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Elevated S-Phase Kinase-Associated Protein 2 Protein Expression in Acute Myelogenous Leukemia: Its Association with Constitutive Phosphorylation of Phosphatase and Tensin Homologue Protein and Poor Prognosis

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ABSTRACT

Purpose: The F-box protein S-phase kinase-associated protein 2 (Skp2) positively regulates the G₁-S phase transition by controlling the stability of several G₁ regulators, such as p27Kip1. However, the clinical significance of Skp2 in patients with acute myelogenous leukemia (AML) remains unknown.

Experimental Design: We examined the clinical and biological significance of Skp2 expression in AML and evaluated the relationship between Skp2 and p27Kip1 expression and phosphatase and tensin homologue (PTEN) phosphorylation.

Results: Western blot analysis showed that high Skp2 expression was observed in 57 (57.6%) cases and significantly correlated with unfavorable cytogenetics ($P = 0.035$) but not with age, white blood cell count, serum lactic dehydrogenase level, and the French-American-British subtype. An inverse correlation was not observed between Skp2 and p27Kip1 expression. However, p27Kip1 protein was preferentially localized to cytoplasm in the high-Skp2-expression group. The cytoplasmic to nuclear ratio of p27Kip1 expression was significantly correlated with the levels of Skp2 expression ($P < 0.001$). The frequency of PTEN phosphorylation was significantly higher in the high-Skp2-expression group compared with the low-Skp2-expression group ($P = 0.035$). The Skp2 overexpression was significantly associated with shorter disease-free survival and overall sur-

vival ($P = 0.0386$ and $P = 0.0369$, respectively). Multivariate analysis showed that Skp2 expression was an independent prognostic factor both in the disease-free survival and overall survival.

Conclusion: These findings suggest that Skp2 expression is an independent marker for a poor prognosis in AML. The presence of a positive correlation between Skp2 and phosphorylated PTEN suggests that an aberration in the PTEN/Skp2 signaling pathway might be operating in AML.

INTRODUCTION

The importance of the G₁-S-phase progression in various tumors has been highlighted by the frequent observations of aberrant regulation of molecules involved in this process. p27Kip1 is a potent inhibitor of the cyclin E–cyclin-dependent kinase-2 and cyclin A–cyclin-dependent kinase-2, which drive the cells from the G₁ to the S phase (1–2). Although p27Kip1 is rarely mutated in human cancers, many studies have shown that the reduced expression of p27Kip1 is frequently observed in various cancers (3–7). Low p27Kip1 levels are associated with the high aggressiveness and reduced survival in many cancers including acute myelogenous leukemia (AML; refs. 3–10).

The expression of p27Kip1 is controlled both at the level of transcription and by multiple posttranslational mechanisms, including the ubiquitin-mediated proteasomal degradation (11–14). S-phase kinase-associated protein 2 (Skp2) is a member of the F-box family of the specific substrate-recognition subunit of the Skp1/Cu11/F-box ubiquitin-protein ligase complexes (15). Skp2 is required for the ubiquitination and subsequent proteasomal degradation of p27Kip1 protein (16–18). High levels of p27Kip1 and free cyclin E expression were observed in the Skp2 knockout cells (19). Recent studies have indicated a possible relationship between Skp2 and its oncogenic potential. Skp2 expression was increased in the transformed cell lines (15) and malignant tumors (9, 20–25). Furthermore, a high Skp2 expression level was significantly correlated with an advanced clinical stage (9, 24) and a poor prognosis in tumors (9, 23, 24, 26, 27). The p27Kip1 level was inversely related to the Skp2 level in various human cancers (9–10, 21–23, 25, 26). Interestingly, this inverse correlation was not observed in a substantial proportion of aggressive cancers, although the high levels of Skp2 expression correlated well with the cell proliferation and disease progression (27–29). Until now, an evaluation of Skp2 protein expression in relation to the p27Kip1 expression and the clinical outcome has not been undertaken in AML.

Skp2 has a complex relationship with the tumor suppressor protein phosphatase and tensin homologue (PTEN; refs. 30–32). By dephosphorylating phosphatidylinositol 3,4,5-triphosphate (PIP₃), PTEN antagonizes the phosphatidylinositol 3-kinase

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(PI3-K)-mediated growth-promoting and antiapoptotic pathways (33–34). A high frequency of *PTEN* mutations has been reported in several human cancers (35). The loss of *PTEN* also predisposes the malignant transformation in a variety of tissues (36). The downstream effectors that mediate the *PTEN*-induced growth arrest have been identified, including Akt (37–38) and p27Kip1 (39). The relationship between Skp2 expression and the loss of *PTEN* is particularly interesting in view of the finding that a deletion of *PTEN* in mouse fibroblasts leads to the increased Skp2 levels with concomitant reductions in the p27Kip1 levels (40). Skp2 expression was inversely correlated with *PTEN* expression in prostate cancer (9). These findings suggest that *PTEN* functions as a negative regulator of the Skp2 pathway. Therefore, it would be necessary to evaluate the functional implication of *PTEN* as a regulator of the Skp2 pathway in AML.

