JBUON 2019; 24(6): 2478-2485
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
Email: editorial_office@jbuon.com

ORIGINAL ARTICLE

The VEGF gene polymorphism in glioblastoma may be a new prognostic marker of overall survival

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Summary

Purpose: *Glioblastoma (GBM) is the most aggressive primary brain tumor. Vascular endothelial growth factor (VEGF) gene polymorphisms and overexpression are involved in high-grade malignant gliomas. The aim of this study was to assess the distribution of +405C>G VEGF gene polymorphism in patients diagnosed by glioblastoma and to test its association with the overall survival (OS).*

Methods: *Patients diagnosed for glioblastoma were randomly selected, and follow-up was conducted for a minimum of 36 months. Tissue paraffin embedded GBM samples were subjected for the VEGF polymorphism detection. The associations of the observed genotypes and clinical data were evaluated.*

Results: *The most frequent single nucleotide polymorphism (SNP) variant was G (72.58%). The GG genotype was proved to have statistically significant longer OS and patient status (alive/dead) compared to CC and CG genotypes (p=0.022 and 0.005, respectively).*

Conclusion: *Our results indicate that +405C>G VEGF gene polymorphism may be used as prognostic genetic marker of OS in GBM patients.*

Key words: *VEGF polymorphism, glioblastoma, overall survival, prognostic marker, SNP, target therapy*

Introduction

Glioblastoma (GBM) is the most common and aggressive primary brain tumor. It accounts approximately for 51% of gliomas, occurs in both men and women, being more frequent in males. GBM can be characterized by immunohistological, molecular and genetic markers. Except for O^6 methylguanine DNA (MGMT) methyltransferase methylation, no statistically significant data about correlation between molecular markers, genetic markers and response to drugs was found regarding overall survival (OS) [1,2]. GBM is highly angiogenic tumor and characterized by evident vascular proliferation [3,4]. Furthermore, antivascular endothelial growth factor antibody (bevacizumab) showed progression-free survival (PFS) benefit in the recurrence setting [5-7]. Vascular endothelial growth factor (VEGF) is a heparin-binding glycoprotein growth factor specific for vascular endothelial cells which is responsible for angiogenesis [8]. VEGF possesses high angiogenic, mitogenic and vascular permeability-enhancing activity specific for endothelial cells [9,10]. The VEGF gene is lo-

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Received: 27/02/2019; Accepted: 02/04/2019

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cated on chromosome 6p21.3 and contains 8 exons. The various VEGF coding region forms are consequences of the posttranscriptional control through alternative splicing. Four main VEGF protein isoforms are known (121,165,189 and 206 amino acids; 34-42 kDa) [11]. So far, 44 single nucleotide polymorphisms (SNPs) with clinical relevance have been detected (ClinVar NCBI database, October 2018) and the majority of those are associated with cancers of colon and others solid tumors (e.g. breast, pancreatic and renal cancers). Four common VEGF polymorphisms (i.e., -2578C>A, -460C>T, +936C>T and +405C>G) are known [12]. Polymorphism +405C > G (rs2010963) [13] has been associated with prostate cancer. In patients with increased levels of VEGF in blood or tumor were associated with distant metastases and worse prognosis also in patients with pancreatic, colorectal and breast carcinomas [14-17]. Beside tumor progression, VEGF plays a role in other conditions, such as type 2 diabetes [18], myocardial infarction [19], Alzheimer's disease [20], and amyotrophic lateral sclerosis (ALS) [21].

Inactivation of the PTEN tumor suppressor gene and overexpression of VEGF protein represent one of the most common events observed in high-grade malignant gliomas. Linhares et al [22] suggested that PTEN acts on VEGF most likely via down regulation of the transcription factor HIF1-alpha and by inhibition of PI3K. Increased PTEN expression also inhibited the growth and migration of glioma-activated endothelial cells in culture. Recently, genomic polymorphisms of VEGF were associated with a worse prognosis for glioma and glioblastoma, especially the VEGF polymorphisms rs3024994, rs2010963 and the homozygous of rs1005230 [23]. Our research group has started to follow-up a group of patients diagnosed for GBM and after 24 months the first results regarding the +405C>G VEGF polymorphism (rs2010963) and OS in GBM patients didn't reveal any significant association [24]. But in the next period first observations of possible association were detected.

