Ki-67 Proliferative Activity in the Tumor Margins as a Robust Prognosis Factor in Glioblastoma Patients

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Abstract

Introduction The infiltrative margin of glioblastomas (GBM) contains proliferative tumor cells difficult to estimate radiologically as they are included in the hyperintense signal of T2 sequences and they remain in the cavity margin after tumor resection. The amount of these cells could determine overall survival (OS) of these patients.

Material and Methods From October 2007 to January 2010, patients whose MRI were suggestive of newly diagnosed, resectable high-grade glioma were operated using fluorescence-guided surgery (FGS). Separate samples were selectively taken from nonfluorescent white matter areas just adjacent to the border of the pale fluorescence and staining was made for Ki-67. OS was analyzed with Kaplan–Meier and Cox regression. Multivariate analysis included the following prognosis variables: age, extent of resection (EOR), O-6-methylguanine-DNA methyltransferase (MGMT) promoter methylation, and performance status index.

Results Sample included 65 patients, comprising 37 men and 28 women, with a median Karnofsky Performance Score (KPS) of 80 (40–100) and mean age of 60 (34–78) years. Mean preoperative tumor volume was 35.8 mL. EOR was 100% in 52 patients (80%), with the lower EOR being 88%. For Ki-67, 39 patients had $<5\%$ and 26 had $\geq 5\%$. OS was 26.8 months (95% confidence interval [CI]: 18.9–28.2) for the Ki-67 low group versus 15.8 months (95% CI: 7.7–18.2) for the Ki-67 high group ($p = 0.002$).

Conclusion Proliferative activity in the normal-looking brain around the resection cavity measured with Ki-67 immunostaining is an important independent prognostic factor for GBM cases with complete resection of enhancing tumor. When complete resection is not reached, this factor is not relevant for prognosis.

Keywords
- proliferative invasive tumor
- Ki-67 prognosis factor
- Glioma extent of resection

Introduction

Glioblastomas (GBM) are the most prevalent form of primary brain tumors with a dismal prognosis, regardless of all forms of therapy available including surgery, radiotherapy, chemotherapy, and immunotherapy.

These tumors frequently have a solid component, shown radiologically as gadolinium enhancing or necrotic in T1 sequences (T1Gd), and a diffuse component, shown as a T2/fluid-attenuated inversion recovery (FLAIR) abnormality.[1] Most histological and molecular information is based on the solid tumor component. The data on the invasive component of the GBM are limited; the studies on GBM have a small number of samples and the parameters are not well defined,[2–4] mainly because the tumor border can infiltrate

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the eloquent areas of the normal brain. It is difficult for neurosurgeons to identify the limit of the normal brain, and it is complicated for pathologists to distinguish the infiltrative tumor cells from the reactive glia.

5-aminolevulinic (5-ALA) fluorescence-guided surgery (FGS) is a surgical technique approved for the resection of GBM.[5] This is the first surgical technique that allows the neurosurgeon to identify the tumor border with the microscope with a very high positive predictive value.[6] There are differences in the fluorescence intensity between cases and between different parts of the same tumor; these differences correlate with the number of tumor cells[7] and allows us to differentiate solid tumors from infiltrative areas in real time.[8]

We reported in a previous study that FGS is useful for obtaining separate samples of the infiltrating cells from the tumor margin.[9] The aim of this study was to analyze the tumor infiltrating margin and look for differences in the number and type of tumor cells that could have a correlation with survival.

Materials and Methods

At our institution, patients whose MR images were suggestive of newly diagnosed, resectable high-grade gliomas were operated on using FGS. Only patients whose tumors were bilateral, multicentric, or had diffuse invasion of eloquent areas in T1Gd were excluded.

Patient management was standard, including oral administration of 20 mg/kg body weight of 5-ALA (Medac, Weller, Germany) 2 to 4 hours before surgery, as previously published. All surgeries were done with a Pentero microscope (Carl Zeiss, Oberkochen, Germany) and Navigation (Brainlab AG, Feldkirchen, Germany).

