

## Continuous low-dose temozolomide and celecoxib in recurrent glioblastoma

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**Abstract** Even after gross tumor resection and combined radiochemotherapy, glioblastomas recur within a few months. Salvage therapy often consists of rechallenging with temozolomide in a dose-intensified schedule. Previously, low-dose metronomic temozolomide in combination with cyclo-oxygenase 2 inhibitors has had a beneficial effect as first-line treatment for glioblastoma. We report our experience with this procedure in recurrent glioblastomas after standard treatment. From June 2007 to April 2009, 28 patients with recurrent glioblastoma received continuous low-dose temozolomide of 10 mg/m<sup>2</sup> twice daily and 200 mg celecoxib. Before therapy the recurrent tumor was resected in 19 of 28 patients. Microvessel density (MVD) was determined by immunohistochemistry in 19 patients, and MGMT promoter methylation status, using the pyrosequencing method, was determined in 17 patients. In 14/28 patients, positron emission tomography with [F-18]-fluoroethyl-L-tyrosine (FET-PET) was performed. Tumor progression was defined by the Macdonald

criteria on MRI every 8–12 weeks or by clinical deterioration. The median time to progression was 4.2 months. Progression-free survival (PFS) after 6 months was 43%. Except for a lymphopenia in one patient, there was no grade 3 or 4 toxicity. PFS did not correlate with MVD or MGMT status. A high FET uptake correlated with tumor control after 6 months under therapy ( $P = 0.041$ ,  $t$ -test). Low-dose continuous temozolomide in combination with celecoxib seems to have activity in recurrent glioblastoma without relevant toxicity. High FET uptake correlated with a better outcome under metronomic therapy.

**Keywords** Glioblastoma · Recurrence · Anti-angiogenesis · Metronomic · FET-PET · MGMT

### Introduction

Despite advances in surgical techniques, radiotherapy, and chemotherapy, the prognosis of glioblastoma patients remains poor. Tumor resection followed by radiotherapy with concomitant and adjuvant temozolomide chemotherapy is the standard primary treatment [1]. Because there is no standard therapy in recurrent glioblastoma, various treatment strategies have been reported. Besides the introduction of new drugs, rechallenging with temozolomide in dose-intensified schedules has been reported and is currently the subject of ongoing trials. Although comparative data are lacking, schedules with reduced dose and prolonged treatment (75–150 mg/m<sup>2</sup> temozolomide) seem to be more efficient than conventional maximum tolerated dose (MTD) regimens [2–4]. In parallel, metronomic low-dose schedules, with daily administration of temozolomide at a reduced dose of down to 1/10 of the MTD, have been shown to exert a beneficial effect on tumor growth [5–8].

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Conceptually, with this metronomic low-dose scheduling of temozolomide, the tumor endothelial cells, and hence glioma angiogenesis, becomes the primary target, promising efficacy even in the light of acquired tumor cell resistance. Previously, we have reported the feasibility and tolerability of continuous or metronomic low-dose temozolomide (20 mg/m<sup>2</sup>/daily), which we combined with a cyclooxygenase-2 inhibitor to enhance its antiangiogenic activity [7, 8]. In this paper we report our experience with continuous low-dose temozolomide and celecoxib (LDTC) in patients with relapsed glioblastoma following standard treatment. In order to identify predictors for treatment response, we analyzed the relationship of O6-methylguanine-methyltransferase (MGMT) promoter methylation status [9], microvessel density, and FET uptake to PFS in the subgroup of patients who underwent recurrent tumor resection and FET-PET investigation.

## Materials and methods

Between June 2007 and April 2009 all patients with histologically proven glioblastoma and diagnosis of recurrent disease, according to the Macdonald criteria [10], a Karnofski performance score of at least 70%, and who gave informed consent for treatment, received continuous low-dose temozolomide in combination with celecoxib. Of note, between August 2008 and January 2009 nine patients with first glioblastoma recurrence were recruited for the SURGE 01-07 trial (EudraCT: 2007-002142-37). Twenty-eight consecutive patients were included in this retrospective analysis. The local ethics committee gave approval for this work (EA2/144/09). All but four patients who received additional nitrosourea-based treatment of previously progressive disease presented with the first tumor recurrence after concomitant and adjuvant temozolomide treatment. For details see Table 1.

