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Crosstalk of Cyclin-dependent kinase inhibitor 1A (CDKN1A) gene polymorphism with p53 and CCND1 polymorphism in breast cancer

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Abstract. – OBJECTIVE: Mutations and polymorphisms in genes of cell- cycle and apoptosis regulatory pathway influence the breast cancer risk. Analysis of single low penetrance mutant alleles may not reflect the precise risk association when analyzed alone.

PATIENTS AND METHODS: A total of 115 DNA samples extracted from breast cancer patients and an equal number of age and sex-matched normal controls were used for polymorphic analysis. Genotyping for p21 rs1801270 and CCND1 rs603965 was done by PCR-RFLP method while AFLP method was used for p53 rs1042522 single nucleotide polymorphism detection. Statistical methods included simple mean±SD and correlation coefficient to analyze the risk of association of p21, p53 and CCND1 SNPs and breast cancer.

RESULTS: Individuals harboring SNPs in p21, p53 and CCND1 genes namely rs1801270, rs1042522 and rs603965, respectively were rendered increasingly susceptible to developing breast cancer when compared with normal controls.

CONCLUSIONS: Our report emphasizes the need of combinational analysis of low-penetrance mutant alleles to assess accurately their association with breast cancer risk. Future case-control studies analyzing gene-environment interactions across different populations may confirm reported risk associations of studied polymorphisms with developing breast cancer.

Key Words:
CDKN1A, p53, CCND1, SNP, Breast cancer.

Introduction

Breast cancer, the most common type of cancer in women resulted in 627,000 deaths in 2018, is impacting developed and developing regions of the world equally[1] with an increase of 30% annually[2,3]. Trends in past breast cancer incidence from 2012 to 2018 shows a yearly increase of almost 6% (from 1655589 to 2069792). However, the future estimates project a reduction of almost 50% in annual breast cancer cases from 2018 to 2040[4,5]. An improved understanding of the genetic mechanisms involved in the onset and progression of breast cancer may prove the key to early diagnosis, better clinical management and prognosis of breast cancer cases.

Cell Cycle Control and Relevance of p21, p53 And CCND1

Loss of control in mammalian cell cycle control is at the center of cellular transformation. The cyclin-dependent kinase inhibitor p21 (CDKN1A), acting simultaneously as a sensor and an effector of many anti-proliferative signals, mediates cell cycle

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progress in tumorigenic milieu[6,7]. Cell cycle progression without repairing the DNA damage leads to uninhibited cellular growth resulting in tumorigenesis[8,9]. Any germ line mutation or change in cell cycle genes or encoded proteins may disable the inherent cell cycle checkpoint mechanism and thus increases cancer risk significantly[8,10]. Defects in cell cycle checkpoint promote cancer onset and impact the efficacy of anticancer treatment[11]. The p53 mediates the tumor suppressor effects by regulating multiple genes involved in growth inhibition or apoptosis[12]. Of these, p21 effects cellular growth arrest at G1 stage in p53-dependent fashion[7,13]. Early evidence[14] suggests that p21 bind to the cyclin-dependent kinases (CDK1 and CDK2) and inhibits their kinase activity, resulting in cell cycle arrest at particular stages and thereby suppresses tumorigenesis.

Additionally, p21 reduces PCNA-dependent DNA polymerase activity, thereby inhibiting DNA replication and also affects various PCNA dependent DNA repair processes negatively by binding to proliferating cell nuclear antigen (PCNA)[15-17]. The changes in p21 gene are significantly associated with the risk of developing many cancers[18-20] but many studies have also reported contradictory results[21-25]. Among them, p21 rs1801270 SNP induces a C to A transversion leading to the addition of amino acid arginine in place of serine. This substitution is located within zinc finger domain and thus affects the DNA binding capacity[26]. Although clinical significance of p21 C93A polymorphism is categorized as "benign" and has a global allelic frequency of C=0.913055 (reference) and A=0.086945 (variant), early reports have shown a frequent occurrence of this SNP among cancer patients[27-30] including breast cancer[19,31]. However, the impact of this polymorphism on tumorigenesis in presence of other SNPs remains to be studied.

The p53 tumor suppressor gene regulate the cell cycle and acts as an important check point in the wake of serious genomic insult. The wt p53 gene harbors rs1042522 SNP at codon 72 in exon 4 which induces the addition of amino acid arginine in place of proline (Arg72Pro)[32]. This substitution disturbs a proline-rich region encompassing residues 64 to 92. This 72 Proline amino acid is located within one of the five critical proline-rich motifs, structurally similar to SH3 binding domain[33] essential for inhibiting uncontrolled proliferation and apoptosis. Polymorphic p53 proteins have some different biochemical and biological properties and the SNP is categorized as benign in terms of clinical significance, with global allelic frequency of wt G=0.29787 and variant C=0.70213, in risk disposition of syndromes like hereditary cancer predisposing syndrome[34] and Li Fraumeni syndrome 1[35]. However, the impact of this polymorphism on efficacy and toxicity response of several drugs like paclitaxel[36], cyclophosphamide and antineoplastic agents[37], fluorouracil[38] and cisplatin[36,39,40] is significant.

