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COMBINATION OF TWO DIFFERENT C174T AND C235T OF THE ANGIOTENSINOGEN GENE MUTATION AND C677T METHYLENETETRAHYDROFOLATE REDUCTASE GENE MUTATION IN PATIENTS WITH CVD IN THE POPULATION OF AZERBAIJAN

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Abstract. We studied two mutations of C174T (Met174Tre) and C235T (Met235Tre) of the angiotensinogen gene and mutation C677T (Ala677Val) of the methylenetetrahydrofolate reductase gene, as well as their combinations among patients with cardiovascular diseases: coronary heart disease, myocardial infarction and hypertension, using a complex of modern molecular genetic diagnostic methods. A high frequency of these mutations has been established in the group of patients with severe forms of cardiovascular disorders. Homozygous and compound conditions were found in people with severe diseases. In particular, the heterozygous state of the mutation was found in people with moderate hypertension. It was also found that the presence of close blood relationship between the parents of patients with cardiovascular diseases increases the homozygotization of mutations of the genes AGT and MTGFR in probands. Consequently, such persons are susceptible to the disease or in other words, have an increased risk of developing diseases of the cardiovascular system. For the first time, the frequencies of these mutations in the Azerbaijani population have been determined, which are mostly consistent with the frequencies described in other populations of the world. The studied genetic markers are of interest as genes presumably associated with a wide range of cardiovascular diseases and can be used in further population and epidemiological studies.
Therefore, the results of molecular genetic studies obtained by us in people with diseases of the cardiovascular system are of great practical importance. Timely prevention by detecting mutations of C174T, C235T of the AGT gene and C677T of the MTGFR gene in patients will allow doctors to carry out qualified treatment of cardiovascular diseases

Keywords: *Genetic polymorphism, gene frequency, phenotypic frequency, genotype, angiotensinogen, methylthetrahydrofolate reductase, polymerase chain reaction, cardiovascular diseases.*

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Abbreviations:

AGT – angiotensinogen
AGTR1 – angiotensin II receptor type 1
ADRB2 – β-2adrenergic receptor
MTGFR - methylenetetrahydrofolate reductase

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1. Introduction

Genetic studies among the population of many countries of the world have shown that every inhabitant of the Earth is heterozygote for four or five mutations, in other words, each practically healthy person is a carrier of four or five recessive genes, each of which has clinical manifestations in a homozygous state.

It is quite common to find a combination of different mutations of two or more genes. As an example, we can show a combination of alpha and beta thalassemia, the genes of which are localized on chromosome 8 and 16, respectively. There is also an example of a simultaneous combination of three genes: the alpha-thalassemia gene, the beta-thalassemia gene and the sickle cell gene. Baigisheva et al. (2019) described clinical and laboratory manifestations of a combination of hemaglobinopathies of sickle cell anemia and alpha-thalassemia, which is most common among Azerbaijanis living in Dagestan. Gene polymorphisms in arterial hypertension (renin-angiotensin-aldosterone system) were studied in different countries (Shakhanova *et al.,* 2018)

In our research, we identified a combination of two different AGT gene mutations with each other and with one MTHFR gene mutation.

The angiotensinogen (AGT) gene is localized in the long arm of the first chromosome at the 1q42-q43 locus and contains five exons 12 kBp long, while its transcript (RNA) is about 1 kBp. The primary protein product of the gene contains 452 amino acids. It is known that this gene is expressed mainly in the liver and is under the control of estrogens, glucocorticoids, thyroid hormones and angiotensin II. It is also synthesized in the brain, large arteries, kidneys and adipose tissue. In the AGT gene, Met-Thr is replaced at position 235 (M235T) and Thr-Met – at position 174 (T174M) (Chang *et al.*, 2010).