## FET-PET

Fourteen patients were studied by FET-PET before resection or treatment of recurrent tumor. The patients adhered to a low-protein diet for 12 h before PET investigation. Image acquisition started 10 min after intravenous injection of 185–220 MBq *O*-(2-[F-18]-fluoroethyl)-L-tyrosine. The PET/CT was performed 10 min p.i., with acquisition of helical CT data immediately before acquisition of 3D emission data (10 min. per bed position; two bed positions/subject) using the Siemens Biograph 16 scanner. PET data were reconstructed iteratively. The maximum standard uptake value (SUV<sub>max</sub>) and the background activity were calculated by measurement of the opposite non-tumor bearing side in unaffected remote brain tissue (SUV<sub>brain</sub>).

**Table 1** Characteristics of the 28 patients

Patient characteristic	Data
Age, years	
Median	56
Range	27–76
Sex	
Male	20 (71%)
Female	8 (29%)
KPS	
Median	80%
Range	70–100%
Number of recurrence before treatment	
1	24
2	4
Prior treatment patients (median number of cycles, range)	
RT/TMZ	24
Radiotherapy completed	24
Concomitant temozolomide completed	23
Adjuvant temozolomide	18 (4 cycles, 1–21)
RT/CCNU + TMZ	1
Radiotherapy completed	1
Adjuvant CCNU	1 (4 cycles)
Salvage temozolomide	1 (6 cycles)
RT/TMZ + PCV	3
Radiotherapy completed	3
Adjuvant temozolomide	3 (7 cycles, 5–7)
Salvage PCV	3 (2 cycles, 1–6)
Time from first operation to treatment (months; first recurrence only)	
Median	8.5 (6.8)
Range	3.2–30.6 (3.2–30.6)
Resection before treatment	
Yes	19
No	9

KPS, Karnofsky performance score; RT, radiotherapy; TMZ, temozolomide; CCNU, lomustine; PCV, procarbazine, CCNU, and vincristine

The relative FET uptake (SUV<sub>ratio</sub>) was determined by dividing SUV<sub>max</sub> by SUV<sub>brain</sub>.

## Microvessel density (MVD) evaluation

Nineteen patients underwent surgical resection of the recurrent tumor. The formalin-fixed and paraffin-embedded tissue was investigated with standard H&E staining and immunohistochemistry using a monoclonal anti-human CD31 (1:100, Dako, Germany) antibody, the secondary antibody, and a streptavidin–biotin peroxidase amplification kit (Ventana, Tucson, AZ, USA). In the area with maximum tumor vessel density a representative digital image was obtained. The percentage of pixels with specific

CD31 staining were determined and counted using Adobe Photoshop 9.0.2 (Adobe Systems, San Jose, CA, USA).

#### Methylation analysis of the MGMT promoter

##### *DNA extraction and bisulfite treatment*

Tissue fragments selected for DNA extraction were pre-examined by histology to document the histopathological appearance of the specimen and exclude contaminating normal or necrotic tissue. Only fragments with a tumor cell content of at least 80% were included. Because of lack of tumor tissue two patients had to be excluded from DNA analysis. DNA was isolated from tumor tissue using the Qi-Amp DNA minikit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. To analyze the methylation status of *MGMT*, genomic DNA (500 ng) of 17 recurrent glioblastomas, was treated with sodium bisulfite using the EZ DNA Methylation Gold kit according to the specified procedure (Zymo Research, Orange, CA, USA). Genomic DNA from white matter of non-tumorous lesions was used as internal control.

##### *Pyrosequencing*

The pyrosequencing methylation assay was performed with the PyroMark MGMT kit (Biotage, Uppsala, Sweden) on a PSQTM96 MA system (Biotage, Uppsala, Sweden), according to the manufacturer's procedure. The PyroMark MGMT kit detects the level of methylation of five CpG sites located in the first exon of the *MGMT* gene. A cytosine not followed by a guanine, and which is therefore not methylated, serves as an internal control for completion of the bisulfite treatment.

##### *Metronomic therapy*

All patients received temozolomide 10 mg/m<sup>2</sup> b.i.d. and celecoxib 200 mg q.d. Blood cell count, and kidney and liver function were checked every two weeks. Toxicity was categorized according to the common toxicity criteria (CTC) version 3.0. MRI scans were performed every 8–12 weeks. Tumor progression was defined either by progression of the contrast-enhancing tumor according to the Macdonald criteria [10] or by clinical deterioration, defined as drop of KPS below 60%. The location of recurrence was defined as “local” if the recurrent tumor was in contact with the residual tumor or cavity after tumor resection, and was defined as “distant” if not.

