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

Mohini A Tupurani [1], **Chiranjeevi Padala** [1], **Kaushik Puranam** [1], **Rajesh Galimudi** [1], **Keerthi Kupsal** [1], **Nivas Shyamala** [1], **Srilatha Gantala** [1], **Ramanjaneyulu Kummari** [1], **Sanjeeva Chinta** [2], **Surekha R Hanumanth** Corresp. [3]

[1] Genetics, Osmania University

[2] Radiation Oncology, MNJ Institute of Oncology Regional Cancer, Hyderabad, Telangana, India

[3] Genetics, Osmania University, Hyderabad, Telangana State, India

Corresponding Author: Surekha R Hanumanth
Email address: surekharanih@gmail.com

Background: Oxidative stress (OS) is a strong characteristic feature in cancer initiation and progression. NADPH oxidase (NOX) dependent free radical production is implicated in oxidative stress among multiple cancers. P22phox is a subunit of NADPH oxidase encoded by the CYBA gene that has functional polymorphisms associated with multiple diseases. 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 breast cancer (BC) development and progression. 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. The Insilco analysis has been performed to predict the effect of single nucleotide polymorphism on the gene regulation using online 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 might aid in increased risk for breast cancer initiation and progression.

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Tupurani Mohini Aiyengar [1], Padala Chiranjeevi [1], Puranam Kaushik [1], Galimudi Rajesh Kumar [1], Kupsal Keerthi [1], Shyamala Nivas [1], Gantala Srilatha [1], Kummari Ramanjaneyulu [1], Chinta Sanjeeva Kumari [2], Hanumanth Surekha Rani [1]*

[1]Department of Genetics & Biotechnology, Osmania University, Hyderabad, Telangana, India
[2]Department of Radiation oncology, MNJ Regional Cancer Centre, Hyderabad, Telangana, India
Corresponding author: H. Surekha Rani
Email address: surekharanih@gmail.com

ABSTRACT

Background: Oxidative stress (OS) is a strong characteristic feature in cancer initiation and progression. NADPH oxidase (NOX) dependent free radical production is implicated in oxidative stress among multiple cancers. P22phox is a subunit of NADPH oxidase encoded by the CYBA gene that has functional polymorphisms associated with multiple diseases. 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 breast cancer (BC) development and progression. 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. The Insilco analysis has been performed to predict the effect of single nucleotide polymorphism on the gene regulation using online 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

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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 (Jezierska-Drutel, Rosenzweig & Neumann, 2013). 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 (Visconti & Grieco, 2009; Fiaschi & Chiarugi, 2012). 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 production (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 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 encoding 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).

The -930 A/G polymorphism (rs9932581) located in the promoter is associated with a higher promoter activity and an increased level of oxidative stress (Ochoa et al., 2008). While, the 242 C/T polymorphism (rs4673) is located in the exon 4 of the CYBA gene, leading to a his72-to-tyr (H72Y) substitution results in altered NADPH oxidase activity (Castaldo et al., 2015). 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. The study followed the Helsinki declaration and was approved by Institutional ethics committee. Patients were enrolled from the department of oncology from August 2013 to August 2017 and during the same time controls subjects were enrolled from the local population. Patients with breast cancer were consecutively recruited from MNJ Regional Cancer Centre, Hyderabad and women with any other cancer or other systemic inflammatory disease were excluded from the case and control group.

All subjects were explained about the purpose of the study and were ensured that the information collected from them would be confidential. Subsequently written informed consent to participate in the study was obtained from each individual. 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, axilliary lymph

node involvement and metastasis were collected via medical records with approval of patients with the help of medical oncologist.

Sample collection

Following an overnight fast 4ml of blood sample was collected by antecubital venipuncture in EDTA vaccutainer from each individual in the morning. Plasma was separated by centrifuging the blood sample at 3000 rpm for 5 min. Blood samples were collected from the patients before any treatment modality such as chemotherapy and surgery.

Plasma MDA levels estimation

Lipid peroxidation, as evidenced by the formation of malondialdehyde (MDA), was assayed by the method described previously (Gavino et al., 1981; Rajesh et al., 2011). Briefly, to 0.5ml of freshly obtained plasma an equal volume of 0.9% saline and trichloroacetic acid (TCA) was added and incubated at 37°C for 20 minutes, and centrifuged for 10 minutes at 3000 rpm. To 1ml of protein free supernatant (TCA extract) 0.25ml of thiobarbituric acid (TBA) was added and the reaction mixture was heated for 60 min at 95°C till a faint pink color develops. After cooling the color intensity was measured at 532 nm with eppendorf UV 240-Spectrophotometer. 1,1,3,3-Tetraethoxypropane(1-100 nmol/ml) was used as the standard. The lipid peroxidation activity was expressed in “nano moles” of MDA equivalents/ml of standard 1,1,3,3-Tetraethoxypropane.

