EGYPTIAN ACADEMIC JOURNAL OF

BIOLOGICAL SCIENCES

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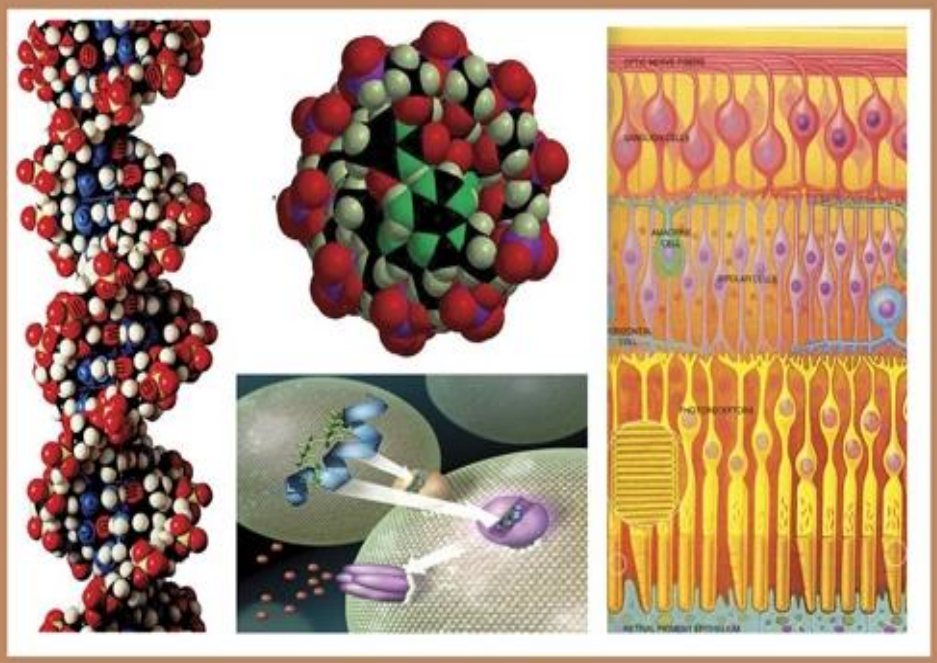


ISSN
2090-0767

WWW.EAJBS.EG.NET

Vol. 12 No. 2 (2020)

Citation: *Egypt.Acad.J.Biolog.Sci. (C.Physiology and Molecular biology) Vol. 12(2) pp157-167(2020)*

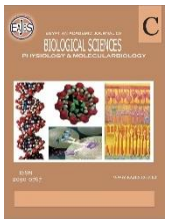
Egypt. Acad. J. Biolog. Sci., 12(2):157-167(2020)

Egyptian Academic Journal of Biological Sciences

C. Physiology & Molecular Biology

ISSN 2090-0767

www.eajbsc.journals.ekb.eg



Association of CCND1 rs603965 Polymorphism with Breast Cancer Risk in North Indian Population

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ARTICLE INFO

Article History

Received:19/10/2020

Accepted:16/12/2020

Keywords:

CCND1; G/A 870 (rs603965); breast cancer; gene polymorphism

ABSTRACT

The potential association of G870A SNP (rs603965) of cyclin D1 gene (CCND1) with susceptibility to breast cancer in the north Indian population was investigated. The study included 230 subjects comprising 115 breast cancer cases and an even number of controls. PCR-RFLP employed for genotyping revealed that the females carrying AA genotype have 2.66- fold increased risk for development of breast cancer in the total cohort (OR 2.66, 95% CI 1.25– 5.67; P= 0.017) compared to GG genotype. Further, a significantly higher risk was also observed in premenopausal women (OR 3.35, 95% CI 1.22– 9.18; P= 0.0310). However, GG and GA genotype are not found associated significantly with breast cancer risk. The results suggest that CCND1 G870A polymorphism influences the genetic susceptibility to breast cancer. Larger studies conducted in diverse populations are needed to ascertain the contribution of these risk alleles to breast cancer risk.

