CKS1B nuclear expression is inversely correlated with p27[Kip1] expression and is predictive of an adverse survival in patients with multiple myeloma

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ABSTRACT

Background

CKS1B is a member of the highly conserved cyclin kinase subunit 1 (CKS1) family that interacts with cyclin-dependent kinases and plays an important role in cell cycle progression. We and others have shown that *CKS1B* amplification located on chromosome 1q21 is an adverse prognostic factor in multiple myeloma, but its relationship with CKS1B nuclear protein expression, is unclear. The aim of this study was to correlate nuclear CKS1B protein immunoreactivity, 1q21 amplification status, p27[Kip1] expression and survival in patients with newly-diagnosed multiple myeloma.

Design and Methods

Nuclear expression of CKS1B and p27[Kip1] was evaluated by immunohistochemistry in decalcified, paraffin-embedded bone marrow biopsies from 94 patients with newly diagnosed multiple myeloma. Clonal plasma cells of the bone marrow aspirates from the same cohort were examined for *CKS1B* gene status by interphase cytoplasmic fluorescence *in situ* hybridization.

Results

Fluorescence *in situ* hybridization detected the 1q21 amplification in 36 (38%) of the 94 patients and immunohistochemistry showed CKS1B protein expression in 37 (39%). Thirty-two (86%) of the 36 amplified (1q21) cases expressed CKS1B and 31 (84%) of the 37 CKS1B immunoreactive cases had amplified 1q21. 1q21 amplification and CKS1B protein expression were strongly correlated (*P*<0.0001). CKS1B protein expression was inversely correlated with p27[Kip1] immunostaining (*P*<0.0001) and was associated with a shorter overall survival (median 44.5 *versus* 89.3 months, *P*<0.0001).

Conclusions

Immunohistochemistry for CKS1B is a simple, rapid method that appears to predict 1q21 amplification and adverse outcome for risk stratification of patients with multiple myeloma.

Key words: multiple myeloma, 1q21, cyclin kinase subunit 1B, immunohistochemistry, fluorescence *in situ* hybridization.

Introduction

Multiple myeloma (MM), the second most common blood malignancy in adults, is a clonal plasma cell disorder characterized by recurrent genetic abnormalities, including chromosomal translocations, ploidy changes and alterations in signaling pathways such as nuclear factor-κB, p53 and Ras.[1] Chromosome 1 abnormalities are the most common structural aberration in MM, being found in up to 48% of abnormal metaphase karyotypes.[2,3] Comparative genomic hybridization indicates that 1q is gained or amplified in approximately 45% of MM cases, with the gains most consistently involving 1q21.[4-7] We and other investigators showed that gains of 1q21 were associated with disease progression and poor survival in MM.[8-11] Moreover, 1q21 gains were rarely detected in monoclonal gammopathy of unknown significance, but the prevalence of this abnormality increased significantly from newly diagnosed MM to relapsed disease and plasma cell leukemia.[9,12] Given that the q21 band of chromosome 1 represents an amplification hot spot, genes within this region may confer an aggressive clinical phenotype.[8,13]

One of the most commonly overexpressed genes mapped at 1q21.3 in MM is *CKS1B*,[8,14] a member of the Cks/Suc1 family of small proteins (9-18 kDa) that interacts with cyclin-dependent kinases (Cdk) and plays an important role in cell cycle progression.[15] CKS1B is an essential cofactor for efficient Skp1-Cul1-F-box protein SKP2 (SCFS^{kp2})-dependent ubiquitination of p27^{Kip1}.[16-18] Thus, elevated expression of CKS1B, representing the rate-limiting component of SCF$^{Skp2-CKS1}$ ubiquitin ligase,[16,17] may lead to inappropriate degradation of p27^{Kip1}, which regulates Cdk2-cyclin E activity and the late restriction point of the G1/S transition of the cell cycle.[19] Emerging evidence suggests that deregulation of the G1/S transition of the cell cycle is a critical step in tumor genesis.[20,21] Consistent with this line of evidence, reduced protein levels of p27^{Kip1} are associated with poor prognosis in many cancers,[19] including MM.[21]

