Expression based Intrinsic Glioma Subtypes are prognostic in low grade gliomas of the EORTC22033-26033 clinical trial.

Y Gao[1], B Weenink[1], MJ van den Bent[2], L Erdem-Eraslan[1], JM Kros[3], PAE Sillevis Smitt[1], K Hoang-Xuan[4], AA Brandes[5], M. Vos[6], F Dhermain[7], R Enting[8], GF Ryan[9], O Chinot[10], M Ben Hassel[11], ME van Linde[12], W P Mason[13], JMM Gijtenbeek[14], C. Balana[15], A. von Deimling[16], Th Gorlia[17], R Stupp[18], ME Hegi[19], BG Baumert[20,21] and PJ French[1].

[1] *De`partment of Neurology, Erasmus University Medical Center, 3000CA Rotterdam*

[2] *Department of Neurology, Daniel Den Hoed Cancer Center, 3075 EA Rotterdam, the Netherlands*

[3] *Department of Pathology, Erasmus University Medical Center, 3000CA Rotterdam*

[4] *APHP Pitié-Salpêtrière, Sorbonne Universités, UPMC, ICM, UMR S 1127, Paris, France*

[5] *Ospedale Bellaria, Bologna, Italy*

[6] *Med Ctr Haaglanden, The Netherlands*

[7] *I. Gustave Roussy, Villejuif, France*

[8] *UMCG and University of Groningen, Groningen, The Netherlands*

[9] *Peter MacCallum Cancer Center, Melbourne, Australia*

[10] *Aix Marseille, Université, APHM La Timone, Marseille, France*

[11] *Centre Eugène Marquis, Rennes, France*

[12] *VU University Medical Center and Academic Medical Center, Amsterdam, Netherlands*

[13] *Princess Margaret Hospital, University of Toronto, Toronto, ON, Canada*

[14] *Radboud University Medical Center Nijmegen, The Netherlands*

[15] *ICO Badalona Hospital, Germans Trias I Pujol, Barcelona, Spain*

[16] *German Cancer Consortium (DKTK) and CCU Neuropathology German Cancer Research Center (DKFZ) and Department Neuropathology, Institute of Pathology, University of Heidelberg, Heidelberg, Germany*

[17] *European Organisation for Research and Treatment of Cancer Headquarters, Brussels,Belgium*

[18] *Neuroscience Research Centre, CHUV, Lausanne, Switzerland*

[19] *Department of clinical Neurosciences, Lausanne University Hospital, Lausanne, Switzerland*

[20] *Depts. of Radiation-Oncology Paracelsus Clinic Osnabrueck and University of Muenster, Germany*

[21] *Maastricht University MedicalCentre and GROW (School for Oncology), Maastricht, The Netherlands*

European Journal of Cancer 94 (2018) 168e178

ABSTRACT

Introduction

The EORTC22033-26033 clinical trial (NCT00182819) investigated whether initial temozolomide (TMZ) chemotherapy confers survival advantage compared to radiotherapy (RT) in low grade glioma patients (LGG). In this study we performed gene expression profiling on tissues from this trial in order to identify markers associated with progression free survival and treatment response.

Methods

Gene expression profiling, performed on 195 samples, was used to assign tumors to one of six intrinsic glioma subtypes (IGS; molecularly similar tumors as previously defined using unsupervised expression analysis) and to determine the composition of immune-infiltrate. DNA copy number changes were determined using OncoScan arrays.

Results

We confirm that IGS-subtypes are prognostic in the EORTC22033-26033 clinical trial. Specific genetic changes segregate in distinct IGS subtypes: most samples assigned to IGS-9 have *IDH*-mutations and 1p19q codeletion, samples assigned to IGS-17 have *IDH*-mutations without 1p19q codeletion and samples assigned to other intrinsic subtypes often are *IDH*-wildtype. A trend towards benefit from RT was observed for samples assigned to IGS-9 (HR for TMZ is 1.90, $P = 0.065$), but not for samples assigned to IGS-17 (HR 0.87, $P = 0.62$). We did not identify genes significantly associated with progression free survival (PFS) within intrinsic subtypes, though follow-up time is limited. We also show that LGGs and GBMs differ in their immune-infiltrate which suggests that LGGs are less amenable to checkpoint inhibitor type immune therapies. Gene-expression analysis also allows identification of relatively rare subtypes. Indeed, one patient with a pilocytic astrocytoma (PA) was identified.

Conclusion

Intrinsic glioma subtypes are prognostic for PFS in EORTC22033-26033 clinical trial samples.

INTRODUCTION

Low grade or grade II gliomas (LGGs) are a heterogeneous set of primary brain tumors that mainly occur in the 4th and 5th decade of life [1,2]. The incidence is relatively low (< 1 per 100.000 persons/year) and, as they are slow growing tumors, patients have a relatively favorable prognosis, especially compared to gliomas of higher grade. Nevertheless, LGGs have the tendency to evolve into gliomas of higher grade, and most patients will ultimately die from the disease [3,4]. Based on their histological appearance, three subtypes of LGG can be distinguished: oligodendrogliomas, astrocytomas and mixed oligoastrocytomas. The current WHO classification has incorporated molecular markers (1p19q codeletion, and mutations in the *IDH1/2* and *ATRX* genes) to delineate astrocytoma and oligodendroglioma, but no longer considers oligoastrocytomas as a separate entity as they cannot molecularly be distinguished from other entities. [2,5]

Treatment options for LGG patients include surgery, radiotherapy (RT) and chemotherapy (or combinations thereof), or a watchful waiting strategy can be adopted [4,6]. Nevertheless, the optimal management of patients with a LGG has remained controversial and only relatively few randomized phase III clinical trials have been performed. Earlier trials focusing on the effect of RT showed no effect of RT dosing on overall survival and, in a separate trial, there was no effect of early vs delayed RT after surgery on overall survival [7-9]. Data from two large randomized clinical trials recently reported on the efficacy of chemotherapy in LGGs. Firstly, the RTOG9802 clinical trial, examining the role of the addition of procarbazine, lomustine and vincristine (PCV) chemotherapy after RT, showed improved survival of this regimen when compared to RT only [10]. Second, the EORTC22033-26033 clinical trial examined the role of RT vs temozolomide (TMZ) chemotherapy and found no difference between the two on progression free survival (PFS) or in quality of life [11,12]. Because of the limited follow-up time, data on overall survival is not available.

Interestingly, correlative molecular marker analysis in the EORCT22033-26033 study identified a subpopulation of patients that benefit from RT: Within the group of patients harboring tumors with an IDH mutation and in which the 1p and 19q chromosomal arms were not codeleted ('*Molecular Astrocytomas*'), an improved PFS was noted when they were treated with RT. No such benefit was observed within the group of IDH-mutated, 1p19q-codeleted tumors ('*Molecular Oligodendrogliomas*') [11]. We have previously shown that gene-expression profiling and subsequent molecular subtyping based on the gene-expression profile (intrinsic glioma subtypes) can identify prognostic subgroups and identify genes and subtypes that are associated with response to treatment [13-15]. In this study we have therefore performed gene expression

profiling, and associated immunophenotyping, of 195/477 samples included in the EORTC22033-26033 clinical trial to identify markers associated with survival and to aid in the identification of patients that benefit most from RT or TMZ treatment.

