RESEARCH ARTICLE

Foxp3 promoter polymorphism (rs3761548) in breast cancer progression: a study from India

Parveen Jahan · V. R. Vinish Ramachander · G. Maruthi · S. Nalini · K. Prasanna Latha · T. S. R. Murthy

Abstract Breast cancer is the most common female neoplasm that drives the transformation of normal mammary epithelial cells into highly malignant derivatives. Forkhead Box Protein3 (Foxp3), a tumor suppressor/immunomodulatory gene, which controls the function of Treg cells and oncogenes is down regulated in breast cancer. The main aim of the present study is to evaluate the potential influence of Foxp3-3279 C>A polymorphism (rs3761548) and -2383 C>T polymorphism (rs3761549) in 202 breast cancer patients and 130 normal healthy women of Indian origin. The genotypes were determined using ARMS-PCR for rs3761548 and PCR-RFLP method for rs3761549 using specific primers. The results revealed lack of association of these two polymorphisms with breast cancer susceptibility. However, with respect to AA genotype of rs3761548, we found highly significant association with the advanced stage (T3-4) of the tumor (OR=3.90; 95 % confidence interval (CI)=1.56–9.70; p =0.03). Stratified data also revealed an association of homozygous mutant genotype with advanced stage of tumor in premenopausal women (OR=4.56; 95 % CI=1.07–19.38; p =0.04) with disease duration of <6 months (OR=6.10; 95 % CI=1.80–20.50; p =0.002) suggestive of modulating effect of rs3761548 in tumor progression. We conclude that Foxp3 rs37161548 has a potential to be a polymorphic marker for tumor progression in premenopausal breast cancer patients in Indian women.

Keywords Foxp3 · Treg cells · Menopausal history · Tumor stage

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Introduction

Breast cancer, a malignant breast neoplasm, is one of the leading causes of female deaths worldwide accounting for 3.1 % annual global increase in developing countries like India [1]. It was estimated that 29 % of the new cancer cases identified in women will be of breast cancer [2]. Etiology of the breast cancer depends on various epidemiological factors; however, genetic susceptibility plays a major role in the causation of the disease as small portion of the exposed individuals develop breast cancer.

Forkhead Box Protein3 (Foxp3), a member of transcription factor winged-helix family is involved in regulating the immune system development and function [3]. It was identified during positional cloning of scurfin, a gene responsible for the X-linked autoimmune diseases in mice and humans [4, 5]. Foxp3 plays an essential role in the generation of regulatory T cells (Tregs) and its functional failure leads to lack of Tregs resulting in lethal autoimmune disorders, however, over expression results in severe immunodeficiency [6]. Foxp3 is considered to be an X-linked tumor suppressor gene as it is known to suppress various types of cancers including breast cancer and several lines of evidence support this. The efficacy

of an immunological response to tumors will decrease if the expression of Foxp3 is upregulated (7). Heterozygous mice for Foxp3 mutation develop spontaneous mammary cancer. Somatic mutations and chromosomal deletions are most frequently observed in breast cancer involving a minimal region of Foxp3 [8, 9].

Molecular studies have shown down regulation of Foxp3 expression in the mammary cancer tissues compared with normal breast epithelial cells. Besides, Foxp3 inhibits the transcription of Human Epidermal Growth Factor Receptor 2(*HER2/ErbB2*), a major oncogene for breast cancer; it also down-regulates S phase kinase protein 2(*Skp2*), which plays an important role in cell cycle regulation thus inhibit tumor growth[8, 9]. Worse overall disease free survival probability was reported in breast tumor, which lacks Foxp3 suggesting that tumor inhibition is dependent on the expression of this transcription factor [10]. Further, it has been shown that Foxp3 expression is enhanced by sex hormones via influencing the proliferation of Treg cells [11]; this may in turn alter disease susceptibility and tumor promotion/destruction. Several studies have been carried out dealing with polymorphisms of Foxp3 promoter region in various diseases including breast cancer in different populations [12]. However, there are no reports from India. In the present study, we evaluated the role of two promoter polymorphisms of Foxp3 gene, -3279 C>A (rs3761548) and -2383 C>T (rs3761549) in breast cancer women of Indian origin. To our knowledge, this is the first study reported in relation to Foxp3 and breast cancer from this region of Asia.

