

Association of CYBA (-930 A/G and 242 C/T) gene polymorphisms with Oxidative stress in Breast Cancer: a case-control study

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Background: Oxidative stress (OS) is a strong characteristic feature in cancer initiation and progression. Reactive Oxygen Species (ROS) produced in excess during OS cause lipid peroxidation, cellular damage, apoptosis, etc. These ROS are also involved in breast tumor development. NADPH oxidase (NOX) dependent free radical production is implicated in oxidative stress in cancers. P22phox is a subunit of NADPH oxidase encoded by the CYBA gene that has functional polymorphisms associated with multiple diseases. **Materials and methods:** We have performed a case-control study on 300 breast cancer patients and 300 healthy individuals as controls to examine the role of CYBA gene -930 A/G and 242 C/T single nucleotide polymorphisms (SNPs) using As-PCR and PCR-RFLP assays and its association with OS as measured by plasma MDA levels. Linkage disequilibrium (LD) plots were generated using Haploviewtool and Multifactor dimensionality reduction (MDR) analysis was applied to assess high-order interactions between the SNPs. Insilco analysis was performed using appropriate tools. **Results:** We have found that genotype frequencies of CYBA gene -930 A/G and 242C/T polymorphism were significantly different between controls and BC patients ($p < 0.05$). The haplotype combination -930G/242C and -930G/242T were associated with increased risk for breast cancer. Further, the MDA levels were higher in the patients carrying -930G/242C and -930G/242T haplotype ($p < 0.001$). Our results have been substantiated by Insilco analysis. **Conclusion:** Results of the present study suggest that -930G/242C and -930G/242T haplotypes of CYBA gene polymorphisms have shown association with higher MDA levels in breast cancer patients, signify that elevated oxidative stress aid in increased risk for breast cancer initiation and progression.

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ABSTRACT

Background: Oxidative stress (OS) is a strong characteristic feature in cancer initiation and progression. Reactive Oxygen Species (ROS) produced in excess during OS cause lipid peroxidation, cellular damage, apoptosis, etc. These ROS are also involved in breast tumor development. NADPH oxidase (NOX) dependent free radical production is implicated in oxidative stress in cancers. P22phox is a subunit of NADPH oxidase encoded by the CYBA gene that has functional polymorphisms associated with multiple diseases. Materials and methods: We have performed a case-control study on 300 breast cancer patients and 300 healthy individuals as controls to examine the role of CYBA gene -930 A/G and 242 C/T single nucleotide polymorphisms (SNPs) using As-PCR and PCR-RFLP assays and its association with OS as measured by plasma MDA levels. Linkage disequilibrium (LD) plots were generated using Haploview tool and Multifactor dimensionality reduction (MDR) analysis was applied to assess high-order interactions between the SNPs. Insilco analysis was performed using appropriate tools. Results: We have found that genotype frequencies of CYBA gene -930 A/G and 242C/T polymorphism were significantly different between controls and BC patients ($p < 0.05$). The haplotype combination -930G/242C and -930G/242T were associated with increased risk for breast cancer. Further, the MDA levels were higher in the patients carrying -930G/242C and -930G/242T haplotype ($p < 0.001$). Our results have been substantiated by Insilco analysis. Conclusion: Results of the present study suggest that -930G/242C and -930G/242T haplotypes of CYBA gene polymorphisms have shown association with higher MDA levels in breast cancer patients, signify that elevated oxidative stress aid in increased risk for breast cancer initiation and progression.

Keywords: Oxidative stress, CYBA, polymorphism, Haplotype, LD, MDR, Insilco analysis

INTRODUCTION

Breast cancer (BC) is one of the most frequent malignant tumors, and its morbidity and mortality rates have been increasing annually worldwide and in developing countries such as, India (Gupta, Shridhar & Dhillon, 2015). The breast cancer etiology is complex, involves dynamic interactions of genetic and environmental factors (Abdulkareem, 2013).

Oxidative stress (OS) is a key risk factor for cancer initiation and progression. OS, resulting from an imbalance between Reactive Oxygen Species (ROS) production and antioxidant defenses, contributes to cell damage, apoptosis, lipid peroxidation and interferes with the body's normal metabolic activity, leading to the occurrence and development of diseases. Malondialdehyde (MDA) is one of the end products of lipid peroxidation and it is also formed as a product of the cyclooxygenase reaction in prostaglandin metabolism. Some of the studies have also provided evidence of the potential role of oxidative stress and lipid peroxidation in breast cancer aetiology (Ray et al., 2000; Gönenç et al., 2001).

Intracellular compartments such as mitochondria, is the major site of ROS (Poyton et al., 2009). Enzymes involved in ROS-generating chemical reactions are peroxidases, Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, NADPH oxidase (NOX), xanthine oxidase (XO), lipoxygenases (LOXs), glucose oxidase, myeloperoxidase (MPO), nitric oxide synthase, and cyclooxygenases (COXs) (Kulkarni, Kuppusamy & Parinandi, 2007).

The oxidation of NADPH to NADP⁺ generates superoxide radical from oxygen, which is catalyzed by NADPH oxidase, the enzyme present in cytoplasmic membrane of phagocytic cells and was described first as an enzyme involved in the generation of reactive oxygen species (ROS) in the phagocytic cells (Rossi & Zatti, 1964). This enzyme comprises two membrane-bound proteins (p22phox and gp91phox), three cytosolic proteins (p67phox, p47phox, and p40phox), and a small G-protein Rac. Gp91phox and p22phox form a heterodimer that is bound to the plasma membrane. The p22phox subunit is coded by the CYBA (cytochrome b245 alpha) gene, which is mapped to chromosome 16q24.3 (Powell et al., 2002). Genetic factors might

regulate NADPH-oxidase-driven O_2^- production. Several polymorphisms in the NADPH oxidase gene have been described, some of which have been associated with increased (San José et al., 2004) or diminished NOX activity (Guzik et al., 2000), as well as reduced ROS generation (Schirmer et al., 2008; Bedard et al., 2009).