We included all consecutive patients with radiological diagnosis of GBM, when a complete resection was planned and there were noneloquent areas around the lesion. Separate samples were selectively taken from the central viable core of the tumor, as shown by bright red fluorescence, from the more peripheral areas of vague, pink fluorescence and from normal-looking, nonfluorescent white matter just adjacent to the border of the pale fluorescence.

Routine hematoxylin and eosin (H&E) stain was done; with independent diagnosis for each sample. The pathologist did not know the fluorescent quality or intensity.

Staining was made for Ki-67, nestin, and sox-2 expression by immunohistochemistry and for epidermal growth factor receptor (EGFR) amplification by fluorescence in situ hybridization assay (FISH), as reported in a previous study.[8] A quantitative measure of Ki-67 was obtained with Automated Cellular Imaging System (ACIS III) from DAKO, Denmark. O-6-methylguanine-DNA methyltransferase (MGMT) promoter methylation analysis was performed by pyrosequencing. The pathological studies were done by one experienced neuropathologist. Clinical and radiological data were collected prospectively in all patients.

Contrast MRI was done $<$48 hours after surgery. The pre- and postoperative images were exported to a work station (Brainlab AG, Feldkirchen, Germany); the preoperative and residual tumor volumes were segmented manually, and measured by the iIPlan Net cranial 3.0 software (Brainlab AG). In both pre- and postoperative images, tumor volume was measured as the T1Gd-enhancing area including any necrosis inside. Extent of resection (EOR) was calculated as 100 $\times$ (preoperative volume – postoperative volume)/preoperative volume.

In a preliminary exploratory phase, we analyzed the influence on survival of the different histological parameters of the solid tumor and the tumor infiltration zone, including Ki-67, cells with EGFR amplification, sox-2 expression, and nestin expression. In that phase, the maximum Ki-67 in the nonfluorescent tissue around the tumor border appeared as a strongly negative prognostic marker. After this finding, we expanded the cohort to study this specific parameter, adding consecutive patients with suspicious GBM at first surgery. All the other histological parameters were not studied again.

All the patients gave their informed written consent for the surgery and biopsy sampling.

Statistical analysis was made on SPSS v.20. Overall survival (OS) was analyzed with Kaplan–Meier (log-rank, Tarone and Breslow) and Cox regression.

Age and EOR were used as quantitative variables, and MGMT promoter methylation was dichotomized as methylated or nonmethylated. Karnofsky Performance Score (KPS) was dichotomized as cases with KPS of 70 or worse versus patients with KPS of 80 or better. For Ki-67, the cases were stratified as $\leq$5% (Ki-67 low) versus $>$5% (Ki-67 high), which are typical reference levels for pathologists.

Results

We analyzed the results of patients from October 2007 to January 2010 in the preliminary exploratory phase; after the first analysis, we added more patients to verify the impact of Ki-67 during 2011 to 2014. Total sample included 65 patients (37 men and 28 women), with a median KPS of 80 (40–100) and mean age of 60 (34–78) years. The three different areas described previously were identified as solid fluorescence (bright red), vague fluorescence (shades of pink), and nonfluorescent tissue (blue) in all cases (►**Fig. 1**).

The status of the MGMT promoter was methylated in 28 patients (43.1%), nonmethylated in 34 patients (52.3%), and not conclusive or could not be done in 3 patients (4.6%).

Mean preoperative tumor volume was 35.8 mL. EOR was 100% in 52 patients (80% of patients achieved a complete resection of the gadolinium-enhancing lesion [CRET]), with the lower EOR being 88%. Mean residual tumor was 0.2 mL in the whole series and when including only the patients with incomplete resections, it was 1.1 mL.

Mortality rate at 1 month after surgery was 0%. New or worsened neurological morbidity 1 month after surgery occurred in five patients (7%).

