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Does the expression of glial fibrillary acid protein (GFAP) stain in glioblastoma tissue have a prognostic impact on survival?

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Abstract

Objective: Several parameters are known to predict the survival of glioblastoma (GB), including extent of resection and *MGMT* promotor methylation. Staining for glial fibrillary acidic protein (GFAP) is a common component of routine histological work-up, but its clinical utility in GB is unclear. The aim of the present study was to analyze the predictive value of quantitative GFAP measurements for survival of patients with GB.

Methods: All subjects in our institutional database of patients with primary GB who underwent surgery between 2011 and 2014 with examination of immunohistochemical staining of GFAP were included. Percentage GFAP staining was measured in 5% increments (5-100%). Univariate and multivariate analyses were performed between GFAP values and survival data. Clinically relevant cut-offs for GFAP staining were identified by receiver operating characteristic (ROC) curves.

Results: The final cohort consisted of 272 GB patients with available quantitative GFAP measurements (mean age, 62 (±11.1) years, 117 females [43%]). Overall survival was 11.4 months (±8.6). Median GFAP value was 70% (range, 5-100%). The ROC curve showed the clinically relevant cut-off for GFAP at 75% (area under the curve: 0.691). Accordingly, GB patients with GFAP ≥75% presented poorer survival on Kaplan-Meier survival estimation (p=0.021). Multivariate analysis adjusted for age, extent of resection, preoperative Karnofsky performance status scale, *IDH1* mutation and *MGMT* methylation status confirmed the independent predictive value of GFAP ≥75% for overall survival (p=0.032). Finally, patients with GFAP ≥75% showed significantly poorer long-term survival than those with GFAP <75%: 5.8% vs 15.2% (p=0.0183) and 0.8% vs 8% (p=0.0076) for 2- and 3-year survival respectively.

Conclusion: Quantitative immunohistochemical assessment of GFAP staining could provide a novel biomarker for overall and especially long-term survival of patients with GB. Prospective multi-center validation of the prognostic value of GFAP for GB survival is needed.

Introduction

Glioblastoma (GB) is the most common malignant brain tumor and is of very poor prognosis [1]. Under standard therapy, median survival ranges from 12.2 to 18.2 months [2]. Despite multiple research efforts, prognosis is still poor, due to early tumor progression and recurrence. However, there are individual differences, with some patients surviving only for a few months and others for years [3]. Research over the last decade revealed prognostic factors for survival: O6-methylguanine DNA methyltransferase (*MGMT*) promoter hypermethylation, and isocitrate dehydrogenase 1 (*IDH1*) mutations[4,5]. The clinical significance of such findings highlights the importance of MGMT promoter methylation assessment, which is the most commonly performed molecular analysis in GB [6].

There are also other histological parameters commonly assessed during routine neuropathological diagnostic work-up of GB tissues, including immunohistochemical staining for glial fibrillary acidic protein (GFAP). The clinical evidence on GFAP staining is mostly based on lipid studies in multiple sclerosis brains [7, 8]. In neuropathological preparations, GFAP is frequently used as a reliable marker of astrocytes and tumors of glial origin. Some studies demonstrated progressive loss of GFAP expression with increasing astrocytoma grade [9-13]. Immunohistochemical staining for GFAP frequently shows abundant staining in GB samples [14]. However, studies investigating the direct influence of GFAP expression in GB cells on patients' survival are lacking. The aim of the present study was to evaluate the prognostic impact of routinely assessed GFAP staining count on GB survival using the data from our institutional GB database.

Methods

Patient population:

All adult patients (≥18 years) operated on for primary GB between January 2011 and December 2014 in our neurosurgical department were eligible for this study. All histological findings in the database were reviewed in accordance with the 2016 Classification of Central Nervous System Tumors of the World Health Organization[1]. Cases with quantitative GFAP measurements were included in the final analysis. The study was performed in accordance with the Declaration of Helsinki and was approved by the local review board of Essen University Hospital (n° 15-6504-BO).

The following patient data were collected for analysis: gender, age, preoperative Karnofsky performance status (KPS), extent of resection, *IDH1* mutation and *MGMT* promoter methylation status, immunohistochemical staining of GFAP, postoperative adjuvant treatment, and overall survival (OS).

