Clinical Cancer Research

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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Abstract

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

Experimental Design: 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 noncomparative reference arm of 54 patients evaluated the assumptions on OS12 in a standard-treated cohort of patients. Prespecified *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% confidence interval (CI), 58.2–82.2] in the temozolomide arm and 69.6% (95% CI, 55.8–79.9) in the temsirolimus arm [hazard ratio (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. *Clin Cancer Res; 22(19); 4797–806. ©2016 AACR.*

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Translational 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 methylguanine-DNA-methyltransferase (MGMT)* promoter unmethylated glioblastoma. Temozolomide could be omitted without detriment in the experimental arm. Efficacy of radiotherapy plus temsirolimus failed to reach the prespecified number of patients alive at 12 months. Prespecified 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.

Introduction

The serine/threonine kinase, mTOR serves as a hub integrating multiple intra- and extracellular cues in cancer cells (1). mTOR is involved in the formation of two multiprotein 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 tumor models (2, 3).

Activation of the PI3K/AKT/mTOR pathway has been associated with reduced survival of glioma patients (4). This signaling pathway has been subjected to a number of single- or multitargeted therapies, in trials for recurrent high grade glioma or GBM, including the mTOR inhibitor rapamycin or its derivatives, the "rapalogs" everolimus (RAD001), deforolimus (AP23573), and temsirolimus (CCI-779), all with negative results (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 (RT; ref. 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 in preclinical models demonstrate that temsirolimus could complement the genotoxic activity of radiotherapy (RT) in the treatment of newly diagnosed glioblastoma. However, combination of temozolomide and temsirolimus plus radiotherapy 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 temozolomide could be safely omitted. To this end patients with tumors with an unmethylated *O6 methylguanine-DNA-methyltransferase (MGMT)* gene promoter were selected for the trial, as they derive little if any benefit from the addition of temozolomide (15). Another aim was to identify biologic factors, that is 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 using quantitative methylation-specific polymerase chain reaction of DNA isolated from macrodissected 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*) × 1,000. This corresponds to the lower bound of the 95% confidence interval (CI) established in a cohort of 602 glioblastoma samples screened in the CENTRIC trial where the cutoff corresponding to the established nadir was at a ratio of 2 that separates methylated from unmethylated (19), as visualized in Supplementary Fig. S1. A minimum of 1,250 copies of *ACTB* were required for a valid result, unless the copy number for methylated *MGMT* was 10 or more, which was scored as *MGMT* methylated.

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

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Randomization and masking. Randomization 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 randomization; PFS was defined as duration from randomization until first observation of PD or death from any cause or censored at last disease assessment without progression or start of second anticancer therapy; OS was defined as time from randomization 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 to 2 months. If progression was confirmed, the date of first observation of tumor progress was used for the analyses.

