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Cytoplasmic Mislocalization of p27Kip1 Protein Is Associated with Constitutive Phosphorylation of Akt or Protein Kinase B and Poor Prognosis in Acute Myelogenous Leukemia

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ABSTRACT

Cyclin-dependent kinase inhibitor p27Kip1 functions at the nuclear level by binding to cyclin E/cyclin-dependent kinase-2. It was shown that Akt or protein kinase B (Akt/PKB)-dependent phosphorylation of p27Kip1 led to the cytoplasmic mislocalization of p27Kip1, suggesting the potential abrogation of its activity. Here, we evaluated the localization of p27Kip1 protein in leukemic blasts in relation to Akt/PKB phosphorylation and clinical outcomes in acute myelogenous leukemia (AML). Western blot analysis of the nuclear and cytoplasmic fractions revealed a heterogenous localization pattern of p27Kip1 in AML. Cytoplasmic mislocalization of p27Kip1 was significantly associated with the constitutive serine^{473} Akt/PKB phosphorylation in AML cells ($P < 0.05$). Transfection of U937 cells with an expression construct encoding the constitutively active form of Akt/PKB resulted in a remarkable increase in the levels of cytoplasmic p27Kip1. Whereas the transfection of U937 cells with a construct encoding dominant-negative Akt/PKB resulted in a recovery of nuclear localization of p27Kip1. Both the disease-free survival and overall survival are significantly shorter in AML cases with high cytoplasmic to nuclear ratio of p27Kip1 localization compared with the cases with low cytoplasmic to nuclear ratio ($P = 0.0353$, $P = 0.0023$, respectively). Multivariate analysis indicated that the cytoplasmic to nuclear ratio of p27Kip1 localization was an independent prognostic variable for both disease-free survival and overall survival ($P = 0.043$, $P = 0.008$, respectively). These findings additionally extend our understanding of the role of p27Kip1 in AML, and buttress the case of p27Kip1 mislocalization as a prognostic indicator and Akt/PKB/p27Kip1 pathway as a ready target for antileukemia therapy.

INTRODUCTION

The cyclin-dependent kinase (CDK) inhibitor p27Kip1 is an important regulator of G_1 progression and negatively regulates cell proliferation. It is expressed highly in G_0, where it binds tightly and inhibits cyclin E/CDK-2 (1–4). The p27Kip1 protein levels are regulated predominantly by ubiquitin/proteasome-mediated proteolysis (4, 5) in a process that requires its association with cyclin/CDK complexes (6, 7) and phosphorylation of the threonine (Thr)^{187} of the p27Kip1 protein (6, 8, 9). The expression of S-phase kinase-associated protein 2, a member of the F-box family of the specific substrate-recognition subunit of Skp1/Cull/F-box (SCF) ubiquitin-protein ligase complexes (10) is required for the ubiquitination and subsequent degradation of p27Kip1 (11–15).

Although p27Kip1 is rarely mutated in human cancers, there are considerable evidences that the inactivation of p27Kip1 is a fundamental step for the development of malignancies (16–19). The reduced expression of p27Kip1, because of increased protein degradation, correlates with the poor prognosis of patients with various types of cancer (16, 17, 19–23). Paradoxically, some tumors may contain elevated levels of p27Kip1 protein (17, 20, 23), suggesting that they have developed other mechanisms to circumvent p27Kip1 growth inhibition. High levels of p27Kip1 might inhibit the efficacy of chemotherapeutic agents, which rely on the interfering with the cell proliferation to trigger a cytotoxic response (18, 24). This opposing effect of p27Kip1 as a prognostic relevance prompts additional efforts to understand the mechanisms involved in the modulation of the p27Kip1 activity and its consequences on the therapeutic outcomes in specific tumors.

In human tumors, p27Kip1 protein itself, or its activity, appears to be lost by increased cytoplasmic mislocalization or sequestration as well as increased degradation (25). In tumors with abundant p27Kip1 expression, the protein is often mislocalized to the cytoplasm (26–28). Because the growth-restraining activity of p27Kip1 depends on its nuclear localization, the cytoplasmic mislocalization can effectively inactivate the p27Kip1 inhibitory activity (26, 29–32). Cytoplasmic p27Kip1 appears to directly correlate with a poor prognosis and advanced tumor grade of esophagus and breast carcinoma (28, 33, 34). These findings suggest that the elucidation of the mechanisms implicated in the regulation of p27Kip1 mislocalization can provide the insight into the aberrant p27Kip1 regulatory pathway and its role in tumor cells.

Akt or protein kinase B (Akt/PKB) is a central component of the phosphatidylinositol 3′-kinase pathway (35–37), a pathway replete with oncogenetically relevant molecules (38). Akt/PKB can down-regulate the p27Kip1 by increasing its proteolysis (39) or repressing its expression through Akt/PKB-mediated phosphorylation of the forkhead transcription factor (40). Recently, a novel mechanism of Akt/PKB-mediated p27Kip1 regulation was demonstrated (34, 41). The p27Kip1 is phosphorylated at Thr^{157} by Akt/PKB both *in vitro* and *in vivo* (33, 41). This Akt/PKB-dependent phosphorylation results in an impairing of nuclear import of p27Kip1 protein, leading to its cytoplasmic mislocalization and abrogation of its cyclin E/CDK2 inhibitory activity (33, 41). The Akt/PKB phosphorylation has been shown to correlate statistically with the cytosolic mislocalization of p27Kip1 in tumor cells (33, 41). These data indicate that Akt/PKB activation may contribute to the tumor-cell proliferation via phosphorylation and cytoplasmic retention of p27Kip1, thus, relieving CDK2 from the p27Kip1-induced inhibition.

