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FoxP3 GENE POLYMORPHISM IS ASSOCIATED WITH BREAST CANCER IN IRANIAN PATIENTS

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Aim: **Breast cancer (BC) is one of the leading causes of cancer death among women. Recent studies have characterized FoxP3 as a marker of regulatory T cells and an X-linked tumor suppressor gene, which is involved in the pathogenesis of BC. Therefore, we investigated the potential influence of three single-nucleotide polymorphisms (SNPs) of *FoxP3* gene on the development of BC in Iranian women.** ***Materials and Methods:*** **The association between *FoxP3* rs2232365, rs3761548 and rs4824747 polymorphisms and BC risk was assessed in 124 BC patients and 198 healthy controls using sequence-specific primers.** ***Results:*** **We identified significant difference of rs3761548 in both allele and genotype frequencies between cases and control groups. Our results showed that individuals carrying *FoxP3* rs3761548 AA genotype had about 4.3-fold increased risk of BC compared with CC carriers. No significant association was found between rs3761548C>A polymorphism and clinical outcome parameters (age of onset, tumor size, lymph nodes metastasis, tumor stage, progesterone receptor status, estrogen receptor status, Ki-67 status, HER-2 status and duration of disease).** ***Conclusion:*** **This study has provided the first genetic data on the *FoxP3* gene polymorphism in south of Iran and proposes the rs3761548 polymorphism of *FoxP3* gene as a risk factor, but not a prognostic marker in the development of BC in Iranian population.**
Key Words: **breast cancer, FoxP3, polymorphism, Treg cells.**

Breast cancer (BC) is the most common cancer among women, comprising approximately one of fourth of female cancers worldwide [1]. Both epidemiological and cell biology studies have documented the contribution of multiple susceptibility loci to the development of BC [2, 3]. Forkhead box protein 3 (FoxP3) gene, which more recently has been implicated in the development of BC [4]. FoxP3, was initially identified as a gene responsible for X-linked autoimmune diseases in mice and humans (immune dysregulation, polyendopathy, enteropathy, X-linked, IPEX), and a master regulator of the development and function of regulatory T cells (Treg) [5]. Subsequent studies have revealed that FoxP3 exerts tumor-suppressor activity in breast and prostate cancers [6, 7]. The role of FoxP3 in the development and metastatic spread of BC is supported by several lines of evidence. First, the broad expression of FoxP3 gene in breast epithelial cells, and its down regulation in the mammary cancer tissues. Second, the high rate of *FoxP3* mutations or deletion in the majority of BC samples [4]. Third, the high incidence of BC in FoxP3 mutant mice. Forth, tumor growth inhibition following transfection of *FoxP3* cDNA into BC cells [8]. FoxP3 is a potent transcriptional repressor of several oncogenes including c-MYC [9], HER-2 and S phase kinase protein 2 (Skp2) [8] which all play an important role in BC development [10, 11]. McInnes *et al.* study [12] showed that part of FoxP3 tumor suppressor activity in normal breast is exerted through direct interaction with SATB1 promoter. Furthermore, FoxP3 also induces miR-7 and miR-155, which target the 3'-UTR of *SATB1* and regulate its expression [12]. Although recent researches have pointed out carcinogenic effects of *FoxP3* gene polymorphisms [13–16], there are few studies addressing the *FoxP3* polymorphisms in BC [17–21]. To evaluate the potential influences of *FoxP3* gene polymorphisms rs2232365, rs3761548 and rs4824747 on BC risk, a case — control study was conducted among Fars ethnic women, living in Hormozgan. To the best of our knowledge, this is the first study reported from this region of Asia in relation to FoxP3 and BC.

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Abbreviations used: BC – breast cancer; PCR – polymerase chain reaction; SNP – single-nucleotide polymorphism; SSP-PCR – sequence-specific primed polymerase chain reaction.

