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Study on association of the polymorphic variants of *ACE* (I/D), *AT2R1* (A1166C), *TNF-* (G308A), *MTHFR* (C677T) genes and their combinations with the risk of development of perinatal pathology and gestation reduction

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Aim. *To study the association of the polymorphic variants of ACE (I/D), AT2R1 (A1166C), TNF-б (G308A), MTHFR (C677T) genes and their combinations with the risk of perinatal pathology and gestation reduction.* ***Methods***. *The polymorphic variants of genes were analyzed by PCR and RFLP in 235 newborns with severe perinatal pathology and 110 clinically healthy term newborns.* ***Results***. *An increased risk of severe perinatal pathology was associated with such genotypes: DD and ID (ACE), 1166AC, 1166CC (AT2R1), 677CT (MTHFR), 308AA and 308AG (TNF-), this risk for homozygotes is almost 2-fold higher than for heterozygotes. Reduction of terms of gestation is associated with the genotype 677TT (MTHFR), and resistance to diseases in the perinatal period – with the genotype II (ACE) and 1166AA (AT2R1), 677CC (MTHFR) and the 308GG (TNF-), particularly when com- bined.* ***Conclusions***. *The identified associations evidence the role of polymorphic variants of ACE, AT2R1, TNF- , MTHFR genes in the development of severe perinatal pathology and can be used for its early prediction with subsequent correction of treatment.*

Keywords: perinatal pathology, polymorphism, ACE, AT2R1, TNF- , MTHFR genes..

Introduction. Perinatal pathology of newborns is a current interdisciplinary problem of modern clinical medicine as it contributes significantly to the infant mortality and incapacitation, increasing both. In perinatal period, which covers antenatal life from week 28 of gestation throughout the first week after birth, in organisms of both fetus and newborn experience some important physiological processes occur aimed at preparation and adaptation to the postnatal life. Quite often these physiological processes are accompanied by the development of pathologies. Perinatal pathology is diagnosed in the newborns, who suffered from perinatal asphyxia and showed clinical symptoms of hypoxic and ischemic brain damage, revealed through the analysis of clinical symptoms and the presence of perinatal risk factors. As for today, there are neither laboratory tests nor methods enabling clear forecast or diagnosis and determination of the severity degree.

The role of genetic factors in the development and course of critical conditions of newborns is studied insufficiently. Scientific literature contains disembodied data about the influence of genetic polymorphism and mutations on the risk of development of perinatal pathology and neonatal syndromes [1–5]. There has never been complex study on polymorphic variants of genes and their combinations in view of pathogenic mechanisms of the development of critical conditions in newborns. For that reason the objective of our research was to study association of polymorphic variants of genes of angiotensin-converting enzyme (*ACE* (I/D)), receptor of angiotensin II type 1 (*AT2R1* (A1166C)), tumor necrosis factor (TNF-б (G308A)), methylentetrahydrofolate reductase (*MTHFR* (C677T)) and their combinations with the risk of development of perinatal pathology and reduction in gestation terms.

Materials and Methods.Two hundred and thirty five newborns, 1–3 days old, with severe perinatal pathology, admitted to the specialized Department of Pathology of Newborns of OKHMADET, Science and Research Specialized Hospital, Kyiv, and Department of Intensive Therapy of Maternity Home, Poltava, were examined. 119 newborns with severe perinatal pathology were born with gestation age of 28–37 weeks, 116 newborns – 38–40 weeks. Throughout the early neonatal period all 235 newborns showed clinical symptoms of perinatal hypoxic and ischemic damage of nervous system, which in 201 newborns (85.53%) was caused by perinatal asphyxia. Out of total of 235, 151 newborns (64.26%) showed the complication of early perinatal period by respiratory distress syndrome (RDS), 69 newborns (29.36%) had necrotic enterocolitis (NEC), 84 newborns (35.74%) had neonatal jaundice. A criterion for the newborn to be studied was the presence of severe perinatal pathology, while congenital malformation, development anomalies, and hereditary diseases were the criteria for exclusion. Standard clinical, instrumental, and laboratory methods were applied by neonatologists in specialized departments to determine the diagnosis. Control group included 110 clinically healthy newborns, born during term labors or physiological labors (Apgar score 8-9) without clinical symptoms of perinatal diseases and let home on 3rd–5th day. DNAs were isolated from newborns' cord and peripheral blood using DNA-sorb-B reagents (Russian Federation) according to the manufacturer's instructions. Polymorphic variants of *ACE* (I/D), *AT2R1* (A1166C), *TNF-б* (G308A), *MTHFR* (C677T) genes were analyzed using the method of polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) [6–8]. The amplification conditions and list of restriction endonucleases are presented in Table 1. Depending on the presence/absence of certain restriction sites in the amplified DNA section, the restriction products varied in length (Table 1). The PCR and RFLP products were detected in 2% agarose gel. The length of restriction fragments was analyzed against a marker DNA.