Pathohistological assessment

O6-methylguanine-DNA methyltransferase (*MGMT)* promoter methylation was assessed by pyrosequencing. Isocitrate dehydrogenase analysis was performed by either immunohistochemistry (IHC) or DNA sequencing; in general, IHC testing for *IDH1-R132H* was the preferred method for all patients, but patients were additionally tested by DNA sequencing when *IDH1-R132H* was non-mutant on IHC.

GFAP staining

Paraffin-embedded brain tumor tissue samples were mounted on 5 μm slides. Specimens were analyzed using the double-label streptavidin biotin method. For detection of glial cells, a solution of anti-glial fibrillary acidic protein (GFAP) was incubated and 3,3'-diaminobenzidine tetrahydrochloride (DAB) was used to prepare substrate-chromogen, resulting in brown cytoplasmic staining. [16] Percentage GFAP staining was measured in 5% increments.

Determination was feasible when enough en-bloc non-necrotic tissue samples were available for further analysis. Figure 1 shows examples of cases with 70% and 95% GFAP.

Data Management and Statistical Analysis

The main study endpoint was OS after GB surgery. Long-term (2- and 3-year) survival was also addressed. GFAP measurements were evaluated as continuous variables and as dichotomized at a clinically relevant cutoff using receiver operating characteristic (ROC) curves. Patient age was assessed as a continuous and as a dichotomous variable (dichotomized at the cohort's mean age). Based on radiological reports, extent of resection was evaluated as a categoric variable: gross-total resection (GTR: removal of ≥95% of the contrast-enhancing tumor mass), subtotal resection (STR, <95%) and stereotactic biopsy (SB). Preoperative KPS was also assessed as a dichotomous variable, with KPS ≤ 70% defined as poor initial clinical condition.

Associations between GFAP values and survival data were first analyzed on univariate and bivariate methods. Continuous variables were addressed with Pearson's linear correlation, Student t or Mann-Whitney U tests, as appropriate. Associations between categoric variables were analyzed on $\chi 2$ or Fisher exact test, as appropriate. Kaplan-Meier survival plots were made for GFAP measurements. Finally, the predictive value of GFAP was tested on multivariate models adjusted for common confounders: age, preoperative KPS, extent of resection, *IDH1*-mutation and *MGMT* promoter status, and postoperative chemoradiotherapy), using linear and binary logistic regression analyses for OS and long-term survival respectively. Missing values were replaced using multiple imputation. Statistical analyses were performed on SPSS software (version 24.0). Differences were regarded as significant at $p< 0.05$.

Results

Patient population

Between 2011 and 2014, 327 patients with histologically confirmed diagnosis of primary GB were treated in our neurosurgical department. In 272 cases (83.2%), quantitative GFAP measurements were taken during histological tissue analysis, and these patients were included in final analysis: mean age 62 [±11.1] years; 117 female [43%]. Preoperative KPS was 80% in 79 patients (29%). Diagnosis was confirmed by stereotactic biopsy in 56 patients [20.6%]; the other 216 cases underwent open surgery (GTR=127 [46.7%], STR=89 [32.7%]). MGMT-promoter status was available in 250 cases. Mean OS was 11.4 months (±11.2). Regarding long-term survival, there were 30 (11.0%) and 13 (4.8%) cases with 2- and 3-year survival, respectively.

Cases excluded from analysis due to absence of GFAP measurements (n=55) did not differ from the final cohort on demographic (mean age=64 years [±11.38], p=0.27; 21 females [38.2%], p=0.51) or survival data (OS=11.9 months [±14.2], p=0.17).

GFAP staining and association with survival

The median GFAP value was 70% (range, 5-100%). There was an inverse linear correlation between GFAP values and OS (p=0.033, r=-0.129): long-term survivors showed lower median GFAP count: 60% vs 70% (p=0.0875) and 50% vs 70% (p=0.0189, see Figure E1 in Online Supplements) for 2- and 3-year survival, respectively. Subsequent ROC curve analyses between GFAP values and long-term outcome parameters showed that the clinically relevant cut-off for GFAP at 75% (area under the curve: 0.589 and 0.691 for 2- and 3-year survival respectively, Figure E2 in Online Supplements). Accordingly, GFAP status was dichotomized for further analysis.