METHODS

Patient samples

Patients were considered eligible in the EORTC22033-26033 trial (clinical trial identifier NCT00182819), if they had been diagnosed with a histologically confirmed supratentorial, diffusely infiltrating grade II glioma (either astrocytoma, oligoastrocytoma or oligodendroglioma) according to the WHO 2006 classification [16]. Patients should present with at least one high-risk feature (age ≥40 years, progressive tumor disease, tumor size >5 cm, tumor crossing the midline, neurological symptoms). Details of the eligibility criteria and the consolidated standards on reporting trials (Consort) flow diagram have been described previously [11]. Patients were registered for the trial at any time after initial diagnosis (allowing for tissue collection and molecular analysis) and randomized at a time-point when treatment was required. A total of 707 patients were registered in the EORTC22033-26066 study of which 477 were randomized to receive either RT or TMZ. For this analysis a clinical cut-off date of 17th of January 2013 was used and the database was locked on 7th of August 2013. *IDH* mutation status and 1p/19q codeletion status were obtained as described in Baumert et al [11]. *MGMT* methylation status was determined using the MGMT-STP27 algorithm based on genome wide methylation array data [17] (Bady et al, submitted). All patients gave written informed consent for correlative molecular analysis.

RNA isolation and array hybridization

Sufficient material for expression analysis was available for 203/477 randomized patients. Eight samples failed QC so that expression profiles from a total of 195 samples are presented here. Of these, RNA was isolated from FFPE tissue blocks (n = 166) or from snap frozen tissue samples (n = 29). RNA extraction, purification and quantification from FFPE and FF material was reported previously [18,19]. Purified RNA (250 ng) was used for labeling and hybridization on DASL beadchips (Illumina, San Diego, CA) that were run by Service XS, Leiden, the Netherlands. Expression data were quantile normalized and corrected for batch effects using preprocessCore (Bioconductor) and ber (R) packages respectively. RNA expression profiles were then assigned to one of six intrinsic molecular subtypes of glioma as previously defined [18], using the ClusterRepro R package [20]. These intrinsic subtypes can be recapitulated on FFPE material with high concordance and using various expression profiling platforms [13,14,19]. SAM analysis was performed on FFPE samples using SAMR, an R package [21]. The SAM approach to identify genes

associated with treatment response is similar to previously reported [14,22]. Expression data are available via NCBI GEO datasets, GSE107850. Analysis of the immune infiltrate was assessed using the immunophenoscore R script (https://github.com/MayerC-imed/Immunophenogram) [23]. Glioblastoma samples of patients treated in the BELOB trial were used for immunophenotype comparison between low and high-grade gliomas[14].

DNA isolation and Genotyping

DNA was extracted for genotyping on a subset of samples using a QIAamp DNA FFPE tissue kit (Qiagen). Genotyping was performed using the OncoScan FFPE assays Kit (Affymetrix, Santa Clara, CA), a platform that allows determing copynumber changes and loss of heterozygosity (LOH) in FFPE samples using molecular inversion probes [24,25]. Copynumber changes were analyzed using Nexus Express for Oncoscan (Affymetrix).

Statistical analysis

Distribution of frequencies were compared between subtypes using the Chi-squared test. A Fishers' exact test was used in case the assumptions for chi-square distribution were violated as indicated in the respective tables. Kaplan–Meier survival curves were compared using the log-rank test using the survival package in R[26]. PFS was calculated from the time of initial diagnosis/surgery to the date of clinical or radiological progression or death (whichever occurred first). The significance of prognostic factors was determined with a multivariate analysis using Cox regression.

RESULTS

Expression data was generated successfully for 195/477 samples and most parameters were balanced between the 'included' (i.e. those with gene expression data) and the 'not included' cohort. However, the 'included' subset contained fewer biopsies, which may be expected due to the limited amounts of tissue available from this type of material (table 1). The included cohort also contained a higher proportion of astrocytomas and *IDH*-wt tumors. Interestingly, both variables are correlated with type of surgery: biopsies are more frequently performed in WHO2006 astrocytomas compared to non-astrocytomas (91/189 [48 %] *v.* 75/287 [26 %], $P < 0.0001$) and biopsies are more often performed on IDH-wt tumors (41/65 [74 %] *v.* 107/326 [33 %], $P < 0.0001$). Despite these differences, progression free survival of included vs not included patients was similar (39.8 vs 43.8 months respectively, supplementary figure 1). Progression free survival of included vs not included was also similar within the molecularly defined subgroups 'IDH-mutated, 1p19q codeleted (molecular olidogendrogliomas)', 'IDH-mutated, 1p19q non-codeleted (molecular astrocytomas)' and 'IDH-wt' (supplementary table 1).

Table 1. Comparison between included vs not included samples

variable		All	Included	Not inc	P
Treatment	RT	240	96	144	0.760
	TMZ	237	99	138	
Type of surgery	Biopsy	189	38	151	<0.001*
	Partial resection	206	116	90	
	Total resection	81	41	40	
	n/a	1	0	1	
Histology	Astrocytoma	167	51	116	0.002
	Oligoastrocytoma	118	51	67	
	Oligodendroglioma	192	93	99	
Performance	0	294	122	172	0.86
	1	165	65	100	
	2	18	8	10	
Gender	Female	202	85	117	0.71
	Male	275	110	165	
IDH mutation status	Mutated	327	166	161	<0.001
	Normal	65	14	51	
	n/a	85	15	70	
1p19q status	Codeleted	117	63	54	0.25*
	Intact	240	112	128	
	n/a	120	20	100	
MGMT methylation	Methylated	113	102	11	1 **
	Unmethylated	7	7	0	
Age	Age (years) mean ± SD	44.6 ± 11.7	43.9 ± 11.1	45.1 ± 12.2	0.31
	< Median	238	101	137	0.55
	> Median	239	94	145	

Abbreviations: RT: radiotherapy; TMZ: temozolomide. Performance: ECOG performance score. Chi squared test comparison between included vs not included samples only; Note on performance score, Chi-square test without performance score 2 also has P<0.001. *: Chi-squared test performed without n/a samples, **: Fishers' exact test.

