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ORIGINAL ARTICLE

Association of Cyclin D1 A870G polymorphism with two malignancies: Acute lymphoblastic leukemia and breast cancer

A. M. L. Bedewy[1], M. H. Mostafa[2], A. A. Saad[3], S. M. EL-Maghraby[1], M. M. L. Bedewy[4], A. M. Hilal[5], L. S. Kandil[6]

[1]Department of Hematology, Medical Research Institute, Alexandria University, Alexandria; [2]Department of Environmental Studies, Institute of Graduate Studies and Research, Alexandria University, Alexandria; [3]Department of Biotechnology, Institute of Graduate Studies and Research, Alexandria University, Alexandria; [4]Department of Pathology, Military Medical Academy, Cairo; [5]Department of Oncology, National Cancer Institute, Cairo University, Cairo; [6]Department of Pharmacology, Pharos University, Alexandria, Egypt

Summary

Purpose: To study the association between Cyclin D1 (CCND1) polymorphic variants and acute lymphoblastic leukemia (ALL) and breast cancer cases and the possibility of having different (CCND1) polymorphic variants in the development of ALL and breast cancer. In addition, to study the association between the different CCND1 polymorphic variants and the response to induction chemotherapy in ALL cases and clinicobiological parameters in breast cancer.

Methods: We evaluated the association of CCND1 G870A polymorphism with ALL risk in 25 ALL patients and 15 healthy controls and with breast cancer risk in 30 newly diagnosed breast cancer female patients and in 25 healthy controls. Restriction Fragment Length Polymorphism (RFLP) polymerase chain reaction (PCR) was used for analysis of G870A polymorphism of CCND1 on anticoagulated whole blood of both the ALL and breast cancer cases and control groups.

Results: The frequency of the AA genotype was significantly increased in the ALL cases while GG genotype was significantly increased in the control group. Furthermore, there was a highly statistically significant association between the A allele in the homozygous state AA and the ALL cases. Furthermore, there was a positive risk of developing ALL when having the AA genotype and the results were highly significant for AA genotype compared to GG genotype. For breast cancer, the results revealed that there was a positive risk association for those carrying the CCND1 A allele in the development of breast cancer.

Conclusion: Homozygosity for CCND1 A allele was associated with ALL patients and was a risk factor for ALL development, while the presence of the A allele, whether in homozygous or heterozygous state was associated with breast cancer cases and was a risk for breast cancer. Homozygosity for CCND1 G allele was associated with the control group.

Key words: acute lymphoblastic leukemia, breast cancer, cyclin D1, RFLP-PCR

Correspondence to: Ahmed M. L. Bedewy, MD.
Department of Hematology, Medical Research Institute, Alexandria University, 165 El Horreyah Ave, Alexandria, Egypt. Tel: +201 000040 511, Fax: +203 5222602, E-mail: dr_ahmed_bedewy@yahoo.com
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Introduction

Cancer continues to be a worldwide killer, despite the enormous amount of research and rapid developments seen during the past decade. According to recent statistics, cancer accounts for about 23% of the total deaths in the USA and is the second most common cause of death after cardiovascular diseases [1].

Molecular epidemiology is an emerging new field that combines highly sensitive molecular techniques for detecting early damages associated with cancer. For this purpose, it is necessary to use biological markers as indicators signaling events in biological systems or samples [2]. One of the main biological markers is the marker of susceptibility, especially in preexisting inherited genetic defects that increase the risk of cancer [3].

Single nucleotide polymorphism (SNP) was defined by genome-wide association studies as a DNA sequence variant. Many of SNPs were strongly associated with diseases in large case-control studies [4].

Cyclin-dependent kinases (CDKs) are protein kinases involved in critical cellular processes, such as cell cycle or transcription, whose activities requires association with specific Cyclin subunits. Cyclins are proteins which act as key controlling elements of the eukaryotic cell cycle and function as allosteric regulatory subunits for the CDKs catalytic subunit [5].

The importance of Cyclin-CDK complexes in cell proliferation is underscored by the fact that deregulation in the function of these complexes is found in virtually the whole spectrum of human tumors and this comes from the fact that tumor-associated alterations in Cyclins help to sustain proliferation independent of external mitogenic or anti-mitogenic signals [6].

CCND1 is a 35-kDa protein which is encoded by 5 exons situated at the region of chromosome band 11q13. In the amino terminus of CCND1 appears a motif Leu - X - Cys - X - Glu (X represents any aminoacid) where pRB (retinoblastoma protein) pocket domain binds. The carboxy terminus inhibits myogenic helix loop helix (HLH) protein function. HLH protein's main action is to remove cells from the cell cycle (halt proliferation), so its inhibition by CCND1 leads the cell to G1 phase of the cell cycle [6].