CKS1B over-expression in MM was detected at the mRNA level by gene expression profiling [8,14] and reverse-transcriptase polymerase chain reaction,[11,22] and at the protein level by western blotting.[22] However, it remains unclear whether CKS1B protein over-expression is detectable by immunohistochemistry in samples from patients with myeloma and whether its expression is correlated with 1q21 gains, p27^{Kip1} expression and clinical outcome. We, therefore, systematically evaluated CKS1B expression in MM patients' samples by immunohistochemistry and investigated the relationship of this expression with 1q21 gains and clinical outcome.

Design and Methods

Patients

A total of 94 MM cases treated with high-dose therapy followed by autologous stem cell transplantation, with bone marrow samples available for fluorescence *in situ* hybridization (FISH) and immunohistochemistry analysis, were retrospectively analyzed in this study. The induction therapy included four or five cycles of vincristine, adriamycin and dexamethasone (VAD) and one course of melphalan 200 mg/m². The median post-transplant follow-up was 27.2 months. No maintenance therapy was given in this cohort. The clinical and laboratory features at diagnosis are summarized in Table 1. The 58 males and 36 females had a median age

of 54 years (range, 30-75 years). The immunoglobulin isotypes were IgG in 46 patients, IgA in 22, IgD in 3, light-chain in 22 and non-secretory in only 1 case.

Interphase fluorescence in situ hybridization

Following Institutional Research Review Board approval, bone marrow aspirates were obtained from patients with active MM prior to autologous stem cell transplantation. As previously described, cytoplasmic light chain immunofluorescence with simultaneous FISH analysis (cIg-FISH) was performed on fixed mononuclear cells on cytospin slides.[10,12] Clonal plasma cells were identified by cytoplasmic light chain immunofluorescence and their nuclear hybridization pattern evaluated on an epifluorescence microscope using an imaging system to determine their 1q21 amplification status (Applied Imaging, Santa Clara, CA, USA).

To assess the *CKS1B* gene status, we used a BAC clone RP11-307C12 (180 kb) that contains the *CKS1B* gene. DNA isolated from this BAC clone was labeled with Spectrum-Orange-dUTP using a nick translation kit (Vysis, Downers Grove, IL, USA). The probe has been extensively tested on mononuclear cells from normal peripheral blood and bone marrow donors and does not cross-hybridize to any other chromosome regions. As an internal control, we combined Spectrum-Orange-labeled *CKS1B* with a Spectrum-Green-labeled chromosome 1 classical satellite (1qh) probe. The normal hybridization pattern was a pair of red and green signals. 1q21(*CKS1B*) gains were defined by the presence of three or more red signals in more than 10% of the clonal plasma cells. At least 200 plasma cells were scored to determine the frequency of 1q21(*CKS1B*) gains.

Immunohistochemistry

Immunohistochemistry was performed as previously described.[23,24] B5-fixed (EMD Chemicals, Gibbstown, NJ, USA) bone marrow biopsies were decalcified, paraffin-embedded, cut in sequential 5 μm–thick sections, mounted and de-waxed. The sections were then boiled in 10 mM citrate buffer, pH 6.0 for 8 min at 120°C, stored overnight in the buffer at room temperature, treated with 10% hydrogen peroxide for 20 min and then incubated overnight at room temperature with one of the primary antibodies; CD138 (P18827; Serotec, Oxford, UK), CKS1B (sc-7238; Santa-Cruz Biotechnology, Santa Cruz, CA, USA) and p27^{Kip1} (610241; BD-Transduction Laboratories) then immunostained by the avidin-biotin-peroxidase method (ABC kit, Vectastain; Vector Laboratories, Burlingame, CA, USA).

