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Prognostic significance of the cell cycle inhibitor p27^{Kip1} in acute myeloid leukemia

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There are few molecular biologic determinants that are prognostic for patients with acute myeloid leukemia (AML). Hence, we examined whether cellular levels of the cyclin-dependent kinase inhibitor p27^{Kip1} in acute myeloid leukemia could be used to predict clinical outcome in AML. Using immunoblot analysis, levels of p27 were assessed in blast cells from 72 AML patients who were registered and treated by the identical chemotherapy protocol. AML cases were classified into three groups on the basis of the percentage of the expression level of p27 compared to a control cell line. AML cases exhibiting p27 expression at low, moderate, and high levels were 43, 9, and 20 cases, respectively. No significant differences in the rates of complete remission (CR) were observed among the three groups. Although the level of p27 expression was not correlated with any other possible prognostic markers, such as age, white blood cell count, chromosome abnormalities, and FAB subclasses, patients with high p27 expression had a significantly increased disease-free survival (DFS) (78% *vs* 19%, $P = 0.004$). We further examined the expression of cyclin E at the protein level in all 72 AML cases. We observed a statistically significant correlation between a high cyclin E level and a high p27 level ($P < 0.005$). However, we failed to find any correlation between the rates of CR or DFS and cyclin E expression. The present study reveals that levels of p27 expression can be one of the useful prognostic molecular markers for AML. *Leukemia* (2000) **14,** 28–33.
Keywords: cyclin-dependent kinase inhibitor; p27; AML; prognosis

Introduction

Progression through the various phases of a eukaryotic cell cycle depends upon the activity of specific cyclin-cyclin-dependent kinase (CDK) complexes.[1,2] The G1-S transition is regulated by several CDKs; CDKs are activated by the binding of cyclins and inactivated by CDK inhibitors. A CDK inhibitor, p27Kip1, binds CDK2 and can interfere with all known G1 cyclin-CDK complexes. p27 is affected by intrinsic and extrinsic factors, such as transforming growth factor-β (TGF-β), cell–cell contact, and elevated cyclic adenosine monophosphate (cAMP) levels causing increased expression of the inhibitor with subsequent arrest in G1 phase.[3–5] In fact, p27 modulation may be an essential component of mitogen-dependent cell cycle entry and exit.[2] Multiple post-translational modification regulates p27 abundance. P27 is degraded by the ubiquitin–proteasome pathway.[6] Translational control also regulates p27 abundance: increased p27 translation rates in arrested *vs* growing cells.[7] In somatic cell lines, p27 has a role in limiting the rate of G1-to-S transition during proliferation.[8,9] Evidence that p27 may be involved in human tumor progression comes from several studies that measured the expression of p27 at the protein level.[10] In primary solid tumors such as breast cancer and colorectal carcinoma, a low p27 level has been revealed to be a poor prognostic marker.[11–15] In hematological malignancies, low p27 expression is significantly associated with a poor prognosis of malignant lymphoma, while an inverse relation has been reported between p27 expression and an overall prognosis in B-CLL.[16,17] There is very little information on the significance of p27 expression in myeloid malignancies. In the present study, we have examined the expression of p27 at the protein level in 72 acute myeloid leukemia (AML) patients who were treated by the identical chemotherapy protocol, and analyzed the statistical significance for disease prognosis.

Based largely on *in vitro* experiments and *in vivo* overexpression studies, CDK inhibitors of the Cip/Kip family were initially thought to interfere with the activities of cyclin D-, E-, and A-dependent kinases. Activation of cyclin E/CDK2 is coupled to the site-specific autophosphorylation and ubiquitine-dependent degradation of cyclin E.[18] These suggest the importance of the accumulated cyclin E protein and confirm the necessity for an analysis of cyclin E expression at the protein level.[19,20] In contrast to disruption of RB or p16 function, which is commonly observed in human cancers, cyclin E amplification is rare and gain-of-function mutations of cyclin E have not been found. Actually, in human cancers, only the expression of cyclin E at the protein level was correlated with poor prognosis, as first demonstrated for breast and colon cancers[11,12] and, more recently, for other tumor types.[19,20]

The present study shows that p27 is also associated with increased disease-free survival (DFS) in AML as the cumulative data indicate that low or absent p27 protein in tumor cells is an important clinical marker of disease progression.

