HAL
open science

Predominant influence of MGMT methylation in non-resectable GBM after radiotherapy plus temozolomide

Niklas Thon, Sabina Eigenbrod, Eva Maria Grasbon-Frodl, Juergen Lutz, Simone Kreth, Gabriele Poepperl, Claus Belka, Hans A Kretzschmar, Joerg Christian Tonn, Friedrich-Wilhelm Kreth

To cite this version:

Niklas Thon, Sabina Eigenbrod, Eva Maria Grasbon-Frodl, Juergen Lutz, Simone Kreth, et al.. Predominant influence of MGMT methylation in non-resectable GBM after radiotherapy plus temozolomide. Journal of Neurology, Neurosurgery and Psychiatry, 2010, 82 (4), pp.441. 10.1136/jnnp.2010.214593 . hal-00578711

HAL Id: hal-00578711

https://hal.science/hal-00578711v1

Submitted on 22 Mar 2011

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Predominant influence of *MGMT* methylation in non-resectable glioblastoma after radiotherapy plus temozolomide

Running title: ***MGMT* methylation in non-resectable glioblastoma**

Niklas Thon, Sabina Eigenbrod, Eva M. Grasbon-Frodl, Juergen Lutz, Simone Kreth, Gabriele Popperl, Claus Belka, Hans A. Kretzschmar, Joerg-Christian Tonn, Friedrich W. Kreth

Department of Neurosurgery (N.T., F.W.K., J.-C.T.), Center for Neuropathology and Prion Research (S.E., H.A.K., E.M.G.), Department of Clinical Radiology (J.L.), Department of Anaesthesiology (S.K.), Department of Nuclear Medicine (G.P.), Department of Radiation Oncology (C.B.), University Hospital Großhadern, Ludwig-Maximilians-Universität München, Marchioninistrasse 15, 81377 Munich, Germany

Correspondence to:

Friedrich W. Kreth, MD

Department of Neurosurgery, Ludwig-Maximilians-Universität München

Marchioninistr. 15, 81377 Munich, Germany

phone: +49-7095-3555, fax: +49-7095-6292

e-mail: fkreth@med.uni-muenchen.de

Previous presentations:

Parts of the study have been presented as an oral presentation at the SNO's 13th Annual Scientific Meeting, Lake Las Vegas Resort, November 22-23, 2008, and as a poster presentation at the 2009 AACR Annual Meeting, April 18-22, 2009, Denver, CO.

Licence for Publication:

The Corresponding Author has the right to grant on behalf of all authors and does grant on behalf of all authors, an exclusive licence (or non exclusive for government employees) on a worldwide basis to the BMJ Publishing Group Ltd to permit this article (if accepted) to be published in JNNP and any other BMJPGL products and sublicences such use and exploit all subsidiary rights, as set out in our licence.

Competing Interest:

None declared.

Abstract:

Patients with non-resectable glioblastoma generally exhibit a poor prognosis even after radiotherapy plus concomitant and adjuvant temozolomide (XRT/TMZ→TMZ). Unfortunately, no data are available concerning the predictive value of O^6-methylguanine-DNA methyltransferase (MGMT) promoter methylation for this important subpopulation. For clarification a prospective study was conducted.

Adult patients with a non-resectable glioblastoma were included. Molecular stereotactic biopsy technique was used for tumour characterization combining histopathological diagnosis with small sample size adjusted methylation-specific PCR (MSP) and sodium bisulfite sequencing. Treatment included XRT (60 Gy in 30 fractions)/TMZ (daily dose of 75mg/m^2) → TMZ (150 to 200mg/m^2 per day for 5 days of every 28-day cycle). Primary end point was progression free survival (PFS). Secondary endpoints were overall survival (OS) and treatment response (TR).

Patients were categorized in the Radiation Therapy Oncology Group (RTOG)-recursive partitioning analysis (RPA) Classes III (N=4), IV (N=12), V (N=28), and VI (N=12). The success rates of MSP and sequence analyses were 100%. The *MGMT* promoter was methylated in 30/56 tumours, which was associated with an increased PFS (median, 56 versus 20 weeks; hazard ratio, 0.15; range, 0.07 to 0.33; $p<0.0001$), higher frequency of TR (93.3% versus 46.2%; $p=0.0008$), and increased OS (median, 104 versus 28 weeks; hazard ratio, 0.18; range, 0.08 to 0.38; $p<0.0001$). Transient perioperative morbidity was 1.8%.

In conclusion, *MGMT* promoter methylation has predominant favourable influence even for the important subpopulation with non-resectable glioblastoma. Molecular stereotactic biopsy technique is safe and effective for predictive evaluation and helps to avoid therapeutic nihilism.

