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ACE GENE INSERTION/DELETION POLYMORPHISM IS ASSOCIATED WITH GLIOBLASTOMA IN AN IRANIAN POPULATION: A CASE-CONTROL STUDY

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Abstract

Background. The insertion/deletion (I/D) polymorphism of the angiotensin-converting enzyme (ACE) gene has recently been reported to be associated with the pathogenesis and development of human cancers. This study aimed to assess the potential association between ACE (I/D) polymorphism and glioblastoma in an Iranian population. **Material and Methods.** This case-control study was conducted on 80 patients with glioblastoma and 80 healthy blood donors as controls. Gap-polymerase chain reaction (Gap-PCR) was used to determine the ACE (I/D) genotypes. PCR products were separated and measured by electrophoresis on a 2 % agarose gel. **Results.** Analysis of demographic data showed a significant difference in the family history of cancer between the case and control groups (p=0.03). The distribution of ACE gene variants including II, ID, and DD genotypes was also calculated, and significant differences were seen in the DD genotype (p=0.03) and D allele (p=0.04) between the glioblastoma cases and controls. **Conclusion.** ACE gene polymorphism was associated with glioblastoma in the study population. Further studies are needed to approve this finding.

Key words: angiotensin-converting enzyme, ACE I/D polymorphism, glioblastoma, gap-PCR.

ПОЛИМОРФИЗМ ВСТАВКИ/ДЕЛЕЦИИ ГЕНА АПФ СВЯЗАН С ГЛИОБЛАСТОМОЙ У НАСЕЛЕНИЯ ИРАНА: ИССЛЕДОВАНИЕ СЛУЧАЙ-КОНТРОЛЬ

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Анатация

Актуальность. Недавно сообщалось, что инсерционно-делеционный (I/D) полиморфизм гена ангиотензин-превращающего фермента (АПФ) связан с патогенезом и развитием рака человека. **Целью исследования** была оценка потенциальной связи между I/D полиморфизмом гена АПФ и глиобластомой у населения Ирана. **Материал и методы**. В исследовании случай-контроль участвовали 80 пациентов с глиобластомой и 80 здоровых доноров в качестве группы контроля. Полимеразная цепная реакция (Gap-PCR) использовалась для определения генотипов I/D полиморфизма гена АПФ. ПЦР-продукты разделяли и измеряли электрофорезом в 2 % агарозном геле. **Результаты.** Анализ демографических данных показал значительную разницу в семейной истории рака между основной и контрольной группами (p=0,03). Было рассчитано распределение вариантов гена АПФ, включая генотипы II, ID и DD, и были обнаружены значительные различия в генотипе DD (p=0,03) и аллеле D (p=0,04) между

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группой больных с глиобластомой и контрольной группой. **Заключение**. Полиморфизм гена АПФ был связан с глиобластомой в исследуемой популяции. Необходимы дальнейшие исследования, чтобы подтвердить эти данные.

Ключевые слова: ангиотензин-превращающий фермент, полиморфизм АПФ I/D, глиобластома, gap-ПЦР.

Introduction

Glioblastoma multiforme (GBM) (so-called glioblastoma), which has been indicated as Grade IV by the WHO, is the most collective and most violent type of malignant brain tumor in adults [1, 2]. It accounts for almost 60 % of entire brain tumors in adults [3]. The annual incidence rate is 3.2 per 100,000 population. The disease can arise at any age including childhood, however, the highest rate is between 55 to 60 years [4, 5]. Additionally, men experience a higher incidence of GBM than women [6]. GBM remains a chronic condition with a dismal prognosis even though several conventional medications are even though several contemporary medications are several contemporary medications available. Almost 14 to 15 months following diagnosis, patients often have an average survival rate [6, 7].