Intention-to-treat analysis included the Stupp protocol for all patients. Three patients did not start adjuvant temozolomide, one denied any additional treatment, one had pulmonary embolism, and one had severe pneumonia during radiotherapy.

OS in this population was 18.7 months (95% confidence interval [CI]: 14.6–22.8).

Univariate analysis of OS showed age, EOR, MGMT promoter methylation, and Ki-67 had significant influence on OS, as shown

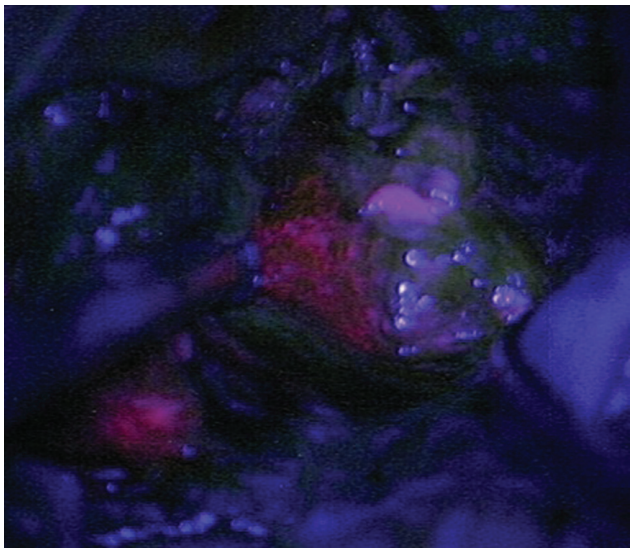

Fig. 1 Glioblastoma fluorescence-guided surgery with 5-aminolevulinic acid showing bright red solid tumor, pink infiltrative areas, and normal-looking blue areas. Due to the low emitted light, these pictures are not of high definition.

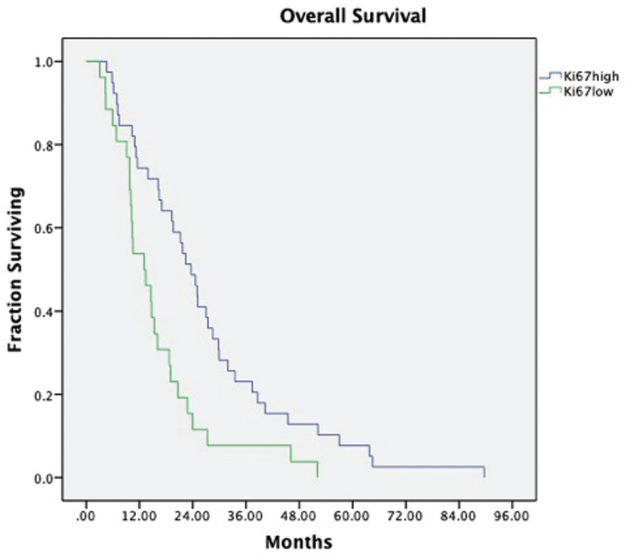

Fig. 3 Kaplan–Meier curve for Ki-67 low patients versus Ki-67 high patients ($p = 0.002$).

in ►**Fig. 2**. KPS showed a clinical difference with significance in some of the statistics. Gender had no impact on OS.

Thirty-nine patients had a Ki-67 index <5%; this group was named Ki-67 low. In 26 patients, it was ≥5%; this group was named Ki-67 high. OS was 26.8 months (95% CI: 18.9–28.2) for Ki-67 low patients versus 15.8 months (95% CI: 7.7–18.2) for Ki-67 high patients (►**Fig. 3**). The difference was statistically significant, with a log-rank test p value of 0.002.

