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Quantification of cyclin D1 and D2 proteins in multiple myeloma identifies different expression patterns from those revealed by gene expression profiling

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IJCB developed and performed qRT-PCR, analyzed data, prepared the figures and wrote the manuscript; IMK conceived the idea, developed and performed CNIA experiments and analyzed protein data; EAR performed CNIA experiments and analyzed protein data; CDR obtained the patients' clinical data; IHI assisted with CNIA experiments; ASS and DQ assisted with qRT-PCR experiments; AMLG and MC assisted with laboratory experiments; MJC, LR, JML, JSM and MVM provided patient samples and clinical data, and were responsible for obtaining informed consent from patients; LAC analyzed the clinical data, supervised the statistical analysis, helped prepare the figures and supervised the whole study; NCG conceived the idea and designed the study, participated in writing the manuscript, supervised

the whole study, and provided funding. All authors critically reviewed and approved the manuscript. # LAC and NCG contributed equally to this work.

- Running Heads:

Quantification of cyclin D1 and D2 proteins in MM

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- Data-sharing statement:

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ABSTRACT

Upregulation of a cyclin D gene determined by expression microarrays is an almost universal event in multiple myeloma (MM), but this finding has not been properly confirmed at the protein level. For this reason, we carried out a quantitative analysis of cyclin D proteins using a capillary electrophoresis nanoimmunoassay in newly diagnosed MM patients.

Exclusive expression of cyclin D1 and D2 proteins was detected in 54/165 (33%) and 30/165 (18%) of the MM patients, respectively. Of note, cyclin D1 or D2 proteins were undetectable in 41% of the samples. High levels of cyclin D1 protein were strongly associated with the presence of t(11;14) or 11q gains. Cyclin D2 protein was detected in all the cases bearing t(14;16), but in only 24% of patients with t(4;14). The presence of cyclin D2 was associated with shorter OS (HR=2.14, p=0.017), although patients expressing cyclin D2 protein, but without 1q gains, had a favorable prognosis.

In conclusion, although one of the cyclins D is overexpressed at the mRNA level in almost all MM patients, in approximately half of the patients this does not translate into detectable protein. This suggests that cyclins D could not play an oncogenic role in a proportion of patients with MM.

ClinicalTrials.gov identifier: NCT01916252

INTRODUCTION

Dysregulation of D-type cyclins has been invoked as an early and unifying oncogenic event in multiple myeloma (MM) and monoclonal gammopathy of undetermined significance (MGUS), which is a premalignant condition[1–3]. Gene expression profiling (GEP) has demonstrated that 98% of patients with MM overexpressed *CCND* mRNAs: *CCND1*, *CCND2* and *CCND3* were overexpressed in about 46%, 41% and 3% of patients, respectively; additionally, *CCND1* and *CCND2* were coexpressed in 8% of patients. *CCND1* and *CCND2* expression was found to be mutually exclusive in almost all cases[1–3]. Thereafter, these results seem to have been confirmed when MM samples were analyzed by RNA-seq[4].

CCND1 mRNA overexpression is attributable in 15-20% of cases to t(11;14), which leads to high levels of cyclin D1. In most of the other MM patients with *CCND1* overexpression, polysomy of chromosome 11 is the probable cause of this *CCND1* dysregulation[5,6]. On the other hand, *CCND2* overexpression apparently arises from mechanisms that are not directly associated with *CCND2* gene abnormalities, but rather are a consequence of the dysregulation of other genes. *MAF* and *MAFB* leucine zipper transcription factors, which are involved in the t(14;16) and t(14;20) translocations, respectively, have been shown to upregulate *CCND2* through their transactivation function, leading to an increase in the rate of cell division and DNA synthesis[7]. Beyond the MAF family, *ZKSCAN3*, a zinc-finger transcription factor, has been described as inducing *CCND2* promoter activity and thereby cyclin D2 upregulation[8]. More recently, our group described that the shortening of *CCND2* 3′UTR by alternative polyadenylation with the consequent loss of miRNA binding sites is also involved in *CCND2* upregulation[9].

Dysregulation of D-type cyclins and their associated pathways is common in both solid and hematological malignancies[10]. A central role of D-type cyclins is the regulation of cyclin-dependent kinases (CDKs), in particular CDK4 and CDK6, to promote cell-cycle progression (G1-S transition) through the phosphorylation and inactivation of the RB tumor-suppressor protein[11–14]. The oncogenic role of cyclin D1 is well established in many tumors, and its amplification and overexpression is generally associated with negative outcomes[15–17]. However, the overexpression of *CCND1*, which is strongly associated with t(11;14) and trisomy 11, does not confer an unfavorable prognosis on patients with MM[3,18]. The function of cyclins D2 and D3 in tumorigenesis has been less thoroughly explored, and their consequences for survival are sometimes mixed[19]. Particularly in MM, overexpression of *CCND2* has been

associated with poor prognosis, probably due to the predominance of high-risk cytogenetic alterations in this group of patients.[20–24].

The overexpression of cyclin D mRNA in almost all MM cases contrasts with the generally low proliferation rate observed in tumor plasma cells[1,3,25]. One possible explanation for this is that cyclins D may perform other functions that are unrelated to cell-cycle progression[13]. Another possibility is that protein levels of cyclins D may not be high or stable enough to trigger cell-cycle transition from G1 to S phase. In this regard, few studies have analyzed the expression of cyclin D proteins, and most of those that have done so used immunohistochemical techniques[26,27]. Although immunohistochemistry provides valuable information about the expression of proteins in the tissue context, the technique is usually semiquantitative and uses arbitrary cutoff levels. The adoption of capillary electrophoresis nanoimmunoassay (CNIA) technology may help overcome these drawbacks, given its capacity to facilitate the quantitative analysis of proteins with high sensitivity and its requirement for only nanogram amounts of sample[28,29]. To shed light on these elusive aspects related to the expression of cyclins D in MM, we carried out a quantitative analysis of cyclin D proteins in a large cohort of newly diagnosed MM (NDMM) patients who were homogeneously treated according to GEM2012 clinical trial. We compared the results with *CCND1* and *CCND2* mRNA levels quantified by qRT-PCR. The impact of cyclin D expression on survival of MM patients was also explored.

METHODS

Patient samples

A total of 165 samples from NDMM patients treated as part of the Spanish Myeloma Group clinical trial GEM2012 (NCT01916252) were included in the study[30], which was approved by the local ethics committee and conducted in accordance with the Declaration of Helsinki. Informed consent was required prior to patient participation in the clinical trial. Patients were treated with six cycles of VRD (bortezomib, lenalidomide and dexamethasone) as induction followed by autologous stem cell transplantation with melphalan 200 *vs.* busulfan-melphalan, and consolidation treatment with two cycles of VRD. $CD138^+$ plasma cells were isolated from bone marrow aspirates using the AutoMACS immunomagnetic separation system (Miltenyi-Biotec, Germany). Plasma cell purity was > 80% in all the cases. All samples were immediately frozen in RLT^+ buffer (Qiagen, Germany) and stored

at -80°C for further analysis, as previously described[29]. RNA, DNA and protein were extracted using an AllPrep DNA/RNA Mini Kit (Qiagen). Proteins were extracted by ice-cold acetone precipitation[28,29].

Cytogenetic analysis by fluorescence *in situ* hybridization (FISH) for detecting *IGH* translocations, 17p deletions, 1q gains and 1p losses was carried out in all patients, as previously described.

