	20 (100.0)	54 (98.2)	112 (84.2)	<.0001
Mutated	0 (0.0)	20 (40.8)	0 (0.0)	1 (1.8)	21 (15.8)	
PTEN						
Normal	9 (100.0)	48 (98.0)	20 (100.0)	43 (78.2)	120 (90.2)	.0022
Mutated	0 (0.0)	1 (2.0)	0 (0.0)	11 (20.0)	12 (9.0)	
Missing	0 (0.0)	0 (0.0)	0 (0.0)	1 (1.8)	1 (0.8)	
NOTCH1						
Normal	9 (100.0)	38 (77.6)	20 (100.0)	53 (96.4)	120 (90.2)	.002
Mutated	0 (0.0)	11 (22.4)	0 (0.0)	2 (3.6)	13 (9.8)	
PIK3CA						
Normal	9 (100.0)	47 (95.9)	19 (95.0)	54 (98.2)	129 (97.0)	.72
Mutated	0 (0.0)	2 (4.1)	1 (5.0)	1 (1.8)	4 (3.0)	

NA, not applicable (the molecular aberration is part of the definition of the molecular class); NL, normal.

Pathology Review

Table 4 summarizes the diagnosis at central pathology review in relation to the molecular classification. At central pathology review, 93 tumors were diagnosed as oligodendroglioma, half of these were “confirmed” as type I tumors in the molecular

classification, while one-third of oligodendroglioma cases were reclassified as type III tumors (glioblastoma). However, 92% of the type I tumors were histopathologically also diagnosed as oligodendroglioma. Most histopathologically diagnosed glioblastomas were diagnosed as type III at molecular classification, but 22% were reclassified as type II. Astrocytomas were

Table 2. Median survival and percentage of patients alive (% OS) at 2 and 5 years and the HR for death in the 3 glioma classes for both the glioma subtype at molecular analysis and at central pathology review

	Number of Patients	Observed Events	Median, y (95% CI)	% OS at 2 y (95% CI)	% OS at 5 y (95% CI)	Hazard Ratio (95% CI)
Molecular subtype						
Type I	49	28	9.53 (5.91, NR)	93.9 (82.2, 98.0)	71.4 (56.6, 82.0)	0.65 (0.33, 1.28)
Type II	20	12	3.07 (2.41, NR)	79.0 (53.2, 91.5)	47.4 (24.4, 67.3)	1.00
Type III	55	54	1.13 (0.96, 1.37)	21.8 (12.1, 33.4)	5.5 (1.4, 13.6)	5.59 (2.90, 10.78)
Pathological subtype						
Oligodendroglioma	93	68	4.50 (2.55, 5.91)	67.4 (56.8, 75.9)	45.7 (35.3, 55.4)	0.91 (0.29, 2.89)
Astrocytoma	4	3	2.98 (1.17, NR)	75.0 (12.8, 96.1)	25.0 (0.9, 66.5)	1.00
Glioblastoma	32	29	1.30 (0.90, 1.50)	31.3 (16.4, 47.3)	12.5 (4.0, 26.2)	2.23 (0.68, 7.32)

Abbreviation: NR, not reached.

rare ($n = 4$). The interrater “agreement” between the histopathological diagnosis and the molecular classification was “fair” (kappa coefficient = 0.33; 95% CI = 0.22, 0.45); overall concordance was 59% (71/121). The histopathological diagnoses at central study review resulted in diagnoses with statistically and clinically clearly different survivals (Table 2, Fig. 1B).

Prognostic Factor Analysis

Entered into a multivariable Cox regression analysis were individual molecular factors significant in univariate analyses and 2 combined factors: (i) the combination of *TERT* mutational status and 1p/19q status and (ii) the presence of 10q loss with either *EGFRamp* or imbalance of 7. In this analysis, compared with absence of *TERT*mut, the presence of *TERT*mut-1p/19q intact ($P < .0001$; HR = 4.04; 95% CI = 2.36, 6.88) and that of *TERT*mut-1p/19q codeleted ($P = .04$; HR = 0.57; 95% CI = 0.33, 0.99) were statistically significant prognostic factors for OS. With bootstrap, *TERT*mut-1p/19q intact was selected in 70.9% of runs, and IDH in 56.4%. With CTREE analysis applied to all molecular factors, IDH was the factor with most significant prognostic information ($P < .001$), with loss of 10q as a second factor ($P = .01$). In multivariable analyses, the prognostic impact of the molecular classification was compared with the prognostic impact of the central study review diagnosis. In these analyses, only the molecular classification was selected (data not shown). The PEV of the survival model including the treatment and the central review diagnosis was smaller than the PEV of the model including the treatment and the molecular classification (PEV = 9.8% vs 35.3%). Only the latter could provide sufficiently precise individual survival predictions according to predefined criteria (PEV > 20%).