INTRODUCTION

Cyclin D1 (or CCND1) gene encodes the regulatory subunit of a holoenzyme that phosphorylates and inactivates the retinoblastoma protein and promotes progression through the G_1-S phase of the cell cycle (Malumbres and Barbacid 2001; Sherr 1996). Of the three D-type cyclins, each of which binds cyclin-dependent kinase (CDK), cyclin D1 overexpression is related to tumorigenesis and cellular metastases in humans (Zhou *et al.*, 1996). Cyclin D1 exerts as a CDK-dependent as well as CDK-independent regulator of the cell cycle (Radu *et al.*, 2003). Cyclin D1 in association with CDK4 regulates the G1/S phase progression by phosphorylating and inactivating the RB protein in turn releasing the transcription factor E2F from RB protein and an eventual DNA synthesis (Radu et al., 2003). However, Cyclin D1 overexpression may lead to the early phosphorylation of RB protein, uncontrolled cell growth and tumorigenesis (Huynh *et al.*, 2004). CCND1 gene is over-amplified in almost 20% of breast cancer and in more than 50% of mammary tumors (Sutherland and Musgrove 2002), supposedly an early event in breast cancer formation (Li *et al.*, 2016).

Five exons of CCND1 encode two major isoforms, cyclin D1a and D1b after alternative splicing. The majority of cyclin D1a isoform mRNA, 4.5 kilobases (kb) in length, with a coding region of only 882 bp, consists of 3'UTR sequences and harbors mRNA destabilizing elements.

The cyclin D1b isoform lacks exon 5 but retains intron 4, which contains a translation stop codon after 99 bp and a polyadenylation signal less than 300 bp 3' from this stop codon. The 1.7-kb cyclin D1b mRNA is found in most tumors and cell lines that express cyclin D1 and encodes a 274 amino acid protein that differs at the C terminus from the 294 amino acid protein encoded by the cyclin D1a mRNA (Betticher *et al.,* 1995; Knudsen *et al.,* 2006). In contrast to cyclin D1a, cyclin D1b is potently transforming in experimental models (Solomon *et al.,* 2003). Of several single nucleotide polymorphisms (SNPs), G870A (rs603965), T223C (rs3862792), G52C (rs2930976) in CCND1 gene, G870A is functional and therefore extensively studied. The relative abundance of the cyclin D1b isoform is affected by G/A single nucleotide polymorphism at the last base of exon 4 (position 870, codon 241), which is the –1 position of the intron 4 splice donor consensus (Knudsen et al., 2006). The G allele is thought to favor correct splicing, whereas the A allele is thought to impair splicing and thereby increase the relative expression of cyclin D1b isoform (Knudsen et al., 2006). In healthy individuals, the G allele is slightly more common, with reported allele frequencies of 51% to 61% (Hosokawa and Arnold 1998). Homozygosity for the A allele is frequently associated with an increased risk of cancer and at times with more aggressive disease.

The alternate transcript produced by the 870A allele lacks exon 5 of CCND1 and contains a PEST-rich region postulated to the target protein for rapid degradation, which is more stable compared to the product of the 870G allele. It has been shown that transcript-b leads to a longer half-life of CCND1, which may bypass the G1/S-checkpoint (Betticher et al., 1995). Several molecular epidemiological studies have been conducted to examine the association between CCND1 G870A polymorphism and breast cancer risk (Ceschi *et al.,* 2005; Forsti *et al.,* 2004; Grieu *et al.,* 2003; Krippl *et al.,* 2003; Onay *et al.,* 2008; Shu *et al.,* 2005; Yu *et al.,* 2008), but the results remain inconsistent. To estimate the overall risk of CCND1 G870A polymorphism associated with breast cancer, a case-control study comprised of 115 breast cancer cases and an equal number of controls was conducted.

MATERAILS AND METHODS

Biological Specimens:

One hundred and fifteen (115) blood samples of Breast Cancer patients were collected from the OT of BRA- Institute Rotary Cancer Hospital (BRA-IRCH), AIIMS, New Delhi. One hundred and fifteen (115) blood samples from unrelated normal healthy women of the same age group and without a family history of cancer were taken as controls for polymorphic studies. Blood was collected directly into BD Vacutainer tube. These controls were recruited from medical indoor patients who were undergoing treatment for conditions such as diabetes, hypertension, etc. Cases of breast cancer were classified and graded according to the World Health Organization (WHO) criteria and staged according to criteria of the International Federation of Gynecolology and Obstetrics (FIGO) (Devitt 1967). The blood sample collected did not compromise the availability of biological material for routine pathology and other tests performed as part of patient care. Prior informed consent was obtained from all subjects. The study was approved and cleared by the Ethics Committee of Jamia Millia Islamia (A Central University), New Delhi, and All India Institute of Medical Sciences, New Delhi, India.

