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GENETIC POLYMORPHISMS OF THE RENIN-ANGIOTENSIN SYSTEM IN BREAST CANCER PATIENTS

L.E. Fishchuk[1,], N.G. Gorovenko[2]*
[1]SI «Institute of Genetic and Regenerative Medicine of NAMS», Kyiv 04114, Ukraine
[2]P.L. Shupik National Medical Academy of Postgraduate Education, Kyiv 04112, Ukraine

***Background*: Breast cancer (BC) is one of the most common cancer pathologies in women. Genetic polymorphism of genes of renin-angiotensin system (RAS) is considered to be associated with cancer development, in particular, with BC. *Aim*: To study the influence of polymorphic variants of genes coding for RAS components, on the risk of BC development in Ukrainian women. *Materials and Methods*: In the study 131 patients with histologically proven diagnosis of BC of I and II stages were enrolled. The control group was composed from 102 women without cancer. Polymorphic variants of *AGT*, *ACE*, *AT2R1* genes were studied with the use of PCR and PCR-RFLP methods. *Results*: It has been revealed that the presence of 1166AC genotype of *AT2R1* gene elevates the risk of BC development nearly 2-fold. The results of analysis for common group and subgroups distributed by age are different. For women from 18 to 35 years old the significant differences were not found. For women from 36 to 54 years old an increased risk of BC development is determined by the presence of D allele of *ACE* gene. Decreased risk of BC development was associated with the presence of combined genotypes *ACE* II/*AGT* 174TT and *ACE* II/*AGT* 235MT. In women older than 54 years an increased risk of BC development was found to be related to the presence of genotypes 235TT of *AGT* gene and 1166AC of *AT2R1* gene. The presence of genotype combinations *AGT* 235TT/*AGT* 174TM and *AGT* 235TT/*AT2R1* 1166AA in women of this age subgroup also significantly increases the risk of BC development. *Conclusion*: These polymorphic gene variants and their associations may be considered as possible prognostic markers of BC development. The results of analysis are different in total cohort and in subgroups distributed by age.**

***Key Words*: breast cancer, renin-angiotensin system, polymorphism, gene.**

Renin-angiotensin system (RAS) is represented by the system of enzymes and hormones which regulate arterial pressure, electrolytic and fluid balance. Renin-angiotensin cascade begins from secretion of renin — aspartyl proteinase enzyme which use angiotensinogen (AGT) as a substrate with the following cleavage of angiotensin I. Then after hydrolysis of angiotensin I by angiotensin I-converting enzyme (ACE) there is generated angiotensin II — octapeptide hormone, potent vasoconstrictor and cell growth stimulator. Biologic functions of angiotensin II are realized upon the binding with specific receptors. It has been revealed that angiotensin II receptor type 1 (AT2R1) take part in realization of main estimated physiologic and pathophysiologic functions of angiotensin II.

In many studies it has been shown that RAS activation directly or indirectly leads to activation of angiogenesis processes [1, 2]. As far as cancer development, progression and metastasis are associated with angiogenesis and proliferative processes, one may suppose that RAS could be related to cancer development.

Recently there have been performed the studies on the relation between genes coding for some RAS components, and the risk of breast cancer (BC) development [3–5]. However, such studies mostly have analyzed not more than two genes; none of such researches were performed in Ukraine.

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*Correspondence: E-mail – medgen@ukr.net
Abbreviations used: ACE – angiotensin converting enzyme; AGT – angiotensinogen; AT2R1 – angiotensin II receptor type 1; BC – breast cancer; CG – control group; MDR – multifactor dimensionality reduction; OR – odds ratio; PCR – polymerase chain reaction; RFLP – restriction fragment length polymorphism; RAS – renin-angiotensin system.

Angiotensingen (*AGT*) gene is localized on long arm of chromosome 1 in 1q42-q43 locus and contains 5 exons. Different genetic *AGT* variants determine different physiologic activity of angiotensin II. In *AGT* gene there have been found more than 30 different polymorphic sites [6], among which M235T and T174M mutations are the most studied and clinically significant. T174M polymorphism (rs4762) is characterized by replacement of threonine to methionine at position 174 of peptide chain caused by single nucleotide replacement of cytosine to thymine at position 521 of *AGT* gene (C521T). M235T polymorphism (rs699) — replacement of threonine to methionine at position 235 of peptide chain caused by single nucleotide replacement of thymine to cytosine at position 704 of angiotensinogen gene (T704C). In T-allele carriers (with M235T polymorphism) and M-allele carriers (with T174M polymorphism) the angiotensinogen level in blood is elevated compared to that in normalcy. For example, with the use of laboratory tests it has been shown that in 235T allele carriers, plasma level of angiotensinogen is by 10–20% higher than that in normalcy [6]. In 1992, complete linkage of M235T and T174M polymorphic variants has been shown [7].

