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RESEARCH ARTICLE

Impact of *TGF-β1* -509C/T and 869T/C polymorphisms on glioma risk and patient prognosis

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Abstract Transforming growth factor beta (TGF-β) plays an important role in carcinogenesis. Two polymorphisms in the *TGF-β1* gene (-509C/T and 869T/C) were described to influence susceptibility to gastric and breast cancers. The 869T/C polymorphism was also associated with overall survival in breast cancer patients. In the present study, we investigated the relevance of these *TGF-β1* polymorphism in glioma risk and prognosis. A case-control study that included 114 glioma patients and 138 cancer-free controls was performed. Single nucleotide polymorphisms (SNPs) were evaluated by polymerase chain reaction followed by restriction fragment length polymorphism (PCR-RFLP). Univariate and multivariate logistic regression analyses were used to calculate odds ratio (OR) and 95 % confidence intervals (95 % CI). The influence of *TGF-β1*-509C/T and 869T/C polymorphisms on glioma patient survival was evaluated by a Cox regression model adjusted for patients' age and sex and represented in Kaplan-Meier curves. Our results demonstrated that *TGF-β1* gene polymorphisms -509C/T and 869T/C are not significantly associated with glioma risk. Survival analyses showed that the homozygous -509TT genotype associates with longer overall survival of glioblastoma (GBM) patients when compared with patients carrying CC+CT genotypes (OR, 2.41; 95 % CI, 1.06–5.50; p=0.036). In addition, the homozygous 869CC genotype is associated with increased overall survival of GBM patients when compared with 869TT+TC genotypes (OR, 2.62; 95 % CI, 1.11–6.17; p=0.027). In conclusion, this study suggests that *TGF-β1*-509C/T and 869T/C polymorphisms are not significantly associated with risk for developing gliomas but may be relevant prognostic biomarkers in GBM patients.

Keywords Glioma · Glioblastoma · Transforming growth factor beta 1 · Single nucleotide polymorphisms · Risk · Prognosis

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Introduction

During the last decades, the incidence and mortality of brain tumors have increased in most developed countries, mainly in the older age groups, with a slightly higher incidence in men than in women [1]. Gliomas, the most common primary tumors of the central nervous system (CNS), account for almost 80 % of brain malignancies [2]. According to their histological characteristics, these tumors can be divided into four main subgroups: astrocytomas, oligodendrogliomas, oligoastrocytomas, and ependymomas (the less common). Glioma tumors can also be divided into four grades of

malignancy according to the World Health Organization (WHO) classification, being glioblastoma (GBM) the most common and biologically aggressive glioma type (grade 4) [3, 4]. Despite the advances in the field of neuro-oncology, the prognosis of glioma patients remains very poor [5], particularly for patients with GBM [6]. Few factors have been associated with increased glioma risk, including hereditary syndromes, such as Li-Fraumeni and Turcot syndromes, neurofibromatosis (type 1 and type 2) and tuberous sclerosis complex [7, 8], familial aggregation [7, 9], and exposure to high doses of ionizing radiation [7, 10, 11]. Some genome-wide association studies have showed that single nucleotide polymorphisms (SNPs) are associated with glioma susceptibility [12, 13]. However, other factors that may contribute to glioma susceptibility require additional investigation.

