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Research Article

Evaluation of the Relationship between Antioxidant Gene Polymorphisms (Endothelial Nitric Oxide Synthase, Myeloperoxidase, Uncoupling Protein 2) and Breast Cancer

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Abstract

Objectives: This study aimed to determine the relationship of breast cancer development with the polymorphisms of endothelial nitric oxide synthase (eNOS), myeloperoxidase (MPO), and uncoupling protein 2 (UCP2) genes.

Methods: The study included 60 breast cancer patients and 70 healthy controls. After exclusion criteria, 37 patients and 70 healthy controls were enrolled into study. The functional variants studied were intron-4 variable number of tandem repeats (VNTR), -G463A, and -866G/A variants for the eNOS, MPO, and UCP-2 genes, respectively. The polymerase chain reaction (PCR) and/or PCR-restriction fragment length polymorphism (RFLP) methods were used for genotyping. The distribution of genotype frequencies of the eNOS, MPO, and UCP2 genes were compared between the breast cancer patients and healthy controls using the Chi-square test.

Results: The BB genotype of the eNOS gene variant (intron-4 VNTR) was associated with a significantly decreased risk of breast cancer (OR=0.56; 95%CI, 0.463–0.676; p=0.001); the AA and AB genotypes were not associated with the risk of breast cancer as reported in our previous work. No significant association was determined between the risk of breast cancer and any genotype of the MPO gene variant. While the AA (OR=8.167; 95% CI, 2.785–23.951; p=0.001) and AG (OR=4.341; 95% CI, 1.679–11.222; p=0.002) genotypes of the UCP2 gene variant were associated with significantly decreased risk of breast cancer, GG genotype of the UCP2 gene variant was associated with significantly increased risk (OR=5.0; 95% CI, 2.207–11.327; p=0.001).

Conclusion: Outcomes of this study revealed that breast cancer was associated with BB genotype of the intron-4 VNTR variant of the eNOS gene and AA, AG, and GG genotypes of the -866G/A variant of the UCP2.

Keywords: Breast cancer, endothelial nitric oxide synthase, myeloperoxidase, uncoupling protein 2

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Breast cancer, which is the most common cancer in females, still remains the leading cause of death for females despite increased survival rates within the last decade with early diagnosis and advancements in treatment methods.[1, 2] The mechanism of the development of breast cancer has not been clearly understood; however, environmental factors and complex genetic changes have been suggested to have a role.[3] Oxidative stress is defined as

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excessive production of reactive oxygen species (ROS) and is one of the most important mechanisms leading to tissue injury.[4] Data on oxidative stress have recently been taken into account for the relationship between ROS genes and breast cancer. Increased ROS levels are considered to be a major factor for cancer development as a result of changes in cell proliferation rates and inhibited apoptosis.[5]

Nitric oxide (NO) is a free radical playing an important role in many metabolic processes such as vasodilation, immune response, and platelet and leukocyte adhesion, yet high concentrations of NO are thought to have an effect on carcinogenesis. Endothelial NO synthesis is catalyzed by endothelial nitric oxide synthase (eNOS) and a functional variant that is responsible for >25% of basal plasma NO production is a variable number of tandem repeats (VNTR, 27 nt) in intron 4.[6]

Myeloperoxidase (MPO) is a lysosomal enzyme found in monocytes, macrophages, and primary granules of neutrophils. MPO participates in biotransformation of specific carcinogens by catalyzing the formation of hypochloric acid. MPO has a role in the destruction of malignant and non-malignant cells.[7] The Guanin-463-Adenine (-G463A) base transition is a common variant within the gene promoter of MPO. The variant A allele is associated with reduced expression of messenger RNA (mRNA) at the SP1 binding site, which in turn results in nearly 25 times less transcription activity as compared with the G allele.[8]

An important mechanism in oxidative stress regulation is the function of uncoupling proteins (UCPs), which are among the mitochondrial membrane proteins. The UCPs control free radical formation. Three UCPs defined in mammalian mitochondria are UCP1, UCP2, and UCP3. Previous studies have shown that UCP2 could cause cancer development and resistance to chemotherapy [9, 10] The role of antioxidant enzymes in cancer has been previously studied and it has been well established that there is a relationship between oxidative damage and malignancy.[11] Furthermore, recently-determined differences in genes encoding antioxidant enzymes have revealed that there might be a relationship between cancer and genetic susceptibility. [11] Accordingly, the objective of this current study was to evaluate the relationship between antioxidant gene polymorphisms (eNOS, MPO, and UCP-2) and breast cancer risk.

