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Original Article

Prognostic value of the preoperative immunological profile in patients with glioblastoma

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Abstract

Background: Aim of our study was to determine the predictive impact of certain serum immunological markers on overall survival (OS) in patients with glioblastoma multiforme (GBM).

Methods: We assayed prospectively values of interleukin 2 (IL-2), immunoglobulin G (IgG), C4, CD3+, CD4+ and CD8+ cells via flow cytometry, enzyme-linked immunosorbent assay (ELISA) and radial immunodiffusion in preoperative sera of adult patients with *de novo* histologically confirmed supratentorial GBM. Kaplan-Meier method and Cox proportional hazards models were used to assess clinical, laboratory, and treatment prognostic factors for OS.

Results: Twenty-six consecutive patients were identified with a mean age of 59.6 years. Median follow up was 12 months. Lower IL-2 values (<7.97 pg/ml vs. ≥7.97 pg/ml, $P = 0.029$) und CD4+ counts (<200 cells/μl vs. ≥200 cells/μl, $P < 0.001$) correlated significantly with a shorter OS. The independent prognostic relevance of CD4 + counts was confirmed by the multivariate analysis (HR = 0.010, 95% CI 0.001-0.226, $P = 0.011$). Further independent prognostic factors for OS were type of resection (resection vs. biopsy) and administration of radiotherapy (yes/no).

Conclusion: Preoperative values IL-2 and CD4+ cells in sera may carry a prognostic impact. Novel diagnostic models prior to histopathological confirmation may be used to predict prognosis of patients with GBM. Future studies should investigate whether targeting immune factors, such as CD4+ and IL-2, may improve the prognosis of patients with GBM.

Key Words: CD4+ cells, glioblastoma multiforme, IL-2, overall survival, serum

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INTRODUCTION

Several studies have been focused on the immunity of glioma, in order to investigate its poor prognosis and to potentially include immunotherapy in its treatment.[3,10,12,13,15,24,26] In a previous study, we have identified significant differences in immunological markers between 49 patients with glioma and 30 healthy

controls, as well as among the patients with different WHO grades of malignancy. In that study we reported that certain immunoglobulins, cytokines, complement and subpopulations of leukocytes differ significantly among patients harboring high and low grade glioma. Thus, preoperative serum evaluation of IL-2 and CD4+ cells was found to represent independent predictors of World Health Organization (WHO) grade in patients with glioma.[13] In an additional work, we analyzed the preoperative phenotype of T cell subpopulation in 17 patients with glioblastoma multiforme (GBM).[15] According to our results, CD4+ cells may display a prognostic tool in those patients, since we recognized lower counts of CD4+ cells as a negative prognostic factor for overall survival (OS) and progression free survival (PFS) in the univariate analysis.

To date, a variety of clinical and treatment parameters have been identified as beneficial prognostic factors for survival in patients with GBM, such as younger age, higher Karnofsky score (KPS), aggressive degree of resection, administration of adjuvant chemotherapy, and radiotherapy.[2,6,18,23,28] Furthermore, some molecular markers, such as methylguanine methyltransferase (MGMT) and IDH-1 status correlate strongly with patients' survival.[16,17] Despite the knowledge of these prognosticators of the survival, the prognosis of those patients remains still dismal. This may rely on the fact that apart from the patients' treatment, we cannot influence the abovementioned parameters, that is, we cannot change the age of the patients or the molecular status of the tumors.

In order to further improve the prognosis of patients with GBM, some novel potential alterable prognostic factors are required. Suitable candidates may be some of the immune parameters of the sera, since they may be easily influenced (i.e. antagonized/substituted/stimulated). To the best of our knowledge, except for the counts of CD4+ cells,[15] no other preoperative immune parameters in sera have been reported as factors to predict OS. In the present study, we investigated whether the previously reported immunological markers, which differ according to WHO grade, may be prognostic significant in patients with GBM.

MATERIALS AND METHODS

Patients

We prospectively examined preoperative fasting morning sera from patients with subsequently histologically confirmed *de novo* supratentorial GBM, operated at the Department of Neurosurgery of the University Hospital of Ioannina between June 1, 2005 and January 31, 2008. Twenty-six patients fit our inclusion criteria (age over 18 years, no previous history of brain tumor, immunological or hematological disease and no previous administration of steroids or antiepileptic drugs for more than 3 days) Median follow up was estimated at 12 months. Mean age was 59.6 years (range 20-78). A total of 18 (69.2%) patients demonstrated preoperative KPS ≥70%. Postoperative KPS ≥70% (see below) was seen in 17 (65.3%) patients. A positive history of epilepsy was present in nine (34.1%) patients. A frontal location of the tumor was seen in 9 (34.1%) cases, whereas the preoperative maximal tumor diameter was over 4 cm in 10 (38.4%) patients [Table 1].

