Research Article

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Flow Cytometric Evaluation of Cell Cycle Regulators (Cyclins and Cyclin-Dependent Kinase Inhibitors) Expressed on Bone Marrow Cells in Patients with Chronic Myeloid Leukemia and Multiple Myeloma

Kronik Miyelositer Lösemi ve Multipl Miyelom Olgularındaki Kemik İliği Kaynaklı Hücrelerde, Hücre Siklusu Düzenleyicilerinin (Siklinler ve Siklin Bağımlı Kinaz İnhibitörleri) Akım Sitometrik Olarak Değerlendirilmesi

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Abstract

Objective: The aim of this study was to use flow cytometry to analyze the expression of cell cycle-regulating elements with low and high proliferative signatures in patients with malignant diseases.

Material and Methods: Cyclin D, E, A, and B, and cyclin-dependent kinase inhibitor (CDKI) p16 and p21 levels were measured via flow cytometry in patients with chronic myeloid leukemia (CML) (n = 16) and multiple myeloma (MM) (n = 13), and in controls (n = 15).

Results: The distributions of the cell cycle S phase were 10, 63%, 6, 72% and 3, 59%; for CML, MM and control patients, respectively. Among all the cyclins expressed during the S phase, cyclin D expression was the lowest in the CML patients. Distribution of cyclins and CDKIs during the G2/M phase was similar in the MM and control groups, whereas cyclin expression was similar during all 3 phases in the MM and CML groups.

Conclusion: Elevated cyclin expression during cell cycle phases in the CML and MM patients was not associated with elevated CDKI expression. This finding may increase our understanding of the mechanisms involved in the etiopathogenesis of hematological malignancy.

Key Words: Chronic myeloid leukemia, Cyclin, Cyclin dependent kinase inhibitor, Flow cytometry, Multiple myeloma

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Özet

Amaç: Bu çalışmanın amacı, yüksek ve düşük proliferasyon hızına sahip malign hastalıklarda, hücre siklusunu düzenleyen faktörlerin flow sitometrik olarak analiz edilmesidir.

Gereç ve Yöntemler: Kronik miyelositer lösemi (KML) (n=16), Multipl Miyelom (MM) (n=13) ve kontrol (n=15) olgularında siklin D, E, A, B ve siklin bağımlı kinaz inhibitörü (SBKİ) p16, p21 düzeyleri akımsitometrik yöntemle ölçülmüş ve değerlendirilmiştir.

Bulgular: KML, MM ve kontrol olgularındaki S evresi dağılımı sırasıyla % 10, 63; % 6, 72 ve % 3, 59 olarak saptanmıştır. KML grubunda siklin D ekspresyonu, S evresindeki diğer siklinlere göre en düşük düzeyde bulunmuştur. G2/M evresinde, MM ve kontrol gruplarındaki siklin ve SBKİ düzeyleri birbirleriyle benzer saptanırken, MM ve KML gruplarının her üç evresindeki siklin ekspresyonları ise paralel bulunmuştur.

Sonuç: Kontrol grubuyla karşılaştırıldığında hasta gruplarında, siklin ekspresyonlarının artışına SBKİ düzeylerinin artışının eşlik etmediği saptanmıştır. Bu bulgu, belki de hematolojik malign hastalıkların etyopatogenezinin açıklanmasına katkıda bulunabilecektir.

Anahtar Sözcükler: Akımsitometri, Kronik miyelositer lösemi, Multipl Miyelom, Siklin, Siklin bağımlı kinaz inhibitörü

Introduction

Tissue homeostasis is dependent on the perfect balance between cell proliferation and cell death [1]. Proliferation of cells occurs following consecutive events and stages. Dysregulated cell cycle control is a fundamental characteristic of cancers [2]. Normal cells only proliferate in response to developmental or other mitogenic signals that indicate a requirement for tissue growth, whereas the proliferation of cancer cells proceeds essentially unchecked [2]. An understanding of the molecular details of cell cycle regulation and checkpoint abnormalities in cancer, and how these control mechanisms can be manipulated could provide insight into potential therapeutic strategies [3].