The rabbit polyclonal anti-CKS1B antibody is raised against amino acids 1-79, enabling recognition of full length CKS1 of human origin. The mouse monoclonal anti-p27^{Kip1} antibody is raised against the 60-amino acid sequence in the N-terminal region, enabling recognition of p27^{Kip1}. Antibodies to CD138, CKS1B and p27^{Kip1} were used at dilutions of 1:800, 1:100 and 1:500, respectively. This incubation was followed by a 30-min incubation with a biotinylated linking reagent (ID Labs, London, ON, Canada) and a subsequent 35-min incubation with horseradish peroxidase-conjugated Ultra Streptavidin labeling reagent (ID Labs). The immunoreactions were visualized with freshly prepared NovaRed Solution (Vector Laboratories); the slides were counterstained with Mayer hematoxylin and evaluated with an Olympus BX50 microscope (Olympus, Melville, NY, USA). Representative images of CD138, CKS1B, and p27^{Kip1} expression in myeloma cells are shown in Figure 1A, B, and C.

Non-neoplastic lymphoid tissue (tonsil) was the positive control for all antibodies.[25] Sections without the primary antibody were the negative controls. Cases with 10% or more CKS1B and 50% or more p27^{Kip1} immunoreactive cells were considered positive.[26]

Two observers, blinded to the clinical outcome of the patients, independently scored the myeloma cell staining. Discrepant cases were re-assessed by both investigators together and a consensus was reached.

Statistical analysis

Statistical evaluations were conducted using Fisher's exact test, the χ^2-test and the non-parametric Wilcoxon's test depending on the nature of the data. Progression-free survival and overall survival were calculated from the transplant date by the Kaplan-Meier method. Differences between survival curves were analyzed by the log-rank test. Multivariate analysis was performed for overall survival using the Cox proportional-hazard model. Results were considered statistically significant if the *P* value was less than or equal to 0.05. The statistical analysis was performed using SPSS 15.0 (SPSS Inc., Chicago, IL, USA).

Results

CKS1B and p27^{kip1} immunohistochemistry

CD138$^+$ myeloma cell aggregates were identified on the CD138-stained slide, the corresponding aggregates found on the CKS1B-stained serial section and the percent CKS1B-immunostained nuclei estimated independently by two observers. Cases were considered positive if CKS1B stained 10% or more of the CD138$^+$ myeloma cell nuclei. In all cases studied, the myeloma cells were CD138 immunoreactive,

but the CKS1B immunoreactivity varied from negative to cases with rare (<2%) CKS1B-immunostained nuclei to cases with more than 10% immunoreactive nuclei. The CKS1B nuclear staining intensity was moderate to strong in most positive cases. Of the 94 cases, 37 (39%) had 10% or more immunohistochemically reactive nuclei (median, 55%; range, 10-95%).

p27^{Kip1} expression varied from cases with less than 5% p27^{Kip1} immunostained nuclei to cases with 90% immunoreactive nuclei (median, 50%; range, 0-90 %). Although cells had a range of staining intensities, moderately to strongly stained nuclei were scored for p27^{Kip1} expression. Of the 94 patient samples, 53 (56%) had 50% or more of immunohistochemically reactive nuclei (p27^{Kip1} positive).

Correlation between CKS1B over-expression and 1q21 gains

Of the 94 MM cases, 36 (38%) had 1q21 gains (10% or more clonal plasma cells harboring three to eight *CKS1B* gene copies) by interphase cIg-FISH analysis (Figure 1D). Among the cases positive for 1q21 gains, the percentage of myeloma cells containing the gains ranged from less than 10% to greater than 90%. CKS1B expression was strongly correlated with 1q21 amplification (*P*<0.0001); 31 (84%) of the 37 cases that were immunohistochemically positive for CKS1B had 1q21 gains, and 31 (86 %) of the 36 cases with 1q21 amplification had CKS1B over-expression.

To confirm that CKS1B over-expression is associated with

Table 1. Myeloma patients' characteristics by CKS1B immunostaining.