The transforming growth factor beta (TGF-β), a multifunctional cytokine, is involved in the regulation of several immunomodulatory processes that play a key role in numerous cellular processes, such as proliferation, differentiation, apoptosis, angiogenesis, tumor progression, and extracellular matrix production [14]. TGF-β has three isoforms, TGF-β1, TGF-β2, and TGF-β3. These three isoforms bind and activate a membrane receptor serine/threonine complex (type I TGF-βRI and type II TGF-βRII). The intracellular signaling is initiated when TGF-βRII phosphorylates TGF-βRI, which in turn phosphorylates the transcription factors Smad2 or Smad3 that consequently bind Smad4. This complex is translocated from the cytoplasm to the nucleus, resulting in the transcriptional activation of TGF-β responsive genes that ultimately mediate the effects of TGF-β at the cellular level [15]. Deregulation of TGF-β signaling has been implicated in cancer, where TGF-β has been demonstrated to have a dual role. It may act as a strong inhibitor of proliferation of normal astrocytes and epithelial cells, being considered a tumor suppressor factor, but in some tumor types, including high-grade glioma, TGF-β acts as an oncogenic factor contributing to cell growth and invasion and decreases host immune responses against tumor [16]. It was also demonstrated that TGF-β activity confers poor prognosis in glioma patients [17, 18]. Several studies have identified *TGF-β1* as a predictive cancer biomarker, particularly focusing on *TGF-β1* genetic polymorphisms [19–22]. In fact, it was demonstrated that polymorphisms in this gene contribute to breast and gastric cancers susceptibility [19, 23]. Additionally, studies demonstrated an association of *TGF-β1* 869T/C polymorphism with overall survival of breast cancer patients [24, 25]. The *TGF-β1* gene is located on chromosome 19q13, and two common polymorphisms of the *TGF-β1* gene have been extensively studied, the -509C/T (rs1800469) and the 869T/C (rs1800470, previously known as rs1982073; T29C and Leu10Pro) [22, 26, 27]. The -509C/T polymorphism is located in the promoter region of *TGF-β1* gene, which may potentially regulate *TGF-β1* transcription. The 869T/C polymorphism is located in exon 1 and could lead to a leucine-to-proline substitution at codon 10 [21, 26]. Some studies demonstrated that the -509T allele is associated with an increased transcriptional activity as compared to -509C allele [28], which leads to a higher serum concentration of TGF-β1 among TT homozygotes than in the CT heterozygotes [29]. Similarly, the 869C allele was associated with high serum concentrations of TGF-β1 [19, 30]. Moreover, some studies showed that -509C/T and 869T/C *TGF-β1* polymorphisms were able to affect TGF-β1 protein expression [31, 32]. Importantly, the circulating levels of this cytokine have been associated with cancer [33–35]. The relevance of *TGF-β1* polymorphisms has not been reported in gliomas. Thus, the aim of this case-control study was to investigate the relevance of *TGF-β1*-509C/T and 869T/C polymorphisms in glioma susceptibility and how specific polymorphic variants may influence the prognosis of patients.

Methods

Study population

In this case-control study, we enrolled 114 glioma patients from Portugal (Hospital of Braga, Braga, and Hospital São João, Porto) diagnosed between 2004 and 2013. The peripheral blood from these subjects was collected. Tumors were classified according to WHO [3], and clinico-pathological features are summarized in Table 1. The control group was randomly selected from blood donors at Hospital of Braga, and it included 138 cancer-free individuals. All subjects were of Caucasian ethnic background. The procedures followed in the present study were in accordance with institutional ethical standards.

Genotyping

Genomic DNA from glioma cases and controls was extracted from peripheral blood leukocytes by proteinase K/chloroform/isopropanol treatment [36]. The purified DNA was used to determine the genotypes of both polymorphisms, using polymerase chain reaction followed by restriction fragment length polymorphism (PCR-RFLP) methods. The PCR primers for -509C/T polymorphism were 5′-CCCGGCTCCATTTCCA GGTG-3′ (forward) and 5′-GGTCACCAGAGAAAGAGG AC-3′ (reverse), and for the 869T/C polymorphism were 5′-CCTCCCCACCACACCAG-3′ (forward) and 5′-CCGCAG CTTGGACAGG-3′ (reverse). The PCR was performed in a total volume of 25 μl containing 50 ng of DNA, 0.5 U of KAPA Taq DNA polymerase (GRiSP), 1× KAPA Taq Buffer A containing $MgCl_2$, 0.2 mM dNTP mix, and 0.8 μM of each primer. For the -509C/T polymorphism, the DNA was initially