The complex biological system known as the Renin-Angiotensin System (RAS) interacts with a wide range of convergent signaling pathways involved in cancer development [8]. The major effector hormone, angiotensin II, is produced as the result of many stages in the RAS pathway (ATII) [9]. It is generally known that the CNS contains this crucial homeostatic mechanism [10]. In CNS disorders, the RAS's critical features are also engaged [9]. Different parts of the brain express renin and its metabolite prorenin differently in neurons, astrocytes, oligodendrocytes, and microglia [11, 12]. Prorenin binds to the prorenin receptor (PRR) to generate rennin [13]. The brain regions responsible for blood pressure regulation and homeostasis express the angiotensin-converting enzyme (ACE), commonly known as ACE1 [14]. The endothelium of the brain contains ACE2 in several areas, including the cortex and brainstem [15]. By transforming ATII into Ang(1-7) _ a ligand for the Mas Receptor (MasR) _ ACE2 participates in the neuroprotective ACE2/ Angiotensin (1-7) (MasR) signaling axis [16]. ACE1 transforms ATI into ATII. Both ATII receptor 1 (AT1R) and ATII receptor 2 (AT2R) bind to ATII [17, 18]. The creation of NF-κB, TGF-1, and increased RAS activity in the TME are all caused by AT1R signaling, which also encourages cellular proliferation, inflammation, and angiogenesis [19]. Cellular growth is inhibited and apoptosis is increased when AT2R is activated by ATII [19]. Pro Renin Receptor (PRR), AT1R, and AT2R are also expressed in GBM along with stemness-related markers [20]. Compared to lower-grade gliomas, GBM has a higher expression of PRR. This increased PRR expression in higher-grade gliomas is significant since the Wnt/β-catenin signaling pathway is thought to be involved in stem cells' self-renewing capacity [21].

Numerous malignancies, including lung, breast, prostate, gastrointestinal, and oral cancers, have been affected by ACE [22–25]. Additionally, it has variable expression in a variety of carcinomas and may have an impact on the growth, migration, angiogenesis, and metastatic mechanisms of tumor cells [26]. In vitro and in vivo tumor progression is inhibited in animal models when ACE activity is inhibited. Similarly, epidemiological studies have suggested that ACE inhibitors may lower the incidence of cancer and mortality [26, 27]. A 21 kb gene with 26 exons and 25 introns, the human ACE gene is found on chromosome 17q23 [28]. On intron 16, the insertion/ deletion (I/D) polymorphism (rs1799752) of the ACE gene is detectable [29]. There are three genotypes that it may take, II, ID, and DD, depending on whether a 287-bp Alu repeat sequence is present (I) or deleted (D) [30–32]. 20 % to 50 % of the variation in ACE expression or activity in blood and tissues among individuals is accounted for by the I/D polymorphism [26]. In comparison to homozygote DD, homozygote II may have plasma ACE levels that are as much as 50 % lower, whereas heterozygote ID has levels that are in the middle [27]. Understanding the pathophysiology, predicting prognosis and response to therapy, and maybe acting as analytical markers is aided by the identification of genetic, epigenetic, and transcriptional anomalies in glioblastoma.

This case-control research was carried out to investigate any potential links between ACE I/D polymorphism and glioblastoma. Given the significant role of ACE in the formation of malignancies, this is the first study of the association between ACE I/D polymorphism and glioblastoma in an Iranian population including ethnicities of Azerbaijanis and Persians which are based on the birth region of individuals.

Material and Methods

Population study

80 paraffin-embedded glioblastoma tumor tissues (totally Grade IV)**,** which had been surgically resected from the patients who had been referred to Imam Khomeini Hospital (Tehran-Iran) from January 2018 to December 2021 were analyzed. The glioblastoma tissues were obtained from the Iran National Tumor Bank, funded by the Cancer Institute of Tehran University of Medical Sciences for Cancer Researches (Tehran, Iran). The histopathologic characteristics of the samples were evaluated and characterized by an experienced pathologist. 11 Patients of all 80 patients received treatments which 8 of them received