Materials and methodology

Study population

We report a hospital-based case–control study carried out with institutional ethics committee clearance (Osmaina University, Hyderabad, India). The present study was conducted on a total of 332 individuals, which include 202 patients with a mean age of 50 ± 11 years recruited from Railway Hospital, E.S.I and Osmania General Hospital, Hyderabad, India and 130 healthy volunteers with a mean age of 47 ± 13 years as controls. Clinical and demographical information such as age, age at onset, tumor stage(T_{1-4}), duration of disease (DOD), i.e. duration of time from the initial clinical symptoms to sample collection, *HER2*, and hormone receptor status (*ER* and *PR*) was collected from the patients' with the help of the oncologist and medical records. As limited information ($n = 54$) was available on *ER*, *PR*, and *HER2* status, we could not include this parameter for analysis. All the subjects were made to understand the reasons for the sample collection, and written consent was obtained before taking the blood samples.

Sampling

Patients According to the International Society for the study of cancer, patients who were diagnosed with lump in the breast and confirmed by fine needle aspiration cytology (FNAC) were recruited. Patients who had other breast diseases like abscess, phyllodes tumor, and fibroids were excluded from the study.

Controls Age-matched healthy women with no family history of cancer, non-alcoholics, and non-smokers were considered for the study.

Molecular analysis

About 2 ml of peripheral blood sample was collected in EDTA vacutainer from all the subjects and stored at 4 °C for further use. Genomic DNA was isolated by salting out method using standard established protocol in our lab [13]. Using appropriate primers genotyping was carried out for Foxp3 rs3761548 [14] and rs3761549 [15]

> Primers for rs3761548
> OF: 5′-GACTTAACCAGACAGCGTAG-3′
> IF: 5′-TTCTGGCTCTC TCCCCAACTGC-3′ (G allele specific)
> IR: 5′-TGAGGGGTAAACTGAGGCCTT-3′ (Aallele specific)
> OR: 5′-CTGGTGTGCCTTTGGTCT-3′

> Primers for rs3761549
> FP: 5′-CTGAGACTTTGGGACCGTAG-3′
> RP: 5′-TGCGCCGGGCTTCATCGACA-3′

Polymerase chain reaction (PCR) were carried out in a final volume of 20 μl reaction mixture containing 2.5 μl of 100 ng DNA, 2.5 μl Mgcl2, 1.25 μl of Taq DNA polymerase (Labpro) and 1 μl of each primer (Bioserve), 2.5 μl dNTPs (Labpro), and 1 μl PCR buffer. PCR conditions were initial denaturation at 94 °C for 5 min followed by 29 cycles of 94 °C for 30 s, annealing at 53.5 °C (rs3761548) and 56.5 °C (rs3761549) for 45 and 30 s, respectively, and extension at 72 °C for 30 s. The final extension was at 72 °C for 5 min and holds at 4 °C. The amplified products were run on 2 % agarose gel containing ethidium bromide at 100 V for 20 min. For rs3761548, A allele specific product showed a band at 209 base pair (bp) and C allele specific band at 397 bp with general product at 564 bp (Fig. 1). PCR amplified product of rs3761549 was of 388 bp, which was digested with *Bsr1* restriction endonuclease; fragment size of 308 and 80 bp for wild type (CC) and 388, 308, and 80 bp for heterozygote (CT) was observed (Fig. 2) (80 bp product was run out and not seen).
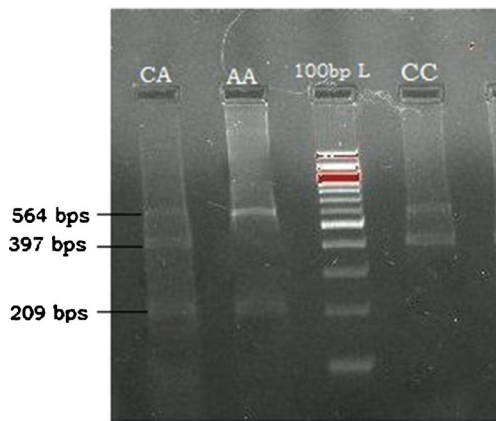
Fig. 1 Gel picture representing genotype distribution of rs3761548 in breast cancer

Statistical analysis

Descriptive statistics were done to calculate percentages, mean, and SD. Chi- square contingency tables were used to compare the allele and genotype frequencies between patients and controls and various subgroups. The risk associated with genotypes was calculated using online odds ratio calculator and logistic regression analysis with 95 % confidence interval (CI). Data analysis was carried out by SPSS version 18 wherever required. Statistical significance was defined as a two-sided p-value <0.05.