The following clinical and treatment parameters were studied as potential prognostic factors; age, gender, tumor location, preoperative maximal tumor diameter and KPS, short-term functional outcome as reflected by KPS at discharge, history of epilepsy, type of resection, temozolomide (TMZ) chemotherapy, and radiotherapy. In addition, we preoperatively evaluated values of immunological parameters, which differ among WHO grades,[13] such as IL-2, IgG, C4, CD3+, CD4+, and CD8+ cells.

The methods applied in our study have been previously described.[13] Briefly summarized, the enzyme-linked immunosorbent assay (ELISA) was used for quantitative detection of human IL-2 and total IgG (Bender MedSystems, Vienna, Austria and IBL, Hamburg, Germany), C4 was quantified by radial immunodiffusion with NOR PARTIGEN© C4 plate (Dade Behring, Marburg, Germany), whereas frequencies of T-lymphocyte subpopulations were determined by flow cytometry using two different fluorochromes (fluorescein isothiocyanate and phycoerythrin).

Table 1: Study group demographics and oncological treatment

Variable	Value (%)
Mean age (range)	59.6 (20-78)
Gender	
Males	13 (50.0)
Location	
Frontal	9 (34.1)
Preoperative seizures	9 (34.1)
Preoperative maximum tumor diameter≥4 cm	10 (38.4)
Preoperative KPS≥70	18 (69.2)
Postoperative KPS≥70	17 (65.3)
Surgery	
Resection	21 (80.7)
Biopsy	5 (19.3)
Radiotherapy	21 (80.7)
Chemotherapy	17 (65.3)
Standard adjuvant therapy*	16 (61.5)
Only radiotherapy	4 (15.3)
No concomitant or adjuvant therapy	5 (19.3)

*Concomitant radiochemotherapy and at least one cycle of monthly (5/28) TMZ chemotherapy

The statistical analyses of the clinical and laboratory data were performed using commercially available software (SSPS version 21.0, IBM Deutschland, Ehningen, Germany) and standard procedures (Fisher's exact test, Cox regression analysis, Kaplan–Meier estimates) were followed. The immunological markers were treated as dichotomized variables with the respective median value as the cut-off. $P \leq 0.05$ were considered to be statistically significant.

OS was defined as the time upon surgery (resection or biopsy) to death or censored at the date of last follow up. The patients' samples and clinical data were collected after their informed consent was obtained in accordance with the tenets of the declaration of Helsinki, and after approval of the study by the Ethics Committee of the Medical Faculty of the University of Ioannina.

RESULTS

Median OS was estimated at 343 days (95% CI 255-431). Surgical resection (gross total or partial resection) was performed in 21 (80.7%) cases, whereas 5 cases (19.3%) had an open or stereotactic biopsy. The standard therapy for our patients consisted of combined radiotherapy (total dose 64 Gy) with TMZ followed by monthly TMZ courses up to 6 cycles or until recurrence.[29] Concomitant radiochemotherapy followed by at least one course of monthly TMZ was administered in 16 (61.5%) patients. Patients who recurred had only supportive care but no further chemotherapy. One patient had no concomitant chemoradiotherapy but radiotherapy followed by TMZ chemotherapy and four patients had only standard radiotherapy without chemotherapy. Five additional patients had only surgery. The reason for no administration of either radiotherapy or chemotherapy in some patients was the deterioration of their performance status after surgery and/or their refusal to receive any further therapy [Table 1].