Table 1 Clinico-pathological features of gliomas and controls

Groups (WHO grade)	Number of cases	Age, year (mean±SD)	Male/female ratio
Controls	138	40.9±12.1	1.2
Gliomas (2–4)	114	58.3±12.9	1.7
Astrocytomas (2–4)	97	58.9±12.7	2.2
Astrocytomas (2–3)	8	52.3±13.3	1
Diffuse astrocytomas (2)	5	54.8±13.8	0.67
Anaplastic astrocytomas (3)	2	55.0±9.9	1
Gliosarcomas (4)	4	61.3±9.1	All males
Glioblastomas (4)	85	59.5±12.7	2.1
Oligodendrogliomas (2–3)	16	53.1±12.3	0.45
Oligodendrogliomas (2)	4	46.3±9.7	All females
Anaplastic Oligodendrogliomas (3)	10	54.7±12.9	0.67

denatured at 95 °C for 7 min, followed by 11 cycles of 95 °C for 30 s, 66–61 °C for 30 s, and 72 °C for 1 min, followed by 30 cycles of 95 °C for 30 s, 61 °C for 30 s, and 72 °C for 1 min. The PCR was finished by a final extension cycle at 72 °C for 8 min. Regarding 869T/C polymorphism, the PCR cycle conditions consisted of an initial denaturation step at 95 °C for 5 min, followed by 9 cycles of 95 °C for 30 s, 68–64 °C for 30 s, and 72 °C for 30 s, followed by 30 cycles of 95 °C for 30 s, 64 °C for 30 s, and 72 °C for 30 s. Finally, the PCR was completed by a final extension cycle at 72 °C for 8 min. After confirmation of an amplified fragment of the expected size (808 bp for -509C/T and 235 bp for 869T/C) on 2 % agarose gel, 8–12 μL of PCR products were digested overnight at 37 °C with the appropriate restriction enzymes. For the -509C/T polymorphism, 10 U of restriction enzyme Bsu36I (New England Biolabs) was used, and for the 869T/C, 5 U of the restriction enzyme MspA1I (Fermentas) was applied. The DNA fragments were resolved on 2 % agarose gel for -509C/T polymorphism and 4 % agarose gel for 869T/C polymorphism and were detected by Greensafe Premium staining (Nzytech). For -509C/T polymorphism, the PCR product (808 bp) with C allele was digested into two fragments (617 and 191 bp), whereas the PCR product with T allele was not digested by Bsu36I. For 869T/C polymorphism,

Table 2 Univariate analysis of the association between -509C/T and 869T/C polymorphisms and risk for each glioma group

Polymorphism	Control	Glioma (WHO grades 2–4)	OR (95 % CI)[a]	Glioblastoma (WHO grade 4)	OR (95 % CI)[a]
TGF-β1-509C/T					
Genotypes					
TT	22	18	–	16	–
CC	54	42	0.95 (0.45–1.98)	28	0.71 (0.32–1.57)
CT	62	54	1.07 (0.52–2.19)	41	0.91 (0.43–1.94)
CC+CT	116	96	1.01 (0.51–2.00)	69	0.82 (0.40–1.66)
Alleles					
T	0.384	0.395	–	0.429	–
C	0.616	0.605	0.97 (0.68–1.39)	0.571	0.85 (0.57–1.25)
TGF-β1 869T/C					
Genotypes					
CC	26	19	–	17	–
TT	48	42	1.20 (0.58–2.47)	30	0.96 (0.45–2.05)
TC	64	53	1.13 (0.57–2.27)	38	0.91 (0.44–1.89)
TT+TC	112	95	1.16 (0.61–2.23)	68	0.93 (0.47–1.84)
Alleles					
C	0.420	0.399	–	0.424	–
T	0.580	0.601	1.09 (0.76–1.56)	0.576	1.10 (0.75–1.64)

[a] Odds ratio (OR) with 95 % confidence intervals (CI)

the PCR product (235 bp) with T allele was digested into four fragments (103, 67, 40, and 25 bp), and the PCR product with C allele was digested into five fragments (91, 67, 40, 25, and 12 bp).