Results

Demographic and clinical information of patients and controls was given in Table 1. The mean age at onset of breast cancer in patients was 49±10.60 years. Of the total patients, 75 (37 %) were premenopausal women, and 127 (63 %) were postmenopausal with a mean age of 41±7 and 56±8 years, respectively. The genotype distribution of Foxp3 rs3761548 and rs3761549 in 202 breast cancer patients and 130 healthy controls was detailed in Table 2.

Genotype distribution of rs3761548

The perusal of Table 2 revealed that the frequencies of C and A alleles were 0.44 and 0.56 in controls and 0.47 and
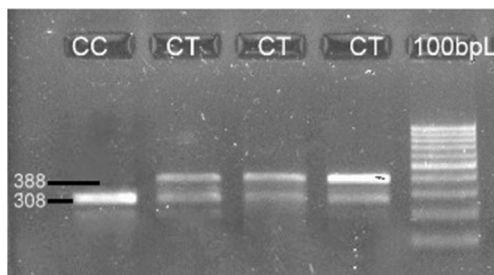

Fig. 2 Gel picture representing genotype distribution of rs3761549 in breast cancer

Table 1 Demographic and clinical information of breast cancer patients and healthy controls

Category	Number (%)	x±SD
Age (years)		
Patients	202	50±11
Controls	130	47±13
Age at onset (years)	202	49±10.6
Menopausal status (years)		
Premenopausal women	75 (37)	41±7.0
Postmenopausal women	127 (63)	56±8.0
Duration of disease		
<6 months	104 (52)	3.31±1.8
7–12 months	65 (32)	11.01±1.8
>12 months	33 (16)	47.81±31.18
Tumor stage		
T_{1-2}	108 (53)	-
T_{3-4}	94 (47)	-
Primary histology		
Ductal carcinoma	200 (99)	-
Lobular carcinoma	2 (1)	-

0.53 in breast cancer patients, correspondingly. The respective distribution of CC, CA, and AA genotypes was observed to be 7, 80, and 13 % in patients and 3, 82, and 15 % in controls (p >0.05). Overall data of patients with respect to early (T_{1-2}) and advanced stage (T_{3-4}) of tumor showed a significant difference in their genotype distribution (χ^2=11.57, p <0.01). Women with homozygous AA genotype were predominated in advanced stage (T_{3-4}) of tumor category (OR=3.90; 95 % CI; 1.56–9.70; p=0.003), and it was women with CA genotype that were elevated in early stages (T_{1-2}) of tumor (OR=0.31; 95 % CI; 0.14–0.63; p=0.001). Upon categorization of patients as pre and post-menopausal groups, premenopausal women revealed similar elevated frequency of AA genotype with advanced stages (OR=4.56; 95 % CI=1.07–19.38; p=0.04) and CA genotype with early stages of tumor (OR=0.19; 95 % CI=0.06-0.66; p=0.008). No such variation was observed in post-menopausal group. With respect to duration of the disease (DOD), the genotypes did not differ between the groups, i.e., patients with DOD of <12 months and >12 months. However, logistic regression analysis showed significant OR values at 95 % CI for DOD along with menopausal status and tumor stage (Table 3).

As we observed a significant risk independently with respect to DOD and stages of tumor in logistic regression analysis with genotypes, it was felt that it would be interesting to test whether there is an influence of the genotypes on the disease progression (tumor stage) with DOD. In our data, the range of disease duration was observed to be quite wide

Table 2 Distribution of rs3761548 and rs3761549 genotypes in patients and controls