The aim of this study was to assess the distribution of +405C>G VEGF gene polymorphism in patients diagnosed by glioblastoma and to test the association of the named polymorphism with the OS after a minimal follow-up of 36 months.

Methods

Patients >18 years old and with diagnosis of GBM were included in this analysis. The study protocol was approved by the local ethics committee. All consecutive patients between 2014 and 2015 were included in the study. All patients had tumor resection and received adjuvant treatment with concomitant temozolomide plus radiotherapy, followed by 6 cycles of temozolomide monotherapy.

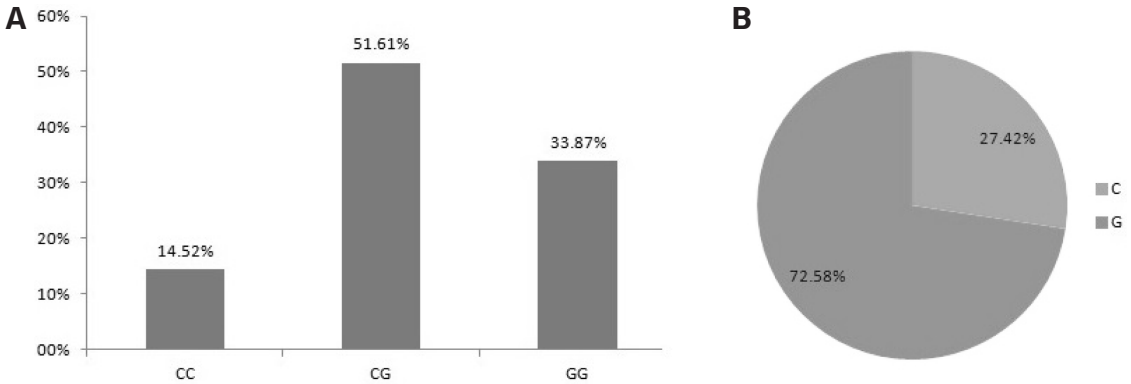

Figure 1. Frequencies of observed genotypes in VEGF gene for all the patients **(A)** and allele frequencies in VEGF gene **(B)**.

Table 1. Descriptive statistics for overall survival in three genotypes of VEGF gene groups

Genotype	*N*	*Mean OS*	*SE*	*95% CI*		*Median OS*	*SE*	*95% CI*		*Min OS*	*Max OS*
				Lower	*Upper*			*Lower*	*Upper*		
CC	9	11.22	3.53	3.06	19.38	6.00	1.49	3.07	8.92	3	36
CG	32	11.88	1.96	7.86	15.89	7.00	1.40	4.25	9.75	2	40
GG	21	16.33	3.03	10.01	22.66	11.00	4.57	2.03	19.97	3	42
Total	62	13.29	1.53	10.22	16.36	7.00	2.15	2.79	11.20	2	42

N: number of patients, OS: overall survival in months, SD: standard deviation, SE: standard error, CI: confidence interval, min – minimum in months, max – maximum in months

Table 2. Multiple comparisons between VEGF genotypes groups considering overall survival

	I	*J*	*Mean difference (I-J)*	*SE*	*p*	*95% CI*	
						Lower	*Upper*
Tukey HSD	CC	CG	-0.653	4.555	0.989	-11.60	10.30
		GG	-5.111	4.809	0.541	-16.67	6.45
	CG	CC	0.653	4.555	0.989	-10.30	11.60
		GG	-4.458	3.390	0.393	-12.61	3.69
	GG	CC	5.111	4.809	0.541	-6.45	16.67
		CG	4.458	3.390	0.393	-3.69	12.61

