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Phase II study of radiotherapy and temsirolimus versus radiochemotherapy with temozolomide in patients with newly diagnosed glioblastoma without *MGMT* promoter hypermethylation (EORTC 26082)

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Running Head: Temsirolimus for newly diagnosed glioblastoma

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M.E.H. has served on advisory boards for MSD, Genentech/Roche, and MDxHealth, and has provided services to Novocure.

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Supplemental Information	

Statement of clinical relevance: The prospective randomized EORTC 26082 trial assessed the tolerability and efficacy of the mechanistic target of rapamycin (mTOR) inhibitor temsirolimus in patients with newly diagnosed, *O6 methlyguanine-DNA-methlytransferase (MGMT)* promoter unmethylated glioblastoma. Temozolomide could be omitted without detriment in the experimental arm. Efficacy of radiotherapy plus temsirolimus failed to reach the pre-specified number of patients alive at 12 months. Pre-specified assessment of activity in the mTOR pathway allows to suggest that one third of patients with phosphorylated mTOR at Ser2448 derive a robust and clinically relevant survival benefit and will be candidates for clinical development of temsirolimus as a targeted therapy in a molecularly defined subgroup.

ABSTRACT

Purpose: EORTC 26082 assessed the activity of temsirolimus in patients with newly diagnosed glioblastoma harboring an unmethylated O6 methlyguanine-DNA-methlytransferase (*MGMT*) promoter.

Patients and Methods: Patients (n=257) fulfilling eligibility criteria underwent central MGMT testing. Patients with *MGMT* unmethylated glioblastoma (n=111) were randomized 1:1 between standard chemo-radiotherapy with temozolomide or radiotherapy plus weekly temsirolimus (25 mg). Primary endpoint was overall survival at 12 months (OS12). A positive signal was considered >38 patients alive at 12 months in the per protocol population. A non-comparative reference arm of 54 patients evaluated the assumptions on OS12 in a standard-treated cohort of patients. Pre-specified post hoc analyses of markers reflecting target activation were performed.

Results: Both therapies were administered per protocol with a median of 13 cycles of maintenance temsirolimus. Median age was 55 and 58 years in the temsirolimus and standard arms, the WHO performance status 0 or 1 for most patients (95.5%). In the per protocol population, 38 of 54 patients treated with temsirolimus reached OS12. The actuarial 1-year survival was 72.2% [95% CI (58.2-82.2)] in the temozolomide arm and 69.6% [95% CI (55.8-79.9)] in the temsirolimus arm [HR=1.16, 95% CI (0.77-1.76), p=0.47]. In multivariable prognostic analyses of clinical and molecular factors phosphorylation of mTORSer2448 in tumor tissue (HR=0.13, 95% CI (0.04-0.47), p=0.002), detected in 37.6%, was associated with benefit from temsirolimus.

Conclusions: Temsirolimus was not superior to temozolomide in patients with an unmethylated *MGMT* promoter. Phosphorylation of mTORSer2448 in the pretreatment tumor tissue may define a subgroup benefitting from mTOR inhibition.

INTRODUCTION

The serine/threonine kinase, mechanistic target of rapamycin (mTOR) serves as a hub integrating multiple intra- and extracellular cues in cancer cells (1). mTOR is involved in the formation of two multi-protein complexes, mTORC1 and mTORC2, that direct cell metabolism, growth, proliferation, survival, and angiogenesis.

Preclinical studies suggested an enhanced activity of mTOR inhibition in PTEN-deficient tumour models (2, 3).

Activation of the PI3K/AKT/mTOR pathway has been associated with reduced survival of glioma patients (4) and this signalling pathway has been subjected to a number of negative single- or multi-targeted therapies including the mTOR inhibitor rapamycin or its derivatives, the ‘rapalogs’ everolimus (RAD001), deforolimus (AP23573), and temsirolimus (CCI-779) (5-9).

The experience with temozolomide (TMZ) teaches that limited activity at recurrence (10) may still relevantly modify the disease in patients with newly diagnosed glioblastoma when combined with radiotherapy (11). Accordingly, mTOR inhibition has been considered an option for patients with treatment-naïve glioblastomas that likely lack some of the mechanisms of resistance acquired at recurrence.

Temsirolimus (Torisel®) has been approved for advanced renal cell carcinoma (12) and relapsed or refractory mantle cell lymphoma (13). Additive effects of temsirolimus plus radiotherapy (RT) in preclinical models demonstrate that temsirolimus could complement the genotoxic activity of RT in the treatment of newly diagnosed glioblastoma. However, combination of TMZ and temsirolimus plus RT was too toxic (14).

Therefore, the rationale of this study was to test the biological effects of mTOR inhibition when combined with ionizing radiation in patients in whom TMZ could be safely omitted. To this end patients with tumors with an unmethylated *O6 methlyguanine-DNA-methlytransferase (MGMT)* gene promoter were selected for the trial, as they derive little if any benefit from the addition of TMZ (15). Another aim was to identify biological factors, i.e.

biomarkers linked to benefit from mTOR inhibition. Temsirolimus may counteract therapy-induced angiogenesis and invasion (16, 17).

PATIENTS AND METHODS

Clinical Trial

Study design and treatment

Patients for EORTC 26082 (NCT01019434) were recruited at 14 study sites in 10 countries in Europe. First, patients were registered after consenting for independent pathology review and central testing of the *MGMT* promoter methylation status by licensed laboratories of MDxHealth (Herstal, Belgium) using quantitative methylation-specific polymerase chain reaction of DNA isolated from macro-dissected formalin fixed paraffin embedded tumor sections (18). Patients were considered *MGMT* unmethylated, applying a safety margin, when the ratio of *MGMT* to the control gene *ACTB* was < 0·6, calculated as (methylated *MGMT*/*ACTB*)×1000. This corresponds to the lower bound of the 95% confidence interval established in a cohort of 602 glioblastoma samples screened in the CENTRIC trial where the cut-off corresponding to the established nadir was at a ratio of 2 that separates methylated from unmethylated. (19) as visualized in **Supplementary Figure S1**. A minimum of 1,250 copies of *ACTB* were required for a valid result, unless the copy number for methylated *MGMT* was ten or more, which was scored as *MGMT* methylated.

Eligible patients (see **Supplementary Information**) were randomly assigned to receive either standard chemoradiotherapy (TMZ/RT→TMZ) (11), or standard fractionated RT with concomitant temsirolimus (standard dose of 25 mg i.v. weekly beginning at day -7 from the start of RT, to be continued until disease progression) (**Figure 1 and Supplement**). The study was conducted according to the Declaration of Helsinki, the International Conference on Harmonisation note for good clinical practice (Topic E6, 1996), and regulatory requirements.

This study was funded by a grant from Pfizer, Berlin, Germany (details on the Role of the Funding Source in the **Supplement**).

Randomisation and masking

Randomisation was performed centrally using an interactive voice response system. Patients were stratified according to age, WHO performance status and baseline steroids. As this was an open-label study, no blinding procedures were applied.

Study endpoints

The primary endpoint was overall survival at 12 months (OS12) to avoid issues around pseudoprogression and generate a timely signal. Secondary endpoints included progression-free survival (PFS), OS, safety and assessment of prognostic and predictive biomarkers.

Outcome measures and statistical analyses

OS12 was defined as the fraction of patients alive at 12 months from randomisation; PFS was defined as duration from randomisation until first observation of PD or death from any cause or censored at last disease assessment without progression or start of second anti-cancer therapy; OS was defined as time from randomisation until death or last visit.

PFS was assessed locally by investigators according to the Macdonald criteria (20), in case of suspected pseudoprogression investigators were advised to continue treatment *per protocol* and repeat imaging after 1-2 months. If progression was confirmed, the date of first observation of tumor progress was used for the analyses.

Adverse events (AEs) were coded according to the Medical Dictionary for Regulatory Activities version 15.0, and their severity was graded according to National Cancer Institute Common Terminology Criteria for Adverse Events version 3.0.

A Fleming one-sample one-stage testing procedure was used in each arm. It was assumed that with OS12 lower or equal to 60% (P_0) the therapeutic activity of temsirolimus (CCI-779) was too low(11). While a OS12 greater or equal to 80% (P_1) implied that the therapeutic activity of temsirolimus (CCI-779) was adequate Type I (α) and II (β) errors were both equal to 5%. Under these hypotheses, a sample size of 54 eligible patients in each arm was required. The decision rule was that if >38 eligible patients were alive at 1 year, it was concluded that the therapeutic activity of temsirolimus was adequate.

All statistical analyses were performed on mature data (median follow-up 32 months) by Thierry Gorlia. The concept of a non-comparative control arm allows for adjustment of the initial assumptions based on contemporary control treatment. The trial would be insufficient to confirmatory declare efficacy. However, statistical comparisons are still valid and useful for hypothesis-generation and exploratory analyses.

The OS12 was also computed in the TMZ/RT→TMZ arm in order to assess the consistency with P0.

Biomarker substudy

Tissue Micro Array, Immunohistochemistry and FISH EGFR

Tissue micro arrays (TMA) were constructed using recipient paraffin blocks with an agarose matrix (21). Immunohistochemical analyses and Fluorescent *In Situ* Hybridization (FISH) were performed in duplicate on sections from 2 replicate TMAs basically as recommended by the manufacturers (see supplemental methods for antibody description, conditions and dilutions; FISH probes). Markers for *post hoc* analyzes of the mTOR pathway were pre-specified in the protocol (phosphorylated S6 ribosomal protein, p-S6RP$^{Ser235/236}$; phosphorylated AKT, p-AKT^{Ser473}; PTEN; phosphorylated AKT1 Substrate 1 (proline-rich), p-PRAS40^{Thr246}; phosphorylated extracellular signal-regulated linase, ERK1/2$^{Thr202/Tyr204}$) or based on a more recent study (phosphorylated p-mTOR^{Ser2448}) (22, 23). Scoring and definition of dichotomization is detailed in the Supplemental Methods.

