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EPIDEMIOLOGY

# Association of single nucleotide polymorphisms (SNPs) in *TNF-LTA* locus with breast cancer risk in Indian population

**Indu Kohaar · Pratibha Tiwari · Rakesh Kumar · Vilas Nasare · Nisha Thakur · Bhudev C. Das · Mausumi Bharadwaj**

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**Abstract** *Purpose* Cytokine milieu of tumor microenvironment affects tumorigenesis in breast cancer. The aim of the present study was to investigate the potential association of functional single nucleotide polymorphisms (SNPs) in *TNF-LTA* locus with breast cancer. *Methods* The study included 127 individuals comprising 40 breast cancer cases (35 sporadic & 5 familial) and 87 individuals of high risk group (with family history of breast cancer) along with 150 healthy controls. PCR-RFLP was employed to analyze *TNFA* promoter polymorphisms at −238 G/A, −308 G/A, −857 C/T, −863 C/A and −1031 T/C along with +252 A/G SNP in *LTA*. The results were further confirmed by direct sequencing. *Results* Significant association was established for *TNFA* −308 G/A and *LTA* +252 A/G polymorphisms with breast cancer versus controls ($P < 0.0001$; OR, 9.53; 95% CI, 4.11–22.13; $P_c < 0.001$) and high risk group versus controls ($P < 0.0001$; OR, 8.27; 95% CI, 4.28–16.0; $P_c < 0.001$) respectively. GGACCT haplotype was found to be positively associated with breast cancer ($P < 0.0001$; OR, 12.17; 95% CI = 5.12–28.92; $P_c < 0.001$) and high risk group ($P$, 0.03; OR, 2.95; 95% CI, 1.20–7.26; $P_c$, 0.005) in relation to controls. While GGGCCT haplotype was significantly related with high risk group in comparison to cancer ($P$, 0.0002; OR, 5.71; 95% CI, 2.18–14.99; $P_c$, 0.003) and controls ($P$, 0.0002; OR, 2.48; 95% CI, 1.55–3.96; $P_c$, 0.003). *Conclusion TNF-LTA* locus could serve as an important biomarker for breast cancer predisposition in Indian population.

**Keywords** *TNF* · *LTA* · SNP · Breast cancer

I. Kohaar · P. Tiwari · V. Nasare · N. Thakur · M. Bharadwaj (✉)
Division of Molecular Genetics & Biochemistry, Institute of Cytology & Preventive Oncology (ICMR), I-7, Sector 39, Noida 201301, India
e-mails: bharadwajm@icmr.org.in; mausumi02@yahoo.com

R. Kumar · B. C. Das
Division of Molecular Oncology, Institute of Cytology & Preventive Oncology (ICMR), I-7, Sector 39, Noida, India

## Introduction

Breast cancer is the third most common cancer in the world but it is the largest cause of deaths in the women of developed countries. Every year there are 1.05 million new cases worldwide which represent over 20% of all malignancies among females [1]. It is the most common non-cutaneous malignancy in women and is second only to lung cancer in mortality rates [2]. In India, breast cancer is the second most common cancer among females, while in the metropolitan cities of Delhi and Mumbai it ranks as the commonest cancer. In 2001 there were ∼80,000 new breast cancer cases in India [3–5].

The etiology of breast cancer is extremely complex and its onset and progression is a multi step process resulting from a series of epigenetic, genetic, endocrine and external environmental factors like infectious agents [6].

The role of genetic factors in epidemiology and pathogenesis of both sporadic breast cancer and familial breast cancer is now well established. Germline mutations in dominant, highly penetrant susceptibility genes such as *BRCA1* and *BRCA2* constitute only a small fraction (∼5%) of patients developing the disease. The majority of multiple case breast cancer families do not segregate mutations in these genes. In addition, genetic linkage studies have failed to identify further major breast cancer genes [7]. These observations have led to the proposal that breast cancer susceptibility is largely 'polygenic': that is, susceptibility is

conferred by a large number of loci, each with a small effect on breast cancer risk [8, 9]. High numbers of tumour-associated macrophages (TAMs) are found to be significantly associated with increased tumour angiogenesis and poor prognosis in invasive carcinoma of the breast [10]. TAM derived pro-inflammatory cytokines, in particular, TNFA and a related cytokine LTA [10–14] play a pivotal role in cellular immunity of the host, thereby constituting important genetically determined host factors in breast carcinogenesis.