CCND1 is overexpressed in several human tumors. Chromosomal translocations, gene amplification and disruption of normal intercellular trafficking and proteolysis are the procedures which lead to accumulation of CCND1 in tumor cell nuclei and eventually to CCND1 overexpression in many tumors [6].

CCND1 is the major D Cyclin in most cell types. All 3 Cyclin D molecules act in late G phase, just before entry into S phase. Many tumors have high CCND1 levels without amplification or mutation of the CCND1 structural gene [7]. Unlike other Cyclins, D-Cyclins are strongly dependent on extracellular mitogenic stimuli [8]. Due to this property they are regarded as mitogenic sensors that relay signals from the extracellular environment to the core cell cycle machinery [9].

CCND1 expression and accumulation are induced by growth factors and occur at multiple levels including increased transcription, translation, and protein stability. Regulation is mediated primarily through Ras (rat sarcoma) signaling pathways [10].

Following its induction by mitogenic growth factors, newly synthesized CCND1 associates with CDK4; the predominant function of the D1/CDK4 enzyme involves the phosphorylation-dependent inactivation of the retinoblastoma protein [11].

Although intragenic somatic mutation of CCND1 in human disease is rare, CCND1 gene translocation, amplification and/or overexpression are frequent events in selected tumor types. A polymorphism of CCND1 that occurs in a splice donor site has been epidemiologically linked to increased cancer risk or poor prognosis in a number of tumor types. Recent functional analyses have revealed that protein product of an alternately spliced transcript, CCND1b, harbors overlapping but distinct functions as compared to full length CCND1 [12].

Acute leukemia is the major pediatric cancer affecting between 30-45 per 1,000,000 children each year [13]. Current protocols for diagnosing and treating ALL have achieved overall cure rates, defined as the absence of disease for at least 10 years, of more than 80% in children, while adults with ALL still have a relatively poor prognosis [14].

The causes of the vast majority of ALL cases are unknown. Among childhood, only ionizing radiation

and certain genetic disorders are known risk factors. Many other risk factors have been suggested but remain under investigation, such as exposure to pesticides, automobile exhaust, certain chemicals such as benzene, non ionizing radiation (e.g., magnetic fields), parental exposures (e.g., cigarette smoking, alcohol consumption and use of some pharmaceuticals), and even parental consumption of certain dietary constituents [15].

The genetic candidates that have been evaluated as susceptibility genes for childhood ALL to date can be broadly delineated into those coding for carcinogen metabolism enzymes, folate metabolism enzymes, DNA repair proteins, and others [13].

Breast cancer is a heterogeneous disease that encompasses several distinct entities with remarkably different biological characteristics and clinical behaviors. Among women, breast cancer remains the most commonly diagnosed cancer. Genetic risk factors contribute to about 5-10% of all cases, 90-95% of them result from somatic mutation and about 5-10% are inherited as a result of germ line mutation in autosomal dominant breast cancer susceptibility genes [16].

The aim of the present work was to study the association between CCND1 genetic polymorphism and the risk of different types of human cancers as compared to control groups. This study investigated the associated risk of CCND1 polymorphism with the development of ALL and breast cancer.

Methods

Patients

This study was carried out on 25 patients with newly diagnosed ALL, who presented to the Hematology Department, Medical Research Institute, University of Alexandria and Elshatby Children hospital, University of Alexandria and 30 patients newly diagnosed with breast cancer, who presented to the Oncology Department, Medical Research Institute, University of Alexandria and National Cancer Institute Outpatient Clinic from January 2010 to June 2011, after obtaining oral informed consent. The diagnosis of ALL was based on complete blood count, bone marrow aspiration and immunophenotyping, while the diagnosis of breast cancer was based on initial mammographic screening and biopsy. The study included age and sex matched control groups, i.e. 15 healthy children for the ALL group and 25 healthy females for the breast cancer group.

All patients were subjected to careful history taking, thorough physical examination, complete blood count, liver and renal function tests, plain chest X-ray and abdominal ultrasound. For ALL patients: bone marrow examination and immunophenotyping by flow cytometry using B and T cell markers (DAKO, Denmark) were performed. For breast cancer patients: mammography and immunohistochemical (IHC) staining for estrogen and progesterone receptors status were carried out on formalin-fixed, paraffin-embedded tumor samples using monoclonal antibodies (Thermo Scientific, USA). The percentage of stained cells and the staining intensity determined the score of positivity (1, 2 or 3) for estrogen (ER) and progesterone receptors (PR) with presence of stain in <1% cells or weak staining implying receptor negative status [17].