CCND1 gene codes for the critical regulatory subunit of the enzyme responsible for phosphorylation and subsequent inactivation of the RB protein, leading to the cell cycle progression from G1 to S phase[41,42]. Among all D-type cyclins binding with cyclin-dependent, kinase (CDK), over expression of CCND1 is at the core of tumorigenesis and metastases in humans[43]. CCND1 regulates cell cycle in CDK-dependent as well as CDK-independent fashion[44]. Normally, progression through G1 to S phase is regulated by phosphorylation and inactivation of the RB protein and beginning the DNA synthesis. A silent G to A substitution at nt870 (rs603965) in exon 4 of CCND1 leads to an alternative protein, transcript-b[45] which phosphorylates and inactivates the RB protein inefficiently when compared with transcript-a[46]. Clinical Significance of this polymorphism is categorized as "Risk-Factor" with a global allelic frequency of G=0.540392 and A=0.459608. The rs603965 increases susceptibility to various cancers such as urinary bladder cancer[47], esophagus and gastric cancer[48], prostate cancer[49], squamous cell carcinoma of the head and neck[50], colorectal cancer[51], cervical cancer[52], multiple myeloma[53], and colorectal cancer at young age[54].

SNPs of p21, p53 and CCND1 genes namely rs1801270, rs1042522 and rs603965, respectively influence in control of cell cycle and the polymorphisms therein increases the breast cancer susceptibility. However, the cumulative effects of these polymorphisms on breast cancer susceptibility are unexplored. The current study elucidates the combinatorial effect of these polymorphisms in breast cancer onset and progression using a retrospective case-control study.

Patients and Methods

Biological Specimens

Blood samples of 115 breast Cancer patients having different grades of Adeno or infiltratory duct carcinoma were collected for polymorphic studies from BRA-IRCH, All India Institute of Medical Sciences, New Delhi, India. Additionally, same number

Table I. Risk associated with different clinicopathological variables.

Clinicopathological variables	No. of Patients	Percentage (%)	Risk ratio	χ^2 significance (*p*-value pearson)
Age Distribution	115			
25-77 years, average 35-50 years				
Age				
< 50	77/ 115	66.95	2.02	< 0.0001
> 50	38/ 115	33		
Menstrual status				
Pre-Menopausal	69/ 115	60	1.5	0.002
Post-Menopausal	46/ 115	40		
Nodal status				
Positive	75/ 115	65.2	1.87	< 0.0001
Negative	40/ 115	34.78		
Histological grading				
PD	62/ 115	53.9	PD v/s MD	
			1.67	0.000
MD	37/ 115	32.17	PD v/s MD+ WD	
			1.16	0.23
WD	16/ 115	13.9		
Histological status				
Invasive Ductular Carcinoma (IDC)	107/ 115	93	13.37	< 0.0001
Invasive Lobular Carcinoma (ILC)	8/ 115	7		
Tumor Size				
pT3 (<15)	68/ 115	59.13	pT3 v/s pT2	
			1.65	0.000
pT2 (<5)	41/ 115	35.65	pT3 v/s pT2+ pT1	
			1.44	0.005
pT1 (<2)	6/ 115	5. 2		
Estrogen Receptor (ER) status				
+ve	42/ 115	36.52	0.57	< 0.0001
-ve	73/ 115	63.48		
Progesterone Receptor (PR) status				
+ve	45/ 115	39.13	0.64	0.0001
-ve	70/ 115	60.87		
Clinical Stage TNM				
III + IV	68/ 115	59.13	III + IV v/s II	
			1.65	0.000
II	41/ 115	35.65	III + IV v/s II+ I	
			1.44	0.005*
I	6/ 115	5.20		

*statistically significant.

of blood samples were collected from healthy women (having no family history of cancer) of matched age group. A prior informed consent was obtained from all participants (Table I).

The study was approved and cleared by the Ethics Committee of Jamia Millia Islamia (A Central University) and All India Institute of Medical Sciences.

Genotype Analysis

DNA isolation from Peripheral Blood was done as described by Sambrook et al[55]. Detailed primer information for the determination of the p21 rs1801270, p53 rs1042522 and CCND1 rs603965 SNP genotypes is given in Table II. Briefly, genotyping of rs1801270 and rs603965

Table II. Primer details.

