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LYMPHOID NEOPLASIA

Targeted sequencing of refractory myeloma reveals a high incidence of mutations in *CRBN* and Ras pathway genes

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Key Points

- The incidence of mutations within the MAPK pathway, the CRBN pathway, and *TP53* is significantly increased in drug-refractory MM.
- Mutations in *CRBN* might contribute to IMiD resistance in drug-refractory MM.

In this study, targeted sequencing to screen 50 multidrug refractory multiple myeloma (rMM) patients was performed by using the Multiple Myeloma Mutation Panel. Patients were pretreated with both immunomodulatory drugs (IMiDs) and proteasome inhibitors (PIs), and 88%, 78%, and 68% were refractory to an IMiD, a PI, or both, respectively. The majority of patients had progressive (82%) or refractory (78%) disease immediately before sampling, with 43% being IMiD refractory and 46% being PI refractory in the most recent line of therapy. Compared with newly diagnosed MM, an increased prevalence of mutations in the Ras pathway genes *KRAS*, *NRAS*, and/or *BRAF* (72%), as well as *TP53* (26%), *CRBN* (12%), and CRBN pathway genes (10%) was observed. Longitudinal analyses performed in 3 patients with *CRBN* mutations at time of IMiD resistance confirmed that these mutations were undetectable at earlier, IMiD-sensitive time points. Furthermore, the functional introduction of these mutations in MM cells conferred lenalidomide resistance in vitro. These data indicate a differential genetic landscape in rMM associated with drug response. (*Blood*. 2016;128(9):1226-1233)

Introduction

Significant efforts have recently been made to better understand the genetic landscape of multiple myeloma (MM), with more than 1000 MM exomes sequenced to date.[1-5] However, most of the data have been obtained from untreated or heterogeneously treated cohorts. Recurrent mutations have been identified only in a limited number of genes, a subset of which seems to affect survival.[1,6,7] Patterns of clonal evolution were identified; however, these findings are still based on a relatively small number of intensively longitudinally studied patients.[2,8-10]

Although the novel agents, namely immunomodulatory drugs (IMiDs) and proteasome inhibitors (PIs) paved the road toward a prolonged survival and durable responses, most MM patients eventually relapse and become drug refractory. The mechanisms underlying resistance to IMiDs, PIs, and corticosteroids, however, remain poorly understood. Cereblon (*CRBN*) was recently identified as being essential for the anti-MM activity of IMiDs.[11-18] To date, the reported incidence of mutations in proteasome subunits, the CRBN pathway, or the steroid receptor is low, and mutations in these genes, except in single case studies[19,20] or in vitro cell line analyses,[20,21] have not yet been identified as a major source of primary or acquired drug resistance in larger MM data sets.

The acquisition or the selection of mutants under the selective pressure of targeted therapy has been shown be a key driver of the development of drug resistance in other hematologic malignancies (eg, BCR-ABL mutations in imatinib-treated chronic myeloid leucemia[22] or *BTK* mutations in chronic lymphocytic leukemia patients treated with the selective inhibitor ibrutinib).[23] Of note, genomic data from large series of homogeneously pretreated patients with refractory MM (rMM) are still missing.

Therefore, in this study, 88 frequently mutated or drug-resistance pathway MM genes with a focus on genes relevant to IMiD and PI interactions were investigated in a cohort of 50 heavily pretreated rMM patients by using our updated disease-specific Multiple Myeloma Mutation Panel (M^3P v3.0). Our approach yielded rapid, robust, and sensitive variant screening and allowed for the detection of small subclonal mutations. Strikingly, we were able to identify mutations in potentially targetable genes and in genes related to drug resistance in the majority of patients.

Patients and methods

Patients

Between November 2013 and April 2015, samples from 50 rMM patients were collected at the University Hospital Heidelberg (Heidelberg, Germany). Samples

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were obtained from bone marrow aspirates in 44 of the patients. In 6 patients with extramedullary disease, tumor DNA was obtained from tissue block (n = 2; patients #1 and #7) and from peripheral blood (n = 4; secondary plasma cell leukemia; #2, #3, #16, and #17). In all but one patient (#30), the corresponding germ line was available for analysis, and in 3 patients with acquired IMiD resistance at tumor sampling (#12, #24, and #32), prior samples from earlier IMiD-sensitive disease were available for comparative analysis. Patients in our study had received a minimum of 2 previous lines of therapy and had been exposed to at least one PI and one IMiD. All patients gave written informed consent to the sampling, analysis of tumor and germ line probes, and collection of clinical data in accordance with the Declaration of Helsinki. Data collection and analysis were approved by the institutional ethics committee (Heidelberg #229/2003, S-152/2010).

