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Quantitative analysis of *CKS1B* mRNA expression and copy number gain in patients with plasma cell disorders

Flavia Stella [a,*], Estela Pedrazzini [a,d], Edgardo Baialardo [b], Dorotea Beatriz Fantl [c], Natalia Schutz [c], Irma Slavutsky [a]

[a] Laboratorio de Genética de Neoplasias Linfoides, Instituto de Medicina Experimental, CONICET-Academia Nacional de Medicina, Buenos Aires, Argentina
[b] Centro de Estudios Genéticos, Buenos Aires, Argentina
[c] Departamento de Clínica Médica, Sección Hematología, Hospital Italiano de Buenos Aires, Argentina
[d] Universidad Nacional del Noroeste de la Provincia de Buenos Aires (UNNOBA), Argentina

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ABSTRACT

In this study, we have examined *CKS1B* gene expression and copy number in a total of 114- patients at diagnosis: 83 with multiple myeloma (MM) and 31 with monoclonal gammopathy of undetermined significance (MGUS). Results were correlated with cytogenetics, FISH and clinical characteristic. Significant *CKS1B* mRNA levels in MM compared to MGUS cases ($p < 0.048$) were detected. In MM, the frequency of 1q21 (*CKS1B*) copy gain was significantly higher in cases with abnormal karyotype compared to patients with normal karyotype ($p = 0.021$). Global analysis showed a positive correlation between *CKS1B* expression and 1q21 copy number ($p < 0.0001$). No association between *CKS1B* mRNA expression and clinical parameters was found. However, a significantly higher level of β2 microglobulin in cases with 1q21 gains than those without ($p = 0.0094$) was observed. Overall survival was shorter in cases with 1q21 gain compared to those with normal 1q21 region ($p = 0.0082$). Our results suggest a role for *CKS1B* in the multiple step process of progression of MGUS to MM and show that *CKS1B* copy gain has a more significant prognostic value than its overexpression. This adverse impact on survival probably reflects the genetic instability associated to chromosome 1q alterations resulting in a more aggressive behavior of the disease.

Introduction

Multiple myeloma (MM) is an aggressive bone marrow (BM) cancer originated from terminally differentiated B cells. The disease is characterized by the clonal expansion of malignant plasma cells within the bone marrow and the presence of a monoclonal immunoglobulin in the serum and/or urine (M protein). These cells constitute the end stage in the multistep transformation process from normal to malignant plasma cells. Clinically, patients usually present osteolytic bone lesions, suppression of normal hematopoiesis and less often, renal failure [1]. MM shows significant heterogeneity with regards to clinical presentation, biologic characteristics, response to treatment, and outcome, with patients evolving in a few weeks and others living for longer than 10 years [2]. MM is almost always preceded by pre-malignant disease phases of monoclonal gammopathy of undetermined significance (MGUS) and asymptomatic or smoldering multiple myeloma (SMM). Both this phases lack the clinical features of myeloma but share some of its genetic features, as chromosomal aberrations, copy number abnormalities, somatic mutations, and epigenetic changes [3].

In the last decades, it was increasingly evident the importance of genetic features of the tumor cells that drives the clinical evolution and drug resistance. Cytogenetic alterations are considered one of the most important prognostic factors in newly diagnosed MM cases, increasingly used in the patient management [4,5]. Among them, chromosome 1 aberrations are one of the most common cytogenetic alterations in MM, and often involve deletions of the short arm (1p) and gains of the long arm (1q) [6–10]. Deletions of 1p have been identified in approximately 7% to 40% of myeloma cases using cytogenetics, fluorescence in situ hybridization (FISH), and comparative genomic hybridization (CGH) [11–13], and a recurrent region of losses at 1p32.3 affecting *CDKN2C* (cyclin-dependent kinase inhibitor 2C) ($p18^{INK4C}$) locus was defined [14,15]. Long arm gains/amplifications, are found in approximately 45% of all MM and in almost all cases with plasma cell leukemia, but they were not detected in MGUS patients, being the 1q21 region the hotspot of such alteration [16,17]. One of the key genes mapped on chromosome 1q21 is *CKS1B* (CDC28 protein kinase regulatory subunit 1B), which encodes for a positive cell cycle regulator that activates cyclin-dependent kinases to promote proliferation and cell cycle progression [18,19]. *CKS1B* is essential for the ubiquitinization of

* Corresponding author at: Laboratorio de Genética de Neoplasias Linfoides, Instituto de Medicina Experimental, CONICET-Academia Nacional de Medicina, J.A. Pacheco de Melo 3081, C1425AUM-Buenos Aires, Argentina. Fax: +54 11 4803 9475.
E-mail address: fla_stella@yahoo.com.ar (F. Stella).

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the inhibitor of the cell cycle *CDKN1B* ($p27^{KIP1}$), which degradation is required for the cellular transition from quiescence cells to the proliferative state [20]. This gene was found overexpressed in a number of human malignancies, including solid tumors as non-small cell lung carcinoma [21], colon [22], breast [23,24], and cervical cancer [25], as well as different mature B-cell lymphoid malignancies [26,27].

