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TITLE: Differential effect of vascularity between long- and short-term survivors with IDH1/2 wild-type glioblastoma

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ABBREVIATIONS:

- CD34: cluster of differentiation 34
- GB: glioblastoma
- HAT: high angiogenic tumor
- HR: hazard ratio
- HTS: hemodynamic tissue signature
- IDH1/2: isocitrate dehydrogenase 1/2
- LTS: long-term survivors
- MGMT: methylation of the O(6)-Methylguanine-DNA methyltransferase
- MRI: magnetic resonance image
- OS: overall survival
- P: p-value
- rCBV: relative cerebral blood volume
- STS: short-term survivors
- TERT: telomerase reverse transcriptase
- Wt: wid-type

ABSTRACT

Introduction: IDH1/2 wt glioblastoma represents the most lethal tumor of the Central Nervous System. Tumor vascularity is associated with overall survival (OS), and the clinical relevance of vascular markers, such rCBV, has already been validated. Still, molecular and clinical factors may have different influence in the beneficial effect of a favorable vascular signature.

Purpose: To analyze the prognostic effect of rCBV for IDH1/2 wt GB patients for long-term survivors (LTS) and short-term survivors (STS). Given that early vascularity may affect the patient's OS in follow-up stages of the disease, we will assess whether a moderate vascularity is beneficial for OS in both groups of patients.

Materials and Methods: Ninety-nine IDH1/2 wt GB patients were divided in a LTS group (OS ≥ 400 days) and a STS group (OS < 400 days). Mann-Whitney test, Uni- and Multiparametric Cox, Aalen's Additive Regression and Kaplan Meier were developed. ONCOhabitats was used to process the MRIs and to calculate the $rCBV_{mean}$ in the High Angiogenic Tumor (HAT).

Results: For the LTS group, we found a significant association between the moderate value of $rCBV_{mean}$ and higher OS ($p = 0.0140$, HR = 1.19; and $p = 0.0085$, HR = 1.22, respectively) and when evaluating its stratification capability ($p = 0.0343$). For the STS group, no association between vascular status and survival was observed. Moreover, no significant differences ($p>0.05$) in gender, age, resection status, chemo-radiation or MGMT promoter methylation status were observed between the LTS and the STS groups.

Conclusion: We have found different prognostic and stratification effect of the vascular marker for the LTS and STS groups. We propose the use of $rCBV_{mean}$ at HAT as a vascular marker clinically relevant for the long-term survivors with IDH1/2 wild-type glioblastoma and may be a potential target for randomized clinical trials focused in this group of patients.

INTRODUCTION

Glioblastoma with isocitrate dehydrogenase 1/2 wild-type (IDH1/2 wt GB) is the most lethal and common tumor of the central nervous system [1, 2] with patient median survival rates of 13-14 months [1, 2]. Inter-patient tumor heterogeneity makes notable differences on the overall survival (OS) of glioblastoma patients, being angiogenesis one of the most relevant processes involved in tumor heterogeneity [3-5].

This process can be studied using non-invasive techniques such as magnetic resonance imaging (MRI) from the time of tumor diagnosis [6-11]. Hence, functional MRI techniques allow researchers and clinicians to study relevant vascular markers with prognostic, predictive and stratification capabilities [6-11]. For that, the Hemodynamic Tissue Signature (HTS) methodology is able to automatically define regions of interest within the tumor and the edema and to calculate vascular markers associated with patient OS [12, 13]. However, despite having demonstrated the robustness of these vascular markers [13], biological and clinical factors can affect the effectiveness of these prognostic image markers.

Different studies have evaluated the molecular differences between LTS and STS groups of GB patients related to tumor angiogenesis, because of their relevance to improve patient prognosis and to decide on the correct therapeutic target [5]. Burgenske *et al*. analyzed the gene expression profile of IDH1/2 wt GB patients and split their patients into LTS and STS to elucidate which variables were associated with differences in survival. Their results showed apparent similarities between the two groups.

Despite those results, it was observed that LTS presented a higher proportion of methylated O-6-methylguanine-DNA methyltransferase (MGMT), and for that group, an enrichment of the genes of sphingomyelin metabolism was detected, which has been related to a decrease in tumor growth and angiogenesis [14]. In addition, Michaelsen *et al.* analyzed the molecular profile of LTS and STS to identify cluster of differentiation 34 (CD34) mRNA level (regulator of GB angiogenesis by promoting new blood vessel networks [16]) as prognostic for GB patient survival [15]. Moreover, clinical factors, such as initial performance score [17], tumor size and location [18]

and completeness of tumor resection [19] may also determine the possibilities of a patient to be a long-term survivor.