These genes are arranged in tandem within the highly polymorphic major histocompatibility complex class III region at chromosome 6p21. Their protein products have related functions, and both bind the same receptor. Several single nucleotide polymorphisms (SNPs) in *TNF-LTA* locus are found to be involved in the modulation of gene expression that affect carcinogenesis [15–20]. Both these genes encode proteins which have cytostatic and cytotoxic effects on certain tumors [21–23]. TNF constitutes a useful immunological biomarker in breast carcinogenesis owing to its elevated levels in circulation along with enhanced TAM derived expression of TNF. This is suggestive of metastatic behaviour of inflammatory breast carcinomas [10, 14]. Therefore, it becomes imperative to identify functional polymorphisms and haplotypes in *TNF-LTA* locus for their role in predisposition towards breast cancer.

However, to the best of our knowledge, no study has been carried out addressing the association of the SNPs in the *TNF-LTA* locus with the susceptibility to breast cancer in Indian population. Therefore, we have investigated these polymorphisms for their role in breast cancer susceptibility by means of an allelic association study, in Indian population.

## Materials and methods

### Subjects

We investigated the association of five SNPs in the promoter region of *TNFA* at −238 (G/A), −308 (G/A), −857 (C/T), −863 (C/A) and −1031 (T/C) along with +252 (A/G) polymorphism of *LTA* in breast cancer patients (both sporadic & familial cases) in a hospital based case–control study. A total of 127 individuals comprising 40 breast cancer cases (35 sporadic & 5 familial) and 87 individuals of high risk group (with family history of breast cancer) belonging to Indo-Aryan ethnicity were employed for the study. The patients were recruited from Lok Nayak Jai Prakash (LNJP) and BRA-Institute Rotary Cancer Hospital (BRA-IRCH), New Delhi, with histopathologically confirmed carcinoma of breast. Blood samples of high risk group were collected from relatives of familial breast cancer patients either from the hospital or from their respective homes. The breast cancer patients and high risk group had a mean age of 49 ± 9.26 and 44.08 ± 9.26 years, respectively. The ethnicity matched control group consisting of 150 healthy women with mean age of 49.4 ± 12.4 years and having no self or family history of any neoplastic disease were from outdoor patients of Department of Gynaecology, Safdarjung hospital, New Delhi, who came for routine checkup. Written consent was obtained from all the participants and the study was carried out in accordance with the principles of Helsinki Declaration and was approved by the Ethics Committee of the Institute.

### Genomic DNA extraction

Genomic DNA was extracted from freshly collected blood of patients and controls by standard method using proteinase K followed by phenol/chloroform/isopropanol treatment [24].

### Analysis of *TNFA* and *LTA* polymorphisms by PCR-RFLP

We used polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) approach to genotype all the loci of the *TNF–LTA* region. −238 G/A & −308 G/A were genotyped by method employed by Jang et al. [25] with some modification [26]. While −857 C/T, −863 C/A & −1031 T/C were genotyped according to Skoog et al. [27]. In case of *LTA* gene, primers [Forward Primer—5′-GCT TCG TGC TTT GGA CTA CC-3′; Reverse Primer—5′-AAG GTG AGC AGA GGG AGA CA-3′] were designed using the software PRIMER 3.0. Fast Digest *NcoI* (MBI Fermentas) was employed for RFLP study of *LTA*.

The RFLP analysis was performed on 10% native polyacrylamide gel.

### DNA sequencing

We sequenced 20% of the patient samples randomly to validate the data generated by PCR-RFLP method. Sequencing reactions were performed according to the conventional dideoxy chain termination method using ABI Prism™ 310 Automated DNA Sequencer (Applied Biosystem, USA).

### Statistical analysis

The data analysis was performed using the computer software Statistical Package for the Social Sciences (SPSS) for Windows (version 12.0). Chi-square test/Fisher's Exact Test (for smaller numbers on subgroup analysis) was used to compare the distributions of *TNFA* and *LTA* polymorphisms between cancer patients, high risk group and

healthy controls. Haplotypes were constructed from genotypes of these six polymorphic markers by using PHASE (http://linkage.rockfeller.edu). LD estimates were determined by Haploview (http://www.broad.mit.edu/mpg/haploview/) [28].