The gene coding for angiotensin I-converting enzyme (*ACE*) is localized on long arm of chromosome 17 in 17q23 locus and contains 26 exons and 25 introns. There are known more than 110 polymorphisms in *ACE* gene, and the most studied one is an insertion-deletion (I/D) polymophism (rs1799752) — the presence or lack of Alu-repeat (284 bp large) in 16th intron. Allele with Alu-repeat is named insertional one (I), and allele lacking this repeat — deletional one (D). There has been found a strong association between

the gene polymorphic variant and ACE level in blood. In a number of studies it has been shown that ACE content in individuals with DD genotype is approximately twice higher than that in individuals with II genotype, while intermediate blood level of this enzyme is found in persons with ID genotype [8, 9]. Presently it remains unclear why polymorphism affects the level of soluble ACE, but it could be possible that genetic regulation of ACE content occurs at transcription level. Some authors suppose that insertion-deletion could be related to altered transcription regulation and/or splicing of *ACE* pre-mRNA [10, 11].

AT2R1 gene is localized on long arm of chromosome 3 in 3q24 locus. There are known more than twenty polymorphic variants of *AT2R1* gene, and the most studied one is A1166C mutation (replacement of adenine to cytosine in 1166 position) in 3' untranslated region. It has been shown that such polymorphism is functionally insignificant, but is tightly linked with T810A variant which is localized in promoter part of *AT2R1* gene and affects transcription factor binding [12]. In individuals with 1166C allele an elevated sensitivity to angiotensin II has been detected [13, 14].

The aim of our study was to analyze the relation between polymorphic variants of *AGT* (T174M, M235T), *ACE* (I/D), and *AT2R1* (A1166C) genes and their combinations and the risk of BC development in Ukrainian women.

MATERIALS AND METHODS

The study protocol was approved by the Bioethics committee of SI "Institute of Genetic and Regenerative Medicine of NAMS", and written informed consent was obtained from all participants.

131 female patients (average age — 43.22±12.99) with histologically proven diagnosis of BC of stages I and II cured in Kyiv City Oncology Center, were enrolled in the study. The control group (CG) was composed from 102 women (average age — 47.95±15.48) without cardiovascular pathologies and cancer.

The BC patients were distributed in three subgroups according to the age of BC development: 1) women of younger age, 18–35 years old; 2) women of middle age, 36–54 years old; 3) women of postmenopausal age, older than 54 years. The control group of women was also distributed in three subgroups dependent on age (Table 1).

Table 1. Distribution of basic research groups into subgroups by age

Groups	n	Average age
18–35 years old		
BC	53	30.6±3.96
CG	33	29.24±4.02
36–54 years old		
BC	52	46.39±5.05
CG	23	45.87±5.78
Older than 54 years		
BC	26	62.58±6.72
CG	46	62.22±6.75

Molecular-genetic study was performed on the samples of venous blood taken from all examined patients and healthy controls. Genomic DNA was isolated from venous blood with the use of commercial kit "DNA-Sorb-B" (CSRI of Epidemiology of Ministry of Health of Russian Federation).

Genotyping of I/D polymorphic variants of *ACE* gene was carried out with the use of allele-specific PCR method, and analysis of polymorphic variants of *AGT* gene (T174M, M235T), and *AT2R1* gene (A1166C) was performed with the use of PCR-RFLP method according to protocols described in the literature [15–18].

The results were analyzed with the use of Statistica 6.0 program. To evaluate the significance of the differences between genotype frequencies in compared groups, standard χ^2 Pearson criterion was used. Association of alleles or genotypes with BC predisposition was evaluated by odds ratio (OR). Frequencies of haplotypes were calculated with the use of "The EH software program" (EH) (Rockefeller University, USA). Testing of the differences in haplotype frequency distribution between groups of patients and control group was done by algorithm proposed by X. Xie and J. Ott [19], and realized in EH program. The differences $p < 0.05$ were considered statistically significant.

Inter-gene interactions were studied with the use of bioinformative method of multifactor dimensionality reduction (MDR) which allows modeling of genomic interactions of high order what is impossible to perform with the use of conventional parametric methods. The basis of the MDR method is a constructive induction algorithm that converts two or more variables or attributes to a single attribute. This process of constructing a new attribute changes the representation space of the data. The end goal is to create or discover a representation that facilitates the detection of nonlinear or nonadditive interactions among the attributes such that prediction of the class variable is improved over that of the original representation of the data. The best model was determined among n-locus models with the use of permutation test which is realized in MDRpt-1.0_beta_2 program and was used for evaluation of significance of these models.

