Clinical Cancer Research

Post-transcriptional Modifications Contribute to the Upregulation of Cyclin D2 in Multiple Myeloma

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Abstract

Purpose: Dysregulation of one of the three D-cyclin genes has been observed in virtually all multiple myeloma tumors. The mechanisms by which *CCND2* is upregulated in a set of multiple myeloma are not completely deciphered. We investigated the role of post-transcriptional regulation through the interaction between miRNAs and their binding sites at 3′UTR in *CCND2* overexpression in multiple myeloma.

Experimental Design: Eleven myeloma cell lines and 45 primary myeloma samples were included in the study. Interactions between miRNAs deregulated in multiple myeloma and mRNA targets were analyzed by 3′UTR-luciferase plasmid assay. The presence of *CCND2* mRNA isoforms different in length was explored using qRT-PCR, Northern blot, mRNA FISH, and 3′ rapid amplification of cDNA ends (RACE)-PCR.

Results: We detected the presence of short *CCND2* mRNA, both in the multiple myeloma cell lines and primary cells. The results obtained by 3′RACE experiments revealed that changes in *CCND2* 3′UTR length are explained by alternative polyadenylation. The luciferase assays using plasmids harboring the truncated *CCND2* mRNA strongly confirmed the loss of miRNA sites in the shorter *CCND2* mRNA isoform. Those multiple myelomas with greater abundance of the shorter 3′UTR isoform were associated with significant higher level of total *CCND2* mRNA expression. Furthermore, functional analysis showed significant *CCND2* mRNA shortening after *CCND1* silencing and an increased relative expression of longer isoform after *CCND1* and *CCND3* overexpression, suggesting that cyclin D1 and D3 could regulate *CCND2* levels through modifications in polyadenylation-cleavage reaction.

Conclusions: Overall, these results highlight the impact of *CCND2* 3′UTR shortening on miRNA-dependent regulation of *CCND2* in multiple myeloma. *Clin Cancer Res; 22(1); 207–17. ©2015 AACR.*

Introduction

Gene expression profiling has revealed that expression of *CCND1*, *CCND2*, or *CCND3* is increased in virtually all multiple myeloma tumors, providing the hypothesis of a potential unifying event in multiple myeloma pathogenesis (1). In addition, D-cyclins have been proposed as molecular therapeutic targets in multiple myeloma (2). Cyclin D2 belongs to the group of D-type cyclin proteins that are cyclically expressed during the cell cycle. In physiologic conditions, cyclins D regulate the transition from G_1–S phase by interaction with cyclin-dependent kinases 4 or 6, which further phosphorylate their substrates (3). One of them is Rb that promotes proliferation by release of E2F transcription factor. *CCND1* is directly activated by t(11;14) and by biallelic dysregulation in patients with multiple myeloma with polysomy 11, and *CCND3* is overexpressed in patients with t(6;14). High levels of *CCND2* are detected in multiple myeloma with t(4;14), t(14;16), and in a set of hyper and nonhyperdiploid multiple myeloma (4). The mechanisms by which *CCND2* is upregulated in these cases are not completely deciphered. Apparently, *CCND2* is not directly induced by *IGH* translocations, although the transcription factor Maf involved in t(14;16) transactivates the *CCND2* promoter (5). Similarly, a pathway of *CCND2* transactivation in multiple myeloma by transcription factor ZKSCAN3 has been described (6).

Nowadays, a large body of evidence indicates that miRNAs are key post-transcriptional regulators of gene expression. In fact, *in silico* algorithms have identified hundreds of predicted miRNAs targeting *CCND2*. Previously published data of our group showed a significant correlation between *CCND2* upregulation and decreased expression of seven miRNAs in multiple myeloma: miR-15a, miR-19a, miR-19b, miR-20a, miR-135b, miR-196b, and miR-320 (7). In this regard, a downregulation of *CCND2* has been demonstrated after transfection of myeloma cells with pre-miRNA-15a and -16, supporting the functional role of both

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

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Translational Relevance

CCND2 is upregulated in most of the multiple myeloma samples without *CCND1* or *CCND3* overexpression, but the molecular background of this observation has not yet been elucidated. This study shows that post-transcriptional modifications play a role in *CCND2* expression regulation in multiple myeloma. Downregulation of specific miRNAs directly targeting *CCND2* could contribute to the overexpression of *CCND2* in a set of multiple myelomas. Moreover, the shortening of *CCND2* 3′UTR by alternative polyadenylation with the consequent loss of miRNA binding sites is also participating in *CCND2* upregulation. In fact, this mechanism seems to be involved in the regulatory network between the three D-cyclins in multiple myeloma. Moreover, in myeloma cells with t(11;14), we observed that DNA methylation may contribute to abolish *CCND2* expression. The understanding of mechanisms involved in the overexpression of cyclin D genes in multiple myeloma could help to design therapeutic strategies focused to disrupt their activation.

miRNAs in regulating cell cycle in multiple myeloma (8). Because miRNA function requires the presence of regulatory sites in 3′ untranslated regions (3′UTRs) of mRNA, another important mechanism for oncogenic activation is the loss of miRNA sites in the mRNAs of oncogenes (9). This process can occur through the use of proximal alternative polyadenylation (APA) signals, which lead to 3′UTR shortening and subsequent loss of miRNA complementary sites (10). Several genes, such as *CCND1*, *CCND2*, *IMP-1*, *DICER*, *CDC6*, and *CYP450*, have been reported to be regulated by this mechanism in cancer cells of different origin (9, 11, 12). Some mantle cell lymphomas (MCL) express a short *CCND1* mRNA isoform, which is correlated with high proliferation of lymphoma cells and decreased overall survival of patients (13). Likewise, a short version of *CCND1* mRNA has been detected in multiple myeloma that harbor t(11;14), but it does not correlate with the cell proliferation rate (14).

This background prompted us to investigate the hypothesis that *CCND2* overexpression in multiple myeloma could be induced by a more general mechanism based on miRNA regulation, regardless of a *CCND2* transactivation by selective transcription factors.

Materials and Methods

Cells and multiple myeloma samples

The human myeloma cell lines, NCI-H929, MM1S, MM1R, and U266 were acquired from the ATCC, whereas RPMI-8226, OPM-2, KMS12BM, KMS12PE, JJN3, and HEK923 from Deutsche Sammlung von Mikroorganismen and Zellkulturen (DSMZ). SKMM2 and XG-1 cell lines were kindly provided by Dr Giovanni Tonon from San Raffaele Scientific Institute, Milan, Italy. Cells were routinely checked for the presence of mycoplasma with a MycoAlert kit (Lonza). Cell line identities have been tested and authenticated by short tandem repeat (STR) analysis with a PowerPlex 16 HS System kit (Promega) and online STR matching analysis (DSMZ institute; ref. 15). The human STR profile database includes data sets of 2,455 cell lines from the ATCC, DSMZ, JCRB, and RIKEN.

Forty-five purified CD138-positive myeloma samples from newly diagnosed patients were included in the study. AutoMACS automated separation system (Miltenyi-Biotec) was used to plasma cell isolation. All patients provided written informed consent in accordance with the Helsinki Declaration, and the research ethics committee of the University Hospital of Salamanca approved the study. The systematic screening for *IGH* translocations using interphase FISH analysis was carried out in all the patients (Supplementary Table S1) as previously described (7).

Transfections

H929, MM1S, and RPMI cell lines were transfected using the nucleofector II system (Lonza) with C-16 program for H929, G-16 for MM1S, and RPMI, and X-001 for JJN3 cells. mirVana miRNA mimic of specified miRNA or mirVana miRNA mimic negative, nontargeting control#1 (Ambion) at 50 nmol/L concentration were used. CCND1-on-targeting smart pool siRNA at 100 nmol/L was used to silence CCND1 and compared with nontargeting smart pool control siRNA (Dharmacon). JJN3 cells were transfected with 1 pmole of each of the plasmids. pCMV6-XL5 control empty plasmid and pCMV6-XL5-Cyclin D3 were purchased from Origene. Plasmid to overexpress cyclin D1, pRc/CMV cyclin D1, was a gift from Bob Weinberg (Whitehead Institute for Biomedical Research, Cambridge, MA) (Addgene plasmid #8962; ref. 16). Transfection efficiency was assessed as previously described (17).