Intrinsic glioma subtypes are prognostic in EORTC22033-26033 trial samples

Expression data from clinical trial samples were assigned to one of six 'Intrinsic Glioma Subtypes' (IGS-9, IGS-16, IGS-17, IGS-18, IGS-22 or IGS-23) as previously defined [17]. As can be expected in LGGs, the majority of samples were assigned to the prognostically favorable subtypes IGS-9 and IGS-17 (n = 74 and 115 respectively, figure 1 and table 2). In concordance with previously published data, samples assigned to IGS-9 predominantly had an *IDH* mutation (68/69), had 1p19q codeletion (45/68) and were diagnosed as oligodendroglioma (52/74, supplementary table 2) [13,18]. Samples assigned to IGS-17 predominantly contained tumors with an *IDH*-mutation (97/108), but 1p19q codeletion was rarely observed (17/102) and tumors were more frequently diagnosed,

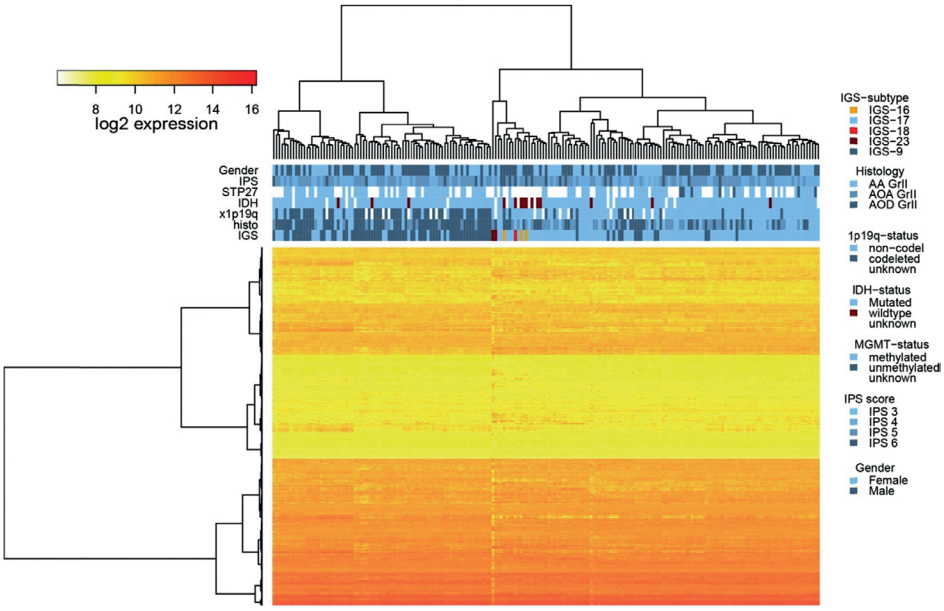


Figure 1. Heatmap showing association of gene-expression with clinical, pathological and other molecular data (*IDH*-mutation status, *MGMT*-promoter methylation status, 1p19q codeletion, intrinsic glioma subtype and immunophenoscore). As can be seen, most patients assigned to IGS-9 have 1p19q codeletion and are diagnosed as oligodendrogliomas.

based on local diagnosis, as astrocytoma or oligoastrocytoma (76/115). In the samples assigned to other molecular subtypes (n = 6), IDH-mutations were infrequent (1/5) as was 1p19q codeletion (1/5).

Patients with most favorable prognosis were those with tumors assigned to IGS-9 (PFS 53 months) or IGS-17 (PFS 40 months), and patients with worst prognosis were those assigned to other molecular subtypes (11.8 months, figure 2). Differences between all groups were significant (logrank $P < 0.0001$), differences between the two most favorable subtypes, IGS-9 and IGS-17, were however, not significant ($P = 0.17$, HR 0.74, 95 % CI [0.49-1.13], though this could be related to limited follow-up time in which relatively few events have occurred. The IGS-'other' subtype remained significant in a multivariate analysis that included other known prognostic factors such as age, type of surgery, histological diagnosis, treatment and performance score (table 3).

Treatment response per IGS-subtype

A trend towards benefit from RT compared to TMZ was observed for samples assigned to IGS-9 (HR for TMZ is 1.90, 95 % CI [0.95, 3.80], $P = 0.065$, figure 3). No such difference was observed for samples assigned to IGS-17 (HR for TMZ vs RT is 0.87, 95 % CI[0.50, 1.51], $P = 0.62$). Too few patients were assigned to other molecular

Table 2. Association between specific genetic changes, histology and IGS-subtype

variable		IGS-9	IGS-17	IGS other	not inc	P
All samples		74	115	6	282	
Treatment	RT	38	54	4	144	0.66
	TMZ	36	61	2	138	
Type of surgery	Biopsy	13	22	3	151	0.87
	Partial resection	46	67	3	90	
	Total resection	15	26	0	40	
	n/a	0	0	0	1	
Histology	Astrocytoma	11	38	2	116	p<0.001
	Oligoastrocytoma	11	38	2	67	
	Oligodendroglioma	52	39	2	99	
Performance	0	58	61	3	172	<0.001
	1	16	46	3	100	
	2	0	8	0	10	
Gender	Female	35	48	2	117	0.55
	Male	39	67	4	165	
IDH status	Mutated	68	97	1	161	0.09
	Normal	1	9	4	51	
	n/a	5	9	1	70	
1p19q status	Codeleted	45	17	1	54	< 0.001
	Intact	23	85	4	128	
	n/a	6	13	1	100	
MGMT	Methylated	45	56	1	11	0.04
	Unmethylated	0	7	0	02	
Age	Age (years) mean ± SD	47.2 ± 10.7	42.1 ± 11.0	38.8 ± 7.5	45.1 ± 12.2	0.013*
	< 44.6 years	25	72	4	137	<0.001
	> 44.6 years	49	43	2	145	

Chi squared test comparison between IGS-9 and IGS-17 only; Note on performance score, Chi-square test without performance score 2 also has P<0.001. IDH-mutation status was done using a Fishers' exact test. *: Anova based on all categories, anova on only IGS-9 and IGS-17: P= 0.002; anova of IGS-9, IGS-17 and IGS-other: P=0.004.

subtypes to assess response to treatment. Our gene expression data therefore provides preliminary evidence that patients with tumors assigned to IGS-9 can benefit from RT whereas no such benefit is observed in tumors assigned to IGS-17. It should be stressed however, that this is a post-hoc analysis performed on a subset of samples included in the EORTC22033-26033 clinical trial.

No genes were found to be significantly associated with progression free survival within molecular subgroups by SAM analysis, also not when subgroups were stratified by treatment. Identification of such genes may require a more mature dataset.

Table 3. Multivariate analysis for PFS

		HR	SE	p	95% CI
Age		0.97	0.01	0.014	0.95-0.99
Type of Surgery	Partial resection vs. Biopsy	0.80	0.27	0.424	0.47-1.37
	Total resection vs .Biopsy	0.76	0.33	0.409	0.40-1.45
Histology	Oligoastrocytoma vs. Astrocytoma	0.87	0.29	0.620	0.49-1.52
	Oligodendroglioma vs. Astrocytoma	0.89	0.24	0.650	0.55-1.45
Treatment	TMZ vs RT	1.41	0.22	0.113	0.92-2.14
Gender	Male vs. Female	1.21	0.21	0.375	0.80-1.82
Performance	ECOG 1 vs. ECOG 0	0.77	0.23	0.267	0.49-1.22
	ECOG 2 vs. ECOG 0	4.49	0.45	0.001	1.87-10.73
IGS-subtype	IGS-9 vs. IGS-17	0.96	0.24	0.864	0.60-1.54
	IGS-other vs. IGS-17	7.40	0.50	0.000	2.75-19.89

n= 195, number of events= 101

Immunophenotyping

Transcriptomic analysis has recently proved a valuable tool in the prediction of response to checkpoint inhibitors in various tumor types. This response prediction is done by a deconvolution of the gene expression data and subsequent cataloguing of the immune infiltrate of the tumor [23]. 'Immunophenotyping' has thus far only been performed on historical and archival samples which makes it difficult to estimate the relevance of potential confounding clinical and patient parameters. We therefore analysed the immune infiltrate of samples included in the EORTC22033-26033 clinical trial. We also included data from the BELOB trial, a randomized phase II trial on recurrent glioblastomas (GBM, $n = 110$, expression data from the initial tumor) to allow comparisons between LGG and GBM [14]. Such comparison is possible as the GBM samples were run on the same platform, and processed in batches alongside the EORTC22033-26033 samples. Any analysis involving clinical parameters were analyzed within the individual clinical trials to avoid potential patient bias.