Characteristics	CKS1B immunohistochemistry		
	Positive, n = 37	Negative, n=57	*P*
Age (years), median (range)	54 (30-73)	54 (31-70)	0.228
Sex (F/M)	16/21	20/37	0.427
International Staging System stage, n (%)			
I	9 (32)	18 (37)	0.563
II	13 (46)	25 (51)	
III	6 (21)	6 (12)	
Bone lesions, n (%)			
None	8 (42)	12 (39)	0.812
Present	11 (58)	19 (61)	
Bone marrow plasmacytosis %, median (range)	60 (15 -90)	65 (17-90)	0.754
Hemoglobin, g/dL, median (range)	115 (82-224)	114 (58-158)	0.330
Calcium level, mmol/L, median (range)	2.35 (1.86 -3.49)	2.30 (1.53-4.21)	0.471
CRP level, mg/L, median (range)	3 (3-19)	4 (3-122)	0.277
β-2 microglobulin, mmol/L, median (range)	277 (111-1700)	266 (91-4910)	0.958
Albumin, g/L, median (range)	36 (25-44)	35 (20-53)	0.869
Creatinine, mol/L, median (range)	90 (33-1759)	88 (38-777)	0.846
Isotypes, n (%)			
IgA	12 (32)	10 (18)	0.245
IgD	1 (3)	2 (4)	
IgG	19 (51)	27 (47)	
Light chains	5 (14)	17 (30)	
Non-secretory	0	1 (2)	
Extramedullary disease			
Absent	16 (84)	29 (88)	0.697
Present	3 (16)	4 (12)	
p27^{Kip1} expression, n (%)			
Negative (<50%)	28 (76)	13 (23)	< 0.0001
Positive (≥50%)	9 (24)	44 (77)	
1q21 amplification (FISH)			
Negative	6 (16)	52 (91)	<0.0001
Positive	31 (84)	5 (9)	

disease progression, we evaluated ten paired MM samples obtained at diagnosis and relapse. Among the diagnostic samples, immunohistochemistry detected three cases positive for CKS1B nuclear expression at diagnosis and all three also showed *CKS1B* amplification by FISH; at relapse, immunohistochemistry stained six cases positive for CKS1B, and all of them harbored *CKS1B* amplifications. There was no discordance in results between these two assays in this subset.

CKS1B protein expression and p27[Kip1] protein levels are inversely correlated

As CKS1B is known to mediate the ubiquitination and proteasomal degradation of p27[Kip1,16,17] we examined the relationship between the protein expression of CKS1B and p27[Kip1] by immunohistochemistry. Twenty-eight (76%) of the 37 CKS1B immunohistochemically-positive cases had low p27[Kip1] expression (<50%), while the other nine of the 37 (24%) CKS1B immunohistochemically-positive cases had high p27[Kip1] expression (≥50%); 44 (77%) of the 57 CKS1B immunohistochemically-negative cases had high p27[Kip1] expression and only 13 (23%) of the 57 CKS1B immunohistochemically-negative cases had low p27[Kip1] expression. There was a strong inverse correlation between CKS1B and p27[Kip1] protein levels (*P*<0.0001) but 13 cases had

low levels of both CKS1B (<10%) and p27[Kip1] and nine cases had high levels of both p27[Kip1] and CKS1B (≥10%).

Correlation of CKS1B over-expression, p27[Kip1] expression, and survival

The median progression-free survival was 21.4 months [95% confidence interval (95% CI): 17.8-25.0] and the overall survival 58.2 months (95% CI: 46.9-69.5). Patients with CKS1B protein over-expression detected by immunohistochemistry had a significantly shorter overall survival than patients without over-expression (44.5 *versus* 89.3 months, *P*<0.0001) (Figure 2A) and a shorter progression-free survival but this did not reach statistical significance (18.7 *versus* 23.6 months, *P*=0.222). Patients with low p27[Kip1] expression had a significantly shorter overall survival than patients with high p27[Kip1] expression (46.7 *versus* 73.5 months, *P*=0.009) (Figure 2B) and a shorter progression-free survival although again this did not reach statistical significance (18.7 *versus* 22.6 months, *P*=0.465).