radiotherapy as neoadjuvant treatment and 3 other patients received adjuvant treatments of chemotherapy and radiotherapy. A total of 80 healthy blood donors with no history of cancer were also collected. The brain tumor patients and the healthy controls were matched for sex and age. Smoking status was categorized as either smoker or non-smoker (defined as fewer than 100 cigarettes smoked in a lifetime). Non-drinkers and drinkers were the categories for drinking status (drinks per day or more than two times a week). Iranians were chosen to participate in both the case and control groups, with 43 Azerbaijanis and 37 Persians making up the case group and 41 Azerbaijanis and 39 Persian blood donors making up the control group. 11 Patients of all 80 patients received treatments. 8 patients received radiotherapy as neoadjuvant treatment. 3 other patients received chemotherapy and radiotherapy at the same time as adjuvant treatment. Before their involvement in the study, the participants gave their written informed permission.

DNA extraction

The paraffin of samples was removed by adding Xylene, mixed carefully with vortex, and then transferred to a thermoblock. Then, the samples were centrifuged. These steps were repeated 3 times. After removing the paraffin from the tissues, absolute ethanol was added to the samples, mixed with vortex, and then washed with a centrifuge device, thereby removing the xylene effect. Afterward, the samples were washed with ethanol (70 %) and centrifuged for 3 minutes. Then the supernatant was poured away (this step was repeated 3 times). Thereafter, cell lysis buffer, proteinase K, protein precipitating agent, and chloroform were added to each sample and centrifuged. Isopropanol was added to the upper phase containing DNA and centrifuged. The supernatant was removed and ethanol (70 %) was added to the settled DNA. Then, RNase-DNase-free distilled water and treatment-1 were added to obtain an optimal density (OD) of 1.8 to 2.

DNA was extracted from the blood samples as follows: After centrifugation, the supernatant (plasma) was removed. RBC lysis buffer was added to each sample and centrifuged to obtain white blood cells (WBC) settled. Cell lysis buffer and SDS 10 % were added to expose all components of the cells, as well as adding RNase A to remove all RNAs in the solution. A precipitating agent was added and after centrifugation, proteins were settled so that the liquid contained only DNA. DNA bundle was observed by adding isopropanol and shaking slowly. After centrifugation, a DNA pellet was obtained. Then the supernatant was removed and DNA was washed with ethanol (70 %). After centrifugation and supernatant removal, TE buffer was added. Finally, the purity and quality of the extracted DNA were measured by Nano-Drop (Thermo Scientific/NANODROP1000 Spectrophotometer), and data were recorded for each sample with an OD of 1.8 to 2.

Gap-PCR

To verify the presence or absence of a 287-bp sequence, primers were designed to completely flank all the 287-bp sequences. Therefore, the mutation in this region could be detected based on the size of the products. Based on the GenBank reference sequence, the PCR primers were designed as forward-5'-CTGGAGACCACTCCCATCCTTTCT-3' and reverse-5'-TGTGGCCATCACATTCGTCAGAT-3'. These primers amplified a 478-bp fragment in the wild type, and the presence of a 191-bp PCR product in the gene indicated a mutation of 287-bp deletion.

The PCR reaction was carried out in a volume of 20 μL, including 3 μL template DNA, 1μL mixed forward and reverse primers, 10 μL PCR Master Mix, and 6 μL nuclease-free distilled water. PCR reaction was defined as follows: initial denaturation at 95°C for 5 min, followed by 34 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 40 s, and extension at 72°C for 40 s, with a final extension at 72°C for 5 min.

Electrophoresis on a 2 % agarose gel was used to identify PCR products. Both the insertion allele (I) and the deletion allele (D) were identified as 478-bp and 191-bp bands, respectively. Every run included the incubation of negative controls (PCR mixture-containing tubes without DNA templates) to check for contamination. A random sample of 15 % of all samples was retested, as well as any sample with an unclear result because of poor yield. Replicate testing turned out with no inconsistencies.

Statistical analysis

A Chi-square (χ^2) test was applied to analyze the genotype distribution between the two groups using the GraphPad Prism software version 8.3.0 for the ACE I/D polymorphism. A Chi-square (χ^2) test was also used to analyze Hardy-Weinberg equilibrium (HWE). The p-value lower than 0.05, was considered significant. The odds ratio (OR) and 95 % confidence interval (CI) were computed using a logistic regression model.