Materials and methods

Patients

The 72 patients in this study were recruited from the Japan Adult Leukemia Study Group (JALSG) treatment protocol AML-92.[21] The genetic analyses were performed as part of the protocols, which procured bone marrow samples prospectively from patients entered in the JALSG treatment studies. None of these samples were included in the samples analyzed in the previous report.[22] The diagnosis of AML was made with bone marrow smears according to the French–American–British (FAB) classification, and confirmed by the central review. Patients with AML-M3 or with a history of preceding myelodysplasia were excluded from these studies.

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JALSG AML-92 is a treatment protocol in which induction chemotherapy consists of daunorubicin (40 mg/m^2 for 4–6 days) and N^4-behenoyl-1-β-D-arabinofuranosyl-cytosine (200 mg/m^2 for 10 days) with or without etoposide (100 mg/m^2 for 5 days). Patients who achieve complete remission (CR) receive three courses of consolidation therapy followed by six cycles of maintenance therapy. The median survival for our 72 patients was 23 months, with a median follow-up time of 43 months (range 22–64 months).

Extraction of whole cell lysate

The 72 AML specimens included 6 M0, 18 M1, 27 M2, 16 M4, 4 M5 and 1 M6 by FAB classification. Blast cells (⩾90%) enriched by Ficoll–Hypaque density gradient and stored in liquid nitrogen were subjected to quick thawing and extraction of whole cell lysate. Viability was assessed by the trypan-blue exclusion test, and every sample contained over 90% viable cells. Whole cell lysates were extracted as reported previously.[23] In brief, the cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.5, 0.3 M NaCl, 1% Nonidet P-40, 0.1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM EDTA) containing 20 μg/ml aportinin, 20 μg/ml leupeptin, 10 mM sodium pyrophosphate, 50 mM sodium fluoride, and 1 mM sodium vanadate. After incubating for 1 h at 4°C, lysates were centrifuged at 17 000 *g* for 20 min. The protein concentration of the lysates was examined with a commercial kit (BioRad Protein Assay, BioRad Laboratories, Hercules, CA, USA).

Western blot analysis

Equal amounts of whole cell lysates (10 μg) were separated by electrophoresis through a 12% SDS polyacrylamide gel. Proteins were electroblotted on to PVDF membranes and probed with monoclonal antibody to p27 or cyclin E (Pharmingen, San Diego, CA, USA). The quality and quantity of the lysates were confirmed by Coomassie blue staining of the gels and reprobing the blots with anti-actin antibody, as we described previously.[22] Immunoreactive proteins were detected using an anti-mouse secondary antibody conjugated to horseradish peroxidase followed by ECL (Amersham, Buckinghamshire, UK). The identical sets of membranes were subjected to SuperSignal ULTRA Chemiluminescent Substrate (Pierce, Rockford, IL, USA) followed by an image analyzer (Molecular Imager System GS-525, BioRad Laboratories). The p27 and cyclin E values were respectively calculated as the ratio of intensity of the band in each sample compared to that in a positive control, U-937 cells, as we described previously.[22] The lysate of this control was obtained from one sampling. To confirm that the immunoblot analysis and signal quantitation were accurate throughout the range of proteins found in the samples, we generated a standard curve by serial dilution of U-937 and compared it to representative samples. The samples were classified into one of the following three groups on the basis of percentage of the expression level of p27 compared to a control cell line, U-937: low (<10%), moderate (10%–19%) and high (⩾20%). Similarly, the samples were also classified into two groups, low (<10%) and high (⩾10%), according to the expression level of cyclin E.

