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Breast Cancer and Cyclin D1 Gene Polymorphism in Turkish Women

İLHAN YAYLIM-ERALTAN[1], ARZU ERGEN[1], UZAY GÖRMÜŞ[1], SOYKAN ARIKAN[2], SEDEN KÜÇÜCÜK[3], ORHAN ŞAHİN[2], NİLÜFER YİĞİT[1], YEMLİHA YİLDİZ[1] and TURGAY İSBİR[1]

[1]*Department of Molecular Medicine, Institute of Experimental Medicine and*
[3]*Institute of Oncology, İstanbul University, Istanbul;*
[2]*Surgery Clinic, Istanbul Research and Teaching Hospital, Istanbul, Turkey*

Abstract. *Background: Cyclin D1 protein plays an important part in regulating the progress of the cell during the G_1 phase of the cell cycle. It has been suggested that G870A polymorphism at the exon4/intron4 splicing region of the CCND1 gene may play a role in tumorigenesis and invasiveness. Patients and Methods: A case-control study was performed to test the association between G870A polymorphisms in the CCND1 gene and breast cancer risk and cancer progression. For this purpose, 38 patients with breast cancer and 64 healthy women controls were included in the study. The CCND1 G870A polymorphisms in our study groups were genotyped by polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP) using peripheral blood samples. Results: A significant difference was found in the distribution of the GG, AG and AA genotypes between the patient group and the control group (p=0.021). A lower risk (odds ratio 0.435, 95% confidence interval 0.223-0.846) was found to be associated with heterozygote AG individuals when compared with homozygote allele carriers in breast cancer. The cyclin D1 A870G genotype was associated with capsular invasion (p=0.02). Conclusion: The risk of breast cancer development and prognosis may be associated with genetic variation in the CCND1 genotype, which may be used as a biomarker for further studies.*

Cyclin D1, a protein encoded by the *CCND1* gene located on chromosome 11q13, is the major cyclin involved in transition of cells from the G_1/S-phase. Overexpression of *CCND1* disrupts normal cell cycle control, possibly promoting the development and progression of cancer (1-5). In up to 20% of breast cancer cases, *CCND1* is amplified and >50% of mammary tumours overexpress it (4, 6). Cyclin D1 mRNA exhibits alternate splicing, and translation of the different transcripts (transcripts a and b) results in protein products with nonidentical –COOH terminal domains and with possible functional differences. Transcript b is a variant transcript reading into intron 4 and skipping exon 5, whereas transcript a is normally spliced (7-9). A common G870A polymorphism at codon 242 on exon 4 in the *CCND1* gene has been shown to modulate splicing of the CCND1 transcript. The dominant A allele preferentially generates the truncated transcript, which encodes a cyclin D1 protein with a longer half-life. The G allele tends to produce the full transcript (9, 10). Several studies have shown that individuals carrying the A870 allele are more likely to have enhanced alternative gene splicing than those carrying the G870 allele. However, the results reported in previous studies are still inconsistent (7, 8, 11, 12).

Many studies found an increased risk for different cancer types and *CCND1* G870A polymorphism (11, 13-17). However, the absence of an association with cancer risk or inverse correlations between cancer risk or prognosis and *CCND1* G870A polymorphism also were reported (18-20). The aim of this study was to investigate possible correlations between CCND1 G870A polymorphism and breast cancer, and specifically, to determine if genetic analysis is capable of identifying patients with breast cancer showing greater tumour aggressiveness and tendency to metastasize.

Patients and Methods

Study participants. CCND1 G870A polymorphism was studied among 38 breast cancer patients (mean age 53.74±11.74 years; age range 31-78 years) and 64 non-malignant healthy females. Controls were selected from surgery polyclinics and clinics from people who had no proven malignant disease or disease history (mean age 55.82±17.66 years; age range 32-87 years). Participants were selected from two teaching hospitals in Istanbul. Each eligible participant, after giving written informed consent, completed a structured self-administered questionnaire in order for us to collect