Age under 60 ($P = 0.011$), resection vs. biopsy ($P = 0.002$), TMZ chemotherapy ($P = 0.005$) and radiotherapy ($P = 0.001$) were identified as beneficial prognostic factors of OS in the univariate analysis (Log rank test). Preoperative KPS ≥ 70% ($P = 0.089$), female gender ($P = 0.058$) were recognized as trends for prolonged OS [Table 2]. The preoperative serum values of immunological parameters, such as IL-2, IgG, C4, CD3+, CD4+ and CD8+ cells, were analyzed as dichotomized variables with their respective median value as the cut-off (medians: IL-2:7.97 pg/ml; IgG: 984 mg/dl; C4: 23.5 mg/dl; CD3+: 808 cells/μl; CD4+: 384 cells/μl, and CD8+: 333 cells/μl). Only lower values of IL-2 (<7.97 pg/ml vs. ≥7.97 pg/ml) were shown to correlate with worse OS ($P = 0.029$) [Figure 1b and Table 2]. Concerning CD4+ cell counts, we defined two additional subgroups with the cut-off value set at 200 cells/μl, a crucial count, which is an established

Table 2: Survival analysis. Effect of clinical, treatment and immune parameters on OS in patients with glioblastomas

Univariate analysis*	Median OS (days)	*P* values
Type of resection (resection vs. biopsy)	386 versus 160	0.002
Age (<60 vs. ≥60)	445 versus 231	0.011
Chemotherapy (yes/no)	418 versus 196	0.005
Radiotherapy (yes/no)	385 versus 163	0.001
CD4+ (≥200 c/μl vs. <200 c/μl)	395 versus 122	<0.001
IL-2 (≥ 7.97 pg/ml vs. <7.97 pg/ml)	412 versus 291	0.029
Sex (male vs. female)	283 versus 423	0.058
KPS (≥70% vs. <70%)	375 versus 279	0.089

Multivariate analysis**	HR	95% CI	*P* values
CD4+ (< 200 c/μl vs. ≥200 c/μl)	0.010	0.001-0.226	0.011
Type of resection (biopsy vs. resection)	0.494	0.264-0.924	0.027
Radiotherapy (no/yes)	0.040	0.004-0.440	0.009

*Log rank test, **Backward stepwise procedure, KPS: Karnofsky score, CI: Confidence interval, HR:Hazard Ratio, OS: Overall survival

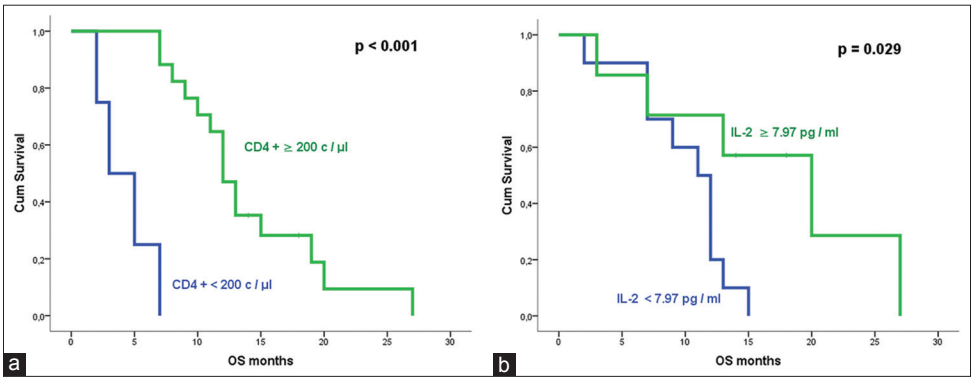

Figure 1: Kaplan-Meier estimates to show the significant influence of (a) CD4+ counts (≥200 c/μl vs. <200 c/μl, *P*<0.001) and (b) IL-2 values (≥7.97 pg/ml vs. <7.97 pg/ml, *P*=0.029) upon OS in patients with GBM

threshold of severe immunosuppression.[31] We identified a powerful prognostic significance of $P < 0.001$, with immunosuppressed patients having a worse OS [Figure 1a].

We used cross tabulation (Fisher's exact test) to investigate potential relations of IL-2 and CD4+ values to other clinical and treatment factors. No differences between the studied subgroups of IL-2 (<7.97 pg/ml vs. ≥7.97 pg/ml) were seen with respect to age, gender, history of epilepsy, location of tumor, preoperative and postoperative KPS, preoperative maximal tumor diameter, type of resection and administration of chemo- and radiotherapy. Similarly, the subgroups of CD4+ cells (<200 cells/μl vs. ≥200 cells/μl) were comparable regarding the clinical parameters. Interestingly, CD4+ counts did not even correlate with preoperative and postoperative KPS. A trend for correlation with preoperative maximal tumor diameter over 4 cm ($P = 0.081$) was found. Positive relations were found to TMZ chemotherapy ($P = 0.006$), whereas radiotherapy and type of resection did not correlate with CD4+ counts.