Methods

Study Population

Sixty patients were planned to participate to current study, however, 23 patients were excluded after exclusion criteria were considered. Therefore, the current study included 37 patients with breast cancer and 70 systemically healthy non-smoking controls for DNA isolation and blood levels evaluation. The patients and controls were selected from the same geographic area. Exclusion criteria were history of malignancy and intake of antibiotics or anti-inflammatory drugs within the last 6 months. Both patients and controls were informed about the aim and methodology of the study and agreed to participate. Informed consent was obtained from all patients and controls before blood sampling. A detailed medical history was obtained and then a whole-body examination was performed. This study was approved by the Local Ethical Committee with 2015/12/01, (13.07.2015) protocol number in terms of the study methods and protocols.

Genotyping

Genotyping was performed for the VNTR variant in the intron-4 of the eNOS gene, the -G463A variant of the MPO gene, and the -866G/A polymorphism of the UCP2 gene. Firstly, the genomic DNA isolation was performed from the peripheral blood mononuclear cells of the patients and healthy controls using the Plus Blood Genomic DNA Purification Kit (GeneMark, Taiwan). The polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) method was used for genotyping MPO (-G463A), eNOS (intron-4 VNTR), and UCP2 (-866G/A) gene variants. For internal quality control, twenty percent of the samples were duplicated to prevent sample or reading errors.

Genotyping of eNOS gene variant (intron-4 VNTR): The intron-4 VNTR variant of the eNOS gene was analyzed by PCR using the following forward (F) and reverse (R) primer sequences: F: 5′-AGGCCCTATGGTAGTGCCTTT-3′ and R: 5′-TCTCTTAGTGCTGTGGTCAC-3′. The resultant PCR products (393 bp and/or 420 bp) were then separated on 4% agarose gel (NuSieve™ GTG™; Lonza Pharma&Biotech, USA). The experimental process was repeated twice for each sample.[12]

Genotyping of MPO gene variant (-G463A): The region carrying the -G463A variant located in the promoter of the MPO gene was amplified by PCR using the following MPO primary chains: F: 5′-CGGTATAGGCACAATGGTGAG and R: 5′-GCAATGGTTCAAGCGATTCTTC. Presence of amplification products was confirmed by gel electrophoresis (2% agarose). The amplified region was then incubated for 16 h with 5 units of AciI enzyme (Thermofisher Scientific, USA)at 37°C and analyzed using gel electrophoresis (3% agarose).[13]

Genotyping of UCP2 gene variant (-866G/A): The -866G/A polymorphism in the promoter region of the human UCP2 gene was determined by the PCR-RFLP method as previously described.[14] The amplified products of 363 bp were

digested with MluI restriction enzyme (MBI Fermentas, St Leon-Rot, Germany) at 65 ºC; the resultant products were two fragments of 291 bp and 72 bp for the A allele or the fragments of 363 bp for the G allele. The insertion variant contains a duplication of the following fragment: 5'CCCTCTTTCCCCACCTCTTCCTTCCGCTCCTTTACCTAC-CACCTT-3'. The polymorphic region was amplified by PCR and the resultant products were two fragments of 457 bp and 502 bp for the deletion and insertion, respectively.[15] The 2% ethidium bromide-stained agarose gels were used for separation of all PCR or digested products and they were visualized under ultraviolet (UV) transilluminator.

Statistical Analysis

Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS, Inc., Chicago, IL, USA; version 13.0) for Windows. Logistic regression analysis was used to determine the statistical significance of the differences between the patient and control groups. Adjusted odds ratios (ORs) were calculated using a logistic regression model that controlled for sex and age and were reported at a confidence interval (CI) of 95%. The Chi-square test and, when needed, Fisher's exact test were used to compare the differences in allele frequencies between the patient and control groups. The estimated and experienced genotype frequencies were calculated by the Hardy-Weinberg equation. The Mann-Whitney U-test was used for statistical comparisons of the groups. A p value of <0.05 was considered statistically significant.

Results

The current study comprised of 37 patients with breast cancer and 70 healthy controls. The demographic and clinical data of patients with breast cancer are summarized in Table 1. The data of the genotype distribution of intron-4 VNTR variant of the eNOS gene was already mentioned by our group in our previous paper.[16] Additionally, in this study, the -G463A variant of the MPO gene, and the -866G/A variant of the UCP2 gene in the patient and control groups were investigated and presented in Table 2.

