ORIGINAL COMMUNICATION

A phase I study of temozolomide and lapatinib combination in patients with recurrent high-grade gliomas

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Abstract We undertook this phase I study to investigate the feasibility of the combination of temozolomide (TMZ) and lapatinib (LP) and to define the maximum tolerated dose (MTD) of LP in patients with relapsed high-grade gliomas. Eligible patients were enrolled in this dose escalation study of LP. TMZ was administered at a fixed dose of 200 mg/m^2 d1–d5 every 28 days. Starting dose of LP was set at 1,000 mg daily continuously, escalated by 250 mg in cohorts of minimum three patients. Translational research investigations were also undertaken in available biopsy material. Between January 2009 and December 2010, 16 patients were entered into the study at three LP levels: 1,000 mg sid (11 patients), 1,250 mg sid (4 patients) and 1,500 mg sid (1 patient). A total of 55 cycles had been delivered. Fourteen patients had stopped treatment because of disease progression, and two because of toxicity. Three patients received 10, 11 and 17 cycles of treatment. Dose-limiting hematological toxicity was observed in 2 patients at the second LP dose level of 1,250 mg sid.

MTD was defined at LP 1,000 mg sid. Median progression-free survival (PFS) and survival were 2.4 and 5.9 months, respectively. EGFR amplification and EGFRvIII expression were not related to PFS. Combination of TMZ and LP is feasible with manageable toxicity. The activity of this combination in patients with recurrent glioblastoma multiforme is further investigated in a recently initiated phase II trial.

Keywords Gliomas · Temozolomide · Lapatinib · Phase I

Introduction

Gliomas are the most common primary central nervous system tumors, with an incidence of about 25,000–30,000 new cases annually. More than half of all gliomas, including glioblastoma multiforme (GBM) and anaplastic astrocytoma (AA), exhibit aggressive clinical behavior. GBM and AA are clinically managed in a similar fashion; however, despite all efforts, their prognosis remains dismal [25]. As recently published data showed, overall survival of patients with newly diagnosed GBM, the most common malignant glioma, was 42.4 % at 6 months, 17.7 % at 1 year, and 3.3 % at 2 years, despite access to state-of-the-art surgery, imaging, radiotherapy and chemotherapy [34].

Current therapeutic strategies include surgical resection followed by radiotherapy and adjuvant temozolomide (TMZ), which contributes to a small but statistically significant survival benefit [30]. However, recurrence inevitably occurs in the majority of patients, followed by death from the disease. Therefore, the need of novel active treatments remains a pressing issue.

Amplification, overexpression and mutations of the EGFR family members are quite common in gliomas, and the concept of targeted treatment has been applied in

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clinical research for some time now [10]. Gefitinib and erlotinib have already been tested in gliomas, and data show that in contrast to the lung cancer pattern of response, amplification and mutations of the EGFR gene are not predictive of response in glioma patients [22, 23, 32]. However, recent data demonstrated that coexpression of EGFRvIII and PTEN by glioblastoma cells is associated with responsiveness to EGFR kinase inhibitors [6].

We undertook a phase I trial of temozolomide and lapatinib, an orally active small molecule that reversibly inhibits EGFR and HER2 tyrosine kinases, to investigate the feasibility of the combination and characterize their toxicity profile in patients with recurrent high-grade gliomas following initial standard treatment.

Available tumor tissues were also profiled for standard genetic glioma markers, as well as for a panel of mRNA targets [24]. The latter were selected taking into account (a) the frequently observed EGFR amplification and the expression of its truncated variant, EGFRvIII in glioblastoma, (b) the EGFR signalling through STAT3/STAT5, which has been proposed as a for therapeutic targeting [21, 26, 37], and (c) the characterization of currently discussed molecular glioma subtypes [4, 33].

Materials and methods

Patient selection

Adult patients with confirmed histological diagnosis of grade IV glioblastoma multiforme or grade III anaplastic astrocytoma, who relapsed after optimal treatment and for whom there were no other standard treatments available, were included. All patients were required to have a WHO performance status of ≤ 2 and be >18 years old with measurable disease at screening, according to RANO criteria [36]. Adequate hematological function (absolute neutrophil count [ULN] $\geq 1.5 \times 10^9$/L, platelet count $\geq 100 \times 10^9$/L and hemoglobin ≥ 9 g/dL), liver function (total bilirubin <1.5 × upper limit of normal [ULN], AST, ALT <2.5 × ULN) and renal function (serum creatinine ≤ 1.25 × ULN or calculated creatinine clearance ≥ 50 mL/min) were mandatory for study entry. Patients were also required to have normal cardiac function (left ventricular ejection fraction within institutional normal range) and in the past 6 months have had no serious cardiac illness or medical condition, including but not confined to: history of documented congestive heart failure, high-risk uncontrolled arrhythmias, angina pectoris requiring antianginal medication, clinically significant valvular heart disease, evidence of transmural infarction on ECG and poorly controlled hypertension (e.g., systolic >180 mmHg or diastolic greater than 100 mmHg). If a patient required anticoagulant therapy, the patient was allowed to remain on study provided that he/she was carefully monitored.