We demonstrated previously that the constitutive phosphorylation of Akt/PKB was observed in a substantial proportion of acute myelogenous leukemia (AML) and associated with a poor prognosis (42). However, the cytoplasmic mislocalization of p27Kip1 protein in relation to Akt/PKB phosphorylation and clinical outcomes has not been evaluated in AML. In this study, the constitutive phosphorylation of Akt/PKB was correlated highly with the cytoplasmic mislocalization of p27Kip1 protein in AML. The induced overexpression of Akt/PKB activity in leukemia cells resulted in a markedly increased localization

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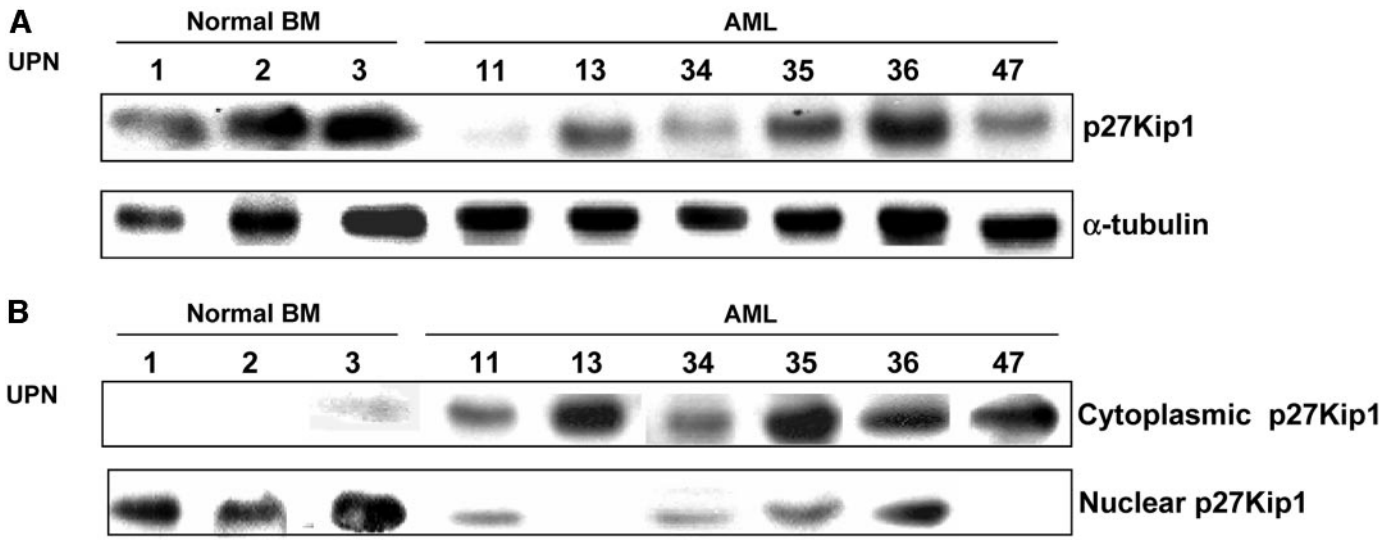

Fig. 1. Western blot analyses of the p27Kip1 protein expression and localization in the representative acute myelogenous leukemia (*AML*) and normal bone marrow (*BM*) samples. *A*, the levels of p27Kip1 protein expression were variable according to the samples. The α-tubulin was used as a control. *B*, the subcellular localization of the p27Kip1 protein was analyzed by Western blotting of the nuclear and cytoplasmic fractions of the cell lysates as described in "Materials and Methods."

of p27Kip1 to the cytoplasm. The pronounced cytoplasmic mislocalization of p27Kip1 was significantly associated with the reduced survival in AML and remained an independent prognostic factor in a multivariate analysis. These findings additionally extend our understanding of the role of p27Kip1 in AML and support the case of p27Kip1 mislocalization as a prognostic indicator and the Akt/PKB/p27Kip1 pathway as a ready target for antileukemia therapy.

MATERIALS AND METHODS

Patients and Treatment. A total of 99 consecutive adults with *de novo* AML who had not received treatment were included in the study. According to the French-American-British classification, 4 patients had M0, 20 M1, 33 M2, 19 M4, 21 M5, and 2 M6 subtype. Patients with AML (M3) were excluded from the study. Eighty patients received induction chemotherapy, comprising of cytarabine (100 mg/m^2/d by continuous infusion for 7 days) and idarubicin (12 mg/m^2/d IV bolus for 3 days). Complete remission was defined as the normalization of blood counts and bone marrow morphology and the disappearance of all signs of leukemia, for at least 4 weeks or longer, in accordance with the recommendations of the National Cancer Institute-sponsored Workshop (43). All of the patients achieving complete remission then received the same two courses of consolidation chemotherapy consisting of cytarabine (1g/m^2/d IV infusion for 2 h every 12 h for 4 days), mitoxantrone (12 mg/m^2/d for 3 days), and VP-16 (100 mg/m^2/d for 2 days) as described previously (44).

Isolation of Leukemic Cells. In conjunction with the institutional review board-approved treatment protocol, bone marrow aspirates were prepared prospectively from the patients before the initiation of chemotherapy. Marrows were sedimented on a Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden) density gradient. After washing the mononuclear cells collected from the upper interface, T-cell depletion was performed using a high-gradient magnetic cell separation system/anti-CD3 monoclonal antibody (Miltenyi Biotech, Auburn, CA) according to the manufacturer's instructions. A morphological evaluation indicated that >95% of the isolated cells were leukemic blasts.

Cell Line. The U937 human leukemia cell line was purchased from the American Type Culture Collection (Manassas, VA). The cells were grown in RPMI 1640 (Life Technologies, Inc., Rockville, MD) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (HyClone Laboratories, Logan, UT), 1% penicillin/streptomycin, sodium pyruvate, and 2 mM L-glutamine (Life Technologies, Inc.).

Antibodies and Reagents. The rabbit polyclonal antibodies against total Akt/PKB and phospho-serine (Ser)^{473} Akt/PKB were purchased from Cell Signaling Technology (Beverly, MA). The mouse monoclonal antibody against p27Kip1 was obtained from Zymed (San Francisco, CA). The rabbit polyclonal antibody against p27Kip1, HRP-conjugated goat-antimouse IgG and HRP-conjugated goat-antirabbit IgG were obtained from Transduction Laboratories (Lexington, KY). The antihuman α-tubulin monoclonal antibody was from Cedarlane (Ontario, Canada). The protein A-agarose bead was from Upstate Biotechnologies (Lake Placid, NY). All of the other culture reagents were purchased from Life Technologies, Inc. unless indicated otherwise.