Gene	SNP	Method	Primers	Enzyme
p21	rs1801270	PCR- RFLP	FP-5′-ATGTCCGTCAGAACCCAT-3′ RP-5′-TGGTCTTCCTCTGCTGTC-3′	BlpI
p53	rs1042522	AFLP	p53Pro+/p53-FP 5′-GCC AGA GGC TGC TCC CCC-3′ p53Pro+/p53-RP 5′-CGT GCA AGT CAC AGA CTT-3′ p53+/p53Arg-FP 5′-TCC CCC TTG CCG TC CCA A-3′ p53+/p53Arg-RP 5′-CTG GTG CAG GGG CCA CGC-3′	
CCND1	rs603965	PCR- RFLP	FP 5′-GTGAAGTTCATTTCCAATCCGC-3′ RP 5′-GGGACATCACCCTCACTTAC-3′	ScrFI

Table III. Frequencies of p21 (C93A) SNP alleles and genotypes in control and breast cancer cases.

Total subjects	Case frequency (n= 115)	Control frequency (n= 115)	Odds ratio (Confidence interval 95%)	*p*-value
Allelic Frequency (Total alleles)				
C	0.91 (210)	0.95 (219)	Ref	0.136
A	0.09 (20)	0.05 (11)	1.896 (0.900-3.992)	
Genotype Frequency (Total genotypes)				
CC	0.83 (95)	0.90 (104)	Ref	0.121
CA	0.17 (20)	0.10 (11)	1.990 (0.918-4.308)	
Premenopausal women	**Patient frequency (n= 69)**	**Control frequency (n= 69)**	**Odds ratio (Confidence interval 95%)**	***p*-value**
Allelic Frequency (Total alleles)				
C	0.91 (126)	0.95 (131)	Ref	0.342
A	0.09 (12)	0.05 (7)	1.782 (0.699-4.535)	
Genotype Frequency (Total genotypes)				
CC	0.83 (57)	0.90 (62)	Ref	0.323
CA	0.17 (12)	0.10 (7)	1.865 (0.704-4.921)	
Premenopausal women	**Patient frequency (n= 46)**	**Control frequency (n= 46)**	**Odds ratio (Confidence interval 95%)**	***p*-value**
Allelic Frequency (Total alleles)				
C	0.91 (84)	0.96 (88)	Ref	0.371
A	0.09 (8)	0.04 (4)	2.095 (0.644-6.784)	
Genotype Frequency (Total genotypes)				
CC	0.83 (38)	0.91 (42)	Ref	0.354
CA	0.17 (8)	0.09 (4)	2.211 (0.649-7.463)	

was determined using a PCR-RFLP method while AFLP method was used to determine rs1042522 genotype.

Statistical Analysis

Chi-square test (χ^2) was used to study the association between breast cancer risk and single nu-

cleotide polymorphisms of p21, p53 and CCND1 genes as well as different clinico-pathological variables, if any, using IBM SPSS Statistics. The *p*-value ≤0.05 was considered significant.

Results

Association of p21 Gene rs1801270 SNP Alleles and Genotypes with Breast Cancer Risk

Analysis of SNP93C and SNP93A alleles in total cohort as well as in premenopausal, and postmenopausal cases, when compared with controls, revealed that neither SNP 93C nor SNP93A p21 genotype increases significant breast cancer susceptibility (Table III).

Relationship of p53 Gene rs1042522 SNP Alleles and Genotypes with the Risk of Breast Cancer

Heterozygous arginine variant of rs1042522 SNP is associated with significant protection against developing breast cancer among total cohort, premenopausal and postmenopausal women. ORs for Arg/Pro (G/C) genotype in total, premenopausal and postmenopausal women were 0.172 (95% CI, 0.097-0.307, *p*-value 0.000), 0.329 (95% CI, 0.162-0.665, *p*-value 0.000) and 0.053 (95% CI, 0.018-0.154, *p*-value 0.000), respectively. Further, GC along with CC genetic model showed significant reduction of breast cancer risk in total and postmenopausal women with ORs= 0.327 and 0.162, respectively (Table IV).

Relationship of CCND1 Gene rs603965 (G870A) Polymorphism Alleles and Genotypes with Breast Cancer Risk

AA genotype of CCND1 was found associated significantly with the breast cancer risk in total and premenopausal women with ORs 2.66 and 3.35, respectively. GG and GA genotype were not associated significantly with the breast cancer risk (Table V).

Association of p21 rs1801270 and p53 1042522 Polymorphism in Combination

Analysis of probable genotypic combinations for p21 rs1801270 and p53 1042522 among total cohort, premenopausal and postmenopausal women revealed that p21 CC genotype is associated with significant protective association against developing breast cancer when present with p53 GC and also with GC+ CC genotype.

A possible risk association of p21 CA in combination with p53 GC genotype with the breast cancer among premenopausal women was observed. However, the small sample size prevented the calculation of statistical significance (Table VI).

Association of p21 rs1801270 and CCND1 rs603965 Polymorphism in Combination

Analysis of every probable combination of p21 rs1801270 and CCND1 rs603965 showed significant association of p21 CC: CCND1 AA with breast cancer risk among total cohort as well as premenopausal subjects with ORs 2.44 and 3.46, respectively. CA:AA genotypic combination was not observed among control subjects (Table VII).

