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GENOMICS. TRANSCRIPTOMICS

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Angiotensin II Type I Receptor—168A/G Polymorphism Is Associated with Increased the Risk of Glioma in Turkish Population

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Abstract—Gliomas are the most common primary tumors of the Central Nervous System. Despite advances in the elucidation of molecular pathogenesis, gliomas still remain incurable. In the study, it was aimed to investigate the possible connection between *ACE* and *AGTR1* polymorphisms with glioma pathogenesis and also the relationship of some angiogenic markers with gliomagenesis. In this respect, 96 glioma patients and 104 healthy controls were included in the study. To determine the effect of genetic polymorphisms on the predisposition of diffuse infiltrative glial tumors in the Turkish population, angiotensin-converting enzyme gene (*ACE*) insertion/deletion, angiotensin II receptor type 1 gene (*AGTR1*) −168A/G, −535C/T, −825T/A, and Vascular Endothelial Growth Factor gene (*VEGF*) +936C/T, −2578C/A polymorphisms were investigated by PCR-RFLP. Allele/genotype frequencies between patients and controls were determined. Besides, relative gene expressions of ACE, AGTR1, and VEGF were detected by real time-PCR, while ACE, VEGF, ET-1, eNOS, and NO levels were measured in both serum and tissue by ELISA. In *AGTR1* −168A/G polymorphism, the risk of glioma in the AA genotype decreased, while increased by 2.27 times in the G allele. Allele frequency and genotype distributions of other polymorphisms were found similar between two groups. Serum levels of ACE, VEGF, eNOS, NO, and tissue levels of ACE, ET-1, eNOS, NO were also different between the patients and controls. *ACE*, *AGTR1*, and *VEGF* expressions in patient group were found significantly higher than in control one. These results provide the first evidence linking −168A/G polymorphism in *AGTR1* gene with glioma risk in the Turkish population.

Keywords: glioma, angiogenesis, *ACE*, *AGTR1*, polymorphism

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INTRODUCTION

Gliomas are the most common primary tumors of the Central Nervous System (CNS) in adults [1]. Although angiogenic activity of gliomas is known to be high, the key mechanisms involved in this activity have not yet been fully elucidated. Recent studies have focused on common polymorphisms that predispose to gliomas or cause them to progress more aggressively. Angiotensin Converting Enzyme (*ACE*) gene polymorphisms have become a new target for cancer research in terms of the relationship cancer pathogenesis, metastasis, and angiogenesis. ACE is an important enzyme in Renin–Angiotensin–Aldosterone System and is found in endothelial cells, lungs, and plasma [2]. Studies have shown that this gene has functional polymorphic Insertion/Deletion (I/D) variability in the intron 16 region of humans with an additional 287 bp part in the I allele [3]. The relevance in solving the connection between *ACE* I/D polymorphism and cancer pathogenesis has increased in recent years. Angiotensin II has two subtype receptors called Angiotensin II Type I Receptor (AGTR1) and Type II Receptor (AGTR2). Many polymorphisms were defined in both coding and uncoding regions of this

Abbreviations: CNS, Central Nervous System; ACE, Angiotensin Converting Enzyme; I/D, Insertion/Deletion; AGTR1, Angiotensin II Type I Receptor; AGTR2, Angiotensin II Type II Receptor; VEGF, Vascular Endothelial Growth Factor; NO, Nitric Oxide; ET-1, Endothelin-1; eNOS, Endothelial Nitric Oxide Synthase; WHO, World Health Organization; PCR-RFLP, Polymerase Chain Reaction Restriction Fragment Length Polymorphism; Touch down-PCR, Touch Down Polymerase Chain Reaction; UV, Ultraviolet; RE, Restriction Enzyme; PBS, Phosphate Buffer; PASS, Power Analysis and Sample Size.

gene at different positions [4]. It has been reported in experimental studies that ACE causes angiogenesis via AGTR1, inhibition of angiogenesis via AGTR2 [5], and figures in tumor cell proliferation and metastasis through the production of Vascular Endothelial Growth Factor (VEGF) [6]. It is thought that *AGTR1* and *AGTR2* polymorphisms might be associated with the risk of cancer.

VEGF is the most active and important proangiogenic factor released by cancer cells for vascularization, means that it is the main inducer of tumor angiogenesis. The role of VEGF in angiogenesis increases vascular permeability, basal membrane, and matrix destruction by inducing Nitric Oxide (NO) release. *VEGF* is a highly polymorphic gene with at least 25 different polymorphisms [7]. There are studies supporting that functional polymorphisms in various positions of *VEGF* can contribute to cancer development. Some of these studies show that certain single nucleotide polymorphisms in *VEGF* and *VEGF*-related genes can affect serum levels resulting of increase the risk of tumors, including gliomas.

Endothelins are found in nerve, glial, and mostly endothelial cells. The concentration of Endothelin-1 (ET-1) has been shown to increase in many tumors [8]. ET-1 is thought to trigger angiogenesis through VEGF as this peptide stimulates the proliferation and migration of endothelial cells [9]. NO takes part in the regulation of systemic blood pressure and plays a vital role in VEGF-induced angiogenesis. VEGF induces NO synthesis due to increased regulation of the endothelial nitric oxide synthase (eNOS) enzyme. Endogenously produced NO also increases VEGF synthesis. Pharmacological inhibition or genetic impairment of eNOS inhibits VEGF-induced angiogenesis [10]. eNOS inhibition or deficiency reduces oxygen-induced neovascularization [11].

Recent studies have been designed on the basis of understanding the molecular mechanisms of cancer and focused on personalized treatment approaches. It has been shown that different levels of ACE are expressed in various types of cancer, and experimental studies have shown that ACE inhibitors reduce tumor growth [12]. In this sense, research has been directed toward the possible relationship between *ACE* and *AGTR1* polymorphisms and tumor formation, progression, and cancer incidence. In the literature, there is no study investigating *ACE* and *AGTR1* polymorphisms together in glioma. So, it was aimed to determine the polymorphisms *ACE* I/D, *AGTR1* –168A/G, –535C/T, –825T/A, *VEGF* +936C/T, –2578C/A and also to investigate the possible association among glioma molecular pathogenesis, polymorphism, and angiogenesis triangle. It is thought that this study will contribute to the studies on glioma treatment due to the evaluation of tissue and serum levels of angiogenic parameters, ACE, VEGF, ET-1, eNOS, and NO together and investigating the relationship with the tumor stage.

EXPERIMENTAL

Patients. Tissue and peripheral blood samples were collected from 96 patients with grade II–III–IV supratentorial glial tumors, hospitalized at the Department of Neurosurgery, Faculty of Medicine, Gazi University. Meanwhile, only peripheral blood samples of 104 healthy controls were gathered. Glioma type and grade were included in Table 1. 30 cases had (World Health Organization) WHO grade II, 16 cases had WHO grade III, and 50 cases had WHO grade IV gliomas. Confirmations of glial tumor type and grade were ensured via a pathological diagnosis based on WHO criteria. The study was approved by the Clinical Research Ethics Committee of Abdurrahman Yurtaslan Ankara Oncology Training and Research Hospital with the decision number 2015-12/189, and all participants provided written informed consent before being included in the study.

Peripheral venous blood of patients and controls was immediately taken into both K_3EDTA tubes for DNA and RNA isolation to determine the polymorphisms and gene expressions, and sterile blood tubes for measurement of serum parameters. Samples were transferred with a cold chain and separated by centrifugation at 4500 rpm for 15 min at 4°C for serum analysis. Subsequently, K_3EDTA and serum tubes were stored at –80°C freezer until analysis. The protocol for the collection of biological materials from groups and the measurement of parameters in these samples is schematized below (Fig. 1).

All glial tumor surgery was performed using the microsurgery technique. For intraoperative examination, tissue samples were frozen at –30°C, and 4–5 μm thick sections were taken in the frozen device and stained with the hematoxylin-eosin staining method. Tissues selected as healthy brain parenchyma to form the control group were separated under the microscope from the tumor-free areas around tumor tissue removed by the surgeon during the operation. Tumor and healthy tissue samples of patients were transferred in liquid nitrogen at –196°C and stored at –80°C until the experimental stage.

Methods. PCR-RFLP and DNA sequence analysis. Genomic DNA isolation from peripheral blood was performed by using a DNA isolation kit (GeneMatrix Quick Blood DNA Purification Kit, EURx Ltd., Gdansk, Poland) according to the manufacturer' s instructions. The concentration and purity of the isolated DNA were analyzed using 1% Agarose Gel Electrophoresis and a NanoDrop ND-1000 spectrophotometer (Thermo Fischer Scientific", USA).

A polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) assay was applied to assess *ACE* I/D, *AGTR1* –168A/G, –535C/T, and

Table 1. Characteristics of the study group

Characteristics		Glioma patients $X \pm SE$	Healthy controls $X \pm SE$
Age, years		49.09 ± 1.33	48.60 ± 1.34
Gender	Female	28	30
	Male	68	74
BMI, kg/m^2		24.28 ± 0.34*	25.57 ± 0.26
Tumor Grade and Type	Grade II	Diffuse astrocytoma ($N = 19$)	–
		Oligodendroglioma ($N = 6$)	–
		Oligoastrocytoma ($N = 5$)	–
	Grade III	Anaplastic astrocytoma ($N = 9$)	–
		Anaplastic oligodendroglioma ($N = 4$)	–
		Anaplastic oligoastrocytoma ($N = 3$)	–
	Grade IV	Glioblastoma (N = 50)	–

*Significant difference from controls, $p < 0.01$. BMI, Body Mass Index.

VEGF +936C/T, −2578C/A genotypes. Additionally, polymorphism of *AGTR1* −825T/A was determined by DNA Sequence Analysis following amplification of the region by PCR and visualization in the gel. For the purification of the PCR product, ExoSAP-IT™ PCR Product Purification Cleanup Reagent (ThermoFisher Scientific) purification enzyme was used. DNA sequencing of *AGTR1* gene −825 region was performed using ABI 3730XL Sanger sequencing device (Applied Biosystems, Foster 35 City, CA) and BigDye Terminator v3.1 Cycle Sequencing Kit in Macrogen Netherlands laboratory using Sanger Sequencing method (Applied Biosystems). The analysis results were compared with the NCBI human genomic sequence database and the results of the study group were evaluated using the FinchTV program. The sequences of patients and controls were overlapped with CLC Sequence Viewer 8.0 program and allele/genotype differences were determined. Based on the GenBank reference sequence, PCR primers were designed and given in Table 2.