Statistic analysis of results was made using Statistica 6 software to calculate standard criterion ч^2 and to define Odds Ratio (OR).

Results and Discussion. While planning this study, we selected some possible candidate genes in accordance with the current views about mechanisms of the development of perinatal pathologies. Analysis of specificities of metabolic processes in fetuses and newborns throughout perinatal period enabled us to distinguish principal pathogenic links, subjected to pathological changes at the development of critical conditions, as well as possible susceptibility-associated genes, presented in Figure 1.

Supporting appropriate level of blood circulation and vascular homeostasis processes is extremely important at the maximal tension of physiological processes during the delivery and adaptation in early neonatal period. The *ACE* and *AT2R1* gene expression products are involved into stabilization of vascular and cell homeostasis, functional state of endothelium, and blood circulation processes [9, 10]. According to the scientific literature data, deletion polymorphism of *ACE* gene (DD-genotype) is known to be associated with an increase in ACE activity, which enhances the tonus of smooth vascular muscles and susceptibility to acute ischemia [11]. Some authors supposed that regulation of soluble ACE level could be connected with disorder in the regulation of transcription or splicing of *ACE* pre-mRNA. As a rule, the presence of genotype II has protective effect on cardiovascular

Table1
Conditions for definition of polymorphic variants of studied genes

Gene	Amplification conditions		Restriction endonuclease	Polymorphism	Length of fragments after amplification and restriction, b.p.
	Annealing, C	No. of cycles			
ACE	58	30	–	*I/D*	490, 190
AT2R1	65	35	*BstDe*I	*A1166C*	352, 238 и 114
MTHFR	70	35	*Hinf*I	*C677T*	301, 176 и 125
TNF-	60	35	*Nco*I	*G308A*	107, 87 и 20

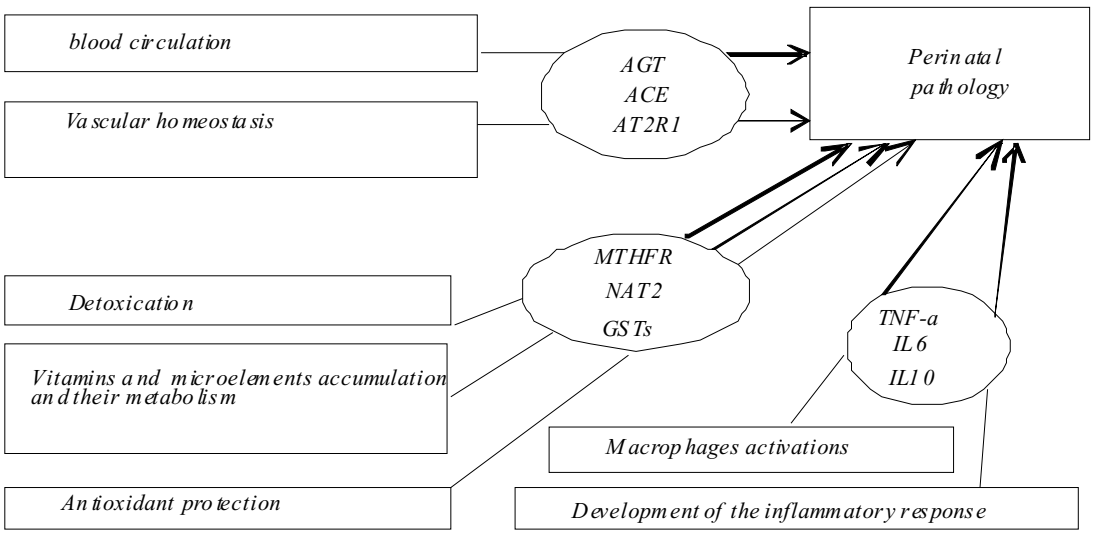

Fig. 1 Possible candidate genes, associated with newborns' hereditary susceptibility to perinatal pathology

events due to the fact that low enzymatic activity does not lead to activation of angiotensin II and inactivation of bradykinin. The *AT2R1* activity also influences the occurrence of dysfunction of vascular endothelium. The carriers of different genotypes of *AT2R1* gene differ in efficiency of binding angiotensin II, which defines functional condition of a blood vessel wall. Scientific literature contains the data about sinergistical impact of *AT2R1* 1166CC genotype and *ACE* DD genotype on the development of pathological changes in cardiovascular system [11].