DNA isolation from Peripheral Blood:

High molecular weight DNA was extracted from blood samples from breast cancer patients and unrelated normal healthy women as described previously (Joseph Sambrook 2001). Briefly, contents of BD Vacutainer tube were transferred into a 50 ml polypropylene conical centrifuge tube and volume was made up to 50 ml with RBC Lysis Buffer. Cells were pellet by centrifuging at 600 x g (approx 1,400 rpm) for 10 minutes in a 4° C centrifuge after

incubation for 10 to 15 minutes at room temperature or for 20 to 30 minutes at 4° C. After carefully decanting the supernatant, 6 ml of Nucleic Acid Lysis Buffer was added and gently vortexed. Subsequently, 300 ul of Proteinase-K was added and pulse vortexed until the pellet was loose and dissociated and the sample was then incubated at 37° C for 48 hours. Afterwards, 2.5 ml of saturated NaCl solution was added and gently mixed and the sample was left at 4° C for 10 minutes before it was centrifuged to pellet the precipitated protein at 4° C for 10 minutes at 1000 X g. The obtained supernatant was added to a tube containing 20 ml of ice-cold 100% ethanol (EtOH) in a 50 ml tube. The sample was allowed to stand upright at room temperature until the emergence of the precipitated DNA. A clean plastic disposable bacterial loop was used to lift the precipitated DNA from the 50 ml tube and transferred to the side of a 1.8 ml microcentrifuge tube. The DNA pellet was washed with 1 ml of ice-cold 70% EtOH by centrifuging it for 2 minutes at 10,000 X g. The supernatant was carefully removed and the pellet was air-dried for 10 to 15 minutes before adding TE buffer to the pellet.

Genotyping of CCND1 polymorphism by PCR-RFLP:

PCR-restriction fragment length polymorphism (PCR-RFLP) approach was employed to genotype the G870A locus (Wang *et al.,* 2002). Briefly, the 167 bp fragment encompassing G to A polymorphic site in the CCND1 exon 4 terminal region using specific primers namely CCND1 exon 4 FP 5′-GTGAAGTTCATTTCCAATCCGC-3′ and CCND1 intron 4 RP 5′-GGGACATCACCCTCACTTAC -3′ was amplified. PCR reactions were carried out in a 25 ul volume containing ~20 ng of genomic DNA, 1x PCR buffer, 0.3 mM of each dNTP (dATP, dCTP, dGTP and dTTP), 1.5mM MgCl2, 50 pmol of each primer and 1.0 unit of Taq DNA polymerase. After confirmation of successful PCR amplification by 2% agarose gel electrophoresis, each PCR product was digested overnight with 2 units of ScrFI enzyme (New England Biolabs Inc., Beverly, MA, USA) at 37°C and was electrophoresed on 10% native polyacrylamide gel. The 167 bp PCR fragment was digested into 145 bp and 22 bp fragments when the ScrFI site was present. The genotype was designated as G or A when the ScrFI restriction site was present or absent, respectively.

Statistical Analysis:

The data were tabulated and analyzed using SPSS software. The mean ±SD was calculated for different groups. Two-way analysis of variance was employed to test for the difference in mean values. Student t-test was employed to compare the mean difference wherever appropriate. A simple correlation coefficient was estimated to quantify the relationship between clinicopathological variables and the status of CCND1 polymorphism.

RESULTS

Clinicopathologic Attributes:

Various clinicopathologic variables like basic demographics and tumor characteristics recorded are represented in table 1. Clinical staging of the tumor was done according to AJCC (American Joint Committee on Cancer) which showed that 6 cases of stage I, 41 of stage II, 64 cases of stage III and 4 were of stage IV. The majority of the patients (55.65%) were in clinical stage III**.**

Genotyping of CCND1 Polymorphism by PCR-RFLP:

PCR-restriction fragment length polymorphism (PCR-RFLP) approach was employed to genotype the G870A locus using specific primers (Wang *et al.,* 2002) (Fig 1).

