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Genotypic and Haplotype analysis of Interleukin-6 and-18 gene polymorphisms in association with Clinicopathological factors in Breast cancer

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Simple Summary: The etiology of breast cancer is multifactorial and heterogeneous; it appears to involve numerous genetic/epigenetic, endocrine, and external environmental factors. The *IL6* and *IL18* network is highly complex and employs an interactive cascade of gene activation and suppression. The present study performed a comprehensive analysis to understand the role of epidemiological, clinic-pathological characteristics and effect of functional SNPs of *IL6* and *IL18,* in breast cancer patients in comparison to controls. Genotype, haplotype, LD, and survival analysis revealed a significant association between epidemiological, clinical variables and the progression of breast cancer. The data suggest genetic polymorphisms of *IL6* (-597G>A, -572C>G & -174G>C) and *IL18* genes (-607C>A, -137G>C & 105A>T) may influence the gene transcription, cytokine secretion and thereby modulate the risk for progression of the disease.

Abstract:

Background: Cytokines are multifunctional glycoproteins playing a vital role in the tumor microenvironment and in the progression of breast cancer. The immune responses in the tumours restrained by pro- and anti-inflammatory cytokine expressions could be influenced by genetic polymorphisms. Hence, the present study evaluated the contribution of *IL6* (rs1800797, rs1800796, and rs1800795), and *IL18* (rs1946518, rs187238, and rs549908) genotypes and its haplotypes with risk and progression of breast cancer in south Indian population. **Methods:** The polymorphisms of *IL6* gene -597G>A, -572G>C, -174G>C and *IL18* gene -607C>A, -137G>C, and 105A>T were genotyped through PCR-RFLP and As-PCR assays in blood DNA of 600 subjects. We have performed haplotype, LD, univariate, multivariate logistic regression and Kaplan-Meier analyses for the obtained data. **Results:** The frequency of AA genotype & A-allele of *IL6* -597G>A, and CC genotype & C allele of *IL6* -174G>C polymorphism was higher in breast cancer patients and was found to be significantly associated with TNM staging, late (advanced) stage, metastasis, etc. While, CG and GG genotypes of *IL6* -572 C>G polymorphism had a protective role against breast cancer. Further, *IL18* -607C>A, -137G>C & 105A>T polymorphisms were found to be associated with lobular carcinoma subtype, PR^{-ve} and $HER2^{+ve}$ breast cancer patients. Perfect LD was observed between all SNPs of *IL6* & *IL18* genes under study; G-C-C, A-G-G and A-C-C haplotype combination of *IL6* genes had conferred 2.09, 2.25 and 4.72 folds risk for breast cancer respectively. In

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survival analysis, we observed that the C allele of rs 1800795 was found to be significantly associated with 5years overall survival in breast cancer subjects. **Conclusions**: Overall, our results suggest the importance of genotypic and haplotype analysis of *IL6*, and *IL18* gene variants in progression and risk identification of breast cancer.

Keywords: Breast Cancer; Haplotype; Single nucleotide polymorphisms; Interleukin-6 and Interleukin-18.

I. Introduction

Breast cancer (BC) a strong interaction between genetic and epigenetic factors cause dynamic changes in the genome leading to uncontrolled cell growth, ability to invade and metastasize [1,2]. Recent experimental evidences implicate the role of cytokines in breast cancer development and progression. Interleukins (*IL*) are a class of cytokines primarily expressed by leukocytes and are low molecular weight protiens, which act on many target cells often in an additive, synergistic or antagonistic manner and are considered as key intercellular mediators that control survival, growth, differentiation, and the effector cell function. In addition, they are also involved in the pathology of BC surveillance system and other complex diseases [3-5].

IL6 and *IL18* belong to the *IL1* cytokine family and are known to regulate the immune reactions, inflammation and promote tumor growth by up-regulating angiogenic and anti-apoptotic proteins [4]. The genes for *IL6* and *IL1*8 are encoded on human chromosome 7p15.3 and 11q23.1 respectively. It has been reported that several important polymorphic sites in the *IL6* and *IL18* genes, including three in the promoter regions (-597G>A, -572C>G & -174G>C) of *IL6* and two in promoter and one in exon regions (-607C>A, -137G>C & 105A>T) of *IL18* play an important role in breast cancer progression [6-8]. Furthermore, the single nucleotide polymorphisms (SNPs) have been considered to be important biomarkers in cancer screening, staging, grading and risk assessment of the disease [9].

Despite the limitations in individual SNP analysis, haplotype blocks in the human genome which are formed as a result of linkage disequilibrium, can be used to identify its association with human diseases like cancer [10]. Hence possible that a haplotype effect, rather than an individual effect of SNPs on clinico-pathological status, might explain some of the prognostic and survival information. The aim of the present study is to perform a comprehensive analysis of the possible prognostic importance of genotypic and haplotypes of the *IL6* gene (-597G>A, -572C>G & -174G>C) and *IL18* gene (-607C>A, -137G>C & 105A>T) polymorphisms with clinicopathological status in susceptibility and progression of breast cancer.

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2. Materials and methods

2.1. Study Design and Participants

A total of 300 breast cancer patients and 300 healthy cancer-free control subjects with similar socio-economic and geographic backgrounds were included in the study after signed written informed consent. The patients with Breast cancer were recruited from the MNJ Institute of Oncology and Regional Cancer Centre (MNJIO&RCC), Hyderabad during the period of 2010-2016. The histo-clinicopathological information of the patients was obtained from their medical records in the hospital. All the samples were coded and maintained confidentially. The study has been approved by the Institutional and Hospital review boards of Osmania University and MNJIO&RCC-Hyderabad, Telangana state, INDIA.

2.2 Polymorphism and SNP Genotyping analysis

Genomic DNA was extracted from peripheral blood using the Bio-serve DNA Blood Mini kit (Bio-Serve, INDIA), according to the manufacturer's instructions [11]. The genotypes of *IL6* (-597G>A, -572C>G and -174G>C) and *IL18* (-607C>A, -137G>C and 105A>T) gene polymorphisms were determined using PCR-RFLP (Polymerase chain reaction-Restriction Fragment Length Polymorphism) and As-PCR (Allele Specific-Polymerase Chain Reaction) methods respectively. PCR primers, RFLP enzymes, size of the PCR-RFLP products and conditions are shown Table 1. As a genotyping quality assessment purpose, we have randomly selected and repeated 10% of the samples. The samples were found to be 100% concordant in two independent assays.

Table 1: Primer sequences and reaction conditions for genotyping *IL6* and *IL18* polymorphisms

Polymorphism (db SNP ID)	PCR Primer sequence	mT (°C)	Amplicon Size (bp)/RE	Product size (bp)
***IL6* -597G>A** (rs1800797)	F: 5′-GGAGTCACACACTCCACCTG-3′ R: 5′-AAGCAGAACCACTCTTCCTTTACTT-3′	61.5	527 *FokI*	GG:527 GA:527+461+66 AA:461+66
***IL6* -572C>G** (rs1800796)	F: 5′-GGAGACGCCTTGAAGTAACTGC-3′ R: 5′-GAGTTTCCTCTGACTCCATCGCAG-3′	55.0	296 *BsrBI*	CC:296 CG: 296+201+95 GG:201+95
***IL6* -174G>C** (rs1800795)	F: 5'-ATGCCAAGTGCTGAGTCACTA-3' R: 5'-TCGAGGGCAGAATGAGCCTC-3'	59.7	230 *NlaIII*	GG:230 GC:230+121+109 CC-121+109
***IL18* -607C>A** (rs1800795)	F: 5'-CTTTGCTATCAT TCCAGGAA-3' R: 5'-TAACCTCATTCAGGACTTCC-3' C allele:5'- GTTGCAGAAAGGTAAAAATTATTAC-3' A allele:5'- TTGCAGAAAGTGTAAAAATTATTAA-3'	*56.5*	As-PCR 306 196	CC:306+196; 306 CA:306+196;306+196 AA-306, 306+196 Common band: 306 C/A allele: 196
***IL18* -137G>C** (rs187238)	F: 5'-CCAATAGGACTGATTATTCCGCA-3' R:5'-AGGAGGGCAAAATGCACTGG-3' G allele: 5'- CCCCAACTTTTACGGAAGAAAAG-3' C allele: 5'- CCCCAACTTTTACGGAAGAAAAC-3'	62.5	*As-PCR* *446* *261*	GG: 446+261; 261 GC: 446+261;446+261 CC: 446;446+ 261 Common band-446 G/C allele: 261

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***IL18* 105A>T (rs549908)**	F: 5'-TGTTTATTGTAGAAAACCTGGAATT-3' R: 5'-CCTCTACAGTCAGAATCAGT-3'	50.0	148 *FokI*	AA: 148,AT: 148+123+25 TT: 123+25

Abbreviations: dbSNP ID, database identifier; single-nucleotide polymorphism (SNP); polymerase chain reaction (PCR); forward primer (F); reverse primer (R); melting temperature (mT); restriction endonuclease (RE); base pair (bp).