The mutations in the *PTEN* gene are very infrequent genetic aberrations in myeloid leukemia (41–42). Recently, our group demonstrated that the COOH-terminal regulatory domain of the *PTEN* protein is constitutively phosphorylated in a substantial proportion of AML cases (43). The phosphorylation of the *PTEN* protein was strongly associated with the activation of the downstream molecules, including Akt and the forkhead transcription factor in AML cells (43). However, the relationship between Skp2 expression and *PTEN* phosphorylation has not been evaluated in AML.

The aim of this study was to examine the expression of the Skp2 protein and evaluate its correlation with the patient characteristics and clinical outcome in AML. This study also examined whether or not Skp2 expression is correlated with the expression and the localization of p27Kip1 protein, and the constitutive phosphorylation of *PTEN* protein in AML cells. We demonstrated for the first time that a high Skp2 expression is observed in a substantial proportion of AML cases and is associated with a poor prognosis. Skp2 expression was also strongly correlated with the mislocalization of the p27Kip1 protein and the constitutive phosphorylation of the *PTEN* protein. These findings might provide additional insight into the molecular pathogenesis and potential therapeutic targets for adult AML.

MATERIALS AND METHODS

Patients and Treatment. A total of 99 consecutive adult patients with *de novo* AML who had not received anti-leukemia treatment were enrolled in the study. According to the French-American-British classification (63), four patients had the M0, 20 patients had the M1, 33 patients had the M2, 19 patients had the M4, 21 patients had the M5 and 2 patients had the M6 subtype (Table 1). Patients with the M3 subtype of AML were excluded from this study. All of the patients were treated according to the first-line induction and consolidation regimens of Severance Hospital. Eighty-four patients received induction chemotherapy comprising cytarabine (100 mg/m²/d, continuous infusion for 7 days) and idarubicin (12 mg/m²/d, i.v. bolus for 3 days). Complete remission was defined as the normalization of the blood counts and bone marrow morphology along with the disappearance of all signs of leukemia, lasting for 4 weeks or longer, in accordance with the recommendations of the National

Cancer Institute-sponsored Workshop (44). All patients achieving complete remission received the same two courses of mitoxantrone-etoposide-cytarabine (MEC) consolidation therapy consisting of cytarabine (1g/m²/d, i.v. infusion for 2 hours, every 12 hours for a total of eight times), mitoxantrone (12 mg/m²/d for 3 days), and (etoposide; 100 mg/m²/d for 2 days), as described previously (45). The patients who relapsed also received a mitoxantrone-etoposide-cytarabine chemotherapy.

Isolation of Leukemic Cells. In conjunction with the institutional review board-approved treatment protocol, bone marrow aspirates were prospectively prepared from the patients before initiating chemotherapy. The bone marrow was sedimented on a Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden) density gradient. After washing the mononuclear cells collected from the upper interface, we performed the T-cell depletion 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 more than 95% of the isolated cells were leukemic blasts.

Antibodies and Reagents. The mouse monoclonal antibodies to Skp2 and p27Kip1 were purchased from ZYMED (San Francisco, CA). The rabbit polyclonal antibodies against *PTEN* and phosphorylated *PTEN* (p*PTEN*) were obtained from Cell Signaling Technology (Beverly, MA). The anti-p*PTEN* antibody detects *PTEN* protein that is phosphorylated at the Ser380/Thr382/Thr383 residues in the COOH-terminal regulatory domain. The antihuman α -tubulin monoclonal antibody was acquired from Cedarlane (Ontario, Canada). The horseradish peroxidase-conjugated goat antimouse IgG and horseradish peroxidase-conjugated goat antirabbit IgG were purchased from PharMingen (San Diego, CA). Unless indicated otherwise, all other culture reagents were purchased from Life Technologies, Inc. (Grand Island, NY).

Cytogenetic Analysis. Chromosomal analysis was performed on the pretreated bone marrow cells. The samples were processed using short-term unstimulated cultures (24–72 h). The clonality criteria and descriptions of the chromosomal aberrations were in accordance with the International System for Human Cytogenetic nomenclature. The patients were divided into three prognostic groups based on the karyotype; the prognostic groups were favorable [*t*(8;21), *inv*(16)], intermediate (normal cytogenetics), and unfavorable (all other abnormalities).