Statistical methods

Differences in the distribution of clinical and biological features among subgroups of patients were analyzed by chi-square test for categorical data and by Wilcoxon rank-sum test for continuous data. Disease-free survival (DFS) for patients who had achieved CR was measured from the day of achieving CR to relapse or death. Patients who underwent bone marrow transplantation during their first CR ($n = 4$) were censored at the time of transplantation. DFS was estimated by the Kaplan and Meier method with 95% confidence intervals (95% CIs) calculated using Greenwood's formula.[24,25] The influence of potential prognostic factors was estimated by Cox's proportional hazard model.[26] The variables selected were age, sex, performance status (PS), peripheral white blood cell (WBC) count, platelet count, presence of Auer rods, FAB classification, myeloperoxidase positivity of blasts, LDH value, number of courses of induction therapy required to obtain CR (one *vs* two), karyotype (favorable: t(8;21), inv(16), other: normal karyotype and any other cytogenetic abnormalities), and the level of expression of p27. A stepwise multivariate approach was used to identify the most important predictor variable with respect to DFS. A P value ⩽0.05, after adjustment for the effects of other variables, was required for retention in the model. Statview Version 5.0 software (Abacus Concepts, Berkeley, CA, USA) was used for the analyses.

Results

Expression of p27 in AML

To investigate the prognostic values of p27 and the possible correlation with other factors, we first examined the relative expression level of p27 gene product compared to actin protein by immunoblot analysis. A representative immunoblot analysis is shown in Figure 1. Immunoblot with anti-actin antibody was performed to confirm the protein integrity. Of 72 AML cases, 43 (59.7%), 9 (12.5%), and 20 (27.8%) presented low, moderate, and high expression of p27, respectively (Table 1). To perform an initial assessment of the potential clinical utility of p27 in AML, we determined the association of p27 level with known prognostic markers of AML. No sig-

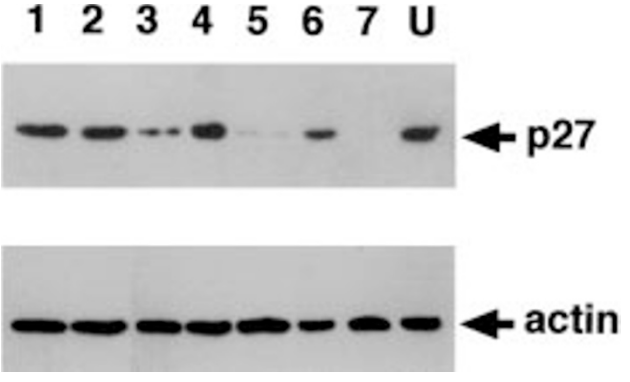

Figure 1 The expression of p27 gene product in AML. Each lane of upper and lower panels shows equal amounts (10 μg) of whole cell lysates from the same AML samples. U, lysate from U-937 (10 μg) used as a control. Upper panel: p27 immunoblot. Lanes 1, 2, 4, and 6 exhibit high, and lane 3 moderate, lanes 5 and 7 low p27 expression, respectively. Lower panel: immunoblot probed with anti-actin antibody.

Table 1 Patient characteristics and p27 expression in 72 patients with AML

	p27: low (n = 43)	*p27: moderate (n = 9)*	*p27: high (n = 20)*	*P value*
Age (years) (median)	16–77 (53)	16–67 (49)	17–75 (49)	NS
WBC (/mm^3) (median)	800–337600 (42500)	2400–129600 (73900)	2300–249200 (21550)	NS
LDH (IU/l) (median)	179–3275 (889)	415–14500 (1435)	109–5320 (600)	NS
FAB classification				NS
M0	5	0	1	
M1	13	2	3	
M2	15	4	8	
M4	8	1	7	
M5	2	1	1	
M6	0	1	0	
Favorable chromosome	4	1	4	NS
MPO positive (>50%)	20	4	9	NS

Range and median values and number of cases are noted.
Differences are calculated with chi-square test for categorical data and Wilcoxon rank-sum test for continuous data.
Favorable chromosome includes t(8;21) and inv(16).
MPO, myeloperoxidase; NS, not significant.

nificant association of p27 was observed with the factors including karyotype, WBC count and LDH level at initial diagnosis (Table 1). Samples categorized to M0 or M1 by FAB classification seemed to be more associated with low p27, whereas samples of M4 with high p27 but no statistical significance were observed among FAB subclasses.