Drugs and several herbal constituents (e.g., bergamontin and glabridin), which are inducers or inhibitors of CYP3A4, were prohibited during and 10 days prior to the initiation of the treatment. Available archived tumor tissue for subsequent translational research studies was mandatory for study entry.

The clinical protocol and the related translational research studies were approved by the Hellenic Cooperative Oncology Group (HeCOG) Protocol Review Committee, the Institutional Review Boards of Papageorgiou and Ioannina Hospitals and the National Organization for Medicines. The trial was included in the Australian New Zealand Clinical Trials Registry (ANZCTR) and allocated the following Registration Number: ACTRN12611000418976. Upon participation in the trial, all patients provided a study specific written informed consent and a separate informed consent for providing biological material for future research studies. The study was conducted according to the Declaration of Helsinki and the guidelines for Good Clinical Practice.

Dosage and dose escalation schema

Patients were enrolled in the phase I study in cohorts of at minimum three patients. Patients were to receive TMZ 200 mg/m^2 once daily for 5 consecutive days in cycles of 28 days. The TMZ dose was fixed at each dose level. The starting dose of lapatinib was 1,000 mg, administered once daily. Three dose levels of lapatinib were planned to be explored (1,000, 1,250 and 1,500 mg). Patients would receive LP/TMZ combination until disease progression or unacceptable toxicity. They were advised to take the LP and TMZ tablets on an empty stomach (either 1 h before or 1 h after meals). Antiemetic prophylaxis with a 5HT$_3$ inhibitor was used for the 5 days of temozolomide administration. Cohorts of three patients received escalating doses of lapatinib until the maximum tolerated dose (MTD) was determined, as recorded in week 4 of the treatment (end of the 1st treatment cycle of the combination, day 28 of the treatment).

The MTD was defined as the dose preceding that at which two of a maximum six patients experienced dose-limiting toxicity (DLT). If one DLT was observed within a cohort, then the cohort was expanded to include three more patients. If no further DLTs were observed, dose escalation proceeded. If a second DLT was observed within the expanded cohort, then the MTD was reached. Patients who developed a DLT were withdrawn from the trial but were offered the option to continue treatment at a lower dose, off-protocol, if they had derived benefit from this therapy. The dose level below the MTD was then expanded. At this dose, a minimum of eight patients had to be treated before that dose was defined to be the recommended dose for a subsequent phase II trial (<30 % DLTs).

Patients continued on their initial dose until the development of unacceptable toxicity or disease progression. Dose escalation between cohorts was done according to the modified continual reassessment method, combined with review of available clinical and laboratory data. The evaluable patient population for the determination of the MTD consisted of patients who had met the minimum safety evaluation requirements of the study. Antitumor efficacy was assessed after two cycles of treatment.

Evaluation of toxicity

Toxicity was graded according to the National Cancer Institute Common Toxicity Criteria. Complete blood count, biochemical analyses and clinical toxicity assessments were performed weekly during all treatment courses. Dose escalation and determination of DLT and MTD were performed on the basis of toxicity occurring during the first cycle of treatment.

Dose limiting toxicity

DLT was defined as any grade 3, grade 4 or life threatening toxicity requiring hospitalization. Specifically a DLT was defined as: ANC $<0.5 \times 10^9$/L (grade 4) for over seven consecutive days, febrile neutropenia [ANC $< 1.0 \times 10^9$/L (grade 3) when accompanied by fever], hemoglobin <6.5 g/dL (grade 4) and grade 4 thrombocytopenia (platelet count $<25 \times 10^9$/L). Any grade 3–4 non-hematological toxicity except alopecia, nausea, vomiting and fever, which could be rapidly controlled with appropriate measures, also consisted a DLT.

Evaluation of response

Patients completing at least two cycles of treatment with at least one follow-up tumor assessment were considered evaluable for response. An initial tumor assessment for all patients was performed within 4 weeks prior to treatment initiation, and thereafter every two cycles while on therapy and every 2 months thereafter until progression of disease. Response was documented using the RANO response criteria for high-grade gliomas [36]. Confirmation of objective responses was required in all cases at a minimum time interval of 4 weeks. Duration of response was calculated from the time the objective response was documented until the date of disease progression. Stable disease was measured from the start of the treatment until the criteria for progression were met.

Translational research

These investigations were undertaken at the Laboratory of Molecular Oncology of the Hellenic Foundation for Cancer Research, Aristotle University of Thessaloniki School of Medicine.