Cell Cycle Analysis. After treatment, the cells were pelleted and fixed in 70% ethanol on ice for 1 hour and resuspended in 1 ml of a 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 10⁶ cells/ml. Cell cycle analysis was performed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) equipped with CellQuest software (Becton Dickinson).

Preparation of Nuclear and Cytoplasmic Fractions. Cells were subfractionated as described previously (46) with minor modifications. 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 MgCl₂, 0.1 mM EGTA, 20 mM NaF, 100 μ M Na₃VO₄, and 0.1% protease inhibitor cocktail (Sigma Chemical, St. Louis, MO)] for 30

minutes at 4°C while rocking. Cells were broken using a Dounce homogenizer (30 strokes), after which nuclei were pelleted by centrifugation (10 min; 3,500 rpm; 4°C). The nuclei-free supernatant was subjected to a second centrifugation at $10,000 \times g$ for 45 minutes at 4°C to separate membrane (pellet) from cytosolic (supernatant) fractions. Nuclear pellets (above) were resuspended in nuclear lysis buffer [10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, and 1% Triton X-100] and were incubated for 1 minute in a sonicating water bath, followed by a 30-min incubation at 4°C while rocking. Twenty milligrams of total cytosolic and nuclear protein were analyzed by Western blot.

Western Blotting. The cells were dissolved in 100 μ l of a SDS-PAGE sample buffer containing β -mercaptoethanol at a final concentration of 3×10^6 cells. The lysates were sonicated for 15 seconds with a Vibra Cell Sonicator, boiled for 10 minutes, and further analyzed by Western blotting. The protein yields were quantified using the Bio-Rad Dc protein assay kit (Hercules, CA) and equivalent amounts of the protein (40 μ g) were applied to the 15% acrylamide gels. The proteins were separated by SDS-PAGE and were transferred to nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ). The membranes were blocked at room temperature with 3% bovine serum albumin in TBST (1 \times Tris-buffered saline, 0.1% Tween 20) for 16 h. After washing twice in TBST, the membranes were incubated with the primary antibodies for 2 hours at room temperature. The membranes were then washed four times in TBST and were incubated with the relevant horseradish peroxidase-conjugated secondary antibodies (1:3000 dilution with 3% bovine serum albumin in TBST) for 1 h. After washing four times in TBST, the reactive proteins were visualized by an enhanced chemiluminescence detection system (Amersham Biosciences). Densitometry was performed using the Molecular Dynamics Imaging system and ImageQuant 3.3 software (Amersham Biosciences) to quantify the relative amounts of the protein detected on Western blots.

Statistical Analysis. To perform the quantitative analysis, the Skp2 protein expression level in the AML cells (L) was evaluated by normalization as follows: corrected L (L_c) = Skp2 (L)/ α -tubulin (L) as determined by Western blot analysis. The patients were divided into two groups (high and low) based on the median L_c value of Skp2 expression in bone marrow mononuclear cells of 10 normal candidates. The comparisons among characteristics of the subgroups were made using a χ^2 test for the binary variables and a Mann-Whitney test for the continuous variables. The linear regression analysis and two-tailed Student t test were used to assess the association between Skp2 and p27Kip1 protein expression. The χ^2 test was used to determine the relationship of Skp2 with pPTEN. The disease-free survival and overall survival probabilities were calculated using the Kaplan-Meier method. The log-rank statistic was used to test the difference in survival times between the groups. In addition to the Skp2 expression, the white blood cell count, age, and cytogenetics were analyzed in the univariate and multivariate analysis. Multivariate analysis was used to test for the independent prognostic significance of variables using the Cox proportional hazards regression model. The patients alive, and still in remission at last follow-up examination, were censored in the analysis. All of the calculations were performed using the SPSS

software, version 11.0.1 (SPSS Inc, Chicago, IL). A P -value of <0.05 was considered significant.

