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Sway of FOXP3 Gene Variants in the Genetic Vulnerability of Asian Indian Women towards Breast Cancer

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Abstract

FOXP3, an X-linked tumor suppressor gene is down regulated in many diseases including breast cancer. The present case-control study was aimed to screen five variants of the FOXP3 gene, rs3761548 C>A, rs3761549 C>T, rs2232365 A>G, rs2232368 G>A, and rs2294021 C>T in 281 breast cancer and 280 healthy women of Asian Indians. Genomic DNA was isolated, genotyping was performed using PCR-RFLP method and the data were subjected to suitable statistics. Women with TT genotype of rs2294021 exhibited twofold increased risk towards breast cancer (OR=2.36, 95% CI 1.20-4.65, p=0.015) whereas CT genotype associated with disease progression (p=0.005) and CC with restricting tumor growth (p=0.01). Furthermore, women with GA genotype of rs2232368 (OR: 4.13; 95% CI: 2.56-6.66; p<0.0001) and AG genotype of rs2232365 (OR: 1.56; 95% CI: 1.0-2.42; p=0.047) were predominant in patients compared to controls. Strong linkage disequilibrium was detected in few combinations of SNPs involving rs3761548, rs2232365, rs2232368 and rs2294021 in healthy women. Haplotype blocks harboring three or more wild type alleles (ACAGC and CCGAC) had increased depiction in controls (p<0.001). We conclude that, rs2294021, rs2232365 and rs2232368 of FOXP3 are allied with augmented risk for breast cancer. In addition CT genotype of rs2294021 implicated in the progression of disease, signifying the role of skewed X inactivation in BC pathogenesis. Investigations involving populations from different geographical regions, X inactivation and tissue expression studies are necessary to realize the role of FOXP3 markers in disease development and promotion which in turn would help in impending therapeutic methods for cancers with FOXP3 flaws.

Keywords: *FOXP3, SNPs, Susceptibility, Progression, X-inactivation*

1. Introduction

Breast cancer (BC) is one of the world's most frequently diagnosed cancers, the second leading cause of mortality in women and known to affect 1 in 26 in developing countries like India [1]. Genetic mutations have been identified as one of the most important factor contributing to BC, with BRCA1, BRCA2, PTEN, CDH1 and TP53 high-penetrance genes involved in DNA repair and response,

contribute to 16-25% of genetic threat [2]. Further, several other low-penetrant genetic variants in combination with environmental factors were known to contribute to increase in individual's risk towards BC [3]. Genes with the functions of immunological surveillance play an important role in immune responses that act by blocking or activating different pathogenic pathways leading to cell proliferation and malignancy. Lack of effective antitumor response has been attributed to numerous escape mechanisms, among which down regulation of immune responses by regulatory T cells (Tregs) has attracted substantial attention in the recent past [4].

FOXP3 (Forkhead Box Protein3), a transcription factor is widely known for its role in the development and function of $CD4^+$ $CD25^+$ Treg cells. Scurfy mutation in the murine FOXP3 was shown to be coupled with cellular infiltration of multiple organs, elevated proinflammatory cytokine levels, and over proliferative $CD4^+$T cells [5]. A similar immune disregulatory condition IPEX (Immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) was seen in humans carrying mutations in the FOXP3 gene [6, 7]. This X-linked gene located on Xp11.23, exhibits duel role of being an immune regulator and a tumor suppressor [8]. The onco-suppressive function of FOXP3 gene was comprehended from the fact that female heterozygous scurfy mice developed spontaneous mammary carcinoma at a high rate [9]. Immunohistochemistry and microarray studies of Liu et al (2009a) showed down regulation of FOXP3 in cancer cells compared to normal breast epithelium [10]. FOXP3 is directly presented to control expression of oncogenes (ERBB2, SKP2) and tumor suppressor genes (p21) by influencing their promoter activity [9, 11].

Genetic variants of the FOXP3 gene are known to alter FOXP3 function besides quantitative and qualitative variation in $CD4^+$ $CD25^+$ Treg cells [12] and have been demonstrated to be associated with numerous diseases [13]. Our previous studies on FOXP3 genetic variant rs3761548 have exposed an association with immunological disorders such as Pre-eclampsia and Vitiligo [14, 15] and BC progression [16]. In the present study, for the first time we aimed to screen a total of five selected polymorphisms of the FOXP3 gene, present in the promoter (rs3761548 C>A, rs3761549 C>T, rs2232365 A>G) and the intronic region (rs2232368 G>A, rs2294021 C>T) for their association with BC risk in Asian Indian women.

2. Materials and Methodology

2.1 Sampling

Blood samples were collected from 281 BC patients and 280 healthy women from the same ethnic group. Patients were recruited from the oncology department of the South Central Railway Hospital, and Soumya Multi Specialty Hospital, Hyderabad, India. Approval for the study was obtained from the Institutional Ethics Committee for Biomedical Research, Osmania University, Hyderabad, India in accordance with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Demographic and clinical information such as age, age at onset (AAO), tumor stage (T_{1-4}), lymph node involvement (LN_0: absence of lymph node; LN_1: presence of lymph node), metastasis (M_0: non-metastatic; M_1: metastatic) and duration of disease (DOD) i.e time gap between initial clinical symptoms to the time of sample collection were elicited from a patient's medical record and through personal interview. Blood samples and written consent were obtained from all the subjects after making them understand the reasons for the sampling.

2.2 Inclusion and Exclusion criteria

Patients with histologically confirmed breast cancer were enrolled in the study. Women with other breast diseases like abscess and fibroids were excluded from the study. Age-matched healthy subjects with no family history of cancers were selected as controls.

2.3 Genotyping

Two milliliters of peripheral blood were collected in EDTA vacutainer and genomic DNA was isolated by standard protocol [17]. Primers were designed (**Table 1**) and genotyping was carried out using PCR-RFLP method in a total volume of the 20µl reaction mixture including 2.5µl of 50-100ng DNA, 2.5µl Mgcl2, 1.25µl of Taq DNA polymerase and 1µl of each primer, 2.5 µl dNTPs and 1µl PCR buffer. The PCR conditions were, initial denaturation at 95°C for 5 min, followed by 30 cycles of final denaturation at 94°C for 30 s, annealing temperature ranging from 50.5°C - 61°C; initial extension at 72°C for 45s and final extension at 72ºC for 5 min. The PCR product was digested with specific restriction endonucleases (**Table 1**) and the banding pattern was visualized upon 2% Agarose gel electrophoresis (**Figure 1**).