Results

Study subjects

The characteristics of 80 brain tumor cases of glioblastoma and 80 control subjects are shown in Table 1. Glioblastoma cases and controls did not differ in terms of gender (p=0.66) or age (p=0.23). The mean age was 55.5 ± 20.01 years for the cases (glioblastoma brain tumors) and 53.74 ± 16.18 years for the controls. Additionally, no significant differences were observed between the cases and controls in smoking (p=0.13) and drinking (p=0.32), while the two groups were significantly different in terms of family history of cancer (p=0.03). The treatment status of cases was in surgery (all 80 cases), surgery + radiotherapy (11 cases), and Surgery + radiotherapy + chemotherapy (3 cases) distribution (Table 1).

Table 1/Таблица 1

Characteristics of cases with glioblastoma and controls

Характеристики пациентов с глиобластомами и группы контроля

Characteristics of cases/ Характеристики пациентов	Cases/ Пациенты с глиобластомами (n=80)	Controls/ Контрольная группа (n=80)	p
Age (Mean ± SD)/Возраст	55.5 ± 20.01	53.74 ± 16.18	0.23
Gender/Пол			
Male/Мужской Female/Женский	43 (53.75 %) 37 (46.25 %)	40 (50 %) 40 (50 %)	0.66
Smoking/Курящие			
Yes/Да No/Нет	32 (40 %) 48 (60 %)	23 (28.75 %) 57 (71.25 %)	0.13
Drinking/Употребляющие алкоголь			
Yes/Да No/Нет	14 (17.5 %) 66 (70.23 %)	19 (23.75 %) 61 (76.25 %)	0.32
Family history of cancer/Отягощенный семейный анамнез			
Yes/Да No/Нет	26 (32.5 %) 54 (67.5 %)	14 (17.5 %) 66 (82.5 %)	0.03*
Treatment status/Лечение			
Surgery/Операция	80 (100 %)	–	
Surgery + Radiotherapy/ Операция + лучевая терапия	11 (13.75 %)	–	
Surgery + Radiotherapy + Chemotherapy/ Операция + лучевая терапия + химиотерапия	3 (3.75 %)	–	

Genotyping and allele distribution

The GAP-PCR products were analyzed by gel electrophoresis (Fig. 1). D and I alleles of the ACE were identified by the existence of 191 bp and 478 bp fragments, respectively. The frequency of the ACE I/D genotype is reported in Table 2. The frequency of the DD genotype was lower in glioblastoma patients than in controls (showing frequencies of 10 % and 25 %, respectively) and conferred a 2.78-fold risk for susceptibility in glioblastoma cases (DD *vs* II genotype, p=0.03). ID genotype frequencies were 47.5 % and 40.0 % in glioblastoma cases and controls, respectively. Also, II genotype frequencies were 42.5 % and 35 % in glioblastoma cases and controls, respectively. The recessive model (ACE II + ID genotypes vs DD) was associated with a 3-fold reduced risk of glioblastoma (OR=3.00, 95 % CI: 1.29–7.01, p=0.01). Similarly, the allelic frequency was analyzed. The frequency of allele D was lower in glioblastoma cases (34 %) compared to the controls (45 %) (OR=1.6, 95% CI: 1.02–2.54; p=0.04) which were significantly different. The frequency of the I allele was 66.25 % and 55 % in cases and controls, respectively (Table 2). The genotype distributions of glioblastoma patients (cases) and healthy blood donors (controls) were in Hardy-Weinberg equilibrium (p>0.05) (Table 3). There were no significant differences in the prevalence of any genotype when the treatment status and ethnicity of the glioblastoma were stratified (Table 4).

