

## LETTERS TO THE EDITOR

Absence of mutations in cereblon (*CRBN*) and DNA damage-binding protein 1 (*DDB1*) genes and significance for IMiD therapy

*Leukemia* (2014) 28, 1129–1131; doi:10.1038/leu.2013.315

Thalidomide and the IMiD immunomodulatory drugs, lenalidomide and pomalidomide, are widely used in the treatment of multiple myeloma (MM), del(5q) myelodysplastic syndromes and other hematologic malignancies, including mantle cell lymphoma. Ito *et al.*<sup>1</sup> recently identified cereblon as a key target of thalidomide. Subsequent studies confirmed cereblon to be a common target for lenalidomide and pomalidomide, and established its essential role in mediating anticancer and immunomodulatory effects of these drugs.<sup>2,3</sup> Cereblon is encoded by the *CRBN* gene on chromosome 3 containing 11 exons, and the fully spliced transcript produces a 51-kDa protein. Cereblon is a component of the cullin ring E3 ubiquitin ligase complex (CRL4<sup>CRBN</sup>) that also contains DNA damage-binding protein 1 (DDB1), cullin (Cul) 4a and regulator of cullins (Roc) 1.<sup>1</sup> E3 ligases attach ubiquitin moieties to specific substrate proteins in the cell that can mark them for proteasomal degradation. The putative role of cereblon within the E3 ligase complex is that of a substrate receptor.

With the discovery of cereblon, as a target of IMiD therapy, there has been considerable interest in defining whether expression of cereblon protein or the presence of *CRBN* mutations will impact clinical responses to these drugs.<sup>2,4–7</sup> There are limited available data on *CRBN* gene mutation in the literature. Originally, a nonsense mutation (R419X) of *CRBN* was described to be associated with autosomal recessive non-syndromic mental retardation.<sup>8</sup> However, the functional link between the mutation in *CRBN* and the onset of mental retardation has not been demonstrated. Sequencing analyses of *CRBN* in MM cells from patients identified a truncating mutation (Q99X) and a point mutation (R283K) in 1 of 30 MM patients.<sup>9</sup> In addition, an A/G polymorphism has been identified at –29 nucleotide from the transcriptional start site of the *CRBN* transcript.<sup>10</sup> So far, mutations in other components of the CRL4 E3 ligase complex (DDB1, Cul4a or Roc1) in MM cell lines or patients have not been described in the limited genome-wide sequencing of MM patients.<sup>11</sup>

Here, we focused on sequencing the exons of *CRBN* with a goal of identifying missense or nonsense mutations with likely functional and clinical consequence. We analyzed IMiD-sensitive, intrinsically IMiD-resistant, as well as isogenic-sensitive or acquired lenalidomide- and/or pomalidomide-resistant MM cell lines. In addition, 90 MM patient samples, including those from 36 lenalidomide-resistant patients, were evaluated for the presence of *CRBN* mutations. As shown in Table 1, we found that the vast majority of IMiD-sensitive cell lines harbored the wild-type *CRBN* gene sequence. Also, none of the three intrinsically resistant cell lines (LP1, RPMI 8226 or JLN3) carried any mutation within the *CRBN* exons. As described previously, all three cell lines express high levels of cereblon transcript and protein.<sup>12</sup> Thus, the lack of mutation in the *CRBN* gene strongly suggests the existence of a

cereblon-independent mechanism(s) of intrinsic resistance to IMiD drugs in the LP1, RPMI 8226 and JLN3 cell lines. In contrast, a heterozygous *CRBN* mutation (D249Y) was detected in the lenalidomide-resistant ANBL-6 cell line, while its sensitive parental line did not harbor the mutation. The location of the mutation suggests that it may impact the binding of cereblon to the drug or interacting partner DDB1. However, it is not clear whether the resistant phenotype in the lenalidomide-resistant ANBL-6 cell line is a direct result of the *CRBN* mutation alone and therefore it requires further evaluation. One copy of *CRBN* gene was shown to be deleted in the MM1S and MM1S.R MM cell lines.<sup>2</sup> However, in our sequencing of the *CRBN* gene in these cell lines, we did not find any missense mutation or single-nucleotide variations (SNVs). SNVs, as opposed to missense or nonsense mutations, are synonymous substitutions of nucleotides that do not change the amino acid at a given codon. In all, we found two SNVs in the KMS-12-BM (rs17027638) and OPM-2 cell lines, respectively. The SNV in OPM-2 has not been described in the public database. Among the isogenic sensitive and resistant pairs of cell lines, no new mutation or polymorphic changes were detected.