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

Demographic, clinical, and biochemical variables are expressed as the mean±SD. All statistical tests were two-sided, a P-value lower than 0.05 was considered statistically significant. For comparison of continuous variables in demographic data between controls and breast cancer patients, Student's *t*-test was performed. 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).

Bioinformatics 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/cgibin/RNAWebSuite/ RNAfold.cgi) online tool (Zuker & Stiegler, 1981). The 3D models for CYBA wild type and variant protein with 242 C/T SNP were generated using homology modeling tool I-TASSER (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 GG genotype was significantly higher and was found to be associated with an 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

146 significantly different between study groups and conferred increased risk for breast cancer
147 compared to A-allele (OR 1.27, 95% CI 1.01-1.6, p=0.035)

148 Table 3, represents the genotype and allele frequency distribution of CYBA 242 C/T
149 polymorphism among the controls and patients with breast cancer. Under the dominant model,
150 carriers of at least one minor allele T (CT+TT) were found to be associated with a significantly
151 increased risk of BC compared to major allele homozygotes (CC) carriers (OR 1.42, 95%CI
152 1.02-1.98, p=0.036). The allelic association revealed that the minor allele T of 242 C/T
153 polymorphism was associated with an increased risk of BC (OR 1.36, 95% CI 1.04-1.78,
154 p=0.02).

155 We further have analysed the haplotype frequencies with respect to CYBA gene polymorphisms
156 in association with risk of breast cancer. Our analysis has revealed a total of 4 haplotypes as
157 shown in Table 4. Comparison of haplotype frequencies between controls and BC patients
158 revealed a significant difference in haplotype frequencies, where -930G/242C and -930G/242T
159 combinations were found to be significantly associated with an increased risk of breast cancer by
160 more than 1.40 fold (95 % CI 1.00–2.07; $p<0.05$) and 1.56 (95 % CI 1.11 – 2.20; $p<0.05$)
161 respectively compared with the common haplotype (-930A/242C).

162 Pairwise LD was computed for CYBA -930 A/G and 242 C/T polymorphism in cases and
163 controls separately. LD plots revealed a moderate LD (D′=56) between the markers in BC
164 patients and a weak LD (D′=31) between the markers in controls as shown in the Figure 1.
165 Further, MDR analysis with respect to CYBA gene polymorphism has shown that 242C/T
166 polymorphism was the best single locus model with a significant risk for breast cancer. The
167 bivariate model showed strong interaction between -930 A/G and 242 C/T polymorphisms as
168 seen in Figure 2.

169 Furthermore, the TFBS analysis with respect to -930 A/G promoter polymorphism has revealed
170 that substitution of A nucleotide by G leads to a loss of C/EBPbeta site as depicted in Figure 3.
171 The comparison of the wild type and variant pre-mRNA secondary structures with respect to 242
172 C/T polymorphism is given in Figure 4, wherein, the stability, as depicted by minimum free
173 energy (MFE) change has revealed that the T-allelic structure had an MFE of -37.61 Kcal/mol
174 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).

The plasma MDA levels were measured in all the subjects in the present study, our results revealed that patients with breast cancer had significantly higher MDA levels (6.84±2.42 nmoles/µl) compared to the control (2±0.69 nmoles/µl) group. Further, MDA levels were stratified with respect to CYBA genotypes, where we found 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

Breast cancer is a common disease worldwide and also one of the leading cause of cancer death in India (Ferlay et al., 2015; Malvia et al., 2017). Breast carcinogenesis involves a cascade of multiple intracellular mechanisms such as genetic alterations and signal transduction pathways (Kurose et al., 2001). However, it also depends on the oxidative stress (OS) and the predominance of endogenous antioxidant system for manifestation of disease. Oxidative stress induces uncontrolled lipid peroxidation, that produce aldehyde end-products, such as free fatty acids, malondialdehyde (MDA) and these products might cause cell injury and death. In addition, cancer initiation and progression have also 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 contrast several reports have been inconsistent, wherein no significant association was observed with respect to smoking and alcohol consumption in breast cancer patients (Byrne, Rockett & Holmes, 2002; Allen et al., 2009; Gathani et al., 2017).