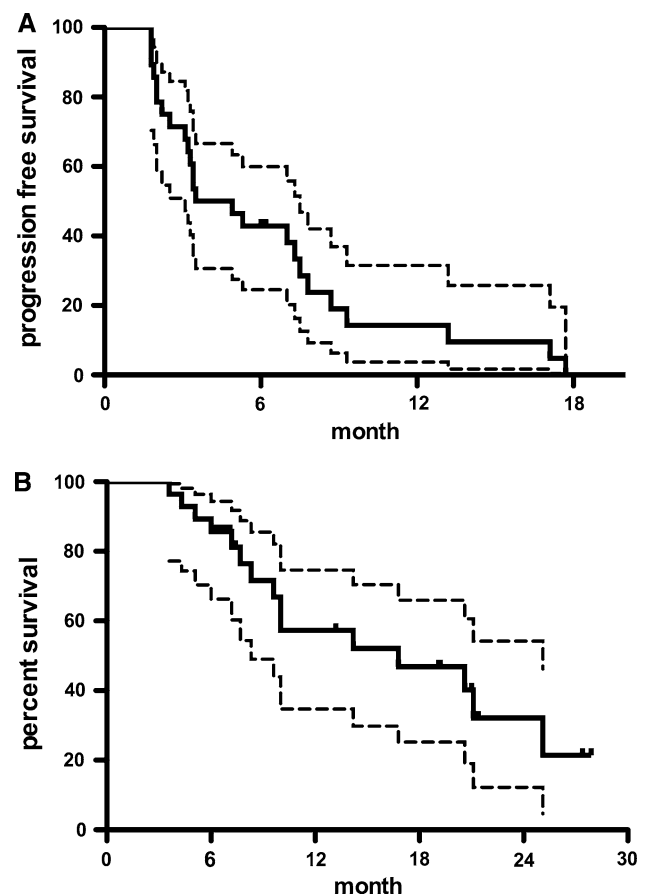
##### *Data analysis*

Progression-free survival (PFS) was the main endpoint in this retrospective analysis, defined as the time from

treatment start to tumor progression. Overall survival (OS) was defined as the time from treatment start to death or last contact. Statistical tests were linear regression, Student's *t*-test, and Mantel Cox log rank test. The statistics were performed with GraphPad Prism version 5.00 for MacOS (GraphPad Software, San Diego, California, USA; [www.graphpad.com](http://www.graphpad.com)).

## Results

Under LDTC 10 patients had progressive disease (35%), 15 patients had stable disease (54%), and three patients had a minor response, i.e., tumor reduction of at least 25%. No remission according to the Macdonald criteria had been observed. During further follow up tumor progression occurred in 25 of 28 patients. The median duration of treatment until progression was 4.2 months. Progression-free survival after six months (PFS6) was 43% (Fig. 1a).



**Fig. 1** **a** Progression-free survival (PFS) of 28 patients with recurrent glioblastoma and continuous low-dose temozolomide in combination with celecoxib (LDTC). The median PFS is 4.2 months and the PFS at 6 months is 43% (dotted line: 95% confidence interval). **b** Overall survival (OS) of 28 patients with recurrent glioblastoma and LDTC. The median OS is 16.8 months and the OS at 6 months is 86%

**Table 2** Further salvage treatment of the 25 patients, with tumor progression

Tumor resection	5
Avastin/irinotecan	7
Conformal radiotherapy	2
PCV	5
Sunitinib	1
Ongoing metronomic low dosed TMZ/Cxb	3
Only palliative	9

Except for the patients receiving only palliative treatment, multiple mentions are possible

The Karnofsky performance status (KPS) at the beginning of therapy was the only clinical predictor for prolonged progression-free survival (KPS  $\leq$  80%: 3.2 months median PFS vs. KPS  $\geq$  90%: 7.8 months median PFS;  $P = 0.029$ , log rank). No significant difference in PFS was found in relation to age  $>55$  years ( $P = 0.815$ , log rank) or resection before therapy ( $P = 0.816$ , log rank). Median overall survival (OS) after first tumor relapse was 16.8 months with an OS after 6 months of 86% (Fig. 1b). KPS reappeared as the only clinical predictive factor for OS, also (KPS  $\leq$  80%: 9.6 months median OS vs. KPS  $\geq$  90%: 25.1 months median OS;  $P = 0.0025$ , log rank). No change in OS was found due to age less than 55 years ( $P = 0.232$ , log rank) and resection of recurrent tumor ( $P = 0.992$ , log rank). After tumor progression under LDTC, 11 of the 25 patients received palliative treatment only. The therapy modalities in the remaining 14 patients are shown in Table 2. Of note, three patients continued LDTC resulting in a clinically stable state for 3–6 months despite progressive disease according to MRI scans.