Keywords: GBM, glioblastoma, *MGMT*, temozolomide, biopsy, epigenetic silencing

Introduction

The incidence of malignant gliomas has increased over the last two decades especially in the elderly subpopulation and currently lies in the range of 5 cases per 100,000.[1] Glioblastoma account for approximately 60-70% of these tumours. Recently, important progress has been achieved in the treatment of this most malignant tumour: 1. Radiotherapy plus concomitant and adjuvant temozolomide (XRT/TMZ→TMZ) has resulted in significant prolongation of both time to progression and survival as compared to XRT alone; This was demonstrated by the results of the prospective randomized trial performed by the European Organisation for Research and Treatment of Cancer (EORTC) and the National Cancer Institute of Canada (NCIC) Clinical Trials Group.[2;3] 2. Epigenetic silencing of the promoter of the gene that encodes O^6-methylguanine-DNA methyltransferase (*MGMT*) in tumour specimens has been identified as a favourable predictive factor of benefit from XRT/TMZ→TMZ; This was shown in a companion retrospective study.[4]

The impact of these findings on clinical practice, however, is still under debate: Subgroup analysis suggests that relative young and/or fit patients harbouring a tumour with a methylated *MGMT* promoter and undergoing resection rather than biopsy benefit most from XRT/TMZ→TMZ.[2] It remains unclear, to which extent unfavourable prognostic factors such as increased age, and/or a deep seated tumour location might outweigh potential benefit resulting from *MGMT* methylation.[5] The important subgroup of patients with non-resectable glioblastoma is particularly concerned: Patients having undergone biopsy only experienced the worst outcome in the EORTC/NCIC trial, which was irrespective of treatment[2] – however, no stratification by the *MGMT* methylation status was done, because this biomarker could not be analyzed from small sized biopsy specimens.[4] In this situation oncologist are confronted with unawareness and uncertainty concerning appropriate treatment strategies for those patients not suitable for open tumour resection. The current prospective

study was conducted to elucidate for the first time the prognosis of patients with non-resectable glioblastoma undergoing XRT/TMZ→TMZ after adjustment for the effects of *MGMT* promoter methylation and other relevant prognostic factors. Characterization of the tumour was performed by molecular stereotactic biopsy technique which combines state of the art histopathological evaluation with small sample size adjusted molecular genetic analysis.[6-8] The current study should not be regarded as an appendix to the EORTC/NCIC trial. Both studies differ in terms of selection criteria and applied treatment strategies.

Methods

Patients

Adult patients (age 18 to 85 years) were eligible if they had i) a supratentorial glioblastoma not suitable for gross total tumour resection (because of an eloquent tumour location and/or significant co-morbidity) with histology being proven by stereotactic biopsy, ii) no severe mass effect of the tumour demanding debulking surgery because of brain stem compression and/ or midline shift, iii) no prior history of surgery, XRT, and/or chemotherapy, iv) a Karnofsky performance score (KPS) ≥ 60,[9] and v) adequate hematologic, renal, and hepatic function [as being defined before].[3] All enrolled patients gave informed consent. The study protocol was approved by the institutional review board (AZ 216/14) of the Ludwig Maximilians University Munich, Germany.

Molecular stereotactic biopsy technique

The still new technique of molecular stereotactic biopsy has been described previously.[6-8] In brief, co-registration of computerized tomography (CT), and magnetic resonance imaging (MRI; including T1- and T2-weighted sequences) served for 3-dimensional (3D) visualization (i-plan stereotaxy®, BrainLAB®, Feldkirchen, Germany) of the tumour and the

simulation of the best trajectory. Serial biopsies were taken along a trajectory representative of the solid tumour. Using micro forceps the maximum amount of tissue per biopsy specimen was 1 mm^3. The number of specimens taken in 1mm steps along the chosen trajectory was in the range of 10-18 samples per tumour. The tissue sampling procedure was guided by intra-operative smear preparations, which were routinely performed by the attending neuropathologist: Only tumour probes next (i.e. 1mm distance) to smear preparations exclusively showing solid vital tumour tissue were used for molecular genetic analysis; A corresponding sample (level +1mm) was taken for paraffin embedding and histopathological examination using standard protocols.[8] Samples chosen for molecular genetic analysis were snap frozen immediately after withdrawal. The described biopsy technique was chosen to minimize the risk of tissue contamination (e.g. by non-neoplastic or necrotic tissue) and more importantly, to recognize contamination, if it occurs. For determination of the *MGMT* promoter methylation status, at least 2 tissue samples were collected from different sites throughout each tumour to test for both the intra-tumoural distribution of the biomarker and the reproducibility of the molecular genetic analysis. The histological diagnosis of all tissue specimens was made according to the 2007 World Health Organization (WHO) classification of tumours of the central nervous system.[10] A CT-scan was done one day after biopsy in all patients and any sequels attributed to the biopsy procedure were classified as morbidity.[11]

Methylation-specific PCR (and sequencing analysis)

Isolation of nucleic acids, bisulfite modification of DNA, methylation-specific PCR (MSP) and sequencing analyses were done as being published in detail before. [8;12] In brief, DNA isolation from tumour specimens was performed using the QIAamp® DNA Micro Kit (Qiagen, Hilden, Germany), DNA recovery from each stereotactic biopsy sample amounted to around 30-60 ng/µl. This step was followed by purification and bisulfite-modification of

DNA according to Mollemann et al..[12] MSP and sequencing analyses were performed using the specific primer pairs as described before.[13-15] “Unmethylated” and “methylated” tumours were defined according to Grasbon-Frodl et al.:[8] Sequencing of bisulfite-modified DNA indicated a methylated promoter when more than half of the CpG sites (13 of 25 CpG sites) were found to be methylated; partial methylation was defined as the cytosine and thymine peaks being equally sized or the cytosine peak being twice as high as compared to the corresponding thymine peak.

