

# The Prognostic Potential of Bone Marrow Cyclin E and P27, in Egyptian Patients with Acute Myeloid Leukemia

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Cyclin E and the cyclin dependent kinase inhibitor P27 are two important regulators of the G<sub>1</sub>-S transition modulating the activity of cyclin – dependent kinases. Aberrations in the cell cycle control are often observed in tumors and might even be mandatory in tumor development. There are few molecular biologic determinants that may be prognostic for patients with acute myeloid leukemia (AML). To investigate the importance of cell cycle defects in AML, the cellular levels of cyclin E and the cyclin-dependent kinase inhibitor P27 (Kip 1) were evaluated in thirty AML patients (11 males and 19 females) diagnosed by standard clinical, morphological and immunophenotypic criteria and staged according to the FAB classification. Using immunoblot analysis, cyclin E and P27 were detected in blast cells of AML patients who were then treated by the standard AML chemotherapeutic protocol and were followed up. With respect to cyclin E, it was detected in 9/30(30%) AML cases among them 13.3% (4/30 cases) exhibited very strong bands while 16.6% (5/30 cases) showed faint bands. Cyclin E was high among M4/M5 cases and low among M3 cases and showed a statistically significant positive correlation with percentage of blast cells, aberrant phenotype and abnormal karyotype at diagnosis. It also showed a significant negative correlation with complete remission (CR) rates. All our AML cases exhibited P27 at low and high levels as seen in 19/30 (63.4%) and 11/30 (36.6%) cases, respectively. P27 showed a statistically significant negative correlation to the percentage of blasts at diagnosis and a significant positive correlation with achievement of CR. A significant negative correlation between P27 and cyclin E ( $P < 0.004$ ) was observed as well. The present study suggested that levels of cell cycle regulators cyclin E and P27 can be used as a useful prognostic molecular markers in AML patients.

Acute myeloid leukemia is clinically and genetically heterogenous disease (Manola, 2009). Investigation on cell cycle regulatory molecules allows a closer approach to the biology of malignant cells, since loss of tissue homeostasis in tumors is related to the imbalance between proliferation and apoptosis (Radosevic *et al.*, 2001). It has been suggested that G<sub>1</sub>/S transition defects are mandatory in tumor development (Hirama and Koeffler, 1995). The G<sub>1</sub>-S transition is controlled by families of highly conserved proteins consisting of cyclin dependent kinases (CDKs) and sets of activating and inhibitory proteins (Sherr and Roberts, 1999). CDKs have their obligatory activating subunits, the cyclins. Five classes of cyclins have been identified in mammalian cells (Erlanson *et al.*, 1998).

Cyclin D1 and E sequentially activate CDKs triggering phosphorylation of key substrates such as retinoblastoma protein (Rbp), thereby initiating DNA replication and passage through the restriction checkpoint (Sherr & Roberts, 1999). Cyclin E production starts in mid G<sub>1</sub> phase and its expression peaks at the G<sub>1</sub>/S transition then decreases as cells proceed through S phase (Iida *et al.*, 1997).

Despite the fact that cyclin E is highly expressed in many tumors, it is not clear if it is a proto-oncogene or not. Interestingly, high cyclin E expression in breast cancer has been associated with increased risk of death (Erlanson *et al.*, 1998). However, the expression of cyclin E in AML was rarely reported (Radosevic *et al.*, 2001).

There are two families of CDK inhibitors negatively regulate the activity of the kinase

complexes contributing to proper control of G1/S transition. The INK family of proteins (P15, P16, P18 and P19) consists of specific CDK inhibitors mainly affecting the cyclin D-CDK4/CDK6 complexes (Erlanson *et al.*, 1998). The other class of inhibitors, the CIP/KIP family (including P21, P27 and P57), has a less selective inhibitory effect on many CDK-complexes with a main activity during G1 phase (Roy *et al.*, 2009). Genes coding for the INK family of CDK inhibitors are often mutated in tumors in contrast to the CIP/KIP family where mutations are rare (Radosevic *et al.*, 2001).

