Association of elevated glial expression of interleukin-1β with improved survival in patients with glioblastomas multiforme

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Object. The aim of this study was to investigate the association of interleukin-1β (IL-1β) expression with improved survival in patients with glioblastomas multiforme (GBMs). Immune and vascular host–tumor interactions play a pivotal role in the control of tumor development, and inflammatory mechanisms may participate in the host’s defense against tumor cells. Expression of proinflammatory cytokines and of inducible nitric oxide synthase (iNOS) has been noted in various types of malignant tumors, raising the possibility that endogenous expression of cytokines and the resulting cytotoxic action of sustained NO production play a role in the control of tumor growth. Indeed, human GBMs express variable amounts of iNOS.

Methods. In this study, the expression of iNOS and of cytokines known to upregulate IL-1β, tumor necrosis factor–α, interferon-γ or downregulate iNOS transcription (IL-10, transforming growth factor [TGF]β1, and TGFβ2) were measured using reverse transcription–polymerase chain reaction with competitor DNA in 39 samples of human GBM. The iNOS level in GBM was positively correlated with IL-1β messenger (m)RNA, but not with the other cytokines tested. Immunocytochemical double labeling revealed that both anti-iNOS immunoreactivity and anti–IL-1β immunoreactivity colocalized with glial fibrillary acidic protein immunoreactivity in GBM. Some macrophage/microglial cells also expressed iNOS, but not IL-1β. Comparison of biological data with clinical parameters indicated that the survival duration was enhanced when levels of IL-1β mRNA were elevated or when levels of TGFβ1 were low, but was independent of the level of iNOS mRNA within the tumor.

Conclusions. Taken together, these data indicate that the proinflammatory cytokine IL-1β produced within GBM by glial-derived cells has a negative impact on tumor growth through a mechanism independent of iNOS induction.

Key Words • glioma • cytokine • interferon • interleukin • nitric oxide • transforming growth factor • tumor necrosis factor

The complex relationships between solid tumors and host tissue provide new insights into tumor biology and the development of alternative therapies. In line with these approaches, attempts have been made to understand better what signals host and tumor cells use to support the complex mechanism of immune and inflammatory host defense against tumor. Indeed, production and release of proinflammatory cytokines within the tumor tissue, either by the tumor cells or by the host-derived stromal cells, may influence tumor growth.

Human brain tumors, especially gliomas, exhibit various patterns of cytokine expression. Under certain circumstances, cytokines can modulate tumor growth, opening the possibility of immunotherapy for human gliomas.

Little is known about the mechanism by which cytokines influence tumor growth, although some authors suggest that NO may have antitumoral properties. Indeed, sustained and elevated tissue levels of NO produced in response to the expression of calcium-independent iNOS have cytotoxic effects. The promoter region of the iNOS gene confers inducibility by various factors, including IL-1β, IFNγ, and TNFα. In turn, other factors such as IL-10, TGFβ, or glucocorticoids attenuate the expression of iNOS. Expression of iNOS mRNA thus reflects the result of the activation of the pro- and antiinflammatory cytokine network. We have previously shown that human GBMs exhibit wide variations in iNOS expression, raising the question of a possible role for endogenous NO in the control of tumor growth.

The present study of human GBM tissue was designed to characterize further the expression of iNOS, to explore the expression of cytokines known to upregulate (IL-1β, TNFα, IFNγ) or downregulate (IL-10, TGFβ1, TGFβ2) iNOS production, and to identify the phenotype of cells expressing iNOS and cytokines within these tumors. Moreover, we attempted to determine whether endogenous cyto-

Abbreviations used in this paper: cDNA = complementary DNA; GBM = glioblastoma multiforme; GFAP = glial fibrillary acidic protein; IFN = interferon; IL = interleukin; iNOS = inducible nitric oxide synthase; mRNA = messenger RNA; NO = nitric oxide; PCR = polymerase chain reaction; RT = reverse transcription; TBS = Tris-buffered saline; TGF = transforming growth factor; TNF = tumor necrosis factor.
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kine and iNOS expression might be associated with either poor or improved clinical outcome in these patients.