►**Table 1** shows demographic and clinical characteristics of patients; the only relevant difference between the Ki-67

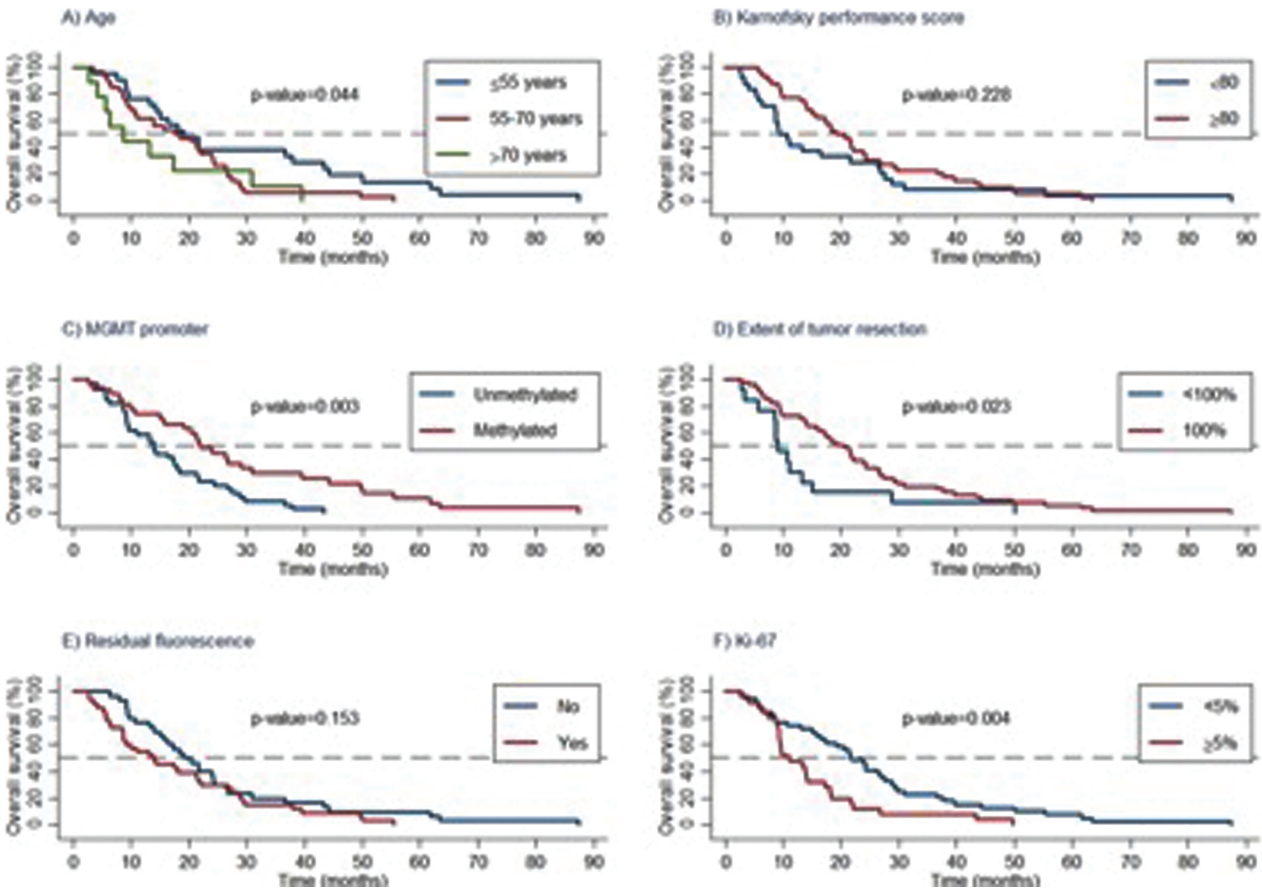

Fig. 2 Univariable survival analysis of the association of age (a), Karnofsky performance score (b), O-6-methylguanine-DNA methyltransferase (MGMT) promoter methylation (c), tumor resection (d), residual fluorescence (e), and Ki-67 (f) with mortality.

