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Circulating Ki-67 Index in Plasma as a Biomarker and Prognostic Indicator in Chronic Lymphocytic Leukemia

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Abstract

Ki-67 is a nuclear antigen that is expressed in all stages of the cell cycle, except G₀, and is widely used as a marker of cellular proliferation in human tumors. We recently showed that elevated levels of Ki-67 circulating in plasma (cKi-67) are associated with shorter survival in patients with acute lymphoblastic leukemia. The study included 194 patients with CLL and 96 healthy control subjects. cKi-67 levels in plasma were determined using an electrochemiluminescent immunoassay. We normalized the cKi-67 level to the absolute number of lymphocytes in the patient's peripheral blood to establish the plasma cKI-67 index. The cKi-67 index showed significant correlation with lymph node involvement and Rai stage ($P = 0.05$). Higher cKi-67 index values were significantly associated with shorter survival. Multivariate Cox proportional hazards regression analysis demonstrated that the association of the cKi-67 index with shorter survival was independent of IgV_H mutation status. In a multivariate model incorporating the cKi-67 index with B2M and IgV_H , only cKi-67 index and B2M levels remained as independent predictors of survival. The results of this study suggest that the plasma cKi-67 index, along with B2M level, is a strong predictor of clinical behavior in CLL

Keywords

cKi-67 Index; Plasma; Chronic Lymphocytic Leukemia

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Authors Contributions

MA, JMB. Conception and design of the study. HK, ZE, WM, CY, AD, HS, SO, MK acquisition of data, or analysis and interpretation of data. JMB, CY, MA. Drafting the article or revising it critically for important intellectual content. JMB, MA. Final approval of the version to be submitted.

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We attest that the authors have no financial conflicts of interest.

INTRODUCTION

B-cell chronic lymphocytic leukemia (CLL) is the most common form of leukemia in adults in the Western hemisphere, and is considered an incurable disease. More than 12,000 new cases are diagnosed annually in the United States alone [1, 2]. Therefore, identifying markers that complement or replace conventional prognostics is necessary. CLL is characterized by the gradual accumulation of growth-arrested (G_0/G_1) clonal mature B-lymphocytes, and flow cytometry analysis has consistently revealed that <5% of B-CLL cells are proliferating at any given time [3]. B-CLL cells are also generally resistant to apoptosis [4]. However, the fraction of proliferating cells increases with disease progression, and increased proliferative activity is associated with more aggressive tumor growth and a decreased disease-free survival period. CLL can transform into a large-cell form of disease (Richter syndrome [5]), but frequently follows a non-aggressive course with occasional observance of large cells with high growth rates. The percentage of large cells may vary, and there are no standards to quantify the level of the proliferation fraction.

Ki-67, a nuclear protein of mammalian cells, is a widely used marker of cell proliferation that is not believed to be secreted or shed from the cell. Ki-67 is preferentially expressed during late G_1 -, S-, M-, and G_2 -phases of the cell cycle, and is absent in the G_0 phase. Ki-67 expression is routinely monitored by immunohistochemistry (IHC) [6-8] in cancer patients, especially when transformation is expected [9-12]. It has been shown that IHC testing of Ki-67 is useful in studying cellular proliferation of CLL, and that CLL with polymorphic morphology is more prone to proliferate than is typical CLL [13].

We recently developed an electrochemiluminescent (ECL) method for sensitive measurement of circulating Ki-67 in plasma (cKi-67). This assay demonstrated significantly higher levels of cKi-67 in patients with newly diagnosed acute lymphoblastic leukemia (ALL) than in healthy control subjects (14). Moreover, elevated plasma cKi-67 was associated with significantly shorter survival in ALL patients. Here we applied the same technology to investigate the association of plasma cKi-67 levels with clinical behavior in a large number of patients with CLL, and found a similar association of cKi-67 index with shorter survival, as well as with lymph node involvement and Rai stage. Our results suggest that the cKi-67 index may be useful as a prognostic indicator in patients with CLL.

