

Protein Kinase CK2 α as an Unfavorable Prognostic Marker and Novel Therapeutic Target in Acute Myeloid Leukemia

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Abstract **Introduction:** Protein kinase CK2 is implicated in cellular proliferation and transformation. However, the clinical and biological significances of CK2 have not been elucidated in acute myeloid leukemia (AML).

Experimental Design: We evaluated the biological significances of catalytic subunit of CK2 (CK2 α) expression in leukemia cell lines and primary leukemic blasts obtained from AML patients.

Results: In this study, the expression of CK2 α was elevated in a substantial proportion of AML. In AML patients with normal karyotype, the disease-free survival and overall survival rates were significantly lower in the CK2 α -high compared with the CK2 α -low AML cases ($P = 0.0252$ and $P = 0.0392$, respectively). An induced overexpression of CK2 α increased the levels of Ser⁴⁷³ phosphorylated (p)-Akt/protein kinase B (PKB), p-PDK1, pFKHR, p-BAD, Bcl-2, Bcl-xL, Mcl-1, and XIAP. Treatment of U937 cell line and primary AML blasts with selective CK2 inhibitor, tetrabromobenzotriazole or apigenin, reduced the levels of these molecules in a dose-dependent manner. CK2 α small interfering RNA treatment also resulted in a down-regulation of p-Akt/PKB and Bcl-2 in U937 cells. Apigenin-induced cell death was preferentially observed in the CK2 α -high leukemia cell lines, HL-60 and NB4, which was accompanied by cytoplasmic release of SMAC/DIABLO and proteolytic cleavage of procaspase-9, procaspase-3, procaspase-8, and poly(ADP)ribose polymerase. An induced overexpression of CK2 α potentially enhanced the sensitivity of U937 cells to the apigenin-induced cell death. Apigenin-induced cell death was significantly higher in CK2 α -high AML compared with CK2 α -low AML ($P < 0.0001$) or normal bone marrow samples ($P < 0.0001$).

Conclusion: These findings strongly suggest protein kinase CK2 α as an unfavorable prognostic marker and novel therapeutic target in AML.

Protein kinase CK2 (formerly casein kinase II) is a highly conserved and ubiquitously expressed serine/threonine kinase (1–3) with a tetrameric structure consisting of two catalytic (α or α' or both) and two regulatory β subunits (2, 4, 5). Not only the holoenzyme but also the isolated catalytic subunits are active as judged from their ability to phosphorylate specific peptide substrates under basal conditions (2, 6). CK2 is a remarkably multifunctional protein kinase with a vast array of more than 300 substrates, many of which are critically involved in the process of cell growth, proliferation, and differentiation (7–14). These include, for example, transcription factors,

oncogenes and tumor-suppressor genes, and proteins involved in the signal transduction (7, 13, 14).

CK2 can also exert antiapoptotic effects through various mechanisms (15–24). For instance, CK2 counteracts apoptosis by protecting Bid from tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced caspase-8-mediated degradation (17, 18). CK2 is also involved in the phosphorylation of several proteins related to apoptosis, including apoptosis repressor with caspase recruitment domain (18, 19), p53 (20), nuclear factor- κ B (21), inhibitor of κ B (22), as well as c-Myc (23). Furthermore, Fas receptor-mediated cell death is regulated by CK2 expression (24).

The level of CK2 seems to be tightly regulated in normal cells, resisting a change in their intrinsic level of CK2 (25). Increasing evidence indicates that CK2 enzyme is a component of regulatory protein kinase networks that are involved in several aspects of cellular transformation and cancer (13, 26–30). Increases in CK2 level and activity has consistently been observed in a variety of human cancers, including mammary gland (26), prostate (27), lung (28), head and neck (29), and kidney cancer (30). Furthermore, the critical role of CK2 α catalytic subunits has been shown in studies conducted with antisense oligodeoxynucleotides targeting CK2 α mRNA in various cell lines (15, 31). Even modest dysregulation of CK2 expression is associated with cellular transformation,

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particularly under conditions that also alter the expression of oncogenes and tumor-suppressor genes (32–34). The crossing of different lines of transgenic mice showed evidence for collaboration between dysregulated expression of CK2 α and *c-Myc* or *Ta1-1* oncogenes in lymphoma development (32). Accelerated lymphomagenesis has also been observed when mice expressing CK2 α in their T cells were crossed with mice deficient in functionally expressed p53 (33). Overexpression of either catalytic isoform of CK2 α exhibited cooperation with H-Ras in the transformation of rat embryo fibroblasts and BALB/c 3T3 cells (35). Evidence supporting an antiapoptotic CK2 function has been obtained in both LNCaP prostate cancer cells (36) and leukemia Jurkat T cells (37). These cell lines have deletions in the phosphatase and tensin homologues on chromosome 10 (PTEN) protein (37–39) PTEN removes phosphate from the D3 position of inositol phospholipid.

Recent studies have provided evidence that CK2 overexpression is not simply associated with proliferative status of transformed cells but additionally reflects the pathobiological characteristics of tumors (1, 29). Elevated levels of CK2 α was highly correlated with metastatic potential, undifferentiated histologic type, and poor clinical outcome (40, 41). These studies further reinforce its tumorigenic potential and role as an antiapoptotic molecule (5). Although CK2 activity has been shown to be elevated in solid tumors, no definite studies are available regarding the nature of CK2 α expression and its biological significance in acute myeloid leukemia (AML). We have previously shown that constitutive phosphorylation of PTEN protein, one of the CK2 substrate, is observed in a substantial proportion of AML cases and is highly associated with poor prognosis (42). We report here, for the first time, that CK2 α is an unfavorable prognostic variable in AML patients with normal karyotype. Because the largest cytogenetic subset of adult AML are patients with normal karyotype, our data revealing CK2 α as an adverse prognostic factor can make it possible to develop a risk-adapted therapeutic strategy in adult AML. Additionally, we report here that a CK2 inhibitor, apigenin, induces apoptosis preferentially in the leukemic blasts obtained from patients with CK2 α -positive AML. In contrast, apigenin-induced cell death was negligible in normal bone marrow cells or CK2 α -negative leukemia cells. These findings suggest that CK2 α expression is an unfavorable prognostic marker in AML with normal karyotype. Our results also suggest the possibility of a pharmacologic window in targeting CK2 α for induction of apoptosis in leukemia cells under conditions that may spare normal bone marrow cells.

Patients, Materials, and Methods

Patients and treatment. A total of 48 primary, untreated AML patients of normal karyotype were included in this study. According to the French-American-British classification, the patient subtypes are as follows: 3 had M₀, 7 had M₁, 20 had M₂, 10 had M₄, 7 had M₅, and 1 had M₆. Pretreatment cytogenetic analyses of bone marrow were done as previously described (43). At least 20 metaphases were analyzed, and the karyotype was normal in each case. Patients received induction chemotherapy with standard dose of cytarabine and idarubicin (42). After achieving complete remission, patients received two cycles of consolidation chemotherapy composed of mitoxantrone, etoposide, and intermediate-dose cytarabine as previously described (43).