I, J: VEGF genotype groups, SE: standard error, p: probability value, CI: confidence interval, HSD: honestly significant difference

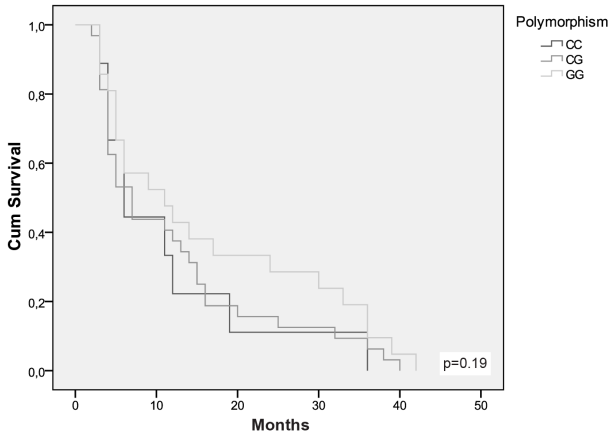

Figure 2. Kaplan-Meier curve showing overall survival of VEGF genotypes.

Paraffine-embadded tissue samples from 66 patients with GBM were subjected to molecular analyses and VEGF polymorphism detection. Genomic DNA was extracted using QIAamp DNA FFPE Tissue Kit (Qiagen Hilden, Germany) following the manufacturer's protocol. DNA extracts were subjected to qualitative and quantitative analysis. DNA concentrations were measured using BioSpec-nano (Shimadzu, Japan), based on the absorbance at 260nm, and the purity estimated by the absorbance ratioA_{260}/A_{280}.

VEGF gene polymorphisms at position +405C>G was evaluated in all patients. VEGF genotypes at position +405C>G were detected using allele-specific PCR. PCR was performed using the following primer pair: 5'-CGA CGG CTT GGG GAG ATT GC-3' and 5'-GGG CGG TGTCTG TCT GTC TG-3'. Reaction was performed in a final volume of 25 μl containing 50-100 ng of DNA, 1xDream Taq Buffer (Fermentas, Lithuania), 2 mM of $MgCl_2$, 0.2 mM of dNTP, 10pM of each primer, and 1.25U Taq polymerase (Fermentas, Lithuania). PCR amplicons were checked using 2% agarose gel electrophoresis.

For the detection of VEGF genotypes, analysis of PCR products was performed by restriction fragment length polymorphism (RFLP-PCR). After amplification, 10 μl of each PCR product was digested with 2U of BsmFI (Fermentas, Lithuania), using the manufacturer's instructions. PCR products were visualised on 4% MetaPhore agarose gels with 10% Roti Stain.

Table 3. Pairwise comparison among VEGF genotypes regarding overall survival and patients' status (alive/dead), inferred by Student's t-test

	CC	*CG*	*GG*
CC	-	0.651	0.091
CG	0.826	-	0.526
GG	0.022	0.005	-

Above: diagonal p values represent significance of OS among all VEGF genotypes; Below: diagonal p values represent significance regarding patients status (alive/dead) after follow-up of 36 months

Table 4. Percent overall survival percent (OS%) after 12, 24 and 36 months in different VEGF genotypes

Polymorphisms	*OS %*		
	12 months	*24 months*	*36 months*
CC	22	11	0
CG	40.6	12.5	6.3
GG	42.9	28.6	9.5

The associations of the analysed genotypes and clinical data have been evaluated. The following data were collected: patient age and gender and OS (months). The results were analyzed using SPSS 17.0 software. For evaluation of differences between groups in OS, t-test was used. The significance of the analyzed VEGF gene polymorphisms was determined using descriptive statistics and presented with their 95% confidence intervals (CI). Furthermore, we applied multi-comparison tests and ANOVA for evaluation of pairwise differences in OS for each genotype. Survival curves were calculated based on the Kaplan-Meier method and log-rank test for comparison of survival among groups. P value <0.05 was considered as statistically significant difference.