## Results

Genotype frequency for all polymorphisms were found to be in concordance with Hardy–Weinberg Equilibrium in both cases and controls ($P > 0.05$) except for *LTA* polymorphisms in high risk group. This might be the consequence of selection pressure and therefore, was not excluded from the study.

The distribution of *TNFA* and *LTA* genotypes and alleles for five *TNFA* promoter SNPs at −238 G/A, −308 G/A, −857 C/T, −863 C/A, −1031 T/C and one intronic SNP +252 A/G of *LTA* in breast cancer cases, high risk group and healthy controls is depicted in Tables 1 and 2.

### *TNFA* promoter polymorphisms and risk of breast cancer (Tables 1 & 2)

A trend for association of *TNFA* −238 polymorphism was observed for both breast cancer cases (*P*, 0.015; OR, 5.12; 95% CI, 1.47–17.76) and high risk group (*P*, 0.027; OR, 3.77; 95% CI, 1.24–11.41) in comparison to controls. However, this significance was lost on applying correction for multiple testing ($P_c$, 0.09 in former and $P_c$, 0.16 in latter). *TNFA* −238 carrier A (GA/AA) genotype distribution revealed statistically insignificant trend from breast cancer cases with 15% (6/40) to high risk group with 11.49% (10/87) and then controls with 3.3% (5/150). No polymorphic homozygous genotype (AA) was found in any of the study groups (data not shown). The frequency of the −238 A allele in the cancer (0.075) and in high risk (0.057) was found to be 4.4 times and 3.4 times more than that of control (0.017), respectively.

Carrier genotype (GA/AA) distribution at −308 G/A locus revealed highly significant association ($P < 0.0001$; OR, 9.53; 95% CI, 4.11–22.13) for breast cancer versus controls and breast cancer versus high risk group (*P*, 0.0002; OR, 5.15; 95% CI, 2.19–12.12) with a frequency of 47.5% (19/40) in cancer, 14.94% (13/87) in high risk group and 8.67% (13/150) in controls. The significance was retained even after applying the criteria for multiple testing ($P_c$ for breast cancer versus controls <0.001; $P_c$ for breast cancer versus high risk group = 0.001). 7.5% (3/40) of the breast cancer cases and 2.3% (2/87) of high risk group were found to be polymorphic homozygous (AA) while it was nil for control group (data not shown). No association was established with respect to −308 G/A polymorphism between high risk group and healthy controls. Minor allele frequency (− 308 A allele) in cancer (0.28) was found to be 6.5 folds higher than that of controls (0.043) while it was found to be two times more than that of high risk group (0.086), thus establishing −308 A allele as major susceptibility allele for breast cancer.

On the contrary, −857 C/T polymorphism was significantly under-represented (*P*, 0.0005; OR, 0.35; 95% CI, 0.19–0.62) in high risk group in comparison to controls with carrier genotype (CT/TT) frequency 25.29% (22/87) in the former and 49.33% (74/150) in the latter. Significant association of this polymorphism with controls was also obtained after using correction for multiple testing ($P_c = 0.003$). The carrier genotype (CT/TT) frequency was also lower in cancer cases (35%, 14/40) than controls but the difference could not attain the limit of statistical significance. −857 TT polymorphic homozygous genotype was found in 6.67% (10/150) of controls, 2.3% (2/87) of high risk group and 5% (2/40) in cancer cases (data not shown). −857 T allele frequency of 0.28 in controls was found to be two times more than the high risk group (0.14) and was comparable to that of cancer (0.21).

On the other hand, the distribution of carrier genotypes for −863 C/A (CA/AA) and −1031 T/C (TC/CC) loci showed no relationship either with susceptibility to or development of breast cancer with a frequency of 32.5% (13/40), 36.78% (32/87), 36% (54/150) for −863 C/A and 52.5% (21/40), 45.98% (40/87), 37.33% (56/150) for −1031 T/C polymorphism in cancer cases, high risk group and controls respectively. Minor allele frequency (−863 A) was found to be similar in cancer and controls (0.21) while it was 0.24 in high risk group. Although, the percentage distribution of carrier genotype and polymorphic homozygous genotype for −1031 T/C polymorphism increased from controls (CC = 10%; 4/40) to high risk (CC = 5.7%; 5/87) and then cancer cases (CC = 10%; 4/40), no significant statistical trend could be established (data not shown). −1031 C allele also showed similar distribution in controls (0.21), high risk group (0.26) and cases (0.31).