To date little is known about the polymorphisms and the relationship between CYBA genotypes and the level of oxidative stress in BC patients. Therefore, the present study was aimed to examine the importance and association of the functional polymorphisms of CYBA (-930 A/G and 242 C/T) with the oxidative stress in BC development and progression.

Materials and Methods:

Study Population

In our study, a total of 600 subjects were enrolled comprising of 300 histopathologically confirmed female patients with breast cancer and the control group included 300 unrelated healthy women with no self-reported history of any cancer. Each subject completed a questionnaire on their demographic characteristics, area of living, lifestyle habits such as tobacco use and alcohol consumption. Clinical characteristics such as tumor size, stage of the cancer, axillary lymph node involvement and metastasis were collected via medical records with approval of patients with the help of medical oncologist.

Ethics Statement

Written informed consent was obtained from every participant before enrolment, and our study was approved by the Ethics committee of MNJ Institute of Oncology and Regional Cancer Centre, Hyderabad.

Sample collection

Whole blood samples (4ml) were collected from the patients (before any treatment modality such as chemotherapy and surgery) and controls in Na-EDTA vacutainers. Plasma was separated by centrifugation and kept at -20°C until analysis.

Plasma MDA levels estimation

Level of lipid peroxidation evidenced by the formation of malondialdehyde (MDA) (nmol/ml) in plasma was determined spectrophotometrically. The MDA levels were analysed by thiobarbituric acid (TBA) method based on the release of color complex due to TBA reaction with MDA as described previously (Rajesh et al., 2011).

Genomic DNA Extraction and Genotyping analysis

Genomic DNA was isolated from whole blood sample using a non-enzymatic method (Miller, Dykes & Polesky, 1988). Polymorphic regions in the CYBA gene were identified by Allele-specific polymerase chain reaction (PCR) and PCR- Restriction fragment length polymorphism (RFLP) assays for -930 A/G and 242 C/T polymorphisms respectively. Cases and controls were randomized during genotyping and 10% of the samples were genotyped in duplicate to assess the genotyping error rate. Concordance of genotypes was 100%.

Statistical analysis

All statistical analyses were performed using the IBM SPSS Statistics version 20. All data are expressed as the mean±SD and a P-value lower than 0.05 was considered statistically significant. Observed genotype frequencies were tested for deviation from Hardy-Weinberg equilibrium with the chi-square goodness-of-fit test (χ^2). Risk estimates were calculated for co-dominant, dominant and recessive genetic models using SNPStats. Odds ratios (OR) and their 95% confidence intervals (CI) were estimated using a univariate analysis. Linkage Disequilibrium (LD) plots were generated using Haploview (v.4.2) software. Multifactor dimensionality reduction (MDR) analysis was performed to identify high-order interaction models that were associated with BC risk using open-source MDR software (v.2.0 beta 8.4).

Bioinformatic analysis

Prediction of presumptive changes in transcription factor binding sites caused by nucleotide alterations in the promoter region was performed with AliBaba software2.1 (<http://gene-regulation.com/pub/programs/alibaba2/index.html>) (Grabe, 2002). Pre-mRNA secondary

structure prediction of 242 C/T polymorphic variants was carried out using Vienna RNAfold webserver (<http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi>) online tool (Zuker & Stiegler, 1981). The 3D structure of CYBA protein with respect to 242 C/T polymorphism was predicted by I-Tasser server (<http://zhanglab.ccmb.med.umich.edu/I-TASSER/>) (Roy, Kucukural & Zhang, 2010).

Results

The baseline/clinical characteristics are summarised in Table 1. In the present study, lifestyle habits such as, mixed diet (Non-vegetarian), habit of smoking and alcohol consumption were found to be associated with breast cancer risk ($p<0.05$).

The genotypic and allele frequency distribution of the CYBA -930 A/G polymorphism is represented in Table 2. In the present study the distribution of the genotypes between cases and controls has shown that under the Co-dominant model, carriers of GG genotype were found to be associated with a significantly increased risk of BC compared to homozygotes AA genotype carriers (OR 2.15, 95%CI 1.16-3.98, $p=0.034$). The allelic distribution has revealed that the prevalence of the G-allele was significantly different between study groups and conferred increased risk for breast cancer compared to A-allele (OR 1.27, 95% CI 1.01-1.6, $p=0.035$).

Table 3, represents the genotype and allele frequency distribution of CYBA 242 C/T polymorphism among the controls and patients with breast cancer. Under the dominant model, carriers of at least one minor allele T (CT+TT) were found to be associated with a significantly increased risk of BC compared to major allele homozygotes (CC) carriers (OR 1.42, 95%CI 1.02-1.98, $p=0.036$). The allelic association revealed that the minor allele T of 242 C/T polymorphism was associated with an increased risk of BC (OR 1.36, 95% CI 1.04-1.78, $p=0.02$). In addition, the estimation of haplotype frequencies with respect to CYBA gene polymorphisms has revealed a total of 4 haplotypes as shown in Table 4. Comparison of haplotype frequencies between controls and BC patients revealed a significant difference in haplotype frequencies, where -930G/242C and -930G/242T combinations were found to be associated with an increased risk of breast cancer (OR 95% CI 1.44, 1.00–2.07; OR 95% CI 1.56, 1.11 – 2.20, respectively with $p<0.05$).

Pairwise LD was computed for CYBA -930 A/G and 242 C/T polymorphism in cases and controls separately. LD plots revealed a moderate LD ($D'=56$) between the markers in BC patients and a weak LD ($D'=31$) between the markers in controls as shown in the Figure 1. Further, MDR analysis with respect to CYBA gene polymorphism has shown that 242C/T polymorphism was the best single locus model with significant risk for breast cancer. The bivariate model showed strong interaction between -930 A/G and 242 C/T polymorphisms as seen in Figure 2.