Results

All analysed patients were Caucasians and their mean age was 57.7±10.2 at the time of diagnosis.

Genotypes of VEGF gene were successfully scored for 62 patients (93.94%). Missing genotypes most probably occurred due to unsatisfied quality

Table 5. Descriptive statistics for overall survival in three genotypes of VEGF gene in gender groups – females and males

Gender	*Genotype*	*N*	*Mean OS*	*SE*	*95% CI*		*Median OS*	*SE*	*95% CI*		*Min OS*	*Max OS*
					Lower	*Upper*			*Lower*	*Upper*		
Female	CC	4	8.00	3.71	0.71	15.29	4.00	1.50	1.06	6.94	3	19
	CG	12	8.91	2.76	3.50	14.33	4.00	0.87	2.30	5.70	2	36
	GG	10	15.80	4.34	7.29	24.3	11.00	4.74	1.70	20.29	3	42
	Total	26	11.42	2.21	7.08	15.76	6.00	1.27	3.50	8.49	2	42
Male	CC	5	13.80	5.74	2.53	25.06	11.00	5.48	0.26	21.73	4	36
	CG	20	13.65	2.64	8.46	18.83	7.00	5.96	0.00	18.68	3	40
	GG	11	16.81	4.43	8.13	18.84	9.00	6.60	0.00	21.94	3	39
	Total	36	14.63	2.10	10.52	18.75	9.00	3.60	1.94	16.05	3	40

N: number of patients, OS: overall survival in months, SD: standard deviation, SE: standard error, CI: confidence interval, min – minimum in months, max – maximum in months

Table 6. Descriptive statistics for overall survival in females and males

Gender	*N*	*Mean OS*	*SE*	*95% CI*		*Median OS*	*SE*	*95% CI*		*Min OS*	*Max OS*
				Lower	*Upper*			*Lower*	*Upper*		
Female	26	9.96	1.73	6.56	13.36	6.00	1.27	3.51	8.49	2	42
Male	36	14.57	2.03	10.59	18.54	11.00	3.04	5.04	16.96	3	40
Total/mean	62	12.66	1.41	9.90	15.43	7.00	1.98	3.11	10.89	2	42