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Table 1: Primers, annealing temperature and a restriction enzyme used in the present study

SNP	Region	Primers	Annealing temperature	Type	Restriction Enzyme	Band size (base pairs)	Reference
rs3761548 C>A	Promoter -3279	FP: 5' GACTTAACCAGACAGCGTAG 3' RP: 5'CTGGTGTGCCTTTGGTCT 3'	50.5°C	PCR-RFLP	Pst1	CC: 373,190 CA:563,373,190 AA:563	Jahan et al., 2013 [15]
rs3761549 C>T	Promoter -2383	FP: 5' - CTGAGACTTTGGGACCGTAG-3' RP: 5'-TGCGCCGGGCTTCATCGACA-3'	56.5°C	PCR-RFLP	Bsr1	CC:308, 80 CT: 388, 308, 80 TT: 388	Owen et al., 2006 [18]
rs2232365 A>G	Promoter -924	FP:5'TTGGACAGGGAAAGAGGAGA3' RP:5'GAAGCTGGATCAGGAGCAGT3'	53.5°C	PCR-RFLP	Pf1FI	AA: 454 AG: 454, 316, 138 GG: 316, 138	Mahabadi et al. 2015 [19]
rs2232368 G>A	Intron 1 -20	FP5-AGGGGTGTGAGAGGGAGACT-3' RP5-TACCTGCTGCTCCAGAGACT-3'	55°C	PCR-RFLP	HiniII	GG:210 GA:210,143,67 AA:143,67	Mahabadi et al. 2015 [19]
rs2294021 C>T	Intron 13	FP: 5'-CACACACAATCCATCCCAGTCACCC-3' RP: 5'-ATCTCCATGCCCTAAGAAGGCCACC-3'	61°C	PCR-RFLP	HaeIII	CC: 216,106,87 CT: 322,216,106,87 TT: 322,87	Han et al., (2010) [20]

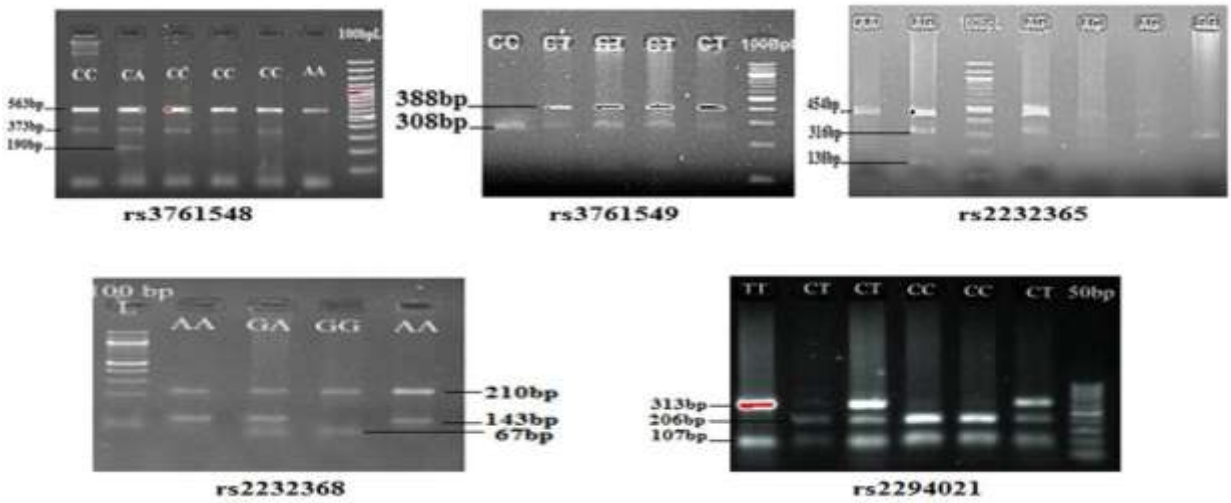

Figure 1 Gel picture representing genotypes of FOX3 polymorphisms rs3761548, rs3761549, rs2232365, rs2232368 and rs2294021in 2% agarose gel electrophoresis

2.4 Statistical analysis

Data analysis was carried out by SPSS version 21 wherever required. A two-sided p-value of <0.05 was considered statistically significant. Descriptive statistics was done to compute percentages. Graphpad online software was used to calculate the mean and standard deviation. Two-sided chi-squared test was performed using quantpsy.org between BC patients and control groups. The Hardy-Weinberg equilibrium (HWE) was tested for SNPs using Court lab-HW calculator. Odds ratio estimates for determining the risk associated with the genotypes and haplotype analysis were carried out by means of SNPStats online software tool. Pairwise linkage disequilibrium (LD) analysis was calculated using Haploview software (version 4.2).

3. Results

3.1 Clinical and demographic characteristics

A case control study in Asian Indian women, including 280 controls and 281 BC patients was carried out to evaluate the association between five SNPs of FOXP3 gene and BC. Demographic and clinical characteristics of BC patients and healthy women are shown in **Table 2**. The age range at onset of BC was 24-85 years with the majority of the patients in >48 years (55.5%).

Table 2: Demographic and Clinical characteristics of breast cancer patients and healthy Volunteers

Category	**Controls (280) N (%), Mean±SD**	**Patients (281) N (%), Mean±SD**	**p value**
1. Age at sample collection (years)	48±13.8	51±10.6	**0.003***
a. ≤48 years	151 (54); 38±6	117 (42); 41±5.6	**0.001***
b. >48 years	129 (46); 60.5±9	164 (58); 58±7	**0.03***
2. Age at onset of BC (years)		50±10.6	
a. ≤48 years	--	125 (44.5);41±5.6	--
b. >48 years	--	156 (55.5);57.6±7.23	
3. BC Stage			
a. T_{1-2}	--	158(56)	--
b. T_{3-4}	--	123(44)	
4. Lymph node involvement			
a. No	--	43(15)	--
b. Yes	--	238(85)	
5. Metastasis			
a. M0	--	243(86)	--
b. M1	--	38(14)	
6. Duration of Disease (DOD)			
a. ≤6 months	--	151(54)	--
b. >6 months	--	130(46)	

3.2 Allele and Genotype distribution

The genotype and allele frequencies of rs3761548 C>A, rs3761549 C>T, rs2232365 A>G, rs2232368 G>A, and rs2294021 C>T in BC patients and healthy controls are summarized in **Table 3**. The estimates of relative risk of disease were adjusted for age for all the SNPs studied. The distributions of the genotypes in both patients and controls were not in accordance with Hardy Weinberg equilibrium ($p<0.05$) this could be owing to excess of heterozygotes that are observed the present study, which may indicate the presence of over-dominant selection.