MATERIALS AND METHODS

Patients and Samples

Peripheral blood samples were collected from 194 adults with a diagnosis of CLL at The University of Texas MD Anderson Cancer Center (Houston, TX). Diagnosis was based on morphologic evaluation of peripheral blood and bone marrow, along with flow cytometry and cytogenetics. Complete clinical and laboratory data were available for each patient, including white blood cell count (WBC), lymphocyte count, platelets, hemoglobin, β_2 -microglobulin (B2M), IgV_H mutation status, Rai stage, and performance status. After separation, plasma was stored at -70°C until use. The study was performed under an IRB-

approved protocol, according to institutional guidelines, after informed consent was obtained.

The normal control group are healthy volunteer. The median age of the normal control is 44 year. The male: female ratio was 0.92.

Reagents

Ninety-six-well small-spot-coated anti-mouse plates, Tris wash buffer, Tris lysis buffer, blocker A, Read Buffer T, and antibody diluents buffer were obtained from Meso-Scale Discovery (MSD; Gaithersburg, MD). Protease inhibitor cocktail set III was from EMD Biosciences (San Diego, CA). Antibodies were obtained from various sources: anti-mouse Ki-67 antibody clone 7B11 from Invitrogen (Carlsbad, CA); anti-rabbit Ki-67 antibody clone H300 from Santa Cruz (Santa Cruz, CA); goat anti-chicken antibody from Rockland (Gilbertsville, PA); anti-rabbit SULFOTAG from MSD; and anti-mouse and rabbit HRP-conjugated antibodies from Bio-Rad Laboratories (Hercules, CA) .

Cell lines

The Jurkat cell line was obtained from ATCC (Manassas, VA) and was maintained in RPMI 1640 supplemented with 10% fetal calf serum (FCS; Hyclone, Tulare, CA), 1 mmol/L L-glutamine, and antibiotics (streptomycin/penicillin). Cells were cultured at 37 °C in a humid atmosphere with 5% CO₂.

Immunoprecipitation

Plasma (500 µL) was incubated with 2 µg of anti-Ki-67 clone H300 or rabbit IgG (Isotype specificity control) in 1 mL lysis buffer for 2 h at 4 °C, followed by incubation with 20 µL protein A/G Sepharose beads (Sigma) for 1 h at room temperature. The supernatants were removed and stored for further Western blot analysis. The beads were then washed 4 times with lysis buffer and Ab:protein complexes were resuspended in loading buffer (100 mM Tris HCl [pH 6.8], 12.5% glycerol, 8% β-mercaptoethanol, 0.1% bromophenol blue, 3% SDS). Immunocomplexes were harvested and subjected to Western blot analysis with appropriate antibodies.

Ki-67 immunoblot analysis

Jurkat cell lysates were prepared in RIPA buffer (10 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 5 mM EDTA) containing protease inhibitors (Complete Protease Inhibitor Cocktail Tablets; Roche Applied Science, Palo Alto, CA). Lysate was normalized to total protein (50 µg) and subjected to immunoprecipitation. The immunoprecipitation product was separated using SDS-polyacrylamide gel electrophoresis (SDS-PAGE; 4% to 20% gradient gels; PIERCE) followed by immunoblot analysis. Primary antibodies included anti-Ki-67, (Cell Signaling, Danvers, MA). Immunodetection was accomplished with the use of HRP-conjugated secondary antibodies and an enhanced chemiluminescence (ECL) method (PIERCE) involving exposure to x-ray film (XAR; Kodak, Sigma).

Circulating Ki-67 ECL assay

This assay employs an ECL-based technology from MSD, an adaptation of enzyme-linked immunosorbent assays (ELISAs), as described previously[14].

Statistics

The Kruskal-Wallis test was used for categorical data. Correlations in plasma and peripheral blood cell samples were determined using Spearman's correlation coefficients. The log-rank test was used to compare Kaplan-Meier survival curves between CLL patients with different plasma levels of cKi-67. Cox proportional hazards models were used for predicting survival and in multivariable analysis. Clinical and biological findings were analyzed for association with survival.