Statistical analysis

Data analysis was performed using SPSS 22.0 software (SPSS, Inc.). Differences in allele and genotype frequencies were compared between glioma patients and cancer-free controls by the chi-square test, and the frequency distribution of age and sex was compared between glioma patients and cancer-free controls by the nonparametric Wilcoxon-Mann Whitney test. Additionally, the chi-square test was used to verify that the observed allele distribution, in the control group, was in Hardy-Weinberg equilibrium. Odds ratio (OR) and 95 % confidence intervals (95 % CI) were estimated by univariate and multivariate logistic regression analyses, adjusted for patients' age (as a continuous variable) and sex, to assess the risk for each glioma type conferred by a particular allele and genotype of each polymorphism. Patient survival curves were assessed by the Kaplan-Meier method for GBM. A Cox regression model adjusted for patients' age (as a continuous variable) and sex was applied to evaluate the effect of the *TGF-β1* genotypes on overall survival. Statistical significance was considered for p values <0.05.

Results

The clinico-pathological features of the controls and cases are summarized in Table 1. For both *TGF-β1*-509C/T and 869T/C polymorphisms, 114 glioma patients and 138 cancer-free control individuals were analyzed. The statistical analysis of age distribution between control and glioma cases showed significant differences ($p \leq 0.001$). Regarding sex distribution, no significant differences were found between controls and cases ($p=0.195$). The genotype and allele frequencies of the *TGF-β1*-509C/T and 869T/C polymorphisms in controls and glioma cases are shown in Table 2. The frequencies of the CC,

Table 3 Multivariate logistic regression analysis of the association between -509C/T and 869T/C polymorphisms and risk for each glioma group

Polymorphism	Control	Glioma (WHO grade 2–4)	OR (95 % CI)[a]	Glioblastoma (WHO grade 4)	OR (95 % CI)[a]
TGF-β1-509C/T					
Genotypes					
TT	22	17	–	15	–
CC	54	40	1.14 (0.45–2.98)	27	0.76 (0.27–2.13)
CT	62	52	1.11 (0.45–2.75)	39	0.82 (0.30–2.21)
CC+CT	116	92	1.13 (0.48–2.63)	66	0.79 (0.31–2.01)
Alleles					
T	0.384	0.394	–	0.426	–
C	0.616	0.606	1.08 (0.68–1.70)	0.574	0.89 (0.53–1.48)
Age			**1.12 (1.09–1.15)**		**1.14 (1.10–1.18)**
Sex					
Male	76	69	–	58	–
Female	62	40	**0.40 (0.20–0.77)**	27	**0.24 (0.11–0.52)**
TGF-β1 869T/C					
Genotypes					
CC	26	17	–	15	–
TT	48	40	1.36 (0.55–3.34)	29	0.89 (0.33–2.41)
TC	64	52	1.12 (0.47–2.66)	37	0.80 (0.31–2.07)
TT+TC	112	92	1.22 (0.55–2.73)	66	0.84 (0.35–2.02)
Alleles					
C	0.420	0.394	–	0.414	–
T	0.580	0.606	1.19 (0.75–1.87)	0.586	1.09 (0.64–1.84)
Age			**1.12 (1.09–1.15)**		**1.14 (1.10–1.18)**
Sex					
Male	76	69	–	58	–
Female	62	40	**0.39 (0.20–0.76)**	27	**0.24 (0.11–0.52)**

[a] Odds ratio (OR) with 95 % confidence intervals (CI), adjusted for age (as a continuous variable) and sex. Bold-faced values indicate significant differences at 5 % level

CT, and TT genotypes of -509C/T were 39.1, 44.9, and 16.0 % in cancer-free controls, and 36.8, 47.4, and 15.8 % in glioma patients, respectively. Regarding the 869T/C polymorphism, the frequencies of the TT, TC, and CC genotypes were 34.8, 46.4, and 18.8 % in controls, and 36.8, 46.5, and 16.7 % in glioma cases, respectively. The distribution of -509C/T and 869T/C allele frequencies in the control group were in Hardy-Weinberg equilibrium (p=0.891 and p= 0.685, respectively).