Cells. Primary leukemic cells were enriched from the diagnostic bone marrow samples of patients with *de novo* AML by Ficoll-Hypaque

(Pharmacia Biotech, Uppsala, Sweden) density gradient centrifugation. Normal bone marrow mononuclear cells were obtained from healthy bone marrow donors. All samples were collected under Severance Hospital Institutional Review Board–approved clinical specimen procurement protocols. Informed consent was obtained in accordance with the Helsinki protocol. After washing the mononuclear cells collected from the upper interface, T-cell depletion was done using a high-gradient magnetic cell separation system/anti-CD3 monoclonal antibody (Miltenyi Biotech, Auburn, CA) according to the manufacturer's instructions. A morphologic evaluation indicated that >90% of the isolated cells were leukemic blasts. U937, K562, HL-60, and NB4 human leukemia cell lines (American Type Culture Collection, Manassas, VA) were maintained in RPMI 1640 supplemented with 25 mmol/L L-glutamine (Cambrex BioScience, Walkersville, MD), 10% (v/v) heat-inactivated fetal bovine serum (HyClone Laboratories, Logan, UT), and 1% penicillin/streptomycin (Life Technologies, Gaithersburg, MD).

Antibodies. The rabbit polyclonal antibody against CK2 α as well as the mouse monoclonal antibody against α -tubulin was obtained from Upstate Biotechnology (Lake Placid, NY). The rabbit polyclonal antibodies against phospho(Ser⁴⁷³)-Akt/PKB, p-PDK1, p-FKHR, p-Bad, XIAP, p53, and Bax as well as the horseradish peroxidase–conjugated goat anti-mouse IgG and horseradish peroxidase–conjugated goat anti-rabbit IgG were purchased from Cell Signaling Technology (Beverly, MA). The rabbit polyclonal antibodies against Mcl-1 and Bcl-xL as well as the mouse monoclonal antibody against Bcl-2 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The mouse monoclonal antibody against poly(ADP)ribose polymerase (PARP), caspase-8, and caspase-9, as well as the rabbit polyclonal antibody against caspase-3, were purchased from PharMingen (San Diego, CA). The mouse monoclonal antibody against cytochrome *c* was obtained from BD Transduction Laboratories (San Jose, CA). The antibody against SMAC/DIABLO was purchased from Delta Biolabs (Campbell, CA).

In vitro CK2 kinase activity. CK2 kinase activity was measured by using a Casein Kinase-2 Assay Kit (Upstate Biotechnologies). Briefly, cell lysates (100 μ g) were tested in a reaction mixture containing 20 mmol/L MOPS (pH 7.2), 25 mmol/L β -glycerol phosphate, 5 mmol/L EGTA, 1 mmol/L sodium orthovanadate, 1 mmol/L DTT, 200 μ mol/L substrate peptide (RRRDDDSDDD), 2 μ mol/L protein kinase A inhibitor peptide [PKI-(6–22)-NH₂], casein kinase 2 (50 ng purified enzyme per assay), and 100 μ Ci [γ -³²P]ATP. The reaction mixtures were incubated and agitated for 10 min at 30°C. Reactions were stopped by adding 20 μ L of 40% trichloroacetic acid. Twenty-five microliters of each sample were then transferred onto phosphocellulose filter paper square P81, and the radiolabeled substrate was allowed to bind to the paper for 30 s. The paper was immersed in 0.75% phosphoric acid and mixed gently on a rotator, followed by washing thrice with 0.75% phosphoric acid for 1 min per wash to reduce background. The radioactivity incorporated into the substrate peptide was determined by scintillation counting.

CK2 inhibitor treatment. The specific inhibitors of CK2, 4,5,6,7-tetrabromobenzotriazole and apigenin, were obtained from Sigma (St. Louis, MO) and dissolved in DMSO (the amount of DMSO added to cell suspension never exceeded 0.5%, v/v). Cells were washed, resuspended at a density of $1 \times 10^6/2$ mL in medium containing 10% fetal bovine serum, and incubated at 37°C in the presence of the various concentration of tetrabromobenzotriazole or apigenin for the indicated duration. Control cells were treated with equal amounts of the solvent. At the end of the incubations, cells were centrifuged, washed, and analyzed as described below.

Annexin V staining. The Annexin V assays were done according to the manufacturer's protocol (PharMingen). Briefly, the cultured cells were collected, washed with Dulbecco's PBS without calcium or magnesium (Cambrex BioScience), and incubated in 100 μ L of a binding buffer containing 5 μ L of Annexin V-FITC. The nuclei were counterstained with propidium iodide. The percentage of apoptotic cells was

Table 1. Patient characteristics according to CK2 α expression in leukemic blasts

	CK2 α expression		P
	Low (n = 32)	High (n = 16)	
Age (y), median (range)	51 (17-66)	50 (15-66)	NS
Sex (male/female)	18/14	5/11	NS
WBC ($\times 10^9/l$), median (range)	28.3 (1.1-597.8)	28.5 (1.4-173.5)	NS
LDH (IU/l), median (range)	1,027 (334-5,310)	846 (317-2,340)	NS
FAB classification			NS
M ₀ /M ₁ /M ₂	20	10	
M ₄ /M ₅	12	5	
M ₆ /M ₇	0	1	
CR rate	80.0% (20/25)	75.0% (9/12)	NS

Abbreviations: FAB, French-American-British classification; LDH, lactic dehydrogenase; CR, complete remission; NS, not significant.

determined using FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA) equipped with Cell Quest software (Becton Dickinson).

Cell cycle analyses. Cells were pelleted, fixed in 70% ethanol at -20°C for 16 h, and resuspended in 1 mL of cell cycle buffer (0.38 mmol/L 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 Modfit LT for Mac V2.0 software (Becton Dickinson).

Transient transfection. For transfection of pCI-CK2 α plasmid, Nucleofector reagents were used. Briefly described, a transfection mixture was prepared by mixing 10 μg of DNA (expression vectors) with 100 μL of human Nucleofector solution (Amaxa Biosystems,

Gaithersburg, MD). pCI-CK2 α was kindly provided by Dr. Gun-Hong Kim (Yonsei University, Seoul, Korea). pCI empty vector was used for control. For transfection of specific-CK2 α small interfering RNA (siRNA), the siRNA of CK2 α (5'-GATGACTACCAGCTGGTTC-3') were purchased from Qiagen, Inc. (Valencia, CA). The cell suspension (2×10^6 leukemia cells) 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 $37^{\circ}\text{C}/5\% \text{CO}_2$ incubator. The cells were harvested after variable times and used for the experiments.

