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FOXP3 Genetic Variation at Position -2383 C/T (rs3761549) in Patients with Breast Cancer

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Abstract

Background: We investigated the possibility of an association between the C-2383T polymorphism (rs3761549) in the promoter region of the FOXP3 gene with breast cancer.

Methods: The study groups consisted of 250 women diagnosed with breast cancer (case group) and 250 healthy women (control group). Polymerase chain reaction-restriction fragment length polymorphism was performed for genotyping.

Results: Breast cancer cases had a C allele frequency of 455 (91%) and T allele frequency of 45 (9%). The control group had a C allele frequency of 468 (93.6%) and 32 (6.4%) for the T allele. The CC genotype was present in 206 (82.4%) patients, 43 (17.2%) patients had the CT genotype, and the TT genotype was observed in 1 (0.4%) patient. In the control group, 219 (87.6%) had the CC genotype, whereas 30 (12%) had the CT genotype, and 1 (0.4%) had the TT genotype. Statistical analysis revealed no significant differences in the distribution of alleles and genotypes between patients and controls ($P>0.05$). There was no significant association between genotype distribution and patients' clinicopathological factors.

Conclusion: Our results do not support an association between the FOXP3 -2383 C/T polymorphism and breast cancer in a population from southern Iran.

Keywords: Breast cancer, FOXP3 gene, Single nucleotide polymorphism, PCR-RFLP

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Introduction

Breast cancer is one of the most common malignancies in women that originates from epithelial cells of breast ducts and lobules.[1] This disease is the second leading cause of cancer deaths among women in the United States.[2] In 2012, nearly 1.7 million women were diagnosed with breast cancer among which 522,000 died from this disease.[3] In Iran, 22 out of 100,000 women are affected with breast cancer.[4] Many proposed breast cancer risk factors include gender, age, race and ethnicity, habits and lifestyle, weight, early menstruation, age at first pregnancy, environmental factors, family background, and hormonal factors (endogenous and exogenous), as well as estrogen and progesterone receptors.[5-8] Studies on twins have shown that around 27% of breast cancer cases are caused by hereditary factors.[9]

Regulatory T cells are a subset of lymphocytes with the $CD25^+$and $CD4^+$phenotypes, and high abundant expression of $FOXP^3$ transcription factor.[10] $FOXP^3$ is a tumor suppressor gene located on the short arm of the X chromosome. It is a member of the Forkhead transcription factor family and possesses a DNA binding domain.[11] Unlike other members of this group, $FOXP^3$ is mainly expressed in regulatory T cells that suppress the immune system. In regulatory T cells, $FOXP^3$ plays a role as a dominant regulator of cell function, growth, and development. This protein is essential for regulatory T cell precursor survival.[12,13] $FOXP^3$ is also expressed by prostate, breast, lung and ovarian epithelial cells. In normal mammary epithelial cells, $FOXP^3$ binds oncogenes and inhibits their expression.[14] Although regulatory T cells suppress immune responses, a tumor escape mechanism is activated. Increased $TCD4^+$ $CD25^+$ cells that express $FOXP^3$ are frequently reported in cancer patients.[15] Increased $FOXP^3$ expression is reported in many tumors.[16]

There are various polymorphisms of the $FOXP^3$ gene.[17,18] Polymorphisms in this gene may alter protein function and, consequently, the function of regulatory T cells. Several studies have shown a relationship between single nucleotide polymorphisms (SNP) in the $FOXP^3$ gene and autoimmune diseases.[19,20] In the present study, we examined the -2383 C/T gene polymorphism (rs3761549) in the promoter region of $FOXP^3$ in women with breast cancer compared to a control group of healthy women.

Subjects and Methods

Subjects

In this case-control study, we recruited 250 patients with breast cancer (cases) and 250 healthy women (controls). Histopathological examination of the excised sample during surgery confirmed that individuals in the case group had breast cancer. This study was approved by the ethics committee of Shiraz University of Medical Sciences and informed consents were obtained from all participants before sample collection. Data on clinicopathological characteristics of the patients were obtained from their medical files (Table 1).