The main characteristics of patients are summarized in Supplementary table 1. This cohort of patients was representative of the whole GEM2012 trial dataset[30].

Capillary electrophoresis nanoimmunoassay

Capillary electrophoresis nanoimmunoassay (CNIA) was performed using the WES system (ProteinSimple, California, EEUU) according to the manufacturer's protocols, and as previously used by our group[28,29,31].

Primary antibodies used in the study under optimized conditions were: rabbit monoclonal cyclin D1 (Abcam [Cambridge, UK], ab134175, dilution 1/50), rabbit monoclonal cyclin D2 (Cell Signaling [Danvers, EEUU], #3741, dilution 1/50) and rabbit monoclonal GAPDH (Cell Signaling, #2118, dilution 1/50). Cyclin D1 and D2 protein peaks were normalized with respect to the GAPDH median area under the peak. Expression of each protein was represented relative to that of GAPDH. A more extensive protocol for relative protein quantification by CNIA has been reported elsewhere [28,29].

Quantitative real-time PCR

RNA concentration and integrity were assessed with an Agilent 2100 Bioanalyzer. Approximately 200 ng of total RNA were reverse-transcribed into cDNA using the SuperScript II First-Strand Synthesis kit (Thermo Fisher, California, EEUU). Gene expression of *CCND1* and *CCND2* were evaluated with TaqMan qRT-PCR assays, Hs00765553_m1 and Hs00153380_m1, respectively (Thermo Fisher). The *PGK1* gene (Hs00943178_g1, Thermo Fisher) was used as the endogenous control. Relative expression was calculated whereby ΔCt = Ct housekeeping gene - Ct target gene.

Statistical analysis

Continuous variables were assessed for normality using the Shapiro-Wilk test. Differences between the experimental groups were analyzed using two-tailed t-tests or Mann-Whitney U tests, as appropriate, for normally and non-normally distributed continuous variables, respectively. The mclust package (v.5.4.10) was used to model these data as a Gaussian mixture in which the optimal number of components would be determined from the Bayesian Information Criterion (BIC) values of the adjusted models. Fisher's exact test was used to evaluate the

association between the resulting categorical variables. The Spearman rank test was used to estimate correlations. Survival curves were depicted using the Kaplan-Meier estimator and were compared with the log-rank test in the survival R package (v.3.3-1). The endpoints included in this survival analysis were time to progression (TTP) and overall survival (OS). The events of interest for TTP were restricted to disease progression and relapse, whereas OS was defined as the time from diagnosis until the date of death from any cause. Values of $p < 0.05$ were considered statistically significant for all tests. Statistical analyses were carried out in R (v.4.2.1).

RESULTS

Expression profile of cyclin D1 and cyclin D2 proteins

Expression of cyclin D1 and cyclin D2 proteins was highly variable among the samples, particularly in the case of cyclin D1. Expression values of cyclin D1 ranged from 0 to 15.05, while those of cyclin D2 varied from 0 to 1.18 (Figure 1A). Excluding non-expressed values, the non-parametric coefficients of variation for cyclin D1 and cyclin D2 were 96% and 70%, respectively.

Patients were divided into four groups based on their cyclin D1 and cyclin D2 protein expression: expression of cyclin D1 exclusively (54 out of 165, 33%); expression of cyclin D2 exclusively (30 out of 165, 18%); coexpression of both proteins (14 out of 165, 8%); no expression of either cyclin D protein (67 out of 165, 41%) (Figure 1B).

The group of MM patients expressing only cyclin D1 contained all the 23 cases with t(11;14) and 55% (21/38) of the cases with 11q13 gains. In other words, cyclin D1 expression was associated with t(11;14) or 11q13 gains in 82% (44/54) of the patients. We next dichotomized the expression of this group of patients by fitting a Gaussian mixture model that differentiated two groups, one with a high level of cyclin D1 expression (cyclin D1 > 0.057), and the other with a low level (cyclin D1 ≤ 0.057) (Figure 1C). Eighteen of the 23 patients (78%) with t(11;14) were classified in the group with high cyclin D1 expression, while only two of the 38 patients (5%) with 11q13 gains were included in that group (Figure 1D). *IGH* translocations other than t(11;14) were rarely found in the group of patients expressing only cyclin D1. In fact, t(4;14) was detected in only two cases that also featured 11q13 gain, which were in turn classified into the low cyclin D1 expression group.

In the group of patients who exclusively expressed cyclin D2, none had t(11;14) as expected, although 11q13 gain was present in three of the 30 patients (10%). The distribution of the other cytogenetic abnormalities in this

group was as follows: t(4;14) and t(14;16) were each present in 13% (4/30) of the cases; 1q gains, and 1p and 17p deletions, were found in 80% (24/30), 27% (8/30) and 20% (6/30) of cases, respectively. t(14;16), 1q gains and 1p deletions were significantly enriched in the group of patients expressing only cyclin D2 compared with the other MM patients (13% *vs.* 0%, $p < 0.001$; 80% *vs.* 40%, $p < 0.001$; 27% *vs.* 10%, $p = 0.03$, respectively). FISH studies yielded normal results in only two of the 30 patients expressing solely cyclin D2. In the same way as for cyclin D1, patients with cyclin D2 expression were dichotomized into two groups, one with high cyclin D2 expression (cyclin D2 > 0.058) and the other with low expression (cyclin D2 ≤ 0.058) (Supplementary Figure 1A). Cytogenetic abnormalities were uniformly distributed throughout the two groups (Supplementary Figure 1B).

In the group of patients coexpressing both cyclins D a preference for the expression of one of them was observed in 10 of the 14 patients (71%) (Figure 1E). Based on the level of expression of each cyclin D, most cases (71%) expressed low levels of cyclin D1 and D2. Two cases each exhibited high levels of expression of cyclin D1 and of cyclin D2. None of the patients belonging to this group showed t(11;14), while 11q13 gain was detected in three patients who expressed low levels of both cyclins D.

Finally, the largest group of patients analyzed (41%) expressed neither of the cyclins D. The distribution of cytogenetic abnormalities analyzed by FISH within this group is summarized and compared with the other three groups of cyclin D expression in Supplementary table 2. Interestingly, more than half of the patients with t(4;14) did not express cyclin D2, whereas all the four samples with t(14;16) did express it.

Expression profiles of *CCND1* and *CCND2* mRNA

Quantifying cyclin D1 and D2 proteins showed a high proportion of MM patients without expression of any of the cyclins D. To gain more insight into this unexpected finding, we evaluated the expression of *CCND1* and *CCND2* at the mRNA level using qRT-PCR in 110 of the 165 samples for which RNA was available.

Expression of *CCND1* and *CCND2* mRNA was quantified in 16 normal plasma cells (NPC) to establish the baseline expression level for both mRNAs in the cohort. *CCND1* and *CCND2* mRNAs were considered to be overexpressed when their expression in MM samples was above the upper 95th percentile expression level in NPC ($\Delta Ct = -5.99$ for *CCND1* and $\Delta Ct = -3.51$ for *CCND2*) (Figures 2A and 2B). According to these criteria, exclusive

overexpression of *CCND1* or *CCND2* was detected in 53% (58 of 110) and 21% (23 of 110) of patients, respectively. Overall, 6% (7 of 110) of the samples simultaneously expressed *CCND1* and *CCND2* at the mRNA level.