In MM, chromosome 1q21 gain was directly correlated with elevated CKS1B protein expression and inversely correlated with $p27^{KIP1}$ immunostaining [28]. In addition, *CKS1B* copy number gain by FISH [8,16,29] as well as mRNA and protein overexpression [28,30,31] has been associated with an unfavorable effect on survival and disease progression in plasma cell disorders. To our knowledge, the association between *CKS1B* copy gain by FISH and gene expression has been scarcely evaluated in MM [29,32], and no data on MGUS transcription profiles were reported. Therefore, here we examined *CKS1B* gene expression by quantitative real time PCR (qRT-PCR) in plasma cell disorders. Furthermore, the relationship between *CKS1B* mRNA expression and FISH copy number was also explored. Results were correlated with laboratory parameters and clinical evolution.

Material and methods

Patients and controls

A total of 114- patients with plasma cell disorders at diagnosis: 83 with MM and 31 with MGUS, were analyzed. Clinico-pathological characteristics of all cases are summarized in Table 1. In addition, 16 patients (six males; mean age: 58 years; range: 41–77 years) were also evaluated during complete remission. The diagnosis was based on standard criteria [33,34]. MM staging was made according to the classification proposed by Durie and Salmon (DS) [35] and the International Staging System [36]. Patients under the age of 65 years and fit were treated with an induction therapy with thalidomide or bortezomib plus hematopoietic stem-cell transplantation. Conventional therapy combined with thalidomide or bortezomib was administered in patients older than 65 years or unfit for high dose treatment. A small number of cases received VAD (vincristine, doxorubicin, dexamethasone) chemotherapy alone. The median of follow-up was 27.3 months (range 1–219 months); 30 patients had died at the time of this study. In a number of MM patients the sample was not enough to obtain high quality RNA leading to differences in the number of cases studied for mRNA expression (68) and those evaluated for cytogenetics and FISH (83). Thirty MM patients had both *CKS1B* expression and FISH studies. All individuals provided their informed consent according to institutional guidelines. The study was approved by the Ethics Committee of our Institution.

Table 1
Clinical characteristic of patients with plasma cell disorders.

Clinical characteristics	MM	MGUS
No. of cases	83	31
Sex (F/M)	40/43	22/9
Mean age (range), years	64.3 (24–86)	70.3 (41–84)
Paraprotein isotype (%)		
IgG	58.7	72
IgA	20.6	7
IgM	4.8	21
Others	15.9	0
Type of light chain (%)		
Kappa	52.4	63
Lambda	47.6	37
DS Stage (%)		
I	15	–
II	24	–
III	61	–
ISS (%)		
I	21	–
II	55	–
III	24	–
BMI (%)		
<20	18.3	100
20–60	45	–
>60	36.7	–
Lytic bone lesions (%)	52.5	–
	Mean (range)	Mean (range)
β_2 microglobulin (μg/ml)	3.1 (0.15–19.5)	0.30 (0.11–0.77)
LDH (U/l)	195 (82–459)	148.7 (94–231)
Hemoglobin (g/dL)	10.7 (5.8–14.5)	12.42 (9–15.8)
Serum albumin (g/dL)	3.35 (1.8–4.8)	3.74 (3–4.4)
Creatinine (g/dL)	1.9 (0.58–11.8)	0.91 (0.46–1.82)
Serum calcium (mg/dL)	9.5 (6.8–14.6)	9.17 (7.8–10.3)
Paraprotein M (g)	3.06 (0.9–9.4)	0.62 (0.18–1.73)

F: Female; M: Male; DS: Durie & Salmon; ISS: International Staging System; BMI: Bone marrow infiltration; LDH: Lactate dehydrogenase.