We next sequenced the *CRBN* gene from 90 MM patients. All samples collected in this study followed institutional procedures for ethical guidelines and informed consent for the analysis. Samples used for the sequencing analysis were either CD138<sup>+</sup> cells isolated from bone marrow aspirates ( $n=36$ ) or bone marrow mononuclear cells (BMMC;  $n=54$ ) with a plasma cell content of at least 20%. The patients were further divided according to disease status: newly diagnosed ( $n=24$ ); relapsed/refractory ( $n=30$ , 19 of whom were heavily pretreated with multiple regimens that included lenalidomide); or relapsed and/or refractory with lenalidomide resistance ( $n=36$ ; clinical criteria for resistance were progression within two cycles or relapse within 6 months of completing the last line of an IMiD-based regimen). In the 24 newly diagnosed patients, two SNVs were detected, and in the 66 relapsed and refractory patients two SNVs were also detected: one of the SNVs was identified in four cases at position 735 (T>C; Y245Y) that was seen previously in the cell lines and the other SNV was found once at 219 (C>T; H73H). Both SNVs have been previously described in public databases (<http://www.ncbi.nlm.nih.gov/projects/SNP/>). Strikingly, we did not find any mutation in the *CRBN* gene in any of the lenalidomide-resistant patient samples.

As DDB1 interaction with cereblon is critical for the E3 ligase function, the *DDB1* gene may potentially harbor mutations of clinical significance. So, we extended our sequencing analysis to *DDB1* to search for the presence of mutations in MM cell lines and patients. Of more than 20 cell lines tested, only a single *DDB1* heterozygous mutation (E303D) was identified in the ANBL-6 parental cell line (Table 1). There is no known functional consequence of this mutation, as ANBL-6 is sensitive to lenalidomide. In the 54 BMMC patient samples tested from newly diagnosed and relapsed and refractory patients, two different SNVs were detected: One of the SNVs occurred six times at

**Table 1.** Summary of mutations and SNVs in *CRBN* and *DDB1* in MM cell lines and patients

<i>IMiD-sensitive MM cell lines</i>	<i>CRBN nucleotide position and change</i>	<i>CRBN amino-acid position and change</i>	<i>DDB1 nucleotide position and change</i>	<i>DDB1 amino-acid position and change</i>
H929 parental, U266, EJM, SKMM2	None		None	
OPM-2	G1209A silent heterozygous	T403T	None	
KMS-12-BM parental	T735C silent homozygous	Y245Y	None	
<i>Intrinsic IMiD-resistant MM cell lines</i>				
LP1	None		None	
RPMI 8226	None		None	
JJN3	None		None	
<i>Paired sensitive and acquired resistant cell lines</i>				
H929 sensitive control	None		None	
H929 4 resistant clones	None		None	
KMS-12-BM sensitive control	T735C silent homozygous	Y245Y	None	
KMS-12-BM LEN-resistant	T735C silent homozygous	Y245Y	None	
KMS-12-BM POM-resistant	T735C silent homozygous	Y245Y	None	
MM1S sensitive	None		None	
MM1S/R10R LEN-resistant	None		None	
ANBL-6 sensitive	None		A909T heterozygous	E303D
	None		C153T silent heterozygous	P51P (rs2230356)
ANBL-6 LEN-resistant	G745T heterozygous	D249Y	A909T heterozygous	E303D
	None		C153T silent heterozygous	P51P (rs2230356)
<i>Patient samples (90)</i>				
Newly diagnosed (24)	T735C silent homozygous, C219T silent heterozygous	2 × Y245Y (rs17027638), 1 × H73H	C153T silent heterozygous, C939T silent heterozygous	2 × P51P (rs2230356), 1 × C313C (rs150106100)
RRMM (30)	T735C silent homozygous	2 × Y245Y (rs17027638)	C153T silent heterozygous	4 × P51P (rs2230356)
LEN-resistant RRMM (36)	None		None	