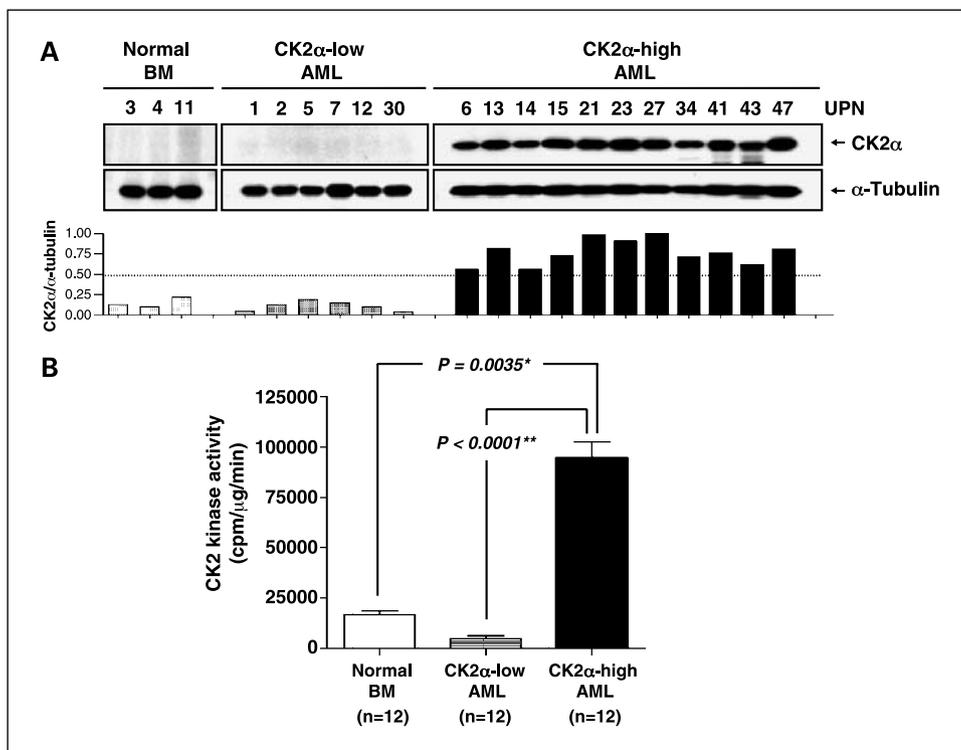
Preparation of mitochondria and cytosolic fraction. Cell fractionation was done by using a Cytosolic/Mitochondria Fractionation Kit (Oncogene Research Products, San Diego, CA). Briefly, cells were washed with ice-cold Dulbecco's PBS and incubated on ice in 100 μL of $1\times$ cytosol extraction buffer containing DTT and protease inhibitors for 10 min. After homogenization, unbroken cells, pieces of large plasma membrane, and nuclei were removed by centrifugation at $700 \times g$ for 10 min at 4°C . The supernatant was centrifuged at $10,000 \times g$ for 30 min at 4°C to collect the cytosolic fraction. Mitochondrial fraction was obtained after dissolving the cell pellet in 100 μL mitochondria extraction buffer containing DTT and protease inhibitors, and by vortexing for 10 s.

Western blot analysis. The cells were dissolved in 100 μL of a SDS-PAGE sample buffer containing β -mercaptoethanol. The lysates were sonicated for 15 s with a Vibra Cell Sonicator, boiled for 10 min, and further analyzed by Western blotting. The protein yields were quantified using the Bio-Rad detergent-compatible protein assay kit (Bio-Rad, Hercules, CA), and equivalent amounts of the protein (20 μg) 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 ($1\times$ TBS with 0.1% Tween 20) for 16 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 were incubated with the relevant horseradish peroxidase-conjugated

Fig. 1. Expression of CK2 α and its kinase activity in primary AML with normal karyotype. **A**, top, constitutive expression of CK2 α in the representative AML samples and normal bone marrow (BM) cells.

Primary leukemic blasts obtained from patients with AML were separated by SDS-PAGE and then analyzed by Western blot analysis. UPN, unique patient number. Note that α -tubulin served as a loading control. Bottom, the ratio between the intensity of the bands corresponding to CK2 α and α -tubulin. AML samples were arbitrary divided into CK2 α -low (CK2 α / α -tubulin <0.50) and CK2 α -high (CK2 α / α -tubulin ≥ 0.50) cases as described in the statistical analysis.

B, *in vitro* CK2 kinase activity in relation to CK2 α expression in AML cells. Intracellular CK2 kinase activity was measured in the representative AML and normal BM samples as described in Materials and Methods. Levels of CK2 kinase activity were significantly higher in the CK2 α -high AML cells compared with the normal BM cells ($*P < 0.005$) and CK2 α -low AML cells ($**P < 0.0001$). Columns, mean; bars, SD.



secondary antibodies (1:3,000 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 done by the Luminescent Image Analyzer LAS-1000 plus and Image Reader LAS-1000 Lite software (Fuji Film Co., Ltd.) to quantify relative amounts of protein detected on the Western blots. Quantification of band intensities was done using TINA software version 2.10e (Raytest Isotopenmessgeraete GmbH, Straubenhardt, Germany).

Statistical analysis. The patients were divided into two groups (CK2 α -low and CK2 α -high) based on the expression level of CK2 α protein in their leukemia cells. The CK2 α -high cases were arbitrary defined as cases showing the ratio of (CK2 α absorbance – background absorbance) versus (α -tubulin absorbance – background absorbance) >0.50. The comparisons among characteristics of the groups were made using a χ^2 test for the binary variables and a Mann-Whitney test for the continuous variables. The disease-free survival and overall survival probabilities were calculated using the Kaplan-Meier method. Log-rank statistics were used to test the difference in survival times between groups. Statistics were calculated by GraphPad Prism software (GraphPad Software, Inc., San Diego, CA) and SPSS software, version 11.0.1 (SPSS, Inc., Chicago, IL). $P < 0.05$ was considered significant.

Results

CK2 α expression in primary AML cells. Western blot analysis revealed that the constitutive expression of CK2 α protein was elevated in 16 (33.3%) of the 48 AML cases of normal karyotype (Table 1). However, all of the normal bone marrow specimens ($n = 12$) revealed negligible degree of CK2 α protein expression (data not shown). Representative Western blot analyses were shown in Fig. 1A. Patients were divided into two groups (CK2 α -high versus CK2 α -low) according to the levels of constitutive expression of CK2 α protein in pretreatment AML samples as described in the statistical analysis. The association of CK2 α expression with various clinical variables was evaluated. As shown in Table 1, there was no significant difference in the patient age, sex, WBC count, lactic dehydrogenase, or French-American-British classification subtype in relation to CK2 α expression.

Prognostic significance of CK2 α expression in AML. Complete remission rates in the CK2 α -high AML cases were similar to that in the CK2 α -low AML cases (75.0% versus 80.0%, $P = 0.617$; Table 1). However, Kaplan-Meier survival analysis showed that disease-free survival rate was significantly lower in the CK2 α -high AML compared with the CK2 α -low AML cases ($P = 0.0252$ by log-rank test; Fig. 2A). The overall survival rate was also significantly lower in the CK2 α -high AML compared with the CK2 α -low AML cases ($P = 0.0392$ by log-rank test; Fig. 2B). The disease-free survival estimates at 3 years for CK2 α -high and CK2 α -low AML patients were 11.4% (SE 10.5%) and 79.9% (SE 10.5%), respectively ($P = 0.0252$, log-rank test; data not shown). The 3-year overall survival estimates for CK2 α -high and CK2 α -low AML cases were 19.3% (SE 11.7%) and 64.8% (SE 14.3%), respectively ($P = 0.0392$, log-rank test, data not shown).