Association of p53 rs1042522 and CCND1 rs603965 Polymorphism in Combination

Analysis of probable groups of p53 rs1042522 and CCND1 rs603965 SNPs showed that p53 GG: CCND1 AA elevated significant breast cancer risk among total cohort with ORs 6.93. GG: AA genotype was not observed in any control postmenopausal subject. Further, p53 GC: CCND1 GA and p53 GC: CCND1 GA+AA was found to provide significant protection against developing breast cancer among total cohort with ORs 0.25 and 0.30, respectively (Table VIII).

Discussion

Breast cancer risk is essentially based on the changes in high-risk susceptibility genes, notably BRCA1 and BRCA2[56]. Importantly, linkage studies suggest the absence of any other high-risk genes[57] indicating the involvement of common lower risk alleles in genes including ATM, CHEK2, BRIP1, PALB2 and CASP8[58-64] and further susceptibility loci[65,66]. Present study sought to elucidate the role of common polymorphisms of cell cycle genes in modulation of breast cancer susceptibility.

Our study is in agreement with the NCRP report 2001 showing more than 80% of breast tumors as invasive ductular carcinoma (IDC). Majority of our patients were <50 years with a mean age of 48.03 years, suggesting breast cancer occurrence at an early age compared to the NCRP report, 2001 (50 to 64 years). This is supported further by detection of breast cancer in pre-menopausal women (60%) in our study. Lack of disease

Table IV. Frequencies of p53 (codon 72) SNP alleles and genotypes in control and breast cancer cases.

Total subjects	Case frequency (n= 115)	Control frequency (n= 115)	Odds ratio (Confidence interval 95%)	*p*-value
Allelic Frequency (Total alleles)				
G	0.64 (147)	0.58 (133)	Ref	0.214
C	0.36 (83)	0.42 (97)	0.774 (0.532 - 1.126)	
Genotype Frequency (Total genotypes)				
GG	0.53 (61)	0.27 (31)	Ref	
GC	0.22 (25)	0.62 (71)	0.172 (0.097-0.307)	<0.001*
CC	0.25 (29)	0.11 (13)	1.113 (0.517-2.483)	0.92
GC+ CC	0.47 (54)	0.73 (84)	0.327 (0.189-0.566)	<0.001*
Premenopausal women	**Patient frequency (n= 69)**	**Control frequency (n= 69)**	**Odds ratio (OR) (95%)**	***p*-value**
Allelic Frequency (Total alleles)				
G	0.64 (89)	0.60 (83)	Ref	0.535
C	0.36 (49)	0.40 (55)	0.831 (0.511-1.351)	
Genotype Frequency (Total genotypes)				
GG	0.50 (35)	0.33 (23)	Ref	
GC	0.28 (19)	0.54 (37)	0.329 (0.162-0.665)	0.008*
CC	0.22 (15)	0.13 (9)	1.09 (0.41-2.91)	1
GC+ CC	0.50 (34)	0.67 (46)	0.486 (0.245-0.963)	0.057
Premenopausal women	**Patient frequency (n= 46)**	**Control frequency (n= 46)**	**Odds ratio (OR) (95% CI)**	***p*-value**
Allelic Frequency (Total alleles)				
G	0.63 (58)	0.54 (50)	Ref	0.295
C	0.37 (34)	0.46 (42)	0.698 (0.388-1.255)	
Genotype Frequency (Total genotypes)				
GG	0.57 (26)	0.17 (8)	Ref	
GC	0.13 (6)	0.74 (34)	0.053 (0.018-0.154)	<0.001*
CC	0.30 (14)	0.09 (4)	1.07 (0.27-4.21)	NC
GC+ CC	0.43 (20)	0.83 (38)	0.162 (0.063-0.418)	0.000*

awareness and hesitation to discuss early symptoms among Indian females are likely to result in late diagnosis as nearly 60% of our patients belonged to late stages of the disease. The problem is further compounded by the paucity of efficient diagnostic tools and screening methods for early detection of breast cancer. We observed lymph node involvement in almost 65% of the cases that often leads to poor prognosis. Early reports associate germline and somatic mutations in cell cycle genes with the onset and development of various cancers[8,67].

We analyzed the role of three key actors namely p21, CCND1 and p53 proteins. p21 and CCND1 are associated with cell cycle progression from G1 to S phase enabling DNA replication and growth after senescence by aiding in overcoming of the restriction point (R)[68,69]. Variations in p21 gene disturb the control of cell propagation and increase cancer risk in humans[18,70]. p53 protein on the other hand enforces a variety of anticancer functions and is aptly considered as a molecular policeman[68]. The SNPs in p21, p53 and CCND1 have been associated individually with breast cancer risk[71,72]. The p21 and p53 SNPs may critically reduce the tumor suppressor activity of p53 and allow cell cycle progression even with compromised genomic integrity after genotoxic dam-

age[73,74]. We examined these important genes of cell cycle pathway to elucidate the association of their common alleles with breast cancer susceptibility. Our study shows potential additive associations between the common p21, p53 and CCND1 polymorphisms and susceptibility to developing breast cancer.