### *LTA* polymorphism and risk of breast cancer (Tables 1 & 2)

+252 A/G polymorphism tends to be related with cancer versus controls (*P*, 0.025; OR, 2.38; 95% CI, 1.17–4.85), but it became insignificant ($P_c = 0.15$) after applying correction for multiple testing. Interestingly, a significant difference of this polymorphism was detected in high risk group versus control ($P < 0.0001$; OR, 8.27; 95% CI, 4.28–16.0) and cancer versus high risk group (*P*, 0.007; OR, 0.29; 95% CI, 0.12–0.67) with carrier genotype (AG/GG) frequency of 60% (24/40), 83.91% (73/87) and 38.67% (58/150) in cancer, high risk group and controls,

**Table 1** Association of single nucleotide polymorphisms in *TNFA* and *LTA* among breast cancer, high-risk group and healthy control subjects

| Genotype | Cancer cases $N = 40$ (%) | High risk group $N = 87$ (%) | Controls $N = 150$ (%) |
|---|---|---|---|
| *TNFA* −238 G/A | | | |
| GA/AA | 6 (15) | 10 (11.49) | 5 (3.33) |
| GG | 34 (85) | 77 (88.51) | 145 (96.67) |
| Odds ratio (95% CI) | 5.12[a] (1.47–17.76) | 3.77[b] (1.24–11.41) | 1.0 |
| *P*-value | 0.015[a] | 0.027[b] | Reference |
| *TNFA* −308 G/A | | | |
| GA/AA | 19 (47.5) | 13 (14.94) | 13 (8.67) |
| GG | 21 (52.5) | 74 (85.06) | 137 (91.33) |
| Odds ratio (95% CI) | 9.53[a] (4.11–22.13) | 1.85[b] (0.82–4.20) | 1.0 |
| *P*-value | <0.0001[a] | 0.20[b] | Reference |
| *TNFA* −857 C/T | | | |
| CT/TT | 14 (35) | 22 (25.29) | 74 (49.33) |
| CC | 26 (65) | 65 (74.71) | 76 (50.67) |
| Odds ratio (95% CI) | 0.55[a] (0.27–1.14) | 0.35[b] (0.19–0.62) | 1.0 |
| *P*-value | 0.15[a] | 0.0005[b] | Reference |
| *TNFA* −863 C/A | | | |
| CA/AA | 13 (32.5) | 32 (36.78) | 54 (36) |
| CC | 27 (67.5) | 55 (63.22) | 96 (64) |
| Odds ratio (95% CI) | 0.86[a] (0.41–1.80) | 1.03[b] (0.60–1.79) | 1.0 |
| *P*-value | 0.08[a] | 0.90[b] | Reference |
| *TNFA* −1031 T/C | | | |
| TC/CC | 21 (52.5) | 40 (45.98) | 56 (37.33) |
| TT | 19 (47.5) | 47 (54.02) | 94 (62.67) |
| Odds ratio (95% CI) | 1.85[a] (0.92–3.75) | 1.43[b] (0.84–2.44) | 1.0 |
| *P*-value | 0.12[a] | 0.24[b] | Reference |
| *LTA* +252 A/G | | | |
| AG/GG | 24 (60) | 73 (83.91) | 58 (38.67) |
| AA | 16 (40) | 14 (16.09) | 92 (61.33) |
| Odds ratio (95% CI) | 2.38[a] (1.17–4.85) | 8.27[b] (4.28–16.0) | 1.0 |
| *P*-value | 0.025[a] | <0.0001[b] | Reference |

CI, Confidence interval; Significance (*P*-value; OR) is assessed by Chi-Square test/ Fisher's Exact Test (using the approximation of Woolf)

OR (95% CI)/*P*-value against control; [a]versus cancer; [b]versus high risk group; *P*-value; OR (95% CI) against high risk group versus cancer is 0.0002; 5.15 (2.19–12.12) for *TNFA* −308 G/A and 0.007; 0.29 (0.12–0.67) for *LTA* +252 A/G