In vitro CK2 kinase activity according to CK2 α expression. We measured the intracellular CK2 kinase activity in the representative AML and normal bone marrow samples as described in Materials and Methods, and analyzed its correlation with the levels of CK2 α expression. As shown in Fig. 1B, the mean value of CK2 kinase activity was significantly higher in the CK2 α -high AML cells ($94,394 \pm 28,813$ cpm/ μ g/min) compared with the

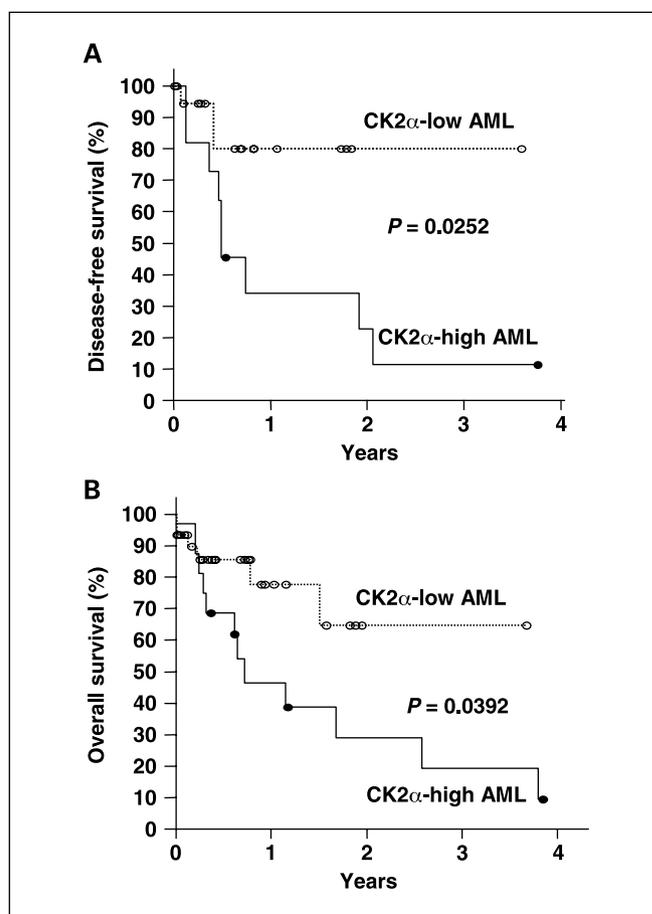


Fig. 2. Prognostic significance of CK2 α expression in primary AML. **A.** Kaplan-Meier survival curves show that disease-free survival rate was significantly lower in the cytogenetically normal CK2 α -high AML compared with the CK2 α -low AML cases with normal karyotype. **B.** overall survival rate was also significantly lower in the CK2 α -high AML cases. Statistical analysis was done by log-rank test. Corresponding P values are indicated.

CK2 α -low AML cells ($4,758 \pm 1,449$ cpm/ μ g/min, $P < 0.0001$) and normal bone marrow samples ($17,969 \pm 713$ cpm/ μ g/min, $P < 0.005$).

Regulation of phosphatidylinositol 3-kinase/Akt/protein kinase B pathway and levels of antiapoptotic proteins by CK2 α . We examined alterations in the level of molecules associated with phosphatidylinositol 3-kinase (PI3K)/Akt/protein kinase B (PKB) signaling pathway and antiapoptotic proteins, in addition to CK2 α , up to 48 h after treatment with selective CK2 inhibitor, tetrabromobenzotriazole or apigenin, in U937 leukemia cell line and primary leukemic blasts obtained from patients. As shown in Fig. 3A, CK2 α expression was down-regulated with either tetrabromobenzotriazole or apigenin treatment in a dose-dependent manner. The level of Ser⁴⁷³-phosphorylated Akt/PKB, which represents the activation of Akt/PKB kinase, was decreased in a dose-dependent manner in U937 cells. A remarkable decrease in the levels of molecules associated with PI3K/Akt/PKB signaling pathway, such as p-PDK1, p-FKHR, and p-Bad, was also observed (Fig. 3A). In contrast, as shown in Fig. 3B, an induced overexpression of CK2 α in U937 cells resulted in an increase in the levels of Ser⁴⁷³-phosphorylated Akt/PKB as well as p-PDK1, p-FKHR, and p-Bad. CK2 α -induced up-regulation of these molecules was

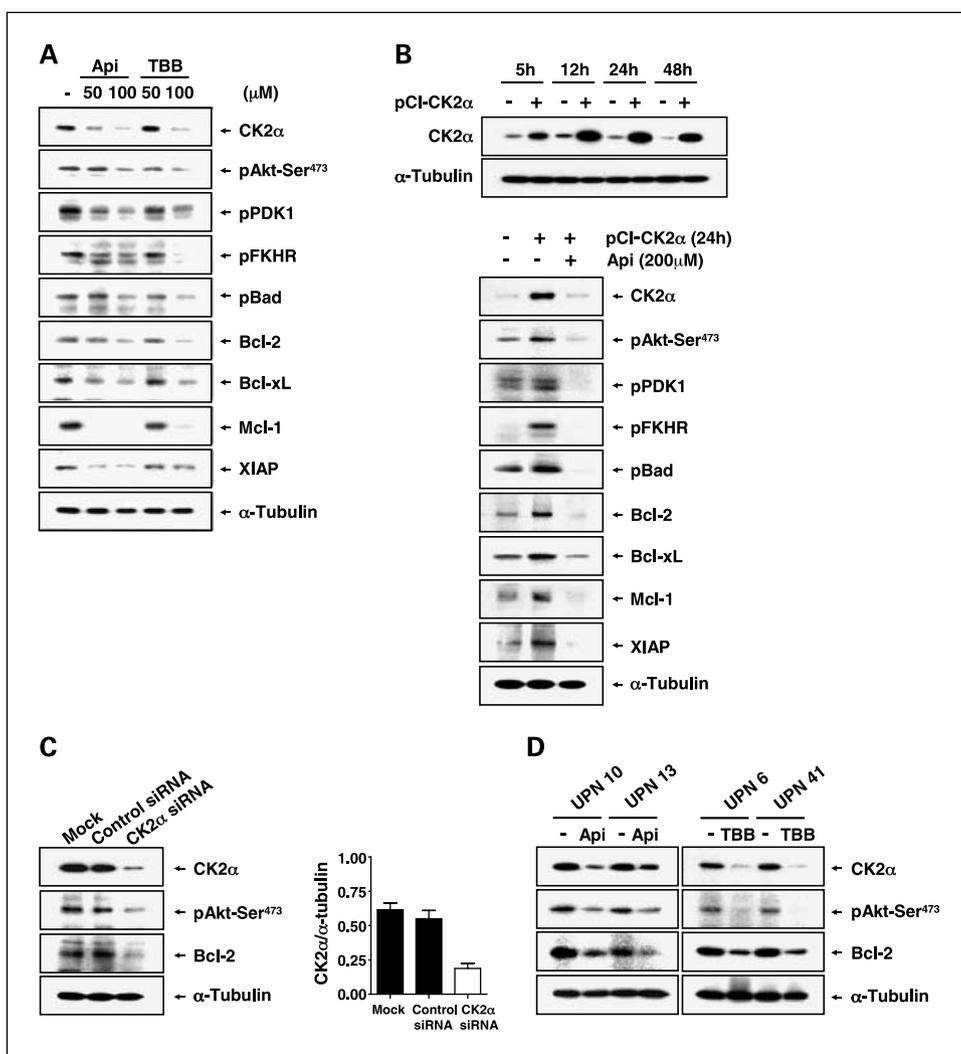
potentially abrogated by apigenin treatment (Fig. 3B, *bottom*). Increase in the levels of antiapoptotic proteins, including Bcl-2, Bcl-xL, Mcl-1, and XIAP, was observed with CK2 α overexpression (Fig. 3B, *bottom*). The CK2 α -induced increase in the levels of these antiapoptotic proteins was also abolished with apigenin treatment. We next evaluated the effect of CK2 α gene silencing on the down-regulation of Ser⁴⁷³ p-Akt/PKB and Bcl-2 in U937 cells. After CK2 α gene silencing, as described in Materials and Methods, the expression level of CK2 α was decreased to 18% of that in the parental cells (Fig. 3C, *right*). Knockdown of CK2 α gene by CK2 α siRNA transfection resulted in a marked down-regulation of Ser⁴⁷³ p-Akt/PKB and Bcl-2 in U937 cells (Fig. 3C, *left*). In primary leukemic blasts obtained from representative AML patients, a notable decrease in the levels of Ser⁴⁷³ p-Akt/PKB and Bcl-2 was also observed after treatment with tetrabromobenzotriazole or apigenin (Fig. 3D).