RESULTS

Skp2 Protein Expression in AML Cells. The expression levels of the Skp2 protein in the AML cells varied among the patients (Fig. 1A). Western blot analysis revealed that Skp2 protein expression was present in 74 (74.7%) of the 99 cases. To perform the quantitative analysis, the Skp2 protein expression level was determined in the AML cells (L) by normalization as follows: $L_c = \text{Skp2 } (L) / \alpha\text{-tubulin } (L)$, as determined by Western blot analysis. In this study, the L_c ranged from 0 to 840.4. In the practical evaluations, a cutoff value (L_c , 0.70) was set for these protein expression levels in the AML cells, because the median L_c level of Skp2 protein expression in the 10 normal bone marrow mononuclear cells was 0.70 (range, 0–1.02). Using this cutoff value, we classified the AML cases into two groups, a

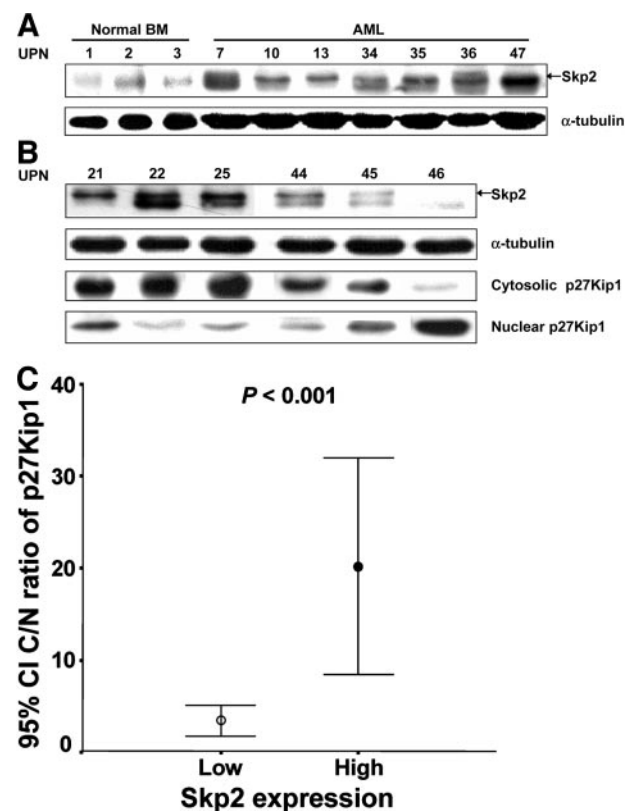


Fig. 1 Western blot analysis of Skp2 and p27Kip1 protein expression in the representative AML and normal bone marrow (BM) samples. **A**, the levels of Skp2 protein expression were variable according to the samples. α -tubulin was used for control of protein concentration on Western blot. UPN represents unique patient number. **B**, the subcellular localization and expression of the p27Kip1 protein in relation to the Skp2 protein expression were analyzed by SDS-PAGE of the nuclear and cytoplasmic fractions of the cell lysates as described in Materials and Methods. **C**, the mean C/N ratio of p27Kip1 protein was significantly higher in the AML cases showing a high Skp2 expression compared with the cases showing a low level of Skp2 expression ($P < 0.001$). CI, confidence interval.

Table 1 Patient characteristics and complete remission (CR) rate according to Skp2 expression levels

| | Skp2 expression* | | P |
|---|------------------|------------------|--------|
| | Low (n = 42) | High (n = 57) | |
| Age (y), median (range) | 45 (15–71) | 44 (15–80) | NS |
| Sex (M/F) | 24/18 | 25/32 | NS |
| WBC ($\times 10^9/L$), median (range) | 15.4 (0.6–238.0) | 23.3 (1.2–253.8) | NS |
| LDH (IU/L), median (range) | 705 (362–7260) | 658 (156–9260) | NS |
| FAB classification | | | NS |
| M0, M1, M2 | 22 | 35 | |
| M4, M5 | 19 | 21 | |
| M6, M7 | 1 | 1 | |
| Cytogenetics | | | 0.035† |
| Favorable | 6 | 4 | |
| Intermediate | 26 | 27 | |
| Unfavorable | 10 | 26 | |
| CR rate | 91.4% (32/35) | 81.6% (40/49) | NS |

Abbreviations: NS, not significant; WBC, white blood cell(s); LDH, lactic dehydrogenase; FAB, French-American-British (classification).

* Skp2 expression [$L_c = \text{Skp2 (L)}/\alpha\text{-tubulin (L)}$], where L is expression level in the AML cells; low, $L_c \leq 0.7$; high, $L_c > 0.7$.

† Unfavorable versus non-unfavorable (favorable and intermediate).

high-expression group [$n = 57$ (57.6%)] and a low-expression group [$n = 42$ (42.4%)].

The association between Skp2 expression and the various clinical parameters in AML were evaluated. As shown in Table 1, there was no significant difference regarding age, sex, white blood cell count, and the French-American-British subtype. A statistically significant correlation was observed between Skp2 expression and unfavorable cytogenetics (χ^2 test, $P = 0.035$). Cell cycle analysis demonstrated that the fraction of cells in the G_0 - G_1 phase was higher in the AML cells showing a high Skp2 expression ($85.2 \pm 18.1\%$) compared with the cells showing a low Skp2 expression ($75.9 \pm 24.0\%$) without statistical significance ($P = 0.435$; Table 2). Likewise, the Skp2 expression levels were not correlated with the fractions of the cells in the G_2 -M or S phase, respectively (Table 2).