The Spearman's rank-order correlation between mRNA and protein expression levels was stronger for cyclin D1 than for cyclin D2 (rho = 0.7 *vs.* rho = 0.53; $p < 0.001$) (Supplementary Figure 2A and 2B).

Almost all the samples that exclusively expressed cyclin D1 protein overexpressed *CCND1* mRNA (40 of 41 samples for which protein and mRNA material was available) (Figure 2C). The highest levels of *CCND1* mRNA were observed in MM patients with t(11;14).

However, when we compared the expression of cyclin D2 at the protein and mRNA levels in the samples for which both molecules were available, we found that 71% (15 out of 21) of the patients exclusively expressing cyclin D2 protein also overexpressed *CCND2* mRNA (Figure 2C). Finally, the 41% of patients who did not express either cyclin D1 or cyclin D2 protein expressed mRNAs at levels lower than those observed in NPC, whereas 59% of those patients expressed the mRNA of at least one cyclin D.

Prognostic effect of cyclin D protein expression

The survival analysis considered only the patients who exclusively expressed cyclin D1 or D2, and compared them with patients who did not express the corresponding cyclin D. Expression of cyclin D1 protein was significantly associated with longer overall survival (OS) (HR [95% CI] = 0.44 [0.22-0.91], $p = 0.022$) (Figure 3A). Conversely, expression of cyclin D2 was significantly associated with shorter OS (HR [95% CI] = 2.14 [1.13-4.05], $p = 0.017$) (Figure 3B). No statistically significant differences were found in the time to progression (TTP) among the patients classified by their cyclin D1 or D2 expression status (Figures 3C and 3D). A positive effect of *CCND1* mRNA overexpression on OS was also observed (Supplementary Figure 3).

Given the significant association between cyclin D2 protein expression and 1q gains, we investigated how this relationship was related to survival. We found that the prognosis of patients with 1q gains was not affected by cyclin D2 protein levels, while cases expressing cyclin D2 exhibited short survival only if they also had 1q gains (Figure 4).

A subsequent survival analysis considering the groups of high and low expression of both cyclins D revealed no significant differences in OS between the two groups (Figure 5A). However, TTP was significantly shorter among

patients with high levels of cyclin D1 (HR [95% CI] = 2.43 [1.06-5.55], p = 0.03), indicating a less favorable prognosis for patients with t(11;14) than for those with 11q gains (Figure 5B). Partitioning the patients into the high and low level cyclin D2 groups revealed no differential association with survival.

DISCUSSION

Upregulation of D cyclins has been considered an early-initiating event in MM pathogenesis since one of the cyclin D genes is known to be overexpressed in almost all MGUS and MM patients[1–3]. These results were based on mRNA quantification using microarrays[1,3,18]. Only limited attempts have been made to validate this overall finding at the protein level; the few studies carried out have only analyzed cyclin D1 protein by immunohistochemistry (IHC) in short series of patients [32–36].

In this study, we quantified cyclin D1 and D2 proteins using CNIA in 165 newly diagnosed MM patients. Cyclin D3 was not included because of the very low frequency of MM cases overexpressing this cyclin D in previous analyses. We observed expression of the two cyclin D proteins, singly or together, in 59% of the patients. These results are in agreement with those of a previous analysis of cyclin D1 and D2 using IHC in almost 100 bone marrow biopsies, in which cyclin D1 protein was detected in 32%, cyclin D2 was found in 18% and both cyclins D were identified in 14% of MM patients [36]. Therefore, we did not detect any cyclin D expression in almost half of the MM samples, even using the CNIA method, which can accurately quantify proteins and is more sensitive than IHC. This finding prompted us to investigate *CCND1* and *CCND2* levels by RT-PCR, using the expression levels of both *CCNDs* in NPC as a cutoff to establish gene overexpression. It has been pointed out that *CCND1* is not expressed in NPC[1,18,37,38], and *CCND2*, is present at very low or null levels in NPC[1,18,39]. We detected *CCND1* and *CCND2* overexpression in 53% and 21% of the patients, respectively, and simultaneous overexpression of *CCND1* and *CCND2* in a small group of patients. *CCND* genes were not expressed at levels above that of NPC in 20% of MM patients. This latter finding contrasts with the previously published results obtained using microarrays and RT-PCR, in which the proportion of MM patients not overexpressing cyclins D did not exceed 8%. Expression microarrays have shown that *CCND1* and *CCND2* genes are both overexpressed in about 40-45% of MM patients, and that the other patients (approximately 11%) simultaneously express *CCND1* and *CCND2* or *CCND3*. These results were corroborated in other series of MM patients assessed using microarrays[1,18]. Moreover, there was a very good concordance between cyclin D expression assessed by microarrays and RT-PCR[18]. The fact that RT-PCR provides a relative quantification of mRNA may largely

explain the differences between the percentage of MM patients who did not express D-cyclin mRNA in our study and in that of Agnelli's group[18].

Protein expression of cyclins D in the present study also showed that cyclin D1 and cyclin D2 were overexpressed in an exclusive manner, and only a small proportion of patients coexpressed both cyclins D, as revealed by mRNA quantification[1,3,18] and protein assays[36].

Our findings confirmed the strong association between the overexpression of cyclin D1 and the presence of t(11;14), as all the cases with this translocation overexpressed cyclin D1 protein, mostly at high levels. The patients overexpressing cyclin D1 at lower levels corresponded mainly to cases with 11q13 gains, although 37% of cases with this abnormality did not express cyclin D1 protein. As with the proteins, patients with high *CCND1* mRNA values had the t(11;14) translocation, and patients with 11q13 gain had intermediate levels of mRNA expression. These results are consistent with previous reports in which high levels and moderate levels of cyclin D1 mRNA were associated with t(11;14) and polysomy 11, respectively[1,3,5,26,40,41].

Overexpression of cyclin D2 may arise from different mechanisms that are not linked to translocations or amplification of *CCND2* gene [7–9]. We found a significant association between cyclin D2 overexpression and t(14;16), 1q gain and 1p deletion, as described in particular in the case of t(14;16)[1,3,7]. However, 53% of patients with the t(4;14) translocation did not express cyclin D2. Even though the correlation between the protein and mRNA for the unique expression of cyclin D2 was weaker than that for cyclin D1, most of the samples expressing cyclin D2 protein also overexpressed *CCND2* mRNA. Six cases expressed cyclin D2 protein but with mRNA *CCND2* levels less than those found in NPC. This could be the result of the protein being generated by insignificant levels of *CCND2* mRNA.

Of the samples without cyclin D protein expression, the levels of mRNA expression of both *CCND1* and *CCND2* were less than the NPC cutoff in almost half of the patients, which explains the absence of protein. However, in the other patients one of the cyclins D was overexpressed at the mRNA level. This discrepancy could be related to post-transcriptional and post-translational modifications, among other possible explanations [42–45]. On the other hand, the greater sensitivity of qRT-PCR compared to the CNIA technique could explain why some cases in which protein expression was not observed, the corresponding mRNA was detected. However, mRNA levels cannot be considered as the final output of gene expression, while proteins are closer to phenotypes and to gene function[46].