MATERIALS AND METHODS

Subjects. In total, 124 female BC patients and 198 healthy female controls who had given informed consent were enrolled. All BC patients had pathologically confirmed primary breast carcinoma, and all were diagnosed and treated at the Shahid Mohammadi Hospital, Bandar Abbas, Hormozgan, Iran. There was no significant difference in age between the patients and controls. The control population was matched with the case population based upon age and ethnicity and had no history of personal and familial malignancy, autoimmune disorders, alcohol consumption and cigarette

smoking. To ensure homogeneity of the genetic background, all subjects resided in the same geographic area (Hormozgan) and were originally from Fars ethnicity. Clinical and demographical information such as age, age of onset, tumor size, nodes involvement, presence or absence of metastasis, cancer stage, duration of disease (the time from diagnosis to sample collection), HER2, estrogen and progesterone receptor status and Ki-67 were collected from the patients' medical records with the help of the oncologist. The study was approved by the Ethics Committee of Hormozgan University of Medical Sciences.

Informed consent. Informed consent was obtained from all individual participants included in the study.

Ethical approval. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Inclusion criteria. Patients were recruited in the study according to the international society for the study of cancer. Those who were diagnosed with bloody discharge, peau d'orange skin, nipple retraction, painless lump in the breast or those who had abnormal mammograms and confirmed by fine needle aspiration cytology, Tru-cut needle biopsy or underwent curative surgery were included in the study.

Exclusion criteria. Patients who had other breast diseases like abscess, phyllodes tumor, and fibroids, history of autoimmune disorders or taking combined hormone replacement therapy, alcoholics and smokers were excluded.

Genotyping. Genomic DNA was extracted from peripheral blood samples of patients and healthy individuals with QIAGEN DNA Extraction kit, Germany. *FoxP3* genotyping for all three single-nucleotide polymorphism (SNPs) rs2232365, rs4824747 and rs3761548 were done by sequence-specific primed polymerase chain reaction (SSP-PCR) assay.

The allele-specific primers used are listed in Table 1. Primers were designed using the Primer Blast and Gene Runner software. PCR reactions were carried out in 20 µl aliquots containing 0.3 µl of *Taq* DNA polymerase (5 unit/µl), 0.4 µl of each dNTP (stock concentration of 10 mM), 0.7 µl of each primer pair (stock concentration of 10 pM), 2 µl PCR buffer 10×, 200–300 ng of genomic DNA and sterile double distilled water. The PCR amplification started with initial denaturation at 95 °C for 5 min and followed for 36 cycles with denaturation at 94 °C for 40 s, annealing at 57 °C for 40 s, extension at 72 °C for 1 min and a final extension at 72 °C for 7 min. The amplified products were separated by electrophoresis on a 2% agarose gel and stained with Gel Red (Sigma). 100 bp DNA ladder (SMOBiO, Taiwan) was used to determine the size of PCR products (Figure).

Table 1. Primer sequences used for SSP-PCR

SNP	Primer sequences	Size of PCR product
rs2232365	F1 5'-CCAGCTCAAGAGACCCCG-3' F2 5'-CCAGCTCAAGAGACCCCA-3' R 5'-GCTATTGTAACAGTCCTGGCAAGTG-3'	426 bp
rs4824747	F1 5'-AGCCACACCTACAGTTTCCTGG-3' F2 5'-AGCCACACCTACAGTTTCCTGT-3' R 5'-CGCTTTCTAGAGGACCAGTT-3'	425 bp
rs3761548	F1 5'-CTGGCTCTCTCCCCAACTGA-3' F2 5'-TGGCTCTCTCCCCAACTGC-3' R 5'-ACAGAGCCCATCATCAGACTCTCTA-3'	332 bp
GAPDH	F 5'-GCAGCCCTGGAGCCTTCA-3' R 5'-TTACCATATACCCAAGGGAGCC-3'	581 bp

Statistical analysis. Data were expressed as the mean ± standard deviation (SD) or number (%). The mean age in cases and controls was compared using Student's test. Genotype frequencies of the three *FoxP3* polymorphisms were tested for deviation from the Hardy — Weinberg equilibrium by using Chi-square (χ^2) test. In cases and control groups, allele and genotyping frequencies were compared using χ^2 test. The disease risk was assessed using odds ratio (OR) and 95% confidence interval (CI). $p < 0.05$ was considered as statistical significance. Statistical analyses were carried out using SPSS release 16 software (SPSS Inc, Chicago, IL, USA).