Association of Skp2 and p27Kip1 Protein Expression in AML. The relationship between Skp2 and p27Kip1 protein expression was examined. Linear regression analysis was used to assess the association between the Skp2 and p27Kip1 expression levels. Overall, there was no inverse correlation between Skp2 and p27Kip1 protein expression ($r = 0.082$, $P = 0.613$; data not shown). The subcellular localization of the p27Kip1 protein with respect to Skp2 expression was then examined. Western blotting of the fractionated cell lysates demonstrated that the subcellular localization of the p27Kip1 was predominantly cytoplasmic in the group with high Skp2 expression level. In contrast, the p27Kip1 protein mainly located in the nucleus in the group with low Skp2 expression (Fig. 1B). To analyze the extent of subcellular localization of the p27Kip1

protein in relation to Skp2 protein expression, we estimated the cytoplasmic to nuclear (C/N) ratio of p27Kip1 protein expression. The C/N ratio of p27Kip1 localization ranged from 0 to 127.21. The mean C/N ratio of p27Kip1 protein of the group showing high levels of Skp2 expression was significantly higher than that of the group showing low levels of Skp2 expression (3.35 ± 1.99 versus 20.11 ± 16.06 , $P < 0.001$; Fig. 1C).

Correlation of Skp2 Expression with PTEN Phosphorylation in AML. The status of PTEN phosphorylation in relation to Skp2 protein expression in the AML cells was next examined. The constitutive phosphorylation of PTEN protein was demonstrated in the majority of AML cases with high Skp2 expression (Fig. 2A). The frequency of PTEN phosphorylation was significantly higher in the group with high Skp2 protein expression (42 cases; 73.7%) compared with the cases with low Skp2 expression (22 cases; 52.4%; $P = 0.035$; Fig. 2B). However, Skp2 expression was not correlated with the levels of PTEN expression itself (Fig. 2B).

Prognostic Significance of Skp2 Expression in AML. As shown in Table 1, the complete remission rate in the high Skp2 expression group was similar to that in the low Skp2 expression group (81.6% versus 91.4%, $P = 0.215$). However, survival analysis using the Kaplan-Meier Method demonstrated that the high Skp2 expression group showed a significantly shorter disease-free survival compared with that of the low Skp2 expression group (Fig. 3A; $P = 0.0386$ by log-rank test). The overall survival rate was also significantly lower in the high Skp2 expression group compared with the low Skp2 expression group (Fig. 3B; $P = 0.0369$). The disease-free survival estimates at 5 years for the patients with or without Skp2 overexpression were 29.4% (SE = 11.6%) and 57.9% (SE = 15.7%), respectively ($P = 0.0386$; Fig. 3A). The overall survival estimates at 5 years for the patients with or without Skp2 overexpression were 15.8% (SE = 6.8%) and 51.9% (SE = 10.8%), respectively ($P = 0.0369$; Fig. 3B). Univariate analysis revealed that neither white blood cell count nor cytogenetics was a prognostic variable in our AML cases (Table 3). However, the Skp2 expression level was a strong prognostic factor for disease-free survival and overall survival. Multivariate analysis also did not

Table 2 Cell cycle analysis according to Skp2 expression levels

| | Skp2 expression | | P |
|---------------|-------------------|-------------------|----|
| | Low (n = 42) | High (n = 57) | |
| G_0 - G_1 | $75.9 \pm 24.0\%$ | $85.2 \pm 18.1\%$ | NS |
| G_2 -M | $11.9 \pm 17.6\%$ | $10.9 \pm 19.9\%$ | NS |
| S phase | $15.7 \pm 22.3\%$ | $8.5 \pm 17.1\%$ | NS |

Abbreviation: NS, not significant.