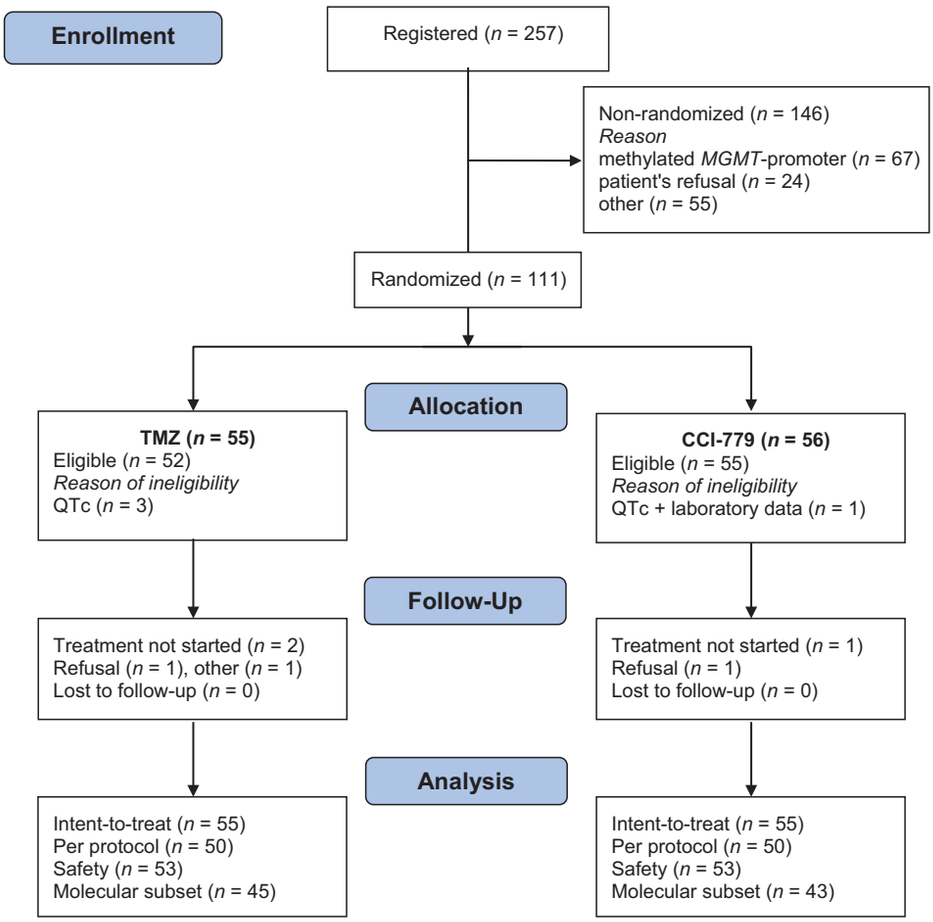


Figure 1.
Supplemented CONSORT diagram of patient disposition.

Adverse events (AE) 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% (P0), the therapeutic activity of temsirolimus (CCI-779) was too low (11). Although a OS12 greater or equal to 80% (P1) 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 to assess the consistency with P0.

Biomarker substudy

Tissue microarray, IHC, and FISH EGFR. Tissue micro arrays (TMA) were constructed using recipient paraffin blocks with an agarose matrix (21). Immunohistochemical analyses and FISH were performed in duplicate on sections from two replicate TMAs basically as recommended by the manufacturers (see Supplementary Methods, for antibody description, conditions, and dilutions; FISH probes). Markers for *post hoc* analyzes of the mTOR pathway were prespecified 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}; refs. 22 and 23). Scoring and definition of dichotomization is detailed in the Supplementary Methods.

Multidimensional marker analysis. The centered score table of the markers containing missing values was analyzed by principal component analysis. Nonlinear 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% to 10%) versus positive (scores 2–5, >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 temozolomide and temsirolimus arms. HRs obtained from univariable Cox models were presented with 95% CI (details in the Supplementary Information).

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 cutoff with a safety margin (Supplementary Fig. S1). The primary reasons for initially registered patients not to continue to randomization 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 ($n = 8$; Fig. 1). A total of 111 patients were randomized from December 2009 to September 2012 and constituted the ITT population: 56 patients were scheduled to receive weekly temsirolimus in addition to standard radiotherapy (temsirolimus arm) and 55 were to receive TMZ/RT→TMZ alone (control arm). In the safety population, that is, patients with at least one dose of drug, there were 53 patients in the temsirolimus and 51 patients in the temozolomide arm.

Median follow-up was 33 (95% CI, 23–37) months in the temsirolimus and 32 (95% CI, 22–40) months in the temozolomide arm. The median duration from operation to randomization 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 because the stratification was PS 0–1 versus 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, because 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 versus without markers assessment (Supplementary Fig. S2 and Supplementary Table S1).

Table 1. Baseline characteristics

	TMZ ($N = 55$) N (%)	Temsirolimus ($N = 56$) N (%)	Total ($N = 111$) 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)

Abbreviation: WHO PS, World Health Organization Performance Status.

Efficacy outcomes

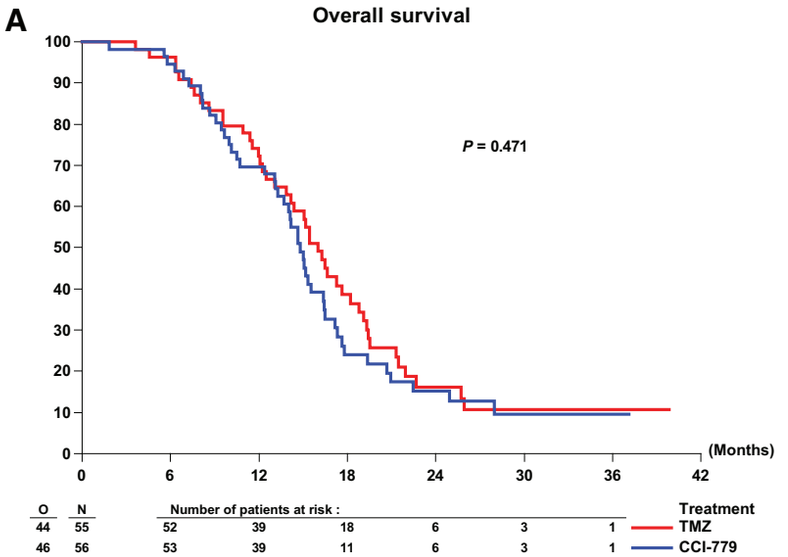
The median duration of radiotherapy was 6.1 weeks in both arms. Main reason for interrupting radiotherapy was technical or administrative (28%). In median, radiotherapy was interrupted 2 days. radiotherapy was completed by >90% of patients. Concomitant treatment was delivered as planned per protocol by >90% of patients in both arms. Patients in the temsirolimus arm received the drug for a median (95% CI) of 16 weeks post radiotherapy (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 hematologic toxicity, 10.6% for AE, 2.1% for other reasons), dose not given during at least one cycle (68%: 6.3% for hematologic toxicity, 34% for nonhematologic toxicity, 58% for other reasons) or treatment delay (58%: 2.1% for hematologic toxicity, 17% for nonhematologic 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; $P = 0.47$; Fig. 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; Fig. 2B). In the per protocol population (Supplementary Information), 38 patients treated with temsirolimus had survived $\geq$1 year. At least 39 patients were needed to reach the targeted drug activity.

