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ARTICLE

Genetic variants related to angiogenesis and apoptosis in patients with glioma

Variantes genéticas relacionadas à angiogênese e apoptose em pacientes com glioma

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ABSTRACT

Background: Glioma, the most common primary malignant brain tumor in adults, is highly aggressive and associated with a poor prognosis. The objectives of this study were to evaluate the association of genetic polymorphisms related to angiogenesis and apoptosis with gliomas, as well as comorbidities, lifestyle, clinical profile, survival and response to treatment (temozolomide [TMZ] and radiotherapy [RT]) in patients with the disease. **Methods:** In a total of 303 individuals, genotypes were performed by real-time PCR, and clinical data, lifestyle and comorbidities were obtained from medical records and questionnaires. The significance level was set at 5%. **Results:** Smoking, alcohol consumption, systemic arterial hypertension, diabetes mellitus and body mass index prevailed among patients, compared to controls ($p < 0.05$). The heterozygous genotype rs1468727 (T/C) and the homozygous genotype rs2010963 (G/G) ($p > 0.05$) were observed in both groups. Lifestyle and comorbidities showed independent risk factors for the disease ($p < 0.0001$, $p = 0.0069$, $p = 0.0394$, respectively). Patients with low-grade gliomas had a survival rate of 80.0 ± 1.7% in three years. For the combination of TMZ+RT, survival was 78.7 ± 7.6% in 20 months, compared to TMZ only (21.9 ± 5.1%, $p = 0.8711$). **Conclusions:** Genetic variants were not associated with gliomas. Specific lifestyle habits and comorbidities stood out as independent risk factors for the disease. Low-grade gliomas showed an increase in patient survival with TMZ+RT treatment.

Keywords: central nervous system; genes, erbB-1; glioblastoma.

RESUMO

Introdução: Glioma, tumor cerebral maligno, é altamente agressivo e associado a mau prognóstico. Os objetivos deste estudo foram avaliar a associação de polimorfismos genéticos relacionados a angiogênese e apoptose em pacientes com glioma, bem como suas comorbidades, hábitos de vida, perfil clínico, sobrevida e resposta ao tratamento (temozolomida [TMZ] e radioterapia [RT]). **Métodos:** 303 indivíduos foram genotipados por PCR em tempo real, e foram coletados dados clínicos, hábitos de vida e comorbidades. Admitiu-se nível de significância para valor $p < 0,05$. **Resultados:** Tabagismo, elitismo, hipertensão arterial sistêmica, diabetes mellitus e índice de massa corporal prevaleceram entre os pacientes, comprados aos controles ($p < 0,05$). O genótipo heterozigoto rs1468727 (T/C) e homozigoto rs2010963 (G/G) ($p > 0,05$) foram observados em ambos os grupos. Tabagismo, elitismo, hipertensão arterial sistêmica, diabetes mellitus e índice de massa corporal apresentaram fatores de risco independentes para a doença ($p < 0.0001$, $p = 0.0069$, $p = 0.0394$, respectivamente). Os pacientes com gliomas de baixo grau apresentaram sobrevida de 80,0 ± 1,7% em três anos. Para a combinação de RT e TMZ, a sobrevida foi de 78,7±7,6% em 20 meses, em comparação com TMZ (21,9 ± 5,1%, $p = 0,8711$). **Conclusões:** As variantes genéticas não estiveram associadas aos gliomas. Hábitos de vida e comorbidades específicas destacaram-se como fatores de risco independentes para a doença. O tratamento com TMZ + RT mostrou aumento na sobrevida dos pacientes.

Palavras-chave: sistema nervoso central; genes erbB-1; glioblastoma.

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Gliomas are the most common type of primary central nervous system tumors and account for 30% of all tumors, 80% of which are considered malignant[1]. Their classification comprises subtypes based on the criteria established by the World Health Organization[2], with emphasis on astrocytomas, oligodendrogliomas, ependymomas and oligoastrocytomas, which differ in grades I-IV[2]. The incidence in the population is 6/100,000 inhabitants per year, with a ratio of 3:2 for men and women, respectively, in the age group between 45 and 70 years. In 2012, 256,000 new cases of gliomas were diagnosed, accounting for 3% of new cases of cancer worldwide[3], with a predicted global increase of 50-60% by 2030[4].