RNA extraction, reverse transcription and real-time quantitative RT-PCR

Total RNA were obtained from BM mononuclear cells of patients, K-562 and Hela cell lines and peripheral mononuclear cells of healthy individuals. The cDNA synthesis was carried out using 1X RT Buffer, 200 U/μL of Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT), 250 ng/μL random primer, 10 mM each dNTP and 1.5 μg/μL of the total RNA. The PCR conditions were: 10 min at 95 °C, 60 min at 37 °C and 10 min at 95 °C. Expression analysis was performed by real-time quantitative PCR (qRT-PCR) in a Rotor-Gene Q (Qiagen) equipment, based on EVAGreen methodology. The PCR reaction was done using 2 μL of cDNA, 10 μL of *Mezcla Real*™ 2x (Biodynamics, Buenos Aires, Argentina) and 10 pmol/μL (0.8 μL) of each primer in a 20 μL final volume. Primers for *CKS1B* were designed for the present study: PF: 5´-CGATCATGTCGCACAAACA-3´ and PR: 5´-GCCAGCTTCATTTCTTTGGT-3´. The PCR conditions were 5 min at 95 °C, followed by 45 cycles at 95 °C for 20 s, 60 °C for 20 s and 72 °C for 45 s, and a holding at 50 °C for 30 s. Each cDNA sample was analyzed in duplicate in parallel using *beta-actin* gene (*ACTB*) as control: PF: 5´-ATGTTTGAGACCTTCAACAC CCC-3´ and PR: 5´-GCCATCTCTTGCTCGAAGTCCAG-3´ [37]. The cycle threshold (Ct) values of target and control genes were computed. Relative gene expression were presented as $2^{(-\Delta\Delta Ct)}$, where $\Delta\Delta Ct = \Delta Ct$ *CKS1B* − ΔCt *ACTB*. The specificity of the PCR products was monitored by dissociation curves with a single peak of each amplicon, and also confirmed by electrophoresis on 2% agarose gel (Supplementary Fig. 1a and b). Standard curves were measured by fivefold duplicated serial dilution of RT templates from K-562 and Hela cell lines.

Cytogenetic analysis

BM cells were processed for cytogenetic analysis by direct method and non- stimulated 72 h culture, in F-12 medium supplemented with 20% fetal calf serum. G- and DAPI-C banding techniques were used. Karyotype abnormalities were described according to the International System of Human Cytogenetic Nomenclature [38].

FISH analysis

For FISH analysis, slides were hybridized with the following locus specific probes: *RB1* at 13q14 band, TP53 at 17p13, *IGH@* at 14q32.3 (LiVE-Lexel, Buenos Aires, Argentina), Dual Color Dual Fusion Translocation Probes LSI CCND1/IGH XT corresponding to t(11;14)(q13;q32), LSI IGH/FGFR3 for t(4;14)(p16;q32) (Vysis-Abbott, Illinois, USA) and *CKS1B*(1q21)/*CDKN2C*(1p32) Amplification Deletion probe (Cytocell LTD, United Kingdom). All probes were used according to the manufacturers' protocols and analyzed as previously described [39]. Chromosome 1q21 (*CKS1B*) gain and 1p32 (*CDKN2C*) deletion were considered as present if they were detectable in 10% or more of evaluated cells. At least 400 cells were scored. Additionally, in cases with complex karyotypes, biotin-labeled whole chromosome painting (WCP) probes for different chromosomes (CAMBIO, Cambridge, UK) and Spectra Vysion WCP

probe (Vysis-Abbott, Illinois, USA) were also used. In each case, a minimum of 10 informative metaphases were analyzed. Image acquisition was performed using Cytovision 3.9 Software (Applied Imaging Corporation, California, USA).

Statistical evaluation

All statistical analyses were performed using GraphPad Prism Version 5.0 (2008). The analysis of mRNA expression data was performed using the Mann–Whitney test. Kendall's coefficient was used to correlate gene expression with clinical variables. Groupwise comparison of the distribution of clinical and laboratory variables was performed with the Student *t* test (for quantitative variables) and the χ^2 or Fisher's exact test (for categorical variables). Receiver operating characteristic (ROC) curve analysis was applied to calculate the expression cut-off value for *CKS1B* gene, with the highest sensitivity and specificity. Overall survival (OS) and event free survival (EFS) were estimated by the Kaplan–Meier method and compared with the log-rank test. For all tests, $p < 0.05$ was regarded as statistically significant.

Results

Cytogenetic and FISH characterization

All 31 MGUS patients showed normal karyotypes (NK) and no lesions using FISH analysis. In MM patients, by conventional cytogenetics 62/83- (74,7%) cases had NK and 20/83- (24.1%) showed abnormal karyotypes (AK), seventeen of them with complex karyotypes. Pseudodiploidy was detected in three cases, hypodiploidy in nine, and hiperdiploidy in eight patients. Chromosome 1 aberrations were the most frequent structural abnormalities, which were identified in thirteen (13/20; 65%) patients with chromosome alterations, with cases with more than one chromosome 1 abnormality. They included: two isochromosomes, eleven translocations (nine unbalanced), one dicentric and three pseudodicentric chromosomes. Other frequent alterations involved chromosome 14 rearrangements, present in seven cases (35%), and 6q deletions observed in six patients (30%). FISH analysis performed on MM samples using *TP53, RB1* and *IGH@* probes showed that 59% of cases had at least one abnormality, 21.7% of them exhibited two and, 13% three alterations. Thus, as a whole taking into account cytogenetics and FISH analysis, 73% of our MM cases showed genetic alterations.