It should also be noted that in mutation carriers: when replacing the cytosine (C) nucleotide with the thymine (T) nucleotide in the 677 position of the MTHFR C677T gene (MTHFR C677T), there is a decrease in enzyme activity to about 35% from the average value, resulting in adverse effects such as DNA methylation deficiency. This mutation leads to resistance of the V factor to the activated protein C due to the binding of homocysteine to the activated V factor. This means that this polymorphism can cause all clinical manifestations of the Leiden mutation. Individuals who inherit this variant of the genotype from both parents are significantly more susceptible (by 14-21%) to diseases of the cardiovascular system (Mitkovskaya *et al.*, 2018). Polymorphic variants of the genes for angiotensin converting enzyme, angiotensinogen and the type 1 receptor gene for angiotensin II were studied as genetic predictors of the development of arterial hypertension (Elkina *et al.*, 2021).

The authors Gardier et al. (2004), Kuznetsova et al. (2004) when searching for associations between C174T and C235T polymorphisms and elevated angiotensinogen levels in blood plasma in patients with hypertension, coronary heart disease, found that individuals with the presence of these alleles and their combinations have significantly increased angiotensinogen levels in blood plasma, besides the women with this genotype has a high risk of developing hypertension.

Silvestrova et al. (2008) for the first time characterized polymorphisms C174T and C235T of the AGT gene in practically healthy Russian men living in the Central region of Russia (Moscow and Podmoskovye). Also, the author, in the study of these polymorphisms, established their relationship with arterial hypertension in Russian men in the Central region of Russia.

Stetskaya et al. (2014) and others studied the association of the T174M polymorphism of the angiotensinogen gene with an increased risk of cerebral stroke in women. Also, the genetic polymorphism of renin-angiotensin-aldosterone system in type 2 diabetes and in combination with arterial hypertension was studied (Saidov *et al.*, 2019). Also, association of angiotensinogen and angiotensin II receptor type I polymorphisms with biomarkers of carbohydrate and lipid metabolism in Dagestan residents with type 2 diabetes and hypertension were studied (Saidov *et al.*, 2021).

Polymorphisms of the AGT, AGTR1 and ADRB2 genes in essential hypertension have also been studied. Their association has been established among the Dagestan population (Saidov *et al.*, 2017). Mulerova et al. (2017) found out the differences in associative relationship of the genotypes of AGT gene with the risk factors of arterial hypertension depending on ethnicity in the inhabitants of Gornaya Shoria.

2. Material and methods

The venous blood of 180 people was used in our study. Of these, 108 were completely healthy, 72 had hypertension of varying severity. In researches, the genomic DNA is isolated from venous blood using ready-made QIAamp genomic DNA and RNA kits (QIAGEN, Germany). Polymerase chain reaction (PCR) is carried out at 95 ^{0} C – 2 min., (95 ^{0} C-30 ^{I}, 58 ^{0} C-30 ^{I}, 78 ^{0} C-2 min. 25-30 cycles), 72 ^{0} C-10 min. and pause at 4^{0} C min. on the amplifier – Professional Thermocycler (Biometra, Germany). The reaction mixture for the polymerase chain reaction consists of: H_2O – 30 mcl, Buffer + Mg_2Cl – 8.2 mcl, 2.5 mcl from each primer (Forward and Reverse), dNTP – 1.25 mcl, Taq polymerase enzyme - 0.63 mcl and DNA - 5 mcl. The following structures of synthetic oligonucleotide primers will be used in the experiments:

Sequence- AGT F1 5^{I}-TGC TTC TGT GTT TTC CCC AGT-3^{I}
Sequence- AGT R1 5^{I}-AGA GAC AAG ACC GAG AAG GAG C-3^{I}
Sequence- AGT F2 5^{I}-GGG CTA AAT GGT GAC AGG GA-3^{I}
Sequence- AGT R2 5^{I}-CCA GAG CCA GCA GAG AGG TTT-3^{I}
Sequence- AGT F3 5^{I}-CCT CAT TCC TGC CCC TGT CT-3^{I}
Sequence- AGT R3 5^{I}-GCT CAG GTG TGT CTA CTC CCC A-3^{I}
Sequence- AGT F4 5^{I}-AGC ACA GAG GTC CTG AGC C-3^{I}
Sequence- AGT R4 5^{I}-CCA AAG TCC AGG AAA GCA C-3^{I}