Collection of clinical data

Clinical data were collected retrospectively from internal digital and external paper patient records. Refractory disease was defined according to International Myeloma Working Group criteria[24] as either progressive disease (PD) under therapy or within 60 days after discontinuation of therapy.

Sample preparation

Mononuclear cells were isolated by density centrifugation (Biocoll Separating Solution; Biochrom GmbH, Berlin, Germany). Magnetic cell separation of CD138$^+$ and CD138$^-$ subsets from the mononuclear cells was performed by using Microbeads CD138-Human and Manual MACS Cell Separation Units (Miltenyi Biotec, Bergisch Gladbach, Germany). Sample purity was assessed by flow cytometry to be >85%. The CD138$^-$ subsets were further cultured to obtain the stroma cells. Sample purity of stroma cells was assessed by flow cytometry to contain <1% CD138$^+$ cells. Sample purity of tissue blocks was estimated by the infiltration rate of clonal plasma cells assessed by immunohistochemistry for CD138. DNA from isolated cells was extracted by using the QIAamp DNA Mini Kit (QIAGEN GmbH, Hilden, Germany). DNA extraction from tissue blocks was performed at the Institute of Pathology (Heidelberg, Germany) as previously described.[25] Germ line controls for sequencing studies were obtained from stroma cells, as described above, or from buccal swabs, which were processed by using the blackPREP Swab DNA Kit (Analytikjena; Biometra, Berlin, Germany) and stored at −20°C.

Targeted sequencing

Targeted sequencing was performed on the personal genome machine (Thermo-Fisher, Waltham, MA) by using 20 ng DNA from flow-sorted CD138$^+$ plasma cells as well as from corresponding germ line samples. IonOneTouch automated systems were used to generate and enrich 400 bp libraries. Our 185-kb customized M^{3}P v3.0 includes 1327 amplicons covering the coding regions of 88 disease-specific genes (supplemental Table 1, available on the *Blood* Web site). We selected key genes in pathways previously identified as being important in MM (MAPK, nuclear factor κB, interleukin-6, cell cycle, MYC) as well as genes known to be expressed in MM and being recurrently mutated in >2% of patients in previously published genome-wide MM studies. Furthermore, we selected genes that provide a potential drug target and genes that have been associated with drug resistance to the most commonly used drug families (IMiDs, PIs, and corticosteroids). Three paired tumor and germ line samples were barcoded by using the Ion Xpress Barcode Adapters, pooled, and sequenced on a 318TM chip by using the Ion PGM Hi-Q Seq Kit (Thermo-Fisher). An average of ~800× depth sequencing coverage was generated per nucleotide. Mutation analysis was performed by using Ion Reporter Software v4.4 (Thermo-Fisher), and further data curation was performed by using the Biological Reference Repository (BioR, Mayo Clinic, Rochester, MN).[26] BioR includes gene annotations that use various database resources, including National Center for Biotechnology Information/Ensembl; University of California, Santa Cruz (UCSC); Single Nucleotide Polymorphism Database (dbSNP); HapMap; 1000 Genomes Project; the Catalogue of Somatic Mutations in Cancer (COSMIC; Wellcome Trust Sanger Institute); SIFT (J. Craig Venter Institute)[27]; and PolyPhen-2 (Harvard Medical School).[28] Germ line variants were filtered out by comparing each tumor sample with its germ line counterpart. We then filtered synonymous variants and variants identified outside exonic and splicing site regions and visualized the remaining calls by using the Integrative Genomics Viewer (Broad Institute, Cambridge, MA). Finally, we eliminated from further analyses the variants that were either supported with unidirectional reads, covered with <20× depth, or found in <3% of reads in the tumor sample. For the sample for which we had no germ line sample available, we applied the same filters; however, we included only those findings that were listed in the COSMIC database and excluded those that were present in dbSNP or in 1000 Genomes Project with a prevalence of more than 1%.

For the comparative analysis of longitudinal samples, we used a specific 400 bp gene panel containing a reduced number of 24 amplicons including *CRBN* and batched 3 CD183 tumor samples on a 318 chip. This allowed us to ensure the maximum sensitivity of the personal genome machine technology (~10^{-3}). All sequencing data are deposited in the Sequence Read Archive database (http://www.ncbi.nlm.nih.gov/sra); study accession number is SRP066263.

Site-directed mutagenesis for functional *CRBN* mutation analysis

To establish mutant *CRBN*-expressing OCI-MY5 cell lines, we created lentivector pCDHPuroCRBN by subcloning wild-type (wt) CRBN from pCDHGFPCRBN and used this pCDHPuroCRBN as a template to introduce CRBN mutations by polymerase chain reaction (see supplemental Data for details). The ligated plasmid was amplified and isolated by using a Miniprep Plasmid Isolating Kit (QIAGEN) and then cloned into lentivector pCDH-CMV-MCS-EF1-Puro (SBI, Mountain View, CA) and packaged into a lentivirus. The lentivirus constructs were then used to infect OCI-MY5 myeloma cells to overexpress mutant CRBN protein. Cell viability was assessed in triplicate by using 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolium bromide (MTT) after a 5-day incubation time with increasing doses of lenalidomide.