In order to genotype the polymorphisms of *ACE* I/D (rs1799752), *AGTR1* −168A/G (rs275653), *AGTR1* −535C/T (rs1492078), *VEGF* +936C/T

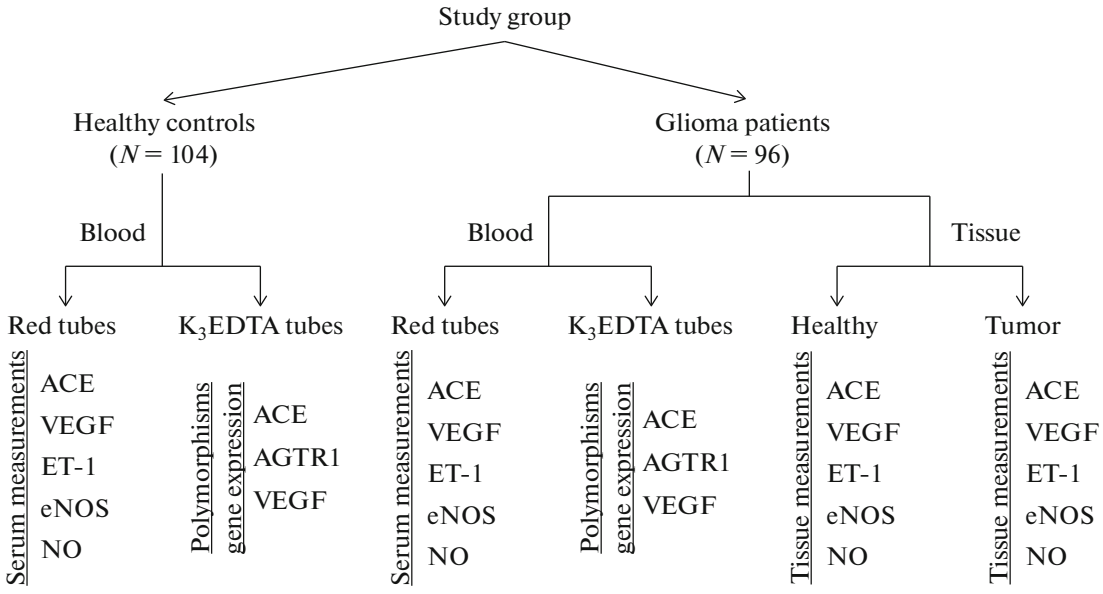


Fig. 1. The protocol for the collection of biological materials from study groups and measurement of parameters in these samples. Blood samples taken from the study group were put into red-capped tubes for serum and K_3EDTA tubes for plasma. ACE, VEGF, ET-1, eNOS, and NO in serum, polymorphism, and gene expression analyses in plasma were performed. On the other hand, tumor and healthy tissue specimens were collected from only glioma patients and were used for tissue measurements of ACE, VEGF, ET-1, eNOS, and NO.

Table 2. Primer sets of gene regions included in the study

	T_m, °C
***ACE* I/D Primers**	67.0
Primer F: 5'-CTG GAG ACC ACT CCC ATC CTT TCT-3' (24 mer)	67.0
Primer R: 5' -GAT GTG GCC ATC ACA TTC GTC AGA T-3' (25 mer)	66.0
***AGTR1* -168A/G Primers**	
Primer F: 5' -GCA ACT TGG GTA GCA TGT CC-3' (20 mer)	60.5
Primer R: 5' -CTC CCC ATC GAT CAC CGC-3' (18 mer)	60.7
***AGTR1* -535C/T Primers**	
Primer F: 5' -GGC ATC AAC CTC ACT TGA CC-3' (20 mer)	60.5
Primer R: 5' -GAG CTG GGG GTA CAG GAG TA-3' (20 mer)	62.5
***AGTR1* -825T/A Primers**	
Primer F: 5'-AAA TTC GGA GCT GCC TCC TC-3' (20 mer)	60.5
Primer R: 5' -GGA CAT GCT ACC CAA GTT GC-3' (20 mer)	60.5
***VEGF* +936C/T Primers**	
Primer F: 5' -ACC CCA GCC TTT GTT TTC CA-3' (20 mer)	58.4
Primer R: 5' -CCC TCC CAA CTC AAG TCC AC-3' (20 mer)	62.5
***VEGF* -2578C/A Primers**	
Primer F: 5' -GAT GGA AAG GGA GGG TTG GG-3' (20 mer)	62.5
Primer R: 5'-GCC AGC ACT AAG GAA CGT CT-3' (20 mer)	60.5

ACE I/D—Angiotensin Converting Enzyme Insertion/Deletion, *AGTR1*—Angiotensin II Type I Receptor, *VEGF*—Vascular Endothelial Growth Factor.

(rs3025039) and *VEGF* –2578C/A (rs699947), the touchdown polymerase chain reaction (Touch down-PCR) was used. Touch down-PCR reaction was performed in a total volume of 25 μL containing 20–150 ng genomic DNA, 5 pmol forward and reverse primers, 0.5 mM total deoxy-ribonucleoside triphosphates dNTPs, 0.5 U of Taq DNA polymerase (Promega), 25 mM $MgCl_2$, 5 μL 5× PCR Buffer. DNA was denatured at 94°C for 3 min, followed by 21 cycles of denaturation at 94°C for 1 min, annealing from 68°C to 59°C for 1 min 45 s and extension at 72°C for 2 min, with a final elongation step of 10 min at 72°C. All PCR products of genotypes were separated by 2% agarose gel electrophoresis, stained with ethidium bromide, and viewed with ultraviolet (UV) light. The sizes of fragments were estimated by comparison with previously known size markers.

The following fragment sizes were used for allele identification on gels, for *ACE* I/D polymorphism, 190 bp (DD), 490 bp (II), both 490 and 190 bp (ID). For determination of the polymorphism of *AGTR1* –168A/G, –535C/T and *VEGF* +936C/T, –2578C/A, the length of gene region amplified by each primer, Restriction Enzyme (RE) used in cutting, and the number and lengths of bands formed at the end of the cutting protocol with RE are given in Table 3. The cutting reaction with appropriate commercial RE's was carried out according to the manufacturer' s protocol (Thermo Scientific), the products obtained were loaded on a 2% gel and results were evaluated.

Gene expressions. PCR primers and appropriate probes were designed considering nucleotide sequences in the NCBI GeneBank for each gene, choosing highly conserved regions, and using the NCBI Primer Designing tool and given in Table 4.

To determine the *ACE*, *AGTR1*, and *VEGF* gene expressions of the samples in the study group, total RNA isolation from blood samples taken into tubes with K_3EDTA was performed with TRIzol manually. RNA was quantified using a NanoDrop, run on 1% agarose gel electrophoresis to check its integrity and quality, then visualized under UV (GeneGenius Gel Imaging system).

The extracted RNA was reverse transcribed to cDNA using the NG dART Reverse Transcription Kit (Eurex, Poland). Briefly, 1000 ng of RNA was mixed with 0.5 μL of oligo (dt) primer (50 pmol/μL), and an appropriate volume of RNase-free water in a 13 μL reaction system. The mixture was denatured in a thermal cycler at 65°C for 10 min, then was taken on ice immediately. In the next step, reverse transcription was performed in a total volume of 20 μL, by adding the Reverse Transcription mixture containing 4.0 μL of Transcriptor Reverse Transcriptase Reaction Buffer (5×), 0.5 μL of RNase inhibitor (40 U/μL), 2 μL of dNTP Mix (10 mM), and 0.5 μL of Transcriptor Reverse Transcriptase Enzyme (20 U/μL), under the specified reaction conditions at 25°C for 10 min, 55°C for 10 min, and 85°C for 5 min. cDNA concentrations and quality were assessed by agarose gel electrophore-

Table 3. Length of the gene regions amplified by primers before restriction, restriction enzymes, and the number of bands and their lengths in each genotype after restriction

Gene	SNP	Lengths before restriction	RE	The number of bands and their lengths in genotypes (base pair)
AGTR1	−168A/G	588 bp	Bfu I	AA (588)/AG (240–348–588)/GG (240–348)
	−535C/T	513 bp	Ssp I	CC (103–410)/CT (103-142-268-410)/TT (240–348)
VEGF	+936C/T	559 bp	CviAII	CC (559)/CT (272–287–559)/TT (272–287)
	−2578C/A	676 bp	Bgl II	AA (258–418)/CA (258–418–676)/CC (676)

Abbreviations as in the Table 2, SNP—Single Nucleotide Polymorphism, RE—Restriction Enzyme.

Table 4. Primer and probe sequences used for amplifications of *ACE*, *AGTR1*, and *VEGF* expressions

***ACE* Gene**
Primer F: 5'-TCG GCC ATG TTG AGC TAC TTC-3'
Primer R: 5'-TCC CCA TGC AGC TCG TTC-3'
Taqman probe: 5' -FAM-AGC CGC TGC TGG ACT GGC TCC- TAMRA-3'
***AGTR1* Gene**
Primer F: 5'-TTG GAA ACA GCT TGG TGG TG-3'
Primer R: 5'-GAC AGC CCA TAG TGG CAA AG-3'
Taqman probe: 5'-FAM-ACA CTG GCC ACA GTC TTC AGC TTC A-TAMRA-3'
***VEGF* Gene**
Primer F: 5'-CAG CTA CTG CCA TCC AAT CG-3'
Primer R: 5'-CAC ACA GGA TGG CTT GAA GA-3'
Taqman probe: 5' -FAM-ACC CTG GTG GAC ATC TTC CAG GAG T-TAMRA-3'
***Housekeeping* Gene (β-Actin)**
Primer F: 5'-TCA CCC ACA CTG TGC CCA T-3'
Primer R: 5'-TCC TTA ATG TCA CGC ACG ATT T-3'
Taqman Probe: 5'-FAM-ATC CTG CGT CTG GAC CTG GCT-TAMRA-3'

Abbreviations as in the Table 2, ACE—Angiotensin Converting Enzyme.

sis and a NanoDrop spectrophotometer. cDNAs were mixed with the Gotaq probe master mixture and centrifuged at 14000 rpm for 1 min. After centrifugation, the plate was covered with sealing foil, and the RT-PCR assay (Roche Light Cycler 480) was performed in optimized temperatures for hydrolysis probes. After the RT-PCR procedure, relative expression values were calculated for each gene according to the $2^{-\Delta\Delta CT}$ method using the peak profiles of all patient and control samples.

Serum/tissue measurements. For tissue homogenization, tumor and adjacent healthy tissues were placed on ice from −80°C and washed with a sufficient volume of cold Phosphate Buffer (PBS) (0.01 M, pH 7.4). At the end of the washing process, tissues were quickly crushed in a mortar containing liquid nitrogen. Then, it was weighed in microcentrifuge tubes and stuck on the ice again. 1 mL PBS was added to each 1 g of tissue samples (tissue weight (g):PBS (mL)). By using an ultrasonic homogenizator, the homogenization process was applied 3 times at 1 min intervals for 15 s by taking care that the tissue did not heat up and proteins were not denatured. After this step, all tissue samples were centrifuged at 5000 *g* for 10 min at 4°C. The supernatant (homogenate) was portioned in small volumes, taken into new microcentrifuge tubes, and the tissue homogenization step was completed. Protein quantification was performed for each tissue using the Bradford method.

The ACE, VEGF, ET-1, and eNOS levels in serum/tissue were measured spectrophotometrically using a sandwich human ELISA assay kit, according to the manufacturer's instructions (Elabscience, USA). Serum/tissue NO levels were evaluated with spectrophotometric assay based on the Griess reagent measuring nitrite and nitrate together.

Statistical analysis. Patient and control sample sizes were calculated with PASS (Power Analysis and Sample Size, version 11, Kaysville, Utah). SPSS (version 22 software, SPSS Inc. Chicago, Illinois, USA) was used for all statistical analyses of the data. Descriptive statistics were presented as mean ± stan-

dard deviation. Normality of the distribution of data was tested by the Kolmogorov-Smirnov test.

Pearson correlation coefficients were calculated for the relationship between serum/tissue levels of ACE, VEGF, ET-1, eNOS, and NO, and gene expressions of *ACE*, *AGTR1*, and *VEGF*. Chi-square (χ^2) analysis was used to determine the difference between patient and control groups in terms of allele/genotype frequency. The value of $p < 0.05$ was considered statistically significant, in all analyses.