Restriction fragment length polymorphism (RFLP) PCR for analysis of G870A polymorphism of CCND1 on peripheral blood of all cases

Blood specimens from all participants were collected into tubes containing EDTA. DNA isolation from anticoagulated whole blood of controls as well as of breast cancer patients and children with ALL was carried out according to Sambrook et al. method [18]. Typically, 1% agarose gel was used for genomic DNA analysis, while 2% gel was used to analyze PCR products and 3% to analyze digested PCR products.

Amplification of CCND1 gene by RCR

PCR technique was used to detect CCND1 gene on chromosome 11q13 using reverse primer (CCND1R-21) with the sequence: TTTCCGTGGCACTAGGTGTC and forward primer (CCND1F`22) with the sequence: AGTTCATTTCCAATCCGCCC and the expected PCR product of 212 base pair (bp) length. Primers were HPLC purified and obtained from Fermentas Chemical Co, USA.

PCR was carried as follows: 15 µl PCR master mix [Fermentas PCR Master Mix (2X)], 3µl DNA template (0.5 µg final), forward and reverse primers 1µl

each (30 pmol) and 10μl nuclease free water with final volume of 30μl. PCR condition consisted of an initial denaturation of 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 sec, 60 °C for 30 sec, and 72 °C for 30 sec, with a final extension of 72 °C for 10 min. The PCR product of 212 bp was detected by agarose gel electrophoresis using 2% agar and base pair marker of 100 bp.

Restriction fragment length polymorphism for the PCR product for detection of CCND1 G870A polymorphism
The resulting 212 base pair (bp) PCR product was digested with the restriction enzyme *Moraxella* species (*Msp*I) (Fermentas) as follows:

15 μl of the resulting PCR product was digested with 2 μl of MspI enzyme (fast digest) and 3 μl enzyme buffer and then completed with nuclease-free water to a final volume of 30 μl, and incubated at 37°C for 30 min.

Digested PCR products were electrophoresed using 3% agarose gel to distinguish between the 175 bp band produced by the digestion of the A allele and the 141 bp band produced by the digestion of the G allele. Heterozygous state yielded both 141 bp and 175 bp bands as previously described by Le Marchand et al. [19].

Statistics
Data were analyzed using SPSS software package version 18.0 (SPSS, Chicago, IL, USA). Quantitative data were expressed using range, mean, standard deviation and median, while qualitative data were expressed as frequencies and percents. Qualitative data were analyzed using chi-square test; also exact tests such Fisher exact test and Monte Carlo test were applied to compare different groups. P value was assumed to be significant at <0.05.

Results

Acute lymphoblastic leukemia patients and control group
The age of the studied patients ranged between 4 to 12 years (mean 7.68 ± 2.51). Among the 25 patients 19 (76%) were males and 6 (24%) females. The age of the control group ranged from 3 to 12 years (mean 6.80 ± 2.88). Among the 15 healthy children 9 (60%) were males and 6 (40%) females.

All patients presented with anemia, 22 (88%) with bleeding, infections were present in 24 patients (96%), splenomegaly and lymphadenopathy were present in all patients, hepatomegaly was present in 20 patients (80%), neurological manifestations were present in 4 patients (16%), while only 2 patients (8%) presented with testicular infiltration.

Table 1 shows the peripheral blood count and bone marrow blast cells percents of the studied ALL cases.

Immunophenotyping showed only 3 patients (12%) with T-ALL, while 22 patients (88%) were identified as B-ALL. Among the B-ALL cases, 4 (16%) were identified as Pro-B, 9 (36%) as Pre-B and 9 (36%) as common B-ALL.

All patients responded to therapy with 19 of them (76%) achieving complete and 6 (24%) partial response.

CCND1 polymorphic variants among the cases were distributed as follows: 9 (36%) were homozygous to the A allele (AA), 12 (48%) were heterozygous to the A allele, while only 4 (16%) were homozygous to G allele (GG).

In the control group only 1(6%) patient was AA, 7 (46.7%) were AG and 7 (46.7%) were GG.

Distribution of CCND1 polymorphic variants (AA, AG, GG) among patients and controls showed statistically significant difference (p=0.040) with significantly increased frequency of AA genotype in cases (p= 0.038) and significantly increased frequency of GG genotype in controls (p=0.035; Table 2).

Figure 1 shows the PCR product of CCND1 of 212 bp as detected by agarose gel electrophoresis using 2% agarose and base pair marker of 100 bp, whereas Figures 2 and 3 show RFLP digested PCR products for analysis of G870A polymorphism of CCND1 using 3% agarose gel electrophoresis for ALL and cancer breast patients, respectively.