DNA analysis

Genomic DNA was extracted from peripheral whole blood by the proteinase K method. Genotyping of rs3761549 SNPs in the FOXP3 gene was carried out by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. The sequence of the primers used for amplification of the target sequence was 5′-CTGAGACTTTGGGACCGTAG-3′ as the forward primer and 5′-TGCGCCGGGCTTCATC-GACA-3′ as the reverse primer.[21] The PCR thermal program was as follows: first denaturation at 94ºC for 5 min, followed by 30 cycles at 94ºC for 30 sec, 60.5ºC for 30 sec, and 72ºC for 30 sec, with a final extension at 72ºC for 10 min. The PCR products were run on 2% agarose gel to visualize the initial bands. Then, 5 µL of PCR products were added to 5 µL of the restriction enzymatic reaction mixture that contained 0.5 µL-10 U/µL of BseNI (BsrI; Fermentas, Lithuania), 1 µL of 10X PCR buffer , and 3.5 µL of dH_2O. The mixture was incubated at 65ºC for 16 h. Finally, the enzyme-

digested products were separated on 2.7% agarose gel by electrophoresis and migrating DNA bands were visualized under a UV transilluminator (Gel Doc, Bio-Rad Laboratories Ltd., Hercules, CA, USA). The resultant bands consisted of 2 fragments of 261 bp and 127 bp for the TT genotype; 3 fragments of 184, 127, and 77 bp for the CC genotype; and 4 fragments of 261,184, 127, and 77 bp for the CT genotype (Figure 1).

Statistical analysis

Deviation from Hardy-Weinberg equilibrium was assessed by the χ^2 test. This test was also used to investigate the differences in genotype and allele distribution between cases and controls. $P<0.05$ was considered significant. All statistical analysis were performed using SPSS 16 for Windows (SPSS GmbH Software, Germany).

Results

Characteristics of the study participants

In this study, we assessed the genotypes of 250 women with breast cancer and 250 healthy women from southern Iran for the FOXP3 gene polymorphism at position C-2383T. Patients and controls were matched in terms of age and sex. Patients had an average age of 50.05±11.81 years and the average age for the control group was 51.50±12.98 years. Distribution of genotypes and controls did not deviate from the Hardy-Weinberg equilibrium.

As illustrated in table 2, the results showed that in the case group the frequency of the C allele was 455 (91%) and for the T allele, it was 45 (9%). In the control group, 468 (93.6%) had the C allele and 32 (6.4%) had the T allele. In the case group, the number of individuals with the CC genotype was 206 (82.4%), whereas there were 43 (17.2%) with the CT genotype, and one (0.4%) had the TT genotype. There were 219 (87.6%) in the control group who had the CC genotype, 30 (12%) had the CT genotype, and one (0.4%) person had the TT genotype. Statistical analysis revealed no significant differences in the distribution of alleles and genotypes between patients and controls ($P>0.05$).

There was no significant association between genotype distribution and patients' clinicopathological factors of tumor type, tumor size, tumor stage, lymph node involvement, calcification, peritumoral vessel invasion, distant metastasis, histological grade, nuclear grade, and tumor necrosis (data not shown, $P>0.05$)

Discussion

Breast cancer is the most common cancer among women. Investigations have suggested a role for genetic factors in predisposing individuals to breast cancer. Genetic factors related to antitumor immunity have been among the top candidates for genetic-disease association studies in recent decades. In the present study, we investigated a single nucleotide genetic variation in the FOXP3 gene at position -2383 C/T (rs3761549) in a population of women from southern Iran diagnosed with breast cancer compared to age-sex matched healthy controls. Statistical analysis revealed no significant associations between the inherited genotypes or alleles and the risk of breast cancer. No significant association was also found between genotype