For the assessment of PTEN protein expression, a tissue microarray (TMA) block, containing two 1.5-mm tissue cores from the tumor area was created from the original blocks using the Beecher Instruments MTA-1 Tissue Arrayer (Beecher Instruments, Sun Prairie, WI). Serial 3-μm-thick TMA sections, mounted on adhesion microscope slides, were cut at the Laboratory of Molecular Oncology of the Hellenic Foundation for Cancer Research, Aristotle University of Thessaloniki School of Medicine. The IHC labeling was performed using the Bond Max™ (Leica Microsystems, Wezlar, Germany) and i6000 (Biogenex, San Ramon, CA) autostainers. Sections were stained with an antibody against PTEN, clone 6H2.1, Dako, Glostrup, DK, at dilution 1:300, for 30 min. DAB (3,3-diaminobenzidine) was used as a chromogen and Harris hematoxylin as a counterstain. The quality of IHC staining was evaluated using internal positive controls (endothelial cells). PTEN staining was scored according to Mellinghoff et al. [16], using a scale of 0–2. Vascular endothelial cells were used as control markers of staining intensity. Tumors with PTEN scores of 0 or 1 were considered to have PTEN protein loss.

Histologically adequate tumor material (routinely processed formalin-fixed paraffin-embedded [FFPE] tissue) was available from 16 patients.

DNA and RNA was extracted upon enrichment in tumor cells, while DNA was also extracted from peripheral blood samples. For DNA extraction, the QIAamp mini kit (Qiagen, Hilden, Germany) was used. Upon overnight tissue fragment lysis with proteinase K at 56 °C, RNA was extracted with TRIZOL-LS (Life Technologies, Paisley, UK) and reverse transcribed with random primers and the Superscript III system (Life Technologies). Commercial reagents were applied according to the instructions of the manufacturers. FFPE-DNA samples were checked for amplification capacity with the BIOMED2 multiplex DNA control PCR assay prior to undertaking molecular investigations. Molecular templates were of inadequate quality in one of the 16 cases; hence, molecular data are reported for 15 tumors only.

MGMT promoter methylation was assessed with the SALSA MS-MLPA KIT ME011 (MRC, Amsterdam, NL, Holland). Normal blood DNA and a reactive lymph node FFPE-DNA sample were used as controls. This method gave informative results, i.e., all peaks present in the non-digested sample with acceptable internal controls, in 10 of the 15 cases (66.6 %), in accordance with FFPE-DNA amplification capacity ($\geq$300 bp for these 10 samples, $<$300 for the remaining 5 samples). The results were evaluated according to [13]: relative peak values $\leq$0.25 were characterized as unmethylated, relative peak values

>0.25 as methylated, and relative peak values >0.75 as completely methylated. The procedure was repeated twice to assess concordance of results (retention in the same category of methylation status).

Chromosomal 1p/19q deletion was investigated with a previously published loss of heterozygocity (LOH) protocol [3]. Briefly, peripheral blood and tumor DNA samples from the same patient were compared for the presence of four microsatellite markers, D1S2845 (1pter), D1S216 (1p31), D1S2696 (1p13) and D19S589 (19q13), with capillary electrophoresis and analyzed with the Gene-Mapper software. Twelve tumors were informative for this parameter.

Mutation analysis for IDH1 exon 2 (including codon for p.R132) and IDH2 exon 4 (including p.R172) was accomplished with dd-sequencing (sense and antisense) in an ABI3130XL genetic analyzer. This approach was informative in 13 of the15 tumors (87 %).

Copy number variation (CNV) was assessed for the *EGFR* (7p12), *MET* (7q31), *ERBB2* (17q21.1) and *CCND1* (11q13) genes. For each gene, two genomic targets were amplified, to ensure informative results. The method involves a duplex real-time PCR reaction including (a) for the target gene, TaqMan® minor groove binding (MGB) probes, FAM™ labeled and (b) for the reference gene, Taqman VIC®-TAMRA™ labeled probes, both assays with unlabeled primers. Because these assays detect tiny parts of the whole gene, we selected two assays for each target gene, located at distant parts of the genomic sequence. The ready-made assays used and their ID numbers (Applied Biosystems/Life Technologies) were: for *EGFR* exon 2, Hs01426560_cn; for *EGFR* exon 10, Hs02822119_cn; for *ERBB2* exon 13, Hs01839367_cn; for *ERBB2* exon 21, Hs00709630_cn; for *CCND1* intron 2 Hs03802861_cn, and exon 3 Hs01133305_cn; and for *MET*, exon 8 Hs02633538_cn, and intron 9 Hs05005398_cn. The TaqMan® Copy Number Reference Assay RNase P was used as endogenous reference. Reactions were run in duplicates in an ABI7900 system. Three normal blood DNA samples were included in each run as calibrator samples, along with a no-template control (NTC). Results (predicted copy numbers [CN]) were obtained automatically with the CopyCaller™ Software v1.0, in comparison to averaged calibrator values upon setting the evaluation threshold at Ct = 33. Z-scores for all accepted samples and CN range for duplicates were <1. No amplification was observed for NTCs. Out of the two assays that were used per target, the more stable one (lower CN range among replicate runs of the three normal samples) was used as a guide in ambiguous cases with more >2 CN difference. Amplification (CN gain) was considered for cases with >3 CN; cases with 2.5–3 CN were considered to be ambiguous (low amplification/polysomy).