RESULTS

Characteristics of the study group were given in Table 1. There was no significant difference between the patient and control groups in terms of age and sex. However, there were significant differences in terms of quetelet index ($p < 0.01$).

Polymorphisms

Distribution of *ACE* I/D, *AGTR1* −168A/G, −535C/T, and −825T/A genotypes, and allele frequency of the study groups were shown in Table 5. Gel images of all SNP's and DNA sequence analysis chromatograms for *AGTR1* −825T/A polymorphism are reported in Supplemantary material. There was no significant difference between glioma and control groups for *ACE* I/D polymorphism ($p > 0.05$), and no deviations from Hardy–Weinberg equilibrium were observed. *ACE* genotypes DD, ID, and II were of almost identical frequency within both groups. Risk assessment demonstrated that there were no significant risk changes for glioma subjects either with the DD genotype/D allele.

Significant difference was found between glioma patients and healthy controls in terms of genotype distribution and allele frequencies for *AGTR1* −168A/G polymorphism ($p < 0.01$, $p < 0.01$, respectively). Compared to the AG reference genotype, glioma risk of individuals with the AA genotype is reduced (odds ratio: 0.44, 95% CI: 0.24 to 0.79, $p < 0.01$). According to these results, the AA genotype is the protective genotype against glioma risk. In other words, the risk of glioma in individuals carrying the G allele is increased by 2.27 times compared to those carrying the A allele (odds ratio: 2.27, 95% CI: 1.41 to 3.66, $p < 0.01$).

In *AGTR1* −535C/T and −825T/A polymorphisms, genotypes, and allele frequencies were not statistically different in both groups. Similarly, allele/genotype frequencies were not associated with glioma risk ($p > 0.05$).

The distribution of *VEGF* +936C/T and −2578C/A genotypes of the study groups were shown in Table 6. There was no significant relationship between patients and controls according to genotype distribution for *VEGF* +936C/T polymorphism in chi-square analysis since there was not enough data for TT and CT genotypes ($p > 0.05$). Logistic regression analysis also could not be performed.

No differences were observed in *VEGF* −2578C/A polymorphism with respect to genotype distribution and allele frequency between study groups in chi-square analysis ($p > 0.05$). There was no significant difference regarding the effect of allele/genotype frequencies on glioma risk ($p > 0.05$).

ELISA and RT-PCR Assays

ACE serum/tissue levels and gene expressions. Serum ACE levels of the glioma group were found to be significantly higher than the control group ($p < 0.01$) (Fig. 2a). ACE levels in tumor tissue decreased as against healthy tissue ($p < 0.01$) (Fig. 2b). The relative *ACE* gene expression of glioma patients was higher than controls ($p < 0.01$) (Fig. 2c).

***AGTR1* gene expressions.** The relative *AGTR1* gene expression of glioma group was higher than control group ($p < 0.05$) (Fig. 3).

VEGF serum/tissue levels and gene expressions. Serum VEGF levels increased in patients compared to controls ($p < 0.01$) (Fig. 4a). On the other hand, there were no significant differences between tumor and healthy tissue in terms of tissue VEGF levels ($p > 0.05$) (Fig. 4b). The elevated relative *VEGF* gene expression was observed in patients than controls ($p < 0.01$) (Fig. 4c).

ET-1 serum/tissue levels. Serum ET-1 levels of glioma patients were found to be higher than healthy controls, though not significant ($p > 0.05$) (Fig. 5a). On the other hand, ET-1 levels of tumor tissue were significantly higher than healthy ones ($p < 0.05$) (Fig. 5b).

e-NOS serum/tissue levels. Serum e-NOS concentration significantly increased in glioma patients as compared with controls ($p < 0.01$) (Fig. 6a). Likewise, in tumor tissue, e-NOS levels were higher than in healthy tissue ($p < 0.01$) (Fig. 6b).

NO serum/tissue levels. Serum NO concentration was found to be higher in the glioma group compared with the control group ($p < 0.01$) (Fig. 7a). Similarly, NO levels were also increased in tumor tissue as compared with healthy tissue ($p < 0.01$) (Fig. 7b).

Serum VEGF levels of II genotype (262.27 ± 78.03 pg/mL) were significantly higher than both ID and DD genotype (139.21 ± 23.15, 160.81 ± 39.28 pg/mL), in *ACE* I/D polymorphism in glioma patients, ($p < 0.05$, $p< 0.05$, respectively). Additionally, serum ET-1/eNOS levels of ID genotype (18.06 ± 3.62, 2.62 ± 0.08 ng/mL) were lower/higher as compared to II genotype (23.09 ± 4.33, 2.31 ± 0.11 ng/mL) ($p < 0.05$, $p < 0.05$, respectively). Serum NO levels of ID genotype (20.66 ± 2.48 μmol/L) were also found significantly higher than DD genotype (14.31 ± 0.96 μmol/L) ($p < 0.05$). Tumor tissue ET-1/eNOS levels of II genotype (19.73 ± 3.37, 4.78 ±

Table 5. *ACE* I/D, *AGTR1* −168A/G, −535C/T, and −825T/A allele/genotype distributions and the relationship of these polymorphisms with GR

Gene	SNP	Genotypes	Glioma group ($N = 96$)	Control group ($N = 104$)	χ^2 test	OR (95% CI)★	p
ACE	I/D (rs1799752)	DD	36 (37.50%)	35 (33.65%)	$\chi^2 = 0.585$ $p > 0.05$	1.25 (0.67–2.34)	>0.05
		ID	41 (42.71%)	50 (48.08%)		1.00 (Reference)	
		II	19 (19.79%)	19 (18.27%)		1.22 (0.57–2.60)	>0.05
		D Allele	113 (58.85%)	120 (57.69%)	$\chi^2 = 0.055$ $p > 0.05$	1.05 (0.70–1.56)	>0.05
		I Allele	79 (41.15%)	88 (42.31%)		1.00 (Reference)	
		Total	96/192 (100%)	104/208 (100%)			
	−168A/G (rs275653)	AA	45 (46.88%)	72 (69.23%)	$\chi^2 = 11.845$ $p < 0.01$*	0.44 (0.24–0.79)	<0.01
		AG	43 (44.79%)	30 (28.85%)		1.00 (Reference)	
		GG$^{\Psi}$	8 (8.33%)	2 (1.92%)		2.79 (0.55–14.08)	ω
		A Allele	133 (69.27%)	174 (83.65%)	$\chi^2 = 11.575$ $p < 0.01$*	1.00 (Reference)	
		G Allele	59 (30.73%)	34 (16.35%)		2.27 (1.41–3.66)	<0.01
		Total	96/192 (100%)	104/208 (100%)			
AGTR1	−535C/T (rs1492078)	CC	25 (26.04%)	34 (32.69%)	$\chi^2 = 1.085$ $p > 0.05$	0.74 (0.39–1.39)	>0.05
		CT	54 (56.25%)	54 (51.92%)		1.00 (Reference)	
		TT	17 (17.71%)	16 (15.39%)		1.06 (0.49–2.32)	>0.05
		C Allele	104 (54.17%)	122 (58.65%)	$\chi^2 = 0.818$ $p > 0.05$	0.83 (0.56–1.24)	>0.05
		T Allele	88 (45.83%)	86 (41.35%)		1.00 (Reference)	
		Total	96/192 (100%)	104/208 (100%)			
	−825T/A (rs275651)	TT	46 (47.92%)	67 (64.42%)	$\chi^2 = 5.549$ $p > 0.05$	0.50 (0.28–0.90)	>0.05
		AT	45 (46.87%)	33 (31.73%)		1.00 (Reference)	
		AA$^{\Psi}$	5 (5.21%)	4 (3.85%)		0.55 (0.14–2.16)	ω
		T Allele	90 (93.75%)	100 (96.15%)	$\chi^2 = 0.607$ $p > 0.05$	1.00 (Reference)	
		A Allele$^{\Psi}$	6 (6.25%)	4 (3.85%)		1.67 (0.46–6.10)	ω
		Total	96/192 (100%)	104/208 (100%)			

★ OR—Odds Ratio was calculated by logistic regression analysis, CI—Confidence interval, GR—Glioma risk, *ACE* I/D—Angiotensin Converting Enzyme Insertion/Deletion, *AGTR1*—Angiotensin II Type I Receptor, SNP—Single Nucleotide Polymorphism.

$^{\Psi}$, $^{\omega}$ Since there is not enough data to analyze in the data set, this genotype/allele was excluded from the chi-square and logistic regression analyses. Genotype and allele frequencies were calculated by chi-square analysis and were found to be statistically significant, $p < 0.01$, $p < 0.01$ respectively.

0.80 ng/mL) were lower than both DD and ID genotype (30.82 ± 3.60, 7.65 ± 0.50 ng/mL and 40.68 ± 8.08, 6.93 ± 0.55 ng/mL) ($p < 0.05$, $p < 0.05$ for ET-1 and $p < 0.01$, $p < 0.01$ for eNOS, respectively). Tumor tissue NO levels of the DD genotype (15.76 ± 1.94 μmol/L) were found to be lower compared to the II genotype (24.42 ± 5.94 μmol/L) ($p < 0.05$). Healthy tissue eNOS levels of DD genotype (2.17 ± 0.55 ng/mL) were lower than ID genotype (3.65 ± 0.64 ng/mL) ($p < 0.05$). It was also obtained that NO levels of healthy tissue of II genotype (12.76 ± 1.17 μmol/L) were lower compared with ID genotype (17.76 ± 1.97 μmol/L) ($p < 0.05$).

In *AGTR1* −168A/G polymorphism in glioma patients, tumor tissue eNOS levels of both AA and AG genotypes (6.81 ± 0.50 and 6.26 ± 0.56 ng/mL) were found lower than GG genotype (9.38 ± 0.47 ng/mL) ($p < 0.05$, $p < 0.05$, respectively). Healthy tissue VEGF levels of the AG genotype (1.07 ± 0.24 ng/mL) and ET-1 levels of the GG genotype (21.47 ± 2.04 pg/mL) were higher than VEGF/ET-1 levels of the AA genotype (0.75 ± 0.31, 16.15 ± 3.10 pg/mL) ($p < 0.05$, $p < 0.05$, respectively). On the other hand, healthy tissue eNOS levels of GG genotype (0.67 ± 0.04 ng/mL) were significantly lower than AG genotype (3.80 ± 0.63 ng/mL) ($p < 0.05$).

No significant differences were found among serum, tumor tissue, and healthy tissue levels of ACE, VEGF, ET-1, eNOS, and NO in terms of genotype in *AGTR1* −535C/T polymorphism in glioma patients ($p > 0.05$).