Study design

Patients were enrolled in the period from March 2006 to August 2008. Patients were categorized according to the Radiation Therapy Oncology Group (RTOG)-recursive partitioning analysis (RPA) classes.[16] The KPS was used for clinical evaluation.[9] Rating on the KPS was done independently by two investigators (NT and FWK). In case of discordantly rated performance scores a third investigator (JCT) was introduced and the most matching score was used thereafter. Histopathological diagnosis and determination of the *MGMT* methylation status were obtained within 5-7 working days. Within 3 weeks after stereotactic biopsy, patients were assigned to receive XRT/TMZ→TMZ: Treatment included XRT (60 gray in 30 fractions)/ TMZ [daily dose of 75 mg/m^2] → TMZ (150 to 200mg/m^2 per day for 5 days of every 28-day cycle). In case of long term compliance, however, TMZ was continued (at the same dose) until tumour progression occurred. At baseline evaluation, 4-6 weeks after XRT/TMZ and every 3 cycles during TMZ maintenance therapy, clinical and neuroradiologic examinations were performed (until last follow up). MRI interpretation was independently done according to the Macdonald criteria [17] by an experienced neuroradiologist (JL), who was blinded for the *MGMT* status and the follow up data of the patient. Treatment response was evaluated after the completion of 3 TMZ cycles or earlier in

case of clinical deterioration. Tumour progression had to be confirmed by further clinical and neuroradiological follow up to exclude any bias by pseudoprogression.[18] Additional metabolic imaging [19] and/or stereotactic re-biopsy were considered necessary, when further (modified) invasive treatment strategies were under consideration. Haematology was performed weekly. Adverse events were defined according to the National Cancer Institute (NCI) Common Toxicity Criteria, version 3.0. The minimum follow up after inclusion of the last patient had to be 6 months.

Statistical analysis

The hazard ratio for tumour progression was in the range of 0.48 in favour of the *MGMT* promoter methylated group in the study of Hegi et al..[4] In the absence of cytoreductive surgery we expected an even more pronounced outcome difference; this assumption was indirectly supported by the study of Chinot et al. [20] who have found highly divergent outcome scores as a function of MGMT expression after neoadjuvant TMZ of non-resectable glioblastoma [median progression free survival (PFS): 5.5 versus 1.9 months]. In the current series a hazard ratio of 0.4 or even less in favour of the *MGMT* promoter methylated group was expected. Assuming exponential survival, we estimated that a sample size in the range of 27 patients or more in each group would be sufficient to have a power of 80% to demonstrate a significant difference in PFS in favour of glioblastoma with a methylated *MGMT* promoter.

The reference point of this study was the date of the diagnosis. Primary endpoint was PFS; Secondary endpoints were treatment response (TR) and overall survival (OS). PFS and OS were analyzed by the Kaplan-Meier method [21] and compared with the two-sided log-rank test. Logistic regression models were used to analyze the association between TR and other variables. The Cox model was fitted to asses the prognostic value of the *MGMT* methylation status and other potential prognostic factors.[22] First, the importance of each variable was

tested univariately. Second, all variables were fitted together. The "BEST" model contained only variables associated with PFS or OS after the adjustment for the effects of the other variables. The distribution of patient- and tumour-related variables between *MGMT* promoter methylated and unmethylated subgroups was analyzed by the chi-squared statistics (for dichotomized variables) and the Wilcoxon test (for continuously scaled variables). $P \leq 0.05$ was considered significant. All calculations were performed using the SAS software package (version 9.1).

Results

Patient characteristics

Out of an overall number of 136 patients that have been newly diagnosed with a glioblastoma in our department between March 2006 and August 2008 a total of 56 consecutive patients (33 men, 23 women) fulfilled inclusion criteria and were enrolled in this study. Patients were categorized into RTOG-RPA class III (4 patients, 7.1%), class IV (12 patients, 21.4%), class V (28 patients, 50.0%), and class VI (12 patients, 21.4%). The median of the age distribution was 62.5 years (range, 23-85 years); 22 (39%) patients were 65 years of age or older. The median KPS was 70; 24 patients (42.9%) experienced a KPS of 70 and 13 a KPS of 60 (23.2%). Forty-eight patients (85.7%) exhibited multi-focal (6 patients) or eloquent (42 patients) tumour locations including involvement of the primary motor/ sensory cortex (10 patients), speech area (8 patients), visual cortex (3 patients), basal ganglia (16 patients), and the corpus callosum (5 patients). Stereotactic biopsy procedures were associated with a transient morbidity of 1.8% (1 patient with transient aphasia). MSP and sequencing analyses always exhibited concordant and reproducible results throughout each tumour (mean number of samples per tumour: 3; range 2-5). The overall frequency of *MGMT* methylated tumours was 53.6% (30/56) including one patient with a partially methylated promoter. Methylated

and unmethylated subpopulations did not differ with respect to patients' characteristics even though patients with a methylated *MGMT* promoter were somewhat younger (Table 1).