Prevalence of 1q21 gain in MM patients

As previously mentioned, chromosome 1 was the most frequently involved in rearrangements in our cohort. Thus, we were interested in determining the presence of 1q21 gain by FISH and its association with karyotypic characteristics. This analysis was performed using the CKS1B/CDKN2C probe in all MM patients with AK and in 24 cases with NK which BM samples were available for additional FISH analysis. A significantly higher percentage of cases with AK (9/20; 45%) showed 1q21 gain (10% or more clonal plasma cells harboring three to ten *CKS1B* gene copies) compared to 12.5% (3/24) patients with NK ($p = 0.021$; OR: 5.73; 95% CI: 1.3–25.6). The distribution of the percentage of cells with 1q21 gain according to normal or abnormal karyotypes is shown in Fig. 1. Among patients with chromosome 1 abnormalities, 69% (9/13) of cases had *CKS1B* abnormal hybridization patterns. Most frequent abnormal clones had 3 to 5 *CKS1B* copies: 75% with three signals, 17% with five and 8% with four. The mean percentage of myeloma cells with *CKS1B* gain was 40% (range 10.5–78.6%). Patients with chromosome 1 alterations but normal 1q21 region showed the following anomalies: del(1)(p33-pter), der(2)t(1;2)(q25;q35), der(X)t(X;1)(q28;q25) [39] and t(1;4)(q21;p14). In addition, seven cases with 1q21 gains (46%) also showed *CDKN2C* (1p32) deletions, with a mean percentage of 51.25% (range: 19.1–80.4%) cells with one signal. Only one case showed deletion of *CDKN2C* without 1q21 gain. In cases with 1q21 gain and

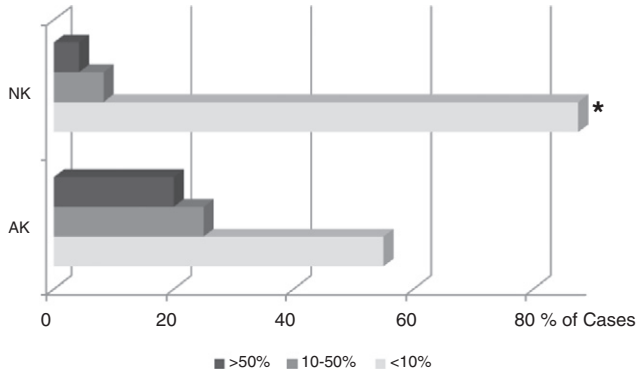

Fig. 1. Distribution of the percentage of cells with 1q21 (*CKS1B*) gain according to normal or abnormal karyotypes (AK). Positive cases for 1q21 gain in patients with normal karyotypes (NK): 8.3% of patients with 10–50% of abnormal cells and 4.2% of cases with ≥50% of abnormal cells; Positive cases for 1q21 gain in patients with AK: 25% of cases with 10–50% of abnormal cells and 20% of patients with ≥50% of abnormal cells (*$p = 0.052$).

1p32 deletion, a positive correlation between the percentage of cells with both abnormalities ($p = 0.014$) was found. Interestingly, one case with NK and multiple *CKS1B* copies at interphase nuclei (9.5% cells) showed in only one metaphase a HSR (Homogeneously Staining Region) totally hybridized with the *CKS1B* probe (Supplementary Fig. 2). This alteration has not been observed by conventional cytogenetics analysis. We also investigated the associations among different genetic alterations. A similar distribution of del(13)(q14), del(17)(p13) and *IGH@* rearrangements (>0.05), was found in patients with and without 1q21 gain. Interestingly, a positive correlation between the percentage of cells with *CKS1B* copy number gain and those with *TP53* deletion ($p = 0.0136$), was detected, indicating the coexistence of both abnormalities in the same clone.

Expression profile of CKS1B mRNA expression and comparison with 1q21 gains

Taking into account these results, we were interested in exploring the expression profile of *CKS1B* in both MM and MGUS patients, and evaluate its relationship with 1q21 gain. *CKS1B* mRNA expression was analyzed in 68 cases with MM at diagnosis and in 31 with MGUS. Mononuclear cells of 17 healthy controls were also evaluated to know the normal value of *CKS1B* expression achieved with our approach (0.13 ± 0.031). Overall, *CKS1B* was expressed in 94% (64/68) MM samples, and in 87% (27/31) MGUS samples. Analysis of data showed significantly higher *CKS1B* mRNA levels in MM (2.57 ± 1.15) than in MGUS cases (0.71 ± 0.17) ($p < 0.048$) (Fig. 2). We also analyzed

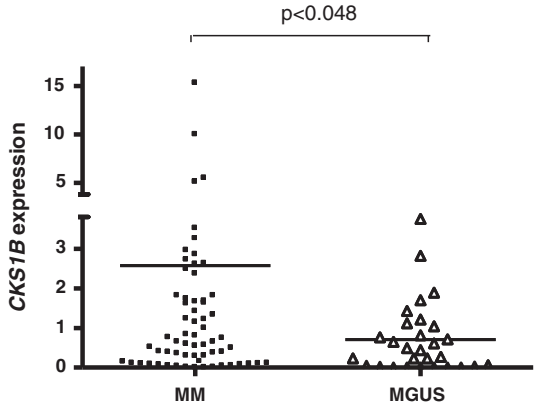

Fig. 2. Scatter plot showing *CKS1B* mRNA expression levels in patients with MM and MGUS. Significant differences: $p < 0.048$.