Tumor tissue eNOS levels of CC and CT genotypes (7.06 ± 0.39 and 6.54 ± 0.72 ng/mL) were found higher compared to the TT genotype (0.28 ±

Table 6. *VEGF* +936C/T and -2578C/A allele/genotype distributions of the study group and the relationship of these polymorphisms with GR

Gene	SNP	Genotypes	Glioma group ($N = 96$)	Control group ($N = 104$)	x^2 test	OR (95% CI)★	p
VEGF	+936C/T (rs3025039)	CC	68 (70.83%)	103 (99.04%)	Could not calculate	0.03 (0.01–0.19)	>0.05
		CT$^{\Psi}$	26 (27.09%)	1 (0.96%)		1.00 (Reference)	ω
		TT$^{\Psi}$	2 (2.08%)	0 (0%)			ω
		C Allele	162 (84.38%)	207 (99.52%)	$\chi^2 = 3\ 2.028$	1.00 (Reference)	
		T Allele$^{\Psi}$	30 (15.62%)	1 (0.48%)	$p < 0.01$	38.33 (5.17–284.09)	ω
		Total	96/192 (100%)	104/208 (100%)			
	-2578C/A (rs699947)	AA	18 (18.75%)	22 (21.15%)	$\chi^2 = 1.560$	0.73 (0.35–1.55)	>0.05
		CA	49 (51.04%)	44 (42.31%)	$p > 0.05$	1.00 (Reference)	
		CC	29 (30.21%)	38 (36.54%)		0.69 (0.36–1.29)	>0.05
		A Allele	85 (44.27%)	88 (42.31%)	$\chi^2 = 0.157$	1.00 (Reference)	
		C Allele	107 (55.73%)	120 (57.69%)	$p > 0.05$	0.92 (0.62–1.37)	>0.05
		Total	96/192 (100%)	104/208 (100%)			

★OR—Odds Ratio was calculated by logistic regression analysis, CI—Confidence interval, GR—Glioma risk, *VEGF*—Vascular Endothelial Growth Factor, SNP—Single Nucleotide Polymorphism.

$^{\Psi, \omega}$ Since there is not enough data to analyze in the data set, this genotype/allele was excluded from the chi-square and logistic regression analyses.

Table 7. Evaluation of serum/tissue parameters according to tumor grade in glioma patients

Tumor grade (N)	Grade II (30)	Grade III (16)	Grade IV (50)
sACE, ng/mL	141.23 ± 17.65	185.73 ± 23.20	193.04 ± 11.55[a]
tVEGF, ng/mL	0.22 ± 0.03	1.35 ± 0.73[b]	1.43 ± 0.09[c, d]
seNOS, ng/mL	2.30 ± 0.09	2.60 ± 0.17[e]	2.55 ± 0.07

[a, b, c, e] Significant difference from grade II, $p < 0.05$, $p < 0.05$, $p < 0.01$, $p < 0.05$.
[d] Significant difference from grade III, $p < 0.01$.
s, Serum, t, Tissue.

0.05 ng/mL) ($p < 0.05$, $p < 0.05$, respectively), in *VEGF* +936C/T polymorphism in glioma patients. Similarly, healthy tissue ACE levels of CC and CT genotypes (392.53 ± 13.29 and 371.90 ± 21.39 ng/mL) were higher than TT genotype (229.57 ± 22.13 ng/mL) ($p < 0.05$, $p < 0.05$, respectively).

In *VEGF* −2578C/A polymorphism in glioma patients, serum VEGF levels of the CA genotype (198.19 ± 31.85 pg/mL) were significantly higher than AA genotype (146.73 ± 80.90 pg/mL) ($p < 0.01$). Tumor tissue ACE levels of the AA genotype (108.83 ± 9.88 ng/mL) were lower as compared to the CA genotype (136.96 ± 9.15 ng/mL) ($p < 0.01$).

According to tumor grade, serum ACE levels of patients in grade IV (193.04 ± 11.55 ng/mL) were found to be higher than grade II (141.23 ± 17.65 ng/mL) ($p < 0.05$). Serum eNOS levels of patients in grade III (2.60 ± 0.17 ng/mL) were also higher than grade II (2.30 ± 0.09 ng/mL) ($p < 0.05$). Tissue VEGF levels of patients in grade VI (1.43 ± 0.09 ng/mL) were found to be significantly higher than grade III (1.35 ± 0.73 ng/mL) and grade II (0.22 ± 0.03 ng/mL) ($p < 0.01$, $p < 0.01$, respectively). Likewise, tissue VEGF levels of patients in grade III were higher than grade II ($p < 0.05$) (Table 7).

Positive correlations between serum ACE and serum eNOS, serum ACE and tumor tissue ET-1, serum ET-1 and serum NO, serum ET-1 and tumor tissue ET-1, serum eNOS and tumor tissue NO, serum NO and tumor tissue ACE, serum NO and tumor tissue NO, tumor tissue ACE and tumor tissue NO, tumor tissue ET-1 and tumor tissue eNOS, and *ACE* gene expression and *AGTR1* gene expression were found, ($r = 0.255$, $p < 0.05$; $r = 0.262$, $p < 0.01$; $r = 0.244$, $p < 0.05$; $r = 0.494$, $p < 0.01$; $r = 0.264$, $p < 0.01$; $r = 0.310$, $p < 0.01$; $r = 0.259$, $p < 0.05$; $r = 0.216$, $p < 0.05$; $r = 0.266$, $p < 0.01$; $r = 0.216$, $p < 0.05$, respectively) while there was a negative correlation between serum VEGF and tumor tissue eNOS, serum NO and tumor tissue eNOS, tumor tissue ACE and tumor tissue eNOS, and tumor tissue eNOS and

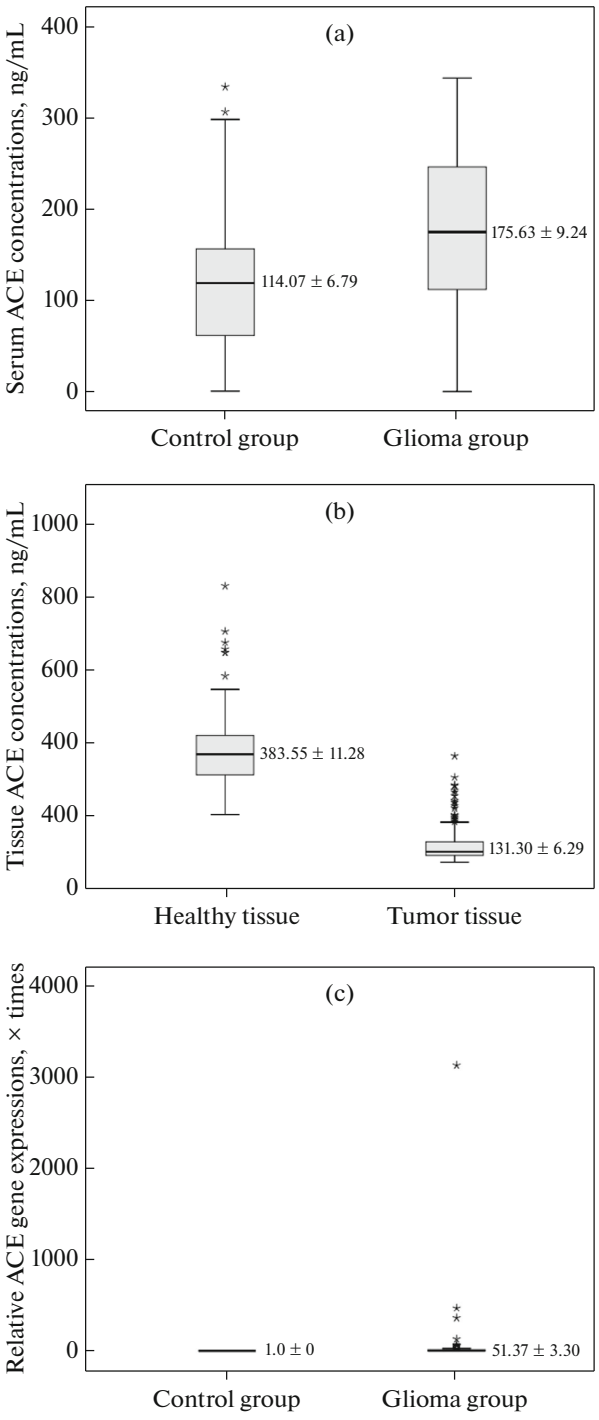


Fig. 2. (a) Serum ACE concentrations of the control and glioma groups; (b) ACE concentrations in healthy tissue and tumor tissue in glioma patients; (c) Relative *ACE* gene expressions of control and glioma group. Lines in the boxes represent the means of the serum ACE and tissue ACE levels and error bars indicate ± standard deviation. The value of $p < 0.05$ was considered statistically significant, in all statistical analyses. Angiotensin Converting Enzyme (ACE). (a) Significant difference from control group, $p < 0.01$; (b) significant difference from tumor tissue, $p < 0.01$; (c) significant difference from the control group, $p < 0.01$.

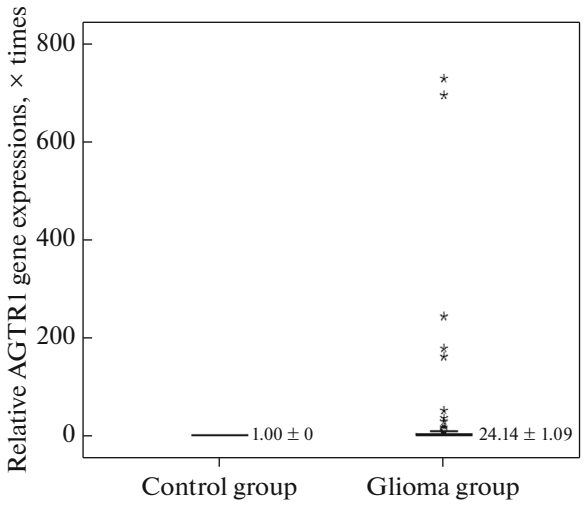


Fig. 3. Relative *AGTR1* gene expressions of control and glioma group. The value of $p < 0.05$ was considered statistically significant, in all statistical analyses. Angiotensin II Type I Receptor (AGTR1) significant difference from the control group, $p < 0.05$.

tumor tissue NO ($r = -0.418$, $p < 0.01$; $r = -0.361$, $p < 0.01$; $r = -0.576$, $p < 0.01$; $r = -0.363$, $p < 0.01$).

DISCUSSION

Gliomas are pretty lethal tumors with both the highest percentage and the fastest growth ratio among primary brain tumors. Although angiogenic activities of advanced-grade gliomas are known to be high, the key mechanisms that play a role in this activity have not been fully elucidated yet.

