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Investigation Of Nitric Oxide Synthase 3 (Nos3) Glu298asp Gene Variation In Breast Cancer Patients

Canan Cacina[1,2], Cem Horozoglu[1,2], Oncu Koc Erbasoglu[1], Bahar Toptas[1], Yemliha Yildiz[1], Soykan Arıkan[3], Seden Kucucuk[4], İlhan Yaylım[1] and Turgay İsbir[5]

[1] *Department of Molecular Medicine, Aziz Sancar Institute of Experimental Medicine, Istanbul University, İstanbul-Turkey*

[2] *Department of Medical Services and Techniques, Istanbul Gelisim University, Istanbul-Turkey*

[3] *Clinic of Surgery, Istanbul Training and Research Hospital, Istanbul-Turkey*

[4] *Department of Radiation Oncology, Institute of Oncology, Istanbul University, İstanbul-Turkey*

[5] *Department of Medical Biology, Faculity of Medicine, Yeditepe University, İstanbul*

[6] *Both authors contributed equally to this work.*

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Corresponding author: *İlhan Yaylim, Istanbul University, Aziz Sancar Institute for Experimental Medicine, Department of Molecular Medicine, Vakif Gureba Cad. Capa 34390 Istanbul Turkey, Tel: +90 0212 6351959, Fax: +90 0212 6351959, e-mail address: ilhanyaylim@gmail.com*

Abstract

The role of Nitric Oxide (NO) in tumor biology is not well understood. However, it has been proposed that NO is a highly reactive free radical and plays important roles in growth, progression or metastasis of tumour. Nitric oxide syntheses (NOSs) are a family of enzymes that catalyzing the production of NO by converting L-arginine to L-citrulline. The aim of present study was to examine Nitric Oxide Synthase 3 (NOS3) Glu298Asp polymorphism in patients with breast cancer. For this purpose, 49 patients with breast cancer and 89 healthy controls were included in the study. The NOS3 Glu298Asp polymorphism was genotyped by PCR-RFLP using peripheral blood samples in our study groups. NOS3 Glu298Asp genotype distribution in the breast cancer patients was statistically different from the healthy control group NOS3 Glu298Asp gene variation T allele frequency was significantly higher in breast cancer patients that of controls. Our finding suggests that NOS3 Glu298Asp might be associated with breast cancer risk.

Key words : Oxidative stress; NOS3; breast cancer.

Introduction

Breast cancer is one of the most common types of cancer in the world and is ranked first among the most commonly diagnosed cancers in women and second among the most commonly cause of death in cancer [1].Known risk factors for breast cancer include family history, age, first gestational age, alcohol consumption, postmenopausal obesity, and ionizing radiation. However, all these factors are insufficient to understand the etiology of breast cancer and its geographical variation [2].The heterogeneity of genes involved in oxidative stress and metabolism of carcinogens can also affect the susceptibility to breast cancer [3].

Free radical nitric oxide (NO) is an important biological mediator that plays a role in various cytostatic and cytotoxic functions and is associated with apoptosis, metastasis and angiogenesis [4].Reactive nitrogen species, derived from NO and NO, by leading to oxidative and nitrosative stress,cause DNA damage and DNA repair enzymes to be inhibited by direct or indirect mechanisms [5].Nitric oxide (NO) is synthesized by Nitric Oxide Synthase (NOS) is forms during L-sitrulinin, L-arginine conversion [6, 7].NO has tumor growth and tumor angiogenesis stimulation and metastasis-activating effects at low concentrations, whereas at higher concentrations, tumor cells play a role in apoptosis processing and tumor growth arrest [8]. Changes in the balance between cell proliferation and apoptosis contribute to the pathogenesis of many diseases. NO can change this balance because it is toxic or protective depending on the concentration, cell type and environmental conditions, it can cause or inhibit apoptosis [9].In endothelial cells, there are two forms of Nitric Oxide Sentetase (NOS), the inducible NOS encoded by the NOS2 gene and the endothelial NOS encoded by the NOS3 gene [10].NOS3 is encoded by a 26 exon gene on chromosome 7 [11].The Glu298Asp variant of NOS3is one of the common polymorphisms seen in this gene and is characterized by glutamate-aspartate exchange (G-T) for codon 298 at nucleotide 894. In some studies it has been concluded that NOS3 Glu298Asp (894G> T) mutant TT variant may be associated with breast cancer [12].