The changes in p21 gene are rarely found associated with human cancers[18] despite the importance of p21 in cell cycle regulation. Polymorphisms of p21 gene, which is under transcriptional control of p53, negatively impacts tumor suppressor function of the p53 pathway and critical cellular processes like growth halt and apoptosis in the wake of genomic damage. The C93A SNP of the p21 gene is linked with developing some human cancers including that of colon[75], soft tissue[76], breast[77], prostate[78] and head and neck[79]. Inconsistent reports[80,81,82,83,84,21] regarding association of Arg allele of the p21 codon 31 polymorphisms with human cancers exist in the literature.

Contradictory reports regarding the association of p21 C93A variants with high cancer risk in humans[70] was an impetus for our further study. We found no association of p21 C93A SNP with an elevated risk of developing breast cancer, in

Table V. Frequencies of CCND1 (G870A) alleles and genotypes in control and breast cancer patients.

Total women	Patient frequency (n= 115)	Control frequency (n= 115)	Odds ratio (Confidence interval 95%)	*p*-value
Allelic Frequency (Total alleles)				
G	0.40 (92)	0.53 (122)	Ref	0.007*
A	0.60 (138)	0.47 (108)	1.694 (1.171-2.451)	
Genotype Frequency (Total genotypes)				
GG	0.20 (23)	0.24 (28)	Ref	
GA	0.40 (46)	0.58 (66)	0.84 (0.43-1.65)	0.75
AA	0.40 (46)	0.18 (21)	2.66 (1.25-5.67)	0.017*
GA+ AA	0.80 (92)	0.76 (87)	1.287 (0.693-2.392)	0. 52
Premenopausal women	**Patient frequency (n= 69)**	**Case Control frequency (n= 69)**	**Odds ratio (OR) (95%)**	***p*-value**
Allelic Frequency (Total alleles)				
G	0.36 (50)	0.52 (72)	Ref	0.011*
A	0.64 (88)	0.48 (66)	1.920 (1.187-3.104)	
Genotype Frequency (Total genotypes)				
GG	0.16 (11)	0.23 (16)	Ref	
GA	0.41 (28)	0.58 (40)	1.01 (0.41-2.52)	0.84
AA	0.43 (30)	0.19 (13)	3.35 (1.22-9.18)	0.031*
GA+ AA	0.84 (58)	0.77 (53)	1.59 (0.687-3.682)	0.391
Premenopausal women	**Patient frequency (n= 46)**	**Case Control frequency (n= 46)**	**Odds ratio (OR) (95% CI)**	***p*-value**
Allelic Frequency (Total alleles)				
G	0.46 (42)	0.54 (50)	Ref	0.302
A	0.54 (50)	0.46 (42)	1.417	
Genotype Frequency (Total genotypes)				
GG	0.26 (12)	0.26 (12)	Ref	
GA	0.39 (18)	0.57 (26)	0.69 (0.25-1.88)	0.63
AA	0.35 (16)	0.17 (8)	2.00 (0.62-6.42)	0.38
GA+ AA	0.74 (34)	0.74 (34)	1.00 (0.400-2.501)	0.80

Table VI. Combined genotypic frequencies of p21 rs1801270 and p53 1042522 among breast cancer cases and controls in Indian females.

Total cohort	Case frequency (n= 115)	Control frequency (n= 115)	Odds ratio (CI 95%)	*p*-value
CC:GG	0.45 (52)	0.21 (24)	Ref	
CC:GC	0.16 (19)	0.58 (67)	0.13 (0.06 – 0.26)	<0.0001*
CC:CC	0.21 (24)	0.11 (13)	0.85 (0.37 – 1.95)	0.86
CC:GC+CC	0.37 (43)	0.70 (80)	0.24 (0.13 – 0.45)	<0.0001*
CA:GG	0.08 (9)	0.06 (7)	0.59 (0.19 – 1.78)	0.51
CA:GC	0.05 (6)	0.03 (4)	0.69 (0.17 – 2.68)	NC
CA:CC	0.04 (5)	- (0)	NC	NC
CA:GC+CC	0.10 (11)	0.03 (4)	1.26 (0.36 – 4.39)	NC
Premenopausal women	**Case frequency (n= 69)**	**Control frequency (n= 69)**	**Odds ratio (OR) (CI 95%)**	***p*-value**
CC:GG	0.42 (29)	0.25 (17)	Ref	
CC:GC	0.22 (15)	0.54 (37)	0.240 (0.115 – 0.502)	0.001*
CC:CC	0.19 (13)	0.12 (8)	0.95 (0.32 – 2.76)	0.86
CC:GC+CC	0.40 (28)	0.65 (45)	0.36 (0.183 – 0.78)	0.014*
CA:GG	0.09 (6)	0.09 (6)	0.58 (0.16 – 2.11)	NC
CA:GC	0.06 (4)	0.01 (1)	2.34 (0.24 – 22.73)	NC
CA:CC	0.03 (2)	- (0)	- - - -	- -
CA:GC+CC	0.09 (6)	0.01 (1)	3.51 (0.38 – 31.74)	NC
Premenopausal	**Case frequency (n= 46)**	**Control frequency (n= 46)**	**Odds ratio (OR) (95% CI)**	***p*-value**
CC:GG	0.5 (23)	0.15 (7)	Ref	
CC:GC	0.09 (4)	0.65 (30)	0.04 (0.01-0.15)	<0.0001*
CC:CC	0.24 (11)	0.11 (5)	0.66 (0.17-2.59)	NC
CC:GC+CC	0.33 (15)	0.76 (35)	0.13 (0.04-0.36)	0.000*
CA:GG	0.06 (3)	0.02 (1)	0.91 (0.08-10.22)	NC
CA:GC	0.04 (2)	0.06 (3)	0.20 (0.02-1.46)	NC
CA:CC	0.06 (3)	- (0)	- - - -	- -
CA: GC+CC	0.11 (5)	0.06 (3)	0.50 (0.09-2.67)	NC

