Open access • Journal Article • DOI:10.1200/JCO.2009.23.0805

Molecular Predictors of Progression-Free and Overall Survival in Patients With Newly Diagnosed Glioblastoma: A Prospective Translational Study of the German Glioma Network — Source link

Michael Weller, Jörg Felsberg, Christian Hartmann, Hilmar Berger ...+14 more authors

Institutions: University of Zurich

Published on: 01 Dec 2009 - Journal of Clinical Oncology (American Society of Clinical Oncology)

Topics: Temozolomide, Glioma, Progression-free survival, Survival rate and Genetic translation

Molecular predictors of progression-free and overall survival in patients with newly diagnosed glioblastoma: a prospective translational study of the German Glioma Network

Weller, M; Felsberg, J; Hartmann, C; Berger, H; Steinbach, J P; Schramm, J; Westphal, M; Schackert, G; Simon, M; Tonn, J C; Heese, O; Krex, D; Nikkhah, G; Pietsch, T; Wiestler, O; Reifenberger, G; von Deimling, A; Loeffler, M

Molecular predictors of progression-free and overall survival in patients with newly diagnosed glioblastoma: a prospective translational study of the German Glioma Network

Abstract

PURPOSE: The prognostic value of genetic alterations characteristic of glioblastoma in patients treated according to present standards of care is unclear. PATIENTS AND METHODS: Three hundred one patients with glioblastoma were prospectively recruited between October 2004 and December 2006 at the clinical centers of the German Glioma Network. Two hundred fifty-eight patients had radiotherapy, 199 patients had temozolomide, 189 had both, and seven had another chemotherapy as the initial treatment. The tumors were investigated for TP53 mutation, p53 immunoreactivity, epidermal growth factor receptor, cyclin-dependent kinase CDK 4 or murine double minute 2 amplification, CDKN2A homozygous deletion, allelic losses on chromosome arms 1p, 9p, 10q, and 19q, O(6)-methylguanine methyltransferase (MGMT) promoter methylation, and isocitrate dehydrogenase 1 (IDH1) mutations. RESULTS: Median progression-free (PFS) and overall survival (OS) were 6.8 and 12.5 months. Multivariate analysis revealed younger age, higher performance score, MGMT promoter methylation, and temozolomide radiochemotherapy as independent factors associated with longer OS. MGMT promoter methylation was associated with longer PFS (relative risk [RR], 0.5; 95% CI, 0.38 to 0.68; $P < .001$) and OS (RR, 0.39; 95% CI, 0.28 to 0.54; $P < .001$) in patients receiving temozolomide. IDH1 mutations were associated with prolonged PFS (RR, 0.42; 95% CI, 0.19 to 0.91; $P = .028$) and a trend for prolonged OS (RR, 0.43; 95% CI, 0.15 to 1.19; $P = .10$). No other molecular factor was associated with outcome. CONCLUSION: Molecular changes associated with gliomagenesis do not predict response to therapy in glioblastoma patients managed according to current standards of care. MGMT promoter methylation and IDH1 mutational status allow for stratification into prognostically distinct subgroups.

Molecular predictors of progression-free and overall survival in patients with newly diagnosed glioblastoma.

A prospective translational study of the German Glioma Network

Michael Weller[1,2], Jörg Felsberg[3], Christian Hartmann[4], Hilmar Berger[5], Joachim P. Steinbach[2,6], Johannes Schramm[7], Manfred Westphal[8], Gabriele Schackert[9], Matthias Simon[7], Jörg C. Tonn[10], Oliver Heese[8], Dietmar Krex[9], Guido Nikkhah[11], Torsten Pietsch[12], Otmar Wiestler[13], Guido Reifenberger[3], Andreas von Deimling[4], Markus Loeffler[5], for the German Glioma Network

[1]Department of Neurology, University Hospital Zurich, Switzerland; [2]Department of General Neurology, University of Tübingen; [3]Institute of Neuropathology, University of Düsseldorf; [4]Department of Neuropathology, Institute of Pathology, University of Heidelberg, and Clinical Cooperation Unit Neuropathology, German Cancer Research Center, Heidelberg; [5]Institute of Medicinal Informatics, Statistics and Epidemiology, University of Leipzig; [6]Dr. Senckenberg Institute of Neuro-Oncology, University of Frankfurt; [7]Department of Neurosurgery, University of Bonn; [8]Department of Neurosurgery, University of Hamburg; [9]Department of Neurosurgery, Technical University Dresden; [10]Department of Neurosurgery, University of Munich LMU; [11]Department of Stereotactic Neurosurgery, University of Freiburg, [12]Institute of Neuropathology, University of Bonn; [13]German Cancer Research Center, Heidelberg, Germany

Acknowledgement: The German Glioma Network is supported by the German Cancer Aid (Deutsche Krebshilfe 70-3163-Wi 3).

Corresponding author: Michael Weller, MD, Department of Neurology, University Hospital Zurich, Frauenklinikstrasse 26, CH-8091 Zürich, Phone: +41 (0) 44 255 5500, Fax: +41 (0) 44 255 4507, E-mail: michael.weller@usz.ch.

Running title: Molecular profiling and outcome in glioblastoma

Abstract

PURPOSE: The prognostic value of genetic alterations characteristic of glioblastoma in patients treated according to present standards of care is unclear.

PATIENTS AND METHODS: 301 glioblastoma patients were prospectively recruited between October 2004 and December 2006 at the clinical centers of the German Glioma Network. 258 patients had radiotherapy, 199 patients had temozolomide, 189 had both, 7 had another chemotherapy as the initial treatment. The tumors were investigated for *TP53* mutation, p53 immunoreactivity, epidermal growth factor receptor (*EGFR)*, cyclin-dependent kinase (*CDK) 4* or murine double minute (*MDM) 2* amplification, *CDKN2A* homozygous deletion, allelic losses on chromosome arms 1p, 9p, 10q and 19q, O^6-methylguanine methyltransferase *(MGMT)* promoter methylation, and isocitrate dehydrogenase (*IDH*)-1 mutations.

RESULTS: Median progression-free (PFS) and overall survival (OS) were 6.8 and 12.5 months. Multivariate analysis revealed younger age, higher performance score, *MGMT* promoter methylation and temozolomide radiochemotherapy as independent factors associated with longer OS. *MGMT* promoter methylation was associated with longer PFS (RR 0.5, 95% CI 0.38-0.68, $p < 0.001$) and OS (RR: 0.39, 95%CI 0.28-0.54, $p<0.001$) in patients receiving temozolomide. IDH-1 mutations were associated with prolonged PFS (RR 0.42, 95% CI: 0.19 - 0.91, $p= 0.028$) and a trend for prolonged OS (RR 0.43, 95% CI 0.15 – 1.19, $p = 0.10$). No other molecular factor was associated with outcome.

CONCLUSION: Molecular changes associated with gliomagenesis do not predict response to therapy in glioblastoma patients managed according to current

standards of care. *MGMT* promoter methylation and *IDH*-1 mutational status allow for stratification into prognostically distinct subgroups.