Table 3: Genotype and allelic distribution for five polymorphisms of FOXP3 gene in the study group

SNP	Genotype and allele Distribution		Yates' χ^2 value (p value)	Group comparison	OR (95% CI)	p value
	Controls (280); N (%)	Patients (281); N (%)				
rs3761548						
Genotype						
CC	39 (14)	26 (9)	2.54 (0.28)	CC vs. CA+AA	0.63 (0.37-1.06)	0.08
CA	210 (75)	220 (78)		CA vs. CC+AA	1.20 (0.81-1.78)	0.37
AA	31 (11)	35 (13)		AA vs. CC+CA	1.14 (0.68-1.91)	0.69
Allele						
C	288 (51)	272 (48)		C vs. A	0.88 (0.70-1.12)	0.31
A	272 (49)	290 (52)		A vs. C	1.13 (0.89-1.43)	0.31
rs3761549						
Genotype						
CC	4 (1)	2 (1)	0.17 (0.68)	CC vs. CT	0.49 (0.09-2.72)	0.45
CT	276 (99)	279 (99)		CT vs. CC	2.02 (0.37-11.1)	0.45
Allele						
C	284 (51)	283 (50)		C vs. T	0.98 (0.78-1.24)	0.9
T	276 (49)	279 (50)		T vs. C	1.01 (0.80-1.28)	0.9
rs2232365						
Genotype						
AA	57 (20)	39 (14)	3.96 (0.14)	AA vs. AG+GG	**0.63 (0.40-0.98)**	**0.04***
AG	221(79)	240 (85)		AG vs. AA+GG	**1.56 (1.0-2.42)**	**0.047***
GG	2 (1)	2 (1)		GG vs. AA+AG	0.99 (0.14-7.12)	1
Allele						
A	335 (60)	318 (57)		A vs. G	0.87 (0.69-1.11)	0.27
G	225 (40)	244 (43)		G vs. A	1.14 (0.90-1.45)	0.27
rs2232368						
Genotype						
GG	63 (23)	21 (7)	35.03 (2e-8)	GG vs. GA+AA	**0.28 (0.16-0.47)**	**$4.76e^{-7*}$**
GA	197 (70)	255 (91)		GA vs. GG+AA	**4.13 (2.56-6.66)**	**$6.62e^{-10*}$**
AA	20 (7)	5 (2)		AA vs. GA+GG	**0.23 (0.09-0.64)**	**0.002***
Allele						
G	323 (58)	297 (53)		G vs. A	0.82 (0.65-1.04)	0.1
A	237 (42)	265 (47)		A vs. G	1.22 (0.96-1.54)	0.1
rs2294021						
Genotype						
CC	59 (21)	48 (17)	6.31 (0.04)	CC vs. CT+TT	0.77 (0.50-1.18)	0.24
CT	208 (74)	204 (73)		CT vs. CC+TT	0.91 (0.63-1.33)	0.70
TT	13 (5)	29 (10)		TT vs. CC+CT	**2.36 (1.20-4.65)**	**0.015***
Allele						
C	326 (58)	300 (53)		C vs. T	0.82 (0.65-1.04)	0.1
T	234 (42)	262 (47)		T vs. C	1.22 (0.96-1.54)	0.1

Adjusted (for age) OR and 95% CI

3.3 rs3761548 C>A polymorphism

The genotype frequencies of CC, CA, AA was found to be 14%, 75%, 11% and 9%, 78%, 13% respectively in controls and patients (**Table 3**). The genotype and allele frequencies did not differ significantly between the two groups (p>0.05).

Further analysis was executed to determine the relationship between rs3761548 C>A polymorphism with demographic and clinical variables such as, age, age at onset (AAO), tumor stage, lymph node involvement, metastasis and duration of disease with tumor stage. Analysis revealed a lack of association with any of the studied variables (p>0.05). (**Data not shown**)

3.4 rs3761549 C>T polymorphism

The frequencies of CC, CT, TT genotypes were similar both in BC cases and controls (1%, 99%, and 0%) and there was a complete absence of mutant homozygous genotype (TT) in our study (**Table 3**). None of the demographic and clinical variables were shown to be influenced by this polymorphism. (**Data not shown**)

3.5 rs2232365 A>G polymorphism

The distributions of AA, AG, GG genotypes were 20%, 79%, 1% in controls and 14%, 85%, 1% in cases which did not vary significantly between patients and controls (p>0.05). However, AG genotype was considerably elevated in patients compared to controls (OR: 1.56; 95% CI: 1.0-2.42; p=0.047) and wild type AA was more in controls (OR: 0.63; 95% CI: 0.40-0.98; p=0.04). Further, no effect of this polymorphism was observed with demographic and clinical variables. (**Data not shown**)

3.6 rs2232368 G>A polymorphism

The percentage genotype distribution in healthy women and BC patients were 23, 70, 7 and 7, 91, 2 for GG, GA, AA genotypes correspondingly (χ^2=37.4, p=1e^{-8}). Women with GA genotype were predominant in patients (OR: 4.13; 95% CI: 2.56-6.66; p=6.62e^{-10}) and GG (OR: 0.28; 95% CI: 0.16-0.47; p=4.76e^{-7}) and AA (OR: 0.23; 95% CI: 0.09-0.64; p=0.002) in controls. A similar trend was seen with respect to the genotypes between the patients and controls of >48 years of age. Additionally, patients with GA genotype (OR: 3.53; 95% CI: 1.84-6.79; p=0.00007) were more in ≤48 years of age group than in controls (**Table 4**). No association was observed between AAO and clinical parameters with this polymorphism. (**Data not shown**)

3.7 rs2294021 C>T polymorphism

The genotype distribution of CC, CT, TT in healthy controls and BC patients were 21%, 74%, 5% and 17%, 73%, 10% respectively (χ^2=9.42, p<0.05). Individuals with TT genotype were significantly more in patients compared to controls (OR=2.36, 95% CI 1.20-4.65, p=0.015) (**Table 3**).

