Title: Multiple Cereblon genetic changes associate with acquired resistance to Lenalidomide or Pomalidomide in Multiple Myeloma

Running title: CRBN changes in IMiD drug resistance in myeloma

Authors

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KEY POINTS

1. Almost a third of myeloma patients have genetic alteration of Cereblon (IMiD binding protein) by the time they are pomalidomide-refractory.
2. Cereblon genetic aberrations are associated with inferior outcome to pomalidomide-based regimes in those already refractory to lenalidomide.

ABSTRACT

Emergence of drug resistance to all available therapies is the major challenge to improving survival in myeloma. Cereblon (CRBN) is the essential binding protein of the widely-used IMiD and novel CelMOD drugs in myeloma, as well as certain PROTACs in development for a range of diseases. Using whole genome sequencing data from 455 patients and RNASeq data from 655 patients, including a newly-diagnosed cohort (n=198 WGS, n=437 RNASeq), a lenalidomide (LEN)-refractory cohort (n=203 WGS, n=176 RNASeq) and a pomalidomide (POM)-refractory cohort (n=54 WGS, n=42 RNASeq), we find incremental increase in the frequency of three CRBN aberrations, namely point mutations, copy loss/structural variation and a specific variant transcript (exon 10-spliced), with progressive IMiD exposure, until almost one third of patients have CBRN alterations by the time they are POM-refractory. We find all 3 CRBN aberrations are associated with an inferior outcome to POM in those already refractory to LEN, including those with gene copy loss and structural variation, which has not previously been described. This is the first comprehensive analysis of CBRN alterations in myeloma patients as they progress through therapy, and the largest dataset. It will help inform patient selection for sequential therapies with CRBN-targeting drugs.

INTRODUCTION

Myeloma remains incurable due to universal emergence of drug resistance. Identification of resistance mechanisms in relapsed/refractory myeloma (RRMM) would facilitate appropriate targeting of novel therapeutics to resistant myeloma cells.

The anti-myeloma therapies IMiDs© and their newer derivatives CELMoDs bind the E3 ligase substrate-recognition adapter protein cereblon (CRBN). CRBN is essential for the therapeutic effect of these drugs in myeloma[1,2]. CRBN mutations have been described as rare events (<1%) in newly-diagnosed (ND) patients[3,4], but are reported at higher frequency (12%) in IMiD-treated patients, although numbers reported are low[5]. Beyond mutation, CRBN function may be impaired by other genomic (structural variation, copy loss) or transcriptomic (epigenetic, RNA splicing/stability) aberrations. Gene expression loss and transcript splice variants have been associated with acquired resistance to IMiDs Lenalidomide (LEN) or Pomalidomide (POM)[6-8]. One-third of LEN-refractory patients go on to respond to POM[9]. However, a systematic analysis of the diversity of CRBN changes associated with acquired resistance to IMiD-based therapies, and their clinical significance, has not been undertaken. We report CRBN changes identified in 454 patients by WGS, and 655 patients by RNASeq, including newly diagnosed (ND), LEN-refractory and POM-refractory cases.

METHODS

DNA and RNA extracted from bead-enriched CD138+ myeloma cells and peripheral blood germline DNA were collected at baseline and relapse from patients recruited into trials CC4074-MM010 (STRATUS, NCT01712789) (entry criteria included LEN-refractory status)[9], CC220-MM001 (NCT02773030)[10] and CC122-ST-001MM2 (NCT01421524) (entry criteria included LEN- and POM-refractory status for both). In all cases clinical annotation data were collected. PFS data were not available for the latter two phase 1/dose finding trials. Illumina whole genome sequencing (WGS) (depth coverage 60/30x tumour/germline), and RNA-Seq was performed. Newly-diagnosed patient data from the IFM/DFCI-2009 trial (NCT01191060)[11] (WGS), and the Myeloma Genome Project[12] (RNA-Seq) were also used. Computational methods are described in Supplementary Methods (summarised Fig. S1).

This analysis is complementary to a landscape analysis of relapsed/refractory myeloma using the same cohorts, the subject of a separate publication (in preparation).

RESULTS/DISCUSSION

We include WGS data from 198 ND, 203 LEN-refractory and 54 POM-refractory patients, and RNASeq of 437 ND, 176 LEN-refractory and 42 POM-refractory patients (summary patient characteristics: Tables S1,S2). Only those with confirmed drug-refractory status were included in the LEN/POM-refractory cohorts, defined as myeloma either nonresponsive or progressing whilst taking the named drug, or progression within 60 days of stopping it.