CKS1B expression in 16 MM patients during complete remission, detecting lower levels (1.48 ± 0.37) than cases at diagnosis. In both pathologies, expression profiles showed a high inter-individual heterogeneity. Thus, for a better analysis, patients were divided into two expression groups: high expression (HE) and low expression (LE), according to the cut-off value obtained using ROC curves analysis (1.62). Upregulation of *CKS1B* was observed in 32.8% of MM and in 14.8% of MGUS patients (Table 2).

CKS1B copy number and mRNA expression were measured in 30 MM cases. Global analysis showed a positive correlation between *CKS1B* expression levels and the percentage of cells with gene copy gain by FISH ($p < 0.0001$; $R^2 = 0.6991$). Fig. 3 shows global distribution of values detected by qRT-PCR and FISH. Interestingly, 81.2% of cases with low *CKS1B* levels had a normal hybridization pattern by FISH, while patients with high *CKS1B* mRNA expression showed variable 1q21 copy number.

Analysis of clinical parameters

Furthermore, we were interested in determining if the expression profile of *CKS1B* and/or copy number had a clinical significance in our cohort. Expression analysis showed significantly higher *CKS1B* mRNA transcripts in younger MGUS patients (less than 60 years) (1.40 ± 0.50), than in elderly MGUS cases (0.44 ± 0.10) ($p = 0.0354$) (Fig. 4a). In MM, we only found a tendency to increased levels of M protein in patients with *CKS1B* HE (4.15 ± 0.83 g) with respect to cases with LE (2.24 ± 0.36 g) ($p = 0.067$) (Fig. 4b). However, when we evaluated *CKS1B* copy number taking into account clinical parameters, a significantly higher level of β2 microglobulin (β2M) in samples with 1q21 gain (5.62 ± 1.5 μg/ml) than in those with normal 1q21 copy number (2.68 ± 1.06 μg/ml) ($p = 0.0094$), was found (Table 3). In addition, although no significant differences were found, most of patients with 1q21 gain (81.8%) were in DS stage III and they also showed the highest mean percentage of bone marrow infiltration (50.5%). Besides, we have evaluated clinical characteristics in MM cases with or without *CKS1B* gene copy gain vs. *CKS1B* HE or LE mRNA levels. Groupwise comparison showed higher β2M levels in patients with 1q21 copy gain with respect to cases with low (2.61 ± 0.8 μg/mL) or high (2.67 ± 0.95 μg/mL) *CKS1B* expression ($p \leq 0.0183$) (Table 3) and significantly increased LDH (lactate dehydrogenase) levels in patients with 1q21 gain (260.5 ± 36.5 U/L) compared to cases with low *CKS1B* expression (201.4 ± 28.3 U/L) ($p = 0.0424$).

No significant differences in OS between patients with *CKS1B* HE and LE levels were found. Nevertheless, the OS was significantly shorter (21.3 month) in cases with 1q21 gain compared to those with a normal hybridization pattern that not reached the median survival (Log-rank $p = 0.0082$) (Fig. 5a). Besides patients with 1q21 gain showed shorter OS than those with *CKS1B* low mRNA levels ($p = 0.028$) (Fig. 5b). EFS (calculated from the date of diagnosis to the first relapse), were longer in cases with a normal 1q21 hybridization pattern (76 months) than patients with 1q21 copy gain (18 months), and cases with HE (38 months), and LE *CKS1B* levels (35 months). We only found significant differences in EFS between the last group and patients with 1q21 extra signals ($p = 0.0296$).

Table 2
CKS1B mRNA expression in MM and MGUS patients.

Group	N° of cases (%)	Gene expression (mean ± ES)
MM	64	2.4 ± 1.08
MM HE	21 (32.8%)	12.0 ± 5.9
MM LE	43 (67.2%)	0.46 ± 0.06
MGUS	27	0.66 ± 0.16
MGUS HE	4 (14.8%)	2.5 ± 0.309
MGUS LE	23 (85.2%)	0.41 ± 0.08

MM: multiple myeloma; MGUS: monoclonal gammopathy of undetermined significance; HE: High expression; LE: Low expression.

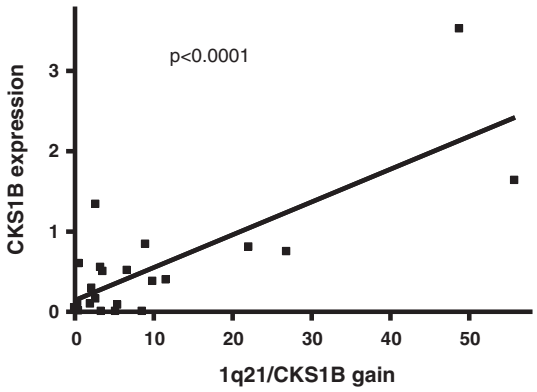

Fig. 3. Positive correlation between *CKS1B* mRNA expression and the percentage of cells with 1q21 gain ($r^2 = 0.6991$; $p < 0.0001$).