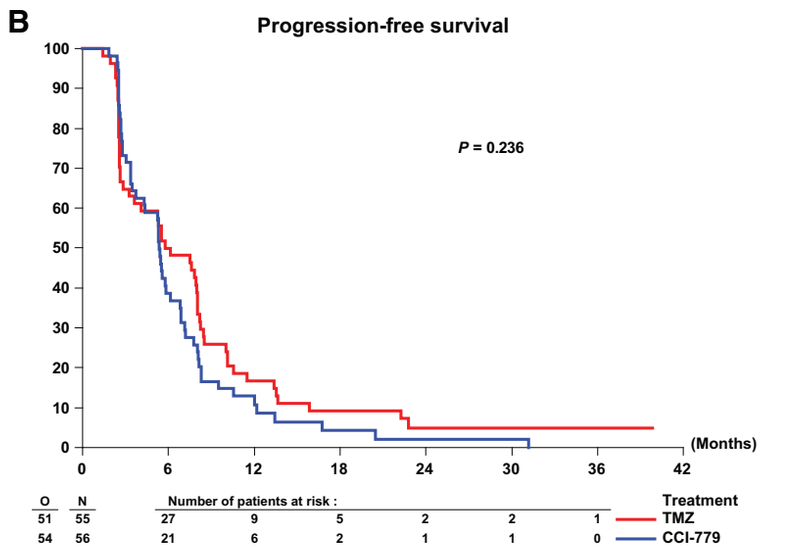
Safety

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 temozolomide arm, severe hematologic 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.



Survival time

Treatment	Patients (N)	Observed events (O)	HR (95% CI)	*P* (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)



Survival time

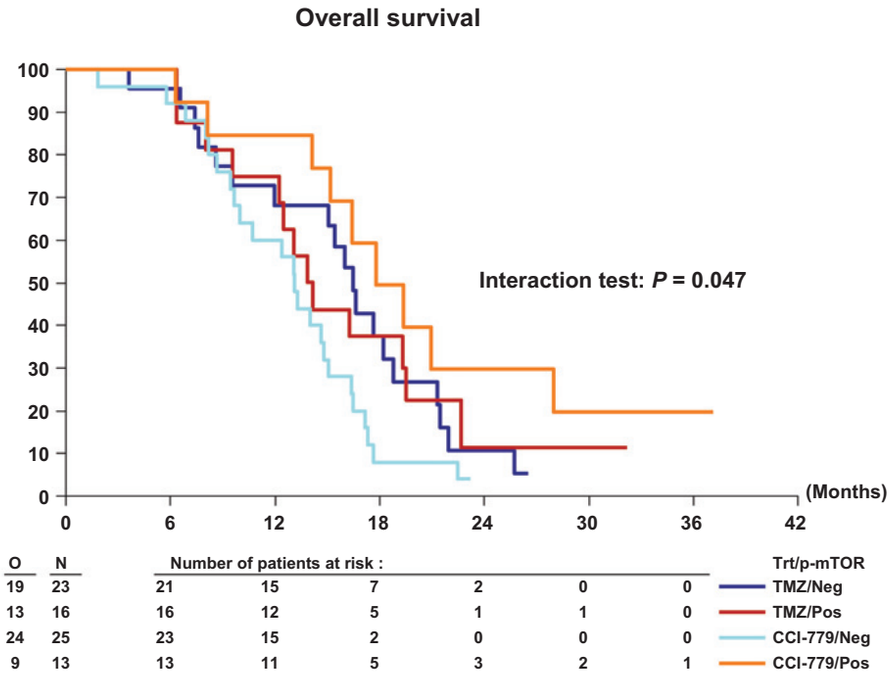
Treatment	Patients (N)	Observed events (O)	HR (95% CI)	*P* (log-rank)	Median (95% CI) (months)	% at 0.5 Year(s) (95% CI)
TMZ	55	51	1.00	0.2358	5.95 (3.25–8.02)	50.00 (36.12–62.39)
CCI-779	56	54	1.26 (0.86–1.86)		5.36 (3.71–6.14)	38.67 (25.96–51.20)

Figure 2.
Principal efficacy outcomes per treatment.