The integrity and amount of isolated genomic DNA, as well as amplicon, i.e. a gene fragment after PCR, is determined by electrophoresis on a 1.7% agarose gel using an electrophoretic apparatus and a power source (Power Pac Basic Gel Doc ^{IM} EZ - Imager, Bio Rad and USA). In electrophoresis, DNA Ladder 100 bp is used as a marker for identification of synthesized DNA fragments. According to modern methods, DNA fragments after two subsequent PCR are purified by using reagents: *Agencourt AMPure XP and SPRI CleanSEQ, Magnetic BEARDS, respectively.* In this case, the nucleotide sequence of each of the four fragments of the AGT gene is determined by sequencing on the GenomeLab CEQ and GeXP (Genetic Analysis Systems) device from Beckman Coulter, USA (Baigisheva *et al*., 2019; Gu *et al.*, 2008).

3. Results and discussion

In thirteen cases, we were able to identify a combination of AGT and MTHFR gene mutations. In seven cases, a combination of the C174T (Met174Tre) of the AGT gene

mutation with the C677T (Ala677Val) of the MTHFR gene mutation, with the genotype C174T AGT/C677T of the MTHFR, was observed. In six cases, a combination of C235T of the AGT gene mutation (Met235Tre) and MTHFR with the genotype C235T of the AGT/C677T of the MTHFR.

Also, in six cases, the compound state of two mutations of the same AGT gene with the C174T (Met174Tre) /C235T (Met235Tre) genotype was identified.

Table 1 shows the results of the identified combined genotypes among the patients with hypertension.

Among patients with mild hypertension, there was no homozygous and compound state of mutations. Homozygous mutations were also not observed in patients with moderate hypertension. All identified homozygotes and compounds were identified in the group with severe hypertension.

Table 1. Results of screening of combined genotypes among the patients with hypertension

Clinical course of the disease	Combination of mutations							
	174/174		174/677		235/677		174/235	
	abs.	%	abs.	%	abs.	%	abs.	%
Soft form, n=18	–	–	–	–	–	–	–	–
Moderate form, n=30	–	–	–	–	–	–	–	–
Severe form, n=24	4	16.67	7	29.17	6	25.0	6	25.0
P_1	0.095		0.013		0.026		0.026	
P_2	0.034		0.002		0.005		0.005	

Note: P_1, P_2 – the reliability of the difference between the relatively mild and moderate forms of hypertension, respectively

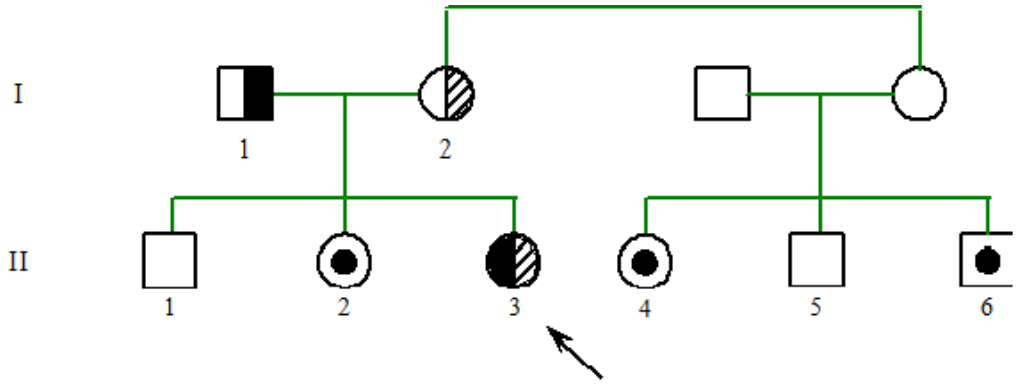

Figure 1. A combination of two C174T (Met174Tre) of the AGT gene mutation and C677T (Ala677Val) of the MTHFR gene mutation, with the genotype C174T of the AGT/C677T of the MTHFR in proband B.N. Proband – II-3, parents of proband – I-1 and I-2, siblings of proband – II-1, II-2, cousins of proband – II-4, II-5 and II-6, parents of german cousins – I-3 and I-4

Figure 1 shows the pedigree of a proband with a double heterozygous state for two different mutations.