rs3761548	CC N (%)	CA N (%)	AA N (%)	χ^2 value (p-value)	C	A	Group comparison	OR (95 % CI)	p-value
Controls (130)	4 (3)	106 (82)	20 (15)	2.89	0.44	0.56	CC versus others	2.5 (0.81–7.7)	0.14
Patients (202)	15 (7)	160 (79)	27 (14)	0.35	0.47	0.53	CA versus others	0.86 (0.37–1.23)	0.67
							AA versus others	0.84 (0.45–1.59)	0.63
Pre-patients(75)	6 (8)	59 (79)	10 (13)	0.05	0.47	0.53	CC versus others	1.14 (0.39–3.34)	1
Post patients (127)	9 (7)	101 (80)	17 (13)	0.97	0.47	0.53	CA versus others	0.95 (0.47–1.91)	1
							AA versus others	0.99 (0.43–2.8)	1
T_{1-2} (108)	6 (6)	95 (88)	7 (6)	11.57	0.50	0.50	CC versus others	1.8 (0.61–5.26)	0.29
T_{3-4} (94)	9 (10)	65 (69)	20 (21)	0.003	0.44	0.56	CA versus others	0.31 (0.14–0.63)	0.001
							AA versus others	3.90 (1.56–9.7)	0.003
Pre-patients (75)									
T_{1-2} (46)	2 (4)	41 (89)	3 (7)	7.78	0.48	0.52	CC versus others	3.52 (0.62–20.0)	0.19
T_{3-4} (29)	4 (14)	18 (62)	7 (24)	0.02	0.42	0.58	CA versus others	0.19 (0.06–0.66)	0.008
							AA versus others	4.56 (1.07–19.38)	0.04
Post patients (127)									
T_{1-2} (64)	5 (9)	53 (82)	6 (9)	1.82	0.45	0.55	CC versus others	0.8 (0.20–3.13)	1
T_{3-4} (63)	4 (6)	48 (76)	11 (18)	0.4	0.49	0.51	CA versus others	0.66 (0.28–1.58)	0.38
							AA versus others	2.06 (0.71–5.92)	0.20
Duration of disease									
<12 months	14 (8)	135 (80)	20 (12)	2.9	0.48	0.52	CC versus others	2.7 (0.32–21.38)	0.4
>12 months	1 (3)	25 (76)	7 (21)	0.23	0.41	0.59	CA versus others	1.27 (0.52–3.06)	0.64
							AA versus others	0.49 (0.19–1.29)	0.16
HWE: Controls-χ^2=55.91; Patients-χ^2=70.26 (p<0.05)									
rs3761549	CC N (%)	CT N (%)	TT N (%)	χ^2 value (p-value)	C	A	Group comparison	OR (95 % CI)	p-value
Controls (130)	2 (2)	128 (98)	0	0.08	0.51	0.49	CC versus CT	1.39 (0.25–7.7)	1
Patients (202)	4 (2)	198 (98)	0	0.76	0.51	0.49	CT versus CC	0.72 (.12–3.9)	1
Pre-patients (83)	1 (1)	82 (99)	0	0.4	0.51	0.49	CC versus CT	2.12 (0.21–20.7)	0.64
Post patients (119)	3 (3)	116 (97)	0	0.5	0.51	0.49	CT versus CC	0.47 (0.04–4.6)	0.64
Stage I & II (113)	3 (3)	110 (97)	0	0.60	0.51	0.49	CT versus CC	0.41 (0.04–4.07)	0.63
T_{3-4} (89)	1 (1)	88 (99)	0	0.44	0.51	0.49	CC versus CT	2.4 (0.24–23.4)	0.63
Pre-patients (83)									
T_{1-2} (49)	1 (2)	48 (98)	0	0.7	0.51	0.49	CT versus CC	–	–
T_{3-4} (34)	0	34 (100)	0	0.4	0.50	0.50	CC versus CT		
Post patient (119)									
T_{1-2} (64)	2 (3)	62 (97)	0	0.2	0.52	0.48	CC versus CT	0.57 (0.05–6.5)	1
T_{3-4} (55)	1 (2)	54 (98)	0	0.6	0.51	0.49	CT versus CC	1.79 (0.15–19.7)	1
Duration of disease									
<12 months	4	165	0	0.7	0.51	0.49	CC versus CT	–	–
>12 months	0	33	0	0.37	0.50	0.50	CT versus CC		
HWE: Controls-χ^2=122.2; Patients-χ^2=186.6 (p<0.05)									

OR odds ratio; *CI* class interval

(1 month to 10 years) and most of our patients, i.e., 84 % fall under the disease duration of 12 months. Hence, for this analysis, we considered only these 169 patients and categorized them into two groups, i.e., patients with disease duration of <6 months (62 %) and >6 months (38 %). Results showed that 29 % of patients with AA genotype progressed to advanced stage of cancer within 6 months of the disease duration (OR=6.10; CI=1.80-20.50; p=0.002) (Table 4).