2.3 Statistical analysis

χ2 (chi-squraed) test and the odds ratio (OR) for allele and genotype frequencies of the studied polymorphism between cases and healthy controls were determined. All the *p* values were two sided, and the level of significance was taken as $p<0.05$. The different statistical tools used to analyse the quantitative and qualitative variables in the present study are IBM SPSS version 20.0, SNPStats (http://bioinfo.iconcologia .net/index.php? module ¼Snp stats)[12], HAPLOVIEW (http://www.broad.mit.edu/mpg/haploview) [13], Generalized Linkage disequilibrium and Graph Pad Prism [14].

2.4 Insilico Analysis

The SNPs in the promoter regions of *IL6*, and *IL18* genes were studied for the presence of transcription factor binding sites using AliBaba2.1 online bioinformatics tool (http://www.gene-regulation. com/pub/programs/alibaba2/index.html). Pre mRNA Secondary structures for *IL18* 105A>T (exon) were identified by using RNAfold WebServer tool originally proposed by Zuker and Stiegler (http://rna.tbi.Univie.ac.at /cgi-bin/RNAWebSuite/RNAfold.cgi) [15]. Protein-protein interaction (PPI) analysis of IL6 and IL18 was performed using Search tool for the Retrieval of Intracting Genes/Protines (STRING 9.1 http://string.embl.de/) [16].

3. RESULTS

3.1 Comparison of demographic characteristics among patients with breast cancer and controls

The detailed demographic and baseline clinicopathological characteristics of study subjects are illustrated in Fig. 1. In the present study the age of the breast cancer patients was within a range of 30–75 years, and their average age at diagnosis was 47.98 years (SD:±10.80) while the age of the controls at recruitment ranged from 35-68 years with a mean age of 46.34 years (SD:±7.97). An interesting finding that there was a significantly greater proportion of subjects are with higher BMI, mixed diet, history of smoking habit and alcohol consumption in the BC group (37.0%, 85.56%, 18.34% and 40.4%) than in the carcinoma-free control group (15.7%, 71.0%, 9.0% and 19.0%) respectively ($p<0.001$), while there was a no significant difference observed in age, area of living and occupation ($p>0.05$) of the patients and controls as shown in Fig.1A. The frequency distributions of age at late menarche >14 years (27.0%), nulliparous (8.34%), lactation (9.0%) and history of parental consanguinity (37.0%) were high in breast cancer patients compared to controls. Moreover, there was no significant difference between the two groups in the frequency distribution in terms of menstrual cycles, and menstruation status

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(p>0.05). The familial incidence of the breast cancer was found to be 15.0% while the same of the other carcinomas was found to be 19% in breast cancer patients as shown in Fig.1B.

The clinicopathological characteristics of breast cancer cases are shown in Fig. 1C. In the present study, breast cancer patients were categorized according to tumor node metastasis (TNM) classification for breast cancer and found that 65.67% are with T_0-T_2 stage, 34.33% are T_3-T_4 stages. Among the breast cancer patients 65.7% have shown about <50mm tumor size. The percentage of histologically confirmed breast cancer patients was 82.33% in ductal carcinoma and 18.0% had lobular carcinoma. Whereas, 73.33% of patients had positive axillary lymph node metastasis and 34.4% had distant metastasis. According to steroid hormonal clinical characteristics, patients were classified as with positive receptor status for ER (59.0%), PR (56.0%,), HER2/neu (54.33%,) and for some of the patients there was a missing data on receptor status (8.67%) respectively.

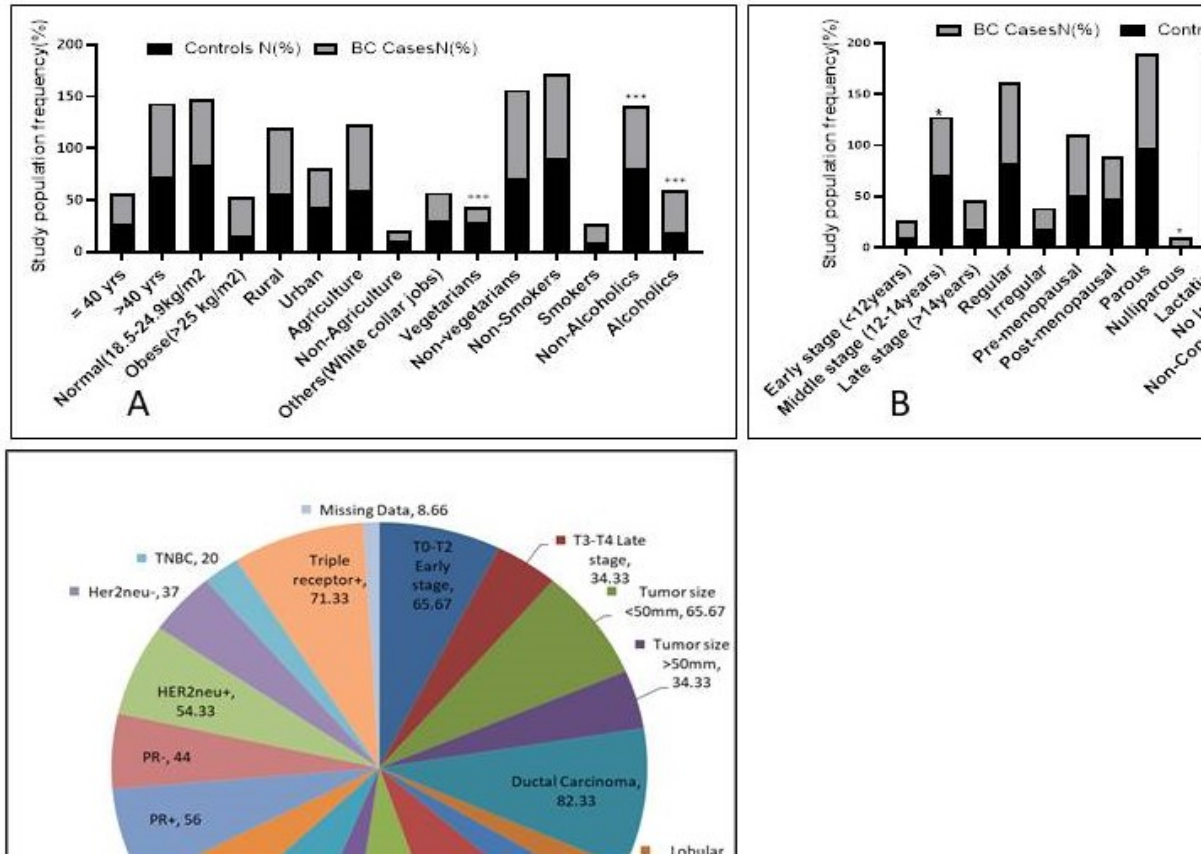

Figure: 1. Distributions of the frequencies study population(Controls N=300 and BC cases N=300): Distributions of the frequencies of epidemiological characteristics controls and BC cases A, Baseline clinical and family history characteristics B, Clinicopathological risk parameters prevalence in the breast cancer cases C.

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3.2 Association Analysis of Alleles and Genotypes with Risk of Breast cancer

The genotypic and allele frequencies of 6 SNPs in the *IL6* (-597G>A,-572C>G, -174G>C) and *IL18* (-607C>A, -137G>C, 105A>T) genes in 300 breast cancer patients and 300 controls groups adjusted with age by multivariate logistic regression analyses are summarized in Table 2. The frequency of A-allele and the GA and AA genotypes of *IL6* -597G>A polymorphism have shown a significant association (p<0.01) with breast cancer. The frequency of G-allele and GG genotype of *IL6* -572C>G polymorphism was found to be less in breast cancer cases when compared to controls. Further analysis revealed that the C allele of *IL6* -174G>C polymorphism was found to be significantly associated with breast cancer patients when compared to the healthy controls.