Figure 2 shows the pedigree of a proband having a combination of C235T

(Met174Tre) of the AGT gene mutation with C677T (Ala677Val) of the MTHFR gene mutation. In this case, the proband had the genotype: C235T AGT/C677T MTHFR.

As can be seen from Figure 1, two different mutations were identified in 48-year-old proband B.N. (II-3): a combination of C174T (Met174Tre) of the AGT gene mutation with the C677T (Ala677Val) of the MTHFR gene mutation, each of which individually has its own clinical manifestations. Proband's parents were not consanguineously related and came from different regions of the Republic. Proband had hypertension from an early age. By the age of 36, he suffered a myocardial infarction.

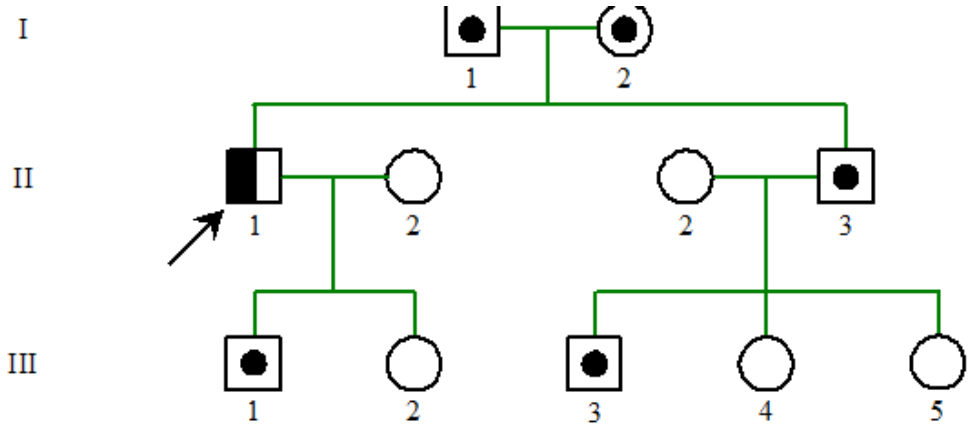

Figure 2. A combination of two C235T (Met235Tre) of the AGT gene mutation and C677T (Ala677Val) of the MTHFR gene mutation with the genotype C235T of the AGT/ C677T of the MTHFR in proband M.I. Proband G.M. (II-1), I-1, I-2 – proband's parents, proband's wife – II-2, proband's brother – II-3, proband's children – III-1 and III-2, proband's brother's children – III-3 III-4 III-5

Clinical and genealogical analysis, considering the anamnestic data, showed that one of the siblings (II-2) and two cousins (II-4, II-6) suffer from hypertension, but none of them suffered a myocardial infarction. It should also be noted that both proband's parents (I-1, I-2) and proband's mother's sister (I-4) suffered from hypertension with various clinical manifestations. No genetic analysis was performed for them to test AGT and MTHFR genes mutations.

In 55-year-old proband G.M. (II-1) diagnosed with coronary heart disease, we identified two different mutations: a combination of the C235T (Met235Tre) of the AGT gene mutation with the C677T (Ala677Val) of the MTHFR gene mutation. The marriage between the proband's parents was an endemic marriage, i.e. both parents came from the same locality.

Proband had hypertension from an early age. By the age of 42, he suffered a myocardial infarction. Clinical and genealogical analysis, considering the anamnestic data, showed that one of the siblings (III-1) and one of the male cousins (III-3) suffer from hypertension, he did not suffer a myocardial infarction. It should also be noted that both proband's parents (I-1, I-2) and proband's father's brother (I-4) suffered from hypertension with various clinical manifestations. No genetic analysis was performed for them to test AGT and MTHFR genes mutations.

The results of genetic screening of C174T mutations of the C174T, C235T alleles of the AGT gene and C677T of the MTHFR gene among patients with cardio-vascular diseases are summarized in Table 2.