The cell division cycle is regulated by fluctuation in cyclin-dependent kinase (CDK) and cyclin pairs activity [4]. CDK activity requires binding to regulatory subunits known as cyclins [5]. CDK-cyclin complexes include 3 interphase CDKs (CDK2, CDK4, and CDK6), a mitotic CDK (CDK1, also known as cell division control protein 2), and 4 different classes of cyclins (A-, B-, D-, and E-type cyclins) [5]. The transition of cells through the early G1 stage of the cell cycle is coordinated by the activity of CDK4 and CDK6 complexes that are formed following the mitogen-dependent expression of D-type cyclins (D1, D2, and D3) [6]. CDK4/6-type-D cyclin complexes phosphorylate and inactivate retinoblastoma family protein (pRb), resulting in the release of E2F transcription factors that control the expression of the genes required for G1/synthesis phase transition and synthesis to S phase progression [4]. Inactivation of pRb facilitates expression of E-type cyclins that bind and activate CDK2 during the late G1 and early S phases. In reference to such studies, CDK2-cyclin A were implicated in committing a cell to the completion of S phase [7].

Despite requiring phosphorylation, CDK-cyclin complexes are kept inactivated by binding to a CDK inhibitor (CDKI). CDK activity is regulated by 2 families of inhibitors: INK4 proteins, including INK4A (p16), INK4B (p15), INK4C (p18), and INK4D (p19), and the Cip and Kip family, which is composed of p21 (Cip1), p27 (Kip1), and p57 (Kip2) [5,8]. In general, when the INK group functions in the genetic pathway containing cyclin D-CDK4/6-pRb and E2F, the Cip/Kip group can inhibit CDK2 kinase and CDK4/6 [9,10].

Recent research indicates that CDK down regulation may result in defective homeostasis in specific tissues and that CDK hyperactivation may facilitate tumor development by inducing unscheduled cell division in stem and progenitor cells [5]. CDKs are targets for cancer therapy; their expression is often perturbed in cases of malignancy and their inhibition can induce apoptosis [11]. Cellular checkpoint integrity is often lost as a result of CDKI inactivation or cyclin overexpression [11].

Multiple myeloma (MM) is a malignant neoplasm that arises from plasma cells of low proliferative potential [12]. Translocations involving the immunoglobulin heavy chain region (IgH) on chromosome 14q32 are an important cytogenetic event in the pathogenesis of various B-cell lymphoid neoplasms such as MM. To date, approximately more than 20 different chromosomal partner regions that

translocate to 14q32 have been identified in MM, of which t(11;14)(q13;q32) is the most common translocation, with a reported frequency of 15%-20% based on fluorescence in situ hybridization (FISH) and conventional cytogenetic analysis [13].

Although myeloma tumors exhibit complex karyotypes, and a variety of structural and quantitative chromosomal abnormalities, these tumors are unified in their ubiquitous targeting of cyclin D genes for overexpression [14]. In all, 54% of myeloma tumors overexpress cyclin D1 (CCND1), 48% overexpress cyclin D2 (CCND2), 3% overexpress cyclin D3 (CCND3), and 8% overexpress both CCND1 and CCND2 [15]. Recently, translocation and cyclin (TC) classification of MM has been introduced; the classification is based on cyclin expression types [16]. Thus, CDK inhibitors have a potential role in the treatment of MM, including PD 0332991, a specific inhibitor of CDK4/6, and seliciclib, UCN-01, P276-00, AT7519, and RGB 286638, non-specific CDK inhibitors [12].

Chronic myeloid leukemia (CML) is a clonal myeloproliferative disorder characterized by a chromosomal translocation (9;22) (q34; q11) that produces the oncogenic Bcr-Abl fusion protein, resulting in a constitutively active tyrosine kinase with high proliferative potential [17]. Despite progress in the treatment of early-stage CML, the accelerated and blastic phases of CML remain a therapeutic challenge; therefore, novel treatment approaches are needed [17]. Few data are available concerning the expression status of such cell cycle regulators as cyclins and CDKIs in CML [18]. Recent data show that low or undetectable expression of CDKI genes is a significant marker for the active phase of the disease; therefore, several new molecules are now being tested—alone and in combination with imatinib—to overcome accelerated and blastic phases. Indirubin is a CDKI that has been used in traditional medicine for hundreds of years and is currently being used in clinical trials for CML [19].