The genotype frequency of *IL18* -607C>A polymorphism did not revealed any significant association with breast cancer .The C-allele of -137C>A gene polymorphism was found to be similar in both controls and breast cancer patients, though the CC genotype was observed to be significantly associated with risk for development of breast cancer (p<0.04). Further TT genotype frequency of *IL18* 105A>T polymorphism was marginally higher in breast cancer cases compared to controls are showed in Table 2. The genotype frequencies of *IL18* -607C>A and *IL18* 105A>T polymorphisms are following Hardy–Weinberg equilibrium in both controls and breast cancer patients (p>0.05), and the *IL6* -597G>A, IL6 -572C>G, *IL6* -174G>C and *IL18* -137G>C polymorphisms have deviated from Hardy–Weinberg equilibrium in both controls and cases (p<0.05). The Hardy–Weinberg equilibrium deviation in controls generally reflects the gene pool of the population studied pointing to gene diversity and fixation of alleles in the population.

Table 2: Distribution of *IL6* and *IL18* genotypes among the breast cancer patients and non-cancer healthy control subjects

Polymorphism	Genotype frequency			Allele frequency	*p*-value	Model	AOA OR(95%CI)	χ2 *p*-value
IL6 -597G>A	GG	GA	AA	G/A				
Controls (n=300)	74.0	17.0	9.0	0.82/0.18	0.01*	Additive	2.17(1.70-2.76)	0.01*
Cases (n=300)	50.3	29.3	20.3	0.65/0.35 OR 2.53(1.93-2.32)		Co-dominant1 Co-dominant2	2.85(1.88-4.32) 3.97(2.38-6.65)	0.01*
HWE(p)						Dominant	3.23(2.26-4.62)	0.01*
Control	<0.01					Recessive	2.93(1.78-4.81)	0.01*
Cases	<0.01					Over-dominant	2.16(1.45-3.22)	0.01*
IL6 -572C>G	CC	CG	GG	C/G				
Controls (n=300)	17.0	59.7	23.3	0.47/0.53	0.03*	Additive	0.67(0.51-0.87)	0.002*
Cases (n=300)	24.0	62.3	13.7	0.55/0.45 OR 0.71(0.57-0.89)		Co-dominant1 Co-dominant2	0.79(0.52-1.20) 0.44(0.26-0.76)	0.007*
HWE(p)						Dominant	0.69(0.46-1.04)	0.07
Control	0.0007					Recessive	0.53(0.34-0.81)	0.003*
Cases	<0.01					Over-dominant	1.16(0.83-1.62)	0.38
IL6 -174G>C	GG	GC	CC	G/C				
Controls (n=300)	56.0	91.0	13.7	0.71/0.29	0.01*	Additive	1.65(1.32-2.06)	0.01*
Cases (n=300)	36.3	40.0	23.7	0.56/0.44 OR 1.91(1.50-2.43)		Co-dominant1 Co-dominant2	1.93(1.33-2.29) 2.52(1.59-4.00)	0.01*
HWE(p)						Dominant	2.11(1.52-2.95)	0.01*

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Control	0.01					Recessive	1.89(1.23-2.91)	0.003*
Cases	0.0015					Over-dominant	1.48(1.05-2.08)	0.026*
IL18 -607C>A	CC	CA	AA	C/A				
Controls(n=300)	60.0	33.7	6.3	0.77/0.23	0.25	Additive	1.11(0.85-1.44)	0.45
Cases(n=300)	55.7	36.7	7.7	0.74/0.26		Co-dominant1	1.15(0.81-1.63)	0.71
			OR 1.16(0.89-1.51)			Co-dominant2	1.14(0.59-2.21)	
HWE(p)						Dominant	1.15(0.83-1.60)	0.41
Control	0.33					Recessive	1.08(0.57-2.07)	0.81
Cases	0.45					Over-dominant	1.13(0.81-1.59)	0.47
IL18 -137G>C	GG	GC	CC	G>C				
Controls(n=300)	49.3	43.7	7.0	0.71/0.29	0.94	Additive	0.98(0.76-1.26)	0.86
Cases(n=300)	53.7	35.3	11.0	0.71/0.29		Co-dominant1	0.73(0.51-1.02)	0.04
			OR 0.95(0.71-1.27)			Co-dominant2	1.43(0.78-2.61)	
HWE(p)						Dominant	0.82(0.59-1.14)	0.24
Control	0.32					Recessive	1.64(092-2.94)	0.09
Cases	0.023					Over-dominant	0.69(0.49-0.96)	0.02*
IL18 105A>T	AA	AT	TT	A/T				
Controls(n=300)	46.7	42.0	11.3	0.68/0.32	0.41	Additive	0.95(0.75-1.20)	0.66
Cases(n=300)	53.3	33.0	13.7	0.7/0.3		Co-dominant1	0.71(0.50-1.01)	0.09
			OR 0.90(0.70-1.15)			Co-dominant2	1.13(0.68-1.90)	
HWE(p)						Dominant	0.80(0.58-1.11)	0.18
Control	0.51					Recessive	1.31(0.80-2.15)	0.28
Cases	0.0021					Over-dominant	0.70(0.75-1.20)	0.03*
Models were adjusted for age, OR-odds ratio; CI-confidence intervals;* *Significant at $p<0.05$								

3.3 Association of genotypes with Clinicopathological risk Factors of Breast cancer

Analyses of association between genotypic and clinico-pathological features adjusted by age with multivariate logistic regression analyses in breast cancer patients revealed that the AA genotype of *IL6* -597G>A polymorphism was found to be associated with TNM staging (T_2-T_4 late stage); GG genotype of *IL6* -572C>G polymorphism was found to be significantly associated with late (advanced) stage of breast cancer patients. In addition, the frequency of AA genotype of *IL6* -597G>A, GG genotype of *IL6* -572C>G and CC genotype of *IL6* -174G>C gene polymorphism were found to be significantly associated with positive metastasis status in breast cancer patients as summarized in Table 2a**.** However, analysis of polymorphic variants (-607C>A, -137G>C and 105A>T) of *IL18* gene polymorphisms with respect to histological subtype revealed that the AA and CA genotype of -607C>A polymorphism and AT+TT genotype of 105A>T polymorphisms had significant association with lobular carcinoma subtype in breast cancer patients. Furthermore, CC and GC genotypes of -137G>C polymorphism was found to be significantly associated with PgR^{-ve} and $HER2^{+ve}$ breast cancer patients respectively (Table 2b).

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Table 2a: Genotype frequencies of *IL6* gene and clinicopathological features of breast cancer patients