Furthermore, the TFBS analysis with respect to -930 A/G promoter polymorphism has revealed that substitution of A nucleotide by G leads to a loss of C/EBPbeta site as depicted in Figure 3. The comparison of the wild type and mutant pre-mRNA secondary structures with respect to 242 C/T polymorphism is given in Figure 4, wherein, the stability, as depicted by minimum free energy (MFE) change has revealed that the T-allelic structure had an MFE of -37.61 Kcal/mol and the C-allelic structure had an MFE of -37.91 Kcal/mol respectively. In addition, an altered 3D structure was also observed corresponding to loss of cavities with respect to variant structure when compared to wild type structure as seen in Figure 5 (Table 5).

Oxidative stress marker plasma MDA levels were measured in all the subjects, our results revealed higher levels in the patients with breast cancer (6.84 ± 2.42 nmoles/ μ l) compared to the control (2 ± 0.69 nmoles/ μ l) group. MDA levels with respect to CYBA genotypes have revealed that individuals with GG genotype of -930 A/G polymorphism had higher MDA levels compared to those with AA genotype. Furthermore, the MDA levels with respect to CYBA gene haplotypes has shown that -930G/242C haplotype combination was associated with higher MDA levels in breast cancer patients compared to other haplotypes at $p < 0.05$ as summarized in Figure 6.

Discussion

Oxidative stress (OS) is defined as an imbalance between production of free radicals and reactive metabolites, which are called reactive oxygen species (ROS), and ROS elimination by protective mechanisms which are referred to as antioxidants. In addition, cancer initiation and progression have been shown to be associated with oxidative stress by causing DNA mutations or inducing DNA damage, genome instability, and cell proliferation (Visconti & Grieco, 2009). It has been

confirmed that oxidative stress is involved in multiple cancers (Srivastava et al., 2009; Wang et al., 2011; Wu et al., 2017).

In the present study a higher frequency of breast cancer patients with habit of smoking and alcohol was observed. Results of several studies have also shown that habit of smoking and alcohol consumption were associated with increased risk for breast cancer (Lew et al., 2009; Reynolds et al., 2009; Luo et al., 2011) as they are more exposed to free radicals leading to oxidative damage to lipids, proteins and DNA that may aid in cancer progression.

Alteration in expression of enzyme system that produces ROS such as NADPH oxidase has been shown to be important susceptibility factor for cancer (Arcucci et al., 2016). The most significant sources of ROS are nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, which include two membrane-bound subunits Nox2 and p22phox. The p22phox encoded by the CYBA gene has several functional polymorphisms.

In view of the above, in this study we attempted to determine the -930 A/G and 242 C/T polymorphisms of CYBA gene that encodes p22phox subunit of NADPH oxidase among controls and patients with breast cancer & their association with oxidative stress.

The -930 A/G functional SNP located at the promoter region in a dual-luciferase reported assay system has revealed that the G allele was found to be associated with a 30% increase in promoter activity. Furthermore, the frequency of the G allele was higher than the A allele in hypertensive individuals (Moreno et al., 2003). Recent large population study on -930 A/G polymorphism has also reported that the GG genotype confers susceptibility for hypertension (Kokubo et al., 2005). Therefore, we investigated the association between this SNP and breast cancer. The G-allele in the present population was significantly higher in breast cancer patients compared to healthy controls conferring a 1.27-fold risk towards breast cancer. Further, the -930 A/G polymorphism has a potential binding site for C/EBP (CCAAT/enhancer-binding protein) transcription factors, and it has been speculated that it might modulate CYBA transcriptional activity (San José et al., 2004). This observation was substantiated by our insilco analysis wherein the substitution of A by G results at -930 position resulted in the loss of repressor C/EBPbeta transcription factor site that might increase transcriptional activity.

The C242T polymorphism has been demonstrated to be related to multiple diseases (Guzik et al., 2000; San José et al., 2008; Vibhuti et al., 2010; Schreiber et al., 2011; Zhou & Zhao, 2015). Results of the present study had showed that individuals with the CT/TT genotype of 242 C/T polymorphism had a 1.42-fold higher risk for breast cancer compared to those with the CC genotype. Our finding was consistent with reports showing significant association with vascular disease (Ito et al., 2000). The C242T polymorphism located in exon 4 encodes a CAC→TAC codon change thus resulting in a non-conservative substitution of His72 for a tyrosine residue that may impair the haem-binding site of the p22phox protein (Tahara et al., 2008; Fu et al., 2016). Insilco analysis substantiates this observation wherein change of histidine residue a candidate for the coordinating ligand of the heme prosthetic group of cytochrome b results in altered 3D structure.

In the present study higher MDA levels were found in breast cancer patients compared to controls, which reflects the increased rate of lipid peroxidation due to oxidative stress in breast cancer. Previous studies have also reported increased levels of MDA in breast cancer patients compared to healthy controls (Gönenç et al., 2001, 2006; Yeh et al., 2005) suggesting that elevated oxidative stress contributes to increased risk for breast cancer development and progression. Further, comparison of MDA levels with respect to CYBA gene haplotypes revealed that -930G/242C and -930G/242T haplotype carriers in the patients with breast cancer showed higher MDA levels than other haplotypes; this could be in line with observation that states presence of G-allele could increase the transcriptional activity, elevating ROS production resulting in oxidative stress in breast cancer patients.

Conclusion

In conclusion, our results suggest that oxidative damage may play an important role in BC patients and the -930G/242C and -930G/242T haplotypes of CYBA gene may predispose to increased oxidative stress. Therefore, more attention should be paid to oxidative stress-related pathological manifestations in BC patients with the risk haplotype. Furthermore, studies on CYBA gene polymorphisms/haplotypes and levels of oxidative stress should be done in a multicenter, multi-ethnic population and with a large number of patients in the future to strengthen our findings.

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Conflict of interest: The authors report no conflicts of interest.

