Proteasome Enzymatic Activities in Plasma as Risk Stratification of Patients with Acute Myeloid Leukemia and Advanced-Stage Myelodysplastic Syndrome

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Abstract

Purpose: Cytogenetic abnormalities are currently the most important predictors of response and clinical outcome for patients with acute myeloid leukemia (AML) or advanced-stage myelodysplastic syndrome (MDS). Because clinical outcomes vary markedly within cytogenetic subgroups, additional biological markers are needed for risk stratification.

Experimental Design: We assessed the utility of measuring pretreatment proteasome chymotrypsin-like, caspase-like, and trypsin-like activities in plasma to predict response and survival of patients with AML ($n = 174$) or advanced-stage MDS ($n = 52$).

Results: All three enzymatic activities were significantly ($P < 0.001$) increased in the plasma of patients with AML and MDS compared with normal controls. Both chymotrypsin-like and caspase-like activities, but not trypsin-like activity, correlated with outcome. Chymotrypsin-like and caspase-like activities, but not trypsin-like activity, predicted response in univariate analysis ($P = 0.002$). However, only chymotrypsin-like activity was independent predictor of response from age grouping (<70 versus ≥70 years), cytogenetics, and blood urea nitrogen in multivariate analysis. Similarly, both chymotrypsin-like and caspase-like activities, but not trypsin-like activity, were predictors of overall survival in univariate analysis ($P < 0.0001$), but only chymotrypsin-like activity was independent of cytogenetics, age, performance status, blood urea nitrogen, and β_2-microglobulin in multivariate Cox regression models. Chymotrypsin-like activity was also a strong independent predictor of survival in patients with intermediate karyotype ($n = 124$).

Conclusions: Measuring plasma chymotrypsin-like activity may provide a powerful biomarker for risk stratification in patients with AML and advanced-stage MDS, including those with normal karyotype.

Acute myeloid leukemia (AML) is a disease with significant morphologic, cytogenetic, and molecular heterogeneity. Management and therapeutic decisions are frequently based on multiple prognostic factors including age, karyotype, presence or absence of *fms-related tyrosine kinase 3 gene* and *nucleophosmin gene* mutations, WBC count, comorbid conditions, underlying dysplasia, and other, less well-defined, factors. Despite the numerous predictive factors available, an effective and reliable means for risk stratification remains elusive. Often, analysis of available prognostic factors fails to answer the most important questions: which patients will not benefit from chemotherapy and which should be considered for stem cell transplantation. Risk stratification is particularly problematic in patients with intermediate cytogenetics, including those with normal karyotype.

Recent studies suggested that gene expression profiling may provide a new means to further refine risk stratification in patients with AML (1). However, poor reproducibility and difficulties in adapting gene expression profiling in clinical laboratories may limit the utility of such approaches. Additional challenges include variability from multiple sources, including post-transcriptional modification, sampling (interindividual variation in the percentage of leukemic cells), and dilution effects from normal cells in the analyzed population of cells.

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Received 11/20/08; revised 2/4/09; accepted 3/8/09; published OnlineFirst 5/19/09.

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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doi:10.1158/1078-0432.CCR-08-3034

Translational Relevance

We show that measuring proteasome enzymatic activities in the plasma provides new biomarkers for the prediction of clinical behavior in patients with acute myeloid leukemia and advanced-stage myelodysplastic syndrome. We show that the three enzymatic activities of the proteasomes (chymotrypsin-like, trypsin-like, and caspase like) are highly elevated in the plasma of patients with acute myeloid leukemia and myelodysplastic syndrome. Using multivariate regression model, we show that levels of the chymotrypsin-like activity is strong predictor of response and survival that is independent of cytogenetic grouping and all other known prognostic factors. We believe that this new biomarker, which is easily and reliably measured in the peripheral blood plasma, can be used to stratify patients for therapeutic approaches (chemotherapy versus transplant or other experimental therapy). The use of plasma levels of proteasome activities as biomarkers is a novel approach and has the potential to be used in other cancers.