Cytogenetic Analysis. Cytogenetic G-banding analysis was performed on pretreated bone marrow cells. The definition of the cytogenetic clone and descriptions of karyotypes followed the International System for Human Cytogenetic Nomenclature. Patients were divided into three prognostic groups based on their karyotype: (*a*) prognostic groups were favorable [t(8;21), inv(16)]; (*b*) intermediate (normal cytogenetics); and (*c*) unfavorable (all other abnormalities).

Cell Cycle Analysis. Leukemic cells were pelleted, fixed in 70% ethanol on ice for 1 h, and resuspended in 1 ml of cell cycle buffer (0.38 mM sodium citrate, 0.5 mg/ml RNase A, and 0.01 mg/ml propidium iodide) at a concentration of 10^6 cells/ml. Cell cycle analysis was carried out using a FACSCalibur flow cytometer equipped with CellQuest software (Becton Dickinson, San Jose, CA).

Transient Transfection. Cells were plated in 12-well plates containing RPMI 1640 supplemented with 10% (v/v) fetal bovine serum and 1% penicillin/streptomycin at a density of 2×10^6 cells/well. The next day, a transfection mixture was prepared by mixing 5 μg of DNA (expression vectors) with 100 μl of human Nucleofector solution (Amaxa Biosystems, Gaithersburg, MD),

Table 1 *Patient characteristics and CR[a] rate according to the C:N ratio of p27Kip1 protein localization*

	C:N ratio of p27Kip1 expression[b]		
	Low (n = 44)	High (n = 44)	P
Age (yr), median (range)	44 (15–75)	44 (20–75)	NS
Sex (male/female)	24/18	25/32	NS
WBC (×10^9/l), median (range)	16.5 (0.6–238.0)	24.7 (1.2–253.8)	NS
LDH (IU/l), median (range)	688 (304–9260)	685 (156–7260)	NS
FAB classification			NS
M0/M1/M2	23	24	
M4/M5	21	18	
M6/M7	0	2	
Cytogenetics			NS
Favorable	6	3	
Intermediate	23	23	
Unfavorable	15	18	
CR rate	95% (38/40)	75% (30/40)	NS

[a] CR, complete remission; C:N, cytoplasmic to nuclear; NS, not significant; WBC, white blood cell; LDH, lactic dehydrogenase; FAB, French-American-British.
[b] C:N ratio of p27Kip1 localization; Low, ≤1.97; High, >1.97.

Table 2 *Cell cycle distribution according to the C:N[a] ratio of p27Kip1 localization*

	C:N ratio of p27Kip1 localization		
	Low (n = 44)	High (n = 44)	P
G_0/G_1	79.4 ± 27.0%	86.4 ± 11.9%	NS
G_2/M	15.9 ± 27.1%	6.6 ± 2.5%	NS
S	7.8 ± 12.3%	14.0 ± 27.7%	NS

[a] C:N, cytoplasmic to nuclear; NS, not significant.

which was then used to transfect 2×10^6 leukemia cells. Cells were transfected with expression constructs encoding the constitutively active form of the Akt/PKB (pcDNA3-myristoylated-Akt/PKB) or dominant negative (DN)-Akt/PKB (pcDNA3-DN-Akt/PKB), which were kindly provided by Dr. Young-Guen Kwon (Yonsei University, Seoul, Korea). The cell suspension was immediately electroporated by a Nucleofector instrument (Amaxa Biosystems), according to the manufacturer's instructions. Immediately after electroporation, the cells were suspended in the complete medium and incubated in a humidified 37°C/5% CO_2 incubator. The cells were harvested 48 h after transfection and used for the experiments. The experiments were repeated at least three times.

Confocal Microscopy and Image Analysis. Confocal microscopy was used to examine the localization of the p27Kip1 protein in AML cells. Cells were fixed, permeabilized, and blocked with 2% BSA. Cells were incubated with the primary antibody against p27Kip1 (1:100 dilution) for 1 h at 37°C and then incubated with the corresponding FITC-conjugated secondary antibody (1:200 dilution). After the cells had been spun down on a slide, the nuclei were counterstained by 4′,6-diamidino-2-phenylindole (Molecular Probes, Eugene, OR) for 1 min. After washing, the slides were mounted in a drop of antifade mounting medium (Vectashield; Vector Laboratories, Burlingame, CA). Antibody labeling was examined by a Bio-Rad 1024-UV confocal system attached to a laser scanning confocal microscope (Leica Lasertechnick GmbH, Heidelberg, Germany). Control experiments, with the omission of the primary antibody, showed negative staining in all of the experiments. Simultaneous FITC or 4′,6-diamidino-2-phenylindole images were captured from the same optical section. The captured images were then pseudocolored as follows: (*a*) blue for 4′,6-diamidino-2-phenylindole; and (*b*) green for FITC. Regions of colocalization appear in cyan, reflecting the additive effect of superimposing the green and blue pixels. Image analysis was performed using the standard system operating software provided with the confocal microscope. All of the illustrations were assembled and processed digitally (Adobe PhotoShop, version 6.0; Adobe Systems Inc., San Diego, CA).

Preparation of Nuclear and Cytoplasmic Fractions. Cells were subfractionated, as described previously (45), with minor modifications. Briefly, cells were pelleted by centrifugation (5 min, 12,000 rpm, 4°C) and incubated in a hypotonic buffer [10 mM HEPES (pH 7.2), 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EGTA, 20 mM NaF, 100 μM Na_3VO_4, and a 0.1% protease inhibitor cocktail; Sigma Chemical, St. Louis, MO] for 30 min at 4°C, with rocking. Cells were broken using a Dounce homogenizer (30 strokes), after which the nuclei were pelleted by centrifugation (10 min, 3,500 rpm, 4°C). The nuclei-free supernatant was subjected to a second 10,000 × *g* centrifugation for 45 min at 4°C to separate the membranes from the cytosolic fractions. The nuclear pellets were resuspended in nuclear lysis buffer [10 nM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, and 1% Triton X-100], incubated for 1 min in a sonicating water bath, then incubated for 30 min at 4°C, with rocking. Twenty μg of total cytosolic and nuclear proteins were analyzed by Western blotting.