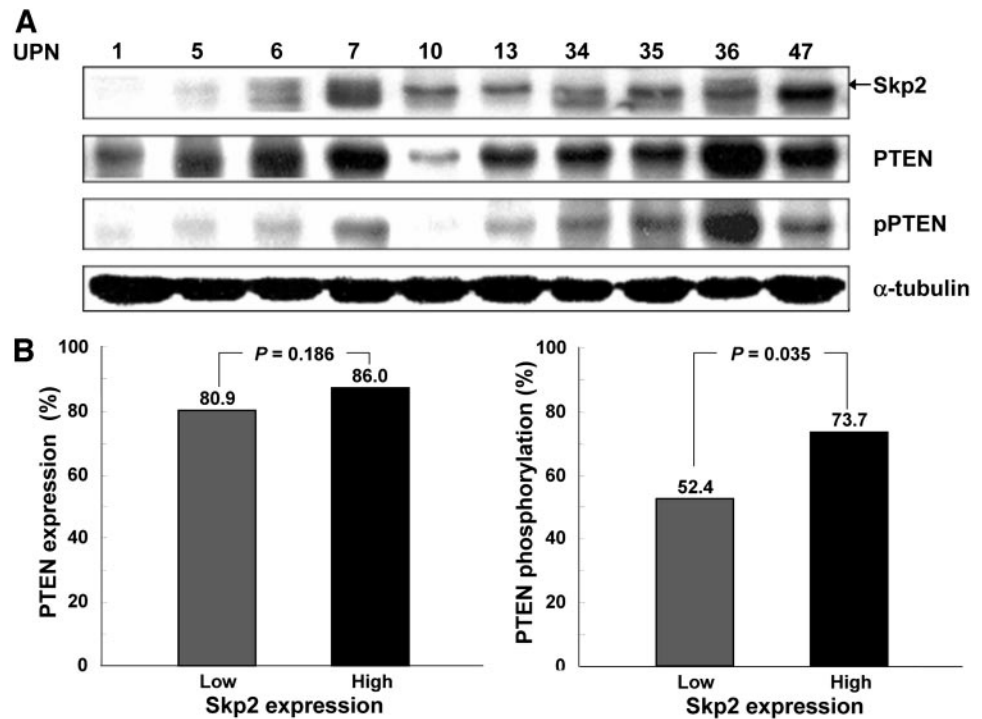


Fig. 2 A, Western blot analysis of Skp2, PTEN, and pPTEN protein expression in the representative AML samples. Levels of protein loading were visualized by stripping the membrane and reprobing with an antibody for α -tubulin. B, the PTEN (left) and pPTEN (right) expression according to levels of Skp2 expression. The frequency of PTEN phosphorylation was significantly higher in the high Skp2 expression group compared with the low Skp2 expression group ($P = 0.035$).

confirm the prognostic value of the white blood cell count nor of cytogenetics in the enrolled AML cases (Table 4). In contrast, Skp2 expression remained as an independent prognostic factor of disease-free survival [relative risk (95% confidence interval) = 2.797 (1.038–7.535), $P = 0.042$], and the overall survival [relative risk (95% confidence interval) = 1.927 (1.040–3.569), $P = 0.037$] (Table 4). These findings suggest that Skp2 expression is an independent marker of a poor prognosis in adult AML.

DISCUSSION

This study demonstrated that the high levels of Skp2 protein expression were observed in a substantial proportion of AML cases and were significantly correlated with a poor prognosis. Furthermore, it was shown for the first time that Skp2

expression was positively correlated with the constitutive phosphorylation of the COOH-terminal regulatory domain of the PTEN protein, along with the cytoplasmic localization of p27Kip1 protein in AML cells.

This study supports a growing body of evidence demonstrating the role of Skp2 in an oncogenic process (21–23, 25, 26). Because Skp2 is required for the ubiquitination and proteasomal degradation of p27Kip1 protein (16–18), the deregulation of Skp2 may contribute to the neoplastic transformation through accelerated p27Kip1 proteolysis. The Skp2-transfected cell lines expressed low levels of p27Kip1, and showed high levels of proliferation activity and of resistance to apoptosis and a high tumor-invasion potential (19, 26). Therefore, a low p27Kip1 expression in malignant cells may be caused by an increased expression of Skp2. Indeed, there was an inverse

Fig. 3 Kaplan-Meier survival curve for disease-free survival and overall survival rate of patients with AML according to the levels of Skp2 protein expression. High levels of Skp2 protein expression were significantly associated with lower disease-free survival (A) and overall survival rates (B; $P = 0.0386$ and $P = 0.0369$, respectively). The log-rank statistic was used to test the difference in survival times between the groups.