In our study, it was aimed to determine genotype and allele frequencies in patient and control subjects in order to investigate the importance of NOS3 gene Glu298Asp polymorphism in breast cancer susceptibility. At the same time, the relationship between the clinical and prognostic parameters of the disease and NOS3 polymorphism was examined in patients with breast cancer.

Materials And Methods

In our study, 49 breast cancer patients were included in the patient group and 89 healthy individuals were included in

the control group. Patients with breast cancer were followed by Istanbul University Oncology Institute Radiation Oncology and Istanbul Training and Research Hospital Surgical Clinic. Pathologically diagnosed and clinical evaluations and sampling of patients with breast cancer who were included in the project were performed by the relevant clinics.

Medical Ethics Committee of Istanbul Medical Faculty approval was obtained for the study. The protocol followed was consistent with the World Medical Association Declaration of Helsinki(Ethical Principles for Medical Research Involving Human subjects).Our work has been supported by the Istanbul University Scientific Research Projects Unit with project number 44022.

Genotyping

DNA isolation was performed by salting out peripheral blood samples from breast cancer patients and healthy subjects [13].5'-AAG GCA GGA GAC AGT GGA TGGA -3 'and 5'- CCC AGT CAA TCC CTT TGG TGC TCA -3' specific primer sequences were used for the amplification of the gene region of NOS3 Glu298Asp gene polymorphism. The PCR reaction mixture contained 50-100 ng of genomic DNA, 1x PCR buffer, 0.2 mM dNTP, 3mM MgCl2, 0.2 mM primer and 0.5 U Taq polymerase (MBI Fermentas, Lithuania). The PCR conditions were determined as first denaturation at 95 °C for 5 minutes, 35 cycles at 94 °C for 1minute, 58 °C for 1minute, 72 °C for 1minute, and a final extension at 72 °C for 5 minutes. Amplification products after PCR were cut with Ban II enzyme. Products obtained after restriction were run on 3% agarose gel electrophoresis and genotyped after UV imaging. After the enzymatic restriction, one fragment having 248 bp was as homozygous (TT), two fragments having 163 and 85 bp was as homozygotes (GG) andthree fragment having 248, 163 and 85 bp was as heterozygous (GT) were evaluated [14].

Statistical Analysis

Statistical analyzes of this study were performed using the SPSS 11 package program. The statistical significance limit was taken as $p < 0.05$. Chi square ($\chi 2$) was used in evaluating the frequency of alleles with NOS3 Glu298Asp genotype and the differences between groups. Student-t test was used for numerical data analysis.

Results

The information about the healthy controls and the patients with breast cancer used in our study are shown in Table 1. There was no statistical significance between the patients with breast cancer and the control group in terms of family history, sex, smoking and other parameters. There was no significant difference in age between the patients and control groups ($p > 0.05$).

80% of the patients with breast cancer who participated in our study were invasive ductal, 8.9% were invasive lobules and 11.1% were combined type. 29% of the patients were premenopausal, and 71% were postmenopausal women. It was detected ER(-) in 15.8% of patients and PR(-) in 13.9% of patients. It was determined vascular invasion in 44.2%, perinodal invasion in 46.3% and capsule invasion in 50% of patients with breast cancer.

Table 1: Information about working group

	Control n=89	Breast Cancer Patients n=49
Sample Numbers(Female/Male)	64/25	49/49
Age Averages (Year)	51.14±16.32	54.02±11.04

The values in the table are given as x + SD. Groups were examined with the significance level student t test.

Considering the results of NOS3 Glu298Asp gene polymorphism, when we examined our study group in terms of genotype distribution, NOS3 Glu298Aspgenotype distribution in patients with breast cancer and control group is seen in Table 2. In our study, the T allele frequency of the NOS3 Glu298Asp variant was higher in the breast cancer patient group than in the control group and the difference was statistically significant ($\chi 2 = 7.229$, $p = 0.007$, OR: 1.228, 95% CI: 1.065-1.415).