The aim of the present study was to use flow cytometry to analyze the expression of nuclear cell cycle-regulating elements in patients with MM and CML, diseases with a low and high proliferation signature, respectively. In normal and hematologically malignant cells partial illumination of the cell cycle—and thus the etiopathology of malignancy—can only be determined via comparison of the quantified changes in the cyclical phases of cyclins and CDKIs in healthy and malignant proliferated cells.

Material and Methods

Patients and their Characteristics

Following ethics committee approval of the study protocol and obtaining written informed consent from each patient, 16 consecutive CML patients, 13 consecutive MM patients, and 15 controls were included in the study. Mean age of the CML patients (9 male and 7 female) was 44.5 ± 8.2 years (rage: 22-59 years), versus 54.5 ± 4.5 years (range: 49-66 years) in the MM patients (8 male and 5 female). Mean age of the controls (7 male and 8 female), who regardless of diagnosis had histopathologically proven normal bone marrow, was 45.8 ± 11.8 years (range: 26-71 years). Table 1 summarizes the characteristics of the patients and controls. Prognosis in the CML patients was determined according to the Sokal Index and staging of the MM patients was based on the International Staging System (ISS) (Table 1) [20,21]. Samples that were obtained for diagnoses were used in this prospective study.

Table 1: Characteristics of the Patients and Controls

Groups	n	Gender (M/F)	Mean age in years (range)	Stage (n)	Prognosis	BM plasma cell
CML	16	9/7	44.5 ± 8.2 (22-59)	Chronic: 15 Blastic: 1	Low: 0 Intermediate: 8 High: 8	-
MM	13	8/5	54.5 ± 4.5 (49-66)	Stage I: 4 Stage II: 6 Stage III: 3	-	n = 9 (≥30%) n = 4 (<30%)
Control	15	7/8	45.8 ± 11.8 (26-71)	-	-	-

Flow Cytometry

Principle

We used specific monoclonal antibodies and flow cytometry to determine cellular expression of cyclins (cyclin A, B, D, and E) and cyclin inhibitors (p16 and p21) throughout the cell cycle.

Preparation of Cells

Mononuclear cells (MNCs) were isolated from selected bone marrow (BM) specimens collected in tubes containing ethylene diamine tetraacetic acid (EDTA) using a density gradient (Ficoll 1.077, Biochrom KG, Berlin, Germany). Following isolation of MNCs, a cell count was performed before the cells were fixed in a methanol-free 1% formaldehyde solution for 15 min at 4 °C. Following a washing step performed with phosphate-buffered saline (PBS) containing 1% fetal calf serum (FCS, Sigma, St. Lois, USA), fixation was repeated with 80% ethanol while vortexing the cells in order to prevent the formation of aggregates. The fixed cells were kept in 80% ethanol at –20 °C until the assay was performed. Before the assay, the cells were washed with wash buffer (WB) at 400 g for 10 min, so as to remove the ethanol, and then incubated with WB containing Triton X-100 for 5 min at 4 °C in order to perforate the cells.

Monoclonal Antibodies

We used the FITC conjugates of the antibodies specific to cyclins A, B, and D, and inhibitor p16 (Pharmingen, USA) and their isotopic controls. The antibodies specific to cyclin E and inhibitor p21 were pure and labeled with a secondary antibody conjugated with FITC (GAM-FITC Pharmingen, USA) (Table 2). We used isotypic controls in order to check for unspecific binding and to set the markers during the evaluation of the results.