Discussion

MM remains an incurable disease with highly heterogeneous clinical outcome that cannot be accounted for by conventional laboratory

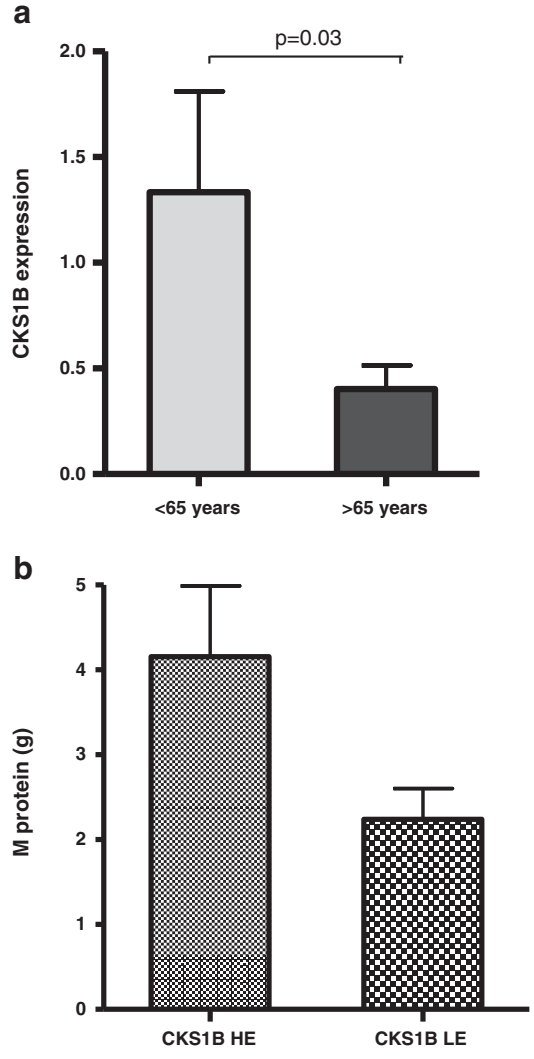

Fig. 4. Association between *CKS1B* mRNA expression and clinical parameters. a) Significant upregulation of *CKS1B* in MGUS patients with <65 years ($p = 0.03$); b) tendency to higher M-protein levels in MM cases with *CKS1B* overexpression ($p = 0.067$). HE: high expression; LE: low expression.

Table 3
Clinical characteristics of patients with and without 1q21 gain and *CKS1B* expression groups.

Clinical characteristics	Normal 1q21	1q21 gain	*CKS1B* LE	*CKS1B* HE
N° of cases	32	13	43	21
Sex (F/M)	17/15	3/10	21/22	10/11
Mean age (range), years	66.5 (36–83)	64.7 (46–86)	65.5 (30–86)	62.8 (24–83)
Paraprotein isotype (%)				
IgG	65	33.4	50	73.7
IgA	20	44.4	29	10.5
IgM	5	11.1	5.2	5.3
Other	10	11.1	15.8	10.5
DS Stage (%)				
I	15	0	12	24
II	10	18.2	26	19
III	75	81.8	62	57
ISS (%)				
I	53	36.4	22	26
II	26	27.2	61	53
III	21	36.4	17	21
β_2 microglobulin (ug/ul)	2.68 ± 1.06	5.62 ± 1.5*	2.61 ± 0.80	2.67 ± 0.95
LDH (U/L)	263.7 ± 54.9	260.5 ± 36.5#	201.4 ± 28.3	180.7 ± 17.5
%IMO (%)	43.9 ± 6.6	50.5 ± 8.6	44.84 ± 4.8	35.85 ± 6.67
Serum albumin (g/dL)	3.6 ± 0.13	3.2 ± 0.14	3.4 ± 0.09	3.2 ± 0.13
Creatinine (g/dL)	1.8 ± 0.5	1.7 ± 0.2	2.07 ± 0.4	1.85 ± 0.5
Serum calcium (mg/dL)	12.95 ± 3.72	9.61 ± 0.42	9.21 ± 0.16	9.78 ± 0.41
M protein (g)	3.56 ± 0.83	3.68 ± 0.79	2.24 ± 0.36	4.15 ± 0.83

* Significant differences with respect to normal 1q21 ($p = 0.0094$), LE ($p = 0.004$) and HE ($p = 0.0183$).
\# Significant differences with respect to LE ($p = 0.0424$).