Correspondence to: Professor Dr. Turgay Isbir, İstanbul Üniversitesi DETAE 34390 P.O. Box 7 Çapa/İstanbul, Turkey. Tel/Fax: +90 2126351959, e-mail: tisbir@superonline.com.tr

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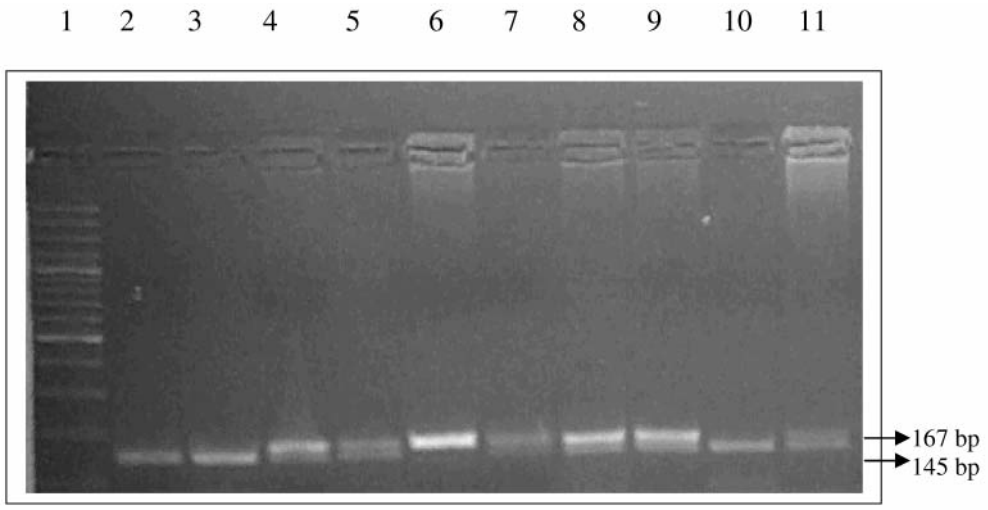

Figure 1. *Direct visualization of PCR-RFLP typing pattern of CCND1 genotypes by ethidium bromide staining. A 167-base pair CCND1 G870A fragment was amplified, cleaved with NciI, and electrophoresed on a 3% agarose gel. Results from ten representative breast cancer patients are shown. Lane1: 50 bp marker; lane 2, 3, 10: GG homozygote; lane 6: AA homozygote; lane 4, 5, 7-9, 11: AG heterozygotes.*

demographic data. Diagnosis of breast cancer by surgical clinics relied upon mammography, ultrasonography, and finally pathological examination. All the breast cancer patients and controls were citizens of the Turkish Republic. Detailed medical history, physical examination, and pathological diagnosis were performed for all patients in the study. The samples were collected before any chemotherapeutic or radiation therapy treatment had been started. Blood samples were taken from patients who had pathological diagnosis and had not undergone blood transfusion. A standardized questionnaire was administrated to collect data concerning age, sex, family history of breast cancer, and family history of any kind of cancer for only 38 breast patients from whom we obtained blood samples. Pathological staging information on all breast cancer diagnoses were confirmed by manual review of the pathology reports and clinical charts. Stage was defined according to the American Joint Committee on Cancer (AJCC) TNM system. Patients were categorised in T1 (invasion ≤2 cm) and T2 (>2 cm, ≤5 cm), and T3 (>5 cm) and T4 (invasion of skin, or chest wall, or both); thus patients were accepted as two different groups (non-advanced and advanced tumour stage patients). Nodal status was categorized as no regional lymph nodes affected (N0) or at least one nodal metastasis. This study protocol was approved by our Local Ethical Committee.

Isolation of DNA. Blood specimens from all participants were collected into tubes containing EDTA. DNA was isolated from the blood leukocytes in 10 ml EDTA by the method of Miller *et al.* based on sodium dodecyl sulphate lysis, ammonium acetate extraction, and ethanol precipitation (21).