The above mentioned mechanisms of support of vascular homeostasis are extremely important for newborns as in perinatal period normal blood circulation is essential in the conditions of short and long term hypoxia as well as in case of resuscitation in critical conditions.

Necessary physiological reorganization of newborn requires optimal metabolic transformations taking into account the fact that occurring hypoxia leads to the imbalance of oxidative and antioxidative processes, which, in its turn, stimulates the synthesis of anti-inflammatory cytokines and assists in changing vascular homeostasis.

Activation of vascular cytokines, including TNF-б, is a response to many stimuli, i.e. hypoxia, intrauterine infection, birth stress [12]. It has been shown that for individuals with AA genotype in 308th position of promoter area of *TNF-б* gene the transcriptional activity of the latter is higher [13], i.e. anti-inflammatory cytokine in these individuals is synthesized in inadequately increased amounts, and it influences rapid increase in the formation of hydrogen peroxide and other free radicals in macrophages and neutrophils, which results in significantly higher oxidative stress. Accordingly, the individuals with 308GG genotype have normal transcriptional activity of the gene, which does not lead to excessive

Table2
Distribution of genotypes of studied genes in mature and premature newborns

Gene	Genotype	Premature patients		Mature patients		^{2}	OR	CI
		n	%	*n*	%			
ACE (I/D)	II	15	12,61	23	19,83	2,26	1,71	0,84–3,48
	ID	69	57,98	60	51,72	0,93	0,78	0,46–1,30
	DD	35	29,41	33	28,45	0,03	0,95	0,54–1,68
*AT2R*1 (A1166C)	1166AA	67	56,30	61	52,58	0,33	0,86	0,51–1,44
	1166AC	36	30,25	47	40,52	2,71	1,53	0,92–2,69
	1166CC	16	13,45	8	6,90	2,75	0,48	0,20–1,16
TNF- G	308GG	51	42,86	50	43,10	0,00	1,01	0,60–1,69
	308GA	55	46,02	59	50,86	0,51	1,20	0,72–2,01
	308AA	13	10,92	7	6,03	1,80	0,52	0,20–1,36
MTHFR (C677T)	677CC	48	40,34	49	42,24	0,09	1,08	0,64–1,82
	677CT	51	42,86	63	54,31	3,08	1,58	0,95–2,65
	677TT	20	16,81	4	3,45	11,43	5,66	1,87–17,1*

*p < 0,05.

production of TNF-б cytokine. The newborns with AA genotype responding to unfavorable course of perinatal period or birth stress will hyperproduce anti-inflammatory cytokine TNF-б, which causes irreversible changes accommodating perinatal hypoxic damage, cardiovascular disorders and apoptosis.

Missense mutation *C677T* in *MTHFR* gene decreases catalytic activity of corresponding enzyme by average 35-70% of norm by changing its thermolability. A number of polymorphic *MTHFR* variants were shown to influence the process of apostasies of chromosomes and antenatal development [14]. Thus, genotypes 677CT and 677TT are associated with the development of defects and other disorders in fetus as a result of occurring deficit of methyl groups, which are necessary for cell proliferation and differentiation, as well as for an increase in the level of homocysteine that causes toxic effect. However, the development of endothelial dysfunction and reduced anti-oxidative protection in newborns with 677CT and 677TT genotypes can be considered as two of the most important mechanisms of realization of unfavorable impact of polymorphic *MTHFR* variants in perinatal period.

Our preliminary analysis showed that the polymorphic gene variants, studied in this work, are the most important for normal course of metabolic processes in perinatal period. Modifications in structures of genes and their functional activity are the key factors in the development of perinatal pathologies and can assist the reduction of gestation term due to the accumulation of functional changes throughout prenatal development, which decrease the adaptation of fetus to antenatal life conditions.

Tables 2 and 3 present the results of our analysis of polymorphic variants of *ACE*, *AT2R1*, *MTHFR*, and *TNF-б* genes. Analysis of frequency of polymorphic variants in premature and mature newborns showed (Table 2) that reduced gestations terms are reliably associated only with homozygous variant 677TT of *MTHFR* ($ч^2$ = 11.43, OR = 5.66 (1.87-17.1)).