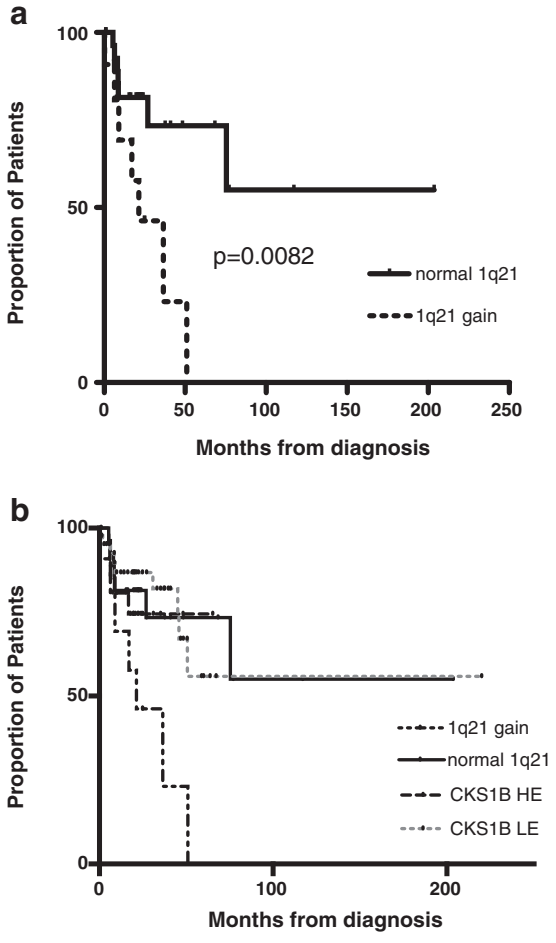

Fig. 5. Kaplan–Meier analysis of overall survival in MM patients stratified by 1q21 gain and *CKS1B* mRNA expression. a) Significant differences between cases with and without 1q21 gain ($p = 0.0082$); b) significant differences between patients with 1q21 gain and low *CKS1B* mRNA expression ($p = 0.036$).

parameters. This clinical heterogeneity may be related to genetic aberrations within the tumor cells, some of them already present in MGUS and SMM [40,41]. Among them, 1q21 gain is one of the most common recurrent chromosomal abnormalities in MM [8,16,29]. This alteration is not specific to MM and can be found in many hematological malignancies and solid tumors [42], but it is highly prevalent in MM and its frequency rises during the course of the disease. In this region maps several genes including *CKS1B* (1q21.3) [9,43], which overexpression was associated with a high rate of proliferation and poor prognosis in MM patients [30,32,44].

In concordance with previous reports [8,45], by conventional cytogenetics our cohort showed chromosome 1 rearrangements as the most frequent structural alteration. By FISH analysis, we have detected 1q21 gain in 27.3% of our MM cases, results comparable to those previously observed [9,13,46,47]. We also found a significantly higher percentage of cases with AK harboring extra 1q21 (*CKS1B*) copies compared to those with only normal metaphases. These findings support an association between 1q gain and genomic instability [48,49], through which clonal evolution and gene amplification, related to disease progression, may occur. Higher frequency of 1q21 gain was observed in relapsed patients (72% of cases), suggesting clonal selection and expansion of myeloma cells with extra copies of 1q21, probably associated to drug resistance [8,29,50]. Additionally, Boyd et al. [51] showed that 1q21 copy gain was an independent adverse prognostic factor for MM patients treated with bortezomib. In concordance, a recent report [52] found that three copies of 1q21 in at least 20% of plasma cells were enough to confer bortezomib resistance, supporting that chromosome 1q21 gain should be considered a high-risk feature in MM.

Furthermore, *CKS1B* mRNA expression was measured in MM and in MGUS patients being, to our knowledge, the first qRT-PCR evaluation in this premalignant disease. Our findings showed higher values in MM compared to the MGUS samples, suggesting a role of this gene in the multiple step process of progression of MGUS to MM [16,30,53]. In addition, MM samples at diagnosis had higher *CKS1B* mRNA levels compared to patients at complete remission of the disease. In this regard, Zhan et al. [30] detected that *CKS1B* mRNA and protein levels were typically high in aggressive primary MM and increase during disease progression, meanwhile, primary disease with low levels of *CKS1B* was usually associated with a more indolent clinical course. It suggests that *CKS1B* expression levels would decrease along with the response to treatment.

Additionally, we found a positive correlation between the percentage of cells with 1q21 gain and *CKS1B* mRNA expression levels. These results support those observed by Fonseca et al. [29] in MM samples and by Mosca et al. [54] in patients with plasma cell leukemia, that detected *CKS1B* gene expression using GEP (genome expression profile) significantly increased in cases with 1q21 gain. Our findings also reinforce the strongly positive associations between *CKS1B* protein and the mRNA expression [30] as well as *CKS1B* protein expression and the chromosome 1q21 amplification [28] previously described.