Introduction

Numerous chromosomal, genetic and epigenetic aberrations occur at more than random frequency in gliomas (1). However, the clinical value of most glioma-associated molecular aberrations in terms of their significance as diagnostic, prognostic or predictive molecular markers has remained unclear (2-5). To date, only two molecular aberrations have been demonstrated to be of clinical significance in prospective clinical trials. Allelic losses on chromosome arms 1p and 19q are associated with a favorable prognosis in patients with oligodendroglial and oligoastrocytic anaplastic gliomas treated with radiotherapy or radiotherapy plus chemotherapy (6-9). *MGMT* promoter hypermethylation is associated with prolonged progression-free and overall survival in glioblastoma patients treated with alkylating agents such as nitrosoureas or temozolomide (10-13). To systematically investigate the prognostic significance of molecular genetic markers in gliomas beyond known clinical prognostic factors, we determined a set of common glioma-associated aberrations in a prospective manner for each glioblastoma patient recruited into the German Glioma Network (GGN).

Patients and Methods

Patients

The patients were prospectively enrolled in the clinical centers of the GGN: Bonn, Dresden, Freiburg, Hamburg, LMU Munich and Tübingen. The GGN is a clinical research network sponsored by the German Cancer Aid (Deutsche Krebshilfe) which in addition to the above-mentioned clinical centers includes associated centers for central neuropathology review in Bonn, molecular neuropathology in Düsseldorf and Heidelberg, and biometry in Leipzig. The GGN aims at enrolling all adult patients with gliomas seen at these centers since October 2004. Fresh-frozen tissue and blood samples are obtained from all patients with microsurgical tumor resection and clinical follow-up information is collected on electronic case report forms (CRF) in regular intervals (www.gliomnetzwerk.de). All activities of the GGN have been approved by the review boards of the participating institutions. Recruitment is ongoing. The data presented here include consecutive patients included until December 2006.

Central reference pathology

Formalin-fixed, paraffin-embedded material of all 301 patients was submitted from the local (neuro)pathologists of the GGN centers for an independent histopathological review to the German Brain Tumor Reference Center in Bonn. Four µm sections were cut and conventional hematoxylin/eosin and silver impregnation (Gomori) stainings and basic immunohistochemical reactions for protein expression including glial fibrillary acidic protein (GFAP; polyclonal rabbit antibody, Dako, Hamburg, 1: 500 dilution), p53 (monoclonal murine, clone DO7, Dako, 1:70 dilution, microwave pretreatment with citrate buffer pH 6.0) and Ki-67 (monoclonal murine,

clone Mib-1, Dako, 1:50 dilution, microwave pretreatment with citrate buffer pH 6.0) were performed. Binding of the first antibody was detected by the avidin-biotin-peroxidase method and diaminobenzidine peroxidase substrate. Staining was graded according to the percentages of stained tumor cells as absent, weak (<30%) or strong (>30%). For correlation with other molecular markers, we compared absent and weak versus strong staining.

All tumors were classified according to the WHO classification of tumors of the central nervous system (14-15). Essential characteristics including the presence or absence of necrosis, pseudopalisading perinecrotic cells, microvascular proliferation, giant cells, oligodendroglial-like, sarcomatous, neuronal or other components, and Ki-67 proliferation index were scored. If the final classification of the tumor was different between local and central reference center, the reference diagnosis overruled the local diagnosis for data analysis.

Extraction of nucleic acids

Tumor samples were immediately frozen in liquid nitrogen and stored at –80° C. From each patient, a peripheral blood sample was drawn and stored at –80° C. Only specimens with an estimated tumor cell content of 80% or more were used for molecular analyses. DNA extraction was carried out using DNA blood and tissue DNA extraction kits (Qiagen, Hilden, Germany). High molecular weight DNA was extracted from peripheral blood leukocytes.

MGMT promoter methylation analysis

MGMT promoter methylation status was determined by methylation-specific PCR (MSP) (16-17), using the following primer sequences to amplify sequences from the

methylated or unmethylated *MGMT* promoter: 5'-GTTTTTAGAACGTTTTGCGTTTCGAC-3' and 5'-CACCGTCCCGAAAAAAAACTCCG-3' or 5'-TGTGTTTTTAGAATGTTTTGTGTTTTGAT-3' and 5'-CTACCACCATCCCAAAAAAAAACTCCA-3'. A172 glioma cells, obtained from American Type Culture Collection, and peripheral blood DNA served as positive and negative controls. A further control reaction without template DNA was performed together with each PCR experiment.

TP53 mutation analysis

To amplify *TP53* exons 5 to 8, PCR was performed in a volume of 10 µl containing 10 ng of DNA, 50 mmol/L KCl, 10 mmol/L Tris-HCl, 200 mmol/L of each dNTP, 0.1% gelatin, 20 pmol of each primer, 1-2 mmol/L $MgCl_2$, and 0.025 U Taq polymerase. The primer sequences are exon 5a 5'-TCAACTCTGTCTCCTTCCTC-3' and 5'-CTGTGACTGCTTGTAGATGG-3'; exon 5b 5'-GTGGGTTGATTCCACACCCC-3' and 5'-AACCAGCCCTGTCGTCTCTC-3'; exon 6 5'-AGGCCTCTGATTCCTCACTG-3' and 5'-AGAGACCCCAGTTGCAAACC-3'; exon 7 5'-GGCCTCATCTTGGGCCTGTG-3' and 5'-GTGTGCAGGGTGGCAAGTGG-3'; exon 8 5'-AATGGGACAGGTAGGACCTG-3' and 5'-ACCGCTTCTTGTCCTGCTTG-3'. The PCR conditions on an automated thermal cycler (Biometra, Göttingen, Germany) were initially 94°C for 3 min followed by 30 cycles at 94°C/30 sec, 57°C/40 sec and extension at 72°C for 40 sec. A final extension step at 72°C for 10 min was added. Single strand conformation polymorphism analysis was performed on a sequencing apparatus (BlueSeq 400. Serva, Marburg, Germany) using 8% and 14% acrylamide gels and electrophoresis at 3 to 6 W for 15 h followed by silver staining of the gels.

Aberrantly migrating bands were excised and DNA was extracted. After reamplification with the same set of primers the PCR products were sequenced on a semiautomated sequencer (ABI 3100, Applied Biosystems, Foster City, CA) using a Taq cycle sequencing kit (Applied Biosystems). Each amplicon was sequenced bidirectionally.