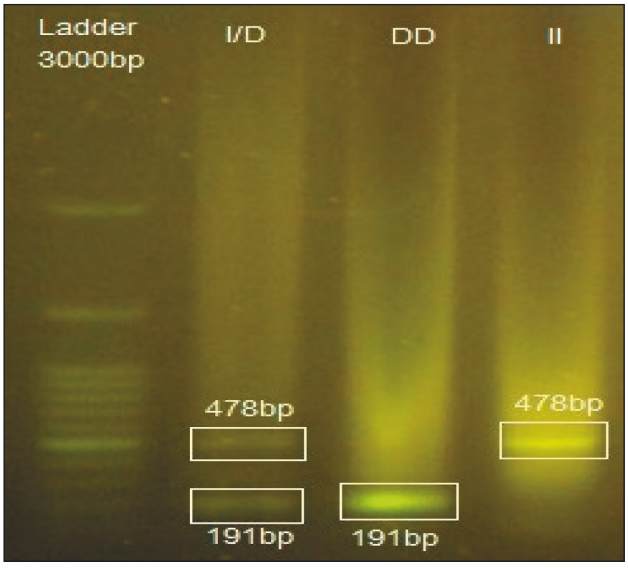

Fig. 1. PCR products of ACE I/D polymorphism. Specific bands are shown for the I/I genotype (478 bp), D/D genotype (191 bp), and I/D genotype (478, 191 bp)

Рис. 1. ПЦР-продукты I/D полиморфизма гена АПФ. Специфические полосы показаны для генотипа I/I (478 bp.), генотипа D/D (191 bp.) и генотипа I/D (478, 191 bp.)

Table 2/Таблица 2

Frequency of ACE I/D genotype in patients with and controls

Частота генотипа АПФ I/D у больных глиобластомой и контрольной группы

Genetic model/ Генетическая модель	Cases/ Больные с глиобластомой	Controls/ Контрольная группа	OR	Confidence interval/ Доверительный интервал	p
		Co–dominant/Co–доминантная			
II	34 (42.5 %)	28 (35 %)	1.00	Reference/ Референтный интервал	–
ID	38 (47.5 %)	32 (40 %)	0.98	0.52 to 2.01	0.94
DD	8 (10 %)	20 (25 %)	2.78	1.02 to 7.37	0.03*
		Dominant/Доминантная			
II	34 (42.5 %)	28 (35 %)	1.00	Reference/ Референтный интервал	–
ID+DD	46 (57.5 %)	52 (65 %)	1.37	0.72 to 2.62	0.33
		Recessive/Рецессивная			
II+ID	72 (90 %)	60 (75 %)	1.00	Reference/ Референтный интервал	–
DD	8 (10 %)	20 (25 %)	3.00	1.29 to 7.01	0.01*
		Over dominant/Сверхдоминантная			
II+DD	42 (52.5 %)	48 (60 %)	1.00	Reference/ Референтный интервал	–
ID	38 (47.5 %)	32 (40 %)	0.73	0.39 to 1.35	0.34
		Allelic Frequency/Аллельная частота			
I	106 (66 %)	88 (55 %)	1.00	Reference/ Референтный интервал	–
D	54 (34 %)	72 (45 %)	1.6	1.02 2.54	0.04*

Table 3/Таблица 3

Hardy–Weinberg Equilibrium (HWE) test for genotypic frequencies in patients with Glioblastoma and the control group

Тест равновесия Харди–Вайнберга (HWE) для генотипических частот у пациентов с глиобластомой и контрольной группы

Genotype/ Генотип	Case/ Пациенты с глиобластомой	HWE/ Тест равновесия Харди–Вайнберга		Control/ Контрольная группа	HWE/ Тест равновесия Харди–Вайнберга	
		χ^2	p		χ^2	p
II	34			28		
ID	38	0.31	0.85	32	2.95	0.23
DD	8			20		

Discussion

Gliomas are a group of cancers that comprise astrocytic tumors (glioblastoma, oligodendroglioma, and astrocytic tumors), oligodendrogliomas, and ependymomas. Mixed gliomas are the most prevalent intraparenchymal tumors of the central nervous system (CNS), and they have a very poor prognosis [33–36]. They characterize 81 % of the malignant tumors and about 26 % of all primary brain and other CNS malignancies. The majority of gliomas (56.6 %) are glioblastoma. Only 5.6 % of individuals with glioblastoma survived five years after diagnosis, according to comparative survival approximations [37].