Sorting leukemic cells or specifically dissecting the tumor can help avoid these problems, but both techniques are difficult and frequently require significant *ex vivo* manipulation, which may affect the internal signaling pathways and the original intracellular protein balance. Analysis of plasma or serum proteins may provide a solution to this dilemma; our search for such a biomarker led us to develop a plasma-based assay for proteasome activity.

The ubiquitin-proteasome system plays a major role in cell cycle regulation and division, cell differentiation, response to stress and extracellular effectors, transcription regulation, and DNA repair (2–5). This system also helps maintain the health of individual cells by recognizing and removing misfolded proteins. Through a complex interaction, ubiquitin is activated at the appropriate time, then binds the protein to be hydrolyzed, and transfers it to the proteasome for degradation. The proteasome itself consists of a large, complex structure with three enzymatic activities (chymotrypsin-like, trypsin-like, and caspase-like), which are responsible for digestion of proteins that are no longer needed (6–8). A recent study indicates that serum levels of circulating proteasomes correlate with outcome in multiple myeloma (9). We have also reported that proteasome activity can be monitored in the plasma and correlates with clinical behavior in patients with chronic lymphocytic leukemia (10).

Here, we used a simple plasma-based assay and measured the three enzymatic activities of proteasome and examined their ability to predict treatment response and survival in patients with AML or advanced-stage myelodysplastic syndrome (MDS). We report that levels of chymotrypsin-like activity in plasma provide reliable prediction of response to standard chemotherapy and survival, even in patients with intermediate or normal cytogenetics, and discuss the implications of these findings for risk stratification.

Materials and Methods

Patients and samples. All samples from patients and healthy volunteers were collected under an internal review board-approved protocol with written informed consent. Patient samples were collected during the period of 2001 and 2003 without selection from newly diagnosed patients before initiating therapy at M. D. Anderson Cancer Center. All patients were newly diagnosed, but majority were referred after diagnosis by their local physician within few days of their diagnosis. Diagnosis of AML and advanced-stage MDS was made at M. D. Anderson Cancer Center and based on examination of peripheral blood and bone marrow samples. Blood counts, flow cytometry, and molecular study data were used for diagnosis. Plasma was separated from EDTA peripheral blood tubes by centrifuging at 1,500 × *g* for 10 min at 4°C. Plasma samples obtained from apparently healthy volunteers were used as controls for each chip. Plasma samples were stored at −70°C from EDTA peripheral blood and stored at −70°C until analysis for chymotrypsin-like proteasome activity. This is a retrospective study, all samples used in this study were frozen, and no fresh samples were used. Both AML and MDS patients were treated at M. D. Anderson Cancer Center with standard therapy based on idarubicin + 1-β-D-arabinofuranosylcytosine with minor variations (± topotecan or fludarabine). All patients with MDS had advance disease and were candidate for chemotherapy. Advanced-stage MDS disease is defined by the presence of severe anemia (<8 g/dL), thrombocytopenia (<50 × 10^9/L), or >10% blasts. Of the MDS patients, 65% had refractory anemia with excess blasts in transformation and can be classified as AML according to the WHO classification and the chronic myelomonocytic leukemia patients had blast count >10%. The remaining 14 patients (Table 1) had a score of 1 to 2 on the International Prognostic Scoring System.

Measurement of proteasome enzymatic activities. Chymotrypsin-like, caspase-like, and trypsin-like activities were assayed by continuously monitoring the production of 7-amino-4-methylcoumarin (AMC) from fluorogenic peptides as described previously (10). Briefly, 45 μL plasma was first mixed with 5 μL of 10% SDS at room temperature for 15 min to activate the plasma. The reaction wells contained 30 μL assay buffer (0.05% SDS in 25 mmol/L HEPES), 10 μL activated plasma, and 10 μL of the prospective fluorogenic peptide-AMC substrate (Suc-LLVY-AMC for chymotrypsin-like, Z-LLE-AMC for caspase-like, and BZ-VGR-AMC for trypsin-like). To measure the release of free AMC with time, the SpectraMax Gemini EM (Molecular Devices) instrument was used with the following parameters: Exi = 380 nm, Emi = 460 nm, read interval = 1 min, and read length = 30 min at 37°C. Enzymatic activities were quantitated by generating a standard curve of AMC (range, 0-8 μmol/L). The slope of the AMC standard curve was then used as a conversion factor to calculate the absolute specific activity of each individual sample as pmol AMC/s/mL plasma.