In the present study, a higher frequency of breast cancer patients with habit of smoking and alcohol was observed. Multiple reports 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 an 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 have investigated the association between this SNP in association with breast cancer risk. In the present population the G-allele was found to be significantly higher in breast cancer patients compared to healthy controls conferring a 1.27-fold risk towards breast cancer. The promoter region SNPs affects gene expression by altering promoter activity, transcription-factor binding, DNA methylation and histone modifications (Deng et al., 2017). Interactions between transcription factors (TFs) and target sites are the main edges of regulatory networks as such interactions determine the expression levels of target genes. Since the -930 A/G polymorphism has a potential binding site for C/EBP (CCAAT/enhancer-binding protein) transcription factors it has been speculated that it might modulate CYBA transcriptional activity (San José et al., 2004). Our insilco analysis transcription-factor binding sites with respect to -930 A/G polymorphic variants revealed that the substitution of A by G results 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 alter the haem-binding site of the p22phox protein (Tahara et al., 2008; Fu et al., 2016). Finding 3D structure of proteins is helpful in predicting the impact of SNPs on the structural level and in showing the degrees of alteration. Our insilco analysis with respect to C242T polymorphism has resulted in an altered 3D structure with a change of histidine residue, a key candidate for the coordinating ligand of the heme prosthetic group of cytochrome b that might cause functional impairment.

MDA is a naturally occurring endogenous product of lipid peroxidation and prostaglandin biosynthesis, but is mutagenic and carcinogenic. The MDA level in blood sample is a good measure of the body ability to handle the oxidative stress. Oxidative stress as measured by an increase in MDA levels was established in gastric, colorectal adenomas, prostate and oral cancer (Bakan et al., 2002; Leuratti et al., 2002; Zhang et al., 2008; Chole et al., 2010). In this study we have also demonstrated an increase in lipid peroxidation due to oxidative stress in breast cancer patients. 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.

There are several limitations in this study. The foremost limitation to our study concerns the use of limited sample size, which prevented us from drawing causal relationships. Owing to its importance as an oxidative stress indicator we have measured MDA levels in our study, however MDA alone is not a sole indicator of oxidative stress. Further we have not directly quantified the NADPH oxidase activity. Further more studies on CYBA gene polymorphisms/haplotypes along

with different oxidative stress markers should be done in a multicenter, multi-ethnic population and with a large number of patients in the future to strengthen our findings.

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 breast cancer patients with the risk haplotype.

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447

Figure 1

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

(A) LD plot of controls (B) LD plot of Cases. D' values are shown in the plot. A value of 100 represents maximum possible linkage disequilibrium.