RESULTS

Demographic characteristics. Table 2 shows the base line characteristics and tumor biological factors of the BC subjects. 124 women suffering from BC and 198 healthy women controls were enrolled in the study. Written informed consent was obtained from all subjects. The mean ages of healthy controls and BC patients were respectively 49.4 ± 5.4 and 50.6 ± 12.0 years which were not significantly different ($p > 0.05$). Age of patients ranged from 27 to 69 years (mean of 48.4 ± 11.7 years) at the time of diagnosis. All subjects were genotyped for three SNPs (rs2232365, rs3761548 and rs4824747) and the SNPs distributions were in Hardy — Weinberg equilibrium. Genotyping of the SNPs in both cases and control groups were successful and appropriate for the analysis. For all three SNPs, 10% of the samples in cases and control groups were selected to repeat PCR amplification five times and obtained the same results.

FoxP3 polymorphism and risk of BC. The SNP selection strategy was based on the literature search of relevant studies. Minor allele frequency of ⩾ 5% was considered for all SNPs according to the 1000 Genomes Project (http://browser.1000genomes.org/) and NCBI dbSNP database (http://www.ncbi.nlm.nih.gov/SNP). Three different genotypes were identified by SSP-PCR analysis as shown in Figure. The upper band in each lane was GAPDH as positive control. Table 3 summarizes the genotype and allele frequencies of the examined polymorphisms in the *FoxP3* gene. Analyses compared case and control groups. Analysis of the SNP rs2232365 and rs4824747 showed no significant differences in the genotypes or allele frequencies between cases and controls ($p > 0.05$). Analysis of the SNP rs3761548 showed significant difference in both allele and genotype frequencies between cases and control groups. The genotypes frequencies of this polymorphism in the case group were AA (27.4%), AC (69.4%) and CC (3.2%). Moreover, there were AA (8.1%), AC