CharacteristicsN=300(%)	*IL6* -597G>A			*IL6* -572C>G			*IL6* -174G>C		
	GG	GA	AA	CC	CG	GG	GG	GC	CC
Stage of the cancer									
Early197 (65.67)	53.8	31.0	15.2	25.9	65.0	9.1	33.5	42.1	24.1
Late103 (34.33)	43.7	26.2	30.1	20.4	57.3	22.3	41.8	35.9	22.3
AOR (95% CIs)	1.0(ref)	1.05(0.59-.86)	2.40(1.32-4.60)	1.0(ref)	1.11(0.61-2.01)	3.10(1.39-6.89)	1.0(ref)	0.68(0.40-1.18)	0.74(0.39-1.39)
p-value		0.012*			0.008*			0.37	
Type of the cancer									
Ductal 247 (82.33)	51.8	30.4	17.8	12.1	62.4	25.5	35.2	40.5	24.3
Lobular53 (17.67)	43.4	24.5	32.1	20.8	62.3	16.9	41.5	37.7	20.8
AOR (95% CI)	1.0(ref)	0.91(0.43-1.92)	1.89(0.91-3.92)	1.0(ref)	0.56(0.25-1.23)	0.39(0.14-1.04)	1.0(ref)	0.80(0.41-1.57)	0.75(0.34-1.68)
p-value		0.16			0.17			0.73	
Axillary Lymph node Status									
Negative 80 (26.67)	45.0	28.8	26.2	31.2	48.0	8.8	32.5	37.5	30.0
Positive 220 (73.33)	52.26	29.54	18.2	21.4	63.2	15.4	37.7	40.9	21.4
AOR (95% CI)	1.0(ref)	0.88(0.48-1.62)	0.59(0.31-1.15)	1.0(ref)	1.54(0.85-2.76)	2.58(1.02-6.06)	1.0(ref)	0.94(0.51-1.12)	0.61(0.31-1.18)
p-value		0.31				0.11		0.3	
EgR Status									
Positive177 (59)	49.7	29.9	20.4	22.6	64.4	13.0	32.5	37.5	30.0
Negative123 (41)	51.2	28.5	20.3	26.0	59.4	14.6	37.7	40.9	21.4
AOR (95% CI)	1.0(ref)	0.92(0.54-1.58)	0.97(0.52-1.80)	1.0(ref)	0.80(0.46-1.39)	0.98(0.45-2.12)	1.0(ref)	0.94(0.51-1.72)	0.61(0.31-1.18)
p-value		0.96			0.67			0.3	
PgR Status									
Positive 168 (56)	50.59	30.35	19.06	20.2	66.7	13.1	36.9	42.3	20.8
Negative 132 (44)	50.0	28.0	22.0	28.8	56.8	14.4	35.6	37.1	27.3
AOR (95% CI)	1.0(ref)	0.93(0.55-1.59)	1.17(0.64-2.15)	1.0(ref)	1.08(0.67-1.76)	0.55(0.211-1.41)	1.0(ref)	0.91(0.54-1.54)	1.36(0.75-2.48)
p-value		0.80			0.67			0.4	
HER2/neu Status									
Positive 163 (54.33)	47.9	30.7	21.4	27.0	58.9	14.1	33.7	39.3	26.4
Negative 111 (37)	55.9	27.9	16.2	20.7	64.9	14.4	39.6	39.6	20.8
AOR (95% CI)	1.0(ref)	0.78(0.45-1.36)	0.65(0.33-1.25)	1.0(ref)	1.43(0.80-2.59)	1.33(0.59-3.00)	1.0(ref)	0.85(0.49-1.47)	0.67(0.35-1.27)
p-value		0.38				0.48			0.47
Metastasis Status									
Positive 103 (34.33)	49.8	33.0	17.2	26.9	63.5	9.6	30.5	43.5	25.9
Negative 197 (66.67	51.5	22.3	26.2	18.4	60.2	21.4	47.1	33.3	19.6
AOR (95% CI)	1.0(ref)	0.65(0.37-1.17)	1.14(0.80-2.69)	1.0(ref)	1.38(0.75-2.54)	3.23(1.44-7.24)	1.0(ref)	0.48(0.28-0.84)	0.48(0.25-0.91)
p-value			0.06#			0.01*			0.01*

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Table 2b: Genotype frequencies of *IL18* gene and clinicopathological features of breast cancer patients

Characteristics N=300 (%)	*IL18* -607C>A			*IL-18* -137G>C			*IL18* 105A>T		
	CC	CA	AA	GG	GC	CC	AA	AT	TT
Stage of the cancer									
Early197 (65.67)	54.8	37.1	8.1	57.9	33.0	9.1	53.8	30.5	15.7
Late103 (34.33)	57.3	35.9	6.8	45.6	39.8	14.6	52.4	37.9	9.7
AOR (95% CIs)	1.0(ref)	0.93(0.56-1.55)	0.82(0.32-2.13)	1.0(ref)	1.54(0.92-2.59)	2.03(0.94-4.37)	1.0(ref)	1.26(0.75-2.13)	0.63(0.29-1.38)
***p*-value**		0.9			0.09			0.22	
Type of the cancer									
Ductal 247 (82.33)	59.1	35.2	5.7	54.7	36.0	9.3	56.3	32.0	11.7
Lobular53 (17.67)	39.6	43.4	17.0	49.1	32.1	18.8	39.6	37.7	22.7
AOR (95% CI)	1.0(ref)	1.93(1.00-3.72)	6.02(2.19-16.55)	1.0(ref)	1.03(0.53-2.02)	2.32(0.98-5.51)	1.0(ref)	1.56(0.79-3.08)	2.60(1.14-5.91)
***p*-value**		0.001*			0.16			0.07#	
Axillary Lymph node Status									
Negative 80 (26.67)	51.2	40.0	8.8	60.0	32.5	7.5	52.5	38.8	8.8
Positive 220 (73.33)	57.3	35.5	7.2	51.4	36.4	12.2	53.6	30.9	15.5
AOR (95% CI)	1.0(ref)	0.79(0.46-1.36)	0.72(0.28-1.91)	1.0(ref)	1.30(0.75-2.28)	1.91(0.74-4.92)	1.0(ref)	0.79(0.45-1.37)	1.74(0.71-4.23)
***p*-value**		0.63				0.31		0.2	
EgR Status									
Positive177 (59)	56.5	35.6	7.9	53.1	37.3	9.6	51.4	36.2	12.4
Negative123 (41)	54.5	38.2	7.3	54.5	32.5	13.0	56.1	28.5	15.4
AOR (95% CI)	1.0(ref)	1.11(0.68-1.81)	0.95(0.39-2.36)	1.0(ref)	0.85(0.51-1.40)	1.32(0.62-2.80)	1.0(ref)	0.72(0.43-1.21)	1.14(0.57-2.27)
***p*-value**		0.89			0.53			0.35	
PgR Status									
Positive 168 (56)	55.4	35.1	9.5	50.6	40.5	8.9	52.4	34.5	13.1
Negative 132 (44)	56.1	38.6	5.3	50.0	15.1	34.9	54.5	31.1	14.4
AOR (95% CI)	1.0(ref)	1.08(0.67-1.76)	0.55(0.21-1.41)	1.0(ref)	0.37(0.20-0.68)	3.94(2.03-7.68)	1.0(ref)	0.86(0.52-1.43)	1.05(0.53-2.10)
***p*-value**		0.36			0.002*			0.8	
HER2/neu Status									
Positive 163 (54.33)	55.2	39.9	4.9	63.2	28.2	8.6	55.8	34.4	9.8
Negative 111 (37)	59.5	30.6	9.9	46.9	41.4	11.7	56.8	29.7	13.5
AOR (95% CI)	1.0(ref)	0.71(0.42-1.20)	1.87(0.71-4.92)	1.0(ref)	1.98(1.17-3.36)	1.84(0.81-4.20)	1.0(ref)	0.85(0.50-1.46)	1.35(0.62-2.94)
***p*-value**		0.13				0.02*			0.54
Metastasis Status									
Positive 103 (34.33)	55.3	37.6	7.1	61.4	29.9	8.7	51.8	33.0	15.2
Negative 197 (66.67	56.3	35.0	8.7	38.8	45.6	15.6	56.3	33.0	10.7
AOR (95% CI)	1.0(ref)	0.91(0.55-1.52)	1.21(0.49-2.96)	1.0(ref)	2.41(1.43-4.07)	2.85(1.32-6.15)	1.0(ref)	0.92(0.54-1.56)	0.64(0.30-1.38)
***p*-value**			0.83			0.001*			0.52

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***p<0.05 is considered to be statistically significant; #Borderline significant.** The odds ratios (ORs) with their 95% confidence intervals (CIs) were estimated by logistic regression models. The adjusted odds ratios (AORs) with their 95% confidence intervals (CIs) were estimated by multiple logistic regression models, after controlling for age, (Estrogen receptor (EgR), Progesterone receptor (PgR), Human epidermal growth factor receptor 2/neu receptor (HER2/neu).

3.4. HapMap analysis of different ethnic groups:

We have compared the allele frequencies of *IL6* and *IL18* in different ethnic groups (Asian-JPT, Asian-HCB, Japanese-JPT, and European CEU) using HapMap dataset and found that the frequency of G allele of -597, the C allele of -572 and C allele of -174; C allele of -607, G allele of -137 and A allele of 105 were similar to that of our population (Fig. 2).

Figure 2. HapMap analysis of IL6 -597G>A, -572C>G, -174G>C and IL18 (-607C>A, -137G>C, 105 A>T) polymorphisms in Breast cancer

3.5 Haplotype Analysis and Estimation of Linkage Disequilibrium (LD)

***IL6* haplotype:** The analysis of *IL6* gene showed eight haplotype groups among which G-G-G haplotype was most frequent, hence was chosen as a reference. Three haplotype (G-C-C, A-G-G and A-C-C) combinations had conferred 2.09, 2.25 and 4.72 folds risk for breast cancer respectively (p<0.05), while G-C-G, G-G-C and A-G-C haplotypes had conferred protection against breast cancer (p<0.05) **(**Table 3). Analysis of clinicopathological variables for haplotypes did not show any significant association with breast cancer **(**Table 4).