Subgroup analysis showed that patients with CKS1B over-expression and low p27[Kip1] expression had the shortest overall survival in comparison to patients with low CKS1B and low p27[Kip1], patients with high CKS1B and high p27[Kip1], or patients with low CKS1B and high p27[Kip1] (42.2 *versus* median not reached, 53.8 months and 73.5 months, respec-
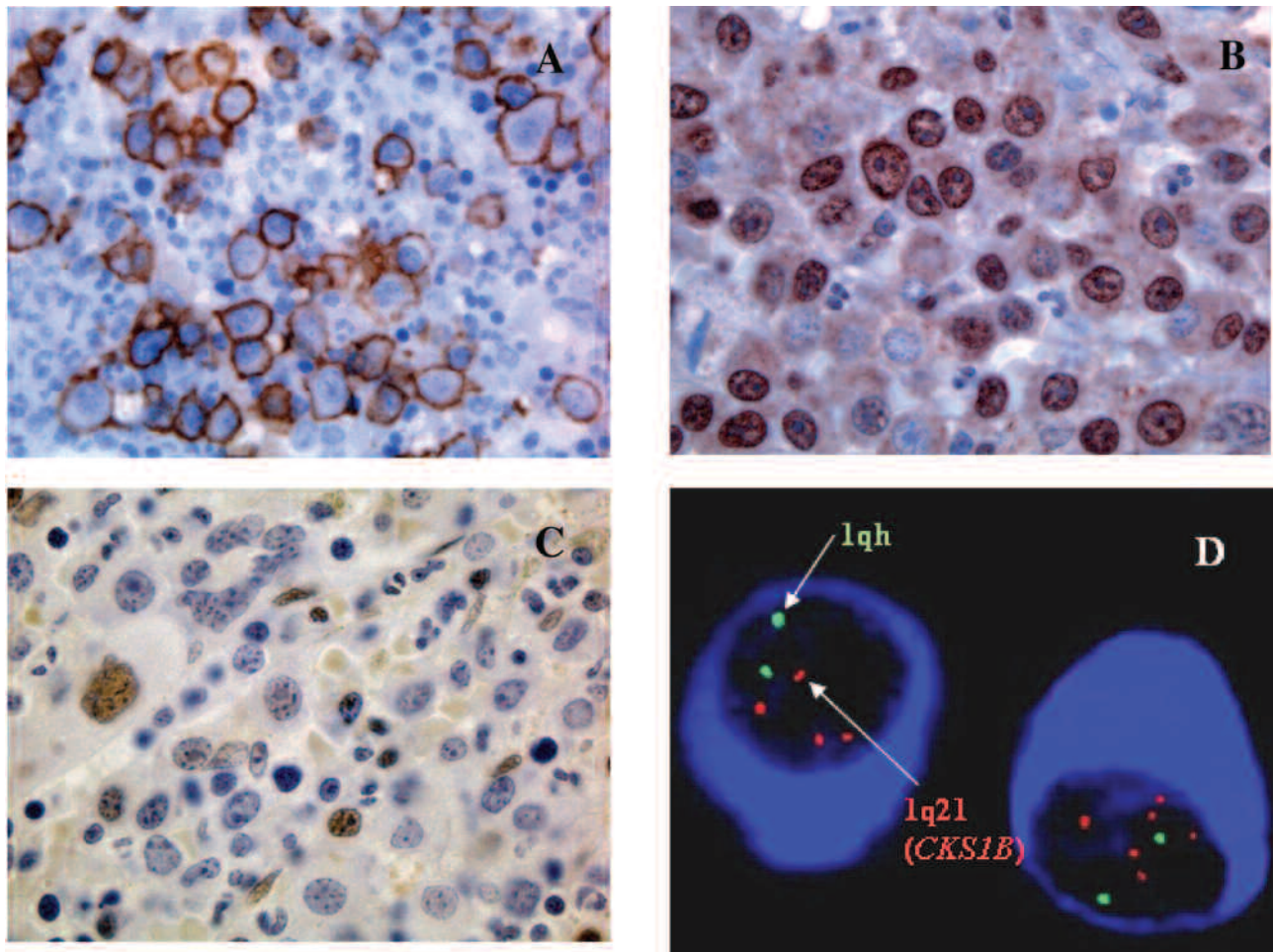