RNA extraction and qRT-PCR analysis

RNA was extracted using an Allprep DNA/RNA kit (Qiagen). The RNA integrity was assessed using Agilent 2100 Bioanalyzer (Agilent Technologies). Mature miRNA expression levels were detected with TaqMan qRT-PCR miRNA assays (Applied Biosystems) and normalized to RNU43 endogenous control using the $2^{-\Delta Ct}$ method. Total RNA (1 μg) was reverse transcribed to cDNA using an iScript cDNA synthesis kit (Bio-Rad). *CCND2* and *CCND1* mRNA expression were also evaluated by Taqman assay qRT-PCR using the respective *GAPDH* Taqman assay as a control. The *CCND2* short and long 3′UTR isoform expression was evaluated by qRT-PCR. The expression of the isoforms was normalized against *GAPDH*. The efficiency of PCR was evaluated by 10-fold change dilutions. qPCRs were performed using iQSYBR Green Supermix and iQ5 real-time PCR system (Bio-Rad). The long 3′UTR versus short 3′UTR *CCND2* mRNA expression was calculated as the percentage of isoform expression. Percentage of LONG 3′UTR = $((2^{-\Delta Ct}\,3'\text{UTR})/(2^{-\Delta Ct}\,\text{CDS}))^{*}100\%$ and percentage of SHORT = 100% − LONG. Sequences of all custom primers used in the study are provided in Supplementary Table S2.

Rapid amplification of cDNA ends (RACE)-coupled sequencing

For the validation of the APA of *CCND2* mRNA, 3′RACE was performed using the FirstChoice RLM-RACE Kit (Ambion). RACE-specific cDNA was synthesized from 1 μg of total RNA and 3′RACE anchor adaptor. Outer 3′RACE adaptor primer, complimentary to the anchored adapter, and the outer-*CCND2* primer were used for first amplification, followed by nested PCR with the inner-3′RACE adaptor primer and the inner *CCND2*-specific primer (Supplementary Table S2). 3′RACE PCR was performed using AccuPrimeTaq DNA Polymerase High Fidelity (Invitrogen). Following gel extraction of correct size bands with the GeneJet Gel Extraction Kit (Thermo Scientific), the PCR product was ligated into plasmid using a pGEMT-Easy kit (Promega). The vector was then sequenced using Applied Biosystems 3130×l Genetic Analyzer.

The sequences were aligned to *CCND2* mRNA sequence (NM_001759.3) using BLASTN algorithm.

Immunoblotting

Whole cell lysates were collected using RIPA buffer (Santa Cruz Biotechnology) containing protease inhibitors and phosphatase inhibitors (Roche). Protein samples (50 μg/lane) were subjected to SDS-PAGE electrophoresis and transferred to 0.45 μm polyvinylidene difluoride membrane using iBlot Dry Blotting System (Invitrogen). The primary antibodies used for immunoblotting were anti-cyclin D2 (Cell Signaling, #3741), anti-cyclin D1 (Abcam ab134175), anti-cyclin D3 (Abcam ab52598), and anti–β-actin-HRP (Sigma-Aldrich) as control for protein loading. The chemiluminescence from horseradish peroxidase-linked anti-rabbit IgG antibody (PierceNet) was detected using Clarity Western ECL Substrate (BioRad). The density of bands was determined using the Image J program.

Luciferase reporter assay

HEK293 cells were transfected with 1 μg of the pGFP and 0.65 pmoles of plasmid with the 3′UTR cloned into pIS1 Cyclin D2 long UTR (Addgene Plasmid 21645) or pIS1 Cyclin D2 short UTR (Addgene Plasmid 21644) and cotransfected with 50 nmol/L miRNA precursor molecule by nucleofection using HEK293 cell line program in the nucleofector II system (LONZA). Cells were collected 24 hours after transfection and Renilla luciferase activity was measured using Dual-Glo Luciferase Assay System (Promega) according to the manufacturer's protocol. GFP was measured using the fluorescence microplate reader Tekan Infinite F500. Renilla luciferase activity was normalized to GFP.

Northern blot

Total RNA (10 μg) was glyoxylated and fractionated in 1.4% agarose gels in 10 mmol/L Na_2HPO_4 buffer (pH 7). After electrophoresis, the gel was blotted onto Hybond-N (Amersham), UV light-cross-linked, and hybridized to ^{32}P-radiolabeled *CCND2* probe that was developed by PCR product gel extraction. Northern blot probe was generated with the same primers used to detect both the short and long *CCND2* isoforms by qPCR (see scheme in Fig. 2A). Loading was monitored by reprobing the filters with *β-actin* cDNA.

mRNA FISH

mRNA FISH was performed using Stellaris FISH probes (Biosearch Technologies) as described previously (18). Forty-eight probes against *CCND2* 3′UTR and 25 against *CCND2* CDS were designed using the Stellaris FISH Probe Designer (Supplementary Table S3). 3′UTR and CDS probes were coupled to Quasar-670 and Quasar-570 dyes, respectively. Cells were visualized with Olympus IX70 FV 500 confocal microscope equipped with 60× oil immersion objective. The microscope settings were adjusted as to observe no signal from the negative control and were kept identical for all the samples measured. The whole sample was examined and the most representative image was taken for analysis, which included only black and white levels adjustments.

Methylation analysis of *CCND2* promoter

Genomic DNA (500 ng) was processed using EpiTect Bisulfite Kits (Qiagen) to obtain bisulfate-treated DNA. To achieve sequencing data concerning DNA methylation, PCR products obtained from bisulfate-treated DNA were ligated into pGEM-Easy plasmid (Promega) and sequenced with the ABI 3100 DNA sequencer using M13 primers. PCR primers to amplify parts of the *CCND2* promoter are provided in Supplementary Table S2.

Statistical analysis

The two-sided Student *t* test was used to analyze differences in experiments. Data are reported as mean values ± SD of at least triplicate determinations. The Mann–Whitney *U* test was used to identify statistically significant differences between the two groups of multiple myeloma. All statistical analyses were conducted using the SPSS 21.0 statistical package (SPSS).

Results

Cyclin D2 and D1 expression in multiple myeloma

We evaluated *CCND2* and *CCND1* mRNA expression level by qRT-PCR in a set of 45 newly diagnosed multiple myeloma. We found that in all the samples with high expression of *CCND1*, the expression levels of *CCND2* were much lower, particularly in those samples with t(11;14), in which *CCND2* transcript was undetectable or barely expressed. On the contrary, most of the samples with the highest levels of *CCND2* displayed lower expression levels of *CCND1*, between 10 and 100 time less (Fig. 1A). The simultaneous expression of both cyclin D genes was an uncommon observation. The predominant overexpression of a single *CCND* gene was also observed in the cell lines (Fig. 1B). Cyclin D2 protein levels assessed by Western blot were noticeable in all the cell lines (Fig. 1C).

miRNAs contribute to Cyclin D2 overexpression in multiple myeloma

We reported in a previous study that the high expression of *CCND2* mRNA in the multiple myeloma samples was correlated with a significant downregulation of some of the following seven miRNAs, miR-15a, miR-19a, miR-19b, miR-20a, miR-135b, miR-196b, and miR-320, depending on the cytogenetic abnormality (7). We updated these miRNA-*CCND2* predictions using six databases (DIANAmT, miRanda, miRWalk, RNAhybrid, PICTAR4, and Targetscan). Because *CCND2* gene shares high homology with *CCND1*, miRNAs targeting *CCND1* were also explored. After selecting the miRNA-target interactions predicted by three out of six databases, 88 and 225 miRNAs were predicted to target *CCND1* and *CCND2*, respectively. Moreover, 164 miRNAs were common for *CCND1* and *CCND2* targets (Supplementary Table S4). Among the seven miRNAs correlated with *CCND2* expression, miR-135b, miR-196b, and miR-320 were predicted to exclusively target CCND2, whereas miR-15a, miR-19a, miR-19b, and miR-20a were predicted to target both *CCND1* and *CCND2*.

Next, the level of miRNAs correlated with *CCND2* expression was evaluated by qRT-PCR in six human myeloma cell lines. Additionally, miR-214 and miR-375, which also targeted *CCND2* and whose expression was downregulated in multiple myeloma (7), were included in qRT-PCR analysis. No expression of miR-15a, 135b, 196b, 214, and 375 was observed in almost all cell lines, although expression of miR-19a, 19b, and 20a was detected in all the cell lines (Supplementary Table S5).

