

Angiotensin-converting enzyme gene insertion/deletion (I/D) polymorphism in Azerbaijan population

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Angiotensin-converting enzyme (ACE) is a key enzyme of the renin-angiotensin-aldosterone system (RAAS), which is directly involved in the regulation of blood pressure. It is assumed that the insertion/deletion (I/D) polymorphism of the gene of this enzyme (ACE gene) appears due to the presence/absence of ~ 287 bp Alu repeats in the 16th intron and is associated with the risk of the development of some diseases, including cardiovascular diseases, various kinds of mental disorders, Alzheimer's disease, gestational diabetes, etc. Given the lack of data on ACE gene I/D polymorphism for the Azerbaijan population, we studied polymorphism of this gene by PCR, using sequence specific pairs of primers (Hace3s and Hace3as (I), ACE-F and ACE-R (II)). DNA samples isolated from 346 individuals were divided into 4 groups: (1) patients with various mental disorders (90 patients); (2) a group of young students involved in various sports (84 male persons); (3) patients with diabetes (28 patients with I type DM (3A subgroup) and 72 patients with II type DM (3B subgroup)); (4) a group of conditionally healthy people of different ages and specialties (72 persons, control). Based on the results of PCR of both primer pairs, the following genotypes were obtained: 16 individuals with genotype II (4.6%, homozygous co-dominants for the I-allele), 101 individuals with genotype DD (29.2%, homozygous co-dominants for the D-allele) and 228 individuals with genotype ID (66.2%, heterozygotes for both alleles). The frequency of occurrence was: $f_I=0.373$, $f_D=0.627$, $N_D:N_I=1.681$. The ratio of separate genotypes within the studied population: ID:DD=2.173; ID:II=14.125; DD:II=6.500. Comparison of the values of the dominant model for the allele D - (DD+ID)/II=20.625 and the recessive model DD/(ID+II)=0.430 relative to the dominant model for the allele I - (II+ID)/DD=1.152 and the recessive model II/(ID+DD)=0.048 indicates that in both models the probability of the D allele to associate with any particular trait is higher than that of the I allele (17.904 and 8.958 times, respectively). These results confirm the literature data on the association of the D allele with many pathologies or diseases. The analysis of the obtained data also revealed a significant correlation ($p \leq 0.01$) of the studied features from the D allele both within groups and between groups.

Keywords: Renin-angiotensin-aldosterone system (RAAS), angiotensin-converting enzyme (ACE), insertion, deletion, hypertension, psychiatric disorders, diabetes mellitus (DM), polymorphism, co-dominant, homozygote, heterozygote, COVID-19

INTRODUCTION

The renin-angiotensin-aldosterone system (RAAS) which include renin inhibitors, angiotensin-converting enzyme (ACE) inhibitors, angiotensin II type 1 receptor antagonists, and mineralocorticoid receptor antagonists, is known as a regulator

of hypertension and fluid as well as electrolyte homeostasis. ACE is a key enzyme in the RAAS that is converting angiotensin I to the vasoactive peptide angiotensin II. Renin (EC 3.4.99.19), the enzyme that catalyzes the proteolytic conversion of angiotensinogen to the decapeptide angiotensin I (Khakoo et al., 2008) Angiotensinogen, a large

globular protein that serves as the substrate for renin (Malikova et al., 2016). Angiotensin II and the angiotensin II receptor, control the transduction of the cellular effects of angiotensin II (Forrester et al., 2018). Binding of the angiotensin II to its receptor mediates vasoconstriction, aldosterone and catecholamine release, as well as fluid consumption, secretion of prolactin, adrenocorticotrophic hormone, and glycogenolysis (Lynch et al., 2018).

