**LETTERS TO THE EDITOR**

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

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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. 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. 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 (CRL4CRBN) that also contains DNA damage-binding protein 1 (DDB1), cullin (Cul) 4a and regulator of cullins (Roc) 1. 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. 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. 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. In addition, an A/G polymorphism has been identified at —29 nucleotide from the transcriptional start site of the CRBN transcript. 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.

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 JJN3) carried any mutation within the CRBN exons. As described previously, all three cell lines express high levels of cereblon transcript and protein. 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 JJN3 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. 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+ 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

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It has been shown by us and others that cereblon levels are predominantly CRBN 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 IMiD exposure could arise by a mutation within the CRBN mutation—and provides the proof of principle that resistance to lenalidomide resistance in the ANBL-6 cell line may be linked to a CRBN amino-acid position change.

**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.

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**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 Thakurta1, AK Gandhi1, MF Waldman1, C Bjorklund1, Y Ning1, D Mendi1, P Schafer1, A Lopez-Girona1, S Lentzsch2, SA Schey1, Y Calle3, R Chelliah3, RZ Orlowski4, A Madan5, H Avet-Loiseau6 and RC Chopra1

1Translational Development, Celgene Corporation, Summit, NJ, USA; 2Department of Medicine, College of Physicians and Surgeons, Columbia University, New York, NY, USA; 3Department of Haemato-oncology, King’s College Hospital and King’s College London, London, UK; 4Department of Lymphoma and Myeloma, The University of Texas MD Anderson Cancer Center, Houston, TX, USA; 5Covance Genomics Laboratory, Seattle, WA, USA and 6Unité de Génomique du Myélome, University Hospital, Toulouse, France

E-mail: rachopra@celgene.com

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

| IMiD-sensitive MM cell lines | CRBN nucleotide position and change | CRBN amino-acid position and change | DDB1 nucleotide position and change | DDB1 amino-acid position and change |
|-----------------------------|-------------------------------------|-------------------------------------|-------------------------------------|-------------------------------------|
| H929 parental, U266, EJM, SKM2 | None | T403T | None | None |
| KMS-12-BM parental | G1209A silent heterozygous | Y245Y | None |

**Intrinsic IMiD-resistant MM cell lines**

| LP1 | None | None | None |
| RPMI 8226 | None | None | None |
| JJN3 | None | None | None |

**Paired sensitive and acquired resistant cell lines**

| H929 sensitive control | None | None | None |
| H929 4 resistant clones | None | 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 |
| MM15 sensitive | None | None | None |
| MM15/R108 LEN-resistant | None | None | None |
| ANBL-6 sensitive | None | None | None |
| ANBL-6 LEN-resistant | G745T heterozygous | D249Y | None |

**Patient samples (90)**

| Newly diagnosed (24) | T735C silent homozygous, C219T silent heterozygous | 2 × Y245Y (rs17027638), 1 × H73H | C153T silent heterozygous, C939T silent heterozygous | 4 × PS1 (rs2230356), 1 × C313C (rs150106100) |
| RRMM (30) | T735C silent homozygous | 2 × Y245Y (rs17027638) | C153T silent heterozygous | 2 × PS1 (rs2230356), 1 × C313C (rs150106100) |

**LEN-resistant RRMM (36)**

| None | 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.
What does MRD in leukemia really mean?

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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.

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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.