Despite the evident efficacy of imaging vascular markers when dealing with clinical challenges such as diagnosis, non-invasive characterization of molecular profile and prediction of prognosis, among other [20], the influence of these markers for LTS and STS has not yet fully assessed. To elucidate this, we analyze in our work the prognostic and stratification capabilities of $rCBV_{mean}$ at the high angiogenic tumor habitat for the LTS and STS groups, independently. We evaluate whether a moderate and functional vascular status is beneficial for overall survival in both groups of patients.

MATERIALS AND METHODS

Patient Information

This is a sub-study of the approved multicenter retrospective clinical trial NCT03439332. For this study, 99 IDH1/2 wt GB patients from five clinical centers were included. Participating centers were Hospital Universitario de La Ribera, Alzira, Spain; Hospital Clinic, Barcelona, Spain; Hospital Universitario Vall d'Hebron, Barcelona, Spain; Azienda Ospedaliero-Universitaria di Parma, Parma, Italy; and Oslo University Hospital, Oslo, Norway.

A Material Transfer Agreement was approved by all the participating centers and an acceptance report was issued by the Ethical Committee of each center. The managing institution (Universitat Politècnica de València, Valencia, Spain) Review Board also approved this study.

The criteria to include patients in this study were: *(a)* adult patients (age >18 years) with histopathological confirmation of IDH1/2 wt GB [2] diagnosed between January 1, 2012, and January 1, 2018; *(b)* access to the preoperative MRI studies, including: pre- and post-gadolinium T1-weighted, T2-weighted, Fluid-Attenuated Inversion Recovery (FLAIR) and Dynamic Susceptibility Contrast (DSC) T2*-weighted perfusion sequences; and *(c)* patients with a minimum survival of 30 days.

The study cohort was divided in two groups as previously reported [21, 22]: LTS were defined as those patients with an OS equal or higher than 400 days and STS were defined as those patients with an OS lower than 400 days. Because this value is close to the median survival of the study population (384 days), it allows for the number of patients in the two groups to be balanced. Patients still alive at readout were considered censored observations. The date of censorship was the last date of contact with the patient or, if not available, the date of the last MRI exam.

Magnetic Resonance Imaging

Standard-of-care MR examinations were obtained for each patient before surgery, including pre- and post-gadolinium-based contrast agent enhanced T1-weighted MRI, as well as T2-weighted, FLAIR T2- weighted, and DSC T2* perfusion MRI. A detailed description of the acquisition parameters used at each institution is shown in supplementary Table S1.

Processing of MRIs and Vascular Markers

We used the HTS method, freely accessible at the ONCOhabitats platform at www.oncohabitats.upv.es, to process MRIs and calculate the vascular biomarkers. The HTS is an automated unsupervised method to describe the heterogeneity of the enhancing tumor and edema tissues and includes four phases: a) preprocessing, b) segmentation, c) DSC quantification and d) hemodynamic tissue signature, which delineates four vascular habitats within the tumor and the edema. All the information related to the development, functionality and validation of the HTS methodology has previously been published [12, 13].

The vascular biomarker used in our study was the mean relative cerebral blood volume ($rCBV_{mean}$) calculated in the HAT habitat, shown to be a relevant prognostic marker in previous studies [23-25].

Statistical Analyses

We described the main demographic, clinical, and molecular variables for the LTS and STS groups and for the entire cohort. Possible differences in the distributions of these variables for the LTS and STS groups were assessed using Mann Whitney tests in Matlab R2017b (MathWorks, Natick,MA). The significance level used in all the statistical analyses was 0.05.

To analyze the time-dependent influence of the vascular biomarker ($rCBV_{mean}$ at HAT) on patient survival, we used Aalen's Additive Regression Model included at the library "survival" for R software. This model allows to plot time-varying effects of covariates on patient survival [26].

To analyze the association between the $rCBV_{mean}$ at HAT and patient survival, we used both Uniparametric and Multiparametric Cox proportional hazard regression analyses with the entire cohort, and independently with LTS and STS groups. The proportional hazard ratios (HRs) with a 95% confidence interval (CI), as well as the associated p-values are reported.