RESULTS AND DISCUSSION

Distribution of genotype frequencies of the studied genes in the group of BC patients and in control group is presented in Table 2.

Table 2. Distribution of genotype frequencies of the studied genes

Gene, genotype	Frequency, n (%)		χ^2	OR (95% CI)
	BC (n = 131)	CG (n = 102)		
***AGT* T174M**				
174TT	95 (72.52)	72 (75.49)	0.26	0.86 (0.47–1.55)
174TM	32 (24.43)	24 (23.53)	0.03	1.05 (0.57–1.93)
174MM	4 (3.05)	1 (0.98)	0.39	3.18 (0.35–28.91)
***AGT* M235T**				
235MM	36 (27.48)	32 (31.37)	0.42	0.83 (0.47–1.46)
235MT	66 (50.38)	55 (53.92)	0.29	0.87 (0.52–1.46)
235TT	29 (22.14)	15 (14.71)	2.07	1.65 (0.83–3.27)
***ACE* I/D**				
II	37 (28.24)	31 (30.39)	0.13	0.90 (0.51–1.59)
ID	53 (40.46)	50 (49.02)	1.70	0.71 (0.42–1.19)
DD	41 (31.30)	21 (20.56)	3.37	1.76 (0.96–3.22)
***AT2R1* A1166C**				
1166AA	72 (64.96)	64 (62.75)	1.43	0.72 (0.43–1.23)
1166AC	47 (35.88)	24 (23.53)	4.13	1.82 (1.02–3.25)
1166CC	12 (9.160	14 (13.73)	1.21	0.63 (0.28–1.44)

Significant difference between BC group and CG has been revealed for 1166AC variant of *AT2R1* gene (χ^2=4.13; OR=1.82; 95% CI 1.02–3.25) what evidences that the presence of this genotype in women increases BC risk nearly 2-fold. The results of the study of S. Namazi et al. [20] have shown that 1166AC genotype is associated with higher TNM stage in BC patients.

According to literature data, 36 years are considered as a critical age for women because exactly from this age BC incidence quickly starts to rise. The large number of BC cases is diagnosed in 36–54 years old women. This could be explained in part by initiation of involution changes in the structure of mammary glands when glandular tissue is replaced by adipose or fibrous tissues that starts approximately at the age of 36 years. Also, starting from 36 years in women quick progression of fertility decrease begins, and this process is caused by hormonal changes: initially imbalance develops, and then acute deficiency of sex hormones — estrogens and progesterone.

An evaluation of influence of candidate gene polymorphism on the risk of BC development in different age subgroups has been performed.

Analysis of distribution of possible genotype variants in BC group and control group for each age subgroup has revealed significant differences only for older subgroups but not for women of younger age (Table 3).

Table 3. Significant differences in the analysis of polymorphic and allele variants of genes in age subgroups of BC patients and control group

Gene	Polymor-	Frequency, n (%)		χ^2	OR (95% CI)
		BC	CG		
36–54 years old					
ACE (I/D)	D allele	57 (54.81)	12 (26.09)	10.59	3.44 (1.60–7.37)
Older than 54 years					
AGT (M235T)	235TT	11 (42.31)	7 (15.22)	5.14	4.09 (1.33–12.51)
AT2R1 (A1166C)	1166AC	12 (46.15)	10 (21.74)	4.67	3.09 (1.09–8.75)

For age subgroup 2 (36–54 years) the presence of D allele of A*CE* gene elevates the risk of BC development, what is in accordance with the data of other authors [5, 21]. W.P. Koh et al. conducted a study among Chinese postmenopausal women in which they found that individuals carrying the II genotype had a significantly reduced risk of BC independently of environmental and other familial risk factors for the disease [22]. On other hand, A. Yaren et al. showed that DD genotype may influence the local tumor growth of BC in premenopausal patients [23].

For women older than 54 years such associations disappear and the risk of BC development is increased in the presence of 235TT genotype of *AGT* gene (OR=4.09; 95% CI 1.33–12.51), and in the presence of 1166AC genotype of *AT2R1* gene (OR=3.09; 95% CI 1.09–8.75) compared to control group. Contrary, A.M. González-Zuloeta Ladd et al. showed that postmenopausal women who were homozygous for the 235M allele of the M235T *AGT* polymorphism had a significantly increased risk for BC [4]. The results of other study showed that A1166C was associated with a lower risk of BC for 1166C carriers [21].

At the next stage of the work we have analyzed the influence of combinations of the studied polymorphic variants of *ACE*, *AGT*, *AT2R1* genes on BC risk in three age subgroups of the BC patients and control group (Table 4). Significant differences have been revealed only for two older aged subgroups but not in younger one (18–35 years).