In the distribution of C174T, C235T, C677T mutations among the patients with various degrees of severity of hypertension, coronary heart disease and myocardial

infarction, the results are presented in Table 3.

Table 4 shows the phenotypic frequencies of established mutations among the patients with cardiovascular diseases.

Among the group of patients with mild hypertension, all mutations were in a heterozygous state and the total frequency of incidence in all patients was 20.82% (AGT mutations – 12.49%; MTHFR mutations – 8.33%). Within the group, the mutation rate was 83.33%

In patients with moderate hypertension, a heterozygous state of mutations was also observed.

Table 2. Phenotypic, genotypic and gene frequencies of C174T, C235T alleles of the AGT gene and C677T of the MTHFR gene among the patients with cardiovascular diseases

	Frequency of the phenotype		Frequency of the genotype (in unit fractions)	Frequency of alleles (in unit fractions)	
	abs.	%			
C174T of the AGT (Met174Tre) mutation					
T/T	3	4.17	0.0417	T	0.1875
C/T	21	29.17	0.2917	C	0.8125
C/C	48	66.67	0.6667		
C235T of the AGT (Met235Tre) mutation					
T/T	9	12.5	0.1250	T	0.25
C/T	18	25.0	0.2500	C	0.75
C/C	45	62.5	0.6250		
C677T of the MTHFR (Ala677Val) mutation					
T/T	–	–	–	T	0.1875
C/T	27	37.5	0.3750	C	0.8125
C/C	45	62.5	0.6250		

Table 3. Number of patients with heterozygous, homozygous and compound genotypes among the patients with cardiovascular diseases

Clinical course of the disease	Genotype of mutation								
	Heterozygote			Homozygote			Compound		
	174/N	235/N	677/N	174/174	235/235	677/677	174/677	235/677	174/235
Soft form, n=18	3	6	6	–	–	–	–	–	–
Moderate form, n=30	6	6	8	–	–	–	–	–	–
Severe form, n=24	12	6	13	3	9	–	7	6	6

Table 4. Phenotypic frequency of mutations among the patients with cardiovascular diseases

Genotype of mutation			Clinical course of the disease				P_1	P_2
			Soft form, n=18	Moderate form, n=30	P_1	Severe form, n=24		
Heterozygote	174/N	abs.	3	6	0.54	12	0.042	<0,05
		%	16.67	20.0		50.0		
	235/N	abs.	2	6	0.35	10	0.043	>0,05
		%	11.11	20.0		41.67		
	677/N	abs.	4	10	0.31	13	0.064	<0,05
		%	22.22	33.33		54.17		
Homozygote	174/174	abs.	0	0	–	3	0.18	0.082
		%	–	–		12.50		
	235/235	abs.	0	1	0.63	8	0.007	0.013
		%	–	3.33		33.33		
	677/677	abs.	0	0	–	0	–	–
		%	–	–		–		
Compound	174/174/ 235/N	abs.	0	0	–	3	0.18	0.082
		%	–	–		12.5		
	174/N/ 677/N	abs.	0	0	–	6	0.026	0.005
		%	–	–		25.0		
	235/235/ 677/N	abs.	0	0	–	6	0.026	0.005
		%	–	–		25.0		
	174/N/ 235/N	abs.	0	0	–	7	0.013	0.002
		%	–	–		29.17		

Note: P_1, P_2 – the reliability of the difference between the relatively mild and moderate forms of hypertension, respectively

In the group of patients with severe hypertension, both heterozygous and compound and homozygous mutations were observed. 24 patients in 31 cases had a heterozygous state (12 with the C174T mutation, 6 cases with the C235T mutation and 13 cases with the C677T mutation), 19 patients had a compound state of mutations, 12 patients had a homozygous state of mutations. Consequently, each of the seriously ill patients had one or another mutation.

Table 5 shows the frequency of alleles in the group of patients with hypertension.

The total frequency of C174T, C235T and C677T alleles in the experimental group, respectively, was: 0,0764-0,1873, 0,1111-0,2498, 0,2292-0,1875 (these are the total frequencies of mutant alleles in all three groups (by severity of the disease), which coincides with the frequency of the mutant allele T in the tables.