A CDK inhibitor P27 kip 1 binds CDK2 and interferes with all known G1 cyclin CDK complexes (Erol *et al.*, 2008). It is affected by intrinsic and extrinsic factors, such as transforming growth factor- $\beta$  (TGF- $\beta$ ), elevated cyclic adenosine monophosphate (cAMP) levels, interferon gamma (IFN- $\gamma$ ) and cell-cell contact all of which increase P27, and hence arrest in G1 phase: or cell cycle exit. Therefore, P27 is known to be triggered by antiproliferative signals and to play a key role in maintaining cells into a G0/G1 – arrested state. It declines as cells progress towards S phases (Sicinski *et al.*, 2007). Its low level of expression has been shown to be correlated with a high proliferative index and an aggressive disease in various tumors and in lymphomas (Erlanson *et al.*, 1998). Independently of its role as a CDK inhibitor, P27 Kip1 promoted stem cell expansion and functioned as a dominant oncogene *in vivo*. Therefore, it is a tumor suppressor by virtue of its cyclin – CDK regulatory function, and also an oncogene through a cyclin-CDK-independent function (Besson *et al.*, 2007).

In primary solid tumors such as breast cancer and colorectal carcinoma, low P27 expression was significantly associated with poor prognosis (Chetty, 2002). In hematologic malignancies, including malignant lymphoma and B-cell chronic lymphocytic leukemia

(CLL), low P27 level has been significantly associated with a poor prognosis (Vrhovac *et al.*, 1998). However, there is very little information on the significance of the level of P27 in myeloid malignancies (Radosevic *et al.*, 2001; Yokozawa *et al.*, 2000).

In the present study, we evaluated the prognostic relevance of expression of cyclin E and P27 at the protein level in AML patients.

## Material and Methods

### Subjects

This study included 30 newly diagnosed AML patients, 19 females and 11 males and their mean age was 35.6 $\pm$ 22.4 years. The patients were enrolled at the Oncology/Hematology clinic, Ain Shams University Hospital. All patients gave written informed consent to participate in the study and the study was approved by Ain Shams Medical Ethics Committee.

AML diagnosis was based on the standard clinical, hematological and immunophenotypic criteria. Patients were classified according to the FAB classification (Bennett *et al.*, 1985). Genetic analysis was performed as part of the protocols of diagnosis and follow up (Rooney, 2001). All patients were subjected to the same treatment protocols except for AML-M3 cases. All patients were followed-up clinically, and by hematological tests (complete blood count (CBC) and bone marrow (BM) examination) which were done routinely every month.

### Laboratory Investigations

CBC was performed using a coulter counter (model T660, Hielach, Florida, USA). Morphology and % of blasts were examined using Leishman-stained smears of peripheral blood samples and bone marrow aspiration. Cytochemical analysis was performed using peroxidase preparations for myeloperoxidase activity of the blasts in peripheral blood and BM smears (Catovsky, 1991).

Lactate dehydrogenase (LDH) enzyme level was measured in serum of AML patients using automated chemistry analyzer Synchron Cx9 (Bechman instrument Inc. Brea, California, USA).

Immunophenotyping was performed on cells obtained from BM specimens by RBCs lysis technique. The flowcytometric immunophenotyping was performed using single and dual colour analysis by means of fluorescein (FITC) and phycoerythrin (PE) labeled

monoclonal antibodies to various CD antigens including (CD34/HLADR, CD19/10, CD2/13, CD7/33, CD14, CD11b, CD5) (Beckman Coulter Inc. Marseille, France) using coulter Epics XL flow cytometer (Beckman Miami, Florida, USA). The results were analyzed against negative matched isotype controls to exclude non specific binding. Markers were considered positive if > 20% of cells were positive for the CD antigen except for CD34 where it is positive if > 10% of cells were positive according to the manufacturer's instructions.