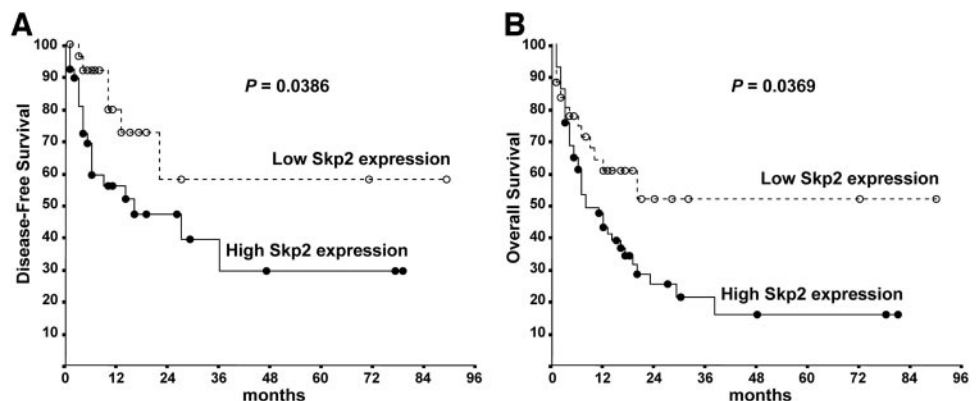


Table 3 Univariate analysis of age, white blood cell count (WBC), cytogenetics, and Skp2 expression status for complete remission (CR) rate, disease-free survival (DFS), and overall survival (OS) in AML patients

| | <i>P</i> * | | |
|--|------------|--------|--------|
| | CR | DFS | OS |
| Age (y) ≤60 vs. >60 | 1.0 | 0.6815 | 0.0126 |
| WBC ($\times 10^9/L$), ≤30 vs. >30 | 0.192 | 0.4519 | 0.8996 |
| Cytogenetics, non-unfavorable vs. unfavorable | 0.744 | 0.6187 | 0.7413 |
| Skp2 expression (<i>Lc</i> value), † low vs. high | 0.184 | 0.0386 | 0.0369 |

* Log-rank test.

† Skp2 expression [$Lc = \text{Skp2 (L)}/\alpha\text{-tubulin (L)}$], where *L* is expression level in the AML cells.

correlation between the p27Kip1 and Skp2 expression in the various human cancers (9–10, 21–23, 25, 26).

However, an inverse correlation between the Skp2 and p27Kip1 protein expression was not observed in our AML cases. This finding was in agreement with the recent reports showing that an inverse correlation between these proteins was not observed in a substantial proportion of aggressive cancers, although high Skp2 level correlated well with the extent of cell proliferation and disease progression (27–29). The lack of an inverse correlation suggests that additional molecular events associated with the tumorigenic properties of Skp2 (22, 25) or the aberrant regulation of the p27Kip1 activity (47) may be operating in AML.

It was suggested that the oncogenic potential of Skp2 is not restricted to p27Kip1 proteolysis (17, 22, 25). The forced expression of Skp2 in the quiescent fibroblasts induced DNA synthesis (17). Skp2 cooperated with *N-Ras* in the tumorigenesis of a T-cell lymphoma in a transgenic mice model (25). Rodent fibroblasts primarily transformed by both Skp2 and *H-Ras* gene transfection could form tumors in nude mice (22). The Skp2-transfected gastric carcinoma cell lines showed significantly higher growth rates, invasion potential, and resistance to an apoptotic induction by chemotherapeutic agents (26). These findings suggest that Skp2 plays an important role in human oncogenesis and in the modulation of the phenotype of malignant tumors. However, the leukemogenic potential of Skp2 itself remains to be determined in AML.

In addition to degradation, p27Kip1 activity appears to be altered by the intracellular localization or sequestration. In tumors with abundant p27Kip1 expression, the protein is often mislocalized to the cytoplasm (48–50). Because the growth-

restraining activity of p27Kip1 depends on its nuclear localization, the mislocalization effectively abrogates the p27Kip1 inhibitory activity (48, 51–54). In fact, cytoplasmic p27Kip1 localization seems to directly correlate with a poor long-term survival and the tumor grade of esophagus and breast carcinoma (50, 55–57). Although an inverse correlation between Skp2 and the p27Kip1 protein was not observed in our AML cases, the high Skp2 level was found to be significantly associated with the extent of cytoplasmic localization of p27Kip1 protein. The C/N ratio of p27Kip1 protein significantly correlated with the level of Skp2 protein expression in AML. These findings suggest that the molecular mechanism, which is involved in the increased expression of Skp2, is also closely associated with the mislocalization of p27Kip1 protein in AML.

The mechanism by which the Skp2 protein expression is increased in AML cells needs to be understood. Skp2 expression is usually cell cycle dependent; it is low in the G_1 phase, increases in the S phase, and declines afterward (16, 19, 58). However, in this study, the cell cycle distribution was not different in relation to Skp2 expression. This finding is in line with the finding that p27Kip1 expression does not seem to reflect the cell cycle status of AML in this study, as many investigators have pointed this out in other types of cancer (59). It was also previously shown that the levels of Skp2 protein are not cell cycle dependent in malignant lymphoma (10). These findings suggest that the dysregulation of the cell cycle cannot explain the aberrant expression of Skp2 protein in AML cells.