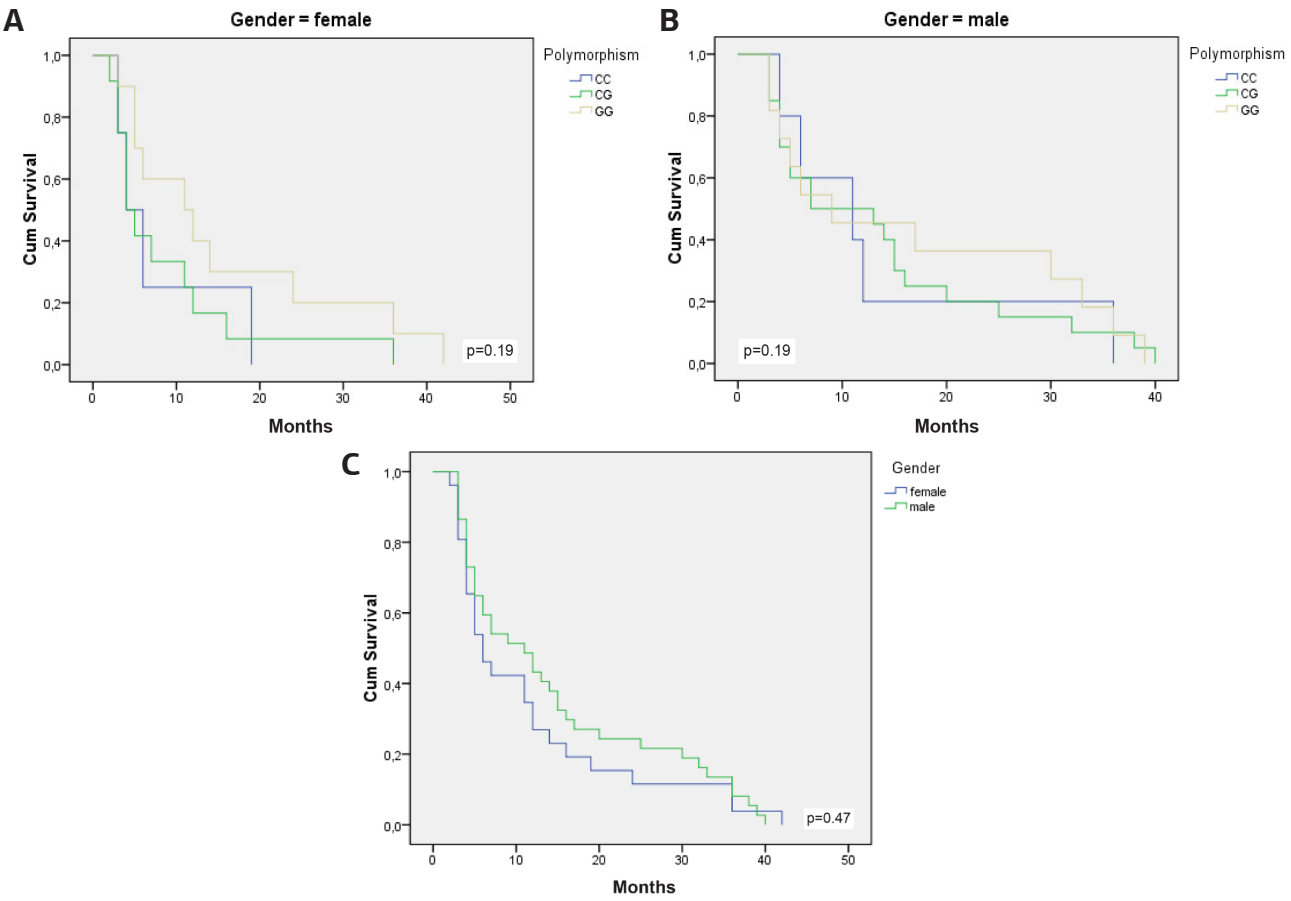

Figure 3. Kaplan-Meier curve showing overall survival of VEGF genotypes in females **(A)** and males **(B)** including VEGF polymorphism, and pairwise comparison of males and females **(C)**.

of DNA extracts, primarily paraffin residues in the samples. All three VEGF genotypes were detected. The most frequent allele (SNP variant) was G (72.58%) (Figure 1).

Descriptive statistics for OS in the three genotypes of VEGF gene were calculated (Table 1) and showed differences in mean and median OS among different genotypes.

Multiple comparisons between VEGF genotypes groups (ANOVA) considering OS as dependent variable didn't show statistical significance (Table 2; Figure 2).

Due to inequality of the number of genotypes in different VEGF genotype groups, we have applied multiple comparison tests using Harmonic Mean Sample Size (15.791) and the performed analysis confirmed lack of statistical significance in OS among genotypes.

Furthermore, t-test was applied as additional test for pairwise comparison among VEGF genotype groups regarding both parameters, OS and patient status (alive/dead) (Table 3). This analysis revealed statistically significant difference between GG genotype (longer survival) and CG and CC, respectively, patients' status (alive/dead).

Graphical representation depicts differences in OS (Figure 2) and reveals that the carriers of the C allele had lower OS. Thus, we analysed the total group for OS in 12, 24 and 36 months, respectively (Table 4).

In order to evaluate differences in OS of GBM patients including VEGF genotype and gender data, we divided the total group in females and males and used descriptive statistics for OS in the three genotypes of VEGF gene in gender groups (Table 5) by constructing Kaplan-Meier curves (Figure 3). The general OS in the gender groups regardless VEGF genotype is presented in Table 6 and Figure 3 and show longer OS in the male group.

We have also analysed female and male groups for percent OS in 12, 24 and 36 months (Table 7) and performed pairwise comparison in OS patient status (alive/dead) for females and males (Table 8), which revealed longer OS in males.