Multidimensional marker analysis

The centered score table of the markers containing missing values was analysed by principal component analysis. Non-linear Iterative Partial Least Squares (NIPALS) algorithm (24) was used to perform singular-value decomposition with missing value and to complete the data. A consensus hierarchical clustering analysis (25) based on Euclidean distance and Ward's algorithm was used to investigate the optimal number of clusters. The association among marker scores was illustrated by network representation based on Spearman correlation. Analyses and graphical representations were performed using R-3.2.0 and the R packages mixOmics, qgraphs (26) and ConsensusClusterPlus.

Statistical analysis

The scores of the P-markers were dichotomized into negative (scores 0, 1, corresponding to 0 to10%) vs positive (scores 2 to 5, >10%). Study stratification factors (age, WHO performance status, baseline steroids) and molecular markers were correlated to OS.

Treatment arms were compared with a log-rank test at 5 % significance. For each of them, PFS and OS were estimated using the Kaplan-Meier (KM) method. Associations of marker profiles with treatment efficacy were presented by Forest Plot and significance was assessed with the test for interaction computed from a Cox model including the treatment, the marker and their interaction term. A 5% significance was used for screening predictive markers. For each factor, univariable survival estimates were calculated using the KM technique in the TMZ and temsirolimus arms. Hazard Ratios obtained from univariable Cox models were presented with 95 % Confidence Intervals (CI) (details in the **Supplement**).

RESULTS

Patients

Overall, 257 patients were registered, screened for eligibility and assessed for *MGMT* promoter methylation status, whereof 28 patients were registered after screening through the CENTRIC trial that selected *MGMT* methylated patients only (19); 190 patients were found to have glioblastoma with an unmethylated *MGMT* promoter applying the cut-off with a safety margin (Figure S1). The primary reasons for initially registered patients not to continue to randomisation were hypermethylated *MGMT* status (n=67), withdrawal of consent (n=24), and other reasons (n=55), including insufficient tumor material (n=30), and AEs after surgery (n=8) **(Figure 1)**. A total of 111 patients were randomised from December 2009 through September 2012 and constituted the ITT population: 56 patients were scheduled to receive weekly temsirolimus in addition to standard RT (temsirolimus arm) and 55 were to receive TMZ/RT→TMZ alone (control arm). In the safety population, i.e. patients with at least one dose of drug, there were 53 patients in the temsirolimus and 51 patients in the TMZ arm.

Median follow-up was 33 (95% CI: 23-37) months in the temsirolimus and 32 (95% CI: 22-40) months in the TMZ arm. The median duration from operation to randomisation was 2.6 weeks (range 0.4–6.1 weeks). Patient baseline and demographic characteristics were well balanced between treatment arms except for the WHO Performance status between PS0 and PS1, which favored the control arm. This is explained since the stratification was PS 0-1 *vs* PS2 (**Table 1**).

In the biomarker cohort (n=88), only one patient sample displayed positive staining for the IDH1-R132H mutant (1/78; 1.3%), an expected low frequency, since 75% of the few *IDH1* mutant glioblastoma are *MGMT* hypermethylated (27). The frequency of *EGFR* amplification was in the expected range (54%, 44/82). There was no difference in baseline characteristics and outcome in patients with *vs* without markers assessment (**Supplementary Figure S2**,

Supplementary Table S1).

Efficacy outcomes

The median duration of radiotherapy was 6.1 weeks in both arms. Main reason for interrupting RT was technical or administrative (28%). In median, RT was interrupted 2 days. RT was completed by >90% of patients. Concomitant treatment was delivered as planned *per protocol* by >90% of patients in both arms. Patients in the temsirolimus arm received the drug for a median (95% CI) of 16 weeks post RT (4.0 – 84.3), with a mean dose intensity of 21.4 (6.3 - 25) mg/week.

Maintenance temsirolimus was administered *per protocol* at a median of 13 weekly cycles. Median relative dose-intensity was 85.6%. Twelve patients had a reduction in dose intensity below 70%, because of dose reduction (19.1%: 6.4% for hematological toxicity, 10.6% for AE, 2.1% for other reasons), dose not given during at least one cycle (68%: 6.3% for hematological toxicity, 34% for non-hematological toxicity, 58% for other reasons) or treatment delay (58%: 2.1% for hematological toxicity, 17% for non-hematological toxicity, 43% for other reasons).

Median OS was 14.8 (13.3-16.4) months in the temsirolimus arm and 16.0 (13.8-18.2) in the control arm (90 deaths; HR, 1.2; 95% CI, 0.8-1.8; p=0.47; **Figure 2A**). The OS12 and OS24 rates did not differ between arms (70%, 72% and 15%, 16%, respectively). Median PFS as assessed by the investigator was 5.4 (95% CI, 3.7-6.1) months in the temsirolimus arm and 6.0 (95% CI, 2.8-8.0) months in the control arm (54 PFS events; HR, 1.26; 95% CI, 0.86–1.86; p=0.24; **Figure 2B**). In the *per protocol* population (see **Supplementary Information**), 38 patients treated with temsirolimus had survived ≥ to 1 year. At least 39 patients were needed to reach the targeted drug activity.

Safety

In the temsirolimus arm severe hematological toxicity was: neutropenia (G3: n=1, 1.9%) and lymphocytopenia (G3: n=9, 16.4%, G4: n=1, 1.8%). In the TMZ arm severe hematological toxicity was: leukopenia G3 (n=2, 3.8%), neutropenia G4 (n=2, 3.8%), lymphocytopenia (G3: n=14, 26.4%, G4: n=2, 3.8%) and thrombocytopenia (G3: n=1, 1.9%, G4: n=1, 1.9%). There was no other severe (G3/4) treatment-related AE with an incidence >5% in either arm.

Molecular correlations with outcome

Markers interrogated for their relevance of targeting the mTOR signaling pathway (22, 23) are visualized in the mTOR KEGG pathway (28) (**Supplementary Figure S3**). Phosphorylated $mTOR^{Ser2448}$ was associated with prolonged OS as evidenced by the significant interaction term between treatment and $p\text{-}mTOR^{Ser2448}$ ($p=0.047$, **Figure 3**). Tumors of 37.6% of the patients scored positive for $p\text{-}mTOR^{Ser2448}$. There was a non-significant trend for longer OS when $p\text{-}mTOR^{Ser2448}$ positive patients received temsirolimus as compared with controls (HR=0.62, 95% CI 0.26-1·47, $p=0.27$). When non-phosphorylated $mTOR^{Ser2448}$ patients received temsirolimus a non-significant decrease in survival was observed compared with controls (HR=1.77, 95% CI 0.95-3.29, $p=0.07$) (**Figure 3**). The median OS in the temsirolimus group was 17.8 months (CI, 14.1-28.0) for patients with $p\text{-}mTOR^{Ser2448}$ positive tumors and 13.1 months (CI, 9.7-15.1) in the negative subgroup ($p=0.007$, Figure 3A). In the RT/TMZ→TMZ control arm the median OS in the $p\text{-}mTOR^{Ser2448}$ positive group was 14.0 months (CI, 9.6-19.6) and 16.5 months (CI, 9.5-18.8) in the $p\text{-}mTOR^{Ser2448}$ negative subgroup ($p=0.999$). For $p\text{-}PRAS40^{Thr246}$, the interaction test with treatment was borderline non-significant ($p=0.07$). The impact of all other markers on survival is illustrated in a forest plot for all other markers in **Supplementary Figure S4**.

A multi dimensional analysis used the full range of the scores of the mTOR-associated markers integrated information for the identification of clinically relevant molecular subgroups and to gain further insights on pathway interactions (**Figure 4**). The two first axes obtained by PCA explained 57·8% of the total inertia. The first axis was mainly explained by $p\text{-}mTOR^{Ser2448}$ and $p\text{-}PRAS40^{Thr246}$. The $p\text{-}S6RP^{Ser235/236}$ mainly contributed to the construction of the second axis (**Figures 4E and F**). PTEN expression played a minor role in the structure of the score table (**Figure 4F**). Subgroups were determined by consensus clustering. We kept the cluster based on two groups (k=2) by default, as no strong indication for the optimal number of clusters was obtained and the sample size is limited (**Supplementary Figure S5**). Cluster 2, highly enriched for p-mTOR^{Ser2448}-positive cases, revealed a strong association with outcome in the temsirolimus treatment group and no difference in the TMZ/RT→TMZ group (**Figure 4**). Significant interaction was observed with treatment (p=0.009): in Cluster 2 the HR was 0.42 (95% CI 0.15-1.13, p=0.08) and in Cluster 1 HR=1.77 (95% CI 0.96-3.25, p=0.06).

In multivariable prognostic analyses of clinical and molecular factors (**Supplementary Table S1**), p-mTOR^{Ser2448} (HR=0.13, 95% CI 0.04-0.47, p=0.002), p-PRAS40^{Thr246} (HR=0.50, 95% CI 0.21-1.18, p=0.12), p-ERK$^{Thr202/Tyr204}$ (HR=2.81, 95% CI 0.97-8.09, p=0.06), but no clinical factor was associated with OS in the temsirolimus arm. The PEV was equal to 14.9% In the TMZ arm, there was a trend for decreased survival in p-AKT^{Ser473} positive patients (HR=3.21, 95% CI 0.89-11.56, p=0.07, PEV=4.5%). None of the models had a PEV larger than 20%.