Table 1. Demographic and clinical characteristics of the patients with breast cancer

	Patients n=37
Age, years, median (minimum-maximum)	59 (32–103)
Female sex, n (%)	37 (100)
Family History, n (%)	
Absent, n	25 (67.6)
Present	12 (32.4)
Menopause, n (%)	
Absent	12 (32.4)
Present	25 (67.6)
Molecular subtypes, n (%)	
Luminal A	21 (56.8)
Luminal B	8 (21.6)
HER2 positive	1 (2.7)
Triple negative	7 (18.9)
Tumor Grade, n (%)	
I	4 (10.8)
II	22 (59.5)
III	11 (29.7)
Metastasis, n (%)	
Absent	6 (16.2)
Present	31 (83.8)

Table 2. Comparison of the frequencies of antioxidant gene variants between the patients with breast cancer and healthy controls

	Genotype	Patients with breast cancer n=37 n (%)	Healthy Controls n=70 n (%)	OR	95% CI	p
eNOS (intron-4 VNTR)	AA	26 (70.1)	33 (47.2)	1.739	0.771–3.922	0.223
	AB	11 (29.9)	15 (21.4)	1.428	0.581–3.509	0.487
	BB	0 (0)	22 (31.4)	0.560	0.463–0.676	0.001
MPO (-G463A)	GG	26 (65)	48 (68.6)	1.272	0.531–3.048	0.590
	GA	14 (35)	22 (31.4)	1.340	0.520–3.450	0.530
	AA	0 (0)	0 (0)			
UCP2 (-866G/A)	AA	12 (29.3)	29 (41.1)	8.167	2.785–23.951	0.001
	AG	13 (31.7)	29 (41.4)	4.341	1.679–11.222	0.002
	GG	16 (39.0)	12 (17.5)	5.000	2.207–11.327	0.001

OR: Odds ratio; CI: Confidence interval; eNOS: Endothelial nitric oxide synthase; VNTR: Variable number of tandem repeats; MPO: Myeloperoxidase; UCP2: Uncoupling protein 2.

eNOS gene variant (intron-4 VNTR): The results of eNOS distrubition was already stated by our latest work.[16] Briefly, the distribution of the frequencies of AA, AB, and BB genotypes for the eNOS gene variant (intron-4 VNTR) were 47.2%, 21.4% and 31.4%, respectively, in healthy controls and 70.1%, 29.9%, and 0%, respectively, in the patients with breast cancer. We demonstrated that BB genotype of the eNOS gene variant (intron-4 VNTR) was associated with a significantly decreased risk of breast cancer (OR=0.56; 95%CI, 0.463–0.676; p=0.001). As reported in our previous paper, no significant associations were found between the risk of breast cancer and other genotypes (p=0.223 for AA genotype and p=0.487 for AB genotype).

MPO gene variant (-G463A): The distribution of the frequencies of GG, GA, and AA genotypes for the MPO gene variant (-G463A) were 68.6%, 31.4%, and 0%, respectively, in healthy controls and 65%, 35%, and 0%, respectively, in the patients with breast cancer. No significant association was found between the risk of breast cancer and any genotype of the MPO gene variant.

UCP2 gene variant (-866G/A): The distribution of the frequencies of the AA, AG, and GG genotypes of the UCP2 gene variant (-866G/A) were 41.1%, 41.4%, and 17.5%, respectively, in healthy controls and 29.3%, 31.7%, and 39%, respectively, in the patients with breast cancer. It was found that AA (OR=8.167; 95% CI, 2.785–23.951; p=0.001) and AG (OR=4.341; 95% CI, 1.679–11.222; p=0.002) genotypes of the UCP2 gene variant (-866G/A) were associated with significantly decreased risk of breast cancer. The GG genotype of the UCP2 gene variant (-866G/A) was associated with significantly increased risk of breast cancer (OR=5.0; 95% CI, 2.207–11.327; p=0.001).

Discussion

The important role of oxygen free radicals in oxidative stress in the pathogenesis of cancer is a well-known issue; however, there are also studies reporting conflicting results about oxidative stress-related genes. In the current study, it was aimed to determine the relationship of the risk of breast cancer with the variants of the eNOS (intron-4 VNTR variant), UCP2 (-866G/A variant), and MPO (-G463A variant) genes.