The United States, compared with other countries, has the highest incidence of primary brain tumors, i.e., about 22,000, and 13,000 deaths per year[5], probably due to improved access to diagnostic imaging, whereas India and the Philippines have the lowest rates[6]. Despite aggressive treatment, the patients show precarious prognoses, with mortality rates higher than 95% between three and five years[5,7,8]. Increasing mortality rates due to this neoplasm can be observed in Colombia and Brazil[9]. In Central and South America, central nervous system tumors were the 11th cause of morbidity and mortality, with 26,000 new cases and 19,000 deaths[3].

The development of gliomas is attributed to interactions between environmental factors, such as lifestyle habits and comorbidities, in addition to genetic factors, such as single nucleotide polymorphisms (SNPs)[4]. Thus, epidemiological studies report mutations in several SNPs as susceptibility factors for gliomas[10,11]. Recent human genome studies have shown increased risk in individuals with variants in the genes for the epidermal growth factor receptor (*EGFR*) and vascular endothelial growth factor (*VEGF*)[10]. However, the mechanisms involved in gliomagenesis need further clarification. Admittedly, angiogenesis is a critical physiological process that results in the growth and progression of various cancers[12]. Additionally, the *EGFR* signaling pathway contributes to many biological processes, including cell cycle progression, metastasis and angiogenesis, which together cause tumor progression[13].

In this context, the objectives of this study were to evaluate the association of gene polymorphisms related to angiogenesis and apoptosis, with gliomas, as well as comorbidities, lifestyle habits, clinical profile, survival and response to treatment in patients with the disease.

METHODS

This was a study with 303 individuals, regardless of gender, ethnicity and age. The study group (SG) comprised 100 patients with gliomas (1–81 years old; 62% males), regardless of the grade of malignancy (Grade I = 7 patients; Grade II = 7 patients; Grade II = 18 patients; Grade IV = 68 patients). Samples of brain tumor tissue were collected from 2003 to 2015 and stored in paraffin-embedded blocks. The control group (CG) comprised 203 individuals without clinical signs of any neoplasia (7–90 years old; 67% males).

All SG patients were selected after histological confirmation from the blocks by the Pathology Department of Hospital de Base (University Hospital of the Medical School of São José do Rio Preto - HB/FAMERP) and their respective clinical and radiological data from medical records. An informed consent document was not required, as the material had already been collected and, therefore, offered no additional risks. The CG individuals were selected from the HB Imaging Department, after skull MRI with a negative diagnosis for gliomas, other cancers or chronic diseases. All CG individuals were informed of the characteristics of the study and confirmed their participation by signing an informed consent form. They also completed a questionnaire with demographics, comorbidities and lifestyle habits, and underwent peripheral blood collection for the analysis of genetic polymorphisms. This study was approved by the Research Ethics Committee CEP/FAMERP (CAAE: 34123314.9.0000.5415).

Genotyping

Genomic DNA from the paraffin-embedded block was extracted by the "ReliaPrep™ FFPE gDNA Miniprep System" (Promega Biotechnology - Brazil). This was performed in five steps: 1) deparaffinization; 2) lysis of cells; 3) RNAse treatment; 4) isolation of DNA; 5) washing and adaptation according to the manufacturer's protocol.

For the CG, the genomic DNA was extracted from a peripheral blood leukocytes sample collected with EDTA, the technique of which consisted of the salting-out method[14], performed in three stages, comprising: 1) lysis of blood cells; 2) deproteinization; 3) DNA precipitation and resuspension, according to the protocol[14].

Concentration and purity were analyzed in a NanoDrop® ND-1000 Spectrophotometer (Thermo Scientific - USA), according to the manufacturer's instructions. The sample absorbance was measured at 260nm/280nm, considering a ratio of 1.8-2.0 as pure. The allele distribution per polymerase chain reaction (PCR) in real time (SNP) was used for genotyping of polymorphisms of *EGFR* and *VEGF*, using TaqMan® SNP Genotyping Assay probes (Applied Biosystems, USA): *EGFR* (rs1468727) - C___2678655_10 and *VEGF* (rs2010963) - C___8311614_10, respectively. Positive and negative control for all reactions was applied with a total volume of 10 μL at a final concentration of 20 ng/μL of DNA. For the reaction mixture, 5μL of TaqMan® Universal PCR Master Mix (Thermo Scientific, USA), 3 μL of DEPC solution, 0.5 μL of TaqMan® SNP Genotyping Assay and 1.5 μL of the DNA sample were added. The samples were processed at 94°C for five minutes, followed by 40 cycles at 94°C for 10 seconds, 60°C for 15 seconds and 72°C for 15 seconds.

