Low $p27^{kip1}$ Expression Is an Independent Adverse Prognostic Factor in Patients with Multiple Myeloma

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ABSTRACT

Purpose: To determine the clinical relevance of $p27^{kip1}$ in multiple myeloma (MM), we examined the relationship between $p27^{kip1}$ expression at diagnosis and clinical as well as laboratory parameters, including response to chemotherapy and overall survival in 74 previously untreated patients with MM.

Experimental Design: Expression of $p27^{kip1}$ was assessed by immunohistochemistry on formalin-fixed, paraffin-embedded bone marrow biopsies. $p27^{kip1}$ expression was classified as low ($\leq 5\%$ $p27^{kip1}$-positive myeloma cells) or high ($> 5\%$ $p27^{kip1}$-positive myeloma cells).

Results: Low $p27^{kip1}$ expression was observed in 23 (31\%) patients. The response rate to standard dose chemotherapy (including vincristine, doxorubicin, and dexamethasone induction before high-dose chemotherapy) was 70\%, with no significant difference between patients with low or high $p27^{kip1}$ expression (83 versus 65\%; $P = 0.1$). Kaplan-Meier analysis of all 74 patients revealed that patients with low $p27^{kip1}$ expression had a significantly shorter overall survival (median, 3.7 years versus 4.7 years; $P = 0.03$) than those with high $p27^{kip1}$ expression. Patients with high $p27^{kip1}$ expression receiving high-dose chemotherapy experienced prolonged overall survival as compared with those with low $p27^{kip1}$ expression (median not yet reached versus 2.9 years; $P = 0.008$). By multivariate Cox regression analysis, low $p27^{kip1}$ ($P = 0.03$), deletion of chromosome 13q14 ($P = 0.02$), and $\beta_2$-microglobulin ($P = 0.01$) were identified as independent adverse prognostic factors for overall survival. According to the number of independent unfavorable prognostic factors present in each patient, low-risk, intermediate-risk, and high-risk patients with different overall survival times were defined (median overall survival, 6.3 versus 4.2 versus 1.8 years; $P < 0.001$).

Conclusions: Low $p27^{kip1}$ expression is an independent adverse prognostic factor in patients with MM. The proposed risk score might be useful for risk-adapted treatment in the future.

INTRODUCTION

Deregulation of cell cycle control is a critical step in the development of human cancers and, therefore, knowledge of the expression of cell cycle regulatory proteins in tumor cells is essential for understanding tumor cell behavior and may be also important for predicting prognosis of cancer patients. Cell cycle progression from G1 to the S-phase of the cell cycle is closely regulated by the formation of cyclin/Cdk complexes (1). Cdk activity is inhibited by Cdk inhibitory proteins including the Cip/Kip family members $p21^{Waf1/Cip1}$, $p27^{kip1}$, and $p57^{kip2}$ (2, 3). These proteins interact with complexes containing cyclin D, E, and A (4–6), and recent data suggest that they exert both positive and negative regulation of Cdk activity at G1–S transition (7–9). Many putative functions have been attributed to $p27^{kip1}$, including potential tumor suppressor gene (10), promoter of apoptosis (11, 12), role in cell differentiation (13, 14), safeguard against inflammatory injury (15), and regulator of drug resistance (16).

Low expression of $p27^{kip1}$ has been shown to be an important prognostic factor in various B-cell hematological malignancies (17–20), but little is known about the prognostic significance of $p27^{kip1}$ in MM. In this study, we therefore determined the clinical relevance of $p27^{kip1}$ expression in unselected patients with MM and established a new prognostic model that included standard prognostic factors and $p27^{kip1}$ status.

PATIENTS AND METHODS

Patients. Eighty-eight consecutive, newly diagnosed patients with MM who had been treated at our institution between 1992 and 2000 were studied. Bone marrow biopsies obtained for diagnostic purposes were used after having obtained informed consent according to institutional guidelines. Fourteen patients had to be excluded because of insufficient number of myeloma cells in the specimens. Main characteristics of the 74 evaluable...
patients and correlations with p27Kip1 status are summarized in Table 1.