Regarding the importance and implications of renin-angiotensin system components in various types of cancers, we analyzed ACE (I/D) gene polymorphism to determine possible association with glioblastoma in an Iranian population. Our results showed that there were significant differences in the frequency of the DD genotype between patients and controls and also showed significant differences in terms of family history of cancer characteristics, between those who took part in glioblastoma research.

ACE I/D polymorphism and glioma in the Chinese population may be associated, according to research that investigated the matter. According to their findings, glioma patients had a considerably lower frequency of the DD genotype than controls (OR=1.61, 95 % CI: 1.12–2.32; p=0.01). However, the D allele's frequency was equivalent in glioma patients and controls. (30.9 *vs*

Table 4/Таблица 4

Stratification analysis of angiotensin–converting enzyme ACE (I/D) polymorphism in glioblastoma
Стратификационный анализ полиморфизма ангиотензин-превращающего фермента АПФ (I/D) при глиобластоме

Variation/ Параметр	Glioblastoma/ Глиобластома	II			ID			DD		
		n	OR 95 % CI	p	n	OR 95 % CI	p	n	OR 95 % CI	p
Treatment /Лечение	80	34			38			8		
Surgery/Операция	80	34	1	–	38	1	–	8	1	–
Surgery + Radiotherapy/ Операция + лучевая терапия	11	4	0.86 0.28–2.71	0.96	5	0.96 0.34–2.72	0.94	2	1.81 0.35–8.33	0.47
Surgery, Radiotherapy + Chemotherapy/ Операция, лучевая терапия + химиотерапия	3	1	0.78 0.05–5.42	0.84	1	0.7 0.05–4.85	0.76	1	3.33 0.23–24.27	0.29
Ethnicity/ Этническая принадлежность	80	34			38			8		
Azerbaijanis/ Азербайджанцы	43	16	–	–	21	–	–	6	–	–
Persians/Персы	37	18	1.31 0.6–2.84	0.51	17	0.94 0.42–2.05	0.88	2	0.39 0.08–1.69	0.25

28.3 %, p=0.1). Conversely, there were no significant differences regarding characteristics between patients and controls in a Chinese population who participated in glioma research [38]. In another study on the Indian population, the ACE DD genotype was highly presented in glioma cases (26.8 %) compared to the controls (10.6 %) (p<0.0001), and a 5-fold risk of tendency was observed in glioma cases. Accordingly, the presence of the D allele was higher in glioma cases than in controls (54 and 25 %, respectively, p<0.0001) [39].

According to population-based case-control research, the ACE DD genotype may have a significant role in the substantial correlation between this polymorphism and the risk of developing glioma in the Algerian population. The main result specified that the recessive model was connected to a 72 % reduced risk of glioma (p<0.001). A significant difference was observed in the family history of cancer (p<0.001) between the patients and controls in this study [40]. In comparison, our study showed a slightly significant difference in mentioned characteristics (p=0.03).

Li et al. found that the ID genotype was linked to a higher frequency of glioma than the DD and II genotypes [41]. Different types of other cancers have been studied frequently to indicate the association between ACE (I/D) genotype and cancer of a specific tissue or organ as discussed by Lian et al. [38].

Lastly, it is noteworthy that the current study is the first in the Middle East to investigate the association between ACE I/D polymorphism and glioblastoma, with regard to the prospective utilization of the RAS components as biomarkers or therapeutic targets in glioblastoma. It should be mentioned that this case-control study only considered the Azerbaijani and Persian ethnicities of Iran, therefore it cannot be claimed to be completely representative of the population of the entire country.

Conclusion

To conclude, our results suggested that the genotype of DD may confer a protective effect on glioblastoma and might be considered in the genetic risk assessment of glioblastoma.

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