In general, LGGs have a slightly lower overall immunophenoscore (IPS, a score derived from immunophenotyping that is associated with response to checkpoint inhibitors in melanomas) than GBMs ($P = 0.004$). Specifically, LGGs score higher on checkpoint (CP) and suppressor cell (SC) populations, but score lower on the effector cell (EC) and antigen processing (MHC) populations. This difference was apparent per IPS score (supplementary figure 2). For example, LGGs and GBMs with IPS score of 4 have an MHC score of respectively 0.86 ± 0.16 and 1.21 ± 0.24 ($P < 0.0001$, $n = 77$ and $n = 25$). Similarly, LGGs with an IPS score of 5 have an SC score of 0.005 ± 0.13 whereas GBMs have a score of -0.29 ± 0.18 ($P < 0.001$).

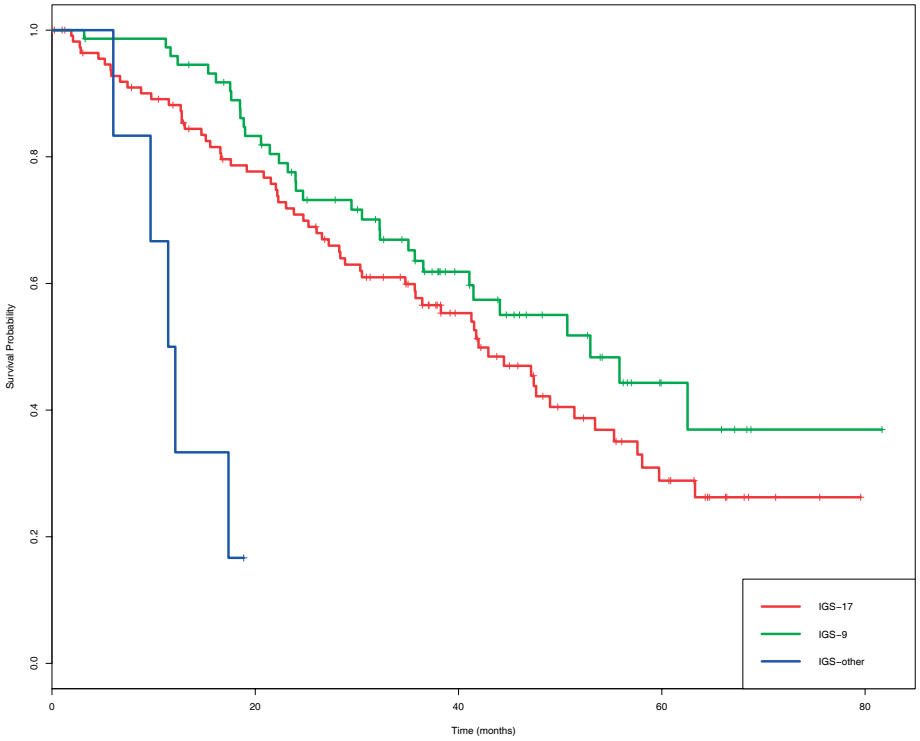


Figure 2. Intrinsic glioma subtypes are prognostic for progression free survival in patients treated within the EORTC22033-26033 clinical trial. Patients with most favorable prognosis were those with tumors assigned to IGS-9 (grey) or IGS-17 (dotted), and patients with worst prognosis were those assigned to other molecular subtypes (black), P<0.0001.

Within the EORTC22033-26033 samples, the Immunophenoscore was significantly correlated to histological subtype of the tumor ($P < 0.001$), presence or absence of IDH mutations ($P = 0.03$), but not to gender, age, MGMT-promoter methylation status, 1p19q codeletion or IGS-subtype (Chi squared test). Within the BELOB trial, correlation between immunophenoscore and IDH-mutations could not be confirmed, though the number of IDH-mutated tumors in that cohort was low ($n = 5$). In fact, the immunophenoscore was not associated with any clinical or molecular parameter in this trial (age, gender, IGS-subtype, treatment, MGMT-promoter or IDH-mutation status). In neither trial, IPS score was associated with outcome (supplementary figure 3). In summary, IPS score appears to be independent of known clinical and prognostic molecular markers. Moreover, the higher IPS score in GBMs suggests that LGGs are less amenable to checkpoint inhibitor type immune therapies.

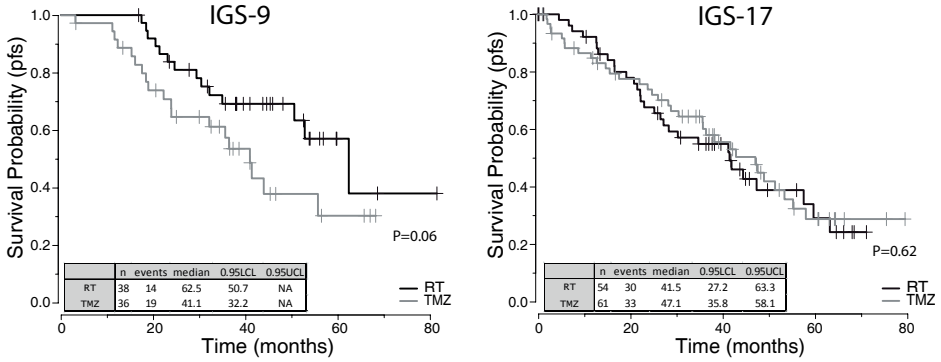


Figure 3. Progression free survival stratified by IGS-subtype and treatment: Samples assigned to IGS-9 show a trend towards benefit from RT compared TMZ (left panel, HR for TMZ is 1.90, P=0.065). No such difference was observed for samples assigned to IGS-17 (right panel, HR for TMZ vs RT is 0.87, P=0.62. Too few patients were assigned to other molecular subtypes to assess response to treatment (not shown).