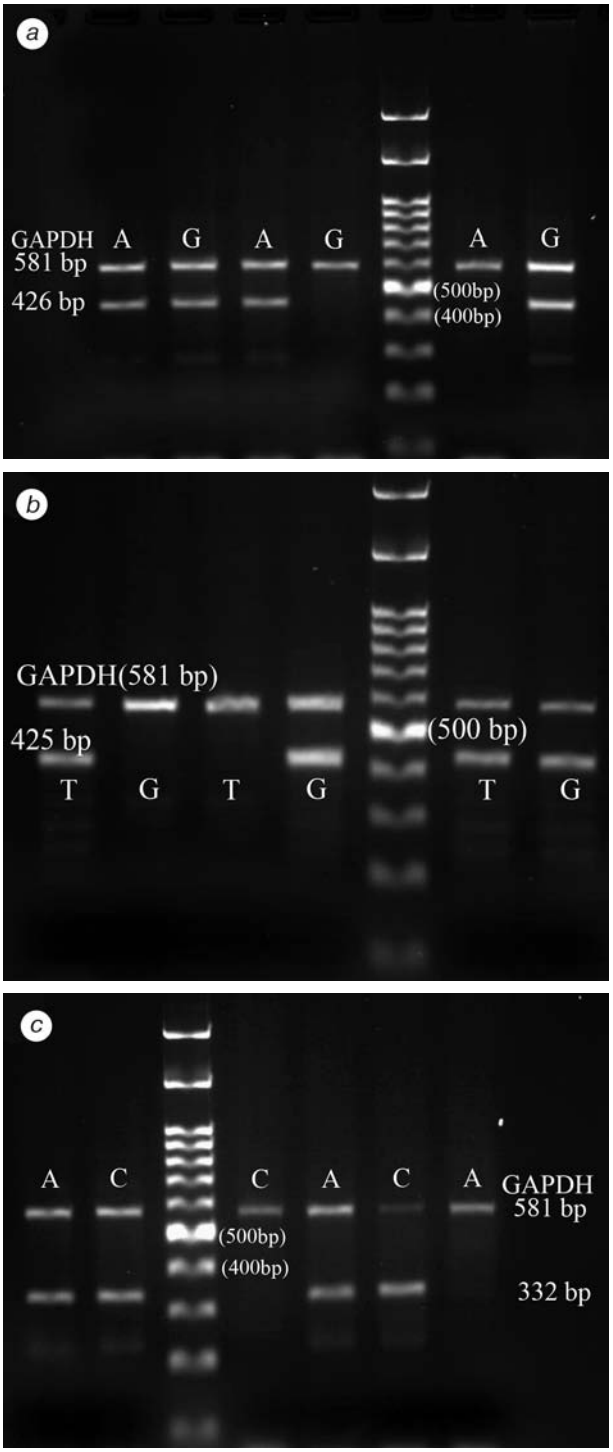

Figure. Gel electrophoresis representing genotypes of FoxP3 polymorphisms. 3′ base of the specific primers were shown in each lane (*a*) rs2232365 polymorphisms; G represents homozygote individuals for G allele; AG represents heterozygote individuals and A represents homozygote individuals for A allele. (*b*) rs4824747 polymorphisms, T shows homozygote individuals for T allele; TG, heterozygote individuals and G homozygote individuals for G allele. (*c*) rs3761548 polymorphisms; A: homozygote individuals for A allele; AC: heterozygote individuals; C: homozygote individuals for C allele. GAPDH was used as internal positive control

(47.5%) and CC (44.4%) in the control group. AA and CC genotypes frequencies were significantly different in cases and controls (AA genotype: OR = 4.3, 95% CI = 1.7–10.7 and $p = 0.002$, CC genotype: OR = 0.06, 95% CI = 0.005–0.65 and $p = 0.02$. The allele frequencies of this polymorphism were A (62.0%), C (38.0%)

Table 2. Clinical and tumor biological characteristics of BC patients

Variable	Case (N = 124)	Control (N = 198)	*p*-value
Age	50.6 ± 12.0	49.4 ± 5.4	0.6

Variable	Case (N=124)
Age, years (mean ± SD)	50.6 ± 12.0
Age at diagnosis, years (mean ± SD)	48.4 ± 11.7
Tumor size, n/%	
⩽ 2 cm	78/62.9
> 2 cm	46/37.1
Stage at diagnoses, n/%	
I	14/11.3
II	32/25.8
III	38/30.6
IV	40/32.2
Progesterone receptor status, n/%	
positive	59/46.8
negative	65/51.6
Estrogen receptor status, n/%	
positive	64/51.6
negative	60/48.4
Ki-67 status, n/%	
positive	2/1.6
negative	122/98.4
HER-2 status, n/%	
positive	42/33.9
negative	82/66.1
Lymph nodes involvement, n/%	
Yes	90 /72.6
No	34/27.4
Metastasis, n/%	
Yes	112/90.3
No	12/9.7
Duration of disease, years (mean ± SD)	3.15 ± 1.40

in the case group and A (31.8%), C (68.2%) in the control group. The frequencies of A and C alleles in the two groups were significantly different (A allele OR = 3.5, 95% CI = 2.2–5.6 and C allele OR = 0.28, 95% CI = 0.18–0.45) (Table 3). The risk of BC in women with A allele was observed to be higher than the controls (62.0% *vs* 31.8%). Finally, allele frequencies were comparable with 1000 Genomes Project (http://browser.1000genomes.org/). The relationship between SNPs and all clinical characteristics were studied. No significant differences were observed (data not shown).