<u>CRBN genetic variants and copy loss increase in incidence at sequential IMiD-refractory states</u>

CRBN mutations were detected in 1/198 (0.5%) ND myeloma patients, 5/203 (2.2%) LEN-refractory patients and 5/54 (9%; 8 mutations detected) POM-refractory patients (Fig. 1a), a significant increase ($p<0.05$) from baseline. Mutation clonal fraction distribution (Fig. 1a) remained variable at LEN/POM-refractory states, and mutations displayed no clustering along the CRBN gene (Fig. 1b), but structural modelling predicts the majority to have a deleterious effect on protein stability or function (Fig. S2).

CRBN gene copy loss occurred in 3/198 (1.5%) ND patients, 16/203 (7.9%) LEN-refractory patients, and 13/54 (24%) POM-refractory patients (Fig. 1c), again a significant increase ($p<0.05$) from baseline. CRBN copy loss was compared to background LOH (loss of heterozygosity) rate using a permutation test with FDR correction. In LEN-refractory patients, CRBN loss was not enriched beyond background LOH. However, in POM-refractory patients, CRBN LOH was significantly enriched ($p=0.006$). The size of the lost region varied widely, but whole chromosome or homozygous loss were not seen (Fig. S3). CRBN gene expression was no different in cases with gene copy loss (Fig. S4a), so how copy loss of CRBN could affect IMiD resistance remains unclear. Additional structural variants (translocations, inversions) of the CRBN gene or promotor region were only found in the LEN-refractory cohort (Fig. 1c); all but one of those identified are highly likely to impair gene function (Fig. 1d).

Exon 10-spliced ('Spliced10') CRBN transcripts increase in incidence at sequential IMiD-refractory states

In a sub-cohort of the cases described here, we previously reported that pre-therapy CRBN expression, as measured by RT-qPCR, did not correlate with subsequent response to POM[13]. We confirmed this lack of correlation by RNASeq (data not shown), although as previously described[7] overall CRBN expression is lower at LEN and/or POM-refractory states (Fig. S4c). However, many splice variants of CRBN have been described[14] including a transcript lacking exon 10, deleting the Lenalidomide/Pomalidomide-binding region[2]. This splice variant is not expected to bind or support IMiD function. We found that the ratio of 'Spliced10'/Full length CRBN transcripts, adjusted for sample purity, was significantly increased in POM-refractory compared to ND or LEN-refractory patients ($p<0.0001$) (Fig. 1e). Full length CRBN gene expression did not significantly vary between cases with high vs. low 'Spliced10' ratio (Fig. S3b).

The ratio of exon 10-spliced (ENST00000424814.5) to full-length (ENST00000231948.8) transcript has previously been proposed to correlate with outcome after IMiD-based regimens[8]. We determined the optimum sample purity-adjusted cut-off for a high 'Spliced10'/full length transcript ratio to be 2.6 (Fig. S5). Using this cut-off, the overall incidence of CRBN alterations in LEN-refractory cases was 20.7%. In POM-refractory cases, it was 29.6% (Fig. 1f), although poorer sample purity in the POM-refractory cohort means high 'Spliced10' incidence may have been under-reported, once ratio adjustment by sample purity was applied (Fig. S6/7; Supplementary Methods).

In LEN-refractory patients, presence of CRBN genetic variations or high 'Spliced10' ratio all individually correlate with PFS.

Next, we examined the relationship between the incidence of CRBN aberrations and PFS, in LEN-refractory patients with both WGS and RNASeq data available (n=174), treated with a POM-based regimen. Overall, PFS was significantly reduced ($p<0.0001$) in LEN-refractory patients with mutation (n=4), copy loss (n=15), structural variation (n=6) or high 'Spliced10' (n=17) (Fig. 2a-b), compared to patients with no CRBN aberration. We found that this association was also present in ND myeloma undergoing induction therapy. Only high 'Spliced10' ratio had sufficient incidence at ND to assess correlation, and it was associated with significantly reduced PFS (Fig. 2c). Induction regimen was too varied to perform sub-

group analysis specifically for IMiD-based induction regimen, but 62.4% (236 out of 378 patients aged <75 for whom sample purity data was available) received regimens containing an IMiD drug. In both cohorts, OS was also significantly reduced in those with CRBN aberration (Fig. 2d,e, aberrations by type Fig. S8). High risk cytogenetics were not different between CRBN aberration and unaffected groups (Table S2).

