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Evaluation of ARTN and PSPN Gene Polymorphisms in Breast Cancer Risk: Insights from a Case-Control Study in Turkish Population

Meme Kanseri Riskinde ARTN ve PSPN Gen Polimorfizmlerinin Değerlendirilmesi: Türk Popülasyonunda Bir Vaka-Kontrol Çalışması

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Abstract: RET is a member of the receptor tyrosine kinase family, which is essential for the normal development and maturation of various tissues. RET signaling promotes changes in the tumor microenvironment that alter the surrounding stroma and cellular composition. Gene polymorphisms of RET and its ligands artemin (ARTN) and persephin (PSPN) are a research focus in cancer pathogenesis, risk, invasion, and metastasis. Although RET polymorphisms have been investigated in various cancers in many ethnicities and populations, there are limited studies in the literature on the effect of ARTN and PSPN polymorphisms on breast cancer risk. The study included 102 breast cancer patients and 102 healthy controls. rs3762422 and rs2304198 polymorphisms were detected by polymerase chain reaction and sequencing methods and genotype distributions were determined. The study groups showed a similar profile in terms of genotype distributions in both variants and no statistically significant difference was found ($p>0.05$, $p>0.05$, respectively). Our findings suggest that ARTN rs3762422 and PSPN rs2304198 polymorphisms are not associated with breast cancer risk in our cohort. In this study, we aimed to investigate and evaluate the possible risk between ARTN gene rs3762422 and PSPN gene rs2304198 variants and breast cancer. To our knowledge, this is the first study investigating these variants in breast cancer, and it may contribute to future research.
Keywords: Breast Cancer, RET, Artemin, Persephin, Single Nucleotide Polymorphism.

Ethics Committee Approval: The study was approved by Afyonkarahisar Health SciencesUniversity Noninterventional Clinical Research Ethical Committee (Decision no: 2011-KAEK-2 Date: 13.05.2022

Informed Consent: The authors declared that it was not considered necessary to get consent from the patients because the study was a retrospective data analysis.

Authorship Contributions: Medical Practices: OK, NK. Concept: TT, OK, NK Design: TT, AG. Data, Collection or Processing: Analysis or Interpretation: TT, OK, NK, OK, NK. Literature Search: TT, AG. Writing: TT, AG.

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Conflict of Interest: The authors declare that they have no competing interests.

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Özet: RET, çeşitli dokuların normal gelişimi ve olgunlaşması için gerekli olan reseptör tirozin kinaz ailesinin bir üyesidir. RET sinyalizasyonu, çevredeki stromayı ve hücre kompozisyonunu değiştiren tümör mikroçevresindeki değişiklikleri teşvik eder. RET ve ligandları artemin (ARTN) ve persefin (PSPN) gen polimorfizmleri, kanser patogenezi, riski, invazyonu ve metastazında bir araştırma odağıdır. RET polimorfizmleri birçok etnik köken ve popülasyonda çeşitli kanserlerde araştırılmış olsa da, literatürde ARTN ve PSPN polimorfizmlerinin meme kanseri riski üzerindeki etkisine ilişkin sınırlı sayıda çalışma bulunmaktadır. Çalışmaya 102 meme kanseri hastası ve 102 sağlıklı kontrol dahil edildi. rs3762422 ve rs2304198 polimorfizmleri polimeraz zincir reaksiyonu ve dizileme yöntemleri ile tespit edildi ve genotip dağılımları belirlendi. Çalışma grupları her iki varyantta da genotip dağılımları açısından benzer bir profil gösterdi ve istatistiksel olarak anlamlı bir fark bulunmadı (sırasıyla $p>0,05$, $p>0,05$). Bulgularımız, ARTN rs3762422 ve PSPN rs2304198 polimorfizmlerinin kohortumuzda meme kanseri riskiyle ilişkili olmadığını göstermektedir. Bu çalışmada ARTN geni rs3762422 ve PSPN geni rs2304198 varyantları ile meme kanseri arasındaki olası riski araştırmayı ve değerlendirmeyi amaçladık. Bilgilerimize göre bu çalışma meme kanserindeki bu varyantları araştıran ilk çalışmadır ve gelecekteki araştırmalara katkıda bulunabilir.
Anahtar Kelimeler: Meme Kanseri, RET, Artemin, Persephin, Tek Nükleotid Polimorfizmi