There was a positive risk of developing ALL with the AA genotype and the results were highly significant for AA genotype compared to GG genotype (p=0.024). However, no significant risk was found for AA genotype when compared to non homozygosity for AA genotype (AG+GG; p=0.060; Table 3).

Distribution of the different polymorphic variants among immunophenotypic categories of ALL patients T, B (Pro B, Pre B, common B) showed no statistically significant association between ALL immunophenotypes and the polymorphic variants

Table 1. Peripheral blood counts and bone marrow blast cells percentage of the studied acute lymphoblastic leukemia cases

Peripheral blood counts and BM blasts (normal range)	*Mean ± SD*	*Range*
Hemoglobin (11-16g/dl)	6.47 ± 1.50	4.42 – 10.57
Red blood cells (4-5.5x 10^6)	2.38 ± 0.53	1.58 – 3.41
Platelets (150-450x 10^3)	32.68 ± 14.07	15.0 – 63.0
White blood cells (4-13x10^3)	8.84 ± 7.71	1.90 – 33.40
BM blasts (0-2%)	64.28 ± 19.09	29.0 – 90.0

BM: bone marrow, SD: standard deviation

Table 2. Comparison of Cyclin D1 polymorphic variants in acute lymphoblastic leukemia in cases and controls

	Cases (N = 25)		*Controls (N = 15)*		x^2, *p-value*
Polymorphic variants	*N*	*%*	*N*	*%*	
AA	9	36.0	1	6.7	0.038
AG	12	48.0	7	46.7	0.935
GG	4	16.0	7	46.7	0.035

For abbreviations see text

Table 3. Assessment of the risk of having the A allele in developing acute lymphoblastic leukemia

	Cases (N = 25)		*Controls (N = 15)*		*OR (95% CI)*	*p-value**
Polymorphic variants	*N*	*%*	*N*	*%*		
AA	9	36.0	1	6.7	15.750 (1.424 – 174.246)	0.024
AG	12	48.0	7	46.7	3.0 (0.642 – 14.023)	0.257
AA + AG	21	84.0	8	53.3	4.594 (1.052 – 20.057)	0.065
GG	4	16.0	7	46.7		
AA	9	36.0	1	6.7	7.875 (0.884 – 70.151)	0.060
AG + GG	16	64.0	14	93.3		

*Fisher's exact test, OR : odds ratio. For other abbreviations see text

Table 4. Comparison of Cyclin D1 polymorphic variants between the immunophenotypic categories in acute lymphoblastic leukemia cases

	Polymorphic variant						*p-value**
	AA (N = 9)		*AG (N = 12)*		*GG (N = 4)*		
	N	*%*	*N*	*%*	*N*	*%*	
Immunophenotyping							
T (N= 3)	1	33.3	1	33.3	1	33.3	0.736
B (N = 22)	8	36.4	11	50.0	3	13.6	
p-value**	1.000		1.000		0.422		
B	8	36.4	11	50.0	3	13.6	
Pro B (N = 4)	1	25.0	3	75.0	0	0.0	
Pre B (N = 9)	5	55.6	3	33.3	1	11.1	0.587
Common B (N = 9)	2	22.2	5	55.6	2	22.2	

* Monte Carlo test, ** Fisher's exact test

Table 5. Comparison of type of response to induction chemotherapy according to Cyclin D1 polymorphic variant in acute lymphoblastic leukemia cases

Response to chemotherapy	*Polymorphic variant*						*p-value**
	AA (N = 9)		*AG (N = 12)*		*GG (N = 4)*		
	N	*%*	*N*	*%*	*N*	*%*	
CR (N = 19)	5	26.3	10	52.6	4	21.1	0.250
PR (N = 6)	4	66.7	2	33.3	0	0.0	
p-value**	0.142		0.645		0.540		

*Monte Carlo test, **Fisher's exact test CR: complete remission, PR : partial remission

Table 6. Clinicobiological parameters of breast cancer patients

Parameters	*N*	*%*
Stage		
II	15	50.0
III, IV	15	50.0
Metastasis		
Metastatic	2	6.7
Non metastatic	28	93.3
Pathology		
IDC	27	90.0
ILC	3	10.0
Menstrual status		
Postmenopausal	13	43.3
Premenopausal	17	56.7

IDC: invasive ductal carcinoma, ILC: invasive lobular carcinoma

(p=0.736 and p=0.587; Table 4).

Distribution of CCND1 polymorphic variants among ALL cases according to type of response to induction chemotherapy showed no statistically significant association between kind of response to chemotherapy and different CCND1 polymorphic variants (p=0.250; Table 5).

