Impact of Multifocality and Molecular Markers on Survival of Glioblastoma

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■ OBJECTIVE: **Several parameters like extent of resection and *MGMT* promotor methylation in glioblastoma (GBM) are known to influence survival. Other elements like multifocality and proliferation indices are not commonly used. The aim of the present study was to analyze routinely and not routinely assessed prognostic markers for survival of patients suffering from GBM in a single center.**

■ METHODS: **Adult cases with GBM operated at our institution were included in this survey. The association of age, Karnofsky performance status (KPS), *MGMT* promotor methylation, *Ki67* proliferation index, *IDH1/2* mutational status, and multifocality on overall survival (OS) was analyzed in univariate and multivariate cox regression models.**

■ RESULTS: **We analyzed 565 patients with a mean age of 62.2 (18–84) years. Median OS was 12.5 months. *MGMT* promoter methylation and *IDH 1/2* mutation were associated with significant better OS ($P < 0.01$). In 48 cases (8.5%), the tumor was localized in both hemispheres, which was associated with a significant worse OS than tumor infiltration of 1 hemisphere ($P = 0.039$). Mean *Ki67* proliferation index increased to 18% when both hemispheres were infiltrated. Multivariate analysis for OS revealed *IDH 1/2* wildtype (adjusted odds ratio [aOR] 4.3), higher age (aOR 4.2), unmethylated *MGMT* promotor (aOR 3.5), preoperative KPS score <70 (aOR 1.9), and multifocality (aOR 2.1) as independent parameters for worse survival.**

■ CONCLUSIONS: **This study confirms well-known parameters like *MGMT promoter* methylation, *IDH 1/2* mutational status, KPS, and age as independent prognostic factors for survival and reveals multifocality as further independent prognostic marker for survival. The dismal prognosis of multifocal involvement is associated with an increasing Ki67 proliferation index.**

INTRODUCTION

Glioblastoma (GBM) is the most common malignant brain tumor and is associated with a dismal prognosis.[1] One can differentiate primary GBM from secondary GBM. Commonly, the former rises de novo in older patients, while the latter develops from low-grade astrocytoma in younger patients. Standard therapy comprises a combination of surgical, radiotherapeutic, and chemotherapeutic approaches.[2] Despite of multiple scientifical efforts in recent years, the prognosis is still dismal which is caused by early tumor progression and recurrence. However, there are interindividual differences with some patients surviving for only a few months and others for years.[3] Functional impairment in the neurooncologic context is often measured by Karnofsky performance status (KPS) in order to classify patients' disability.[4] Research over the past decade has revealed prognostic survival factors like age, clinical performance status, and *O6-alkylguanine DNA methyltransferase* (MGMT) promotor hypermethylation, which are recognized as standard prognostic factors.[5-7]

On the other hand, there are further parameters, which could be assessed routinely like proliferation markers and multifocality. But to the best of our knowledge, these parameters have never been

Key words
- Glioblastoma
- Molecular markers
- Multifocality
- Overall survival

Abbreviations and Acronyms
3D: Three-dimensional
aOR: Adjusted odds ratio
EOR: Extent of resection
GBM: Glioblastoma
IDH: Isocitrate dehydrogenase
KPS: Karnofsky performance status
MGMT: Methylguanine methyltransferase
OS: Overall survival rate
ST: Slice thickness
TE: Echo time
TR: Repetition time

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investigated as independent survival markers. Proliferation markers like *Ki-67* are mainly expressed in activated cell cycle correlating to tumor proliferation. A significant correlation between these proliferation markers and tumor grading has been revealed.[8]

In clinical routine, one can recognize different paths of tumor propagation. Although some tumors are characterized by single-area affection, others are located in multiple areas, even spreading to the contralateral brain.

The aim of the present study was to elucidate further independent survival prognosticators to differentiate the interindividual difference in survival and their mutual dependence.

METHODS

This study was performed in accordance with the Declaration of Helsinki and was approved by the local ethics committee of the University Hospital Essen 15-6504-BO and 15-6505-BO. Informed consent was obtained.