Identification of pilocytic astrocytoma in 22033 clinical trial samples

Three out of the 195 samples were assigned to IGS-16 in the EORTC-22033 dataset. A hallmark of IGS-16 is that pilocytic astrocytomas (PAs, gliomas with favorable prognosis) are almost always assigned to this specific subtype [18]. However, this expression based intrinsic subtype does not only contain PAs; other histological subtypes of gliomas (including GBMs) may also be assigned to IGS-16. As it is sometimes difficult to distinguish between pilocytic and grade II astrocytomas by histology, it is therefore possible that one or more of the three EORTC 22033 samples assigned to IGS-16 are actually PAs [27]. Additional genetic testing to determine diagnosis is therefore required.

We therefore screened for typical genetic hallmarks of PAs (i.e. tandem duplication of 7q34 involving the BRAF locus [28 29]) in samples assigned to IGS-16. Genotyping arrays were used to determine the genetic changes in these samples. One of the three samples assigned to IGS-16 indeed showed the characteristic tandem duplication on 7q34, and a lack of other genetic changes (figure 4). The centromeric breakpoint lies within the BRAF locus. Of note, 12 samples from the TCGA dataset (combined LGG and GBM) are also assigned to IGS-16 [30-32], and analysis of the genotype confirms that one of these samples (TCGA-HT-7691) harbors a BRAF-KIAA1549 gene-fusion (and no other notable mutations and/or copy number aberrations). Genotyping analysis therefore indicates that at least one sample of the EORTC22033 clinical trial (and one TCGA sample in other datasets) can molecularly be classified as a pilocytic astrocytoma.

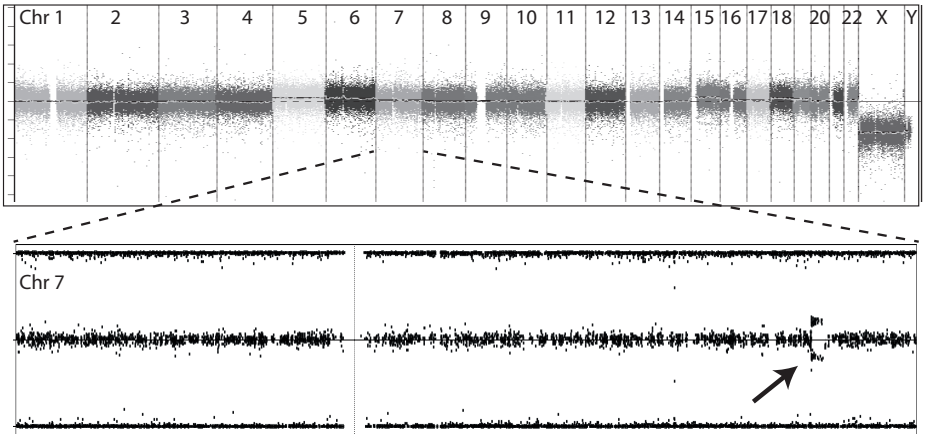


Figure 4. Identification of a pilocytic astrocytoma in a sample of the EORTC22033-26033 clinical trial. Three out of the 195 samples were assigned to IGS-16, a subtype to which pilocytic astrocytomas are assigned. In one of these samples we identified classical hallmark genetic changes of PAs: a tandem duplication on 7q34 (lower panel) and a marked absence of other genetic changes (upper panel). The centromeric breakpoint lies within the *BRAF* gene between 140.5 and 140.57 Mb, the Q-terminal breakpoint lies between 142.65 and 143.03 Mb.

DISCUSSION

In this study we have performed gene-expression profiling on samples of patients that were treated within the EORTC22033-26033 clinical trial. We show that intrinsic glioma subtypes show overlap with histological and molecular subtypes of glioma and that the IGS- subtypes are prognostic for PFS. Our data are in line with other studies that demonstrated the prognostic significance of gene-expression-based molecular subtypes in gliomas, but is only the second to be performed on randomized phase III clinical trial material [13,18,31,33,34]. We confirm earlier observations that specific genetic changes segregate in defined IGS-subtypes [13,14,18].

The randomized phase III clinical trial EORTC22033-26033 was initiated to optimize treatment for LGG patients. The overall trial result demonstrated equal efficacy of RT *v.* TMZ monotherapy in LGG patients both on PFS and quality of life [11,12]. Interestingly, correlative molecular marker analysis provided evidence for reduced benefit from TMZ in patients with *IDH*-mutated, 1p19q intact tumors. Our gene expression data did not support this observation: we show that samples assigned to IGS-17, of which most are *IDH*- mutated and 1p19q intact, have equal benefit from RT and TMZ.

The difference in the predictive effect between IGS-subtype and *IDH*-mutant, 1p19q codeleted tumors (despite a large degree of overlap in samples), may be explained by the fact that IGS subtyping probes a different type of tumor characteristic and thus

is not identical to molecular marker analysis of 1p19q and *IDH*-status. Analysis of DNA markers such as 1p19q and *IDH*-status do not take epi-genetic or phenotypic variability (such as those associated with tumor grade) into account. Alternatively, our gene-expression analysis only examined a subset of tumors, and those contained fewer biopsies and fewer astrocytomas compared to the entire dataset. The difference in the predictive effect between IGS-subtype and 1p19q (intact or codeleted) in *IDH*-mutant tumors may however, at least in part, also be explained by an incorrect determination of molecular markers. For example, IGS-subtyping has a degree of error due to e.g. intratumoral heterogeneity or effects of RNA-quality on tumor classification [35,36]. Alternatively, the various methods to determine 1p19q codeletion also do not always give concordant results [37,38].

Several older trials have also analyzed the efficacy of alkylating chemotherapy in 1p19q intact low grade gliomas. One trial demonstrated efficacy of procarbazine, CCNU and vincristine (PCV) monotherapy in LGG, and the efficacy was not associated with 1p/19q codeletion (though numbers for correlative marker analysis were small) [39]. A separate trial that examined the efficacy of the addition of chemotherapy to RT also showed that both LGG tumors with and without 1p19q codeletion responded to TMZ, though tumors with 1p19q codeletion showed a higher response rate [40]. Since 1p19q codeletion is associated with histological subtype, data from the recently published RTOG9802 trial also confirm the efficacy of combined RT + PCV treatment: efficacy was observed in all histological subtypes. Similar data, but on grade III gliomas, confirm the efficacy of PCV chemotherapy in both 1p19q codeleted and non codeleted tumors [41-43] [44]. Data from these trials and data obtained in the current study therefore suggest that alkylating chemotherapy is effective in IDH-mutated, 1p19q intact gliomas, though it is possible that the response duration is shorter than in 1p19q codeleted gliomas.

Checkpoint inhibitors have recently gained attention as novel therapeutic agents in various cancer types including GBMs [45-48]. Since only a subset of patients benefit from these treatments, identification of (bio-) markers associated with response is of clinical relevance. The mutational load, i.e. the number of mutations that lead to a neo-epitope of the tumor, has been coined as predictive response marker. However, analysis of the tumors' immune infiltrate, which can be done by a deconvolution of the gene expression data, also can identify tumors likely to respond [23]. Such 'Immunophenotyping' has thus far only been performed on historical and archival samples which makes it difficult to estimate the relevance of potential confounding clinical and patient parameters. Our gene expression data from the EORTC22033-26033 and BELOB clinical trials therefore can help determine the relevance of immunophenotyping in glioma samples. Our data show that in neither trial, IPS score was associated with outcome and that the IPS

score appears to be independent of known clinical and prognostic molecular markers. Nevertheless, GBMs in general have a higher IPS score and score higher on the effector cell and antigen processing populations than LGGs (even within defined IPS scores). These data suggest that LGGs are less amenable than GBMs to checkpoint inhibitor type immune therapies, which is in concordance with findings from other groups [49].

