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The clinical significance of cereblon expression in multiple myeloma

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Abstract

Cereblon (CRBN) mediates immunomodulatory drug (IMiD) action in multiple myeloma (MM). We demonstrate here that no patient with very low *CRBN* expression responded to IMiD plus dexamethasone therapy. In 53 refractory MM patients treated with pomalidomide and dexamethasone, *CRBN* levels predict for decreased response rates and significant differences in PFS (3.0 vs. 8.9 months, $p < 0.001$) and OS (9.1 vs. 27.2 months, $p = 0.01$) (lowest quartile vs. highest three quartiles). While higher *CRBN* levels can serve as a surrogate for low risk disease, our study demonstrates that low *CRBN* expression can predict resistance to IMiD monotherapy and is a predictive biomarker for survival outcomes.

Keywords

Multiple myeloma; Cereblon; Gene expression profiling; Immunomodulatory drugs; Biological markers

1. Introduction

Immunomodulatory drugs (IMiDs) are key components of treatment for multiple hematologic malignancies [1]. The first drug in this class, thalidomide, was originally used as a sedative in pregnant women and became infamous for its teratogenic effects. Early studies implicated thalidomide-induced oxidative stress and anti-angiogenesis as possible mechanisms of these malformations [2, 3]. Recently, Ito et al. revealed in a landmark paper that cereblon (*CRBN*) is the primary target of thalidomide teratogenicity [4]. Thalidomide binds *CRBN* to inhibit the function of the E3 ubiquitin ligase complex (composed of *CRBN*, DDB1, and Cul4). This complex regulates DNA repair, replication and transcription, and its inhibition by thalidomide causes teratogenic effects by preventing degradation of proteins

that play a crucial role in embryonic limb development (e.g. down regulation of fibroblast growth factor 8) [4]. Drug-induced downstream effects of *CRBN* inhibition include cell cycle arrest with up regulation of the cyclin-dependent kinase inhibitor p21^{WAF-1} [5] and down regulation of interferon regulatory factor 4 (IRF4), a MM cell survival factor that targets critical genes like *MYC*, *CDK6* and *CASP3* [6–8].

We recently demonstrated that *CRBN* is also required for the anti-MM action of the thalidomide derivatives lenalidomide and pomalidomide, thus more accurately referred to collectively as “cereblon inhibitors” [9]. Furthermore, we observed that *CRBN* expression decreases in MM patients that developed resistance to lenalidomide therapy [9, 10]. Conversely, loss of *CRBN* expression did not affect response to other agents, such as bortezomib, dexamethasone, and melphalan [4]. Recent studies have observed a positive association between *CRBN* and response with thalidomide maintenance and upfront lenalidomide and dexamethasone therapy [11, 12]. Furthermore, we and others have observed *CRBN* mutations in relapsed and refractory patients, supporting the key role of *CRBN* in the response to IMiDs [13]. However, many patients with low *CRBN* levels have no mutation evident, thus transcriptional or post-transcriptional factors (e.g. regulation by microRNA) may influence *CRBN* gene expression and responsiveness to IMiD therapy [14].

Since IMiDs are also effective in myelodysplasia, chronic lymphocytic leukemia and some non-Hodgkin lymphomas, we assume that *CRBN* inhibition is the inherent mechanism of action in all of these malignancies [15–17]. This inhibition is also possibly implicated in the increased incidence of secondary malignancies when IMiDs are used for extended periods of time with, or following, alkylating agent therapy.

In the current study, we analyze *CRBN* gene expression levels in a cohort of homogeneously treated MM patients in order to examine the relationship of *CRBN* expression level with clinical outcomes following IMiD therapy.

2. Methods

2.1. Patient population

We screened the University of Arkansas Medical School (UAMS) gene expression profiling (GEP) database of MM patients treated on the Total therapy 2 (TT2) and Total therapy 3 (TT3) combination therapy regimens. This included 176 patients that received TT2 without thalidomide (TT2–), 175 patients that received TT2 with thalidomide (TT2+), and 441 MM patients treated with TT3. TT3 involves the following combination therapy: induction with bortezomib, thalidomide, dexamethasone, cisplatin, doxorubicin, cyclophosphamide, and etoposide (VTD-PACE) followed by tandem autologous stem cell transplantation with melphalan conditioning, followed by 3 years of maintenance that includes bortezomib, thalidomide, and dexamethasone. Thalidomide was substituted by lenalidomide in many TT3 patients.

