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High expression of cereblon (CRBN) is associated with improved clinical response in patients with multiple myeloma treated with lenalidomide and dexamethasone

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- lenalidomide;
- multiple myeloma

Summary

Cereblon (CRBN) has recently been identified as a target for immunomodulatory drugs (IMiDs) and its downregulation has been linked to resistance to lenalidomide. Here, we studied CRBN expression by real time polymerase chain reaction in 49 bone marrow samples of newly diagnosed patients with multiple myeloma treated with lenalidomide and dexamethasone. Median CRBN expression was 3·45 in patients who achieved complete response, and 3·75, 2·01, 0·78, and 0·70 in those with very good partial response, partial response, stable disease and progressive disease respectively. CRBN expression levels correlated significantly with response to lenalidomide treatment ($r = 0·48$; $P < 0·001$). Among established prognostic parameters, only beta-2-microglobulin correlated with cereblon ($r = 0·66$; $P < 0·001$). A close association of CRBN with interferon regulatory factor 4 (IRF4) ($P < 0·001$) and with CTNNB1 ($P < 0·001$) was found. Overall, a statistically significant association between baseline CRBN expression and response in MM patients treated with lenalidomide is shown. CRBN expression is closely associated with IRF4, which is an important target of IMiD therapy.

The immunomodulatory drugs (IMiDs), thalidomide, lenalidomide and pomalidomide exert significant activity in the treatment of multiple myeloma (MM) (Singhal et al, 1999; Dimopoulos et al, 2007, 2009; Van Rhee et al, 2008; Galustian & Dalgleish, 2009; Lacy, 2011; Schey &

Ramasamy, 2011). However, the precise molecular mechanisms of action of lenalidomide and other IMiDs are still not completely understood (Anderson, 2005; Vallet et al, 2008; Quach et al, 2010; Tageja, 2011). Recently, cereblon (CRBN) has been identified as the primary target of thalidomide teratogenicity (Ito et al, 2010, 2011), and is highly expressed in human brain and various other tissues. CRBN forms an E3 ubiquitin ligase complex with damaged DNA binding protein 1 (DDB1) and Cullin-4 (CUL4) which are important for regular cell function (Chang & Stewart, 2011). Thalidomide directly binds and inactivates CRBN (Chang & Stewart, 2011). It was postulated, that CRBN might not only be a direct target for teratogenicity, but also for anti-myeloma activity. In fact, CRBN knocked-down human MM cell lines (HMCLs) have been shown to be highly resistant to lenalidomide and pomalidomide, in contrast to other therapeutic agents (Zhu et al, 2011; Lopez-Girona et al, 2012). Thus, CRBN seems to be an essential requirement for IMiD effects and could therefore be associated with response to treatment with lenalidomide in MM patients. Downregulation of CRBN expression was noted at the time of drug resistance in eight out of nine myeloma patients (Zhu et al, 2011).

A recent study reported that accumulation of CTNNB1 (ß-catenin) during treatment with lenalidomide might be another cause of drug resistance (Bjorklund et al, 2011). Exposure of plasma cells to lenalidomide activated the Wnt/ß-catenin pathway and its downstream targets such as cyclin D1 and MYC (Bjorklund et al, 2011).

To date, information on CRBN expression and its impact on clinical outcome and the association of CRBN with other clinical and prognostic parameters in a representative patient sample is lacking. Moreover, downstream targets of CRBN have not yet been defined (Chang & Stewart, 2011). One putative downstream target of CRBN is interferon regulatory factor 4 (IRF4) (Zhu et al, 2011; Lopez-Girona et al, 2012). IRF4 expression is essential for myeloma cell survival (Shaffer et al, 2008, 2009), has been shown to correlate with prognosis in MM patients (Heintel et al, 2008), and is probably an important target for IMiD therapy (Li et al, 2001; Lopez-Girona et al, 2011).

Here we measured CRBN expression by real time polymerase chain reaction (PCR) in seven cell lines and in 49 bone marrow (BM) samples of patients with MM (i) to correlate CRBN expression levels with response to lenalidomide-based treatment, (ii) to analyse the association of CRBN with clinical and prognostic parameters and (iii) to evaluate the association of CRBN with IRF4 and CTNNB1.

Patients and methods

Patient characteristics

Forty-nine previously untreated patients with multiple myeloma, 16 from Department of Internal Medicine I, Center for Oncology and Haematology, Wilhelminenspital, Vienna, 29 from the Division of Haematology of the University of Torino, and four from the Division of Haematology and Haemostaseology, Department of Medicine I, Medical University of Vienna, were included in this retrospective analysis. The median age of patients was 59 years (range: 37–85 years). Sixteen patients were International Staging System (ISS)-stage I, 18 were stage II, and 15 were stage III.