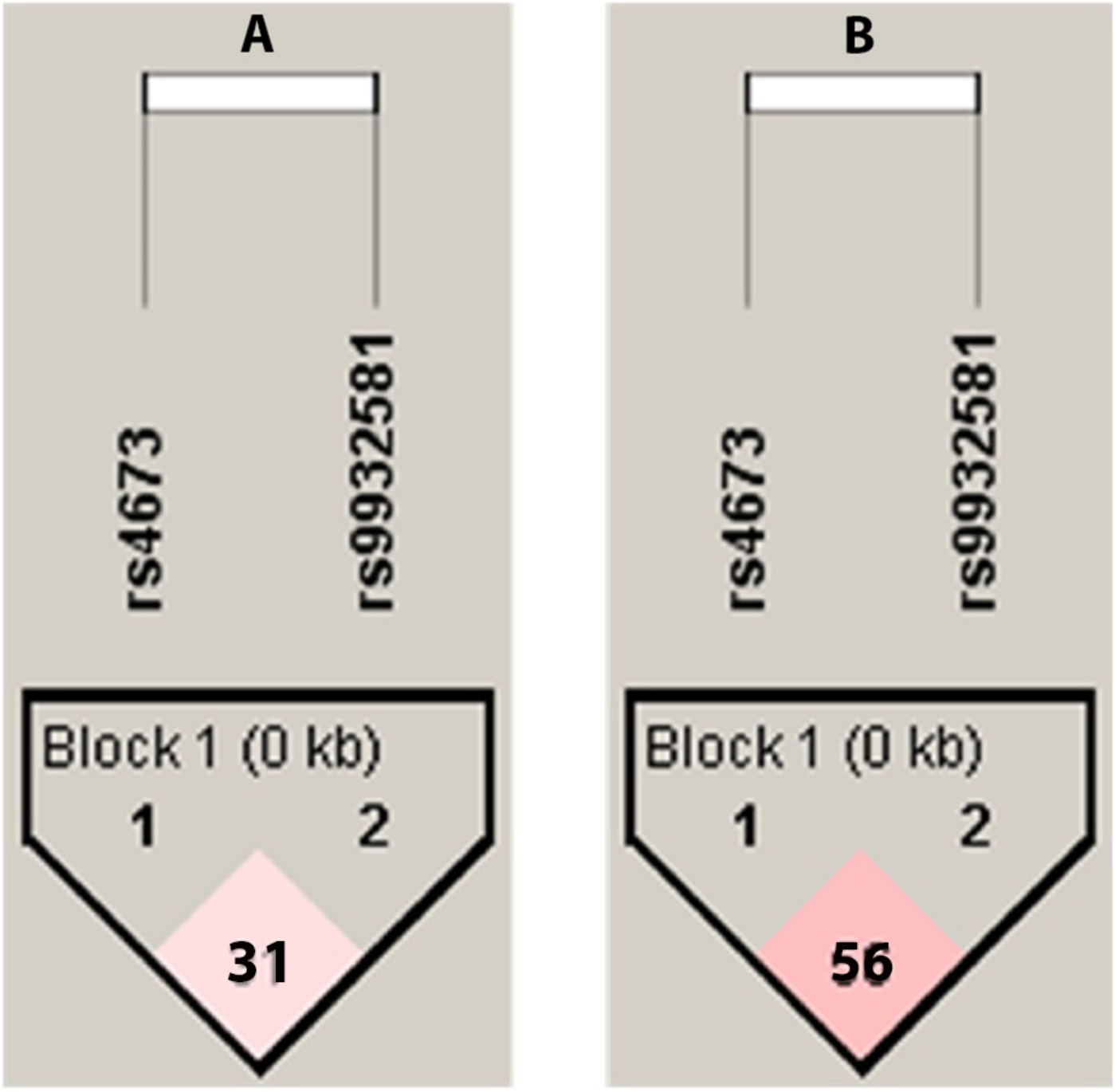

Figure 2

Multifactor dimensionality reduction (MDR) analysis of CYBA gene polymorphims in association with breast cancer

(A) Univariate and (B)Bivariate analysis- In each block, the dark and light bars represent the number of cases and number of controls with that particular genotype, respectively. Dark and light backgrounds of the block represent a positive and negative association with breast cancer, respectively. (C) Interaction dendrogram- The interaction dendrogram was used to confirm, visualize, and interpret the interaction model. The colours used to depict the degree of synergy, ranging from red (highest information gain) to blue (highest information redundancy). Our analysis has revealed a synergistic interaction between SNPs (gain of information).