Polymerase chain reaction (PCR) for CCND1 gene. Template DNA (0.5-1.0 μg) was used in a PCR under sterile conditions. A concentration of 0.4 μmol/l of each primer was used for the reaction. The forward primer was 5'GTGAAGTTCATTTCC AATCCGC-3' and the reverse primer was 5'GGGACATCACCCTC ACTTAC-3' in a volume of 25 μl containing 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.4), 0.16 mM each of deoxnucleotide triphosphate (MBI Fermentas, Vilnius, Lithuania), and 1 unit of Taq polymerase (MBI Fermentas, Vilnius, Lithuania).The reaction mixture was initially denatured at 94˚C for 5 minutes, followed by 35 cycles with denaturation steps at 94˚C for 45 seconds, annealing at 55˚C for 45 seconds, and extension at 72˚C for 45 seconds. The PCR programme was completed by a final extension cycle at 72˚C for 5 minutes. The PCR product exhibited a 167 base pair fragment. PCR products (10 ml) were digested with 15U NciI (MBI Fermentas) at 37˚C for 3 hours, and visualized by electrophoresis on 3% agarose containing 0.5 mg/ml ethidium bromide. The 167 bp PCR product generated is not cut by NciI if the A allele is present, whereas the product from the G allele is cut to produce fragments of 145 and 22 bp (Figure 1). *CCND1* G870GA polymorphism was typed by visualization under ultraviolet light and photographing with a Polaroid camera. The *CCND1* G870A alleles were identified in each sample (22). The allele types were determined as follows: a single 167 bp fragment for the AA genotype, two fragments of 22 and 145 bp for the GG genotype, and three fragments of 22, 145 and 167 bp for the AG genotype.

Statistical analysis. All statistical analyses were carried out using SPSS version 7.5 for Windows (SPSS Inc, Chicago, USA). Numeric values were analysed by Student's *t*-test. Differences in characteristics between breast cancer patients and controls, as well as disparities in genotype and allele frequencies, were assessed with the chi-square test. *CCND1* G870A allele frequencies were estimated by gene counting methods. Odds ratios (ORs) and 95% confidence intervals (95% CI) were calculated to estimate the risk for breast cancer. The threshold for significance was $p<0.05$.

Results

The characteristics of the study population are summarized in Table I. The frequencies of AA, AG, and GG genotypes were 28.1%, 48.4% and 23.4%, respectively, in healthy

Table I. *Characteristics of patients with breast cancer.*

	N	%
No. of patients	38	
Age distribution		
≤45 years	9	23.7
>45 years	29	76.3
Smoking status		
Yes	8	21.1
No	30	78.9
Alcohol consumption		
No	38	100.0
Family history of any kind of cancer		
Yes	14	36.8
No	24	63.2
Family history of breast cancer		
Yes	11	28.9
No	27	71.1
Histological subtype		
Ductal	29	76.3
Lobular	3	7.9
Mucinous	1	2.6
Medullary	1	2.6
Other	4	10.5
T stage		
T1	8	21.1
T2	18	47.4
T3	9	23.7
T4	3	7.9
Lymph node status		
N0	8	21.1
N1	18	47.4
N2	11	28.9
N3	1	2.6
Distant metastasis (+)	4	10.5

controls, and 39.5% , 21.1% , and 39.5% , respectively, in the patient group. With the use of the χ^2 test, the difference of the distribution of three *CCND1* G870A genotype frequencies between breast cancer patients and controls were statistically significant, but not significant in allele frequencies, respectively (p=0.021; p=0.746). Our results indicate that individuals carrying the AG genotype have a decrease in the risk for development of breast cancer (OR 0.435, 95% CI, 0.223-0.846) (Table II).

Homozygous G allele presence in breast cancer patients with any kind of cancer in their relatives was higher than in those without (OR 1.959, 95% CI: 0.906-4.236), but this was not statistically significant (p=0.089). The same relationship was also observed between patients with homozygous G allele with familial breast cancer and those without (OR 2.148 95% CI:1.032-4.471) (p=0.073). The distribution of *CCND1* G870A genotypes was not significantly different between stage T3-T4 and T1-T2 tumors (p=0.394).