Prognostic value of p27 expression level

CR rates of patients in the groups with low, moderate, and high expression of p27 were 74.4% (32/43), 77.8% (7/9), and 75% (15/20), respectively, accounting for 75% of the overall CR rate. This suggests that the levels of p27 expression did not correlate with the response to remission induction therapy in these patients. We thus analyzed the DFS among patients who achieved CR according to p27 expression. Estimated DFS rates at 4 years were 78% (95% CI = 56%–99%), 43% (95% CI = 6%–80%), and 13% (95% CI = 0%–32%) for high (⩾20%), moderate (10%–19%), and low (<10%) p27 groups, respectively ($P = 0.014$ by logrank test) (Figure 2a). When the patients were divided into two groups using 20% as the cut off value, DFS were 78% (95% CI = 56%–99%) for high, p27 group (⩾20%), and 19% (95% CI = 0%–38%) for either low, or moderate, p27 group (<20%), respectively ($P = 0.004$ by logrank test) (Figure 2b). When using 10% as the cut off value, patients with low, p27 expression (<10%) also had a significantly reduced DFS ($P = 0.010$ by logrank test) (Figure 2c). The minimal overlap of the CIs reflects the strong association of p27 expression with clinical outcome in patients with AML. These results lend support to our suggestion of a significant correlation between p27 expression and the prognosis in AML and give us the rationale to arbitrarily use 20% of positive control as the cut off for survival analysis. We thus use 20% as the cut off for multivariate analysis of DFS. A multivariate analysis from potential prognostic factors including karyotype, age, LDH value, and WBC count, demonstrated that p27 status was an independent prognostic factor in predicting DFS with significance (relative risk = 3.85, 95% CI = 1.12–12.5; $P = 0.032$).

Association of p27 expression with cyclin E expression

The level of cyclin E in each sample was similarly analyzed by immunoblot analysis. Of 72 AML cases, 52 (72.2%) and 20 (27.8%) patients presented low and high expression of cyclin E, respectively (data not shown). CR rates of patients with low and high expression of cyclin E were 73.1% and 80.0%, respectively, suggesting that the level of cyclin E did not correlate with the CR rate. Of 54 patients who achieved CR, the estimated DFS at 4 years was 25.2% (95% CI; 36.9–88.9%) and 62.9% (95% CI; 8.4%–42.0%) for patients with low (<10%) and high (⩾10%) expression of cyclin E, respectively ($P = 0.063$, logrank test). No statistical significance of DFS was observed with the difference of cyclin E expression. Interestingly, a significant correlation was observed between the cyclin E level and p27 level ($P < 0.0005$) (Figure 3).

Discussion

We have already reported some data on p27 expression at the protein level in a smaller number of AML cases (37 cases). 48.6% of the cases exhibited high p27 expression, however, the analyzed samples were chosen according to cyclin E expression to determine the correlation of p27 with cyclin E.[22] It was virtually impossible to draw any definitive conclusion on the significance of p27 expression in AML from the previous study probably due to the small number of patients treated with various chemotherapy protocols. The patient series in the present study seemed to represent a nonbiased material of AML based on general patient characteristics and survival rates. All patients analyzed in the present study were registered in the identical treatment protocol, JALSG AML-92, in which 655 patients with AML had been enrolled with a median follow-up of 44 months. The CR rate of the whole 655 patients was 76%, and the 4-year DFS of CR cases was 35%, respectively.[21] These are equivalent to the data obtained from the present 72 patients (75% CR rate and 34% 4-year DFS). In addition, no differences in the distribution of patient characteristics including age, LDH, FAB, and chromosomal abnormalities were observed between our present series and