GFAP values were ≥75% in 121 patients (44.5%). Multivariate linear regression analysis showed significant association between GFAP ≥75% and OS (p=0.039) adjusted for preoperative KPS, extent of surgery, *IDH1*- mutation and *MGMT*-methylation status (Table 1).

GB patients with GFAP ≥75% likewise presented poorer Kaplan-Meier survival estimates (p=0.021, Figure 2).

There was an association between GFAP cutoff and long-term outcome. Patients with GFAP ≥75% showed significantly lower long-term survival: 5.8% vs 15.2% (p=0.018) and 0.8% vs 8% (p=0.008) for 2- and 3-year survival, respectively (Figure 3). Finally, multivariate binary logistic regression analysis for predictors of 2- and 3-year survival confirmed an independent association between GFAP ≥75% and long-term survival (p=0.037/p=0.018, Table 2).

Due to poor clinical condition after surgery and/or refusal, 41 patients (15.1%) did not receive any postoperative adjuvant treatment and were referred to best supportive care. In the remaining cases, standard chemoradiotherapy was performed with temozolomide according to the STUPP protocol. No significant association was found between restriction of postoperative treatment and GFAP status (19 [46.3%] vs 102 [44.2%] for GFAP ≥75%, p=0.87).

Discussion

To the best of our knowledge, the prognostic value of GFAP staining for GB survival has not previously been analyzed. The present large single-center series of primary GB demonstrated a strong association between the characteristics of GFAP staining in GB cells and clinical outcome. We identified a clinically relevant cutoff, whereby GFAP greater than or equal to 75% was independently associated with GB survival, OS and long-term survival.

GFAP staining: historical development and clinical application

The clinical evidence on GFAP staining is mostly based on lipid studies in multiple sclerosis brains [8,17]. Astrocytes (astroglia) are characterized by the presence of this unique structural protein, isolated and specified by Eng in 1969. GFAP is a key intermediate filament (IF) III protein responsible for the cytoskeleton structure of glia cells and for maintaining their mechanical strength, as well as supporting neighboring neurons and the blood-brain barrier [7]. There are 10 isoform variants, GFAP-α (Isoform 1) being the predominant isoform in brain and spinal cord, but also found in the peripheral nerve system [18]. GFAP-δ, also called GFAP-ε, (Isoform 2) is preferentially expressed by neurogenic astrocytes in the subventricular zone [19]. The gene for GFAP is localized in human chromosome 17q21. Mutations in the GFAP gene have been identified in a few disease states such as Alexander's disease, and in glioma-like tumors in some Alexander's disease patients [20,21].

Evidence for GFAP staining in neuro-oncology: a new prognostic marker for GB?

GFAP is frequently used for visualization of astrocytes and tumors of glial origin. Some previous studies analyzing GFAP staining in high-grade glioma cells reported lower GFAP expression in giant cell glioma [9, 10]. Similarly, Rutka et al. reported progressive loss of GFAP production with increasing malignancy in astrocytoma cells [13].

Recent investigations found no somatic mutations of GFAP in genome-wide GB sequencing, although GFAP expression was reduced in primary GBs, xenograft specimens and GB cell lines

[22, 23]. Takeuchi et al. also demonstrated that GFAP-positive tumor cells had low proliferative potential on an immunohistochemical double-labeling method [16]. In recent studies, Berendsen and van Bodegraven et al. observed differential expression of GFAP which was inconsistent with the degree of astrocytoma malignancy[25.26]. All these studies addressed possible alterations in GFAP expression depending on the malignancy of the analyzed glial tumors.

However, GFAP staining might also have clinical value as a prognostic marker for glial tumor. Brehar et al. described a potential clinical implication of GFAP-δ, which was associated with greater tumor invasiveness in cerebral astrocytoma[27]. This might be in line with the present findings of an association between percentage GFAP staining in GB cells and patient survival: the higher the GFAP value in GB tissues, the poorer the outcome. In particular, ≥75% immunohistochemical staining of GFAP was strongly associated with the overall and especially with long-term survival, independently of the main confounders (age, preoperative KPS, extent of surgery, IDH1-mutation and MGMT-methylation status). Statistical assessments on univariate, multivariate and survival analysis confirmed this correlation. This association between GFAP staining and GB survival might reflect greater destruction of cellular membranes in GB tissue, resulting in more strongly enhanced immunohistochemical staining. On the other hand, the previously reported loss of GFAP production with increasing malignancy [13] could be attributed to higher cell mitosis rates, which in turn might be associated with better response to radiation and/or chemo-therapy.