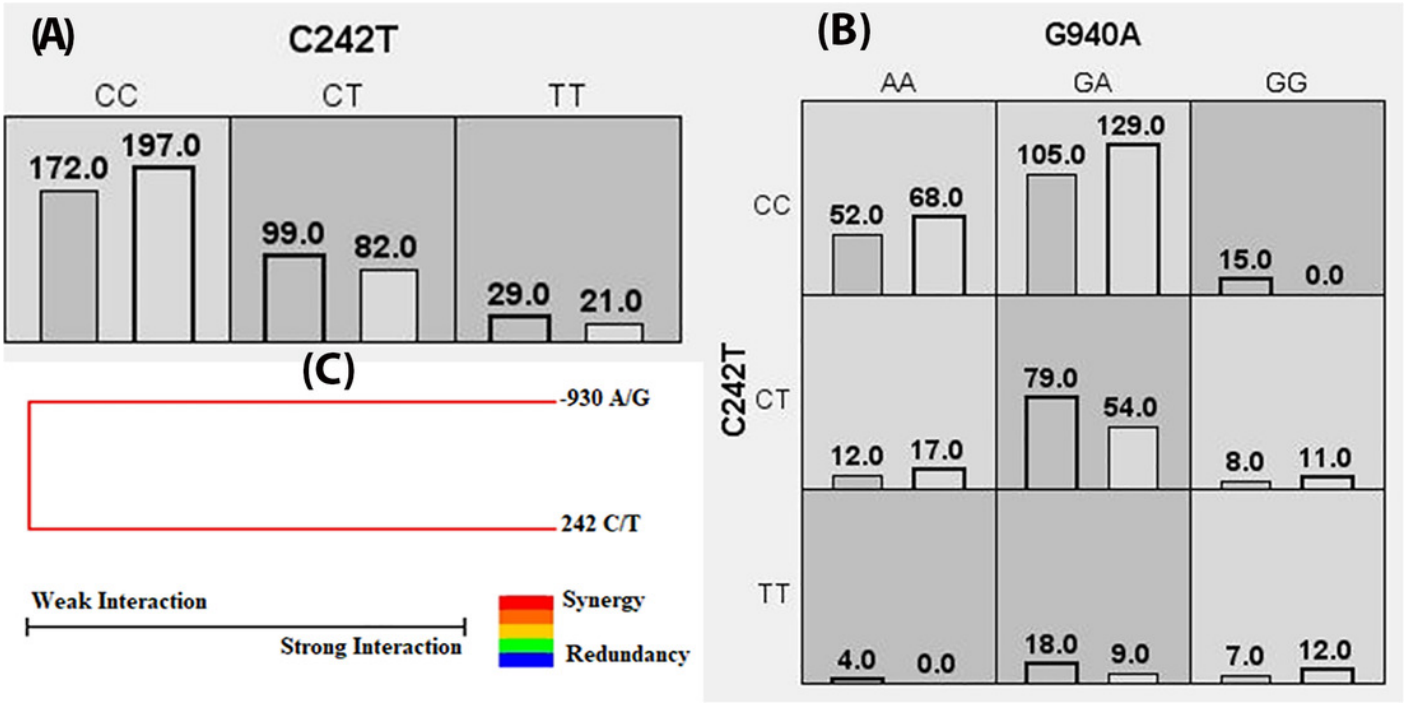

Figure 3

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

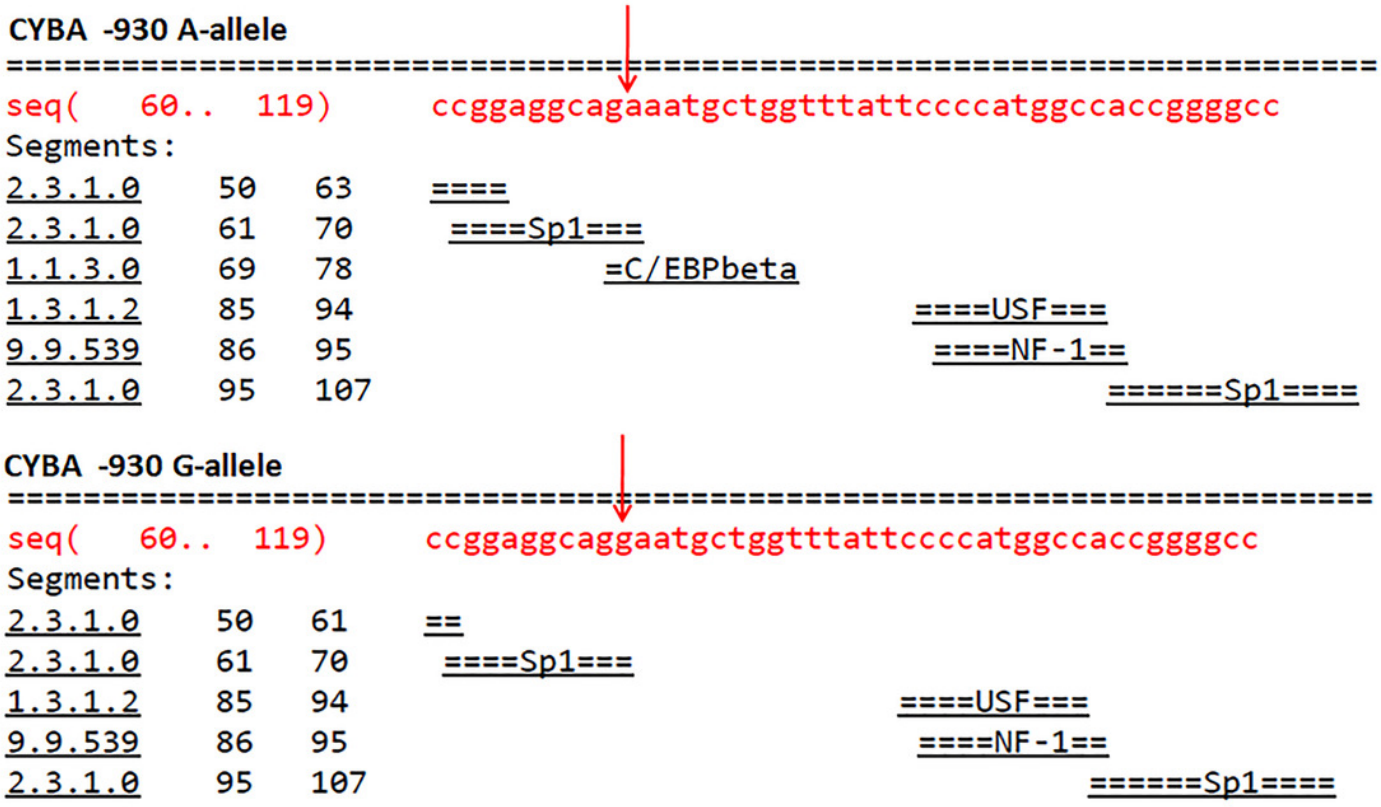

Figure 4

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

Predicted minimal free energy based RNA structure of (A) major (C-allele) and (B) minor (T-allele) alleles of 242C/T polymorphism using the RNA fold program in the Vienna RNA package (Zuker algorithm). Structure colours encode base-pair probabilities and arrow denotes the location of