The median number of cycles of lenalidomide-based treatment after BM aspiration was 4 (range: 3–37). Lenalidomide was given in combination with dexamethasone in all patients with a starting dose of 25 mg/d on days 1–21 in a 28-day cycle. Dexamethasone was administered at a dose of 40 mg on days 1–4 and 15–18 in a 28-day cycle, or 40 mg (once) weekly, or 20 mg (once) weekly. Responses were assessed according to the International Myeloma Working Group criteria (Durie et al, 2006).

BM samples of eight donors without a malignant haematological or other malignant disease were used as controls.

Cell lines

The human myeloma cell lines (HMCLs) U266, KMS-12-BM, OPM-2, NCI-H929, MM.1S, SK-MM-1, and RPMI8226 were obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and cultivated in RPMI-1640 medium supplemented with 10% heat-inactivated foetal bovine serum, 2 m mol/l L-glutamine and 1× penicillin/streptomycin (all reagents were obtained from Gibco, Life Technologies Inc., Paisley, UK).

Real time PCR

BM biopsy was performed after informed consent was obtained and before treatment with lenalidomide and dexamethasone was started. RNA and cDNA were prepared from HMCLs and BM samples using the RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) and M-MuLV reverse transcriptase (NEB GmbH, Frankfurt, Germany). Real time PCR was performed with the ABI Prism 7000 Sequence Detector (Applied Biosystems, LifeTech Austria, Vienna, Austria) according to the manufacturer's instructions as previously described (Heintel et al, 2004). PCR was carried out in a 25 µl reaction volume using 1 µl of cDNA with CRBN (Hs00372271_m1, exons 8–9), IRF4 (Hs 00180031_m1, exons 8–9), CTNNB1 (Hs 00355049_m1, exons 14–15) specific primers (Assays-on-Demand Gene Expression system; Applied Biosystems), and TaqMan® Universal Master Mix. ACTB was used as an endogenous control. All samples were run in duplicates. The Delta Delta CT method was used for relative quantification.

Cell sorting

BM mononuclear cells of six MM patients and four healthy donors were first incubated with 20 µl/10^7 cells with monoclonal antibody (anti-CD138)-tagged magnetic microbeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) for 20 min at 4°C followed by a washing step with magnetic-activated cell sorting (MACS) buffer. Antibody-labelled, MACS buffer-washed cells were then layered on Mini-MACS separation columns (Miltenyi Biotec GmbH) and sorted immunomagnetically following the manufacturer's instructions.

Statistical analyses

Correlations (Spearman and Pearson), multivariate analysis, and survival analysis (Cox regression analysis) were performed using SPSS statistics 17.0. P-values < 0·05 were considered to be statistically significant.

Results

CRBN expression in normal BM, normal plasma cells, and HMCLs

CRBN expression of normal BM of eight healthy donors was used as a control and was arbitrarily set at 1. Median CRBN expression of normal, CD 138^+ sorted plasma cells was 1·80-fold relative to normal BM (range: 1·61–2·58; N = 4). In the seven human myeloma cell lines tested (U266, KMS-12-BM, OPM-2, NCI-H929, MM.1S, SK-MM-1, RPMI8226), a higher expression of CRBN compared to normal BM was found (range: 2·34–10·32-fold relative to normal BM). Exposure of

the cell lines with lenalidomide for 72 h revealed variable results with either no change or a slight downregulation of CRBN expression. In contrast, treatment with bortezomib resulted in upregulation of CRBN, which was most prominent in the U266 cell line and lowest in KMS-12-BM (data not shown). Exposure to dexamethasone in different concentrations (1–1000 n mol/l) did not significantly change CRBN expression in two cell lines tested.

CRBN expression in $CD138^+$ and $CD138^-$ sorted BM samples of MM patients

Analysis of different cell compartments showed lower median CRBN expression in $CD138^-$ sorted BM cells (1·22; range: 0·70–5·03, N = 6), but a higher median CRBN expression in corresponding $CD138^+$ sorted cells (4·06; range: 1·23–5·01, N = 6) (P = 0·06). These results indicate that CRBN expression is higher in malignant plasma cells, although this difference was not significant and the ranges overlap. However, low CRBN expression levels were detectable in $CD138^-$ cells representing BM microenvironment.