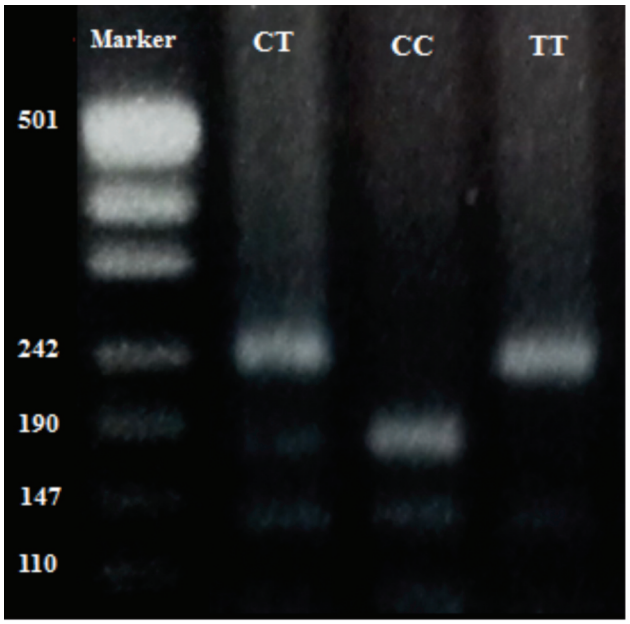

Figure 1. Genotyping of the C-2383T polymorphism in the promoter region of the FOXP3 gene in breast cancer patients by using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Left to right: PUC19 marker, CT (261, 184, 127, and 77 bp), CC (184, 127, and 77 bp), and TT (261 and 127 bp).

Table 1. Breast cancer patients' characteristics

Clinicopathological features		Total	Statistics N (%)
Gender	Female:	250	250 (100)
Tumor type	IDC:	231	212 (84.8)
	Other:		12 (4.8)
	No tumor:		7 (2.8)
	Unknown*:		19 (7.6)
Tumor size	<2 cm:	195	80 (32)
	≥2 to 5 cm:		98 (39.2)
	>5 cm:		17 (6.8)
	Unknown:		55 (22)
Stage	I:	181	42 (16.8)
	IIa:		53 (21.2)
	IIb:		28 (11.2)
	IIIa:		30 (12)
	IIIc:		27 (10.8)
	IV:		1 (0.4)
	Unknown:		69 (27.6)
Histological grade	Well-differentiated:	163	45 (18)
	Moderately differentiated:		71 (28.4)
	Poorly differentiated:		47 (18.8)
	Unknown:		87 (34.8)
Nuclear grade	ell-differentiated:	61	W 19 (7.6)
	Moderately differentiated:		18 (7.2)
	Poorly differentiated:		24 (9.6)
	Unknown:		189 (75.6)
Tumor necrosis	Positive:	175	102 (40.8)
	Negative:		73 (29.2)
	Unknown:		75 (30)
Calcification	Present:	188	71 (28.4)
	Absent:		117 (46.8)
	Unknown:		62 (24.8)
Peritumoral vessel invasion	Positive:	152	100 (40)
	Negative:		52 (20.8)
	Unknown:		98 (39.2)
Axillary lymph node involvement	Positive:	176	95 (38)
	Negative:		81 (32.4)
	Unknown:		74 (29.6)
Distant metastasis at the time of diagnosis	Negative:	181	180 (72)
	Positive:		1 (0.4)
	Unknown:		69 (27.6)

*Known cases with breast cancer whose tumor type was not found in the medical records. IDC: Invasive ductal carcinoma

distribution and patients' clinicopathological factors.

Located in the promoter region of the FOXP3 gene, the C to T exchange at the -2383 position is a potential candidate for the modification of promoter activity, and has been reported to be associated with different immune-related diseases. Despite the potential of this SNP in the FOXP3 gene promoter region, and consistent with our study, Jahan et al. did not find an association between the rs3761549 polymorphism (-2383 C/T) and breast cancer in an Indian population.[22] In the same study, no association was found between another FOXP3 polymorphism, rs3761548, and breast cancer.[22] Jiang and Ruan investigated both rs3761549 and rs3761548 SNPs and did not find any association between the investigated SNPs and the risk of breast cancer in

Table 2. Allele and genotype frequencies in the patient and control groups

C-2383T polymorphism		Patients (N, %)	Controls (N, %)	*P*-value	Odds ratio (OR)	95% CI* for OR
Allele frequency	C	455 (91)	468 (93.6)	0.15	0.69	0.42-1.13
	T	45 (9)	32 (6.4)			
Genotype frequency	CC	206 (82.4)	219 (87.6)	0.13	0.66	0.39-1.12
	CT	43 (17.2)	30 (12)	0.13	1.52	0.89-2.6
	TT	1 (0.4)	1 (0.4)	-	-	-

*CI: Confidence interval

a Chinese population.[23] In the same study, however, they showed a positive relation between the 3761549 SNP and 3761548 SNP with two other malignancies, hepatocellular carcinoma and lung cancer.[23] These findings, in concordance with our study, collectively suggested that 3761549 SNP could not be considered a risk locus for breast cancer.