CK2 α and cell cycle distribution. Up to 48 h after transfection of U937 cells with pCI-CK2 α , cell cycle analysis was done as described in Materials and Methods. As shown in Fig. 4A, CK2 α overexpression induced an alteration in the cell cycle distribution. The proportion of cells at the G₀-G₁ fraction at 24 h after transfection was significantly decreased in the pCI-CK2 α -transfected cells ($33.05 \pm 1.60\%$) compared with the

mock-transfected ($44.08 \pm 0.22\%$, $P < 0.005$) or pCI-empty vector-transfected cells ($47.94 \pm 0.96\%$, $P < 0.005$). We next evaluated the alteration in the level of p53, which induces G₀-G₁ phase arrest. The levels of p53 decreased with an induced overexpression of CK2 α in U937 leukemia cell line (Fig. 4B).

CK2 inhibitor exerts the apoptosis-inducing effect selectively on the CK2 α -high leukemia cells. To determine the differential effect of CK2 inhibitor on induction of apoptosis in leukemia cells according to the CK2 α expression, we first analyzed the effects of apigenin on various leukemia cell lines. As shown in Fig. 5A, CK2 α expression was variable according to the leukemia cell lines. After 24-h treatment with apigenin, the apoptotic cell death was markedly increased in HL-60 ($72.1 \pm 3.2\%$) and NB4 ($84.4 \pm 4.5\%$) cell lines, both expressed relatively higher levels of CK2 α (Fig. 5B). Cell cycle analysis revealed that the cells in the sub-G₁ fraction were markedly increased in HL-60 cells ($47.0 \pm 0.1\%$) and NB4 cells ($50.5 \pm 0.9\%$) with apigenin treatment (Fig. 5C). In contrast, any notable increase in the extent of apoptosis was not observed in U937 ($2.2 \pm 0.7\%$) and K562 ($2.5 \pm 0.7\%$) leukemia cell lines, both of which are characterized by low levels of CK2 α expression (Fig. 5A and B). The frequency of the cells in the sub-G₁ fraction was not altered with apigenin treatment

Fig. 3. CK2 α -mediated regulation of PI3K/Akt/PKB pathway and antiapoptotic proteins in leukemia cells. **A**, effects of selective CK2 inhibitor, apigenin or tetrabromobenzotriazole (TBB), on the PI3K/Akt/PKB pathway and antiapoptotic proteins in U937 leukemia cells. Western blot analysis revealed that the levels of Ser⁴⁷³ p-Akt/PKB, p-PDK1, pFKHR, pBAD, Bcl-2, Bcl-xL, Mcl-1, and XIAP protein were decreased after 24-h treatment of U937 cells with either of apigenin or tetrabromobenzotriazole in a dose-dependent manner. **B**, cell lysates from pCI-CK2 α -transfected U937 cells were separated by SDS-PAGE and subjected to immunoblotting with CK2 α antibody (*top*) and the antibodies indicated (*bottom*). The levels of molecules associated with PI3K/Akt/PKB pathway and antiapoptosis were increased with induced CK2 α overexpression. However, these effects were abolished with apigenin treatment (*bottom*). **C**, U937 leukemia cells were transfected with CK2 α siRNA as described in Materials and Methods. Mock and control siRNA transfection were done for control experiments. After CK2 α gene silencing, the expression level of CK2 α was decreased to 18% of that in the parental cells (*right*). Columns, mean of three independent experiments; bars, SD. Knockdown of CK2 α by CK2 α siRNA resulted in a marked down-regulation of p-Akt/PKB and Bcl-2 (*left*). **D**, in primary leukemic blasts obtained from representative AML patients, a notable decrease in the levels of p-Akt/PKB and Bcl-2 was observed after 24-h treatment with tetrabromobenzotriazole (100 μ mol/L) and apigenin (100 μ mol/L).



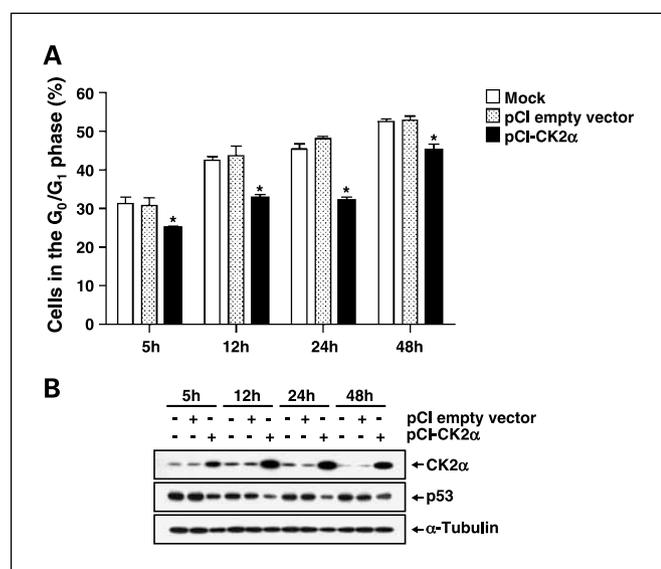


Fig. 4. Effect of CK2 α on the cell cycle distribution in U937 leukemia cells. **A**, after U937 cells were transfected with pCI-CK2 α construct (10 μ g), cell cycle analysis was done at the indicated times using a FACSCalibur flow cytometer. Mock and pCI empty vector transfection were done for control experiments. The proportion of cells at the G₀-G₁ phase was significantly decreased in the pCI-CK2 α -transfected U937 cells compared with the mock- or empty vector-transfected cells ($*P < 0.005$). Columns, mean value of three independent experiments; bars, SD. **B**, CK2 α -transfected U937 cell lysates were separated by SDS-PAGE and subjected to immunoblotting using antibodies against CK2 α and p53 at the indicated times. CK2 α overexpression was accompanied with the decrease in the level of p53. Note that α -tubulin served as a loading control.