Relevant toxicity according to CTC grade 3 occurred in one of 28 patients (3.5%) with lymphopenia of 258/nl. In this case temozolomide was continued and cotrimoxazol was added as PCP-prophylaxis. Lymphocyte count spontaneously recovered to more than 400/ $\mu$ l after two weeks. CTC grade 2 toxicity appeared as lymphopenia and thrombocytopenia in one patient each. Infections presented as facial zoster, tooth infection, and multiple soft tissue abscesses in one patient each. All toxicities were reversible. One patient with signs of allergy to celecoxib omitted celecoxib after two weeks and had early tumor progress after 4.9 months. The temozolomide was very well tolerated with no nausea or vomiting despite the absence of antiemetic medication in all patients.

#### Patterns of recurrence

Out of 25 patients with tumor progression, in all but two patients the tumor recurrence was revealed by MRI scan. In

one patient, the tumor progression was based on CT findings, in another one on clinical deterioration. The location of the recurrent tumor was “local” in 15/23, “distant” in 3/23, and both in 5/23 patients. Thus, distant recurrence occurred in 35% of the patients (Fig. 2).

#### FET-PET

The median SUV<sub>ratio</sub> of the 14 patients was 3.8 (range from 2.5 to 7.5). Patients with stable tumor at six months had a higher mean FET uptake than patients with early tumor progression under LDTC (SUV<sub>ratio</sub> 5.1 vs. 3.7, respectively;  $P = 0.041$ , *t*-test, Fig. 3).

#### Histological evaluation and MGMT promoter methylation

The median microvascular density was 3% (range 0.4–8%). Vascular morphology appeared very heterogeneous, as shown in Fig. 4. There was no significant correlation between vascular density and PFS under therapy ( $P = 0.215$ , *t*-test, Fig. 5). Likewise a vascular density of more than 3.5% did not correlate with better course under LDTC ( $P = 0.594$ , *t*-test). However, FET uptake correlated with microvascular density ( $R^2 = 0.4265$ ,  $P = 0.0155$ , Fig. 6).