Survival analysis showed that OS was significantly shorter for patients expressing cyclin D2 protein, while high levels of cyclin D1 protein were associated with prolonged OS. These results are consistent with those previously published, which demonstrate a significantly better prognosis for the patients who expressed high levels of cyclin D1 protein detected by immunohistochemistry than for those with low or null levels of cyclin D1 expression[27,47]. Overexpression of *CCND1* mRNA has also been associated with better prognosis[3]. The different effect on OS depending on the levels of cyclin D1 and cyclin D2 was not observed for TTP in the present series, indicating the effectiveness of VRD induction and ASCT consolidation in all patients independently of the level of expression of cyclin D proteins. However, the strong association between the expression of cyclin D1 and t(11;14) and polysomy 11, and between the expression of cyclin D2 and the presence of high-risk cytogenetic abnormalities suggests that the differences in survival for each cyclin D are related to cytogenetic abnormalities rather than to cyclin D expression[3,39]. In fact, patients with cyclin D2 protein expression but without 1q gains had a favorable prognosis.

When the survival analysis partitioned the cyclin D1 expression into high and low levels, we found that patients with high levels of cyclin D1 protein had significantly shorter TTP than did those with low levels, although this difference was not maintained during the subsequent course of the disease, since OS was similar for both groups. The strong association between lower cyclin D levels and 11q13 gains indicates a more favorable outcome for MM patients with 11q13 than for those with t(11;14). This is consistent with the findings of earlier studies[48,49].

In summary, no cyclin D1 or D2 protein expression was detected in about half of the MM patients, in whom 41% of the cases could be explained by very low mRNA levels (values less than the NPC cutoff). The discrepancy between cyclin D protein abundance and mRNA levels in the other cases may be related to post-transcriptional and post-translational mechanisms. In any case, our data demonstrate that D cyclin proteins are not universally expressed in MM. While we found that cyclin D1 was overexpressed in almost all cases with t(11;14) and 11q13 gain, cyclin D2 was not detected in the majority of the MM patients not expressing cyclin D1. Although GEP had demonstrated dysregulation of one of the D cyclins at the mRNA level in almost all MMs, this increased expression does not culminate in the production of more protein, especially in the case of cyclin D2. This suggests that cyclins D could not play an oncogenic role in a proportion of patients with MM. On the other hand, it remains to be determined whether the high levels of cyclin D present in approximately 60% of patients with MM are always functionally relevant. In terms of the prognostic impact of cyclins D, our results support that the relationship of their

overexpression with the prognosis of patients with MM is driven more by genetic alterations associated with cyclin D1 and D2 upregulation than by their dysregulation *per se*.

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FIGURE LEGENDS

Figure 1. Cyclin D protein expression in 165 samples from newly diagnosed MM (NDMM) patients.

Cyclin D1 and D2 protein expression were measured by CNIA technology (simple western blotting). Data were normalized relative to GAPDH protein expression. **A)** Protein expression levels of cyclin D1 and D2. **B)** Pie chart showing percentage of cyclin D1 and D2 protein expression in the patient cohort. **C)** Distribution of cyclin D1 protein expression in the two groups generated after dichotomization using a Gaussian mixture model. Patients with cyclin D1 expression ≤ 0.057 and > 0.057 were classified as "Low" and "High", respectively. **D)** Comparison of the distribution of cyclin D1 expression between patients harboring t(11;14) or 11q13 gains (***, $p < 0.001$). E) Coexpression of cyclin D1 and D2 in NDMM patients.

Figure 2. *CCND1* and *CCND2* mRNA expression analysis.

A) Comparison between *CCND1* mRNA levels of MM patients and those of normal plasma cells (NPC). **B)** Comparison between *CCND2* mRNA levels of MM patients and those of NPC. Cutoff point for *CCND1* and *CCND2* mRNA overexpression was the 95th percentile (red line) of NPCs. **C)** Distribution of *CCND* mRNA expression group (normal expression or overexpression) by cyclin D protein type.

Figure 3. Impact of cyclin D1 and cyclin D2 protein expression on survival of MM patients.

A and B) Kaplan-Meier curves of overall survival (OS) by cyclin D1 and cyclin D2 protein expression group, respectively. **C and D)** Kaplan-Meier curves of time to progression (TTP) by cyclin D1 and cyclin D2 protein expression group, respectively. Log-rank (Mantel-Cox) test *p* values are shown.

Figure 4. Survival analysis of the combination of cyclin D2 expression with 1q gain abnormality in MM patients.

A and B) Kaplan-Meier curves of OS and TTP, respectively, in patients with 1q gains according to the presence or absence of cyclin D2 protein. **C and D)** Kaplan-Meier curves of OS and TTP, respectively, in patients expressing cyclin D2 protein according to the presence or absence of 1q gains. Log-rank (Mantel-Cox) test *p* values are shown.

Figure 5. Survival analysis by dichotomized cyclin D1 protein expression groups (high and low).

A and B) Kaplan-Meier curves for OS and TTP, respectively. Log-rank (Mantel-Cox) test *p* values are shown.