In addition, the stratification capability of the $rCBV_{mean}$ at HAT was evaluated with the Kaplan–Meier test. The analyses were performed with the entire cohort, as well as independently with the LTS and STS groups. For all tests, we evaluated the capability of the $rCBV_{mean}$ at HAT to stratify the population into *moderate vascular* and *high vascular* groups and we analyzed if these two *vascular groups* presented different survival rates. We define *moderate* and *high vascular* as the two groups of patients generated by dividing a population using the optimum cutoff threshold according the vascular marker. We calculate the optimum vascular cutoff threshold using the $rCBV_{mean}$ at HAT and determined by the C-index method, previously used in [13]. The *moderate-vascular* group included patients with an $rCBV_{mean}$ lower than the calculated cutoff, and the *high vascular* group included patients with an $rCBV_{mean}$ higher than the cutoff.

The log-rank test was used to determine any statistical differences between the estimated survival functions of the vascular groups. The optimal threshold, the number of patients included in each vascular group, the median OS rates of each group, the estimated C-index and the p-value are reported.

RESULTS

Description of the entire Cohort, and the Long- and Short-Term Survivors groups

A total of 99 IDH1/2 wt GB patients conformed the entire cohort of the study. This population was divided into **I)** the LTS group, which includes 45 patients (7 censored), and **II)** the STS group, which includes 54 patients (8 censored). The information related to the entire cohort and the LTS and STS groups is summarized in Figure 1.

Table 1 summarizes the most relevant demographic, clinical and molecular features of the entire cohort and patients included in the LTS and STS groups.

No significant differences ($p>0.05$) in gender, age, resection status, chemo-radiation, methylation of the MGMT promoter methylation status, or $rCBV_{mean}$ at HAT were observed between the LTS and the STS groups (Mann Whitney tests).

Differences between LTS and STS and the effect of rCBV on patient survival

While the studied variables of the LTS and the STS groups displayed similar distributions (Table 1), we found a different effect of $rCBV_{mean}$ at HAT on the patients' OS in the LTS group compared to the STS group. A significant negative association between the $rCBV_{mean}$ level and OS was found for the LTS group ($p = 0.0140$). but not for the STS group ($p = 0.3543$). Results of the Uniparametric Cox Analysis are shown in Table 2. The highest HR (1.19) found for the LTS group, imply an increase of one unit in the $rCBV_{mean}$ at HAT will equal a 19% higher risk of exitus. This result suggests that for the LTS group, patients with lower $rCBV_{mean}$ in the HAT habitat, had significant longer survival. For the entire cohort, the beneficial effect of having moderate vascularity in the HAT habitat was borderline significant, yet it did not reach statistical significance.

Table 3 depicts the results of the Multiparametric Cox analyses, including the MGMT methylation status as a covariable. We did not have the MGMT methylation status information of 41 patients, so for those cases we used a mean imputation method. Collectively, combining MGMT methylation status and $rCBV_{mean}$ at HAT were significantly associated with OS for the entire cohort. Patients with lower $rCBV_{mean}$ at HAT and methylated MGMT had longer OS. Again, the influence of the combination of these two variables on OS is higher for the LTS group compared with the entire population (HR: 1.22 vs. 1.10 for the $rCBV_{mean}$; and 2.68 vs. 1.80 for the MGMT methylation status). Additionally, the statistical power of the results for the long-term survivors was the highest (with the lowest p-values). None significant result was found for the STS group when analyzing the association between the HAT $rCBV_{mean}$ and the MGMT methylation status with the OS.

The effect of the vascularity on OS is also shown using the Aalen's Additive Regression Model. Figure 2 shows the marked incremental effect of both MGMT methylation status and $rCBV_{mean}$ at HAT on OS from 400 days after diagnosis. Again, the influence of the $rCBV_{mean}$ at HAT on OS is revealed for the LTS group. In addition, the patient baseline conditions (represented with the intercept) starts to be relevant from 400 days after diagnosis, but causing a beneficial effect on survival.

The results of the Kaplan–Meier analysis are summarized in Table 4, including estimated optimal cutoff thresholds, number of patients per vascular group, estimated C-index, median OS calculated per each group, and log-rank test results (p-values).

We found a significant stratification capacity of the $rCBV_{mean}$ when analyzing the whole cohort (n = 99 patients) and when analyzing the LTS group (n = 45 patients). However, the stratification in vascular groups related with survival was more robust when we analyzed the LTS group, which yielded the highest C-Index or AUC (0.690). Additionally, for the LTS group, we found the greatest difference in OS between the *Moderate Vascular Group* and the *High Vascular Group*

(2.9 months). For the entire cohort, the test yielded a difference of only 1.1 months between these vascular groups. For the STS group, the OS was similar for the *moderate* and the *high vascular* groups and the log rank test did not yield significant results.