According to the prediction algorithms, miR-15a contains three; miR-19a, miR-19b, and miR-320 have two; and miR-20a, miR-135b, miR-196b, miR-214, and miR-375 contain one putative binding site in the *CCND2* 3′UTR. The locations of

seed sites in the 3′UTR of *CCND2* is presented in Fig. 2A. To determine whether *CCND2* was a direct target of those miRNAs, we carried out luciferase reporter assays in HEK293 cells with a reporter plasmid vector containing the 3′UTR of *CCND2*. Data from our experiments revealed that luciferase activity of *CCND2* 3′UTR luciferase reporter in the cells cotransfected with miR-15a, 19a, 19b, 20a, 135b, 196b, 214, 320, and 375 was significantly lower than that of the cells transfected with nontargeting control miRNA or with miR-155, which does not target *CCND2* (Fig. 2B).

Given the validation of miRNAs-*CCND2* interaction for the miRNAs explored, we analyzed the effect of ectopic expression of the miRNAs downregulated in the multiple myeloma cell lines on cyclin D2 protein. We found a significant decrease of cyclin D2 protein level in miR-15a, 135b, 196b, 214, and 320 transfected MM1S cells. A dramatic downregulation of cyclin D2 level was observed in RPMI cells transfected with miR-214, and to a small extent with miR-15a and miR-196b. This effect on cyclin D2 protein was correlated with the downregulation of mRNA observed in RPMI for miR-15a and miR-214 (Supplementary Fig. S1). Conversely, we only noted an attenuated decrease of cyclin D2 in the H929 cells transfected with miR-214 and miR-135b (Fig. 2C and D). The ectopic expression of miRNAs could result in nonphysiologic concentrations inside the cells, which could induce off-target effects leading to false positive results. In order to evaluate the miRNA overexpression after transfection, we quantified the level of miR-15a and miR-196b in the H929 and MM1S cell lines (Supplementary Fig. S2). The range of miR-15a and miR-196b overexpression was not higher than the expression levels of other miRNAs physiologically present in the multiple myeloma cells (Supplementary Table S5). In spite of the lower miR-15a expression in MM1S than in H929 cell line, the decrease of cyclin D2 protein was higher in MM1S cells. Although the upregulation of miR-196b after ectopic transfection was similar in both cell lines, the decrease of cyclin D2 protein was stronger in MM1S than in H929 cell line. To further rule out the possibility that off-target effects were influencing the results, the concentration of miRNA in each transfection was lowered to 10 nmol/L in MM1S cells. The effect was similar to 50 nmol/L concentrations (Fig. 2C and D for densitometric analysis).

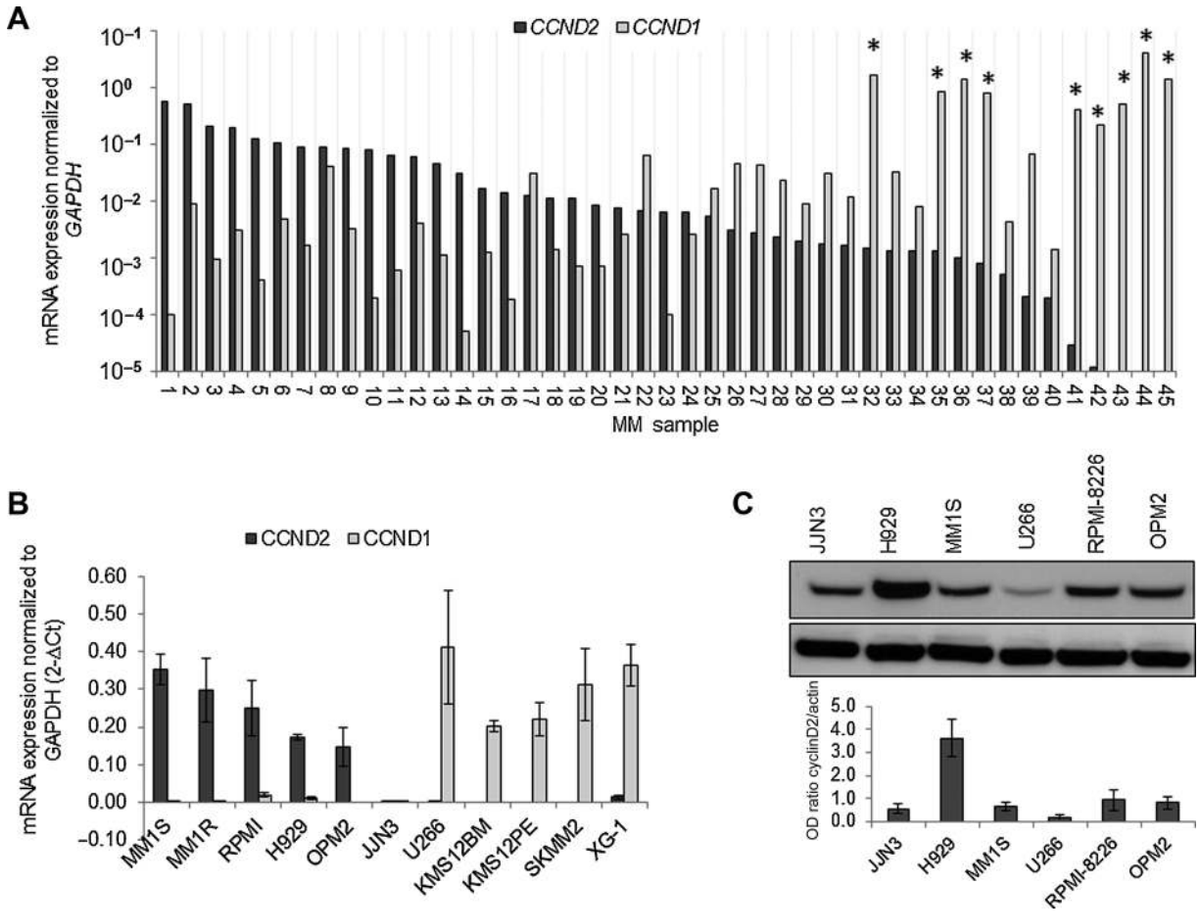

Figure 1.
Cyclin D1 and D2 expression in multiple myeloma (MM). mRNA level of *CCND1* and *CCND2* measured by qRT-PCR with Taqman assays and normalized to *GAPDH* in multiple myeloma samples, presented as $2^{-\Delta Ct}$ on log scale; cells with t(11;14) are indicated by asterisks (A) and in myeloma cell lines (B). Western blots showing cyclin D2 protein in cell lines (C) with the densitometric measurements of cyclin D2 level compared with actin (mean ± SD). PCRs were performed in duplicate for each multiple myeloma sample and in triplicate for cell lines.