In 47 patients, treatment consisted of standard dose chemotherapy, either a melphalan-based regimen (melphalan plus prednisone or vincristine, melphalan, cyclophosphamide, and prednisone with or without carmustine) in 36 patients or VAD in 11 patients. The remaining 27 patients were treated by high-dose chemotherapy, followed by either autologous (n = 24) or allogeneic (n = 3) stem cell transplantation. In the autologous group, high-dose melphalan was preceded by three to six cycles of VAD as induction chemotherapy and one cycle of high-dose cyclophosphamide (n = 19) or ifosfamide, epirubicin, and etoposide (n = 5) followed by granulocyte colony-stimulating factor and peripheral blood stem cell collection. High-dose melphalan consisted of melphalan 200 mg/m² in 12 patients, melphalan 140 mg/m² and total body irradiation in 9 patients, and three cycles of melphalan 100 mg/m² in 3 patients. Patients receiving allogeneic stem cell grafts were also treated by VAD before transplantation. Four patients with stage I disease were treated with chemotherapy because of a symptomatic disease.

**Immunohistochemistry.** Sequential biopsy sections were stained according to Giemsa and immunostained for CD138 and p27Kip1. Among hematopoietic cells, CD138 is selectively expressed by normal and neoplastic plasma cells (23), and delicate membrane staining for CD138 is achieved by immunohistochemistry. p27Kip1 immunostaining was performed on formalin-fixed, paraffin-embedded bone marrow biopsies as described previously (24). CD138 immunostaining was done according to the instructions of the manufacturer.

Briefly, sections were deparaffinized and endogenous peroxidase activity was blocked by incubation in 0.06% hydrogen peroxide for 10 min at room temperature. After boiling for 10 min in 10 mM citrate buffer (pH 6.0) for antigen retrieval, the tissues were preincubated for 20 min in normal serum (1:50; Dako, Glostrup, Denmark) before a 60-min incubation with the p27Kip1 monoclonal antibody (clone 57, antibody used at 1.25 μg/ml; Transduction Laboratories, Lexington, KY) or with the CD138 monoclonal antibody (clone B-B4, dilution 1:40; Serotec Ltd., Oxford, United Kingdom). Antibody binding was detected by the avidin-biotin-peroxidase method. Bound peroxidase was developed with 3,3′-diaminobenzidine (Dako) in the case of CD138. Staining of myeloma cells was examined on formalin-fixed, paraffin-embedded bone marrow biopsies as described previously (25). In addition, negative controls without the primary antibody were performed as described above. Staining of myeloma cells was examined independently by two observers without prior knowledge of the clinical outcome of the patients. Areas containing sheets of myeloma cells, as demonstrated by CD138 staining, were chosen for the evaluation of p27Kip1 immunoreactivity. At least 200 myeloma cells/case were evaluated and the result expressed as the percentage of p27Kip1 labeled nuclei. Discrepant cases were reassessed together by both investigators and a consensus was reached.

**FISH.** Chromosome 13q14 was studied by FISH with a DNA probe specific for the retinoblastoma gene 1 locus as described previously (26).

**Statistical Analysis.** Associations of p27Kip1 expression with clinical as well as laboratory parameters were assessed by the χ² test or Mann-Whitney U test. Spearman rank correlation coefficient rₛ was used as a measure of correlation between...
p27\textsuperscript{Kip1} and Ki-67 expression. Survival probabilities were calculated with the product limit method according to Kaplan-Meier (27). Overall survival time was defined as the period between the time of diagnosis and the time of death. Survival times of patients still alive were censored with the date of last follow-up. Differences between survival curves were analyzed by the log-rank test. Cox proportional hazards regression models were used to assess the independent effects of p27\textsuperscript{Kip1} expression on survival (28). All P values are results of two-sided tests. The SPSS 10.0 statistical software (SPSS Inc., Chicago, IL) was used for calculations.

RESULTS

p27\textsuperscript{Kip1} Expression in MM at Diagnosis. p27\textsuperscript{Kip1} expression was immunohistochemically determined in 74 previously untreated patients with MM. p27\textsuperscript{Kip1} immunostaining was nuclear and ranged from 0 to 90% of the myeloma cells. An example for p27\textsuperscript{Kip1} immunohistochemistry is shown in Fig. 1. Comparisons of p27\textsuperscript{Kip1} expression with clinical parameters, including response to chemotherapy, were performed with p27\textsuperscript{Kip1} expression as a dichotomized variable classified as low (≤5% p27\textsuperscript{Kip1}-positive myeloma cells) or high (>5% p27\textsuperscript{Kip1}-positive myeloma cells). Low p27\textsuperscript{Kip1} expression was observed in 23 (31%) patients.