Furthermore, there was no statistically significant association of A allele either in heterozygous (AG) or homozygous state (AA) with the kind of response to chemotherapy in ALL cases (p=0.540).

There was no positive risk association of being homozygous to the A allele and the response to induction chemotherapy in ALL cases (p=0.142).

Breast cancer patients and control group

The age of the studied patients ranged between 29 to 76 years (mean 52.60 ± 11.17).

Table 7. Estrogen and progesterone receptor status of breast cancer patients

	N	*%*
ER		
-ve	2	6.7
+ve	28	93.3
+	2	7.1
++	12	42.9
+++	14	50.0
PR		
-ve	2	6.7
+ve	28	93.3
+	2	7.1
++	22	78.6
+++	4	14.3
ER / PR		
-ve	2	6.7
+ve	28	93.3
+ / +	2	7.1
++ / ++	12	42.9
+++ / ++	10	35.7
+++ / +++	4	14.3

ER: estrogen receptor, PR: progesterone receptor

The control group was age-matched with the cases, their age ranging from 26 to 75 years (mean 48.16 ± 12.82).

As shown in Table 6, 15 (50%) of the patients were identified as stage II while the rest were either stage III or IV. Only 2 (6.7%) had metastatic disease. Biopsy showed that almost 90% (n=27) of the patients had infiltrating ductal carcinoma (IDC) and the rest infiltrating lobular carcinoma (ILC). Seventeen (56%) were premenopausal and 13 (43.3%) postmenopausal.

Only 2 (6.7%) patients were ER/PR negative, while among the 28 ER/PR positive patients the degree of positivity differed (Table 7).

Table 8. Comparison of Cyclin D1 polymorphic variants between breast cancer patients and the control group

Polymorphic variants	*Cases (N = 30)*		*Controls (N = 25)*		*p-value**
	N	*%*	*N*	*%*	
AA	8	26.7	4	16.0	1.000
AG	18	60.0	11	44.0	0.237
GG	4	13.3	10	40.0	0.032
p-value**		0.111			

*Fisher's exact test, **Monte Carlo test

Table 9. Comparison of Cyclin D1 polymorphic variants (presence or absence of A allele) between breast cancer patients and the control group

Polymorphic variants	*Cases (N = 30)*		*Controls (N = 25)*		x^2, *p-value*
	N	*%*	*N*	*%*	
AA + AG	26	86.7	15	60.0	0.024
GG	4	13.3	10	40.0	

Table 10. Assessment of the risk of having A allele on developing breast cancer

Polymorphic variants	*Cases (N = 30)*		*Controls (N = 25)*		*OR (95% CI)*	*p-value**
	N	*%*	*N*	*%*		
AA	8	26.7	4	16.0	5.0 (0.942 – 26.530)	0.113
AG	18	60.0	11	44.0	4.091 (1.028 – 16.277)	0.055
AA + AG	26	86.7	15	60.0	4.333 (1.155 – 16.258)	0.032
GG	4	13.3	10	40.0		

*Fisher's exact test, OR: odds ratio

Distribution of different CCND1 polymorphic variants among breast cancer patients and controls showed no statistically significant difference, however the frequency of having the GG genotype was higher in the controls than in the cases when compared to the distribution of the other genotypes (p= 0.032; Table 8). Furthermore, there was statistically significant association of A allele either in heterozygous (AG) or homozygous state (AA) in breast cancer patients and its absence (GG) in the control group (p=0.024) as shown in Table 9.

Positive risk of developing breast cancer when having A allele (AA+AG) was registered compared to GG genotype (p=0.032; Table 10). However, when homozygous A allele (AA) was compared to the non-homozygous A allele (AG+GG) no statistically significant association was found (p=0.514).

No statistically significant association between the ER/PR status and the CCND1 polymorphic variants was detected (p=1).

Distribution of different CCND1 polymorphic variants among breast cancer cases with different ER/PR status showed no statistically significant association of A allele either in heterozygous (AG) or homozygous state (AA) or its absence (GG) (p=1).

Comparison of the different CCND1 polymor-

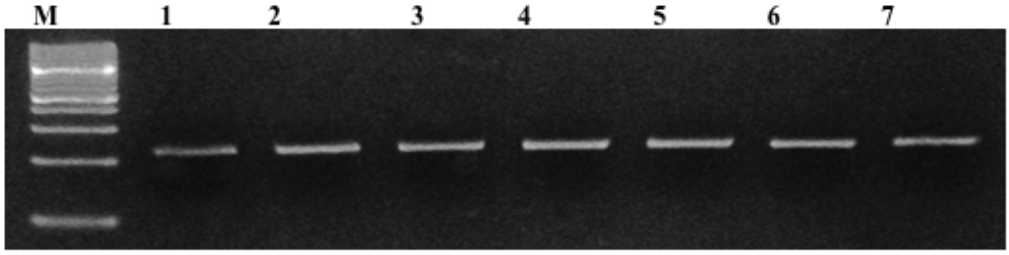

Figure 1. Agarose gel (2%) electrophoresis for PCR products. Lanes 1-7 represent PCR products for ALL patients (212 bp). Lane M represents 100bp ladder base pair marker.