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Table 3: Frequencies of *IL6* Haplotypes (-597 G>A, -572 C>G and -174 G>C) in healthy controls and breast cancer patients

Possible haplotype	Controls No (%)	Patients No (%)	Adjusted OR(95%CI)	*p*- value
GGG	0.317	0.287	1.0(ref)	
GCC	0.112	0.251	2.09(0.134-3.24)	0.0011*
GCG	0.264	0.095	0.41 (0.25 - 0.68)	<0.001*
AGG	0.053	0.145	2.25 (1.23 - 4.12)	<0.0086*
ACC	0.014	0.164	4.72 (2.30 - 9.67)	<0.0001*
GGC	0.130	0.009	0.07 (0.03 - 0.20)	<0.0001
ACG	0.076	0.034	0.61 (0.32 - 1.16)	0.13
AGC	0.030	0.005	0.26 (0.07 - 0.99)	0.048

Global haplotype association *p*-value: <0.0001*, Adjusted (for age); OR and 95%CI. *p*<0.05 significance

Table 4: Frequencies of IL-6 Haplotypes (-597 G>A, -572 C>G -174 G>C) and clinicopathological variable of breast cancer patients

Clinicopathological Variables	Haplotype	Variable presence		Adjusted OR(95%CI)	*p*- value
Stage of the cancer		Early State	Late Stage		
	GCC	2.96	0.184	0.62(0.34-1.12)	0.12
	ACC	0.154	0.180	0.99(0.58-1.70)	0.98
Type of the cancer		Ductal	Lobular		
	GCC	0.276	0.175	0.62(0.29-1.31)	0.21
	ACC	0.157	0.189	0.90(0.47-1.71)	0.74
Axillary Lymph node Status		Positive	Negative		
	GCC	0.263	0.257	0.70(0.37-1.37)	0.26
	ACC	0.217	0.141	0.52(0.29-0.92)	0.025
EgR Status		Positive	Negative		
	GCC	0.236	0.288	1.15(0.68-1.95)	0.61
	ACC	0.169	0.153	0.91(0.54-1.53)	0.73
PgR Status		Positive	Negative		
	GCC	0.243	0.277	1.25(0.73-2.12)	0.41
	ACC	0.154	0.172	1.22(0.73-2.02)	0.45
HER2/neu Status		Positive	Negative		
	GCC	0.245	0.296	1.05(0.61-1.79)	0.87
	ACC	0.204	0.097	0.56(0.32-1.01)	0.054
Metastasis Status)		Positive	Negative		
	GCC	0.259	0.189	0.62(0.35-1.10)	0.11
	ACC	0.293	0.137	0.73(0.42-1.28)	0.88

Global haplotype association *p*-value: <0.0001*, Adjusted (for age); OR and 95%CI. *p*<0.05 significance

***IL18* haplotype:** None of the haplotypes of *IL18* gene revealed any association with Breast cancer (Table 5). However, the analysis of clinicopathological variables with haplotypes revealed that A-G-A and A-C-T haplotypes to be associated with histological type of breast cancer (ductal) ($p<0.019$). In addition, we also found strong association of C-C-T haplotype with axillary lymph node and HER2/neu receptor status while C-C-A with metastasis status respectively as shown in Table 6.

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Table 5: Frequencies of *IL18* Haplotypes (-607 C>A, -137G> C and 105A>T) in healthy controls and breast cancer patients

Possible haplotype	Controls No (%)	Patients No (%)	Adjusted OR(95%CI)	*p*- value
CGA	0.406	0.37	1.00(ref)	--
CGT	0.185	0.169	0.98(0.66-1.46)	0.94
CCA	0.117	0.144	1.31(0.83-2.07)	0.25
AGA	0.084	0.124	1.51(0.90-2.52)	0.12
ACA	0.069	0.599	0.97(0.54-1.73)	0.91
CCT	0.059	0.056	0.08(0.59-1.97)	0.82
AGT	0.035	0.050	0.65(0.83-3.27)	0.15
ACT	0.042	0.025	0.64(0.28-1.46)	0.29

Global haplotype association *p*-value: <0.0001*, Adjusted (for age); OR and 95%CI. *p*<0.05 significance

Table 6: Frequencies of *IL18* Haplotypes (-607 C>A, -137G>C 105A>T) and clinicopathological variables of breast cancer patients

Clinicopathological Variables	Haplotype	Variable presence		Adjusted OR(95%CI)	*p*- value
Stage of the cancer		Early State	Late Stage		
	CCA	0.144	0.129	1.54(0.84-2.81)	0.16
Type of the cancer		Ductal	Lobular		
	AGA	0.115	0.152	3.00(1.20-7.49)	0.019*
	ACT	0.041	0.87	18.90(3.51-101.71)	0.001*
Axillary Lymph node Status		Positive	Negative		
	CCT	0.056	0.009	7.82(1.03-59.29)	0.04*
EgR Status		Positive	Negative		
	CCT	0.052	0.063	1.30(0.59-2.86)	0.52
PgR Status		Positive	Negative		
	CCT	0.047	0.065	1.31(0.59-2.93)	0.51
HER2/neu Status		Positive	Negative		
	CCT	0.027	0.072	2.77(1.04-3.70)	0.02*
Metastasis Status)		Positive	Negative		
	CCA	0.109	0.210	2.00(1.08-3.70)	0.027*
	CCT	0.056	0.056	1.03(0.43-2.47)	0.94

Global haplotype association *p*-value: <0.0001*, Adjusted (for age); OR and 95%CI. *p*<0.05 significance
Abbreviations: CI = confidence interval; OR = odds ratio; SNP = single nucleotide polymorphism.

Linkage disequilibrium (LD) of SNPs

The SNP *IL6* -597G>A was in strong LD with *IL6* -572C>G, *IL6* -174G>C, *IL18* -607C>A and *IL18* -137G>C. While, the SNPs of *IL6* -572 C>G, *IL6* -174G>C also showed a strong LD with *IL18* -607C>A and *IL18* -137G >C. Similarly, the SNPs *IL18* -607C>A and *IL18* -137G >C have also shown a strong LD with 105A>T with Pearson's correlation coefficient (D) of in red colour Table 7.

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Table 7. Linkage disequilibrium analysis between the Six Interleukin SNPs analyzed in Breast cancer patients.

SNPs	*IL6*-597G>A	*IL6*-572C>G	*IL6*-174G>C	*IL18*-607C>A	*IL18*-137G>C
***IL6*-597G>A**	-	-	-	-	-
***IL6*-572C>G**	1.0	-	-	-	-
***IL6* -174G>C**	1.0	0.971	-	-	-
***IL18*-607C>A**	1.0	1.0	1.0	-	-
***IL18*-137G>C**	1.0	1.0	1.0	0.928	-
***IL18* 105A>T**	0.974	0.953	0.978	1.0	1.0

D values 1.0 (high grade of linkage disequilibrium) in red; D^l values D^l <1.0 in yellow.

3.6 Cox regression analysis of SNPs

Multivariable Cox regression analysis showed that the adjusted risk of primary endpoint for breast cancer patients with GC of *IL6* -174G>C polymorphism was 2.19-folds and GC heterozygote and CC homozygote of *IL18* -137G>C gene polymorphisms to be 14.29 and 9.55 folds higher risk than non-carriers (p<0.01). The study of COX regression analysis showed a decreased survival frequency in patients with breast cancer with AT heterozygote and TT homozygote of *IL18* 105A>T gene polymorphism (p<0.05) (Table 8).