All 15 tumor samples were informative for CN evaluation with this approach.

Relative mRNA expression was assessed with RTQ-PCR, using premade Taqman-MGB assays in an ABI7900HT system under default conditions. Samples were tested in 10 µl reactions and were run in duplicates. The assays used were: EGFR, Hs00193306_m1; DARPP32 (PPP1R1B), Hs00259967_m1; STAT1, Hs01014005_m1; STAT3, Hs01047580_m1; STAT5A, Hs00234181_m1; and STAT5B, Hs00560035_m1. EGFRvIII expression was assessed with a custom designed Taqman-MGB assay by using Primer Express® v1.3, spanning exons 1^8 (sequencing validated amplicon length: 102 bp; primers/probe available upon request). Exclusion criteria for RQ analysis were endogenous control (GUSB, assay 4333767F) CT values higher than 36 for each duplicate; and deltaCTs higher than 0.5 per duplicate pair. According to this evaluation, all 15 tumor samples (100 %) were found eligible for further analysis. Relative quantification (RQ) was assessed in a linear mode as (40 dCT) [9], whereby dCT = (avg. CT target) − (avg. CT GUSB).

Statistical analysis

The primary endpoint for the phase I study was to determine the MTD of LP when given in combination with TMZ. Patient demographics, tumor characteristics and lab data (IHC and mRNA) were presented in frequency tables using descriptive statistics. Associations between binary variables were examined using Fisher's exact test, while the Mann–Whitney test was used to compare group of cases in terms of continuous variables. Progression-free survival (PFS) was calculated from date of study entry to the date of documented tumor progression or death from any cause. Survival was calculated from the date of study entry to the date of death from any cause. Alive patients or patients lost to follow-up were censored on the last date they were known to be alive. Survival and PFS were estimated by the Kaplan–Meier method. Toxicity is presented in a frequency table according to grade, along with the corresponding percentages (N %).

Results

Between January 2009 and December 2010, 16 patients (9 males and 7 females) with a median age of 60.3 years entered the study at three LP levels: 1,000 mg sid (11 pts), 1,250 mg sid (4 pts) and 1,500 mg sid (1 pt). At the time of the analysis, a total of 68 cycles had been delivered with a median cycles per patient value of 2.5 (range: 1–17). All patients eventually discontinued treatment, two due to adverse events and the other 14 patients because of disease progression. Patient characteristics are shown in Table 1.

Table 1 Patient demographics and tumor characteristics

N = 16	Median (range)
Age (at study entry)	60.3 (43–79)

N = 16	N (%)
Dose group	
1st level	11 (68.7)
2nd level	4 (25.0)
3rd level	1 (6.3)
Gender	
Men	9 (56.3)
Women	7 (43.7)
Performance status (ECOG)	
0	8 (50.0)
1	5 (31.3)
2	3 (18.7)
Type of initial surgery	
Total excision	4 (25.0)
Subtotal excision (75–99 % of the tumor)	8 (50.0)
Biopsy (<75 % of the tumor)	4 (25.0)
Location	
Temporal	7 (43.6)
Parietal	3 (18.8)
Occipital	3 (18.8)
Frontal	3 (18.8)
Hemisphere	
Right	10 (62.5)
Left	6 (37.5)
Number of sites	
1	14 (87.5)
2	2 (12.5)
Histology type	
Glioblastoma multiforme	14 (87.5)
Anaplastic astrocytoma	2 (12.5)
Post-operative treatment	
Chemo-radiotherapy	16 (100)

Table 2 Dose limiting toxicities during the first course of treatment

	Regimen	DLT
Dose level 1 (11 patients)	TMZ/LP 1,000 mg	No
Dose level 2 (4 patients)	TMZ/LP 1,250 mg	Neutropenia grade 3 and leukopenia grade 4 (1 patient)
		Thrombocytopenia grade 4 (1 patient)
Dose level 3 (1 patient)*	TMZ/LP 1,500 mg	No

* Protocol violation

DLT dose limiting toxicities, *LP* lapatinib, *TMZ* temozolimide

Dose limiting toxicity

Hematological toxicity was the only DLT toxicity seen in two patients, at the second dose level, during the first cycle of their treatment (Table 2). Specifically, the first patient experienced grade 3 neutropenia and grade 4 leukopenia and the second patient had grade 4 thrombocytopenia. Treatment was discontinued in both patients. The first patient had a rapid disease progression and died a week after discontinuation of treatment, while the second patient died because of disease progression 8 months later, without receiving any further treatment. Neither was considered to be treatment-related death. No non-hematological DLT toxicity was reported. Febrile neutropenia requiring hospitalization was observed in one patient at the expanded first dose level. This episode occurred at the second cycle of treatment.