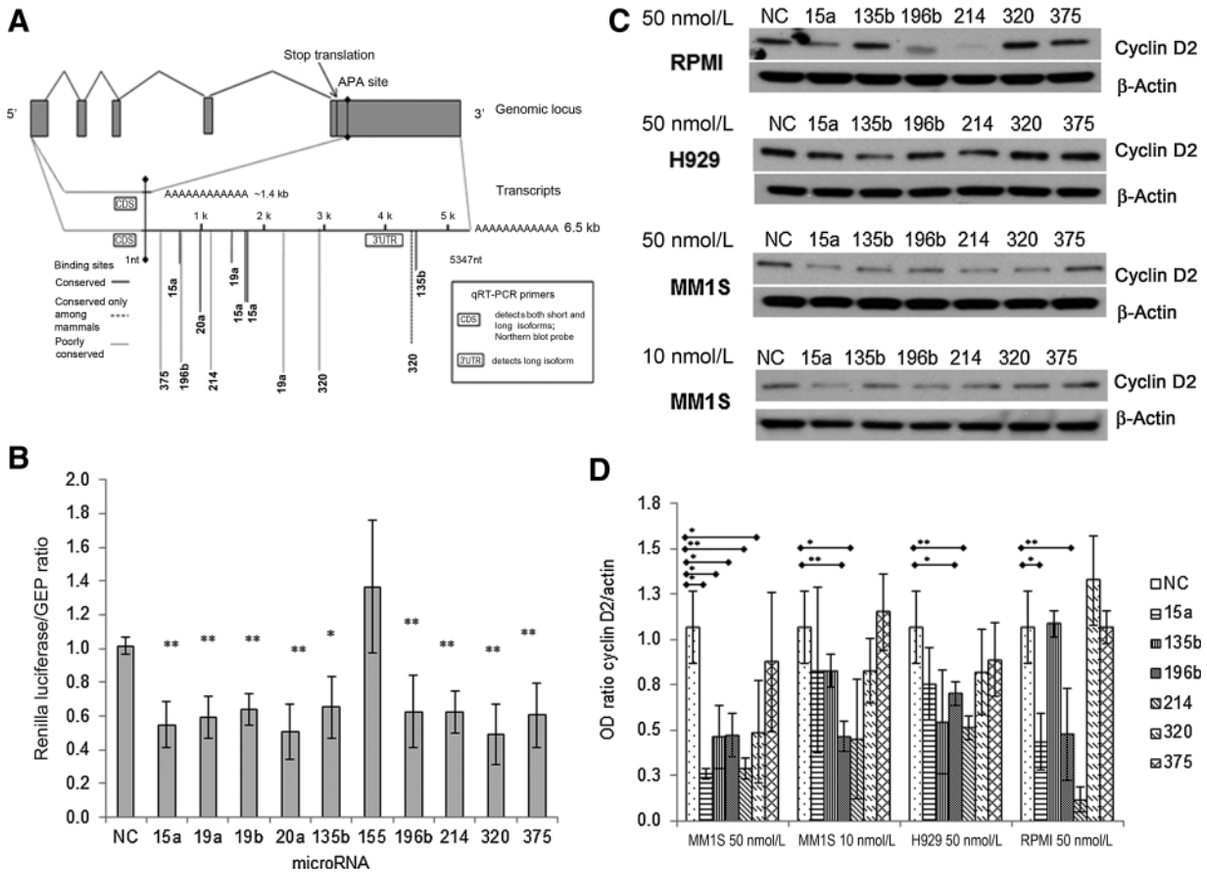

Figure 2.
miRNAs regulate *CCND2* expression in multiple myeloma (MM). Schematic diagram of the cyclin D2 locus that encodes transcript: alternative three polyadenylation sites can result in the expression of a short (1.4 kb) and a long (6.5 kb) *CCND2* mRNA. Location of primers for qRT-PCR locations is also indicated (A). Luciferase activity in HEK923 cells cotransfected with different miRNAs, plasmid encoding GFP and plasmid containing luciferase, and 3′UTR of *CCND2* long isoform; experiments were performed in triplicate and are presented as mean ± SD; luciferase activity was normalized to GFP fluorescence (B). Cyclin D2 protein after transfection with miRNAs at 50 nmol/L in RPMI, H929 MM1S and at 10 nmol/L in MM1S cell lines by Western blot (C) and densitometric analysis (D). Three independent experiments were performed and representative blots are shown. The densitometry is presented as mean ± SD. *, $P < 0.05$; **, $P < 0.01$.

Cyclin D2 exhibits two mRNA isoforms in multiple myeloma

Although the aforementioned results suggest the possible role of miRNA downregulation in cyclin D2 overexpression in multiple myeloma, some of the findings were, to a certain extent, contradictory. Thus, miR-19a, 19b, and 20a that were shown to interact with *CCND2* 3′UTR *in vitro* did not induce downregulation of cyclin D2 in the multiple myeloma cell lines expressing these miRNAs. On the other hand, the ectopic expression of miRNAs 15a, 135b, 196b, 214, and 320 downregulated in multiple myeloma cell lines and whose interaction with *CCND2* at its 3′UTR was demonstrated by luciferase assays, did not provoke a decrease in cyclin D2 mRNA and protein level in all the cell lines explored. These unexpected findings prompted us to explore the possibility that *CCND2* post-transcriptional repression induced by miRNAs could be interfered. It has been reported in several cancer cell lines that *CCND2* was subject to changes in 3′UTR length by means of APA with the consequent modification of 3′UTR repressive elements (9).

In order to investigate the presence of a full-length *CCND2* mRNA and a shorter isoform in myeloma cells, two qRT-PCR assays were designed: one for the detection of both the long and the short isoforms, and the second for targeting the sequence at 3′UTR that was lost in the truncated form (Fig. 2A). The percentage of the two *CCND2* mRNA isoforms was calculated in 31 multiple myeloma primary samples that expressed *CCND2* mRNA. Most of them exhibited both *CCND2* isoforms, although only long form was detected in five samples (Fig. 3A). The short mRNA of *CCND2* was also detected in the six multiple myeloma cell lines expressing *CCND2* (Fig. 3B).

To validate the PCR results, Northern blot was carried out in multiple myeloma cell lines. As shown in Fig. 3C, the presence of two isoforms of *CCND2* mRNA, the full-length mRNA and its shorten isoform, was observed in multiple myeloma cell lines with cyclin D2 protein. The same approach was performed in three multiple myeloma samples with adequate amount of RNA. Both *CCND2* isoforms were detected in one patient (MM#1), although only the full-length isoform was present in the other (Fig. 3C). Nested 3′RACE PCR using *CCND2*-specific forward primer and oligo-T anchor reverse primer generated the expected size product for the short 3′UTR isoform (Fig. 3D). Sequencing of this PCR product verified the presence of proximal poly(A) site (AATAAA) and the polyA tail, which demonstrated that this

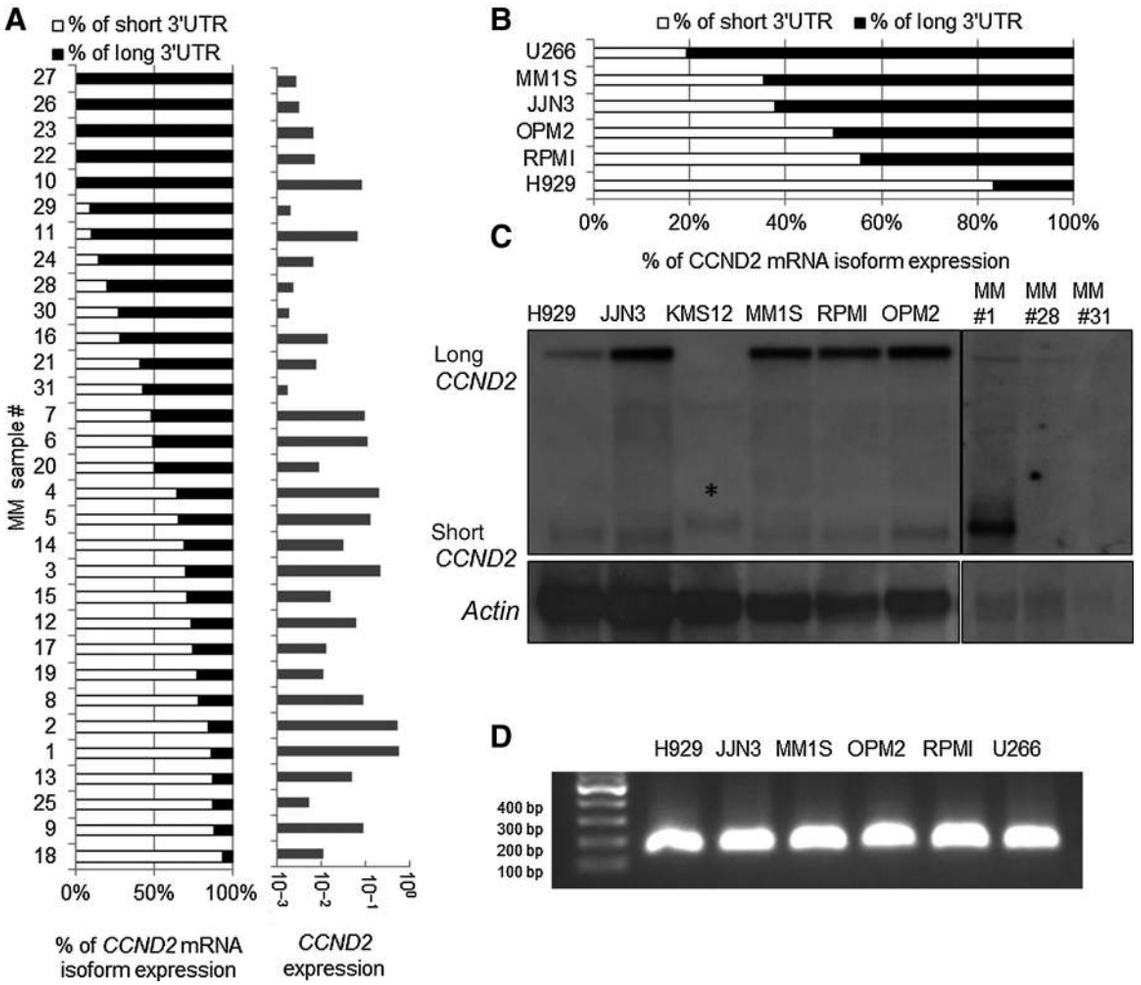

Figure 3.
Cyclin D2 exhibits two mRNA isoforms in multiple myeloma (MM). qRT-PCR evaluation of full-length 3′UTR and short 3′UTR *CCND2* expression in 31 multiple myeloma is shown in the left panel. Right, mRNA level of *CCND2* measured by qRT-PCR with Taqman assays; same data as in Fig. 1A are shown for the sake of better understanding. (A) and 6 multiple myeloma cell lines (B). Northern blot for full-length and short 3′UTR of *CCND2* in multiple myeloma cell lines and in three samples from multiple myeloma patients (C); the region of the probe used is marked in Fig. 2A. Unspecific band present in the KMS12 cell line probably corresponds to *CCND1*. 3′RACE-nested PCR product in the multiple myeloma cell lines obtained using *CCND2*-specific forward and oligo(T) anchor-specific reverse primers (D).