ACE is known to have an important role in tumor cell proliferation, cell adhesion, migration, angiogenesis, and metastasis, and performs this role through VEGF production. Although there are a few studies examining the effect of *ACE* I/D polymorphism on pathogenesis and risk of glioma, conflicting results are reported. Lian et al. [13] assessed the potential relationship between *ACE* I/D polymorphism and glioma. They found that the frequencies of II, ID, and DD genotypes were 48.0, 42.1, 9.9% and D allele was 30.9% in patients and 49.7, 43.9, 6.4 and 28.3% in controls, respectively. According to genotype distributions, they declared that the DD genotype was found to be higher in patients than in controls [13]. Similar results obtained by Pandith et al. [14]. It was shown that the DD genotypes were highly presented in glioma cases 26.78% as against only 10.63% in controls [14]. In another study, both D allele frequency and DD genotype were highly presented in glioma [15]. On the other hand, Li et al. reported genotype frequencies of DD, ID, and II as 9.21, 51.97 and 38.82% in glioma and 16.08, 43.72 and 40.20% in controls, respectively [16]. The allelic frequencies of D and I alleles were 35.20 and 64.80% among the patients, while 37.94 and

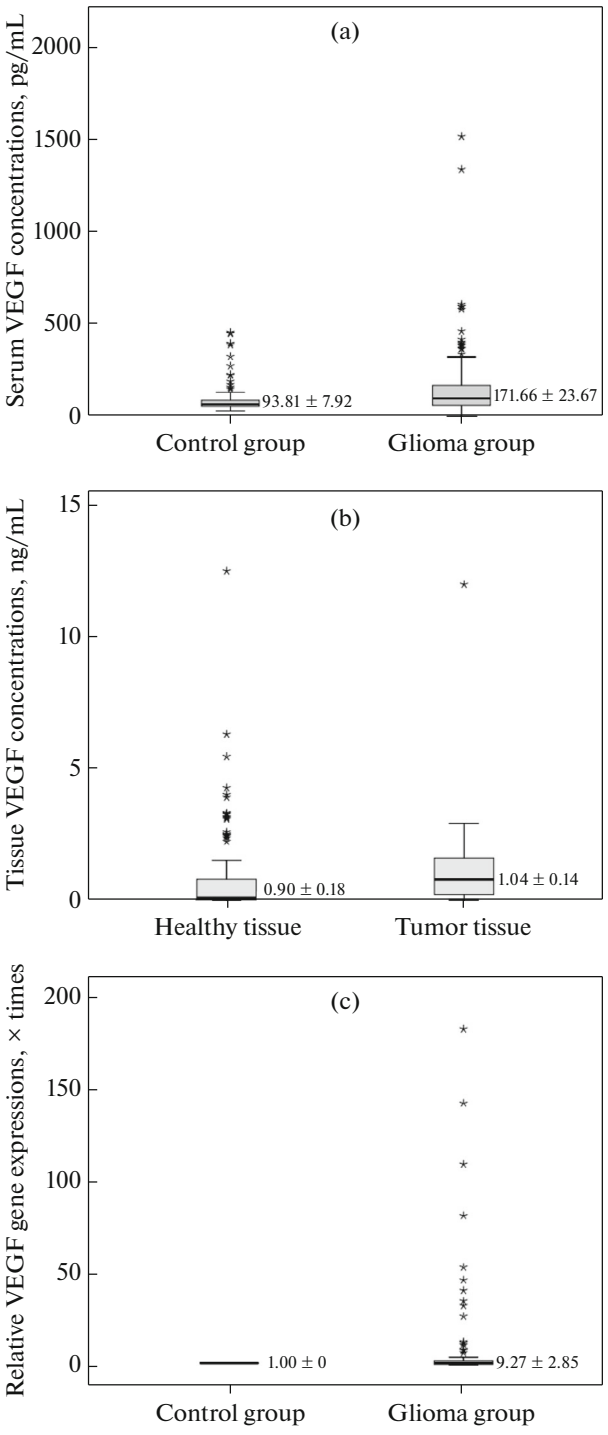


Fig. 4. (a) Serum VEGF concentrations of the control and glioma groups; (b) VEGF concentrations in healthy tissue and tumor tissue in glioma patients; (c) Relative *VEGF* gene expressions of control and glioma group. Lines in the boxes represent the means of the serum VEGF and tissue VEGF levels and error bars indicate ± standard deviation. The value of $p < 0.05$ was considered statistically significant, in all statistical analyses. Vascular Endothelial Growth Factor (VEGF). (a) significant difference from control group, $p < 0.01$; (b) statistically similar results with healthy tissue, $p > 0.05$; (c) significant difference from control group, $p < 0.01$.

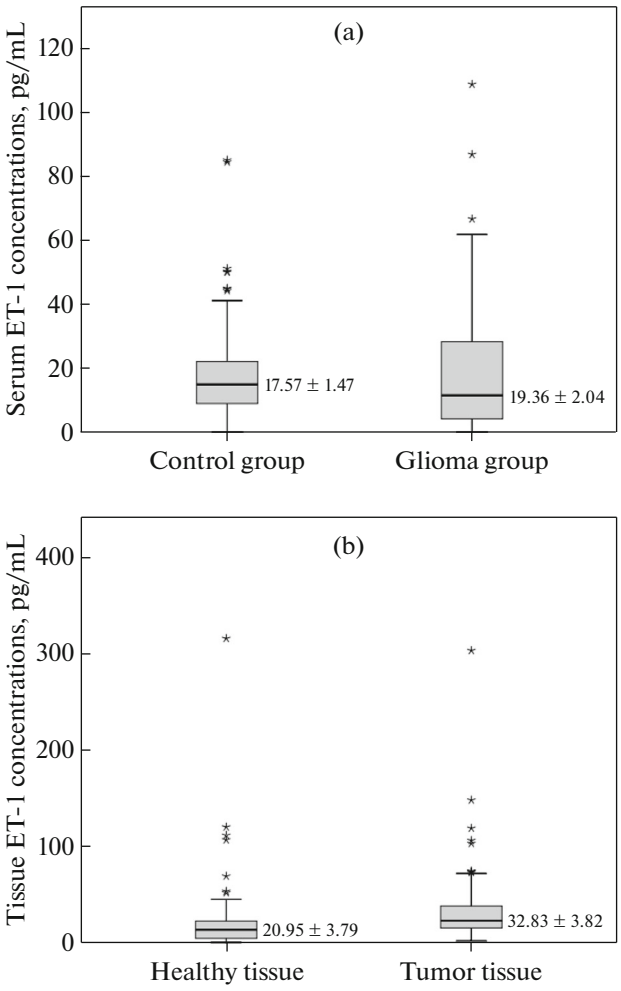


Fig. 5. (a) Serum ET-1 concentrations of the control and glioma groups; (b) ET-1 concentrations in healthy tissue and tumor tissue in glioma patients. Lines in the boxes represent the means of the serum ET-1 and tissue ET-1 levels and error bars indicate ± standard deviation. The value of $p < 0.05$ was considered statistically significant, in all statistical analyses. Endothelin-1 (ET-1). (a) statistically similar results with the control group, $p > 0.05$; (b) significant difference from tumor tissue, $p < 0.05$.

62.06% were in controls, respectively. Contrary to the previous studies, authors noticed that the ID genotype was associated with an increased risk of glioma compared with the DD genotype [16]. In a meta-analysis, interestingly, it was not found an association between *ACE* I/D polymorphism and glioma risk. Also, they suggested that *ACE* I/D polymorphisms might not be involved in glioma progression [17]. In our study, DD genotype was determined 37.5 versus 33.6%, II was 19.8 versus 18.3% and ID was 42.7 versus 48.1% in patients and controls, respectively. There was no significant relationship between patients and controls in terms of *ACE* I/D genotype distribution and allele frequencies. The originality of the study stems from both the type of glial tumor that contains and the popula-

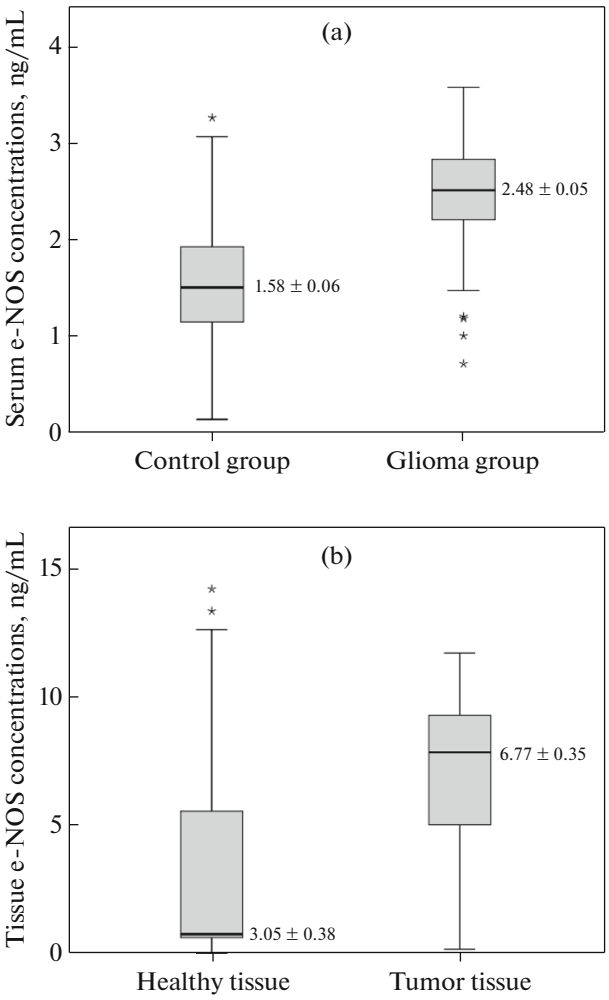


Fig. 6. (a) Serum e-NOS concentrations of the control and glioma groups; (b) e-NOS concentrations in healthy tissue and tumor tissue in glioma patients. Lines in the boxes represent the means of the serum e-NOS and tissue e-NOS levels and error bars indicate ± standard deviation. The value of $p < 0.05$ was considered as statistically significant, in all statistical analyses. Endothelial Nitric Oxide Synthase (e-NOS). (a) significant difference from control group, $p < 0.01$; (b) significant difference from tumor tissue, $p < 0.01$.

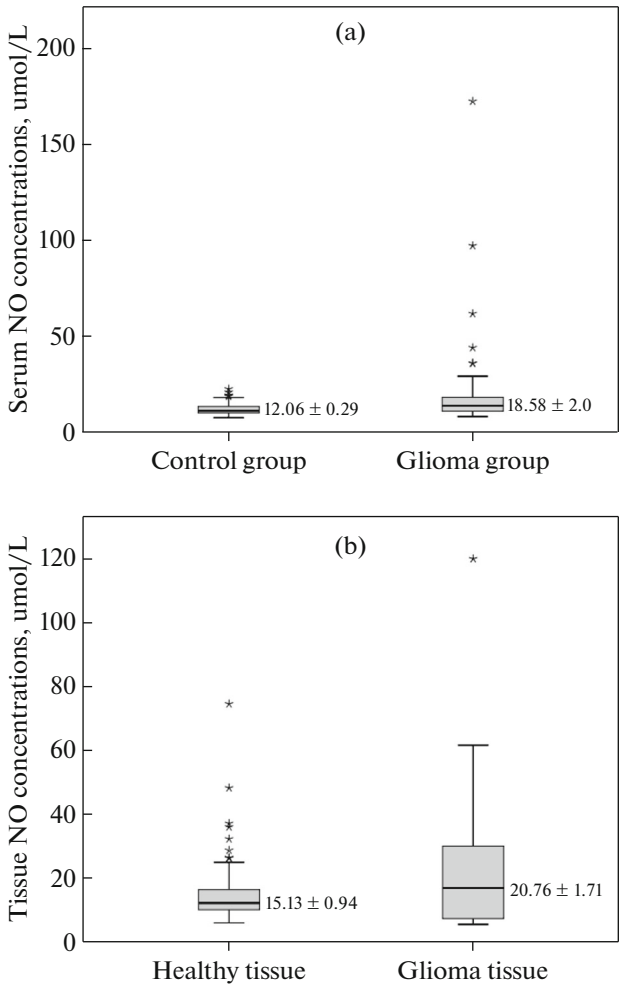


Fig. 7. (a) Serum NO concentrations of the control and glioma groups; (b) NO concentrations in healthy tissue and tumor tissue in glioma patients. Lines in the boxes represent the means of the serum NO and tissue NO levels and error bars indicate ± standard deviation. The value of $p < 0.05$ was considered statistically significant, in all statistical analyses. Nitric Oxide (NO). (a) significant difference from the control group, $p < 0.01$; (b) significant difference from tumor tissue, $p < 0.01$.

tion. The results were compatible with the study of Sun et al.. It is thought that this different distribution in the *ACE* I/D genotype may be due to gene-gene, gene-environment interactions, or population difference that constitutes the study group.