($9.3 \pm 0.2\%$ in U937 cells and $3.5 \pm 1.5\%$ in K562 cells, respectively; Fig. 5C).

Apigenin treatment resulted in a proteolytic cleavage of PARP, and procaspase-9, procaspase-3, and procaspase-8 in HL-60 and NB4 leukemia cell lines, indicating that apigenin-induced cell death occurred in a caspase-dependent manner (Fig. 5D, left). Treatment of HL-60 and NB4 cell lines with apigenin resulted in a cytosolic release of SMAC/DIABLO protein and mitochondrial translocation of Bax protein (Fig. 5D, right). In contrast, apigenin-induced cleavages of PARP, procaspase-9, procaspase-3, procaspase-8, as well as cytosolic release of SMAC/DIABLO and mitochondrial translocation of Bax, were very negligible in U937 and K562 leukemia cell lines (Fig. 5D, right and left).

To clarify the relevance of CK2 α expression in the CK2 inhibitor-induced cell death, U937 cells were transfected with pCI-CK2 α vector. As shown in Fig. 5E, an induced overexpression of CK2 α remarkably enhanced the sensitivity of U937 leukemia cells to the apigenin-induced cell death at 48 h after transfection ($11.0 \pm 1.2\%$ in parental cells versus $34.6 \pm 2.3\%$ in pCI-CK2 α -transfected cells, $P = 0.0001$). Empty vector transfection did not affect the apigenin-induced cell death (Fig. 5E). These findings potentially indicate that the extent of apigenin-induced leukemia cell death depends on the levels of CK2 α expression in leukemia cells.

We next examined whether the selective apoptosis-inducing effect of apigenin in the CK2 α -high leukemia cell lines can also be documented in primary leukemic blasts obtained from patients with AML. As shown in Fig. 6A, a substantial degree of apoptosis was not observed in leukemic blasts obtained from CK2 α -low AML cases after 24-h treatment with apigenin.

Apigenin-induced cell death was also negligible in bone marrow mononuclear cells obtained from healthy donors. However, the levels of apoptosis were markedly increased in the leukemic blasts obtained from CK2 α -high AML cases with apigenin treatment (Fig. 6A). The proportion of apoptotic cells 24 h after apigenin treatment was significantly higher in the CK2 α -high AML cases ($59.6 \pm 9.0\%$) compared with the CK2 α -low AML ($11.1 \pm 5.8\%$, $P < 0.0001$) and normal bone marrow mononuclear cells ($13.9 \pm 6.7\%$, $P < 0.0001$; Fig. 6A). Apigenin-induced cell death in the CK2 α -high AML cases was accompanied by proteolytic cleavage of procaspase-8, procaspase-9, procaspase-3, and PARP (Fig. 6B, left), as well as cytosolic release of cytochrome *c* and SMAC/DIABLO, and mitochondrial translocation of Bax protein (Fig. 6B, right).

Discussion

In this study, we found that protein kinase CK2 α is an independent prognostic marker in AML with normal karyotype. In addition, the inhibition of CK2 α seems to be a novel approach for targeted therapy for AML. There have been accumulating evidences indicating that CK2 is a component of regulatory protein kinase networks involved in various aspects of cellular transformation and cancer. In this respect, abnormally high levels of CK2 protein have been observed in a number of human cancers (26–30, 44). Overexpression of CK2 not only indicates tumor cell proliferation but also reflects the pathological characteristics of the tumors (1, 29). Elevated levels of CK2 α is highly correlated with metastatic potential, undifferentiated histologic grade, and poor clinical outcome (40, 41), further reinforcing its tumorigenic potential and role as an antiapoptotic molecule (5). Although a striking induction of CK2 was observed in leukemia-like disease of cattle (45), CK2 expression and its biological implication have not been evaluated to date in human AML.

In our examination, the levels of constitutive CK2 α expression were very low in normal bone marrow specimens. However, the elevated level of CK2 α protein was observed in a substantial proportion of AML cases with normal karyotype. A previous report showed that CK2 α levels did not correlate with CK2 enzymatic activity in prostate cancer cell lines (46). However, the levels of CK2 α expression were highly correlated with CK2 catalytic activity in our AML samples, suggesting that the correlation between the level of CK2 α expression and CK2 catalytic activity may be different according to cell types. The mechanism responsible for regulation of CK2 remains controversial, with conflicting views on whether CK2 is constitutively active or activated in response to certain stimuli. A number of distinct mechanisms, including assembly, covalent modification, phosphorylation, and regulatory interactions with nonprotein small molecules (5), may all contribute to the pathologic regulation of CK2 in leukemia cells. A complete understanding of CK2 regulation is critical to gain insight into the pathophysiological roles of CK2 in leukemia.

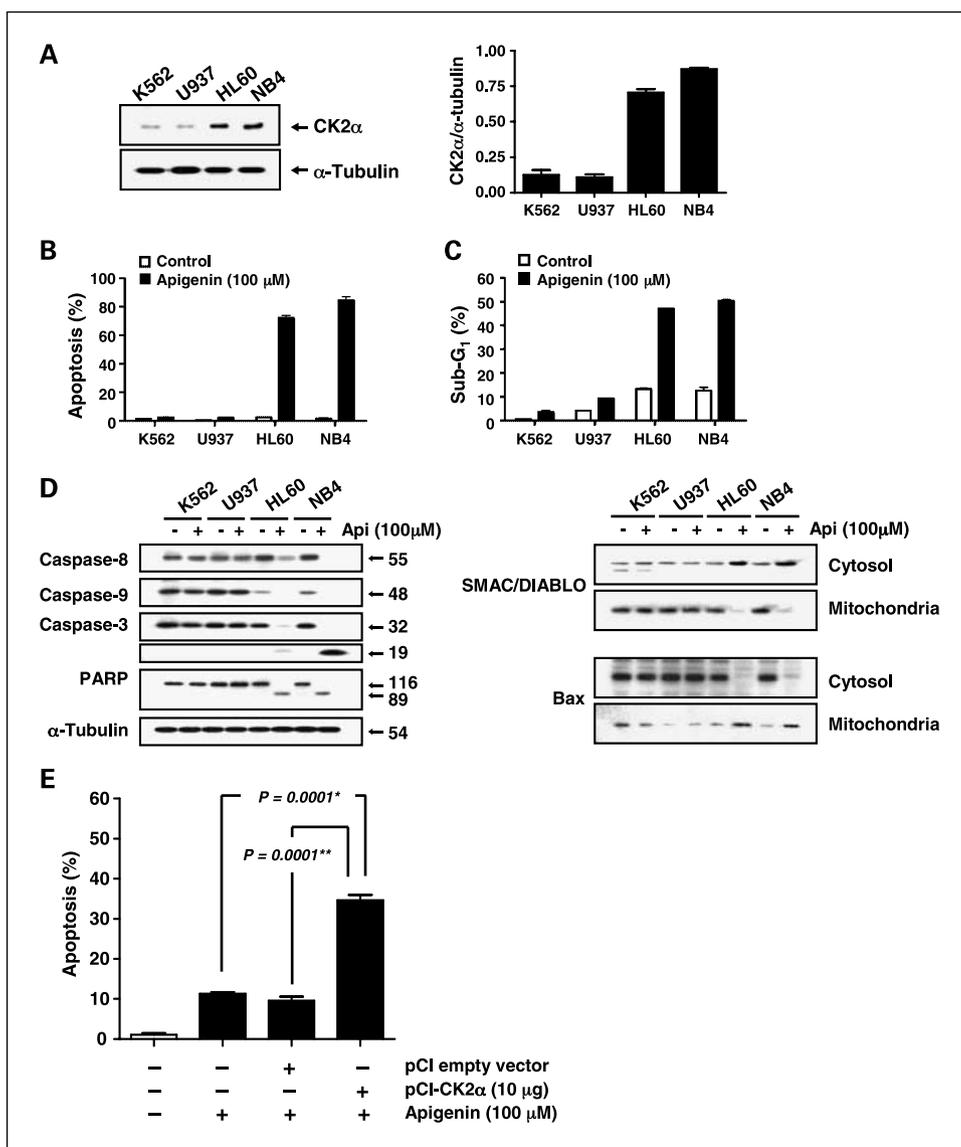
It is noteworthy to observe that, for the first time, the levels of CK2 α expression is an important prognostic factor in AML with normal karyotype. Chromosomal abnormalities have provided a powerful tool to stratify AML patients into different prognostic risk groups. However, $\sim 45\%$ of adults with *de novo* AML have normal chromosome and therefore lack chromosomal markers (47, 48). This heterogeneous cohort of patients