Table 1. Characteristics and Clinical Outcome of Patients with Glioblastoma according to the Methylation Status of the *MGMT* Promoter

		Unmeth (N=26)	**Meth** (N=30)	P- value
Characteristics				
Age (years)	median	64.5	58.5	n.s.
	< 50	7 (26.9%)	10 (33.3%)	
	50-60	3 (11.6%)	6 (20.0%)	
	> 60	16 (61.6%)	14 (46.7%)	
KPS	median	70	70	n.s.
RTOG-RPA	III	1 (3.8%)	3 (10.0%)	
	IV	6 (23.1%)	6 (20.0%)	
	V	12 (46.2%)	16 (53.3%)	
	VI	7 (26.9%)	5 (16.7%)	
Sex	male	15 (57.7%)	18 (60.0%)	n.s
	female	11 (42.3%)	12 (40.0%)	.
Tumour volume (ml)	mean (SD)	43.7 (±24.9)	38.8 (±31.2)	
Tumour side	right	10 (38.5%)	14 (46.7%)	n.s.
	left	16 (61.5%)	16 (53.3%)	
Tumour location	lobar	3 (11.5%)	5 (16.7%)	n.s.
	eloquent	21 (80.8%)	21 (70.0%)	

	multifocal	2 (7.8%)	4 (13.3%)	
Outcome				
Treatment response				0.0008
	Partial response	3 (11.6%)	17 (56.7%)	
	Stable disease	9 (34.6%)	11 (36.6%)	
	Progression	14 (53.8%)	2 (6.7%)	
Progression free survival				<0.0001
	Median (weeks)	20	56	
	Rate at 6 mo	34.6%	83.3%	
	Rate at 12 mo	0%	54.4%	
	Hazard ratio (95% CI)		0.15 (0.07-0.33)	
Overall Survival				<0.0001
	Median (weeks)	28	104	
	Rate at 6 mo	57.7%	90.0%	
	Rate at 12 mo	13.2%	64.8%	
	Hazard ratio (95% CI)		0.18 (0.08-0.38)	

Abbreviation:

SD, standard deviation; n.s., not significant; mo, month; CI, confidence interval

One patient was lost to follow up 24 weeks after biopsy without experiencing tumour progression at this time. Two patients had to be discontinued from concomitant TMZ after 3 and 4 weeks, respectively, because of grade 3 haematological toxicity and lung embolism. Adjuvant TMZ was not initiated in 3 patients because of low clinical performance (KPS<60) after XRT/TMZ, which was associated with a decline in the mental status (disorientation and confusion). TMZ maintenance therapy caused grade 1/2 toxicity in 12 patients. As a

consequence of grade 3 toxicity adjuvant TMZ had to be interrupted for 4 weeks in 2 patients and to be discontinued after 2 cycles in 1 patient (Table 2).

Table 2. Toxicity Symptoms*

	Number of patients (%) (N=56)	
NCI Grade	1 – 2	3 – 4
Myelosuppression	4 (7)	3 (5.4)
Thrombosis	m.v.	4 (7)
Nausea	9 (16)	m.v.
Infection	5 (9)	m.v.
Mental status**	7 (13)	7 (13)
Other***	4 (7)	1 (2)

Abbreviations:
NCI, National Institute of Cancer Common Toxicity Criteria; N, number of patients
m.v., missing value
* one patient can suffer from multiple symptoms
** incl. cognitive impairment, disorientation, confusion, depression, psychosis
*** incl. hypocortisolism, apoplex, cardial dysrhythmias, pruritus

Adverse events were more often seen in elder patients (median age of patients with/without adverse events: 70 versus 57.5 years; $p<0.03$). The application of more than 6 TMZ cycles (15 patients with a median number of 9 TMZ cycles) was not associated with increased toxicity. However, late grade 2 hematotoxicity was seen in 2 patients after the 11th and 14th cycle of TMZ, respectively. MRI-based suspicion of tumour progression was additionally verified by metabolic imaging [O-(2-[18F]fluoroethyl)-L-tyrosine (FET) positron emission tomography (PET)] in 20 patients and/or re-biopsy in 13 patients; the latter always revealed an unchanged *MGMT* promoter methylation status.

Clinical outcome

The median follow up time was 48 weeks (range: 24-144) for the survivors. Forty-one out of 56 patients exhibited tumour progression and 34 patients had died. Death was tumour-related in all patients. Kaplan-Meier estimates for PFS and OS of the whole study population are presented in Figure 1. Both primary and secondary end points were in favour of *MGMT* promoter methylated tumours (Table 1): *MGMT* promoter methylated tumours exhibited superior median PFS (56 versus 20 weeks) and superior median OS (104 versus 28 weeks); PFS after 6 (12) months was 83.3% (54.4%) for *MGMT* promoter methylated tumours and 34.6% (0%) for those with an unmethylated *MGMT* promoter ($p<0.0001$) (Figure 2); The corresponding 6 (12) months survival rates were 90.0% (64.8%) and 57.7% (13.2%), respectively ($p<0.0001$) (Figure 2). Among patients with *MGMT* promoter methylation the unadjusted hazard ratio for disease progression and death was 0.15 (95% confidence interval: 0.07-0.33) and 0.18 (95% confidence interval: 0.08-0.38), respectively.

Treatment responders showing partial response or stable disease (three months after treatment) were more often seen in case of a methylated *MGMT* promoter (28/30 *MGMT* methylated tumours versus 12/26 unmethylated tumours; $p=0.0008$). Treatment responders were younger and experienced a longer OS than non-responders (median: 72 versus 18 weeks; $p<0.0001$) (see Table 3).