Figure 1

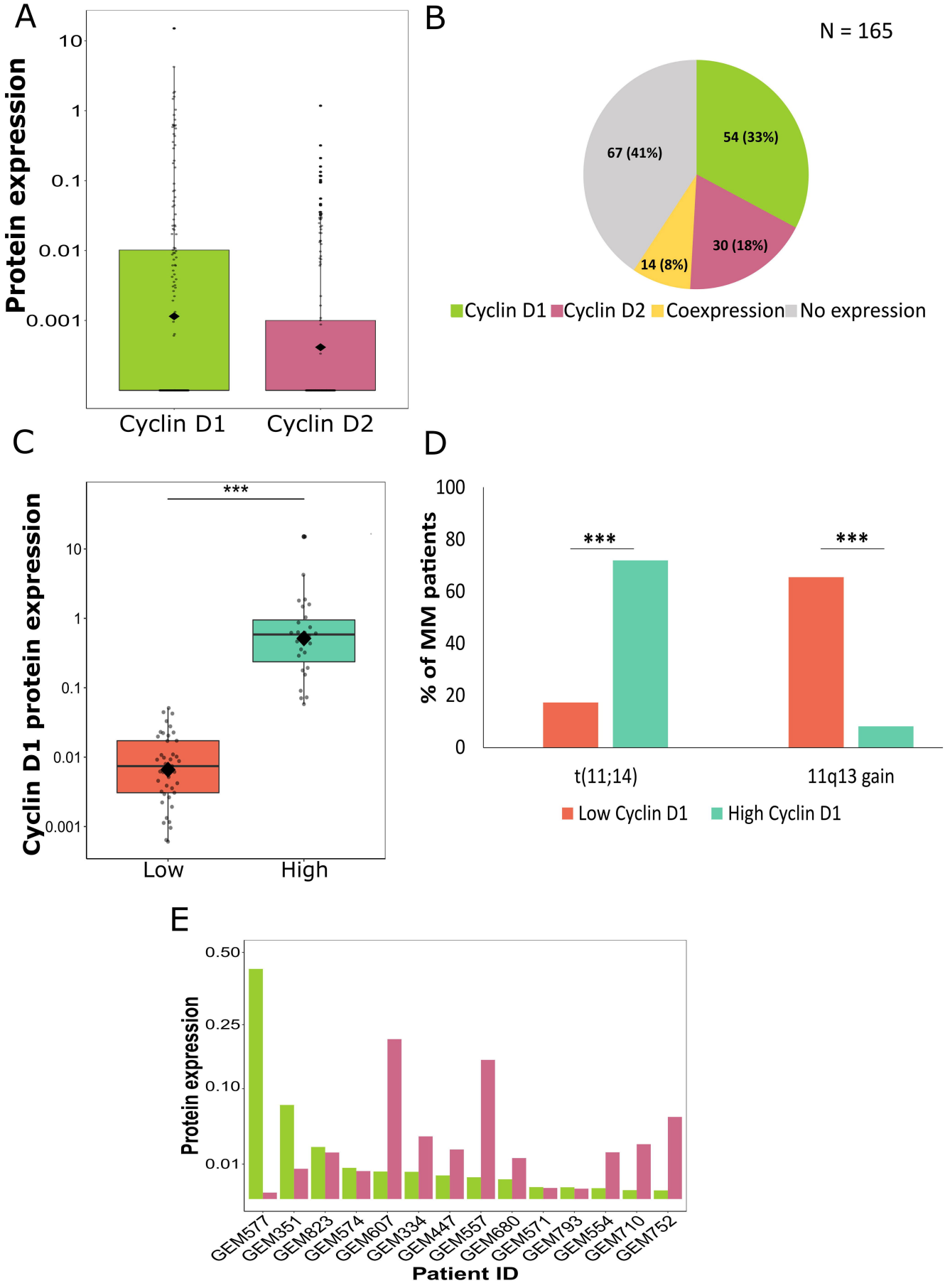

A
Protein expression
10
1
0.1
0.01
0.001
Cyclin D1
Cyclin D2
B
N = 165
54 (33%)
30 (18%)
14 (8%)
67 (41%)
Cyclin D1
Cyclin D2
Coexpression
No expression
C
Cyclin D1 protein expression
***
10
1
0.1
0.01
0.001
Low
High
D
% of MM patients
100
80
60
40
20
0
***
***
t(11;14)
11q13 gain
Low Cyclin D1
High Cyclin D1
E
Protein expression
0.50
0.25
0.10
0.01
GEM577
GEM351
GEM823
GEM574
GEM607
GEM334
GEM447
GEM557
GEM680
GEM571
GEM793
GEM554
GEM710
GEM752
Patient ID

Figure 2

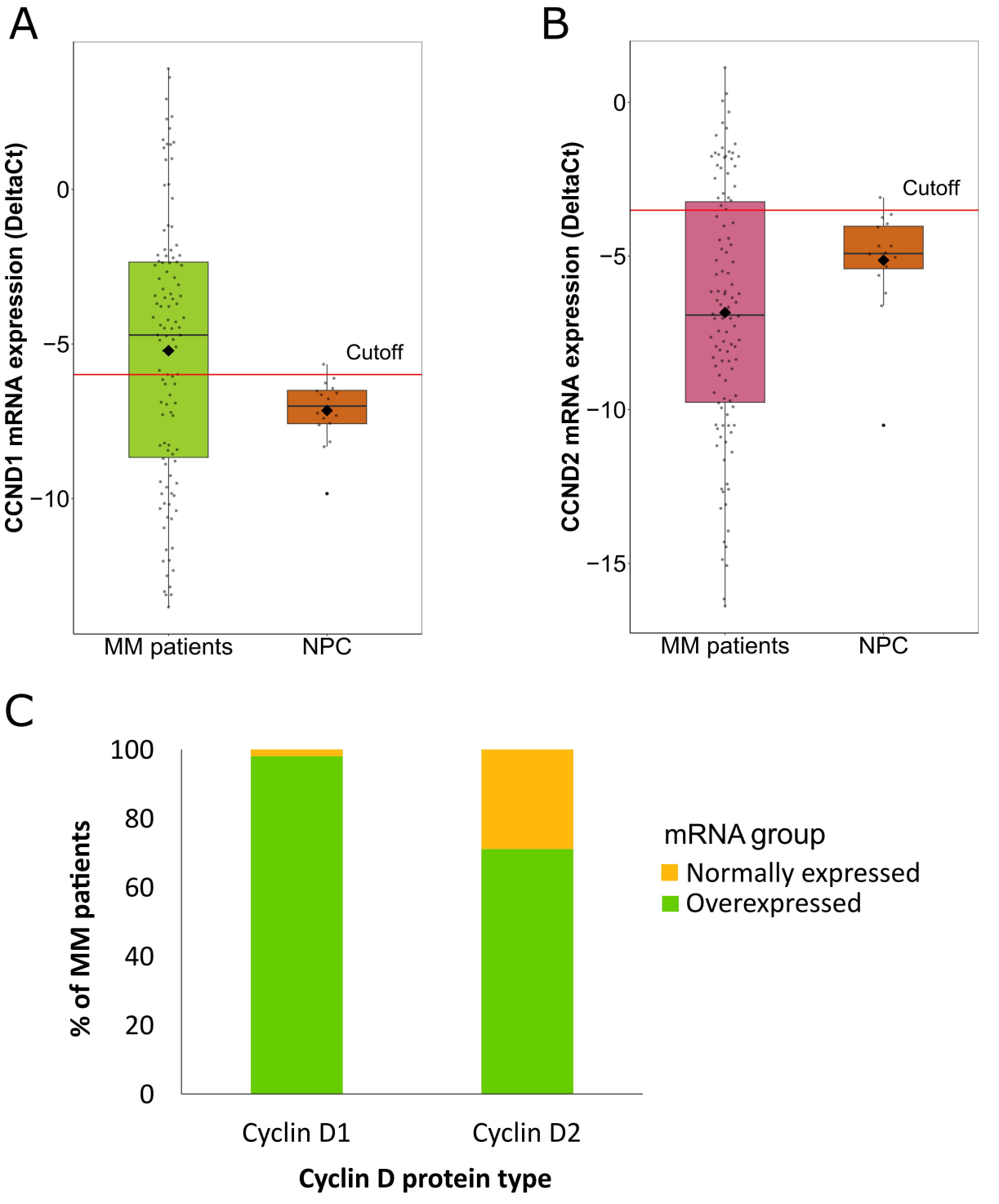

A
CCND1 mRNA expression (DeltaCt)
0
−5
−10
Cutoff
MM patients
NPC
B
CCND2 mRNA expression (DeltaCt)
0
−5
−10
−15
Cutoff
MM patients
NPC
C
% of MM patients
100
80
60
40
20
0
Cyclin D1
Cyclin D2
Cyclin D protein type
mRNA group
Normally expressed
Overexpressed