Western Blotting. The cells were dissolved in 100 μl of SDS-PAGE sample buffer containing β-mercaptoethanol to a final concentration of 3×10^6 cells. Lysates were sonicated for 15 s with a Vibra Cell Sonicator, boiled for 10 min, and additionally analyzed by Western blotting. The protein yields were quantified using the Bio-Rad Dc protein assay kit (Bio-Rad, Hercules, CA) and equivalent amounts of protein applied to 15% acrylamide gels. The proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Amersham Biosciences, Sunnyvale, CA). The membranes were blocked with 3% BSA in Tris-buffered saline-Tween (TBST; 1 × Tris-buffered saline, 0.1% Tween 20) for 2 h. After washing twice in TBST, the membranes were incubated with the primary antibodies for 2 h at room temperature. The membranes were then washed four times in TBST and incubated with the relevant horseradish peroxidase-conjugated secondary antibodies (1:3000 dilution with 3% BSA in TBST) for 30 min. After washing four times in TBST, the reactive proteins were visualized with an enhanced chemiluminescence detection system (Amersham Biosciences). Densitometry was performed by the Molecular Dynamics Imaging system and ImageQuant 3.3 software (Amersham Biosciences) to quantify relative amounts of protein detected on the Western blots.

Statistical Analysis. The patients were divided into two groups (high and low) in relation to the median value of the cytoplasmic to nuclear ratio (C:N) of the p27Kip1 protein localization. Comparisons among the characteristics of the groups were made using a chi-square test for the binary variables and a Mann-Whitney test for the continuous variables. The disease-free survival (DFS) and overall survival (OS) probabilities were calculated using the Kaplan-Meier method. The log-rank statistic was used to test for the difference in survival times between the groups. In addition to the p27Kip1 protein localization, the WBC count, age, and cytogenetics were analyzed in the

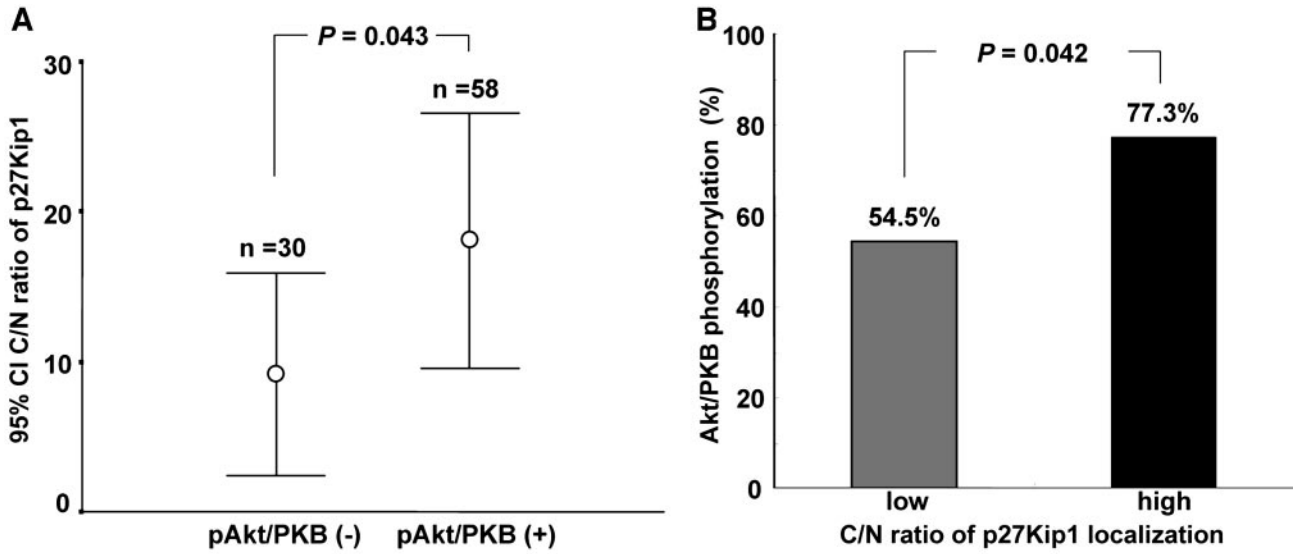

Fig. 2. *A*, the levels of cytoplasmic to nuclear ratio (*C:N*) of the p27Kip1 protein localization in relation to the constitutive Akt or protein kinase B (*Akt/PKB*) phosphorylation in acute myelogenous leukemia cells. The mean value of the C:N ratio of the p27Kip1 protein localization (○) was significantly higher in the pAkt/PKB-positive compared with the pAkt/PKB-negative group (P = 0.043; *B*) The constitutive Akt/PKB phosphorylation, according to the C:N ratio of p27Kip1 protein localization, in acute myelogenous leukemia cells. The frequency of Akt/PKB phosphorylation was significantly higher in the high C:N ratio group compared with the low C:N ratio group (P = 0.042).