Figure 1. (A) CD138^{+} myeloma cells in a myeloma patient. (B) Nuclear CKS1B over-expression in myeloma cells. (C) low p27[Kip1] expression in myeloma cells from a patient with CKS1B over-expression (D) clg-FISH detected multiple signals indicating 1q21 (*CKS1B*) amplifications in myeloma cells.

tively, *P*<0.0001).

CKS1B over-expression was not associated with any of the routine clinical laboratory parameters listed in Table 1. Except for CKS1B and p27[Kip1], on univariant analysis, none of the parameters emerged as a significant prognostic factor for survivals in this cohort.

Discussion

As one of the most common genetic abnormalities in myeloma is 1q21 gains with CKS1B over-expression, which is associated with an adverse prognosis, a robust assay could have clinical utility. We, therefore, investigated CKS1B over-expression by immunohistochemistry in a large cohort of patients with newly diagnosed multiple myeloma and found a strong correlation between nuclear CKS1B protein expression and 1q21 gains. Importantly, the survival of CKS1B-immunoreactive patients was significantly shorter than that of CKS1B non-immunoreactive patients, suggesting CKS1B over-expression detected by immunohistochemistry may serve as a prognostic marker for myeloma.

In this series, 55% of the MM cases were negative for both CKS1B over-expression and 1q21 gains, 33% were positive for both CKS1B over-expression and 1q21 gains, 5.3% were negative for CKS1B over-expression but positive for 1q21 gains, and 6.4% were positive for CKS1B over-expression but negative for 1q21 gains. Immunohistochemical staining had an 84% sensitivity and 91% specificity for predicting 1q21 gains. Thus, CKS1B over-expression detected by immunohistochemistry may be regarded as a surrogate for 1q21 gains, particularly in centers where FISH is not available.

That five (14%) of our 36 cases with 1q21 gains were negative for CKS1B over-expression is unexpected. Three (60%) of these five cases had four or more copies of *CKS1B*, compatible with those concomitant cases, whereas 20 (65%) of 31 had four or more copies. It is possible that cryptic mechanisms block CKS1B expression at transcriptional or translational levels and account for the discrepancy between the CKS1B protein and 1q21 status. The discrepancy could also be due to an increase in protein turnover from a mechanism such as ubiquitination.

Another discrepancy was that six (16%) of our 37 cases with CKS1B over-expression had a normal 1q21 status. Of these six cases, CKS1B was expressed in a median of 30% of MM cells (range, 20-40%); whereas those with concomitant 1q21 gains expressed CKS1B in a median of 50% of MM cells (range, 15-80%). This is unlikely to be due to non-specific staining of the polyclonal anti-CKS1B antibody as the antibody was validated in six human myeloma cell lines with 1q21 amplifications by western blotting and in four normal bone marrow samples with normal 1q21 status by FISH. We found that the anti-CKS1B antibody only reacted with 1q21-amplification-positive MM cell lines and detected the band at a molecular weight of 9 kDa, corresponding to CKS1B (*data not shown*). Alternatively, over-expression of CKS1B may be due to an increase in transcriptional or translational activity or an increase in protein stability.