We further screened GEP levels from 148 MM patients from Mayo Clinic treated with IMiDs with or without steroids, 77 of them had only an IMiD plus dexamethasone treatment, of which 53 were homogeneously treated in two prospective clinical trials with pomalidomide and dexamethasone [18, 19]. The first trial included 35 relapsed or refractory patients that received pomalidomide 2 mg daily, continuously on a 28 day cycle, and dexamethasone 40 mg weekly. The second trial included 35 relapsed or refractory patients that received pomalidomide 4 mg daily, continuously on a 28 day cycle, as well as dexamethasone 40 mg weekly. 53 of the 70 patients on these two trials were successfully analyzed for *CRBN* expression prior to therapy initiation.

2.2. Gene expression profiling (GEP)

RNA was isolated from marrow CD138 positive plasma cells using RNeasy Plus Mini Kit (Qiagen). GEP was performed from total RNA using the Affymetrix U133Plus2.0 array. All technical steps were performed by the MicroArray facility at the Mayo Clinic Gene Expression Core following the manufacturer's protocol. Microarrays were scanned with an Affymetrix Scanner 3000 and data normalization was performed using Expression Console (Affymetrix) and the Robust Multi-array Average (RMA). Additional databases were used for comparative expression studies, including datasets from different stages of plasma cell neoplasm (GSE6477 and GSE5900), pre-treatment MM (GSE2658 and <http://www.broadinstitute.org/mmgp/home>), relapse MM (<http://www.broadinstitute.org/mmgp/home>), and other B cell lymphomas (Marginal Zone (GSE35348), Follicular and Diffuse Large Cell Lymphomas (GSE12195) and multiple cell lines established from hematological malignancies and solid tumors (source: Broad-Novartis Cancer Cell Line Encyclopedia, <http://www.broadinstitute.org/ccle/home>).

Intensity values were median normalized across the entire cohort for subsequent analyses. A median normalized *CRBN* expression <0.8 was defined 'a priori' as 'very low *CRBN* expression' to be used for determining the response rate of the cohort.

In order to evaluate progression free survival and overall survival, we also determined the quartile cut-off points for *CRBN* median normalized expression (for all MM patients) to be 0.889, 1.027 and 1.211, respectively, with a goal of comparing the lowest quartile and the higher three quartiles of *CRBN* expression.

2.3. Response criteria

Response was assessed according to the International Myeloma Working Group criteria [20]. A positive response to therapy in this study required a partial response or better.

2.4. Statistical analysis

For the comparison of gene expression levels between groups, we used Fisher two-tailed test (comparison between 2 groups) and Kruskal–Wallis test (3 or more groups). Differences in overall survival (time from registration to death) and progression free survival (time from registration to the earlier of disease progression or death) were estimated using Kaplan Meier methods and log-rank statistics. Optimal cutoff points for survival analyses were determined using the Contal and O'Quigley method.

3. Results

3.1. Cereblon gene expression

We compared *CRBN* expression levels across different human tissues and malignancies. Overall, *CRBN* expression tended to be higher in cell lines originated from hematologic malignancies, including MM, compared to solid tumors (Fig. 1).

Furthermore, we found that normal B cells and low grade B-lymphoid cell tumors (marginal zone lymphoma, lymphoplasmacytic lymphoma and follicular lymphoma) share similar *CRBN* levels with MM. Interestingly, levels in diffuse large cell B-cell lymphoma (DLBCL) were significantly lower ($p < 0.0001$, Kruskal–Wallis test), but without any significant differences between the activated B-cell (ABC) and the germinal center B-cell (GCB) types (data not shown).

We did not find differences in *CRBN* expression levels between normal plasma cells, MGUS, smoldering and symptomatic MM (Fig. 2A). But when we compared the *CRBN*

expression level across genetic MM subclasses, the TC (translocation/cyclin D) groups “D1” and “None” showed higher expression levels ($p < 0.0001$) compared to the remaining groups (Fig. 2B) [21]. Conversely, the TC group 4p16 ($t(4; 14)$) was associated with lower *CRBN* expression ($p = 0.03$).

When we looked at differences in *CRBN* expression based on the ploidy status, the hyperdiploid MM group showed higher expression than the non-hyperdiploid group (Fig. 2C, $p < 0.001$). This suggests that a component of higher *CRBN* expression is copy-number dependent, as *CRBN* is located on chromosome 3 and trisomy 3 is commonly, but not universally, found in hyperdiploid MM [22].