polymorphism. The mountain plot is a XY -graph that represents a secondary structure including MFE structure, the thermodynamic ensemble of RNA structures (pf), and the centroid structure in a plot of height versus position. “mfe” represents minimum free energy structure; “pf” indicates partition function; “centroid” represents the centroid structure

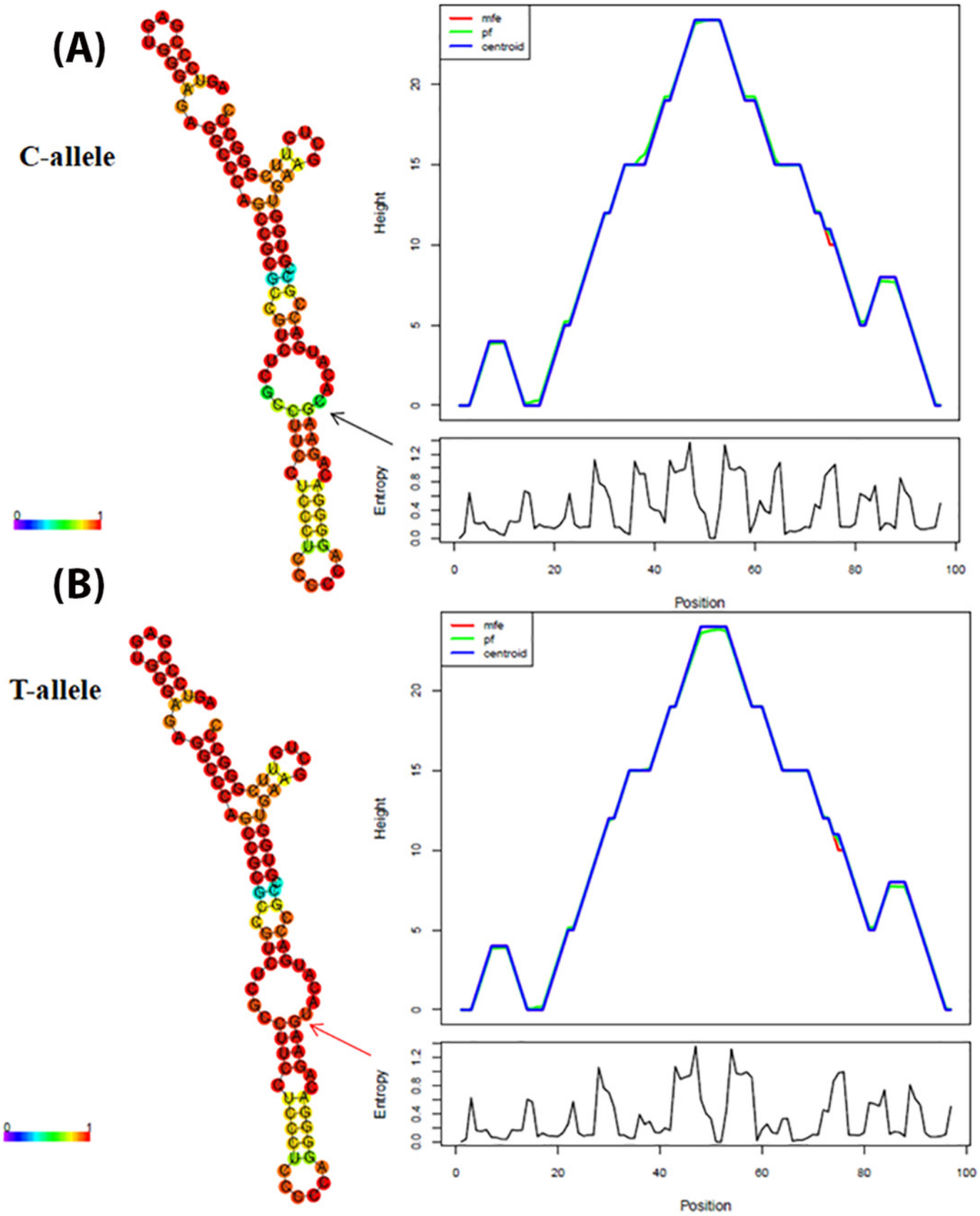
(A)
C-allele
mfe
pf
centroid
Height
Entropy
Position
(B)
T-allele
mfe
pf
centroid
Height
Entropy
Position