Determination of EGFR, CDK4, MDM2 and CDKN2A gene dosages

EGFR amplification was demonstrated by real-time PCR using the ABI PRISM 5700 sequence detection system (Applied Biosystems, Foster City, CA, USA). The *EGFR* gene dosage was normalized to the dosage of the marker *D2S1743* at 2q21.2. The following oligonucleotide primers were used: *EGFR*, 5'CACTTGCCTCATCTCTCACCATC-3' and 5'-GACTCACCGTAGCTCCAGAC-3' (110 bp); *D2S1743*, 5'-CATGACTGCGAGCCCAAGATG-3' and 5'-CAGGTGGTGTCATCAGAATCAG-3' (131 bp). Gene dosages of *MDM2*, *CDK4* and *CDKN2A* were determined by duplex PCR assays, using the following primers: 5'-GCGATGAATTGATGCTAATGAATG-3' and 5'-CAGGATCTTCTTCAAATGAATCTG-3' for a 99 bp *MDM2* amplicon; 5'-GACTGCTACCTTATATCCCTTC-3' and 5'-CTCCCATGTTGGTCACTTAC-3' for a 102 bp *CDK4* amplicon and 5'-GAAGAAAGAGGAGGGGCTG-3' and 5'-GCGCTACCTGATTTCAATTC-3' for a 338 bp *CDKN2A* amplicon. The reference locus was *APRT* (5'-CTGGAGCACCTGCTCTCTGC-3' and 5'-GCCCTGTGGTCACTCATACTGC-3', 211 bp amplicon). Positive controls included a glioblastoma with *MDM2* and *CDK4* amplification previously demonstrated by Southern blot analysis (18) and the glioblastoma cell line U118MG which carries a homozygous *CDKN2A* deletion. DNA extracted from non-neoplastic brain tissue and peripheral blood leukocytes served as

constitutional reference. PCR products were separated on 3% agarose gels, and the ethidium bromide–stained bands were recorded using the Gel-Doc 1000 system (BioRad, Hercules, CA). Quantitative analysis of the signals obtained for the target and the reference genes was performed with the Molecular-Analyst software (BioRad). Increases in the target (*CDK4* and *MDM2*) to reference gene ratio of more than 5-fold of the ratio obtained for the constitutional DNA were considered as gene amplification. Decreases in the target (*CDKN2A*) to reference gene ratio of equal to or less than 0.3-fold of the ratio obtained for the constitutional DNA were considered as homozygous deletion.

Microsatellite analyses

The following microsatellite loci were investigated for loss of heterozygosity in each tumor: 1p: *D1S1608* (1p36.31), *D1S548* (1p36.31), *D1S1592* (1p36.13), *DS1161* (1p35.2) and *D1S1184* (1p31.3); 9p: *D9S171* (9p21.3), *D9S168* (9p23) and *D9S162* (9p22.1); 10q: *D10S541* (10q23.31), *D10S209* (10q26.12) and *D10S212* (10q26.3); 19q: *D19S433* (19q12), *D19S431* (19q12), *D19S718* (19q13.2), *D19S559* (19q13.32) and *D19S601* (19q13.41). PCR amplification of these microsatellite markers was carried out as reported (19-20). The PCR products were separated by electrophoresis on denaturing polyacrylamide gels and visualized by silver staining. The allele patterns were assessed for allelic imbalance (9, 20).

IDH-1 mutation analyses

IDH-1 mutations were assessed as previously described (21).

Statistical analyses

Statistical analysis was done using the statistical software R. The Mann-Whitney U, Chi-square and Fisher's exact tests were used to test for association of clinical variables and molecular markers. The log-rank test was used to test for survival differences among groups. The false discovery rate method (22) was used to adjust p-values for multiple testing of correlations between molecular markers and their associations with progression-free survival (PFS) and overall survival (OS). PFS was calculated from the day of first surgery until tumor progression, death or end of follow-up. OS was calculated from the day of first surgery until death or end of follow-up. The effect of each single molecular marker on PFS and OS was investigated using the Cox proportional hazards model adjusting for the major clinical prognostic factors age at diagnosis (< 60 versus $\geq$ 60 years), Karnofsky performance score (KPS) (< 80 versus $\geq$ 80), extent of resection (total versus nontotal), MGMT promoter methylation status and adjuvant therapy (radiotherapy alone versus radiotherapy plus temozolomide). The multivariate model was applied to all patients who had complete information on the five clinical factors named above and who received radiotherapy alone or radiotherapy plus temozolomide as their first-line therapy. The models presented are calculated without taking interactions terms into account. Variants of the multivariate model including interactions between age, MGMT and temozolomide were evaluated in a sensitivity analysis, but did not show qualitatively different results regarding the prognostic value of the molecular markers. Radiation Therapy Oncology Group (RTOG) recursive partitioning analysis (RPA) classes were assessed as described (23).

Results

Clinical parameters

The study population comprised 291 patients with glioblastoma, 8 with giant cell glioblastoma and 2 with gliosarcoma, for a total of 301 patients. The median follow-up was 29.4 months and 238 patients have died. Clinical patient characteristics are summarized in Table 1 (left column). There were no significant differences for any of these parameters between male and female patients, except for a trend to younger age in male patients (51% versus 39% < 60 years in male and female patients, p=0.045). The median ages for patients treated with radiotherapy plus temozolomide versus radiotherapy alone were 58.5 (interquartile range, IQR: 48.4-65.4) and 65.4 (IQR: 56.9-71.1) years. The median KPS for patients treated with radiotherapy plus temozolomide versus radiotherapy alone were 90 (IQR: 80-90) and 80 (IQR: 70-87.5). The median age was 72.1 (IQR: 63.5-76.7) years for patients receiving no adjuvant treatment, and their median KPS was 70 (IQR: 62.5-90).

The median PFS for all patients was 6.8 months (95% CI: 6.2-7.3). The median OS was 12.5 months (95% CI: 11.1-14.3). The prognostic relevance of the clinical parameters age, KPS and extent of resection is summarized in Table 2, as is the prognostic impact of RTOG RPA scores. A significant impact of extent of resection became only apparent when patients with a complete resection were compared with patients with either subtotal or partial resection or open biopsy pooled. The extent of resection was determined by early (<72 h) postoperative MRI or CT (n=199) or documented without reference to the imaging modality (n=102). Estimates were available for all patients. Tumor localization defined as frontal, temporal, parietal, occipital or other was not associated with PFS or OS (data not shown).

Kaplan-Meier estimates for PFS and OS by primary treatment are shown in Figure 1A,B. The PFS data for patients receiving radiotherapy alone (n=64) may be overestimated since 30% of these patients had no documented progression prior to death whereas this was the case for only 6% of the patients starting on temozolomide radiochemotherapy. Hence, follow-up documentation may have been less vigorous in patients managed with radiotherapy alone.

Molecular parameters

Glioblastoma-associated molecular aberrations were observed with the following frequencies: *TP53* mutation 15%, *EGFR* amplification 40%, *CDK4* amplification 10%, *MDM2* amplification 6%, *CDKN2A* homozygous deletion 19%, allelic losses on chromosome arms 1p 21%, 9p 41%, 10q 58%, 19q 19%, and 1p+19q 8% (Table 3). None of these aberrations was associated with the clinical parameters age, KPS or extent of resection (data not shown) or PFS or OS (Fig. 2, Supplementary Table 1). This was true for all patients as well as for the subpopulations receiving radiotherapy alone, radiotherapy and temozolomide or any of these therapies after correcting for multiple testing. Various associations of these molecular parameters were confirmed. A complete overview of the pairwise associations between these molecular markers and MGMT status is given in Supplementary Table 2.