Consequently, in the experimental group, the lowest values were obtained for the C174T (C677) allele and the highest – for the C677T allele.

Table 5. Frequency of mutant alleles of the AGT and MTHFR in the group of patients with hypertension

Genotype of mutation		Clinical course of the disease		
		Soft form, n=18	Moderate form, n=30	Severe form, n=24
Heterozygote	174/N	0.1667	0.2000	0.5000
	235/N	0.1111	0.2000	0.4167
	677/N	0.2222	0.3333	0.5417
Homozygote	174/174	0.0000	0.0000	0.1250
	235/235	0.0000	0.0333	0.3333
	677/677	0.0000	0.0000	0.0000
Compound	174/174/235/N	0.0000	0.0000	0.1250
	174/N/677/N	0.0000	0.0000	0.2500
	235/235/677/N	0.0000	0.0000	0.2500
	174/N/235/N	0.0000	0.0000	0.2917

Consequently, the experimental results obtained during the examination of patients with hypertension, coronary heart disease and myocardial infarction are well complied with the results described in the literature.

The analysis of literature also confirms the results obtained by us in the experimental group, in the group of patients with cardiovascular diseases. According to the literature, the analysis of the C235T polymorphism of the angiotensinogen gene showed a strict correlation between the C235T allele and various forms of hypertension, mainly in European populations and in Japanese. At the same time, there was a lack of this association among African-Americans. It has also been shown that the option C235T is an independent risk factor for the development of myocardial infarction and coronary heart disease in Europeans, whereas in Japanese it was found that there was no association of this polymorphism with coronary heart disease (Chang *et al*., 2010).

Consequently, the options C235T and T174M have a certain pathogenetic effect, however these options cannot be considered significant mutations, since their effect is very different among the representatives of different ethnic groups. The effect of these polymorphic variations can be determined by their linkage disequilibrium with some pathogenetic options of the AGT gene or on the other hand, it may manifest itself only against the background of a certain population-specific genetic background. It is also impossible to exclude the high frequency of consanguineous marriages in the studied population.

According to the results of our research, the frequency of consanguineous marriages in the experimental group exceeded the same fact in the control group.

The results of the frequency of consanguineous marriages among parents of probands and persons in the control group are presented in Table 6.

Table 6. The frequency of consanguineous marriages and the inbreeding coefficient in parents of probands and persons in the control group

Examined	Experimental group of 72 people		Control group of 108 people		Difference validity
	abs.	%	abs.	%	
Cousin	6	8.33	4	3.70	>0,05
Twice removed	9	12.50	8	7.41	>0,05
Fourth generation	3	4.17	6	5.56	>0,05
Inbreeding coefficient	F= 0,0195		F= 0,0109		

The frequency of cousin marriages in the experimental group was 2.25 times higher than those in the control group. Twice removed relationship in the experimental group were about two times (1.7) higher. The number of fourth generation relationship in the control group exceeded the values obtained in the experimental group by 1.39%. Consequently, the inbreeding coefficient in the experimental group (F – 0.0195) was significantly higher than those in the control group (F – 0.0109).

We tried to determine the frequency of consanguineous marriages for each group of patients with cardiovascular diseases, to determine the type of consanguineous marriage and also to calculate the inbreeding coefficient. The results are presented in Table 7.

Table 7. Types and frequencies of consanguineous marriages and inbreeding coefficient in various groups of patients with cardiovascular disorders

Examined	Soft form, n=18		Moderate form, n=30		Severe form, n=24		Total n=72	
	abs.	%	abs.	%	abs.	%	abs.	%
Cousin	0	–	3	10.00	3	12.50	6	8.33
Twice removed	2	11.11	3	10.00	4	16.67	9	12.50
Fourth generation	2	11.11	1	3.33	0	–	3	4.17
Total	4	22.22	7	23.33	7	29.17	18	25.0
Coefficient of inbreeding	F=0,0104		F=0,0198		F=0,0260			