In the subgroup of 1p/19q codeleted tumors, the presence of *CIC*, *FUBP1*, *NOTCH*, and *TP53* mutations or copy number alterations of 7 and 10q did not have an impact on outcome (univariate analysis; Fig. 2A–E). In the relatively small group of astrocytoma patients ($n = 20$), the presence of *ATRX* mutations had no impact on outcome ($P = .87$; Fig. 2F).

Predictive Factors for Benefit of PCV Chemotherapy

In the present subgroup the addition of PCV to RT was associated with improved survival ($P = .03$; HR = 0.65; 95% CI = 0.44,

0.97). With the information available from NGS and from epigenetic changes in 78 cases, only 3 factors showed statistically significant predictive value for improved outcome after PCV: *MGMT*-STP27 ($P = .025$), *MGMT* status in combination with *IDH* mutational status ($P = .05$), and imbalance of 7 (Fig. 3). The significance in the small group with imbalance of 7 was heavily confounded by 3 long-term survivors with combined 1p/19q loss. In this dataset, neither 1p/19q codeletion, *IDH* mutational status, nor molecular subtype was found to be predictive (test for interaction $P = .78$, .42, and .64, respectively). None of the other factors was correlated to improved benefit from PCV.

Multiple Correspondence Analysis

MCA summarized the molecular markers into 2 uncorrelated factorial dimensions, explaining 38% of factor variation. These 2 dimensions are built on 3 separate groups of patients characterized by the association of the following markers:

- Group 1: *CIC*mut, *FUBP1*mut, 1p/19q loss, *IDH*mut, *Notch1*-mut, 10q no loss, Chr 7 normal, *PIK3CA*, *CIMP*+, *EGFR* normal
- Group 2: *EGFRamp*, *CIMP*-, *PTEN*mut, *MGMT* unmet, *IDH*wt, *EGFR*mut, 10q loss, 1p/19q no loss, Chr 7 imbalance, *CIC*wt
- Group 3: *H3F3A*mut, *ATRX*mut, *TERT*wt, *TP53*mut, *CIMP*+, *MGMT* met, Chr 7 imbalance, 1p/19q no loss, *CIC*wt

Discussion

The present dataset on locally diagnosed anaplastic oligodendroglioma with extensive follow-up data shows the power of targeted NGS for the routine molecular classification of diffuse glioma. Despite the long time in paraffin (>10 y), 96% of the tumor samples could be successfully analyzed; and of those, 95% could be identified molecularly as either type I (oligodendroglioma), type II (astrocytoma), or type III (glioblastoma) ($n = 124$) or as a childhood glioblastoma (presence of an *H3F3A* mutation, $n = 2$).⁴⁰ The survival rates of patients with these molecularly defined tumor classes were markedly different and consistent with expectations, although in this small group the median survival of 3.1 years and the 5-year survival of 47% in the type II group were not statistically significantly different from the median survival of 9.5 years and the

Fig. 1. Kaplan–Meier overall survival (A) of the three molecular defined diffuse glioma classes; and (B) of the pathological subtypes as diagnosed by the central pathology reviewer.