Correlation with Clinical and Laboratory Parameters. The major clinical and laboratory findings of the patients grouped according to low or high p27\textsuperscript{Kip1} expression are summarized in Table 1. Seventy-four patients were evaluable for analysis of clinical and laboratory parameters and follow-up data. Patients with low or high p27\textsuperscript{Kip1} expression did not differ significantly in age and sex, stage, immunoglobulin subtype, percentage of bone marrow myeloma cells, creatinine, hemoglobin, β\textsubscript{2}-microglobulin, C-reactive protein, lactate dehydrogenase, deletion of chromosome 13q14, and Ki-67 expression. It is of particular interest that we found no correlation of p27\textsuperscript{Kip1} expression with the proliferation marker Ki-67 (r\textsubscript{s} = −0.1, P = 0.4; Fig. 2).

Response to Chemotherapy and Survival. Response to standard dose induction chemotherapy (including VAD before high-dose treatment) was evaluated in 74 patients. The overall response rate was 70%. Patients with low p27\textsuperscript{Kip1} expression had a response rate of 83%, whereas in patients with high p27\textsuperscript{Kip1} expression the response rate was 65%, but this difference did not reach the level of statistical significance (P = 0.1). A similar result (80 versus 64%; P = 0.3) was observed when only patients receiving VAD chemotherapy (n = 38) were analyzed. Twenty-seven patients were again evaluated for response after high-dose chemotherapy. At this time point, the
complete response rate (stringently defined as normal bone marrow and absence of the paraprotein by immunofixation) was 30%. The complete response rates were not significantly different between patients with low or high p27Kip1 expression (38 versus 26%; P = 0.7).

The median follow-up of the total study population was 3.9 years, and the maximum follow-up was 7.4 years. Thirty-four patients died (14 patients with low p27Kip1 expression, 20 patients with high p27Kip1 expression). High-dose treatment was equally distributed among patients with low and with those with high p27Kip1 expression (35 versus 37%; P = 0.8). The median overall survival of all 74 patients was 4.2 years. Patients with low p27Kip1 expression had a significantly shorter overall survival than those with high p27Kip1 expression (Fig. 3). Kaplan-Meier estimate of the median overall survival was 3.7 years for patients with low p27Kip1 expression, whereas for patients with high p27Kip1 expression, it was 4.7 years (P = 0.03). In the subgroup of patients receiving high-dose chemotherapy, a similar difference in overall survival times was observed between patients with low or high p27Kip1 expression (median overall survival 2.9 years versus not reached; P = 0.008).

Analysis of Prognostic Factors. The clinical and laboratory parameters used in the univariate analyses were age, sex, stage, immunoglobulin subtype, percentage of bone marrow myeloma cells, creatinine, hemoglobin, β2-microglobulin, C-reactive protein, lactate dehydrogenase, deletion of chromosome 13q14, and Ki-67 expression. In the univariate Cox regression analysis, the only variables significantly associated with shorter overall survival were β2-microglobulin (P = 0.004), deletion of chromosome 13q14 (P = 0.004), and low p27Kip1 expression (P = 0.03; Table 2). We performed a multivariate Cox regression analysis of prognostic factors that included the parameters statistically significant in the univariate analyses. In this analysis, β2-microglobulin (P = 0.01), deletion of chromosome 13q14 (P = 0.02), and low p27Kip1 expression (P = 0.03) were identified as independent prognostic factor for poor overall survival (Table 2).

On the basis of the results of the multivariate analysis, we used the three independent prognostic factors associated with poor clinical outcome [high β2-microglobulin (≥3 mg/liter, median value), deletion of chromosome 13q14, and low p27Kip1 expression] to define a risk score. Dependent on the number of poor prognostic factors present in each patient, three groups of patients were defined. Low-risk patients (with no poor prognostic factors), intermediate-risk patients (one or two unfavorable prognostic factors), and high-risk patients (three unfavorable prognostic factors) had significantly different overall survival times (Fig. 4). The median overall survival of low-risk, intermediate-risk, and high-risk patients was 6.3, 4.2, and 1.8 years, respectively (P < 0.001; Fig. 4). In the subgroup of patients who received high-dose chemotherapy, this difference in overall survival between low, intermediate, and high-risk patients was again observed (median overall survival not yet reached, 4.7, and 2.8 years; P = 0.002). Of particular note, all 6 low-risk patients were still alive at the time of the analysis.