Our gene expression analysis has also identified one patient treated within EORTC22033-26033 with the hallmark genetic change of PAs: tandem duplication of 7q34. It is important to identify such patients as they have better prognosis and require a different treatment than diffuse low grade gliomas[50]. We identified this patient based on the assignment of the tumor to IGS-16. However, IGS-16 does not only contain PAs: a few samples (of other histological subtypes with associated poorer prognosis) are also assigned to IGS-16, which necessitates additional molecular testing. The PA identified in the EORTC22033-26033 clinical trial also highlights difficulties to distinguish this tumor type by histology[27]. In addition to the EORTC22033-26033 trial sample, we also identify a PA sample in the TCGA dataset. Additional genomic testing of samples assigned to IGS-16 therefore may be warranted.

To summarize, gene-expression profiling of samples included in the EORTC22033-26033 clinical trial confirmed the prognostic relevance of IGS subtyping. We failed to find evidence for differential treatment benefit in one or more specific molecular subgroups. IGS-subtyping has also identified one PA in the EORTC22033-26033 clinical trial and one in the TCGA database.

Disclosure of Potential Conflicts of Interest

MJvdB has received grants from Roche and Abbvie, and personal fees from Roche, Abbvie, Merck AG, Novocure, Cavion, Bristol-Myers Squibb, Novartis, and Actelion. BT acknowledges financial support from NCIC-CTG, during the conduct of the study. OC reports grants, personal fees and non-financial support from Roche, and personal fees from Ipsen and AstraZeneca. RS received non-financial support from Novocure; and honoraria from Roche, Merck KGaA, MSD, Merck, and Novartis. BGB reports personal fees from Merck Sharp & Dohme (MSD). MEH has received grants from Orion, service fees from Novocure, and has served on advisory board from BMS, and received non-financial support from MDxHealth. The other authors declare that they have no conflict of interest.

Role of the Funding Source

The financial sponsors of the study had no role in the study design and in the collection, analysis and interpretation of data.

REFERENCES

1. Ostrom QT, Gittleman H, Fulop J, et al. CBTRUS Statistical Report: Primary Brain and Central Nervous System Tumors Diagnosed in the United States in 2008-2012. *Neuro-oncology.* 2015;17 Suppl 4:iv1-iv62.
2. Louis DN, Perry A, Reifenberger G, et al. The 2016 World Health Organization Classification of Tumors of the Central Nervous System: a summary. *Acta Neuropathol.* 2016;131(6):803-820.
3. Sanai N, Chang S, Berger MS. Low-grade gliomas in adults. *J Neurosurg.* 2011;115(5):948-965.
4. Soffietti R, Baumert BG, Bello L, et al. Guidelines on management of low-grade gliomas: report of an EFNS-EANO Task Force. *Eur J Neurol.* 2010;17(9):1124-1133.
5. Sahm F, Reuss D, Koelsche C, et al. Farewell to oligoastrocytoma: in situ molecular genetics favor classification as either oligodendroglioma or astrocytoma. *Acta Neuropathol.* 2014;128(4):551-559.
6. Ryken TC, Parney I, Buatti J, Kalkanis SN, Olson JJ. The role of radiotherapy in the management of patients with diffuse low grade glioma : A systematic review and evidence-based clinical practice guideline. *J Neurooncol.* 2015;125(3):551-583.
7. Karim AB, Maat B, Hatlevoll R, et al. A randomized trial on dose-response in radiation therapy of low-grade cerebral glioma: European Organization for Research and Treatment of Cancer (EORTC) Study 22844. *International journal of radiation oncology, biology, physics.* 1996;36(3):549-556.
8. van den Bent MJ, Afra D, de Witte O, et al. Long-term efficacy of early versus delayed radiotherapy for low-grade astrocytoma and oligodendroglioma in adults: the EORTC 22845 randomised trial. *Lancet.* 2005;366(9490):985-990.
9. Shaw E, Arusell R, Scheithauer B, et al. Prospective randomized trial of low- versus high-dose radiation therapy in adults with supratentorial low-grade glioma: initial report of a North Central Cancer Treatment Group/Radiation Therapy Oncology Group/Eastern Cooperative Oncology Group study. *J Clin Oncol.* 2002;20(9):2267-2276.
10. Buckner JC, Shaw EG, Pugh SL, et al. Radiation plus Procarbazine, CCNU, and Vincristine in Low-Grade Glioma. *N Engl J Med.* 2016;374(14):1344-1355.
11. Baumert BG, Hegi ME, van den Bent MJ, et al. Temozolomide chemotherapy versus radiotherapy in high-risk low-grade glioma (EORTC 22033-26033): a randomised, open-label, phase 3 intergroup study. *Lancet Oncol.* 2016;17(11):1521-1532.
12. Reijneveld JC, Taphoorn MJ, Coens C, et al. Health-related quality of life in patients with high-risk low-grade glioma (EORTC 22033-26033): a randomised, open-label, phase 3 intergroup study. *Lancet Oncol.* 2016;17(11):1533-1542.
13. Erdem-Eraslan L, Gravendeel LA, de Rooi J, et al. Intrinsic molecular subtypes of glioma are prognostic and predict benefit from adjuvant procarbazine, lomustine, and vincristine chemotherapy in combination with other prognostic factors in anaplastic oligodendroglial brain tumors: a report from EORTC study 26951. *J Clin Oncol.* 2013;31(3):328-336.
14. Erdem-Eraslan L, van den Bent MJ, Hoogstrate Y, et al. Identification of patients with recurrent glioblastoma who may benefit from combined bevacizumab and CCNU therapy, a report from the BELOB trial. *Cancer Res.* 2016;76(3):525-534.
15. French PJ, Swagemakers SMA, Nagel JHA, et al. Gene expression profiles associated with treatment response in oligodendrogliomas. *Cancer Res.* 2005;65(24):11335-11344.
16. Louis DN. Molecular pathology of malignant gliomas. *Annual review of pathology.* 2006;1:97-117.