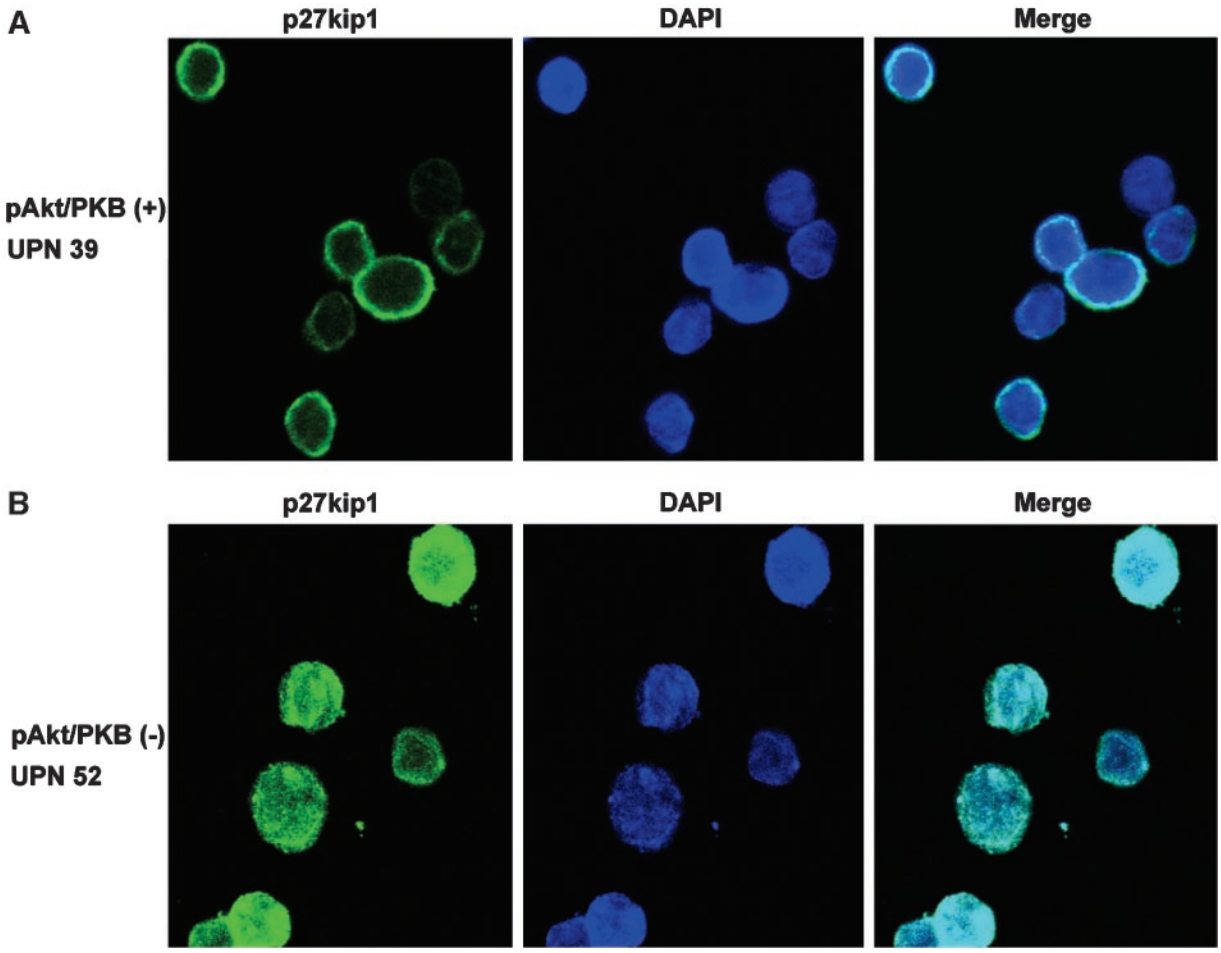

Fig. 3. Confocal microscopic analysis of the p27Kip1 protein localization in acute myelogenous leukemia (AML) cells in relation to the constitutive Akt or protein kinase B (Akt/PKB) phosphorylation. *A,* A representative pAkt/PKB-positive AML specimen was stained for p27Kip1 by FITC (*green*) as described in "Materials and Methods." The confocal image demonstrated the preferential localization of the p27Kip1 protein to the cytoplasm. Nuclear counterstain was carried out with 4′,6-diamidino-2-phenylindole (*DAPI*) staining (*blue*). *Cyan* represents the merged images. *B,* nuclear staining of the p27Kip1 protein was evident in the representative AML case, where no Akt/PKB phosphorylation was seen.

univariate and multivariate analysis. A multivariate analysis was used to test for the independent prognostic significance of the variables using the Cox proportional hazards regression model. Patients alive and still in remission at their last follow-up examination were censored in the analysis. A $P < 0.05$ was used to indicate statistical significance. All of the calculations were performed using the SPSS software, version 11.0.1 (SPSS Inc., Chicago, IL).

RESULTS

The p27Kip1 Protein Expression in AML Cells. Western blot analysis demonstrated that the p27Kip1 protein was expressed to a variable degree in 88 (88.9%) of 99 AML cases (Fig. 1*A*). To perform the quantitative analysis, the expression level of p27Kip1 protein was evaluated in the AML cells (*L*) by normalization as follows: *Lc* = p27Kip1 (*L*)/α-tubulin (*L*) as determined by Western blot analysis. In this study, the *Lc* ranged from 0 to 68.3 (median, 8.08). There was no association between the cellular levels of the p27Kip1 protein and the various clinical parameters such as age, French-American-British classification, WBC count, lactic dehydrogenase level, cell-cycle distribution, cytogenetics, complete remission rate, DFS, and OS (data not shown).

Subcellular Localization of p27Kip1 Protein in AML Cells. Next, the subcellular localization of the p27Kip1 protein was examined in the leukemia cells obtained from 88 AML patients. Western blot analysis of the fractionated cell lysates demonstrated that the subcellular localization of the p27Kip1 protein was different, according to the patients (Fig. 1*B*). To perform the quantitative analysis, the relative levels of the p27Kip1 expression at the subcellular compartment were represented as the C:N ratio of the p27Kip1 protein levels as shown in the following data: C:N = p27Kip1(C)/p27Kip1(N) as determined by the Western blot analysis. In this study, the C:N ratios ranged from 0 to 127.21 (median, 1.97). For the practical evaluations, the median value was used as a cutoff value for the levels of subcellular localization of the p27Kip1 in the AML cells. Using this value, the AML cases were classified into two groups; the high and low C:N ratio groups ($n = 44$ for both; Table 1). Next, the association between the C:N ratio of the p27Kip1 localization and various clinical parameters in AML were analyzed. As shown in Table 1, the C:N ratio of the p27Kip1 localization was not correlated with age, gender, WBC count, lactic dehydrogenase level, French-American-British classification, or cytogenetics. Cell cycle analysis demonstrated that the fraction of cells in the G_0/G_1 phase were not different between the AML cells, showing either a high or low C:N ratio of the p27Kip1 localization (86.4 ± 11.9 and 79.4 ± 27.0%, respectively; Table 2). Likewise, the C:N ratio of the p27Kip1 localization was not correlated with the fractions of the cells in the G_2-M or S-phase (Table 2).