MGMT promoter methylation was detected in 44% of the patients and conferred superior PFS and OS (Supplementary Table 1). The *MGMT* status was not associated with the clinical parameters age, KPS or extent of resection, nor with the first-line treatment administered. Moreover, *MGMT* promoter methylation status was not associated with any of the molecular parameters tested here (Supplementary Table 2). *MGMT* promoter methylation did not translate into a significant prolongation

of PFS in patients receiving radiotherapy alone as their first-line treatment. In contrast, *MGMT* promoter methylation was strongly associated with longer PFS and OS in patients receiving temozolomide (Fig. 1C,D). We also tested for interactions between *MGMT* promotor methylation and single molecular factors regarding PFS and OS, but did not find any relevant interaction (data not shown). *IDH*-1 mutations were found in 16 of 286 patients tested in our data set. They were associated with prolonged PFS and OS on univariate analysis (Supplementary Table 1) and correlated positively with p53 immunoreactivity, but negatively with EGFR amplification and 10q loss (Supplementary Table 2).

Multivariate analyses for outcome

We established multivariate survival models for PFS and OS including the prognostic factors age at diagnosis, KPS, extent of resection, *MGMT* promoter methylation status and adjuvant therapy. These analyses were restricted to the two patient populations receiving either radiotherapy alone or radiotherapy plus temozolomide as their first-line treatment with full information on all clinical parameters (229 patients, Table 1). The models were designed to take each of the factors into account without considering interaction terms.

Independent prognostic factors for PFS were age and *MGMT* status, and for OS were age, KPS, *MGMT* status and type of adjuvant therapy (Table 4). These multivariate models for PFS and OS using age, KPS, extent of resection, *MGMT* status and adjuvant therapy were subsequently used as adjustments for estimating the relative risk associated with the effect of single molecular parameters besides *MGMT*. Relative risk estimates and confidence intervals are summarized in Supplementary Fig. 1. Among the eleven molecular markers investigated, only IDH-1

mutation status was associated with prolonged PFS (RR 0.42, 95%CI 0.19 – 0.91, p= 0.028) and a trend for improved OS (RR 0.43, 95% CI 0.15 – 1.19, p = 0.10). All other markers were not associated with PFS or OS.

In a sensitivity analysis, we investigated whether more extended multivariate models also considering possible two-way interactions between age, *MGMT* status and treatment modality - radiotherapy only or radiotherapy plus temozolomide - would impact this conclusion. Although we found some indications for interactions, e.g. of age and *MGMT* status (data not shown), all estimates on the molecular markers remained very similar to those shown in Supplementary Fig. 1 with a general tendency to shrink towards the relative risk of 1. Hence, the estimates shown are the least conservative.

Discussion

The German Glioma Network (GGN) aims at generating a data base for the diagnosis, treatment and follow-up of glioma patients at several large academic centers as well as a tissue bank for translational research. This study focused on the prognostic significance of established molecular markers in glioblastoma patients enrolled in the first 27 months of funding. The major strengths include the following: (i) clinical data were collected prospectively, (ii) patients were enrolled within a short time frame, (iii) clinical data were obtained in the temozolomide era which makes them particularly relevant for contemporary neuro-oncology, (iv) we studied not only overall, but also progression-free survival, and both endpoints stratified by treatment. We confirm the major therapy-independent prognostic factors age and KPS. Extent of resection was only of prognostic value when completely resected patients were compared with all other patients (Table 2) and on univariate analysis only (Table 4). In contrast, all molecular aberrations attributed a role in the development of glioblastoma, including *TP53* mutation, *EGFR* amplification, *CDK4* amplification, *MDM2* amplification, *CDKN2A* homozygous deletion, LOH 1p, LOH 9p, LOH 10q, LOH 19q or 1p/19q codeletion, were not associated with PFS and OS (Supplementary Table 1, Fig. 2). When controlled for age, neither of the parameters examined had a prognostic impact in the Boston (*TP53*, *EGFR*, *CDKN2A*, LOH 1p, 10q, 19q) (2) or Duke (*TP53*, *EGFR*, *CDKN2A*, *PTEN*) (4) series. An association of LOH 10q with poorer survival was identified in the Zurich series (3) whereas *EGFR* amplification was associated with better survival in the Paris series (5) (Table 3). As a result of the EORTC NCIC trial (24), temozolomide is now the chemotherapeutic agent most commonly used in patients with newly diagnosed glioblastoma (Table 1,

Fig. 1A,B). We confirm that *MGMT* promoter methylation is a strong predictor of prolonged progression-free and overall survival in patients receiving temozolomide (Fig. 1C,D). Similarly, in a French series, *MGMT* promoter methylation assumed only predictive significance when patients received adjuvant temozolomide (25).

This study may also have clarified the controversial interrelation of *MGMT* promoter methylation and p53 status. p53 immunoreactivity suggestive of *TP53* mutations has been linked to low *MGMT* mRNA expression (26), but did not correlate with *MGMT* promoter methylation (25). We observed that abrogation of wild-type p53 function in cultured glioma cells greatly attenuated sensitivity to temozolomide whereas a p53 mimetic small molecule enhanced temozolomide sensitivity (27). The extensive analysis here suggested a trend that patients with *TP53* mutations derived more benefit from temozolomide chemotherapy than patients with *TP53* wild-type tumors, but overall no significant association between *MGMT* promoter methylation and p53 status became apparent (data not shown).

Future approaches likely to yield novel predictors of response to therapy and outcome include global analyses of gene expression profiling (4, 28) and genomic (29) and epigenetic (30) profiling. High throughput analyses have recently resulted in the identification of mutations in the active site of the *IDH*-1 gene as a novel prognostic marker in gliomas (21,31-32). The present study supports the hypothesis that within the group of primary glioblastomas, *IDH*-1 mutations are rare and define a prognostically favorable subgroup (Fig. 2).

In summary, we demonstrate here that genetic changes commonly associated with the pathogenesis of gliomas do not predict response to therapy in glioblastoma patients managed according to current standards of care. *MGMT* promoter

methylation status is clinically the most relevant molecular parameter and should be included as a stratification factor in all future trials in glioblastoma.