Assay

Following distribution of 100 mL of cells (10^6 cells $tube^{-1}$) in each tube containing specific antibodies or isotypic controls, cells were incubated for 16 h at 4 °C. At the end of the first incubation secondary antibodies were added to the number 7, 8, and 9 tubes, which contained the pure antibodies, followed by incubation for 1 h at room temperature. During this period all other tubes were kept at 4 °C. Following incubation, cells were washed with 2 mL of WB (spun at 400 g for 5 min). Upon elimination of unbound material, the tubes were incubated with 0.5 mL of propidium iodide for 10 min (PI; 10 mg mL^{-1}, Medac, Germany) in order to label cellular DNA.

The tubes were maintained at 4 °C until data acquisition, which was performed within 1 h of the final incubation.

Table 2: Pipetting Scheme

Tube	Primary Antibody		GAM-FITC	PI (10 mg mL^{-1})
1	Cyclin A isotypic control-FITC	20 µL	-	0.5 mL
2	Cyclin A-FITC	20 µL	-	0.5 mL
3	Cyclin B isotypic control-FITC	20 µL	-	0.5 mL
4	Cyclin B	20 µL	-	0.5 mL
5	Cyclin D isotypic control-FITC	20 µL	-	0.5 mL
6	Cyclin D	20 µL	-	0.5 mL
7	Cyclin E isotypic control (pure)	10 µL	10 µL	0.5 mL
8	Cyclin E (pure)	10 µL	10 µL	0.5 mL
9	P21 (pure)	10 µL	10 µL	0.5 mL
10	P16 isotypic control-FITC	20 µL	-	0.5 mL
11	P16-FITC	20 µL	-	0.5 mL

Cell Acquisition

Data acquisition was performed using a FACS Calibur flow cytometry instrument (Becton Dickinson San Jose, CA, USA) after daily checks were made using Calibrites and CENs (Chicken Erytocyte Nucleus). We collected and analyzed the data for each tube using CellQuest software.

We detected and recorded signals that originated from FSC, SSC, FL_1, FL_2, $FL_{2Width,}$ and FL_{2Area}. We also performed cell cycle analysis using ModFit software (Verity Software House, ME, USA).

Analysis

Before cell cycle analysis, aggregated cells were eliminated using the FL_{2Width}-FL_{2Area} distribution of cells. All analyses were performed in this selected single cell population. Upon the definition of G0/G1, S, and G2/M phases based on the $FL2_{Area}$ parameter that expressed the DNA content of the cell, the level of expression of cyclins A, B, D, and E, and inhibitors p21 and p16 was evaluated separately for each phase. We also analyzed the cell cycle phases using ModFit software, which uses a different mathematical approach to fit the $FL2_{Area}$ curve.

Statistics

Kruskal-Wallis analysis was used for comparison of cyclin and inhibitor expression levels during each phase of the cell cycle. When a statistically significant difference was established between these two patients and one control groups, the multiple comparisons test was used to investigate the each group.

Apart from this, analysis of cyclin and inhibitor data was performed within each group separately depending on changes of each phase using the Wilcoxon signed-rank test.

Table 3: Cell cycle Phase Distribution in the Control and Patient Groups

Groups	Phase (%)		
	G0/G1	S*	G2/M
CML	90.01	**10.63**	0.74
MM	91.80	**6.72**	1.73
Control	89.0	**3.59**	1.7

(P = 0.536)