The present findings conflict with previous reports of GFAP staining as a marker of glioma malignancy. However, our study focused on survival of patients with primary GB, whereas previous studies predominantly investigated various GFAP patterns in low- and high-grade glial tumor. In addition, we performed quantitative measurement of GFAP staining based on the percentage scale, whereas other studies used a simple dichotomous assessment differentiating

"high" versus "low" GFAP expression. Nevertheless, the present results need to be confirmed in a large prospective series.

Study Limitations

This study was limited by its retrospective design, affecting the quality and accuracy of the collected data. In particular, molecular genetic marker data (IDH1 mutation and *MGMT* methylation status) were partially missing and had to be replaced in the statistical analysis by multiple imputation. Furthermore, quantitative GFAP measurements were not always performed in all patients in due time, and several cases without GFAP count (16.8%) had to be excluded from analysis, incurring some selection bias. However, there were no differences in demographic and clinical characteristics between included and excluded patients. Finally, non-computerized quantitative assessment of GFAP staining incurred an additional risk of bias regarding interobserver reliability. Accuracy could be improved by using computer image analysis programs, limiting observer bias and increasing the sensitivity and throughput of immunohistochemistry [28]. Nevertheless, our study presents the first evidence for the potential prognostic value of GFAP staining in GB patients.

Conclusion

Routine immunohistochemical assessment of GFAP with quantitative measurements might become a novel biomarker for overall and especially long-term survival of patients with GB. Prospective multi-center validation of GFAP as a marker for GB survival is needed.

FIGURES

Figure 1: Tissue samples with different GFAP percentages (A: 70%, B: 95%)

RC – regression coefficient, CI – confidence interval, EOR – Extent of resection, RCT – adjuvant radiation/chemotherapy

Table 2: Multivariate binary logistic regression analysis for predictors of long-term survival

Parameter	**2-year survival**				**3-year survival**			
	aOR	**95% CI**		**p-value**	**aOR**	**95% CI**		**p-value**
Age ≥ 62 years	0.72	0.28	1.82	0.483	0.54	0.12	2.38	0.416
GFAP ≥ 75%	0.37	0.14	1.00	**0.049**	0.06	0.01	0.64	**0.020**
KPS ≤ 70%	0.50	0.12	2.17	0.356	0.43	0.03	6.11	0.529
MGMT	5.96	2.25	15.81	<0.001	4.07	0.83	19.94	0.083
IDH1	5.74	0.91	36.41	0.06	22.15	2.19	224.43	0.009
EOR (SB vs STR vs GTR)	2.48	1.15	5.36	0.02	4.58	1.14	18.46	0.032
RCT	3.37	1.10	10.28	0.03	4.40	0.54	35.61	0.165

aOR – adjusted odds ratio, CI – confidence interval, EOR – Extent of resection, RCT–adjuvant radiation/chemotherapy