When assessing the allele frequencies of the *TGF-β1* -509C/T polymorphism by univariate analysis, we found that the C allele was not significantly associated with a higher risk for glioma (OR, 0.97; 95 % CI, 0.68–1.39; Table 2). Additionally, using TT genotype as reference, the OR analysis showed that the CC, CT, and combined CC+CT genotypes were not significantly associated with increased risk for glioma (OR, 0.95; 95 % CI, 0.45–1.98 for CC; OR, 1.07; 95 % CI, 0.52–2.19 for CT; OR, 1.01; 95 % CI, 0.51–2.00 for CC+CT; Table 2). Evaluating the *TGF-β1* 869T/C polymorphism by univariate analysis, the T allele was not significantly associated with a higher risk for glioma (OR, 1.09; 95 % CI, 0.76–1.56; Table 2). Using CC genotype as reference, the OR analysis showed that the TT, TC, and combined TT+TC genotypes were not significantly associated with increased risk for glioma (OR, 1.20; 95 % CI, 0.58–2.47 for TT; OR, 1.13; 95 % CI, 0.57–2.27 for TC; OR, 1.16; 95 % CI, 0.61–2.23 for TT+TC; Table 2). Taking into account that GBM were the most frequent subtype in our series (n=85), we also compared the control group with GBM cases. Using similar analysis, a lack of association between both *TGF-β1* -509C/T and 869T/C allele or genotype variants and risk for developing GBM was observed (Table 2). Moreover, for both polymorphisms, a multivariate logistic regression model adjusted for sex and age as a continuous variable (Table 3) was applied. As expected, increased age was associated with increased risks for developing glioma and GBM. Similarly, female gender was associated with decreased risks (Table 3). Consistent with the results observed by the univariate analysis, no associations between each polymorphic variant and risk for developing gliomas or GBMs were found (Table 3).

We then evaluated whether these *TGF-β1* polymorphisms may have an impact in patients' survival. To do so, we focused exclusively in GBM patients with available survival data (n= 44), as glioma grade is a strong influencer of survival, precluding an analysis in the whole glioma dataset. Regarding -509C/T polymorphism, the Cox model showed that GBM patients carrying the TT genotype had significantly increased overall survival compared to those with the CC+CT genotypes (OR, 2.41; 95 % CI, 1.06–5.50; Table 4; p=0.036, Fig. 1a). Moreover, patients with CT genotype alone presented a shorter overall survival when compared to those carrying TT genotype (OR, 2.72; 95 % CI, 1.12–6.65; Table 4; p= 0.028, Fig. 1b). No significant differences in overall survival were found in GBM patients with *TGF-β1* -509CC versus TT genotypes (Table 4). Concerning the survival analysis for the *TGF-β1* 869T/C polymorphism, the Cox regression model demonstrated that TT+TC genotypes were significantly associated with shorter survival in GBM patients, as compared to the CC genotype (OR, 2.62; 95 % CI, 1.11–6.17; Table 4; p= 0.027, Fig. 1c). These results were further supported when we compared patients with TC genotype with patients carrying the CC genotype (OR, 2.71; 95 % CI, 1.12–6.54; Table 4; p= 0.027, Fig. 1d). No significant differences in overall survival were found in GBM patients with *TGF-β1* 869CC versus TT genotypes (Table 4).

Discussion

Gliomagenesis is a complex and poorly understood process in which genetic and environmental factors play critical roles. Several studies have suggested that SNPs are the most common sources of human genetic variation, and they may contribute to individual's susceptibility to cancer, including glioma [37]. So far, SNPs of several genes have been studied and identified as putative biomarkers for glioma susceptibility. Some examples include genes encoding proteins involved in

Table 4 Multivariate COX regression analysis of the association between -509C/T and 869T/C polymorphisms and survival in grade 4 gliomas

Polymorphism	Number of cases	OR (95 % CI)[a]
TGF-β1 -509C/T		
Genotypes		
TT	12	–
CC	16	2.09 (0.83–5.31)
CT	16	**2.72 (1.12–6.65)**
CC+CT	32	**2.41 (1.06–5.50)**
Age		1.02 (0.99–1.05)
Sex		
Male	31	–
Female	13	1.13 (0.52–2.45)
TGF-β1 869T/C		
Genotypes		
CC	12	–
TT	14	2.43 (0.88–6.70)
TC	18	**2.71 (1.12–6.54)**
TT+TC	32	**2.62 (1.11–6.17)**
Age		1.01 (0.99–1.04)
Sex		
Male	31	–
Female	13	1.28 (0.57–2.89)