As expected, in the group of patients with severe hypertension, the highest rates of cousin and twice removed types of marriages were observed, 12.5% and 16.67%, respectively, with an inbreeding coefficient of F = 0.0260. The lowest values of the above-described frequencies were observed for the group with mild hypertension: twice removed relationship with the same frequency - 11.11% with an inbreeding coefficient of F = 0.0104. The frequency of the inbreeding coefficient in the group of patients with mild hypertension was almost 2.5 times lower than in the group of patients with severe hypertension. The average values of frequencies of cousin, twice removed and fourth generation marriages were obtained in a group of patients with moderate hypertension with an inbreeding coefficient of F – 0.0198.

An analysis of the results presented in Tables 5, 6 and 7 shows that the presence of

a close consanguineous relation between the parents of patients with cardiovascular diseases increases the homozygotization of the AGT and MTGFR genes mutations in probands.

Consequently, such persons are susceptible to the disease, or in other words, they have an increased risk for diseases of the cardiovascular system.

The establishment of an association of a specific mutation of specific gene with a disease and the subsequent assessment of individual genetic risk are significant for the development of a differentiated approach to the prevention and treatment of this pathology and its complications, depending on the hereditary predisposition of a particular patient.

4. Conclusion

By applying a complex of molecular-genetic methods, including polymerase chain reaction, the phenotypic, genotypic and gene frequencies of C174T (Met174Tre), C235T (met235Tre) of the AGT gene mutations and C677T (Ala677Val) of the MTHFR gene mutations were identified and established both in the control group and in the group of patients with cardiovascular diseases.

Detected polymorphisms in the groups were distributed by severity of CVD as follows: in case of the C174T of the AGT gene mutation in Soft form of hypertension the phenotype frequency is 4.16 %, in Moderate form of hypertension – 8.33 %, in Severe form of hypertension – 20.82% (16.6% – heterozygotes, 4.16 % – homozygotes), the frequency of the mutant allele in Soft form of hypertension is 0.0208, Moderate form of hypertension – 0.0416, Severe form of hypertension – 0.1249 in unit fractions (as we see the frequency of the mutant allele in severe form of hypertension is six times higher than in moderate form); in case of the C235T of the AGT gene mutation in Soft form of hypertension and Moderate form of hypertension, the frequency of the phenotype is 8.33%, in Severe form of hypertension is 20.83% (8.33% – heterozygotes, 12.5% – homozygotes), the frequency of the mutant allele in Soft form of hypertension and Moderate form of hypertension, respectively, is 0.0416 and in Severe form of hypertension – 0.1666, which is four times higher; in case of C677T of the MTHFR gene mutation in Soft form of hypertension the frequency of the phenotype is 8.33%, in Moderate form – 11.11 %, in Severe form – 18.6 %, the frequency of the mutant allele is 0.0417, 0.0556 and 0.0903 in unit fractions, respectively. As it can be seen, according to the above results, the highest frequencies of phenotypes and frequencies of mutant alleles were detected in the group of patients with severe hypertension.

Combinations of the following mutations were revealed in the experimental group: seven cases of combination of C174T of the AGT gene mutation with the C677T of the MTHFR gene mutation – 9.72%, six cases of combination of C235T of the AGT gene mutation with C677T of the MTHFR gene mutation – 8.33% and six cases of combination of C174T and C235T of the AGT gene mutation – 8.33%. All cases of homozygous mutations and their combinations were found in a group of patients with severe hypertension.

The highest frequencies of cousin (12.5%) and twice removed types of marriages (16.7%) with an inbreeding coefficient of F = 0.0260 were found in the group of patients with severe hypertension. The lowest values of the above-described frequencies and almost 2.5 times lower inbreeding coefficient (F=0.0104) were obtained for a group of patients with a mild course of the disease.

Timely molecular and genetic studies conducted to prevent diseases of the cardiovascular system in case of detection of C174T, C235T of the AGT gene mutations and C677T of the MTHFR gene mutations in patients, both individually and in combined forms, which are a risk group, will allow physicians to carry out their qualified treatment.

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