Conventional cytogenetic tests were done using G-banding technique (ISCN, 1995). Briefly, metaphase preparations were obtained by cell culture of BM specimens on RPMI 1640 media (Biochrom, Berlin, Germany) containing 10% fetal calf serum (FCS), (Al Bardisi, Egypt from GIBCO, Grand Island, New York USA) and 10 µl penicillin/ streptomycin antibiotics (Seromed, Berlin). Set up of direct (within 2 hours of the preparation) and short term (24- and 48-hour) cultures were done with and without phytohemagglutinin under complete aseptic conditions. Cells were treated by colcemid 0.1 µg/ml (Biochrom, Berlin, Germany) for 90 minutes at 37°C prior to harvesting. Harvested cells were resuspended in chilled Carnoy's fixative and at least 10 slides were prepared for each sample. Proper slide aging and banding/counter staining was then performed using trypsin/Geimsa preparation. Slides were subsequently examined for karyotypic abnormalities.

Western Blotting technique adopted by Towbin *et al.*, (1979) was used for the detection of cyclin E and P27. Mononuclear cells were isolated from BM specimens using Ficoll Hypaque density gradient. Whole cell protein extracts were prepared by lysing  $4 \times 10^6$  mononuclear cells in RIPA buffer (10 mM tris HCl pH 7.5, 150 mM NaCl, 1% Nondipet-40, 0.1% sodium dodecyl sulfate (SDS), 1% deoxycholate, 10 µg/ml leupeptin and 10 µg/ml aprotinin) for 30 min on ice. Lysates were then centrifuged at 12,000 rpm for 15 min and supernatants collected, aliquoted and stored at -20°C till time of electrophoresis. Equal volumes of cell lysates (25 µl) were prepared in 2X SDS gel-loading buffer (25µl) (100mM tris HCl, pH 6.8; 4% SDS; 20% (v/v) glycerol; 1% (w/v) bromophenol blue) and heated at 95-100°C for 3 minutes. The samples (10µl) were loaded per each well of 12% SDS-polyacrylamide gel. A prestained low-range molecular weight (Mw) marker (7 µl) (supplied by BIORON, Germany) was included in a separate well to allow relative estimation of Mw of the target proteins. Electrotransfer on nitrocellulose sheets (Amersham Pharmacia Blotech UK Limited, Amersham place little

chalfon Buckinghamshire England HP7 9NA) was followed using a mini-transblot cell according to manufacturer's instructions (Scie-plas MVI-DC, U.K). The membranes were then blocked for 1 h in 5% (w/v) non fat dry milk in phosphate buffer saline (PBS), pH 7.5 at room temperature. The membranes then were incubated for 2 hours with mouse monoclonal antibody (mAb) against human P27 (R&D systems, Inc., Minneapolis, USA) and cyclin E 13A3:sc-56310 mouse mAb (Santa Cruz Biotechnology, Inc., 2145 Delaware Avenue Santa Cruz, California, USA) at concentration of 0.5 µg/ml (each mAb in a separate run). Nitrocellulose membranes (NC) were washed for 20 minutes with rocking (2 wash cycles) in buffer containing 10 mM tris PH 8.0, 200 mM NaCl and 0.1% Tween 20 to remove unbound materials. NC membranes were incubated with labeled-anti-mouse alkaline phosphatase (Oncogene Research Products 650 Albany Street Boston, MA02118) diluted 1: 1000 in 2% non-fat dry milk in PBS at room temperature for 30 min with rocking.. After three washings using buffer, reactions were detected by adding 10 ml freshly prepared chromogenic, substrate mixture [ nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate]. The colored reaction, which appeared as purple to dense blue bands, if any, were then washed with distilled water to stop reaction.

#### Statistical Analysis

SPSS (Statistical Program for Social Science) statistical software package (V. 17.0, Echosoft Corp., USA, 2008) was used for data analysis. Quantitative variables were reported as mean  $\pm$  SD, qualitative variables as numbers and percentages. Spearman rank correlation test was used to correlate categorical parameters. The probability level of  $\leq 0.05$  was considered significant (Clinton Miller & Rebecca Knopp, 1992).

## Results

Thirty AML patients were included in this study. Age mean $\pm$ SD was 35.6 $\pm$ 22.4 years. The patients (based on morphology of the cells, cytochemistry, and immunophenotyping) were classified according to the FAB into AML- M0 (n=3), AML-M1/M2 (n=14), AML-M3 (n=8), AML-M4/M5 (n=4) and M7 (n=1).

Immunophenotyping analysis revealed that 26.6% (n=8/30) of patients showed aberrant expression of CD markers; namely CD7 in

13.3% (n=4) of patients, aberrant CD19 and CD4, each in 6.7% (n=2).