Table 3 Foxp3 rs3761548 genotype with the adjusted odds ratio in relation to clinical features of breast cancer

Cateogory	OR (95 % CI)	p-value
1. Age at onset	1.75 (0.78–3.96)	0.178
2. Menopausal status	0.35 (0.14–0.89)	0.0269
3. Tumor stage	3.14 (1.13–8.76)	0.0283
4. Duration of disease	2.47 (1.10–5.54)	0.0277

OR odds ratio, *CI* class interval

Genotype distribution of rs3761549

The frequency of C and T alleles was similar in both patients and controls (51 and 49 %). The frequency of CC, CT genotypes was in the order of 2 and 98 % in patients and 2 and 98 % in controls, respectively. TT genotype was found to be completely absent in this study. There was no difference observed between genotype frequencies of the patients and controls ($p > 0.05$). Further, there was a lack of variation observed with respect to menopausal status between patients

and controls ($p > 0.05$). The data of patients with regard to early ($T_{1–2}$) and advanced tumor stage ($T_{3–4}$) also showed no variation in their genotype distribution ($p > 0.05$). No difference was observed between the genotype distribution in pre and postmenopausal groups in relation to tumor stage. We found no influence of the genotypes on the duration of disease (DOD) also (Table 2).

Discussion

Immune system plays a crucial role at various stages of tumor development influencing its destruction or promotion. When cancerous cells escape intrinsic tumor suppressor mechanism, immune system as an extrinsic tumor suppressor eliminates them and limits their growth [16, 17]. Continuous supply of Tregs is essential for the immune system to work properly, and diminished supply results in its functional deficiency. Tregs require transcription factor Foxp3, a member of forkhead/winged-helix family for its development and function. It was recognized to be a candidate gene for autoimmune diseases

Table 4 Distribution of rs3761548 and rs3761549 genotype in relation to duration of disease (DOD) and tumor stage

rs3761548	CC N (%)	CA N (%)	AA N (%)	χ^2 value (p-value)	C	A	Group comparison	OR (95 % CI)	p-value
DOD (n=169)									
<6 months (104)	9 (9)	79 (76)	16 (15)	3.4	0.47	0.53	CC versus others	1.13 (0.36–3.55)	1
>6 months (65)	5 (8)	56 (86)	4 (6)	0.17	0.51	0.49	CA versus others	0.5 (0.22–1.17)	0.1
							AA versus others	2.77 (0.88–8.69)	0.08
DOD <6 months(104)									
$T_{1–2}$ (63)	6 (10)	53 (84)	4 (6)	10.02	0.52	0.48	CC versus others	0.75 (0.17–3.18)	0.74
$T_{3–4}$ (41)	3 (8)	26 (63)	12 (29)	0.006	0.39	0.61	CA versus others	0.33 (0.13–0.82)	0.02
							AA versus others	6.10 (1.80–20.5)	0.002
DOD >6 months (65)									
$T_{1–2}$ (35)	1 (3)	32 (91)	2 (6)	2.57	0.49	0.51	CC versus others	5.23 (0.55–49.6)	0.17
$T_{3–4}$ (30)	4 (13)	24 (80)	2 (7)	0.27	0.53	0.47	CA versus others	1.5 (0.34–6.6)	0.72
							AA versus others	1.18 (0.16–8.91)	1
rs3761549	CC N (%)	CT N (%)	TT N (%)	χ^2 value (p-value)	C	T	Group comparison	OR (95 % CI)	p-value
DOD (169)									
<6 months (104)	1 (1)	103 (99)	0	2.31	0.50	0.50	CC versus CT	4.98 (0.50–48.9)	0.3
>6 months (65)	3 (5)	62 (95)	0	0.13	0.52	0.48	CT versus CC	0.2 (0.02–1.97)	0.2
DOD <6 months(104)									
$T_{1–2}$ (68)	0 (0)	68 (100)	0	1.9	0.50	0.50	CC versus CT	–	–
$T_{3–4}$ (36)	1 (3)	35 (97)	0	0.17	0.51	0.49	CT versus CC		
DOD>6 months (65)									
$T_{1–2}$ (29)	2 (7)	27 (93)	0	0.61	0.53	0.47	CC versus CT	0.38 (0.03–4.48)	0.58
$T_{3–4}$ (36)	1 (3)	35 (97)	0	0.43	0.51	0.49	CT versus CC	2.5 (0.22–30.1)	0.58

OR odds ratio, *CI* class interval

because of its role in immunoregulation that was based on the involvement of Foxp3 mutation in IPEX [18]. Several members of Fox family such as FoxO, FoxM, and FoxG1 apart from Foxp3 are involved in tumerogenesis [19]. A mouse with Foxp3 mutation exhibit spontaneous breast cancer indicating it to be an X-linked tumor suppressor gene for breast cancer [20]. Down regulation of Foxp3 gene contributes to immune suppression and makes anti-tumor response inactive [21]. The oncogenes that are under the control of tumor suppressor gene Foxp3 get activated and influence the cell proliferation resulting into malignant cells.