[a] Odds ratio (OR) with 95 % confidence intervals (CI), adjusted for age (as a continuous variable) and sex. Bold-faced values indicate significant differences at 5 % level

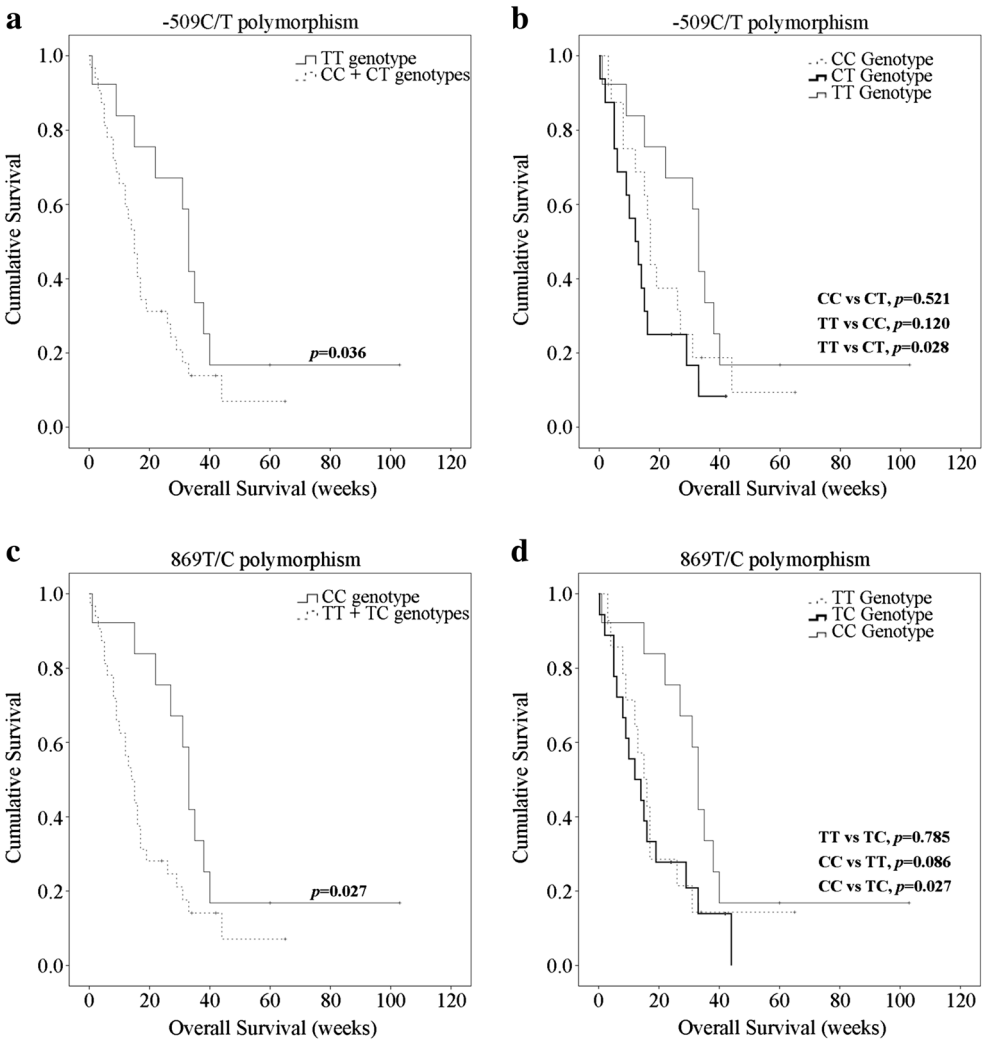

Fig. 1 Effect of *TGF-β1*-509C/T and 869T/C polymorphisms in the survival of glioblastoma patients. Kaplan-Meier overall survival curves for *TGF-β1* -509C/T (**a**, **b**) and 869T/C (**c**, **d**) polymorphisms. In the -509C/T polymorphism, Cox regression analysis showed that the group of glioblastoma patients harboring CC+CT genotypes (**a**, $p=0.036$) or patients with CT genotype (**b**, $p=0.028$) had statistically significant shorter overall survivals when compared to patients with TT genotype. Regarding the 869T/C polymorphism, the group of glioblastoma patients with TT+TC genotypes (**c**, $p=0.027$) or patients with TC genotype (**d**, $p=0.027$) had significantly shorter overall survivals than patients with CC genotype. *Tick marks* indicate censored data

DNA repair pathways (*MGMT*, *PRKDC*, *ERCC1*, *XRCC1*, *APEX1*, *TP53*, *PARP1*, and *LIG1*) [38–41], cancer metabolism (*GST*, *CYP2D6*, *SOD2*, *SOD3*, *GPX1*, and *NOS1*) [42, 43], growth pathways [44, 45], among others [46, 47]. Many association studies on the *TGF-β1* polymorphisms have been conducted in several types of cancer, including lung [27], prostate [20, 26], gastric [21], hepatocellular [22], and breast cancers [19, 24, 30, 48]. To the best of our knowledge, this is the first study to evaluate the *TGF-β1*-509C/T and 869T/C polymorphisms in glioma patients. This is particularly relevant as these two polymorphisms have been reported to affect TGF-β1 protein expression and influence the structure and function of TGF-β1 peptide which may contribute to cancer [31, 32].

Using both univariate and multivariate statistical analyses, our results showed that none of the *TGF-β1*-509C/T and 869T/C polymorphisms are significantly associated with glioma susceptibility. These data fit well with previous studies in other tumor types in which 869T/C was not associated with breast cancer risk [48], and -509C/T polymorphism was not associated with an increased risk of colorectal cancer [49]. While in our dataset, we included solely patients of Caucasian background, future studies should evaluate how these *TGF-β1* polymorphisms may have relevance in other ethnic backgrounds, as previously suggested for many other polymorphisms [50–53].