Conventional cytogenetic analysis performed showed that 23.3% (n=7/30) patients had abnormal karyotype namely, Philadelphia chromosome, t (8;21), deletion of long arm of chromosome 9 (9q-), t (15;17) in two patients, monosomy 5 and supernumerary marker chromosome.

Thirty percent (n=9/30) of the study patients achieved complete remission (CR) after initial induction therapy. Twelve patients required more intensive therapeutic regimen either because they failed to go into remission or achieved only partial remission after initial induction therapy. Nine patients died during the course of therapy.

#### Expression of Cyclin E Protein in AML Patients

Immunoblot analysis showed that level of cyclin E protein expressed were detected in cells obtained from 30% (n=9/30) patients; of these 13.3% (n=4) displayed very strong bands and 16.6% (n= 5) had faint bands (Figure 1).

The percentage of cases with higher levels of cyclin E expression was observed in M4/M5 (n=3/4) than in M1/M2 and M3 blasts (n=4/14 and n=1/8, respectively) (Table 1).

Cyclin E expression showed a significant positive correlation with % of blast cells at diagnosis, aberrant phenotype and abnormal karyotype ( $P < 0.05$ ) while there was no significant correlation found between cyclin E expression and TLC, Hb level and LDH level ( $P > 0.05$ ) (Table 2).

CR rate by conventional chemotherapy was lower in patients with than without cyclin E positive as identified by immunoblot analysis (11.1% (n=1/9) versus 38.1%; n=8/21, respectively) (Table 2). Moreover, there was statistical significant difference between

positive and negative cyclin E expression (at the protein level) and the overall survival (Figure 3).

#### Expression of P27 Protein in AML Patients

P27 protein was expressed in all samples and its level of expression was variable. In AML patients, presented with low (63.3%, n=19/30) and high expression (36.6%; n=11/30) of P27 (Figure 2). Low levels of P27 were associated with M0/M1 cases except 1 case with AML-M1 showing low P27 whereas high levels of P27 was predominant in cases with M3 (Table 1).

To investigate the prognostic values of P27, we examined the association of P27 with known prognostic markers. No significant correlation was observed between P27 and TLC, LDH level, abnormal karyotype or aberrant CD markers, while a significant negative correlation was observed with the % blast cells and Hb level at diagnosis (Table 2).

High level of P27 expression was observed in cases with aberrant CD19, while low levels were observed in cases with aberrant CD7 or CD4 expression but these findings were not statistically significant ( $P > 0.05$ ).

The CR rate was in AML patients with high and low levels of P27 expression was 45.5% (n=5/11) versus 21% (n=4/19), respectively (Table 2).

Multivariate analysis for the potential prognostic factors including karyotypes, age, LDH value and TLC demonstrated that P27 status was an independent prognostic factor in predicting induction treatment success. However, there was a statistical significant difference between low and high P27 regarding the overall survival (Figure 4).

Table 1. Expression of Cyclin E and P27 among AML FAB Subtypes.

FAB subtypes	Cyclin E expression		P 27 expression	
	Positive	Negative	Low	High
M0 (n=3)	1*	2	3	0
M1/M2 (n=14)	4(n=2)*	10	10	4
M3 (n=8)	1	7	3	5
M4/M5(n=4)	3(n=1)*	1	2	2
M7 (n=1)	0	1	1	0
Total (n=30)	9	21	19/	11

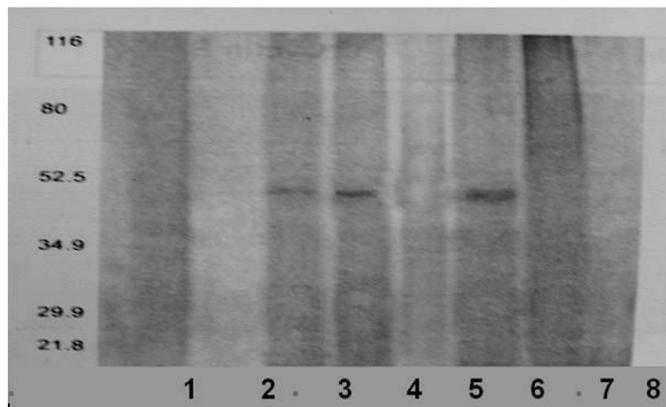
\* Four cases showed cyclin E bands of high intensity