Results

Patient characteristics and previous therapies

Baseline characteristics of the patient cohort are described in Table 1. Cytogenetic results were available in 39 patients (78%) and included gain 1q21 (>2 copies), deletion 17p, and translocations t(4;14) and t(14;16) in 62%, 33%, 13%, and 8% of screened patients. Overall, 82% of patients carried at least 1 adverse cytogenetic aberration (Figure 1). Patients received a median of 5 prior therapies before tumor sampling (range, 2 to 15 prior therapies). All patients were exposed to at least 1 IMiD and 1 PI, and 88%, 78%, and 68% of patients were refractory to at least 1 IMiD, PI, or PI/IMiD, respectively. At time of sampling, 82% of patients had PD and 78% were refractory to the last line of treatment. The median time between PD and sampling was 12 days (range, 0 to 164 days). Previous therapies and refractory status immediately before sampling are described in Table 1 and displayed in Figure 1.

Detected mutations with M^{3}P

In our cohort of 50 patients, we detected 136 mutations, resulting in an average mutation incidence of 2.7 per patient (range, 0 to 13 mutations), of which there were 114 missense (84%), 9 nonsense (7%), 1 stop lost (1%), 11 frameshift deletions (8%), and 1 frameshift insertion (1%), and 6 were located in splice donor or acceptor sites (4%). We found mutations in at least 1 of the selected M^{3}P genes in 98% of patients, and at least 1 mutation was identified in 47 (53%) of the 88 investigated genes (Figure 1). Multiple mutations within the same gene were observed in 5 patients, 1 of whom harbored 4 mutations within the steroid receptor *NR3C1* (variant reads [VRs], 7% to 17%). Other genes harboring multiple mutations in a single patient included *BRAF* (VRs, 54%/41%), *CUL4B* (VRs, 19%/6% and 5%/5%), *NFKB2* (VRs, 26%/6%), and *TP53* (VRs, 18%/8% and 14%/8%) (supplemental Table 2). PolyPhen2,[29] SIFT,[27] and PROVEAN[30] were applied to estimate the

Table 1. Baseline patient characteristics and previous treatments

Characteristic	No.	%
Baseline	(n = 50)	
Sex		
Male		52
Female		48
Age, years		
Median at diagnosis (range)	58.8 (38.1-73.5)	
Median at sampling (range)	65.5 (44.7-85.3)	
Median time from initial treatment to sampling, years (range)	4.8 (1.4-13.8)	
Heavy chain		
IgG		60
IgA		22
Light chain disease		18
Light chain		
κ		66
λ		34
Median hemoglobin, g/dL (range)	10.6 (7.0-14.6)	
Median No. of thrombocytes per microliter (range)	104.0 (15.0-302.0)	
Median LDH, U/L (range)	230.5 (124.0-2294.0)	
Median GFR, mL/min (range)	69.4 (8.0-148.0)	
Salmon and Durie stage at diagnosis		
IIA		6
IIB		0
IIIA		78
IIIB		16
Cytogenetic analyses performed		78
Cytogenetic aberrations	(n = 39)	
Gain 1q21 (>2 copies)		62
Deletion 17p		33
t(4;14)		13
t(14;16)		8
At previous treatments	(n = 50)	
Median No. of previous lines of therapy (range)	5 (2-15)	
Prior exposure class		
PI		100
IMiD		100
Cytotoxic agent		94
HDT		90
Prior exposure agent		
Bortezomib		100
Carfilzomib		18
Thalidomide		34
Lenalidomide		100
Pomalidomide		48
Refractory class		
PI		76
IMiD		88
PI + IMiD		66
Refractory agent		
Bortezomib		72
Carfilzomib		16
Thalidomide		14
Lenalidomide		82
Pomalidomide		40
PD prior to sampling		82
Refractory to last therapy		78
Refractory to last therapy agent	(n = 39)	
Bortezomib		33
Carfilzomib		13
Lenalidomide		20
Pomalidomide		23
Bendamustine		8
Monoclonal CD38 antibody		3

Laboratory values at the time of sampling are shown.

GFR, glomerular infiltration rate; HDT, high-dose therapy; IgG, immunoglobulin G; LDH, lactate dehydrogenase; PD, progressive disease.

functional impact of each mutation. In total, predictions for 115 mutations were generated and in 93% of cases a damaging, potentially damaging, or probably damaging impact was assessed by at least 1 of the algorithms. The mutational spectrum observed was broad, with a significant number of small subclonal mutations; VRs did not reach 10% in more than a quarter (26%) of all mutations, and almost half (45%) of the mutations identified were below a VR of 25% (supplemental Table 2).