Table 1. Clinicopathologic attributes

Clinicopathological variables	No. of Patients	Percentage (%)
Age Distribution 25-77 years, average 35- 50 years	115	
Age		
< 50	77/ 115	66. 95
> 50	38/ 115	33
Menstrual status		
Pre- Menopausal	69/ 115	60
Post- Menopausal	46/ 115	40
Nodal status		
Positive	75/ 115	65. 2
Negative	40/ 115	34. 78
Histological grading		
PD	62/ 115	53. 9
MD	37/ 115	32. 17
WD	16/ 115	13. 9
Histological status		
Invasive Ductular Carcinoma (IDC)	107/ 115	93
Invasive Lobular Carcinoma (ILC)	8/ 115	7
Tumor Size		
pT1 (<2)	6/ 115	5. 2
pT2 (<5)	41/ 115	35. 65
pT3 (<15)	68/ 115	59. 13
Estrogen Receptor (ER) status		
+ve	42/ 115	36.52
-ve	73/ 115	63.48
Progesterone Receptor (PR) status		
+ve	45/ 115	39.13
-ve	70/ 115	60.87
Clinical Stage TNM		
I	6/ 115	5. 20
II	41/ 115	35. 65
III + IV	68/ 115	59. 13

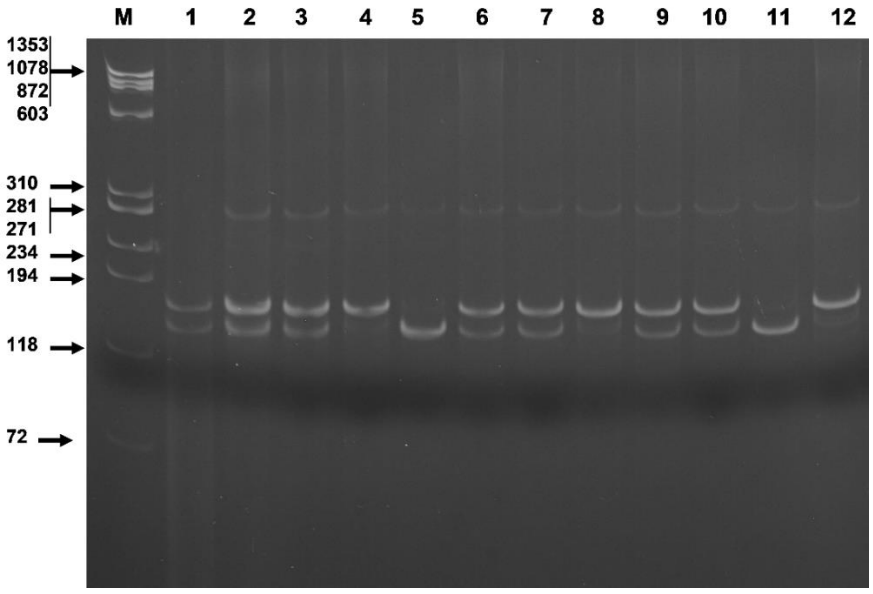

Fig 1 Restriction fragment length polymorphism analysis of the CCND1 G870A genotypes in Breast cancer. Ethidium bromide-stained 10% native PAGE gel showing RFLP analysis of CCND1 G870A genotypes in Breast cancer patient samples. M is Φx174 /*Hae* III-digested molecular weight marker. Lane 4, 8 and 12- Homozygous GG; Lane 1, 2, 3, 6, 7, 9, 10- Heterozygous GA; Lane 5 and 11- Homozygous AA

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

AA genotype of CCND1 is associated significantly with the breast cancer risk in total and premenopausal women with ORs 2.66 and 3.35, respectively. However, GG and GA genotype are not associated significantly with breast cancer risk (Table 2).

Table 2. Allelic and genotypic frequencies of CCND1 (G870A) gene polymorphism in case control and breast cancer patients

Total women	**Patient frequency (n= 115)**	**Control frequency (n= 115)**	**Odds ratio (Confidence interval 95%)**	**p- value**
Allele Frequency (Total number of alleles)				
G	0.40 (92)	0.53 (122)	Ref	0.007*
A	0.60 (138)	0.47 (108)	1.694 (1.171 – 2.451)	
Genotypic Frequency (Total number of 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% CI)**	**p- value**
Allele Frequency (Total number of alleles)				
G	0.36 (50)	0.52 (72)	Ref	0.011*
A	0.64 (88)	0.48 (66)	1.920 (1.187 – 3.104)	
Genotypic Frequency (Total number of 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
Postmenopausal Women	**Patient Frequency (n= 46)**	**Case control Frequency (n= 46)**	**Odds ratio (OR) (95% CI)**	**p- value**
Allele Frequency (Total number of alleles)				
G	0.46 (42)	0.54 (50)	Ref	0.302
A	0.54 (50)	0.46 (42)	1.417	
Genotypic Frequency (Total number of 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