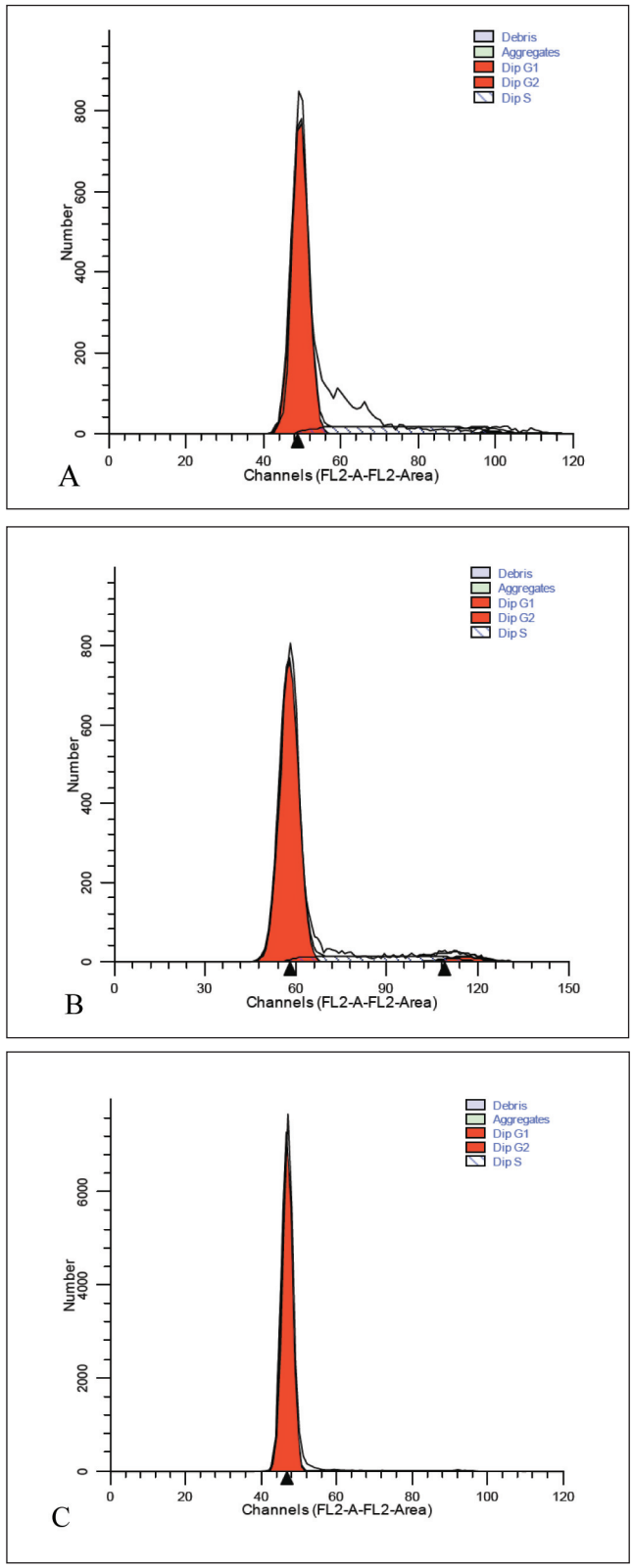

Figure: Distribution of the cell cycle phases analyzed using ModFit and CellQuest software (sample cases: **A:** CML; **B:** MM; **C:** control).

Results

Cell Cycle Phase Evaluation

The distribution of the cell cycle phases based on analysis using ModFit and CellQuest software is shown in Table 3. There was a trend towards a higher percentage of cells in the S phase for the CML group, while the values were not significantly different (P = 0.536).

DNA analysis of the control samples showed an abundance of G0/G1 phase cells, as expected; however, the CML and MM patients had more cells in the S phase (Figure), although the number of cells in the S phase was similarly low in the MM and control groups. As the number of control cells in the G2/M phase was insufficient, cyclin and CDKI measurements could not be performed in this group.

Distribution of cell Cycle Regulators in the Control Group According cell Cycle Phases

Comparison of the cell cycle-regulating elements cyclin A, B, D, and E, and CDKIs p16 and p21 in the control group showed that cyclin D was expressed most frequently during the G0/G1 phase; the other cyclins were present in 33% of the cells, and the cyclin E level was very low. Both CDK inhibitors (p16 and p21) were detectable, though p16 was expressed at a higher level. During the S phase all cyclins, except for cyclin E, were present in 66% of the cells. The relationship with p16 and p21 in the S phase was similar to the G0/G1 phase. No cells in the G2/M phase were detected in the control group.

Cell Cycle Regulators in the Patients

CML Patients

Expression of cyclins A, B, D, and E, and CDKIs p21 and p16 during the G0/G1 and S phases were similar in the CML patients and controls (P > 0.05); however, this was due to the similarity in the change observed from the G0/G1 to S phase in the controls. Cyclin D had the lowest level of expression of all the cyclins during the S phase, unlike in the control group (P < 0.05).