The four major components of the RAAS (renin, angiotensinogen [angiotensins I and II], angiotensin-converting enzyme, and the AT receptors) are important components of vascular diseases (De Mello et al., 2017; Lynch et al., 2018). ACE was the first discovered in the 1950s by Skeggs and colleagues. This enzyme was able to convert angiotensin I to angiotensin II (called hypertensin I and hypertensin II at that time) in horse plasma. ACE was originally called "converting enzyme". Later Phillips and colleagues identified an enzyme in human blood, designated kininase II, which was able to degrade bradykinin (Phillips et al., 2018). Converting enzyme and kininase II was later found to be the same enzyme and today the enzyme is referred to as angiotensin-converting enzyme. ACE is a dipeptidyl carboxypeptidase that exists in 2 isoforms. Somatic ACE is induced in different tissues and cell types including the cardiovascular system, lungs, kidneys, etc, whereas testicular ACE only can be found in sperm cells. Both isoforms have a hydrophobic trans-membrane domain and a short cytoplasmic fragment (De Mello et al., 2017). The ACE gene is located in chromosome 17 (17q23 region) and contains a polymorphism based on the presence (insertion) or absence (deletion) of a 287 base-pair (bp) fragment on 16th intron of ACE gene. Based on the polymorphism there are three genotypes; ID, II, DD which are classified as I and D alleles which are termed as insertion or deletion, respectively (Turgut et al., 2004).

ACE I/D polymorphism associated to the level of ACE in plasma and with higher risk for cardiovascular diseases. Three ACE genotypes – II, ID, and DD, have different correlation with the percentage of hypertension, myocardial hypertrophy, diabetes mellitus, psychological disorders and

other diseases. The D allele has been connected with an increased risk of developing various pathological processes, such as coronary heart disease and ventricular hypertrophy. While various cardiac disorders appear to have a worse prognosis in individuals homozygous for the D allele, the I allele has been associated with increased endurance performance in athletes (Goessler et al., 2016). Polymorphisms of the ACE gene was found to be involved in bipolar disorders as well as in schizophrenia (Song et al., 2015). Bipolar disorders are severe early-onset diseases that comprise psychiatric conditions characterized by recurrent mood changes from depression to mania. Their prevalence is high, possibly as much as 5%, in the US population (Dal Mas et al., 2019). The presence of the I/D genotype of the ACE gene contribute to an increase of ACE plasma activity, which could be a predictor in schizophrenia diagnosis (Dal Mas et al., 2019; Gadelha et al., 2015).

The results of the polymorphism of the ACE gene in American 45 male football athletes vs 72 non-athletes showed a greater frequency of the D allele in athletes with comparison in non-athletes (Santoro et al., 2019). Moreover, a significant difference ($p \leq 0.05$) in the genotypic distribution of the athletes was composed of a higher number of the DD genotype as compared to the control group. However, the association of the I/D polymorphism of the ACE in sports abilities have been debated.

ACE gene I/D polymorphism is also associated with the development of diabetes mellitus (basically type II DM), gestational diabetes and various comorbidities in different world populations (Purnamasari et al., 2012; Pan et al., 2016; Mir-Feizi et al., 2018; Pirozzi et al., 2018; Shen et al., 2019).

The new coronavirus COVID-19 (or Severe Acute Respiratory Syndrome SARS-CoV-2) which activated by transmembrane proteins (for example by transmembrane protease serine 2 (TMPRSS2)) mainly penetrates into the cells (endocytosis) through a receptors by binding to membran ACE-2 widely expressed in cardiac cells including endothelial cells, smooth muscle cells in the myocardial vasculature and in cardiac myocytes (Fig. 1, for detailed see: Abbasi et al., 2020).