It has been described that TGF-β1 contributes to cell growth, angiogenesis, and invasion, is highly active, and confers poor prognosis in high-grade glioma patients [16–18]. Therefore, it is conceivable that patients carrying the T allele of the -509C/T polymorphism and patients with the C allele of the 869T/C polymorphism may have reduced cancer survival, since both these alleles are associated with an elevated *TGF-β1* levels. Contrarily, in our study, GBM patients carrying TT genotype of the -509C/T polymorphism and patients with CC genotype of 869T/C polymorphism presented longer overall survival. This is in agreement with a previous work where it has been shown that breast cancer patients carrying the CC genotype of the 869T/C polymorphism presented a longer overall survival [24]. Therefore, the TT genotype of

the -509C/T polymorphism and the CC genotype of the 869T/C polymorphism have the potential to be used as predictive marker of better survival in patients with GBM. Additionally, taking into account that -509T allele has been suspected to increase the transcription of *TGF-β1*, patients that present this variant may be more suited for an anti-TGF-β1 monoclonal antibody therapy (Metelimumab) [54]. It remains, however, to be seen if -509C/T and 869T/C polymorphisms are in a linkage disequilibrium and if it is functionally relevant. For instance, it has been shown that these two *TGF-β1* polymorphisms (-509C/T and 869T/C) are in strong linkage disequilibrium in breast cancer patients, although it remains to be determined which of the two polymorphisms is functionally significant and affect survival [25].

In conclusion, this study shows that *TGF-β1*-509C/T and 869T/C polymorphisms do not confer susceptibility to develop glioma but may have an impact in the survival of GBM patients. Specifically, the *TGF-β1*-509TT and 869CC genotypes can be used as predictive markers of improved survival. In the future, additional studies with larger datasets will be needed to extend and validate these novel findings.

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Conflict of interest None.

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