Table 3. Methylation data and molecular glioma classification

	Subtype				Total (N = 133) N (%)	P
	No Type (N = 9) N (%)	Type I (N = 49) N (%)	Type II (N = 20) N (%)	Type III (N = 55) N (%)		
CIMP						
CIMP-	3 (33.3)	0 (0.0)	0 (0.0)	32 (58.2)	35 (26.3)	<.0001
CIMP+	3 (33.3)	23 (46.9)	16 (80.0)	1 (1.8)	43 (32.3)	
Missing	3 (33.3)	26 (53.1)	4 (20.0)	22 (40.0)	55 (41.4)	
MGMT-STP27						
Unmethylated	3 (33.3)	0 (0.0)	2 (10.0)	14 (25.5)	19 (14.3)	.0005
Methylated	3 (33.3)	23 (46.9)	14 (70.0)	19 (34.5)	59 (44.4)	
Missing	3 (33.3)	26 (53.1)	4 (20.0)	22 (40.0)	55 (41.4)	

Table 4. Review pathology diagnosis in the 3 molecularly defined glioma groups

	Molecularly Defined Glial Subtype				Total (N = 133) N (%)	P
	No Type (N = 9) N (%)	Type I (N = 49) N (%)	Type II (N = 20) N (%)	Type III (N = 55) N (%)		
Central pathology review						
Astrocytoma	0 (0.0)	0 (0.0)	3 (15.0)	1 (1.8)	4 (3.0)	<.0001*
Oligodendroglioma	8 (88.9)	45 (91.8)	10 (50.0)	30 (54.5)	93 (69.9)	
GBM	0 (0.0)	1 (2.0)	7 (35.0)	24 (43.6)	32 (24.1)	
Missing	1 (11.1)	3 (6.1)	0 (0.0)	0 (0.0)	4 (3.0)	

Abbreviation: GBM, glioblastoma multiforme. *Kappa 0.33 (0.22–0.45), overall concordance: 59% (71/121).

5-year survival of 71% observed in the type I group. The currently available studies consistently show that at the molecular level, true mixed glioma tumors do not exist, despite occasional observations.^{10–12} Moreover, the clinical relevance of describing a mixed phenotype is unclear, other than prompting for molecular analysis.

The prognostic significance of the molecular classification was found to be superior to the significance of the histopathological classification. Within the type I tumors, the presence of *CIC*, *FUBP*, *NOTCH*, *TP53*, and other abnormalities did not have an impact on overall outcome of 1p/19q codeleted tumors or on outcome to PCV chemotherapy. Others have shown that in 1p/19q codeleted oligodendrogliomas, contrast enhancement and the presence of necrosis and microvascular proliferation are associated with 9p loss and with increasing chromosomal copy number alterations.^{41,42}

Our data suggest that NGS is not only of major prognostic significance, but also more sensitive and specific than classical single factor analysis. For our molecular classification, we used a handful of molecular abnormalities that have recently been identified as pivotal for the diffuse gliomas of the 3 predominant lineages: *IDH* mutations, 1p/19q codeletion, *TERT* mutations, *EGFR* amplification, and imbalance of Chr 7 in combination with LOH 10q. These genetic alterations are highly specific for 3 different diffuse glioma lineages. The validity of this classification is supported by both the prognostic factor analysis and by the unsupervised MCA. In the present series,

we conducted several prognostic factor analyses, and the most relevant prognostic factors identified were the presence of *IDH* mutations, *TERT* mutations in relation to 1p/19q status, and 10q LOH. This underscores that the characteristic genetic abnormalities that underlie our molecular classification carry major prognostic information. Moreover, in unsupervised MCA, the same 3 groups of molecular lesions were identified, with *H3F3A* mutated tumors clustering with the astrocytoma.

In previous publications, somewhat different criteria have been suggested for the various molecularly defined subsets of diffuse glioma, and minimum molecular requirements for each diagnosis have not yet been established. For instance, it is unclear whether loss of 1p/19q is sufficient for the molecular diagnosis of type I, or oligodendroglioma; whether *ATRX* mutations are critical for the diagnosis of type II, or astrocytoma; and whether type III, or glioblastoma, can be best defined as gain of Chr 7 and loss of 10 or as the presence of *TERT* mutations in the absence of 1p/19q codeletion. In fact, when considering only mutations of the *IDH* and *TERT* genes or only *IDH* mutations, 1p/19q status, and LOH 10q with imbalance of Chr 7, very similar prognostic classifications are obtained.