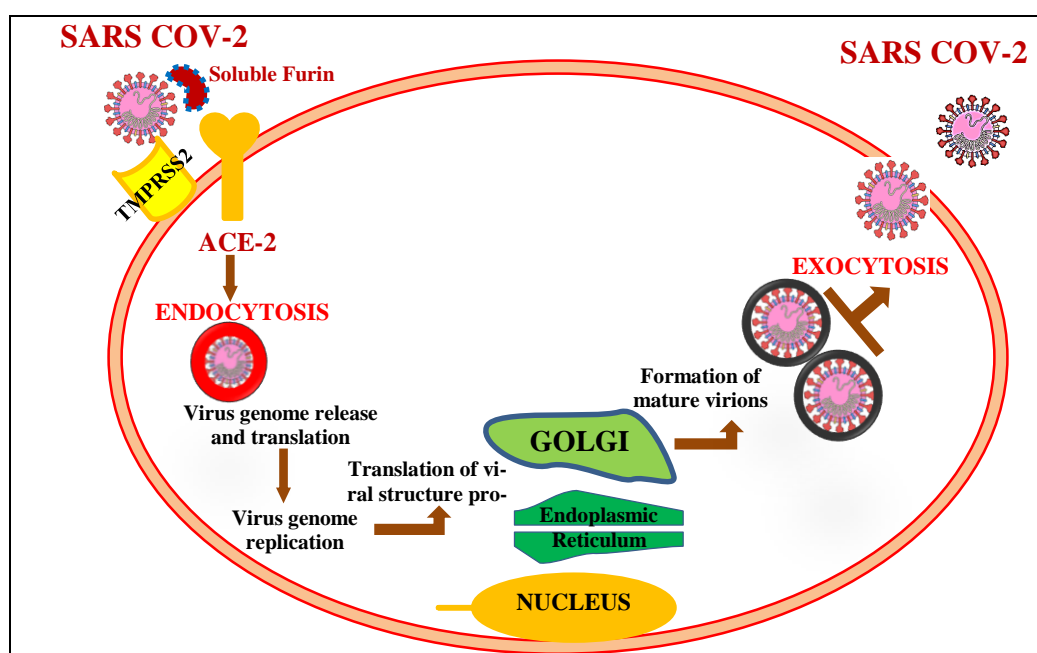


Fig. 1. The simplified scheme of the processes at the invasion of SARS-CoV-2.

Almost all cells, particularly cells of the epithelium and diaphragm of the lungs, which are more sensitive and accessible to the penetration of coronavirus, contain many of such receptors belonging to various forms and classes of ACE. In the way to produce an approaches for the treatment and prevention of infected by coronavirus patients, it is required to know all possible mechanisms of endocytosis that are directly or indirectly associated with both ACE receptor and ACE gene polymorphism (Das et al., 2020).

Coronavirus infection has a particularly high risk of fatalities with co-presence a number of autoimmune and chronic diseases (diabetes mellitus (I and II types), hypertension, chronic obstructive pulmonary disease, cardiovascular disease, cancer, etc.) as well as in seasonal respiratory diseases (influenza, inflammation, etc.) (Abbasi et al., 2020; Bosso et al., 2020; Brojakowska et al., 2020; Cure E., Cumhuri Cure M., 2020; Devaux et al., 2020; Guo et al., 2020; Hussain et al., 2020; Li et al., 2020; Lippi et al., 2020; Othman et al., 2020; Öztürk et al., 2020; South et al., 2020; Yamamoto et al., 2020; Zhang et al., 2020). The investigation of I/D polymorphism of ACE gene may help to understand molecular mechanisms of coronavirus infection. However, I/D polymorphism of ACE gene in the Azerbaijan population has not been

studied yet. Therefore, in this work, we study the ACE gene I/D polymorphism in the Azerbaijan population with various primers to clarify the association of these polymorphism with different diseases.

MATERIALS AND METHODS

Population Studied. The fresh blood samples were collected on a voluntary basis from 346 citizens of the republic in different ages and with different professional activities. The studied population sample include following groups:

- (1) 84 male athletes engaged in various sports;
- (2) 90 mental patients with various diagnoses (24 female, 66 male);
- (3) 100 patients with diabetes mellitus (28 patients I type DM (11 male and 17 female); 72 patients II type DM (21 male and 51 female));
- (4) 72 conditionally healthy individuals (control group, 42 female, 30 male).