Magnetic Resonance Imaging Scans

Patients received a preoperative magnetic resonance imaging scan to determine tumor extension and localization. The preoperative and postoperative magnetic resonance imaging protocol included a transversal T1-weighted dark-fluid-sequence (repetition time [TR] 2000 ms, echo time [TE] 13 ms, slice thickness [ST] 5 mm); 3-dimensional (3D)-fluid-attenuated inversion recovery-sequence (TR 5000 ms, TE 395 ms, ST 1 mm) with 3D-reconstructions; a transversal diffusion-weighted-imaging-sequence (TR 7900 ms; TE 101 ms; slice thickness 5 mm); a transversal susceptibility-weighted imaging-sequence (TR 26 ms, TE 20 ms, ST 2 mm); contrast-enhanced 3D-MPRAGE in sagittal orientation (TR 1790, TE 2.67 ms, ST 1 mm); and contrast-enhanced T1 in transversal orientation (TR 162 ms, TE 5.05 ms, ST 5 mm) with 3D-reconstructions. As contrast agent 1 mmol/10 kg body weight Dotarem was used (Guerbet, Sulzbach/Taunus, Germany). Tumor localization was determined on the basis of contrast-enhanced T1-weighted sequences on axial and coronal images.

Study Population

All adult patients (≥18 years) operated on for GBM between January 2006 and December 2014 in a single institution were evaluated. Inclusion criteria were a histopathologic diagnosis of GBM, according to the World Health Organization (WHO), and the potential of follow-up of at least 24 months.[1]

Patients' data were analyzed with special respect to gender, age, and preoperative and postoperative KPS. Depending on the extent of resection (EOR), operative procedures were divided in gross total resection, with a reduction of the contrast-enhanced tumor mass ≥95%; subtotal resection, where residual contrast-enhancing tumor mass is >5%; and stereotactic biopsy using Leksell stereotactic System (Elekta, Stockholm, Sweden).

Histology was confirmed in all patients, and neuropathologic specimen analysis was performed according to the World Health Organization classification of central nervous system tumors.[1]

Multifocality was divided into 3 groups with glioblastoma infiltration in a singular lobe, infiltration of >1 lobe within 1 hemisphere, and tumor infiltration of both hemispheres (Figure 1). Whenever possible, *MGMT* promoter status, *IDH* 1/2 mutation status, and *Ki67* proliferation indexes were assessed.

Immunohistochemistry

Proliferation of tumor cells was assessed via immunohistochemistry using *Ki67*-antibody (Zytomed, MSK018, dilution 1:250). For detecting isocitrate dehydrogenase (*IDH*) 1/2 mutation in gliomas, immunohistochemistry using an *anti-IDH1-R132H* mutation-specific antibody (Dianova, clone H09, dilution 1:150) was performed. Following the diaminobenzidine reaction, slides were counterstained with hematoxylin.

Sanger Sequencing of IDH1 and IDH2

All cases that were negative for the *IDH1-R132H* mutation by immunohistochemistry were interrogated by Sanger sequencing for *IDH1* exon 4 (codon 132) and *IDH2* exon 4 (codon 172). Therefore DNA extraction from formalin-fixed paraffin embedded tissue was performed using the RSC DNA formalin-fixed paraffin embedded kit (Maxwell) according to the manufacturer's

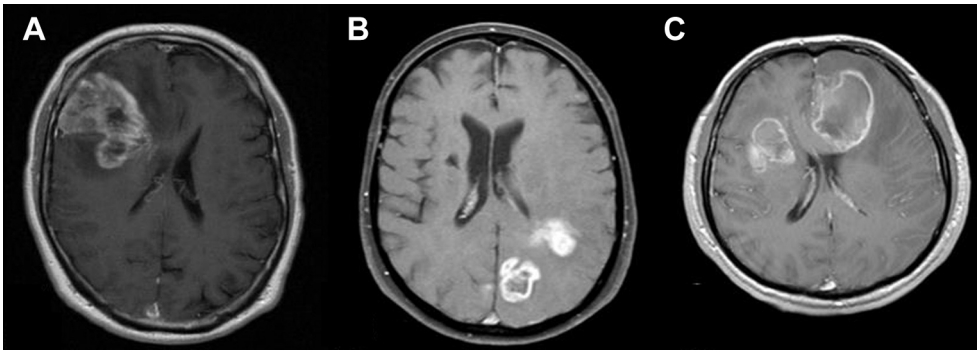

Figure 1. Multifocality in glioblastoma with single lobe infiltration (**A**), tumor infiltration of multiple lobes on 1 hemisphere (**B**), and bihemispheral infiltration (**C**).