Statistical methods. Clinical and biological characteristics were analyzed for their association with response and survival using log-rank test and multivariate Cox proportional hazards models. Estimates of survival curves (from the time of referral to M. D. Anderson Cancer Center) were calculated according to the Kaplan-Meier product-limit method (11). All patients were newly diagnosed. However, most patients were referred to M. D. Anderson Cancer Center after diagnosis by their local physician and the possibility of few days lapsing before arriving to M. D. Anderson Cancer Center cannot be ruled out. Survival times were compared by means of the log-rank test (12). Cox proportional hazards regression models (13) were used to assess the relationship between patient characteristics and survival, with goodness-of-fit assessed by martingale residual plots and likelihood ratio statistics. Univariate and multivariate Cox proportional hazard models were developed. Predictive variables in the Cox proportional hazards regression model were reviewed to assess the need for transformation based on smoothed martingale residual plots. Predictive variables with P values < 0.10 for the univariate Cox proportional hazards model were included in a

Table 1. Characteristics of patients with AML or MDS

Characteristics	AML (*n* = 174)	MDS (*n* = 52)
Median (range) age	64 (17-84)	65 (23-75)
Performance status, *n* (%)		
0-1	131 (75)	51 (98)
2-4	43 (25)	1 (2)
Cytogenetics, *n* (%)		
Favorable	11 (6)	0
Unfavorable	66 (38)	25 (48)
Intermediate	97 (56)	27 (52)
Median (range) WBC count × 10^9/L	5.6 (0.4-228.0)	2.85 (0.8-148.0)
Median (range) hemoglobin, g/dL	7.8 (3.4-13.1)	7.7 (2.2-11.0)
Median (range) platelets × 10^9/L	50 (6-377)	41.0 (10-270)
French-American-British classification, *n* (%)		
M_{0-2}	106 (61)	
M_3	3 (2)	
M_{4-5}	47 (27)	
M6/M7	18 (10)	
RARS		2 (4)
RAEB		12 (23)
RAEB-T		34 (65)
CMML		4 (8)

Abbreviations: RARS, refractory anemia with ring sideroblasts; RAEB, refractory anemia with excess blasts; RAEB-T, refractory anemia with excess blasts in transformation; CMML, chronic myelomonocytic leukemia.

multivariate model. We attempted to estimate optimal cut points for various covariates in this analysis. Because this dichotomization was based on an optimal cut-point search, we adjusted the *P* value using the method of Schulgen et al. (14). All computations were carried out on a Dell PC using the Windows NT operating system in SAS using standard SAS procedures (SAS Institute).

Nomograms for overall survival and response were developed as described by Kattan et al. (15) using patient characteristics found to be predictive of these two outcomes in the Cox proportional hazards model for survival and logistic regression for response. Overall survival time and response were also predicted via the use of a Visual Basic for Applications computer application developed within Microsoft Excel. This application was developed as a clinical aid and can be considered a computer-based analog to the nomogram. The core construction was based on the Cox proportional hazards model given in the multivariate analysis. To develop Visual Basic for Applications, we first obtained base hazard rate and parameter estimates using the SAS phreg procedure (for overall survival) and SAS logistic procedure (for response). Estimates from these models were then used to obtain estimated survival and response probabilities given the patient's covariates. The program makes use of statistical models to create a graphical representation of a given patient's predicted survival curve.

Results

Characteristics of patients. The 97 control subjects ranged in age from 22 to 72 years, with a median of 47. Complete clinical data for AML and MDS patients were recorded at the time of diagnosis at M. D. Anderson Cancer Center (Table 1). Patients with advanced-stage MDS were treated with AML therapy. There was also no significant difference in response or survival between the AML and the MDS patients. Few AML patients had good cytogenetics [inv16, t(8;21), or t(15;17)] and about one-third had poor cytogenetics (−5, −7, and complex abnormalities); the majority of the AML and MDS patients had intermediate cytogenetics (diploid and other cytogenetics). Because all MDS patients had aggressive disease, only the AML classification of cytogenetics was used. Most of the MDS patients had refractory anemia with excess blasts in transformation. Few had chronic myelomonocytic leukemia with increased blasts (>10%; Table 1).