Further analysis on demographic data has shown that TT genotype was considerably more in >48 years patients compared to controls of the same age group (OR: 3.05, 95% CI 1.10-8.47, p=0.03) (**Table 4**). A significant difference was observed between early (T_{1-2}) and advanced tumor stage (T_{3-4}) with respect to the genotypes. The frequency of CC genotype was higher in early tumor stage (OR: 0.3; 95% CI: 0.14-0.61; p=0.0007) whereas, CT genotype in advanced tumor stage (OR: 5.3; 95% CI: 0.2.61-10.62; p=4.57e^{-7}). Metastatic cases presented augmented frequency of the CT genotype in relation to non-metastatic cases (OR: 3.4; 95% CI: 1.16-9.94; p=0.026) and CC genotype was significantly elevated in lymph node negative cases compared to lymph node positive cases (OR: 0.46; 95% CI: 0.22-0.97; p=0.04) (**Table 5**). In addition to the above analysis, the tumor stage was scrutinized with duration of disease (DOD) to understand the genotype dependent progression of the disease. The range of the DOD was 1 month to 10 years, with the comparatively more number of patients under less than 6 months of DOD, therefore, we categorized them into DOD ≤6months and DOD >6months. The results showed that CT individuals progressed to an advanced stage, irrespective of the DOD [≤6 months: T_{1-2} vs. $T_{3-4;}$ OR: 3.30; 95% CI: 1.40-7.78; p=0.005 and >6 months, OR: 2.5; 95% CI: 1.07-5.83; p=0.036)] whereas CC genotype appears to delay the spread of the disease in ≤6 months of disease duration [DOD ≤6 (OR: 0.29; 95% CI: 0.10-0.79; p=0.01)] (**Table 5**).

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3.8 Linkage Disequilibrium (LD) Analysis using Haploview

LD is the non-random association of alleles at two or more loci and the measure of LD is D′ (LD coefficient). The D′ is 1 in the absence of recombination, which determines the strength of LD. **Table 6** shows the D′ values between 10 SNP combinations obtained from five polymorphisms studied in normal healthy controls and BC cases. Based on the D′ values obtained in the present study, it may be specified that, rs2294021 and rs2232365 (0.72 vs. 0.54), rs376158 and rs2232365 (0.76 vs. 0.62), rs2232368 and rs2232365 (0.92 vs. 0.67) combinations showed the higher D′ value in controls in relation to patients.

3.9 Haplotype analysis

Haplotype analysis (**Table 7**) revealed that frequency of ACAGC and CCGAC haplotypes were significantly lower in patients than in controls (OR=0.46, 95% CI 0.27-0.80, p<0.001 and OR=0.12, 95% CI 0.03-0.55, p<0.001).

Table 4: Genotype and allelic distribution of age group ≤48 years and >48 years in rs2232368 G>A and rs2294021 C>T polymorphisms

SNP	Genotype and allele Distribution		Yates' χ^2 value (p value)	Group comparison	OR (95% CI)	p value
	Controls	Patients				
rs2232368 ≤48 years	Genotype GG: 36 (24) GA: 102 (67) AA: 13 (9) Allele G: 174 (58) A: 128 (42)	Genotype GG: 10 (9) GA: 103 (88) AA: 4 (3) Allele G: 123 (53) A: 111 (47)	13.51 (0.0012)	GG vs. GA+AA GA vs. GG+AA AA vs. GA+GG G vs. A A vs. G	0.37 (0.12-1.18) **3.53 (1.84-6.79)** 0.37 (0.12-1.18) 0.81 (0.58-1.15) 1.22 (0.87-1.73)	0.13 **0.00007*** 0.12 0.24 0.24
rs2232368 >48 years	Genotype GG: 27 (21) GA: 95 (74) AA: 7 (5) Allele G:149 (58) A: 109 (42)	Genotype GG:11 (7) GA:152 (91) AA:1 (2) Allele G: 174 (53) A: 154 (47)	17.57 (0.0001)	GG vs. GA+AA GA vs. GG+AA AA vs. GA+GG G vs. A A vs. G	**0.27 (0.13-0.57)** **4.53 (2.24-9.18)** **0.11 (0.01-0.88)** 0.83 (0.59-1.15) 1.21 (0.87-1.68)	**0.0003*** **0.00000*** **0.02*** 0.27 0.27
rs2294021 >48 years	Genotype CC: 30 (23) CT: 94 (73) TT: 5 (4) Allele C: 154 (60) T: 104 (40)	Genotype CC: 31 (19) CT: 115 (70) TT: 18 (11) Allele C: 177 (54) T: 151 (46)	4.28 (0.12)	CC vs. CT+TT CT vs. CC+TT TT vs. CC+CT C vs. T T vs. C	0.77 (0.43-1.35) 0.87 (0.52-1.45) **3.05 (1.10-8.47)** 0.79 (0.57-1.10) 1.26 (0.90-1.76)	0.38 0.69 **0.028*** 0.18 0.18

Table 5: Genotype and allelic distribution of rs2294021 C>T polymorphism of Foxp3 gene with respect to clinical parameters