Author Contributions

Mohini Aiyengar Tupurani conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, wrote the paper, prepared figures and/or tables. Chiranjeevi Padala, Kaushik Puranam analyzed the data, contributed reagents/materials. Rajesh Kumar Galimudi, Keerthi Kupsal, Nivas Shyamala, Srilatha Gantala, Ramajaneyulu Kummari, Sanjeeva Kumari Chinta, contributed materials, analyzed the data and reviewed drafts of the paper. Surekha Rani Hanumanth designed the experiments, analyzed the data and reviewed drafts of the paper.

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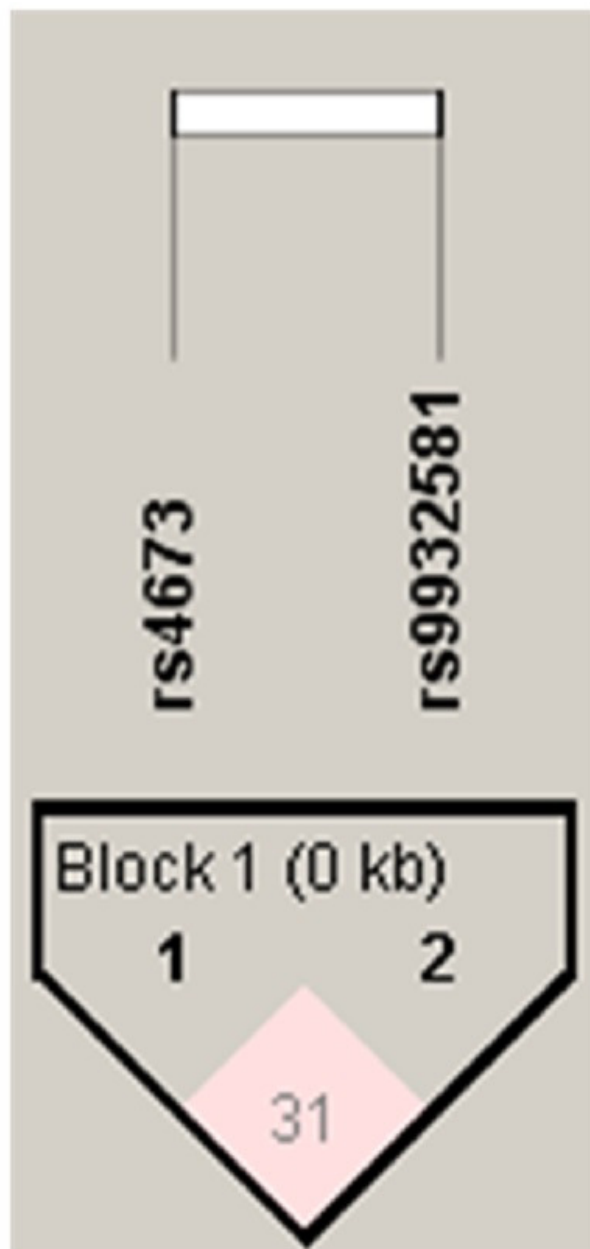
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Figure 1

Plot of pair-wise linkage disequilibrium analysis of SNPs of CYBA genes in controls and BC patients