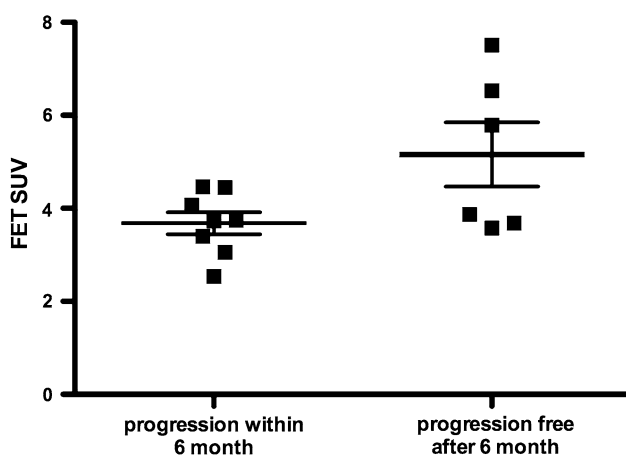
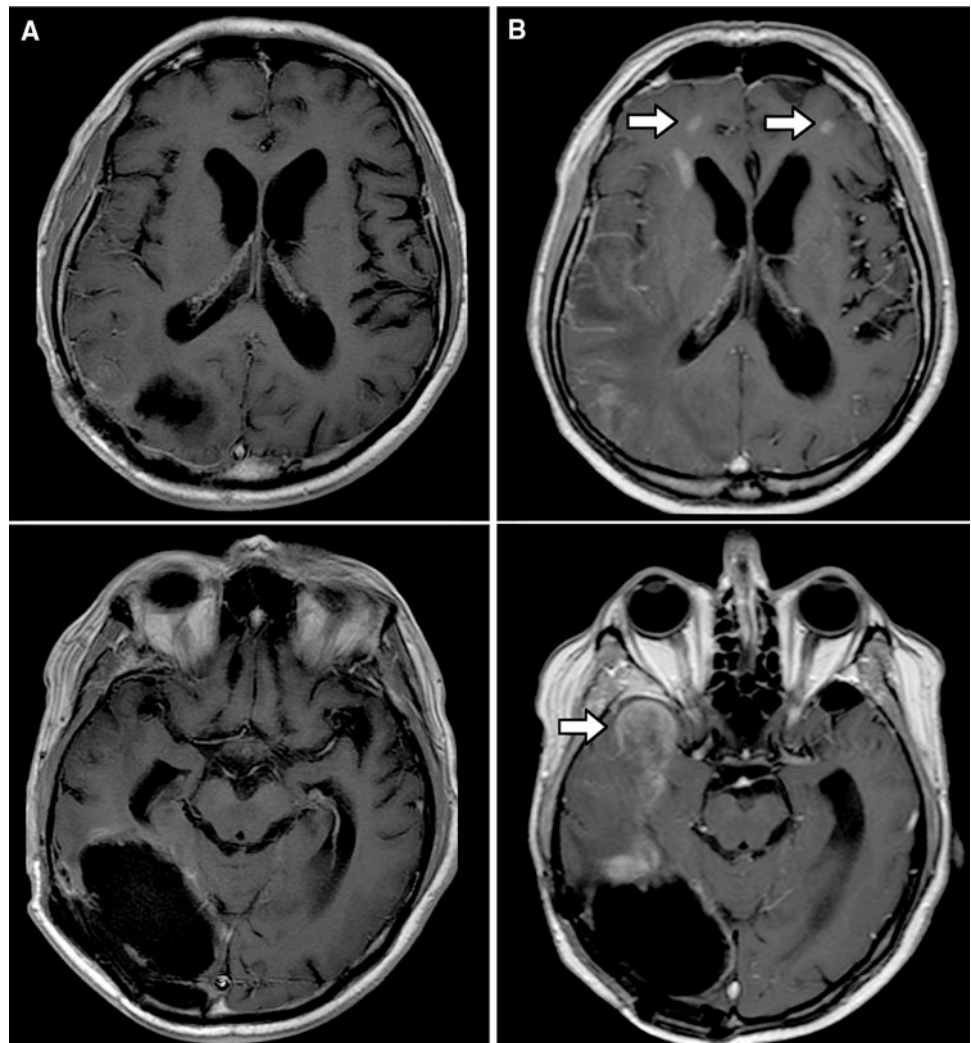
The MGMT promoter was methylated in 8/17 (47%) patients. Methylation of the MGMT promoter did not correlate with a prolonged time to progression ( $P = 0.436$ , log rank, Fig. 7) or prolonged overall survival ( $P = 0.512$ , log rank).

#### Discussion

Although radiotherapy combined with concomitant and adjuvant temozolomide has become standard first-line therapy for glioblastoma patients [1], a standard therapy for tumor recurrence is lacking. Evaluating new dosing schedules and substances in the absence of a standard treatment, giving a potential control arm for phase III trials, mainly phase II trials and single armed series have been reported in treatment of recurrent glioblastoma. Because overall survival (OS) depends on further salvage treatment [11], and median progression-free survival (PFS) depends on MRI timing, the PFS after six months (PFS6) has become a common surrogate end point for comparison of treatment efficiency.

In this retrospective analysis we report a PFS6 of 43% for patients with recurrent glioblastoma after administration of metronomic low-dose temozolomide in combination with celecoxib. Although all patients in our series were pretreated with temozolomide, we observed a better PFS than the 21% when applying a 5 in 28 days cyclic schedule