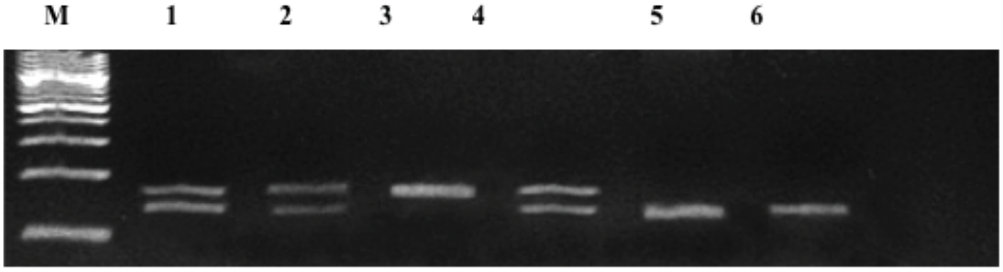

Figure 2. Agarose gel (3%) electrophoresis for RFLP digested PCR products. Lane 3 represents restricted PCR product of an ALL patient with AA genotype (175 bp). Lanes 1,2,4 represent restricted fragments of PCR products of ALL patients with AG genotype (175,141 bp). Lanes 5, 6 represent restricted PCR products of ALL patients with GG genotype (141bp).

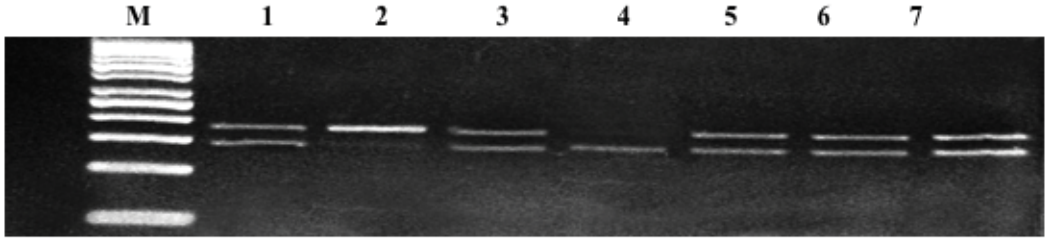

Figure 3. Agarose gel (3%) electrophoresis for RFLP digested PCR products. Lane 2 represents restricted PCR product of a breast cancer patient with AA genotype (175 bp). Lanes 1,3,5,6,7 represent restricted fragments of PCR products of a breast cancer patient with AG genotype (175,141 bp). Lane 4 represents restricted PCR products of a breast cancer patient with GG genotype (141 bp). Lane M represents 50bp ladder base pair marker.

phic variants and breast cancer patients' menstrual status at diagnosis showed no statistically significant association (p=0.096). Furthermore, there was no statistically significant association of A allele either in heterozygous (AG) or homozygous state (AA) or its absence (GG) with menstrual status at diagnosis (p=0.290).

Also, no statistically significant association was shown between the stage of disease and the CCND1 polymorphic variants (p=0.095). Furthermore, there was no statistically significant association of A allele either in heterozygous (AG) or homozygous state (AA) or its absence (GG) with different breast cancer stages at diagnosis (p=0.598).

Discussion

Cancer, in common with many other diseases, has both genetic and environmental components to its etiology. It is caused by both intrinsic factors (such as inherited mutations, hormones, and immune conditions) and environmental/acquired factors (such as tobacco, diet, radiation, and infectious organisms) [1].

The relevance of cell cycle deregulation in human cancer is being widely investigated and it affects mainly cyclin dependent kinase (CDK)/cyclin functions. Deregulation of these proteins mediates the three basic cell cycle defects, namely unscheduled proliferation, genomic instability (GIN) and chromosomal instability (CIN) [20-22].

In cancer cells, constitutive nuclear accumulation of active CCND1/CDK4 complexes can be achieved through one of several mechanisms, one of which is alternative splicing of the CCND1 transcript that resulted from a common G870A polymorphism of CCND1 that occurs in a splice donor site where the A allele was hypothesized to reduce the efficacy of the splice donor site and favor production of the alternate transcript encoding CCND1b which lacks PEST motif containing exon 5. PEST motif is critical for the degradation of CCND1; thus, transcript b (A allele) has shown to have a longer half-life than the transcript a (G allele) encoded protein [12].