accordance with an early study conducted using cases of invasive breast carcinomas[23] and primary breast cancer[85]. However, frequent observation of Arg allele in breast cancer cases than controls among Indian females is noteworthy and needs to be studied. Alterations in p53 gene, including rs1042522 Arg72Pro polymorphism of exon 4, are the most commonly associated mutations found in human cancers. The variants differ in biochemical properties, transcription control modulation, signaling DNA repair or apoptosis thereby reducing genomic instability and suppressing uncontrolled cellular proliferation[86-89]. Frequent observation of Arg or Pro alleles in cases of breast cancer is reported by some studies while others have shown no preferential retention of these alleles[90-93]. Similarly, studies reporting the association of rs1042522 SNP with breast cancer risk have remained discordant[80,94,95]. Conflicting reports from Indian population showing an association of Arg72 variant with the cancer of oral cavity[96] and Pro72 variant with urinary bladder[97] and breast cancer[98] exist in the literature. This discrepancy might be because of reports from different ethnicities, for example, Syeed et al[98] studied ethnically diverse Kashmiri population. Furthermore, gene environment interactions might also have modified the effect of TSP53 variants. We observed p53 heterozygous arginine variant as protective against developing breast cancer in all cases. These findings are in agreement with early studies showing that codon 72 Arginine has a protective effect owing to an increased apoptotic potential induced by G allele[87,99-101]. Therefore, G allele possibly serves as a risk allele for breast cancer development in Indian ethnicity. Although

G/G homozygous genotype have been shown to elevate breast cancer risk in Caucasians and Turk ethnicities[90,95], few studies conducted in Japanese populations have shown the association of C/C genotype with an increased risk of breast cancer in ER-positive postmenopausal women[101,102].

CCND1 play a critical role in breast cancer etiology. G870A (rs603965) SNP leads to the formation of Cyclin D1 transcript-b harboring a PEST-rich region by modulating the splicing at the exon 4-intron 4 boundaries. The alternate transcript bestows a longer half-life of CCND1 and can also evade the G1/S- checkpoint of cell cycle[45]. Multiple molecular epidemiological reports regarding the association of rs603965 with breast cancer susceptibility return inconclusive findings[103]. However, involvement of CCND1 is strongly suggested by the over expression of CCND1 gene found in almost 20% of breast cancer and 50% of mammary tumors[104,105], that seems like an early event in breast cancer formation[106]. Our study found association of A/A genotype of rs603965 SNP with increased risk of developing breast cancer in total cohort and premenopausal cases. Our findings corroborate with many early reports suggesting the relationship of this SNP with susceptibility to developing cancers of urinary bladder[47], esophagus and stomach[48], prostate[107], head and neck[50], colon[51] and cervix[108]. Several reports from India also suggest a link between rs603965 SNP and an elevated risk for developing cancers of cervix[109], prostate[110], urinary bladder[111] and esophagus[112].

Multiple reports have shown the association of polymorphic variants of p21 at codon 31,

Table VII. Combined genotype frequency of p21 rs1801270 and CCND1 rs603965 among breast cancer cases and controls.