**Materials and Methods**

**Tumor Specimens**

Tumor tissue specimens were collected from 39 consecutively treated patients. All patients had been treated with corticosteroids (1–2 mg/kg/day methylprednisolone). The diagnosis of GBM was made using the histological classification of the World Health Organization. For each tumor, some samples were immediately frozen and stored at −80°C under RNase-free conditions, whereas others were processed for paraffin embedding. All subsequent experiments were conducted in a blinded fashion. Clinical data including age at diagnosis, sex, performance status using the Order scale, treatment modalities, and duration of survival (from date of histological diagnosis to death) were prospectively recorded.

**Reverse Transcription–Polymerase Chain Reaction Studies**

The RNA extraction was performed according to Chomyczynski and Sacchi. The RNA yield was quantified spectrophotometrically from the absorbance at 260 nm, and RNA integrity was checked by electrophoresis on a 1% agarose gel containing 3% formaldehyde. The RT was performed in a final volume of 20 µl from 1 µg of denatured template RNA incubated at 37°C for 1 h in the presence of random hexamers (2.5 µM), recombinant RNase inhibitor (25 IU), deoxyxynucleoside triphosphate (500 µM), RT buffer, and Moloney murine leukemia virus RNAse-H (RT) (20 IU). The cDNA was amplified by the PCR by using primers for specific iNOS (sense: GTGCATCGACCTGGGCTGGA; antisense: CACTGCAGGCGCTGTC), TGFβ (sense: GCCCTGGACACC-ACTATTGCT; antisense: AGGCTCCAAATGTAGGGGCAG), IL-1β (sense: GGATATGGACGACCAACAAGTTG; antisense: ATGTACCAGTTGGGAACTG), TNFα (sense: ACAAGCCTTGATGCGACATGT; antisense: AAAGATGACCTGCCCCAGACT), IFNγ (sense: GAGAGCCTAACATTGCTCCTTCT; antisense: ATAGTCTGGTGTGACAGACAGA), IL-10 (sense: ATGGTCCAGATCTCCCGAGA; antisense: AAATCGATGACACCGCGCGTA), TGFβ2 (sense: GCCCTGGACACCAACTATTGCT; antisense: AGGCTCCAAATGTAGGGGCAG), TGFβ3 (sense: GAGAGCCTAACATTGCTCCTTCT; antisense: CCTTCGTCCTCCGCGGTCG), and β-actin (sense: GTGCACAGGGGCTGACCG, 269 bp (TGFβ2), 158 bp (β-actin). The results were quantified after two control steps, as follows: 1) β-actin amplification in a separate tube as a control for RNA extraction and RT, 2) coamplification of standard competitor DNA for each pair of primers as a control for PCR amplification. Standard DNA for iNOS amplification was constructed and cloned in the pT7-blue vector and Sacchi. The RNA yield was quantified spectrophotometrically from the absorbance at 260 nm, and RNA integrity was checked by electrophoresis on a 1% agarose gel containing 3% formaldehyde.

**Immunocytochemical Studies**

Paraffin-embedded adjacent cryostat sections (3 µm) were placed on gelatin-coated slides and processed for double immunocytochemical labeling by using a labeling kit, whereas some sections were processed for hematoxylin and eosin staining. Briefly, paraffin was removed by successive incubations in toluene (2 × 5 minutes), 100% alcohol (5 + 3 minutes), 95% alcohol (5 + 3 minutes), and distilled water (5 minutes). Slides were pretreated by immersion in a 20°C target retrieval solution (pH 6) progressively heated to 96°C and maintained for 20 minutes at this temperature, left at room temperature for at least 20 minutes, and then washed in 1× TBS buffer. Endogenous peroxidase activity was blocked by a 3-minute incubation in the appropriate reagent; slides were then rinsed in distilled water for 1 minute and washed in 1× TBS for 5 minutes. A 3-minute incubation in TBS containing 3% bovine serum albumin, followed by 2 × 5 minutes of washing in TBS containing 0.1% Tween 20, was used to block nonspecific labeling. As the first primary antibody, rabbit polyclonal antiserum directed to human IL-1β (dilution 1:200 in Dako buffer) was incubated overnight at 4°C, then for 15 minutes at room temperature, and then washed for 3 × 5 minutes. The amplification polymer coupled to alkaline phosphatase was incubated for 30 minutes at room temperature and subsequently washed for 10 and 2 × 5 minutes. The 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium alkaline phosphatase substrate was added for 30 minutes in a dark environment, the reaction was stopped by distilled water, and the slides were washed for 2 × 5 minutes. Unbound primary antibodies were blocked by a 3-minute incubation in the corresponding buffer, followed by washing for 2 × 5 minutes.