Genetic polymorphisms in the FOXP3 promoter region may alter its expression. Several single nucleotide polymorphisms in this region including rs3761548 and rs3761549 have been reported in different ethnic groups [12] dealing with various human diseases.

In our study, the evaluation of distribution of these two promoter polymorphisms of Foxp3 gene in Indian women has revealed lack of association with breast cancer. In support to our study, similar observations were made by Raskin et al. (2009) in Israeli population [22] and Zheng et al. (2013) in Han Chinese population [7] with respect to rs3761548 and breast cancer. Andre et al. (2011) reported no relation with endometriosis/idiopathic infertility [23] and Parket al. (2005) for Crohn's disease [24]. However, several other studies have shown association between rs3761548 and different disorders such as Psoriasis [25], Allergic Rhinitis [26], Graves' disease [27], unexplained recurrent spontaneous abortions [28], and our recent two studies on Preeclampsia [29] and Vitiligo [13].

A number of reports have been published with respect to rs3761549 and various human pathologies. In accordance to our results, no association was found with breast cancer and Graves' disease by others [7, 14]. However, a study by Zahra Mojtahedi et al. (2013) in Iran population has shown a connection between this polymorphism of Foxp3 with metastatic colorectal cancer[30]. An investigation connected with endometriosis has also revealed an association in Brazilian women [31]. This SNP showed no influence either on the susceptibility or on the progression of the disease in our study.

The significant risk obtained in relation to stage of the tumor in overall data with respect to rs3761548 genotypes could be explained on the basis of influence of homozygous mutant genotype on the progression of tumor. The functional importance of this SNP is loss of binding with E47 and C-Myb transcription factor leading to defective transcription of Foxp3 [32]. The decreased expression of Foxp3 in our patients with AA genotype might have over expressed the downstream oncogenes and helped in metastatic process/ tumor progression. It is evidenced from the study of Mahmoud, S.M et al.,(2010) who had reported that loss of Foxp3 contributes to over expression of *HER2* in breast cancer patients [33]. Zuo et al., (2007a) demonstrated that Foxp3 directly represses *Skp2* expression in human and mouse mammary epithelial cells [8]. The preponderance of heterozygous (CA) individuals of rs3761548 in early stages of tumor in our study reflects that the optimal level of Foxp3 expression may hinder the tumor progression. Stratified data on tumor stage for pre and post- menopausal groups have shown AA as a risk genotype for fast progression in the former group but not in the latter group, suggesting the influence of the hormones. Heather et al.,(2006) observed an association between premenopausal estrogen levels and breast cancer risk [34]. In addition, Polanczyk MJ et al. (2006) and Prieto GA et al., (2006) have reported that estrogens promote immunotolerance by increasing Treg compartment via augmenting Foxp3 expression [11, 35]. As Foxp3 has dual function of tumor suppression and immunomodulation, it is difficult to delineate which of the two functions of this molecule is influencing the progression of the disease in our patients.

In conclusion, Foxp3 single nucleotide promoter polymorphisms rs3761548and rs3761549 may not be playing a role in predisposing the Indian women to breast cancer. However, we report a significant impact of rs3761548 on tumor progression in younger patients. In addition, homozygous mutant genotype was observed to be influencing the tumor progression by promoting it to advanced stage within 6 months of onset of clinical symptoms, indicating the potential of this SNP to serve as a marker for tumor progression in younger women. The relative role of Foxp3 as tumor suppressor or immuno-modulator in the tumor progression needs to be elucidated. The limitation of our study is lack of information on ER and HER2 status of the tumor to correlate with genotypes. This is the first study pertaining to the above SNPs among Indian women in relation to breast cancer. To test our hypothesis, large replicative studies are warranted in different ethnic groups. The generated information might help in better therapeutic interventions and drug development.

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