Table 1. Study Population with Mean Age in Years, Median Overall Survival (OS) in Months, *MGMT* Promotor Methylated Tumors and Extent of Surgical Resection with Gross Total Resection (GTR), Subtotal Resection (STR), and Stereotactic Biopsy (SB)

Glioblastoma	Number = 565
Men	324 (57%)
Women	241 (43%)
Age	62.2 years (±11.2)
OS	12.5 months
MGMT methylated	160
GTR	255 (45%)
STR	136 (24%)
SB	174 (31%)

instructions. The following oligonucleotide primers were used for amplifying the sequence for codon 132 of the *IDH1* gene: *IDH1*-215F 5′-GAGAAGAGGGTTGAGGAGTTCAAG-3′ and *IDH1*-446R 5′-TCTGGGCCATGAAAAAAAAAAC-3′. The polymerase chain reaction primers for amplification of the sequence for codon 172 of the *IDH2* gene were *IDH2*-137F 5′-AACTATCCGGAACATCCTGGG-3′ and *IDH2*-360R 5′-AGTCTGTGGCCTTGTACTGCAG-3′.

MGMT Promoter Methylation Analyses

For MGMT promoter methylation analysis, DNA was isolated from paraffin sections of GBM. *MGMT* promoter methylation was analyzed by methylation-specific polymerase chain reaction. The primer sequences used to detect unmethylated *MGMT* promoter sequences were 5-TGT GTT TTT AGA ATG TTT TGT GTT TTG AT-3 and 5-CTA CCA CCA TCC CAA AAA AAA ACT CCA-3. The primer sequences used to detect methylated MGMT promoter sequences were 5-GTT TTT AGA ACG TTT TGC GTT TCG AC-3 and 5-CAC CGT CCC GAA AAA AAA CTC CG-3 [49].