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1. Introduction

Breast cancer (BCA) is the most common cancer in women according to the World Health Organization, affecting 2.1 million women each year and causing the highest number of cancer-related deaths among women (1). REarranged during Transfection (RET) is the signaling receptor of a multimolecular complex, that binds growth factors of the glial cell line-derived neurotrophic factor (GDNF) family of neurotrophic factors. RET signaling is also mediated by the binding of soluble proteins of GDNF family members, including artemin (ARTN), persephin (PSPN), neurturin (NRTN), and GDNF. RET activation has been shown to increase proliferation, progression, and metastasis in various cancer types (2).

Neurotrophic factors are polypeptides that perform their functions by binding to specific cell surface receptors. Neurotrophic factors are divided into three groups: classical neurotrophins, GDNF ligands, and neuroprotective cytokines. ARTN and PSPN, which belong to the family of GDNF ligands, spread their oncogenic effects by activating several signaling pathways through the RET, causing cell survival, proliferation, differentiation, and migration (3). The protein complex formed by the binding of ARTN to its coreceptor GFRα3 and PSPN to its coreceptor GFRα4 activates the transmembrane receptor RET (2).

ARTN, promotes sensory neuron survival, peripheral nerve homeostasis and exhibits structural features of a dimeric and cysteine knot motif-like molecule that resembles distant members of the TGF-β superfamily (4). ARTN plays a role in breast carcinoma progression by increasing endothelial cell proliferation, migration, and invasion. Using xenograft experiments, ARTN-overexpressing breast carcinoma cells were found to induce tumor formation via increased microvessel density combined with increased VEGF-A expression (5). Genetic studies show that upregulation of ARTN increases resistance of breast carcinoma cells to trastuzumab. Gene silencing of ARTN increases the efficacy of trastuzumab (6).

PSPN, which is approximately 40% identical to GDNF and NRTN, was identified as a novel neurotrophic factor by polymerase chain reaction. PSPN, like GDNF and NRTN, supports the survival of ventral midbrain dopaminergic neurons in culture and prevents their degeneration after 6-hydroxydopamine treatment *in vivo* (7).

Activation of RET increases proliferation, invasion and metastasis in various cancer types. Functional polymorphic variants, mutations and increased expression of RET have been reported to influence oncogenic functions. In addition to RET mutations, RET polymorphisms, which are genetic alterations, have also been reported to be associated with increased risk of disease (8). However, previous studies have shown that SNPs analyzed in different ligands of RET may be related to cancer risk. Some studies on many polymorphic variants of RET in various cancers such as thyroid carcinoma, sporadic medullary thyroid carcinoma (sMTC), desmoplastic melanoma, lung cancer (9-12), but there is limited literature on the polymorphisms of ARTN and PSPN ligands that provide RET activation.

There are very limited studies on polymorphisms of RET ligands in breast cancer. The aim of our study, which focuses on this gap in the literature to investigate whether there is a relationship between ARTN and PSPN polymorphisms and BCA risk.

2. Materials and Method

The study consists of 102 patients diagnosed with BCA and 102 control group, who applied to Gazi University Faculty of Medicine Hospital and Dr. Abdurrahman Yurtaslan Ankara Oncology Training and Research Hospital General Surgery Outpatient Clinics. The study groups consisted of women between the years of 18-88. Both BCA and control groups are matched in terms of age (53.26±1.27; 52.02±1.34, respectively), body mass index (BMI) (27.50±0.45; 27.69±0.48, respectively), menouposal status before/after (48/62; 57;53 respectively), age of first menarche (13.05±0.13; 12.99±0.14, respectively) and family BCA history in patients (yes/no; 25/57). Women with BCA were selected from those who had not received radiotherapy or chemotherapy for any malignancy before. In addition, the control group consisted of women without any systemic disease. Participants were informed about the study and gave their consent. Afyonkarahisar Health Sciences University Clinical Research Ethics Committee approval was obtained for the study in accordance with the Declaration of Helsinki (approval no: 292).