Proteasome activities in plasma of patients with AML and MDS. All three enzymatic activities (chymotrypsin-like, caspase-like, and trypsin-like) were significantly higher in AML and MDS patients than in control subjects ($P < 0.001$; Table 2). The median for chymotrypsin-like activity was 2.0 pmol/s/mL in AML and 1.4 pmol/s/mL in MDS compared with 0.8 pmol/s/mL in controls. The difference between AML and MDS groups was not significant ($P = 0.62$). The median for trypsin-like activity was 2.5 pmol/s/mL in AML and 2.1 pmol/s/mL in MDS compared with 0.8 pmol/s/mL in control and the difference between AML and MDS groups was not significant ($P = 0.2$). As for caspase-like activity, the median was 3.6 pmol/s/mL in AML and 2.1 pmol/s/mL in MDS compared with 0.9 pmol/s/mL in control,

Table 2. Higher levels of proteasome activities in AML and MDS (pmol/s/mL)

Variable	AML (*n* = 188)	MDS (*n* = 58)	Normal (*n* = 97)	*P* vs normal	*P* AML vs MDS
Chymotrypsin-like	2.0 ± 1.9	1.4 ± 0.9	0.8 ± 0.4	<0.0001	0.62
Trypsin-like	2.5 ± 2.5	2.1 ± 2.6	0.8 ± 1.1	<0.0001	0.2
Caspase-like	3.6 ± 3.5	2.1 ± 1.2	0.9 ± 0.6	<0.0001	0.0006

Table 3. Spearman rank (R value) correlations between proteasome activities and various laboratory findings

	Age	B2-M	WBC	% PB blasts	% PB lymphocytes	Platelets	HGB	BUN	Creatinine	LDH
AML										
Trypsin-like	0.03	−0.03	0.17	0.09	−0.14	0.20	0.03	−0.10	−0.17	0.14
Caspase-like	0.02	0.39	0.41	0.33	−0.43	0.07	−0.09	0.13	0.16	0.59
Chymotrypsin-like	0.05	0.30	0.15	0.14	−0.16	−0.10	−0.05	0.22	0.24	0.33
MDS										
Trypsin-like	0.03	−0.01	0.04	−0.18	−0.02	0.17	0.03	−0.10	−0.08	0.08
Caspase-like	−0.04	0.36	0.44	0.31	−0.38	0.02	−0.08	−0.14	−0.10	0.44
Chymotrypsin-like	0.01	0.29	0.16	0.27	−0.16	0.03	−0.08	−0.02	0.00	0.16

Abbreviations: B2-M, β_2-microglobulin; PB, peripheral blood; HGB, hemoglobin; LDH, lactate dehydrogenase.

but the difference between MDS and AML was significant ($P = 0.0006$; Table 2). There was significant direct correlation between caspase-like and chymotrypsin-like activities in AML ($R = 0.55$) and MDS ($R = 0.51$). In contrast, there was negative correlation between trypsin-like and chymotrypsin-like activities in AML ($R = -0.57$) and MDS ($R = -0.60$). There was no correlation between trypsin-like and caspase-like activities in AML ($R = 0.21$) and MDS ($R = 0.17$).

Clinical correlates of proteasome activities. As shown in Table 3, there was significant correlation between levels of chymotrypsin-like and caspase-like activities and β_2-microglobulin in AML and MDS. Only caspase-like activity correlated with WBC in both AML and MDS. Lactate dehydrogenase correlated with caspase-like activity in both AML and MDS and only with chymotrypsin-like activity in AML. Blast count in peripheral blood correlated with caspase-like activity in both AML and MDS and with chymotrypsin-like activity in MDS. Interestingly, lymphocyte count in peripheral blood correlated negatively with caspase-like activity.

Correlation with response. Response to therapy was similar in the AML and MDS patients (54% versus 53%, respectively). Overall, only 53% of the AML and MDS patients responded to therapy. Because AML and advanced-stage MDS patients had similar outcomes, we pooled these groups for subsequent analyses.