Total cohort	Case frequency (n= 115)	Control frequency (n= 115)	Odds ratio (CI 95%)	*p*-value
CC:GG	0.16 (19)	0.22 (25)	Ref	
CC:GA	0.32 (37)	0.50 (58)	0.83 (0.40 – 1.73)	0.777
CC:AA	0.34 (39)	0.18 (21)	2.44 (1.09 – 5.42)	0.044*
CC:GA+AA	0.66 (76)	0.69 (79)	1.26 (0.64 – 2.48)	0.61
CA:GG	0.03 (4)	0.0.03 (3)	1.75 (0.35 – 8.78)	NC
CA:GA	0.08 (9)	0.07 (8)	1.48 (0.48 – 4.55)	0.68
CA:AA	0.06 (7)	- (0)	NC	NC
CA:GA+AA	0.14 (16)	0.07 (8)	2.63 (0.93 – 7.42)	0.11
Premenopausal women	**Case frequency (n= 69)**	**Control frequency (n= 69)**	**Odds ratio (OR) (CI 95%)**	***p*-value**
CC:GG	0.12 (8)	0.22 (15)	Ref	
CC:GA	0.36 (25)	0.49 (34)	1.37 (0.50 – 3.75)	0.70
CC:AA	0.35 (24)	0.19 (13)	3.46 (1.16 – 10.31)	0.044*
CC:GA+AA	0.71 (49)	0.68 (47)	1.95 (0.75 – 5.03)	0.24
CA:GG	0.04 (3)	0.01 (1)	5.62 (0.5 – 63.28)	NC
CA:GA	0.04 (3)	0.09 (6)	0.93 (0.18 – 4.78)	NC
CA:AA	0.09 (6)	- (0)	NC	NC
CA:GA+AA	0.13 (9)	0.09 (6)	2.81 (0.73 – 10.77)	0.23
Premenopausal	**Case frequency (n= 46)**	**Control frequency (n= 46)**	**Odds ratio (OR) (95% CI)**	***p*-value**
CC:GG	0.24 (11)	0.22 (10)	Ref	
CC:GA	0.26 (12)	0.52 (24)	0.45 (0.15 – 1.36)	0.25
CC:AA	0.33 (15)	0.17 (8)	1.70 (0.50 – 5.72)	0.57
CC:GA+AA	0.59 (27)	0.69 (32)	0.76 (0.28 – 2.08)	0.79
CA:GG	0.02 (1)	0.04 (2)	0.45 (0.03 – 5.81)	NC
CA:GA	0.13 (6)	0.04 (2)	2.72 (0.44 – 16.74)	NC
CA:AA	0.02 (1)	- (0)	NC	NC
CA:GA+AA	0.15 (7)	0.04 (2)	3.18 (0.53 – 19.05)	NC
CA: GC+CC	0.11 (5)	0.06 (3)	0.50 (0.09-2.67)	NC

Table VIII. Combinational genotypic frequencies of p53 rs1042522 and CCND1 rs603965 among breast cancer cases and controls.

Total cohort	Case frequency (n= 115)	Control frequency (n= 115)	Odds ratio (CI 95%)	*p*-value
GG:GG	0.13 (15)	0.11 (13)	Ref	
GG:GA	0.19 (22)	0.13 (15)	1.27 (0.47 – 3.42)	0.82
GG:AA	0.21 (24)	0.03 (3)	6.93 (1.69 – 28.44)	0.009*
GG:GA+AA	0.40 (46)	0.16 (18)	2.21 (0.88 – 5.56)	0.141
GC:GG	0.03 (4)	0.10 (12)	0.28 (0.07 – 1.11)	0.127
GC:GA	0.12 (14)	0.41 (47)	0.25 (0.09 – 0.66)	0.008*
GC:AA	0.06 (7)	0.10 (12)	0.50 (0.15 – 1.66)	0.406
GC:GA+AA	0.18 (21)	0.51 (59)	0.30 (0.12 – 0.75)	0.016*
CC:GG	0.03 (4)	0.03 (3)	1.15 (0.21 – 6.14)	NC
CC:GA	0.09 (10)	0.03 (4)	2.16 (0.54 – 8.58)	0.43
CC:AA	0.13 (15)	0.05 (6)	2.16 (0.65 – 7.21)	0.329
CC:GA+AA	0.22 (25)	0.08 (10)	2.16 (0.76 – 6.15)	0.23
Premenopausal	**Case frequency (n= 69)**	**Control frequency (n= 69)**	**Odds ratio (OR) (CI 95%)**	***p*-value**
GG:GG	0.12 (8)	0.12 (8)	Ref	
GG:GA	0.19 (13)	0.17 (12)	1.08 (0.30-3.80)	0.84
GG:AA	0.20 (14)	0.04 (3)	4.66 (0.95-22.79)	0.10
GG:GA+AA	0.39 (27)	0.22 (15)	1.8 (0.56-5.77)	0.48
GC:GG	0.04 (3)	0.10 (7)	0.42 (0.08-2.27)	NC
GC:GA	0.15 (10)	0.36 (25)	0.4 (0.11-1.36)	0.24
GC:AA	0.09 (6)	0.09 (6)	1.00 (0.22- 4.46)	0.69
GC:GA+AA	0.24 (16)	0.45 (31)	0.51 (0.16-1.63)	0.40
CC:GG	- (0)	0.01 (1)	NC	NC
CC:GA	0.07 (5)	0.04 (3)	1.66 (0.29-9.44)	NC
CC:AA	0.15 (10)	0.06 (4)	2.5 (0.54-11.41)	0.40
CC:GG+AA	0.22 (15)	0.10 (7)	2.14 (0.50-8.09)	0.42
Premenopausal	**Case frequency (n= 46)**	**Control frequency (n= 46)**	**Odds ratio (OR) (95% CI)**	***p*-value**
GG:GG	0.15 (7)	0.15 (5)	Ref	
GG:GA	0.20 (9)	0.06 (3)	2.14 (0.37-12.19)	NC
GG:AA	0.22 (10)	- (0)	NC	NC
GG:GA+AA	0.41 (19)	0.06 (3)	4.52 (0.84-24.11)	NC
GC:GG	0.02 (1)	0.11 (5)	0.14 (0.01-1.63)	NC
GC:GA	0.09 (4)	0.48 (22)	0.12 (0.02-0.62)	NC
GC:AA	0.02 (1)	0.13 (6)	0.11 (0.01-1.32)	NC
GC:GA+AA	0.11 (5)	0.61(28)	0.12 (0.02-0.56)	NC
CC:GG	0.09 (4)	0.04 (2)	1.42 (0.18-11.08)	NC
CC:GA	0.11 (5)	0.02 (1)	3.57 (0.31-40.77)	NC
CC:AA	0.11(5)	0.04 (2)	1.78 (0.24-13.21)	NC
CC:GA+AA	0.22 (10)	0.06 (3)	2.38 (0.42-13.38)	NC