DISCUSSION

Cancer caused almost 17% of the total deaths globally, in 2018 (Siegel *et al.*, 2018). The most common cancer types in men include lung, prostate, colorectal, stomach and liver. Female cancer predominantly included breast, colorectal, lung, cervical and thyroid cancer. The cancer incidence is growing globally, however, the impact is largest in developing countries owing to poor

diagnostic and treatment facilities resulting in almost 70% of the cancer deaths in low- and middle-income countries, in 2008 (Ferlay *et al.,* 2010). Globally, breast cancer is by far the most frequent cancer among women with an estimated 1.38 million new cancer cases diagnosed in 2008 (23% of all cancers) and ranks second overall (10.9% of all cancers). It is now the most common cancer both in developed and developing regions with around 690,000 new cases estimated in each region (population ratio 1: 4) (Ferlay *et al.,* 2010). There are an estimated 4.4 million women alive who have had breast cancer diagnosed within the last five years (compared with just 1.4 million survivors- males or females- from lung cancer). By an estimate, 1.5% of the US female population is survivors of breast cancer (Hewitt *et al.,* 1999).

The development of breast cancer has been associated with several well-recognized clinico- epidemiological risk factors which include advancing age (over 45 years of age), obesity and lack of physical activity, late menopause, early menarche, diet, alcohol, folate intake, smoking, benign breast diseases, oral contraceptives, radiation and hormone replacement therapy (HRT) (Sun *et al.,* 2017). In the present study, the age of patients ranged from 25- 77 years with a mean age of 48.03 years and the majority of cases were in the age group of <50 years which is near to the lower limit of the susceptible age group (50- 64 years) According to menstrual status, in our study, breast cancer was found more in pre-menopausal women (60%). Lack of awareness about the disease and the hesitation to discuss the same leads to the late diagnosis of the disease. This is evident in the study as ~60% of patients belonged to clinical stage III or IV. Another contributing factor is a paucity of high-quality diagnostic tools and screening methods for the early detection of breast cancer. Lymph node positivity in ~65% of the cases observed suggest late diagnosis and probably poor prognosis of the disease.

Largely, breast cancer risk is explained by high-risk susceptibility genes, notably BRCA1 and BRCA2 (Yoshida and Miki 2004). Linkage studies suggest that other high-risk genes are unlikely to exist (Antoniou and Easton 2006) and most of the aggregation is likely to be due to common lower risk alleles. Lower risk susceptibility alleles in ATM, CHEK2, BRIP1, PALB2 and CASP8 (2004; Canzian *et al.,* 2010; Erkko *et al.,* 2007; Meijers-Heijboer *et al.,* 2002; Rahman *et al.,* 2007; Seal *et al.,* 2006; Thompson *et al.,* 2004; Turnbull *et al.,* 2012) have been identified, and new susceptibility loci have been detected through genome-wide studies (Michailidou *et al.,* 2017; Shan *et al.,* 2012; Siddiq *et al.,* 2012; Zhang *et al.,* 2014). Therefore, present study was undertaken to ascertain whether common polymorphisms in the cell cycle genes are associated with breast cancer risk.

Since the cell cycle governs the proliferation and growth of cells, germ-line alterations in the cell cycle genes and their products could predispose to tumors (Golias *et al.,* 2004). The cell cycle pathway has been extensively reviewed (Malumbres and Barbacid 2001). Germ-line and somatic mutations within the genes (Lukas *et al.,* 1997; Zheng *et al.,* 2001) and changes in expression of their protein products (Geradts and Ingram 2000; Woloschak *et al.,* 1996) have been reported in several cancers. The cell cycle can be broken down into distinct phases (Howard and Pelc 1951). This study focuses on CCND1 involved in the G1 to S transition. This protein is involved in surmounting the 'restriction point' (R): a critical period within the cell cycle at which cells shift from senescence to becoming committed to DNA replication and cell growth (Pardee 1989).