In our previous publication [3] we have shown that at all types of perinatal pathologies this genotype was predominantly (5 to 7 times) present in premature newborns whereas in the general group of newborns the increased frequency of 677TT genotype was shown to be unreliable, which confirms again the association of this genotype with reduced gestation term.

Table 3
Distribution of frequencies of genotypes by ACE, AT2R1, MTHFR and TNF-б in newborns with perinatal pathology and in control

Locus	Patients		Control		Statistic parameters			
	n	%	*n*	%	^{2}	OR	95 % CI	p
				ACE (I/D)				
Genotype								
II	38	16,17	51	46,36	35,68	0,22	0,13–0,37	<0,001
ID	129	54,89	44	40,00	6,65	1,83	1,15–2,89	<0,01
DD	68	28,94	15	13,64	9,6	2,58	1,40–4,76	<0,01
				AT2R1 (A1166C)				
Genotype								
1166AA	128	54,47	84	76,36	15,16	0,37	0,22–0,62	<0,01
1166AC	83	35,32	24	21,82	6,38	1,96	1,16–3,31	<0,05
1166CC	24	10,21	2	1,82	7,58	6,14	1,42–26,47	<0,05
				MTHFR (C677T)				
Genotype								
677CC	97	41,28	68	61,82	12,67	0,43	0,27–0,69	<0,05
677CT	114	48,51	37	33,63	6,74	1,86	1,16–2,98	<0,05
677TT	24	10,21	5	4,55	3,13	2,39	0,89–6,44	>0,05
				TNF- (G308A)				
Genotype								
308GG	101	42,98	80	72,73	16,45	0,28	0,17–0,46	<0,01
308AG	114	48,51	28	25,45	26,59	2,86	1,67–4,55	<0,001
308AA	20	8,51	2	1,82	5,62	5,02	1,15–21,89	<0,05

Due to the fact that no statistically significant differences have been revealed in distribution of polymorphic variants of *ACE*, *AT2R1*, and *TNF-б* in mature and premature sick newborns (Table 2), in our further work the results of genotyping were compared for the integrated group of all children with perinatal pathology and the control, while for *MTHFR* gene an additional analysis was performed].

Table 3 shows that for all studied polymorphic variants of *ACE*, *AT2R1*, *TNF-б*, *MTHFR*, except for one (677TT genotype of *MTHFR* gene), we revealed statistically reliable differences in frequencies of genotypes, i.e.: 1) increased frequency in the group of sick (DD, ID of *ACE* gene; 1166CC, 1166AC of *AT2R1*; 677CT of *MTHFR*; 308AA, 308AG of *TNF-б*), which testifies to the association of these genotypes with increased risk of the development of perinatal pathologies, in case of homozygotes, the risk being almost twice as high as that for heterozygotes; 2) reduced frequency in the sick compared to the control (II of *ACE* gene; 1166AA of *AT2R1*; 677CC of *MTHFR*; 308GG of *TNF-б*), which characterises protective effect of the aforementioned genotypes. High reliability of the results obtained confirms the participation of selected genes in pathogenesis of severe perinatal pathology.

The separate analysis of the polymorphic variants of *MTHFR* gene in mature and premature newborns

Table 4
Analysis of significant combinations of studied genotypes of ACE, AT2R1, MTHFR, and TNF-a genes

Genotype combination	Patients, *n* = 235		Control, *n* = 110		Statistic parameters			
	n	%	*n*	%	^{2}	OR	95 % CI	p
				ACE/TNF-a				
ID/AG	75	31,9	13	11,82	15,93	3,5	1,84–6,64	< 0,05
II/GG	31	13,19	42	38,18	28,05	0,25	0,14–0,42	< 0,05
				ACE/MTHFR				
DD/CT	39	16,6	6	5,45	8,2	3,45	1,41–8,41	< 0,05
ID/CT	59	25,11	17	15,45	4,06	1,83	1,01–3/33	< 0,05
II/CC	20	8,51	37	33,64	34,3	0,18	0,10–0,34	< 0,001
				ACE/AT2R1				
II/AA	13	5,52	50	45,45	56,74	0,07	0,04–0,14	< 0,001
				TNF-a/MTHFR				
AG/CT	59	25,11	12	10,91	9,24	2,74	1,40–5,34	< 0,05
GG/CC	42	17,87	52	47,27	32,67	0,24	0,14–0,39	< 0,001
				TNF-a/AT2R1				
AG/AC	35	14,89	7	6,36	5,1	2,58	1,11–6,00	< 0,05
GG/AA	51	21,70	63	57,27	42,85	0,21	0,13–0,34	< 0,001
				ACE/AT2R1/MTHFR/TNF-				
II/AA/CC/GG	6	2,55	30	27,27	48,99	0,07	0,03–0,17	< 0,001