PTEN regulates the ubiquitin-dependent Skp2-mediated p27Kip1 degradation (40). A deletion of PTEN in mouse fibroblasts led to increased levels of Skp2 with concomitant reductions in the p27Kip1 levels (40). Skp2 was down-regulated by the expression of the wild-type PTEN or by exposure to the PI3-K inhibitor in the human glioblastoma cell line U87MG (40). Skp2 expression is inversely correlated with PTEN expression in prostate cancer cells (9). These findings suggest that PTEN functions as a negative regulator of the Skp2 pathway. However, it has been reported that mutations in the *PTEN* gene are very infrequent genetic aberrations in myeloid leukemia (41–42). Loss of heterozygosity analysis and polymerase chain reaction single-strand conformation polymorphism (PCR-SSCP) of the entire coding region showed that none of the AML cases had a loss of heterozygosity or a mutation (41). We observed no correlation between the PTEN and Skp2 expression levels in AML cells.

It was shown that the phosphorylation of the serine and threonine residues in the COOH-terminal regulatory domain of PTEN protein regulates both PTEN stability and PTEN enzy-

Table 4 Multivariate analysis of disease free survival (DFS) and overall survival (OS) in AML patients

| | DFS | | OS | |
|--|----------|---------------------|----------|----------------------|
| | <i>P</i> | RR (95% CI) | <i>P</i> | RR (95% CI) |
| Age (y), ≤60 vs. >60 | 0.371 | 1.653 (0.550–4.968) | 0.010 | 2.174 (1.203–3.936) |
| WBC ($\times 10^9/L$), ≤30 vs. >30 | 0.495 | 0.749 (0.326–1.720) | 0.786 | 0.9270 (0.538–1.598) |
| Cytogenetics, non-unfavorable vs. favorable | 0.754 | 0.873 (0.374–2.041) | 0.920 | 1.030 (0.581–1.826) |
| *Skp expression (<i>Lc</i> value), low vs. high | 0.042 | 2.797 (1.038–7.535) | 0.037 | 1.927 (1.040–3.569) |

Abbreviations: RR, relative risk; CI, confidence interval.

* Skp2 expression [$Lc = \text{Skp2 (L)}/\alpha\text{-tubulin (L)}$], where *L* is expression level in the AML cells.

matic activity (60–61). The PTEN phosphorylation keeps the PTEN stable but makes it less active toward its substrate, PIP₃ (62). Recently, we demonstrated that PTEN protein is constitutively phosphorylated in a substantial proportion of AML cases (43). PTEN phosphorylation was significantly associated with Akt phosphorylation and the activation of Akt downstream (43). The underlying mechanism for the constitutive PTEN phosphorylation in AML remains unclear. Because it was demonstrated that a key enzyme regulating the phosphorylation of the COOH-terminal regulatory domain of PTEN seems to be the protein kinase casein kinase 2 (62), the casein kinase 2 activity of the AML cells in relation to PTEN phosphorylation needs to be evaluated. In this study, PTEN phosphorylation was strongly associated with the levels of Skp2 expression, but not with p27Kip1 protein. Because the phosphorylation of the PTEN protein is highly associated with Akt phosphorylation (43), PTEN phosphorylation is meant to be closely involved in the regulation of Skp2 expression via the PI3-K/Akt pathway. If the specific kinase that regulates PTEN phosphorylation is identified, the direct contribution of PTEN phosphorylation to the Akt/protein kinase-B-mediated Skp2 expression will be understood.

This study demonstrated that the high Skp2 protein expression is significantly associated with poor prognostic features in AML. The complete remission rate was not different according to the levels of Skp2 expression. However, the disease-free survival and overall survival were significantly shorter in AML cases showing high levels of Skp2 expression compared with the cases with low levels of Skp2 protein. Multivariate analysis using the Cox proportional hazard model revealed that Skp2 protein expression level is an independent prognostic factor for both disease-free survival and overall survival in the whole cohort.

In conclusion, we demonstrated for the first time that Skp2 overexpression is an independent prognostic factor in AML. In a majority of AML cases showing high levels of Skp2 expression, the p27Kip1 protein was preferentially localized to the cytoplasm, suggesting that an aberrant regulatory pathway is operating in the Skp2-mediated p27Kip1 proteolysis in AML. In addition, the finding that Skp2 expression is significantly correlated with the constitutive phosphorylation of the PTEN protein suggests that the pPTEN-Skp2 axis may be a promising therapeutic target for adult AML.

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