has variable clinical outcomes. Little is known about the underlying molecular mechanisms contributing to the clinical heterogeneity of AML with normal karyotype. Recently, it was shown that *BAALC* (brain and acute leukemia, cytoplasmic) expression (49, 50), mutation of *CEBPA* (51, 52), and *FLT3-ITD* (53, 54) categorize normal karyotype AML into clinically distinct subgroups. Given that intensive treatments, such as allogeneic hematopoietic stem cell transplantation, are associated with high treatment-related mortality, the elucidation of novel prognostic markers can lead to the development of risk-adapted therapeutic strategies in karyotypically normal AML patients. In this study, the significantly reduced disease-free survival and overall survival rates observed in CK2 α -high AML cases with normal karyotype indicates that the level of CK2 α expression may be one of the potential markers for predicting the prognosis of AML patients with normal karyotype. Because the largest cytogenetic subset of adult AML consists of patients with normal karyotype, our data revealing CK2 α as an adverse prognostic factor makes possible the development of a risk-adapted therapeutic strategy in adult AML.

However, the function of CK2 α protein in contributing to a more chemoresistant phenotype in AML has yet to be identified. We observed that the cell fraction in G₀-G₁ phase was markedly decreased with induced CK2 α overexpression in U937 cells, suggesting that CK2 α renders leukemia cells a growth advantage. Because p53 induces the G₀-G₁ arrest, we observed the alteration in the level of p53 induced by CK2 α overexpression. As expected, the levels of p53 were decreased with CK2 α overexpression in U937 leukemia cell line.

CK2 also plays a crucial role in ensuring cell survival by counteracting apoptosis through various mechanisms (5, 16, 36, 37, 55). For example, CK2 protects BID from TRAIL-induced caspase-8-mediated degradation (17). Fas receptor-mediated cell death is also regulated by CK2 expression (24). Additional support for the role of CK2 in controlling cell survival and death comes from data showing that CK2 targets several proteins involved in apoptosis; that is, apoptosis repressor with caspase recruitment domain (18, 19), p53 (20), nuclear factor- κ B (21), inhibitor of κ B (22), as well as c-Myc (23). CK2 promotes nuclear factor- κ B-mediated

Fig. 5. Differential effect of CK2 inhibitor on the apoptosis of leukemia cells in relation to CK2 α expression. **A**, constitutive expression of CK2 α protein is variable according to the leukemia cell lines (*left*). Columns, mean value of CK2 α / α -tubulin ratio of three independent experiments; bars, SD (*right*). **B**, after 24-h treatment with apigenin, the apoptotic cell death was markedly increased in HL-60 and NB4 cell lines, both expressed relatively higher levels of CK2 α . In contrast, any notable increase in the extent of apoptosis was not observed in U937 and K562 cell lines, both of which are characterized by low levels of CK2 α expression. **C**, cell cycle analysis revealed that cells in the sub-G₁ fraction were also markedly increased in HL-60 cells and NB4 cells with apigenin treatment. **D**, after 24-h treatment of leukemia cell lines with apigenin, whole-cell extracts (*left*) or cytosolic/mitochondrial fractions (*right*) were subjected to Western blot analysis. The proteolytic cleavage of caspase-8, caspase-9, caspase-3, and PARP was evident in HL60 and NB4 cell lines (*left*). Cytosolic release of SMAC/DIABLO and the translocation of Bax protein into mitochondria were shown in HL60 and NB4 cell lines (*right*), indicating that the mitochondrial apoptotic pathway was executed. **E**, an induced overexpression of CK2 α remarkably enhanced the sensitivity of U937 leukemia cells to the apigenin-induced cell death. The level of apoptosis induced by 48-h treatment with apigenin was significantly increased in the pCI-CK2 α -transfected U937 cells, compared with the untransfected cells (* $P = 0.0001$) or empty vector-transfected U937 cells (** $P = 0.0001$). Columns, mean of three independent experiments; bars, SD.