ONLINE SUPPLEMENTS

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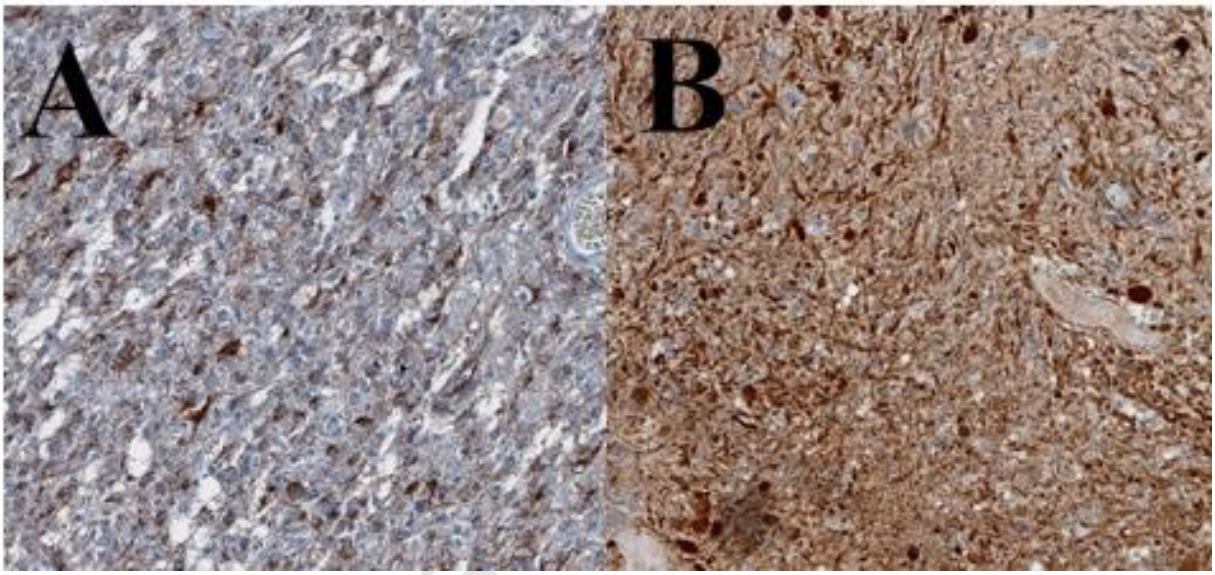


The immunohistochemical images were provided by the Institute of Neuropathology of the University Hospital of Essen

Figure 2: Kaplan-Meier survival curve showing survival pattern and time in GB patients with GFAP ≥75% versus <75%.