Figure 5

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

The 3D structures of the CYBA 242 C/T polymorphic variants were modeled on I-TASSER server. The left box (A) displays the wildtype structures and the right box (B) exhibits the relevant variant structure.

**Note: Auto Gamma Correction was used for the image. This only affects the reviewing manuscript. See original source image if needed for review.*

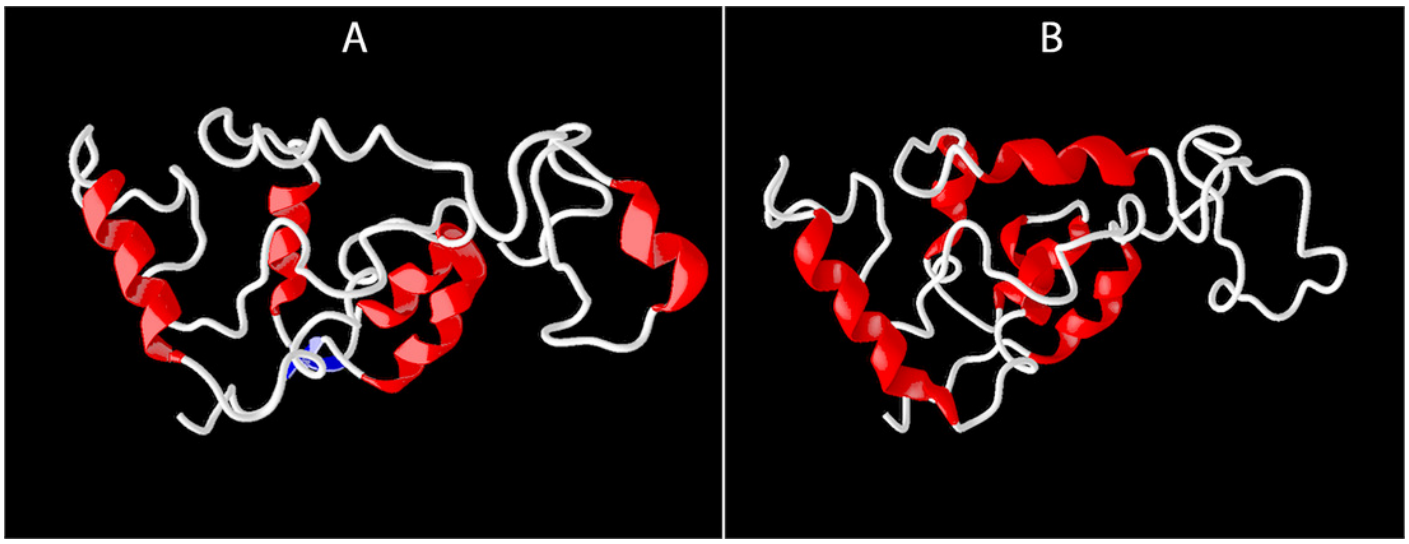

Figure 6

MDA levels in controls and breast cancer patients

(A) Malondialdehyde (MDA) levels in the control group and breast cancer patients (B) MDA levels with respect to CYBA polymorphic genotypes and (C) MDA levels with respect to CYBA gene haplotypes in controls and breast cancer patients