This is the first attempt to quantify the range of defects in CRBN, the molecular target of IMiDs and CELMoDs. Our analysis confirms the rarity of CRBN mutations in newly-diagnosed myeloma. As patients receive therapy, the incidence of mutation and other genetic alterations increases with the acquisition of LEN and POM-refractory states. All alteration types are associated with adverse clinical outcome, irrespective of CRBN gene expression. Interestingly, the drug binding region of the protein is not favoured in these mutations, which occur along the length of the protein. We describe structural variants in the CRBN gene for the first time, and show association with adverse outcome.
Taken together, direct CRBN genetic alterations occur in nearly 30% of RRMM patients treated with IMiDs, making it the single most clinically significant contributor to clinical resistance. The novel CELMoDs iberdomide and CC92480 are currently in clinical trial in the LEN/POM-refractory myeloma population (NCT02773030, NCT03374085). It is premature to ascertain the impact of CRBN dysregulations on the efficacy of CELMoDs at present, although analysis of data from ongoing trials may reveal this in time. This initial focused analysis excluded CRBN-independent genomic and transcriptomic features of resistance, nor did we explore the underlying mechanisms that cause such CRBN aberrations and result in drug resistance, which will be the subject of future analyses.

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AUTHORSHIP CONTRIBUTIONS

SG: Designed research question, analyzed and interpreted the data and wrote the manuscript. NA-P, FT, PD and MO: Data analysis, statistical design and data interpretation.

PC: Generated supplementary figure 2. KT, MH and DR: Curated and maintained clinical trial datasets. EF, NB, PN, KR and PV: Assisted with study design and manuscript. EF, FT: oversight and management of resources (data generation, infrastructure, processing) AT: Designed research question, oversaw scientific direction, and assisted with manuscript.

CONFLICT OF INTEREST DISCLOSURES

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FIGURE LEGENDS

Figure 1. The incidence of mutation, copy Loss, structural variation or high Exon 10-spliced transcript of *CRBN* increases with sequential IMiD-refractoriness

(a) The incidence of CRBN mutations (LH y-axis), and their cancer clonal fraction (CCF, RH y-axis) at ND, LEN and POM refractory states. Significance detected by Fisher's exact test to detect equality of proportions (non-parametric).

(b) Amino acid changes resulting from the mutations in CRBN gene found at each state. CCF: cancer clonal fraction of each mutation. *Mutations from same patient.

(c) The incidence of copy loss or structural variants (LH y-axis), and their CCF (RH y-axis, copy loss only) at ND, LEN and POM refractory states. Significance detected by Fisher's exact test to detect equality of proportions (non-parametric).

(d) Diagrammatic representation of site of structural variant breakpoints identified at LEN-refractoriness.

(e) Change in ratio of Exon 10-spliced: full-length transcripts between ND, LEN and POM refractory states: ratio expressed as log2(exon 10-spliced: full length transcript count) x tumour sample purity. Significant difference detected by Kruskal-Wallis one-way ANOVA (non-parametric).

(f) Landscape of total and co-incidence of four types of CRBN aberration in LEN and POM-refractory cases analysed.

Figure 2: The incidence of mutation, copy loss, structural variation or high Exon 10-spliced transcript of *CRBN* correlates with outcome to Pomalidomide in a LEN-refractory cohort.

High Exon 10-spliced transcript correlates with outcome to induction therapy in newly-diagnosed myeloma.

(a) Progression free survival of those who carried the four types of CRBN aberration versus those unaffected, in the LEN-refractory cohort exposed to POM-based therapy.

(b) Progression free survival of those carrying ≥ 1 of any type of CRBN aberration versus those unaffected, in the LEN-refractory cohort exposed to POM-based therapy.

(c) Association of high Exon 10-spliced CRBN transcript with PFS in the ND cohort undergoing induction therapy.

(d) Overall survival of those carrying ≥ 1 of any type of CRBN aberration versus those unaffected, in the LEN-refractory cohort exposed to POM-based therapy.

(e) Association of high Exon 10-spliced CRBN transcript with OS in the ND cohort undergoing induction therapy.

Comparison of survival curves by log-rank testing in all cases.