Table 1 Demographic and clinical characteristics of patients

	Total	Ki-67 <5%	Ki-67≥5%	*p* value
N	64	39	25	
Age (y)	59.8 (9.8)	59.7 (9.1)	59.9 (11.0)	0.933
Men, *n* (%)	37 (57.8)	22 (56.4)	15 (60)	0.777
KPS, median (min, max)	80 (40, 100)	80 (50, 100)	80 (40, 100)	0.129
MGMT promoter methylation,[a] *n* (%)	27 (44.3)	21 (56.8)	6 (25)	0.015
Preoperative T1Gd volume (mL)	36.2 (24.6)	38.5 (25.4)	32.5 (23.3)	0.341
Postoperative T1Gd volume (mL)	0.2 (0.8)	0.3 (1)	0.1 (0.3)	0.930
EOTR (%)	99.4 (1.9)	99.4 (2)	99.5 (1.7)	0.945
Residual fluorescent tissue, *n* (%)[b]	33 (52.4)	19 (48.7)	14 (58.3)	0.458

Abbreviations: EOTR, extent of tumor resection; KPS, Karnofsky performance score; Max, maximum; MGMT, O-6-methylguanine-DNA methyltransferase; Min, minimum; T1Gd, T1-weighted gadolinium enhanced.
Note: Values are expressed as mean (standard deviation) unless otherwise stated.
[a]Three patients with unknown MGMT promoter methylation (2 in the Ki-67 <5% group and 1 in the Ki-67 ≥ 5% group).
[b]One patient with unknown residual fluorescence in the Ki-67 ≥ 5% group.

high and Ki-67 low groups was the number of patients with methylation of MGMT promoter (39 vs. 26 patients).

Multivariate analysis was done with a Cox regression model including all the variables significant in the univariate analysis (age, EOR, MGMT methylation status, and Ki-67) and a different model including KPS due to its known influence even if it was marginally significant in univariate analysis in this sample. In both models, Ki-67 retained significance with a strong effect on OS. ►**Table 2** shows multivariate-adjusted risk of mortality, showing a strong effect of Ki-67 with adjusted hazard ratio of 1.9.

A separate analysis of the impact of Ki-67 high or Ki-67 low group on OS was done for EOR (100 vs. <100%) and for MGMT (patients with MGMT methylated or unmethylated). OS was higher in all the Ki-67 low subgroups. The difference was remarkable and significant in those patients with 100% EOR, was clinically relevant with a tendency toward statistical significance in both methylated and unmethylated MGMT subgroups, and was very low and nonsignificant for patients with <100% EOR.

Discussion

The goal of surgery in GBM has been defined as CRET, as the EOR of this component has shown a consistent increase in OS in large series of patients.[10–12]

The concept of supramarginal resection has emerged looking for the potential benefit of resection further than T1Gd volume.

Resection of a significant part of T2 abnormal volume showed impact on OS in a retrospective large series review.[13] Resection with safe margin on partial lobectomy samples also showed an increase in OS.[14]

Amino acid PET identifies a different tumor margin than T1Gd and sometimes beyond it[15] with a small series suggesting a better OS with the resection of these additional areas.[16,17]

FGS with 5-ALA also shows a border beyond the gadolinium limits. A series of patients with CRET found that patients without residual pale fluorescence tissue lived longer than those with some residual fluorescence.[18]

Table 2 Multivariable-adjusted risk of mortality according to Ki-67 levels

	Ki-67 < 5%	Ki-67 ≥ 5%	
N	39	25	
Time at risk (person-months)	995.5	376	
Crude incident mortality rate (per 100 person-months)	3.9	6.6	
Median OS (95% CI) in months	21.7 (15.7–27.5)	11.1 (9.3–17.3)	
HR (95% CI)			*p* value
Age adjusted	1 (reference)	2.02 (1.18–3.44)	0.010
Multivariable adjusted[a]	1 (reference)	1.89 (1.07–3.33)	0.029

Abbreviations: HR, hazard ratio; OS, overall survival; 95% CI: 95% confidence interval.
[a]Additionally adjusted for O-6-methylguanine-DNA methyltransferase (MGMT) promoter methylation and extent of tumor resection. Unknown values in MGMT promoter methylation were treated as a separate category.