Furthermore, the present study revealed a highly significant association between A allele either in heterozygous (AG) or homozygous state (AA) and cases. In addition, there was a positive risk of developing ALL with AA genotype and the results were highly statistically significant for AA genotype compared to GG genotype. However, no significant risk was found for AA genotype when compared to non homozygosity for AA genotype (AG+GG).

These observations were in concordance with those of Hou et al.[23],who studied 183 ALL cases and 190 both age- and sex-matched healthy controls, and found that CCND1 A allele was more frequent in the ALL group than in the control group.

To the best of our knowledge no other reports are published to date concerning the CCND1 polymorphic variants and ALL. However, many published reports studied the questionable association between CCND1 polymorphic variants and the risk of developing cancer in various body organs. One of these reports is the study of Le Marchand et al. [19], who conducted a population-based case-control study in the multiethnic population of Hawaii and found that CCND1 A allele may be associated with colorectal cancer, and particularly with forms of the disease that result in severe morbidity and mortality. Moreover, Marsit et al. found that the variant AA CCND1 genotype was associated with increased relative risk of head and neck squamous cell carcinoma [24].

In the present series, there was a positive risk of developing ALL when having the AA genotype and the results were highly significant for AA genotype compared to GG genotype (p=0.024). However, no significant risk was found for AA genotype when compared to non homozygosity for AA genotype (AG+GG). These findings were also confirmed by the study of Hou et al. [23], where AA genotype constituted significantly higher risk of ALL when compared to GG or AG+GG genotypes.

In the present work, the distribution of the different CCND1 polymorphic variants among the immunophenotypic categories of ALL patients (T, B [Pro B, Pre B, and Common B]) showed no statistically significant association with the polymorphic variants. Furthermore, the association between the CCND1 polymorphic variants in the form of presence of A allele (AA and AG genotypes) vs. its absence (GG genotype) and the immunophenotypic results of ALL cases was investigated and showed no significant association. However, in the study of Hou et al. [23], patients with T-ALL tended to present AA more frequently than B-ALL patients, although the difference was only slightly significant (p=0.047). This could be attributed to the fact that the number of subjects involved in the present study was relatively small.

In the current study, the distribution of the different CCND1 polymorphic variants among ALL cases according to the kind of response to induction chemotherapy showed no significant association. Furthermore, there was no significant association of the A allele either in heterozygous (AG) or homozygous (AA) state with the kind of response to induction chemotherapy in ALL cases. However, patients who

partially responded to chemotherapy were all carrying A allele either (AA=66.7% and AG=33.3%) while all patients carrying GG genotype achieved complete disease remission. This is in agreement with the study of Hou et al. [23] in which the genotype distribution in ALL patients was significantly different between completely remitted (CR) and non completely remitted (NCR) patients. NCR patients tended to present the AA genotype more frequently than CR patients.

In the current study, TNM stage II had 50% of breast cancer patients, stage III had 43.3% and 6.6% had stage IV disease. Among the 30 patients 93.3% had non metastatic disease and almost 90% were identified as IDC while the rest had ILC.

Multiple large scale studies evaluated the CCND1 polymorphic variants and breast cancer patients from different ethnic groups; different ethnicity descents were categorized as Asian and Caucasian. The risk of breast cancer associated with the CCND1 polymorphic variants was estimated for each study by odds ratio (OR) together with 95% confidence intervals (95% CI).

In the study of Shu et al. [25], 82.3% of patients had TNM stages from 0 to II and in the study of Ceschi et al. [26] almost 70% of the cases presented at stage II or less. This reflects that high education, and health awareness, including mammographic screening and laboratory investigations, contribute to early detection of breast cancer.

In the present series, the frequency of having the GG genotype was higher in the control group than in cases when compared to the distribution of the other genotypes. Furthermore, there was a statistically significant association between the A allele either in heterozygous (AG) or homozygous state (AA) and breast cancer patients.

In agreement with the present study, Yu et al. [27] found that the frequency of GG genotype was higher in the control group, while the frequency of A allele either in homozygous (AA) or heterozygous (AG) state was higher in cases. On the other hand, Krippl et al. [28] reported that CCND1 genotype frequencies were similar among patients and controls.

Abramson et al. [29] analyzed CCND1a (which is postulated to be correlated to the G allele) and CCND1b (which is postulated to be correlated to the A allele expression [21] in a cohort of women with early-stage breast carcinoma. They noticed that AA genotype was found in 22% of the cases, in 4% of the AG genotype and in 22% of the GG genotype of the cases.