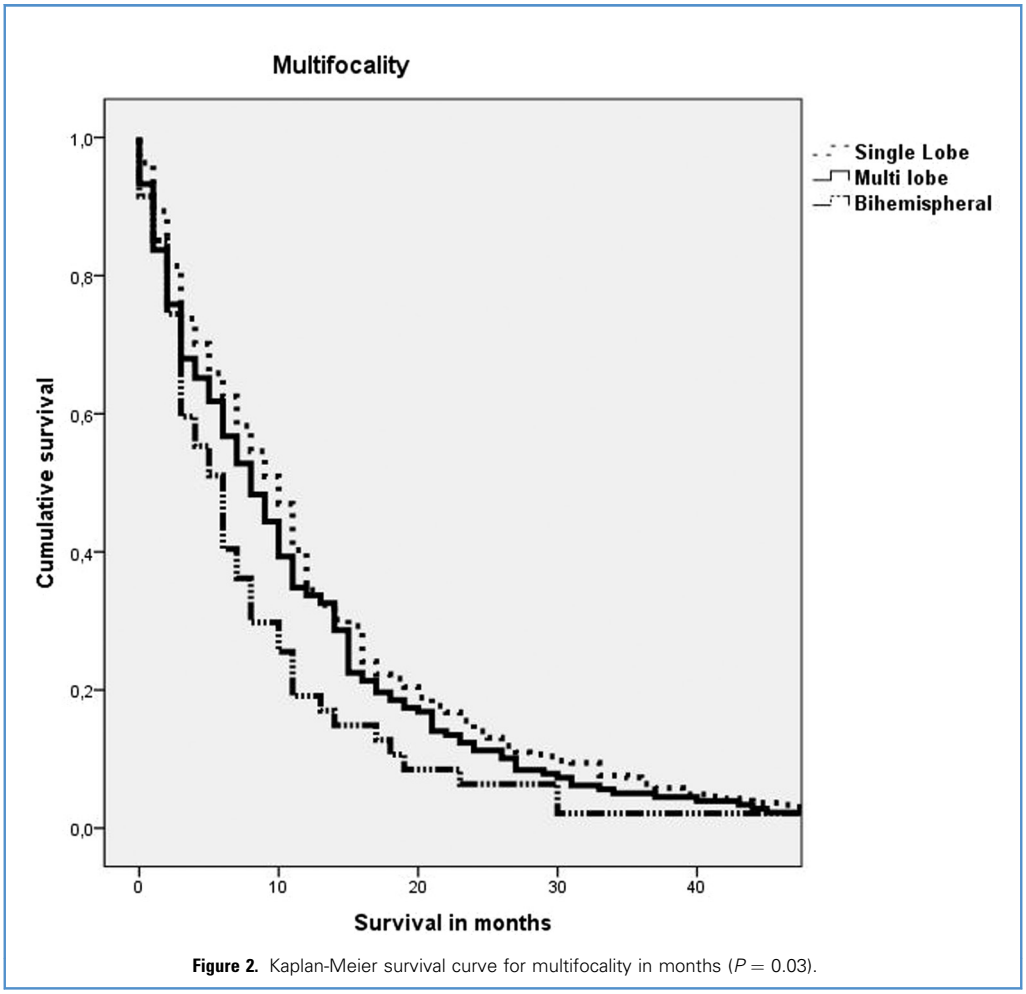

Figure 2. Kaplan-Meier survival curve for multifocality in months ($P = 0.03$).

Table 2. Multivariant Cox Regression Analysis with Adjusted Odds Ratio (aOR) with Significant Parameters for Worse Survival

Coefficients of Survival

	Unstandardized Coefficients		Standardized Coefficients		
Model	**B**	**Standard Error**	**Beta**	**aOR**	***P* Value**
(Constant)	16.250	6871		2.365	0.019
Age in years	−0.273	0.066	−0.284	−4.170	0.000
KPS preoperative	0.108	0.056	0.114	1.933	0.041
MGMT promotor methylation	4.994	1.420	0.209	3.518	0.001
Ki67 index	−0.064	0.071	−0.056	−0.908	0.365
IDH 1/2 mutational status	16.048	3.732	0.275	4.300	0.000
EOR	4.279	1.960	0.138	2.183	0.030
Multifocality bihemispheral	−4.453	2.149	−0.095	−2.072	0.039

These are higher age, lower Karnofsky performance scale (KPS), unmethylated MGMT promotor, higher Ki67 index, IDH 1/2 wildtype, lower extent of surgical resection (EOR), and multifocal tumor localization.

Statistical Analysis

Statistical analyses were performed with the software SPSS (version 24.0). Patients' survival as the study end point was assessed as overall survival (OS) and 2 years' survival. Differences between the groups were compared using the log-rank test. Comparisons of clinical variables between different subtypes (*IDH* 1/2 status and multifocality) were made using the Kruskal-Wallis test for continuous variables and the Pearson chi square test for categorical variables.

The association between study end points and prognostic markers were evaluated using Kaplan-Meier survival plot. Well-established parameters for survival, such as age, MGMT promotor methylation, sex, KPS, and operation modality were analyzed for the 2 years' survival by univariate and stepwise multivariate Cox regression model based on log-rank or Wilcoxon test analysis. Adjusted odds ratio (aOR) and 95% confidence intervals (CI) were calculated from the Cox regression model, including all factors for multivariate analysis. Differences were regarded as significant at $P < 0.05$.

RESULTS

A total of 565 (324 male/241 female) patients were included in this study. The mean age was 62.2 years (±11.2) with a mean OS of 12.5 months (±15). Evaluation of *MGMT* promoter methylation could be assessed in 420 (74.3%) patients. Methylation of *MGMT* promoter was present in 160 (38%) of them and revealed with 15.7 moths (±16) a higher OS than the unmethylated group with 11 months (±11). EOR comprises gross total resection in 255 (45.1%), followed by stereotactic biopsy in 174 (30.8%) and subtotal resection in 136 (24.1%) cases (Table 1).

Multifocality and Survival

Tumor location in a singular lobe was present in 334 (59.1%), a multifocal incidence on 1 hemisphere in 183 (32.4%), and an additional infiltration to the contralateral side in 48 (8.5%) cases. Although patients with infiltration of a single lobe had a mean OS of 13.5 months (±16), this value revealed 11.4 months (±12.5) in patients with multifocal infiltration on 1 hemisphere. Individuals with infiltration of the contralateral side showed a mean OS of 9.3 (±15.5) months (Figure 2). In Cox regression analysis, a statistical significant association between multifocality and survival could be determined with $P = 0.03$.

IDH 1/2 Mutational Status

IDH 1/2 mutational status could be analyzed in 240 patients, whereof 10 cases had an *IDH* 1/2 mutation. Patients harboring *IDH* 1/2 mutation were significantly younger (44.7 vs. 63.6 years, $P < 0.01$). Mean OS was 32 months versus 12 months in *IDH* 1/2 mutated cases. Besides the Kaplan-Meier survival curve, the multivariate analysis via Wilcoxon test revealed *IDH* 1/2 wildtype as an independent prognostic factor for decreased 2 years' survival (aOR 4.4, $P = 0.013$). All *IDH* 1/2 mutated GBMs revealed an ipsilateral tumor infiltration.