Statistical analysis

For the comparative analysis between the groups, the t-test and Fisher's exact test or Chi-square test were used for

quantitative and categorical variables, respectively, as well as logistic regression to identify independent risk factors for the disease. In the evaluation of the Hardy-Weinberg equilibrium, the observed and expected genotype distribution was performed using the Chi-square test. P-values of < 0.05 were considered statistically significant, analyzed using the StatsDirect and GraphPad Prism software.

RESULTS

Regarding the polymorphism analysis (Table 1), the heterozygous genotype (T/C) prevailed in both groups for *EGFR* (SG: 86%, CG=88%, p = 0.7236). The same occurred for the wild-type allele (T) (0.57; 0.56, respectively, p = 0.8674). For *VEGF*, the wild-type homozygous genotype (G/G) was found in both groups (SG = 49%; CG = 44.8%, p = 0.5738), as well as the G allele (0.70 and 0.65, respectively; p = 0.3748).

The Hardy-Weinberg equilibrium analysis showed similarities between the genotype distributions observed and expected for *VEGF*-rs2010963 (SG: χ^2 = 0.1082; p = 0.74211 and CG: χ^2 = 1.4395; p = 0.23021), which did not occur for *EGFR*-rs1468727 (SG: χ^2 = 56.9098; p = 0.00000 and CG: χ^2 = 126.264; p = 0.00000).

Lifestyle and comorbidities are shown in Table 2. Smoking and alcohol consumption prevailed in the SG (39% and 47%, respectively), compared with the CG (24.6% and 16.7%; p = 0.0088; p = 0.0001, respectively). The same occurred for systemic arterial hypertension (SG: 55.0% versus CG: 27.1%; p = 0.0001) and diabetes mellitus (22.0% versus 8.4%, respectively; p = 0.0011), whereas overweight or obesity were similar between the groups (56.0% versus 62.2%, respectively, p = 0.6421).

The genotype distribution (wild-type homozygote versus risk allele genotypes) was also assessed according to the histologic tumor grades. In this case, the risk genotype for both polymorphisms was highlighted. In the analysis of logistic regression, systemic arterial hypertension and diabetes mellitus were identified as independent risk factors for gliomas (p = 0.0001; p = 0.0069 and p = 0.0394, respectively).

In the logistic regression equation, the genotypes with at least one risk allele were considered as follows: (logit Y = -1.29206 +0.225365 smoking +1,376 alcohol consumption +0.786414 systemic arterial hypertension +0.822675 diabetes mellitus -0.272917 *EGFR* -0.403889 *VEGF*). In this case, there was no significance for the risk and smoking genotypes.

The Kaplan-Meier actuarial curve evaluated the survival of patients with gliomas (Grade II-IV) considering the period immediately following the day of the patient's surgery. The comparison between low grade (II) and high grade (III-IV) gliomas showed 80 ± 1.7% free of event/death for low-grade gliomas in three years compared to high grade gliomas (12.9 ± 4.6%), but with no significant difference (p = 0.2689; Figure A). The analysis of the total sample showed 16.7 ± 5% of patients free of event/death in three years (Figure B).

Regarding the response to treatment (Figure C), the combination of temozolomide (TMZ), considered as the gold standard for treatment of gliomas, particularly high-grade gliomas and radiotherapy (RT) as adjuvant therapy (TMZ +

Table 1. Distribution of genotype and allele frequencies of the polymorphisms *EGFR*-rs1468727 and *VEGF*- rs2010963 in patients with gliomas (SG) and individuals without the disease (CG).

Model	Polymorphism EGFR rs1468727 T > C	SG		CG		*p SGxGC
	Genotype	N	%	N	%	
Wild homozygous	T/T	14	14	24	12	0.7236
Heterozygous	T/C	86	86	179	88	0.7236
Mutant homozygous	C/C	0	0	0	0	N/C
	Total	100	100	203	100	
	Allele	N	Freq. Abs.	N	Freq. Abs.	
	T	114	0.57	227	0.56	0.8674
	C	70	0.43	179	0.44	
	Total	200	1	406	1	
Model	**Polymorphism VEGF rs2010963 G > C**	**SG**		**CG**		***p SGxGC**
	Genotype	N	%	N	%	
Wild homozygous	G/G	49	49	91	44.8	0.5738
Heterozygous	G/C	41	41	84	41.4	0.9497
Mutant homozygous	C/C	10	10	28	13.8	0.4514
	Total	100	100	203	100	
	Allele	N	Freq. Abs.	N	Freq. Abs.	
	G	139	0.70	266	0.65	0.3748
	C	61	0.30	140	0.35	
	Total	200	1	406	1	

*Chi-square and Fisher's tests with a significance level of p < 0.05; SG: study group; CG: control group; N: number of individuals; Abs. Freq.: absolute frequency; EGFR: epidermal growth factor receptor; VEGF: vascular endothelial growth factor; NC: not calculated.