D' values are shown in the plot

LD plot Controls



LD plot BC patients

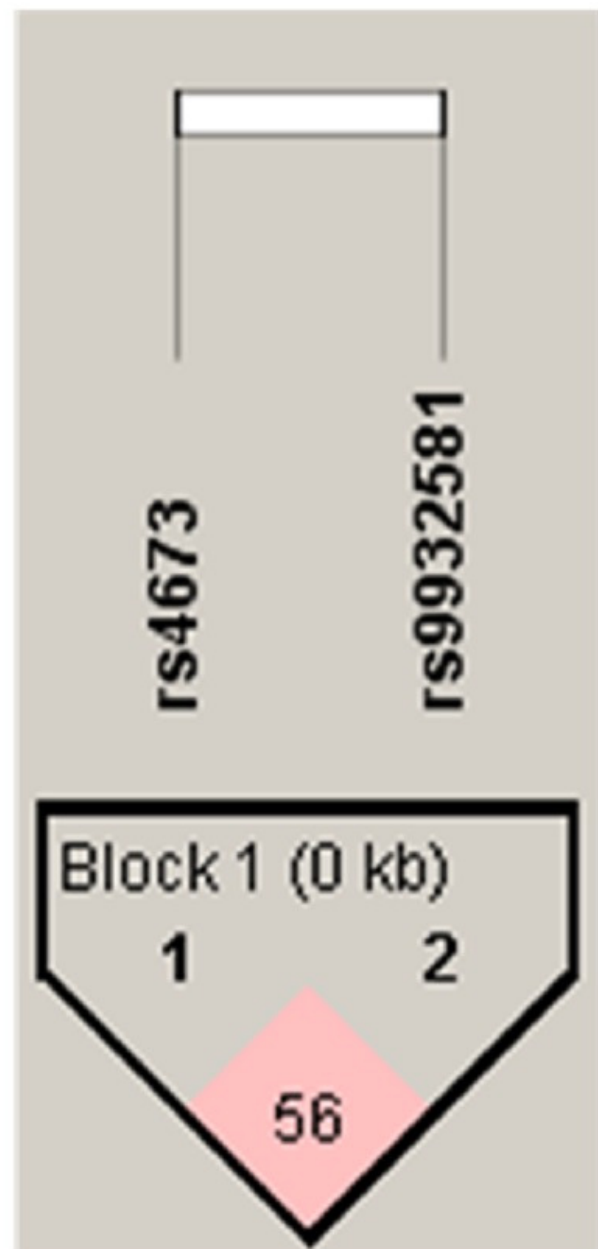


Figure 2

MDR analysis of CYBA gene polymorphisms in association with breast cancer

A) Univariate and Bivariate analysis

B) Interaction dendrogram

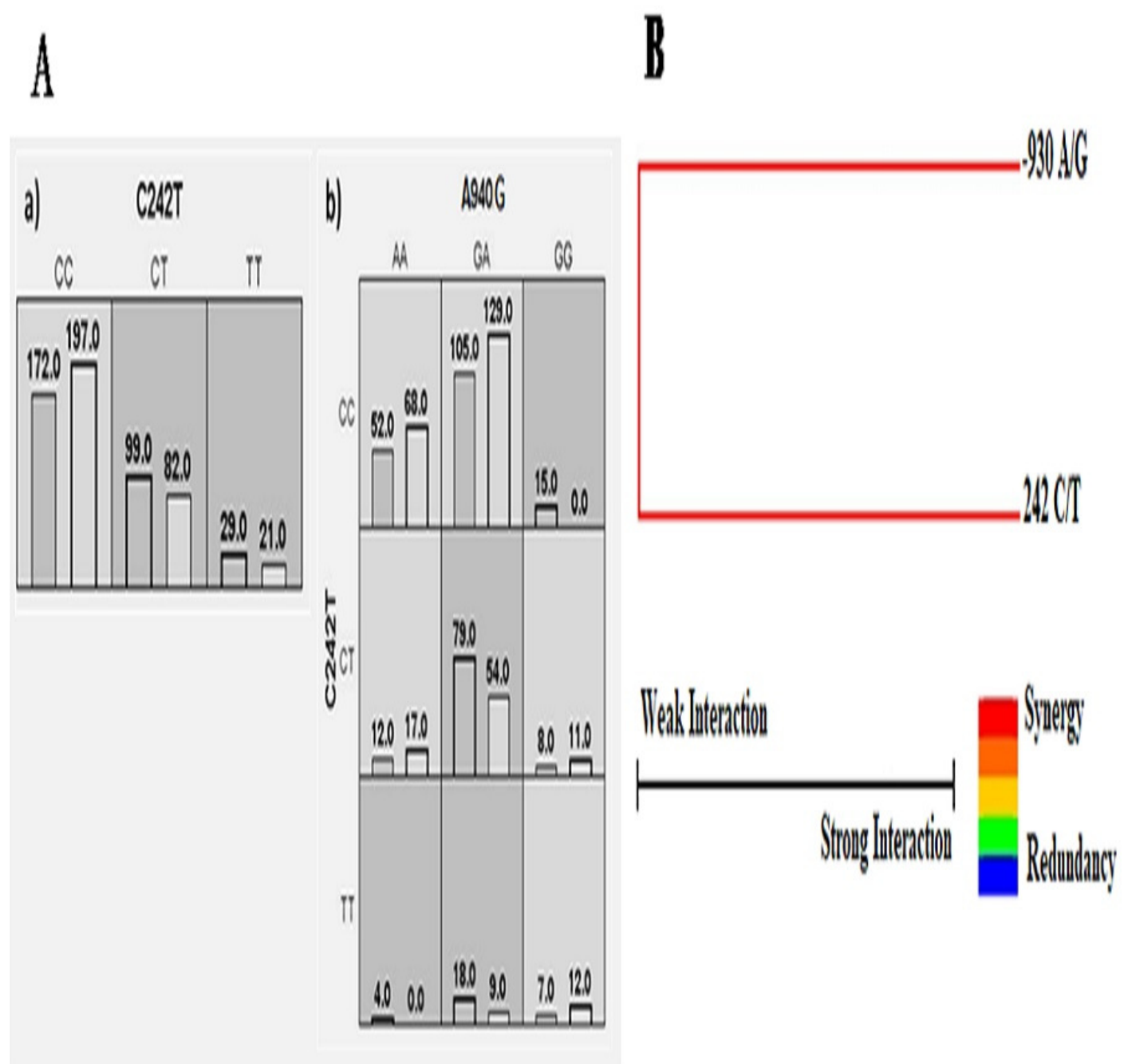


Figure 3

Effect of the CYBA -930 A/G polymorphism on transcription factor binding sites

CYBA -930 A-allele

seq(60.. 119) ccggaggcagaaatgctggtttattcccatggccaccggggcc

Segments:

<u>2.3.1.0</u>	50	63	=====	
<u>2.3.1.0</u>	61	70	=====Sp1=====	
<u>1.1.3.0</u>	69	78		<u>=C/EBPbeta</u>
<u>1.3.1.2</u>	85	94		<u>=====USF=====</u>
<u>9.9.539</u>	86	95		<u>=====NF-1=====</u>
<u>2.3.1.0</u>	95	107		<u>=====Sp1=====</u>