Table 2. The significance of cyclin E and P27 protein expression in relation to the standard prognostic parameters

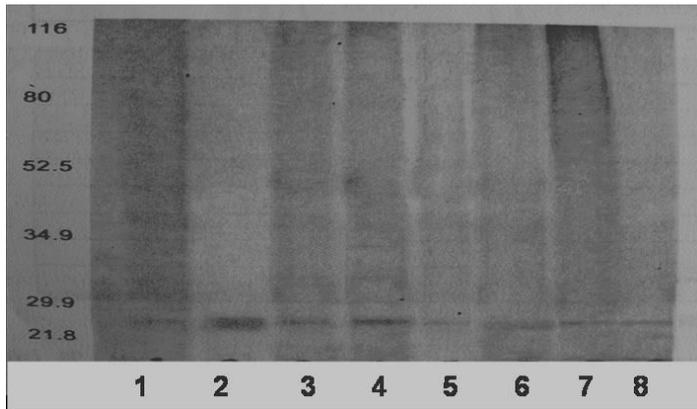
Parameter	Cyclin E expression		P value	P 27 expression		P value
	Negative (n=21)	Positive (n=9)		Low (n=19)	High (n=11)	
Age (mean±SD) in years	35.6±12.6	35.8±16.3		36.9±12.4	33.5±15.7	
% Total Leucocytes Count ( $10^3/\mu\text{l}$ )	98.1	84.7		82.3	78.6	
Hemoglobin (g/dl)	9.7	8.8		10.2	8.5	< 0.05
Blast cells%	70	90	< 0.05	82	69	< 0.05
Lactate dehydrogenase (U/L)	1052	920		880	600	
Aberrant CD% of Patient	19	33.3	< 0.05	26.3	18.8	
Abnormal karyotype% of patient	19	44.4	< 0.05	15.7	18.8	
CR rate %	38.1	11.1	< 0.001	21	45.5	< 0.001