CRBN expression in BM samples of patients with MM

Median CRBN expression in 49 unsorted BM samples of MM patients was 1·71-fold relative to normal BM (range: 0·31–28·74). Thirty-five patients (71%) had higher CRBN expression than normal BM, while in four patients CRBN was not detectable. Of note, no correlation was found between CRBN levels and the percentage of plasma cell BM infiltration (Pearson correlation: r = 0·14; P = not significant [NS]).

CRBN expression and clinical response

An initial analysis was performed on BM samples from patients treated at two institutions in Vienna. In this cohort, CRBN levels correlated significantly with response to lenalidomide and dexamethasone (learning series). We therefore decided to validate these results in an independent patient cohort (validation series). Twenty-nine additional patient samples derived from the Division of Haematology of the University of Torino were analysed. Data regarding response to therapy were blinded during evaluation of CRBN expression. A statistically significant correlation between CRBN and clinical response became evident in the Vienna cohort as well as in the Torino cohort. After establishing the validity of our learning series, we analysed the whole cohort of 49 uniformly treated and newly diagnosed patients together. Of the entire group, six patients (12%) achieved complete response (CR), 7 (14%) very good partial response (VGPR), 24 (49%) partial response (PR), 10 (20%) had stable disease (SD), and 2 (4%) had progressive disease (PD). Median CRBN expression was 3·45 in patients with CR, 3·75 in patients with VGPR, 2·01 in patients with PR, 0·78 in patients with SD, and 0·70 in patients with PD (Table 1). Of note, only non-responding patients (SD, PD) had a median CRBN expression that was lower than normal BM. CRBN levels were slightly higher in patients with VGPR than in those with CR, but this difference was not significant. In responding patients (CR, VGPR, PR) significantly higher CRBN expression levels compared to non-responding patients (SD, PD) were noted (r = 0·48; P < 0·001, Fig 1A).

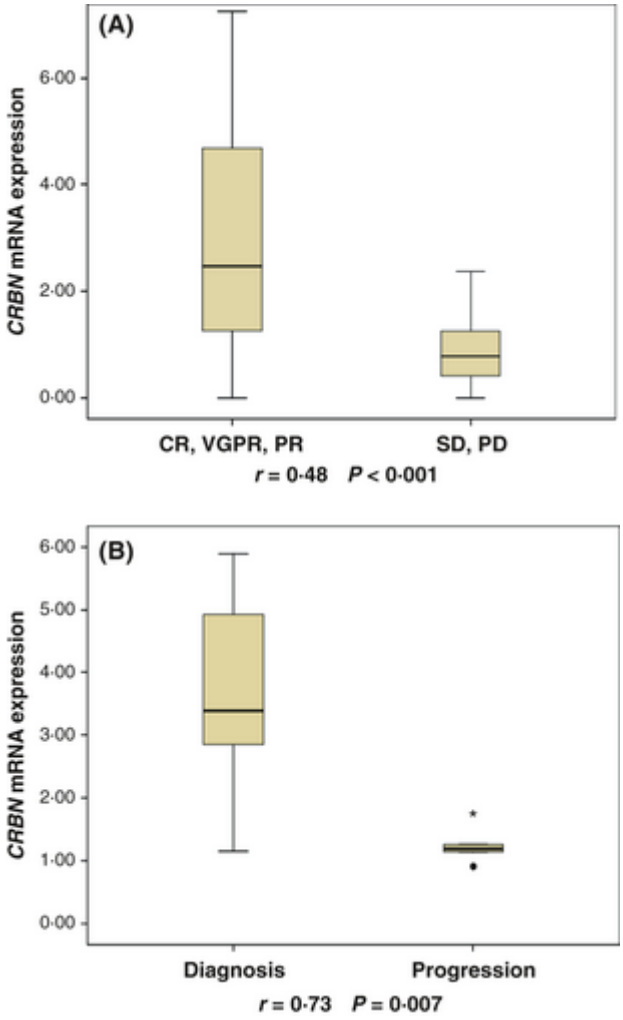

Figure 1. (A) Clinical response according to CRBN expression in newly diagnosed MM patients treated with lenalidomide and dexamethasone (N = 49; r = 0·48, P < 0·001). (B) CRBN expression in six MM patients at diagnosis and time of progression. Median CRBN at diagnosis: 3·38; range: 1·15–5·90; median CRBN at progression: 1·19; range: 0·91–1·75; r = 0·73; P = 0·007. MM, multiple myeloma; CR, complete response; VGPR, very good partial response; PR, partial response; SD, stable disease; PD, progressive disease.