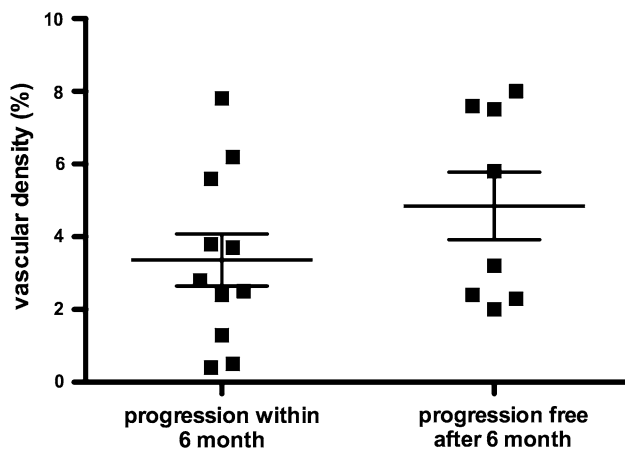
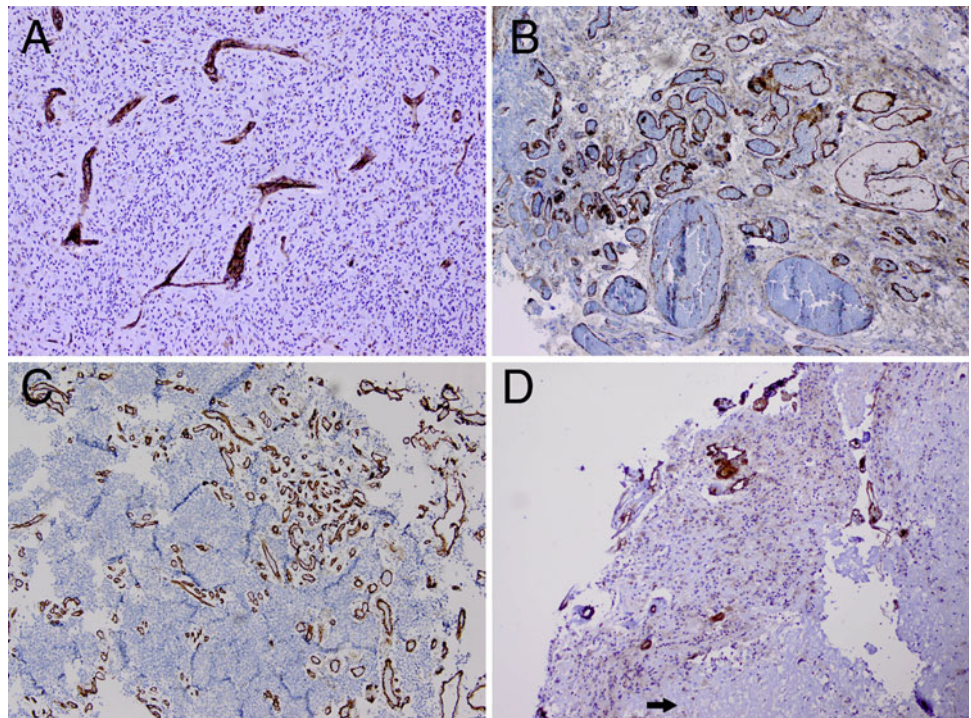
**Fig. 2** Sixty-five-year-old male patient after resection of recurrent glioblastoma. MRI scan with gadolinium enhancement at the beginning (a) and three months after continuous low-dose temozolomide in combination with celecoxib (b). Note the combined local and distant (arrows) pattern of tumor recurrence



**Fig. 3** Scatter dot plot depicting FET standard uptake value (SUV) of the recurrent glioblastoma in correlation with MRI findings after six months of treatment. Patients with high FET uptake tend to have a stable disease after six months ( $P = 0.041$ ;  $t$ -test; lines: mean values with standard error of mean)

with 200 mg/m<sup>2</sup> temozolomide in a prospective trial with chemo-naïve patients [4]. Wick et al. presented a one-week-on/one-week-off schedule resulting in a PFS6 of 44–48% for a total of 85 glioblastoma patients [2, 12], but with 14.4% of the patients experiencing grade 4 toxicity. A PFS6 of 30.3% was achieved when the treatment was prolonged to 21 of 28 days with 75 mg/m<sup>2</sup> temozolomide [3]. However, this regimen led to grade 3 lymphopenia in 24.2% of the patients. Perry et al. rechallenged glioblastoma patients with 50 mg/m<sup>2</sup> continuous temozolomide and reported a PFS6 of 57% in 14 patients at first recurrence after concomitant and adjuvant temozolomide chemotherapy. Again, the only severe toxicity was grade 3 lymphopenia in 10% of the patients [12]. As there is no sufficient treatment in patients with recurrent glioblastoma the toxicity should be a major issue in future approaches. The procedure reported in our series reveals CTC grade 3 toxicity consisting of lymphopenia in 1/28 patient only. Of note, lymphopenia, especially, seems to be related to

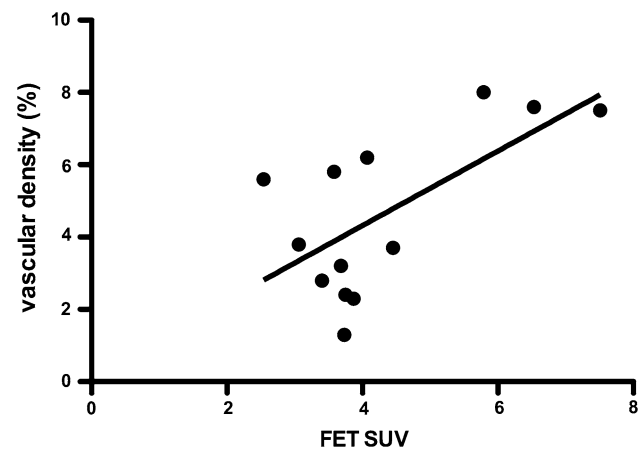
**Fig. 4** Immunohistochemical staining of endothelial cells of recurrent glioblastoma (anti-CD31-antibody, 200× magnification). The variety of findings include low microvascular density with endothelial proliferation (a), thin endothelium with prominent vessel lumina (b), high microvascular density with fine and delicate vessel walls (c), and focally cell-free tissue (arrow) with residual vital tumor cells (d)