In another study, Raskin et al. did not show an association between breast cancer in Jewish women with the rs3761548, rs2294020, and rs5906761 polymorphisms, the other SNPs of FOXP3 gene.[24] Although the findings of our study did not verify an association between 3761549 SNP with breast cancer, later studies collectively suggested that other SNPs in the FOXP3 gene might be considered as breast cancer risk factors.

The discrepancies in findings that pertained to the rs3761549 SNP might also arise from the differences in molecular pathology of the diseases. Mojtahedi et al. reported an association between rs3761549 SNP with colorectal cancer in a population from southern Iran.[21] Despite no association between rs3761549 SNP with breast cancer in a study by Jiang and Ruan, this group reported an association between the rs3761549 SNP and increased risk of hepatocellular carcinoma and lung cancer.[23] Associations of 3761549 SNP with other non-malignant disorders have been reported and include chronic hepatitis B in a Chinese population,[25] autoimmune thyroid diseases (Graves and Hashimoto's thyroiditis) in a Japanese population,[26] and endometriosis in a population from Brazil.[27]

Breast cancer is a multifactorial disease and a variety of genetic and environmental factors may participate in its molecular pathology. Different immune-related genes may have a role in the incidence of this disease. Not all risk factors and interactions between genes or between the environment and genes involved in breast cancer have been identified. Our data collectively suggested a lack of any association between the 3761549 SNP in the FOXP3 gene with breast cancer in a population from southern Iran. Other SNPs in the FOXP3 gene might be considered as breast cancer risk factors. This research area would merit additional investigations.

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Conflict of Interest

None declared.

References

1. Ferlay J, Héry C, Autier P, Sankaranarayanan R. In: Li C, editor. Breast cancer epidemiology. Springer: New York, NY; 2010. p.1-19.
2. Kelsey JL. Breast cancer epidemiology: summary and future directions. *Epidemiol Rev.* 1993;15(1):256-63.