References

1. Reifenberger G, Collins VP: Pathology and molecular genetics of astrocytic gliomas. J Mol Med 82:656-670, 2004.

2. Batchelor TT, Betensky RA, Esposito JM, Pham LD, Dorfman MV, Piscatelli N, Jhung S, Rhee D, Louis DN: Age-dependent prognostic effects of genetic alterations in glioblastoma. Clin Cancer Res 10:228-233, 2004

3. Ohgaki H, Dessen P, Jourde B, Horstmann S, Nishikawa T, Di Patre PL, Burkhard C, Schuler D, Probst-Hensch NM, Maiorka PC, Baeza N, Pisani P, Yonekawa Y, Yasargil MG, Lutolf UM, Kleihues P: Genetic pathways to glioblastoma: a population-based study. Cancer Res 64:6892-6899, 2004

4. Rich JN, Hans C, Jones B, Iversen ES, McLendon RE, Rasheed BK, Dobra A, Dressman HK, Bigner DD, Nevins JR, West M: Gene expression profiling and genetic markers in glioblastoma survival. Cancer Res 65:4051-4508, 2005

5. Houillier C, Lejeune J, Benouaich-Amiel A, Laigle-Donadey F, Criniere E, Mokhtari K, Thillet J, Delattre JY, Hoang-Xuan K, Sanson M: Prognostic impact of molecular markers in a series of 220 primary glioblastomas. Cancer 106:2218-2223, 2006

6. Cairncross JG, Berkey B, Shaw E, Jenkins R, Scheithauer B, Brachman D, Buckner J, Fink K, Souhami L, Laperierre N, Mehta M, Curran W: Phase III trial of chemotherapy plus radiotherapy compared with radiotherapy alone for pure and mixed anaplastic oligodendroglioma: Intergroup Radiation Therapy Oncology Group Trial 9402. J Clin Oncol 24:2707-2714, 2006

7. van den Bent MJ, Carpentier AF, Brandes AA, Sanson M, Taphoorn MJ, Bernsen HJ, Frenay M, Tijssen CC, Grisold W, Sipos L, Haaxma-Reiche H, Kros JM, van Kouwenhoven MC, Vecht CJ, Allgeier A, Lacombe D, Gorlia T: Adjuvant procarbazine, lomustine, and vincristine improves progression-free survival but not

overall survival in newly diagnosed anaplastic oligodendrogliomas and oligoastrocytomas: a randomized European Organisation for Research and Treatment of Cancer phase III trial. J Clin Oncol 24:2715-2722, 2006

8. Wick W, Weller M, for the Neurooncology Working Group of the German Cancer Society: NOA-04 randomized phase III study of sequential radiochemotherapy of anaplastic glioma with PCV or temozolomide. J Clin Oncol, ASCO Proceedings, #LBA2007, 2008

9. Felsberg J, Erkwoh A, Sabel MC, Kirsch L, Fimmers R, Blaschke B, Schlegel U, Schramm J, Wiestler OD, Reifenberger G: Oligodendroglial tumors: refinement of candidate regions on chromosome arm 1p and correlation of 1p/19q status with survival. Brain Pathol 14:121-130, 2004

10. Esteller M, Garcia-Foncillas J, Andion E, Goodman SN, Hidalgo OF, Vanaclocha V, Baylin SB, Herman JG: Inactivation of the DNA-repair gene MGMT and the clinical response of gliomas to alkylating agents. N Engl J Med 343:1350-1354, 2000

11. Hegi ME, Diserens AC, Gorlia T, Hamou MF, de Tribolet N, Weller M, Kros JM, Hainfellner JA, Mason W, Mariani L, Bromberg JE, Hau P, Mirimanoff RO, Cairncross JG, Janzer RC, Stupp R: MGMT gene silencing and benefit from temozolomide in glioblastoma. N Engl J Med 352:997-1003, 2005

12. Herrlinger U, Rieger J, Koch D, Loeser S, Blaschke B, Kortmann RD, Steinbach JP, Hundsberger T, Wick W, Meyermann R, Sommer C, Bamberg M, Reifenberger G, Weller M: Phase I/II trial (UKT-03) of CCNU plus temozolomide chemotherapy in addition to radiotherapy as first-line therapy for glioblastoma. J Clin Oncol 24:4412-4417, 2006

13. Glas M, Happold C, Rieger J, Wiewrodt D, Bähr O, Steinbach PJ, Wick W, Kortmann RD, Reifenberger G, Weller M, Herrlinger U: Long-term survival of

glioblastoma patients treated with radiotherapy and lomustine plus temozolomide. J Clin Oncol 27:1257-1261, 2009

14. Kleihues P, Burger PC, Collins VP, Newcomb EW, Ohgaki H, Cavenee WK: Glioblastoma. In: Kleihues P, Cavenee WK. WHO classification of tumours of the nervous system. IARC Press, Lyon, France, 29-39, 2000

15. Kleihues P, Burger PC, Aldape KD, Brat DJ, Biernat W, Bigner DD, Nakazato Y, Plate KH, Giangaspero F, von Deimling A, Ohgaki H, Cavenee WK: Glioblastoma. In: Louis DN, Ohgaki H, Wiestler OD, Cavenee WK. WHO classification of tumours of the central nervous system. IARC Press, Lyon, France, 33-49, 2007

16. Möllemann M, Wolter M, Felsberg J, Collins VP, Reifenberger G: Frequent promoter hypermethylation and low expression of the MGMT gene in oligodendroglial tumors. Int J Cancer 113:379-385, 2005

17. Krex D, Klink B, Hartmann C, von Deimling A, Pietsch T, Simon M, Sabel M, Steinbach JP, Heese O, Reifenberger G, Weller M, Schackert G, for the German Glioma Network: Long-term survival with glioblastoma. Brain 130:2596-2606, 2007

18. Reifenberger G, Reifenberger J, Ichimura K, Meltzer PS, Collins VP: Amplification of multiple genes from chromosomal region 12q13-14 in human malignant gliomas: preliminary mapping of the amplicons shows preferential involvement of CDK4, SAS, and MDM2. Cancer Res 54:4299-4303, 1994

19. Kaulich K, Blaschke B, Nümann A, von Deimling A, Wiestler OD, Weber RG, Reifenberger G: Genetic alterations commonly found in diffusely infiltrating cerebral gliomas are rare or absent in pleomorphic xanthoastrocytomas. J Neuropathol Exp Neurol 61:1092-1099, 2002

20. Hartmann C, Mueller W, Lass U, Kamel-Reid S, von Deimling A: Molecular genetic analysis of oligodendroglial tumors. J Neuropathol Exp Neurol 64:10-14,

2005

21. Balss J, Meyer J, Mueller W, Korshunov A, Hartmann C, von Deimling A: Analysis of the IDH1 codon 132 mutation in brain tumors. Acta Neuropathol 116:597-602, 2008

22. Benjamini Y, Hochberg Y: Controlling the false discovery rate: a practical and powerful approach to multiple testing. J Royal Statist Soc Series B 57:289-300, 1995

23. Mirimanoff RO, Gorlia T, Mason W, Van den Bent MJ, Kortmann RD, Fisher B, Reni M, Brandes AA, Curschmann J, Villa S, Cairncross G, Allgeier A, Lacombe D, Stupp R: Radiotherapy and temozolomide for newly diagnosed glioblastoma: recursive partitioning analysis of the EORTC 26981/22981-NCIC CE3 phase III randomized trial. J Clin Oncol 24:2563-2569, 2006

24. Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJB, Belanger K, Brandes AA, Cairncross JG, Marosi C, Bogdahn U, Curschmann J, Janzer RC, Ludwin S, Gorlia T, Allgeier A, Lacombe D, Eisenhauer E, Mirimanoff RO, on behalf of the European Organisation for Research and Treatment of Cancer (EORTC) Brain Tumor and Radiotherapy Groups and National Cancer Institute of Canada Clinical Trials Group (NCIC CTG): Radiotherapy plus concomitant and adjuvant temozolomide for patients with newly diagnosed glioblastoma. N Engl J Med 352:987-996, 2005