In the next grouping, we divided patients in two age groups: ≤64 and ≥65 years and examined the distribution of VEGF genotypes in different age groups in relation to OS (Table 9, Figure 4). The mean OS in age groups regardless of VEGF genotype is presented using descriptive statistics and Kaplan-Meier curve (Table 10, Figure 4). The independent sample test comparison in OS and age (≤64 and ≥65) (Student's t-test) was significant for intergroup comparison (p=0.015).

Table 7. The percent of alive female and male patients (OS%) after 12, 24 and 36 months

Gender	*OS %*		
	12 months	*24 months*	*36 months*
Female	26.9	11.5	3.8
Male	43.2	21.6	8.3

Table 8. Pairwise comparison in overall survival (OS) of all patients and OS in patients' status (alive/dead) after follow-up for females and males, as inferred by Student's t-test. Numbers represent p values

	Females	*Males*
Females	-	0.318
Males	0.034	-

Above, diagonal p values represent significance of OS among all VEGF genotypes; Below, diagonal p values represent significance regarding patients status (alive/dead) after follow-up of 36 months

Discussion

In order to evaluate whether VEGF polymorphisms has an impact on OS in GBM patients, the distribution of +405C>G VEGF gene polymorphism was screened and the association of named poly-

Table 9. Descriptive statistics for overall survival in three genotypes of VEGF gene in age groups ≤64 and ≥65

Age (years)	*Genotype*	*Mean OS*	*SE*	*95% CI*		*Median OS*	*SE*	*95% CI*	
				Lower	*Upper*			*Lower*	*Upper*
≤64	CC	16.75	7.11	2.81	30.69	6	5.70	0.00	22.20
	CG	18.19	3.13	12.05	24.32	15.00	2.00	11.08	18.92
	GG	19.50	4.39	10.89	28.11	12.00	2.59	6.90	17.09
	Total	18.50	2.36	13.87	23.13	14.00	2.26	9.56	18.44
≥65	CC	9.00	2.52	4.06	13.93	11.00	5.71	0.00	22.20
	CG	7.57	1.90	3.84	11.29	7.00	1.79	3.48	10.51
	GG	15.50	5.18	5.34	25.65	6.00	7.35	0.00	20.40
	Total	10.81	2.24	6.41	15.24	7.00	0.99	5.05	8.94