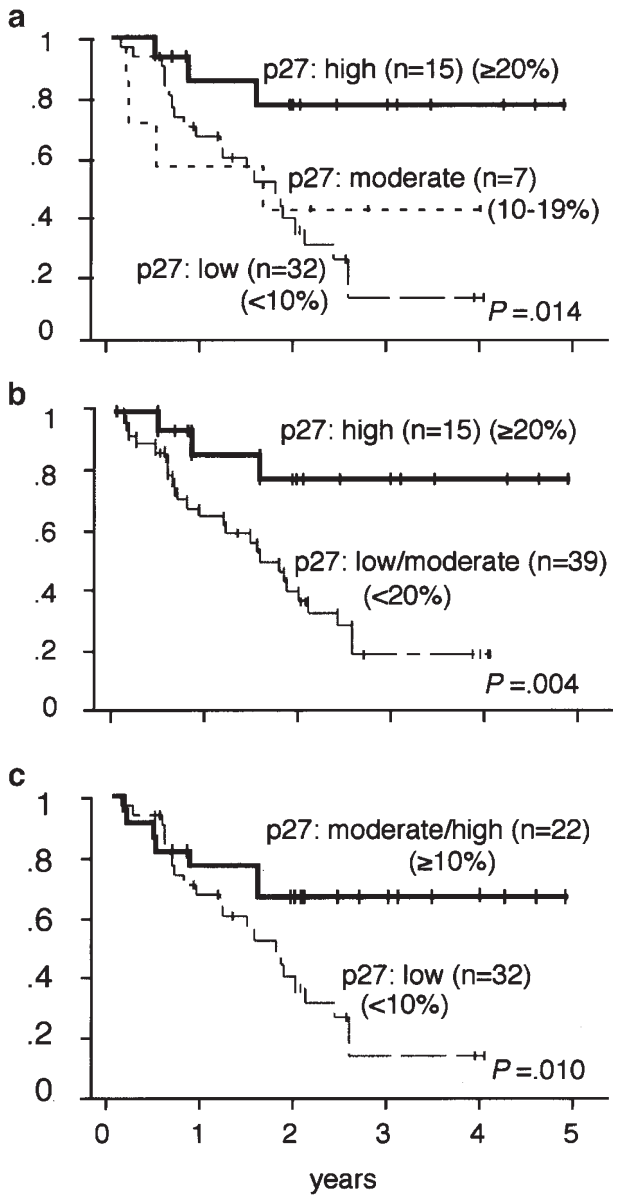

Figure 2 DFS for 54 CR patients based on the level of p27 expression in (a) low (n = 32), moderate (n = 13), and high (n = 9) by the cut off of 10% and 20%; (b) low (n = 32) and moderate/high (n = 22) by the cut off of 20%; (c) the classification into two groups by the cut off of 10%: The P value was based on the logrank test.

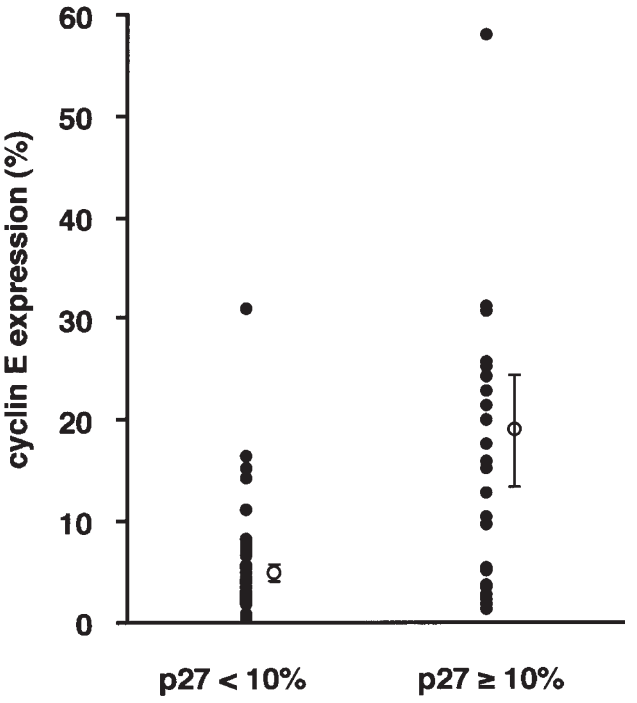

Figure 3 Cyclin E expression in AML cells according to p27 expression. The expression of cyclin E was analyzed by immunoblot analysis. Relative intensities of cyclin E level were classified into two groups according to the levels of p27 expression. Error bars show standard errors. The samples with low p27 expression (<10%) showed significantly lower cyclin E expression than those with moderate or high p27 (⩾10%) ($P < 0.0005$).

the whole 655 patients. Our patient series exhibited a statistically significant association between either low or moderate p27 expression and poor prognosis by univariate analysis despite the limited number of cases analyzed ($P = 0.004$, cut off 20%). When additional factors were introduced by multivariate analysis, the prognostic impact decreased and exhibited marginal significance ($P = 0.032$). This may partly indicate an association between p27 and other prognostic factors, but a large-scale study must further clarify the impact.