Most common toxicities

Hematological toxicity was the most common side effect observed. Eleven patients (68 %) developed hematological toxicity of any grade. Other common side effects, either clinical or laboratory, were minimal and are all depicted in the Appendix Table. Grade 3 CNS toxicity was observed in two patients, and although it was initially reported as a serious adverse event, after assessment it turned out to be disease related. Almost all side effects resolved to at least grade 1, either spontaneously or after temporary (less than 2 weeks) withdrawal of study medications.

Overall study treatment was well tolerated. Most patients discontinued treatment due to disease progression. In the expanded first cohort, one patient experienced 2 DLTs (febrile neutropenia grade 3 and thrombocytopenia grade 3) in the second cycle of treatment. Since DLTs were observed in less than 30 % of patients in the expanded first cohort, TMZ and LP 1,000 mg sid is the recommended dose for further phase II studies evaluating the combination.

Dose escalation and maximum tolerated dose

Three patients were entered in the first dose level (LP 1,000 mg sid). No DLT toxicity was observed, and therefore the study moved forward to the second dose level (LP 1,250 mg sid), where three patients were initially entered. One patient had a DLT and subsequently one more patient entered the same dose level. A second DLT was reported. However, just before the end of the second dose level, one patient was dosed at the next dose level. This case was a protocol violation. Since the first dose level was the MTD, as per protocol design, 8 more patients were entered at this level to further characterize the toxicity profile of the drug combination.

Table 3 Histologic, genotypic and phenotypic characteristics of the tumors examined with respect to patient outcome

| | | | | | | LOH | MLPA | IHC | CNV qPCR | RNA (cDNA), qPCR | | | | | | |
Sample ID	gender	age	GRADE^	no of cycles	PFS (mo)	1p/19q deletion	MGMT methyl	PTEN protein	EGFR gene status	EGFR	EGFRvIII	STAT1	STAT3	DARPP32	STAT5A	STAT5B
GM001T	F	62	IV	1	0.95	partial	UM	non info	amplified							
GM002T	F	58	IV	2	1.64	no	UM	pos	non-amp							
GM014T	M	50	IV	1	0.95	no	noninfo	neg	non-amp							
GM005T	M	43	IV	4	3.54	no	UM	neg	amplified							
GM003T	F	67	IV	4	2.3	no	UM	neg	non-amp							
GM006T	M	63	II/III/IV	3	2.82	partial	noninfo	non info	non-amp							
GM010T	M	55	IV	10	8.98	no	UM	non info	non-amp							
GM009T	M	58	IV	11	10.33	non info	UM	pos	amplified							
GM007T	F	49	II/III/IV	17	17.25	no	METH	neg	amplified							
GM016T	M	76	IV	4	4.49	non info	UM	neg	non-amp							
GM004T	F	45	III/IV	2	1.77	no	UM	pos	amplified							
GM015T	F	79	III/IV	3	4.46	no	noninfo	pos	amplified							
GM011T	F	73	III/IV	2	2.43	no	noninfo	neg	amplified							
GM012T	M	55	IV	1	1.64	non info	noninfo	pos	amplified							
GM013T	F	65	IV	1	2.36	no	METH	pos	amplified							

^ some glioblastomas included lower grade areas (histologically secondary glioblastomas)

LOH loss of heterozygocity, *MLPA* multiplex ligation-dependent probe amplification, *IHC* immunohistochemistry, *CNV qPCR* gene copy number assessment with Taqman PCR, *RNA* relative expression with Taqman PCR, *heatmap* clustered relative quantification values with JMP v.8 software (embedded) with red = high and green = low expression, non info non informative results, *UM* unmethylated
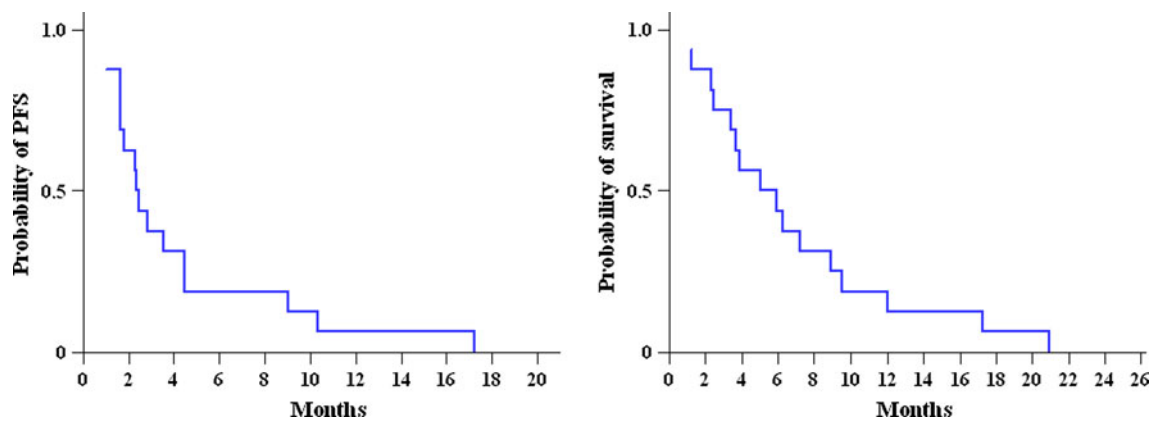
Fig. 1 Kaplan–Meier curves for PFS (*left*) and survival (*right*) in all patients