isoform was generated by polyadenylation. Moreover, the sequence was uniquely aligned to *CCND2* mRNA 3′UTR (NM_001759.3; Supplementary Fig. S3).

Both *CCND2* mRNA isoforms are present in the same cell

The quantification of full-length and short mRNA of *CCND2* by qRT-PCR approach showed that most of the patients and all the cell lines displayed both the transcripts. In order to find out if the presence of both short and long *CCND2* 3′UTR occurs in the same cell, or rather there are subpopulations of cells with short and long 3′UTR, two-color mRNA FISH was performed. The coding sequence of *CCND2* and the 3′UTR fragment lost in the short isoform were differentially stained (Supplementary Fig. S4). Consistent with the qRT-PCR results, H929 cells showed brighter fluorescence of *CCND2* CDS than JJN3 cells, indicating the predominance of short 3′UTR in H929 cells. Apparently, all the cells showed uniform and homogenous staining and different subpopulations with more abundance of one of the isoforms were not identified. Although the number of cells in patients was much lower, the two multiple myeloma samples showed similar results. As it was expected, KMS12BM cells did not express detectable levels of *CCND2* mRNA.

Multiple myeloma samples with predominant shorter *CCND2* mRNA displayed higher *CCND2* expression levels

To determine if the short 3′UTR isoform of *CCND2* could be responsible for higher expression of *CCND2* coding region in multiple myeloma cells, we analyzed the *CCND2* expression by qRT-PCR in all multiple myeloma samples. Then, the 31 multiple myeloma samples expressing detectable level of *CCND2* were grouped regarding predominant expression of one out of the two isoforms different in length. Thus, those multiple myeloma

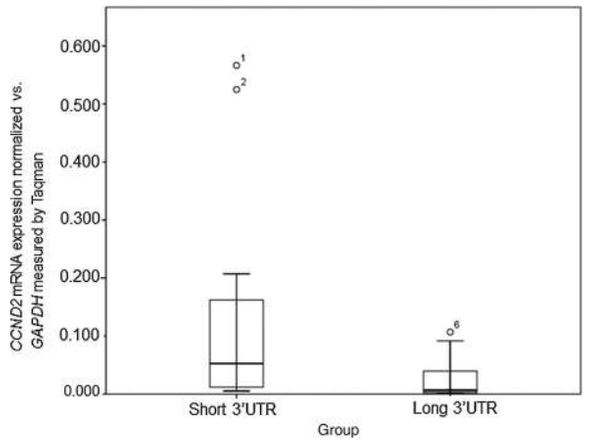

Figure 4.
Multiple myeloma samples with shorter *CCND2* mRNA displayed higher *CCND2* expression levels. Total *CCND2* mRNA levels in multiple myeloma samples according to the predominance of short 3′UTR or full-length 3′UTR. CCND2 expression was assessed by qRT-PCR using the Taqman assay, normalized to *GAPDH*, and calculated by the $2^{-\Delta Ct}$ method. Mann–Whitney *U* test, $P = 0.002$.

samples whose percentage of long isoform was detected above 50% were considered as samples with predominant long 3′UTR, and those whose long isoform percentage was lower than 50% were grouped as samples with predominant short 3′UTR. The statistical analysis revealed that multiple myeloma samples with predominant short 3′UTR expressed significantly higher levels of *CCND2* than those with long 3′UTR (Fig. 4).

Because one of the functional consequences of the shortening of *CCND2* 3′UTR is the loss of miRNA sites (9), we investigated if this possibility could be the reason of the escape of *CCND2* from miRNAs validated in the present study. To confirm that the form of cyclin D2 with truncated 3′UTR was not to be regulated by miRNAs that repress the full-length form, luciferase reporter assays in HEK923 cells with a plasmid containing the short 3′UTR isoforms of *CCND2* was performed. The results showed that none of the miRNAs downregulated the luciferase activity at the same level as for the luciferase reporter assay with long isoform, suggesting that the higher level of *CCND2* in multiple myeloma with shorter 3′UTR was due to inefficient downregulation by miRNAs (Supplementary Fig. S5)

Cyclin D2 is regulated by cyclin D1 and cyclin D3

Among the 10 multiple myeloma with intermediate *CCND1* levels and detectable *CCND2* expression, the prevalent form of the latter was the long isoform in 80% of the cases (8/10), although only 20% (2/10) of multiple myeloma with high *CCND1* expression exhibited short isoform of *CCND2* mRNA. Additionally, by using U266 cells that coexpress *CCND1* and *CCND2*, we confirmed previous observations indicating that the *CCND1* silencing led to increased cyclin D2 protein levels (Fig. 5A and Supplementary Fig. S6A for densitometric quantification; ref. 19). Likewise, *CCND2* mRNA levels were found to be increased 72 hours after *CCND1* silencing (Supplementary Fig. S6B). One possibility raised by these findings was that polyadenylation/cleavage reaction of *CCND2* mRNA could be modulated by cyclin D1. Accordingly, we found a shortening of *CCND2* mRNA after *CCND1* silencing (Fig. 5B).

In order to confirm this finding and to find out whether *CCND3* could also be involved in the regulation of *CCND2* expression, the JJN3 cell line was nucleofected with plasmids containing *CCND1* and *CCND2*. We have observed that both cyclin D1 and cyclin D3 overexpression resulted in the decrease of cyclin D2 at the protein and mRNA levels (Fig. 5C and Supplementary Fig. S6C–S6E). Moreover, this effect was accompanied by an increase in the proportion of long *CCND2* mRNA isoform (Fig. 5D). Overall, these results suggest a mechanism of partial cross-regulation of D-cyclins in multiple myeloma.

The promoter of *CCND2* is methylated in cells with t(11;14)

Although *CCND1* might have a role in the downregulation of *CCND2* through APA-mediated shortening of *CCND2* 3′UTR, this mechanism is unlikely to be active in those multiple myeloma with t(11;14), which lack *CCND2* expression. Therefore, we hypothesized that epigenetic silencing, such as DNA methylation, could be involved in the abrogation of *CCND2* expression in multiple myeloma cells with high level of *CCND1* because of t(11;14). To test this possibility we analyzed two regions of *CCND2* promoter that contain CpG islands located −642 nt (25 CpG) and −19 nt (20 CpG) before transcription start site (TSS). A clear methylation pattern of the CpG island more proximal to the TSS of CCND2 was identified in cell lines carrying t(11;14) (Fig. 6A). Thus, KMS12BM and KMS12PE, which are derived from the same patient, displayed a similar pattern of methylation with 54% and 44% of the CpG island methylated, respectively (Fig. 6B and C). For the remaining two cell lines, SKMM2 and XG-1, methylation of the distal part of CpG island was observed (26% and 19%). In contrast, cells with strong *CCND2* expression, like H929 or MM1S, displayed 3.6% and 2.1% of methylated CpG island, respectively, and U266 coexpressing *CCND1* and *CCND2*, only had 3.6% of methylation.