Table II. *Genotypes and allele frequencies for CCND1 G870A in breast cancer patients and controls.*

	Patients, n=38	%	Controls, n=64	%	p-Value
Genotype					
AG	8	21.1	31	48.4	0.006
AA+GG	30	78.9	33	51.6	
Alleles					
G	38	50	67	52.3	0.746
A	38	50	61	47.6	

p-Value obtained by chi-square test.

Breast cancer patients carrying the *CCND1* GG genotype had a 1.7-fold increased risk for lymph node metastasis but this was not statistically significant (p=0.440). Periganglionar invasion was present in 17 (47.4%) cases and 22 (57.9%) cases had angiolymphatic invasion but these parameters were also not found to have any statistically significant meaning for different genotypes. The *CCND1* genotypes were furthermore not associated with TNM stage or age (data not shown). Capsular invasion in homozygous G patients was higher when compared with A allele carriers and this difference was statistically significant (p=0.02) (Table III).

Discussion

Cyclin D1, an important cell cycle regulator located on chromosome 11q13, is overexpressed in several types of human cancer including esophageal, squamous head and neck, non-small cell lung, hepatocellular, bladder, colon, prostate and breast (23-30). In the breast, cyclin D1 protein plays a role in both normal mammary development and malignant transformation (13, 25). Cyclin D1 is one of the most commonly overexpressed oncogenes in breast cancer, with 45-50% of primary ductal carcinomas overexpressing this oncoprotein (4). In particular, alterations in the *CCND1* gene may be a fundamental and early step in breast cancer progression (31-33). The G870A polymorphism has been examined in association with breast cancer risk and progression in many epidemiological studies. It has been proposed that the 870-A variant leads to the alternative splice variant b, which misses exon 5 and has a longer half-life than the common transcript a (8, 9). Contrary to this, Howe and Lynas have reported that GG homozygous individuals produce more transcript b, while AA homozygous ones have more transcript a (12). Findings from previous studies regarding the association between the *CCND1* genotype and breast cancer are inconsistent.

In our study, we found a significant difference in the genotype distribution between patients and controls, suggesting that *CCND1* G870A polymorphism is associated

Table III. *Association of CCND1 G870A polymorphism with clinicopathological features of breast cancer.*

CCND1 genotype	AA+AG N (%)	GG N (%)	OR[#]	95 % CI	*p*-Value	GG+AG N (%)	AA N (%)	OR[##]	(95% CI)	*p*-Value
Family history of breast cancer										
Yes	4 (36.4)	7 (63.6)	2.148	1.032-4.471	0.073	9 (81.8)	2 (18.2)	1.578	0.998-2.494	0.145
No	19 (70.4)	8 (29.6)	-	-	-	14 (51.9)	13 (48.1)	-	-	-
Family history of any kind of cancer										
Yes	6 (42.9)	8 (57.1)	1.959	0.906-4.236	0.089	10 (71.4)	4 (28.6)	1.319	0.804-2.164	0.294
No	17 (70.8)	7 (29.2)	-	-	-	13 (54.2)	11 (45.8)	-	-	-
T stage										
T3+T4	6 (50.0)	6 (50.0)	1.440	0.666-3.132	0.481	7 (58.3)	5 (41.7)	0.948	0.538-1.670	0.851
T1+T2	17 (65.4)	9 (34.6)	-	-	-	16 (61.5)	10 (38.5)	-	-	-
Lymph node status										
N(+)	17 (56.7)	13 (43.3)	1.733	0.488-6.160	0.440	19 (63.3)	11 (36.7)	1.267	0.602-2.667	0.687
N(–)	6 (75.0)	2 (25.0)	-	-	-	4 (50.0)	4 (50.0)	-	-	-
Distant metastasis (+)										
Yes	3 (75.0)	1 (25.0)	0.607	0.106-3.474	1.000	2 (50.0)	2 (50.0)	0.810	0.293-2.234	1.000
No	20 (58.8)	14 (41.2)	-	-	-	21 (61.8)	13 (38.2)	-	-	-
Periganglionar invasion										
(+)	8 (44.4)	10 (55.6)	2.222	0.936-5.274	0.054	12 (66.7)	6 (33.3)	1.212	0.725-2.026	0.463
(–)	15 (75.0)	5 (25.0)	-	-	-	11 (55.0)	9 (45.0)	-	-	-
Capsule invasion										
(+)	8 (42.1)	11 (57.9)	2.750	1.062-7.121	0.020	13 (68.4)	6 (31.6)	1.300	0.769-2.197	0.319
(–)	15 (78.9)	4 (21.1)	-	-	-	10 (52.6)	9 (47.4)	-	-	-
Angiolymphatic invasion										
(+)	13 (59.1)	9 (40.9)	1.091	0.486-2.447	0.832	15 (68.2)	7 (31.8)	1.364	0.773-2.404	0.258
(–)	10 (62.5)	6 (37.5)	-	-	-	8 (50.0)	8 (50.0)	-	-	-