Table 2. Distribution of comorbidities and lifestyle habits in patients with gliomas (SG) and individuals without any signs of the disease (CG).

Variables	SG (N =100)		CG (N = 203)		p-value
	N	%	N	%	
Lifestyle habits					
Smoking	39	39	50	24.6	0.0088
Alcohol consumption	47	47	34	16.7	0.0001
Comorbidities					
Systemic arterial hypertension	55	55	55	27.1	0.0001
Diabetes mellitus	22	22	17	8.4	0.0011
BMI ≥ 25 kg/m^2	56	56	60*	62.2	0.6421

*Chi-square test; SG: study group; CG: control group p: significance level < 0.05; N: number of individuals; BMI: body mass index; *60=60/98.

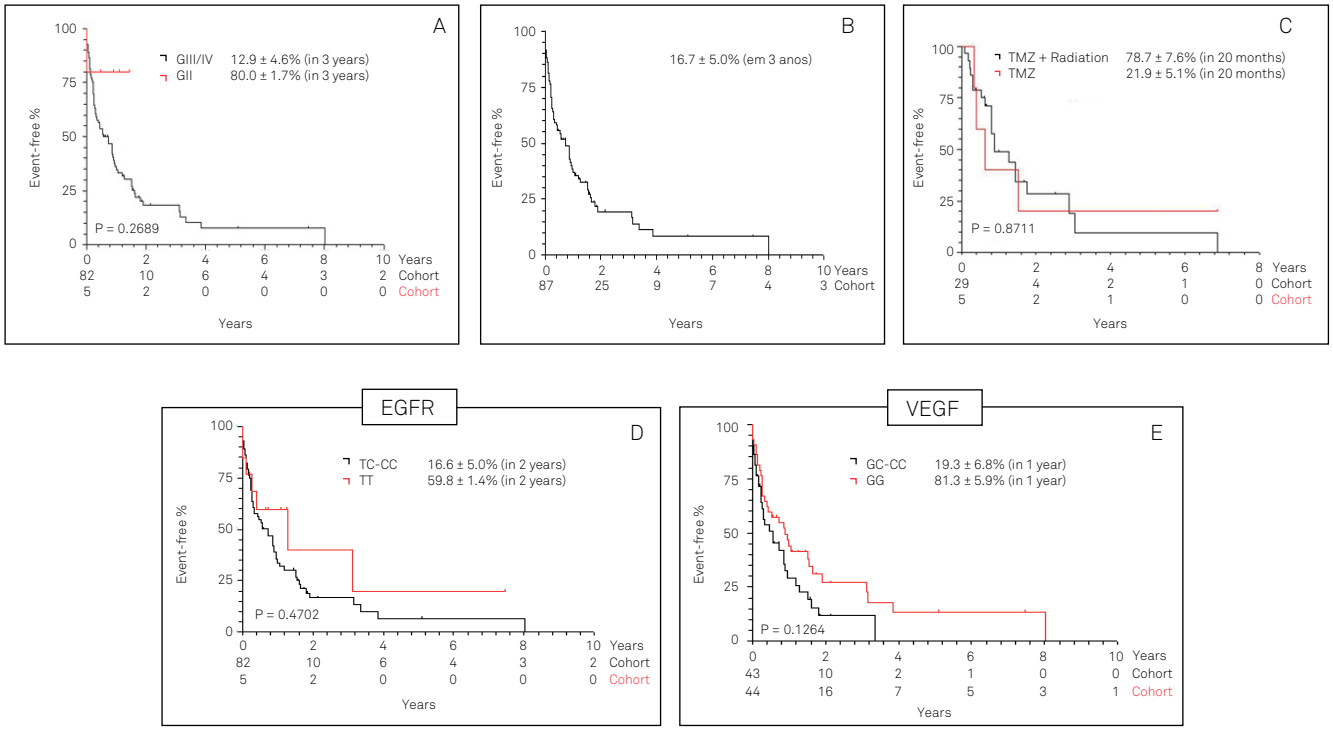

Figure. Kaplan-Meier Curve for analysis of event-free survival (death) in patients with gliomas: A) Analysis according to severity of disease; GII: grade II; GIII: grade III; GIV: grade IV; B) Total group, regardless of disease severity; C) Survival analysis in patients with gliomas receiving treatment with temozolomide (TMZ) and radiotherapy; D) EGFR (*Epidermal growth factor receptor*) = genotype TT and TC/CC and E); VEGF (*Vascular endothelial growth factor*) = genotype GG and GC/CC; Test Log Rank.