Discussion

CCND2 expression has previously been shown to be upregulated in most of multiple myeloma samples without *CCND1* or *CCND3* overexpression (1). The mechanisms leading to *CCND2* dysregulation are not well characterized in some multiple myeloma cases. In contrast to other D-cyclins, no aberrations involving the *CCND2* locus have been detected that could explain its overexpression in multiple myeloma. Up to now, only transcriptional mechanisms have been described to enhance *CCND2* expression in multiple myeloma. Thus, MAF and MAFB transcription factors directly target *CCND2* in about 7% of tumors (5). In addition, the transcription factor ZKSCAN3 has also been described to participate in *CCND2* regulation in multiple myeloma (6). The present study shows that other mechanisms like modifications in the normal post-transcriptional regulation of *CCND2* could participate in the upregulation of *CCND2* in a group of multiple myeloma.

Our results confirmed that ectopic transfection of multiple myeloma cells with several miRNAs (miR-15a, 135b, 196b, 214, and 320) downregulated in multiple myeloma, decreased the level of cyclin D2 by directly targeting *CCND2* 3′UTR. However, this effect was not observed in all the cell lines. This fact together with the inability of miR-19a, 19b, and 20a to downregulate *CCND2* in those multiple myeloma cell lines expressing these miRNAs, suggested the possible disruption of miRNA target sites. In fact, we detected the presence of short *CCND2* mRNA,

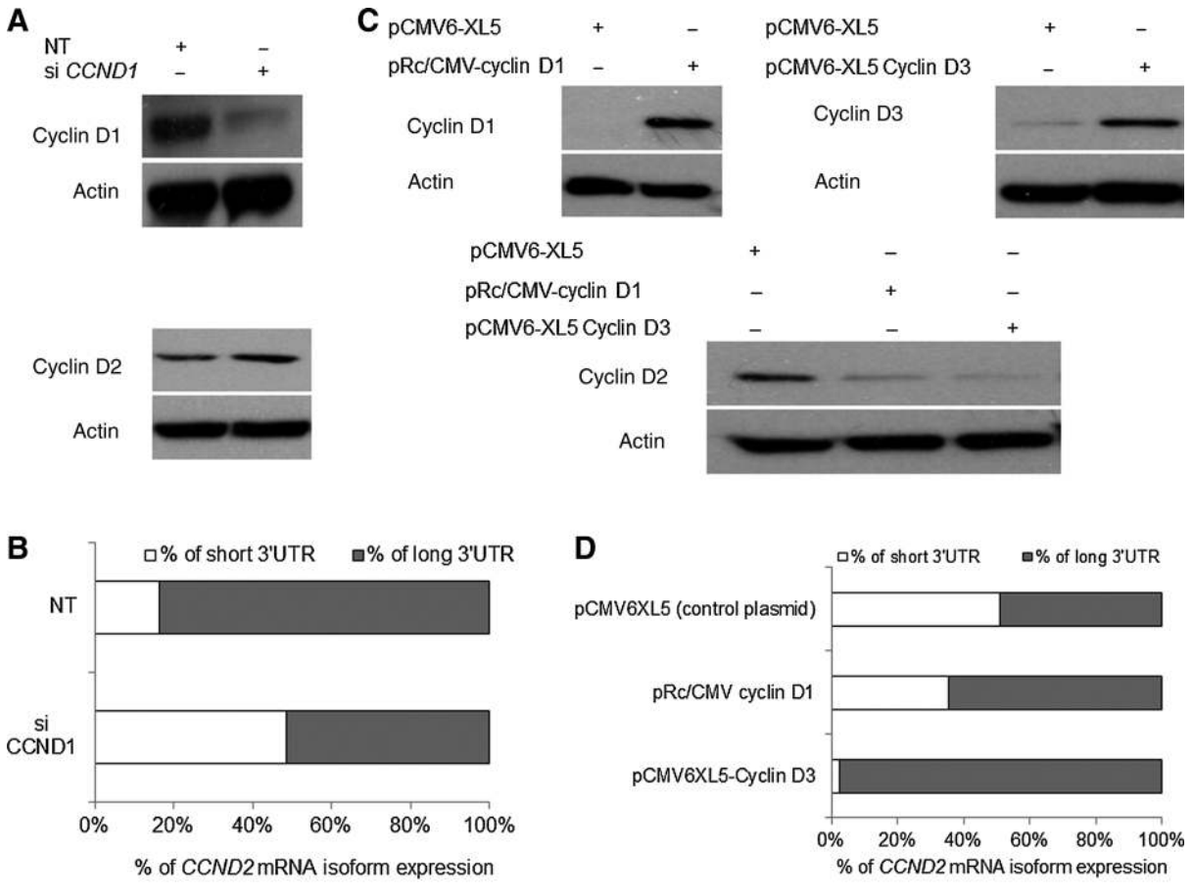

Figure 5.
Cyclin D2 protein level is controlled by cyclin D1 and D3. Cyclin D2 protein levels 72 hours after transfection with *CCND1* siRNA in the U266 cell line by Western blot (A). Proportion between short and long *CCND2* 3′UTR after *CCND1* silencing (B). Western blot experiments were made in duplicate and PCR experiments were repeated at least three times. Cyclins D protein level 48 hours after transfection with indicated plasmid by Western blot in the JJN3 cell line (C). Proportion between the short and long *CCND2* 3′UTR after *CCND1* or *CCND3* overexpression in the JJN3 cell line (D).

both in multiple myeloma cell lines and primary cells, using four different methodological approaches: qRT-PCR, Northern blot, mRNA FISH, and 3′RACE PCR with product sequencing.

The short *CCND2* isoform was observed by qRT-PCR in the majority of patients and in all multiple myeloma cell lines expressing *CCND2*. This finding was confirmed by Northern blot results. Interestingly, the H929 cells in which the longer *CCND2* 3′UTR was less predominant compared with other multiple myeloma cell lines, barely displayed modification of cyclin D2 protein expression after miRNAs transfection. The level of cyclin D2 protein was higher in the H929 cell line than in the other cell lines, despite the *CCND2* mRNA expression was lower than in MM1S, MM1R, or RPMI-8226. It is possible that other regulatory mechanisms, like translational and post-translational modifications participate in this surprising finding. The abundance of each *CCND2* mRNA isoform was also assessed by two-color mRNA FISH designed to discriminate the two mRNA different in length. This approach also enabled us to notice that no subpopulation of cells distinguishable by the load of one isoform with respect to another was present. Apparently, clonal heterogeneity identified in other genomic context of multiple myeloma was not observed in the abundance of short and long *CCND2* isoforms (20, 21). It has been shown for several genes, such as *CCND1*, *CCND2*, *IMP-1*, *DICER*, *CDC6*, or *CYP450*, the shortening of their 3′UTR region (9, 11, 12, 22). Genomic deletions of the 3′UTR or point mutations in the 3′UTR region are genetic events that can cause the expression of truncated transcripts through premature cleavage and polyadenylation signals (9). Nevertheless, a large fraction of human genes use alternative cleavage and polyadenylation (APA) to generate mRNA transcripts that differ in the length of their 3′UTR (23). The results obtained by RACE experiments in the multiple myeloma cell lines support the idea that changes in *CCND2* 3′UTR length are explained by APA. Moreover, the recent genome-wide analyses of hundreds of multiple myeloma samples using massively parallel sequencing failed to detect genomic deletions or mutations in *CCND2* (24–26). The functional consequences of 3′UTR shortening is the mRNA stabilization due to the loss of miRNA sites and regulatory elements located in the 3′UTR (9, 10, 23). Accordingly, the luciferase assays using plasmids harboring the truncated *CCND2* mRNA strongly confirmed the loss of miRNA sites in the shorter *CCND2* mRNA isoform. The short 3′UTRs lacking miRNA-binding sites have been associated