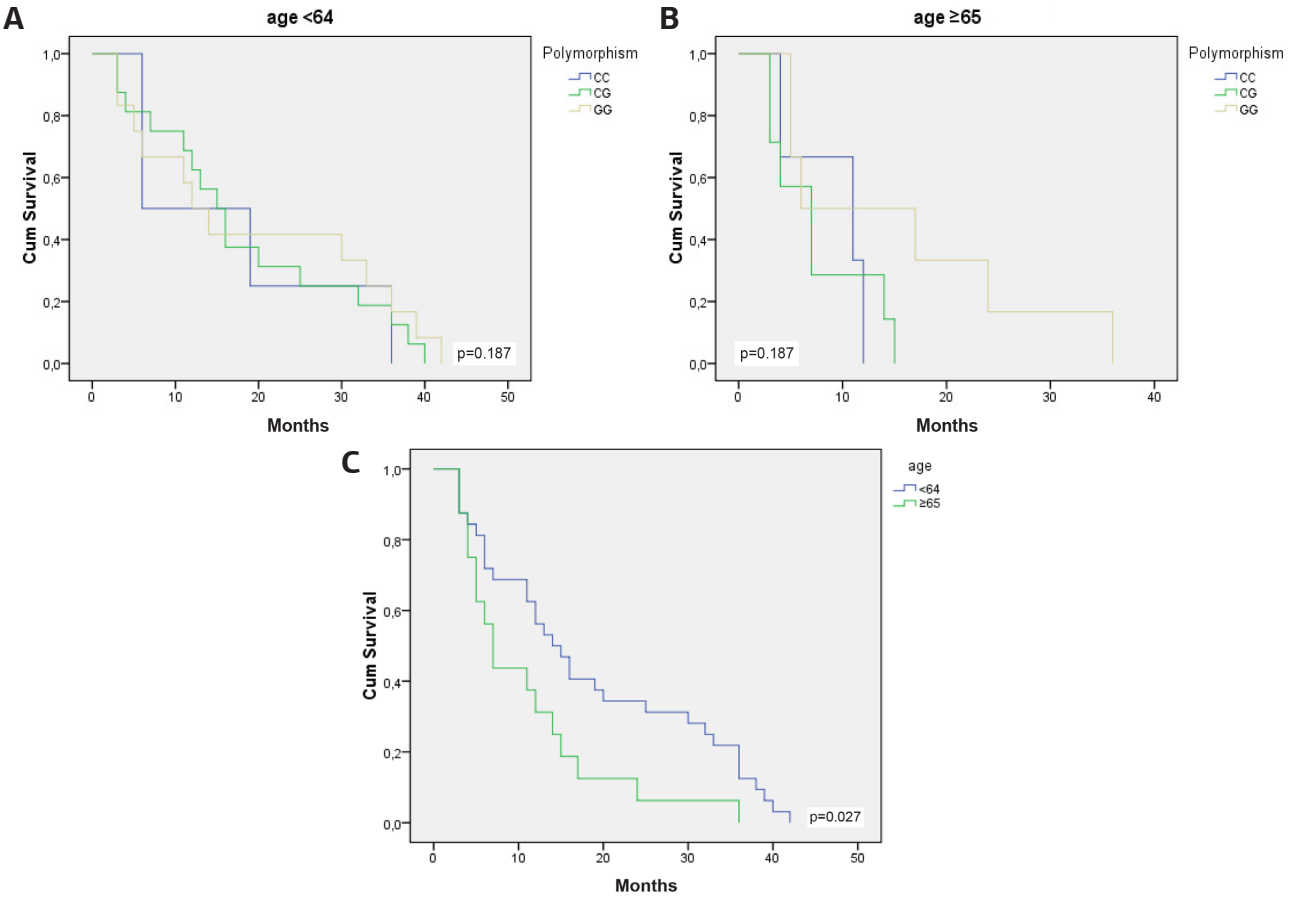

Figure 4. Kaplan-Meier curve showing overall survival in the ≤64 years group **(A)** and ≥65 years group **(B)** of VEGF genotypes and overall survival in ≤64 and ≥65 age groups **(C)**.

Table 10. Descriptive statistics for overall survival in ≤64 and ≥65 years age groups

Age, years	*Mean OS*	*SE*	*95% CI*		*Median OS*	*SE*	*95% CI*	
			Lower	*Upper*			*Lower*	*Upper*
≤64	18.30	2.30	13.80	22.80	14.00	1.79	10.48	17.52
≥65	10.81	2.25	6.41	15.21	7.00	0.99	5.05	8.94
Total/mean	15.86	1.77	12.38	19.33	12.00	1.40	9.26	14.74

morphisms with the OS and patient status (alive/dead) was assessed.

The most frequent allele (SNP variant) was G (72.58%). The genotype distribution was as follows in the total patients group: 14.52%, 51.61% and 33.87% for CC, CG and GG, respectively. Not many studies have dealt with population distribution of the named polymorphism. Among few, +405C>G VEGF gene polymorphism was analysed in healthy UK population (12.9%, 45.2% and 42.0%, respectively) [25] and healthy Hungarian population (4.3%, 30.1% and 65.6%, respectively) [26]. To the best of our knowledge there are not many published data about the distribution of +405C>G VEGF alleles or genotypes in GBM. Actually, we are aware of the results from the study of Linhares et al [23] who detected GC (75.6%) and CC (24.4%), and no GG genotypes. But, we must stress that our study group included almost double number of patients (62 vs. 37) and that we have analysed Serbian population. Considering our data, it seems that in the studied GMB patient group there is a higher percentage of C allele carriers as compared to the healthy populations studied, but since there are no population-genetic data in the named VEGF polymorphism in Serbian or regional populations, these results may also have occurred by chance.