**Fig. 5** Scatter dot plot depicting vascular density of the recurrent glioblastoma in correlation with MRI findings after six months of treatment. Vascular density does not correlate with tumor control under LDTC. ( $P = 0.215$ ;  $t$ -test; lines: mean values with standard error of mean)

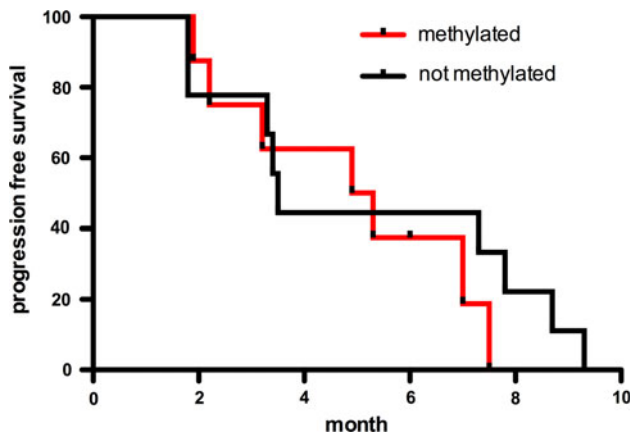
prolonged or metronomic dosing schedules [3, 12–14]. On the other hand, in alternating, high-dose temozolomide procedures, leukopenia and thrombopenia seemed to be the major dose-limiting [2, 4] or even fatal [15] events. However, we did not observe any CTC grade 3 or 4 leukopenia or thrombopenia when treating with metronomic low-dose temozolomide.

In our series only three patients had minor tumor regression and none had remission according to the Macdonald criteria, similar to the low response rate in the first



**Fig. 6** Scattergram showing the correlation of FET SUV<sub>ratio</sub> with microvessel density ( $R^2 = 0.4265$ ;  $P = 0.0155$ )

line administration of metronomic therapy [7]. On the other hand, high tumor response rates of 34–57% are reported when treating recurrent glioblastoma with antibodies against vascular endothelial growth factor (VEGF), i.e., bevacizumab combined with irinotecan [16, 17]. However, the PFS6 of 42–46% in the latter studies is similar to 43% in our series. As the effect of VEGF, in particular, causes contrast enhancement in glioblastomas, it is questionable whether the response in terms of Macdonald criteria always mirrors biological tumor response [18]. Although celecoxib can downregulate VEGF via blocking COX-2, our findings seem to suggest this pathway is not as efficient as using a VEGF antibody.



**Fig. 7** Progression-free survival (PFS) of 17 patients with recurrent glioblastoma and continuous low-dose temozolomide in combination with celecoxib (LDTC) with methylated and non-methylated MGMT promoter. Methylation of the MGMT promoter has no effect on progression-free survival ( $P = 0.436$ , log rank)

One important pitfall in interpreting treatment of recurrent glioblastoma is the possible treatment of pseudoprogression [13, 14]. As reported by Chamberlain and colleagues, most frequently within three months up to 13.7% of patients treated with combined radiochemotherapy reveal progressive contrast enhancement without histological evidence of tumor recurrence [13]. Besides the histological evaluation of tumor progression, FET-PET has proved to discriminate tumor recurrence from therapy-related changes [15]. In our series, 19 of 28 patients received resection of the recurrent tumor, and tumor recurrence could be confirmed histologically. In 13 of these 19 patients FET-PET also indicated tumor recurrence. In one patient the recurrent tumor was proved by FET-PET only. Of the remaining eight patients without confirmation of recurrence, five patients revealed tumor progression later than six months after radiotherapy. Interestingly, two of the remaining three patients with early progression again progressed early after eight weeks of LDTC. On the other hand in one patient of our series with progressive contrast enhancement three months after initiation of radiotherapy the FET-PET excluded tumor progression, thus adjuvant treatment was continued for another four months. We conclude that the control rate observed in this series is unlikely to be because of treatment of pseudoprogression. This estimate is well supported by subgroup analysis of the 20 patients with proved recurrence resulting in a median PFS of 5.1 month and a PFS6 of 45%.