Table 8: Cox regression analysis of SNPs for *IL6* (-597G>C,-572C>G and -174G>C) and *IL18* (-607,-137G>C and 105 A>T) studied on 5 years median survival rate in breast cancer patients

Polymorphism	Genotypes (5 years survival)	HR[a] (95%CI)	*p*- value
IL6 -597G>A	GG (n=122)	1.00(ref)	--
	GC (n=75)	0.67(0.39-1.12)	0.13
	CC (n=49)	0.45(0.23-0.78)	0.19
IL6 -572C>G	CC (n=53)	1.00(ref)	--
	CG (n=157)	1.82(0.92-3.58)	0.81
	GG (n=36)	2.06(0.92-3.58)	0.77
IL6 -174G>C	GG (n=89)	1.00(ref)	--
	GC (n=103)	2.19(1.15-4.18)	0.017*
	CC (n=54)	1.20(0.61-2.34)	0.58
IL18 -607C>A	CC (n=135)	1.00(ref)	--
	CA (n=89)	0.76(0.35-1.64)	0.47
	AA (n=22)	1.12(0.21-2.43)	0.58
IL18 -137G>C	GG (n=126)	1.00(ref)	--
	GC (n=88)	14.29(6.25-31.30)	0.01*
	CC (n=32)	9.45(3.75-23.81)	0.01*
IL18 105A>T	AA (n=141)	1.00(ref)	--
	AT (n=71)	0.59(0.34-1.02)	0.05*
	TT (n=34)	0.51(0.24-1.07)	0.05*

***Significant at p<0.05, [a]-Hazardous Risk**

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3.7 Kaplan-Meier 5 year's survival analysis of Breast Cancer Patients with respect to *IL6* and *IL18* gene polymorphisms

Furthermore, Kaplan Meier analysis was carried out to know the influence of the genotypes on 5 years median survival rate, and the results showed that the patients with GA heterozygote of *IL6* -597G>A, and TT homozygote of *IL18* 105A>T, polymorphism has borderline significant association and CC homozygote of *IL6* -174G>C and CC homozygote of *IL18* -137G>C polymorphism has reduced median survival rate compared to that of GG homozygotes (p<0.05) (Table 9 and Fig 3 A-F).

Table 9: Kaplan-Meier 5 years survival analysis of Breast Cancer Patients with respect to *IL6* and *IL18* gene polymorphisms

Variable	N (%)	Death (n%)	HR(95%CI)	Mean±SEM	Median	*p*- value
IL6 (-597G>A)						
GG	122(49.60)	48(39.34)	1.00(ref)	50.47±1.56	57.0	
GA	75(30.48)	20(26.66)		55.06±1.52	39.0	0.08#
AA	49(19.91)	11(22.44)		57.54±1.22	51.0	
IL6 (-572C>G)						
CC	53(21.54)	10(18.86)	1.00(ref)	56.06±1.76	52.0	
CG	157(63.82)	54(34.39)		52.34±1.24	57.0	0.13
GG	36(14.63)	15(41.66)		53.26±2.52	50.0	
IL6 (-174G>C)						
GG	89(36.17)	40(44.94)	1.00(ref)	49.84±1.79	46.0	
GC	103(41.86)	27(26.21)		54.69±1.37	57.0	
CC	54(21.95)	12(22.22)		56.41±1.66	52.0	0.006*
IL18(-607 C>A)						
CC	135(54.87)	37(27.40)	1.00(ref)	54.87±1.09	57.0	
CA	89(36.17)	34(38.20)		50.77±1.85	48.0	0.26
AA	22(8.94)	8(36.36)		53.77±1.85	30.0	
IL18 (-137 G>C)						
GG	126(51.21)	7(5.55)	1.00(ref)	59.94±0.60	45.0	
GC	88(35.77)	59(67.04)		45.80±1.86	54.0	0.001*
CC	32(13.00)	13(40.62)		50.88±3.04	44.0	
IL18 (105 A>T)						
AA	141(57.31)	54(38.29)	1.00(ref)	51.12±1.38	57.0	
AT	71(28.86)	17(23.94)		56.04±1.44	56.0	0.07#
TT	34(13.82)	8(23.52)		56.31±2.24	30.0	

[a]Log Rank *p*- values by mantle COX regression statistically significant at p<0.05 , [#]border line significant,

(Mean±SEM -5 years survival in months)

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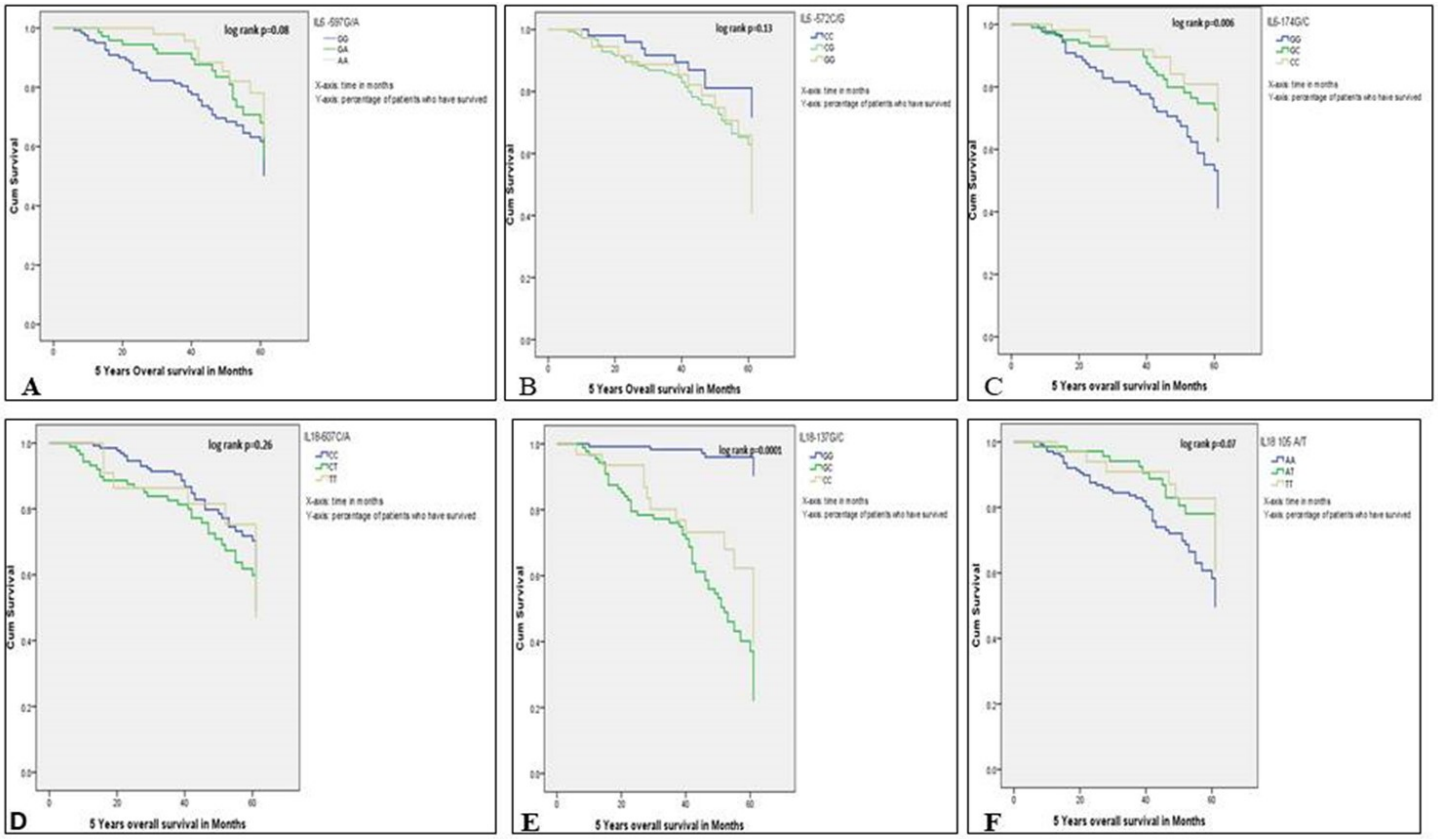

Figure 3. Kaplan-Meier Curves of 5-Year Survival among the Breast cancer patients: SNPs for A. *IL6*-597G>C; B. *IL6* -572C>G; C. *IL6* -174G>C; D. *IL18* -607C>A; E. *IL*18-137G>C and F. *IL*18 105 A>T gene polymorphisms.

4. **Transcription factor binding sites (TFBS) and Pre-mRNA secondary structures predictions**

The prediction of transcription factor binding sites (TFBSs) for *IL6* gene (-597G>A, -572C>G and -174G>C) polymorphisms revealed that G-allele of -597G>A polymorphism has NF-1, Sp1 and AP-2alph sites whereas A-allele has TBP, YY1 and NF-1. Further it is revealed that the C-allele of -572C>G polymorphism has binding site for Sp1, whereas G-allele has loss of Sp1 binding site. Furthermore, G allele of 174G>C polymorphism has no binding site while C -allele has binding site for NF-1 respectively as depicted in Figure 4. Similarly, the *IL18* gene -607C>A polymorphism has shown that C allele has binding sites for C/EBP beta, MEB-1 sites whereas A allele has binding sites for C/EBP beta, HOXA4, MEB-1 sites. Further, the prediction of transcription factor binding sites of -137G>C polymorphism has shown that G allele has binding site for Nf-kappa B whereas C allele has an additional binding site for C/EBP beta and NF1 sites as respectively depicted in Fig. 4. The pre-mRNA secondary structures of *IL18* 105A>T polymorphism had shown that T-allele (-77.65 kcal/mol) has higher entropy and is less stable than A-allele (-76.08 kcal/mol) as deduced from the minimum free energy was shown in Fig. 4.