Our statistical analyses revealed statistically significant differences among different VEGF genotypes in GBM patients considering the patient status (alive/dead). The Kaplan-Meier curves depicted a trend for longer (although not significant) OS in GG genotype as compared with the other two genotypes (also confirmed by Student t-test,

p=0.022, compared to CC genotype, p=0.005 compared to CG genotype). The statistical significance was very high regarding the patient status (alive/dead), since it is clear that GG genotype is related with longer survival rate. Graphical representation obtained by construction of the Kaplan-Meier curve (Figure 2) supported the idea to test for the OS percentage (OS%) after 12, 24 and 36 months in different VEGF genotypes (Table 4). The highest OS% occurs in GG genotypes. Considering the fact that all patients were consecutively recruited regardless the therapy applied, we can confirm our observation that GG VEGF genotype is characterized with the highest OS rate.

Since GBM occurs more frequently in males, we have tested the influence of gender together with the VEGF polymorphism on OS and by dividing patients in two groups (male and female). As for the total group, according to our findings (Tables 5 and 6, Figure 3) higher OS for the GG VEGF genotype was observed in comparison with CC and CG genotypes for both, females and males. Statistical significance difference was found in patients' status (alive/dead) between females and males, in favor to males (Tables 7 and 8, Figure 3). Several reasons could be speculated for this finding. It might be that in general diagnosis in females is performed later due to late visits to clinicians when the disease progression is more advanced. We believe that this difference is not directly related to the VEGF genotype, since similar number of females and males were genotyped for GG genotype variant of +405C>G VEGF polymorphism.

By grouping patients in two age groups (≤64 and ≥65) statistical significance was found in mean OS for GG genotype in both groups. According to available literature data, significant differences were found in mean OS between similar age groups of GBM diagnosed patients as in our study [2,27], but to the best of our knowledge testing of differences regarding +405C>G VEGF gene polymorphism has not been investigated in different age groups. The association between +405C>G VEGF gene polymorphism and OS in GBM patients was tested in research involving 225 patients and showed that the presence of CC genotype under therapy with the anti-VEGF drug bevacizumab leads to a long period without progression (28.3 versus 19 weeks with GG genotype) [26], but they didn't register significant difference in survival rate (40.6 vs. 36.2 weeks). In patients who did not receive treatment with bevacizumab, there was no difference in PSF and OS. Another research conducted on 69 patients showed that the presence of G allele shortens the period without progression in patients with incomplete tumor resection. Furthermore, authors consider that the G allele, observed together with resection, can be considered as independent prognostic marker for OS [25]. The latest study [23] strongly suggested that different VEGF SNPs highly increase the risk of developing gliomas and glioblastomas, and that several VEGF SNPs seem to be prognostic markers of survival in glioma and glioblastoma patients. Among those, +405C>G VEGF gene polymorphism (rs2010963) was named as the one which in heterozygous state reduces OS. Some authors stated that this data need confirmation by independent studies, to further prove that VEGF SNPs are potential glioblastoma biomarkers [23].

In conclusion, our results suggest that +405C>G VEGF gene polymorphism (rs2010963) may be used as prognostic genetic marker of OS in GBM patients, as well as potential target for anti-VEGF therapy. Our study included larger number of patients and we strongly believe that the results confirm the important role of this VEGF polymorphism as biomarker, with relevance for anti-angiogenic therapy.

Acknowledgements

The authors acknowledge the financial support of the Provincial Secretariat for Science and Technology Development within the project "Multiparametric structural and metabolic imaging of the intratumorous bioarchitectonics in the function of the diagnosis and therapy improvement in patients with malignant lung and central nervous system tumors"; Grant No. 142-451-2433/2018.

Conflict of interests

The authors declare no conflict of interests.

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