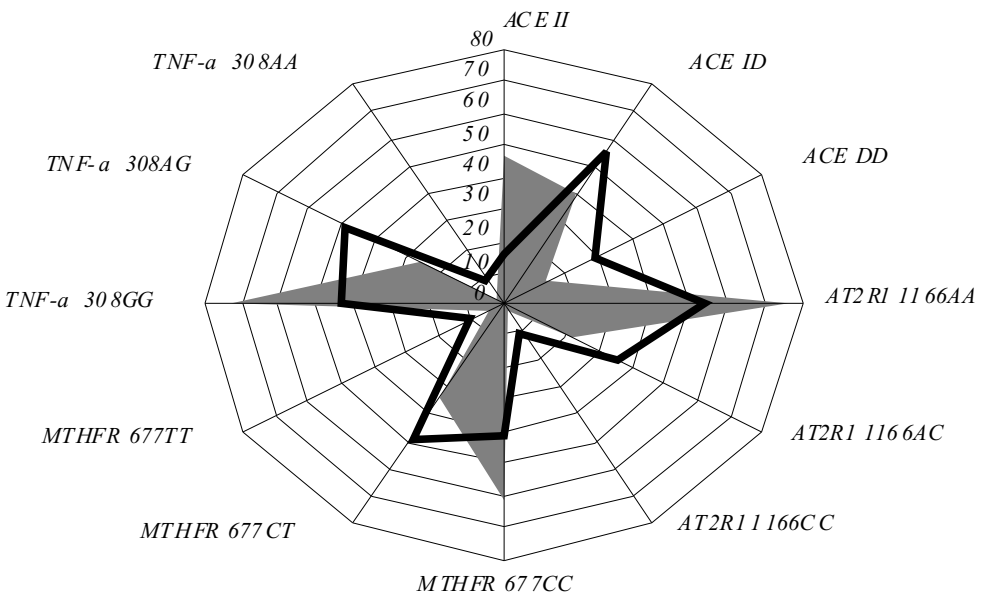

Fig. 2 Comparative analysis of distribu- tion of frequencies of genotypes for stu- died genes: grey – control; white – newborns with peri- natal pathology

with perinatal pathologies compared with the control demonstrated that genotype 677TT had increased frequency only for premature newborns, 677CT - for mature newborns, whereas the frequency of 677CC genotype was reliably decreased equally in mature and premature newborns. (Noteworthy fact is that generally in the group of sick (Table 3) the increase in frequency of 677TT genotype was unreliable.) It means that the presence of T-allele in homozygous or heterozygous condition is also a risk factor in the development of perinatal pathologies regardless of gestation age.

Fig.2 illustrates revealed by us multidirectional influence of homozygotes with different allele variants of each of four genes on the development of perinatal pathology (unfavorable effect of homozygosis for one allele and protective - for homozygotes on the other allele).

We have analyzed all possible combinations of polymorphic variants of studied genes, and some combinations were detected in some patients, whereas the others were not revealed at all. Table 4 presents only those combinations of studied genes, the frequencies of which were reliably different for sick and healthy newborns.

Comparison of the data in Tables 3 and 4 revealed the presence of potentiating effect of combination of some polymorphic variants in newborns' genotypes on the development of perinatal pathologies: 1) increased risk for combination in ID genotype of *ACE* gene and genotype AG of *TNF-б* gene variant (at the combination of mentioned polymorphic variants of OR increases to 3.5 compared with the presence of only one of these variants – OR equals 1.83 and 2.86 respectively). The risk increases also for the combination of DD variant of *ACE* gene and CT variant of *MTHFR* gene (OR=3.45 for the combination of variants against 2.58 and 1.86 respectively for separate polymorphic variants); 2) significantly reduced risk of perinatal pathology at the combination of polymorphic variants with proven by our study protective action: the most significantly reduced risk (14.23 times) at the combination II of *ACE* gene and AA variants of *AT2R1* gene, which finds its confirmation in decrease in OR down to 0.07 against 0.22 and 0.37 for certain corresponding variants at high probability ($ч^2$=56.74, p<0.001) and II of *ACE*/CC of *MTHFR* (5.45 times), GG of gene *TNF-б*/AA of *AT2R1* (4.84 times), GG of *TNF-б*/CC of *MTHFR* (4.12 times), GG *TNF-б*/II of *ACE* (3.97 times).