Efficacy

Half of the patients received more than two cycles of the study regimens. One patient, treated at the second dose level, had early disease progression in the first cycle and therefore treatment had to be stopped. One patient who developed DLT at the second dose level, resulting in discontinuation of the treatment due to the observed toxicity, also had rapid disease progression and died 8 months later without receiving any further treatment. All eight patients who received more than two cycles of the study drugs were treated at the first dose level. There was one partial response documented, while five patients had stable disease. Three patients received treatment for 10, 11, and 17 cycles, with a PFS of 9, 10 and 17 months, respectively (Table 3). Median survival for all patients was calculated to be 5.0 months (range: 1.15–20.95), while median PFS was 2.4 months (range: 0.95–17.25) (Fig. 1).

Translational analysis results

MGMT promoter methylation was observed in 2 of the 10 informative tumors and was considered to be incomplete; in both cases, only the 193 bp probe was found methylated with peak values of 0.43 and 0.64, respectively. No complete 1p/19q deletions were observed in the 12 informative tumors, although partial deletions were observed in two cases (Table 3). None of the tumors examined harbored *IDH1* or *IDH2* mutations or copy number gains for *ERBB2*, *MET* and *CCND1*.

EGFR gene copy number gains, corresponding to gene amplification, were observed in 9 of the 15 tumors (60 %) (Table 3). EGFRvIII expression was considered to be positive (RQ > 30) in 7 tumors and occurred in strong association with *EGFR* gene amplification, since all EGFRvIII positive tumors were *EGFR* amplified (Fisher's exact test, $p = 0.007$). In some EGFRvIII positive tumors

(cases GM007 and GM013 in Table 3), *EGFR* amplification could only be evaluated with the exon 10 assay; in these cases, CNV test results were negative with the exon 2 assay. In the same line, in 3 additional tumors, the predicted CN for exon 2 appeared distinctly lower than that for exon 10. Such discrepancies can be explained, considering that exon 2 is within the area deleted in the rearranged *EGFR* gene that expresses the truncated vIII variant. It thus appears that the application of *EGFR* CNV testing for distinct areas of the gene may indicate EGFRvIII expression, which might be interesting to validate in larger sample series for screening purposes.

EGFR mRNA expression was generally high in the GBM cases examined and was strongly positively associated with *EGFR* gene amplification (Mann–Whitney test, $p = 0.009$). STAT1 and STAT3 mRNA expression was also high, STAT5B intermediate, while DARPP32 and STAT5A mRNA expression was relatively low (Table 3). Neither *EGFR* gene amplification nor EGFRvIII expression were associated with relative transcript levels of the *DARPP32/STAT* genes.

Profiling of all mRNAs examined (Table 3) revealed distinct subgroups among the small number of tumors in this study. In three tumors, all transcripts were barely detectable (green cluster in Table 3), 5 tumors were *EGFR* amplified/EGFRvIII positive and expressed low *DARPP32/* all *STAT* genes (orange cluster), while 6 tumors were mostly EGFRvIII negative and expressed relatively high DARPP32/STAT5 and low STAT3 mRNA (blue cluster). Interestingly, patients with tumors in the blue cluster had longer PFS when compared to all other patients; the three patients who received the highest number of treatment cycles were also included in this group. However, numbers were too small to perform statistical analyses.

PTEN protein expression was assessed in 13 cases; in three cases biopsy material was inadequate. PTEN protein expression was observed in seven cases, whereas five cases had PTEN loss. *EGFR* amplification was observed in five cases with PTEN protein expression and three cases with PTEN loss. From the seven EGFRvIII positive tumors, four expressed PTEN (Table 3).

Discussion

The survival outcomes for high-grade gliomas remain poor [34]. Failing initial treatment with surgery and chemoradiotherapy with TMZ, patients with recurrent tumors have limited therapeutic options and dismal prognosis [34]. Targeting the vascular endothelial growth factor and epidermal growth factor pathways has recently emerged as an increasingly interesting option in the treatment of recurrent disease [8, 28]. However, the employment of EGFR

tyrosine kinase inhibitors in the treatment of recurrent gliomas has met limited utility. Despite the fact that EGFR is frequently overexpressed or mutated in glioblastoma tumor cells, response rates to single-agent EGFR tyrosine kinases, such as gefitinib and erlotinib, range between 0 and 15 % [8]. PTEN activity is necessary for EGFR tyrosine kinase inhibitors to be active, and PTEN mutations, which are common in these tumors, likely contribute to the limited activity observed with this class of agents to date [7].