Table 1. Quality of response and CRBN expression in 49 newly diagnosed MM patients treated with lenalidomide and dexamethasone

Quality of response	*N*	Median *CRBN* expression	Range
CR	6	3·45	0·81–7·26
VGPR	7	3·75	0·60–28·74
PR	24	2·01	0–6·70
SD	10	0·78	0–2·37
PD	2	0·70	0·31–1·09

MM, multiple myeloma; CR, complete response; VGPR, very good partial response; PR, partial response; SD, stable disease; PD, progressive disease.

The association of CRBN with response was still significant after adjustment for known prognostic markers [percentage of BM infiltration, ISS stage, ß-2-microglobulin, albumin, haemoglobin, lactate dehydrogenase, age, and the presence of high risk cytogenetic aberrations (t(4;14), del13, and del17p)], and treatment centre (Torino or Vienna) ($P = 0{\cdot}002$).

Cox regression analysis did not reveal a significant association between CRBN expression and progression-free survival (PFS), or overall survival (OS) in our patient cohort.

Correlation of CRBN expression and myeloma response with prognostic factors

Among established prognostic parameters including ISS stage, β-2-microglobulin, albumin, haemoglobin, and cytogenetic aberrations (t(4;14), del13, and del17p), only β-2-microglobulin correlated highly significant with CRBN ($r = 0{\cdot}66$; $P < 0{\cdot}001$). In contrast to CRBN expression which was highly correlated with response to lenalidomide-dexamethasone treatment, no correlations of β-2-microglobulin, albumin, ISS stage, haemoglobin, percentage of BM infiltration (BM aspirate and BM biopsy), and cytogenetic aberrations with response to lenalidomide and dexamethasone therapy were noted. Moreover, no association of dexamethasone dosage and response and/or CRBN expression was noted.

CRBN expression after lenalidomide therapy in BM samples

In five patients BM analysis was repeated after prolonged exposure to lenalidomide. Results revealed a heterogeneous expression pattern of CRBN: mRNA levels were stable in one patient, slightly reduced in another patient and increased in three patients. One of the latter patients had a very high CRBN expression at baseline and during lenalidomide therapy. He achieved a PR at the time of repeated testing (22 months after start of lenalidomide), and finally after 7 months of further continued therapy, the patient achived a CR. Lenalidomide treatment was short and amounted for only 4 months in the patient with significant reduction of CRBN levels.

In six patients responding to lenalidomide and dexamethasone, BM for RNA isolation was available at the time of diagnosis and at documentation of progressive disease. In all of them CRBN expression was significantly lower at time of progressive disease compared to the baseline values

(median CRBN at progression: 1·19; range: 0·91–1·75, median CRBN at diagnosis: 3·38; range: 1·15–5·90; r = 0·73; P = 0·007) (Fig 1B).

Correlation of CRBN with IRF4 and CTNNB1

A highly significant correlation was observed between the expression of CRBN and IRF4 (r = 0·57; P < 0·001), (Fig 2A). A similar significant correlation between both parameters was also obtained in 6 $CD138^{+}$ sorted primary myeloma cell samples (r = 0·79; P = 0·03). Furthermore, expression of CRBN was significantly correlated with that of CTNNB1 (r = 0·7; P < 0·001, Fig 2B). In three patients, CTNNB1 was measured before start of lenalidomide therapy and after continuous exposure. CTNNB1 was found to be up-regulated, unchanged, and down-regulated in each one of these three patients.

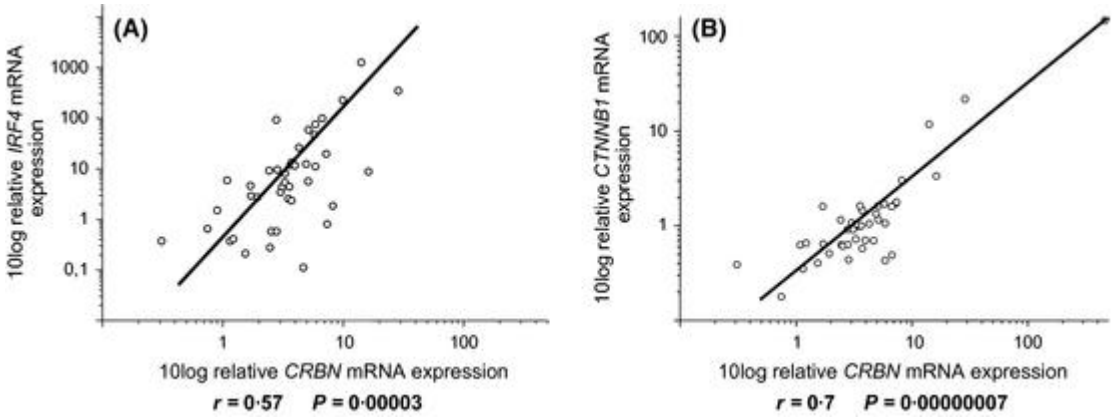

Figure 2. (A,B) Correlation of CRBN with IRF4 expression in MM patients (N = 43; one patient with IRF4 of 0 is not represented in this logarithmic scale) and correlation of CRBN with CTNNB1 expression in MM patients (N = 41).