3. Tao Z, Shi A, Lu C, Song T, Zhang Z, Zhao J. Breast cancer: Epidemiology and etiology. *Cell Biochem Biophys*. 2015;72(2):333-8. doi: 10.1007/s12013-014-0459-6.
4. Mousavi SM, Montazeri A, Mohagheghi MA, Jarrahi AM, Harirchi I, Najafi M, et al. Breast cancer in Iran: an epidemiological review. *Breast J.* 2007;13(4):383-91.
5. McPherson K, Steel CM, Dixon JM. ABC of breast diseases. Breast cancer-epidemiology, risk factors, and genetics. *BMJ*. 2000;321(7261):624-8.
6. Collaborative Group on Hormonal Factors in Breast Cancer. Menarche, menopause, and breast cancer risk: individual participant meta-analysis, including 118 964 women with breast cancer from 117 epidemiological studies. *Lancet Oncol.* 2012;13(11):1141-51. doi: 10.1016/S1470-2045(12)70425-4.
7. Colditz GA, Rosner BA, Chen WY, Holmes MD, Hankinson SE. Risk factors for breast cancer according to estrogen and progesterone receptor status. *J Natl Cancer Inst.* 2004;96(3):218-28.
8. Key TJ, Verkasalo PK, Banks E. Epidemiology of breast cancer. *Lancet Oncol*. 2001;2(3):133-40.
9. Colditz GA, Rosner BA, Speizer FE. Risk factors for breast cancer according to family history of breast cancer. For the Nurses' Health Study Research Group. *J Natl Cancer Inst.* 1996;88(6):365-71.
10. Bandukwala HS, Wu Y, Feuerer M, Chen Y, Barboza B, Ghosh S, et al. Structure of a domain-swapped FOXP3 dimer on DNA and its function in regulatory T cells. *Immunity*. 2011;34(4):479-91. doi: 10.1016/j.immuni.2011.02.017.
11. Zuo T, Wang L, Morrison C, Chang X, Zhang H, Li W, et al. FOXP3 is an X-linked breast cancer suppressor gene and an important repressor of the HER-2/ErbB2 oncogene. *Cell*. 2007;129(7):1275-86. Erratum in: *Cell*. 2008;134(3):546.
12. Zheng Y, Rudensky AY. FOXP3 in control of the regulatory T cell lineage. *Nat Immunol.* 2007;8(5):457-62.
13. Fontenot JD, Rasmussen JP, Williams LM, Dooley JL, Farr AG, Rudensky AY. Regulatory T cell lineage specification by the forkhead transcription factor foxp3. *Immunity*. 2005;22(3):329-41.
14. Zhang HY, Sun H. Up-regulation of FOXP3 inhibits cell proliferation, migration and invasion in epithelial ovarian cancer. *Cancer Lett.* 2010;287(1):91-7. doi: 10.1016/j.canlet.2009.06.001.
15. Liu VC, Wong LY, Jang T, Shah AH, Park I, Yang X, et al. Tumor evasion of the immune system by converting $CD4^+CD25^-$ T cells into $CD4^+CD25^+$ T regulatory cells: role of tumor-derived TGF-beta. *J Immunol.* 2007;178(5):2883-92.
16. Tan B, Anaka M, Deb S, Freyer C, Ebert LM, Chueh AC, et al. FOXP3 over-expression inhibits melanoma tumorigenesis via effects on proliferation and apoptosis. *Oncotarget*. 2014;5(1):264-76.
17. Floess S, Freyer J, Siewert C, Baron U, Olek S, Polansky J, et al. Epigenetic control of the FOXP3 locus in regulatory T cells. *PLoS Biol.* 2007;5(2):e38.
18. Bennett CL, Christie J, Ramsdell F, Brunkow ME, Ferguson PJ, Whitesell L, et al. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat Genet.* 2001;27(1):20-1.
19. Oda JM, Hirata BK, Guembarovski RL, Watanabe MA. Genetic polymorphism in FOXP3 gene: imbalance in regulatory T-cell role and development of human diseases. *J Genet*. 2013;92(1):163-71.
20. d'Hennezel E, Ben-Shoshan M, Ochs HD, Torgerson TR, Russell LJ, Lejtenyi C, et al. FOXP3 forkhead domain mutation and regulatory T cells in the IPEX syndrome. *N Engl J Med*. 2009;361(17):1710-3. doi: 10.1056/NEJMc0907093.
21. Mojtahedi Z, Erfani N, Haghshenas MR, Hosseini SV,Ghaderi A. Association of FOXP3/Scurfin germline polymorphism (C-2383T/rs3761549) with colorectal cancer. *Ann Colorectal Res*. 2013;1(1):12-6. doi: 10.17795/acr-11478.
22. Jahan P, Ramachander VR, Maruthi G, Nalini S, Latha KP, Murthy TS. FOXP3 promoter polymorphism (rs3761548) in breast cancer progression: a study from India. *Tumour Biol*. 2014;35(4):3785-91. doi: 10.1007/s13277-013-1501-9.
23. Jiang LL, Ruan LW. Association between FOXP3 promoter polymorphisms and cancer risk: A meta-analysis. *Oncol Lett.* 2014;8(6):2795-99.
24. Raskin L, Rennert G, Gruber SB. FOXP3 germline polymorphisms are not associated with risk of breast cancer. *Cancer Genet Cytogenet*. 2009;190(1):40-2. doi: 10.1016/j.cancergencyto.2008.12.005.
25. Chen Y, Zhang H, Liao W, Zhou J, He G, Xie X, et al. FOXP3 gene polymorphism is associated with hepatitis B-related hepatocellular carcinoma in China. *J Exp Clin Cancer Res.* 2013;32:39. doi: 10.1186/1756-9966-32-39.
26. Inoue N, Watanabe M, Morita M, Tomizawa R, Akamizu T, Tatsumi K, et al. Association of functional polymorphisms related to the transcriptional level of FOXP3 with prognosis of autoimmune thyroid diseases. *Clin Exp Immunol*. 2010;162(3):402-6. doi: 10.1111/j.1365-2249.2010.04229.x.
27. André GM, Barbosa CP, Teles JS, Vilarino FL, Christofolini DM, Bianco B. Analysis of FOXP3 polymorphisms in infertile women with and without endometriosis. *Fertil Steril.* 2011;95(7):2223-7. doi: 10.1016/j.fertnstert.2011.03.033.