These differences between the LTS and the STS groups in the efficacy of vascular markers are also illustrated with the Kaplan Meier curves for both groups (LTS and STS) in Figure 3. For the STS group, the survival curves of the *high vascular* and *moderate vascular* groups are overlapping indicating no apparent differences in survival time for patients included in the STS group, independently of their $rCBV_{mean}$ at HAT. However, for the LTS group, the vascular marker is capable to stratifying survival according the level of $rCBV_{mean}$ in the HAT habitat (Table 4).

DISCUSSION

This study of the differential effect of vascularity in long- and short-term survivors with IDH1/2 wild-type glioblastoma is based on the data from multicenter clinical trial NCT03439332 [13] and included 99 patients with IDH1/2 wt GB. We found that the beneficial effect of having a moderate $rCBV_{mean}$ can only be observed in patients surviving more than 400 days i.e., those included in the LTS group. We did not find an association between the $rCBV_{mean}$ biomarker and patient OS for the STS group. These results are compatible with previous studies in which the effect of vascularity was evaluated [8, 12, 13]. In our study we went a step further to analyze whether the beneficial effect of having moderate vascularity in the HAT habitat is constant along time and if it is present in both long- and short-survival groups.

A possible explanation for the association of vascularity with survival in long-term survivors may rely be how the influence of vascularity increase significatively after approximately 400 days from diagnosis (Figure 2). This implies that from that time, LTS patients with a moderate vascular signature will have longer survival than those patients with a high vascular signature. By contrary, the STS group is highly conditioned by general evolved conditions of the patient, and tumor vascularity does not represent an influent covariable which affects survival.

For the LTS, vascularity marks the tumor behaviour, so the $rCBV_{mean}$ image marker can be used as an accurate prognostic biomarker. In fact, we found now a much higher HR than that reported in our previously study which included both wild and mutant IDH1/2 GB (HR: 1.19 vs. 1.05, respectively) [13]. This may suggest that the prognostic value of this vascular biomarker is more accurate and clinically relevant for IDH1/2 wt LTS group, rather than the entire population of GB patients.

Moreover, the different influence of tissue vascularity on survival between the LTS and STS groups may be also influenced by several clinical factors [27, 28]. Comorbid conditions, such as cardiovascular or pulmonary diseases [27], or early deaths caused by treatment complications,

could hide the effect of vascularity on OS in the STS group, whereas for the LTS group, the significant effect of the vascularity becomes apparent revealing a strong association with patient OS. Comparing the distributions of main demographics (age and gender) and clinical characteristics (type of resection, completeness of standard treatment), we did not find significant differences in these variables between the groups.

Moreover, we found that patients with the methylated MGMT promoter benefit most from treatment with temozolomide [29], an integral component of the standard treatment for GB patients [30]. We analyzed the distributions of patients with methylated and non-methylated MGMT in each group, as well as the completeness of the Stupp's treatment. No significant differences were observed in this regard, suggesting that these variables do not impact the observed effect of vascularity on survival. Other molecular factors, however, might also influence our results. The influence of telomerase reverse transcriptase (TERT) promoter mutation on survival of GB patients has been suggested by several authors. More than 90% of GB are IDH1/2 wt; among them, about 80% have mutations on TERT promoter, which confers a worse prognosis. In two studies [31, 32], the negative impact of TERT promoter mutations on survival of patients with IDH1/2 wt GB, becomes visible only after approximately 400 days of evolution; before this time point, survival curves overlapped. Thus, the distribution of TERT mutations in our patient series could help understand the different influence of vascular biomarkers on the LTS and STS groups. Of note, the molecular profiles of IDH1/2-wt and TERT mutation have been associated with the classical and mesenchymal subtypes [31, 33], in which the latter is associated with active angiogenesis [33, 34].

Our study has some limitations. Firstly, a more detailed clinical information on completion of treatment, comorbidities and treatment complications would have helped to explain our results. Moreover, MGMT promoter methylation status was unknown in more than one third of patients, and other molecular features, such as TERT promoter mutation status, was not recorded. Thus, for future studies, we aim to analyze the association between the molecular profile of tumors with

the imaging markers and the effect of vascularity on survival. The combined information may provide a better understanding of the influence of vascular biomarkers on the evolution of glioblastoma.