Ras pathway—*KRAS/NRAS/BRAF*

The incidence of mutations in the RAS pathway in our refractory cohort was quite high, with 36 patients (72%) having at least 1 mutation in *NRAS*, *KRAS*, and/or *BRAF*, significantly exceeding previously published frequencies in newly diagnosed patients (Figure 1).[1-4] We observed 9 *BRAF*-mutated patients (18%), with 4 (8%) having the mutation at known activating and druggable site p.Val600Glu.[31,32] In 4 patients, kinase-inhibiting *BRAF* mutations were identified, known to paradoxically activate the MAPK pathway via c-RAF (Gly466Val,[33] Gly469Arg,[34] and Asp594Asn[35] [present in 2 patients]). Two patients showed additional activating mutations in *NRAS* and *KRAS*, respectively, and 1 patient harbored 2 *BRAF* mutations (Lys499Asn and Glu695Lys) (supplemental Table 2). Finally, 2 patients (4%) with *RASA2* mutations and 1 patient with an activating mutation in *PTPN11* were identified; both of the genes had potentially activating effects on the MAPK pathway.[36,37]

TP53

We saw an increased incidence of *TP53* mutations in our patient cohort (26%), more commonly found in patients with del17p (8 [62%] of 13) compared with patients without the deletion (4 [15%] of 26) (Figure 1). Two patients harbored 2 mutations within the gene. The majority of mutations were found within subclonal ranges (9 of 15 mutations: 16% VRs or less; supplemental Table 2).

CRBN-associated mutations

Of note, the cereblon pathway, important for the anti-MM action of IMiDs,[12-14,17] was found mutated in almost one-quarter of patients (22%). This includes *CRBN* (12%), *CUL4B* (6%), *IRF4* (4%), and *IKZF1* (2%) (Figure 1; supplemental Figure 1). All *CRBN* mutations found in our cohort were detected in patients with IMiD-refractory disease (patients #5, #12, #24, #32, #34, and #38; Figure 1) as was the case for mutant CUL4B (#6, #15, and #38) and IKZF1 (#2). The mutation found in *IKZF1* (p.Ala152Thr) is located inside a sequence of 30 amino acids known from a previous study to be essential for lenalidomide sensitivity.[17] All CRBN mutations occurred at critical sites, potentially having an impact on CRBN-IMiD interactions by either truncating the protein (p.Ile393Metfs*10 [#5], p.Pro241Argfs*10 [#38], p.Gln327* [#12], and splicing acceptor c.551-2T>C [#34]; supplemental Table 2) or by being located within the thalidomide-binding domain (p.Phe381Cys [#24] and p.Pro411His [#32]) as illustrated in Figure 2.[38,39]

Furthermore, the cumulative time on IMiD treatment among the 6 *CRBN*-mutant patients (median, 1146 days; range, 154 to 1912 days) was longer compared with CRBN wt patients (median, 479 days; range, 40 to 2182 days; $P = .02$) .

In 3 *CRBN*-mutated patients with acquired resistance to IMiDs (#12, #24, and #32), earlier tumor samples collected before refractory lenalidomide or pomalidomide treatment were available (Figure 3; supplemental Figure 2). In all 3 patients, the mutation was undetectable at the earlier time point, even with an increased sequencing depth.