The selection of SNPs to be studied was determined by screening previous studies and checking SNP databases such as Ensembl. Blood samples were taken from patients and controls in purple capped (K_3EDTA) tubes and were stored at -20°C until DNA isolation. DNA isolation from blood was

performed using EURx, GeneMATRIX Quick Blood DNA Isolation kit. Polymerase chain reaction (PCR) product was obtained by using thermal cycler. Appropriate primers were determined by searching the literature to detect SNPs belonging to the ARTN gene (OMIM 603886) rs3762422 and PSPN gene (OMIM 602921) rs2304198 regions. Primers required for polymorphism analyzes ARTN rs3762422, F-5'-GGAGGGGGACAGACCTAGAC-3', R-5'-GGGCTGGAGAAGAATTAGGG-3' and primers for PSPN rs2304198, F-5'-AGGGTCCCTGGAAGTCCTTA-3', R-5'-CGTAAATCAGGGGGTGTCTG-3' was synthesized.

Both of regions were amplified on the thermal cycler using PCR buffer 1x, $MgCl_2$ 1.3 mM, dNTP mix 0.2 mM, F primer 0.3 µM, R primer 0.3 µM, Taq DNA polymerase 2 U and DNA 20-150 ng. SNPs sequencing were performed on the obtained PCR products to detect. The PCR products were purified according to the kit procedure using the MAGBIO "HighPrep™ PCR Clean-up System" (AC-60005) purification kit for the single band samples obtained during the purification step. After purification, ABI 3730XL Sanger sequencing device (Applied Biosystems, Foster City, CA) and BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) were used for Sanger Sequencing samples. The obtained peaks were displayed in the Finch TV program and results were evaluated (15).

a. Statistical Analysis

SPSS version 26.0 for Windows (SPSS Inc, Chicago, IL, USA) was used in the analysis of the data obtained in the study. The independent sample t-test was used to compare the means of quantitative variables according to paired groups and for groups of more than two, used One Way ANOVA. Pearson's chi-square analysis and Fisher's Exact test were used to compare frequency comparisons in qualitative variables and control-patient distributions according to genotype frequencies. Logistic regression analysis was used to examine genotype differences.

3. Results

Polymorphism analysis was performed in the ARTN gene rs3762422 and PSPN gene rs2304198 regions in the patient and control groups. The relationship of G691S, L769L, and S904S genotype distributions with BCA risk was investigated.

No significant difference was found between the patient and control groups in terms of age, BMI, menopausal status, age at first menarche, family history of BCA ($p>0.05$). Sample sizes of BCA patients' clinical characteristics groups and subgroups are given in Table 1.

Table 1. Sample sizes of BCA patients' clinical characteristics groups and subgroups

				ARTN rs3762422		PSPN rs2304198	
			N	**Wild +Homozygote**	**Heterozygote**	**Wild +Homozygote**	**Heterozygote**
Histological	Ductal Carcinoma In situ		12	9	3	10	2
	Invasive Ductal Carcinoma		67	32	35	52	14
	Invasive Lobular Carcinoma		8	3	5	8	0
	Mixed Type		9	7	2	6	2
	Other		6	3	3	4	2
Molecular	Luminal A		23	9	14	18	5
	Luminal B		58	32	26	47	9
	Her2		5	5	0	3	2
	Bazal Type		5	2	3	5	0
Receptor Status	**ER**	Positive	82	42	40	65	17
		Negative	10	7	3	8	2
	PR	Positive	15	9	6	13	2
		Negative	76	39	37	60	14
	HER2/neu	Positive	62	33	29	52	10
		Negative	26	14	12	20	6
Tumor Grade	1		16	6	10	11	5
	2		36	21	17	27	9
	3		35	19	16	32	3

Following the obtaining of ARTN rs3762422 and PSPN rs2304198 PCR products in the study groups, it was confirmed that the correct PCR product was obtained as a result of the electrophoresis process in 2% gel. PCR products of each patient and control were sequenced using the Sanger Sequencing method. Then, the obtained sequences were examined one by one with the Finch TV program and the analysis results were recorded.