The second primary antibodies consisted of either Kp1, which was directed to the macrophage/microglial marker CD68 (murine monoclonal anti-human CD68, diluted 1:300), or an anti-GFAP antiserum (rabbit polyclonal anti-gow GFAP, diluted 1:1000). The cells were incubated for 30 minutes at ambient temperature, washed for 3 × 5 minutes, incubated for 30 minutes with the amplification polymer coupled to peroxidase, and then washed for 10 and 2 × 5 minutes. The 3-amino-9-ethylcarbazole chromogen peroxidase substrate was incubated for 8 minutes in the dark, the reaction was stopped by distilled water, the slides were washed for 15 minutes, and then mounted with the appropriate medium and dried for 10 minutes before microscopic observation. In this experiment, slides were labeled for either IL-1β alone, IL-1β and Kp1, or IL-1β and GFAP. Control reactions performed without primary antiserum resulted in an absence of labeling. The same protocol was used for immunocytochemical double labeling with a first primary antibody directed to iNOS (polyclonal rabbit anti-iNOS, diluted 1:150).

**Statistical Analysis**

Normal distribution of the values used in regression models (iNOS, cytokines, duration of survival) was obtained by logarithmic transformation. Correlation between these numerical values was tested using a simple linear regression model. After splitting the sample into two categories by the median value for each parameter (high or low value), a log-rank test for equality of survivor functions was used to compare survival between the categories. Multivariate analysis was performed using the Cox proportional hazards model, which allowed us to assess, after adjustment for the other factors, the influence on patient’s survival of factors entered in the model.
The pT7-blue vector was obtained from Merck Eurolab, Fontenay-sous-Bois, France. The deoxycytidine triphosphate was acquired from Amersham International, Buckinghamshire, UK. The Trio-thermoblock was supplied by Biometra, Göttingen, Germany. The laser densitometer (Phosphorimager model SI) and ImageQuant software were purchased from Molecular Dynamics (now Amersham Pharmacia Biotech, Les Ullis, France). The following were obtained from Dako Corp., Glostrup, Denmark: Envision immunocytochemical labeling kit (K1395), retrieval solution (S1700), reagent (S2001), buffer (S0809), alkaline phosphatase substrate (K0598), CD68 (# M0814), GFAP (# Z334), 3-amino-9-ethylcarbazole chromogen peroxidase substrate (K3461), and medium (S3025). The rabbit polyclonal antiserum (LP 712) was acquired.

Fig. 1. Photomicrographs of GBM cells. Upper: Immunocytochemical staining for IL-1β immunoreactivity shows that staining in GBM was restricted to the cytoplasm of some large-sized cells. Center: Double labeling indicated that these IL-1β–positive cells frequently exhibited GFAP immunoreactivity in a pericytoplasmic pattern, sometimes with numerous processes, suggestive of their astrocytic phenotype. Lower: In contrast, IL-1β never colocalized with the macrophage/microglial marker CD68. Bar = 50 μm.

Fig. 2. Photomicrographs of GBM cells. Upper: Immunocytochemical labeling for iNOS. In most cases, iNOS immunoreactivity labeled the cytoplasm of large-sized cells, but some small-sized cells were also labeled. Center and Lower: Immunocytochemical double staining indicated that iNOS immunoreactivity colocalized with the astrocytic marker GFAP in the large-sized cells (center) but also with the macrophage/microglial marker CD68 in a few small-sized cells (lower). Bar = 50 μm.
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from Genzyme, Cambridge, MA. The first primary antibody (# N32030) was purchased from BD Transduction Laboratories, Erembodegem, Belgium.

**Results**

This study included tissue from 39 GBMs diagnosed according to the histological classification of the World Health Organization. Multiple samples (between two and four) were collected from the dense tumor area in these patients, letting us assess the low intranidividual changes of the measured variables (in any case, intrindividual variance was significantly lower than interindividual variance, that is, p = 0.0012 for iNOS). The median survival duration in our sample (mean age 53 years, 76% male) was 340 days (mean 423 days).