As it can be seen from Table 4, the maximal potentiating effect is inherent to the combination II of *ACE*/AA variant of *AT2R1* as even the increase in the number of protective homozygous variants up to 4 in individual genotype has influence neither on the level of risk (OR=0.07) nor on the degree of reliability (OR=0.001).

Rating of genotype/phenotype correlation based on the results of this study will be presented in a separate publication.

The results obtained on distribution of polymorphic variants of investigated genes and their combinations as well as the results of statistical analysis testify to the presence of genetic susceptibility to the development of critical conditions in newborns in early prenatal period and reduction of gestation period.

Genetic susceptibility to perinatal pathology in newborns, revealed by us, along with subsequent investigations and developments will accommodate the creation of personified protocols of prophylactics and treatment of critical conditions.

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Изучение ассоциации полиморфных вариантов генов *ACE* (I/D), *AT2R1* (A1166C), *TNF-* (G308A), *MTHFR* (C677T) и их комбинаций с риском развития перинатальной патологии и сокращением сроков гестации

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Резюме

Цель. Изучить ассоциацию полиморфных вариантов генов ACE (I/D), AT2R1 (A1166C), TNF- (G308A), MTHFR (C677T) и их комбинаций с риском развития перинатальной патологии и сокращением сроков гестации. ***Методы****. Полиморфные варианты генов анализировали методами полимеразной цепной реакции и полиморфизма длины рестрикционных фрагментов у 235 новорож-*

денных с тяжелой перинатальной патологией и 110 клинически здоровых доношенных новорожденных. ***Результаты****. Повы- шенный риск развития тяжелой перинатальной патологии обусловлен генотипами DD и ID (ACE), 1166AC, 1166CC (AT2R1), 677CT (MTHFR), 308AA и 308AG (TNF-), причем для гомозигот он почти в 2 раза выше, чем для гетерозигот. Сокращение сроков гестации связано с генотипом 677TT (MTHFR), а резистентность к возникновению заболеваний в перинатальном периоде – с генотипом II (ACE) и 1166AA (AT2R1), 677CC (MTHFR) и 308GG (TNF-), особенно при их сочетании.* ***Выводы****. Выявленные ассоциации свидетельствуют о роли полиморфних вариантов генов ACE, AT2R1 и TNF- , MTHFR в развитии тяжелой перинатальной патологии и могут быть использованы для раннего прогнозирования ее возникновения с последующей коррекцией тактики лечения.*

Ключевые слова: перинатальная патология, полиморфизм, гены ACE, AT2R1, TNF- , MTHFR

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Вивчення асоціації поліморфних варіантів генів *ACE* (I/D), *AT2R1* (A1166C), *TNF-* (G308A), *MTHFR* (C677T) та їхніх комбінацій з ризиком розвитку перинатальної патології і скороченням термінів гестації

Резюме

Мета*. Вивчити асоціацію поліморфних варіантів генів ACE (I/D), AT2R1 (A1166C), TNF- (G308A), MTHFR (C677T) та їхніх комбінацій з ризиком розвитку перинатальної патології і скороченням термінів гестації.* ***Методи****. Поліморфні варіанти генів аналізували методами полімеразної ланцюгової реакції та поліморфізму довжини рестрикційних фрагментів у 235 новонароджених з важкою перинатальною патологією та в 110 клінічно здорових доношених новонароджених.* ***Результати****. Підвищений ризик розвитку важкої перинатальної патології обумовлений генотипами DD і ID (ACE), 1166AC, 1166CC (AT2R1), 677CT (MTHFR), 308AA і 308AG (TNF-), причому для гомозигот він майже вдвічі вищий, ніж для гетерозигот. Скорочення термінів гестації пов'язане з генотипом 677TT (MTHFR), а резистентність до виникнення захворювань в перинатальному періоді – з генотипом II (ACE), 1166AA (AT2R1), 677CC (MTHFR) і 308GG (TNF-), особливо при їхньому поєднанні.* ***Висновки****. Виявлені асоціації свідчать про роль поліморфних варіантів генів ACE, AT2R1, TNF- і MTHFR у розвитку важкої перинатальної патології і можуть бути використані для раннього прогнозування її виникнення з подальшим коригуванням тактики лікування.*

Ключові слова: перинатальна патологія, поліморфізм, гени ACE, AT2R1, TNF- , MTHFR.

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