Figure 3

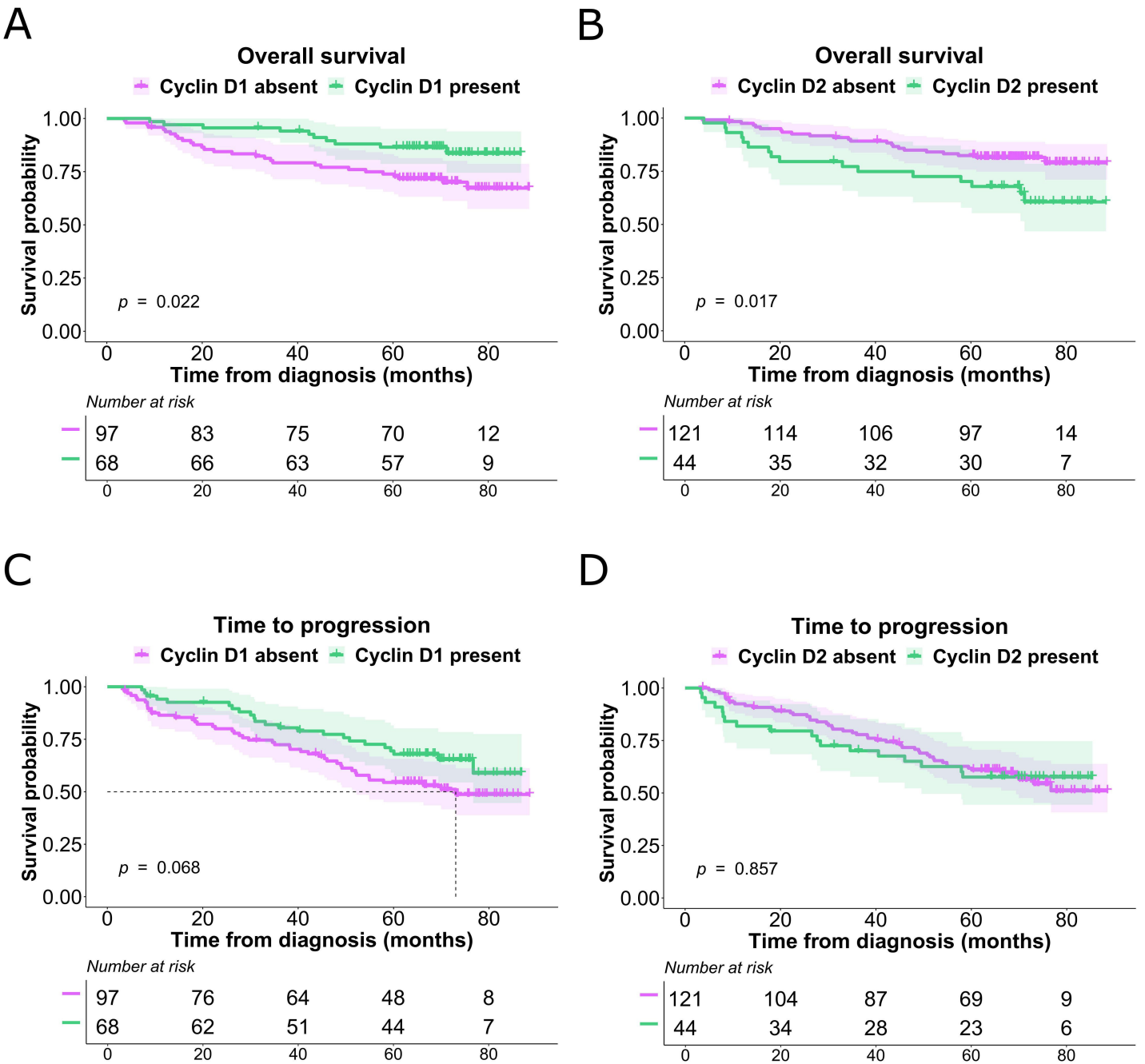

A
Overall survival
Cyclin D1 absent
Cyclin D1 present
Survival probability
1.00
0.75
0.50
0.25
0.00
p = 0.022
0
20
40
60
80
Time from diagnosis (months)
Number at risk
97 83 75 70 12
68 66 63 57 9
B
Overall survival
Cyclin D2 absent
Cyclin D2 present
p = 0.017
Time from diagnosis (months)
Number at risk
121 114 106 97 14
44 35 32 30 7
C
Time to progression
Cyclin D1 absent
Cyclin D1 present
p = 0.068
Time from diagnosis (months)
Number at risk
97 76 64 48 8
68 62 51 44 7
D
Time to progression
Cyclin D2 absent
Cyclin D2 present
p = 0.857
Time from diagnosis (months)
Number at risk
121 104 87 69 9
44 34 28 23 6

Figure 4

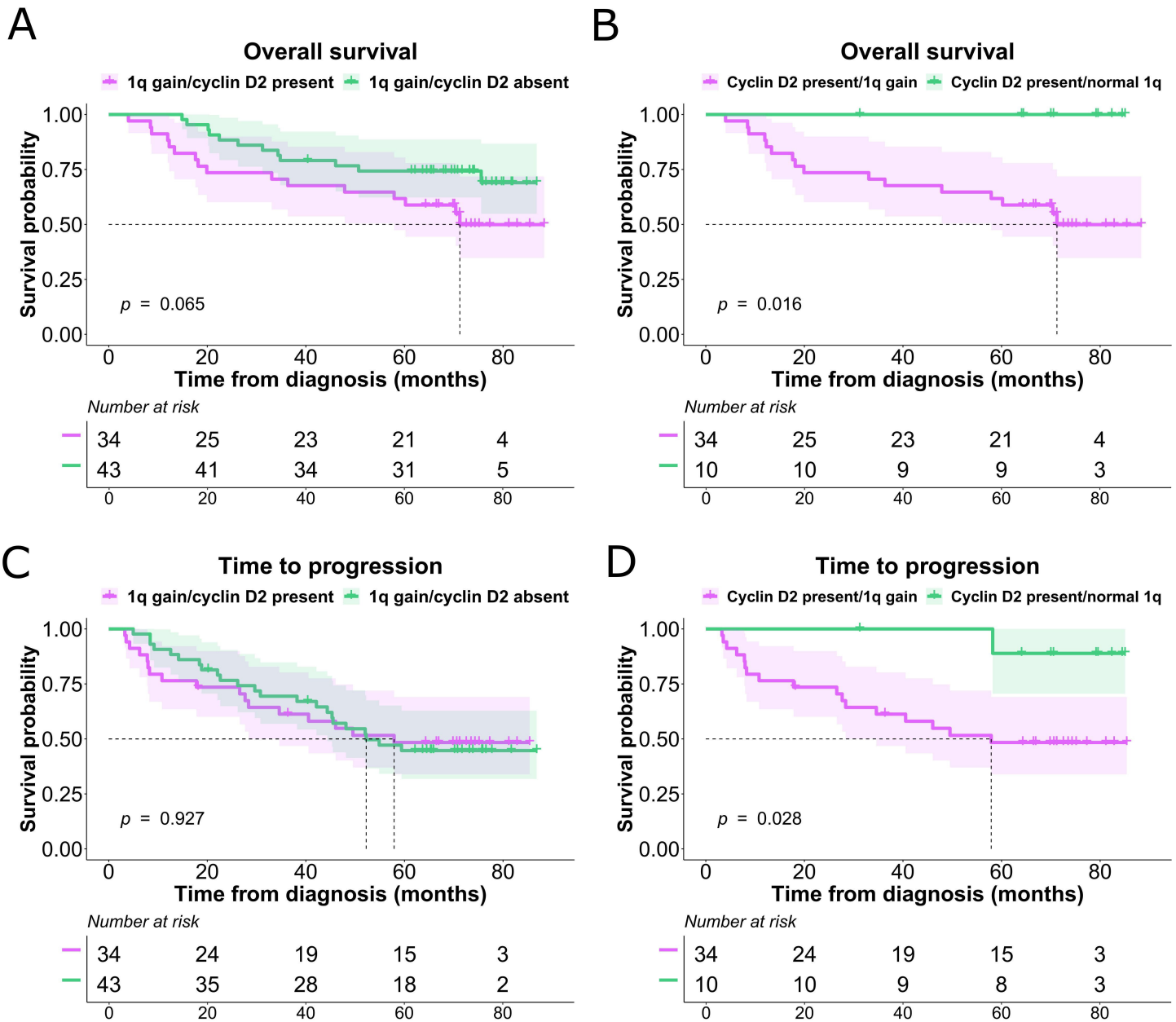
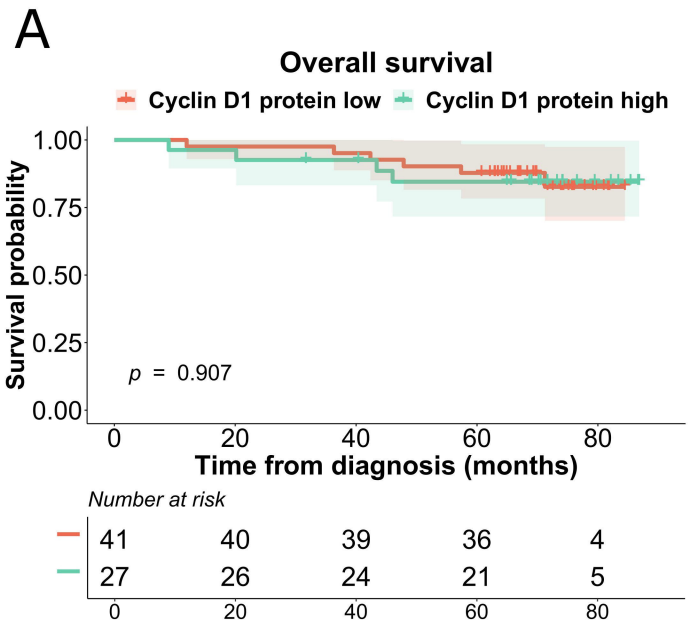
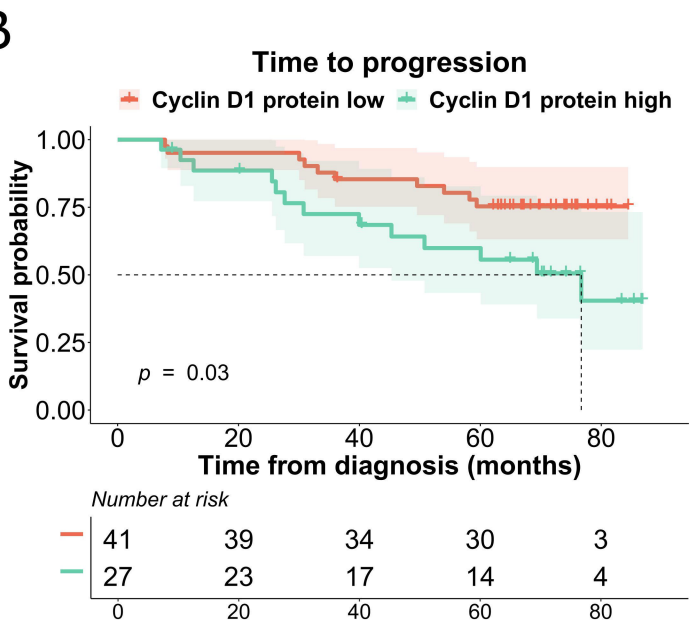