Correlation between Constitutive Akt/PKB Phosphorylation and p27Kip1 Localization. The constitutive phosphorylation of Ser^{473} Akt/PKB was demonstrated in 58 (58.6%) of 99 cases (Fig. 2*A*). Cell cycle analysis showed that neither the fraction of cells in the G_0/G_1 phase, G_2-M phase, or S phase were different, according to the

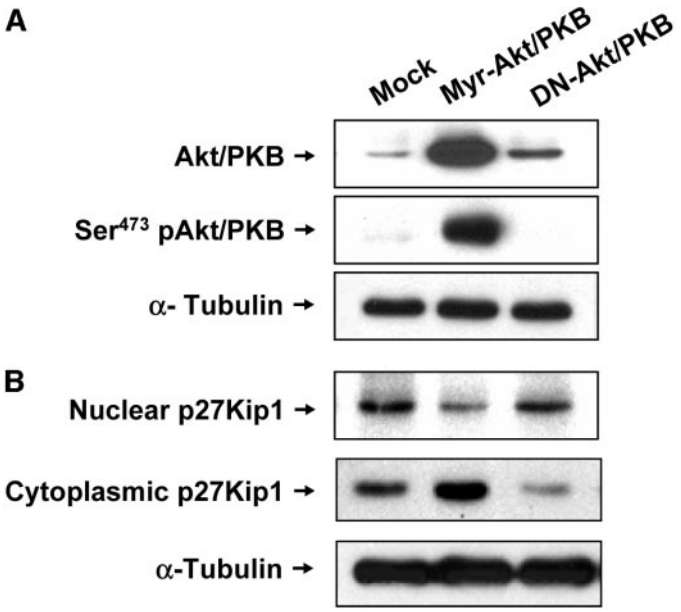

Fig. 4. Effects of myristoylated Akt or protein kinase B (*Myr-Akt/PKB*) and DN-Akt/PKB transfection on the subcellular localization of the p27kip protein in the U937 cells. The U937 cells were transiently transfected with Myr-Akt/PKB or DN-Akt/PKB constructs as described in "Materials and Methods." *A*, the total cell lysates were analyzed by Western blotting, using antibodies against total Akt/PKB and Ser^{473} pAkt/PKB. *B*, Myr-Akt/PKB-transfected and DN-Akt/PKB-transfected U937 cells were subjected to the subcellular fractionation. Equal amounts of proteins (20 μg) from the nuclear and cytoplasmic fractions were separated by SDS-PAGE and then probed with the anti-p27kip1 antibody. The α-tubulin was used as a loading control.

phosphorylation of Akt/PKB (data not shown). Next, the association of the phosphorylation of Akt/PKB and localization of the p27Kip1 protein was examined in the AML cells. Akt/PKB phosphorylation was observed more frequently in the high C:N ratio group compared with the low C:N ratio group (77.3% *versus* 54.5%, $P = 0.042$; Fig. 2*B*). The mean value of the C:N ratio of the p27Kip1 localization was significantly higher in the pAkt/PKB-positive AML cases compared with the pAkt/PKB-negative AML cases (18.15 $\pm$ 4.2 *versus* 9.2 $\pm$ 3.3, $P = 0.043$; Fig. 2*A*). The confocal microscopic analysis revealed the p27Kip1 protein to be preferentially localized to the cytoplasm in a pAkt/PKB-positive AML case (Fig. 3*A*). In contrast, the nuclear staining of the p27Kip1 protein was evident in a pAkt/PKB-negative AML case (Fig. 3*B*).

The p27Kip1 Mislocalization Induced by Akt/PKB Overexpression in Leukemia Cells. To investigate whether the active Akt/PKB directly affects the localization of the p27Kip1 protein in leukemia cells, the U937 leukemia cells were transfected using the pcDNA3-myristoylated-Akt/PKB or pcDNA3-DN-Akt/PKB expression vectors (Fig. 4*A*). As shown in Fig. 4*B*, the expression of the p27Kip1 protein was preferentially localized to the cytoplasm of the myristoylated-Akt/PKB-transfected U937 cells. In contrast, the DN-Akt/PKB-transfected U937 cells revealed a reduced level of the p27Kip1 in the cytoplasm, but increased the p27Kip1 levels in the nuclei.

The p27Kip1 Localization as a Prognostic Variable in AML. The complete remission rate of the patients with the high C:N ratio of the p27Kip1 localization was lower than that with the low C:N ratio (75% *versus* 95%), but without statistical significance ($P = 0.116$; Table 1). A survival analysis using the Kaplan-Meier method demonstrated that the high C:N ratio group had a significantly shorter DFS than the low C:N ratio group (Fig. 5*A*; $P = 0.0353$ by log-rank test). The OS rate was also significantly lower in the high C:N ratio group compared with the low C:N ratio group (Fig. 5*B*; $P = 0.0023$). The DFS estimates at 5 years for patients with a high or low C:N ratio of p27Kip1 localization were 19.0 (SE = 14.6%) and 58.2% (SE = 12.4%), respectively ($P = 0.0353$). The OS estimates at 5 years for patients with a high or low C:N ratio were 10.8 (SE = 8.4%) and 52.6% (SE = 11.2%), respectively ($P = 0.0023$). The univariate analysis revealed that the C:N ratio of the p27Kip1 localization was a strong prognostic factor both of the DFS and OS (Table 3). A multivariate analysis of the covariates in the Cox regression model demonstrated that the C:N ratio of the p27Kip1 protein localization remained as an independent prognostic factor of the DFS [relative risk (95% confidence interval) = 2.326 (0.989–5.466), $P = 0.043$] and the OS [relative risk (95% confidence interval) = 2.399 (1.255–4.585), $P = 0.008$] (Table 4).