DISCUSSION

This randomized, open label phase II trial investigating the mTOR inhibitor temsirolimus in combination with RT for patients with low probability of benefit from the TMZ-based radiochemotherapy failed to demonstrate the targeted outcome. Neither PFS nor OS demonstrated a signal of relevant activity in the total trial population (**Figure 2**). Safety and tolerability of temsirolimus in combination with standard RT were non-concerning and the trial is an example that temozolomide can be safely omitted in patients with *MGMT* unmethylated glioblastoma. The trial proposes $mTOR^{Ser2448}$ phosphorylation as a biomarker for benefit from mTOR inhibition. These results need further confirmation, and a trial to prospectively assess the relevance of this putative biomarker is underway (NCT Neuro Master Match, *EudraCT 2015-002752-27*).

The good outcome data in both arms of the trial prompted a comparison with the EORTC26981-22981/NCIC CE3 trial. The comparison with our pivotal TMZ/RT→TMZ vs RT trial (EORTC26981-22981/NCIC CE3) (29) was favourable in all aspects supporting the principal rational to design trials for patients with *MGMT* unmethylated glioblastoma and withhold TMZ in the experimental arm (**Supplementary Results**). Biases in favor of EORTC 26082 may have been patient selection, and the lower number of patients on steroids (30). Bevacizumab was administered in about 45% of the patients in both arms of EORTC 26082. The OS of the EORTC 26082 arms is comparable to the outcome in the control arms of trials with selection of *MGMT* unmethylated patients, with 13.4 months in the CORE trial (95% CI 12.2-14.3) with a bevacizumab use at recurrence of 22% (31) and 17.3 months (95%CI 14.8-20.4 months) in the GLARIUS trial with cross over to bevacizumab of 60% (32).

The EORTC 26082 trial aimed at not withholding TMZ from any patient with an equivocally methylated *MGMT* promoter by applying a *MGMT* cut-off with a safety margin. This prompted an adaption also in the GLARIUS trial (32) with similar design and therefore demarcates an evolution from the S039 trial with enzastaurin (33). Two randomized phase III trials in elderly patients with newly diagnosed glioblastoma further support a strictly predictive effect of the *MGMT* status for benefit from TMZ (34, 35). However, we cannot completely exclude a small baseline effect of TMZ despite the *MGMT* unmethylated state (11). Hence, withholding TMZ outside trials and elderly patients with unmethlylated *MGMT* promoter is not advocated by the present data. In the temsirolimus arm 59% (n=33) of the patients received TMZ after treatment discontinuation, and 26% of TMZ patients (n=14) were re-challenged with TMZ, not being aware of the recent data from the DIRECTOR trial that re-challenge with TMZ might be relevant only for patients with a methylated *MGMT* promoter (36).

The choice of temsirolimus for patients with unmethylated glioblastoma was based on preclinical data already highlighting that not every tumor responds to the treatment (37) as well as a response may be only transient because of the overt feedback resistance mechanisms (22, 38).

Molecular analyses of prespecified principal components of the EGFR-PI3-K/mTOR/AKT pathway were performed. EORTC 26082 provides first evidence that p-mTOR^{Ser2448} and – to a lesser extent - p-PRAS40^{Thr246} may serve as decisive biomarkers for the treatment of patients with newly diagnosed glioblastoma with an unmethylated *MGMT* promoter. Phosphorylation of mTOR^{Ser2448} has been shown to be targeted and blocked by rapamycin, a major metabolite of temsirolimus (39), while phosphorylated PRAS40^{Thr246} (substrate of AKT1) relieves inhibitory function on mTORC1 (40). The survival curves may even suggest that there is a detrimental effect of temsirolimus in p-mTOR^{Ser2448} negative tumors (**Figures 3 and 4**). Previous trials testing temsirolimus at recurrence had focused on the PTEN status with a PTEN deficiency as a prerequisite for response (22) or on other downstream mTOR targets, e.g. p-S6RP$^{Ser235/236}$, which was neither associated with outcome in biomarker analyses of patients with recurrent glioblastoma receiving temsirolimus (6, 38) nor in this study. It cannot be excluded that glioblastomas treated at recurrence may have changed mTOR pathway activity as compared to tumor specimen used for marker analyses obtained at the first resection (41). Also, "paradoxical" activation of AKT by elimination of negative feedback downregulating survival signaling has been postulated as potential resistance mechanism to mTOR inhibition in previous trials, based on the analyzes of paired tumor specimen taken before and after treatment (22, 38). Interestingly, trials in other diseases did not provide predictive biomarkers (12, 13).

The limitations of EORTC 26082 are the relatively small sample size of this non-comparative phase II trial. For the biomarker analyses using IHC only a limited number of tumor tissue samples from the ITT cohort were available. The findings should be validated by evaluation of previous trials in particular in those treating newly diagnosed glioblastoma patients (42) and the randomized phase II study RTOG-0913. Ongoing trials using mTOR inhibitors may need to take into account a potentially detrimental effect in patients with an unphosphorylated $mTOR^{Ser2448}$. Given the ongoing efforts of biomarker-driven basket trials for patients with newly diagnosed glioblastoma, the concept of mTOR inhibition using the marker predictive in this study, $p\text{-}mTOR^{Ser2448}$ is incorporated into the design of a future study.

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This report has been presented in part as abstract 2003 at ASCO 2014 by W. Wick.

CONTRIBUTORS

The concept of the trial was developed by W.W. in collaboration with the T.G., G.P., M.E.H., R.S. and the EORTC Brain Tumor Group. The concept of the biomarker analyses was developed by M.E.H. in collaboration with T.G, P.B. and W.W.

Study material: W.W., M.P., M.J.v.d.B., M.J.B.T., A.A., M.W., P.R., M.C., J.-S. F., M.W., R.S., D.R., C.M., S.V., A.W., Ki.H., Kr.H., G.P. recruited patients to the study, were involved in data collection and provided administrative support.

The biomarker data were generated and evaluated by P.B., M.-F.H, B.L. and M.E.H. Reference pathology was performed by B.L.

The statistical analyses were performed by T.G. and P.B.

The article was written by W.W. and M.E.H. with support from all co-authors.

All authors reviewed and approved the manuscript.

FIGURE LEGENDS

Figure 1. Supplemented CONSORT diagram of patient disposition.

Figure 2. Principal efficacy outcomes per treatment.

Figure 3. Overall survival according to phosphorylated mTOR stratified by treatment. (**A**) Kaplan-Meier curves shown represent patients separated by the phosphorylation status of mTOR^{Ser2448} (Pos, positive; Neg, negative) stratified for the two treatment arms CCI-779/RT and TMZ/RT$\rightarrow$TMZ (TMZ). The interaction test was significant p=0.047). (**B**) Representative glioblastoma samples negative or positive for p-mTOR^{Ser2448} expression.

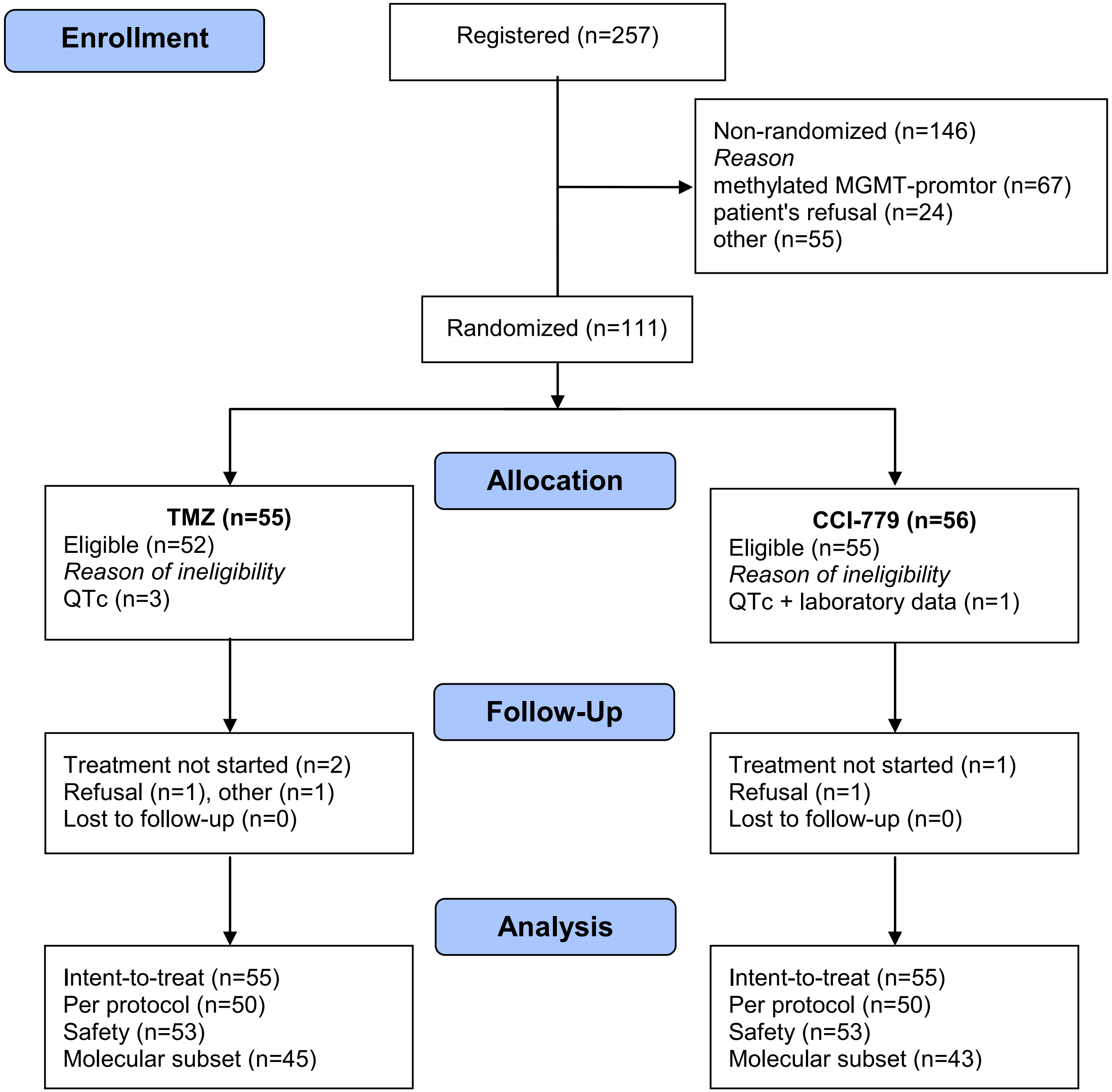
Figure 4. Multidimensional analysis of m-TOR associated markers. The associations among markers in the mTOR pathway are illustrated by "The network representation" based on Spearman correlations between scores (**A**). (**B)** The glioblastoma subgroups based on mTOR pathway markers are visualized in a heatmap of the score table obtained after reconstruction using Non-linear Iterative Partial Least Squares (NIPALS). The rows were ordered by the first axis of the PCA. The columns are ordered by the consensus classification (k=2; clusters 1, blue; cluster 2, red) and are annotated for absence or presence of mutated IDH1^{R132H} (positive, red; negative, grey; unknown; white), and the *EGFR* status (amplified dark green, non-amplified, green; unknown, white). The association between OS and consensus classification for two groups (k=2) (cluster 1, blue; cluster 2, red) is illustrated by Kaplan-Meier representation for patients randomized to CCI-779 **(C)** and TMZ **(D)**. The p-value is given for each KM. The patients **(E)** and m-TOR-associated markers **(F)** were projected onto the two first components of the principal component analysis (PCA). Inertia ellipses and stars visualize the separation of the patients into the two groups obtained from consensus clustering (cluster 1, blue; cluster 2, red) **(E)**.