A
Overall survival
1q gain/cyclin D2 present
1q gain/cyclin D2 absent
Survival probability
1.00
0.75
0.50
0.25
0.00
p = 0.065
0
20
40
60
80
Time from diagnosis (months)
Number at risk
34 25 23 21 4
43 41 34 31 5
B
Overall survival
Cyclin D2 present/1q gain
Cyclin D2 present/normal 1q
p = 0.016
Number at risk
34 25 23 21 4
10 10 9 9 3
C
Time to progression
p = 0.927
Number at risk
34 24 19 15 3
43 35 28 18 2
D
Time to progression
p = 0.028
Number at risk
34 24 19 15 3
10 10 9 8 3

Figure 5


A
Overall survival
Cyclin D1 protein low
Cyclin D1 protein high
Survival probability
1.00
0.75
0.50
0.25
0.00
p = 0.907
0
20
40
60
80
Time from diagnosis (months)
Number at risk
41
40
39
36
4
27
26
24
21
5
0
20
40
60
80


B
Time to progression
Cyclin D1 protein low
Cyclin D1 protein high
Survival probability
1.00
0.75
0.50
0.25
0.00
p = 0.03
0
20
40
60
80
Time from diagnosis (months)
Number at risk
41
39
34
30
3
27
23
17
14
4
0
20
40
60
80

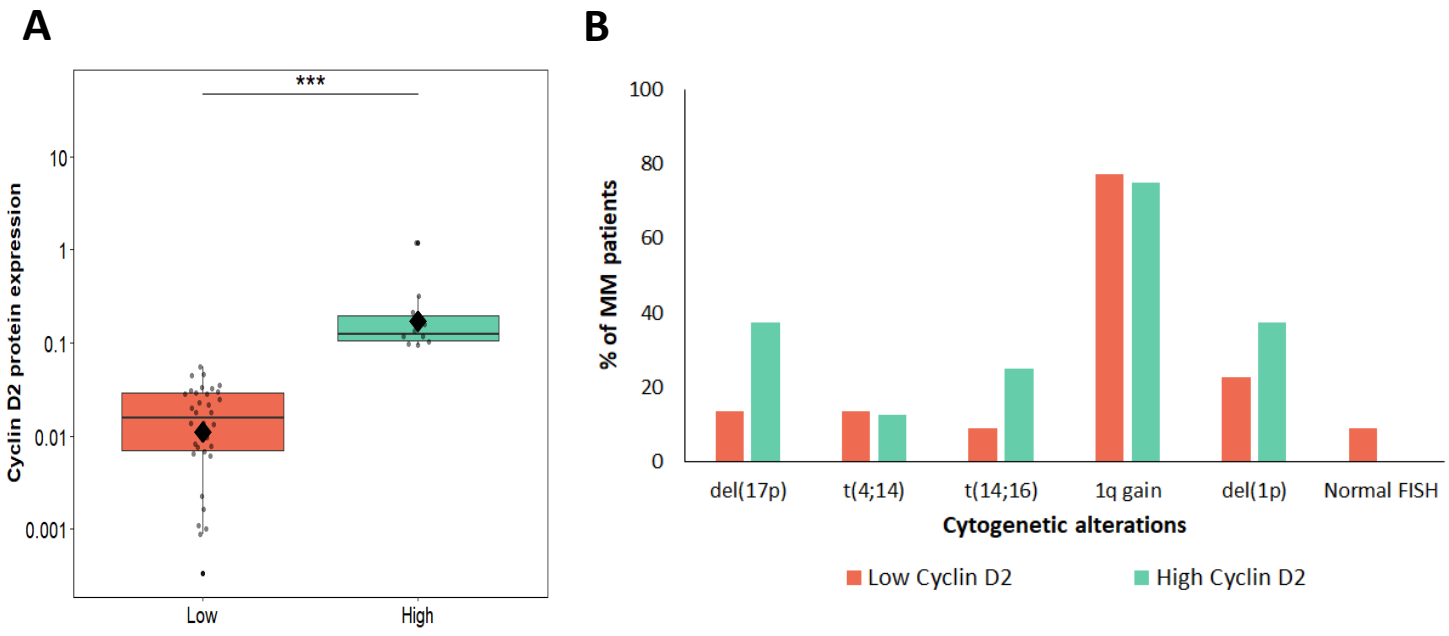
SUPPLEMENTARY INFORMATION.

Supplementary Table 1 Patient characteristics (n = 165).