Significant predictors of response in univariate logistic regression modeling were cytogenetic grouping, blood urea nitrogen (BUN), percentages of blasts and lymphocytes in peripheral blood, and both caspase-like and chymotrypsin-like activities as continuous variables (Table 4). There was no correlation between trypsin-like and response. Multivariate models based on the univariate results yielded only three independent predictors of nonresponse: chymotrypsin-like activity (continuous), age (dichotomized by age 70 years), and cytogenetic grouping (Table 2). Ability of caspase-like activity to predict response was redundant to that of chymotrypsin-like and was not independent in the multivariate model. Identical results were obtained when the AML patients and MDS patients were analyzed as separate groups (data not shown).

Using the three independent covariates detected in the multivariate model, we developed a nomogram (Fig. 1A) to predict the odds of nonresponse for individual patients. Comparison

Table 4. Logistic regression modeling results for predicting response in patients with AML and advanced-stage MDS

Variable	Odds ratio (95% confidence interval)	P
Univariate		
β_2-Microglobulin	1.07 (0.98-1.18)	0.122
Platelet count	0.999 (0.995-1.004)	0.818
WBC count	1.01 (0.995-1.014)	0.334
Age (<70 vs >70 y)	1.92 (1.06-3.48)	0.032
Cytogenetics (unfavorable vs other)	2.20 (1.28-3.78)	0.004
Performance status (<2 vs >2)	1.65 (0.86-3.19)	0.135
% Blasts in blood	1.01 (1.00-1.02)	0.043
% Monocytes in blood	1.00 (0.98-1.02)	0.877
% Lymphocytes in blood	0.99 (0.98-1.00)	0.040
Hemoglobin	0.88 (0.75-1.03)	0.120
% Blasts in bone marrow	1.01 (0.99-1.02)	0.363
% Monocytes in bone marrow	1.02 (0.98-1.06)	0.315
% Lymphocytes in bone marrow	1.01 (0.98-1.04)	0.558
BUN	1.04 (1.01-1.07)	0.014
Creatinine	0.95 (0.76-1.20)	0.671
Chymotrypsin-like activity	1.41 (1.14-1.76)	0.002
Caspase-like activity	1.21 (1.07-1.36)	0.002
Multivariate		
Chymotrypsin-like activity	1.44 (1.15-1.81)	0.002
Cytogenetics	2.36 (1.34-4.16)	0.003
Age (<70 vs ≥70 y)	2.03 (1.08-3.79)	0.028

of nomogram-predicted outcomes versus actual outcomes in 126 test patients yielded an area under the curve of 0.70 (Fig. 1B).

When we limited the analysis to patients with intermediate cytogenetic abnormalities (n = 124), response was associated with age group, percent of peripheral blood lymphocytes, BUN, and chymotrypsin-like and caspase-like activities as a continuous variable (each $P < 0.01$) but not with peripheral blood blasts or performance status. Chymotrypsin-like activity, but not caspase-like activity, predicted response when we considered only patients with poor cytogenetics (n = 91; $P = 0.02$).

Predictors of survival. During the follow-up period, 134 (77%) patients with AML and 44 (84%) with advanced-stage MDS died. Univariate Cox regression modeling was used to determine overall hazard ratios for survival in the combined group of MDS and AML patients. Unfavorable cytogenetics, performance status <2, age <70 years, and higher values of β_2-microglobulin, BUN, and both chymotrypsin-like and caspase-like activities were associated with increased risk of death (Table 5). Trypsin-like activity did not correlate with survival.

In multivariate Cox proportional hazards models for survival incorporating the above covariates, cytogenetics, age, and performance status grouping as well as BUN level and chymotrypsin-like activity as continuous variables were independent prognostic factors for survival (Table 5). Identical results were obtained when AML and MDS patients were considered separately (data not shown).

We also used the four independent variables identified in the multivariate analysis to develop a nomogram to predict survival for individual patients (Fig. 1C). Based on the total points, the 2- and 5-year probabilities of survival can be estimated in addition to median survival. Comparison of nomogram-predicted survival versus actual survival in 126 test patients yielded an area under the curve of 0.69 (Fig. 1D).