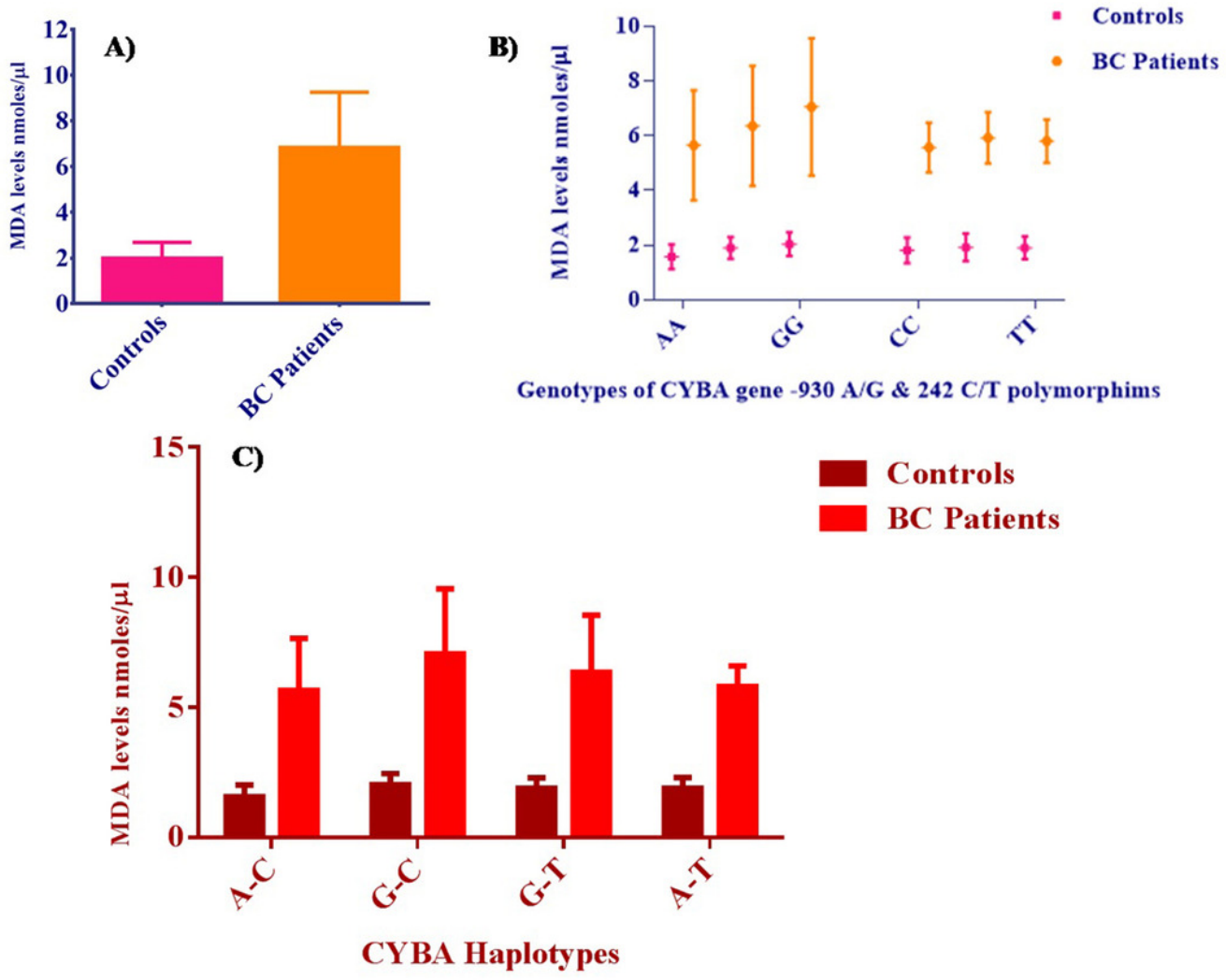

Table 1(on next page)

Baseline characteristics of controls and breast cancer cases

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

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

1

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	
	C/T	82 (27.3)	99 (33)	1.38 (0.97-1.98)	0.1
	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	
	T	124 (0.21)	157 (0.26)	**1.36 (1.04-1.78)**	**0.02**
HWE(p)		0.16	**0.027**		

1

Table 4(on next page)

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

[a]*Order 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

1

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	--

1