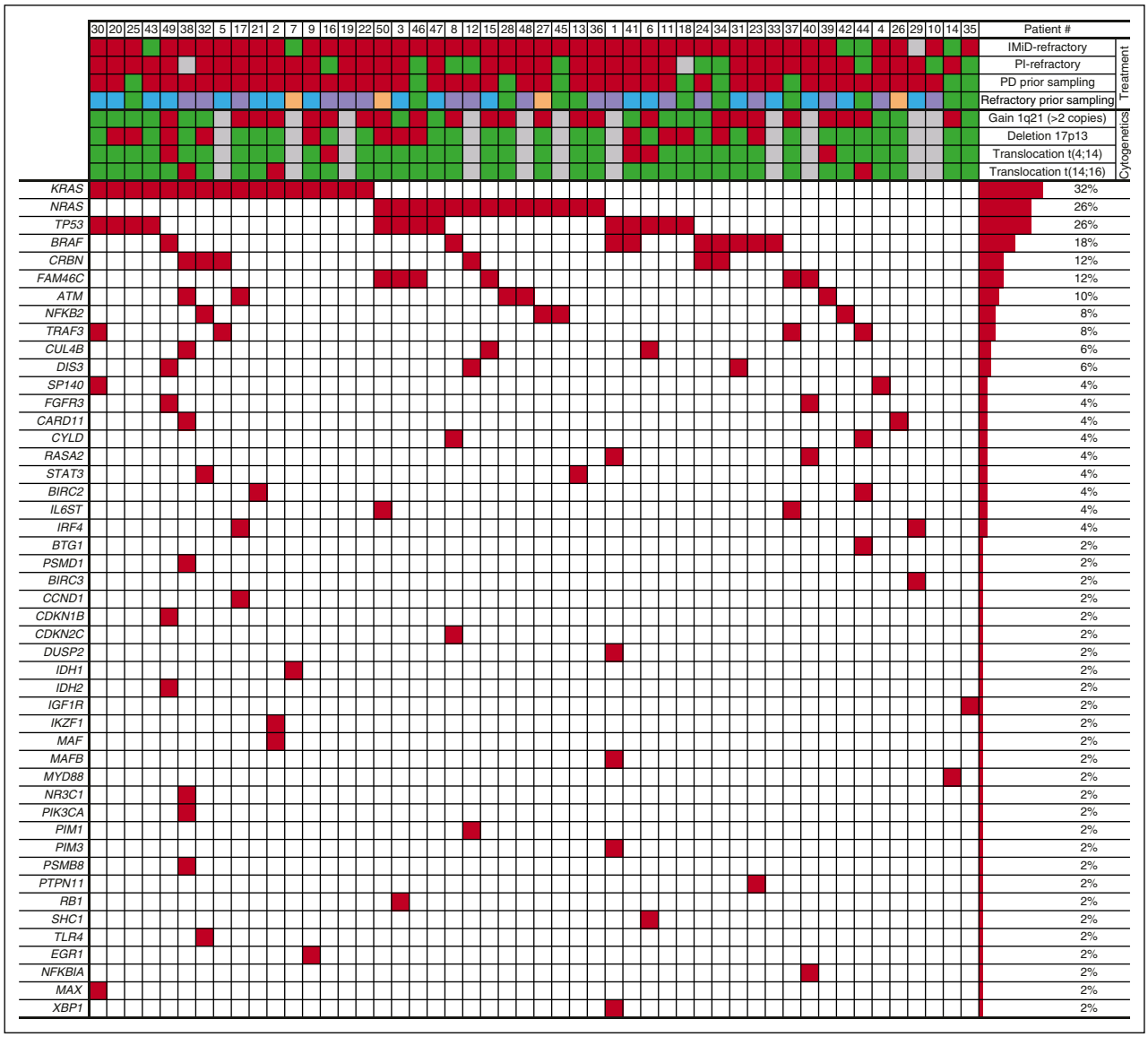

Figure 1. Genomic mutations, adverse cytogenetic aberrations, and treatment characteristics of 50 relapsed/refractory MM patients. All patients are identified by individual numbers. Refractory status to IMiDs or PIs at any time before sampling is shown as well as PD and refractory (IMiD, PI, other drug classes) status immediately before sampling. Information on the adverse cytogenetic aberrations gain 1q21 (>2 copies), deletion (17p), and translocations t(4;14) and t(14;16) are depicted. Genes mutated in at least 1 patient are displayed. Genes (from top to bottom) and patients (from left to right) are sorted according to gene mutation frequencies. Treatment characteristics and cytogenetic aberrations are color-coded: red, yes; green, no; gray, unknown/not available. Refractory drugs immediately prior to sampling are also color-coded: purple, IMiD refractory; blue, PI refractory; orange, other drug classes (including cytotoxic agents and antibodies); green, not refractory to treatment immediately prior to sampling.

To assess the impact of these 3 *CRBN* mutations on protein function and sensitivity to IMiD treatment, the mutations were introduced into the myeloma cell line OCI-MY5. OCI-MY5 is characterized by a very low expression of wt *CRBN*, rendering the cell line IMiD resistant. When wt *CRBN* is introduced and overexpressed, OCI-MY5 regains IMiD sensitivity.[12] We introduced and overexpressed all 3 *CRBN* mutations into OCI-MY5 in independent experiments, as well as the wt CRBN (positive control) and the viral vector alone (negative control). Cell viability was assessed by using an MTT assay (in triplicate) after a 6-day treatment with increasing doses of lenalidomide (Figure 4). The OCI-MY5 wt cells regained lenalidomide sensitivity, whereas the OCI-MY5 cells containing the *CRBN* mutants or the viral vector alone remained lenalidomide resistant. The expression of exogenous wt and mutated CRBN was detected by an immunoblotting assay using an anti-CRBN antibody.