Molecular correlations with outcome

Markers interrogated for their relevance of targeting the mTOR signaling pathway (22, 23) are visualized in the mTOR KEGG pathway (ref. 28; Supplementary Fig. S3). Phosphorylated mTOR^{Ser2448} was associated with prolonged OS as evidenced by the significant interaction term between treatment and p-mTOR^{Ser2448} ($P = 0.047$; Fig. 3). Tumors of 37.6% of the patients scored positive for p-mTOR^{Ser2448}. There was a nonsignificant

A



Survival time			Nonparametric		Cox model	
Treatment/p-mtor	Patients (N)	Observed events (O)	Median (95% CI) (Months)	% at 2 Year(s) (95% CI)	HR (95% CI)	*P* (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* = 0.041

B

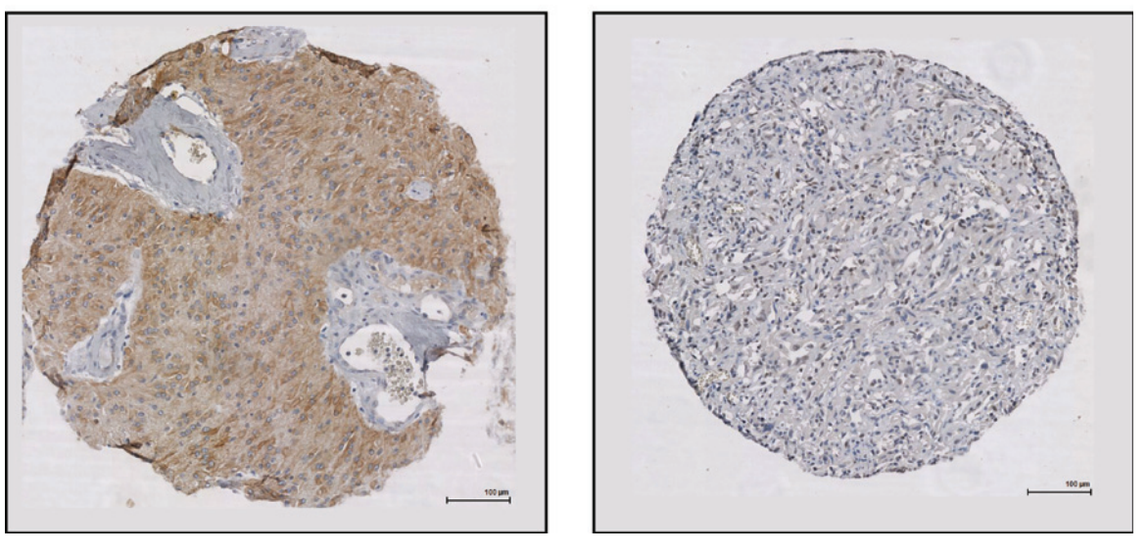


Figure 3.

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→TMZ (TMZ). The interaction test was significant ($P = 0.047$). **B,** representative glioblastoma samples negative or positive for p-mTOR^{Ser2448} expression.

trend for longer OS when p-mTOR^{Ser2448} positive patients received temsirolimus as compared with controls (HR 0.62; 95% CI, 0.26–1·47; $P = 0.27$). When nonphosphorylated mTOR^{Ser2448} patients received temsirolimus, a nonsignificant decrease in survival was observed compared with controls (HR 1.77; 95% CI; 0.95–3.29; $P = 0.07$; Fig. 3). The median OS in the temsirolimus group was 17.8 months (95% CI, 14.1–28.0) for patients with p-mTOR^{Ser2448} positive tumors and 13.1 months (95% CI, 9.7–15.1) in the negative subgroup ($P = 0.007$; Fig. 3A). In the RT/TMZ→TMZ control arm, the median OS in the p-mTOR^{Ser2448} positive group was 14.0 months (95% CI, 9.6–19.6) and 16.5 months (95% CI, 9.5–18.8) in the p-mTOR^{Ser2448} negative subgroup ($P = 0.999$). For p-PRAS40^{Thr246}, the interaction test with treatment was borderline nonsignificant ($P = 0.07$). The impact of all other markers on survival is illustrated in a forest plot for all other markers in Supplementary Fig. S4.