Table 3. Characteristics of Patients with Glioblastoma stratified for Treatment Response

	Non-responder (progression)	**Responder** (regressive + stable disease)	P-value

	(N=16)	(N=40)	
Characteristics			
Median age (years)	68.5	59.0	0.04
MGMT methylated	2 (12.5%)	28 (70.0%)	0.0008
MGMT unmethylated	14 (87.5%)	12 (30.0%)	
Outcome			
Overall Survival			<0.0001
Median (weeks)	18	72	
Rate at 6 mo	37.5%	90.0%	
Rate at 12 mo	0%	58.6%	

Abbreviations: mo, months; N, number of Patients

The survival advantage, however, was less pronounced for those with an unmethylated *MGMT* promoter (median: 44 weeks versus 104 weeks, p<0.001). At the time of tumour progression, treatment concepts included second-line chemotherapy (n=16), re-irradiation (n=4), tumour debulking due to significant space occupying effects (n=2), stereotactic brachytherapy (n=3), a combination of these treatment modalities (n=8), and palliative care (n= 23). Median survival after progression was longer for methylated tumours (3.7 months versus 1.9 months); the difference, however, was not significant.

Prognostic and predictive factors

Univariately, *MGMT* promoter methylation, younger age, higher KPS scores, and RTOG-RPA classes III+IV were positively correlated with both increased PFS and OS. In the "BEST" prognostic Cox models only *MGMT* promoter methylation status (OS: p<0.0001; PFS: p<0.0001), younger age (PFS: p= 0.02), and higher KPS scores (OS: p=0.02) remained

statistically significant (Table 4).

Table 4. Prognostic Factors (univariate and multivariate models)

	PFS	OS
	P-value (Hazard ratio/95% CI)	
Univariate		
MGMT	<0.0001 (0.15/ 0.07-0.33)	<0.0001 (0.18/ 0.08-0.38)
Age	0.01 (1.03/ 1.01-1.06)	0.009 (1.04/ 1.01-1.08)
KPS	0.01 (0.95/0.92-0.99)	0.003 (0.94/0.90-0.98)
RTOG	0.04 (1.57/1.02-2.43)	0.01 (1.85/1.13-3.04)
Location of tumour	n.s.	n.s.
Volume of tumour	n.s.	n.s.
Site of tumour	n.s.	n.s.
Multivariate		
MGMT	<0.0001 (0.16/ 0.07-0.35)	<0.0001 (0.20/ 0.09-0.44)
Age	0.02 (1.03/1.0-1.06)	n.s.
KPS	n.s.	0.02 (0.95/0.91-0.99)

Abbreviations: CI, confidence interval; n.s., not significant

The adjusted hazard ratio of 0.2 (95percent confidence interval, 0.09-0.43) of *MGMT* promoter methylation for PFS was consistent with the unadjusted hazard ratio of 0.15 (95percent confidence interval, 0.07-0.33). Logistic regression analysis identified *MGMT* promoter methylation as the only predictor for TR (partial response or stable disease; p=0.0008); The prognostic influence of age (which was detected in one-variable models) was lost.

Discussion

Currently, patients with non-resectable glioblastoma undergoing XRT/TMZ→TMZ are left with uncertainties concerning their prognosis; Moreover, they are unlikely to be entered into randomized clinical trials, addressing molecular biomarkers such as *MGMT* methylation for patient stratification. Difficulties to perform molecular genetic analysis from small sized biopsy specimens have so far contributed to these barriers in knowledge and scientific evaluation.[4] Here, we show in a prospective consecutive series of 56 non-resectable glioblastoma patients, that by means of the still new technique of molecular stereotactic biopsy even this somewhat neglected patient population can be addressed: Overall survival for patients of this study cohort was similarly poor as compared to those undergoing biopsy only in the EORTC/NCIC trial.[2] Outcome measurements, however, became highly divergent after stratification for *MGMT* promoter methylation: Whereas patients harbouring a glioblastoma with an unmethylated *MGMT* promoter experienced both extremely short PFS and OS, those with a methylated promoter exhibited surprisingly long PFS and OS. Time to progression was similar to that reported by Hegi et al.[4] after cytoreductive surgery plus XRT/TMZ→TMZ. Survival after tumour progression, however, was shorter in the current series. One may speculate that the relatively large tumour volumes, which were not reduced by open surgery, have led to a relatively delayed diagnosis of tumour progression (using the Macdonald criteria [17]). It should be further noted that a considerable number of patients received more than 6 cycles TMZ in our series, which indicate an important difference to the EORTC/NCIC trial; Accordingly, any comparative analysis should be avoided at this moment.

Early tumour progression was associated with poor outcome in all patients of this series. Apparently, pseudoprogression seems to be not a frequent event in non-resectable glioblastoma patients undergoing XRT/TMZ. Partial response or tumour control (three

months after XRT/TMZ), was a strong predictor for a more favourable outcome and not unequivocally related to the *MGMT* promoter status: 93.3% of the *MGMT* methylated and, noteworthy, 46.2% of the unmethylated tumours exhibited partial response/stable disease after XRT/TMZ→TMZ. Survival, however, was significantly longer in case of a methylated *MGMT* promoter. Thus, for treatment responders with an unmethylated *MGMT* promoter, the benefit of XRT/TMZ→TMZ as compared to XRT alone continues to be unclear. Future studies are required that further elucidate the molecular network of epigenetic silencing of the *MGMT* promoter and *MGMT* gene expression in correlation with clinical course and prognosis.[23]