PI resistance–associated mutations

Only limited data are currently available regarding mutations in proteasomal subunits associated with resistance to PI-based therapies in MM. Thus, only a limited number of genes coding for proteasomal subunits were selected for our current M[3]P design (supplemental Table 1). Mutations of *XBP1* or PI subunits (*PSMB8* and *PSMD1*) were found in 2 patients (4%) in our cohort, with 1 plasma cell leukemia patient carrying a mutation in both *PSMB8* and *PSMD1*. The response of the latter patient to PIs was unavailable. However, another patient with a p.L314Ffs*38 *XBP1* had known PI resistance at the time of tumor sampling.

Other genes with potential therapeutic implications

Besides *BRAF*, other mutations in potentially actionable genes were identified in a subset of patients, including *IDH1/2*, and *FGFR3* (Figure 1). IDH mutations are frequently found in glioma[40] or acute

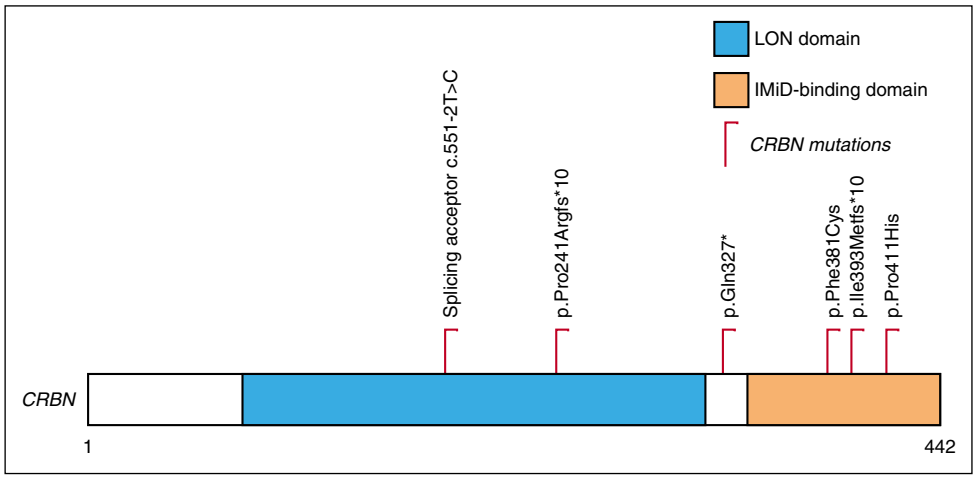

Figure 2. Localization of mutations within the *CRBN* gene. *CRBN* mutations were found in 6 patients. All mutations found in our cohort occurred at critical sites, and they had the potential to impact CRBN-IMiD interactions by truncating, affecting splicing, or being located within the IMiD-binding site of *CRBN*. Each mutation is described at the corresponding site of the CRBN protein. CRBN, cereblon; LON, long undivided filaments upon ultraviolet irradiation (homo-oligomeric ATP-dependent protease domain).

myeloid leukemia.[41] An *IDH1*-activating mutation at amino acid position 132 (p.Arg132His) found in 1 patient provides a druggable target, provided that it is of functional relevance in MM, and specific inhibitors for activating *IDH1* mutations are in advanced clinical testing.[42,43] FGFR3 has been identified as a druggable target, and we identified an activating p.Arg248Cys mutation[44,45] and an activating read-through stop lost mutation *809Cys[46] in our cohort. Various specific FGFR3-inhibiting agents are in clinical and preclinical investigation in MM. The most promising results have been obtained in solid tumors.[47,48] Furthermore, 1 FGFR3 mutation is present in a t(4;14) patient (#49), whereas the translocation status is unknown in the second patient (#40). However, whether the sequelae conferred by the translocation in general or whether specific FGFR3 mutations are essential for successful anti-FGFR3 treatment remains to be confirmed.

Discussion

Despite the fact that more than 1000 MM genomes have already been sequenced, the mutational landscape of drug-refractory MM remains largely unknown, and our understanding of drug resistance mechanisms to the most commonly used drug families is limited. We have therefore designed and established a disease-specific MM gene panel (M^3P) for rapid and sensitive variant screening in key MM genes. Our panel includes a subset of disease-specific key MM genes that we used to screen a selected cohort of 50 drug-refractory MM patients. We provide evidence that M^3P sequencing can generate valuable information within potentially meaningful timeframes for clinical decision-making in the vast majority of rMM patients.

Mutations in potentially targetable genes (*MAPK*, *FGFR3*, and *IDH1*) were detectable in 76% of patients, whereas mutations in genes affecting the prognosis as described in previous studies of newly diagnosed MM (*TP53*, *EGR1*, and *IRF4*)[1] were identified in 32% of patients. Mutations in genes potentially associated with drug response (*CRBN* pathway, *XBP1*, proteasome subunits) were identified in 24% of patients.