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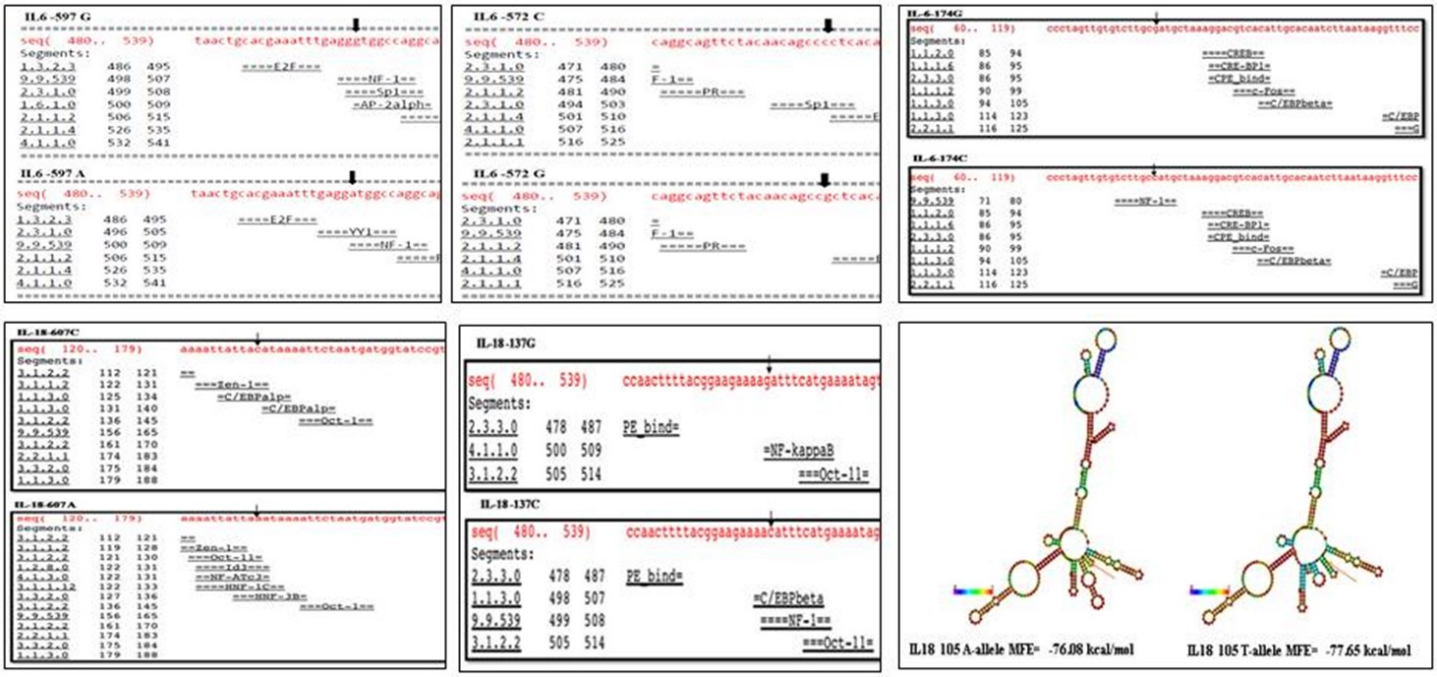

Figure 4 . Effect of the IL-6(-597G>A,-572C>G and-174G>C) and IL-18 -607C>A, and -137G>C polymorphisms on transcription factor binding and IL-18 105A>T Pre-mRNA secondary structure analysis respectively.

5. **Protein- protein interaction (PPI) analysis**

For protein interaction data, the present study utilized a human PPI dataset from the Search Tool for the Retrieval of Interacting genes/proteins. The obtained image contained several colored lines supporting their interactions, the more lines between two peptides and the more evidence to support their relationship. Our analysis revealed that the *IL6* protein is found to be directly interacting with *IL18*, *MMP1, 3, 9* and *TIMP1* genes, while, *MMP1* and *MMP9* proteins are strongly interacting with each other and are co-expressed along with *TIMP1* proteins as shown in Fig.5.

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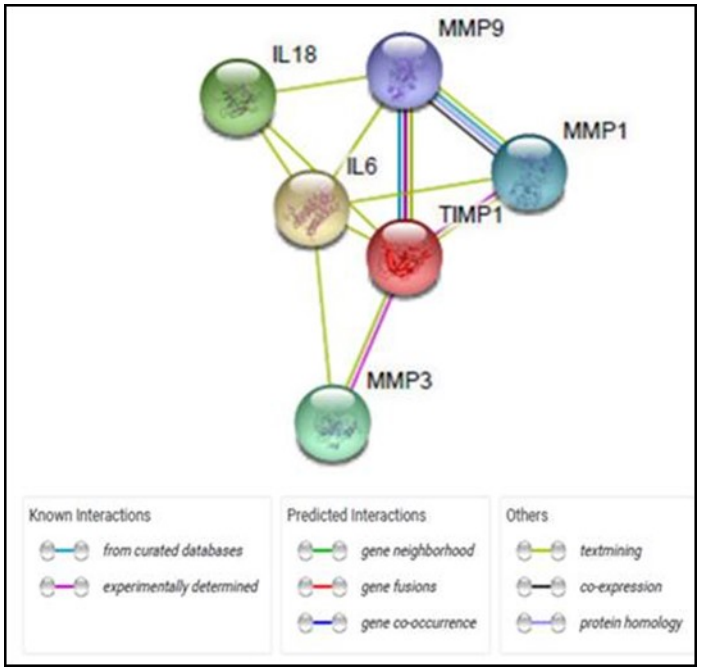

Fig.5 Protein -Protein interaction for *IL6* and *IL18* genes by String analysis.

4. DISCUSSION

Multiple molecular mechanisms are known to be involved in the development of breast cancer. Inflammation within the tumor microenvironment, one of the key mechanisms of carcinogenesis has been correlated with increased invasiveness and poor prognosis in many types of cancer including breast cancer [17]. Different cytokines are known to have diverse roles in breast cancer initiation and metastasis [18]. The cytokines are critical mediators of the inflammatory response [4]. The present study aimed to evaluate the association of *IL6* (-597G>A, -572C>G, -174G>C) and *IL18* (-607C>A, -137G>C, 105A>T) gene polymorphic variants with the epidemiological and clinicopathological characteristics in south Indian Breast cancer patients. The genetic variations of *IL6* gene are known to influence the transcriptional activity by affecting the expression, leading to the susceptibility and progression of breast cancer [19]. Three single-nucleotide polymorphisms -597G>A, -572C>G and -174G>C have been identified in the promoter region of the *IL6* gene, which might have an effect on IL6 transcription and plasma *IL6* levels in multiple human diseases [20].

IL6 -597G>A polymorphism was reported to be associated with serum *IL6* levels and as a susceptibility factor in several diseases such as multiple myeloma [21], rheumatoid arthritis [22], Sepsis [23], cervical cancer [24] and chronic obstructive pulmonary disease [25]. The genotype and allele frequency distribution of *IL6* -597G>A polymorphism in controls and breast cancer patients revealed that the AA genotype and A-allele frequency was higher in breast cancer patients compared to controls and has conferred 3.97 and 2.53 folds risk for breast cancer

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respectively. Several groups have shown an association between AA genotype *IL6* -597G>A polymorphism with elevated serum *IL6* levels and incidence of chronic inflammation with poor survival in multiple cancers. *IL6* -572C>G polymorphism is another important promoter polymorphism associated with serum IL6 levels and susceptibility to several diseases like OSCC [26], hypertension [27], T2DM [28]. It has also been suggested that individuals carrying the C allele of IL6 -572C>G polymorphism have an increased risk for breast cancer. Similarly, the genotype and allele frequency distribution of *IL6* -572C>G polymorphism in controls and breast cancer patients revealed that the CG and GG genotypes had a protective role against breast cancer. Further, G allele frequency was high in controls and was associated with reduced risk, indicating C-allele carriers are at an increased risk for breast cancer.