**Levels of iNOS and Cytokine mRNA**

Levels of transcripts encoding iNOS in GBM showed scattered values, as previously reported.16 Significant amounts of mRNAs encoding IL-1β were detected in the 39 GBM samples (2.23 ± 0.51 [mean ± standard error of the mean], PCR 30 cycles). In contrast, only a minority of tumors (eight of 39) expressed TNFα, and at low levels when compared with IL-1β mRNA values (1.11 ± 0.39, PCR 30 cycles). No mRNAs encoding IFNγ were detected under these experimental conditions (30 PCR cycles). Moderate amounts of IL-10 mRNA were detected in nearly all tumor samples (33 of 39) (0.74 ± 0.12, PCR 30 cycles), and levels of TGFβ2 mRNA were elevated in 38 of 39 tumor samples (2.69 ± 0.25, PCR 30 cycles). The expression of TGFβ2 mRNA (detected in 37 of 39 tumors) was far greater, however, than that of any other cytokine because its detection only required 20 PCR cycles (0.26 ± 0.03, PCR 20 cycles).

**Immunocytochemical Localization of iNOS and IL-1β in GBM**

Tumor tissue samples were collected from the dense tumor area. Most of the cells among these tumor samples consisted of large-sized cells with numerous processes exhibiting pericytoplasmic GFAP immunoreactivity, although the level of staining varied from cell to cell. In contrast, only a few scattered and small-sized cells were positive for the macrophage/microglial marker CD68. Immunoreactivity for IL-1β was present only in the cytoplasm of large-sized cells with processes, and frequently colocalized with GFAP-IR (Fig. 1). In contrast, IL-1β never colocalized with CD68. In most cases, immunoreactivity for iNOS was present in large-sized cells and also frequently colocalized with GFAP-IR, although in some cases iNOS immunoreactivity was detected in small-sized cells and colocalized with the macrophage/microglial marker CD68 (Fig. 2).

**Correlation Between iNOS and Cytokine mRNA Levels**

Simple linear regression analysis showed that iNOS mRNA levels did not significantly correlate with IL-10, TGFβ2, or TGFβ3 mRNA levels (however, TGFβ3 mRNAs exhibited a significant positive correlation with IL-10 mRNAs; \( r = 0.455; p = 0.005 \)). In the few samples expressing TNFα, levels of its mRNA did not correlate with iNOS expression. In contrast, as shown in Fig. 3, there was a highly significant positive correlation between IL-1β and iNOS mRNA levels (\( r = 0.494; p = 0.0014 \)).

**Correlation Between Clinical Data and iNOS or Cytokine mRNA Levels**

Simple regression analysis showed a significant positive correlation between survival and IL-1β mRNA level (\( r = 0.421, p = 0.0077 \)), and a similar but nonsignificant trend for iNOS mRNA (\( r = 0.296, p = 0.068 \)). As shown in Fig. 4, the log-rank test for equality of survivor functions confirmed that survival was positively influenced by the levels of IL-1β mRNA (\( p = 0.0053 \)). The levels of iNOS (\( p = 0.19 \)), IL-10 (\( p = 0.60 \)), TGFβ2 (\( p = 0.14 \)), and TGFβ3 (\( p = 0.12 \)) mRNA had no significant influence on outcome, although survival tended to increase when iNOS levels were higher or TGFβ2 levels were lower. Survival was not significantly influenced by chemotherapy (\( p = 0.43 \)), sex (\( p = 0.19 \)), age (\( p = 0.19 \)), or by the functional status of patients (\( p = 0.53 \)). Obviously, patients previously treated with radiotherapy (six of 39) had a worse outcome (\( p = 0.02 \)).

Multivariate analysis with the Cox proportional hazards model also indicated that, when cytokines were not taken into account, survival duration tended to increase with higher NO2 mRNA levels, but without significant association (\( p = 0.2 \)). The IL-1β mRNA levels were significantly associated with an increased survival duration: a one-log increase in IL-1β mRNA was associated with a 59% decrease in death risk (\( p = 0.02 \) after adjustment for iNOS mRNA). When iNOS and the cytokines (IL-1β, IL-10, TGFβ2, TGFβ3) were entered in the model, the positive association between survival duration and IL-1β mRNA level was strengthened (88% decrease in death risk for a one-log increase in IL-1β mRNA, \( p = 0.002 \) after adjustment for iNOS and the other cytokines). Survival was also nega-
tively associated with TGFβ2 mRNA (120% increase in death risk for a one-log increase in TGFβ2 mRNA, p = 0.04 after adjustment for iNOS and the other cytokines), whereas the other cytokines (IL-10, p = 0.19; TGFβ1, p = 0.27) had no significant influence on survival. Finally, the iNOS mRNA level was no longer associated with an improved survival after adjustment for IL-1 and the other cytokines (p = 0.83) (Table 1).