Molecular stereotactic biopsy turned out to be a safe (transient morbidity of as low as 1.8% in this series) and precise diagnostic procedure. The highly controlled sampling technique ensured the use of only vital tumour tissues for subsequent molecular analyses, and excluded bias of the results by non-neoplastic tissue contaminations. Handling of nucleic acids as well as routine setup for MSP and sequencing analyses was successfully adapted to the small tissue samples containing limited amounts of nucleic acids.[8] Due to these affords, there were no drop-outs due to difficulties in determining the *MGMT* promoter status: The success rates of MSP and sequencing were 100% and both methods exhibited 100% concordant findings throughout each tumour investigated. The latter indicates a homogenous intra-tumoral distribution of the biomarker. The overall frequency of tumours harbouring a methylated *MGMT* promoter was 53.6% which was slightly higher than in the study of Hegi et al. (*MGMT* promoter methylated tumours: 45%) [4]; the difference between these studies was statistically not significant and might have occurred by chance. However, the exact assignment of each biopsy specimen within the stereotactically defined tumour space, the determination of its relation to tumour necrosis or non-neoplastic tissue by alternating histopathological and molecular evaluation within 1mm-steps, the simultaneous application

of two molecular genetic methods at different sites of each tumour, and the exclusive use of cryopreserved specimens indicate important differences to the study of Hegi et al.[4].

We further demonstrate that the *MGMT* status after XRT/TMZ→TMZ remained unchanged, which was seen in 13 patients undergoing stereotactic re-biopsy because of tumour progression. Both the detected intra-tumoural homogeneous distribution of the *MGMT* promoter methylation status and its unchanged pattern before and after XRT/TMZ→TMZ suggests that *MGMT* promoter methylation may be a rather early event in tumour genesis.

In summary, the current series describes for the first time the powerful treatment effects of XRT/TMZ→TMZ for patients with non-resectable glioblastoma in case of a methylated *MGMT* promoter; It underscores that non-resectable tumours should not be associated per se with a worse prognosis as compared to those undergoing open tumour resection. The demonstrated high diagnostic sensitivity and specificity of MSP and sequence analysis in combination with stereotactic biopsy technique might help to encourage the further development and implementation of molecular genetic analysis into biopsy procedures of daily clinical practice.

Acknowledgements

Michael Ruiter is gratefully acknowledged for help with MSP and sequencing analysis. We further thank Barbara Stetter, Margit Deschner, and Anja Ewert for their support with management of the patient database. Funding did not influence collection, analysis and interpretation of the data, writing of the manuscript, and the decision to submit the paper for publication.

Reference List

1. Wen PY, Kesari S. Malignant gliomas in adults. *N Engl J Med* 2008;**359**:492-507.

2. Stupp R, Hegi ME, Mason WP, van den Bent MJ, Taphoorn MJ, Janzer RC, Ludwin SK, Allgeier A, Fisher B, Belanger K, Hau P, Brandes AA, Gijtenbeek J, Marosi C, Vecht CJ, Mokhtari K, Wesseling P, Villa S, Eisenhauer E, Gorlia T, Weller M, Lacombe D, Cairncross JG, Mirimanoff RO. 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.

3. Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ, Belanger K, Brandes AA, Marosi C, Bogdahn U, Curschmann J, Janzer RC, Ludwin SK, Gorlia T, Allgeier A, Lacombe D, Cairncross JG, Eisenhauer E, Mirimanoff RO. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N Engl J Med* 2005;**352**:987-96.

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

5. Gorlia T, van den Bent MJ, Hegi ME, Mirimanoff RO, Weller M, Cairncross JG, Eisenhauer E, Belanger K, Brandes AA, Allgeier A, Lacombe D, Stupp R. Nomograms for predicting survival of patients with newly diagnosed glioblastoma: prognostic factor analysis of EORTC and NCIC trial 26981-22981/CE.3. *Lancet Oncol* 2008;**9**:29-38.

6. Kreth S, Heyn J, Grau S, Kretzschmar, H.A:, Egensperger R, Kreth FW. Identification of valid endogenous control genes for determining gene expression in human glioma. *Neuro-Oncology* 2010;**12**:570-9.

7. Thon N, Eigenbrod S, Grasbon-Frodl EM, Ruiter M, Mehrkens JH, Kreth S, Tonn JC, Kretzschmar HA, Kreth FW. Novel Molecular Stereotactic Biopsy Procedures Reveal Intratumoral Homogeneity of Loss of Heterozygosity of 1p/19q and TP53 Mutations in World Health Organization Grade II Gliomas. *J Neuropathol Exp Neurol* 2009;**68**:1219-28.

8. Grasbon-Frodl EM, Kreth FW, Ruiter M, Schnell O, Bise K, Felsberg J, Reifenberger G, Tonn JC, Kretzschmar HA. Intratumoral homogeneity of MGMT promoter hypermethylation as demonstrated in serial stereotactic specimens from anaplastic astrocytomas and glioblastomas. *Int J Cancer* 2007;**121**:2458-64.

9. Karnofsky, D.A., Abelmann, W.H. C, L.S. The use of the nitrogen mustards in the palliative treatment of carcinoma: with particular reference to bronchogenic carcinoma. *Cancer* 1948;**1**:634-56.

10. Louis DN, Ohgaki H, Wiestler OD, Cavenee WK, Burger PC, Jouvet A, Scheithauer BW, Kleihues P. The 2007 WHO classification of tumours of the central nervous system. *Acta Neuropathol* 2007;**114**:97-109.