CYBA -930 G-allele

seq(60.. 119) ccggaggcaggaatgctggtttattcccatggccaccggggcc

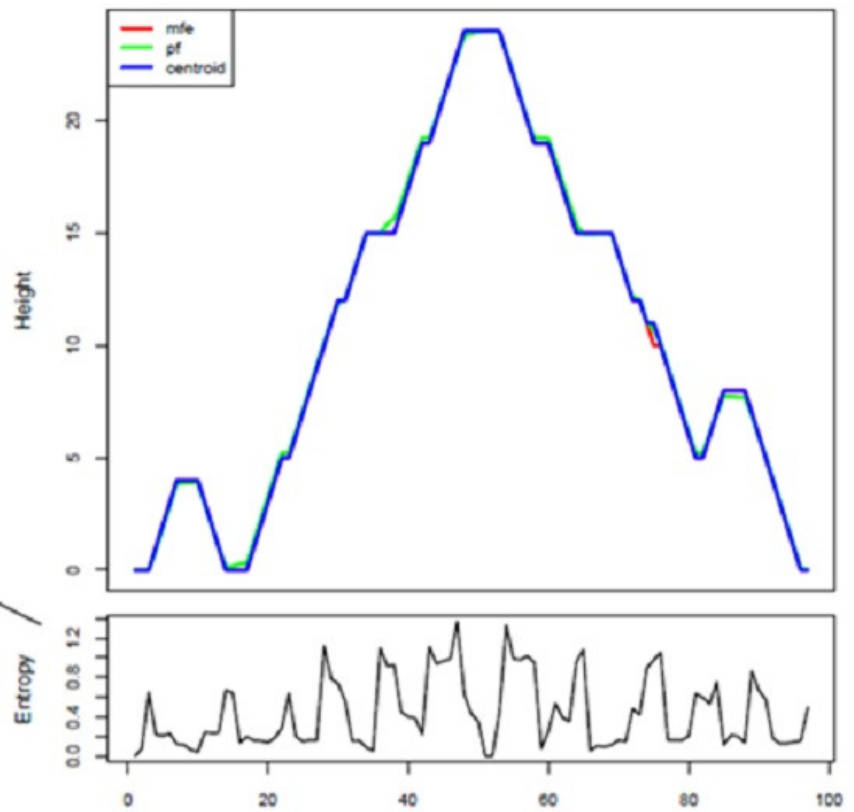
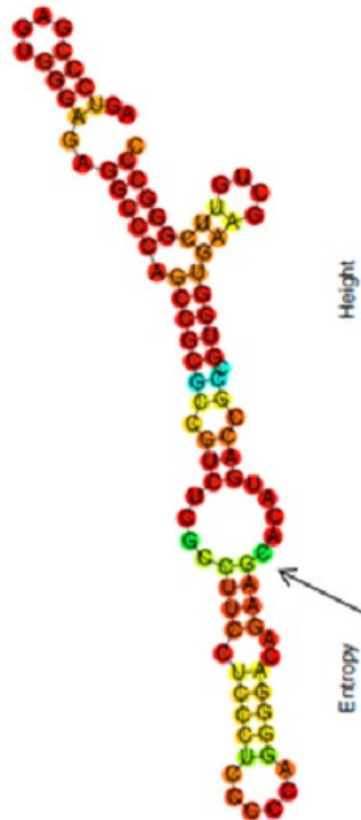
Segments:

<u>2.3.1.0</u>	50	61	==	
<u>2.3.1.0</u>	61	70	=====Sp1=====	
<u>1.3.1.2</u>	85	94		<u>=====USF=====</u>
<u>9.9.539</u>	86	95		<u>=====NF-1=====</u>
<u>2.3.1.0</u>	95	107		<u>=====Sp1=====</u>

Figure 4

Computational analysis of CYBA 242 C/T polymorphism based pre-mRNA secondary structures