DNA isolation procedures. DNA from 200 µl blood samples was isolated using “Diatom™ DNA Prep 200” kit (Izogen, Russian Federation) on manufacturer protocols. DNA samples were stored at -80°C. The concentrations and purity of the DNA samples were determined spectrophotometrically in Epoch™ Microplate Spectrophotometer (BioTek,

Aglient, USA) using Gene5 software. DNA samples were diluted individually before PCR.

Detection of I/D Polymorphism of ACE gene. ACE polymorphism on 16th intron was determined by polymerase chain reaction (PCR) using two pairs of specific primers: Hace3s (5'-GCCCTGCAGGTGTCTGCAGCATGT-3') and Hace3as (5'-GGATGGCTCTCCCCGCCTTGTC TC-3') (Castellano et al., 1995), ACE-F (5'-CTG-GAGACCACTCCCATCCTTTCT-3') and ACE-R (5'-GATGTGGCCATCACATTCGTCA GAT-3') (Rigat et al., 1992).

The obtained DNA fragments were electrophoresed in a 1.5% agarose gel and visualized by ethidium bromide staining. The sizes of fragments were estimated by comparison with previously known molecular weight markers M100. The polymorphism detected by PCR was evident as a 490-bp and 597 bp fragments in the presence of the insertion (I allele) and as a 190-bp and 319 bp product in the absence of the insertion (D allele). Each sample found to have the D/D genotype was subjected to a second PCR amplification with insertion-specific primers (5a: 5'-TGGGAC-CACAGCGCCCGCCACTAC-3' and 5c: 5'-TCGCCAGCCCTCCCATGCCCA TAA-3') in order to avoid D/D mistyping (Shanmugam et al., 1993).

RESULTS AND DISCUSSION

Primers Hace3s and Hace3as (Figure 2) revealed an insertion-specific 597 bp fragment in 16 samples, deletion-specific 319 bp fragment in 104 samples and insertion-deletion fragments of both types in 226 samples. Similar results were observed with the primer pair ACE-F and ACE-R (Figure 3), which yielded the corresponding 190 (with deletion) and 490 (with insertion) b.p. fragments.

Interestingly, the primer pair ACE-5a and ACE-5c, which synthesizes a specific 335 bp fragment, gave a positive result in only one case. Thus, we show that the allelic forms of the ACE gene for the Azerbaijan population of 346 individuals, consists of 66.2% of the ID, 29.2% of the DD and 4.6% carriers of the II genotype. Genotypes revealed among studied groups shown in Table 1.

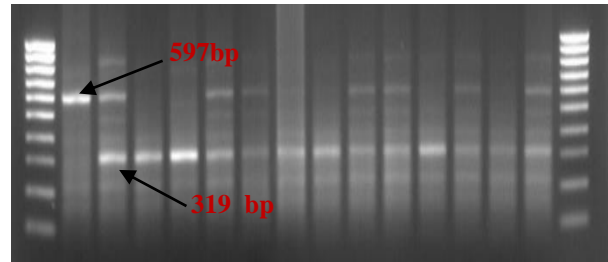


Fig 2. The amplification results with the Hace3s and Hace3as primers

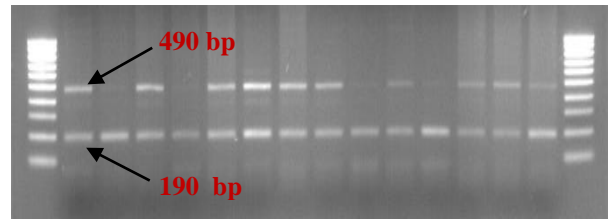


Fig 3. The amplification results with the ACE-F and ACE-R primers

Groups, gender (M – male; F - female)		Genotypes			
		II	ID	DD	
1	M	6	45	15	
	F	3	18	3	
	Total:	9	63	18	
2	M	-	63	21	
	F	-	-	-	
	Total:	0	63	21	
3	I type DM (3A)	M	-	9	2
		F	1	9	7
	II type DM (3B)	M	-	12	9
		F	-	33	18
	Total:	1	63	36	
4	M	-	15	12	
	F	6	21	18	
	Total:	6	36	30	
Total on studied population	M	6	144	59	
	F	10	81	46	
		16	225	105	

Note: – indicates the absence of given genotype.