Angiotensin II AGTR1 receptor has been reported to be associated with tumor formation, apoptosis, metastasis, and angiogenesis. In a study, authors suggested that selective blockage of AGTR1 induced apoptosis and diminished tumoral growth, cell proliferation, and angiogenesis in rats with glioma [18]. *AGTR1* polymorphism has been studied on different regions in various cancers except for diffuse infiltrative glial tumors. In the study performed by Koh et al. [19], *AGTR1* −168A/G, −535C/T, −825T/A polymorphisms were investigated in breast cancer. Genotypic distributions were demonstrated as 86.2% for AA and 13.8 for AG+GG genotypes in cases versus 81.7 and 18.3% in controls for *AGTR1* −168A/G polymorphism, 72.9% for CC and 27.1 for CT+TT genotypes in cases versus 66.8 and 33.2% in controls for *AGTR1* −535C/T polymorphism and 83.5% for TT and 16.5 for TA+TT genotypes in cases versus 77.9 and 22.1% in controls for *AGTR1* -825T/A polymorphism. They concluded that the women carrying GG or GA, TT or CT, and AA or AT genotypes have a lower risk of breast cancer compared to AA, CC, or TT genotypes [19]. In our study, −168A/G, −535C/T, and −825T/A regions of *AGTR1* polymorphisms were

evaluated in glioma for the first time. *AGTR1* −168A/G genotype distribution for AA, GG, and AG was found as 46.88, 8.33 and 44.79% in the patient group, while 69.23, 1.92 and 28.85% in the control group. For *AGTR1* −535C/T genotype distribution for CC, TT, and CT was calculated as 26.04, 17.71 and 56.25% in patients, while 32.69, 15.39 and 51.92% in controls. Similarly, *AGTR1* −825T/A genotype distribution for TT, AA, and AT was obtained as 46.88, 8.33, and 44.79% in the patient group, while 69.23, 1.92 and 28.85% in the control group. In our study, the AA genotype was found to be significantly associated with lower glioma risk regarding *AGTR1* −168A/G gene region. The risk of glioma in individuals with the G allele was increased by 2.27 times compared to the A allele. This study is the first to examine *AGTR1* polymorphism in glioma. In the evaluation of *AGTR1* −535C/T and 825T/A gene region, it was determined that there was no relationship between the groups in terms of genotype distribution and allele frequencies.

There are studies supporting that various polymorphisms of *VEGF* may contribute to the development of cancer. Polymorphisms in different regions of *VEGF* and susceptibility to glioma were evaluated in the Chinese population. In *VEGF* +936C/T polymorphism, genotype distribution of CC, CT, and TT were declared 69.8, 26.6 and 3.6% in case, and 70.6, 27.6, and 1.9% in controls, respectively. It was reported that TT carriers showed nearly 2-fold elevated glioma risk [20]. The results obtained by Li et al. were supported by another study on glioma by Jiang et al. [20, 21]. In the study, TT genotypes were significantly higher in glioma [21]. Similarly, Zhang et al. suggested that people who carry the TT genotype were 3.13 times more likely to develop glioma than carry the CC genotype and the T allele increased susceptibility to glioma compared to the C allele [22]. Linhares et al. aimed to clarify the role of *VEGF* +936C/T and −2578C/A polymorphisms in glioma susceptibility [23]. In the study, CC, CT, and TT genotype frequencies for *VEGF* +936C/T were calculated as 76.63, 19.63 and 3.74% in glioma, 75.34, 21.92 and 2.74% in glioblastoma, and 77.62, 20.28 and 2.10% in controls, respectively. CC, CA, and AA genotypes frequencies for *VEGF* −2578C/A found 25.23, 49.54 and 25.23% in glioma, 26.03, 49.31 and 24.66% in glioblastoma, and 31.47, 51.75 and 16.78% in controls, respectively. Contrary to the study by Zhang et al. [22], they suggested that both polymorphisms were not associated with increased risk for glioma and glioblastoma [23]. When *VEGF* +936C/T genotypes were evaluated in the Turkish population, CC, TT, and CT genotypes were found as 70.83, 2.08 and 27.09% in glioma. In controls, CC was 99.04%, CT was 0.96% and TT was 0%. Also, the relationship between *VEGF* −2578C/A polymorphism and risk of glioma was evaluated and genotype distributions were as follows, AA, CC, and CA were 18.75, 30.21 and 51.04% in patients, while 21.15, 36.54 and 42.31% in controls. It was determined that there was no significant relationship between the two groups in terms of both *VEGF* +936C/T and −2578C/A genotype distribution and allele frequencies. Besides some studies reported that increased risk of having TT genotype in +936 C/T polymorphism in glioma, our present results agree with previous reports of Linhares et al. [23].

Controversial results have been reported related to *ACE* gene expression in different cancers. Elevated expression of ACE protein and mRNA were shown in human glioblastoma vessels, while decreased in non-tumoral tissue vessels [24]. These findings were subsequently supported by the study of Bradshaw et al. in glioblastoma tissues in humans [25]. Similarly, increased expression of *ACE* was shown in laryngeal [26] and colorectal cancers [27]. Contrary to these studies, Mehranfard et al. found a decrease in the expression of *ACE* in colorectal cancer tissues [28]. In our study, *ACE* gene expression was measured not only in glioblastoma but also in other diffuse infiltrative glial tumors and it was found to be significantly higher in the glioma group compared to controls. This finding supports the idea that the *ACE* gene has a possible role in glial tumor cell proliferation and gliomagenesis. The obtained results seem to be compatible with the *ACE* expression studies of Juillerat-Jeanneret et al. and Bradshaw et al. [24, 25]. Although it has been reported that there is an increase in *ACE* expression in most cancer, conflicting studies are showing that serum ACE levels increase or decrease in various types of cancer. To the best of our knowledge, there are no studies of serum ACE concentrations in glioma. Ibiş et al. analyzed serum ACE levels in pancreas cancer and did not find differences compared to controls [29]. In the study of newly diagnosed epithelial ovarian cancer conducted by Beyazit et al. [30], ACE levels in patients were found to be higher than controls, nevertheless, according to tumor stage did not differ [30]. However, serum ACE levels were found low in patients with hepatocellular carcinoma [31]. In our study, serum ACE levels in glioma were found to be higher than controls. Our results are consistent with the work of Beyazit et al. Moreover, serum levels of ACE in grade IV glioma were found to be significantly higher compared with those in grade II, and it is thought that this increase may be due to an increase in *ACE* expression in glioma. ACE levels in different tumor tissue were also controversial. Ohta et al. examined ACE activity in tissues from the normal pancreas, pancreatic ductal, colon, and hepatocellular cancer, and they did not find any difference [32]. In other respects, Procházka et al. and Danilov et al. reported a decrease in ACE levels in tumor tissue compared to healthy's [33, 34]. In our study, tissue ACE levels were found to be significantly lower than those of adjacent tissue. These results appear to be consistent with the study of Danilov et al. and Procházka et al. From a clinical point of view, independent from *ACE* gene I/D polymorphism in glioma, increased *ACE* gene expression caused an

increase in serum ACE levels. But tissue levels of ACE were reduced. It is thought that ACE may be removed from the tissue as a result of its rapid binding to cell receptors or extracellular matrix and entering the blood circulation. Increase in the serum level of ACE may increase the glial tumor aggressiveness.

The expression of the *AGTR1* receptor in healthy brain tissue and its role in cerebral vascular regulation was described by Ganong [35]. Arrieta et al. [36] showed a strong expression of both *AGTR1* and *AGTR2* at 52 and 44%, respectively, in astrocytoma. Additionally, they suggested that the presence of these receptors was associated with the grade of malignancy [36]. Another study by Arrieta et al. notified that expression of *AGTR1* was increased in malignant breast tissue compared to benign breast tissue, and the increase was associated with angiogenesis and cellular proliferation [37]. The expression of *AGTR1* was evaluated in a different type of glial tumors for the first time with the study of the Turkish population and in accordance with the studies performed by Arrieta et al. the relative *AGTR1* expression of the glioma group was found to higher than the control group [36].

Enhanced *VEGF* expression in the periphery of many tumors was demonstrated both in tumor tissue in comparison with normal tissue. Since the first report of elevated *VEGF* expression in glioblastoma by Plate et al. [38], it has also been reported in other CNS tumors, including astrocytomas, hemangioblastomas, meningiomas, and metastatic brain tumors [39–41]. Our findings of higher relative *VEGF* expression in glioma support the earlier observations. The increase in *VEGF* expression in glioma patients is thought to be responsible for the increase in serum VEGF levels. Serum VEGF concentrations were measured as high in patients with glioma, meningioma, metastatic tumor, and other CNS tumors before and after surgery in comparison to controls, but no significant difference was found. The highest serum VEGF levels were observed in early-grade glial tumors [42]. Likewise, in the studies by Chiorean et al. and Rafat et al., serum VEGF was found to be significantly higher in glioblastoma patients compared to controls [43, 44]. In our study, serum VEGF levels were enhanced in glioma patients parallel to *VEGF* expression. Our results were in agreement with the work of Nowacka et al., Chiorean et al., and Rafat et al. [42–44]. Serum, tissue, and cyst fluid VEGF concentrations were measured in patients with different CNS tumors. Tissue VEGF levels were observed higher in glioblastoma when compared with other types of tumors and normal brains [45]. Correlatively, the mean levels of tissue VEGF were measured in glioma and reported nearly 11-fold higher in high-grade than low-grade [46]. In our study, no significant difference was found between glioma tissue and healthy tissue in terms of VEGF levels, though it was higher in glioma. In addition, tissue VEGF levels were observed to increase from grade II to grade IV by positively correlating with the clinical grade of gliomas. The result was compatible with the study of Schmidt et al. [46].

ET-1 is known as a powerful mitogenic and angiogenic peptide in carcinogenesis, thereby facilitating tumor growth. Synthesis of ET-1 in vitro in glioblastoma cell lines and expression of ET-1 in vivo has been demonstrated [47], but no studies were reported on the serum and tissue levels in glioma to the best of our knowledge. Increased serum levels of ET-1 have been detected in many cancers. Serum ET-1 levels in patients with colon cancer were determined as higher than controls. Authors also reported that increases in ET-1 showed a positive correlation with advanced tumor stage [48]. Similarly, enhanced serum/tissue ET-1 concentrations were also found in several types of cancers [49–51]. Also, Alanen et al., showed that immunoreactivity for ET-1 in breast ductal carcinoma in situ specimens was higher than normal tissue [52]. In our study, although the serum ET-1 level increased in the patient group, no significant difference was found. However, ET-1 levels in glioma tissue were elevated compared to healthy tissue. Serum and tissue ET-1 levels were determined for the first time in the study.

eNOS is in close association with the VEGF signaling pathway. Serum eNOS were measured in glioma, other CNS tumors, cerebral metastases, and healthy donors, and augmented levels were reported in glioma than any of the other groups. However, no differences were found according to tumor grade [53]. This result was consistent with increased expression of eNOS in glioma tissue [54]. Enhanced eNOS expression and plasma NO levels in tumor tissue of mice transfected with lung cancer cells were reported. After tumor-bearing mice were treated with an injection of eNOS agonist, a significant decrease was observed in tumor tissue eNOS expression and plasma NO compared to pre-treatment [55]. In our study, serum/tissue eNOS of the glioma patients were found to be higher than the controls. Unlike the study of Zheng et al. [53], serum levels of eNOS were found to be significantly higher in grade III compared to grade II. Higher eNOS levels were seen in serum and tumor tissue is consistent with the study by Zheng et al.; Pan et al.; Mei et al. [53–55]. This result can be interpreted as eNOS being activated in the process of gliomagenesis. In CNS, various cell types such as neurons, endothelial, microglial, and astroglial cells produce NO and it can act as a neurotransmitter, depending on its cellular source, amount and production site. It was shown that serum NO was raised in breast cancer patients compared with controls [56]. This finding was in accordance with the study conducted by Ratajczak-Wrona et al., where serum NO was higher in squamous cell carcinoma rather than control [57]. Elevated serum levels of NO were also seen in tissue levels in oral squamous cell carcinoma [58, 59]. In our study, the serum/tissue NO level of the glioma group was found to be higher than the control group. These increases

were in agreement with the research of Meena et al., Ratajczak-Wrona et al., Ghosh et al., and Sangle et al. [56–59].