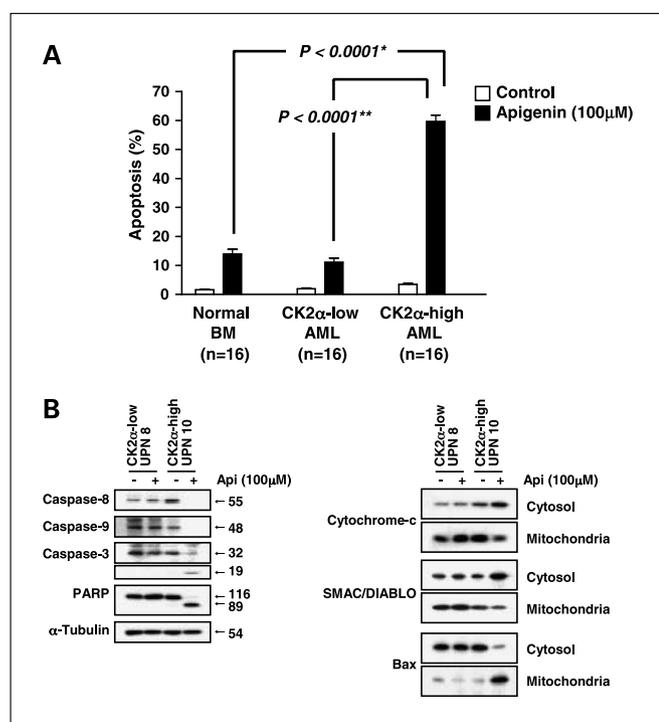


Fig. 6. Levels of CK2 inhibitor – induced apoptosis of primary AML cells according to CK2 α expression. **A**, primary leukemic blasts obtained from representative AML patients were treated with apigenin (100 μ mol/L) for 24 h; then, the apoptotic cells were measured using flow cytometry. The apigenin-induced apoptosis was significantly increased in the CK2 α -high AML cells compared with the normal BM cells ($*P < 0.0001$) or CK2 α -low AML cells ($**P < 0.0001$). Columns, mean of three independent experiments; bars, SD. **B**, after 24-h treatment with apigenin (100 μ mol/L), the processing of caspase-8, caspase-9, caspase-3, and PARP cleavage was markedly increased in the representative CK2 α -high AML cells but was negligible in the representative CK2 α -low AML cells (*left*). The release of cytochrome *c* and SMAC/DIABLO into the cytosol, and the translocation of Bax into the mitochondria, were evident by apigenin treatment in the CK2 α -high AML cells but were negligible in the representative CK2 α -low AML cells (*right*).

expression of Bcl-xL, whereas cancer cells with constitutive activation of CK2 exhibit a high Bcl-xL/Bid ratio (17). Apoptosis induced by either heat shock or radiation is blocked by CK2 (56–58). CK2 also facilitates repair of chromosomal DNA single-strand breaks (59). In this study, we showed that CK2 α overexpression resulted in up-regulation of molecules associated with PI3K/Akt/PKB pathway, such as Ser⁴⁷³ p-Akt/PKB, p-PDK1, p-FKHR, and p-Bad, and antiapoptotic molecules such as Bcl-2, Bcl-xL, Mcl-1, and XIAP in U937 cells.

Because CK2 α is associated with the suppression of apoptosis, dysregulation of cell proliferation, and poor clinical outcome, CK2 may also serve as a molecular target for novel therapeutic approaches. Antisense oligodeoxynucleotide-induced down-regulation of CK2 subunits resulted in a significant degree of cell death in prostate adenocarcinoma and head and neck squamous cell carcinoma (60). In a similar vein, inhibitors of CK2 have been reported to trigger apoptosis and to increase the susceptibility of cancer cells to chemotherapeutic agents and apoptotic stimuli (15, 17, 37). In this study, we observed that treatment of U937 leukemia cells with selective CK2 inhibitor, tetrabromobenzotriazole or apigenin, resulted in a marked down-regulation of molecules associated with cell proliferation and antiapoptosis in a dose-dependent manner. The molecules that are down-regulated include CK2 α ,

Ser⁴⁷³ p-Akt/PKB, p-PDK1, p-FKHR, p-Bad, Bcl-2, Bcl-xL, Mcl-1, and XIAP. It was also of great importance to observe that apigenin treatment potentially abolished the CK2 α overexpression-induced increases in levels of molecules associated with PI3K/Akt/PKB pathway and antiapoptotic molecules described above. Knockdown of CK2 α gene by CK2 α siRNA transfection also resulted in a marked down-regulation of Ser⁴⁷³ p-Akt/PKB and Bcl-2 in U937 cells, indicating that levels of Ser⁴⁷³ p-Akt/PKB and Bcl-2 are directly regulated by CK2 α . These findings were not limited to the leukemia cell line. In primary leukemic blasts obtained from representative AML patients, a notable decrease in the levels of Ser⁴⁷³ p-Akt/PKB and Bcl-2 was also observed after treatment with CK2 inhibitors.

Surprisingly, we found that the extent of CK2 inhibitor-induced cell death is significantly different according to levels of CK2 α in leukemia cells. Apoptosis was markedly increased by apigenin treatment in HL60 and NB4 leukemia cell lines, both of which constitutively expressed high levels of CK2 α . In contrast, apigenin-induced apoptosis was negligible in U937 and K562 leukemia cell lines that express low levels of CK2 α . The cell death induced by this agent was typically accompanied with cytoplasmic release of SMAC/DIABLO and proteolytic cleavage of procaspase-9, procaspase-3, procaspase-8, and PARP in HL-60 and NB4 cell lines. To prove that the apoptosis-inducing effect of apigenin is dependent on CK2 α expression in leukemia cells, the effect of apigenin was also evaluated in U937 cells transfected with pCI-CK2 α . Although CK2 α expression was positively correlated with the expression of antiapoptotic proteins, an induced overexpression of CK2 α remarkably enhanced the sensitivity of U937 leukemia cells to the apigenin-induced cell death. Empty vector transfection did not affect the apigenin-induced cell death. These findings potentially indicate that the extent of apigenin-induced leukemia cell death critically depends on the levels of CK2 α expression in leukemia cells.

The preferential apoptosis-inducing effects of apigenin on CK2 α -high leukemia cells were also observed in primary leukemia cells obtained from patients with AML. The proportion of apoptotic cells after apigenin treatment was significantly higher in CK2 α -high AML cases compared with CK2 α -low AML cases. This observation is in line with the recent finding that inhibition of Akt/PKB kinase with the small-molecule API-2 resulted in suppression of cell growth and induction of apoptosis in Akt/PKB-overexpressing cancer cells (61). API-2 potentially inhibited tumor growth in nude mice of human cancer cells in which Akt/PKB is aberrantly expressed/activated but not of those cancer cells in which it is not (61).

As expected, the apigenin-induced apoptosis was negligible in our normal bone marrow samples. All these findings suggest that CK2 inhibition potentially induces apoptosis selectively in CK2 α -high AML cells, whereas normal hematopoietic stem cells escape apoptosis. In another study, whereas CK2 α oligodeoxynucleotide mediated tumor cell death in a dose- and time-dependent manner, normal and benign cells showed a relative resistance to antisense CK2 α oligodeoxynucleotide treatment (62). These findings indicate that CK2 inhibitor potentially induces apoptosis selectively in the CK2-positive AML cells while sparing normal hematopoietic stem cells.

Taken together, we show here that elevated CK2 α expression is an unfavorable prognostic marker in AML with normal

karyotype. At the cellular level, CK2 α increases the levels of antiapoptotic proteins. However, CK2 α -overexpressing leukemia cells are also very sensitive to CK2 inhibitor-induced apoptosis, with significant down-regulation of antiapoptotic molecules. Our findings that the leukemia cells harboring high levels of CK2 α are more susceptible to CK2 inhibition-induced cell death and that normal bone marrow cells are relatively insensitive to CK2 inhibitor provide new hope for the

applicability of CK2-targeted therapy in AML. Furthermore, recent reports that inhibition of CK2 results in enhanced apoptosis in cancer cells after TRAIL (17, 63), ionizing radiation damage (58), or 6-thioguanine treatment (64) likely suggests the potentiality of CK2-targeted therapy as a combination therapy for AML. We are now investigating whether CK2 inhibition renders leukemia cells sensitive to conventional chemotherapeutic agents.

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Protein Kinase CK2 α as an Unfavorable Prognostic Marker and Novel Therapeutic Target in Acute Myeloid Leukemia

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