In exon 4, CCND1 harbors a silent G to A substitution at nt870 (rs603965). Betticher *et al.,* in 1995 (Betticher *et al.,* 1995) demonstrated that CCND1 G870A produces an alternative protein, transcript-b, which has been suggested to have a longer half-life than transcript- a. Several studies also reported that transcript-b is a poor catalyst of RB phosphorylation or inactivation and markedly enhances cell transformation activity compared to transcript-a (Solomon *et al.,*

2003). The G870A polymorphism has been studied as a risk factor in various cancers such as urinary bladder cancer (Wang *et al.,* 2002), esophagus and gastric cancer (Zhang *et al.,* 2003), prostate cancer (Wang *et al.,* 2003), squamous cell carcinoma of the head and neck (Zheng *et al.,* 2001), colorectal cancer (Lewis *et al.,* 2003) and cervical cancer (Ni *et al.,* 2011). Few reports from India demonstrate an association between 870AA genotype and an increased risk for the development of cervical (Thakur *et al.,* 2009), prostate (Mandal and Mittal 2012), urinary bladder (Gangwar and Mittal 2010) and esophageal (Hussain *et al.,* 2011) cancer.

Over-expression of CCND1 may disrupt normal cell cycle control, and subsequently promote the development of human cancers (Zhou *et al.,* 1996). CCND1 amplification and overexpression have been frequently found in breast cancer (Barnes and Gillett 1998; Chung *et al.,* 2014; Elayat *et al.,* 2011; Lundgren *et al.,* 2008), suggesting that CCND1 may play an important role in the etiology of breast cancer.

Several molecular epidemiological studies have been conducted to examine the association between CCND1 G870A polymorphism and breast cancer risk (Akhter *et al.,* 2019; Ceschi *et al.,* 2005; Lu *et al.,* 2009; Thakur *et al.,* 2018; Yu *et al.,* 2008; Zhang *et al.,* 2016), but the results remain inconsistent. In the present study on the association between CCND1 G870A and risk of breast cancer, A/A genotype in CCND1 found associated significantly with the breast cancer risk in total and premenopausal women with ORs 2.66 and 3.35, respectively, which is in corroboration with several studies showing that CCND1 870A was associated with increased risk of many other types of cancers, such as urinary bladder cancer (Wang et al., 2002), esophagus and gastric cancer (Zhang *et al.,* 2003), prostate cancer (Wang *et al.,* 2003), squamous cell carcinoma of the head and neck (Zheng et al., 2001), colorectal cancer (Xie *et al.,* 2017) and cervical cancer (Ni *et al.,* 2011). We also found many recent corroborating reports from India suggesting an association between 870AA genotype and an increased risk for the development of cervical (Thakur *et al.,* 2018), prostate (Mandal and Mittal 2012), urinary bladder (Gangwar and Mittal 2010) and esophageal (Hussain *et al.,* 2011) cancer.

However, G/G and G/A genotype were not associated significantly with the breast cancer risk in the present study. The current study elucidates that the A- allele of CCND1 G870A polymorphism is associated with elevated breast cancer risk, but the effect seems confined to homozygous AA carriers.

Conclusion

The incidence of breast cancer is rising steadily primarily driven by advancing age, obesity, lack of physical activity, late menopause, early menarche, diet, alcohol, smoking, benign breast diseases, oral contraceptives, radiation and hormone replacement therapy (HRT). Current study shows that A/A genotype in CCND1 gene is associated significantly with the breast cancer risk in the total cohort and premenopausal women. However, G/G and G/A genotype are not associated significantly with the breast cancer risk. Considering the cancer heterogeneity, many more gene variants are needed for this risk score to be informative in predicting breast cancer risk.

Abbreviations: CCND1, cyclin D1; SNP, single nucleotide polymorphism; ATM, ataxia telangiectasia mutated; CHEK2, Checkpoint kinase 2; BRIP1, BRCA1 Interacting Protein C-Terminal Helicase 1; PALB2, Partner And Localizer Of BRCA2; CASP8, Caspase 8; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; OR, odds ratio.

Acknowledgments

The author thankfully acknowledges all the subjects included in the study.

Conflict of Interests

The author declares no conflict of interests.

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