In a previous study aimed to determine the relationship between MPO genotypes and breast cancer, although not statistically significant, a reduction by 13% was reported in the risk of developing breast cancer in individuals with at least one A allele (GA and AA genotypes).[17] In the same study, it was reported that among premenopausal women, those having GA or AA genotype of the MPO gene had a 43% reduction in the risk of developing breast cancer and that there was no association between genotype and breast cancer in postmenopausal women.[17] In a study conducted in China, the AA genotype of the MPO gene was found to be associated with a reduced risk of developing breast cancer.[18] In another study of 502 patients diagnosed with breast cancer, MPO -G463A polymorphisms were shown to be associated with the development of breast cancer.[19] A 2017 meta-analysis of 60 studies investigated the relationship between the MPO -G463A polymorphism and the risk of cancer development.[20] While this meta-analysis included 25 studies of lung cancer, it included only 5 studies focusing on breast cancer; accordingly, the subgroup analyses performed according to the cancer type revealed a significant association for gastrointestinal and lung malignancies but no association for breast cancer. Although our study did not declare any significant relationship between MPO polymorphism and breast cancer risk, further investigations are needed.

The relationship between eNOS polymorphism and the risk of cancer development has been studied in literature and also in our previous paper.[16] A meta-analysis investigated the relation of cancer risk with eNOS polymorphisms (-786T>C, 894G>T, and intron 4A/B).[21] Accordingly, both intron 4A/B and 786T>C polymorphisms of the eNOS gene were found to be significantly associated with overall cancer risk; the subgroup analyses revealed a stronger association with the risk of prostate cancer for the eNOS intron 4A/B polymorphism and with the risks of prostate, bladder, and breast cancers for the eNOS 786T>C polymorphism.[21] Additionally, no significant association was found for the eNOS 894G>T polymorphism in overall cancer risk; however, it was reported to be significantly associated with the risk of breast cancer based on the subgroup analyses.[21] Moreover, in that particular meta-analysis, the evaluation of pathological subtypes revealed that while eNOS 786T>C polymorphism was associated with infiltrating ductal carcinoma and other carcinomas, eNOS 894G>T polymorphism was associated only with invasive ductal carcinoma.[21] Another meta-analysis investigated the eNOS E298D and eNOS 786T>C polymorphisms to evaluate the role of eNOS in the risk of cancer and reported these two polymorphisms to be associated with a decrease in the risk of breast cancer development.[22] In our previous study, the eNOS intron-4 VNTR polymorphism was investigated and it was found that the BB genotype was associated with a significantly decreased risk of breast cancer and the other genotypes (AA and AB) were not significantly associated with the risk of breast cancer.[16]

Another molecule that regulates the effects of oxygen free radicals is UCP2. Some studies have reported that UCP-2 might play a role in carcinogenesis. Over-expression of

UCP2 can protect the cell from apoptosis[23] and has been frequently detected in brain and ovarian cancers.[24] In addition, UCP2 is important for prognosis of such cancers. A high expression of UCP2 has been found to be associated with poor prognosis in patients with estrogen receptor-positive breast cancer.[25] In the current study, it was demonstrated that the AA and AG genotypes were associated with a decreased risk of breast cancer among UCP2 -866G/A polymorphism. In contrast, the GG genotype was associated with an increased risk of breast cancer.

The small sample size and the lack of a clear and comprehensive evaluation of the additional clinical parameters can be considered as the limitations of the current study. However, this study is a good starting point to investigate the effects of such genes in the breast cancer development risk of individuals who have corresponding polymorphisms. Different findings from previous pre-clinical in vitro studies have been reported focused on genes that play roles in the formation and metabolism of oxygen free radicals associated with increased risk of cancer development. Those findings should be supported with such clinical evaluation of patients.

In the current study, the AA, AG, and GG genotypes of the UCP2 -866G/A variant and the BB genotype of the eNOS intron-4 VNTR variant were found to be associated with the risk of breast cancer. On the other hand, none of the genotypes of the MPO -G463A variant were associated with the risk of breast cancer. Further studies are required to determine the role of these gene polymorphisms in the diagnosis and in risk determination of the disease.

Disclosures

Ethics Committee Approval: This study was approved by the Local Ethical Committee with 2015/12/01, (13.07.2015) protocol number in terms of the study methods and protocols.

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Conflict of Interest: The authors declare that there is no conflict of interest regarding the publication of this paper.

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