**Table 2** Frequency distribution of minor alleles for different SNP loci in *TNFA-LTA* region among breast cancer, high-risk group and healthy control subjects

| Minor allele | Cancer cases $N = 40$ | High risk group $N = 87$ | Controls $N = 150$ |
|---|---|---|---|
| *TNFA* −238 A | 0.075 | 0.057 | 0.017 |
| *TNFA* −308 A | 0.28 | 0.086 | 0.043 |
| *TNFA* −857 T | 0.21 | 0.14 | 0.28 |
| *TNFA* −863 A | 0.21 | 0.24 | 0.21 |
| *TNFA* −1031 C | 0.31 | 0.26 | 0.21 |
| *LTA* +252 G | 0.3 | 0.43 | 0.22 |

respectively. This significance was also observed after applying correction factor ($P_c < 0.001$ for high risk group versus control; $P_c = 0.04$ for high risk group versus cancer). +252 GG (polymorphic homozygous) was found to be 6% (9/150) in control, 2.3% (2/87) in high risk group while it was nil for cancer cases (data not shown). Minor allele frequency (+252 G allele) was 1.4 and 2 folds higher in cancer (0.3) and high risk group (0.43) than controls (0.22).

## Linkage disequilibrium

Combined analysis of both the patient and control groups revealed that complete linkage disequilibrium was established between −308 G/A and −857 C/T (D' = 1.0, $r^2 = 0.03$) and also between −857 C/T and −1031 T/C polymorphic loci (D' = 1.0, $r^2 = 0.00$). Haplotype analysis using Statistical software, PHASE, showed the presence of 16 haplotypes in 40 cancer, 87 high risk group and 150 healthy controls (Table 3). However, only 12 haplotypes were present at frequencies above 1% in the study population. Four haplotypes—AGGCCT (135/554; 24.37%), AGGTCT (99/554; 17.87%), GGGCCT (93/554; 16.79%) and AGGCAC (83/554; 14.98%) were the most frequent.

**Table 3** Distribution frequencies of *TNF-LTA* haplotypes among breast cancer, high risk group and healthy control subjects