MM Patients

Distribution of cyclins during the G0/G1, S, and G2/M phases was similar in the MM and CML patients. Except for the G2/M phase, a similar pattern of cyclin and CDKI expression was observed in the controls and MM patients. Tables 4 and 5 summarize the results of the comparisons of cyclin and CDKI expression patterns during the cell cycle phases in the 2 patient groups and the control group.

Discussion

The present study used flow cytometry to analyze the expression of cyclins A, B, D, and E, and CDKIs p21 and p16 in patients with CML and MM, and controls. Expression of cyclins D and E is expected in eukaryotes during the G1 phase [22]. When passing through the R checkpoint cyclin type D is expressed [23]; thus, the expression of cyclin D as the major cyclin occurs during the G0/G1 phase [24]. Comparison of the G0/G1 and S phases in the

Table 4: Percentage of Cells Expressing Cyclins and CDKIs, According to Phase

G0/G1	Cyclin A	Cyclin B	Cyclin D	Cyclin E	p21	p16
CML	26	21.5	85.5	28.5	26.5	67.5
Control	31	37	85	10	41	63
MM	40	40	90	33	35	58
S	**Cyclin A**	**Cyclin B**	**Cyclin D**	**Cyclin E**	**p21**	**p16**
CML	82	71	45.5	10.5	36.5	76.5
Control	67	65	62	10	27	59
MM	63	65	70	40	22	61
G2/M	**Cyclin A**	**Cyclin B**	**Cyclin D**	**Cyclin E**	**p21**	**p16**
CML	76.5	70	67	10	20.5	67
Control	-	-	-	-	-	-
MM	72	66	78	24.5	35	62

Table 5: Comparison of Cyclins and CDKIs Between Phases in the Patient Groups

Group	Cyclin/CDKI	G0/G1-S		G0/G1-G2/M		S-G2/M
CML	A	P < 0.001	G0/G1 < S	P < 0.05	G0/G1 < G2/M	-
	B	P < 0.001	G0/G1 < S	P < 0.05	G0/G1 < G2/M	-
	D	P < 0.001	G0/G1 > S	-		-
	E	P < 0.05	G0/G1 > S	-		-
	p21	-		-		-
	p16	P < 0.05	G0/G1 < S	P < 0.05	G0/G1 < G2/M	-
Group	**Cyclin/CDKI**	**G0/G1-S**		**G0/G1-G2/M**		**S-G2/M**
MM	A	P < 0.01	G0/G1 < S	P < 0.05	G0/G1 < G2/M	-
	B	P < 0.05	G0/G1 < S	P < 0.01	G0/G1 < G2/M	-
	D	P < 0.01	G0/G1 > S	P < 0.05	G0/G1 > G2/M	-
	E	-		-		-
	p21	P < 0.05	G0/G1 > S	-		-
	p16	-		-		-
Group	**Cyclin/CDKI**	**G0/G1-S**				
Control	A	P < 0.01		G0/G1 < S		
	B	P < 0.05		G0/G1 < S		
	D	P < 0.01		G0/G1 > S		
	E	-				
	p21	-				
	p16	P < 0.05		G0/G1 > S		

present study showed that D-type cyclin expression was lower during the S phase (P < 0.01). According to the literature, cyclin E plays a role in the transition of cells from the G1 to S phase [25-27].

In the present study cyclin E expression in the control group during G0/G1 and S phases did not differ significantly. Flow cytometry may not be the ideal technique for differentiating early and late cell phases; thus, the finding that cyclin E expression during the S phase was similar to that during the G0/G1 phase might have been because the cells that were measured were in the early S phase. Cyclins A and B, which are referred to as mitotic cyclins, are initially produced following the start of the S phase, and then promote the subsequent phases of the cell cycle [8,28,29]. As the expression of cyclin A begins during the late G1-early S phase, cyclin B expression occurs during the late S phase and peaks through the G2 phase [30,31].

It is well known that cyclin A is responsible from the continuation of the S phase and DNA replication at this phase, expression of cyclin B triggers the end of the G2 phase and initiation of mitosis [32-34].