Table Baseline characteristics

	TMZ (N=55)	Temsirolimus (N=56)	Total (N=111)
	N (%)	**N (%)**	**N (%)**
Age			
median	57.7	54.9	55.7
range	24.4 - 76.0	28.2 - 74.7	24.4 - 76.0
Sex			
male	36 (65.5)	35 (62.5)	71 (64.0)
female	19 (34.5)	21 (37.5)	40 (36.0)
Extent of resection			
open biopsy	1 (1.8)	3 (5.4)	4 (3.6)
resection	54 (98.2)	53 (94.6)	107 (96.4)
Corticosteroids			
no	37 (67.3)	40 (71.4)	77 (69.4)
yes	18 (32.7)	16 (28.6)	33 (29.7)
WHO PS (0-4)			
0	40 (72.7)	32 (57.1)	72 (64.9)
1	14 (25.5)	20 (35.7)	34 (30.6)
2	1 (1.8)	4 (7.1)	5 (4.5)

Abbreviations: TMZ, temozolomide; WHO PS, World Health Organization Performance Status

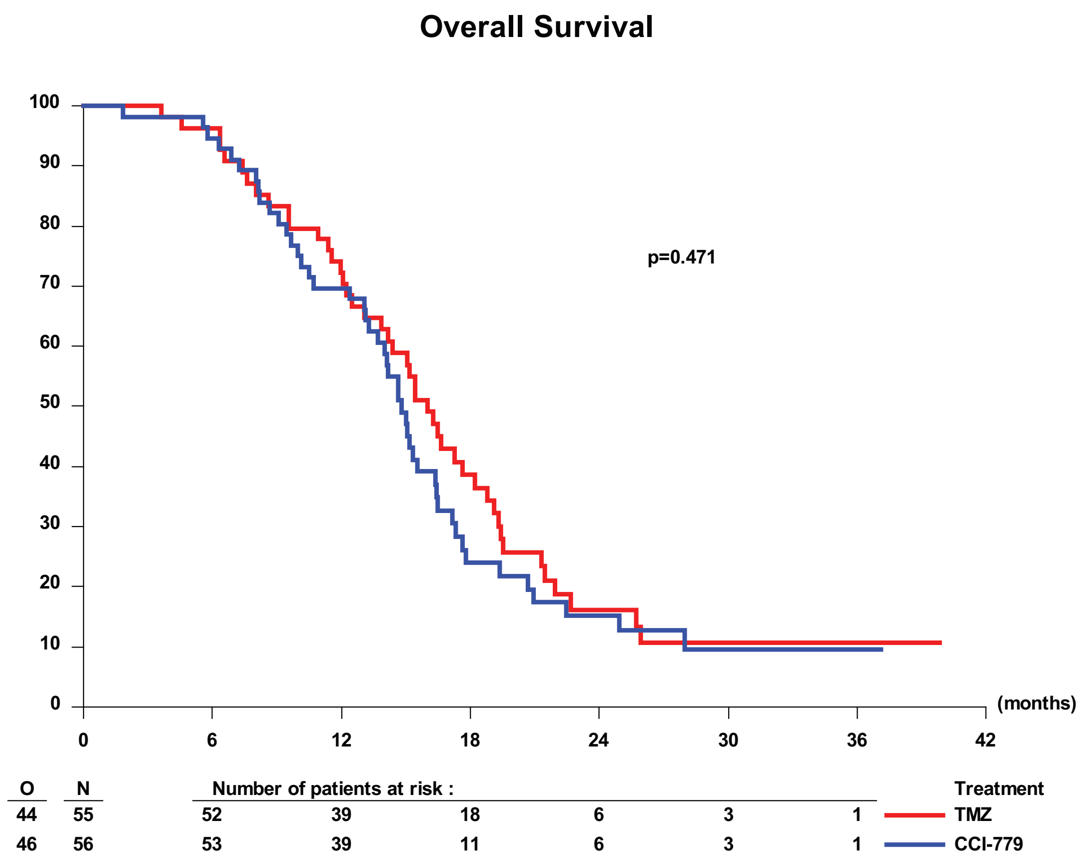
Figure 1



Enrollment
Registered (n=257)
Non-randomized (n=146)
Reason
methylated MGMT-promtor (n=67)
patient's refusal (n=24)
other (n=55)
Randomized (n=111)
Allocation
TMZ (n=55)
Eligible (n=52)
Reason of ineligibility
QTc (n=3)
CCI-779 (n=56)
Eligible (n=55)
Reason of ineligibility
QTc + laboratory data (n=1)
Follow-Up
Treatment not started (n=2)
Refusal (n=1), other (n=1)
Lost to follow-up (n=0)
Treatment not started (n=1)
Refusal (n=1)
Lost to follow-up (n=0)
Analysis
Intent-to-treat (n=55)
Per protocol (n=50)
Safety (n=53)
Molecular subset (n=45)
Intent-to-treat (n=55)
Per protocol (n=50)
Safety (n=53)
Molecular subset (n=43)

Figure 2A

A

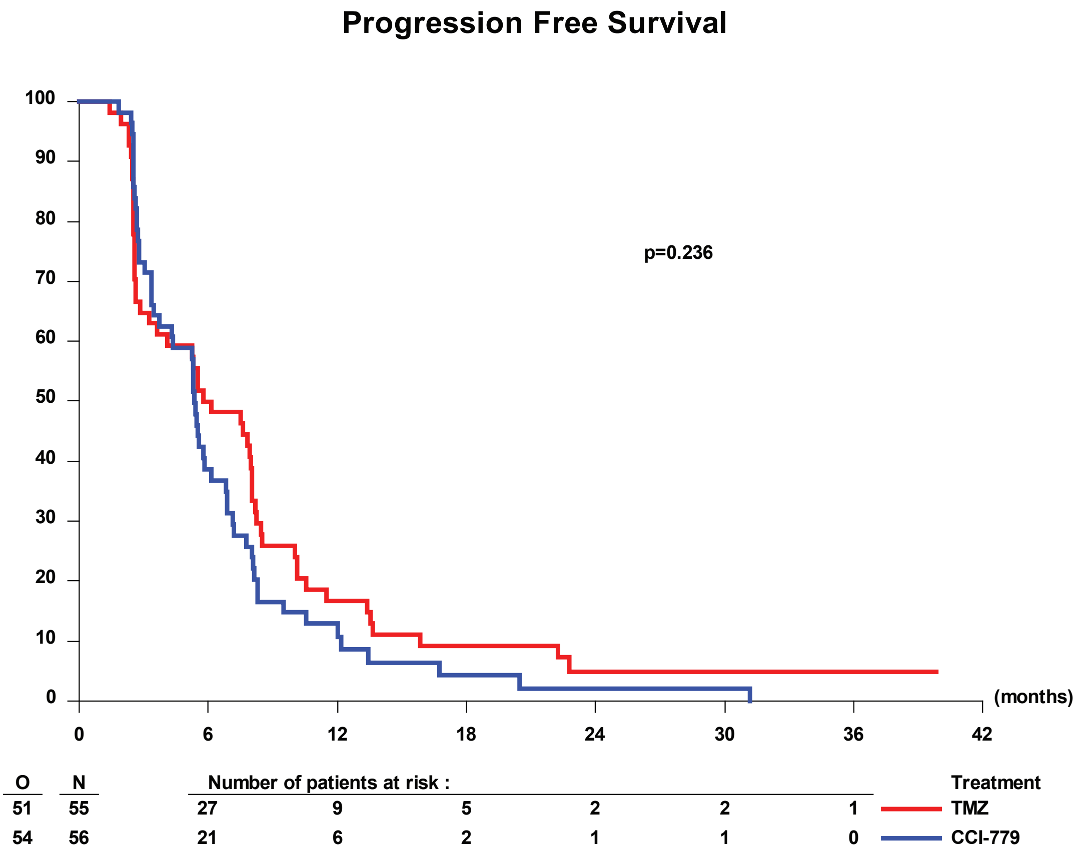


Survival Time

Treatment	Patients (N)	Observed Events (O)	Hazard Ratio (95% CI)	P-Value (Log-Rank)	Median (95% CI) (Months)	% at 1 Year (95% CI)
TMZ	55	44	1.00	0.4708	16.03 (13.83, 18.20)	72.22 (58.22, 82.22)
CCI-779	56	46	1.16 (0.77, 1.76)		14.78 (13.27, 16.39)	69.64 (55.79, 79.91)