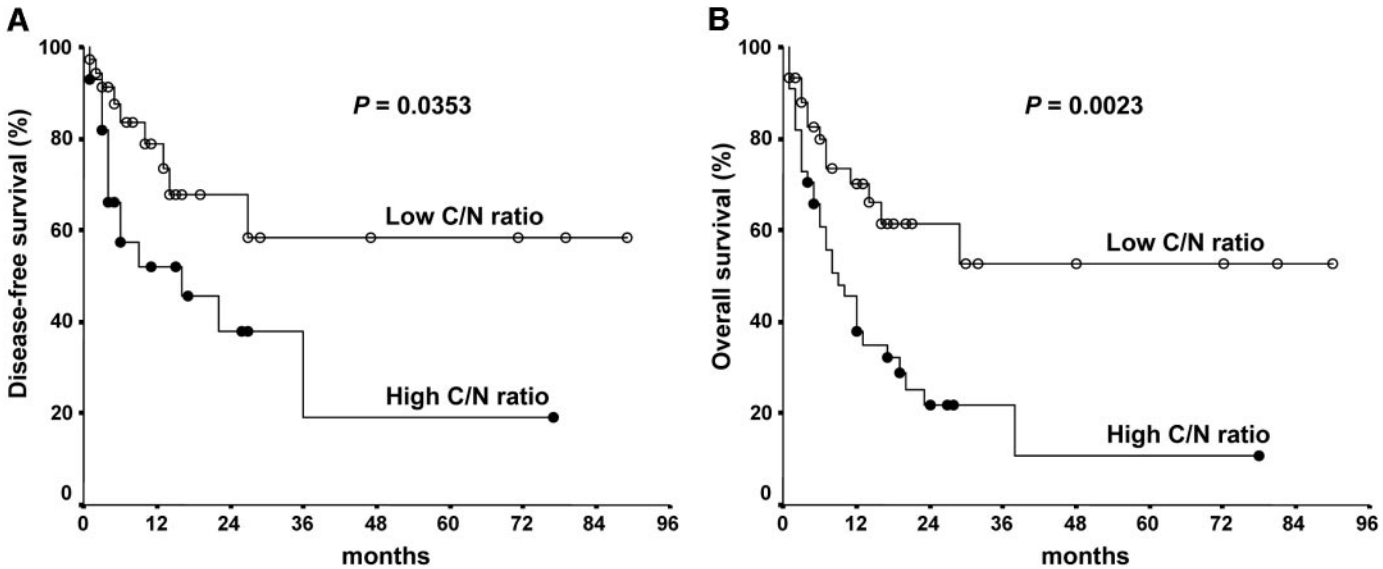

Fig. 5. Kaplan-Meier survival curves for the disease-free survival and overall survival rates of patients with acute myelogenous leukemia, according to the cytoplasmic to nuclear ratio (*C:N*) ratio of the p27Kip1 localization. The acute myelogenous leukemia cases with the high C:N ratio of the p27Kip1 localization had significantly lower disease-free survival (*A*) and overall survival rates (*B*) compared with the cases with the low C:N ratio ($P = 0.0353$ and $P = 0.0023$, respectively). The log-rank statistic was used to test for the difference in survival times between the groups.

Table 3 *Univariate analysis of age, WBC,[a] cytogenetics, and the C:N ratio of p27Kip1 localization for CR rate, DES, and OS in AML patients*

	P[b]		
	CR	DFS	OS
Age (yr)			
≤60 *vs.* >60	0.3300	0.7068	0.1100
WBC ($\times 10^9$/l)			
≤30 *vs.* >30	0.0200	0.5353	0.9890
Cytogenetics			
Non-unfavorable[c] *vs.* unfavorable	1.000	0.8231	0.8659
p27Kip1 localization (C:N ratio)			
Low *vs.* high	0.116	0.0353	0.0023

[a] WBC, white blood cell; C:N, cytoplasmic to nuclear; CR, complete remission; DFS, disease-free survival; OS, overall survival; AML, acute myelogenous leukemia.
[b] Log-rank test.
[c] Favorable plus intermediate prognostic group.

DISCUSSION

In this study, the cytoplasmic mislocalization of the p27Kip1 protein was demonstrated in a substantial proportion of the AML cases. The p27Kip1 mislocalization to the cytoplasm was highly correlated with the constitutive phosphorylation of Akt/PKB in the leukemic cells and with a poor prognosis in AML. However, the mere cellular levels of the p27Kip1 protein were not correlated with the clinical outcome. This study supports a growing body of evidences suggesting that the cytoplasmic p27Kip1 may play a role as an oncoprotein with antiapoptotic properties (26–28, 46).