This preliminary study provides a snapshot of the relationship of *ACE* I/D, *AGTR1* –168A/G, *AGTR1* –535C/T, *AGTR1* –825T/A, *VEGF* +936C/T, and *VEGF* –2578C/A polymorphisms with glioma pathogenesis in Turkish population and the role of some potential angiogenic parameters in serum and tissue in diagnosis. In conclusion, it was found that the risk of glioma was lower in the AA genotype and higher in the G allele as 2.27 fold in *AGTR1* –168A/G polymorphism. There were also increases in serum ACE, VEGF, eNOS, and NO levels, tissue ACE, ET-1, eNOS and NO levels, and relative *ACE*, *AGTR1*, and *VEGF* gene expressions in glioma. The presence of high tumor risk in G allele in *AGTR1* polymorphism, and increased expression of it shed light on the important role of this gene in the diagnosis and treatment of glial tumors. These results should be supported by new studies to be carried out in this field.

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AUTHOR CONTRIBUTION

TT: Conceptualization, Formal Analysis, Investigation, Roles/Writing—Original Draft.

BÖ: Resources, Roles/Writing—Original Draft.

ÖHE: Conceptualization, Methodology, Resources, Supervision, Writing—Review & Editing.

AMK: Resources.

İIG: Investigation, Resources, Validation, Writing—Review & Editing.

MB: Resources.

AG: Conceptualization, Formal analysis, Funding Acquisition, Investigation, Methodology, Project Administration, Supervision, Writing—Review & Editing.

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DATA AVAILABILITY

The datasets generated during and analyzed during the current study will be available from the corresponding author upon reasonable request.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was approved by the Clinical Research Ethics Committee of Abdurrahman Yurtaslan Ankara Oncology Training and Research Hospital with the decision number 189 dated December, 2015. All procedures involving people comply with the ethical standards of the institutional and/or national committee for research ethics and the 1964 Helsinki Declaration and its subsequent changes or comparable ethical standards. Informed written consent was achieved from all subjects included in the study according to the ethical standards of the Ethics Committee.

CONFLICT OF INTEREST

The authors of this work declare that they have no conflicts of interest.

REFERENCES

1. Louis D.N., Perry A., Reifenberger G., von Deimling A., Figarella-Branger D., Cavenee W.K., Ohgaki H., Wiestler O.D., Kleihues P., Ellison D.W. 2016. The 2016 World Health Organization classification of tumors of the central nervous system: A summary. *Acta Neuropathol.* **131** (6), 803–820. https://doi.org/10.1007/s00401-016-1545-1
2. Studdy P.R., Lapworth R., Bird R. 1983. Angiotensin-converting enzyme and its clinical significance—a review. *J. Clin. Pathol.* **36** (8), 938–947. https://doi.org/10.1136/jcp.36.8.938
3. Rigat B., Hubert C., Alhenc-Gelas F., Cambien F., Corvol P., Soubrier F. 1990. An insertion/deletion polymorphism in the angiotensin I-converting enzyme gene accounting for half the variance of serum enzyme levels. *J. Clin. Invest.* **86** (4), 1343–1346. https://doi.org/10.1172/JCI114844
4. Duncan J.A., Scholey J.W., Miller J.A. 2001. Angiotensin II type 1 receptor gene polymorphisms in humans: Physiology and pathophysiology of the genotypes. *Curr. Opin. Nephrol. Hypertens.* **10** (1), 111–116. https://doi.org/10.1097/00041552-200101000-00017
5. Munzenmaier D.H., Greene A.S. 1996. Opposing actions of angiotensin II on microvascular growth and arterial blood pressure. *Hypertension*. **27** (3Pt2), 760–765. https://doi.org/10.1161/01.hyp.27.3.760
6. Uemura H., Ishiguro H., Nakaigawa N., Nagashima Y., Miyoshi Y., Fujinami K., Sakaguchi A., Kubota Y. 2003. Angiotensin II receptor blocker shows antiproliferative activity in prostate cancer cells: A possibility of tyrosine kinase inhibitor of growth factor. *Mol. Cancer Ther.* **2** (11), 1139–1147.
7. Watson C.J., Webb N.J., Bottomley M.J., Brenchley P.E. 2000. Identification of polymorphisms within the vascular endothelial growth factor (VEGF) gene: Correlation with variation in VEGF protein production. *Cytokine*. **12** (8), 1232–1235. https://doi.org/10.1006/cyto.2000.0692
8. Salani D., Taraboletti G., Rosanò L., Di Castro V., Borsotti P., Giavazzi R., Bagnato A. 2000. Endo-

thelin-1 induces an angiogenic phenotype in cultured endothelial cells and stimulates neovascularization in vivo. *Am. J. Pathol.* **157** (5), 1703–1711. https://doi.org/10.1016/S0002-9440(10)64807-9

9. Kaur B., Khwaja F.W., Severson E.A., Matheny S.L., Brat D.J., Van Meir E.G. 2005. Hypoxia and the hypoxia-inducible-factor pathway in glioma growth and angiogenesis. *Neuro Oncol.* **7** (2), 134–153. https://doi.org/10.1215/S1152851704001115
10. Fukumura D., Gohongi T., Kadambi A., Izumi Y., Ang J., Yun C.O., Buerk D.G., Huang P.L., Jain R.K. 2001. Predominant role of endothelial nitric oxide synthase in vascular endothelial growth factor-induced angiogenesis and vascular permeability. *Proc. Natl. Acad. Sci. U. S. A.* **98** (5), 2604–2609. https://doi.org/10.1073/pnas.041359198
11. Brooks S.E., Gu X., Samuel S., Marcus D.M., Bartoli M., Huang P.L., Caldwell R.B. 2001. Reduced severity of oxygen-induced retinopathy in eNOS-deficient mice. *Invest. Ophthalmol. Vis. Sci.* **42** (1), 222–228.
12. Asgharzadeh F., Hassanian S., Ferns G., Khazaei M., Hasanzadeh M. 2018. The therapeutic potential of angiotensin-converting enzyme and angiotensin receptor inhibitors in the treatment of colorectal cancer: Rational strategies and recent progress. *Curr. Pharm. Des.* **24** (39), 4652–4658. https://doi.org/10.2174/1381612825666190111145140
13. Lian M., Jiang H., Wang H., Guo S. 2015. Angiotensin-converting enzyme insertion/deletion gene polymorphisms is associated with risk of glioma in a Chinese population. *J. Renin. Angiotensin. Aldosterone Syst.* **16** (2), 443–447. https://doi.org/10.1177/1470320313495910
14. Pandith A.A., Qasim I., Zahoor W., Shah P., Bhat A.R. 2018. ACE I/D sequence variants but not MTHFR C677T, is strongly linked to malignant glioma risk and its variant DD genotype may act as a promising predictive biomarker for overall survival of glioma patients. *Gene*. **639**, 62–68. https://doi.org/10.1016/j.gene.2017.10.013
15. Benenemissi I., Sifi K., Sahli L., Semmam O., Abadi N., Satta D. 2019. Angiotensin-converting enzyme insertion/deletion gene polymorphisms and the risk of glioma in an Algerian population. *Pan. Afr. Med. J.* **32**, 1–7. https://doi.org/10.11604/pamj.2019.32.197.15129
16. Li J., Sima X., Zhao N. 2013. Studies on association between ACE I/D polymorphism and glioma. *Chin. J. Neurosurg. Dis. Res.* **12**, 106–108.
17. Sun M., Fang Y., Ma S., Gao X., Sun Y. 2020. The genetic polymorphisms of angiotensin converting enzyme insertion / deletion and glioma susceptibility: A meta-analysis. *J. Renin Angiotensin Aldosterone Syst.* **21** (4), 1470320320963939. https://doi.org/10.1177/1470320320963939
18. Arrieta O., Guevara P., Escobar E., García-Navarrete R., Pineda B., Sotelo J. 2005. Blockage of angiotensin II type I receptor decreases the synthesis of growth factors and induces apoptosis in C6 cultured cells and C6 rat glioma. *Br. J. Cancer*. **92** (7), 1247–1252. https://doi.org/10.1038/sj.bjc.6602483
19. Koh W.P., Yuan J.M., Van Den Berg D., Lee H.P., Yu M.C. 2005. Polymorphisms in angiotensin II type 1 receptor and angiotensin I-converting enzyme genes and breast cancer risk among Chinese women in Singapore. *Carcinogenesis*. **26** (2), 459–464. https://doi.org/10.1093/carcin/bgh309
20. Li R., Zhao Y., Fan W., Chen H., Chen Y., Liu Y., Chen G., Zhou K., Huang F., Mao Y., Zhou L., Lu D., Shugart Y.Y. 2011. Possible association between polymorphisms of human vascular endothelial growth factor A gene and susceptibility to glioma in a Chinese population. *Int. J. Cancer*. **128** (1), 166–175. https://doi.org/10.1002/ijc.25306
21. Jiang H., Lian M., Xie J., Li J., Wang M. 2013. Three single nucleotide polymorphisms of the vascular endothelial growth factor (VEGF) gene and glioma risk in a Chinese population. *J. Int. Med. Res.* **41** (5), 1484–1494. https://doi.org/10.1177/0300060513498667
22. Zhang J., Yang J., Chen Y., Mao Q., Li S., Xiong W., Lin Y., Chen J., Ge J. 2016. Genetic Variants of VEGF (rs201963 and rs3025039) and KDR (rs7667298, rs2305948, and rs1870377) are associated with glioma risk in a Han Chinese population: A case-control study. *Mol. Neurobiol.* **53** (4), 2610–2618. https://doi.org/10.1007/s12035-015-9240-0
23. Linhares P., Viana-Pereira M., Ferreira M., Amorim J., Nabiço R., Pinto F., Costa S., Vaz R., Reis R.M. 2018. Genetic variants of vascular endothelial growth factor predict risk and survival of gliomas. *Tumour Biol.* **40** (3), 1010428318766273. https://doi.org/10.1177/1010428318766273
24. Juillerat-Jeanneret L., Celerier J., Bernasconi C.C., Nguyen G., Wostl W., Maerki H.P., Janzer R.C., Corvol P., Gasc J.M. 2004. Renin and angiotensinogen expression and functions in growth and apoptosis of human glioblastoma. *Br. J. Cancer*. **90** (5), 1059–1068. https://doi.org/10.1038/sj.bjc.6601646
25. Bradshaw A.R., Wickremesekera A.C., Brasch H.D., Chibnall A.M., Davis P.F., Tan S.T., Itinteang T. 2016. Glioblastoma multiforme cancer stem cells express components of the renin–angiotensin system. *Front. Surg.* **3**, 51. https://doi.org/10.3389/fsurg.2016.00051
26. Han C.D., Ge W.S. 2016. Up-regulation of angiotensin-converting enzyme (ACE) enhances cell proliferation and predicts poor prognosis in laryngeal cancer. *Med. Sci. Monit.* **22**, 4132–4138. https://doi.org/10.12659/msm.896933
27. Zhang K., Mao T., He Z., Wu X., Peng Y., Chen Y., Dong Y., Ruan Z., Wang Z. 2019. Angiotensin I-converting enzyme gene plays a crucial role in the pathology of carcinomas in colorectal cancer. *Artif. Cells Nanomed. Biotechnol.* **47** (1), 2500–2506. https://doi.org/10.1080/21691401.2019.1626402
28. Mehranfard D., Perez G., Rodriguez A., Ladna J.M., Neagra C.T., Goldstein B., Carroll T., Tran A., Trivedi M., Speth R.C. 2021. Alterations in gene expression of renin-angiotensin system components and related proteins in colorectal cancer. *J. Renin Angiotensin Aldosterone Syst.* **2021**, 9987115. https://doi.org/10.1155/2021/9987115