Figure 2B

B

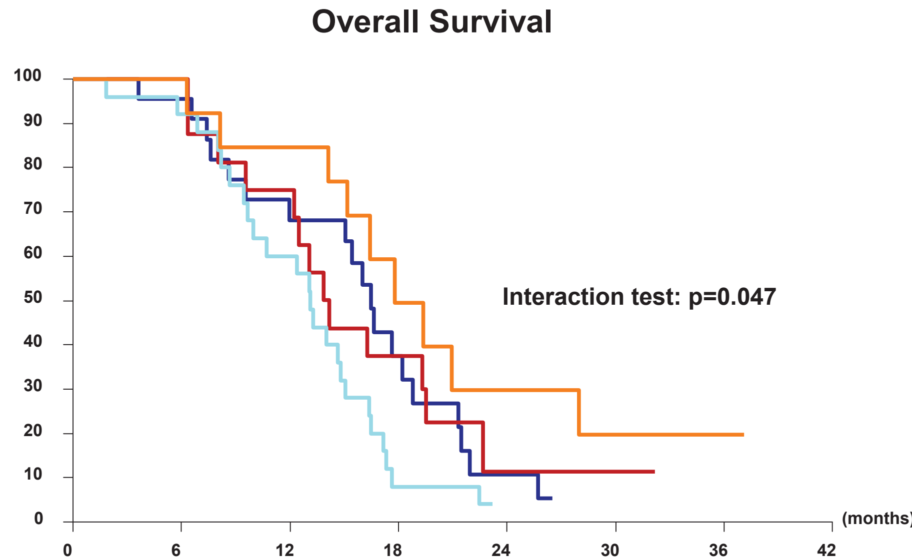


Survival Time

Treatment	Patients (N)	Observed Events (O)	Hazard Ratio (95% CI)	P-Value (Log-Rank)	Median (95% CI) (Months)	% at 0.5 Year(s) (95% CI)
TMZ	55	51	1.00	0.2358	5.95 (3.25, 8.02)	50.00 (36.12, 62.39)
CCI-779	56	54	1.26 (0.86, 1.86)		5.36 (3.71, 6.14)	38.67 (25.96, 51.20)

Figure 3

A



O	N	Number of patients at risk :						Trt/p-mTOR
19	23	21	15	7	2	0	0	TMZ/Neg
13	16	16	12	5	1	1	0	TMZ/Pos
24	25	23	15	2	0	0	0	CCI-779/Neg
9	13	13	11	5	3	2	1	CCI-779/Pos

Survival Time			Non-parametric		Cox model	
treatment/p-mtor	Patients (N)	Observed Events (O)	Median (95% CI) (Months)	% at 2 Year(s) (95% CI)	Hazard Ratio (95% CI)	P-Value (Score test)
TMZ/p-mTOR Neg	23	19	16.46 (9.53, 18.79)	10.7 (1.8, 28.7)	1.00	0.042 (df=3)
TMZ/p-mTOR Pos	16	13	14.01 (9.56, 19.55)	11.3 (0.9, 36.4)	0.99 (0.49, 2.01)	
CCI-779/p-mTOR Neg	25	24	13.11 (9.66, 15.08)	4.0 (0.3, 17.0)	1.71 (0.93, 3.14)	
CCI-779/p-mTOR Pos	13	9	17.77 (14.09, 27.99)	29.7 (7.4, 56.8)	0.59 (0.26, 1.32)	
					Log-rank test:	p-value=0.041

B

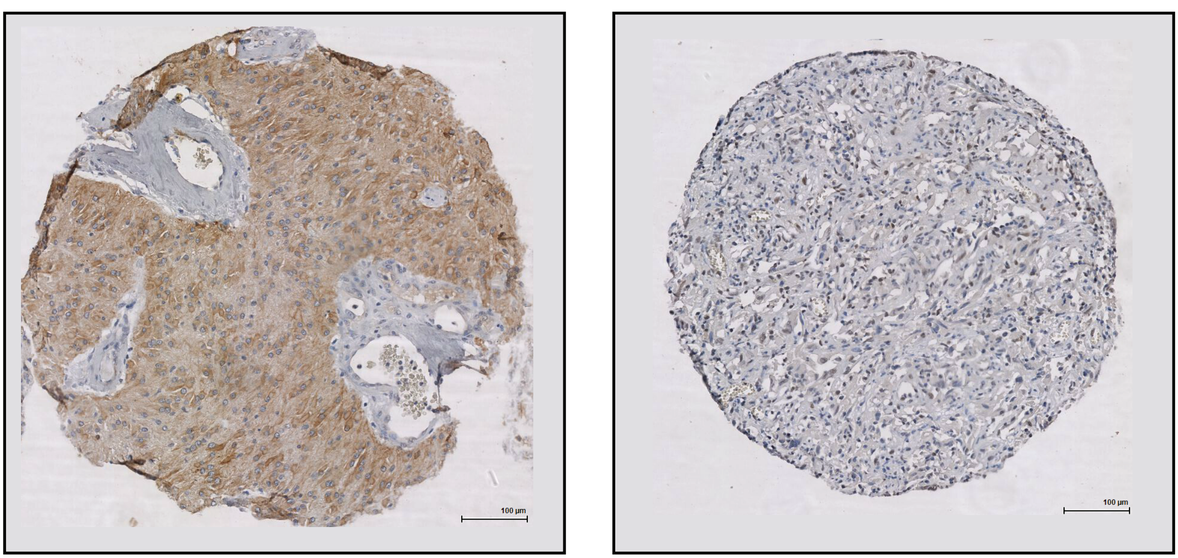
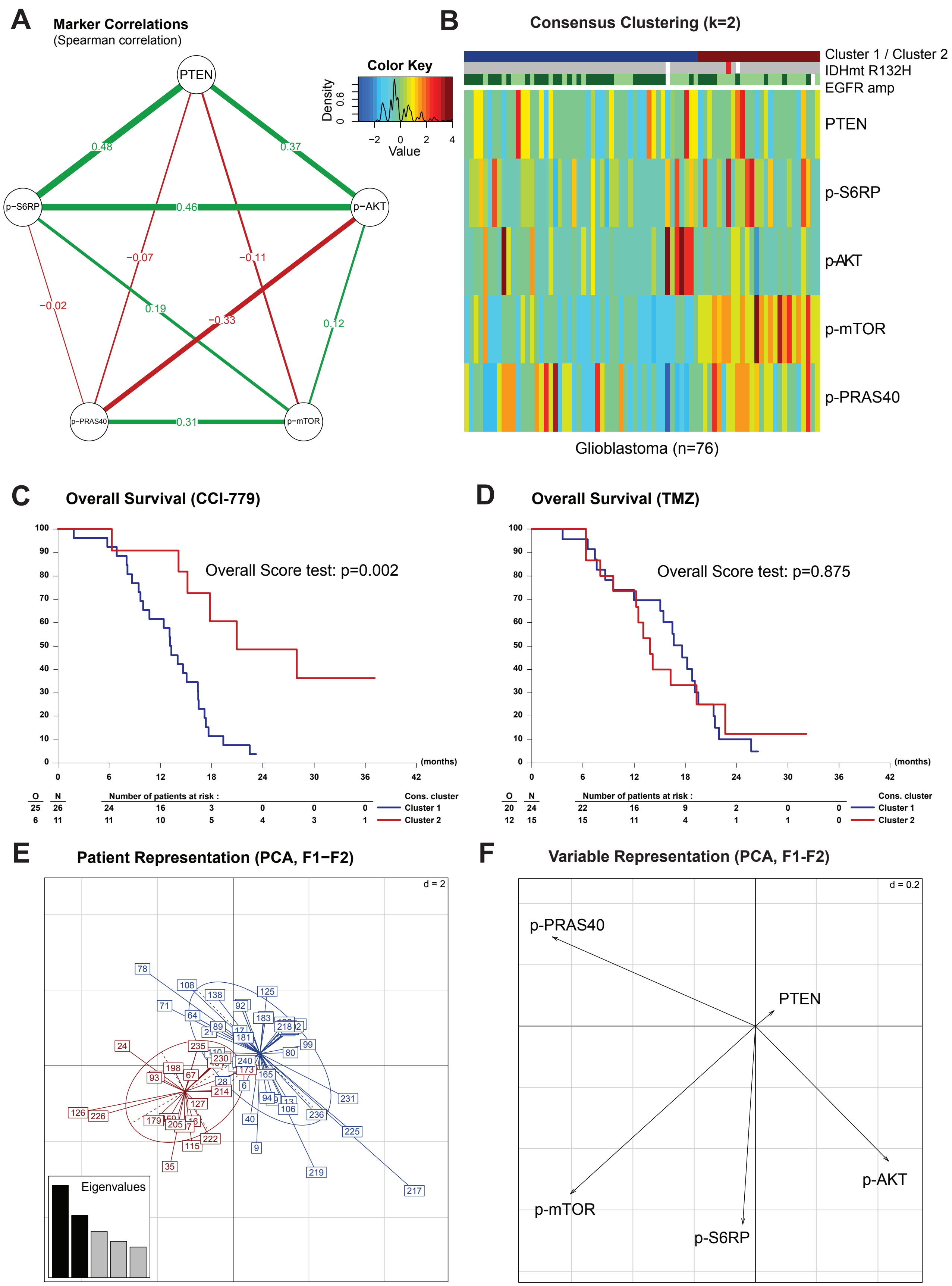