Another functional polymorphism in the promoter region of the *IL6* gene at position -174G>C has been reported to be associated with diseases, such as OSCC [26], Ovarian [29], Cervical and breast cancer [30,31]. Earlier studies by Lagmay et al., have shown that the CC genotype of *IL6* promoter -174G>C polymorphism to be associated with elevated high-risk for neuroblastoma [32] and prostate cancer [33]. A recent study suggested that individuals carrying the -174 C allele have an elevated risk for breast cancer [34]. The genotype and allele frequency distribution of *IL6* -174G>C polymorphism in controls and breast cancer patients revealed that the frequency of CC genotype and C allele were significantly elevated in breast cancer cases and conferred 2.52 and 1.91 fold risk for breast cancer development respectively.

The *IL18* gene promoter -607C>A and -137G>C polymorphisms and 105A>T polymorphism in exon 4 are found to influence the expression and may affect the blood plasma level of *IL18* [35]. *IL18* gene (-607C>A, -137G>C and 105A>T) polymorphisms have been associated with susceptibility to several diseases like CHD, Systemic lupus erythematosus (SLE), esophageal squamous cell carcinoma [36], prostate [37], colorectal [38], ovarian cancer [39], nasopharyngeal (rare type of head and neck) carcinoma [40] and breast cancer [41]. In contrary, no significant association was found when *IL18* -607C>A, *IL18* -137G>C gene polymorphisms were studied in patients with Crohn disease or ulcerative colitis and other cancers. Recently, similar results were found in patients with oral cavity cancer where there was no significant association of *IL18* -607C>A polymorphism even after adjusting for age [42]. Similarly, in the present study the genotype and allele frequency distribution of *IL18* -607C>A polymorphism in controls and breast cancer patients revealed that there was no significant association of *IL18* -607C>A polymorphism with breast cancer.

Another single nucleotide polymorphism in the promoter region of the *IL1*8 gene at position -137 G>C has been reported to be associated with a variety of diseases, such as Chronic Lymphocytic and Chronic Myelogenous [43], cervical [44], breast cancer [41] and unfavourable clinical outcome in patients affected by high-risk neuroblastoma, trauma inflammation etc [45]. In the present study, the genotype and allele frequency distribution of *IL18* -137G>C polymorphism did not revealed any significant association with breast cancer. However, the frequency of CC genotype was slightly high in breast cancer patients compared to controls. The studies by Stassen et al., and Farjadfar et al., showed the relationship between *IL18* gene exonic variants and predisposition to various

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malignant, viral and autoimmune diseases and experiments by have demonstrated that the 105A>T polymorphism of the *IL18* gene may be associated with the pathogenesis of asthma in Japanese patients [46,47]. However in the present study there was no significancant association between *IL18* 105A>T polymorphism and breast cancer in different genetic models studied.

Over expression of *IL6* and *IL18* genes have been found to be positively associated with the clinicopathological characteristics of several malignancies [26,48,49]. In the present study SNPs of *IL6* (-597G>A, 572C>G and 174G>C) and *IL18* (607C>A, 137G>C and 105A>T) genes were correlated with clinicopathological variables for their association in breast cancer progression and susceptibility. Our results revealed a significant association of AA genotype of *IL6* -597 and GG genotype of *IL6* -572 with TNM stage of breast cancer and CC genotype of *IL6* -174 with metastasis, indicating the association of AA, GG and CC genotype with features of tumour phenotype in progression of breast cancer. The CA and AA genotypes of 607C>A and AT & TT of 105A>T of *IL18* polymorphisms, were found to be significantly associated with histological subtype (lobular) breast cancer. The GC and CC genotypes of *IL18*-137G>C polymorphism were found to be significantly associated with PR, HER2/neu, and metastasis status. *IL6* (-597G>A, -572C>G, and -174G>C) and *IL18* (-607C>A, -137G>C and 105A>T) SNP marker combinations exhibited complete LD. The present study revealed a strong association in each of the study groups.

The current available data suggest that the identification of patterns of genetic variations, in the form of haplotypes rather than single or point variations, may present a more promising approach [50-53]. In the present study haplotype analysis of *IL6* gene polymorphisms revealed a significant association with risk for breast cancer development by 2.09 folds for G-C-C haplotype, 2.25 folds for A-G-G haplotype and 4.72 folds for A-C-C haplotype respectively, while G-C-G, G-G-C and A-G-C haplotypes conferred protection against breast cancer. The haplotype containing both risk alleles (A allele of -597G>A, G allele of -572C>G and C allele of -174G>C) conferred enhanced risk for breast cancer. These results are in agreement with individual SNP analysis. The analysis of haplotype groups of *IL18* gene polymorphisms did not revealed any significant association with breast cancer susceptibility but the combined effect of *IL18* gene polymorphisms (-607C>A, -137G>C 105A>T) with clinicopathological variables of haplotypes showed a significant association with ductal and lobular carcinoma, Further, our results showed that the association of haplotypes with axillary lymph nodes (C-C-T), HER2neu (C-C-T) and metastasis (C-C-A) status in breast cancer patients. In addition to above haplotype analysis we have also analysed the allele frequency distribution of *IL6* -597G>A, 572C>G and 174G>C and *IL18*-607C>A, 137G>C and 105A>T in various populations along with Indians by HapMap and found that A allele of -597, G allele of -572, C allele of -174, A allele of -607, C allele of -137 and T allele of 105 were significantly associated with BC. In addition, the 5 years cox-regression survival analysis found a significant association with IL6 -174G>C and IL18-137G>C polymorphisms. The functional polymorphisms in *IL6* and *IL18* could lead to altered gene expression, consequently creating imbalance in the vital cytokines system that results in excessive tumor progression and breast

cancer development. To our best knowledge, present study is first to report the combined effect of three polymorphisms and haplotypes (A-C-C, A-G-G and G-C-C) along with the clinicopathological parameters which showed a significant association of IL6 polymorphism with BC. Overall, our results revealed that the polymorphisms in the promoter region of *IL6*, and *IL18* genes when correlated with clinicopathological characteristics and survival rate have shown significant effects on the risk and progression of breast cancer, substantiated by in-silico analysis.

Conclusions

The study is focussed to understand the clinical importance of *IL6* and *IL18* gene variants in progression of breast cancer and to help in identifying individuals at risk of developing breast cancer. Interleukins are considered as promising targets for therapy due to their strong involvement in tumor pathology and progression at both molecular and clinicopathological levels. Therefore, understanding cancer subtypes, functional epigenomics, transcriptomic programs and signalling pathways regulating *ILs* expression is critical for developing breast cancer therapeutics, not only to treat but also to prevent cancer. Further such a comprehensive approach may reveal highly prominent candidate molecular markers in future for breast cancer diagnosis and prognosis.

Limitation of the study: To the best of our knowledge this is the first study reporting on the combined effects of SNPs of *IL6* and *IL18* genes in correlation with clinicopathological variables along with LD, survival rate and In-silico analysis. However, our study has several limitations. Firstly, a small study that was analysed in South Indian population, because we restricted the study subjects to individuals of South Indian ethnicity; it is uncertain whether these results can be generalized to other populations. Second, there were few patients with missing data on hormonal receptor status, which may bias the results indicating an association with advanced disease status. Third, our LD and survival analysis included only 600 samples and this may have limited the power of the pooled results. Therefore, collaborative studies on different populations are necessary to corroborate our findings.

Author Contributions:

Chiranjeevi Padala: Concept and design; provision of study materials, methodology; data collection; analysis; validation and interpretation; article writing review; editing and final approval of the article. **Kaushik Puranam, Nivas Shyamala, Keerthi Kupusala and Ramanjaneyulu, Kishore kumar Gundapaneni Rajesh Kumar Galimudi, and Mohini Aiyengar Tupurani**: Resources and writing–review and editing; Collection and assembly of data and methodology**. Aparna Suryadevera and Sanjeeva kumari Chinta**: Provision of BC patients' blood samples; data collection; analysis and interpretation. **Surekha Rani Hanumanth**: Concept and design, Supervision; Administrative support; data analysis and interpretation; article writing, editing, and final approval of the article.

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