RT) increased survival in patients (78.7 ± 7.6% in 20 months), compared to those given only TMZ (21.9 ± 5.1% in 20 months). However, no significant difference (p = 0.8711) was observed.

The four grades of gliomas were used for genetic analysis, but only the grade II, III and IV tumors for the survival analysis. We observed that there were similarities between the risk genotype and the wild-type genotype for both polymorphisms (p > 0.05; Figures D and E). However, there was a decrease in survival in the presence of risk alleles for these polymorphisms.

DISCUSSION

In this study, genetic variants related to intracellular signaling and angiogenesis were analyzed, aiming to evaluate its association with glioma. The association between *EGFR*-rs1468727 polymorphism and gliomas was not confirmed, which was also found in another case study[13], but is in disagreement with other authors[15,16]. In this case, there was a prevalence of the heterozygous genotype (T/C) in both groups, which was also reported in a Chinese population[15]. It is worth pointing out the association of *EGFR* polymorphisms with lung, breast and esophageal cancer[12,13,17-20]. The *VEGF*-rs2010963 polymorphism was not associated with gliomas, as in another study[21], disagreeing with studies in Chinese and French populations[20,21]. References of *VEGF*-rs2010963 polymorphism in gliomas are limited in the literature; therefore, this is a pioneer study with a Brazilian population.

Additionally, we observed the absence of the Hardy-Weinberg equilibrium, which suggests, among other

variables, the impact of evolutionary factors that can alter genotype frequencies and the criteria used in group randomization[17]. The typical mixed-race Brazilian population should be highlighted, as this may have contributed to the divergence in the Hardy-Weinberg equilibrium[18].

Admittedly, environmental factors are relevant in the pathogenesis of gliomas, including smoking and alcohol consumption[7,15], as well as systemic arterial hypertension and diabetes mellitus[22]. In this context, the correlation of lifestyle and comorbidities with the production of free radicals triggering inflammatory processes and apoptosis can be pointed out, and this may lead to the proliferation and evolution of gliomas[7,8,23].

The genotype distribution (wild-type homozygote versus risk allele genotypes) was also assessed according to the histologic tumor grades. In this case, the risk genotype for both polymorphisms was highlighted. However, there were similarities in the distribution of genotypes in the four histological grades for both polymorphisms analyzed (data not shown), in agreement with a study carried out in a Caucasian population[23]. However, studies with a larger sample size should be performed. Notably, the regulation of the EGFR pathway plays an important role in the progression of gliomas, and several SNPs may be correlated with the risk of high-grade gliomas, mainly glioblastomas[15], as well as different types of cancer in humans[12,15].

Systemic arterial hypertension and diabetes mellitus were identified as independent risk factors for gliomas, thus are considered possible predictors of the disease, in agreement with other studies[24].

In relation to survival of patients with gliomas (Grade II-IV), it can be pointed out that high-grade tumors show aggressive behavior, characterized by increased neoplasia, mitosis and increased progression, compared with low-grade gliomas[7], in agreement with a study by Zhang et al[5].

Regarding the response to treatment, the results presented in this study agree with a review study[25], which found a relationship between TMZ + RT therapy combination and an increase in survival in patients with glioma[7,8].

There was a decrease in survival in the presence of risk alleles for the polymorphisms analyzed, which concurs with a study conducted in a Chinese population[7]. Studies correlating the genetic variants with the survival of patients with gliomas are scarce in the literature, especially in the Brazilian population, imposing limitations on the discussion.

In conclusion, genetic variants of EGFR and VEGF are not associated with gliomas. However, alcohol consumption, systemic arterial hypertension and diabetes mellitus stand out as independent risk factors for the disease. There is no correlation between the presence of mutant alleles, morphological grades and clinical profile in our opinion. It can be pointed out that low-grade gliomas provide an increase in patient survival, as does TMZ + RT treatment.

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