17. Bady P, Sciuscio D, Diserens AC, et al. MGMT methylation analysis of glioblastoma on the Infinium methylation BeadChip identifies two distinct CpG regions associated with gene silencing and outcome, yielding a prediction model for comparisons across datasets, tumor grades, and CIMP-status. *Acta Neuropathol.* 2012;124(4):547-560.
18. Gravendeel LA, Kouwenhoven MC, Gevaert O, et al. Intrinsic gene expression profiles of gliomas are a better predictor of survival than histology. *Cancer Res.* 2009;69(23):9065-9072.
19. Gravendeel LA, de Rooi JJ, Eilers PH, van den Bent MJ, Sillevis Smitt PA, French PJ. Gene expression profiles of gliomas in formalin-fixed paraffin-embedded material. *Br J Cancer.* 2012;106(3):538-545.
20. Kapp AV, Tibshirani R. Are clusters found in one dataset present in another dataset? *Biostatistics (Oxford, England).* 2007;8(1):9-31.
21. Tusher VG, Tibshirani R, Chu G. Significance analysis of microarrays applied to the ionizing radiation response. *Proc Natl Acad Sci U S A.* 2001;98(9):5116-5121.
22. van den Bent MJ, Gravendeel LA, Gorlia T, et al. A hypermethylated phenotype is a better predictor of survival than MGMT methylation in anaplastic oligodendroglial brain tumors: a report from EORTC study 26951. *Clin Cancer Res.* 2011;17(22):7148-7155.
23. Charoentong P, Finotello F, Angelova M, et al. Pan-cancer Immunogenomic Analyses Reveal Genotype-Immunophenotype Relationships and Predictors of Response to Checkpoint Blockade. *Cell Rep.* 2017;18(1):248-262.
24. Foster JM, Oumie A, Togneri FS, et al. Cross-laboratory validation of the OncoScan(R) FFPE Assay, a multiplex tool for whole genome tumour profiling. *BMC medical genomics.* 2015;8:5.
25. Hardenbol P, Baner J, Jain M, et al. Multiplexed genotyping with sequence-tagged molecular inversion probes. *Nat Biotechnol.* 2003;21(6):673-678.
26. *A Package for Survival Analysis in S* [computer program]. Version 2.382015.
27. Korshunov A, Meyer J, Capper D, et al. Combined molecular analysis of BRAF and IDH1 distinguishes pilocytic astrocytoma from diffuse astrocytoma. *Acta Neuropathol.* 2009;118(3):401-405.
28. Jones DT, Kocialkowski S, Liu L, et al. Tandem duplication producing a novel oncogenic BRAF fusion gene defines the majority of pilocytic astrocytomas. *Cancer Res.* 2008;68(21):8673-8677.
29. Hasselblatt M, Riesmeier B, Lechtape B, et al. BRAF-KIAA1549 fusion transcripts are less frequent in pilocytic astrocytomas diagnosed in adults. *Neuropathol Appl Neurobiol.* 2011;37(7):803-806.
30. Cancer Genome Atlas Research N, Brat DJ, Verhaak RG, et al. Comprehensive, Integrative Genomic Analysis of Diffuse Lower-Grade Gliomas. *N Engl J Med.* 2015;372(26):2481-2498.
31. Ceccarelli M, Barthel FP, Malta TM, et al. Molecular Profiling Reveals Biologically Discrete Subsets and Pathways of Progression in Diffuse Glioma. *Cell.* 2016;164(3):550-563.
32. Brennan CW, Verhaak RG, McKenna A, et al. The somatic genomic landscape of glioblastoma. *Cell.* 2013;155(2):462-477.
33. Li A, Walling J, Ahn S, et al. Unsupervised analysis of transcriptomic profiles reveals six glioma subtypes. *Cancer Res.* 2009;69(5):2091-2099.
34. Phillips HS, Kharbanda S, Chen R, et al. Molecular subclasses of high-grade glioma predict prognosis, delineate a pattern of disease progression, and resemble stages in neurogenesis. *Cancer Cell.* 2006;9(3):157-173.
35. Sottoriva A, Spiteri I, Piccirillo SG, et al. Intratumor heterogeneity in human glioblastoma reflects cancer evolutionary dynamics. *Proc Natl Acad Sci U S A.* 2013;110(10):4009-4014.

36. Parker NR, Hudson AL, Khong P, et al. Intratumoral heterogeneity identified at the epigenetic, genetic and transcriptional level in glioblastoma. *Sci Rep.* 2016;6:22477.
37. Horbinski C, Nikiforova MN, Hobbs J, et al. The importance of 10q status in an outcomes-based comparison between 1p/19q fluorescence in situ hybridization and polymerase chain reaction-based microsatellite loss of heterozygosity analysis of oligodendrogliomas. *J Neuropathol Exp Neurol.* 2012;71(1):73-82.
38. Jha P, Sarkar C, Pathak P, et al. Detection of allelic status of 1p and 19q by microsatellite-based PCR versus FISH: limitations and advantages in application to patient management. *Diagn Mol Pathol.* 2011;20(1):40-47.
39. Buckner JC, Gesme D, Jr., O'Fallon JR, et al. Phase II trial of procarbazine, lomustine, and vincristine as initial therapy for patients with low-grade oligodendroglioma or oligoastrocytoma: efficacy and associations with chromosomal abnormalities. *J Clin Oncol.* 2003;21(2):251-255.
40. Kaloshi G, Benouaich-Amiel A, Diakite F, et al. Temozolomide for low-grade gliomas: predictive impact of 1p/19q loss on response and outcome. *Neurology.* 2007;68(21):1831-1836.
41. Cairncross G, Wang M, Shaw E, et al. Phase III trial of chemoradiotherapy for anaplastic oligodendroglioma: long-term results of RTOG 9402. *J Clin Oncol.* 2013;31(3):337-343.
42. Cairncross JG, Wang M, Jenkins RB, et al. Benefit from procarbazine, lomustine, and vincristine in oligodendroglial tumors is associated with mutation of IDH. *J Clin Oncol.* 2014;32(8):783-790.
43. van den Bent MJ, Brandes AA, Taphoorn MJ, et al. Adjuvant procarbazine, lomustine, and vincristine chemotherapy in newly diagnosed anaplastic oligodendroglioma: long-term follow-up of EORTC brain tumor group study 26951. *J Clin Oncol.* 2013;31(3):344-350.
44. Dubbink HJ, Atmodimedjo PN, Kros JM, et al. Molecular classification of anaplastic oligodendroglioma using next-generation sequencing: a report of the prospective randomized EORTC Brain Tumor Group 26951 phase III trial. *Neuro-oncology.* 2016;18(3):388-400.
45. Bouffet E, Larouche V, Campbell BB, et al. Immune Checkpoint Inhibition for Hypermutant Glioblastoma Multiforme Resulting From Germline Biallelic Mismatch Repair Deficiency. *J Clin Oncol.* 2016.
46. Larkin J, Chiarion-Sileni V, Gonzalez R, et al. Combined Nivolumab and Ipilimumab or Monotherapy in Untreated Melanoma. *N Engl J Med.* 2015;373(1):23-34.
47. Sundar R, Cho BC, Brahmer JR, Soo RA. Nivolumab in NSCLC: latest evidence and clinical potential. *Therapeutic advances in medical oncology.* 2015;7(2):85-96.
48. Eggermont AM, Chiarion-Sileni V, Grob JJ, et al. Prolonged Survival in Stage III Melanoma with Ipilimumab Adjuvant Therapy. *N Engl J Med.* 2016;375(19):1845-1855.
49. Berghoff AS, Kiesel B, Widhalm G, et al. Correlation of immune phenotype with IDH mutation in diffuse glioma. *Neuro-oncology.* 2017.
50. Collins VP, Jones DT, Giannini C. Pilocytic astrocytoma: pathology, molecular mechanisms and markers. *Acta Neuropathol.* 2015;129(6):775-788.