DISCUSSION

Characterization and identification of *FoxP3* gene polymorphisms which affect human phenotype, especially leading to risks of complex disorders is one of the current issues in medical genetics. In this study, we investigated the association of three *FoxP3* variants, rs2232365, rs3761548 and rs4824747 in 124 patients with BC and 198 healthy controls and found a significant difference in **rs3761548**C>A SNP, AA and CC homozygous genotype and alleles distribution between case and control groups (Table 3). Recent data suggested significant associations between **rs3761548**C>A and various types of cancer including colorectal [22], thyroid [23], lung [24, 25] and hepatocellular cancers [26]. Our results showed 4.3 fold increased risk of BC in AA genotype of *FoxP3* rs3761548 polymorphism. This is in line with Lopes *et al.* [20], AL-Hajaj and AL-Battat [21] findings in Brazil population and Iraqi population, respectively. Lopes *et al.* [20] analyzed *FoxP3* rs3761548 polymorphism in triple negative BC patients and suggested that individuals who had inherited both copies of this allelic variation (AA genotype) had a higher susceptibility for developing triple negative BC than individuals

Table 3. Allele and genotype frequencies in subjects with BC (cases) and control groups

SNP rs2232365	Cases (n = 124)	Controls (n = 198)	*p*-value	OR (95% CI)
Genotypes, n (%)				
AA	24 (19.4)	44 (22.2)	0.6	–
AG	62 (50.0)	90 (45.4)	**0.52**	–
GG	38 (30.6)	64 (32.4)	0.8	–
Alleles, n (%)				
A	110 (44.4)	178 (44.9)	0.8	–
G	138 (55.6)	218 (55.1)	0.7	–
SNP rs4824747	Cases (n = 124)	Controls (n = 198)	*p*-value	OR (95% CI)
Genotypes, n (%)				
GG	8 (6.5)	32 (16.2)	0.8	–
TG	82 (66.1)	130 (65.6)	0.53	–
TT	34 (27.4)	36 (18.2)	0.27	–
Alleles, n (%)				
G	98 (39.5)	194 (49.0)	0.16	–
T	150 (60.5)	202 (51.0)	0.07	–
SNP rs3761548	Cases (n = 124)	Controls (n = 198)	*p*-value	OR (95% CI)
Genotypes, n (%)				
AA	34 (27.4)	16 (8.1)	**0.002**	**4.3 (1.7–10.7)**
AC	86 (69.4)	94 (47.5)	0.2	
CC	4 (3.2)	88 (44.4)	**0.02**	**0.06 (0.005–0.65)**
Alleles, n (%)				
A	154 (62.0)	126 (31.8)	**0.0001**	**3.5 (2.2–5.6)**
C	94 (38.0)	270 (68.2)	**0.0001**	**0.28 (0.18–0.45)**

with other genotypes. Jahan *et al.* study [17] in Indian women revealed a highly significant association between AA genotype of rs3761548, with the advanced stage (T3–4) of the tumor. They also showed AA as a risk genotype for fast progression in the premenopausal group [17]. However, our findings are not in accordance with the studies which revealed lack of association between rs3761548 polymorphism and BC [18, 19, 27].

The significant association between rs3761548 and increased risk of BC could be justified on the basis of the rs3761548 location in the *FoxP3* promoter region.