MAPK mutations provide a broad potential target for advanced MM patients. BRAF or MEK inhibitors are already in clinical testing and seem promising.[31,32,49,50] *NRAS* and *KRAS* mutations occurred mostly within known activating hotspots of the gene, and 4 of 9 *BRAF* mutations were druggable p.Val600Glu. However, *BRAF* mutations with paradoxical activation of the pathway via c-raf were also found in 4 patients, and in 1 of them (patient #41), 2 simultaneous *BRAF* mutations were identified: 1 at amino acid position 499 that has been suggested to be ERK activating and has been described in cardiofaciocutaneous syndrome,[51] and 1 in p.Glu695Lys, located in the protein kinase domain within the activator loop, previously reported in skin cutaneous melanoma.[52] In addition, a Gly503Arg mutation in *PTPN11,* another gene of the RAS pathway that has been reported as being frequently mutated in RAS-opathies (juvenile myelomonocytic leukemia, adult acute myelogenous leukemia, gastric cancer, glioblastoma, and anaplastic large cell lymphoma) was identified.[36,37,53] This gene is thought to play a role in acquired resistance to targeted therapy[54] and therefore represents a potential drug target. *PTPN11/SHP2* inhibitors have already been identified, and compounds are in preclinical testing.[55] Furthermore, *RASA2* was found to be mutated in 2 patients. Mutations in this gene have been described in various malignancies, including colorectal, skin, lung, and endometrial cancers; however, the biological function of the gene has not yet been fully deciphered, and it is hypothesized to be a RAS inhibitor.[56] Of note, 1 of the patients (#1) with a Val600Glu *BRAF* mutation harbored an additional nonsense mutation in *RASA2*, which may contribute to further MAPK activation in this patient.

The most remarkable finding of our study is the identification of direct mutations in *CRBN* in 12% of patients and mutations in the CRBN pathway in almost one-quarter (22%) of the cohort. Although a role of mutant *IRF4* in IMiD resistance remains to be established, *CRBN* is the central mediator of IMiD action, and its expression is essential for the anti-MM effects of IMiDs.[12-16] Notably, all *CRBN*-mutated patients (n = 6) and 10 (91%) of 11 of the CRBN pathway–mutated patients were unresponsive to IMiD-based treatment at the time of tumor sampling (the IMiD-refractory status of 1 patient was unknown) suggesting an association of these mutations with IMiD response. Thus far, *CRBN* mutations are largely absent in the publically available MM sequencing data that, of note, include mostly untreated patients. For example, within 511 of the most recently analyzed patients in the Multiple Myeloma Research Foundation (MMRF) CoMMpass trial, only 1 *CRBN* mutation (p.Pro85Ser) was identified (https://research.themmrf.org/). Similarly, in 349 untreated M^3P-sequenced patients at the Mayo Clinic, we identified only 1 *CRBN* mutation (p.Asn316Lys) located in close proximity to the IMiD-binding site of the gene. Of interest, this patient did not respond to either lenalidomide induction or subsequent pomalidomide treatment, suggesting a potential impact of that mutation on the patient's IMiD response. Although primary and secondary resistance to IMiDs is observed in MM patients, mutations in this gene or pathway as the underlying cause for IMiD resistance have not yet been described in the disease apart from a single case report of a highly