Lapatinib is a dual-action tyrosine kinase inhibitor, with activity in both HER2/ERBB2 and EGFR receptors. LP in combination with capecitabine is an active regimen in metastatic breast cancer patients who express the HER2/ new protein [20]. The clinical use of LP in recurrent gliomas has so far been limited. Thiessen and colleagues reported the results of a phase I/II trial of LP monotherapy in recurrent GBM; treatment with single-agent LP did not show significant activity in GBM patients and the trial was terminated early because of lack of efficacy [31].

Rechallenge with TMZ at the time of tumor progression has previously been reported in small case series. Alternative dosing schedules have been used in the form of metronomic administration, which showed remarkable efficacy in GBM patients due to an antiangiogenic effect [15, 29]. The rationale for TMZ rechallenge in previously TMZ-treated patients includes not only the potential antiangiogenic action of continuously delivered drug, but also the potential to overcome TMZ resistance via the MGMT repair pathway [17]. Nevertheless, in our study, MGMT promoter methylation was observed in only 2 out of the 10 tumors assessed and was considered to be incomplete.

This is the first reported trial of LP and TMZ in recurrent high-grade gliomas, as of date. In our study, sixteen patients were treated at two dose levels. The combination of TMZ at standard dose and LP at 1,000 mg sid seems to be feasible with a favorable toxicity profile in the pretreated population. Hematological toxicity was the only DLT observed, which was easily manageable. Approximately one-third of the patients had clinical benefit from the treatment. A phase II trial has recently started recruitment in order to assess the efficacy of this combination.

Several genes, including *ERBB2, MET, CCND1* and *EGFR,* are altered in gliomas [2]. These alterations tend to occur in a defined order during the progression to a high-grade tumor. Loss or mutation of *PTEN* and amplification of *EGFR* are predominant characteristics of higher-grade tumors [16]. Combined allelic loss of 1p/19q is characteristic of oligodendrogliomas and of better prognosis, while partial losses may be observed in astrocytic tumors as well [18]. 1p/19q loss and mutations of the isocitrate dehydrogenase genes (*IDH1 and 2*) are early phenomena in glial tumor development and progression [14].

IDH mutations mark astrocytic and oligodendrocytic gliomas with a better prognosis, being more frequent in grade II/III and clinically secondary glioblastomas while seldom in primary glioblastomas [35, 38]. In GBM, co-expression of PTEN and EGFRvIII is associated with better response to EGFR TKIs [16], while *MET and CCND1* overexpression is associated with shorter PFS and adverse prognosis.

In the present high-grade glioma series, no *ERBB2, MET or CCND1* gene gains were observed. Further, consistent with the astrocytic morphology of the tumors, only parital 1p and 19q losses were observed, which are not related to patient prognosis, while all patients including those with histologically secondary glioblastomas had IDH-negative disease. Hence, we cannot comment on a possible effect of these markers on patient outcome.

In line with a high incidence of *EGFR* gene alterations in glioblastomas, the majority of the tumors examined exhibited *EGFR* copy gains. *EGFR* amplification is observed in half of primary glioblastomas and is associated with poor prognosis [11, 12, 27], while half of *EGFR*-amplified tumors harbor the truncated *EGFRvIII* mutant, the absence of which may be associated with a better prognosis in GBM [19]. Herein we show that EGFR mRNA expression was pronounced in cases with *EGFR* gene copy gains corresponding to *EGFR* amplification; in addition, 7 out of 9 *EGFR* amplified tumors also expressed *EGFRvIII*. However, these EGFR parameters were individually not associated with a benefit from LP treatment in our series.

Three patients received 10 or more cycles of treatment with a remarkable disease control for several months; two had *EGFR* gene amplification, and in the third case the *EGFR* gene was not amplified. From the two *EGFR* amplified cases, one was PTEN positive and EGFRvIII negative (11 months PFS) and the second one was found PTEN negative and EGFRvIII positive (17 months PFS). In the *EGFR* non-amplified case, EGFRvIII was negative with PTEN expression being not informative (9 months PFS). Nevertheless, due to the small number of studied cases, it is unclear whether these findings are associated with response to treatment and disease outcome. Similar to our results, in the study by Thiessen et al. [31], only three out of 16 cases had co-expression of EGFRvIII and PTEN, but it was also unclear whether this co-expression contributed to disease outcome.

