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Potential implication of genetic polymorphisms and Torque teno virus in sporadic breast cancer

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Abstract

The complex genetic susceptibility for breast cancer includes highly penetrating and low-penetrating genetic markers. eNOS (7q36), ACE (17q23) and AGTR1 (3q24) are candidate loci for breast cancer.

The aim of this study was to test the potential association of eNOS 4ab, ACE ID, AGTR1 A1166T polymorphisms and Torque teno virus (TTV) infections with sporadic breast cancer in Romania.

For this case-control study were selected women with sporadic breast cancer (n=100) and healthy women (n=100). The eNOS 4ab, ACE ID, AGTR1 A1166T polymorphisms and presence of TTV DNA were assessed by PCR-based protocols.

The polymorphisms' distribution was in accordance with Hardy-Weinberg equilibrium. No significant association with breast cancer was found for investigated polymorphisms considered individually or in combinations of two or three markers (p>0.05). TTV infections were more frequent in cancer patients than in control (84% vs. 60%, p=0.00016). A preferential distribution of TTV in patients with sporadic breast cancer, especially in those carrying eNOS bb genotype (OR=4.88, 95%CI: 2.01-11.9, p=0.00025) or ACE I variant (OR=4.07, 95%CI: 1.85- 8.97, p=0.0003), was observed.

This study revealed a preferential distribution of TTV infections in women with sporadic breast cancer, carriers of eNOS bb genotype or ACE I variant.

Key words: *ACE ID*, *AGTR1 A1166T*, *eNOS 4ab*, TTV, sporadic breast cancer

Introduction

The complex genetic susceptibility for breast cancer includes highly penetrating and low-penetrating genetic markers. Highly penetrating mutations (e.g. mutations in BRCA genes) are mostly detected in cases which exhibit strong familial aggregation of breast cancer whereas low-penetrating markers are considered to be more important for sporadic forms of disease [1]. Some candidates low-penetrance susceptibility loci are the *endothelial nitric oxide synthase* (*eNOS*, 7q36), *angiotensin converting enzyme* (*ACE*, 17q23) and *angiotensin II type I receptor* (*AGTR1*, 3q24) [2]. They have been detected in different normal [3-6] and tumor cells [7,8] from breast. The eNOS is the main source of NO under physiological conditions. The role of eNOS and NO in tumorigenesis is still poorly understood [9,10]. The effects of eNOS on proliferation, cell survival and angiogenesis [11] may promote tumor formation. It also may inhibit tumor initiation and maintenance [12] and may regulate apoptosis in cultured human breast cancer cells [13]. A high density of eNOS positive

microvessels in the normal tissue surrounding the tumor is a favorable prognostic indicator in premenopausal breast cancer patients [11].

ACE and AGTR1 are the two key elements of renin-angiotensin system (RAS). ACE is the rate-limiting enzyme in the conversion of angiotensin I to angiotensin II and it is involved in degradation of other biomolecules (e.g. the bradykinin, substance P, enkephalins). Angiotensin II is a powerful vasoconstrictor, a pro-inflammatory [14] and pro-oxidant [15] molecule that may induce cellular toxicity and apoptosis. AGTR1 is an important mediator of angiotensin II biological effects. RAS may be involved in etiology of breast cancer by different mechanisms. Thus, it has been proposed that angiotensin II may determine hypertrophy and hyperplasia (by means of AGTR1) [16-18], breast cell proliferation [19], regulation of tumor angiogenesis [2,20] and tumor metastasis [21]. In some studies, pharmacological inhibition of RAS components has the potential to reduce the risk of cancer [22-24].

eNOS 4ab is a 27 bp repeat polymorphism in intron 4 of *eNOS* gene and has variants with four (variant "*a*") or five (variant "*b*") tandem repeats that may influence the gene activity [25]. The insertion/deletion of a 287-bp *Alu* sequence in intron 16 of the *ACE* gene (*ACE ID, rs4646994*), was correlated with the inter-individual variability of ACE plasma circulating level and its activity [26]. The homozygotes for the insertion have lower plasma level than homozygotes for deletion, while *ID* carriers present intermediary levels [27]. The AGTR1 A1166C *(rs5186)* is a SNP located in the *3'UTR* of the gene. Although the *eNOS 4ab, ACE ID* and *AGTR1 A1166C* are mapped in non-coding regions and have unclear functional significance, they are tested as genetic marker in linkage disequilibrium with putative disease causing markers located nearby.

Torque teno virus (TTV) is a novel emerging virus whose disease inducing potential has not been established. Nevertheless, TTV genome was detected in patients with different types of cancer including breast [28-30].

The aim of this case-control study was to test the potential association of *eNOS 4ab, ACE ID, AGTR1 A1166T* polymorphisms and TTV infections with sporadic breast cancer.

Materials and Methods

Subjects. Women with sporadic breast cancer (n=100, 66.1±7.2 years, range: 57-84 years) were selected if they had surgically- and histologically-confirmed disease and had no secondary malignancy. All patients reported no heredo-collateral history of cancer. Patients were selected from Coltea Hospital (Bucharest).