Test used: Spearman rank correlation coefficient

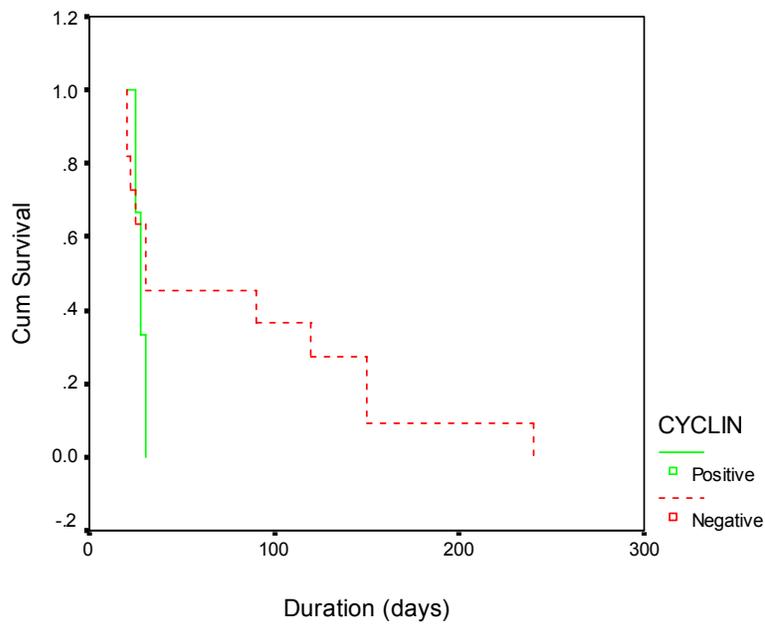
P < 0.05 Significant



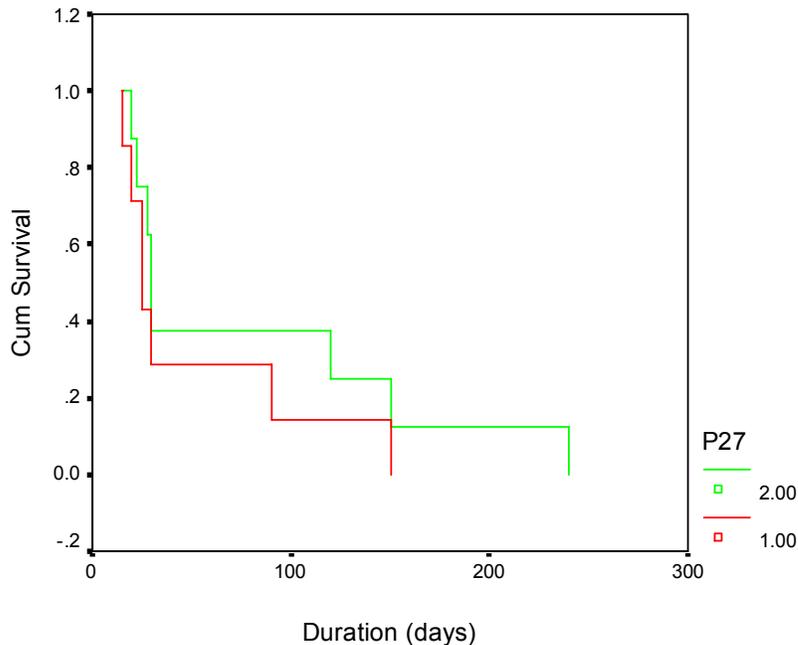
**Figure 1.** Cyclin E expression by Western Blot analysis showing positive Cyclin E bands (at 50 KDa) in lanes 3, 4 and 6. Lane 6 shows a strong band.



**Figure 2.** P27 expression by Western Blot analysis showing positive P27 expression in all lanes (at 27 KDa) with high expression at lanes 2, 4 and 6 and low expression at lanes 1, 3, 5, 7 and 8.



**Figure 3.** Kaplan Meier curve for Cyclin E. (Log rank =2;  $P < 0.05$ )



**Figure 4.** Kaplan Meier curve for P27. (Log rank =0.9;  $P>0.05$ )

## Discussion

Acute myeloid leukemia, a clonal hematological malignancy of myeloid cell line, is a heterogenous disease characterized by many different genetic defects (Mondal *et al.*, 2008).

In the present study we analyzed the protein expression of cell cycle regulators, cyclin E and P27 in blasts obtained from cases with AML. We attempted to assess the correlation of either or both of these two markers with the various known prognostic markers in AML.

In this study, levels of cyclin E protein were observed in one third of studied AML cases. Our data corroborated previous findings indicating that enhanced cyclin E expression does not only reflect the proliferation and differentiation status of CD34 marrow cells but in fact may be closely associated with leukemogenesis (Iida *et al.*, 1997). In addition, cyclin E expression was significantly higher among M4/M5 cases and

lower among M1/M2 and M3 cases as reported elsewhere attributing possible high correlation of cyclin E expression with monocytic leukemia in particular (Yokozawa *et al.*, 2000). The CR rate of patients with enhanced cyclin E expression were lower than in patients with low expression among M4 and M5 patients (Iida *et al.*, 1997). Furthermore, cyclin E expression showed a significant positive correlation with poor prognostic parameters including % blast cells, abnormal karyotype and aberrant phenotype and a significant negative correlation with CR rates. Event free survival was also previously observed to be longer in the cyclin E negative group than the cyclin E positive group (Radosevic *et al.*, 2001).

In this study, identified levels of cyclin E expression in AML reflect a more progressive disease that translates into an unfavorable outcome. Its expression might not only be associated with proliferation but rather indicative of a more malignant phenotype and

more likely to be linked to the induction of chromosomal instability. These biological functions of cyclin E were previously related to a poor prognosis when high cyclin E levels were found (Yasmeen *et al.*, 2003).

The expression of cyclin E2 has been confirmed, for the first time, to positively correlate with the survival rates in acute leukemia patients, implicating the poor prognosis of the disease (Wang *et al.*, 2006). The link between cyclin E and poor prognosis is well established in breast and lung cancer but is likely to be observed in other cancers as well (Yasmeen *et al.*, 2003; Muller-Tidow *et al.*, 2001). This is explained by the fact that a link between oncogenesis and cyclin has been made with the discovery of inappropriate expression of cyclins in tumors (Besson *et al.*, 2007).