Related to further work, image-based vascular biomarkers have proven their ability to guide anti-angiogenic therapy for glioblastoma [35], but this therapy only benefits specific populations of glioma patients [35]. Lui TT *et al.* [36] used MRI features to define a subgroup of GB patients with higher angiogenic activity and better response to the antiangiogenic treatment. Based on the main results of our study, it would be worth studying whether only the group of LTS with high values of $rCBV_{mean}$ at HAT would be the best responders to antiangiogenic treatment.

In conclusion, in our study we found that a moderate $rCBV_{mean}$ level in the HAT habitat is associated with prolonged survival in patients with glioblastoma, particularly for IDH1/2 wt GB patients that survive more than 400 days. For the long-surviving group, vascular $rCBV_{mean}$ image marker can differentiate patients with moderate vascularity and longer OS from patients with high vascularity and shorter OS. However, this association between vascularity and patient survival was not found for patients that did not survive more than 400 days.

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TABLES

Table 1: Demographic, clinical and molecular features of the patients included in the study (whole cohort) and for each group (long- and short-term survivors). P-values resulting from the Mann Whitney test are also included. N: number of patients; RT-CT: radio-chemotherapy.

	Long-Term Survivors	**Short-Term Survivors**	**Entire cohort**	**p-values (Mann Whitney test)**
N (%)	45 (45.5)	54 (54.5)	99 (100)	-
% Females	31.1%	35.2%	33.3%	0.6732
Mean Age at Diagnosis	59	61	60	0.5043
Type of resection (N)				
- Total	22	18	40	0.1191
- Sub-total	17	25	42	0.3978
- Biopsy	6	10	16	0.4909
- Unknown	0	1	1	-
RT-CT (N)				
-Complete	24	23	47	0.2909
-Incomplete	2	6	8	0.2308
-Unknown	19	25	44	-
MGMT methylation status (N)				
-Methylated	14	11	25	0.2248
-Unmethylated	14	19	33	0.6732
-Unknown	17	24	41	-

Table 2: Uniparametric Cox analysis of the association between the vascular marker ($rCBV_{mean}$ at HAT) and the survival for the entire cohort and for the long- and short-term survivors groups.

Variables	Long-term survivors (n = 45pts)		Short-term survivors (n = 54pts)		Entire cohort (n = 99pts)	
	HR [95% CI]	p-value	HR [95% CI]	p-value	HR [95% CI]	p-value
HAT $rCBV_{mean}$	1.19 [1.04, 1.38]	**0.0140***	1.06 [0.94, 1.20]	0.3543	1.09 [1.00, 1.20]	0.0601

Table 3: Multiparametric Cox analysis of the association between the vascular marker ($rCBV_{mean}$ at HAT) and the survival for the entire cohort, the long-term group and short-term survivors group.

Variables	Long-term survivors (N = 45 patients)		Short-term survivors (N = 54 patients)		Entire cohort (N = 99 patients)	
	HR [95% CI]	p-value	HR [95% CI]	p-value	HR [95% CI]	p-value
HAT $rCBV_{mean}$	1.22 [1.05, 1.42]	**0.0085***	1.05 [0.92, 1.19]	0.4777	1.10 [1.00, 1.21]	**0.0468***
MGMT methylation status	2.68 [1.15, 6.26]	**0.0230***	0.45 [0.18, 1.13]	0.0898	1.80 [1.01, 3.22]	**0.0471***

Table 4: Kaplan Meier and Log-rank test results for the vascular marker ($rCBV_{mean}$ at HAT) and the overall survival for the entire cohort and the long- and short-term survivors groups (LTS and STS, respectively).

Group	Cut-off threshold	Patients per group	AUC (c-index)	Median OS per group	Δ OS (months)	P-value (log-rank test)
rCBVmean HAT		**[Moderate, High]**		**[Moderate, High]**		
Entire cohort	6.30	[35, 64]	0.605	[13.8, 12.1]	1.1	**0.0275***
LTS	8.97	[33, 12]	**0.690**	**[18.7, 15.8]**	**2.9**	**0.0343***
STS	6.32	[16, 38]	0.415	[10.6, 9.6]	1.0	0.5149

FIGURE LEGENDS:

Figure 1: Distribution of patients that conforms the entire study cohort and both long- and short-term survivors.

Figure 2: Curves of Aalen's Additive Regression Model that illustrate the incremental effect of the variables MGMT methylation status (in green), the $rCBV_{mean}$ (in blue) and the intercept (in grey) at over time.

Figure 3: Kaplan Meier curves for the STS group (dotted purple lines) and for the LTS group (solid green lines).