Healthy women (n=100, 67±6.9 years, range: 57-83 years) were recruited after a clinical examination from subjects who visited one medical clinic for routine healthy check-up. They were matched for age and birth place with patients.

The study was performed in accordance with the Declaration of Helsinki. Informed consent was obtained from all subjects before selection.

Genotyping methods. Genomic DNA was extracted from 0.25 ml of peripheral blood drawn on EDTA using a commercial kit (AxyPrep™ Blood Genomic DNA Miniprep Kit, Axygen Biosciences, California) according to the manufacturer's recommendation. *ACE ID* [31] and *eNOS 4ab* [32] polymorphisms were genotyped by PCR and *AGTR1 A1166C* [33] by restriction of amplicons with endonuclease *Dde* I according to protocols described previously with slight changes. The presence of *ACE I* variant was tested in a second independent PCR with a set of primers specific for the insertion in an attempt to avoid miss-genotyping of *ID* as *DD* due to the preferential amplification of the *D* variant [31]. Using this

method the correct heterozygous status for one sample was considered after testing all samples considered as *ACE DD* after first round of PCR. TTV infection was tested in a two rounds heminested PCR protocol as described previously [34].

The reaction mixture (15 µl) included *Taq* polymerase buffer (1X), MgCl2 (1.5mM), dNTP mix (0.4 mM), primers (0.4 µM), template DNA (10-100ng) and Taq DNA polymerase (1 unit, Promega). PCR program consist in the initial denaturation for 2 minutes at 95°C, thirty cycles of: 1 minute at 95°C, annealing conditions (**Table 1**), and 1 minute at 72°C, followed by final elongation at 72°C for 2 minutes.

Table 1. Sequence of primer pairs used for PCR, annealing temperature and size of polymorphism variants.

Gene	Polymorphism	Primer sequence	Annealing (^{0}C / sec)	Size of variants (bp)
ACE	*ID*	5' ctggagaccactcccatcctttct 3' 5' gatgtggccatcacattcgtcagat 3'	58 / 45	D: 477 I: 190
	confirmation of *I* variant	5' tgggaccacaggcgcccgccactac 3' 5' tcgccagccctcccatgcccataa 3'	67 / 45	I: 335 D: -
AGTR1	*A1166T*	5' cgactactgcttagcata 3' 5' gcaccatgttttgaggtt 3'	68 / 60	A: 546 C: 435+111
eNOS	*4ab*	5' aggccctatggtagtgcctt 3' 5' tctcttagtgctgtggtcac 3'	58 / 60	b:420 a: 393
TTV infection	First round	5' acwkmcgaatggctgagttt 3' 5' rgtgrcgaatggywgagttt 3'	55 /30	128
	Second round	5' acwkmcgaatggctgagttt 3' 5' ccccttgactbcggtgtgtaa 3'	55 / 30	113

Statistical analysis. PowerMarkerv3.25 was used to calculate summary statistics, deviation from Hardy Weinberg Equilibrium, and significance of marker distribution between groups [35]. Difference in genotypes and allele distribution between cases and control groups were examined with chi^2 test or Fisher exact test. Odds ratio (OR) with 95% confidence interval was used to estimate the statistical risk to develop the disease conferred by each genotype. All statistical tests were two tailed, and $p<0.05$ indicated statistical significance. Yates correction was used when appropriate, or the p value was adjusted using the Bonferroni correction when multiple hypotheses were tested.

Multifactor Dimensionality Reduction (MDR) analysis was performed with MDR software package (v.2.0.beta 8.4) [36]. Best models with possible combinations of the investigated markers were considered based on 10-fold cross-validation and maximum testing accuracy.

The combinations of alleles were constructed and analyzed using SHEsis software [37].

Results and Discussions

Data regarding the association of polymorphisms in *ACE, AGTR1* and *eNOS* genes and breast cancer are conflicting. In this case-control study we tested the association between sporadic breast cancer, three genetic polymorphisms and TTV infections in Romanian population.

The major finding of this study was that the infections with TTV were more frequent in patients with breast cancer than in control (84% vs. 60%, p=0.00016, OR=3.5, 95% CI: 1.79-6.82). The age of patient and control women selected for this study was similar ($p>0.05$) and cannot explain this preferential distribution of virus in patients with breast cancer. Other factors related to genetic background, immunity or treatment of patients may explain this result.

The distribution of genotypes for *ACE ID, AGTR1 A1166C* and *eNOS ab* polymorphisms in patients and control lots are in accordance with the Hardy-Weinberg equilibrium (**Table 2**). Analysis of each marker individually and of combinations of two markers reveals no significant association with breast cancer (p>0.05).