In this study, we also sought to understand the relationship between CKS1B and p27[Kip1] expression in MM and the prognostic impact of the expression. Immunohistochemistry revealed a strong, but not absolute, correlation between CKS1B and p27[Kip1] protein expression. As expected from its role in regulating p27[Kip1] protein turnover, CKS1B protein expression was inversely correlated with levels of its target protein p27[Kip1]. We found that 77% of our cases had an inverse correlation between CKS1B and p27[Kip1] protein expression, consistent with the report by Zhan *et al.*, who identified an inverse correlation between CKS1B and p27[Kip1] protein expression in 67% of their 34 MM cases by western blot analysis.[22] However, some cases lacked such a relationship. This variable expression suggests that there are CKS1B-mediated, p27[Kip1]-independent downstream pathways in a subset of MM. This hypothesis is further supported by a recent study that found that CKS1B can mediate myeloma cell survival and disease progression through activation of MEK/ERK and JAK/STAT3 signaling pathways.[27]

On subgroup analysis, we found that patients with both CKS1B over-expression and low p27[Kip1] expression (likely mediated by a p27[Kip1]-dependent pathway) had the worst survival (median overall survival, 42.2 months). In contrast, the subgroup with CKS1B over-expression but high p27[Kip1] (likely mediated by a p27[Kip1]-independent pathway) tended to have a longer survival (median overall survival, 53.8 months; *P*=0.169). This observation suggests that the p27[Kip1]-dependent pathway may confer a more aggressive course of MM. The lack of a statistically significant difference in the overall survival between these two subgroups could be due to the relatively limited number of patients. Thus, prospec-
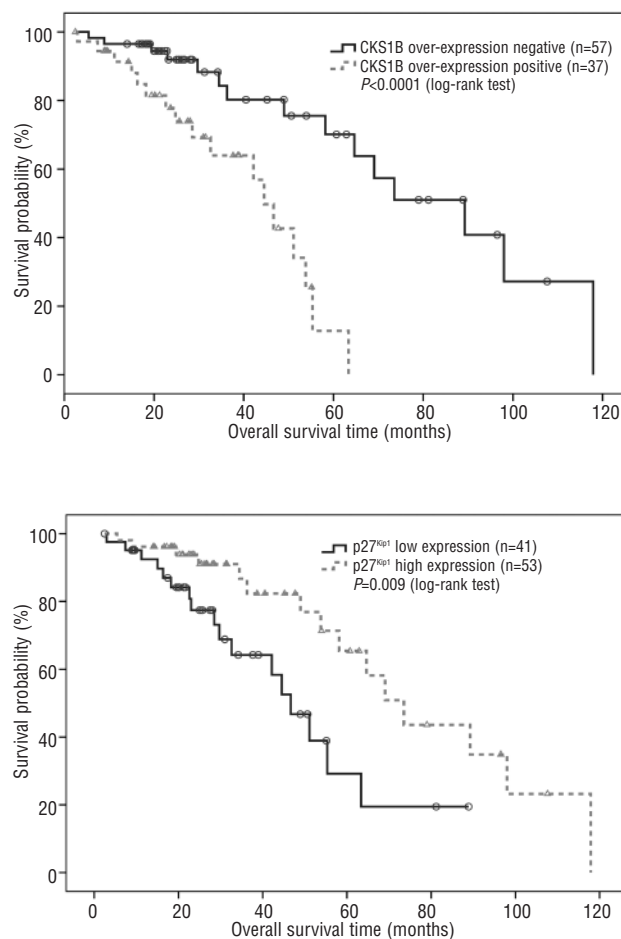

Figure 2. (**A**) Overall survival in relation to CKS1B over-expression detected by immunohistochemistry. (**B**) Overall survival in relation to p27[Kip1] expression detected by immunohistochemistry.

tive studies of larger cohorts are necessary to clarify the role of p27^{Kip1}-dependent and -independent pathways in the prognosis of patients with MM. In addition, functional studies are required to elucidate the role of CKS1B and its downstream and upstream regulatory pathways in the pathogenesis of MM.

In conclusion, paraffin immunohistochemistry reliably detects CKS1B over-expression in MM. As paraffin immunohistochemistry is routinely available, robust and inexpensive, CKS1B immunostaining can be readily adopted in clinical practice to identify myeloma patients with an adverse prognosis who may be candidates for risk-adapted therapies.

Authorship and Contributions

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