ID heterozygous can be observed incorrectly by the PCR method (Shanmugam et al., 1993). Thus, the initial amplification of the shorter D allele during the studies leads to incorrect classification of approximately 4–5% of ID genotypes as DD. An additional PCR-based amplification reaction developed to confirm all DD genotypes obtained from the initial standart PCR to prevent any ID genotype be-

ing misidentified. This confirmatory results obtained in the classification of ACE polymorphism by the insertion-specific PCR method. The combined use of standard and confirmatory PCR methods used in numerous studies investigating the association of DD genotype with the diseases. Based on the numerous literature data (Gatt et al., 2015, Mengesha et al., 2019; Shen et al., 2019; Zhang et al., 2019) on the meta-analysis of the ACE gene polymorphism, the models in which the alleles D and I are dominant or recessive can be observed. The dominant model of the D allele $(DD+ID)/II=20.625$, the recessive model of the D allele $DD/(ID+II)=0.430$; The dominant model of the I allele $(II+DD)/DD=1.152$, the recessive model of the I allele $II/(ID+DD)=0.048$ (table 2). Apparently, the dominant and recessive association of the allele D is greater than that of the allele I (17.904 and 8.958 times, respectively). The results are in agreement with the above literature.

Table 2. The dominant and recessive models revealed alleles on separate groups and at the population level

Groups	Models			
	Dominant		Recessive	
	D	I	D	I
1	8.0	1,5	0.250	0.125
2	-	1	0.333	0
3	99.0	1.029	0.538	0.010
4	11.0	1.2	0.714	0.091
Total at the population level	20.625	1.152	0.430	0.048

There are many association studies showing influence of ACE I/D polymorphism on the onset of diabetes mellitus (Al-Saikhhan et al., 2017, Pirozzi et al., 2018, Mirfeizi et al., 2018, Aggarwal et al., 2016, Ohkuma et al., 2019). However, recent findings do not support this statement. For instance, several studies done on Japanese and Caucasian population indicate the association of the DD genotype with risk of type 2 diabetes while in Gujarati population (India) this association was not found (Doshi et al., 2015). This result was confirmed in other studies in different ethnic groups, both in patients with and without nephropathy (Arfa et al., 2008, Eroglu et al., 2008, Van-Valken-god et al., 2008). Thus, the usage of ACE polymorphism as an independent factor responsible for diabetes is questionable. However, we can not rule

out the other effective genetic factors and environmental factors on the possible role of ACE in the onset of diabetes (Cassis et al., 2019).

Based on the frequency distribution of ACE genotypes showed that ID and II genotypes are frequent in high performance endurance athletes while DD genotype is mainly found among low and middle performance endurance athletes (Hadi et al., 2019). It has been observed that I allele with phenotypes related more to strength than to endurance in 1,027 teenagers (Moran et al. 2006). It suggests a more complicated role for the ACE gene in human physical performance than previously described. Thus, a modest influence of ACE gene on physical performance is clear in general population. Now, the challenge is to clarify the mechanism of ACE influence on performance related phenotypes.