Proliferation Index

Tumor proliferation assessed by Ki67 was variable reaching from 0% to 70% with a median of 9.17 (±11.3). No associations were found between *MGMT* promoter methylation or *IDH* 1/2 mutation and proliferation rate.

Proliferation Index and Multifocality

The highest Ki67 index was found in patients with infiltration of both hemispheres with a mean of 17.5% (±10.5) in comparison with singular lobe or single-hemisphere involvement with 6.9% (±10.3) ($P < 0.01$).

Multivariate Analysis

Well-known survival markers including age, KPS, *MGMT* promoter methylation status, and EOR were analyzed by multivariate Cox

regression on the basis of Wilcoxon test analysis to reveal their independence and impact. *IDH* 1/2 status, Ki67 index, and multifocality were included as well (**Table 2**). *IDH* 1/2-wildtype was a strong negative predictor for survival in comparison with *IDH* 1/2 mutation with aOR of 4.3 ($P < 0.01$), followed by older age (aOR 4.2, $P < 0.01$), *MGMT* promoter methylation (aOR 3.5, $P < 0.01$), and EOR (aOR 2.2, $P = 0.03$). Tumor infiltration of the contralateral side was also associated with a worse prognosis with aOR of 2.1 ($P = 0.04$). Ki67 proliferation index was not independently associated with the OS in the multivariate analysis ($P = 0.4$).

DISCUSSION

Despite the dismal prognosis of GBM, clinical course and survival can be quite variable. Therefore the knowledge of prognostic markers allowing an early estimation of patients' survival is mandatory for clinicians. EOR, KPS, age, and *MGMT* promoter methylation are recognized parameters with impact on survival.[5,9] This could be confirmed in our series, as all parameters revealed to be independent survival markers with aOR of 4.1 for age older 70 years ($P < 0.01$), 3.5 for unmethylated *MGMT* promoter ($P < 0.01$), 1.9 for KPS below 70 ($P = 0.04$), and 2.2 for EOR ($P = 0.03$).

Beyond these markers, there are further prognostic features, which are assessed in routine diagnostic like *IDH* 1/2 mutational status, proliferation index, and multifocality. We wanted to elucidate their role and impact as independent prognostic markers as well.

IDH 1/2 mutational status was a strong, independent prognosticator for survival. Patients harboring the *IDH* 1/2 wildtype had a >4-fold worse outcome in comparison with bearers of the *IDH* 1/2 mutated gene. This is in line with multiple investigations.[6,10] The positive effect of mutated GBM might be in consensus with the fact that IDH 1/2 mutation is sufficient to establish the glioma hypermethylator phenotype.[11]

About 59% of GBMs in our series revealed a singular location, which was significantly associated with prolonged survival (13.5 months) in comparison with GBM infiltrating more than 1 lobe (11.4 months) or the contralateral hemisphere (9.3 months). The latter group of bihemispheric tumor infiltration revealed a 2.1-fold worse 2-years' survival in comparison with singular-located GBM in 1 hemisphere. This finding is in line with the study of Lorimer et al.,[12] where tumor spread on a single hemisphere is a significant marker for better survival. According to Adeberg, the incidence of bihemispheric spread is significantly higher in GBM short-term survivors.[13] Interestingly, in line with our results there is a positive association between multifocality and increasing proliferation measured by *Ki67*, which was highest in infiltration of both hemispheres. It is well known that increasing values of *Ki67* labeling index are associated with higher grade of malignancy.[14] Nevertheless, to date the prognostic value of Ki67 has revealed conflicting results.[15-17] A recent report by Gzell et al[18] detected Ki67 in reoperated GBM as a survival marker.

There are limitations of this study. The quantitative method used to determine *Ki67* index and EOR might be subject to bias, as it was determined by individuals. Moreover, the retrospective study design should be confirmed in prospective trials.

CONCLUSION

The results of the present study confirm well-known parameters like *MGMT promoter* methylation, *IDH* 1/2 mutational status, KPS, and age as independent prognostic factors for survival, and they reveal *Ki67* index, as well as multifocality as further independent prognostic markers for survival.

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