No significant difference was found in terms of BCA risk in the ARTN rs3762422 in the patient and control groups (p>0.05, OR: 0.790, CI: 0.456-1.369). Likewise, both BCA and control groups do not differ in allele frequencies (p>0.05, OR: 0.809, CI: 0.427-1.533) (Table 2). Chromatogram images of the ARTN rs3762422 SNP are given in Figure 1A.

Table 2. ARTN rs3762422 allele frequencies and genotype distributions of the study groups

		Control (n=102)	BCA (n=102)	x^2 Testi	OR (95% CI)	p
Allele Frequencies	**A**	95 (61.3%)	91 (61.1%)	x^2=0.470 p=0.68	1 (reference)	-
	T	60 (38.7%)	58 (38.9%)		0.809 (0.427-1.533)	0.515
Genotype Distribution	**AA+TT**	48 (47.1%)	54 (52.9%)	x^2=0.706 p=0.401	1 (reference)	-
	TA	54 (52.9%)	48 (47.1%)		0.790 (0.456-1.369)	0.553

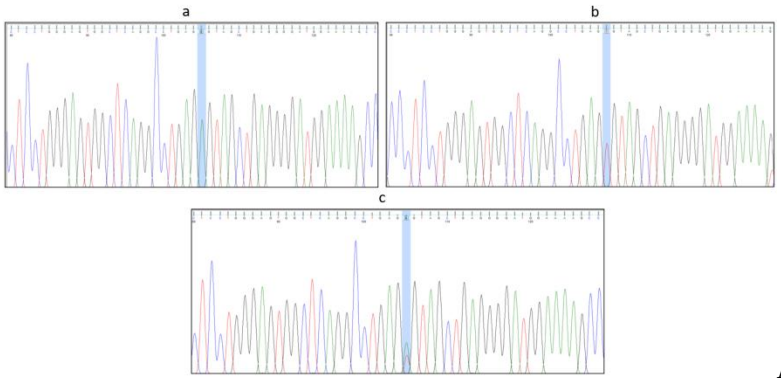
BCA: Breast cancer, OR: Odds Ratio was calculated by logistic regression analysis, CI: Confidence interval

Similarly, no difference was found in terms of genotype distributions for the PSPN rs2304198 (p>0.05, OR: 0.792, CI: 0.405-1.549). In the same way, allele frequencies of study groups do not indicate significance (p>0.05, OR: 0.475, CI: 0.434-1.607) (Table 3). Chromatogram images of the PSPN rs2304198 SNP are given in Figure 1B.

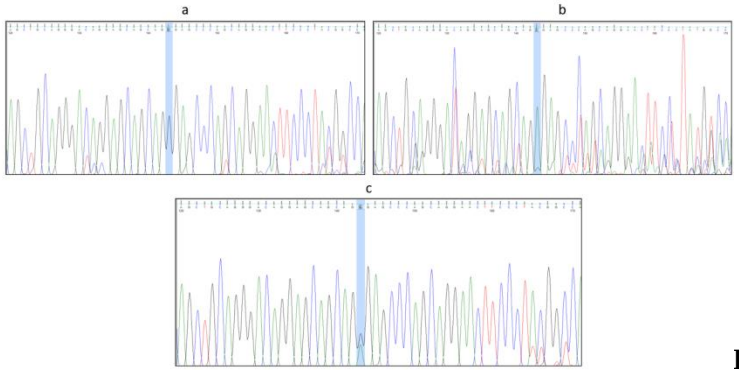
Table 3. PSPN rs2304198 allele frequencies and genotype distributions of the study groups

		Control (n=102)	BCA (n=102)	x^2 Testi	OR (95% CI)	p
Allele Frequencies	**G**	100 (78.7%)	99 (80.5%)	x^2=0.645 p=0.73	1 (reference)	-
	A	27 (21.3%)	24 (19.5%)		0.475 (0.434-1.607)	0.590
Genotype Distribution	**GG+AA**	78 (76.5%)	80 (78.4%)	x^2=0.466 p=0.495	1 (reference)	-
	GA	24 (23.5%)	22 (21.6%)		0.792 (0.405-1.549)	0.749

BCA: Breast cancer, OR: Odds Ratio was calculated by logistic regression analysis, CI: Confidence interval