A multidimensional analysis used the full range of the scores of the mTOR-associated markers integrating information for the identification of clinically relevant molecular subgroups and to gain further insights on pathway interactions (Fig. 4). The two first axes obtained by PCA explained 57.8% of the total inertia. The first axis was mainly explained by p-mTOR^{Ser2448} and p-PRAS40^{Thr246}. The p-S6RP$^{Ser235/236}$ mainly contributed to the construction of the second axis (Fig. 4E and F). PTEN expression played a minor role in the structure of the score table (Fig. 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 Fig. 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 (Fig. 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, the HR was 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 temozolomide 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 radiotherapy for patients with low probability of benefit from the temozolomide-based radiochemotherapy failed to demonstrate the targeted outcome. Neither PFS nor OS demonstrated a signal of relevant activity in the total trial population (Fig. 2). Safety and tolerability of temsirolimus in combination with standard radiotherapy were nonconcerning 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 versus RT trial (EORTC26981-22981/NCIC CE3; ref. 29) was favorable in all aspects, supporting the principal rational to design trials for patients with *MGMT* unmethylated glioblastoma and withhold temozolomide 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 with 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 crossover to bevacizumab of 60% (32).

The EORTC 26082 trial aimed at not withholding temozolomide 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 temozolomide (34, 35). However, we cannot completely exclude a small baseline effect of temozolomide despite the *MGMT* unmethylated state (11). Hence, withholding temozolomide 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 temozolomide after treatment discontinuation, and 26% of temozolomide patients ($n = 14$) were rechallenged with temozolomide, not being aware of the recent data from the DIRECTOR trial that rechallenge with temozolomide 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) and 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), whereas 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 (Figs. 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, for example 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