We observed median overall survival of 16.8 months in 28 consecutive glioblastoma patients after at least first tumor relapse. These data are superior to overall survival after treatment of first recurrence of glioblastoma with alternating temozolomide schedule (11.1 and 7.8 months [3, 4]), procarbazine (5.9 months [4]), bevacizumab/irinotecan

(8.7–11.5 months; 94/119 patients at first relapse [16, 17]), and PCV (9.8 months [18]). Besides the LDTC itself, the high median survival achieved in our series might be because of the consequent salvage treatment as proposed previously [11] and specified in Table 2. One might speculate whether there is a long-term alkylating effect, even after stopping temozolomide [19].

In our series only three of 26 patients (12%) had local tumor control with solely distant progression. This is clearly less than 39% or 62.5% in our previous reports after administration of low-dose metronomic temozolomide with COX-2 inhibitors as first-line therapy [7, 8]. Possibly the adjuvant radiotherapy in first-line treatment causes the higher local control, whereas none of the patients in our current series had radiotherapy parallel to treatment. As five of 26 patients in our study had combined local and distant recurrence (Fig. 2), the total distant recurrence rate is 35%, which is approximately the same as findings in first-line treatment. Thus, the biological activity of the metronomic chemotherapy seems to be similar, even if temozolomide was previously given in an alternating manner. After antiangiogenic treatment with bevacizumab and irinotecan distant recurrence as high as 61% was reported [16]. Then again, after non-antiangiogenic therapy 4% distant recurrence occurred and, interestingly, not even local carmustine application could achieve better local control [20].

In contrast with low-dose metronomic temozolomide in combination with COX-2 inhibitors as first-line treatment [7], vascular density did not seem to be a significant predictive characteristic for treatment response in our series of recurrent glioblastoma. Confirming our findings, Hau et al., also, found no correlation between vascular density and response to antiangiogenic therapy in recurrent glioblastoma [21]. In our series the histological findings of recurrent glioblastomas seemed to be highly heterogeneous with areas of radio and/or tumor necrosis, almost cell free gliotic areas, vital tumor, actinic-induced vascular proliferation, and inflammation. Because all patients received radiation therapy after the first operation, which itself reduces vascular density [22], these findings were to be expected.

It has been reported that even the lack of solid tumor in histological specimens after resection of recurrent glioblastoma did not correlate with clinical outcome [23]. The common histological examination of recurrent glioblastoma is, also, probably highly prone to sampling errors and does not seem to mirror whole tumor vascularity sufficiently.

In our series, MGMT methylation status had no prognostic impact. Except for the small sample size and the retrospective setting, these data are of high value. First, the tissue samples were obtained immediately before the metronomic treatment. Thus, selection of methylated tumor

cells or induction of MGMT by previous radiochemotherapy can be ruled out [24]. Second, the pyrosequencing method used in our work has shown to be reliably and highly predictive for standard high-dose temozolomide administration [25]. But in our series MGMT promoter methylation failed to predict a favorable PFS. This could be explained by the fact that metronomic therapy preferentially acts on endothelial cells [7], where MGMT expression might be unresisted, even if the MGMT analysis of the tumor reveals a methylated promoter [26]. On the other hand, prolonged dosing of temozolomide may deplete MGMT and overcome the effect of an unmethylated MGMT promoter [27, 28]. However, the role of MGMT depletion by continuous low-dose temozolomide, especially in endothelial cells, has not yet been elucidated.

Fourteen patients were studied by FET-PET before LDTC. Surprisingly, the patients with higher FET uptake had a longer PFS under LDTC. This seems not to be in line with previous studies, which uniformly reported a high FET uptake to be associated with malignant lesions and poor prognosis [29–31]. One possible explanation of our findings is that FET uptake correlates with vascular density, as shown in our series (Fig. 6) and previously in non-contrast-enhancing gliomas [32]. Therefore, a high FET uptake might be predictive of response to therapy with potential antiangiogenic activity [7, 33]. The role of FET uptake as a predictor of response to antiangiogenic treatment should be evaluated in future prospective studies.

## Conclusions

Low-dose continuous temozolomide in combination with celecoxib seems to have activity against recurrent glioblastoma without relevant toxicity. High FET uptake correlated with a better outcome under metronomic therapy.

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