Additional support for the prognostic value of the immune parameters in patients with GBM is given by the multivariate analysis. The Cox proportional hazard models (Backward stepwise procedure) identified CD4+ counts (<200 cells/μl vs. ≥200 cells/μl; HR = 0.010, 95% CI 0.001-0.226, $P = 0.011$), type of resection (biopsy vs. surgical resection: HR = 0.494, 95% CI 0.264-0.924, $P = 0.027$) and administration of radiotherapy (no/yes: HR = 0.040, 95% CI 0.004-0.440, $P = 0.009$) as independent prognostic factors of OS [Table 2].

DISCUSSION

Despite recent advances in diagnostic and therapy, the prognosis of patients with GBM still remains poor. The knowledge of clinical and molecular prognostic factors for these patients may recognize patients with a presumably better prognosis and therefore may permit the individualized therapy but may not improve dramatically the patients' prognosis. This may rely on the fact that these factors are inalterable, that is, the attendant physician cannot alter for instance the age or gender of the patient as well as the MGMT/IDH1 status of the tumor.

Novel prognostic markers, which values and function may be altered in favor of the patient during adjuvant therapy, are needed in order to further affect his outcome. Some of the immunological parameters may act indeed as such prognostic markers, since they could be depleted or stimulated during the patients' therapy.[11] Furthermore, they could be assayed using simple and standard laboratory methods, such as flow cytometry, radial immunodiffusion or ELISA. However, immunological factors in serum follow a circadian rhythm and their values are also prone to changes depending on specific medication, for instance cortisone, antiepileptic, and antibiotic medications.[7,9] Thus, the latter limitations should be always considered when analyzing the immunological profile of patients with GBM. Of note, also the type of diet should be carefully considered for the design of such studies, since specific diet, such as omega 3/omega 6 ratio, use of conjugated linoleic acid (CLA) oil or certain nutraceuticals, may alter the values of the immune parameter as well.[5] The latter was not feasible in our study due to lack of data. We carried out a prospective study, analyzing the prognostic relevance of the preoperative immunological profile in 26 patients with GBM. We have taken consideration of known limitations and potential sources of bias and excluded from our study patients with an administration of steroids for over 3 days. Administration of steroids is believed to cause imbalances of patients' leukocytes counts, reducing mainly the CD4+ counts. The time to occur for these biochemical imbalances remains unclear, due to paucity of large series to investigate these changes, but a period of less than 4 days on steroids is regarded to be relatively safe.[7] The majority of patients in our study received steroids only for one day, in a dose ranging from 4 to 12 mg/day (dexamethasone T1/2 = 36-54 h) indicating no significant influence of steroid administration in our findings. Similarly, patients who received antiepileptic medication for more than 3 days prior to blood collection have been excluded from our study. None of the patients in our cohort received antibiotics at the time of drawing blood. In addition, a circadian rhythm for values of immune parameters has been reported.[9] In order to overcome potential bias, our fasting blood samples were collected between 7:30 and 8:30 am, prior to morning dexamethasone dose and were processed within 30 min of collection for optimal accuracy by flow cytometry assays. In addition, we excluded, from our study, patients with history of hematological or immunological disorders who could have abnormalities of lymphocytes counts. Following stringent selection criteria, our results give some evidence for a prognostic value of the preoperative immunological profile in patients with GBM.

We have previously reported various immunological markers in sera, which differ among patients harboring high and low grade gliomas. Preoperative values of IL-2 and CD4+ were estimated as independent predictors of WHO grade. Lower values of CD4+ cells and IL-2 have been associated with a greater WHO histological grade.[13] In our present study, both IL-2 and CD4+ values have been recognized as prognostic factors for OS in patients with GBM in the univariate analysis.