A



B

Figure 1. A. *ARTN rs2304198 SNP chromatograms a) AA b) TT c) TA* ***B.*** *PSPN rs2304198 SNP chromatograms a) GG b) AA c) GA*

4. Discussion and Conclusion

Neurotrophic factors are important regulatory proteins and they promote the survival, proliferation, and differentiation of neurons by inducing intracellular signalling through specific receptors. The well-characterised GDNF ligand family, consists of members: GDNF, NRTN, ARTN, and PSPN. Their signalling is mediated by a receptor complex consisting of the signal transduction module receptor tyrosine kinase RET and the ligand-binding co-receptor GFRα. ARTN has been shown to bind to GFRα3 and PSPN to GFRα4 (3). Activation of RET increases proliferation, invasion, and metastasis in various types of cancer. It is reported that functional polymorphic variants, mutations, and increased expression of RET have an effect on oncogenic functions. RET signaling is mediated by binding of soluble proteins of the GDNF family, including ARTN and PSPN. There are studies on many polymorphic variants of RET in various cancers, but there is limited literature on ARTN rs3762422 and PSPN rs2304198 polymorphisms that provide RET activation (9-12). In our study, the effect of single nucleotide polymorphisms on ARTN rs3762422 and PSPN rs2304198 genes, which are RET ligands, on BCA risk was investigated.

It has been reported in various studies that polymorphisms in RET ligands may change the course of some diseases. The effect of GDNF variants on Multiple endocrine neoplasia type 2A disease has been investigated (13). The effects of ARTN rs3762422 and PSPN rs2304198 SNPs on disease risk were evaluated in a study consisting of 135 sporadic medullary thyroid carcinoma (sMTC) patients and 533 healthy controls. The ARTN rs3762422 variant showed a significant association with sMTC (OR:1.89; 95% CI, 1.26-2.82; P=0.003). However, no statistical significance was found for the PSPN rs2304198 variant (14). In a study involving 142 Hirschsprung's disease patients and 99 controls, ARTN rs3762422 and PSPN rs2304198 SNPs were analysed and their disease predisposition was evaluated. The results showed that the differences in the frequencies and distributions of all polymorphic variants were not statistically significant when the patient group was compared with the controls. Therefore, it was concluded that the variants do not predispose to Hirschsprung's disease (15). In our previous study, we examined RET G691S, L769L, S904S, and NRTN IVSI-663 polymorphisms. We found that they did not affect the disease risk for G691S, L769L, and S904S. However, we found that RET ligand NRTN, like ARTN and PSPN, IVSI-663 polymorphism genotype distribution, statistically significant difference was found in the AA genotype in the heterozygous GA genotype reference and it demonstrates there could be an association with a 0.43 times lesser BCA risk due to the AA genotype. BCA risk was examined in terms of allele frequency, the G allele could demonstrate an association more the risk of BCA 1.54 times (16). In our knowledge, we examined the SNPs of ARTN and PSPN that have been examined first in the literature in terms of BCA risk. There are points where the lack of significance in the results is consistent with other studies. This may be due to the effect of working with a specific population with a small number of samples. More precise information can be obtained in studies specific to BCA subgroups.

The data obtained in our previous study showed that it is important to examine the effect of RET ligands such as ARTN and PSPN on cancer progression mediated by RET activation. In recent years, research on cancer types has focused on RET. Thus, RET ligands such as ARTN and PSPN have started

to attract attention as an important topic in cancer studies. Further studies are needed on the effect of rs3762422 and rs2304198 variants on BCA risk.

This study has some limitations. First of all, the relatively limited sample size reduces the statistical power and limits the generalizability of the results. Secondly, genetic polymorphisms and potential gene-gene interactions could not be evaluated in this study. Thirdly, since the BCA subgroup distribution of the patient individuals included in the study was heterogeneous, the SNP analysis specific to these groups was limited because there were not enough numbers in the subgroups.

In conclusion, we think that further studies should be conducted in larger populations and with different mutations and variants to re-evaluate the effects of epistasis analysis on BCA risk. Investigating how ARTN and PSPN gene polymorphisms affect cancer risk may be an important step for treatment. Further investigations are required to identify the protein expressions ARTN and PSPN.

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