Regardless of more extensive resection, recurrence of GBM is still the norm. The invasive component of diffuse gliomas is described radiologically as the hyperintense area in T2/FLAIR sequences and contains variable degrees of tumor edema and infiltrative tumor cells, but neither the exact extent nor its behavior is well characterized

In this series, we have focused on the tissue that has no fluorescence; therefore, it remains even after a complete fluorescence resection. We found that it mostly contains isolated tumor cells, as have been demonstrated by other authors before.[1] Our data indicate that the number of proliferative tumor cells in this area of the brain is predictive of survival independent of the previously known prognostic factors (age, KPS, and MGMT). Patients with $\geq$5% proliferative cells in the residual margin of the tumor fared markedly worse, with a difference of 10 months in median OS. The statistical analysis showed this difference was limited to the cases with complete resection. It suggests that the characteristics of residual invasive tumor are important only when the solid component of the tumor is no longer present. If there is residual solid tumor, it will cause recurrence, as is already known.[19]

Both the Ki-67 high and Ki-67 low subgroups showed similar prognostic parameters, except the MGMT promoter methylation; there were more cases with methylation in the Ki-67 low group. It could be argued that this unbalance is the reason for better survival in the Ki-67 low group; however, a separate analysis of methylated and unmethylated cases and multivariate analysis showed that this was not the reason. Patients with lower Ki-67 lived longer in both groups of methylation. Our hypothesis for the association is that the methylated cases are intrinsically less invasive tumors.

Some authors have studied in detail the molecular profiles of the remaining cells after a resection, and they have found that these cells are different from solid tumors, with less proliferative potential and more motility.[4] Our data on cell profiling are more limited, but suggest the amount of proliferative cells at the border of resection is relevant for prognosis.

Previous studies have also highlighted the difficulty of sampling the invasive part; FGS with 5-ALA has been proven to be an efficient method for this technique, and differences between stem cells generated from fluorescent areas and those from nonfluorescent areas have been found.[20]

Previous analyses have failed to show the importance of Ki-67 as a prognostic factor in GBM.[21] A very important difference with our data is that we focused only on the normal-looking tissue around the tumor, not on the solid part. Our previous analysis of Ki-67 on the solid tumor, not shown here, agreed with these data. Other parameters were studied in the infiltrative tissue in a retrospective pilot series, but just Ki-67 was significant, so a prospective study was performed with just Ki-67. The results of Ki-67 in the infiltrative tumor should be seen as information about the tissue that remains after a complete resection, and it could be a measure of the invasiveness of the tumor or the proliferative activity on the residual tumor.

Kim et al in 1993 studied the proliferative activity in the resection margin using proliferating cell nuclear antigen (PCNA),[22] which is a proliferation marker associated with the cell cycle as Ki-67. The authors obtained samples from the cavity walls after tumor excision in different types of tumors and found that GBM were the tumors with the highest PCNA scores. They considered this a new technique for estimating the proliferative rate of gliomas and concluded that it could be useful in estimating the "biological" EOR and in predicting the recurrence patterns of gliomas. Our study follows the same concept but is limited to just GBM patients and the conclusions are more robust as it is a prospective study that correlates the histological findings with survival. The conclusions are quite similar in terms of the usefulness of the proliferation scores in the resection margins.

Although this is a prospective study, the sample size is still small; more multicentric studies with a larger sample size are needed to validate these results.

Conclusion

Proliferative activity in the normal-looking brain around the resection cavity measured with Ki-67 immunostaining is an important independent prognostic factor for GBM cases with CRET, with 10 months longer survival for patients with Ki-67 $<$5% in the tumor margin. When complete resection is not reached, this value is not relevant for prognosis. These data confirm that the main objective of GBM surgery is maximal safe resection.

Conflict of Interest
None declared.

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