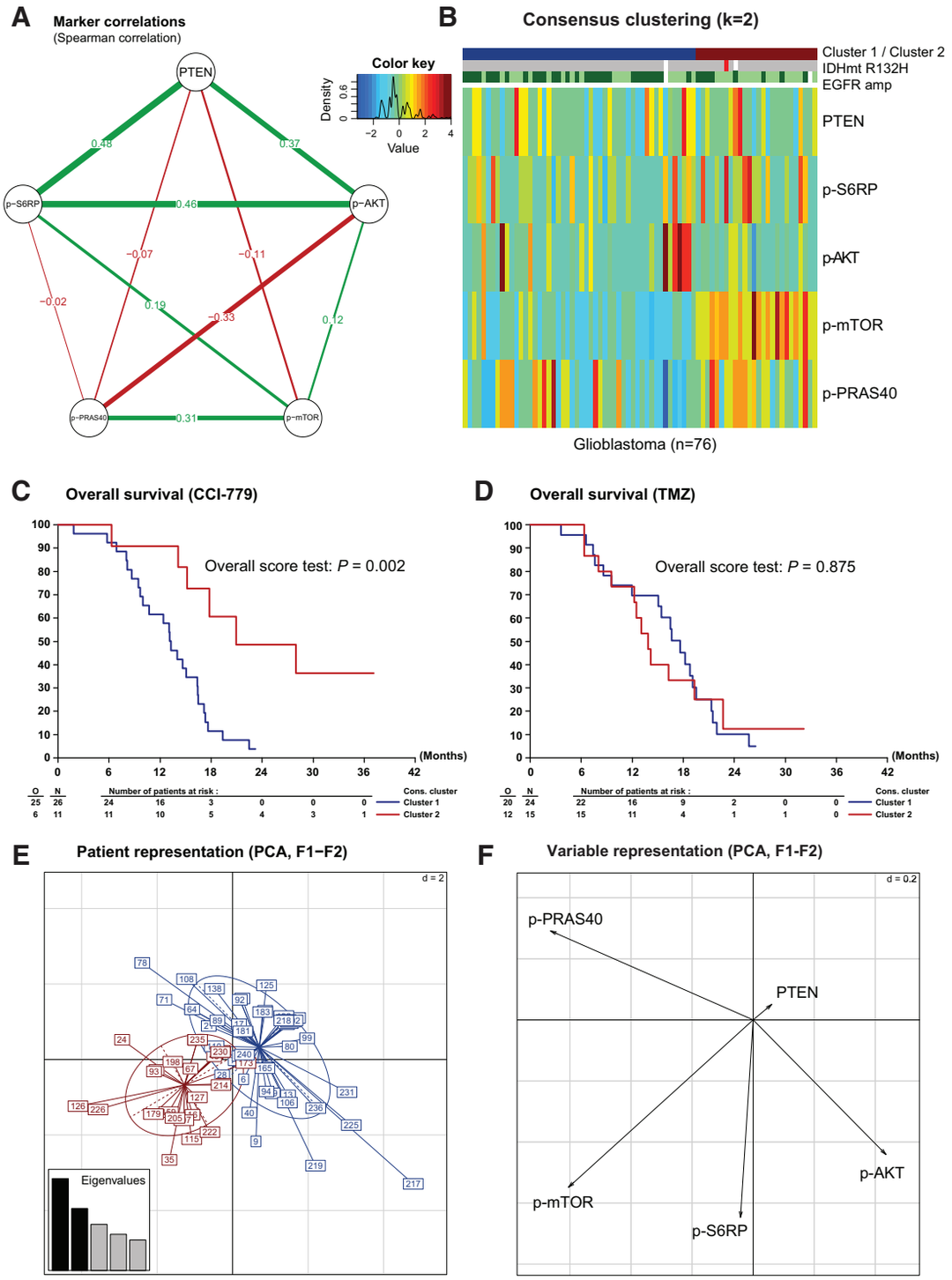


Figure 4.

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 NIPALS. The rows were ordered by the first axis of the PCA. The columns are ordered by the consensus classification ($k = 2$; cluster 1, blue; cluster 2, red) and are annotated for absence or presence of mutated $IDH1^{R132H}$ (positive, red; negative, gray; unknown, white), and the *EGFR* status (amplified, dark green; nonamplified, 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 temozolomide (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**).

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 noncomparative 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-$mTOR^{Ser2448}$ is incorporated into the design of a future study.

Disclosure of Potential Conflicts of Interest

W. Wick reports receiving speakers bureau honoraria from Bristol-Myers Squibb, MSD, and Roche; and is a consultant/advisory board member for MSD, Novocure, and Roche. M.J. van den Bent reports receiving other commercial research support from Abbvie, and Roche; and is a consultant/advisory board member for Abbvie, Actelion, Blue earth Diagnostics, Bristol-Myers Squibb, Cavion, Merck Ag, Novartis, and Roche. M.J.B. Taphoorn is a consultant/advisory board member for Hoffmann-La Roche. P. Roth reports receiving speakers bureau honoraria from Novartis, and is a consultant/advisory board member for MSD, Molecular Partners, and Roche. K. Homicsko reports receiving commercial research grants from Roche, and is a consultant/advisory board member for Amgen, and Roche. G.A. Pesce is a consultant/advisory board member for MSD. M.E. Hegi is a consultant/advisory board member for MDxHealth. No potential conflicts of interest were disclosed by the other authors.

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References

1. Brennan CW, Verhaak RG, McKenna A, Campos B, Noushmehr H, Salama SR, et al. The somatic genomic landscape of glioblastoma. Cell 2013;155:462–77.
2. Neshat MS, Mellinghoff IK, Tran C, Stiles B, Thomas G, Petersen R, et al. Enhanced sensitivity of PTEN-deficient tumors to inhibition of FRAP/mTOR. Proc Natl Acad Sci U S A 2001;98:10314–9.
3. Podsypanina K, Lee RT, Politis C, Hennessy I, Crane A, Puc J, et al. An inhibitor of mTOR reduces neoplasia and normalizes p70/S6 kinase activity in Pten+/- mice. Proc Natl Acad Sci U S A 2001;98:10320–5.