11. Kreth FW, Muacevic A, Medele R, Bise K, Meyer T, Reulen HJ. The risk of haemorrhage after image guided stereotactic biopsy of intra-axial brain tumours--a prospective study. *Acta Neurochir (Wien)* 2001;**143**:539-45.

12. Mollemann M, Wolter M, Felsberg J, Collins VP, Reifenberger G. Frequent promoter hypermethylation and low expression of the MGMT gene in oligodendroglial tumors. *Int J Cancer* 2005;**113**:379-85.

13. Esteller M, Garcia-Foncillas J, Andion E, Goodman SN, Hidalgo OF, Vanaclocha V, Baylin SB, Herman JG. Inactivation of the DNA-repair gene MGMT and the clinical response of gliomas to alkylating agents. *N Engl J Med* 2000;**343**:1350-4.

14. Clark SJ, Statham A, Stirzaker C, Molloy PL, Frommer M. DNA methylation: bisulphite modification and analysis. *Nat Protoc* 2006;**1**:2353-64.

15. Frommer M, McDonald LE, Millar DS, Collis CM, Watt F, Grigg GW, Molloy PL, Paul CL. A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. *Proc Natl Acad Sci U S A* 1992;**89**:1827-31.

16. Curran WJ, Jr., Scott CB, Horton J, Nelson JS, Weinstein AS, Fischbach AJ, Chang CH, Rotman M, Asbell SO, Krisch RE, . Recursive partitioning analysis of prognostic factors in three Radiation Therapy Oncology Group malignant glioma trials. *J Natl Cancer Inst* 1993;**85**:704-10.

17. 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.

18. Brandes AA, Franceschi E, Tosoni A, Blatt V, Pession A, Tallini G, Bertorelle R, Bartolini S, Calbucci F, Andreoli A, Frezza G, Leonardi M, Spagnolli F, Ermani M. MGMT promoter methylation status can predict the incidence and outcome of pseudoprogression after concomitant radiochemotherapy in newly diagnosed glioblastoma patients. *J Clin Oncol* 2008;**26**:2192-7.

19. Popperl G, Kreth FW, Herms J, Koch W, Mehrkens JH, Gildehaus FJ, Kretzschmar HA, Tonn JC, Tatsch K. Analysis of 18F-FET PET for grading of recurrent gliomas: is evaluation of uptake kinetics superior to standard methods? *J Nucl Med* 2006;**47**:393-403.

20. Chinot OL, Barrie M, Fuentes S, Eudes N, Lancelot S, Metellus P, Muracciole X, Braguer D, Ouafik L, Martin PM, Dufour H, Figarella-Branger D. Correlation between O6-methylguanine-DNA methyltransferase and survival in inoperable newly diagnosed glioblastoma patients treated with neoadjuvant temozolomide. *J Clin Oncol* 2007;**25**:1470-5.

21. Kaplan, E., Meier P. Non parametric estimation for incomplete observation. *J Am Stat Assoc* 1958;**53**:457-81.

22. Cox DR. Regression model and life-tables. *J Roy Stat Soc Ser B* 1972;187-220.

23. Everhard S, Tost J, El AH, Criniere E, Busato F, Marie Y, Gut IG, Sanson M, Mokhtari K, Laigle-Donadey F, Hoang-Xuan K, Delattre JY, Thillet J. Identification of regions correlating MGMT promoter methylation and gene expression in glioblastomas. *Neuro Oncol* 2009.

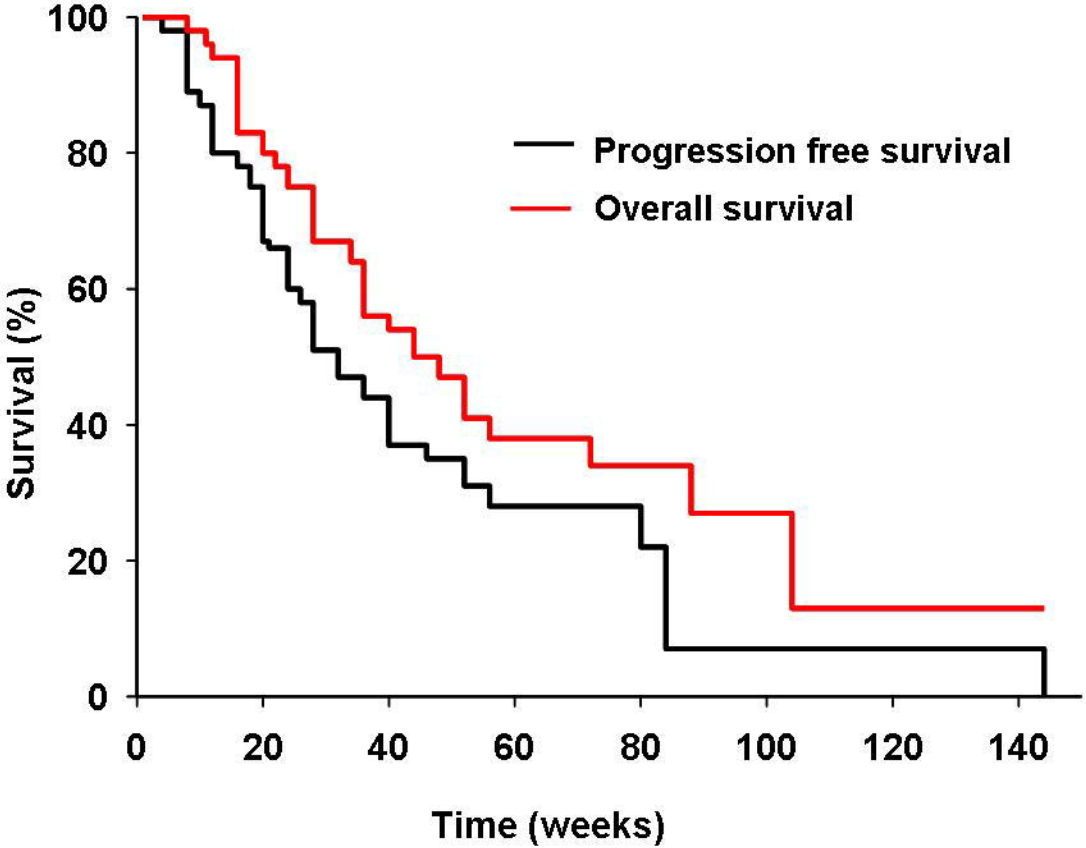
Figure Legends:

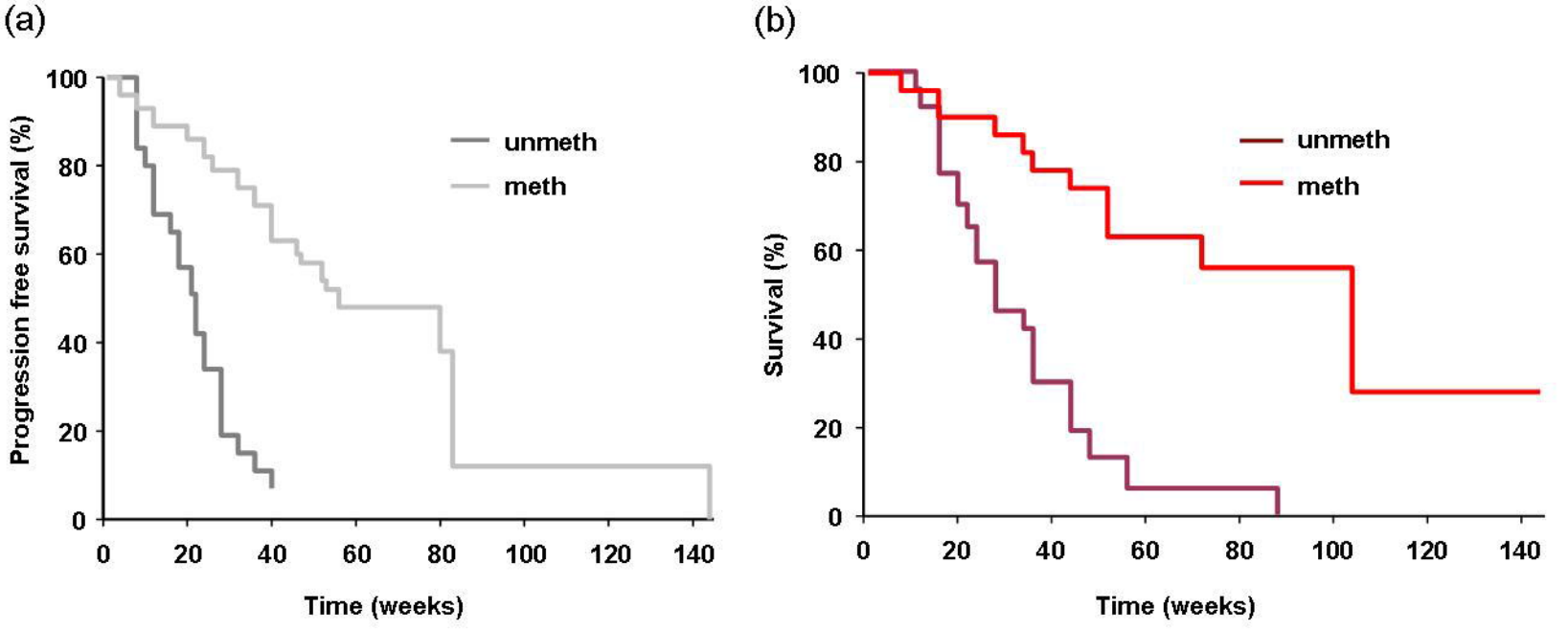
Figure 1:

Kaplan-Meier estimates of progression free survival and overall survival among patients with glioblastoma who were diagnosed by stereotactic biopsy and prospectively treated with TMZ/XRT→TMZ.

Figure 2:

Kaplan-Meier estimates of progression free survival (a) and overall survival (b) among patients with glioblastoma stratified for the *MGMT* promoter methylation status who were diagnosed by stereotactic biopsy and prospectively treated with TMZ/XRT→TMZ.


Progression free survival
Overall survival
Survival (%)
100
80
60
40
20
0
0
20
40
60
80
100
120
140
Time (weeks)


(a)
Progression free survival (%)
100
80
60
40
20
0
0
20
40
60
80
100
120
140
Time (weeks)
unmeth
meth
(b)
Survival (%)
100
80
60
40
20
0
0
20
40
60
80
100
120
140
Time (weeks)
unmeth
meth