In the present study, in accordance with the literature, expression of cyclins during the S phase in the control group was significantly higher than that during the G0/G1 phase (cyclin A: P < 0.01; cyclin B: P < 0.05). As most of the cells in the control group were resting, the G2/M phase could not be observed. CDKI p16 expression in the control group was significantly higher during the G0/G1 phase than during the S phase (P < 0.05), whereas CDKI p21 did not follow this pattern.

In the present study's CML group expression of cyclin D, which belongs to the G1 cyclins group, was maximal during the G0/G1 phase, as expected. Cyclin D expression during the S phase was lower than that during the G0/G1 phase ($P < 0.001$). Based on data from the literature, cyclin D has an evident expression during the G0/G1 phase in CML, but can be higher during the later phases of the cell cycle [35-40].

Expression of the other G1 type cyclin in the present study—cyclin E—did not differ from that of cyclin D. Maximal expression of cyclin E was observed during the G0/G1 phase, which was higher than that during the S phase ($P < 0.05$). In the present study cyclin E expression during the last phase was significantly lower than during the G1 phase, which is agreement with Gong et al.'s results [38]. Cyclin E expression in the present study's CML group was comparable to that reported by Qin et al. [41].

In the present study expression of cyclin A—a mitotic cyclin—was similar in the CML and control groups. Cyclin A expression was higher during the S phase than during the G0/G1 phase ($P < 0.001$), and was higher during the G2/M phase than during the G0/G1 phase ($P < 0.05$), which is in agreement with Paterlini et al. [39]. Koeffler et al. compared the expression of cyclin A1 in normal and leukemic hematopoietic cell lines using RT-PCR and reported that this cyclin was overexpressed in the leukemic lines [42]. Kramer et al. used RT-PCR and reported that cyclin A1 was present in 84 of 113 CML patients [32].

In the present study expression of the other mitotic cyclin—cyclin B—was similar to that of cyclin A throughout the cell cycle. Cyclin B expression was significantly higher during the S phase than during the G0/G1 phase ($P < 0.001$), and was higher during the G2/M phase than during the G0/G1 phase ($P < 0.05$). Cyclin B expression during the S and G2/M phases did not differ significantly, as expected. Gorczyca et al. [31] reported cyclin B1 overexpression during the S phase fraction and in accordance with the present study although cyclin B1 expression was not observed during the S and G2/M phases in lymphocytes administered phytohemagglutinin to stimulate proliferation, its expression was high during all 3 phases in tumoral samples. In the present study cyclin B expression was observed during the first phase in the CML patients, which is similar to the results Ma et al. reported in acute leukemia patients [43].

The expression of p16 in the present study differed between the controls and patients; it occurred during both the G0/G1 and S phases in the control group, but during all 3 phases in the CML group, and its level of expression in the CML group during the S phase was significantly higher than that in the control group ($P < 0.05$). When the cyclin D levels of the G0/G1 and S phases were examined, there was no statistically significant difference between the CML and control groups.

In addition, while cyclin D expression was significantly higher during the G0/G1 phase than during the S phase in the CML group, expression of its inhibitor (p16) was lower during the G0/G1 phase than during the other phases, suggesting an imbalance that facilitates leukemic progression. Hirose et al. did not observe any p16 expression despite the fact that cyclin D1 and CDK4 expression was observed in 16 of the 17 lines they examined [44].

In the present study p21 was expressed at a similar level during all 3 phases in the CML patients; however, more importantly cyclin A expression was higher during the S phase in the control group than in the CML group ($P < 0.01$), whereas p21 expression was similar. This finding suggests another imbalance between cyclins and CDKIs that facilitates malignant cellular proliferation. Cyclin D expression in MM patients has been studied extensively [45-48]; however, findings concerning the phase during which its expression is highest are inconclusive. In some studies p16 protein was observed in mature cell lines, whereas cyclin D1 was highly expressed in immature cells [49].