CONCLUSION

We investigated the insertion/deletion (I/D) polymorphism of the angiotensin-converting enzyme (ACE) gene in the Azerbaijani population among 346 people referring to different age groups, non-relatives and different activities. Based on the results of the PCR method the recessive and dominant model of both allele was performed. According to the models the D allele is highly associated with the any particular trait than that of the I allele. This finding suggests that the ID polymorphisms of the ACE gene may play an important role susceptibility to schizophrenia in Azerbaijan population. Moreover, the results obtained from the athletes also revealed high susceptibility of ID or DD polymorphism of ACE gene to different sport performances. Additionally, the role of ACE gene polymorphism in the risk of diabetes was researched and ID genotype was mainly found among patients with type 1 and 2 diabetes. However, these findings require repetition in larger samples. Since the features and localization of functional polymorphisms of the ACE gene have not yet been fully understood, our study confirmed that most of them are closely associated with I/D polymorphism. Thus, the physiological significance of ACE polymorphism should be understood as its relationship with the levels of ACE expression in plasma.

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Azərbaycan populyasiyasında angiotensin çevirən fermentin (AÇF) geninin insersiya/delesiya (I/D) polimorfizmi

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Angiotenzin-çevirən ferment renin-angiotenzin-aldosteron sisteminin (RAAS) açar fermentlərindən olub qan təzyiqinin tənzimlənməsində bilavasitə iştirak edir. Güman edilir ki, bu fermenti kodlaşdıran genin (ACE geni) 16-cı intronunda uzunluğu ~287 n.c. olan Alu təkrarlarının olması/olmaması ilə şərtlənən insersiya/delesiya (I/D) polimorfizmi bəzi xəstəliklərin yaranma riski ilə, o cümlədən ürək-damar xəstəlikləri, müxtəlif tip psixi pozuntular, Alzheimer xəstəliyi, hestasion şəkərli diabetlə və s. assosiasiya təşkil edir. Azərbaycan populyasiyası üçün ACE geninin I/D polimorfizmi üzrə məlumatların olmadığını nəzərə alaraq tərəfimizdən bu genin polimorfizmi spesifik praymer cütlərindən (Hace3s və Hace3as (I), ACE-F və ACE-R (II)) istifadə etməklə PZR metodu ilə tədqiq edilmişdir. Tədqiqatın obyektini 4 qrupa bölünmüş 346 nəfərin qanından ayrılan DNT nümunələri olmuşdur: (1) müxtəlif psixi pozuntuları olan xəstələr (90 nəfər); (2) idmanın müxtəlif növləri ilə məşğul olan gənc idmançılar (84 nəfər tələbə); (3) müxtəlif tipli şəkərli diabetli xəstələr (28 nəfər I tip ŞD (3A subqrupu) və 72 nəfər II tip ŞD (3B subqrupu)); (4) müxtəlif yaşlara və ixtisaslara malik şərti sağlam insanlar (72 nəfər, nəzarət qrupu). Hər iki praymer cütü ilə II genotipli 16 nəfər (4.6%, I-allelə görə homozigot ko-dominantlar), DD genotipli 101 nəfər (29.2%, D-allelə görə homozigot ko-dominantlar) və ID genotipli 228 nəfər (66.2%, hər iki allelə görə heterozigotlar) aşkar edilmişdir. Rastgəlmə tezlikləri: $f_I=0,373$, $f_D=0,627$, $N_D:N_I=1,681$. Populyasiya daxilində ayrı-ayrı genotiplərin nisbətləri: ID:DD=2,173; ID:II=11,125; DD:II=6,500. D-allelinə görə dominant modelin (DD+ID)/II=20,625 və resessiv modelin DD/(ID+II)=0,430 qiymətlərinin I allelinə görə dominant (II+ID)/DD=1,152 və resessiv II/(ID+DD)=0,048 modellərin qiymətləri müqayisəsi onu göstərir ki, həm dominant, həm də resessiv model üzrə D allelinin hər hansı bir müəyyən əlamətlə (genetik) əlaqəsi ehtimalı I allelinə nisbətən daha yüksəkdir (uyğun olaraq 17.904 və 8.958 dəfə). Bu nəticələr bir çox patologiyalar və ya xəstəliklərlə məhz D allelinin assosiasiya təşkil etməsi haqqında ədəbiyyat məlumatlarını təsdiq edir. Alınan nəticələrin analizi həm qruplar daxilində, həm də qruplar arasında tədqiq olunan əlamətlərin D allelindən asılılığının statistik etibarlı şəkildə ($P \leq 0.01$) korrelyasiya etdiyini aşkar etmişdir.