29. Ibiş M., Yüksel O., Yilmaz G., Köklü S., Yilmaz F.M., Ertuğrul I., Uçar E., Altiparmak M.E. 2008. Serum angiotensin converting enzyme levels in pancreatic diseases. *Hepatogastroenterology*. **55** (86–87), 1814–1817.
30. Beyazit F., Ayhan S., Celik H.T., Gungor T. 2015. Assessment of serum angiotensin-converting enzyme in patients with epithelial ovarian cancer. *Arch. Gynecol. Obstet.* **292** (2), 415–420. https://doi.org/10.1007/s00404-015-3661-x
31. Kardum D., Huskic J., Fabijanic D., Banic M., Buljevac M., Kjundzic M., Loncar B. 1999. Activity of serum angiotensin-converting enzyme as a tumor marker of hepatocellular carcinoma. *Eur. J. Gastroenterol. Hepatol.* **11** (11), 1209–1213. https://doi.org/10.1097/00042737-199911000-00004
32. Ohta T., Amaya K., Yi S., Kitagawa H., Kayahara M., Ninomiya I., Fushida S., Fujimura T., Nishimura G., Shimizu K., Miwa K. 2003. Angiotensin converting enzyme-independent, local angiotensin II-generation in human pancreatic ductal cancer tissues. *Int. J. Oncol.* **23** (3), 593–598
33. Procházka J., Krepela E., Sedo A., Viklický J., Fiala P. 1991. Aminopeptidases and angiotensin I-converting enzyme activities in primary human lung tumors and lung parenchyma. *Neoplasma*. **38** (5), 501–508.
34. Danilov S.M., Kadrev A.V., Kurilova O.V., Tikhomirova V.E., Kryukova O.V., Mamedov V.N., Kamalov D.M., Danilova N.V., Okhobotov D.A., Gayfullin N.M., Evdokimov V.V., Alekseev B.J., Kost O.A., Samokhodskaya L.M., Kamalov A.A. 2019. Tissue ACE phenotyping in prostate cancer. *Oncotarget*. **10** (59), 6349–6361. https://doi.org/10.18632/oncotarget.27276
35. Ganong W.F. 1984. The brain renin–angiotensin system. *Annu. Rev. Physiol.* **46**, 17–31. https://doi.org/10.1146/annurev.ph.46.030184.000313
36. Arrieta O., Pineda-Olvera B., Guevara-Salazar P., Hernández-Pedro N., Morales-Espinosa D., Cerón-Lizarraga T.L., González-De la Rosa C.H., Rembao D., Segura-Pacheco B., Sotelo J. 2008. Expression of AT1 and AT2 angiotensin receptors in astrocytomas is associated with poor prognosis. *Br. J. Cancer*. **99** (1), 160–166. https://doi.org/10.1038/sj.bjc.6604431
37. Arrieta O., Villarreal-Garza C., Vizcaíno G., Pineda B., Hernández-Pedro N., Guevara-Salazar P., Wegman-Ostrosky T., Villanueva-Rodríguez G., Gamboa-Domínguez A. 2015. Association between AT1 and AT2 angiotensin II receptor expression with cell proliferation and angiogenesis in operable breast cancer. *Tumour Biol.* **36** (7), 5627–5634. https://doi.org/10.1007/s13277-015-3235-3
38. Plate K.H., Breier G., Weich H.A., Risau W. 1992. Vascular endothelial growth factor is a potential tumour angiogenesis factor in human gliomas in vivo. *Nature*. **359** (6398), 845–848. https://doi.org/10.1038/359845a0
39. Pietsch T., Valter M.M., Wolf H.K., Von Deimling A., Huang H.J., Cavenee W.K., Wiestler O.D. 1997. Expression and distribution of vascular endothelial growth factor protein in human brain tumors. *Acta Neuropathol.* **93** (2), 109–117. https://doi.org/10.1007/s004010050591
40. Samoto K., Ikezaki K., Ono M., Shono T., Kohno K., Kuwano M., Fukui M. 1995. Expression of vascular endothelial growth factor and its possible relation with neovascularization in human brain tumors. *Cancer Res.* **55** (5), 1189–1193.
41. Li J.T., Yan Q., Yu H.L. 2009. Expression of VEGF and NGF in gliomas of human. *Sichuan Da Xue Xue Bao Yi Xue Ban*. **40** (3), 408–411.
42. Nowacka A., Smuczyński W., Rość D., Woźniak-Dąbrowska K., Śniegocki M. 2018. Serum VEGF-A concentrations in patients with central nervous system (CNS) tumors. *PLoS One*. **13** (3), e0192395. https://doi.org/10.1371/journal.pone.0192395
43. Chiorean R., Berindan-Neagoe I., Braicu C., Florian I.S., Leucuta D., Crisan D., Cernea V. 2014. Quantitative expression of serum biomarkers involved in angiogenesis and inflammation, in patients with glioblastoma multiforme: Correlations with clinical data. *Cancer Biomark.* **14** (2–3), 185–194. https://doi.org/10.3233/CBM-130310
44. Rafat N., Beck G.C., Schulte J., Tuettenberg J., Vajkoczy P. 2010. Circulating endothelial progenitor cells in malignant gliomas. *J. Neurosurg.* **112** (1), 43–49. https://doi.org/10.3171/2009.5.JNS081074
45. Takano S., Yoshii Y., Kondo S., Suzuki H., Maruno T., Shirai S., Nose T. 1996. Concentration of vascular endothelial growth factor in the serum and tumor tissue of brain tumor patients. *Cancer Res.* **56** (9), 2185–2190.
46. Schmidt N.O., Westphal M., Hagel C., Ergün S., Stavrou D., Rosen E.M., Lamszus K. 1999. Levels of vascular endothelial growth factor, hepatocyte growth factor/scatter factor and basic fibroblast growth factor in human gliomas and their relation to angiogenesis. *Int. J. Cancer*. **84** (1), 10–18. https://doi.org/10.1002/(sici)1097-0215(19990219)84:1<10::aid-ijc3>3.0.co;2-1
47. Stiles J.D., Ostrow P.T., Balos L.L., Greenberg S.J., Plunkett R., Grand W., Heffner R.R.Jr. 1997. Correlation of endothelin-1 and transforming growth factor β1 with malignancy and vascularity in human gliomas. *J. Neuropathol. Exp. Neurol.* **56** (4), 435–439. https://doi.org/10.1097/00005072-199704000-00012
48. Abdel-Gawad I.A., Hassanein H.M., Bahgat N.A., Abdel Sattar M.A., El-Sissy A.H., Altaweel M.A., Helal A.M. 2008. Study of endothelin-1 and vascular endothelial growth factor in patients with cancer colon. *J. Egypt. Natl. Canc. Inst.* **20** (3), 216–223.
49. Yildirim Y., Gunel N., Coskun U., Sancak B., Bukan N., Aslan S., Cetin A. 2008. Serum big endothelin-1 levels in female patients with breast cancer. *Int. Immunopharmacol.* **8** (8), 1119–1123. https://doi.org/10.1016/j.intimp.2008.03.023
50. Teng X.J., Liu R., Zhang Z.X., He J.F., Shen Z.X. 2008. Correlation of preoperative plasma levels of big endothelin-1 to prognosis of gastric carcinoma. *Ai Zheng*. **27** (1), 88–91.
51. Pfab T., Stoltenburg-Didinger G., Trautner C., Godes M., Hocher B. 2004. The endothelin system in Morris hepatoma-7777: An endothelin receptor antagonist inhibits growth in vitro and in vivo. *Br. J. Pharmacol.* **141** (2), 215–222. https://doi.org/10.1038/sj.bjp.0705601

52. Alanen K., Deng D.X., Chakrabarti S. 2000. Augmented expression of endothelin-1, endothelin-3 and the endothelin-B receptor in breast carcinoma. *Histopathology*. **36** (2), 161–167. https://doi.org/10.1046/j.1365-2559.2000.00795.x

53. Zheng P.P., Hop W.C., Luider T.M., Sillevis Smitt P.A., Kros J.M. 2007. Increased levels of circulating endothelial progenitor cells and circulating endothelial nitric oxide synthase in patients with gliomas. *Ann. Neurol.* **62** (1), 40–48. https://doi.org/10.1002/ana.21151

54. Pan J.W., Zhan R.Y., Tong Y., Zhou Y.Q., Zhang M. 2005. Expression of endothelial nitric oxide synthase and vascular endothelial growth factor in association with neovascularization in human primary astrocytoma. *J. Zhejiang Univ. Sci. B.* **6** (7), 693–698. https://doi.org/10.1631/jzus.2005.B0693

55. Mei K., Cai X.H., Du L., Chen Y.F., Huang S., Chen J., Yin X.D., Zhang Z.X., Zhao X., Zhou C.Y., Yu J.R. 2010. Basic Research Effect of nitric oxide derived from endothelial nitric oxide synthase (eNOS) on tumor angiogenesis. *Chin. J. Cancer*. **29** (1), 32–37. https://doi.org/10.5732/cjc.009.10246

56. Meena S.K., Kumar R., Sairoz. 2017. Serum nitric oxide and peroxynitrite in breast cancer patients. *Int. J. Curr. Res.* **9** (8), 55725–55727.

57. Ratajczak-Wrona W., Jablonska E., Marcinczyk M., Grabowska Z., Piotrowski L. 2022. Role of p38 MAPK pathway in induction of iNOS expression in neutrophils and peripheral blood mononuclear cells in patients with squamous cell carcinoma. *J. Oral. Maxillofac. Surg.* **67** (11), 2354–2363. https://doi.org/10.1016/j.joms.2009.04.030

58. Ghosh R., Castelino R.L., Babu S.G., Banerjee B. 2021. Estimation of salivary and tissue nitric oxide levels in oral squamous cell carcinoma: A biochemical study. *Eur. J. Ther.* **27**, 26–31.

59. Sangle V.A., Chaware S.J., Kulkarni M.A., Ingle Y.C., Singh P., Pooja V.K. 2018. Elevated tissue nitric oxide in oral squamous cell carcinoma. *J. Oral. Maxillofac. Pathol.* **22** (1), 35–39. https://doi.org/10.4103/jomfp.JOMFP_27_16

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