4. Chakravarti A, Zhai G, Suzuki Y, Sarkesh S, Black PM, Muzikansky A, et al. The prognostic significance of phosphatidylinositol 3-kinase pathway activation in human gliomas. J Clin Oncol 2004;22:1926–33.
5. Faivre S, Kroemer G, Raymond E. Current development of mTOR inhibitors as anticancer agents. Nat Rev Drug Discov 2006;5:671–88.
6. Galanis E, Buckner JC, Maurer MJ, Kreisberg JI, Ballman K, Boni J, et al. Phase II trial of temsirolimus (CCI-779) in recurrent glioblastoma multiforme: a North Central Cancer Treatment Group Study. J Clin Oncol 2005;23:5294–304.
7. Doherty L, Gigas DC, Kesari S, Drappatz J, Kim R, Zimmerman J, et al. Pilot study of the combination of EGFR and mTOR inhibitors in recurrent malignant gliomas. Neurology 2006;67:156–8.
8. Kreisl TN, Lassman AB, Mischel PS, Rosen N, Scher HI, Teruya-Feldstein J, et al. A pilot study of everolimus and gefitinib in the treatment of recurrent glioblastoma (GBM). J Neurooncol 2009;92:99–105.
9. Reardon DA, Desjardins A, Vredenburgh JJ, Gururangan S, Friedman AH, Herndon JEII, et al. Phase 2 trial of erlotinib plus sirolimus in adults with recurrent glioblastoma. J Neurooncol 2010;96:219–30.
10. Yung WK, Albright RE, Olson J, Fredericks R, Fink K, Prados MD, et al. A phase II study of temozolomide vs. procarbazine in patients with glioblastoma multiforme at first relapse. Br J Cancer 2000;83:588–93.
11. Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJB, et al. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med 2005;352:987–96.
12. Motzer RJ, Hudes GR, Curti BD, McDermott DF, Escudier BJ, Negrier S, et al. Phase I/II trial of temsirolimus combined with interferon alfa for advanced renal cell carcinoma. J Clin Oncol 2007;25:3958–64.
13. Hess G, Herbrecht R, Romaguera J, Verhoef G, Crump M, Gisselbrecht C, et al. Phase III study to evaluate temsirolimus compared with investigator's choice therapy for the treatment of relapsed or refractory mantle cell lymphoma. J Clin Oncol 2009;27:3822–9.
14. Sarkaria JN, Galanis E, Wu W, Dietz AB, Kaufmann TJ, Gustafson MP, et al. Combination of temsirolimus (CCI-779) with chemoradiation in newly diagnosed glioblastoma multiforme (GBM) (NCCTG trial N027D) is associated with increased infectious risks. Clin Cancer Res 2010;16:5573–80.
15. Hegi ME, Diserens AC, Gorlia T, Hamou MF, de Tribolet N, Weller M, et al. MGMT gene silencing and benefit from temozolomide in glioblastoma. N Engl J Med 2005;352:997–1003.