	Genotype Distribution		**Yates χ^2 value (p value)**	**Group comparison**	**OR (95% CI)**	**p value**
	A; N (%)	**B; N (%)**				
$^{A}T_{1-2}$ (160) $^{B}T_{3-4}$ (121)	Genotype CC: 38 (24) CT: 102 (64) TT: 20 (12) Allele C: 178 (56) T: 142 (44)	Genotype CC: 10 (8.3) CT: 102 (84.3) TT: 9 (7.4) Allele C: 122 (50) T: 120 (50)	13.77 (0.001)	CC vs. CT+TT CT vs. CC+TT TT vs. CC+CT C vs. T T vs. C	**0.3 (0.14-0.61)** **3.05 (1.69-5.48)** 0.56 (0.25-1.28 0.81 (8-1.13) 1.23 (0.88-1.72)	**0.0007*** **0.0001*** 0.23 0.23 0.23
$^{A}LN_0$ (43) $^{B}LN_1$ (238)	Genotype CC: 12 (28) CT: 26 (60) TT: 5 (12) Allele C: 50 (58) T: 36 (42)	Genotype CC: 36 (15) CT: 178 (75) TT: 24 (10) Allele C: 250 (53) T: 226 (47)	3.61 (0.16)	CC vs. CT+TT CT vs. CC+TT TT vs. CC+CT C vs. T T vs. C	**0.46 (0.22-0.97)** 1.94 (0.98-3.82) 0.85 (0.31-2.37) 0.79 (0.50-1.27) 1.25 (0.78-1.99)	**0.04*** 0.06 0.78 0.35 0.35
$^{A}M_0$ (245) $^{B}M_1$ (36)	Genotype CC: 45 (19) CT: 172 (70) TT: 28 (11) Allele C: 262 (53) T: 200 (47)	Genotype CC: 3 (8) CT: 32 (89) TT: 1 (3) Allele C: 38 (53) T: 34 (47)	4.08 (0.13)	CC vs. CT+TT CT vs. CC+TT TT vs. CC+CT C vs. T T vs. C	0.40 (0.12-1.37) **3.4 (1.16-9.94)** 0.22 (0.03-1.68) 0.85 (0.52-1.40) 1.17 (0.71-1.93)	0.16 **0.026*** 0.14 0.61 0.61
DOD ≤6M $^{A}T_{1-2}$ (102) $^{B}T_{3-4}$ (49)	Genotype CC: 29 (28) CT: 62 (61) TT: 11 (11) Allele C: 120 (59) T: 84 (41)	Genotype CC: 5 (10) CT: 41 (84) TT: 3 (6) Allele C: 51 (52) T: 47 (48)	6.68 (0.03)	CC vs. CT+TT CT vs. CC+TT TT vs. CC+CT C vs. T T vs. C	**0.29 (0.10-0.79)** **3.30 (1.40-7.78)** 0.54 (0.14-2.03) 0.76 (0.47-1.23) 1.31 (0.81-2.13)	**0.01*** **0.005*** 0.39 0.32 0.32
DOD >6M $^{A}T_{1-2}$ (58) $^{B}T_{3-4}$ (72)	Genotype CC: 9 (15.5) CT: 40 (69) TT: 9 (15.5) Allele C: 58 (50) T: 58 (50)	Genotype CC: 6 (8) CT: 61 (85) TT: 5 (7) Allele C: 73 (51) T: 71 (49)	3.18 (0.2)	CC vs. CT+TT CT vs. CC+TT TT vs. CC+CT C vs. T T vs. C	0.49 (0.16-1.48) **2.5 (1.07-5.83)** 0.15 (0.13-1.29) 1.03 (0.63-1.68) 0.97 (0.59-1.58)	0.27 **0.036*** 0.15 1 1
Adjusted (for age) OR and 95% CI *A: Non-risk group, B: Risk group*						

4. Discussion

FOXP3 is an X-linked tumor suppressor gene in BC that plays a dual role in both tumor initiation and progression [9]. In tumor tissues, FOXP3 functions as a tumor suppressor and in the immune system, aids tumor evasion. This suggests that a functional balance for the FOXP3 gene exists between different tissues and immune system, and when this balance is disturbed, it is likely to contribute to tumorigenesis and metastasis. As FOXP3 is located on the X chromosome (Xp11) a genetic/epigenetic single-hit results in inactivation of this gene and a complete loss of activity in males [21]. Fundamentally, almost all tumor suppressor genes are autosomal, however, evidence concerning abnormalities in the X chromosome, including LOH, skewed inactivation, and selective loss, have been reported in BC [21-25].

Genome-wide analysis of FOXP3$^+$ Treg cells indicated that FOXP3 can bind to about 700 genes and several miRNAs [26]. Studies have demonstrated that, as a transcriptional factor, FOXP3 inhibits tumor cell growth by both repressing the oncogenes Erbb2, Skp2, cMyc [9, 11, 27] and inducing the tumor suppressor gene p21 [10]. Based on the above facts, we have investigated the potential influence of five genetic polymorphisms (rs3761548, rs3761549, rs2232365, rs2232368 and rs2294021) of the FOXP3 gene in BC susceptibility and progression in an Asian Indian population.

Of the five SNPs investigated three SNPs rs2294021, rs2232365 andrs2232368 showed an association with the BC risk.

rs2294021 has been explored by many researchers in diverse autoimmune disorders and cancers with mixed results [18, 28, 29]. We observed that the TT genotype of this polymorphism imparted a twofold increased risk towards BC compared to other genotypes. Our result is supported by Zhang et al., (2013) who reported that the FOXP3 rs2294021 C allele enhanced FOXP3 promoter transcription activity, resulting in elevation of its expression level, which in turn increased the number of Tregs and inhibited T-cell proliferation. Conversely, in breast tissue, C allele enhanced FOXP3 expression could suppress the BC oncogenes such as HER2 and SKP2 [30]. Further, CT genotype was shown to be associated with BC risk in Han Chinese population and suggested the probable role of skewed X-chromosome inactivation [20, 30].

Table 6: Linkage disequilibrium and D′values in controls and patients

Sl. No	SNP combination	D′ values in controls	D′ values in Patients
1	rs2294021 and rs3761549	0.72*	0.85*
2	rs2294021 and rs3761548	0.47	0.47
3	rs2294021 and rs2232368	0.65	0.75*
4	rs2294021 and rs2232365	0.72*	0.54
5	rs3761549 and rs3761548	0.77*	0.70*
6	rs3761549 and rs2232368	0.96*	0.97*
7	rs3761549 and rs2232365	0.98*	0.78*
8	rs3761548 and rs2232368	0.69	0.71*
9	rs3761548 and rs2232365	0.76*	0.62
10	rs2232368 and rs2232365	0.92*	0.67
*=strong LD			

rs2232368 is another intronic SNP, where the GA genotype has shown a fourfold elevated risk toward BC, while the GG and AA genotypes conferred protection against BC. Insilico analysis of rs2232368 revealed that it harbors a cryptic splice site. Further characterization of this SNP will help in understanding its role in BC as there are no functional studies carried out so far for this polymorphism. Though there are reports available on the association of this SNP with other disorders [19, 31] ours is the first study to explore the association of this SNP in BC.