Abbreviations: CRBN, cereblon; DDB1, DNA damage-binding protein 1; IMiD, immunomodulatory drug; LEN, lenalidomide; MM, multiple myeloma; POM, pomalidomide; RRMM, relapsed/refractory multiple myeloma; SNV, single-nucleotide variation.

nucleotide position 153 (C>T; P51P) and the other once at nucleotide position 939 (C>T; C313C). Both SNVs have been described in the dbSNP database (<http://www.ncbi.nlm.nih.gov/projects/SNP/>).

There are a number of implications of the sequencing analyses presented here. Although the number of cell lines and patients tested is limited, our findings suggest that incidence of *CRBN* and *DDB1* gene mutations in MM cell lines and patients are rare and will have a limited impact on defining resistance to IMiD therapy. In the three intrinsically IMiD-resistant cell lines that clearly express detectable levels of cereblon, the absence of *CRBN* and *DDB1* mutations suggest that potential cereblon-independent mechanisms of resistance exist. We did, however, detect a mutation in the lenalidomide-resistant ANBL-6 cell line. Thus, the development of lenalidomide resistance in the ANBL-6 cell line may be linked to a *CRBN* mutation and provides the proof of principle that resistance to IMiD exposure could arise by a mutation within the *CRBN* gene. Finally, unlike for ATP-dependent kinase inhibitors, where there is precedence for development of drug resistance due to selection of mutant forms of the drug targets, this does not appear to be a common mechanism for IMiD therapy.

It has been shown by us and others that cereblon levels (measured by gene expression or protein assays) are lower compared with pretreatment levels when cell lines or patients become resistant to lenalidomide or pomalidomide.<sup>2,13</sup> It is likely that epigenetic, transcriptional and/or posttranscriptional mechanisms—rather than *CRBN* mutation—are predominantly involved in the development of *CRBN*-dependent mechanisms of resistance to IMiD-based therapy. Therapeutic intervention to overcome IMiD resistance may have to address the consequences arising from these alternative mechanisms.

#### CONFLICT OF INTEREST

Anjan Thakurta, Anita K Gandhi, Michelle F Waldman, Chad Bjorklund, Yuhong Ning, Derek Mendy, Peter Schafer, Antonia Lopez-Girona and Rajesh Chopra are employees

of Celgene Corporation. Suzanne Lentzsch receives research funding from Celgene Corporation, is a consultant for Onyx Corporation and Celgene Corporation, and has received honoraria from Novartis. Steve A Schey is a consultant for and receives honoraria from Celgene Corporation. Robert Z Orlowski is a consultant for and receives honoraria from Abbott Laboratories, Array BioPharma, Bristol-Myers Squibb Company, Celgene Corporation, Millennium Pharmaceuticals and Onyx Pharmaceuticals; and receives research funding from Celgene Corporation, Millennium Pharmaceuticals and Onyx Pharmaceuticals. The remaining authors declare no conflict of interest.

#### ACKNOWLEDGEMENTS

This study, as well as editorial assistance from Stacey Garrett, PhD (MediTech Media), was supported by Celgene Corporation.

#### AUTHOR CONTRIBUTIONS

Experiments conceived by AT, RC, DM, ALG, SL, SS, HAL, and PS; experiments conducted by AG, MFW, CB, DM, ALG, RC, and AM; data analysis by YC, AM, AT, AG, RC, and YN; manuscript written by AT and RC; reviewed by SS, SL, YC, PS, YN, RO, CB, and HAL.