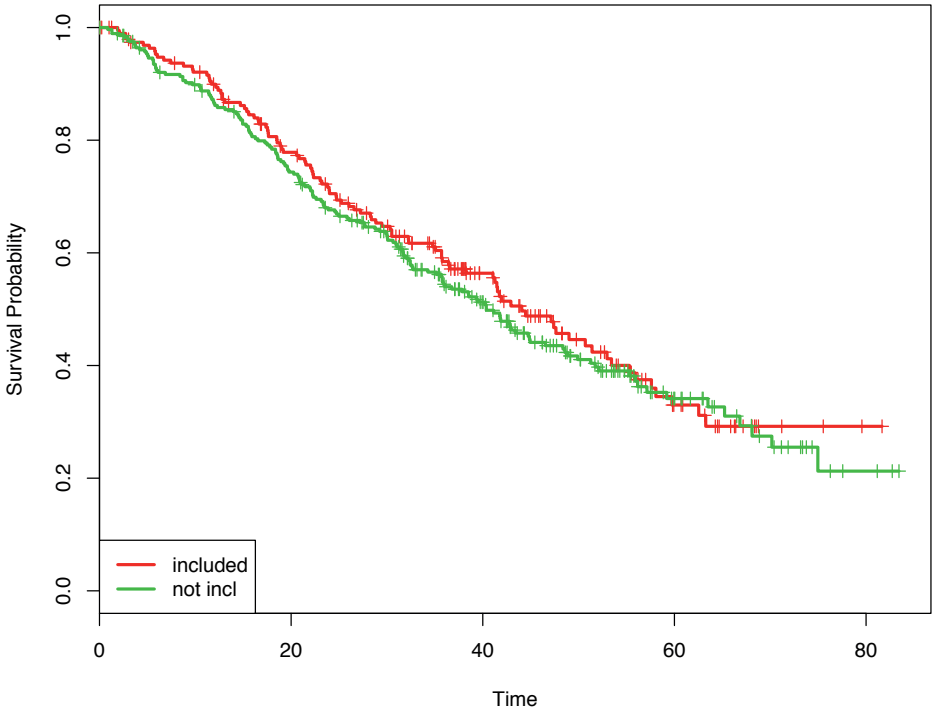
Supplementary Table 1. Survival per molecular subtype included vs not included

Molecular subset	inc/not inc	n	events	median	0.95LCL	0.95UCL
all patients	included	195	101	43.5	37.7	52.7
	not included	282	161	39.8	35.3	47.7
IDH-mutated, 1p19q codeleted	included	60	26	55.1	35.3	NR
	not included	44	15	NR	41.2	NR
IDH-mutated, 1p19q intact	included	92	47	46.5	40.5	56.8
	not included	73	37	48.2	34.7	NR
IDH-wt	included	14	10	16.6	9.6	NR
	not included	51	42	20.6	15.1	27.3

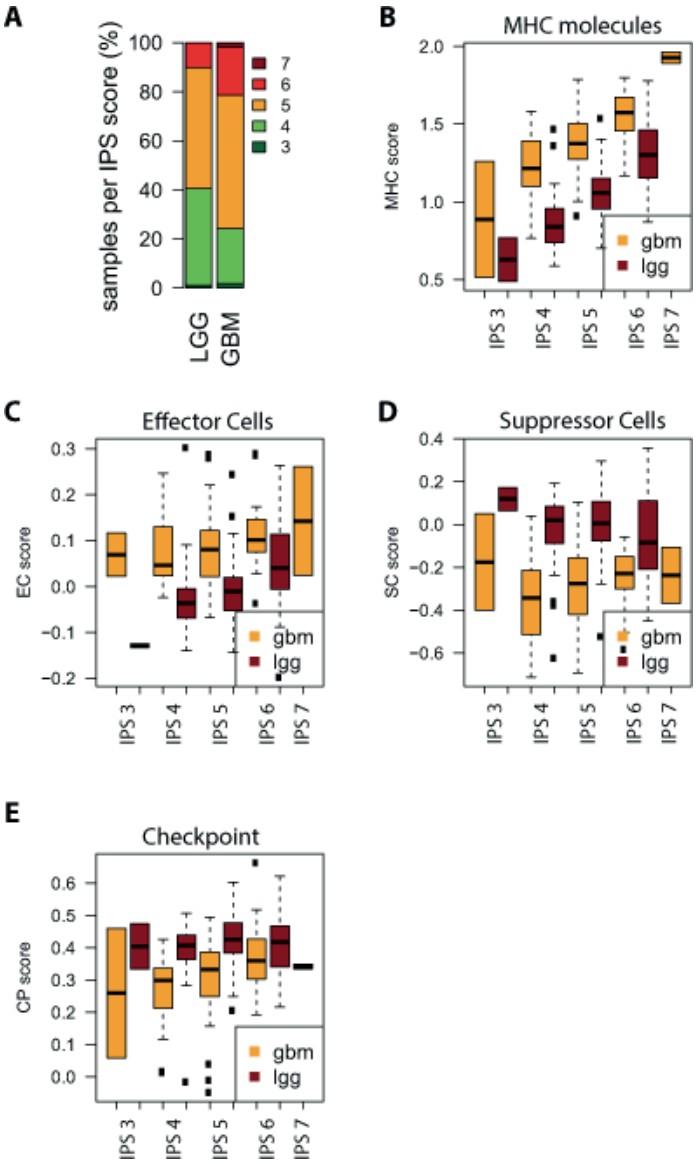
NR: not reached

Supplementary Table 2. correlation of molecular markers with IGS-subtype

IDH status	1p19q status	IGS-9	IGS-17	IGS-other	not included
IDH mutated	1p19q	43	17	0	44
	no codel	20	71	1	73
	undetermined	5	9	0	44
IDH wt	1p19q	0	0	1	6
	no codel	0	7	3	32
	undetermined	1	2	0	13
IDH undetermined	1p19q	2	0	0	4
	no codel	3	7	0	23
	undetermined	0	2	1	43



Supplementary Figure 1. Survival between 'included' and 'not-included' (i.e. those in which gene expression analysis was performed or not). No differences between included and not included were identified, also not when stratified by molecular subgroup (IDH-mutation and 1p19q codeletion, not shown).



Supplementary Figure 2. Immunophenotypes in 22033 and BELOB clinical trials. A: distribution of IPS scores in the EORTC22033-26033 (LGG) and BELOB (GBM) clinical trials. As can be seen, GBMs tend to have higher IPS scores than LGGs. Other figures represent the immune infiltrate per IPS score stratified by the EORTC22033-26033 (LGG) and BELOB (GBM) trials. As can be seen, GBMs have more MHC expression (B) and higher effector cell population (C) per IPS score. In contrast LGGs have higher suppressor and immunemodulator (D and E).