Considering only patients with intermediate cytogenetic abnormalities (n = 124), survival correlated with performance status ($P < 0.001$), β_2-microglobulin level ($P < 0.001$), BUN level ($P = 0.001$), and both chymotrypsin-like and caspase-like activities ($P < 0.001$) in univariate analysis. In multivariate analysis, however, only chymotrypsin-like activity, β_2-microglobulin

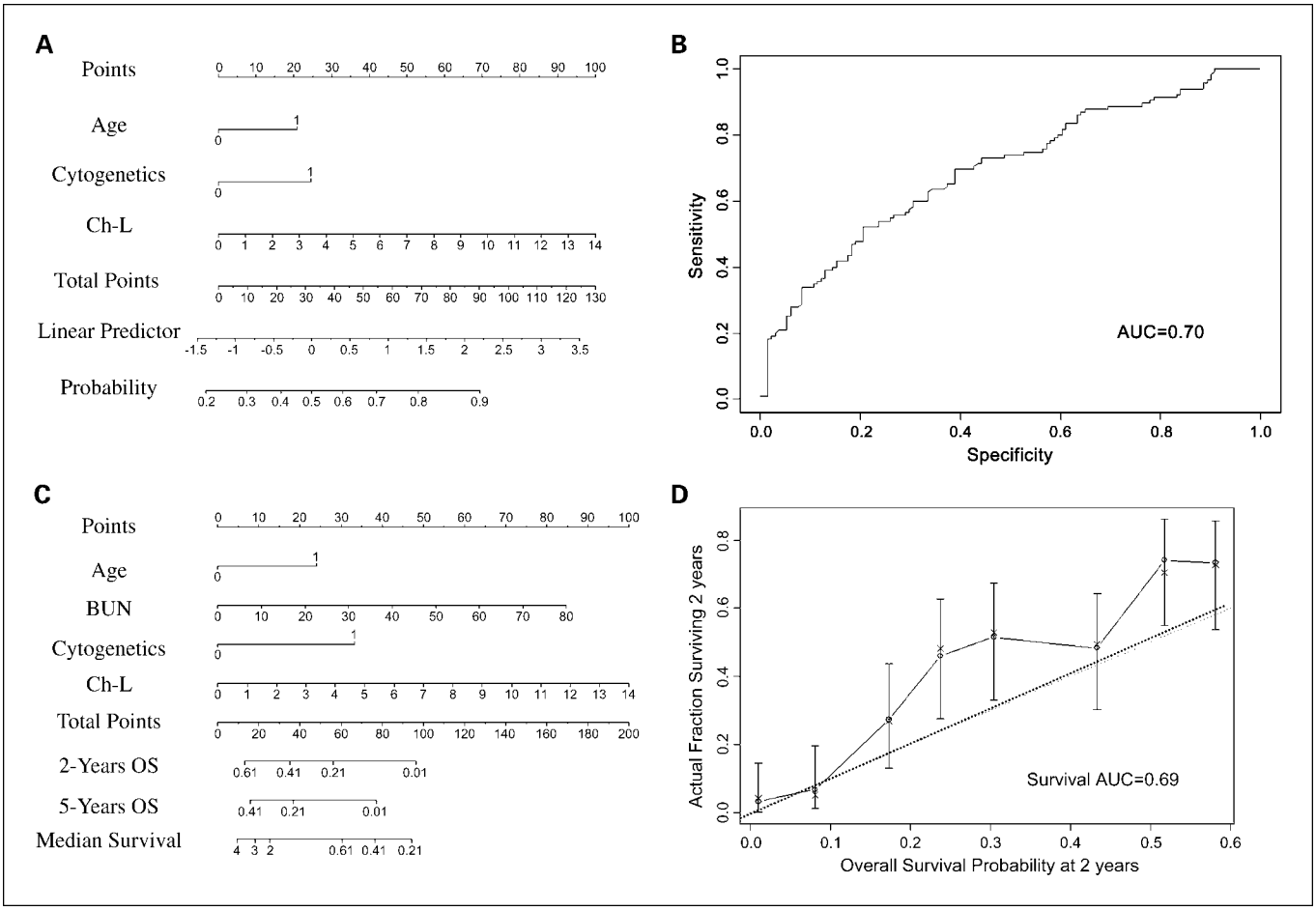

Fig. 1. Estimating probability of nonresponse (*A* and *B*) and survival (*C* and *D*) in previously untreated patients with AML and/or advanced-stage MDS. *A,* nomogram for nonresponse, which is used by totaling the points identified on the top scale for each independent variable. The age is dichotomized as <70 (0 points). Cytogenetics are also dichotomized (24 points, unfavorable; 0 point, intermediate). Chymotrypsin-like proteasome activity is scored on a continuous scale. The total is then used to determine the estimated probability of nonresponse. *B,* receiver operating characteristics curve generated from 126 patients, in which nomogram-predicted outcomes were retrospectively compared with actual outcomes. *C,* nomogram for survival. Interpretation is similar, but poor cytogenetics are scored as 33 and BUN levels are scored on a continuous scale. The probability of 2- and 5-year survival is shown. *D,* calibration curve for 2-year survival based on a retrospective comparison of nomogram-predicted versus actual outcomes for 126 patients. *Solid line,* performance of present nomogram with 95% confidence intervals; *dashed line,* actual survival of the patients.

Table 5. Logistic regression modeling results for predicting survival in patients with AML and advanced-stage MDS

Variable	Hazards ratio (95% confidence interval)	P
Univariate		
β_2-Microglobulin	1.07 (1.04-1.10)	<0.001