Discussion

Cytokine Expression Patterns

Patterns of cytokine expression have been studied in central nervous system tumors. In agreement with other reports, significant amounts of IL-1β mRNA were found in all tumor samples in our GBM series. Although the exact role of IL-1β in tumor growth remains to be elucidated, this cytokine may be involved in growth inhibition and in the direct induction of apoptosis, as well as the expression of adhesion molecules.

It is well demonstrated that experimental intracerebral injections of IL-1β induce an inflammatory reaction and a massive neutrophil infiltration. A very interesting point is the age dependence of the amount of neutrophils observed after IL-1 injection (the younger the animal, the higher the infiltration). It is noteworthy that age is one of the most powerful prognostic factors of malignant gliomas.

Tumor necrosis factor–α has been proposed as a treatment for human gliomas, although its in vitro effect on cell proliferation is highly dependent on the astrocytic cell line. Endogenous TNFα mRNA expression was an inconsistent feature, however, in our GBM series. Other groups have found similar profiles of TNFα expression and have localized its synthesis in various cell types. Although IFNγ mRNAs have been found in some GBMs, we failed to detect its expression in our series, despite the fact that iNOS expression by astrocytic cells in vitro has been reported to require IFNγ. We found significant levels of IL-10 mRNA in most of our GBM specimens, in agreement with previous reports. Whereas TGFβ3 expression was moderate, TGFβ2 mRNA was highly expressed in our GBM samples.

Cytokines and iNOS Induction

Despite this high level of TGFβ2 expression, its action as a downregulator of iNOS expression, and its well-documented immunosuppressive effects in GBM, there was no correlation between TGFβ2 mRNA and iNOS expression in our samples. Whereas TGFβ3 and IL-10 have also been reported to inhibit iNOS expression, we found no correlation between iNOS and IL-10 or TGFβ2 mRNA levels. Tumor necrosis factor–α was inconsistently present and IFNγ was absent in our tumor samples; therefore, IL-1β appeared to be one of the main determinants of iNOS expression because it showed a positive correlation with iNOS mRNA. These results are in agreement with previously reported data showing that IL-1 can induce NOS expression in human astrocytes.

Cellular Localization of IL-1β and iNOS

Immunocytochemical double labeling showed that IL-1β is colocalized with the astrocytic marker GFAP, whereas iNOS colocalizes with either GFAP or CD68, a macrophage/microglial marker. Our data are in agreement with other reports indicating that, in human glioma, IL-1β has a glial-derived origin, whereas infiltrating macrophages express IL-1 Type I receptors (although macrophage production of IL-1 has also been reported). On the other hand, many cell types are able to express iNOS in response to appropriate stimulation. Most of the data on glial iNOS expression have been obtained in vitro from rat C6 glioma and various human glioma cell lines. In these cell lines, glial cells have been shown to express iNOS in response to various mixtures of cytokines, including IFNγ.

Fig. 4. Graph showing survival curves of patients according to the levels of IL-1β mRNA in their samples of GBM cells (Kaplan–Meier and log-rank test, p = 0.0053). The median survival duration was 250 days in the group with low IL-1β mRNA levels, and 385 days in the group with elevated IL-1β.
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Other cell types within the tumors, however, have been shown to express iNOS with appropriate stimulation. Our results with GBM specimens, which included glioma cells, reactive astrocytes, stroma, neovessels, activated microglia, and infiltrating macrophages, indicated that iNOS expression was associated with both astrocytic/glioma cells and macrophage/microglial cells. This result is in agreement with a recent report about iNOS expression in human gliomas in which iNOS immunoreactivity is demonstrated either within activated (CD45-positive) macrophages or tumor cells. Nevertheless, although these authors described a perivascular distribution for iNOS immunoreactive cells, iNOS-positive cells in our specimens had no particular pattern of distribution, as already described for activated macrophage or microglial cells in GBMs. Indeed, macrophage or microglial expression of iNOS is not specific for tumor processes. The same cell types are involved in iNOS production during various neurological disorders including experimental allergic encephalitis and viral encephalitis. Further analysis of biopsy or postmortem samples for quantification of the macrophage infiltration would probably help to determine whether iNOS levels parallel the level of intratumoral macrophage infiltration. This is unlikely, however, because the vast majority of iNOS-positive cells in our samples were large-sized ones mainly consisting of GFAP-positive cells.