Tablo 2: Genotype frequencies of study groups

	Control N(%) n=89	Breast Cancer Patients N(%) n=49	p value
NOS3 Glu298Asp			
GG	25(28.1)	2(4.1)	0.024
GT	34 (38.2)	22 (44.9)	
TT	30(33.7)	25(51.0)	
ALLELE			
G	84(47.2)	26(26.5)	
T	94(52.8)	72(73.5)	0.007

The values in the table are given as sample number and percentage. The differences between the groups are shown by the Kikare test.

No statistically significant difference was found between the genotype distribution of NOS3 Glu298Asp variation and parameters such as age, sex, family history, smoking status of the patients. Given the demographic data of patients with breast cancer, the percentages of detected genotype distributions are shown in Table 3.

An interesting finding was that all of the patients with estrogen receptor ER(-) had T allele of theGlu298Asp variant ($\chi 2 =: 0.439$; $p = 0.980$; OR: 1.074; 95% CI: 0.973-1.186). In our study, all patients with progesterone receptor PR(-) from patients with breast cancer were carrying the TT genotype.

In patients with lymph node involvement positive (N) positive examined in the study, the possibility of carrying the NOS3 Glu298Asp TT genotype was increased but there was no statistically significant difference ($p = 0.697$). At the same time,

Table 3: NOS3 Glu298Asp genotype distribution according to the characteristics of patients with breast cancer

	NOS3 Glu298Asp (%)			
	GG	**GT**	**TT**	**p değeri**
Age				
45<	3.1	42.4	54.5	p>0.05
45≥	9.1	45.5	45.5	
Family cancer story				
Present	8.3	50	41.7	p>0.05
Absent	3.8	42.3	53.8	
Smoking				
Yes	12.5	50	37.5	p>0.05
No	3	44.1	52.9	

Table 4: Analysis of NOS3 Glu298Asp gene polymorphism according to pathologic data of patients with breast cancer

Pathological data	**NOS3 Glu298Asp (%)**		
	GG	**GT**	**TT**
Tumor size			
T1	0	44.4	55.6
T2	0	52.9	47.1
T3	0	33.3	66.7
T4	50	50	0
N stage			
N0	11.1	55.6	33.3
N1	0	50	50
N2	9.1	36.4	54.5
N3	0	0	100
Distant metastasis			
Present	0	33.3	66.7
Absent	5.6	47.2	47.2
ER			
Positive	6.9	51.7	41.4
Negative	0	33.3	66.7
PR			
Positive	7.1	60.7	32.1
Negative	0	0	100
Perinodal invasion			
Present	5.9	41.2	52.9
Absent	0	52.6	47.4
Vascular invasion			
Present	8.7	43.5	47.8
Absent	0	50	50

ER: Estrogen receptor, **PR:** Progesterone receptor

had T allele of theGlu298Asp variant was observed in these patients at a higher rate and no statistically significant data was obtained ($\chi 2 = 1.064$, $p = 0.381$). Table 4

In our patient group,T allele frequency of the Glu298Asp variant decreased in T3+T4tumor stage patients, but statistical significance was not found. In our study, patients with T3+T4 breast cancer increased in G allele frequency, this data in patients with breast cancer did not reach statistical significance ($\chi 2=:0.023$;$p=0.880$).According to the distant metastasis presence in the patient group, when the NOS3 Glu298Asp gene variation was examined, the Gallele was found in patients without distant metastasis at a higher rate than those with distant metastasis, the difference was not statistically significant. In addition, vascular invasion was observed more frequently in patients with breast carcinoma carrying the GG genotype, but a statistically significant data could not be achieved. However, no significant correlation was found between clinical and prognostic parameters and genotype and allele distribution of NOS3 Glu298Asp polymorphism in our patient group ($p> 0.05$).