Figure 4


A
Marker Correlations
(Spearman correlation)
PTEN
p−S6RP
p−AKT
p−PRAS40
p−mTOR
0.48
0.37
0.46
−0.07
−0.11
−0.02
0.19
−0.33
0.12
0.31
Color Key
Density
0 0.6
−2 0 2 4
Value
B
Consensus Clustering (k=2)
Cluster 1 / Cluster 2
IDHmt R132H
EGFR amp
PTEN
p-S6RP
p-AKT
p-mTOR
p-PRAS40
Glioblastoma (n=76)
C
Overall Survival (CCI-779)
Overall Score test: p=0.002
100 90 80 70 60 50 40 30 20 10 0
0 6 12 18 24 30 36 42 (months)
O N Number of patients at risk : Cons. cluster
25 26 24 16 3 0 0 0 Cluster 1
6 11 11 10 5 4 3 1 Cluster 2
D
Overall Survival (TMZ)
Overall Score test: p=0.875
100 90 80 70 60 50 40 30 20 10 0
0 6 12 18 24 30 36 42 (months)
O N Number of patients at risk : Cons. cluster
20 24 22 16 9 2 0 0 Cluster 1
12 15 15 11 4 1 1 0 Cluster 2
E
Patient Representation (PCA, F1−F2)
d = 2
Eigenvalues
F
Variable Representation (PCA, F1-F2)
d = 0.2
p-PRAS40
PTEN
p-AKT
p-mTOR
p-S6RP

SUPPLEMENTARY INFORMATION TO

Phase II study of radiotherapy and temsirolimus versus radiochemotherapy with temozolomide in patients with newly diagnosed glioblastoma without MGMT promoter hypermethylation (EORTC 26082)

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Supplementary Patients and Methods

MGMT Testing

In brief, DNA was isolated from formalin-fixed, paraffin-embedded tumour samples using macro-dissected sections; DNA was modified with sodium bisulfite and subjected to quantitative methylation-specific PCR using β-actin as a reference gene (*ACTB*).[1]

Key eligibility criteria

Patients aged ≥18 years with newly diagnosed, histologically confirmed supratentorial glioblastoma (WHO Grade IV), centrally determined unmethylated *MGMT* status, and with an Eastern Cooperative Oncology Group performance status (ECOG PS) of 0 or 1 were eligible. Additional inclusion criteria were: written informed consent; available tumour tissue from surgery or open biopsy (stereotactic biopsy was not allowed) for *MGMT* promoter methylation status analysis and central pathology review; gadolinium-enhanced (Gd) MRI performed within 48 hours post surgery, or alternatively, Gd-MRI performed before randomisation; stable or decreasing steroid doses for ≥5 days prior to randomisation; and adequate haematological, renal, and liver function. Key exclusion criteria were prior chemotherapy within the last 5 years, prior RT of the head, treatment with other investigational agents 30 days before first dose of temsirolimus, and prior systemic antiangiogenic therapy; history of coagulation disorder associated with bleeding or recurrent thromboembolic events; presence of QTc prolongation >450/470 msec (males/females); placement of Gliadel® wafers at surgery; history of malignancy within the last 5 years (except curatively treated cervical carcinoma *in situ* or basal cell carcinoma of the skin); clinically manifest cardiovascular insufficiency (NYHA III, IV) or myocardial infarction during the past 6 months, and uncontrolled arterial hypertension.

Patients randomized into the trial constituted the intention-to-treat population (n=55 control arm; n=56 temsirolimus arm).

Patients having received at least one trial-specific treatment and fulfilling the basic eligibility criteria constituted the per-protocol-population (n=50 control arm; n=54 temsirolimus arm). Reasons for exclusion from the per-protocol population were no treatment (n=3), and QTc or laboratory value deviations in the baseline criteria that should have prevented inclusion into the trial (n=5). One patient fulfilled two reasons not to be counted for the per-protocol-population.

The safety population excluded only patients that never received any study-specific therapy (n=3) and resulted in 53 patients in the control arm and 55 patients in the temsirolimus arm.

Treatment

Each treatment with temsirolimus was to be preceded by supportive medication with a histamine H2-receptor antagonist. RT consisted of 3D conformal radiotherapy and was given at 2 Gy per fraction, 5 days/week, for up to 6 weeks and to a total dose of 60 Gy; TMZ 75 mg/m^2 was administered orally 7 days/week throughout RT, thereafter, starting 4 weeks after the end of RT (week 11) TMZ 150–200 mg/m^2 was administered for 5 consecutive days every 4 weeks for 6 cycles. Temsirolimus was to be continued until disease progression (PD) or unacceptable toxicity. Crossover from the control to the temsirolimus arm was not allowed. Temsirolimus was administered as 30-minute infusion starting 2 hours before RT; TMZ was given orally at least 1 hour before RT.

Biomarker substudy

Immunohistochemistry was performed basically as recommended by the manufacturers using a heat antigen retrieval procedure (citrate buffer) using the following antibodies and respective dilutions: Phospho-S6 Ribosomal Protein (Ser235/236; 1:400; #2211; Cell Signaling Technology [CST]), Phospho-AKT (Ser473; 1:50; D9E, #4060, CST), Phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204; 1:600; #4370, CST), Phospho-mTOR (Ser2448; 1:100; 49F9, #2976, CST), Phospho-PRAS40 (Thr246; 1:25, #2997, CST), PTEN (1:50, 138G6, #9559, CST), EGFR (1:50; DAKO M7239), and $IDH1^{R132H}$ (1:25; clone H14; Dianova, Hamburg, Germany). The scoring was performed blinded to outcome data. Percentage of tumor cells with any level of positive staining were scored as follows: p-S6RP, p-AKT, p-ERK: invalid, absent or inappropriate tissue, 0 = no positive cells, 1 = 1 - 10%, 2 = 11% - 30%, 3 = 31% - 50%, 4 = 51% - 80%, and 5 = 81% - 100%; p-mTOR, p-PRAS40, PTEN: invalid, absent or inappropriate tissue, 0 = no positive cells, 1 = 1% - 10%, 2 = 11% - 50%, 3 = 51 – 80%, 4 = 81% - 90%, 5 = 91% - 100%. For PTEN presence of vascular staining was used as internal control. For marker analyses the scores were dichotomized into negative (scores 0, 1, corresponding to 0 to10%) versus positive (scores 2 to 5, >10%). EGFR was evaluated according to the Hirsch score, and $IDH1^{R132H}$ was considered positive when cytoplasmic expression was detected.[3,4] FISH for EGFR amplification was performed using Vysis LSI EGFR SpectrumOrange /CEP7 SpectrumGreen Probes (Abbott Molecular, Des Plaines, IL, USA). Tumors with a ratio >2 of the Average EGFR/Average CEP7 were classified as amplified.[3]

Role of the funding source

This study was funded by an academic grant from Pfizer, Berlin, Germany. Study design, data analysis, and data interpretation were performed collaboratively by the principal investigator, the study team and EORTC. The Steering Committee of the EORTC Brain Tumor Group oversaw the study. The principal investigator (WW) had full access to and reviewed all data, and had final responsibility for the decision to submit for publication. Data collection was performed by the investigators with monitoring performed by the EORTC; the database remained blinded to primary outcome variables for all parties including molecular marker analyses until final analysis.

Statistical considerations

For multivariable prognostic analysis, Cox models including the three clinical stratification factors, the P-markers and EGFR amplification were computed in each treatment arm. Forward stepwise method was used to select the most significant factors. Because of limited sample size, this screening was done at a relaxed 15% significance level. Results are interpreted taking this limitation into account. To assess model goodness of fit, the Schemper Percentage of Explained Survival Variation (PEV) was calculated. A PEV of at least 20% was considered a minimum requirement for sufficiently precise predictions. Primary OS12 analysis was performed in the *per protocol* population (i.e. eligible patients who started randomized treatment). All outcome analyses were performed on the intention-to-treat (ITT) population. For multivariable analyses, only samples with all molecular markers assessed were used. Safety was assessed on patients who started randomized treatment.

SAS version 9.4 (SAS Institute Inc., Cary, NC, United States of America [USA]) was used for all analyses. The percentage of explained survival variation (PEV) was computed using the SAS macro RELIMPCR.

Supplementary Results

The median OS of 14·8 and 16·0 months observed in the temsirolimus and the TMZ arms, respectively, prompted us to investigate, how the OS in EORTC 26082 compared to the *MGMT* unmethylated EORTC 26981 subpopulation. This is relevant as one of the *caveats* of trials restricted to patients with *MGMT* unmethlylated glioblastoma is potential undertreatment by leaving out TMZ in the experimental group. Consistent with reports on enzastaurin[28] or bevacizumab[29], this was not the case in EORTC 26082. Looking at comparable trial populations (**Supplementary Table 2**), PFS showed no difference for any comparison between arms of EORTC 26082 and 26981. OS shows a significant improvement in the comparison of either arm of EORTC 26082 with the control arm of EORTC 26981 with a HR= 0·45 (0·30-0·67, p<0·0001) for RT/TMZ→TMZ and HR= 0·53 (0·36-0·79, p=0·0015) for RT/temsirolimus. However, there was only a trend in the comparison between either arm of EORTC 26082 and the RT/TMZ→TMZ arm of the EORTC 26981 trial (data not shown).