Table 2. The distribution of *ACE ID, AGTR1 A1166C* and *eNOS 4ab* polymorphisms in the study groups

Genotype	Breast cancer	Healthy Control
ACE DD	32	25
ACE ID	48	52
ACE II	20	23
MAF (ACE I)	0.44	0.49
Hardy-Weinberg (p value)	0.8	0.69
AGTR1 1166 AA	49	48
AGTR1 1166 AC	44	47
AGTR1 1166 CC	7	5
MAF (AGTR1 1166 C)	0.29	0.285
Hardy-Weinberg (p value)	0.49	0.13
eNOS bb	63	65
eNOS ba	33	32
eNOS aa	4	3
MAF (eNOS a)	0.205	0.19
Hardy-Weinberg (p value)	0.9	0.69
TTV present*	84	60
TTV absent	16	40

* p=0.00016, O.R.=3.5, 95% CI: 1.79- 6.82

MDR analysis was applied to determine the potential epistatic interaction that may confer high or low-risk for development of sporadic breast cancer. We found small percentages of the entropy explained by the investigated polymorphisms (0.07-0.44%) considered independently or by the interaction between combinations of two polymorphisms (maximum 0.56%). TTV had the largest independent effect (5.28%). The analysis did not identify an interaction model significantly associated with breast cancer (**Figure 1**).

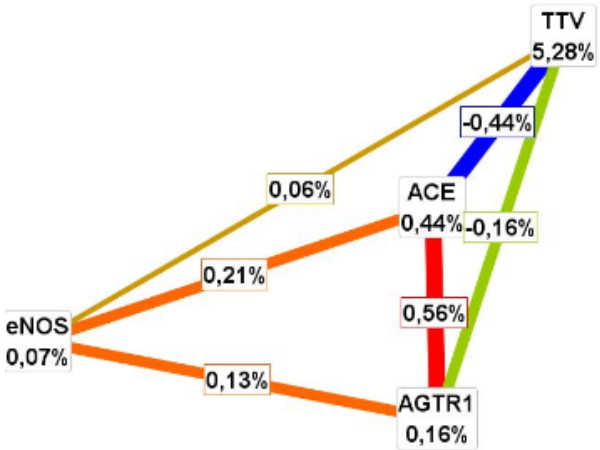

Figure 1. Interaction graph for sporadic breast cancer (TTV has the largest univariate effect 5.28%).

The presence of TTV infections is not similarly distributed in patients and controls with the same genotype. After applying Bonferroni corrections for multiple tests, the differences remain significant only for carriers of the *eNOS bb* genotype and of *ACE I* or *AGTR1 A* allele ($p<0.003$). However only the presence of TTV infection in carriers of *eNOS bb* genotype (OR=4.88) and in carriers of the *ACE I* allele (O.R=4.07) increases statistical risk above the value obtained for TTV alone (OR=3.5) (**Table 3**).

Table 3. The distribution of TTV infections in patients and controls with the same genotype.

Combinations of two risk factors	Lots					
	Patients	Controls	Patients	Controls	Patients	Controls
	eNOS bb		*ACE (DI or II)*		*AGTR1 (AA or AC)*	
TTV present	55	38	57	42	78	58
TTV absent	8	27	11	33	15	37
	OR=4.88, 95%CI: 2.01-11.9, p=0.00025		OR=4.07, 95%CI: 1.85- 8.97, p=0.0003		OR=3.32, 95%CI: 1.66-6.61, p=0.00047	

The combinations of two or three variants of the tested polymorphisms were inferred with SHEsis software for patients and controls. None of the combinations were significantly associated with breast cancer. This finding is in agreement with the result provided by MDR analysis, which indicated no significant interaction between polymorphisms in relation with breast cancer.

Individual analysis of *ACE ID, AGTR1 A1166C* and *eNOS 4ab* polymorphisms revealed no association between markers and sporadic breast cancer in our lots. Although a synergism between *ACE ID* and *eNOS 4ab* polymorphisms in relation to disease is possible, the present study require to be reconfirmed in larger studies. However, our results indicated a preferential distribution of TTV infections in patients with sporadic breast cancer, especially in those carrying *eNOS bb* genotype or *ACE I* variant.

A similar result for *ACE ID* and breast cancer has been detected in Turkey [38] and Iran [39]. The mean age of subjects from these studies (41.6±7.1 years and 49.49±9.69 years respectively) is significantly lower compared with those from our lot (66.1±7.2 years). *ACE ID* has been described to change the disease risk in other studies: *ACE I* exhibits a modest association with disease in a multiethnic cohort [40] or appears as protective in samples from Singapore [41-43]; *ID* is protective in Brazil [44], whereas DD increases the disease risk in samples from Rotterdam [45,22]. Two meta-analyses regarding the association between *eNOS* polymorphism (e.g. *4ba* and *G894T*) and breast cancer provided no evidence for association with risk of breast cancer [46,47]. Difference in genetic background, environment and study design (e.g. age range, method of ascertainment, or selection) may explain the discordant results obtained in different studies. The distribution of *ACE I* (49%), *AGTR1 1166 C* (28.5%) and *eNOS 4a* (19%) variants in our healthy control lot was similar with values reported for other Caucasian populations [48-52].

Conclusions

This study revealed a preferential distribution of TTV infections in patients with sporadic breast cancer, carriers of *eNOS bb* genotype or *ACE I* variant.

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