| S. No. | Haplotype (*LTA* +252/*TNFA* −238/*TNFA* −308/*TNFA* −857/*TNFA* −863/*TNFA* −1031) $N = 554$ (%) | Cancer $N = 80$ (%) | High risk $N = 174$ (%) | Control $N = 300$ (%) |
|---|---|---|---|---|
| 1 | AGGCCT (135; 24.37%) | 11 (13.75) | 40 (22.99) | 84 (28) |
| | Odds ratio 95% (CI) | 0.41[a] (0.21–0.81) | 0.77[b] (0.50–1.18) | 1.0 |
| | *P*-value | 0.013[a] | 0.28[b] | Reference |
| 2 | AGGCCC (19; 3.43%) | 5 (6.25) | 1 (0.57) | 13 (4.33) |
| | Odds ratio 95% (CI) | 1.47[a] (0.51–4.26) | 0.13[b] (0.02–0.98) | 1.0 |
| | *P*-value | 0.67[a] | 0.02[b] | Reference |
| 3 | AGGCAT(15; 2.71%) | 0 | 2 (1.15) | 13 (4.33) |
| | Odds ratio 95% (CI) | 0.13[a] (0.01–2.25) | 0.26[b] (0.06–1.15) | 1.0 |
| | *P*-value | 0.08[a] | 0.06[b] | Reference |
| 4 | AGGCAC (83; 14.98%) | 14 (17.5) | 26 (14.94) | 43 (14.33) |
| | Odds ratio 95% (CI) | 1.27[a] (0.65–2.46) | 1.05[b] (0.62–1.78) | 1.0 |
| | *P*-value | 0.59[a] | 0.96[b] | Reference |
| 5 | AGGTCT (99; 17.87%) | 15 (18.75) | 16 (9.20) | 68 (22.67) |
| | Odds ratio 95% (CI) | 0.79[a] (0.42–1.47) | 0.35[b] (0.19–0.62) | 1.0 |
| | *P*-value | 0.55[a] | 0.0003[b] | Reference |
| 6 | AGGTAT (7; 1.26%) | 1 (1.25) | 3 (1.72) | 3 (1.0) |
| | Odds ratio 95% (CI) | 1.25[a] (0.13–12.22) | 1.74[b] (0.35–8.70) | 1.0 |
| | *P*-value | 1.0[a] | 0.67[b] | Reference |
| 7 | AGACCT (9; 1.62%) | 3 (3.75) | 1 (0.57) | 5 (1.67) |
| | Odds ratio 95% (CI) | 2.30[a] (0.54–9.83) | 0.34[b] (0.04–2.94) | 1.0 |
| | *P*-value | 0.47[a] | 0.42[b] | Reference |
| 8 | AAGCCT (3; 0.54%) | 0 | 0 | 3 (1.0) |
| | Odds ratio 95% (CI) | 0.53[a] (0.03–10.33) | 0.24[b] (0.01–4.75) | 1.0 |
| | *P*-value | 1.0[a] | 0.30[b] | Reference |
| 9 | AAGCCC (14; 2.53%) | 5 (6.25) | 8 (4.60) | 1 (0.33) |
| | Odds ratio 95% (CI) | 19.93[a] (2.29–173.27) | 14.41[b] (1.79–116.27) | 1.0 |
| | *P*-value | 0.002[a] | 0.002[b] | Reference |
| 10 | AAGCAC (2; 0.36%) | 1 (1.25) | 1 (0.57) | 0 |
| | Odds ratio 95% (CI) | 11.34[a] (0.46–281.25) | 5.20[b] (0.21–128.35) | 1.0 |
| | *P*-value | 0.21[a] | 0.37[b] | Reference |
| 11 | GGGCCT (93; 16.79%) | 5 (6.25) | 48 (27.59) | 40 (13.33) |
| | Odds ratio 95% (CI) | 0.43[a] (0.16–1.14) | 2.48[b] (1.55–3.96) | 1.0 |
| | *P*-value | 0.12[a] | 0.0002[b] | Reference |
| 12 | GGGCAC (13; 2.35%) | 0 | 8 (4.60) | 5 (1.67) |
| | Odds ratio 95% (CI) | 0.33[a] (0.02–6.10) | 2.84[b] (0.91–8.83) | 1.0 |
| | *P*-value | 0.59[a] | 0.11[b] | Reference |
| 13 | GGGTCT (18; 3.25%) | 0 | 5 (2.87) | 13 (4.33) |
| | Odds ratio 95% (CI) | 0.13[a] (0.01–2.25) | 0.65[b] (0.23–1.90) | 1.0 |
| | *P*-value | 0.08[a] | 0.58[b] | Reference |
| 14 | GGACCT (41; 7.40%) | 20 (25) | 13 (7.47) | 8 (2.67) |
| | Odds ratio 95% (CI) | 12.17[a] (5.12–28.92) | 2.95[b] (1.20–7.26) | 1.0 |
| | *P*-value | <0.0001[a] | 0.03[b] | Reference |
| 15 | GGACAC (1; 0.18%) | 0 | 1 (0.57) | 0 |
| | Odds ratio 95% (CI) | | 5.20[b] (0.21–128.35) | 1.0 |
| | *P*-value | 0.21[a] | 0.37[b] | Reference |
| 16 | GAGCCT (2; 0.36%) | 0 | 1 (0.57) | 1 (0.33) |
| | Odds ratio 95% (CI) | 1.24[a] (0.05–30.76) | 1.73[b] (0.11–27.83) | 1.0 |
| | *P*-value | 1.0[a] | 1.0[b] | Reference |

CI, Confidence interval; Significance OR is assessed by Chi-Square test/Fisher's Exact Test (using the approximation of Woolf)

OR (95% CI)/*P*-value against control; [a]versus cancer; [b]versus high risk group

*P*-value; OR (95% CI) against high risk group versus cancer is 0.01; 0.09 (0.01–0.76) for AGGCCC; 0.0002; 5.71 (2.18–14.99) for GGGCCT; 0.0003; 0.24 (0.11–0.52) for GGACCT

*TNF-LTA* haplotypes and risk of breast cancer (Table 3)