DISCUSSION

In this study, low p27Kip1 expression in myeloma cells of patients with MM was predictive for shortened overall survival. Multivariate analyses demonstrated that the effect of p27Kip1 expression was independent of β2-microglobulin and deletion of chromosome 13q14. The impact of p27Kip1 expression on overall survival was seen in the total study population but also in patients receiving high-dose chemotherapy. In this subgroup, patients with high p27Kip1 expression experienced also prolonged overall survival as compared with those with low p27Kip1 expression. Thus, our data underline the importance of cell cycle regulators for clinical outcome of MM patients. We additionally defined a risk-score according to the number of adverse prognostic factors present in each patient that allowed us to discriminate patients with different overall survival. This risk-score is an extension to a previously published score that included only β2-microglobulin and deletion of chromosome 13q14 (29).

In a variety of tumors, an inverse relationship between p27Kip1 expression and proliferative capacity has been observed. However, distinct entities of B-cell malignancies appear to
increased p27Kip1 levels, and this increase in p27 Kip1 levels was associated with resistance to anthracyclins or alkylating agents (33). This adhesion also in the extracellular matrix, protects cells from apoptosis initiated by mechanisms (30, 31) or an altered apoptosis (32). The Cdk inhibitors p21Waf1 and p27 Kip1 may represent a new class of inhibitors that may play an important role in signal transduction that regulates cell cycle regulation.

MM is presently an incurable disease, and a major reason for this fact is resistance of myeloma cells to anticancer drugs. Several drug resistance mechanisms have previously been studied for their clinical relevance in MM, including drug transport mechanisms (30, 31) or an altered apoptosis (32). The Cdk inhibitors p21Waf1 and p27Kip1 may represent a new class of drug resistance genes. It has been recently reported that adhesion of myeloma cell lines to fibronectin, a component of the extracellular matrix, protects cells from apoptosis initiated by anthracyclins or alkylating agents (33). This adhesion also increased p27Kip1 levels, and this increase in p27Kip1 levels was causally related to induction of cell adhesion-mediated drug resistance (34). Activation of specific cell adhesion molecules may play an important role in signal transduction that regulates p27Kip1 expression, resulting in the inhibition of drug-induced apoptosis. Although p27Kip1 expression was not significantly associated with response in our study, a higher overall response rate to standard dose chemotherapy and a higher complete response rate after high-dose chemotherapy was observed for patients with low p27Kip1 expression. However, progression-free survival tended to be longer in patients with high p27Kip1 expression as compared with patients with low p27Kip1 expression (median, 2.5 versus 1.4 years; P = 0.1). In the subgroup of patients treated with high-dose chemotherapy, progression-free survival was significantly longer in patients with high p27Kip1 expression than in patients with low p27Kip1 expression (median, 3.5 versus 2.3 years; P = 0.004).

It is of particular clinical interest that patients with poor outcome after high-dose chemotherapy can be identified by low p27Kip1 expression. High-dose melphalan followed by autologous stem cell transplantation has become standard of care for patients with MM. There is now increasing evidence that a subgroup of patients with favorable prognostic features, most notably absence of a chromosome 13 deletion, benefits most from this treatment approach (35, 36). According to the prognostic score reported in our present study, we were able to identify patients with prolonged survival after high-dose therapy. This favorable subgroup was characterized by high p27Kip1 expression, low β2-microglobulin, and a normal chromosome 13q by FISH. On the other hand, patients with risk factors, including low p27Kip1 expression, experience poor outcome even after high-dose therapy, which implies that novel, innovative treatment strategies need to be developed for this group of MM patients with high-risk features.

Proteasome inhibitors represent a potential new anticancer therapy. These agents inhibit the degradation of mult ubiquitinated target proteins, including p27Kip1 (37). After treatment of transformed human fibroblasts with a proteasome inhibitor, p27Kip1 levels increased and apoptosis was induced (37). Therefore, treatment of high-risk MM patients with a proteasome inhibitor may increase p27Kip1 levels and induce apoptosis in MM cells, resulting in improved outcome in these patients. Along this line, PS-341, which is a potent and selective inhibitor of the proteasome, was shown to inhibit proliferation and the growth of human MM cell lines and patient MM cells resistant to doxorubicin, mitoxantrone, melphalan, and dexamethasone (38). PS-341 was also shown to have antitumor activity in some patients with chemotherapy refractory MM and is therefore additionally evaluated in ongoing clinical trials (39). In our study, we defined a subgroup of high-risk patients despite intensive therapy who may benefit from this novel treatment.
strategy. However, this possibility needs to be proven in future studies.

In conclusion, low p27Kip1 expression is an independent unfavorable prognostic factor associated with poor clinical outcome in patients with MM. Thus, p27Kip1 and/or the proposed risk score may be useful for the selection of patients for specific treatments and modulation of p27Kip1 expression may be a potential therapeutic strategy to improve clinical outcome in patients with MM in the future.

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