Supplementary References

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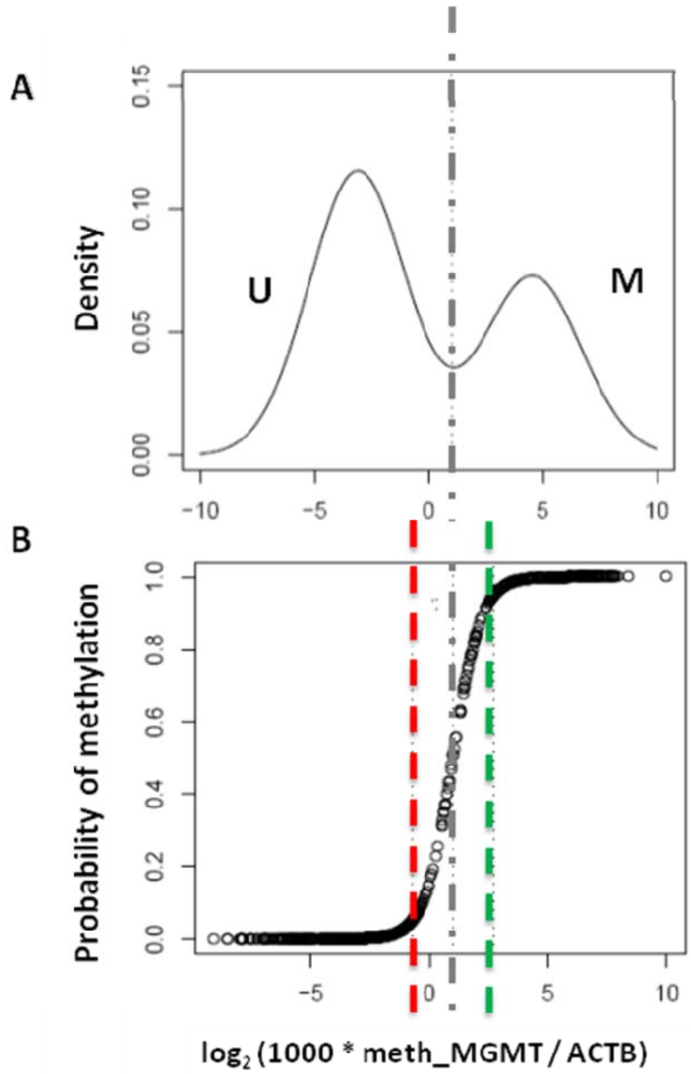
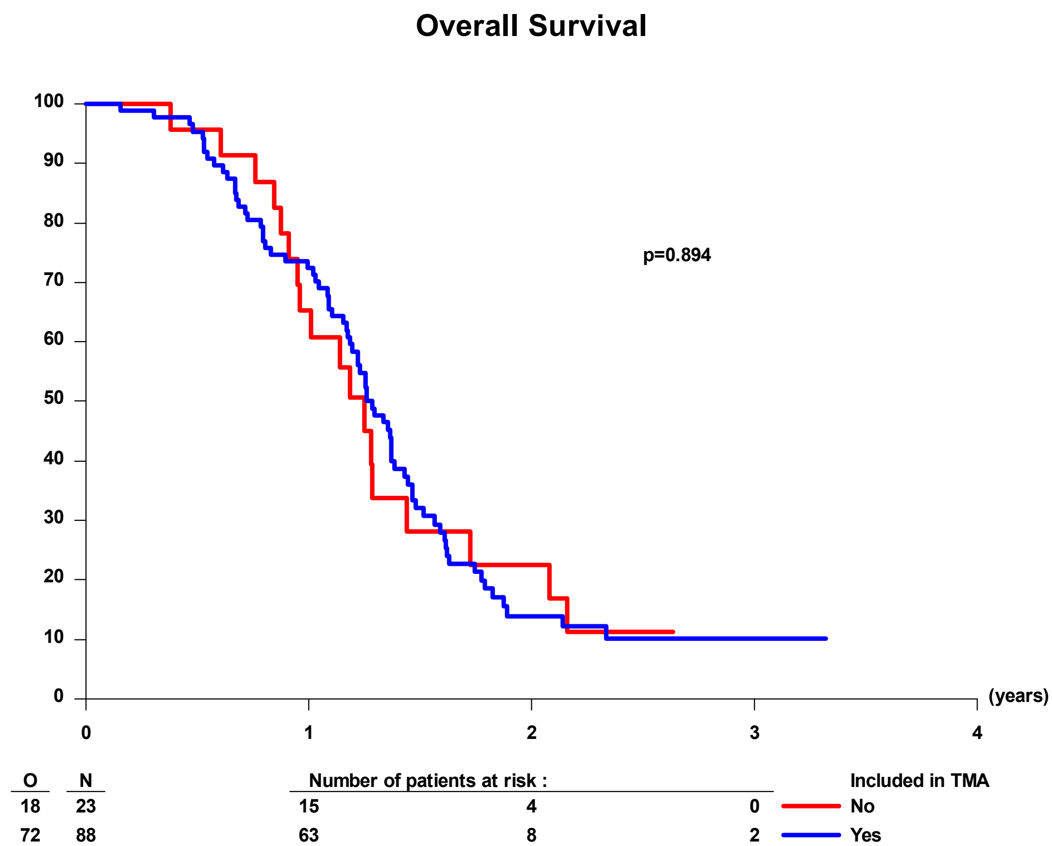
Supplementary Figures

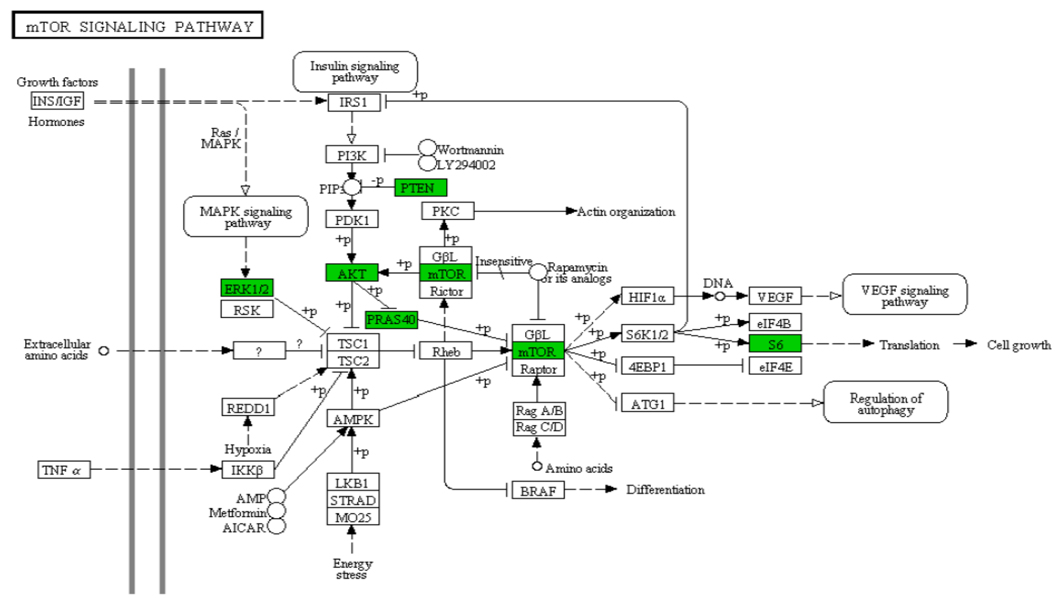


Supplementary Figure S1. Definition of MGMT cut-off with a safety margin. Density plot **(A)**, and posterior probability plot **(B)** for the classification into *MGMT* promoter methylated (M) or unmethylated (U) tumors obtained by fitting a mixture model to the average log_2(1000 * meth_MGMT/ACTB) for 602 glioblastoma samples. A gray **dashed line** represents the optimal cut-off according to the selected model (log_2 ratio= 1) corresponds to a **ratio value of 2**. The thresholds for lower bound of the 95% posterior probability for class U, indicated by a red dashed line (log_2 ratio= -0.75) corresponds to a **ratio value of 0.6**, which has been defined as the **cut-off with a safety margin**. The upper bound 95% posterior probability for class M, is indicated by a green dashed line (log_2 ratio= 2.72) corresponds to a **ratio value**

of 6.59. The region between is often referred to as “gray zone”, since it is associated with higher uncertainty.[1]

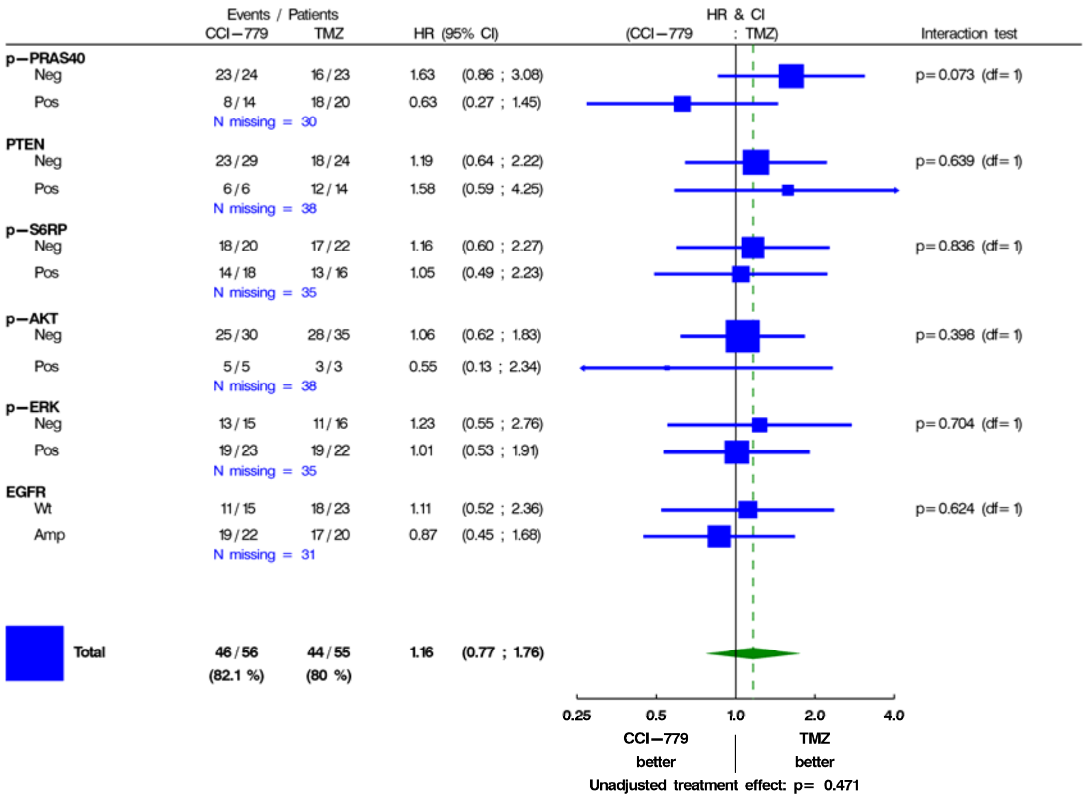


Supplementary Figure S2. Comparison of Overall Survival in patients with *vs* without markers assessments.