Three haplotypes were found to be under-represented in cases or high risk group with respect to controls—AGGCCT in cancer versus controls (*P*, 0.013; OR, 0.41; 95% CI, 0.21–0.81), AGGCCC and AGGTCT in high risk group versus controls with *P* values of 0.02 (OR, 0.13; 95% CI, 0.02–0.98) and 0.0003 (OR, 0.35; 95% CI, 0.19–0.62), respectively. However, statistical significance was retained only for AGGTCT (with minor allele T for −857C/T) in controls versus high risk group ($P_c = 0.005$) after applying correction for multiple testing ($P_c = 0.21$ for AGGCCT in cancer versus controls; $P_c = 0.32$ for AGGCCC in high risk group versus controls). On the other hand AGGCCC haplotype (with minor allele C for −1031T/C) was found to be associated (*P*, 0.01; OR, 0.09; 95% CI, 0.01–0.76) with cancer in comparison to high risk group but the $P_c$ value of 0.16 made the association insignificant. AAGCCC haplotype (with minor allele A for −238G/A and minor allele C for −1031 T/C) was significantly associated with cases versus controls (*P*, 0.002; OR, 19.93; 95% CI, 2.29–173.27) and also high risk group versus controls (*P*, 0.002; OR, 14.41; 95% CI, 1.79–116.27) even after applying correction for multiple testing ($P_c = 0.03$ for both groups). However, GGGCCT (with minor allele G for +252 A/G) was the most frequent haplotype in high risk group when compared to either cancer (*P*, 0.0002; OR, 5.71; 95% CI, 2.18–14.99) or controls (*P*, 0.0002; OR, 2.48; 95% CI, 1.55–3.96) with $P_c$ values of 0.003 in both study groups. Interestingly, GGACCT haplotype (with minor allele A for −308 G/A) in particular, was found to be positively associated with cancer versus controls ($P < 0.0001$; OR, 12.17; 95% CI, 5.12–28.92), high risk group versus controls (*P*, 0.03; OR, 2.95; 95% CI, 1.20–7.26) and also with cancer versus high risk group (*P*, 0.0003; OR, 0.24; 95% CI, 0.11–0.52). But the association was found to be statistically significant after multiple testing for cancer versus control ($P_c < 0.001$) and cancer versus high risk group ($P_c = 0.005$) and not for high risk group versus control ($P_c = 0.48$). Hence, GGACCT and GGGCCT haplotypes can be considered as important risk/susceptibility haplotypes specifically for breast cancer and high risk group respectively in Indian population.

## Discussion

Breast cancer is the second most common malignancy among women, next to cervix cancer in Indian population [4]. However, in urban areas it has overtaken the cervical cancer and thus, has become the most prevalent cancer [3–5]. India carries 13% of global burden of breast cancer and the continuing rise in breast cancer incidence, both globally as well as in Indian perspective, has created an urgent need to develop strategies for prevention. Etiology of breast cancer is quite complex whereby host genetic factors play a key role. Therefore, it is important to evaluate the role of different biomarkers in breast cancer susceptibility for the better understanding of the disease etiology, which may contribute towards treatment and early detection of the cancer. *TNF* and *LTA* polymorphisms are reported to be associated with the inflammatory and immunomodulatory diseases including cancer [15–19].

In the present study, a significant association was established, individually for *TNFa* −308 G/A ($P < 0.0001$; $P_c$, 0.001) SNP with 9.5 folds increased risk to breast cancer than controls and 5 folds increased risk (*P*, 0.0002; $P_c$, 0.001) in comparison to the high risk group. Therefore −308 A allele acts as a major susceptibility allele exclusively for cancer. Interestingly, *LTA* +252 A/G polymorphism was found to be associated with high risk group versus controls ($P < 0.0001$; $P_c$, 0.001) with 8 folds increased risk and also with high risk group versus breast cancer (*P*, 0.007; $P_c$, 0.04), thereby establishing +252 A/G polymorphism as characteristic feature of high risk study group. Though the polymorphic allele for both −238 G/A and −1031 T/C loci in *TNFA* promoter showed, individually, a trend of increasing order from controls to high risk group and then to cancer, but statistical significance could not be attained. −863 C/A polymorphism revealed similar genotype distribution between all the study groups.