The advantage of assessing more mutations and copy number alterations is that the presence of consistent molecular findings in one tumor yields more certainty of the molecular diagnosis, adding to the specificity of the diagnosis. Indeed, the mutual exclusivity of certain findings (eg, *ATRX* and *TERT* mutations, *IDH* mutations, *EGFR* amplifications) and the

A *CIC* mutation in 1p/19q codeleted

Overall Survival

B *FUBP1* mutation in 1p/19q codeleted

Overall Survival

C 10q loss in 1p/19q codeleted

Overall Survival

D *TP53* mutation in 1p/19q codeletion

Overall Survival

E *NOTCH* mutations in 1p/19q codeleted

Overall Survival

F *ATRX* mutations in astrocytoma

Overall Survival

Fig. 2. (A–E) Impact on OS in molecularly defined oligodendroglioma (1p/19q codeleted) of (A) *CIC* mutations, (B) *FUBP1* mutations, (C) LOH 10q, (D) *TP53* mutations, (E) *NOTCH* mutations; and (F) OS of astrocytoma with and without *ATRX* mutations. Red curves: tumors with wt genes or normal copy numbers. Blue curves: tumors with mutations or copy number alteration.

Fig. 3. Forest plot with HR for benefit to adjuvant PCV chemotherapy for the most relevant potential predictive molecular factors (*IDH*, 1p/19q codeletion, CIMP status, *MGMT* methylation as determined with Illumina 450 platform, and Chr 7 status).

presence of findings that usually occur together (eg, *CIC* mutations in 1p/19q codeleted cases, *TP53* mutations in *IDH* mutated but 1p/19q intact tumors, *TERT* and *IDH* mutations in 1p/19q codeleted tumors) help to strengthen the classification of individual tumors. Our data therefore also give some guidance on the minimal requirements for a molecular classification of diffuse glioma. Virtually all 1p/19q codeleted tumors have in addition both *IDH* mutations and *TERT* mutations. In recent discussions, the issue has been raised of how to classify 1p/19q codeleted tumors without oligodendroglial morphology. The available data suggest, however, that the absence of *TERT* and *IDH* mutations in 1p/19q codeleted cases may in fact be more relevant, as these are uncommon. Similarly, nearly all *IDH*mut but 1p/19q intact tumors had *TP53* mutations, and the single *TP53*wt tumor had an *ATRX* mutation. *ATRX* mutations were less frequent (65%) in this group, similar to the findings of others.^{43,44}

The percentage of tumors with 1p/19q codeletion was clearly higher than previously obtained with FISH, with a strong correlation with specific other abnormalities (*TERT* mutations, *IDH* mutations). Others have found similar high rates of *IDH* and *TERT* mutations in tumors with 1p/19q codeletion.^{17,25,45} Taken together, this suggests that NGS is also more sensitive than our previous diagnostics with FISH.

The potential advantage of the molecular classification is not limited to improved prognostication, but also to improved

prediction of outcome to therapy. We again explored factors related to the prediction of OS benefit from PCV chemotherapy. Both the North American RTOG 9402 study and the EORTC 26951 study give evidence that benefit from PCV chemotherapy is not limited to the group of patients with 1p/19q codeleted tumors. In the RTOG 9402 study, in 74% of cases an *IDH* mutation was found, and *IDH* mutations appeared to identify patients with benefit from PCV. Previous work on the EORTC 26951 study suggested that CIMP—but in particular *MGMT* promoter methylation assessed with the Illumina H450 Beadchip—was strongly predictive for improved outcome after PCV, whereas *IDH* status was insufficient to identify patients benefiting from PCV chemotherapy.^{14,29} With the limited number of cases present in this cohort of patients, the presence of *IDH* mutations was again not predictive of benefit from PCV, but (and with even fewer cases) *MGMT* promoter methylation was. This is in line with our earlier reports.¹⁴ A difference between RTOG 9402 and EORTC 26951 is that in the EORTC dataset, only 50% of patients had *IDH* mutations, and in the present analysis 41% of the tumors were reclassified as type III, or glioblastoma. In RTOG 9402, 74% of the examined cases had an *IDH* mutation.⁴⁶ Taken together, the data suggest that the strongest predictive factor for benefit is *MGMT* promoter methylation, but 95% of *IDH* mutated tumors with *MGMT* status available had *MGMT* promoter methylation. This appears to reconcile the observed differences between the North American

and European studies.^{13,14,29,46} Of note, the analyses of both EORTC 26951 and RTOG 9402 on IDH and MGMT (but also this NGS analysis) are of a retrospective nature. The ongoing CATNON study (“Concurrent and/or Adjuvant TMZ for 1p/19q Nondeleted Tumors”) on temozolomide chemotherapy in 1p/19q intact grade III tumors will allow further clarification of the optimal predictive marker.