and p53 at codon 72, with an increased cancer risk. However, limited studies have explored their cumulative effect on susceptibility of developing breast cancer. We investigated every probable genotypic combination of p21 and p53 variants in total cohort as well as in premenopausal and postmenopausal cases to elucidate any possible interaction between p21 codon 31 and p53 codon 72 polymorphisms modulating the breast cancer risk. A protective association of CC genotype of p21 SNP (rs1801270) and GC genotype of p53 SNP (rs1042522) detected in total cohort of cases including premenopausal and postmenopausal females underlines the importance of combinatorial analysis as CC or CA genotype of p21 gene showed no associa-

tion with susceptibility to breast cancer risk when studied alone. Our findings contrast a recent report probing the role of same SNPs of p53 and p21 gene in breast cancer risk among a German population[113]. Nevertheless, another study[114] showed that combinations of p21 and p53 SNPs are strongly associated with elevated susceptibility to developing cervical cancer.

On further analysis we found that p21 CC genotype along with GC and CC genotype of p53 gene was strongly associated with decreased susceptibility to breast cancer across the whole cohort of premenopausal and postmenopausal women. Contrary, CC genotype of p21 SNP (rs1801270) along with AA genotype of CCND1 SNP (rs603965) was found to increase risk of developing breast cancer risk, significantly. Similarly, GG genotype of p53 SNP (rs1042522) in combination with AA genotype of CCND1 SNP (rs603965) increased risk of developing breast cancer among total cohort and premenopausal females. However, GC genotype of p53 gene in combination of GA and GA+AA genotype of CCND1 gene were found to decrease susceptibility to breast cancer in total cohort.

Gene mutations having different levels of penetrance can increase the disease susceptibility greatly. Studies have shown that mutation of high-penetrance susceptibility gene (like BRCA1, BRCA2, p53, CDH1, PTEN) increases cancer risk significantly[9,13]. Although mutations/changes located in low penetrance genes contribute less to disease susceptibility, however, they may contribute significantly to the risk in combination with other low or high penetrance genes[72,74]. Identification of low- and moderate- penetrance gene mutations is equally important for cancer prevention, surveillance, and management.

Presence of additional common epigenomic changes, intra-tumor heterogeneity in a polygenic and multifactorial disease like cancer makes it imperative to assess risk factors in a holistic manner despite their apparently low influence on susceptibility[14]. Our study revealed the increased risk for developing breast cancer associated with gene variants with low, moderate and high susceptibility effects by analyzing multiplicative gene-gene interactions. These findings may prime the development of wide-ranging polygenic breast cancer risk models by including gene–environment interactions. Future analysis may elucidate the relative modifications in susceptibility associated with heterogeneity present in a particular ethnicity and help in developing preventative or therapeutic measures among people at high-risk.

Conclusions

Alterations in cell- cycle genes and genes involved in apoptosis pathway significantly contribute to onset and progression of breast tumorigenesis. Combinatorial analyses of SNPs present in p21, p53 and CCND1 genes namely rs1801270, rs1042522 and rs603965, respectively, revealed significant association with susceptibility to developing breast cancer. Current findings elucidate the combined role of low-penetrance mutant alleles of key cell cycle and tumor suppressor genes in elevated risk of breast cancer. However, further case-control studies considering gene-environment interactions conducted in diverse ethnicities will confirm putative associations between currently studied polymorphisms and breast cancer risk.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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