Platelet count	0.999 (0.996-1.001)	0.347
WBC count	1.00 (0.997-1.007)	0.486
Age (<70 vs ≥70)	2.09 (1.51-2.89)	<0.001
Cytogenetics (unfavorable vs other)	2.30 (1.70-3.13)	<0.001
Performance status (<2 vs ≥2)	2.14 (1.51-3.03)	<0.001
Peripheral blood blasts	1.00 (0.997-1.01)	0.372
Peripheral blood monocytes	1.00 (0.99-1.01)	0.870
Peripheral blood lymphocytes	0.997 (0.99-1.00)	0.289
Hemoglobin	0.96 (0.89-1.05)	0.397
Bone marrow blasts	1.00 (0.995-1.007)	0.772
Bone marrow monocytes	1.00 (0.98-1.02)	0.894
Bone marrow lymphocytes	1.01 (0.998-1.03)	0.092
BUN	1.05 (1.03-1.06)	<0.001
Creatinine	1.00 (0.91-1.11)	0.939
Chymotrypsin-like activity	1.21 (1.11-1.33)	<0.001
Caspase-like activity	1.12 (1.07,1.18)	<0.001
Multivariate		
Cytogenetics (unfavorable vs other)	2.35 (1.72-3.22)	<0.001
Age (<70 vs ≥70 y)	2.00 (1.44-2.79)	<0.001
Performance status (<2 vs >2)	1.844 (1.29-2.63)	0.001
BUN*	1.026 (1.01-1.04)	0.001
Chymotrypsin-like activity*	1.20 (1.10-1.32)	<0.001

*Used as continuous variables.

level, and performance status remained as independent predictors of survival. Chymotrypsin-like and caspase-like activities were also strong predictor of survival when only patients with poor cytogenetics (n = 91; P = 0.002 and 0.01, respectively). Similarly, chymotrypsin-like and caspase-like activities correlated strongly with survival in patients with normal karyotype (n = 84; P = 0.001 and 0.01, respectively).

Univariate models for event-free survival showed similar findings, with age, performance status, and cytogenetic grouping in addition to β_2-microglobulin, BUN, and both chymotrypsin-like and caspase-like activities as strong predictors (see Table 1 in Supplementary Appendix to this article); all but β_2-microglobulin remained as significant predictors of event-free survival in multivariate analysis (see Table 1 in Supplementary Appendix).

