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

Irena Misiewicz-Krzeminska1,2,3, María E. Sarasquete1,2, Carolina Vicente-Dueñas2,4, Patryk Krzeminski1,2, Katarzyna Wiktorska3, Luis Antonio Corchete1,2, Dalia Quwaider1,2, Elizabeta A. Rojas1,2, Rocio Corral2,5, Ana A. Martin2, Fernando Escalante6, Abelardo Bárez7, Juan Luis García1,2, Isidro Sánchez-García2,4, Ramón García-Sanz1,2,5, Jesús F. San Miguel8, and Norma C. Gutiérrez1,2,5

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; 1–11. ©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 G1–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...
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, 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-Biotech) 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 (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 pmol 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−ΔΔCt 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−ΔΔCt/2−ΔΔCt MPs) × 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 electrophoresis 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 3130xl 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 pSil Cyclin D2 long UTR (Addgene Plasmid 21645) or pSil 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 Na2HPO4 buffer (pH 7). After electrophoresis, the gel was blotted onto Hybond-N (Amersham), UV light-cross-linked, and hybridized to 32p-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 (DIANAmI, miRanda, miRWalk, RNAhybrid, PICTar, 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
several 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 non-targeting 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 S3). 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 $2^{-\Delta\Delta C_T}$ 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.
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 poly(A) tail, which demonstrated that this...
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 samples with predominant shorter CCND2 mRNA displayed higher CCND2 expression levels.
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 HEK293 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 were 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 ZSCAN3 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,
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 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).
with increased expression of different genes at both the mRNA and the protein level (9, 11, 12, 22). In fact, the shorter \( \text{CCND2} \) 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 \( \text{CCND2} \) mRNA expression in those multiple myeloma with greater abundance of the shorter 3'UTR isoform, although it is still an open question whether \( \text{CCND2} \) mRNA shortening is the cause or the consequence.

The short form of \( \text{CCND1} \) 3'UTR has been previously reported in multiple myeloma with t(11;14) (1, 14). The existence of this isoform was correlated with higher \( \text{CCND1} \) mRNA level but not with differences in survival (14). Short \( \text{CCND1} \) mRNAs are also observed in MCL whose genetic hallmark is t(11;14). The mechanisms underlying the truncation of \( \text{CCND1} \) 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 \( \text{CCND1} \) 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 \( \text{CCND1} \) and \( \text{CCND2} \) 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 \( \text{CCND2} \) mRNA shortening after \( \text{CCND1} \) silencing suggest that cyclin D1 could downregulate \( \text{CCND2} \) level by modification of polyadenylation/cleavage reaction. Moreover, the increased proportion of long \( \text{CCND2} \) isoform after cyclin D1 or cyclin D3 overexpression along with simultaneous decrease of cyclin D2 protein level in the JIN3 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); 17β-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 mRNA 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.

Authors' Contributions


Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Misiewicz-Krzeminska, P. Krominski, K. Wiktorska, R. Corral, A.A. Martin, F. Escalante, A. Bárez, I. Sánchez-García, N.C. Gutiérrez.


Writing, review, and/or revision of the manuscript: I. Misiewicz-Krzeminska, C. Vicente-Dueñas, P. Krominski, I. Sánchez-García, R. García-Sanz, J.F. San Miguel, N.C. Gutiérrez.

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A.A. Martin.

Study supervision: J.F. San Miguel, N.C. Gutiérrez.

Acknowledgments

The authors thank Isabel Isidro, Teresa Prieto, and Vanesa Gutiérrez for their technical assistance in the purification of multiple myeloma samples and María Ángeles Hernández for the help in mRNA FISH assays.

Grant Support

This study was partially supported by Instituto de Salud Carlos III (Fondo de Investigaciones Sanitarias: PI08/0568 and PI13/00111), Asociación Española Contra el Cancer (AECC, GCB120981SAN), "Gerencia Regional de Salud, Junta de Castilla y León" (GRS 702/A/11, BIO/SAA57/13 and BIO/SA53/14) grants, the Spanish Myeloma Network Program (RD12/0063/0058) and the INNOCAM-PLUS Program (CE110-1-001). I. Misiewicz-Krzeminska was supported by Instituto de Salud Carlos III (PS09/01897). M.E. Sarasquete was supported by Contrato Miguel Servet (CP13/00080). Research in the I. Sánchez-Garcia group was supported by FEDER and MINECO (SAF2012-32810, and Red de Excelencia Consolider Oncobio SAF2014-57791-REDC), Instituto de Salud Carlos III (Pie14/00066), NIH (R01 CA109335-04A1) grants, the Spanish Myeloma Network Program (RD12/0063/0058) and the INNOCAM-PLUS Program (CE110-1-001). I. Misiewicz-Krzeminska was supported by “Miguel Servet” Grant (CP14/00802 AES 2013-2016) from the Instituto de Salud Carlos III (Ministerio de Economía y Competitividad). I. Sánchez-Garcia is an API lab of the EuroSyStem project and a partner within the Marie Curie Initial Training Network DECIDE.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received October 30, 2014; revised August 7, 2015; accepted August 14, 2015; published OnlineFirst September 4, 2015.

References


15. STR matching analysis [access date 26/02/2015]. Available from: www.dsmz.de/fp/cgi-bin/str.html


33. Takagaki Y, Seipel RT, Peterson MI, 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.


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

Irena Misiewicz-Krzeminska, María E. Sarasquete, Carolina Vicente-Dueñas, et al.

Clin Cancer Res  Published OnlineFirst September 4, 2015.

Updated version  Access the most recent version of this article at: doi:10.1158/1078-0432.CCR-14-2796

Supplementary Material  Access the most recent supplemental material at: http://clincancerres.aacrjournals.org/content/suppl/2015/10/02/1078-0432.CCR-14-2796.DC1

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.