To supplement further our understanding of the contribution of these genetic variants to breast cancer, six-locus haplotypes were constructed and their distribution was compared in the cancer, high risk group and the control population. AAGCCC (with minor alleles *TNFA* −238 A & *TNFA* −1031 C) and GGACCT (with minor alleles *LTA* +252 G & *TNFA* −308 A) were found to increase the risk of breast cancer with *P* values of 0.002 ($P_c$, 0.03) and <0.0001 ($P_c < 0.001$) respectively when compared to controls. Haplotypes AAGCCC (with minor alleles *TNFA* −238 A & *TNFA* −1031 C) and GGGCCT (with minor allele *LTA* +252 G) reported association specifically for high risk group than controls with *P* values 0.002 ($P_c$, 0.03) and 0.0002 ($P_c$, 0.003) respectively as well as high risk group versus cancer for GGGCCT (*P*, 0.0002; $P_c$, 0.003). While AGGTCT (with minor allele −857 T) haplotype exhibited protective role (*P*, 0.0003; $P_c$, 0.005) with respect to high risk category versus controls. Additionally, GGACCT (with minor alleles *LTA* +252 G & *TNFA* −308 A) haplotype was also found to be positively related (*P*, 0.0003; $P_c$, 0.005) with cancer versus high risk group. Overall, GGACCT haplotype emerged as a major "Risk Haplotype" which showed a statistically significant trend from controls to high risk and then to breast cancer while GGGCCT haplotype act as a marker for high risk group.

Our study is in concordance with the study on Tunisian population in relation to −308 polymorphism [29, 30] and with other two studies from USA and Poland with respect to −238 G/A SNP [14] in breast cancer. −1031 T/C, −308 G/A and −238 G/A SNPs were also found to be more frequent in invasive breast carcinoma in Croatian women [31]. Similar findings were obtained by Smith et al. [32] who found a non-significant trend for association between the *TNFA* −308 GG genotype and breast cancer in UK women. A study in Korean population also reported the *LTA* +252 AG/GG genotype with an increased risk for breast cancer, which further supports our findings [33]. However, few contradictory reports are also available. Evaluation of common genetic variation across the *TNF–LTA* locus revealed lack of an association for *TNFA* −308G/A with the development of breast cancer in Caucasian population (Ontario breast cancer registry, Canada) [34] as well as Italian population [35]. Our finding with respect to −308 polymorphism was also found to be inconsistent with study on US and Poland populations [14]. Similar findings were reported from Iranian patients [36] and Caucasian population of the northern Netherlands [37] for *TNFA* 308 and *LTA* +252 polymorphisms and Korean population for −1031 (T/C), −863 (C/A), −857 (C/T) and −308 (G/A) SNPs. In addition, no association was found between the −308G/A and the −238G/A in *TNF* locus and susceptibility to breast cancer in North European population [38].

Polymorphisms in *TNF-LTA* locus also exhibit heterogeneity in different world populations [26, 39]. Present study associated *TNFA* −308 A and *LTA* +252 G alleles with breast cancer susceptibility while −857 T allele seemed to have protective effect with respect to controls. On the other hand, *LTA* +252 G allele was found to be characteristic feature of high risk group.

−238 G/A and −308 G/A promoter polymorphisms of *TNF* are shown to be associated with the TNF expression both in vivo and in vitro [18–20]. *TNFA* −857T, −863A and −1031 C are also found to increase TNF promoter activity [40] and lipopolysaccharide-induced TNFα production [41], although contradictory findings have also been reported [42, 43]. The *LTA* +252G allele has been linked to increased lymphotoxin-α production by phytohemagglutinin-activated mononuclear cells in vitro [18]. In fact, the *LTA* +252 G allele was also found to have higher TNFα secretary capacity than +252 A allele [44], as well as higher circulatory concentrations of TNF α [45].

It has been shown that dysregulation and overproduction of TNFα could be involved in cancer development and progression. The tumor-promoting functions of TNF-α may be mediated by its ability to induce proangiogenic functions, to promote the expression of matrix metalloproteinases (MMP) and endothelial adhesion molecules, and to cause DNA damage via reactive oxygen, improves interaction between stroma and malignant cells within the extracellular matrix, and induces a growth-promoting hormone milieu [46]. However, these effects may depend on multiple factors, such as treatment by estrogen and the expression of members of the epidermal growth factor receptor family. TNF-α activity vary under different physiological conditions and in a cell-type-dependent manner which contributes to its paradoxical role [47].

In conclusion, this study established for the first time, an association for *TNF-LTA* locus with susceptibility to breast cancer in Indian population with Indo-Aryan ethnicity. Individual polymorphisms together with haplotype analysis revealed statistically significant differences between the disease groups and controls. Thus, *TNF-LTA* locus could serve as an important biomarker for breast cancer predisposition. Future studies with larger sample size taking into account different grades of breast cancer lesions along with linkage analysis with flanking HLA region is warranted to validate the findings.

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