Beyond the above markers that are usually examined in association with EGFR inhibition, herein we show that all three patients with a relatively more favorable PFS expressed *DARPP32* and *STAT5A/B* mRNA; by contrast, those with the worst registered PFS did not express these genes. STAT5 proteins may be downstream targets of *EGFRvIII* [5], while *DARPP32 (PPP1R1B)* is a key molecule marking neuronal differentiation [1]. According to a proposed molecular glioma subtyping [33], gliomas with the most favorable outcome belong to the proneural subtype (oligodendrocytic profile, IDH-positive disease), followed by the neural, the classical, and the most aggressive mesenchymal subtypes. In this sense, *DARPP32* mRNA may be a marker of the neural subtype, which is characterized by an expression profile resembling neurons and cultured astrocytes. It should be noted that all these data were determined in primary tumors, i.e., before radiation and temozolomide, with the status of these markers being unclear in glioblastoma tissues after progression upon standard treatment. Nevertheless, it seems worth further investigating whether *DARPP32* and *STAT5* expression interferes with lapatinib efficiency or whether it is an intrinsic characteristic of glioblastomas with a more favorable prognosis.

LP combined with capecitabine chemotherapy is efficaceous in HER2-expressing metastatic breast cancer patients. There is also evidence that this combination has remarkable efficacy in the hard-to-treat subpopulation with brain metastases. It has been hypothesized that LP can theoretically cross the blood–brain barrier and, as such, may exert its activity. Nevertheless, only modest activity has been documented with single agent LP in intracranial disease, however this was augmented by combining LP with capecitabine. In our study we did show a modest activity in a limited number of patients with recurrent gliomas treated with the LP and TMZ combination. In line with this, we could speculate that the amount of active drug that had crossed the blood–brain barrier was uncertain, and thus it is unclear whether the optimal drug concentration had reached the tumor. Applying pharmacokinetic studies of LP in CSF in future clinical trial designs, although difficult to carry out, may help to answer these questions.

In conclusion, combination of TMZ and LP seems to be feasible and well tolerated with manageable, predominantly hematological toxicity in patients with recurrent high-grade gliomas. The activity of this combination in patients with recurrent GBM is currently under investigation in a recently initiated phase II trial.

Appendix

See Table 4.

Table 4 Frequency table of adverse events (AEs)

	Grade I		Grade II		Grade III		Grade IV		Grade V	
	N	%	N	%	N	%	N	%	N	%
Blood/bone marrow										
Hemoglobin	3	19	4	25	3	19				
Leukocytes			3	19	1	6	3	19		
Neutrophils	1	6	2	13	1	6	3	19		
Platelets	4	25	2	13	2	13	3	19		
Cardiac										
Palpitations	1	6								
Hypertension			1	6						
Constitutional symptoms										
Feeling of burning	1	6								
Muscle weakness	1	6								
Fatigue	2	13	2	13	1	6	1	6		
Fever			1	6						
Dermatology/skin										
Acne			1	6						
Hyperpigmentation			1	6						
Pruritus	1	6								
Rash	4	25	2	13						
Urticaria	1	6								
Anorexia	2	13								
Constipation	1	6	1	6						
Diarrhea	4	25	2	13						
Dry mouth	1	6								
Flatulence			1	6						
Discomfort	1	6								
Mucositis (clinical exam)			1	6						
Nausea	1	6	3	19						
Vomiting	1	6	2	13						
Infection										
Febrile neutropenia					1	6				
Flu	1	6								
Blood					1	6				
Infection with normal ANC			1	6	1	6				
Bladder (urinary)			1	6						
Metabolic/laboratory										
Alkaline phosphatase	3	19								
ALT	4	25	2	13						
AST	4	25								
Bilirubin	5	31	2	13						
Cholesterol	1	6								
GGT					2	13				
Hyperglycemia	1	6	2	13	3	19				
Hyperkalemia	1	6								
Hypertriglyceridemia	2	13								
Hyperuricemia	1	6			1	6				
Hypoalbuminemia	3	19	1	6						
Hypoglycemia	1	6								

Table 4 continued

	Grade I		Grade II		Grade III		Grade IV		Grade V	
	N	%	N	%	N	%	N	%	N	%
Hypokalemia	3	19	1	6						
Hyponatremia	2	13								
LDH	1	6								
Neurology										
Confusion					1	6				
Dizziness	1	6			2	13				
Memory impairment			1	6						
Mood alteration Anxiety			1	6						
Brain edema					1	6				
Insomnia			1	6						
Instability	1	6								
Neuropathy-sensory	1	6								
Seizures			1	6						
Somnolence	3	19			2	13				
Speech impairment					1	6				
Syncope (fainting)					1	6				
Tremor	1	6								
Ocular/visual										
Blurred vision	1	6								
Diplopia					1	6				
Watery eyes	1	6								
Pain										
Abdomen NOS	1	6								
Bone	1	6								
Joint	1	6								
Neck	1	6								
Head/headache	2	13	2	13	1	6				
Pulmonary/upper respiratory										
Cough	1	6								
Hiccoughs	1	6								
Embolism									1	6
Feeling of choke	1	6								
Nasal congestion	1	6								
Renal/genitourinary										
Cystitis	1	6								
Incontinence, urinary	1	6								
Sexual/reproductive function										
Bloody vaginal secretion	1	6								

The worst grade of each AE per patient is reported

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