C-allele



T-allele

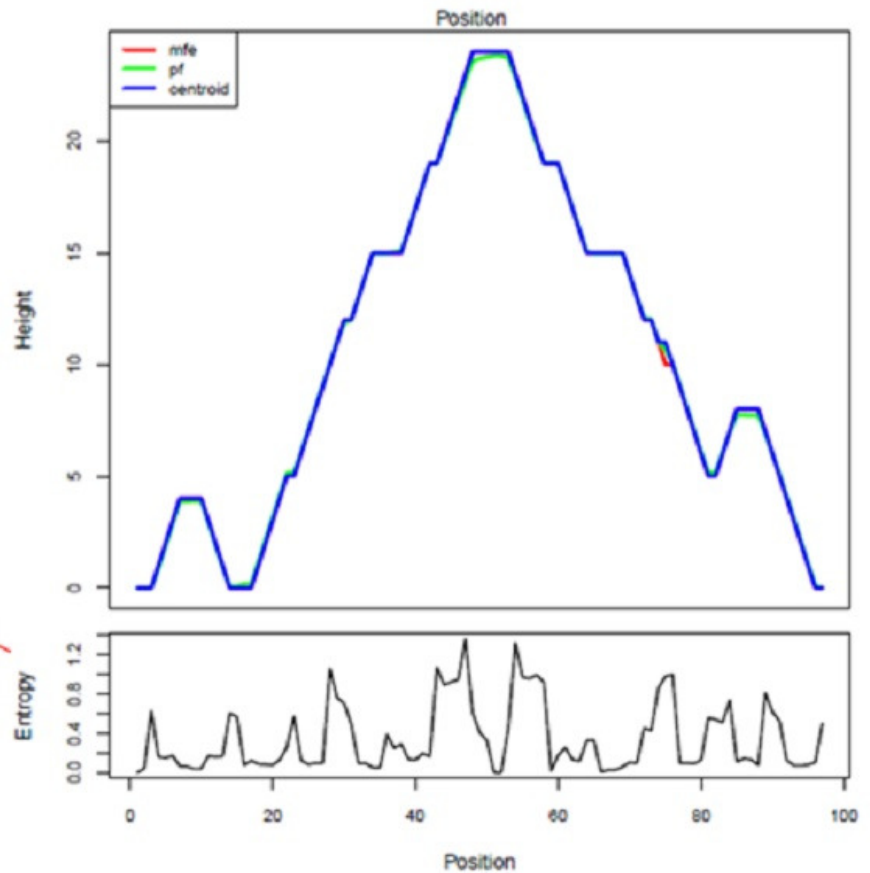


Figure 5

3D structures of CYBA 242 C/T polymorphic variants as predicted by I-TASSER

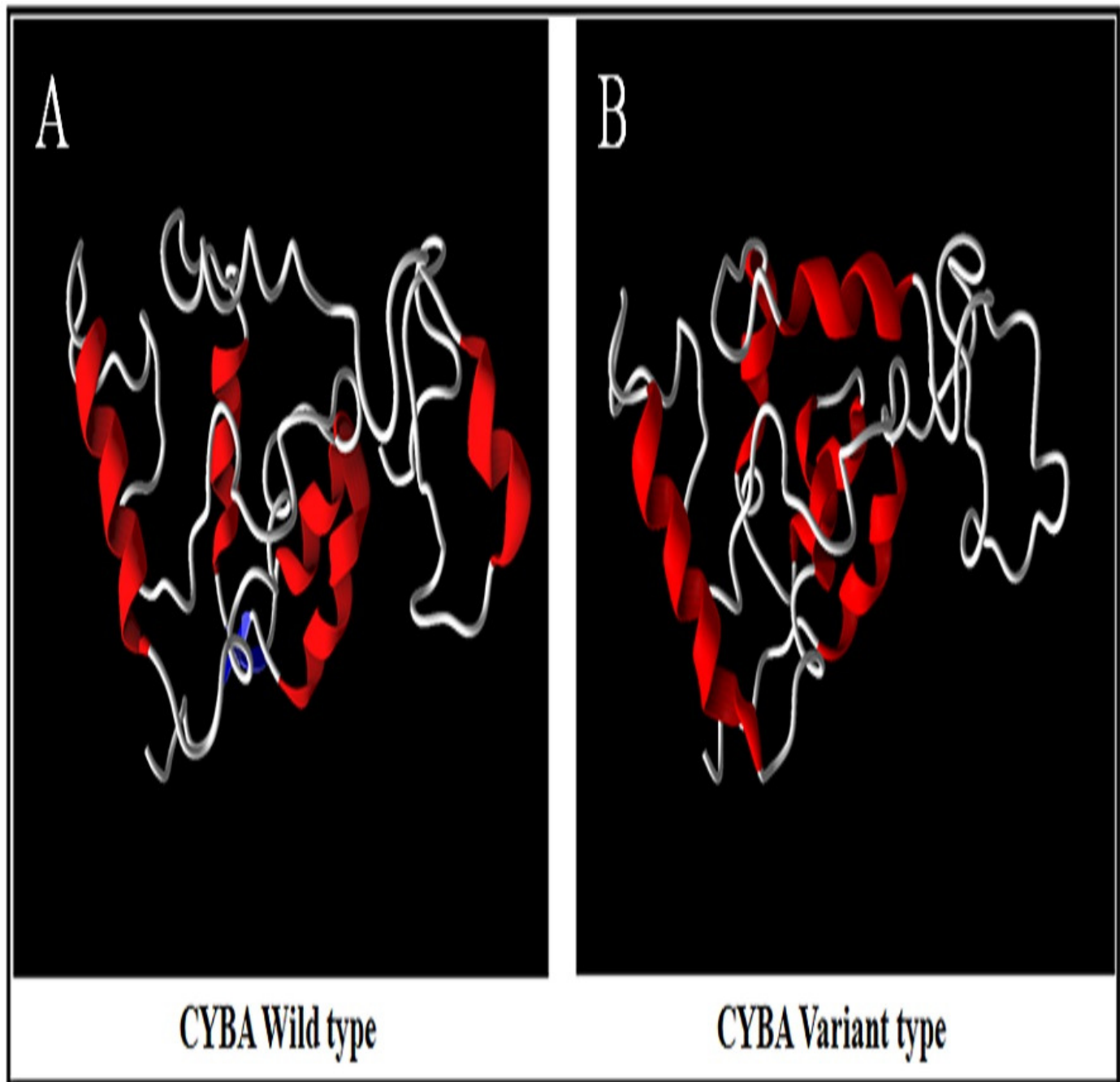


Figure 6

MDA levels in controls and breast cancer patients

MDA levels with respect to CYBA polymorphic genotypes and haplotypes in controls and breast cancer patients