25. Criniere E, Kaloshi G, Laigle-Donadey F, Lejeune J, Auger N, Benouaich-Amiel A, Everhard S, Mokhtari K, Polivka M, Delattre JY, Hoang-Xuan K, Thillet J, Sanson M: MGMT prognostic impact on glioblastoma is dependent on therapeutic modalities. J Neurooncol 83:173-179, 2007

26. Rolhion C, Penault-Llorca F, Kemeny JJ, Kwiatkowski F, Lemaire JJ, Chollet P, Finat-Duclos F, Verrelle P: O^6-methylguanine-DNA methyltransferase gene (MGMT)

expression in human glioblastomas in relation to patient characteristics and p53 accumulation. Int J Cancer 416:416-420, 1999

27. Hermisson M, Klumpp A, Wick W, Wischhusen J, Nagel G, Roos W, Kaina B, Weller M: O^6-methylguanine DNA methyltransferase and p53 status predict temozolomide sensitivity in human malignant glioma cells. J Neurochem 96:766-776, 2006

28. Liang Y, Diehn M, Watson N, Bollen AW, Aldape KD, Nicholas MK, Lamborn KR, Berger MS, Botstein D, Brown PO, Israel MA: Gene expression profiling reveals molecularly and clinically distinct subtypes of glioblastoma multiforme. Proc Natl Acad Sci U S A 102:5814-5819, 2005

29. The Cancer Genome Atlas Research Network: Comprehensive genomic characterization defines human glioblastoma genes and core pathways. Nature 455:1061-1068, 2008

30. Kim TY, Zhong S, Fields CR, Kim JH, Robertson KD: Epigenomic profiling reveals novel and frequent targets of aberrant DNA methylation-mediated silencing in malignant glioma. Cancer Res 66:7490-7501, 2006

31. Parsons DW, Jones S, Zhang X, Lin JC, Leary RJ, Angenendt P, Mankoo P, Carter H, Siu IM, Gallia GL, Olivi A, McLendon R, Rasheed BA, Keir S, Nikolskaya T, Nikolsky Y, Busam DA, Tekleab H, Diaz LA Jr, Hartigan J, Smith DR, Strausberg RL, Marie SK, Shinjo SM, Yan H, Riggins GJ, Bigner DD, Karchin R, Papadopoulos N, Parmigiani G, Vogelstein B, Velculescu VE, Kinzler KW: An integrated genomic analysis of human glioblastoma multiforme. Science 321:1807-1812, 2008

32. Yan H, Parsons DW, Jin G, McLendon R, Rasheed BA, Yuan W, Kos I, Batinic-Haberle I, Jones S, Riggins GJ, Friedman H, Friedman A, Reardon D, Herndon J, Kinzler KW, Velculescu VE, Vogelstein B, Bigner DD: IDH1 and IDH2 mutations in

gliomas. N Engl J Med 360:765-773, 2009

Table 1. Patient characteristics.

	All patients (n=301)		**Patients used for multivariate survival modelling** (n=229)	
Age at diagnosis (years)				
Median	61.5		60.1	
Range	19.2-86.6		19.2-86.6	
Age classes				
18-39 ys	27	9%	23	10%
40-49 ys	41	14%	33	14%
50-59 ys	70	23%	58	25%
60-70 ys	92	31%	79	34%
70-80 ys	59	20%	33	14%
80-90 ys	12	4%	3	1%
Gender				
Male	182	60%	144	63%
Female	119	40%	85	37%
KPS				
90-100	122	(41%)	105	(46%)
70-80	128	(43%)	107	(47%)
<70	30	(10%)	17	(7%)
Missing	21	(7%)	0	(0%)
Surgery				
Total resection	136	45%	117	51%

Subtotal resection	108	36%	83	36%
Partial resection	27	9%	21	9%
Biopsy	27	9%	8	3%
No data	3	1%	0	0%
Review diagnosis				
Glioblastoma	291	97%	220	96%
Gliosarcoma	2	1%	2	1%
Giant cell glioblastoma	8	3%	7	3%
First-line therapy				
Radiotherapy alone	64	21%	53	23%
Radiotherapy plus Temozolomide	189	63%	176	77%
Temozolomide alone	10	3%	0	0%
Other Chemotherapy alone	2	1%	0	0%
Radiotherapy plus other chemotherapy	5	2%	0	0%
No therapy	31	10%	0	0%
RTOG RPA classes				
RPA III	31	10%	27	12%
RPA IV	102	34%	94	41%
RPA V	34	11%	27	12%
RPA VI	6	2%	3	1%
Missing	128	43%	78	34%

Table 2. Clinical parameters, PFS and OS

	N	Median PFS (months)	P Value (Logrank test)	Median OS (months)	P Value (Logrank test)
All patients	301	6.8		12.5	
Age					
< 60 years	138	9.5	<0.001	19.3	<0.001
≥ 60 years	163	5.8		9.6	
KPS					
≥ 80	208	7.2	<0.001	14.9	<0.001
< 80	72	5.4		7.6	
Surgery					
Total resection	136	7.2	0.03	15.0	0.002
No total resection	162	6.3		10.2	
RTOG RPA Score					
III	31	10.2	0.006	17.9	<0.001
IV	102	7.3		18.3	
V	34	6.3		8.7	
VI	6	3.2		5.5	

Table 3. Comparison of the frequency (%) of molecular genetic alterations in glioblastoma

Reference	Patients (n)*	Time of diagnosis	*TP53* mutation	*EGFR* ampli-fication	*CDK4* ampli-fication	*MDM2* ampli-fication	*CDKN2A* homo-zygous deletion	LOH 1p	LOH 9p	LOH 10q	LOH 19q	*PTEN* mutation	*MGMT* gene promoter methy-lation
Batchelor et al. (2)	140	1990-2001	19	36	Nd	nd	33	13	nd	74	24	nd	nd
Ohgaki et al. (3)	715	1980-1994	31	34	Nd	nd	31	nd	nd	69	nd	24	nd
Rich et al. (4)	41	Nd	27	41	Nd	nd	45	nd	nd	Nd	nd	37	nd
Houillier et al. (5)	220	1997-2005	nd	34	7	6	36	19	47	Nd	nd	nd	nd
This study	301	2004-2006	15	40	10	6	19	21	41	58	19		44

*commonly no full data sets were available for all patients

Table 4. Multivariate analysis of PFS and OS (n=229 patients receiving either radiotherapy and temolozomide or radiotherapy alone and data for all factors available)

Factor	**Relative risk of progression (95% CI)**	**P value**	**Relative risk of death (95% CI)**	**p value**
Age				
< 60 years	1.00		1.00	
≥ 60 years	1.63 (1.23-2.17)	0.00063	2.19 (1.59-3.01)	$1.5*10^{-6}$
KPS				
≥ 80	1.00		1.00	
< 80	1.32 (0.92-1.88)	0.13	1.59 (1.10–2.28)	0.013
Resection				
No total resection	1.00		1.00	
Total resection	0.91 (0.69-1.20)	0.50	0.76 (0.56-1.03)	0.076
***MGMT* promoter**				
Unmethylated	1.00		1.00	
Methylated	0.51 (0.38-0.68)	$6*10^{-6}$	0.39 (0.28-0.54)	$1.2*10^{-8}$
Adjuvant therapy				
Radiotherapy alone	1.00		1.00	
Radiotherapy plus temozolomide	0.87 (0.63-1.21)	0.41	0.62 (0.43-0.88)	0.0082