16. Abdollahi A, Lipson KE, Han X, Krempien R, Trinh T, Weber KJ, et al. SU5416 and SU6668 attenuate the angiogenic effects of radiation-induced tumor cell growth factor production and amplify the direct anti-endothelial action of radiation *in vitro*. Cancer Res 2003;63:3755–63.
17. Wild-Bode C, Weller M, Rimner A, Dichgans J, Wick W. Sublethal irradiation promotes migration and invasiveness of glioma cells: implications for radiotherapy of human glioblastoma. Cancer Res 2001;61:2744–50.
18. Vlassenbroeck I, Califice S, Diserens AC, Migliavacca E, Straub J, Di Stefano I, et al. Validation of real-time methylation-specific PCR to determine O6-methylguanine-DNA methyltransferase gene promoter methylation in glioma. J Mol Diagn 2008;10:332–7.
19. Stupp R, Hegi ME, Gorlia T, Erridge SC, Perry J, Hong YK, et al. Cilengitide combined with standard treatment for patients with newly diagnosed glioblastoma with methylated MGMT promoter (CENTRIC EORTC 26071–22072 study): a multicentre, randomised, open-label, phase 3 trial. Lancet Oncol 2014;15:1100–8.
20. Macdonald DR, Cascino TL, Schold SC Jr, Cairncross JG. Response criteria for phase II studies of supratentorial malignant glioma. J Clin Oncol 1990;8:1277–80.
21. Yan P, Seelentag W, Bachmann A, Bosman FT. An agarose matrix facilitates sectioning of tissue microarray blocks. J Histochem Cytochem 2007;55: 21–4.
22. Cloughesy TF, Yoshimoto K, Nghiemphu P, Brown K, Dang J, Zhu S, et al. Antitumor activity of rapamycin in a Phase I trial for patients with recurrent PTEN-deficient glioblastoma. PLoS Med 2008;5:e8.
23. Hegi ME, Diserens AC, Bady P, Kamoshima Y, Kouwenhoven MC, Delorenzi M, et al. Pathway analysis of glioblastoma tissue after preoperative treatment with the EGFR tyrosine kinase inhibitor gefitinib—a phase II trial. Mol Cancer Ther 2011;10:1102–12.
24. Wold H.Estimation of principal components and related models by iterative least squares. Multivariate analysis: New York: Academic Press; 1966. p. 391–420.
25. Monti S, Tamayo P, Mesirov J, Golub T. Consensus clustering: A resampling-based method for class discovery and visualization of gene expression microarray data. Machine Learning 2003;52:91–118.
26. Epskamp S, Cramer AOJ, Waldorp LJ, Schmittmann VD, Borsboom D. qgraph: network visualizations of relationships in psychometric data. J Stat Soft 2012;48:1–18.
27. Bady P, Sciuscio D, Diserens AC, Bloch J, van den Bent MJ, Marosi C, et al. MGMT methylation analysis of glioblastoma on the Infinium methylation BeadChip identifies two distinct CpG regions associated with gene silencing and outcome, yielding a prediction model for comparisons across datasets, tumor grades, and CIMP-status. Acta Neuropathol 2012;124: 547–60.
28. Luo W, Brouwer C. Pathview: an R/Bioconductor package for pathway-based data integration and visualization. Bioinformatics 2013;29:1830–1.
29. Stupp R, Hegi ME, Mason WP, van den Bent MJ, Taphoorn MJ, Janzer RC, et al. Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial. Lancet Oncol 2009;10:459–66.
30. Taal W, Oosterkamp HM, Walenkamp AM, Dubbink HJ, Beerepoot LV, Hanse MC, et al. Single-agent bevacizumab or lomustine versus a combination of bevacizumab plus lomustine in patients with recurrent glioblastoma (BELOB trial): a randomised controlled phase 2 trial. Lancet Oncol 2014;15:943–53.
31. Nabors LB, Fink KL, Mikkelsen T, Grujicic D, Tarnawski R, Nam DH, et al. Two cilengitide regimens in combination with standard treatment for patients with newly diagnosed glioblastoma and unmethylated MGMT gene promoter: results of the open-label, controlled, randomized phase II CORE study. Neuro Oncol 2015;17:708–17.
32. Herrlinger U, Schäfer N, Steinbach JP, Weyerbrock A, Hau P, Goldbrunner R, et al. The randomized, multicenter glarius trial investigating bevacizumab/irinotecan vs. standard temozolomide in newly diagnosed, MGMT-non-methylated glioblastoma patients: final survival results and quality of life. Neuro-Oncology 2014;16:ii23–ii4.
33. Wick W, Steinbach JP, Platten M, Hartmann C, Wenz F, von Deimling A, et al. Enzastaurin before and concomitant with radiation therapy, followed by enzastaurin maintenance therapy, in patients with newly diagnosed glioblastoma without MGMT promoter hypermethylation. Neuro Oncol 2013;15:1405–12.
34. Wick W, Platten M, Meisner C, Felsberg J, Tabatabai G, Simon M, et al. Temozolomide chemotherapy alone versus radiotherapy alone for malignant astrocytoma in the elderly: the NOA-08 randomised, phase 3 trial. Lancet Oncol 2012;13:707–15.
35. Malmstrom A, Gronberg BH, Marosi C, Stupp R, Frappaz D, Schultz H, et al. Temozolomide versus standard 6-week radiotherapy versus hypofractionated radiotherapy in patients older than 60 years with glioblastoma: the Nordic randomised, phase 3 trial. Lancet Oncol 2012; 13:916–26.
36. Weller M, Tabatabai G, Kastner B, Felsberg J, Steinbach JP, Wick A, et al. MGMT promoter methylation is a strong prognostic biomarker for benefit from dose-intensified temozolomide rechallenge in progressive glioblastoma: The DIRECTOR trial. Clin Cancer Res 2015; 21:2057–64.
37. Weiler M, Pfenning PN, Thiepold AL, Blaes J, Jestaedt L, Gronych J, et al. Suppression of proinvasive RGS4 by mTOR inhibition optimizes glioma treatment. Oncogene 2013;32:1099–109.
38. Wen PY, Chang SM, Lamborn KR, Kuhn JG, Norden AD, Cloughesy TF, et al. Phase I/II study of erlotinib and temsirolimus for patients with recurrent malignant gliomas: North American Brain Tumor Consortium trial 04–02. Neuro Oncol 2014;16:567–78.
39. Chiang GG, Abraham RT. Phosphorylation of mammalian target of rapamycin (mTOR) at Ser-2448 is mediated by p70S6 kinase. J Biol Chem 2005;280:25485–90.
40. Wiza C, Nascimento EB, Ouwens DM. Role of PRAS40 in Akt and mTOR signaling in health and disease. Am J Physiol Endocrinol Metab 2012;302: E1453–60.
41. Kim H, Zheng S, Amini SS, Virk SM, Mikkelsen T, Brat DJ, et al. Whole-genome and multisector exome sequencing of primary and post-treatment glioblastoma reveals patterns of tumor evolution. Genome Res 2015; 3:316–27.
42. Ma DJ, Galanis E, Anderson SK, Schiff D, Kaufmann TJ, Peller PJ, et al. A phase II trial of everolimus, temozolomide, and radiotherapy in patients with newly diagnosed glioblastoma: NCCTG N057K. Neuro Oncol 2015;17:1261–9.