Açar sözlər: *Renin-angiotensin-aldosteron sistemi (RAAS), angiotenzin-çevirən ferment (AÇE), insersiya, delesiya, hipertenziya, psixi pozuntular, şəkərli diabet (ŞD), polimorfizm, ko-dominant, homozigot, heterozigot, COVID-19*

Инсерционный/делеционный (I/D) полиморфизм гена ангиотензин-превращающего фермента (АПФ) в Азербайджанской популяции

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Ангиотензин-превращающий фермент (АПФ) является ключевым ферментом ренин-ангиотензин-альдостероновой системы (РААС), которая непосредственно участвует в регуляции артериального давления. Предполагается, что инсерционный/делеционный (I/D) полиморфизм гена этого фермента (гена ACE), благодаря наличия/отсутствия в 16-м интроне Alu-повторов длиной ~287 п.н., ассоциирован с риском развития некоторых заболеваний, включая сердечно-сосудистые заболевания, различные виды психических расстройств, болезнь Альцгеймера, гестационный диабет и др. Учитывая отсутствие данных по I/D-полиморфизму гена ACE для Азербайджанской популяции мы

изучали полиморфизм этого гена методом ПЦР с использованием конкретных пар праймеров (Насе3s и Насе3as (I), ACE-F и ACE-R (II)). Объектом исследования служили образцы ДНК, выделенные у 346 человек, разделенных на 4 группы: (1) пациенты с различными психическими расстройствами (90 пациентов); (2) группа юных студентов, занимающихся различными видами спорта (84 человека мужского пола); (3) больные сахарным диабетом (28 пациентов с СД I типа (подгруппа 3А) и 72 пациента с СД II типа (подгруппа 3В)); (4) группа условно здоровых людей разного возраста и специальностей (72 человека, контроль). Обе пары праймеров идентифицировали 16 лиц с генотипом II (4,6%, гомозиготные ко-доминанты по I-аллелю), 101 человек с генотипом DD (29,2%, гомозиготные ко-доминанты по аллелю D) и 228 лиц с генотипом ID (66,2%, гетерозиготы по обоим аллелям). Частота встречаемости составила: $f_I=0,373$, $f_D=0,627$, $N_D:N_I=1,681$. Соотношение отдельных генотипов в исследуемой популяции: ID:DD=2,173; ID:II=14,125; DD:II=6.500. Сравнение значений доминантной модели для аллеля D - $(DD+ID)/II=20,625$ и рецессивной модели $DD/(ID+II)=0,430$ относительно доминантной модели для аллеля I - $(II+ID)/DD=1,152$ и рецессивной модели для аллеля I - $II/(ID+DD)=0,048$ указывает на то, что как в доминантной, так и в рецессивной моделях вероятность ассоциации аллеля D с конкретным признаком выше, чем у аллеля I (в 17,904 и 8,958 раза соответственно). Эти результаты подтверждают литературные данные об ассоциации именно аллеля D со многими патологиями или заболеваниями. Анализ полученных данных также выявил достоверную корреляцию ($P \leq 0,01$) исследуемых признаков аллеля D как внутри групп, так и между группами.

Ключевые слова: Ренин-ангиотензин-альдостероновая система (РААС), ангиотензин-превращающий фермент (АПФ); инсерция, делеция; гипертония, психические расстройства, сахарный диабет (СД), полиморфизм, кодоминантный, гомозиготный, гетерозиготный, COVID-19