If we consider that these cells expressing IL-1β (and iNOS) frequently displayed signs of mitosis and have been observed in the dense tumor area, they likely correspond to glioma cells rather than reactive astrocytes. Indeed, this point is highly relevant to the understanding of tumor physiology and host–tumor interactions: if these are glioma cells, IL-1β turns out to be a tumor-derived rather than a host-derived substance.

Nitric Oxide and Tumor Development

Despite its well-documented cytotoxic effects, the role of NO in tumor generation and growth or tumor suppression remains to be determined. For instance, the metastatic capacity of murine melanoma cells has been shown to be inversely related to iNOS expression, and transfection of these cells with a constitutively expressed iNOS gene eliminates metastases. On the other hand, iNOS expression has been shown to be directly related to the malignancy of tumors. Nitric oxide could act as an endogenous mutagen, altering the function of the p53 tumor suppressor protein (which, in turn, downregulates iNOS transcription), or it could act through the enhancement of vascular endothelial growth factor expression in GBMs. In striking contrast with the antitumor roles of NO described earlier, NO has been proposed to be an important mediator of tumor growth, and these aspects have been recently reviewed.

Cytokines and Clinical Outcome

It does not appear that iNOS is a main determinant of tumor development and clinical outcome according to our study, in contrast with IL-1β and TGFβ.

Although expressing Class I and II major histocompatibility complex antigens as well as tumor-specific antigens, gliomas escape from host immune surveillance because they appear to have immunosuppressive properties that have been attributed to the production of high levels of TGFβ, whose coding gene has been cloned from a human GBM. The robust expression of TGFβ is a characteristic feature of GBM. This process appears to be crucial for glioma growth in animal models, because blockade of TGFβ expression by administration of antisense DNA into the tumor or by gene transfer results in the eradication of the glial tumor. The immunosuppressive effect of TGFβ is suspected to enable tumor development, and the multivariate analysis supports this idea, demonstrating an adverse impact of TGFβ expression on patient’s survival.

In contrast, endogenous production of IL-1β within the tumor is associated with improved survival. This could suggest that an upstream event directs both IL-1β expression and improved outcome through independent mechanisms. On the other hand, this association could also reveal that IL-1β production has a negative effect on tumor growth through an unknown mechanism. Indeed, iNOS induction by IL-1β and subsequent sustained NO production would have been proposed as a causative link between IL-1β expression and a slowed tumor development. This idea is not supported, however, by our data. Indeed, IL-1β is probably the main signal triggering iNOS transcription within these tumors. Nonetheless, the low correlation between iNOS mRNA and survival, which could be observed when iNOS was considered alone, no longer persists when IL-1β (and the other cytokines) are taken into account in a multivariate model. The iNOS levels have no real influence, independent of IL-1β, on a patient’s survival, and iNOS is therefore unlikely to represent an effector downstream to IL-1β that is responsible for the improved outcome (other substances, such as prostaglandin E2, may play a role downstream to cytokines in the modulation of antitumor host defense). Similar conclusions have been reported in a human tumor cell line. In vitro, IL-1 inhibits the proliferation of glioma cells.

Treatment strategies targeting stromal components or immune host defense have recently been investigated to improve the efficacy of anticancer therapies. Further work is required, however, to understand which mechanism, upstream or downstream to glial cytokine expression, has significant impact on tumor growth and clinical outcome in GBM.

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| Variable | Hazard Ratio† | z Value | p > |z| (95% CI) |
|----------|--------------|---------|-----|----------|
| iNOS     | 1.082 ± 0.404| 0.211   | 0.833 (0.521–2.245) |
| IL-1β    | 0.222 ± 0.107| −3.119  | 0.002 (0.086–0.572) |
| IL-10    | 2.034 ± 1.110| 1.301   | 0.193 (0.619–5.926) |
| TGFβ1    | 0.607 ± 0.273| −1.109  | 0.267 (0.251–1.466) |
| TGFβ2    | 2.222 ± 0.864| 2.054   | 0.040 (1.037–4.762) |

* The Cox proportional hazards model was used to calculate each variable. Abbreviation: CI = confidence interval.
† Values are expressed as the mean ± standard error of the mean.
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