From the data of this Table one may conclude that in group 2 (36–54 years old women) combination of genotypes *ACE* II/*AGT* 174TT is associated with highly significant protective effect in BC development. However, if in this genotype combination polymorphic variant II of *ACE* gene is replaced by DD, the risk of BC development increases. These facts allow to suppose that an impact of polymorphic variant I/D of *ACE* gene in BC development is more potent than protective effect of genotype 174TT of *AGT* gene.

In the oldest age group of women (> 54 years) genotype 235TT of *AGT* gene has the strongest impact in the risk of BC development (see Tables 3 and 4). This risk elevates in the presence of combined genotypes *AGT* 235TT/*AGT* 174TM — from 4.09 fold to 5.28 fold, and up to 4.67 fold in the presence of combination *AGT* 235TT/*AT2R1* 1166AA. Why do genotypes 235TT and 174TM of *AGT* gene which are responsible for elevated level of angiotensinogen, increase the risk of BC development? It is known that high AGT levels are capable to decrease angiogenesis and suppress tumor growth. This could be explained by the low degree of estrogens in females at postmenopausal period — estrogens may regulate angiotensinogen expression and affect its level.

Table 4. The genotype combinations which affect BC risk in the studied age subgroups

Genes	Genotype combination	BC, n (%)	CG, n (%)	χ^2	OR (95% CI)
From 36 to 54 years					
ACE (I/D), *AGT* (M235T)	II/235MT	6 (11.54)	8 (34.78)	4.25	0.24 (0.07–0.82)
ACE (I/D), *AGT* (T174M)	II/174TT	11 (21.15)	11 (47.83)	5.47	0.29 (0.10–0.84)
ACE (I/D), *AGT* (T174M)	DD/174TT	16 (30.77)	0 (0.00)	7.26	-
Older than 54 years					
AGT (M235T), *AGT* (T174M)	235TT/174TM	7 (26.92)	3 (6.52)	4.20	5.28 (1.23–22.65)
AGT (M235T), *AT2R1* (A1166C)	235TT/1166AA	8 (30.77)	4 (8.70)	4.35	4.67 (1.25–17.49)

At the next stage of our study we have calculated the frequencies of haplotypes of *AGT* gene (4 possible variants of haplotypes) in age subgroups of women with the use of EH program. The analysis of calculated haplotype frequencies did not reveal significant differences between studied age subgroups of BC patients and control group.

Also we have performed a modeling of interaction between studied genes in BC patients with the use of MDR method which allows perform simultaneous analysis of many polymorphic gene variants selecting combinations with the highest pathogenetic significance. For evaluation of interactions between polymorphic gene variants with the use of MDR method we have used exhaustive search algorithm which evaluates all possible combinations of the studied DNA-markers in relation to the risk of BC development (Table 5).

Table 5. Models for interlocus interactions of candidate BC genes in women calculated with the use of MDR program at integrated search regimen

Number of loci in a model	Combination of loci in a model	Cross-validation consistency, %	Testing balancing accuracy, %
	From 18 to 35 years		
2	*ACE* (I/D)/*AT2R1* (A1166C)	90	50.29
	From 36 to 54 years		
2*	***ACE* (I/D)/*AGT* (M235T)**	**100**	**78.09**
4	*ACE* (I/D)/*AT2R1* (A1166C)/ *AGT* (T174M)/*AGT* (M235T)	100	60.95
	Older than 54 years		
2	*AT2R1* (A1166C)/*AGT* (M235T)	100	67.47

*The best model (p = 0.05) among n-locus models tested in 1000 simulations with the use of MDRpt-1.0_beta_2 program.

Among proposed models of inter-gene interactions in BC patients from three age subgroups only one model get through permutation test — two-locus model of *ACE* (I/D)/*AGT* (M235T) interaction, which is characterized by 100% reproducibility and 78.09% precognition accuracy. This model correctly predicted in 69% cases probability of occurrence of BC development and in 74% cases correctly classified healthy women from 36 to 54 years old.

An analysis of obtained data allows us to conclude that the character of interactions of polymorphic variants of *ACE* (I/D) and *AGT* (M235T) genes in age subgroup from 36 to 54 years possesses an expressed synergic effect in BC development — *AGT* (M235T) enhanced the action of *ACE* (I/D) by 9.29%.

In conclusion our study has shown that polymorphic variants of RAS genes and their associations may be considered as possible prognostic markers of BC risk; the results of analysis are different for general cohort and subgroups distributed by age, that's why in such studies one should take into account patient's age as an important parameter.

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