RESULTS

The characteristics of patients are listed in Table 1. These characteristics are representative of CLL patients.

Detection of cKi-67 in CLL and normal plasma samples by Western blot

We first used immunoprecipitation followed by immunoblotting to determine whether Ki-67 is present in plasma samples from CLL patients and healthy individuals. cKi-67 was detected by immunoblot in CLL patient samples but was also detectable at low levels in normal plasma samples (Fig 1A). Immunoprecipitation of Ki-67 from Jurkat extracts served as a positive control, while immunoprecipitation with anti-IgG isotype control antibody served as a negative control. These data demonstrate that cKi-67 protein is present and measurable in the plasma of both CLL patients and healthy controls.

Measurement of plasma cKi-67 by ECL-based assay

Having established that cKi-67 can be detected in plasma of patients with ALL, we used the ECL-based assay to measure plasma levels of cKi-67 in samples from normal control subjects and patients with CLL. The assay specificity, sensitivity and accuracy have been previously reported [15]. Figure 1 shows the levels of Ki-67 protein as detected by immunoprecipitation and Western blot (panel A) and as detected by the ECL assay (panel B). The ECL-based assay demonstrated significantly higher absolute cKi-67 levels in CLL patients than in normal control subjects (Fig 2).

Clinical and laboratory correlations of plasma cKi-67 levels

Absolute plasma cKi-67 levels in CLL patients did not correlate significantly with WBC, platelet count, hemoglobin level, B2M level, *IgV_H* mutation status, Rai stage, or performance status. Nor were absolute cKi-67 levels significantly associated with survival, as a continuous variable or with cut-off points (data not shown).

Clinical and laboratory correlations of plasma cKi-67 index

Since most IHC data demonstrate that the size of the fraction of Ki-67-positive cells is important, we also determined the proliferation fraction in our samples. To achieve this, we

normalized the absolute plasma cKi-67 level to the number of circulating lymphocytes as determined by CBC to obtain the cKi-67 index (U/1000 cells). This also ensured that the cKi-67 level truly reflected proliferation rather than simple disease volume. The variation between patients in cKi-67 index is shown in Figure 3.

Unlike absolute cKi-67 levels, the cKi-67 index showed significant correlation with multiple clinical and laboratory variables, including WBC count, lymphocyte count, and percent bone marrow lymphocytes (Table 2). A Wilcoxon paired test showed correlation of the cKi-67 index with Rai stage but not *IgV_H* mutation status ($P = 0.62$) or performance status ($P = 0.71$). The cKi-67 index also correlated with spleen enlargement ($P = 0.007$) and number of lymph node sites ($P < 0.001$).

The cKi-67 index correlated with overall survival when it was used as a continuous variable as well as when used with a cut-off point. As a continuous variable, cKi-67 index was significantly associated with survival in a Cox regression model ($P = 0.002$). The cKi-67 index was also a predictor of survival when a cut-off point (1200 U/1000 cells) was used ($P = 0.005$; log rank test); patients with cKi-67 index values above this cutoff had shorter survival than those with lower values (Fig. 4). The association of cKi-67 index with survival was independent of the *IgV_H* mutation status (Table 3, Model 1). Moreover, in a multivariate model incorporating the cKi-67 index with B2M and *IgV_H*, only cKi-67 index and B2M remained significant predictors of survival (Table 3, Model 2).

As expected survival correlated with Rai stage ($P < 0.0001$). In multivariate analysis incorporating Rai stage and cKi-67 index, cKi-67 was strong predictor of survival independent of Rai stage ($P = 0.0006$). There was no correlation between LDH levels and survival in this group of patients.