Cyclin D1 expression was at its peak during the G0/G1 phase in the present study's MM group, as expected. Cyclin D1 expression was significantly higher during the G0/G1 phase than during the S phase in the MM group ($P < 0.01$), and was higher during the G0/G1 phase than during the G2/M phase ($P < 0.05$). Expression of cyclin D1—a G1 cyclin—during the S and G2/M phases did not differ significantly. A study by Sonoki et al. included 20 patients with plasma cell malignancies that were analyzed using the Northern blot. Cyclin D1 expression was observed in 6 of their 17 MM cases and all 3 plasma cell leukemia cases [45]. Pruneri et al. reported that the rate of cyclin D1 overexpression was 25% among 48 MM patients [46]. Hoechtlen-Vollmar et al. [50] reported cyclin D1 expression in 19 of 50 MM cases, which is similar to the present study's findings. The researchers also reported that beta-2 microglobulin and cyclin D1 amplification can be used together to predict duration of survival. Based on the present study's results and those previously published, cyclin D1 expression is highest during the first phase and gradually decreases during the subsequent phases.

In the present study's MM group p16 expression occurred at a similar level during all 3 phases of the cell cycle. CDKI p16 expression the in MM group was simi-

lar to that in the control group. The level of expression of p16 did not follow the overexpression of cyclin D during the G0/G1 phase, which was another imbalance between cyclins and CDKIs. Kawano et al. compared immature myeloma cell lines with mature myeloma and normal plasma cells, and reported that p16 expression was detected in the mature myeloma cells despite the absence of cyclin D1. Cyclin D1 was the dominant protein in the immature myeloma cells, whereas p16 was primarily expressed in normal plasma cells and mature myeloma cells. According to the researchers, p16 amplification was responsible for the loss of long-term proliferation in some of the cell lines [49].

Although maximal expression of cyclin E occurred during the G0/G1 phase in the present study, its expression did not differ significantly between the G0/G1 and the S phases, the G0/G1 and the G2/M phases, or the S and G2/M phases. To date, cyclin E has not been observed in MM. Cyclin A expression was higher during both the S ($P < 0.01$) and G2/M ($P < 0.05$) phases than during the G0/G1 phase for MM group.

There wasn't a significant difference in cyclin A expression between the S and G2/M phases. Urashima et al. reported that p21 had widespread expression in MM cell lines, independent of p53 [51]. They also reported that p21 expression increased following exposure to dexamethasone and downregulated by interleukin-6. During the resting period (G1 arrest) expression of p21 was significantly higher, but decreased during proliferation. Similarly, we observed an increase in cyclin A expression and a decrease in p21 expression during the S phase, as compared to the G0/G1 phase; however, in the control group cyclin A expression was significantly higher during the S phase than during the G0/G1 phase, whereas p21 expression did not differ. It can be an explanation to why proliferation is increased when compared with the controls. Expression of the other mitotic cyclin—cyclin B—was similar to that of cyclin A in the present study. Similarly, cyclin B expression was higher during the S ($P < 0.05$) and G2/M ($P < 0.01$) phases than during the G0/G1 phase, and there wasn't a significant difference between the S and G2/M phases. The S phase fraction percentages in the CML and MM groups did not differ significantly than those in the control group. As expected, the highest S phase rate was observed in the CML group, followed by the control group, and the lowest rates were noted in the MM group, but there was no statistically significant difference.

CML and MM present exhibit variable degrees of proliferation. It is noteworthy that in the present study we did not isolate plasma cells in the MM patients and that the findings reflect changes observed in bone marrow. Cyclins and their inhibitors in the present study exhibited different properties during the cell cycle phases checkpoints in the CML and MM patients. Additionally, among the cyclins we examined during different phases of the cell cycle, despite the finding that some of them exceeded the normal range, cyclin inhibitors not associating this increase may contribute the mechanisms effective in the etiopathogenesis.

In conclusion, the present findings indicate that synthetic CDKIs may be a promising new treatment for CML and MM.

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Conflict of Interest Statement

The authors of this paper have no conflicts of interest, including specific financial interests, relationships, and/or affiliations relevant to the subject matter or materials included.

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