In the current study, there was a positive risk of developing breast cancer for those having the A allele either in homozygous (AA) or heterozygous (AG) state when compared to GG genotype. However, homozygosity for the A allele (AA) when compared to GG genotype and when compared to non homozygosity for A allele (AG+GG) showed no significant risk association.

In concordance with our findings, Shu et al. [25] showed that the A allele, either in homozygous (AA) or heterozygous (AG) state when compared to GG genotype was only weakly associated (borderline significance) with the risk of breast cancer ,while the AA genotype when compared to GG genotype was not related with positive risk of developing breast cancer. This shows a possible oncogenic effect for the A allele which is maintained in both homozygous and heterozygous states.

This is also in agreement with the results of Yu et al. [27] who showed that there was a positive risk of developing breast cancer when having the A allele either in homozygous (AA) or heterozygous (AG) state when compared to GG genotype. Moreover, no positive risk association was found when AA genotype was compared to non homozygosity for A allele (AG+GG).

Krippl et al. [28], showed that no risk of developing breast cancer for those having the A allele either in homozygous (AA) or heterozygous (AG) state, while there was a borderline risk association between AA genotype and breast cancer (OR =1.25).

In the study of Onay et al. [30], homozygosity for the A allele (AA) when compared to GG genotype showed a positive risk of developing breast cancer in both the Ontario and the Finland samples. However, the presence of the A allele (AA+AG) when compared to GG genotype showed no positive risk association in both Ontario and Finland populations.

Positive risk of developing breast cancer when having the A allele either in homozygous (AA) or heterozygous (AG) state is a common finding among the

discussed studies which included large-scale studies, like Shu et al. and Yu et al. studies and small-scale studies like ours. Nevertheless, the power of homozygosity for the A allele in pertaining a positive risk of breast cancer is a point of discordance between studies in which the study of Onay et al. [30] agreed with this finding, while the studies of Yu et al. [27] and Shu et al. [25] and our study didn't show significant association between homozygosity for the A allele and the risk of breast cancer development.

In the present study, comparing between the different CCND1 polymorphic variants and the Estrogen ER/PR status in breast cancer patients showed that 80% of the cases having the A allele were ER/PR positive. This is in concordance with Abramson et al. [29] study in which 74% of breast cancer cases carrying the A allele were ER/PR positive. On the other hand, the study of Shu et al. [25] reported that only 30% of the cases carrying the A allele were ER/PR positive. Obviously, the rationalization of these discrepant results represents a real difficulty. However, further extrapolation of the confounding factors governing the expression of these hormonal receptors might explain this discordance.

In the present study, comparison between the different CCND1 polymorphic variants and breast cancer patients' menstrual status at diagnosis showed that about 94% of the premenopausal patients and 76% of the postmenopausal patients had the A allele ($p>0.05\%$). Similar results were described by Shu et al. [25] and Yu et al. [27].

However, in the study of Abramson et al. [29] 39% of the premenopausal breast cancer patients were carrying the A allele. As discussed above, these results drew attention towards considering whether other confounding pathobiologic factors might stand behind these unexplained discrepancies.

In the current work, distribution of the different CCND1 polymorphic variants among different stages of breast cancer patients showed that almost 93.3 % of patients with TNM stage III and IV carried the A allele, while 80% with TNM stage II carried this allele ($p>0.05\%$).

Similar results were described by Shu et al. and Ceschi et al. In the study of Shu et al. [25], 76.6% of women with TNM stages III and IV carried the A allele, while 81.9% with TNM stages 0- II carried the A allele. In the study of Ceschi et al. [26], 76.5% of patients with TNM stages > II (advanced stage disease) were carrying the A allele, while 79% with TNM stages 0-I carried the A allele.

In recent years, accumulating evidence from large numbers of studies has implicated that the CCND1 G/A870 polymorphism is a modulator of cancer risk and prognosis. Moreover, these studies presented evidence of associated risk of the A allele with the development of different types of cancer.

The present study supports these findings in that homozygosity for CCND1 A allele was associated with ALL patients and was a risk factor for ALL development, while the presence of the A allele, whether homozygous or heterozygous, was associated with breast cancer cases and was a risk factor for breast cancer development. Homozygosity for CCND1 G allele was associated with the control group .

We recommend expansion of further studies to include more patients and healthy controls in order to acquire firm data concerning the CCND1 polymorphic variants and evaluate patients' disease free survival and overall survival in relation to the CCND1 polymorphic variants. In addition, we invite future research efforts to validate the association of CCND1 polymorphic variants with human malignancies.

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