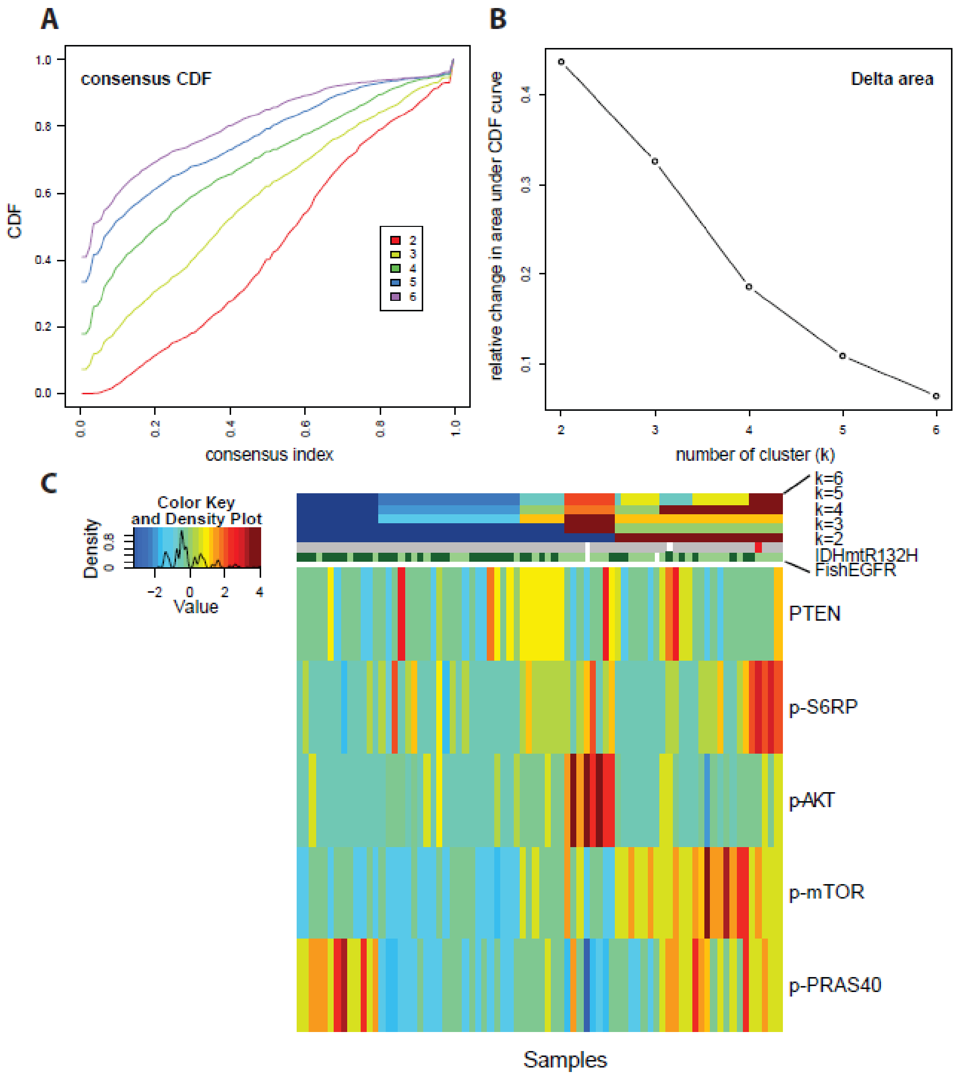


Data on Kegg graph rendered by R package pathview

Supplementary Figure S3. Visualization of markers analyzed in the mTOR signaling pathway from KEGG. The markers are identified by green boxes and the representation was obtained using the R package pathview from the Bioconductor project.[5] We determined phosphorylation of mTOR at serine 2448 (p-mTOR^{Ser2448}) which has been shown to be targeted and blocked by rapamycin, a major metabolite of temsirolimus.[6] Furthermore, phosphorylated S6 ribosomal protein (S6RP$^{Ser235/236}$), a direct target of the mTOR effector S6 kinase 1, phosphorylation of AKT^{Ser473}, expression of PTEN, and phosphorylation of AKT1 Substrate 1 (Proline-Rich) at Thr246 (p-PRAS40^{Thr246}) were assessed. PRAS40^{Thr246} is phosphorylated by AKT1. The latter relieves inhibitory function on mTORC1.[7] In addition the *EGFR* amplification status (not indicated) and phosphorylation of ERK1/2$^{Thr202/Tyr204}$ that have been postulated as potential markers for resistance to inhibition of the PI3K/AKT/mTOR pathway were determined.[8]



Supplementary Figure S4. Forest plot molecular markers



Supplementary Figure S5.

Complete Graphical summary of consensus cluster analysis based on the matrix obtained by the reconstitution of the data in using Non-linear Iterative Partial Least Squares (NIPALS) algorithm. The two first graphics (A et B) were used to determine the optimal cluster number. **(A)** displays the cumulative distribution functions (CDF) of the consensus for each number of clusters (k=2,…, 6). The Delta Area plot **(B)** represents the relative change in the area under the CDF curve comparing k and k-1. Because no strong rupture was detected in this graphic, we kept the cluster based on two groups (k=2) by default. **(C)** Display of the heatmap of the score table obtained after NIPALS reconstruction. The rows were ordered by the first axis of the PCA. The consensus classifications, and status of expression of the mutant IDH1^{R132H} and amplification of *EGFR* were added as supplementary information. Abbreviations: mTOR phosphorylated at serine 2448 (p-mTOR^{Ser2448}); S6 ribosomal protein phosphorylated at serine 235 and 236, p-S6RP$^{Ser235/236}$; AKT phosphorylated at serine 473, p-AKT^{Ser473}; phosphatase and tensin homologue, PTEN; of AKT1 Substrate 1 (Proline-Rich) phosphorylated at threonin 246 (p-PRAS40^{Thr246})

Supplementary Table S1

Patient's characteristics of biomarker cohort			
	Included on TMA		
Biomarker cohort, (No/Yes)	No (N=23)	Yes (N=88)	Total (N=111)
	N (%)	N (%)	N (%)
Age			
Median	58.3	55.4	55.7
Range	24.4 - 73.6	27.4 - 76.0	24.4 - 76.0
Age (class)			
<50yrs	5 (21.7)	24 (27.3)	29 (26.1)
>=50yrs	18 (78.3)	64 (72.7)	82 (73.9)
Sex			
male	16 (69.6)	55 (62.5)	71 (64.0)
female	7 (30.4)	33 (37.5)	40 (36.0)
Last method			
open brain biopsy	0 (0.0)	4 (4.5)	4 (3.6)
resection	23 (100.0)	84 (95.5)	107 (96.4)
Patient taking anti-epileptic drug			
no	9 (39.1)	29 (33.0)	38 (34.2)
yes, non-EIAED only	12 (52.2)	56 (63.6)	68 (61.3)
yes, EIAED switched	2 (8.7)	3 (3.4)	5 (4.5)
Currently on corticosteroids			
no	16 (69.6)	61 (69.3)	77 (69.4)
yes, stable/decreasing dose	7 (30.4)	26 (29.5)	33 (29.7)
yes, increasing dose	0 (0.0)	1 (1.1)	1 (0.9)
WHO performance status (0-4)			
0	17 (73.9)	55 (62.5)	72 (64.9)
1	4 (17.4)	30 (34.1)	34 (30.6)
2	2 (8.7)	3 (3.4)	5 (4.5)

Supplementary Table S2

Baseline characteristics at randomization					
	EORTC 26981 (*MGMT* unmethylated only)		EORTC 26082		
	RT (N=58)	TMZ (N=65)	TMZ** (N=55)	CCI-779** (N=56)	Total (N=111)
	N (%)	N (%)	N (%)	N (%)	N (%)
Age					
Median	54.5	53.0	57.7	54.9	55.7
Range	30.0 - 69.0	22.0 - 70.0	24.4 - 76.0	28.2 - 74.7	24.4 - 76.0
N obs	58	65	55	56	111
Sex					
male	37 (63.8)	38 (58.5)	36 (65.5)	35 (62.5)	71 (64.0)
female	21 (36.2)	27 (41.5)	19 (34.5)	21 (37.5)	40 (36.0)
Extent of resection					
open brain biopsy	2 (3.4)	3 (4.6)	1 (1.8)	3 (5.4)	4 (3.6)
resection	56(96.6)	62(95.4)	54 (98.2)	53 (94.6)	107 (96.4)
currently on corticosteroids					
no	17 (29.3)	20 (30.8)	37 (67.3)	40 (71.4)	77 (69.4)
yes	41 (70.7)	45 (69.2)	18 (32.7)	16 (28.6)	33 (29.7)
WHO performance status (0-4)					
0	17 (29.3)	28 (43.1)	40 (72.7)	32 (57.1)	72 (64.9)
1	35 (60.3)	33 (50.8)	14 (25.5)	20 (35.7)	34 (30.6)
2	6 (10.3)	4 (6.2)	1 (1.8)	4 (7.1)	5 (4.5)

** there is an imbalance between arms for WHO PS.
Stratification by WHO PS (0,1 vs 2) did not work properly. WHO PS 2 accounts for less than 5%.

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