Low p27 expression has been associated with a poor prognosis in tumors such as breast cancer,[11,13] lung cancer,[27–29] colorectal cancer,[12] gastric cancer,[30] and prostate cancer,[14] suggesting that downregulation of p27 seems to be a general phenomenon in malignancies associated with tumor progression. In this study, however, no inverse correlation was observed between p27 expression and WBC count at diagnosis. Low p27 does not necessarily imply a stepwise-increased cell growth of AML blasts in association with high WBC count. Consistent with this, p27 expression was not correlated with S-phase fraction by the same analysis as we used previously (data not shown).[22] The expression of p27 seems not to reflect the cell cycle in AML, as many investigators have pointed out in other types of cancer.[16]

The level of cyclin E expression did not affect the prognosis in AML patients in the present study. Our previous report showed that high cyclin E expressors had a worse prognosis.[22] This analysis indicates that the cases with high cyclin E conversely tend to show better DFS, although no statistical significance was observed. This discrepancy may partly reflect the fact that our former study included a higher percentage of M4/M5 of FAB, which subclasses tended to accompany high expression of cyclin E and poor prognosis. Otherwise, the sample number analyzed may not be enough to detect the significance, if any, of cyclin E expression on DFS in AML. In solid tumors, high cyclin E has been reportedly associated with poor prognosis.[11,12,19,20] The reason why cyclin E in AML plays different roles, if any, from those in solid tumors remains unknown. One simple explanation is that the altered expression of cyclin E occurs as a merely bystander effect and is not involved in characteristics of AML. Another is that the effect of high cyclin E expression may be inhibited by high p27 expressed simultaneously in AML. Finally the seeming significance in event-free survival may be partly regarded as due to the small sample number analyzed. Taking into account the fact that the present cases analyzed were treated with a strict protocol, the level of cyclin E expression may not be an important prognostic marker.

In the previous study, a very close correlation was observed between high cyclin E and high p27 ($P < 0.001$) from the small number of cases examined ($n = 37$). The association is now confirmed statistically ($P < 0.0005$) by the present study. The abundance of cyclin E and p27 proteins is thought to be regulated by both translational and post-translational pathways, and less commonly at the level of transcription.[2,6] 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, there are few reports which examined the correlation between cyclin E and p27 expression in primary human tumors. The present data, which are consistent with our previous report, indicate the correlation of cyclin E expression with p27 expression in *de novo* AML. It has been reported that, in breast cancer cell lines, the levels of p27 were associated with the levels of cyclin D1 and E.[31] Deregulated expression of p27 throughout the cell cycle was speculated to be responsible for it. The mechanism underlying the upregulation of p27 in cyclin E overexpressors still remains to be elucidated. Another possible explanation may be derived from clinical samples showing that protein levels of p27 are closely associated with the degradation activity of p27.[12] Although a study of 15 human colorectal carcinomas exhibited no correlation between p27 degradation activity and cyclin E degradation activity, it is still possible that ubiquitin–proteasome pathway(s) controlling both degradation activities may be responsible for the simultaneous high expression. Accordingly, we simultaneously tried to detect the p21 expression from the part of lysates analyzed in the present study. Probably due to the degradation by proteasome,[32–34] we were unable to detect p21 expression in any samples analyzed by conventional immunoblot analysis with two different commercially available anti-p21 antibodies (data not shown). In any case, the mechanism and relevance of the potential correlation between p27 and cyclin E expression in AML remain to be elucidated in more detail.

Although the sample size analyzed in the present study is relatively small, our patient series seemed to represent non-biased AML based on general patient characteristics and outcome. The data from our series of AML patients treated with an identical protocol suggest that p27 expression may be an important parameter that could be included in a future panel of prognostic markers for AML. Using the same methods described here, large-scale analysis of p27 expression among patients enrolled in a unified protocol will help to better define how p27 expression should be used in managing patients with AML.

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