DISCUSSION

Quantitation of Ki-67 by IHC is becoming a widely used assay for diagnosis and monitoring of multiple cancers. Ki-67 has been reported to be particularly useful as a prognostic indicator because it is believed to reflect the cell growth fraction in tumors, most notably in prostate and breast carcinomas [16-20]. High levels of proliferation and high levels of Ki-67 staining have been reported in patients with ALL, and particularly in patients with Burkitt lymphoma [21]. Slow accumulation of CLL cells in vivo was considered to be due to defective apoptosis rather than proliferation. However, recent data based on isotope incorporation studies suggest significant continuous proliferation of CLL cells in vivo, and similar observations have been made for human memory B cells [22]. These studies not only confirm that CLL is a disease of accumulation with different associated levels of proliferation, but also indicate that proliferation increases with disease progression [23, 24].

Determination of the Ki-67 index using IHC-based assays is useful in establishing the proliferating fraction of various neoplasms. The correlation between low Ki-67 index and histologically low-grade tumors is strong [9, 11, 25-35]. Our recent report [36] demonstrated that plasma cKi-67 levels can be used as a biomarker in ALL, and that the only functional difference between the plasma cKi-67 assay and the IHC Ki-67 index assay is that the

former lacks normalization to the number of tumor cells. Plasma levels of cKi-67 could reflect variation in not only tumor mass, but also in levels of tumor proliferation and aggressiveness. In the present study, we took into account the fact that CLL is usually first characterized by the presence of lymphocytosis [37]. Therefore, to establish a growth fraction using free cKi-67, which is generated by leukemic cells, we normalized the cKi-67 level to the absolute number of lymphocytes in circulation, yielding the cKi-67 index (unit cKi-67/1000 circulating lymphocytes/microliter of plasma). Our findings demonstrated that the cKi-67 index can be used as an independent prognostic marker in patients with CLL. While it is likely that cKi-67 reflects the growth fraction of not only circulating CLL cells, but also lymph nodes and other tissues such as bone marrow, spleen, and liver, using peripheral blood lymphocytes is the easiest means for measuring tumor mass in patients with CLL. Future studies based on normalizing cKi-67 to other tumor markers, such as CD20 [38], may provide more precise normalization.

We and others have previously reported that plasma from patients with leukemia is enriched with leukemia-specific nucleic acid and cellular proteins [39]. This plasma enrichment presumably derives from the more rapid turnover of leukemic (relative to normal) cells and the resulting release of cellular proteins and nucleic acids into circulation [40-44]. B2M is a protein that is sometimes shed from cell surfaces into blood serum [45] and has a strong prognostic value in CLL. In a multivariate Cox proportional hazards regression analysis, we demonstrated a correlation of cKi-67 index with survival that was independent of B2M and *IgV_H* mutation status. Moreover, in a multivariate model incorporating the cKi-67 index, B2M, and *IgV_H*, only the cKi-67 index and B2M remained significant predictors of survival. Hence, determination of the cKi-67 index and B2M level in plasma may be useful for predicting clinical behavior of CLL. Whether these markers alone will replace the need for determination of *IgV_H* mutation status requires additional study.

Antisense treatment directed against Ki-67 mRNA has been found to inhibit proliferation and tumor growth in vitro and in vivo [46, 47]. Moreover, studies analyzing tissue sections have shown that sulindac sulfide inhibits the proliferation of colon cancer cells by reducing expression of the proliferation markers PCNA and Ki-67 [48]. Hence, a quantitative cKi-67 assay could be useful to monitor drug efficacy in clinical trials, without the need for biopsy. Taken together, our findings strongly suggest the potential usefulness of the cKi-67 index as a prognostic indicator in patients with CLL, and warrant further studies on the biologic function and prognostic and therapeutic significance of cKi-67 in CLL.

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Figure 1.

Detection of circulating Ki-67 protein in plasma of CLL patients and control subjects. A) Jurkat extracts (100 μ g, lane 1; positive control) and diluted plasma from 4 CLL patients and 4 normal control subjects (1:5 in lysis buffer, lanes 2-9) were subjected to immunoprecipitation with anti-Ki-67 antibody and analyzed by SDS-PAGE/immunoblotting using anti-Ki-67. Antibody detection was accomplished by enhanced chemiluminescence with exposure to x-ray film. B) Immunoprecipitation products were also evaluated using the Meso Scale Discovery (MSD)-based enzyme immunoassay.