As the promoter region is the site for transcription factor binding, polymorphisms in this region of *FoxP3* gene may potentially change the binding specificity of transcription factors and modify the kinetics of transcription initiation, which result in *FoxP3* gene expression alteration [28]. There is a good agreement between the above theory and the earlier reports about significant number of the *FoxP3* genes mutations [29], dysregulated *FoxP3* expression in BC cells [30] and effects of promoter region polymorphisms on *FoxP3* expression levels. Bassuny *et al.* [31] evaluated the promoter/enhancer activity of the intron with (GT)n polymorphism by dual luciferase reporter assay and showed that the polymorphisms in the promoter region of the *FoxP3* resulted in changing enhancer activity. According to Bassuny *et al.* [31], the intron zero region with (GT)15 dimorphism had a stronger enhancer activity; therefore, this allele of the FoxP3 gene may directly alter the expression level of the FoxP3 gene in the cells. Intriguingly there are direct evidences of **rs3761548**C>A SNP significant effect on *FoxP3* expression. Shen *et al.* research [32] in psoriatic patient have demonstrated that the A allele is associated with a significant reduction in luciferase activity compared with the C allele. The functional importance of A allele is loss of binding with E47 and C-Myb transcription factor, which leads to defective transcription of FoxP3 gene [32]. It is noteworthy that experimental evidences showed that the loss of nuclear FoxP3 contributed to HER2 [33, 34] and SKP2 overexpression in BC samples [8]. About 20% and 50% of invasive BCs overexpress HER2 [35] and SKP2 [36] respectively. The study of Martin *et al.* [37] showed that the high expression of these two oncogenes is related with poor prognosis in patients with BC. On the other hand due to *FoxP3* critical locus on the X chromosome and X-chromosome unisomy, only one single genetic hit could suppress its expression or function [38, 39].

Considering the above statements and our observation about the high association between mutant allele (Table 3) of *FoxP3* promoter, it seemed logical that the mutated *FoxP3* released the repressed oncogenes which subsequently overcame the tumor suppressor proteins such as HER2 [4], SKP2 [8] or down regulated tumor suppressors such as p21 [40] and initiated development of aggressive tumors [37, 41, 42] including BC [30, 37]. However, the prognostic significance of the SNP rs3761548 was not confirmed by this study. In our sample, we did not observe any associations between **rs3761548**C>A polymorphism and clinical outcome parameters, considering age at diagnoses, tumor size, lymph nodes metastasis, tumor stage, progesterone receptor status, estrogen receptor status, Ki-67 status, HER2 status and duration of disease (data not shown).

These results were in accordance with the results of Lopes *et al.* [20], Ramachander *et al.* [27] and Zheng *et al.* [19] who examined the risk of BC related to *FoxP3* rs2294021 polymorphism with stratification by age, age at menarche, menstrual history, body mass index, family history of cancer, pathological type, clinical stage, estrogen receptor status, and progesterone receptor status and did not find significant association between rs3761548 and clinical outcome. However, in contrast to our results, Jahan *et al.* [17] found highly significant association between AA genotype of rs3761548 and the advanced stage (T3–4) of the tumor. The study Ramachander *et al.* [27] on rs2294021:C>T showed a significant

difference between early (T1–2) and advanced tumor stage (T3–4), duration of disease, lymph node involvement and metastasis. Zheng *et al.* [19] determined the relationship between the age at onset of BC and rs2294021:C>T genotypes and showed that the risk of rs2294021CT genotype was more pronounced in younger and premenopausal patients.

Taken together, this study has provided the first genetic data on the *FoxP3* gene polimorfism in population in south of Iran and confirmed the critical role of allele A at SNP rs3761548 in breast carcinogenesis. Although this study somehow confirmed the intriguing interplay between the oncogenes and FoxP3 in cell-cycle regulation and carcinogenesis but the limited sample size may produce relative risk estimates lacking adequate precision. Hence, extended analyses with larger sample size should be carried out from different ethnic origins. A better understanding of FoxP3 polymorphism in BC pathogenesis and progression may highlight many researchable issues including FoxP3 clinical implications

AUTHOR CONTRIBUTIONS

N. Naderi developed the concept, and prepared the manuscript, F. Arabpour and M. Norouzian performed experimentation, A. Shafizad performed examination on patients, M. Rahimzadeh analyzed data, prepared figures and tables and carefully read the manuscript. All authors read and approved the final article.

CONFLICT OF INTEREST

Authors declare that they had no conflict of interest.

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