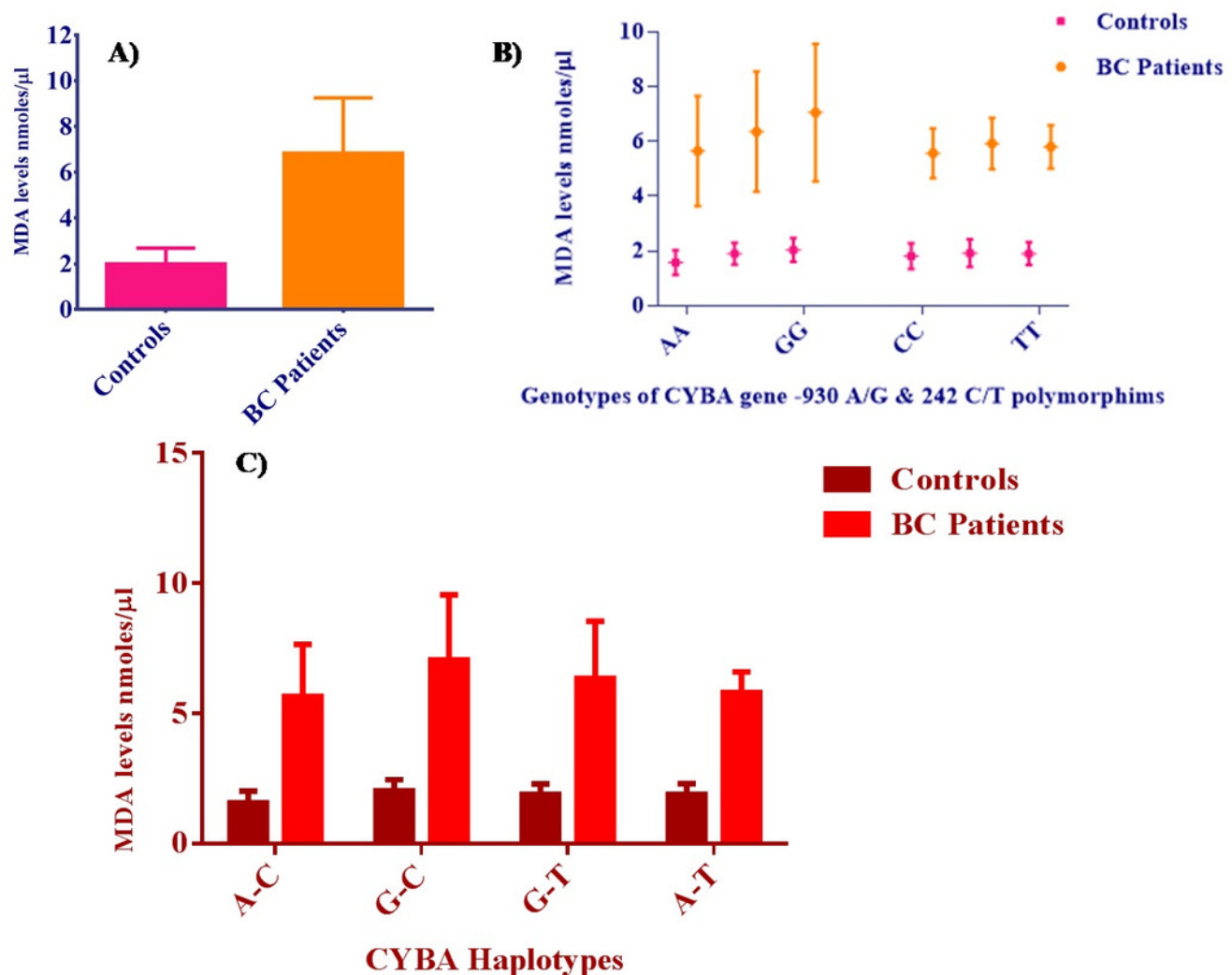


Table 1(on next page)

Baseline characteristics of controls and breast cancer cases

OR, odds ratio, CI, Class interval * p-value by Student's ttest (continuous variables); χ^2 test (categorical variables).

Characteristics	Controls N (%)	Cases N (%)	OR (95% CI)	<i>p</i> ^a
Age (years)	46.34±7.97	47.98±10.8	-	0.034
Lifestyle habits				
Vegetarian Diet	87 (29)	43 (14.34)		
Non-vegetarian Diet	213 (71)	257 (85.56)	2.44 (1.62- 3.67)	<0.005
Non-smokers	273 (91)	245 (81.66)		
Smoker	27 (9)	55 (18.34)	2.27 (1.38-3.71)	0.0004
Non-alcoholics	243 (81)	179 (59.6)		
Alcoholics	57 (19)	121 (40.4)	2.88 (1.99- 4.16)	<0.001

1

Table 2(on next page)

Distribution of genotype and allele frequencies of CYBA -930 A/G polymorphism in controls and breast cancer patients

χ^2 *p-value* <0.05 is considered statistically significant

Model	Genotype	Controls N (%)	Cases N (%)	OR (95% CI)	χ^2 p-value
Co-dominant	A/A	85 (28.3)	62 (20.7)	1.00	0.034*
	A/G	192 (64)	202 (67.3)	1.44 (0.98-2.11)	
	G/G	23 (7.7)	36 (12)	2.15 (1.16-3.98)	
Dominant	A/A	85 (28.3)	62 (20.7)	1.00	0.029*
	A/G-G/G	215 (71.7)	238 (79.3)	1.52 (1.04-2.21)	
Recessive	A/A-A/G	277 (92.3)	264 (88)	1.00	0.074
	G/G	23 (7.7)	36 (12)	1.64 (0.95-2.85)	
Over dominant	A/A-G/G	108 (36)	98 (32.7)	1.00	0.39
	A/G	192 (64)	202 (67.3)	1.16 (0.83-1.62)	
Log-additive	--	--	--	1.46 (1.09-1.94)	0.0094
Allele	A	362 (0.6)	326 (0.54)	1.00	0.035*
	G	238 (0.4)	274 (0.46)	1.27 (1.01-1.6)	
HWE(p)		<0.0001	<0.0001		

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Table 3(on next page)

Distribution of genotype and allele frequencies of CYBA 242 C/T polymorphism in controls and breast cancer patients.

χ^2 *p*-value <0.05 is considered statistically significant

Model	Genotype	Controls N (%)	Cases N (%)	OR (95% CI)	χ^2 p-value
Co-dominant	C/C	197 (65.7)	172 (57.3)	1.00	0.1
	C/T	82 (27.3)	99 (33)	1.38 (0.97-1.98)	
	T/T	21 (7)	29 (9.7)	1.58 (0.87-2.88)	
Dominant	C/C	197 (65.7)	172 (57.3)	1.00	0.036
	C/T-T/T	103 (34.3)	128 (42.7)	1.42 (1.02-1.98)	
Recessive	C/C-C/T	279 (93)	271 (90.3)	1.00	0.24
	T/T	21 (7)	29 (9.7)	1.42 (0.79-2.55)	
Over dominant	C/C-T/T	218 (72.7)	201 (67)	1.00	0.13
	C/T	82 (27.3)	99 (33)	1.31 (0.92-1.86)	
Log-additive	--	--	--	1.31 (1.02-1.68)	0.036
Allele	C	476 (0.79)	443 (0.74)	1.00	0.02
	T	124 (0.21)	157 (0.26)	1.36 (1.04-1.78)	
HWE(p)		0.16	0.027		

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Table 4(on next page)

Haplotype frequencies of CYBA -930 A/G and 242 C/T polymorphisms between Controls and BC patients

^aOrder of SNPs in CYBA gene haplotypes: -930 A/G, 242 C/T; OR-Odds ratio, CI-Class interval; * Interactive Chi-Square p-value<0.05 is statistically significant

Haplotype ^a	Overall (N=600)	Controls (N= 300)	Cases (N=300)	OR (95% CI)	p-value
A-C	0.5116	0.547	0.4752	1.00	---
G-C	0.2542	0.2463	0.2632	1.44 (1.00 – 2.07)	0.05
G-T	0.1724	0.1503	0.1503	1.56 (1.11 – 2.20)	0.011
A-T	0.0617	0.0563	0.0682	1.40 (0.75 - 2.59)	0.29

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Table 5(on next page)

Cavity differences between the structures of CYBA 242C/T polymorphic variants

Cavity	Volume	
	Wild Type	Variant Type
1	53.248	132.09
2	51.2	28.67
3	22.01	17.92
4	19.96	13.312
5	18.94	--

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