Figure 2.

Circulating Ki-67 (cKi-67) levels in plasma from normal control subjects (n=96) and chronic lymphocytic leukemia (CLL) patients (n=194). cKi-67 levels were significantly lower in CLL patients. P value was calculated using Krustal Wallis ANOVA by rank.

Figure 3.
cKi-67 index values in 194 patients with chronic lymphocytic leukemia (CLL) arranged from the lowest to the highest.

Figure 4.

Kaplan-Meier plot showing survival of CLL patients when patients were dichotomized according to the level of cKi-67 index. Patients with cKi-67 index higher than 1200 (U/1000) had significantly shorter survival. Of 128 total number of patients with low cKi-67 index, 28 patients died. In contrast 25 patient died of the 64 patients with high cKi-67. (Abbreviation, E refers to death and N to total number)

Table 1

Patient Characteristics.

Characteristic	%
Male	68
Rai III-IV	25
Splenomegaly	28
Hepatomegaly	4
Lymph nodes	65

	Median (range)
Age	61 (34 - 84)
WBC ($\times 10^3/\mu\text{L}$)	21.25 (1.4- 321)
HGB (g/dL)	13 (3.3-16.8)
B2M (mg/L)	3.1 (1.4-18.1)
Platelets ($\times 10^6/\text{L}$)	177 (4-511)

Abbreviations: HGB: Hemoglobin; WBC: White Blood Cell Count; B2M: $\beta 2$ microglobulin.

Table 2

Spearman Rank order Correlations of Plasma Circulating Ki-67 Index with Other Laboratory Variables

Variable	Valid - N	Spearman - R	t(N-2)	P
HGB (g/dL)	194	0.022	0.310	0.756
PLT ($\times 10^6/L$)	194	0.165	2.325	0.021
WBC ($\times 10^6/L$)	194	-0.865	-23.900	<0.001
Lymphocytes (%)	194	-0.717	-14.288	<0.001
B2M (mg/L)	194	-0.113	-1.575	0.116
BM Cellularity (%)	189	-0.401	-6.001	<0.001
BM-Lymphocytes (%)	194	-0.525	-8.567	<0.001
Creatinine (mg/dL)	193	-0.113	-1.575	0.116
IgG (mg/dL)	184	0.009	0.125	0.900
IgA (mg/dL)	183	0.095	1.296	0.194
IgM (mg/dL)	184	0.154	2.115	0.035
Protein (g/dL)	58	-0.096	-0.726	0.470
CD11/CD22 (%)	194	-0.111	-1.551	0.122
CD11C (%)	194	-0.152	-2.140	0.033
CD22(%)	194	-0.099	-1.384	0.167

Abbreviations: HGB: hemoglobin; PLT: platelet count; WBC: white blood cell count; B2M: β 2 microglobulin; BM: Bone Marrow; Ig, immunoglobulin.

Table 3

Multivariate Cox proportional Hazard Regression Models for Predicting Survival of Patients with Chronic Lymphocytic Leukemia

Model #1						
	Beta	Standard - Error	t-value	exponent - beta	Wald - Statist.	p
cKi-67 index	0.926	0.4118	2.250	2.5267	5.0642	0.024
IgVH	-1.152	0.5413	-2.129	0.3158	4.5330	0.033
Model #2						
cKi-67 Index	1.0120	0.4147	2.440	2.7513	5.954	0.015
IgVH	-0.8000	0.5505	-1.453	0.4493	2.111	0.146
B2M	0.2868	0.0623	4.599	1.3322	21.151	<0.001

Abbreviations: cKi-67: circulating Ki-67 protein in plasma; B2M: beta 2-microglobulin.