3.2. No IMiD response in patients with very low Cereblon levels

One hundred and forty-eight patients with MM at Mayo Clinic were identified as having had *CRBN* expression measured by GEP prior to initiation of chemotherapy. Seventy-one patients were excluded from further analysis because IMiD therapy was combined with bortezomib or an alkylating agent, or because there was insufficient follow up data. As a result, 77 patients who received single agent IMiD therapy alone in combination with dexamethasone were available for analysis. Of these 77 patients, 53 patients received pomalidomide 2 mg or 4 mg daily combined with low dose dexamethasone 40 mg weekly as part of two clinical trials performed at Mayo Clinic [23]. Of the remaining 24 patients, 19 received lenalidomide with or without dexamethasone, while 5 patients received thalidomide with or without dexamethasone.

We were struck by the lack of clinical response in patients at the lower range of *CRBN* expression. When looking at all 77 patients with single agent IMiD therapy, 14 patients had *CRBN* median normalized expression <0.8 , and none of these patients responded to treatment. Response rates were 40% for *CRBN* expression of 0.8–0.9, and 49% for *CRBN* expression >0.9 .

3.3. Cereblon expression levels predict response and survival outcomes in pomalidomide clinical trials

In order to further study the correlation between *CRBN* expression level and clinical survival outcomes, we next analyzed only the homogeneously treated subgroup of 53 MM patients, refractory to both lenalidomide and bortezomib, that received pomalidomide (CC4047) and dexamethasone on two phase II clinical trials at Mayo Clinic [18,19]. Overall response rates for all patients on this study were 49% in the Pomalidomide 2 mg cohort and 43% in the pomalidomide 4 mg cohort with no statistically significant differences in overall survival at 6 months between the trials (78% vs. 67%, respectively) [23].

In the two trials, 53 of the 70 patients had *CRBN* expression levels determined prior to initiation of therapy and were used for our final analysis. We utilized the quartile cut-off points for *CRBN* median normalized expression (0.889, 1.027 and 1.211 for all MM patients) with a goal of determining differences between progression free survival and overall survival between the lowest quartile and the higher three quartiles of *CRBN* expression. Here we saw a clear trend of improved response rates in patients having higher *CRBN* expression levels by quartile; however this trend was not statistically significant $p = 0.0857$ (Fig. 3). The differences in progression free survival (PFS) (3.0 months vs. 8.9 months, $p = 0.0006$, Fig. 4A) and overall survival (OS) (9.1 months vs. 27.2 months, $p = 0.01$, Fig. 4B) were highly significant in patients with *CRBN* expression in the lowest quartile (median normalized *CRBN* expression <0.889) compared to patients with *CRBN* expression in the highest three quartiles (median normalized *CRBN* expression ≥ 0.889). PFS and OS differed even more profoundly when the lowest quartile was compared to the

highest quartile (PFS: 3.0 months vs. 16.8 months, $p = 0.0001$, OS: 9.1 months vs. not reached, Fig. 4C+D).

3.4. *CRBN* expression and survival in multi-agent combination therapies

In order to investigate any association between *CRBN* expression and survival in patients treated with IMiD containing multi agent combination chemotherapies, we screened the University of Arkansas Medical School (UAMS) gene expression (GEP) database of MM patients treated on the Total therapy 2 (TT2) and Total therapy 3 (TT3) regimens.

As a continuous variable, *CRBN* expression did not correlate with survival in any of the protocols. Even with identifying the optimal cutoffs based on all TT2 (not shown) and TT3 data, the results showed no significant differences (Fig. 5).

4. Discussion

Our group recently demonstrated *CRBN* expression to be an indispensable requirement for the activity of immunomodulatory drugs (IMiDs), more accurately referred to as “cereblon inhibitors” [9]. Additionally, we confirmed lower *CRBN* expression levels in patients that had acquired resistance during IMiD therapy and in lenalidomide resistant MM cell lines [9]. Here we describe differences in *CRBN* expression levels between solid tumor and hematologic malignancy with generally higher levels in hematologic malignancies. MM patients showed relatively high *CRBN* levels, but no differences were seen within different stages of the disease progression from normal B cells to active MM. However, statistically significant differences could be demonstrated within genetic subtypes, in particular lower levels in high risk $t(4;14)$ and higher levels in hyperdiploid compared to non-hyperdiploid patients (Fig. 2B). This observation needs to be considered in respect to our PFS and OS data, since *CRBN* expression is copy-number dependent. Thus, high *CRBN* expression levels might be an surrogate for low risk disease, as trisomy 3 (*CRBN* is located on chromosome 3p) is commonly found in hyperdiploid MM, a lower risk subgroup [22]. Nevertheless, there are differences between hyperdiploid and non-hyperdiploid MM that go beyond the trisomy 3 status. When we compared hyperdiploid and non-hyperdiploid patients that do not bear trisomy 3, we still found a small but significant difference in *CRBN* expression levels (Fig. 2D), suggesting that trisomy 3 is a major but not the only factor contributing to higher *CRBN* levels in hyperdiploid MM.