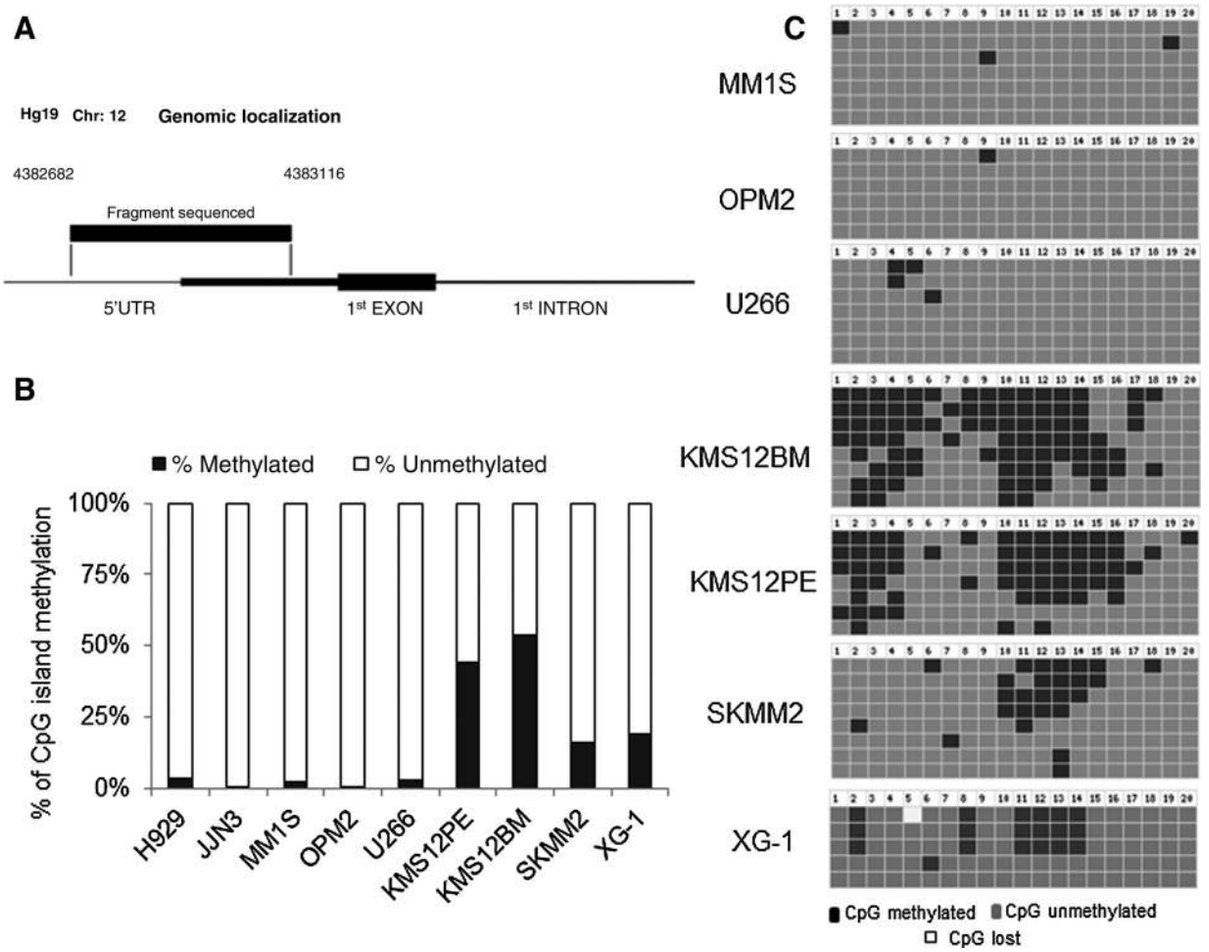

Figure 6.
Methylation of *CCND2* promoter. Genomic location for analyzed CpG island (A). Percentage of methylated island in analyzed clones for each cell line (B). Representation of methylated and unmethylated CpG clones within the CpG island in the MM1S, OPM2, U266, KMS12BM, KMS12PE, SKMM2, and XG-1 cell lines (C).

with increased expression of different genes at both the mRNA and the protein level (9, 11, 12, 22). In fact, the shorter *CCND2* mRNA has been found to increase protein expression in cell lines from various tissues (9). However, there are evidences that support the thesis that increased transcription of genes is positively correlated to the relative usage of proximal poly(A) site (27). Here, we observed a significant higher level of overall *CCND2* mRNA expression in those multiple myeloma with greater abundance of the shorter 3′UTR isoform, although it is still an open question whether *CCND2* mRNA shortening is the cause or the consequence.

The short form of *CCND1* 3′UTR has been previously reported in multiple myeloma with t(11;14) (1, 14). The existence of this isoform was correlated with higher *CCND1* mRNA level but not with differences in survival (14). Short *CCND1* mRNAs are also observed in MCL whose genetic hallmark is t(11;14). The mechanisms underlying the truncation of *CCND1* 3′UTR in MCL are both genomic deletions of the 3′UTR region and premature polyadenylation signal creation by point mutations (28). In contrast to multiple myeloma, the presence of short *CCND1* 3′UTR was correlated with poor survival of MCL patients (13).

D-type cyclins are highly homologous proteins and there is a growing body of evidence that the functions of the D cyclins are mostly exchangeable. During mouse development, the three D-type cyclins are expressed following an often mutually exclusive pattern and their function may be tissue specific (29, 30). In keeping with this observation, simultaneous overexpression of *CCND1* and *CCND2* in multiple myeloma is an infrequent finding (1, 31). Because the 3′UTR length can be considered as a mechanism of gene expression regulation, we investigated if it can be involved in a potential cross-regulation between cyclin D1 and D2. It was previously observed in U266 cells, which express both cyclins D, that when cyclin D1 was silenced the level of cyclin D2 increased (19) and our results ascertain these data. Similar results have also been reported in MCL (32). Although the explanation of this effect is probably unknown, our results showing a *CCND2* mRNA shortening after *CCND1* silencing suggest that cyclin D1 could downregulate *CCND2* level by modification of polyadenylation/cleavage reaction. Moreover, the increased proportion of long *CCND2* isoform after cyclin D1 or cyclin D3 overexpression along with simultaneous decrease of cyclin D2 protein level in the JJN3 cell line further supports the

significance of this mechanism in the regulation of cyclin D2 expression. The signals that provoke mutual exclusion of cyclins D during development and in adult tissues remain elusive. Studies with mouse models point out the existence of a negative feedback loop, in which the dominant D-type cyclin represses the expression of remaining D-cyclins. These studies revealed that the molecular mechanism leading to cyclins D repression functions differently in distinct tissues, in some of them by modification of mRNA level and in others by possible post-transcriptional mechanisms (30). Here, we provide for the first time data about a possible mechanism that can induce the downregulation of *CCND2* in *CCND1* and *CCND3* overexpressing multiple myeloma cells. Different signals provoking 3′UTR shortening have been described in several cell types: for example, activation of T and B lymphocytes results in increased usage of proximal polyadenylation sites (10, 33); 17-β estradiol(E2) induces APA to activate proto-oncogenes in breast cancer cells (12); and E2F transcription factors mediate enhanced APA usage in human cellular models (34).

Because *CCND2* expression is undetectable in myeloma cells with t(11;14), we extended our investigation to explore if DNA methylation might play a role in abolishing *CCND2* expression. We observed that the CpG island more proximal to *CCND2* TSS was highly methylated just in multiple myeloma cell lines with t(11;14). Several studies have indicated that methylation of *CCND2* is responsible for absence of its mRNA and protein in breast, prostate, lung, and hepatocellular cancers (35–38).

In summary, this study demonstrates that post-transcriptional modifications play a role in *CCND2* expression regulation in multiple myeloma. Downregulation of specific miRNAs directly targeting *CCND2* contributes to overexpression of *CCND2* in a set of multiple myeloma. Moreover, the shortening of *CCND2* 3′UTR by APA with the consequent loss of miRNA binding sites is also participating in *CCND2* upregulation. In fact, this mechanism seems to play a decisive role in the regulatory network between *CCND1* and *CCND2* in multiple myeloma. Further studies are needed to unravel the molecular basis of some of these findings, and to define among the mechanisms reported so far, which are the most critical that determine the expression of *CCND2* in myeloma.

Disclosure of Potential Conflicts of Interest

J.F. San-Miguel is a consultant/advisory board member for Bristol-Myers Squibb, Celgene, Janssen, Millennium, MSD, Novartis, and Onyx. No potential conflicts of interest were disclosed by the other authors.