We would also like to determine if the expression profile of *CKS1B* gene had a clinical significance. In MGUS, higher *CKS1B* mRNA levels in <65 years patients compared to older cases were observed. In this aspect, a strong correlation between young age and overexpression of CKS1B by immunohistochemistry was observed in breast cancer, supporting the notion that this pathology has more aggressive tumor biology in young patients than in older ones [23]. In MM, we found a trend to elevated M-protein levels in cases with high expression, suggesting that *CKS1B* could have a possible association with the amount of secreting tumor cells being a probable tumoral marker in this pathology. On the other hand, the evaluation of *CKS1B* citomolecular status according to prognostic factors showed that 1q21 copy gain was associated with higher LDH and β2M levels. The positive association between 1q21 gain by FISH and β2M levels was also observed by others [12,54]. In addition the study published by Nemec et al. [55] evidenced a higher proportion of relapsed patients among cases with 1q21 gain compared to those lacking this alteration. In concordance with these findings, our series showed relapse and/or disease progression in 77.8% of MM patients with 1q21 gain compared to 30% of cases with normal 1q21 (data not shown). Interestingly, when the survival of patients with 1q21 copy gain and *CKS1B* expression was compared, cases with increased 1q21 had the worst prognosis. However, no association between *CKS1B* expression and outcome was observed, which is in concordance with in vivo studies [27] showing that *CKS1B* is required for tumor cell proliferation but not sufficient to induce hematopoietic malignancies. Thus, 1q21 copy gain appears to be a more significant prognostic factor than its overexpression. Consistent with this, integrated analysis of high-resolution CGH array and GEP identified a minimal region (10 Mb) of DNA copy number change on 1q21 [42], containing a large number of candidate genes including *CSK1B*, and others: *BCL9, MUC1, PDZK1* and *MCL1* [7,56–60]. More recently, Nemec et al. [61] detected 27 genes differentially expressed in MM cases with 1q21 gain by FISH, among them they found abnormal higher levels of genes involved in G-protein associated signal transduction and microtubules activity (i.e., *UCHL1*, *GPR63*, *TUBB4*, *KIF21B*). It could explain the greater prognostic significance associated with 1q21 gain.

In our cohort, we also found a similar prevalence of additional FISH abnormalities (*IGH@* rearrangements and *TP53* and *RB1* deletions) in cases with and without abnormal 1q21 copy number. It became important for the analysis of the association between 1q21 copy gain and prognosis excluding the influence of other well known high-risk factors, such as FISH lesions. The literature showed a higher prevalence of 13q deletion and t(4;14) in MM patients with 1q21 gain and no differences in the frequency of *TP53* deletion [29,50,65]. Boyd et al. [51] found a strong positive association between 1q21 gain and adverse *IGH@* translocations: t(4;14), t(14;16) and t(14;20). In addition, different reports have shown that high risk MM cases frequently had 1q21 gain together with adverse *IGH@* translocations and *TP53* deletion, suggesting that additional genetic abnormalities significantly worsen the poor prognosis of 1q21 gain [51,65]. Nevertheless, we also found a positive correlation between the percentage of cells with *CKS1B* gain and *TP53* deletion, indicating the coexistence of both alterations in the same clone. Tumor suppressor protein *TP53* has an important role in promoting apoptosis, senescence or cell cycle arrest in response to DNA damage, while *TP53* deletion or mutations may either predispose cells to DNA damage or allow cellular survival [66]. According to our results, Rother et al. [67] detected an inverse association between *CKS1B* and *TP53* expression in different cell lines suggesting that *TP53* downregulates *CKS1B* gene resulting in reduced mRNA and protein expression.

Besides, we found an association between 1q21 gain and 1p deletions, particularly 1p32.3 region where *CDKN2C* gene is located, as well as a positive correlation between the percentages of cells with both abnormalities. In this aspect there are discordant results; Chang et al. [62] found association between 1q21 gain and 1p21 deletions in relapsed/refractory MM patients, meanwhile no correlation between 1q21 gain and 1p31–32 deletions was observed [63]. Chromosome 1p deletion has also been associated to adverse clinical outcome in MM patients [13,46,51,62,63], and other authors have identified deletions of *CDKN2C* at 1p32.3 in myeloma cell lines [14]. *CDKN2C* gene belongs to the INK4 family of cyclin-dependent kinases (cdk) inhibitors which interacts preferentially to the cdk4/6 preventing G1 progression. Terminal differentiation of B cells into plasma cells is dependent on G1 cell cycle arrest, which is temporally correlated with its increased expression [64]. More studies will be necessary to evaluate the association between both alteration and their clinical significance, and to define the effect on OS of the accumulation of these markers.

In conclusion, although our series is not so large, our study showed a correlation between *CKS1B* mRNA expression and 1q21 copy gain, as well as a poor clinical outcome for patients with this alteration, indicating that 1q21 gain is a more significant prognostic factor than *CKS1B* overexpression. This adverse impact on survival probably reflects the genetic instability associated to chromosome 1q alterations resulting in a more aggressive behavior of the disease. Prospective studies on large patient cohorts are required to confirm the role of 1q21 gain in MM patients.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bcmd.2014.05.006.

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