We also demonstrated that *CRBN* expression levels were similar across normal B-cells and different B-cell malignancies, except in diffuse large B cell lymphoma (DLBCL) where *CRBN* expression was found to be significantly lower [9]. As the ABC and GCB subtypes of DLBCL differ significantly in response to IMiD therapy, we expected to find lower *CRBN* expression levels in the less responsive GCB subtype however, we did not find significant differences based on analysis of published datasets [24].

As *CRBN* expression is required for IMiD action, we hypothesized a correlation between *CRBN* expression levels and survival. Indeed, when we focused on a homogeneously treated cohort of 53 MM patients from Mayo Clinic treated with single agent pomalidomide and dexamethasone, we could clearly demonstrate a significant correlation between low *CRBN* expression and decreased survival outcomes. We further determined a clear trend of decreased response at lower *CRBN* expression levels. Indeed when we looked at MM patients treated with any single agent IMiD and dexamethasone, no patient with median normalized *CRBN* expression less than 0.8 responded to therapy. Our findings are supported by recent studies that show low *CRBN* to be associated with decreased response to thalidomide maintenance or upfront therapy with lenalidomide [11,12].

These findings suggest that *CRBN* expression serves as a predictive biomarker for resistance to IMiD therapy when determined prior to therapy. According to our analysis from the UAMS GEP database, the negative impact on OS and PFS due to low *CRBN* levels might be overcome by use of combination therapies. Consequently, consideration of combination or alternative therapies absent an IMiD may be warranted in MM patients presenting with low *CRBN* expression levels. Further studies are necessary to determine the optimal treatment strategy for these patients with low *CRBN* expression.

In summary, this study demonstrates a clinical correlation between *CRBN* expression level, response to IMiDs and survival. Low *CRBN* expression showed statistically and clinically significant decreases in PFS and OS, and no patient with median normalized *CRBN* expression less than 0.8 responded to IMiD therapy. *CRBN* expression is therefore a potential predictive biomarker for response and survival in MM when quantified prior to therapy with single agent IMiD and dexamethasone.

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Fig. 1. Cereblon expression across tumor cell lines. Higher expression of *CRBN* is observed in hematologic malignancies compared to solid tumors. (Source: Broad-Novartis Cancer Cell Line Encyclopedia). These data suggest that leukemias and neuroblastoma will be relatively sensitive to IMiDs while solid tumors will be relatively resistant.

Fig. 2.

(a)–(d) *CRBN* expression in MM and its precursors. (A) *CRBN* median normalized expression is similar in (MM) and its precursors; (NPC, normal plasma cells; MGUS, monoclonal gammopathy of undetermined significance; and SMM, smoldering myeloma). (B) *CRBN* expression in MM TC genetic subtypes reveals lower expression in *t*(4; 14) patients. (C) *CRBN* expression analysis revealed higher expression in hyperdiploid MM ($p < 0.001$). This increase is not only explained by copy number dependency of *CRBN* expression (shown in D).

Fig. 3. Association between CRBN expression and response in 53 pomalidomide treated patients. We see a strong trend with a p value of 0.0857 (Fisher's exact (2-tailed) analysis) between the first vs. the third and fourth quartile and a high R^2 in the exponential trendline analysis.

Fig. 4. *CRBN* expression correlates with PFS (A+C) and OS (B+D). (A+B) the lowest quartile of *CRBN* expression vs. the higher 3 quartiles (median PFS 3.0 vs 8.9 months, $p = 0.0006$; median OS 9.1 vs 27.2 months, $p = 0.01$). (C+D) the lowest quartile vs. the highest quartile (median PFS 3.0 vs 16.8 months, $p = 0.0001$; median OS 9.1 vs NR months, $p = 0.005$).

Fig. 5.

Influence of *CRBN* expression on survival data in multidrug treatment (TT 3, University of Arkansas). There are no statistically significant associations, but a trend for shorter survival in the lowest quartile of *CRBN* expression. No association was seen in TT 2 (data not shown).