p-Value was obtained by chi-square test. [#]Odds ratio for genotype was calculated as GG *vs*. AA+AG; [##]odds ratio for genotype was calculated as AG+GG *vs*. AA.

with susceptibility to breast cancer. Our results are consistent with previous findings suggesting that *CCND1* genotype is associated with breast cancer development (11, 34, 35). Recent large-scale case-control or case-cohort studies of breast cancer reported that there was no association between *CCND1* G870A polymorphism and breast cancer risk (18-20). Yu *et al*. (11) indicated that *CCND1* G870A polymorphism makes a significant contribution to breast cancer in China, with preponderance of breast cancer in young women. Their results showed that the AG and AA subgroup were at increased risk for developing breast cancer compared with those with the GG genotype. Recently, Lu *et al*. (36) conducted a meta-analysis on the association between G870A polymorphism and the risk of breast cancer and showed that there was an increased risk of breast cancer for carriers of variant 870A allele in Caucasians but not in an Asian population. They suggested that different genetic backgrounds and environmental exposures might also contribute to the ethnic difference. Although we were unable to find a significant result both for homozygous G or A alleles, our results indicate that individuals carrying the AG genotype have a decreased risk for the development of breast cancer. A few studies have examined the GA genotype separately from the AA genotype and principally found no significant GA genotype association with cancer risk (10, 13, 14). Ceschi *et al*. (34) reported that the heterozygous *CCND1* GA genotype significantly reduced the breast cancer risk in all individuals when compared with the GG genotype. Several studies showed that the *CCND1* GA genotype was linked to differential overall and disease-free survival in patients with ovarian and colorectal cancer (37, 38). Molecular heterosis has been described in which individuals heterozygous for a specific genetic polymorphism show stronger effects than those homozygous for either allele (39).

A number of studies have linked the *CCND1* GG genotype to increased cancer risk or reduced survival (40-43) but controversial results have been reported regarding the role of *CCND1* genotypes in cancer development (13, 16). In this study, we found that breast cancer patients with GG genotype whose relatives had any kind of cancer history have a 2.148-fold times higher predisposition to breast cancer than those with AA and AG genotype do (p=0.073). Additional work is necessary to further characterize the molecular reasons for the increased risk seen in individuals with a positive family history. It would be interesting to study whether or not a specific distribution of *CCND1* G870A polymorphism can

also be observed in breast cancer and to test linkage in high-risk families. In our breast cancer group, the distribution of *CCND1* G870A genotypes were not significantly different between stages T3-T4 and T1-T2 tumors. Looking at lymph node metastasis, although not significant, breast cancer patients carrying the *CCND1* GG genotype had a 1.7-fold increased risk compared with those with the AA and AG genotypes. Extranodal extension was considered to be present when tumor was seen outside the lymph node capsule (44). An important finding in our study was the elevated risk for capsule invasion in breast cancer patients who carried the GG genotype.

It is possible that these conflicting results in part reflect the many different mechanisms through which deregulated expression of *CCND1* can occur in cancer. Functional studies in the future may help to explain the conflicting experimental findings and influence of *CCND1* genotypes on tumor behavior in different cell types (45). The relation between the *CCND1* G870A polymorphism and expression of splice variants a and b remains unclear and deserves further research.

In conclusion, our study suggested that the *CCND1* heterozygote genotype may be benefical and may affect breast cancer risk in Turkish women.

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