Characteristics	**Patients (n)=165)**
Age, median (range)	59 (31-65)
Group, n (%)	
Grupo A - Melphalan 200 mg/m^2	80 (49)
Grupo B - Busulfan/Melphalan	85 (51)
Sex, n(%)	
Male	87 (52)
Female	78 (48)
M-protein type, n (%)	
IgG	105 (64)
IgA	37 (22)
Light chain	21 (13)
IgD	0 (0)
Non-secretory	2 (1)
ISS stage, n (%)	
I	52 (31)
II	67 (41)
III	43 (26)
Missing	3 (2)
ISS-R stage, n (%)	
I	74 (45)
II	65 (39)
III	12 (7)
Missing	14 (9)
ECOG performance status, n (%)	
0	67 (41)
1	66 (40)
2	21 (12)
3	8 (5)
Missing	3 (2)
Hemoglobin (g/dL), median (range)	10.7 (6.7-15.5)
Creatinine (mg/dL), median (range)	0.9 (0.4-2)
B2-microglobulin (mg/L), median (range)	3.9 (1.5-17.4)
Elevated lactate dehydrogenase, n (%)	
Yes	19 (12)
No	141 (85)
Missing	5 (3)
Plasmacytoma, n(%)	
Yes	33 (20)
No	132 (80)
Cytogenetics, n (%)	
t(11;14)	23 (17)
11q13 gain	38 (51)
t(4;14)	17 (13)
t(14;16)	4 (3)
del(17p)	18 (12)
del(1p)	21 (14)
1q gain	77 (49)
Follow-up (months): median (range)	69 (9.4-88.5)

ECOG, Eastern Cooperative Oncology Group; ISS, International Staging System; ISS-R, Revised International Staging System.

Supplementary Figure 1. A) Distribution of cyclin D2 protein expression in the groups generated after dichotomizing the samples. Patients with cyclin D2 expression ≤ 0.058 and > 0.058 were classified as "Low" and "High", respectively (***, $p < 0.001$). B) Percentage of patients with chromosomal abnormalities by cyclin D2 protein-expression groups. None of the contrasts between low and high levels of Cyclin D2 within the cytogenetic groups were statistically significant ($p > 0.05$), as demonstrated by the Mann-Whitney U test or Fisher's exact test, as appropriate.

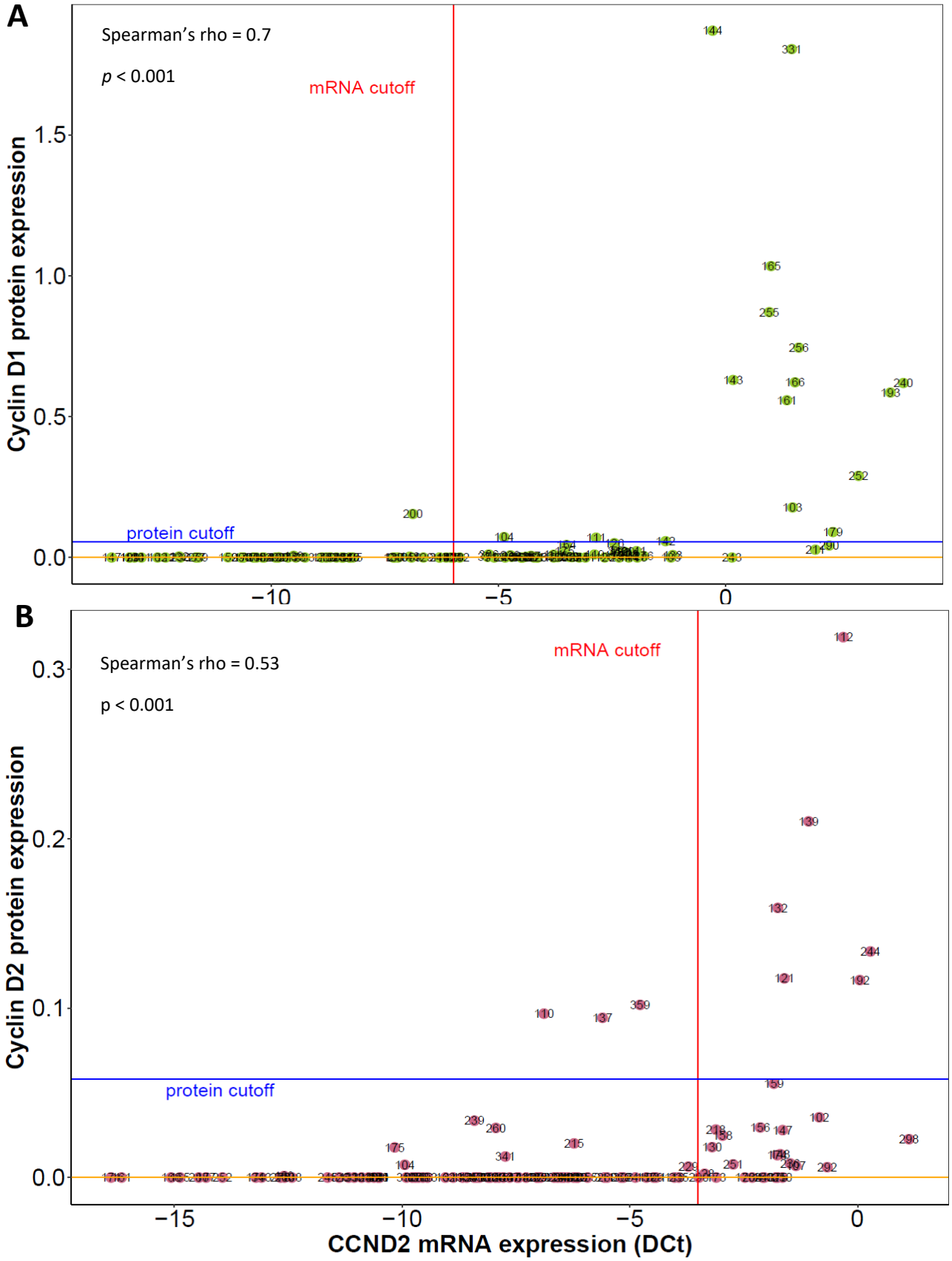


Supplementary Table 2. Distribution of cytogenetic abnormalities by cyclin D-expression groups.

Cytogenetic alteration	Cyclin D1*	Cyclin D2*	Coexpression*	No expression*	Total**
t(11;14)	23 (100)	0 (0)	0 (0)	0 (0)	23
11q13 gain	21 (55)	3 (8)	3 (8)	11 (29)	38
t(4;14)	2 (12)	4 (24)	2 (12)	9 (53)	17
t(14;16)	0 (0)	4 (100)	0 (0)	0 (0)	4
del(17p)	3 (17)	6 (33)	1 (6)	8 (44)	18
del(1p)	4 (19)	8 (38)	3 (14)	6 (29)	21
1q gain	16 (21)	24 (31)	10 (13)	27 (35)	77

*n (%); **n

Supplementary Figure 2. Spearman's correlation between protein and mRNA. A and B) Scatter plot showing protein and mRNA expression for cyclin D1/*CCND1* and cyclin D2/*CCND2*, respectively. The mRNA cutoff (red) divides the samples into normal expression (to the left of this line) and overexpression (to the right). For proteins, the orange line indicates "no protein expression", while the blue line separates samples with low and high protein expression.



Supplementary Figure 3. Survival analysis of *CCND1* and *CCND2* mRNAs. A and B) Kaplan-Meier curves of OS and TTP, respectively, according to *CCND1* mRNA expression. Log-rank (Mantel-Cox) test *p* values are shown.