Reduced values of the proinflammatory cytokine IL-2 in patients with GBM have been well documented in the literature.[13] However, to the best of our knowledge, this is the first study to show a potential prognostic relevance of preoperative IL-2 serum values in those patients. Lower IL-2 values (< median: 7.97 pg/ml) have been found in the univariate analysis to correlate significantly with a shorter OS ($P = 0.029$). We also found no significant differences between our IL-2 studied subgroups with respect to other clinical and treatment parameters. Since the positive role of IL-2 (Th1 immune response) against gliomagenesis is known, that is, IL-2 mediates the infiltration of immune cells into the tumor mass as well as activates the lytic process,[32] several groups have tried to substitute IL-2 directly in the tumor bed in an animal glioma model.[21,27] Spagnolo *et al.* treated mice with GL261 intracerebral glioma by intratumor injections of allogeneic cells modified to secrete IL-2. These mice survived significantly longer than mice in various control groups.[27] Some groups administrate i.v. IL-2 fused to antibodies directed against tumor-associated antigens, in order to deliver IL-2 selectively to the tumor bed.[1,25] Pedretti *et al.* stated that U87MC GBM xenografted BALB/c nude mice treated by combination of i.p TMZ and i.v F16/IL-2 (i.e. IL-2 fused to the human antibody fragment F16) showed a significantly longer survival compared with TMZ monotherapy.[25] To conclude, IL-2 values may possess a diagnostic and prognostic role in the gliomagenesis. Furthermore, IL-2 substitution or activation in the tumor site may also have some therapeutic consequence.

In our series of 26 patients with GBM, also CD4+ values have been identified as prognosticators of survival. Patients with CD4+ counts <200 cells/μl showed significant shorter OS ($P < 0.001$) than the remaining patients. In principle, the current study validated our previous reported results.[15] In that study, we analyzed the preoperative phenotype in sera of T cell subpopulation in a series of 17 patients with GBM and found lower CD4+ counts (< median = 225 cells/μl) to correlate with a poorer OS and PFS in the univariate analysis. However, our current study additionally confirmed the independent prognostic significance of CD4+ cell counts also by the multivariate analysis. To date, CD4+ counts have been correlated with higher rates of complications during the adjuvant therapy but not significantly with survival in patients with gliomas. Hughes *et al.* assayed postoperative CD4+ counts in peripheral blood during radiotherapy in patients with WHO grade III and IV gliomas receiving high dose steroids and found patients with counts of CD4+ <200 cells/μl to experience a higher hospitalization (41% vs. 9%, $P < 0.01$) and infection rate (23% vs. 4%, $P < 0.05$).[19] CD4+ counts have been reported as prognostic factors also in other solid tumors. For instance, Milasiene *et al.* demonstrated that higher preoperative CD4+ counts in sera were beneficial prognostic factors of OS in colorectal and in gastric cancer patients of stage III ($P = 0.021$ and $P = 0.011$, respectively).[22]

In summary, preoperative CD4+ cell counts may serve as diagnostic and prognostic markers for patients with GBM. However, not only the quantity but also more importantly the quality of the T cell subpopulations may reflect the immunocompetence of the patients.[8] Our study, has not investigated for the function of T cells (T helper cells/cytotoxic T cells /T regulatory cells). In our opinion though, our results still identify total CD4+ cells as potential therapeutic targets in patients with glioblastomas.

The prognostic relevance of a significant aspect of the gliomas immunity, such as the family of microglia/macrophages has not been evaluated in the present study. These cells comprise the largest population of the tumor infiltrating cells within the glioma tissue. A shift from M1 (active/cytotoxic/proinflammatory phenotype)[4] toward M2 (antiinflammatory/immunosuppressive phenotype)[4] macrophages population has been well documented in both glioma tissue and peripheral blood monocyte cells.[20] Our study, though, investigates for the prognostic impact of many other important aspects of gliomas immunity, such as representative members of immunoglobulins, cytokines, complement, and T cell subpopulations.

Of note, our prospective study is susceptible to some limitations. We should concede that our limited population may not allow for far-reaching conclusions. However, our survival analysis also identified, similar to larger series, the same known prognostic factors, that is, age, type of resection, and administration of radiotherapy [Table 2].[2,6,18,23,28] Even though the number of our patients was small, our study group demographics did not differ from the reported large series of GBM [Table 1].[2,6,14,18,23,28,30] Apart from the limited population, we could not evaluate, due to lack of data, some interesting clinical and treatment factors, such as extent of resection (gross total resection vs. partial resection vs. biopsy) or eloquence. We could also not estimate other outcome parameters, such as time to progression. However, our results still give some evidence that higher preoperative values of IL-2 and CD4+ cell counts may affect beneficially the prognosis in patients with GBM.

In conclusion, our results demonstrated that preoperative values of certain immune parameters in sera may carry a prognostic impact. Novel diagnostic models prior to histopathological confirmation may be used to predict prognosis of patients with GBM. Future studies should investigate whether targeting immune biomarkers, such as CD4+ and IL-2, may improve the prognosis of patients with GBM.

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