It is also possible that small mutations of cyclin E gene itself are responsible for this overexpression. Minella and his coworkers (2008) reported that cyclin E (T74A T393A) mutation disrupted cyclin E periodicity and caused cyclin E to continuously accumulate as cells reentered the cell cycle from quiescence. *In vivo*, this mutation greatly increased cyclin E activity and caused proliferative anomalies.

P27 is a potent-cyclin dependent kinase inhibitor P27<sup>+</sup>/cyclin E<sup>-</sup> phenotype was found to be expressed in bone marrow biopsies in most cases of AML (Zola *et al.*, 2007). In the present study, all studied AML cases were positive for P27 but various levels of P27 expression were identified, as observed in another study (Manola, 2009). Notably, more than one-third of study AML cases exhibited higher levels of P27 expression. A significant negative correlation was observed between P27 expression and % of blast cells and Hb level at diagnosis both being known poor prognostic markers in AML. In addition, low P27 expression was associated with low CR rates and hence poor prognosis. Similarly, Lu and his coworkers (2004) found that in acute

leukemia, the effective rate (CR and PR) of initial chemical therapy in group of high P27 expression was higher than that in group of low P27 expression. Moreover, they found that the survival time in the former group was longer than in the later one. Other studies also demonstrated a statistically significant association between either low or moderate P27 expression and poor prognosis (Manola, 2009; Yokozawa *et al.*, 2000).

CD44, a cell surface molecule present on AML cells, ligation stabilizes the cyclin-dependent kinase inhibitor P27 protein, resulting in P27 accumulation and resulting in increased association with cyclin E/Cdk2 complexes and inhibition of their kinase activity (Gadhoun *et al.*, 2004). Considering that elevated expression of P27 is a factor of good prognosis in AML, these results provide a basis for developing CD44- targeted therapy in AML.

Low P27 expression has been associated with poor prognosis in solid tumors such as breast cancer, lung cancer, colorectal cancer, gastric cancer, prostate cancer and ovarian cancer suggesting that down regulation of P27 seems to be a general phenomenon in malignancies associated with tumor progression (Muller-Tidow *et al.*, 2001; Catzavelos *et al.*, 1997; Abukhdei & Park, 2008; Cote *et al.*, 1998; Rosen *et al.*, 2005; Chandramohan *et al.*, 2008). Some authors suggest that the supplementation of the cellular CDK inhibitors by pharmacological counterparts is a very promising therapeutic option (Wesierska-Gadek & Krystof, 2009).

In the present study, by excluding cases of AML M4 and M5 which were shown to express both cyclin E and P27, we obtained a negative correlation between cyclin E and P27 ( $P < 0.004$ ). This may explain why other similar studies who included all FAB subtypes in their analysis did not report a negative correlation for cyclin E and P27 (Iida *et al.*, 1997; Yokozawa *et al.*, 2000).

The explanation of the presence of high levels of both markers in monocytic leukemia may possibly be due to the close association of degradation activity with the level of each protein. The ubiquitin-proteasome pathway(s) controlling both degradation activities may be responsible for the simultaneous high expression (Zola *et al.*, 2007; Porter *et al.*, 1997).

The negative correlation of cyclin E and P27 identified in our study may be explained by the concept that P27 has been regarded as a negative regulator of cyclin E/CDK2 where the conventional view obtained from experimental models has been that CDK inhibitors oppose the action of CDKs in order to enforce cell cycle arrest. Increased cyclin E and decreased P27 induce CDK activation and promote cell growth. However, only few reports examined the correlation between cyclin E and P27 expression in primary human tumors (Zola *et al.*, 2007; Gadhoum *et al.*, 2004).

In conclusion, this study suggested that low P27 and high cyclin E expression could be indicative of poor disease outcome in AML cases, thereby might be additional prognostic parameters in AML disease. However, there seem to be unique characteristics for each subtype of AML particularly M4/M5 and M3 cases that may warrant further investigation.

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