A Thakurta<sup>1</sup>, AK Gandhi<sup>1</sup>, MF Waldman<sup>1</sup>, C Bjorklund<sup>1</sup>, Y Ning<sup>1</sup>, D Mendy<sup>1</sup>, P Schafer<sup>1</sup>, A Lopez-Girona<sup>1</sup>, S Lentzsch<sup>2</sup>, SA Schey<sup>3</sup>, Y Calle<sup>3</sup>, R Chelliah<sup>3</sup>, RZ Orlowski<sup>4</sup>, A Madan<sup>5</sup>, H Avet-Loiseau<sup>6</sup> and R Chopra<sup>1</sup>

<sup>1</sup>Translational Development, Celgene Corporation, Summit, NJ, USA;

<sup>2</sup>Department of Medicine, College of Physicians and Surgeons, Columbia University, New York, NY, USA;

<sup>3</sup>Department of Haemato-oncology, King's College Hospital and King's College London, London, UK;

<sup>4</sup>Department of Lymphoma and Myeloma, The University of Texas MD Anderson Cancer Center, Houston, TX, USA;

<sup>5</sup>Covance Genomics Laboratory, Seattle, WA, USA and

<sup>6</sup>Unité de Génomique du Myélocome, University Hospital, Toulouse, France

E-mail: rachopra@celgene.com

## REFERENCES

- Ito T, Ando H, Suzuki T, Ogura T, Hotta K, Imamura Y *et al.* Identification of a primary target of thalidomide teratogenicity. *Science* 2010; **327**: 1345–1350.
- Zhu YX, Braggio E, Shi CX, Bruins LA, Schmidt JE, Van Wier S *et al.* Cereblon expression is required for the anti-myeloma activity of lenalidomide and pomalidomide. *Blood* 2011; **118**: 4771–4779.
- Lopez-Girona A, Mendy D, Ito T, Miller K, Gandhi AK, Kang J *et al.* Cereblon is a direct protein target for immunomodulatory and antiproliferative activities of lenalidomide and pomalidomide. *Leukemia* 2012; **26**: 2326–2335.
- Broyl A, Kuiper R, van Duin M, van der Holt B, el Jarari L, Bertsch U *et al.* High cereblon expression is associated with better survival in patients with newly diagnosed multiple myeloma treated with thalidomide maintenance. *Blood* 2013; **121**: 624–627.
- Heintel D, Rocci A, Ludwig H, Bolomsky A, Caltagirone S, Schreder M *et al.* High expression of cereblon (CRBN) is associated with improved clinical response in patients with multiple myeloma treated with lenalidomide and dexamethasone. *Br J Haematol* 2013; **161**: 695–700.
- Lode L, Amiot M, Maiga S, Touzeau C, Menard A, Magrangeas F *et al.* Cereblon expression in multiple myeloma: not ready for prime time. *Br J Haematol* 2013; **163**: 282–284.
- Schuster SR, Kortuem KM, Zhu XY, Braggio E, Shi CX, Bruins LA *et al.* The clinical significance of cereblon in multiple myeloma. *Leuk Res*; e-pub ahead of print 5 September 2013; doi:10.1016/j.leukres.2013.08.015.
- Higgins JJ, Pucilowska J, Lombardi RQ, Rooney JP. A mutation in a novel ATP-dependent Lon protease gene in a kindred with mild mental retardation. *Neurology* 2004; **63**: 1927–1931.
- Egan JB, Kortuem KM, Kurdoglu A, Izatt T, Aldrich J, Reiman R *et al.* Extramedullary myeloma whole genome sequencing reveals novel mutations in Cereblon, proteasome subunit G2 and the glucocorticoid receptor in multi drug resistant disease. *Br J Haematol* 2013; **161**: 748–751.
- Sardnal V, Rouquette A, Kaltenbach S, Bally C, Chesnais V, Leschi C *et al.* A G polymorphism in the CRBN gene acts as a biomarker of response to treatment with lenalidomide in low/int-1 risk MDS without del(5q). *Leukemia* 2013; **27**: 1610–1613.
- Chapman MA, Lawrence MS, Keats JJ, Cibulskis K, Sougnez C, Schinzel AC *et al.* Initial genome sequencing and analysis of multiple myeloma. *Nature* 2011; **471**: 467–472.
- Gandhi AK, Avet-Loiseau H, Waldman M, Thakurta A, Aukerman SL, Chen G *et al.* Detection and quantification of cereblon protein and mRNA in multiple myeloma cell lines and primary CD138<sup>+</sup> multiple myeloma cells. *Blood* 2012; **120**: (abstract 4043).
- Gandhi AK, Mendy D, Waldman M, Chen G, Rychak E, Miller K *et al.* Measuring cereblon as a biomarker of response or resistance to lenalidomide and pomalidomide requires use of standardized reagents and understanding of gene complexity. *Br J Haematol*. e-pub ahead of print 28 October 2013, doi:10.1111/bjh.12622.