Supplementary Table 1. Molecular genetic parameters, PFS and OS in all evaluable patients (univariate estimates)

		N	PFS (months)	P Value* (Logrank test)	OS (months)	P Value* (Logrank test)
TP53 mutation	Yes	45	6.0	0.47	9.3	0.46
	No	247	6.9		12.9	
P53 immunoreactivity	Negative	169	6.5	0.22	11.7	0.03
	<30% Positive	63	7.1		13.2	
	>=30% Positive	44	7.1		21.6	
EGFR amplification	Yes	121	7.1	0.65	13.3	0.98
	No	174	6.3		11.8	
CDK4 amplification	Yes	29	7.1	0.86	10.2	0.69
	No	262	6.7		12.9	
MDM2 amplification	Yes	19	7.9	0.5	11.6	0.48
	No	272	6.7		12.7	
CDKN2A homozygous deletion	Yes	56	6.6	0.89	10.7	0.35
	No	234	6.8		13.3	
LOH 1p	Yes	62	5.4	0.38	12.9	0.63
	No	217	6.8		12.3	
LOH 9p	Yes	123	5.5	0.06	10.7	0.06
	No	145	7.3		13.6	
LOH 10q	Yes	175	6.3	0.02	11.7	0.006
	No	80	7.5		17.9	
LOH 19q	Yes	58	6.5	0.23	14.2	0.08
	No	221	6.8		11.7	
1p/19q codeletion	Yes	24	6.7	0.46	17.0	0.2
	No	257	6.7		12.2	
MGMT promoter methylation	Yes	133	7.5	<0.001	18.9	<0.001
	No	162	6.3		11.1	
IDH-1 mutation	Yes	16	16.2	<0.001	30.2	0.002
	No	270	6.5		11.2	

* p values unadjusted for multiple testing

Supplementary Table 2. Correlation of molecular markers (upper right triangle: p-values [Fisher's exact test]; lower left triangle: odds ratios for concurrent presence of markers)

p-value / Odds ratio	*TP53* mutation	p53 immuno-reactivity	*EGFR* amplification	*CDK4* amplification	*MDM2* amplification	*CDKN2A* homozygous deletion	LOH 1p	LOH 9p	LOH 10q	LOH 19q	*MGMT* promoter methylation	*IDH-1* mutation
TP53 mutation		$1.3*10^{-6}$ +	0.18	0.78	0.33	0.21	0.84	0.87	0.26	0.07	0.74	0.3
p53 Immunoreactivity	6.1+		0.0013+	1	1	0.79	0.09	0.03	0.24	0.11	0.26	0.005+
EGFR amplfication	0.6	0.4+		0.69	0.33	0.02	0.31	0.01+	0.004+	0.37	0.81	0.0003+
CDK4 amplification	1.2	1.0	1.2		$2.8*10^{-12}$ +	0.002+	0.05	0.0008+	0.01+	0.22	0.05	1
MDM2 amplification	0.3	0.9	1.7	46+		0.03	0.37	0.036	0.56	0.55	0.81	1
CDKN2A homozygous deletion	1.6	0.8	2.1	0+	0		0.009+	$2.2*10^{-10}$ +	$9.3*10^{-6}$ +	0.007+	0.07	0.7
LOH 1p	0.8	0.6	1.3	0.3	0.4	2.4+		0.005+	0.008+	0.0003+	0.55	0.2
LOH 9p	1.1	0.6	1.9+	0.2+	0.3	9.6+	2.4+		$6.9*10^{-7}$ +	0.22	0.62	0.8
LOH 10q	1.6	0.7	2.3+	5.4+	1.6	8.8+	2.7+	4.3+		0.01+	0.02	0.0006+
LOH 19q	2.0	1.7	0.8	0.4	1.5	2.6+	3.3+	1.5	2.9+		0.36	1
MGMT promoter methylation	1.2	1.3	1.1	0.4	0.9	1.8	1.2	1.2	0.5	1.4		0.02
IDH-1 mutation	2.1	4.9+	0+	0.6	0.6	0.6	0.2	0.8	0.1+	1.1	4.0	

+ siginificant at p<0.05 after correction for multiple testing (adjusted p-values not shown); p53 immunoreactivity: >30% versus neg or <30%

Figure legends

Fig. 1. **Clinical course by treatment and MGMT promoter methylation status.** PFS (A) and OS (B) by primary treatment. 64 patients received radiotherapy alone, 183 patients radiotherapy plus temozolomide, 17 patients some other treatment, and 31 patients no adjuvant treatment after surgery. C, D. PFS (C) and OS (D) by *MGMT* promoter methylation status and treatment.

Fig. 2. **OS by molecular markers**: no significant association with *TP53* mutation, *EGFR* amplification, *CDK4* amplification, *MDM2* amplification, *CDKN2A* homozygous deletion, LOH 1p, LOH 9p, LOH 10q, LOH 19q or 1p/19q codeletion, but major prognostic role for *IDH*-1 mutations.

Supplementary Fig. 1. **Forrest plot of hazard ratios** for all molecular markers, corrected for age (< 60 versus $\geq$ 60 ys), KPS ($\geq$ 80 versus < 80), extent of resection (total versus other) and chemotherapy containing temozolomide, for PFS (A) and OS (B). Only patients treated with radiotherapy alone or radiotherapy plus temozolomide were considered.