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References

1. Bergsagel PL, Kuehl WM, Zhan F, Sawyer J, Barlogie B, Shaughnessy J Jr. Cyclin D dysregulation: an early and unifying pathogenic event in multiple myeloma. Blood 2005;106:296–303.
2. Tiedemann RE, Mao X, Shi CX, Zhu YX, Palmer SE, Sebag M, et al. Identification of kinetin riboside as a repressor of CCND1 and CCND2 with preclinical antimyeloma activity. J Clin Invest 2008;118:1750–64.
3. Chiles TC. Regulation and function of cyclin D2 in B lymphocyte subsets. J Immunol 2004;173:2901–7.
4. Chesi M, Bergsagel PL. Many multiple myelomas: making more of the molecular mayhem. Hematology Am Soc Hematol Educ Program 2011; 2011:344–53.
5. Hurt EM, Wiestner A, Rosenwald A, Shaffer AL, Campo E, Grogan T, et al. Overexpression of c-maf is a frequent oncogenic event in multiple myeloma that promotes proliferation and pathological interactions with bone marrow stroma. Cancer Cell 2004;5:191–9.
6. Yang L, Wang H, Kornblau SM, Graber DA, Zhang N, Matthews JA, et al. Evidence of a role for the novel zinc-finger transcription factor ZKSCAN3 in modulating Cyclin D2 expression in multiple myeloma. Oncogene 2011;30:1329–40.
7. Gutierrez NC, Sarasquete ME, Misiewicz-Krzeminska I, Delgado M, De Las Rivas J, Ticona FV, et al. Deregulation of microRNA expression in the different genetic subtypes of multiple myeloma and correlation with gene expression profiling. Leukemia 2010;24: 629–37.
8. Roccaro AM, Sacco A, Thompson B, Leleu X, Azab AK, Azab F, et al. MicroRNAs 15a and 16 regulate tumor proliferation in multiple myeloma. Blood 2009;113:6669–80.
9. Mayr C, Bartel DP. Widespread shortening of 3′UTRs by alternative cleavage and polyadenylation activates oncogenes in cancer cells. Cell 2009;138:673–84.

10. Sandberg R, Neilson JR, Sarma A, Sharp PA, Burge CB. Proliferating cells express mRNAs with shortened 3′ untranslated regions and fewer microRNA target sites. Science 2008;320:1643–7.
11. Hamaya Y, Kuriyama S, Takai T, Yoshida K, Yamada T, Sugimoto M, et al. A distinct expression pattern of the long 3′-untranslated region dicer mRNA and its implications for posttranscriptional regulation in colorectal cancer. Clin Transl Gastroenterol 2012;3:e17.
12. Akman BH, Can T, Erson-Bensan AE. Estrogen-induced upregulation and 3′-UTR shortening of CDC6. Nucleic Acids Res 2012;40: 10679–88.
13. Rosenwald A, Wright G, Wiestner A, Chan WC, Connors JM, Campo E, et al. The proliferation gene expression signature is a quantitative integrator of oncogenic events that predicts survival in mantle cell lymphoma. Cancer Cell 2003;3:185–97.
14. Slotta-Huspenina J, Koch I, Richter M, Bink K, Kremer M, Specht K, et al. Cyclin D1 positive multiple myeloma: predominance of the short, 3′UTR-deficient transcript is associated with high cyclin D1 mRNA levels in cases with t(11;14) translocation, but does not correlate with proliferation rate or genomic deletions. Leuk Res 2008; 32:79–88.
15. STR matching analysis [accesion date 26/02/2015]. Available from: http://www.dsmz.de/fp/register.php
16. Hinds PW, Mittnacht S, Dulic V, Arnold A, Reed SI, Weinberg RA. Regulation of retinoblastoma protein functions by ectopic expression of human cyclins. Cell 1992;70:993–1006.
17. Misiewicz-Krzeminska I, Sarasquete ME, Quwaider D, Krzeminski P, Ticona FV, Paino T, et al. Restoration of microRNA-214 expression reduces growth of myeloma cells through positive regulation of P53 and inhibition of DNA replication. Haematologica 2013;98:640–8.
18. Raj A, van den Bogaard P, Rifkin SA, van Oudenaarden A, Tyagi S. Imaging individual mRNA molecules using multiple singly labeled probes. Nat Methods 2008;5:877–9.
19. Tchakarska G, Le Lan-Leguen A, Roth L, Sola B. The targeting of the sole cyclin D1 is not adequate for mantle cell lymphoma and myeloma therapies. Haematologica 2009;94:1781–2.
20. Melchor L, Brioli A, Wardell CP, Murison A, Potter NE, Kaiser MF, et al. Single-cell genetic analysis reveals the composition of initiating clones and phylogenetic patterns of branching and parallel evolution in myeloma. Leukemia 2014: 10.1038/leu.2014.13.
21. Keats JJ, Chesi M, Egan JB, Garbitt VM, Palmer SE, Braggio E, et al. Clonal competition with alternating dominance in multiple myeloma. Blood 2012;120:1067–76.
22. Li D, Gaedigk R, Hart SN, Leeder JS, Zhong XB. The role of CYP3A4 mRNA transcript with shortened 3′-untranslated region in hepatocyte differentiation, liver development, and response to drug induction. Mol Pharmacol 2012;81:86–96.
23. Lianoglou S, Garg V, Yang JL, Leslie CS, Mayr C. Ubiquitously transcribed genes use alternative polyadenylation to achieve tissue-specific expression. Genes Dev 2013;27:2380–96.
24. Lohr JG, Stojanov P, Carter SL, Cruz-Gordillo P, Lawrence MS, Auclair D, et al. Widespread genetic heterogeneity in multiple myeloma: implications for targeted therapy. Cancer Cell 2014;25:91–101.
25. Egan JB, Shi CX, Tembe W, Christoforides A, Kurdoglu A, Sinari S, et al. Whole-genome sequencing of multiple myeloma from diagnosis to plasma cell leukemia reveals genomic initiating events, evolution, and clonal tides. Blood 2012;120:1060–6.
26. Chapman MA, Lawrence MS, Keats JJ, Cibulskis K, Sougnez C, Schinzel AC, et al. Initial genome sequencing and analysis of multiple myeloma. Nature 2011;471:467–72.
27. Elkon R, Ugalde AP, Agami R. Alternative cleavage and polyadenylation: extent, regulation and function. Nat Rev Genet 2013;14:496–506.
28. Wiestner A, Tehrani M, Chiorazzi M, Wright G, Gibellini F, Nakayama K, et al. Point mutations and genomic deletions in CCND1 create stable truncated cyclin D1 mRNAs that are associated with increased proliferation rate and shorter survival. Blood 2007;109:4599–606.
29. Carthon BC, Neumann CA, Das M, Pawlyk B, Li T, Geng Y, et al. Genetic replacement of cyclin D1 function in mouse development by cyclin D2. Mol Cell Biol 2005;25:1081–8.
30. Ciemerych MA, Kenney AM, Sicinska E, Kalaszczynska I, Bronson RT, Rowitch DH, et al. Development of mice expressing a single D-type cyclin. Genes Dev 2002;16:3277–89.
31. Suzuki R, Kuroda H, Komatsu H, Hosokawa Y, Kagami Y, Ogura M, et al. Selective usage of D-type cyclins in lymphoid malignancies. Leukemia 1999;13:1335–42.
32. Klier M, Anastasov N, Hermann A, Meindl T, Angermeier D, Raffeld M, et al. Specific lentiviral shRNA-mediated knockdown of cyclin D1 in mantle cell lymphoma has minimal effects on cell survival and reveals a regulatory circuit with cyclin D2. Leukemia 2008;22:2097–105.
33. Takagaki Y, Seipelt RL, Peterson ML, Manley JL. The polyadenylation factor CstF-64 regulates alternative processing of IgM heavy chain pre-mRNA during B cell differentiation. Cell 1996;87:941–52.
34. Elkon R, Drost J, van Haaften G, Jenal M, Schrier M, Vrielink JA, et al. E2F mediates enhanced alternative polyadenylation in proliferation. Genome Biol 2012;13:R59.
35. Henrique R, Costa VL, Cerveira N, Carvalho AL, Hoque MO, Ribeiro FR, et al. Hypermethylation of Cyclin D2 is associated with loss of mRNA expression and tumor development in prostate cancer. J Mol Med 2006; 84:911–8.
36. Evron E, Dooley WC, Umbricht CB, Rosenthal D, Sacchi N, Gabrielson E, et al. Detection of breast cancer cells in ductal lavage fluid by methylation-specific PCR. Lancet 2001;357:1335–6.
37. Hawes SE, Stern JE, Feng Q, Wiens LW, Rasey JS, Lu H, et al. DNA hypermethylation of tumors from non-small cell lung cancer (NSCLC) patients is associated with gender and histologic type. Lung Cancer 2010;69:172–9.
38. Moribe T, Iizuka N, Miura T, Kimura N, Tamatsukuri S, Ishitsuka H, et al. Methylation of multiple genes as molecular markers for diagnosis of a small, well-differentiated hepatocellular carcinoma. Int J Cancer 2009; 125:388–97.