This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivs 3.0 Unported License. To view a copy of this license, visit <http://creativecommons.org/licenses/by-nc-nd/3.0/>

## What does MRD in leukemia really mean?

*Leukemia* (2014) **28**, 1131 doi:10.1038/leu.2013.318

The term minimal residual disease (MRD) is a leftover from an era when techniques to measure residual leukemia were rather insensitive by today's standards. In acute myeloid leukemia, for example, the major definition of a complete remission in the 1970s was based on light microscopy and required the levels of blood cells to be normal and the bone marrow to contain fewer than 5% immature myeloid cells (blasts). However, there was no way to know whether these bone marrow cells were normal or leukemic. An important development was the advent of monoclonal antibodies and fluorescence-activated cell sorting that allowed the hematologist to make this distinction with reasonable accuracy. With this advance, some persons previously classified as having MRD could be shown to have many residual leukemia cells whereas others had none.

In the 1980s, complete response in chronic myeloid leukemia (CML) was defined as the absence of bone marrow cells with the Ph-chromosome, namely complete cytogenetic response. However, the sensitivity of this criterion depended on how many metaphases were studied. For example, if only 20 or 30 metaphases were examined there was a substantial likelihood of not detecting any leukemia in a sample where the Ph chromosome positive cells were 15% or lower.

Identification of the BCR-ABL fusion gene led to the design of a reverse transcriptase (RT)-PCR that could detect low levels of CML-specific transcripts. This proved useful for detecting low levels of leukemia in persons previously classified as having achieved a complete cytogenetic remission despite the fact that transcript numbers may not directly equate to numbers of residual leukemia cells. Measuring transcripts has, however, proved

valuable in monitoring responses in CML treated with tyrosine kinase inhibitors. Molecular methods are gaining an increasingly important role in monitoring persons with other forms of leukemia.

It must be apparent from this brief résumé that number of residual leukemia cells detected after treatment depends on the efficacy of the treatment and on sensitivity of the technique used to detect them. Consequently, use of the term MRD has no precise meaning for a given person if these parameters are not specified. Put otherwise, what precisely is the meaning of 'minimal' in minimal residual leukemia without these parameters?

We propose that if the term is MRD is used in the future, there should be qualifier indicating the methodology (for example, RT-PCR, genomic PCR or immune phenotyping) and the sensitivity of the assay (for example  $10 < E-3$  or  $< 10E-4$ ). The acronym MRD could then refer to 'measurable residual disease' in a given patient (or better perhaps be dropped altogether).

### CONFLICT OF INTEREST

The authors declare no conflict of interest.

### ACKNOWLEDGEMENTS

This work was supported by funding from the NIMR Biomedical Research Centre.

JM Goldman\* and RP Gale  
*Haematology Research Centre, Division of Experimental Medicine,  
 Department of Medicine, Imperial College London, London, UK  
 E-mail: robertpetergale@gmail.com*  
 \*Deceased.