Discussion

Proteasomes are intracellular complexes, and their presence in the plasma as functional structures is intriguing. Based on multiple articles, we speculate that their presence in plasma results from turnover of tissue cells (bone marrow) and not necessarily circulating tumor cells (16–22). Data suggest that leukemic cells have high turnover rates and do not go through the routine programmed cell death and cleaning of the cell debris by the reticuloendothelial system, especially in patients with hematopoietic disease (16). Thus, plasma is enriched by tumor-specific products and is less affected by the dilution effect of normal residual cells than are cell samples obtained from bone marrow (17–23). However, further studies are needed to fully understand the mechanisms responsible for the presence of the leukemic cells proteasomes in plasma. Circulating proteasome levels have been measured in serum and plasma samples by ELISA techniques and are elevated in patients with various types of malignant diseases (9). Recent work showed that serum proteasome levels were significantly elevated during active disease in patients with multiple myeloma and decreased significantly in post-therapy samples of responders but not nonresponders (9).

In this study, we not only confirm that proteasome enzymatic activities can be measured in the plasma of patients with AML and advanced-stage MDS but also show that these activities, especially chymotrypsin-like activity, can be used as a unique tumor marker for predicting therapeutic response and survival. There is significant overlap between chymotrypsin-like and caspase-like activities and their levels correlate with each other but differ significantly from trypsin-like activity in AML and MDS. Chymotrypsin-like activity overshadows the biological value of caspase-like activity in predicting clinical behavior in AML and MDS patients. Our data show that chymotrypsin-like activity of proteasomes is a strongest independent marker of all previously well-established prognostic markers in AML and MDS. To date, cytogenetics, age, and performance status remain the most important prognostic factors in patients with AML or advanced-stage MDS. However, a significant proportion of patients have intermediate cytogenetic abnormalities, which are associated with highly variable outcomes. Additional markers are thus needed to distinguish aggressive from less aggressive disease in this group of patients. Multiple markers have been reported to be relevant for predicting clinical behavior for this group of patients, including *fms-related tyrosine kinase 3 gene* mutations, *nucleophosmin gene* mutations, *brain and acute leukemia gene* expression, *CCAAT/enhancer-binding protein α gene* mutation, and gene expression profiling (24). However, most are

not well-established or require sophisticated or cost-prohibitive technology (25). In contrast, the chymotrypsin-like proteasome activity assay described herein is relatively simple and reproducible and can be performed on peripheral blood plasma.

Moreover, our findings suggest that chymotrypsin-like activity is an independent marker for predicting response to standard chemotherapy when combined with cytogenetics and age grouping. The nomogram developed in this study for prediction of nonresponse is a promising tool; prospective clinical trials are needed to evaluate the utility of this approach for guiding treatment decisions.

Chymotrypsin-like activity can be used to predict overall and event-free survival. The data presented here show that chymotrypsin-like activity is a strong predictor of survival independent of cytogenetics, age grouping, β_2-microglobulin level, and performance status. The second nomogram developed in this study holds potential for use in predicting overall survival for individual patients at 2 and 5 years. Again, prospective studies are needed to verify the predictive values of these nomograms.

Together, our findings indicate that measurement of proteasome chymotrypsin-like activity in plasma provides a powerful biomarker for predicting response and survival in patients with AML or advanced-stage MDS. The demonstration that the ubiquitin-proteasome system is particularly important in this group of patients not only provides valuable information on the biology of AML and advanced-stage MDS but also opens the door for potential therapeutic approaches that incorporate proteasome inhibitors that specifically target the most important enzymatic activity of the proteasome in AML. Preclinical studies showed encouraging effects of the proteasome inhibitor bortezomib on AML cells (26). However, clinical trials showed that, unlike multiple myeloma, bortezomib when added to the standard combination therapy idarubicin and cytarabine in treating AML did not significantly improve response rate (27). Although proteasome enzymatic activities are increased in various diseases, direct comparison of the profile of activities between various diseases is needed. Differences in the enzymatic profile between multiple myeloma and AML may explain the difference in the response to bortezomib. These information need to be considered in the context of the new generation of proteasome inhibitors that target different enzymatic activities and further studies are clearly needed to fully take advantage of the information provided in this article in treating patients with AML.

The data presented here have the potential to allow us to stratify individual patients for therapeutic approaches. Patients with a high probability of not responding to standard chemotherapy should be given the choice of stem cell transplant or other clinical trials that investigate new therapeutic approaches. The value of such stratification should be investigated in prospective clinical trials and data on overall survival and quality of life should guide future decisions for the use of this approach.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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