It is noteworthy that 41% of the sequenced tumors were from a molecular perspective glioblastoma, and would not have been eligible if molecular criteria would have been used. The recent studies on molecular profiling of low-grade and grade III glioma showed quite a similar result using a similar molecular classification; here 15%–20% of grades II and III gliomas were shown to have a glioblastoma-like profile.^{47,48} With all recent data, the strength of molecular glioma classification has been proven beyond doubt, leaving the details of such classification to be decided upon. Targeted NGS did not resolve all diagnostic issues, however. Some technical difficulties were encountered. First, *TERT* promoter analysis had to be redone using a PCR technique after initial experiments failed to show good results for this gene. This appears to be due to the fact that this promoter region is highly enriched for G and C nucleotides, preventing optimal DNA amplification. Targeted NGS in the presence of high-level *EGFR* amplification leads in many cases to an insufficient coverage of other genomic regions, due to the relatively high number of primer pairs we designed in the *EGFR* region. We are currently revising the *TERT* and *EGFR* part of the panel. Then, this classification considers only lineage, not grade. Many studies have shown that tumors with more anaplastic features tend to have more genetic abnormalities, but in general tumor grade does not seem to be conferred by single mutations. In this dataset, the presence of other single molecular alterations was not correlated to outcome in 1p/19q codeleted tumors. Others have shown that in 1p/19q codeleted oligodendroglioma, contrast enhancement and the presence of necrosis and microvascular proliferation are associated with 9p loss and with increasing chromosomal copy number alterations.^{41,42} With the present panel, similar mutations may be observed in histologically defined low-grade astrocytoma and secondary glioblastoma with *IDH* mutations. Thus, the grading of tumors is likely to remain relevant in molecularly classified diffuse glioma.

We also did not assess germline SNP alterations, and some abnormalities that we picked up could have been germline variants or unknown SNPs. Also, molecular heterogeneity within tumors is a potential source of error for any molecular classification schedule. The present NGS approach assumes that the molecular abnormalities used for the classification are present in all cells of the tumor, and indeed the current data suggest that 1p/19q codeletion, imbalance of Chr 7, LOH 10q, and mutations in the *IDH*, *ATRX*, and *TERT* genes are early events in the genesis of glial tumors or have shown to be relatively stable across the tumor. The consistency of results in various recent molecular studies are supportive for this assumption, as is the supportive evidence from other chromosomal aberrations that are consistent with the main findings (eg, *CIC* mutations almost exclusively in 1p/19q codeleted tumors, *EGFR* amplification in *IDH*wt and *TERT* mutated tumors). Lastly, targeted NGS addresses DNA mutations and copy number alterations, but not epigenetics. Our present data suggest that for outcome to

chemotherapy, the methylation status of the *MGMT* promoter is the single most important factor. At the end of the day, more than one diagnostic platform may actually be required to collect all the clinically relevant information.

To conclude, our study shows that targeted NGS allows a reliable and clinically relevant classification of glial tumors, which gives more robust prognostic information compared with classical histopathology. The data support the transition to a molecular classification of these tumors, although how this should be implemented in everyday practice remains the subject of discussions. Whether this is best accomplished by a multilayer diagnosis or by primarily a molecular classification probably warrants more rounds of discussion.⁴⁹ Our data, however, strongly argue for a primary molecular classification, to which diagnosis the histopathological grade should be added. Also, this targeted NGS approach is only effective in tumors of diffuse glial lineage, and will not yield a diagnosis in unrelated tumors, that is, not characterized by any of these mutations. Furthermore, epigenetics is not addressed with this approach, and this is also of high clinical relevance for the management of diffuse glioma. It is clear, though, that once implemented in standard-of-care routine, molecular diagnostics will greatly improve our understanding of the outcome of our patients.

Supplementary Material

Supplementary material is available at *Neuro-Oncology Journal online* (<http://neuro-oncology.oxfordjournals.org/>).

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