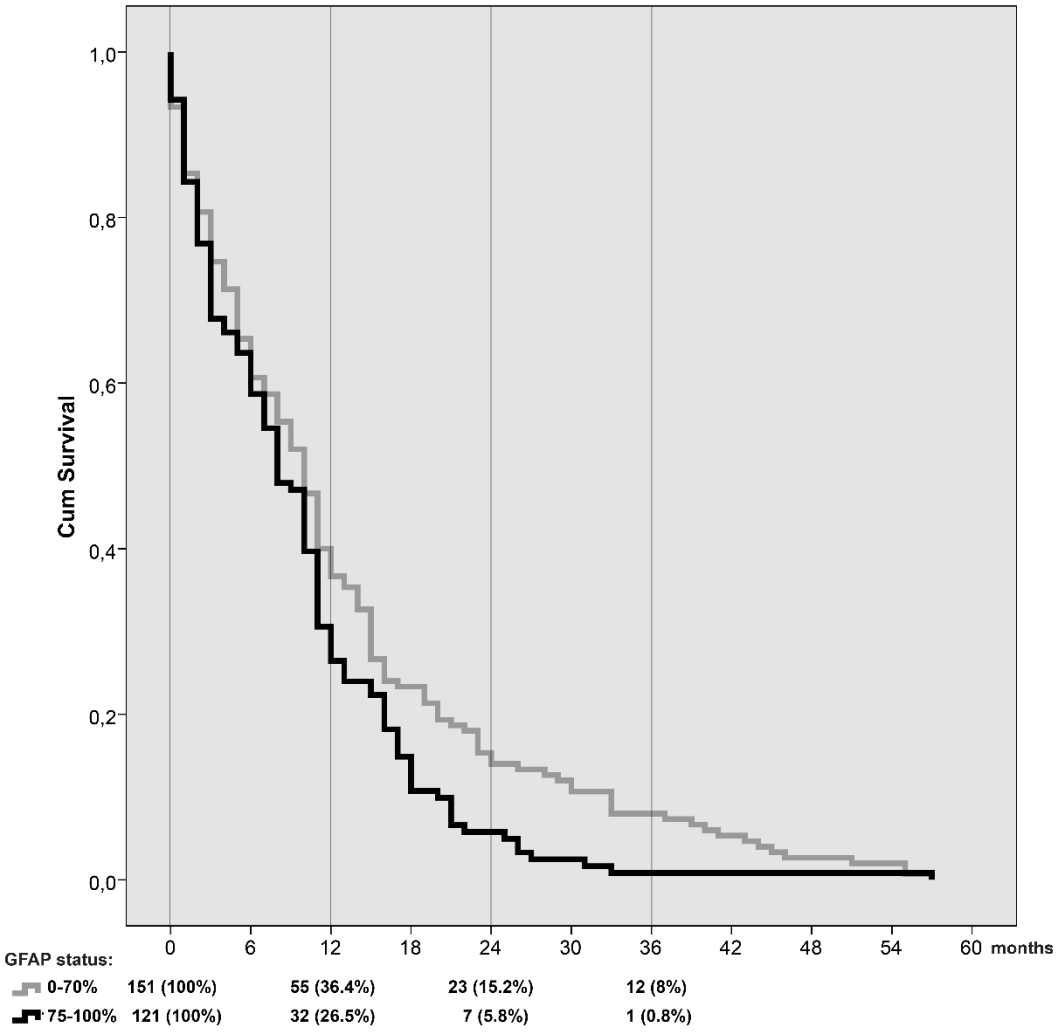


Figure 3: Long-term survival in GB patients with different GFAP staining patterns

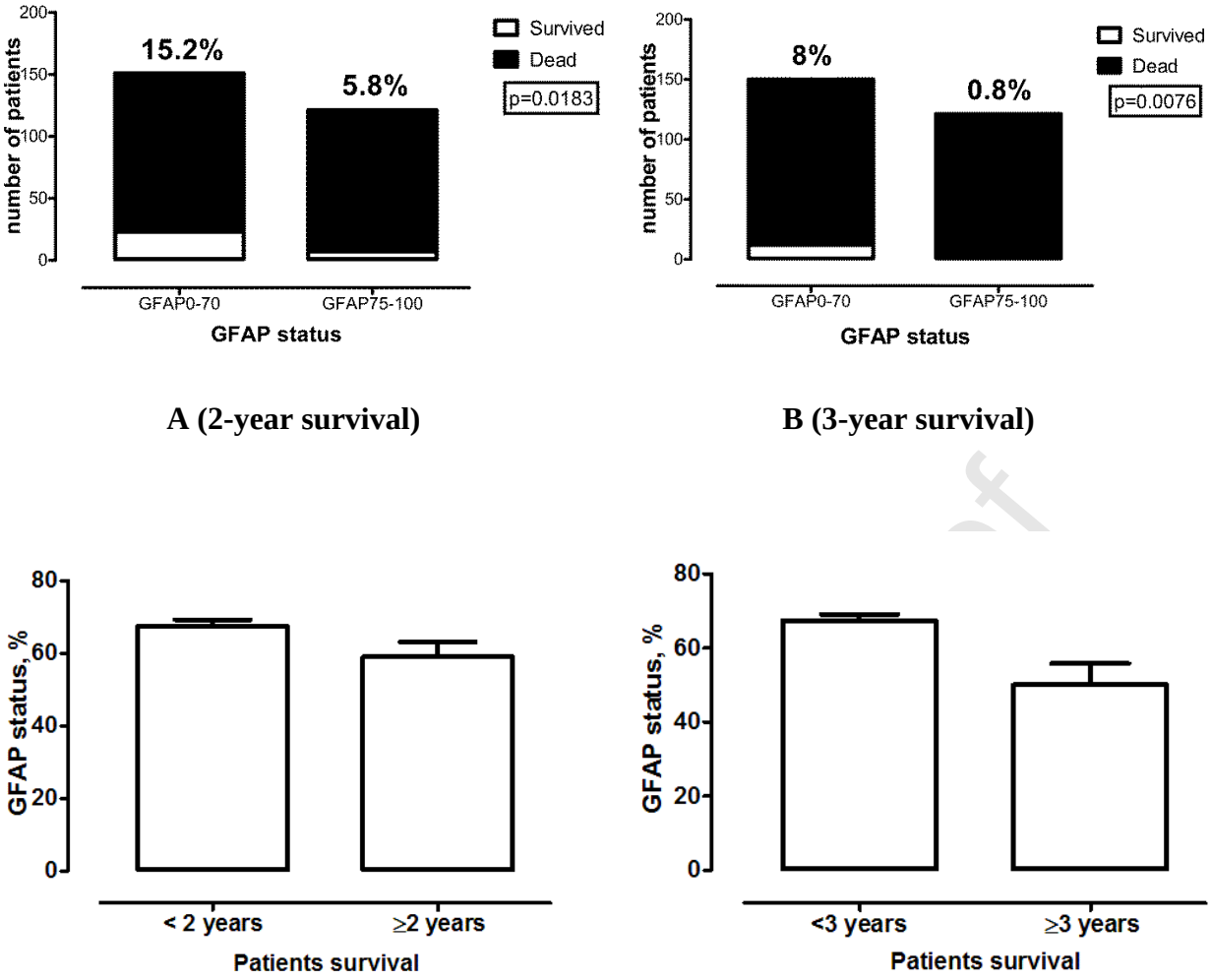
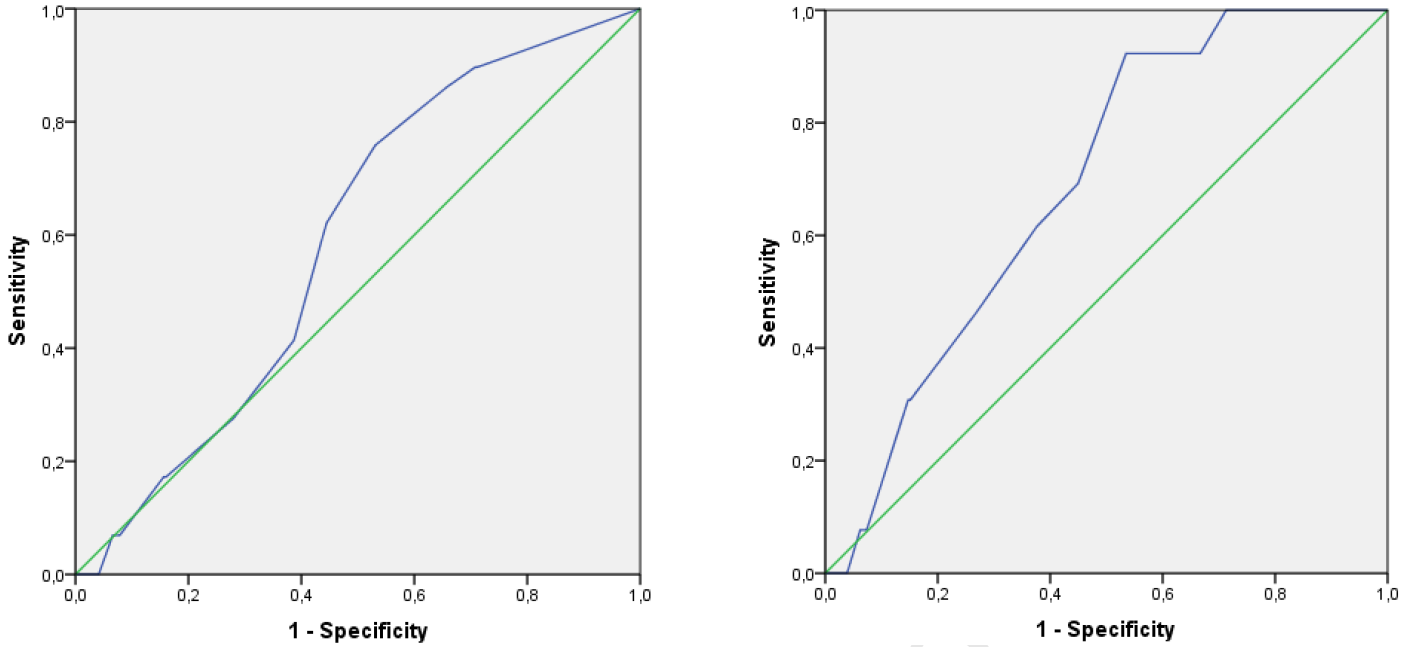


Figure E1: GFAP values of GB patients with different long-term survival patterns



A (2-year survival) **B (3-year survival)**

Figure E2: ROC curves for correlation between GFAP values and 2- and 3-year survival

TABLES

Table 1: Multivariate linear regression analysis of predictors of OS

Parameters	**RC**	**95% CI**		**p-value**
Age ≥ 62 years	-3.15	-5.59	-0.72	0.011
GFAP ≥ 75%	-2.40	-4.70	-0.11	**0.040**
KPS ≤ 70%	-0.46	-3.64	2.72	0.773
MGMT	4.10	1.78	6.42	0.001
IDH1	9.06	-6.91	25.03	0.210
EOR (SB vs STR vs GTR)	3.24	1.76	4.71	<0.001
RCT	5.32	3.61	7.04	<0.001