Figure 1

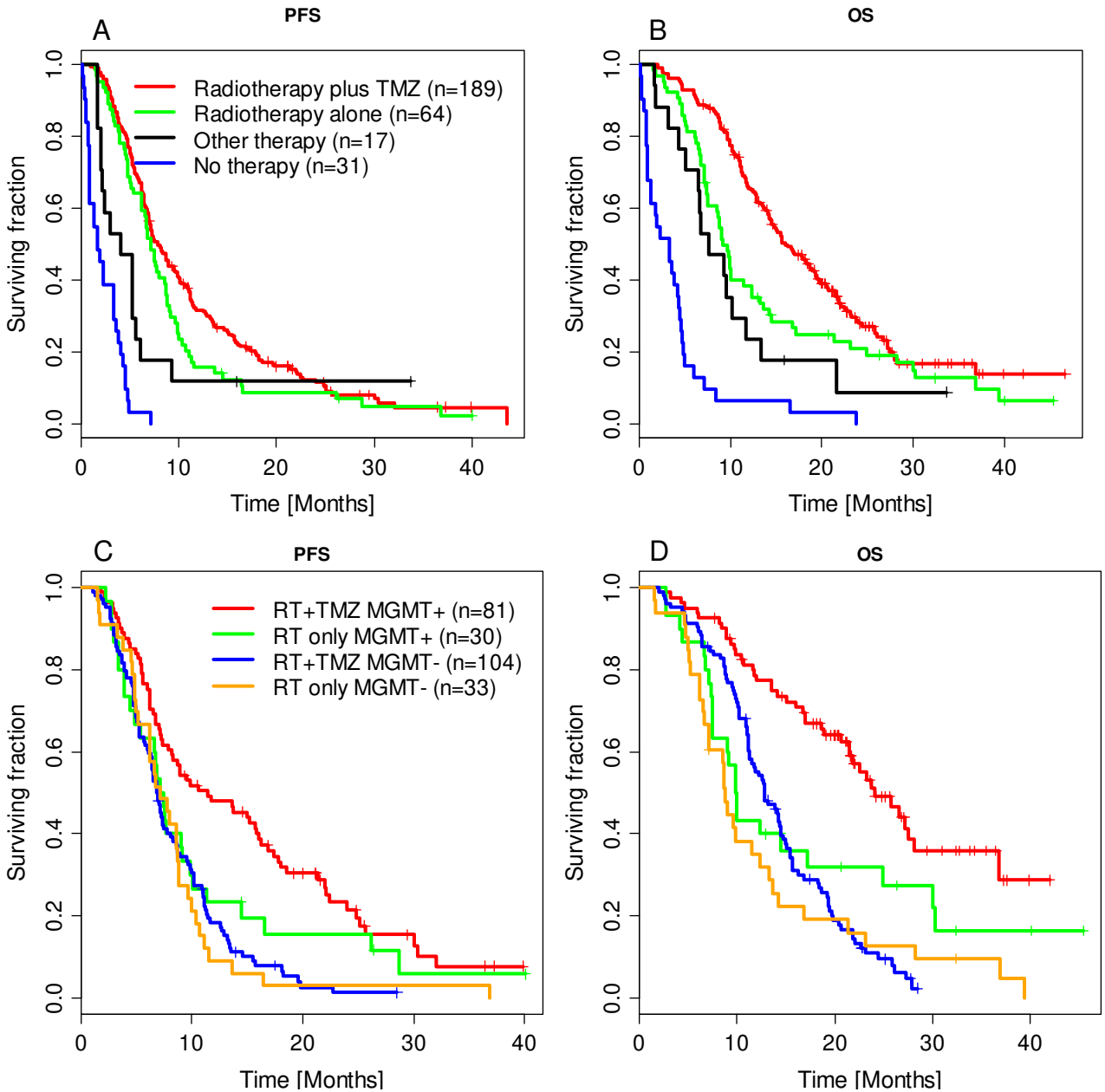
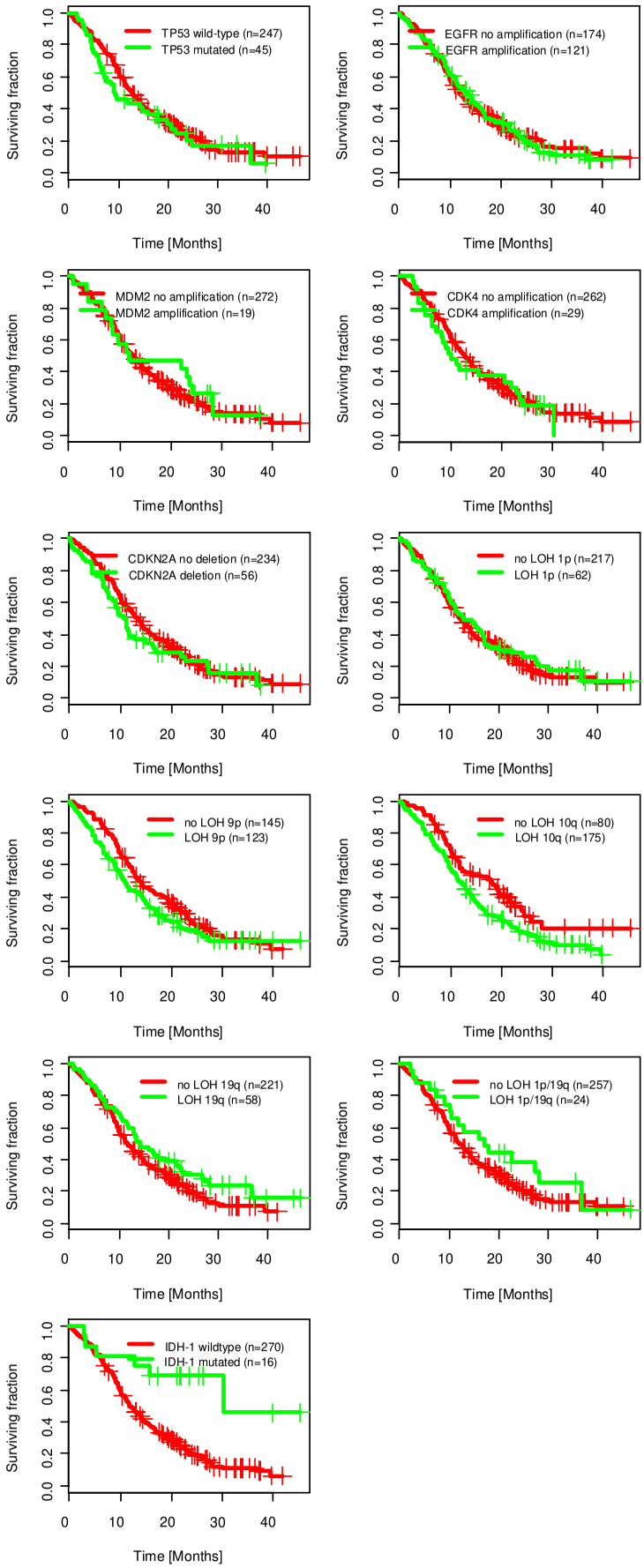

A
PFS
Radiotherapy plus TMZ (n=189)
Radiotherapy alone (n=64)
Other therapy (n=17)
No therapy (n=31)
Surviving fraction
Time [Months]
B
OS
Surviving fraction
Time [Months]
C
PFS
RT+TMZ MGMT+ (n=81)
RT only MGMT+ (n=30)
RT+TMZ MGMT- (n=104)
RT only MGMT- (n=33)
Surviving fraction
Time [Months]
D
OS
Surviving fraction
Time [Months]

Figure 2


TP53 wild-type (n=247)
TP53 mutated (n=45)
EGFR no amplification (n=174)
EGFR amplification (n=121)
MDM2 no amplification (n=272)
MDM2 amplification (n=19)
CDK4 no amplification (n=262)
CDK4 amplification (n=29)
CDKN2A no deletion (n=234)
CDKN2A deletion (n=56)
no LOH 1p (n=217)
LOH 1p (n=62)
no LOH 9p (n=145)
LOH 9p (n=123)
no LOH 10q (n=80)
LOH 10q (n=175)
no LOH 19q (n=221)
LOH 19q (n=58)
no LOH 1p/19q (n=257)
LOH 1p/19q (n=24)
IDH-1 wildtype (n=270)
IDH-1 mutated (n=16)
Surviving fraction
Time [Months]

Supplementary Figure 1