The promoter SNP rs2232365 (AG genotype) showed a 1.56 fold increased risk towards BC susceptibility whereas the AA genotype had a sway in protecting the women from developing the disease. A putative DNA binding site for transcription factor GATA-3 (GATA binding protein-3) exists for rs2232365, would help in the maintenance of Th1/Th2 cytokine profile depending on the mRNA expression of GATA-3 [32]. The polymorphism in this marker may perhaps alter gene expression by shifting the binding of the transcription factor and eventually leading to FOXP3 mRNA instability [13]. Diverse studies dealing with this SNP had given varied results [18, 19, 28, 29, 33-38]. Nonetheless, the current study is the first one to report the link between rs2232365 and BC predisposition in Asian Indians.

rs3761548 and rs3761549 are the most extensively studied SNPs of FOXP3 in various human diseases [14, 15, 19, 28, 29, 31, 33, 34, 36-43]. In the present study rs3761548 and rs3761549 polymorphisms showed lack of association individually with BC. These two SNPs was dealt with us previously as a preliminary study with small sample size that showed no association [16] and the results remained the same

even after the extended sample size as shown in the table 4. On the contrary, AA genotype of rs3761548 has been shown to be positively associated with the risk of BC in Brazilian and Iraqi population [44, 45]. The meta-analysis by Jiang *et al* (2014) reported a lack of association of FOXP3 rs3761548 (C>A) and rs3761549 (C>T) polymorphisms with the breast cancer risk in Asian population [46]. The discrepancy and similarity in the results could be due to sample size and ethnicity variation among the studies.

Table 7: Haplotype analysis of five polymorphisms of FOXP3 gene in the present study

S.No	rs3761548	rs3761549	rs2232365	rs2232368	rs2294021	Frequency	Controls	Patients	OR (95% CI)	p value
1	A	C	G	G	C	0.33	0.0014	0.35	1.00	---
2	C	T	A	A	T	0.27	0.0024	0.31	1.38 (0.43-4.41)	0.59
3	C	T	A	A	C	0.08	0.06	0.09	1.14 (0.33-3.96)	0.84
4	A	C	A	G	C	0.07	0.086	0.046	**0.46 (0.27 - 0.80)**	**0.006***
5	C	T	A	G	T	0.06	NA	0.031	0.34 (0.09 - 1.23)	0.1
6	A	T	A	A	T	0.05	NA	0.053	1.06 (0.29 - 3.88)	0.92
7	A	C	G	G	T	0.03	NA	0.038	1.59 (0.65 - 3.87)	0.31
8	C	C	G	G	C	0.026	NA	0.038	0.74 (0.30 - 1.85)	0.53
9	C	C	G	A	C	0.02	NA	0.0046	**0.12 (0.03 - 0.55)**	**0.007***
10	C	T	A	G	C	0.01	0.02	NA	0.00 (-Inf - Inf)	1
Rare	*	*	*	*	*	0.0538			1.08 (0.49 - 2.37)	0.86
Adjusted (for age) OR and 95% CI **Global haplotype association p-value: <0.0001**										

In the context of progression of the disease, of the five SNPs studied, rs2294021 not only showed an association with the susceptibility towards BC, but also in the progression of the disease. A fivefold increased risk of progression was shown by the CT genotype, whereas the CC genotype was found to restrict tumor growth. Somatic inactivation of the normal allele in the tumor tissue due to X-chromosomal inactivation could be one of the explanations for our observation as suggested by Liu *et al* (2015) [47]. Medema *et al* (2007) reported that in female carriers of a mutant FOXP3 allele, skewing (skewed X chromosome inactivation) selects for expression of the mutant allele during tumorigenesis in breast epithelial cells to promote BC [48]. Though, the biological function and significance of FOXP3 in BC progression remains unclear, clinically nuclear FOXP3 expression is inversely correlated with BC progression. FOXP3 significantly inhibits adhesion, invasion and metastatic process of BC cells by suppressing CD44, activation of which persuades cell motility, stimulation of cell survival responses, and promotion of cell adhesion [49-50]. In addition, Liu *et al* (2015) through chromatin immunoprecipitation (CHIP) sequencing identified that FOXP3 induced miR146 a/b prevent tumor cell proliferation and enhance apoptosis by inhibiting NFκB activation, which can inhibit apoptosis in cancer cells by inducing antiapoptotic factors like BCL2 and BCL2L1, leading to tumor suppression in breast cancer cells. Thus the tumor suppressor function of FOXP3 is linked with NFκB inactivation through miR-146a/b [51]. The association of high producing CC genotype of rs2294021 FOXP3 polymorphism in our present study with restricted progression of tumor compared to CT and TT genotypes could be elucidated based on the above explanation.

Estrogens influence the expression of FOXP3 gene [52], a known risk factor for BC [53]. In our study, we detected that women in >48 years of age group with variant TT genotype (rs2294021) showed an increased risk of BC when compared to their normal counterparts. The plausible explanation for this could be the interaction between the polymorphism (T allele of rs2294021 is associated with loss of tumor repressor activity) and the estrogen levels (the postmenopausal condition is known to have lower estrogen levels). Tai *et al* (2008) experimental results of enhanced expression of FOXP3 in mouse model that was injected with E2 indirectly supports the present observation [54].

Furthermore, a strong LD existed among most of the SNP combinations in the studied population. However, the combination of rs2294021 and

rs2232365 (D′ value: 0.72 vs. 0.54), rs3761548 and rs2232365 (D′ value: 0.76 vs. 0.62), rs2232368 and rs2232365 (D′ value: 0.92 vs. 0.67) appear to have a greater protective role against BC in Asian Indians. In addition, we noted a significant association of two haplotypes (ACAGC and CCGAC) that harbored at least three or more wildtype alleles of the five SNPs with healthy controls, indicating their role in conferring protection against BC. Similar studies considering diverse geographical areas need to be carried out to understand the protective and predisposing role of these SNPs in BC.

5. Conclusion

Foxp3 rs2294021, rs2232365 and rs2232368 SNPs appear to be meaningfully associated with increased risk for BC. The TT genotype of rs2294021 was inclined towards the genetic predisposition to BC, whereas the CT genotype was shown to be involved in the progression, and CC in restricting the tumor growth. The involvement of two different genotypes in the susceptibility (TT) and progression (CT) may propose that, the T allele of rs2294021 which is associated with down regulation of FOXP3 in homozygous condition, might be involved in the initial stages of BC, whereas inactivation of the C allele in tumors with heterozygous genotype, may help in the spread, through up regulation of oncogenes and adhesion molecules at the time of the ongoing process of the disease. Strong linkage disequilibrium was observed for few combinations of SNPs involving rs3761548, rs2232365, rs2232368 and rs2294021 in controls. Further, protective haplotypes ported the wild type alleles, advocated the importance of these gene variants in shrinking the risk of BC in our population, however, required to be confirmed through investigations with larger samples involving populations from various geographical regions. X inactivation and tissue expression studies are necessary to realize the role of FOXP3 markers in disease development and promotion which in turn would help in impending therapeutic methods for cancers with FOXP3 flaws.