Discussion

Although the role of nitric oxide in tumor biology has not been fully elucidated, it has been suggested to play a role in many physiological and pathophysiological processes involving vasodilatation, neurotransmission, the immune system and cancer [12, 15].Endothelial nitric oxide synthase (NOS3) and neuronal nitric oxide synthase (NOS1) are calcium-dependent enzymes that maintain low levels of basal NO production. In contrast, inducible nitric oxide synthase (NOS2) produces high levels of NO in the presence of appropriate physiological stimulus [16, 17].All three is forms are present in the heart, and NOS1 is present in sympathetic nerve endings, NOS3 in vascular endothelial cells, and NOS2 in active macrophages and myocytes [17]. It has been suggested in various studies that NO regulates different cancer events such as angiogenesis, apoptosis, cell cycle, invasion and metastasis. On the other hand, NO also emerges as a potential anti-oncogenic agent [18].Nitric oxide is thought to induce apoptosis at high concentrations, while it is known to exhibit anti-apoptotic effects at low concentrations [19].

Studies have reported NOS gene expression in breast cancer tissues and breast carcinoma cell lines. There was NO increase in blood of breast cancer patients and higher NOS activity in invasive breast tumors compared to benign or normal breast tissue. Furthermore, NOS activity was found to be higher in advanced stage breast carcinomas [18]. Mortensen et al. have shown that NOS3 is present in both breast cancer cells and endothelial cells, and that the high density of NOS3 in normal tissue around breast cancer is associated with statistically significantly longer survival [19]. Martin et al. have shown that NOS3 is expressed in breast cancer tumors and is negatively associated with histologic grade and lymph node status [20].

In our study, there was a statistically significant difference in the distribution of NOS3 Glu298Asp gene polymorphism between patients with breast cancer and the control group ($p = 0.024$).However, the frequency of T allele carriers of Glu298Asp

variant was found to be statistically significantly higher in the breast cancer group than in the control group (p = 0.007).When the age factor is considered, it is determined that the GG genotype of NOS3 Glu298Asp variant is higher than the other genotypesin the patients with breast cancer, especially 45 years and younger. Although our study did not reach statistical significance, lymph node metastasis was found to be more positive in patients with breast cancer carrying TT genotypes of Glu298Asp variant. Another interesting finding is the TT genotype of all breast cancer patients with estrogen or progesterone receptor negative. Studies conducted between NOS3 Glu298Asp polymorphism and cancer risk are available in the literature, but the results are debatable [10, 21– 30].

In a study involving 502 breast cancer patients and 505 healthy individuals, Yang et al. did not find a significant association between Glu298Asp polymorphism and breast cancer [21].For breast cancer, Ghilardi et al. reported that there was no association between NOS3 gene polymorphism and breast cancer risk in studies involving 71 patients and 91 controls in Italy [10].Similarly, Royo et al. [22] and Lu et al. [23] did not find any association between NOS3 gene polymorphism and breast cancer in their studies. In a study involving 269 patients with breast cancer and 244 control groups in Austria, Hefler et al. reported that NOS3 Glu298Asp polymorphism was associated with a 2fold increased risk [24]. The findings of our study are also consistent with the studies results of Hefler et al.

In a study involving 360 colorectal cancer patients and 550 healthy individuals in Spain, no significant difference in Glu298Asp genotype distribution and allele frequency between patient and control group was reported [25].In a study conducted by Medeiros et al. in a Portuguese population, no association was found between prostate cancer risk and Glu298Asp gene polymorphism [26].In another study, Cheon et al. reported that NOS3 gene locus in lung cancer may affect plasma NO levels, and there may be a relationship between NOS3 polymorphism and lung cancer development [27], but there are studies reporting that this is the opposite correlation [10].

As a result of our study, we concluded that NOS3 Glu298Asp polymorphism may be associated with breast cancer risk. Our study is the first study to examine the NOS3 gene variant carrier in Turkish breast cancer cases and it is unique in terms of reveal the allele frequency of the related variant in the Turkish population. Despite the data obtained, the limitation of our work is the small sample size. Extensive studies in larger sample groups are needed to elucidate the molecular mechanisms related to oxidative stress and to confirm the findings. However, in addition to examining other SNPs belonging to the NOS3 gene in the future, it is aimed to reveal its effects by examining the levels of NOS3 gene expression.

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Conflict of interest

The authors declare that there is no conflict of interest in the preparation and publication of this article.

Ethical Approval

Medical Ethics Committee of Istanbul Medical Faculty approval was obtained for the study. The protocol followed was consistent with the World Medical Association Declaration of Helsinki (Ethical Principles for Medical Research Involving Human subjects).

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