Much less is known about the localization of p27Kip1 protein during the cell cycle progression. To inhibit cyclin E/CDK2, p27Kip1 needs to be imported into the nucleus (3). As cells progress along the cell cycle, the p27Kip1 protein shuttles between the nucleus and cytoplasm (47). The cytoplasmic redistribution of p27Kip1 induced by mitogenic stimulation is dependent on the phosphorylation of the Ser^{10} residue (47). Recently, it was demonstrated that p27Kip1 phosphorylation induced by the oncogenically activated kinase Akt/PKB disables the nuclear localization capacity of p27Kip1 (33, 34, 41). Akt/PKB can directly phosphorylate p27Kip1 at a Thr^{157} residue within the nuclear localization signal both *in vitro* and *in vivo* (33, 41). This Akt/PKB-dependent phosphorylation results in an impairing of its nuclear import, leading to cytoplasmic retention of the p27Kip1, abrogation of its CDK2 inhibitory activity, and cell cycle progression (41). The Akt/PKB phosphorylation in primary human breast cancer statistically correlated with the cytoplasmic localization of the p27Kip1 protein (41). An inhibition of the Akt/PKB activation with the phosphatidylinositol 3′-kinase inhibitor LY294002 or by the overexpression of DN Akt/PKB allele allows return of the p27Kip1 to the nucleus, resulting in an inhibition of CDK2 activity (33, 34, 41). As with Ser^{10}, Thr^{157}-phosphorylated p27Kip1 is detected almost exclusively in the cytoplasmic compartment (34, 41). Whereas Ser^{10} phosphorylation promotes the nuclear export of the p27Kip1, Thr^{157} phosphorylation seems to impair its import into the nuclei (33). The complex relationship between the p27Kip1 localization and degradation still remains to be addressed. Previous experimental evidence suggests that at least in breast cancer cells, the proteolysis and mislocalization of p27Kip1 occur through separate pathways (33). Although the p27Kip1 phosphorylation at the Ser^{10} or Thr^{157} residue was not evaluated in this study, the close correlation between the cytoplasmic mislocalization of p27Kip1 and the constitutive Akt/PKB phosphorylation suggests that an Akt/PKB-mediated Thr^{157} phosphorylation mechanism is present in a substantial proportion of AML cases. In this study, the induced Akt/PKB activation resulted in a remarkable increase in the levels of cytoplasmic p27Kip1 in the U937 leukemia cells. Transfection of the U937 cells with a DN Akt/PKB construct resulted in a recovery of the nuclear localization of the p27Kip1 protein. These findings strongly suggest that Akt/PKB activation is one of the crucial mechanisms determining the localization of the p27Kip1 protein in leukemia cells. However, because the Akt/PKB phosphorylation was not observed in a certain proportion of AML cases showing the cytoplasmic mislocalization of p27Kip1 in this study, the contribution of other intracellular oncogenic pathways to the cytoplasmic mislocalization and dysregulation of p27Kip1 protein cannot be excluded.

Whatever the mechanism is, it is important to understand the biological significance of the cytoplasmic mislocalization of p27Kip1 in AML cells. The export of p27Kip1 from the nucleus to the cytoplasm serves to remove the inhibitory activity of p27Kip1 against cyclin E/CDK2 (34, 41). This allows an activation of an increasing number of cyclin E/CDK2 complexes, which are then free to phosphorylate the p27Kip1. The transfection studies suggested that the relocalization of the p27Kip1 from the nucleus to the cytoplasm was sufficient to sustain the cellular proliferation (34, 41, 48). Another issue that needs to be addressed is whether the cytoplasmic mislocalization of the p27Kip1 observed in cancers merely contributes to abrogate its function or induces the p27Kip1 to acquire new cytoplasmic functions. The p27Kip1 was demonstrated to inhibit the drug-induced apoptosis (49), although it is uncertain whether the nuclear and cytoplasmic p27Kip1 differ in their efficiencies to protect the cells from apoptosis.

Only recently, the cytoplasmic mislocalization of p27Kip1 in tumor cells has been identified as a mechanism whereby cancer cells promote carcinogenesis in humans (28). Displacement of p27Kip1 into the cytoplasm contributes to the anchorage-independent growth of human transformed fibroblasts by maintaining high cyclin/CDK activity in the nucleus (31). Cytoplasmic mislocalization of p27Kip1 has been reported for a number of human malignancies (26–28, 33, 34, 41). It appears that the preferential location of p27Kip1 in the cytoplasm of tumor cells is predictive of a more aggressive clinical behavior (33). It was shown that the patients with Barrett's adenocarcinoma or breast carcinoma, which presented the cytoplasmic p27Kip1, showed decreased overall survivals compared with the

Table 4 *Multivariate analysis of DFS[a] and OS in AML patients*

	DFS		OS	
	P	RR (95% CI)	*P*	RR (95% CI)
Age (yr)				
≤60 *vs.* >60	0.974	0.982 (0.320–3.007)	0.010	1.374 (0.686–2.753)
WBC ($\times 10^9$/l)				
≤30 *vs.* >30	0.790	0.790 (0.334–1.866)	0.786	1.003 (0.548–1.836)
Cytogenetics				
Non-unfavorable *vs.* favorable	0.940	1.032 (0.448–2.378)	0.920	1.046 (0.565–1.937)
p27Kip1 localization (C:N ratio)				
Low *vs.* high	0.043	2.326 (0.989–5.466)	0.008	2.399 (1.255–4.585)

[a] DFS, disease-free survival; OS, overall survival; AML, acute myelogenous leukemia; RR, relative risk; CI, confidence interval; WBC, white blood cell; C:N, cytoplasmic to nuclear.

patients who present with nuclear p27Kip1 (28, 41). In this study, the WBC count or cytogenetics, which had been generally considered as a prognostic factor in adult AML, was not significantly associated with prognosis. These findings were in agreement with the previous report, which revealed that neither WBC nor cytogenetics was an independent prognostic factor in adult AML (50). Instead, we demonstrate for the first time that the p27Kip1 mislocalization is the strong prognostic factor in AML cases, whereas the total cellular levels of p27Kip1 do not have any prognostic value. The patients with high C:N ratio of the p27Kip1 localization showed a significantly shorter DFS and OS compared with the patients with low C:N ratio of p27Kip1 localization. The multivariate analysis revealed that the C:N ratio of the p27Kip1 localization remained an independent prognostic factor both the DFS and OS.

In conclusion, the cytoplasmic mislocalization of the p27Kip1 protein was observed in a substantial proportion of the AML cases. The mislocalization, not the cellular levels, of the p27Kip1 protein was highly associated with the constitutive phosphorylation of Akt/PKB and a poor prognosis in AML. Although the biological functions of cytoplasmic p27Kip1 protein and the genetic alterations that cause its mislocalization to the cytoplasm should be evaluated additionally, these findings suggest that the cytoplasmic mislocalization of p27Kip1 is an independent prognostic factor in AML, and the Akt/PKB-p27Kip1 pathway may be a ready target for antileukemia therapy.

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