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Reference

[1] Priyanka Yadav, D P Jaroli. Breast Cancer: Awareness and Risk Factors in College-going Younger Age Group Women in Rajasthan. Asian Pacific J Cancer Prev.; 11:319-322,(2010).

[2] Dossus L and Benusiglio PR. Lobular breast cancer: incidence and genetic and non-genetic risk factors. Breast Cancer Research; 17:37. DOI: 10.1186/s13058-015-0546-7, (2015).

[3] Mavaddat N, Pharoah PD, Michailidou K, Tyrer J, Brook MN et al. Genome-wide association analysis of more than 120,000 individuals identifies 15 new susceptibility loci for breast cancer. Nat Genet.; 47(4): 373–380,(2015).

[4] Beyer M, Schultze JL. Regulatory T cells in cancer. Blood.; 108: 804–811,(2006).

[5] Brunkow ME, Jeffery EW, Hjerrild KA, Paeper B, Clark LB et al. Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. Nature Genet.; 27: 68-73,(2001).

[6] Bennett CL, Brunkow ME, Ramsdell F, O'briant KC, Zhu Q et al. A rare polyadenylation signal mutation of the Foxp3 gene (Aauaaa–>Aaugaa) leads to the IPEX syndrome. Immunogenetics.; 53:435-439,(2001).

[7] Wildin RS, Ramsdell F, Peake J, Faravelli F, Casanova JL et al. X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. Nature Genet.; 27: 18-20,(2001).

[8] Redpath M , Xu B , Kempen LCV , Spatz A. The dual role of the X-linked FoxP3 gene in human cancers. J Moleular oncology.; 5:156-163,(2011).

[9] 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. ; 129:1275–1286,(2007b).
[10] Liu R, Wang L, Chen G, Katoh H, Chen C, Liu Y, et al. FOXP3 up-regulates p21 expression by site-specific inhibition of histone deacetylase 2/histone deacetylase 4 association to the locus. Cancer Res. ; 69:2252–9,(2009a).
[11] Zuo T, Liu R, Zhang H, Chang X, Liu Y, et al. FOXP3 is a novel transcriptional repressor for the breast cancer oncogene SKP2. J Clin Invest. ; 117:3765–3773,(2007a).
[12] Jahan P, Tippisetty S and Komaravalli PL. FOXP3 is a promising and potential candidate gene in generalised vitiligo susceptibility. Front. Genet.; 6:249,(2015).
[13] 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. ; 92(1):163-71,(2013).
[14] Jahan P, Sreenivasagari R, Goudi D, Komaravalli PL, Ishaq M. Role of Foxp3 Gene in Maternal Susceptibility to Pre-eclampsia – A Study From South India. Scandinavian Journal of Immunology. ; 77:104–108,(2012).
[15] Jahan P, Cheruvu R, Tippisetty S, Komaravalli PL, Valluri V et al. Association of FOXP3 (rs3761548) promoter polymorphism with nondermatomal vitiligo: A study from India. J Am Acad Dermatol. ; 69(2):262-6,(2013).
[16] Jahan P, Ramachander VRV, Maruthi G, Nalini S, Latha KP, Murthy TS. Foxp3 promoter polymorphism (rs3761548) in breast cancer progression: a study from India, Tumour Biol. 35(4):3785-91, 2014.
[17] Tippisetty S, Ishaq M, Komaravalli PL, Jahan P. Angiotensisn converting enzyme (ACE) gene polymorphism in vitiligo: protective and predisposing effects of genotypes in disease susceptibility and progression. Eur J Dermatomal.; 21(2):173–7, (2011).
[18] Owen CJ, Eden JA, Jennings CE, Wilson V, Cheetham TD, Pearce SH. Genetic association studies of the FOXP3 gene in Graves' disease and autoimmune Addison's disease in the United Kingdom population. J Mol Endocrinol.; 37:97–104,(2006).
[19] Mahabadi FN, Zarei S, Fatemi R, Kamali K, Pahlavanzadeh Z et al. Association study of forkhead box P3 gene polymorphisms with unexplained recurrent spontaneous abortion. Journal of Reproductive Immunology.; 110, 48–53,(2015).
[20] Han X, Bao G, Zhou Y. Foxp3 rs2294021 polymorphism contributes to the susceptibility to breast carcinoma. Chinese-German J Clin Oncol.; 9(5):253–257,(2010).
[21] Spatz A, Borg C, Feunteun J. X-chromosome genetics and human cancer. Nat. Rev. Cancer.; 4:617-629,(2004).
[22] Piao Z, and Malkhosyan SR. Frequent loss Xq25 on the inactive X chromosome in primary breast carcinomas is associated with tumor grade and axillary lymph node metastasis. Genes Chromosomes Cancer.; 33:262–269,(2002).
[23] Roncuzzi L, Brognara I, Cocchi S, Zoli W, Campani GA. Loss of heterozygosity at pseudoautosomal regions in human breast cancer and association with negative hormonal phenotype. Cancer Genet. Cytogenet.; 135:173–176,(2002).
[24] Kristiansen M, Knudsen GP, Maguire P, Margolin S, Pedersen J, et al. High incidence of skewed X chromosome inactivation in young patients with familial non-BRCA1/ BRCA2 breast cancer. J. Med. Genet. 2005; 42:877–880.
[25] Richardson AL, Wang ZC, De Nicolo A, Lu X., Brown M, Miron A, Liao X, Iglehart JD et al. X chromosomal abnormalities in basal-like human breast cancer. Cancer Cell. ; 9:121–132,(2006).
[26] Zheng Y, Josefowicz SZ, Kas A, Chu TT, Gavin MA, Rudensky AY. Genome-wide analysis of Foxp3 target genes in developing and mature regulatory T cells. Nature.; 445:936–40,(2007).
[27] Wang L, Liu R, Li W, Chen C, Katoh H, et al. Somatic single hits inactivate the X-linked tumor suppressor FOXP3 in the prostate. Cancer Cell.; 16:336–46,(2009).
[28] Saxena D, Misra MK, Parveen F, Phadke SR, Agrawal S. The transcription factor Forkhead Box P3 gene variants affect idiopathic recurrent pregnancy loss. Placenta.; 36: 226-231,(2015).
[29] Wu Z, You Z, Zhang C, Li Z, 1 Su X, Zhang X, Li Y. Association between

Functional Polymorphisms of Foxp3 Gene and the Occurrence of Unexplained Recurrent Spontaneous Abortionina Chinese Han Population. Clinical and Developmental Immunology. Volume 2012, Article ID 896458, 7 pages,(2012).