Discussion

Our study shows a significant correlation between high CRBN expression and response to lenalidomide-dexamethasone in a uniformly treated patient cohort; a finding that was primarily detected in patients at one institution and subsequently confirmed in patients attending the Torino centre. This observation, obtained in 49 patients, supports the important role of CRBN as a central mediator of responsiveness to lenalidomide and orchestrator of a range of subsequent cellular events such as induction of the cytokines, interleukin-2 and tumour necrosis factor-α in human T cells (Zhu et al, 2011; Lopez-Girona et al, 2012). Similar findings showing a close correlation between CRBN expression determined by protein tissue array analysis of BM and response to lenalidomide, PFS and OS have recently been reported (Klimowicz et al, 2012). Additional support for the relevance of our findings comes from thalidomide maintenance therapy in the HOVON-65/GMMG-HD4 trial. Increased CRBN gene expression was associated with long PFS in patients on thalidomide therapy, while no association was noted in those on bortezomib maintenance (Broyl et al, 2012). In our study, CRBN was the only parameter significantly associated with response to lenalidomide among established prognostic parameters including cytogenetic aberrations, but did not correlate with PFS or OS in our patient cohort.

Our study revealed a close correlation between CRBN and the transcription factor IRF4 and with CTNNB1. IRF4 has previously been shown to be essential for myeloma cell survival (Shaffer et al, 2008). Previous investigations showed that knockdown of CRBN by CRBN shRNAs in myeloma cell lines resulted in down regulation of IRF4 (Zhu et al, 2011; Lopez-Girona et al, 2012). Similarly, when myeloma patients were treated with IMiDs, down regulation of IRF4 was observed (Li et al, 2001; Lopez-Girona et al, 2011). These data point to a close functional relationship between IRF4 and CRBN which is supported by our finding of a close correlation between both genes also in $CD138^+$ primary myeloma cells as well as in unsorted BM cells of MM patients.

High expression of CTNNB1, an integral cell-cell adhesion adaptor protein and transcriptional co-regulator may confer drug resistance (Bjorklund et al, 2011; Chang & Stewart, 2011). Unexpectedly, CTNNB1 expression was found to closely correlate with CRBN indicating that elevated baseline CTNNB1 levels are unlikely to be accountable for lenalidomide resistance. Exposure to lenalidomide resulted in increased CTNNB1 expression in only one of three patients investigated. As a caveat, by using real time PCR for evaluation of CTNNB1, posttranscriptional regulations of this protein cannot be detected.

One of the limitations of this study is the retrospective nature of our analysis. Another limitation concerns the use of unsorted BM instead of CD 138^+ selected myeloma cells for evaluation of CRBN expression. However, while we show that CRBN expression might be higher in malignant plasma cells, we can-not exclude a possible CRBN-mediated lenalidomide effect on CRBN expressing $CD138^-$ microenvironmental cells. Although this should be considered when interpreting these results, we show a significant association between CRBN expression and response to lenalidomide and dexamethasone therapy in previously untreated patients with MM. In addition, CRBN expression correlated closely with IRF4, most probably another important target of lenalidomide. Prospective studies in larger series of patients are needed to confirm our data indicating that CRBN expression may serve as a potential marker for individualized selection of lenalidomide therapy in patients with MM.

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Authorship and disclosure

Daniel Heintel and Heinz Ludwig designed the study, Daniel Heintel, Arnold Bolomsky and Sabine Pfeifer conducted the PCR studies, Daniel Heintel made the statistical calculations, Heinz Ludwig, Alberto Rocci, Simona Caltagirone, Heinz Gisslinger, Niklas Zojer, Ulrich Jäger, and Antonio Palumbo provided patient probes and patient data, Martin Schreder provided clinical information. All authors participated in manuscript writing and all approved the manuscript. Heinz Ludwig, Antonio Palumbo and Ulrich Jäger received honoraria and financial support for clinical studies from Celgene. Antonio Palumbo received grants from Programma di ricerca di Rilevante Interesse Nazionale (PRIN) 2009.

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