[30] Zheng J, Deng J, Jiang L, Yang L, You Y et al. Heterozygous Genetic Variations of FOXP3 in Xp11.23 Elevate Breast Cancer Risk in Chinese Population via Skewed X-Chromosome Inactivation. Human Mutation.; 34(4):619–628,(2013).

[31] Andre GM, Barbosa CP, Teles JS, Vilarino FL, Christofolini DM, Bianco B. Analysis of FOXP3 polymorphisms in infertile women with and without endometriosis. J of Fertility and Sterility.; 95(7):2223-2227,(2011).

[32] Chakir H, Wang H, Lefebvre DE, Webb J, Scott FW. T-bet/GATA-3 ratio as a measure of the Th1/Th2 cytokine profile in mixed cell populations: predominant role of GATA-3. J Immunol Methods.; 278(1-2):157-69,(2003).

[33] Song QH, Shen Z, Xing XJ, Yin R, Wu YZ, You Y, et al. An association study of single nucleotide polymorphisms of the FOXP3 intron-1 and the risk of Psoriasis vulgaris. Indian J Biochem Biophys.; 49(1):25-35,(2012).

[34] Gorycka AP, Jurkowska M, Giemza AF, Prochnicka KR, Manczak M, et al. Genetic Polymorphisms of Foxp3 in Patients with Rheumatoid Arthritis. The Journal of Rheumatology.; 42:2,(2014).

[35] Vitiello GAF, Guembarovski RL, de Oliveira CEC, Amarante MK, Perim AL, Watanabe MAE. Involvement of Transforming Growth Factor Beta-1 (TGFβ1) Cytokine and FOXP3 Transcription Factor Genetic Polymorphisms in Hematological Malignancies. Braz. Arch. Biol. Technol.; 58(4):553-561,(2015).

[36] Park O, Grishina I, Leung P, Gershwin M, Prindiville T. Analysis of the Foxp3/Scurfin gene in Crohn's disease. Annals of the New York Academy of Sciences.; 1051:218–228,(2005).

[37] Zhang L, Zhang Y, Desrosiers M, Wang C, Zhao Y, Han D. Genetic association study of FOXP3 polymorphisms in allergic rhinitis in a Chinese population. Human Immunology.; 70(11):930–934,(2009).

[38] Hassannia H, Abediankenari S, Ghaffari J. FOXP3 and TGF-β Gene Polymorphisms in Allergic Rhinitis. Iran. J. Immunol.; 8 (4):218-225,(2011).

[39] Chen X, Gan T, Liao Z, Chen S, Xiao J. Foxp3 (-/ATT) Polymorphism Contributes to the Susceptibility of Preeclampsia. PLoS ONE.; 8 (4): e59696,(2013).

[40] D'amico F, Skarmoutsou E , Marchini M, Malaponte G, Caronni M et al. Genetic polymorphisms of FOXP3 in Italian patients with systemic sclerosis. Immunol Lett. ,(2013).;http://dx.doi.org/10.1016/j.imlet.2013.05.006

[41] Jiang W, Zheng L, Xu L, Zhang Y, Liu X, et al. Association between FOXP3, FOXE1 Gene Polymorphisms and Risk of Differentiated Thyroid Cancer in Chinese Han Population. Mol Biol.; 4: 131,(2015). doi:10.4172/2168-9547.1000131

[42] Haghighi FM, Ghayumi MA, Behzadnia F, Erfani N. Investigation of FOXP3 genetic variations at positions -2383 C/T and IVS9+459 T/C in Southern Iranian patients with lung carcinoma. Iran J Basic Med.; Sci. 18:465-47,(2015).

[43] Chen Y, Zhang H, Liao W, Zhou J, He G et al. FOXP3 gene polymorphism is associated with hepatitis B-related hepatocellular carcinoma in China. Journal of Experimental & Clinical Cancer Research. 32:39. http://www.jeccr.com/content/32/1/39,(2013).

[44] Lopes LF, Guembarovski RL, Guembarovski AL, Kishima MO, Campos CZ. FOXP3 Transcription Factor: A Candidate Marker for Susceptibility and Prognosis in Triple Negative Breast Cancer. BioMed Research International. Volume 2014, Article ID 341654, 7 pages,(2014). http://dx.doi.org/10.1155/2014/341654

[45] AL-Hajaj MAM. FOXP3 gene promoter polymorphism in breast cancer patients in Basra city / Iraq. International Journal of Innovations in Engineering and Technology (IJIET). 5(4). ISSN: 2319 – 1058,(2015).

[46] Jiang LL and Ruan LW. Association between FOXP3 promoter polymorphisms and cancer risk: A meta‑analysis. Oncology Letters. 8: 2795-2799,(2014).

[47] Liu R, Kain M, Wang L. Inactivation of X-linked tumor suppressor genes in human cancer. Future Oncol.8(4): 463–481,(2015). doi:10.2217/fon.12.26.

[48] Medema RH, Burgering BM.The X factor: skewing X inactivation towards cancer. Cell. 129:1253–1234,(2007).

[49] Zhang C, Xu Y, Qiang Hao, Wang S, Li H et al. FOXP3 suppresses breast cancer metastasis through downregulation of CD44. International Journal of Cancer.137 (6): 1279–1290,(2015).

[50] Nandi A, Estess P, Siegelman MH. Hyaluronan anchoring and regulation on the surface of vascular endothelial cells is mediated through the functionally active form of CD44. J Biol Chem. 275:14939-48,(2000).

[51] Liu R, Liu C, Chen D, Yang W, Liu X et al. FOXP3 Controls an miR-146/NF-kB Negative Feedback Loop That Inhibits Apoptosis in Breast Cancer Cells. Cancer Res.75(8),(2015). doi: 10.1158/0008-5472.CAN-14-2108

[52] Polanczyk MJ, Hopke C, Huan J, et al. Enhanced FoxP3 expression and Treg cell function in pregnant and estrogen-treated mice. J Neuroimmunol.170: 85–92,(2005).

[53] Colditz GA, Rosner BA, Chen WY, et al. Risk factors for breast cancer according to estrogen and progesterone receptor status. J Natl Cancer Inst.96: 218–228,(2004).

[54] Tai P, Wang J, Jin H, Song X, Yan J, Kang Y, et al. Induction of regulatory T cells by physiological level estrogen. J Cell Physiol. 214:456–464,(2008).