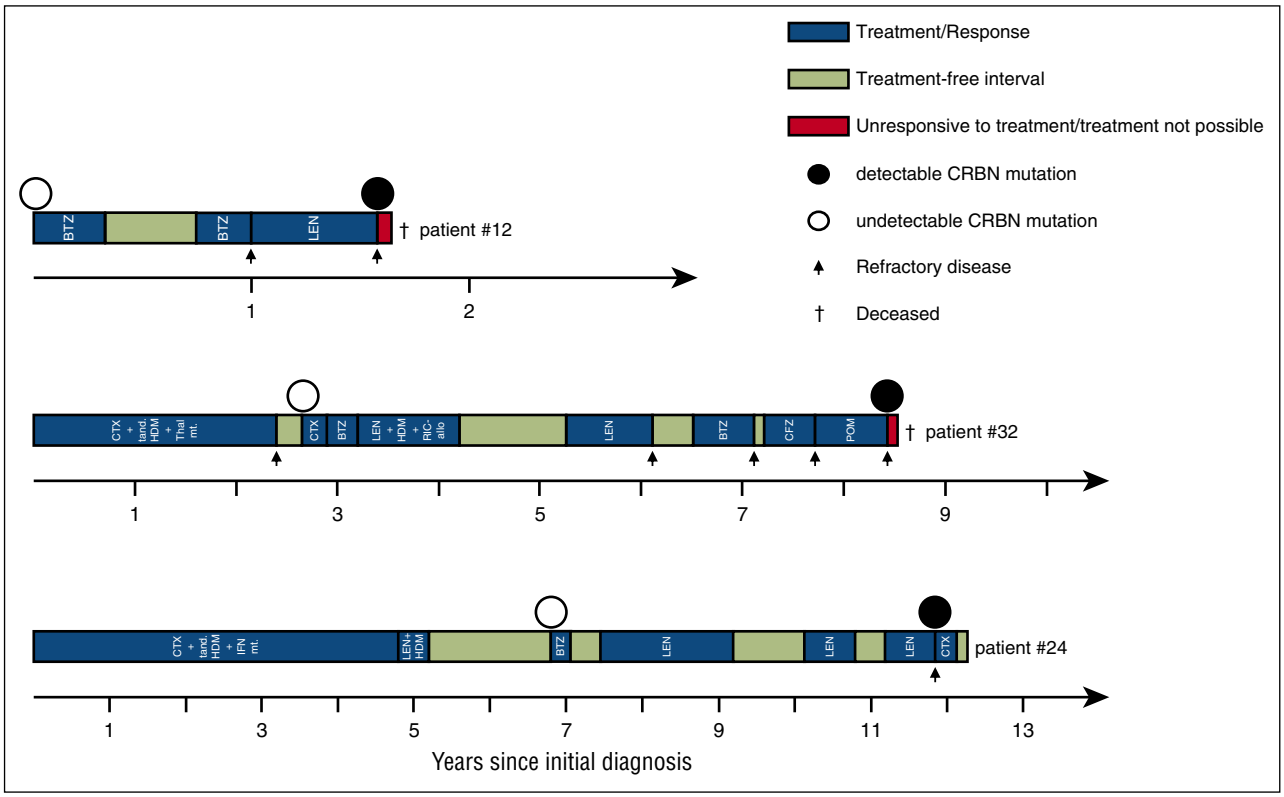

Figure 3. Clinical course and treatment history of 3 MM patients with acquired IMiD resistance. CD138-purified tumor samples were obtained after progression to an IMiD-based treatment and from earlier IMiD-responsive disease stages in each patient. A mutation in CRBN was identified at IMiD-resistant disease stage (black circles). This mutation, however, was undetectable at earlier IMiD-sensitive disease stages in all 3 patients (white circles), suggesting a correlation with the acquired IMiD resistance in these patients. Patient #12 (CRBN mutation Q327TER) was a 72-year-old female diagnosed with MM immunoglobulin G kappa (IgG κ). She was initially treated with bortezomib (BTZ), rapidly relapsed, and died as a result of PD during lenalidomide (LEN) treatment (detection of CRBN mutation). Patient #32 (CRBN mutation P411H) was a 44-year-old female diagnosed with MM IgG κ. She was treated with several chemotherapy agents, high-dose melphalan (HDM), and novel agents, including BTZ, carfilzomib (CFZ), LEN, and pomalidomide (POM), and she died during PD from POM-refractory disease (detection of CRBN mutation). Patient #24 (CRBN mutation F381C) was a 64-year-old male diagnosed with MM IgA λ. Initial treatments included tandem (tand.) HDM and interferon (IFN) maintenance. After a long disease course with several short retreatments including BTZ, LEN, and HDM, the disease became lenalidomide-refractory (detection of CRBN mutation) and conventional chemotherapy was begun. Black arrows indicate refractoriness to the indicated treatment. CTX, chemotherapy; mt., maintenance; RIC-allo, reduced-intensity conditioning allogeneic stem cell transplantation; Thal, thalidomide.

drug-resistant patient.[19] The increased incidence of *CRBN* and *CRBN* pathway–associated mutations in our cohort might be explained by cohort selection. In addition, the targeted sequencing approach allowed us to screen with increased sensitivity for variants and, in fact, 3 of the 6 CRBN mutations in our cohort were identified with VRs below 10% of the reads. In these 3 patients with subclonal *CRBN* mutations, we had earlier tumor samples available. All 3 patients had responded to IMiD treatment after earlier sampling (Figure 3) and even with increased sequencing sensitivity, we were unable to detect any *CRBN* mutations in the earlier, IMiD-sensitive phase of their disease. In addition, our data on the exposure to IMiDs before the acquisition of CRBN mutations indicated that the development of CRBN mutations might be correlated with long-term exposure to IMiDs, but this has to be taken with great caution because of the limited number of patients in this analysis. Regrettably, no prior samples were available from the other 3 patients with mutant CRBN, and larger studies on sequential assessments in patients under IMiD treatment are needed.

To further investigate the functional effect of these mutations on the response to lenalidomide, we introduced them into OCI-MY5 cells and confirmed that all 3 mutations caused in vitro IMiD resistance. These findings support a causal relationship between the appearance of these mutations and the lack of response to IMiDs in the patients. Tumor heterogeneity and the impact of subclonal somatic mutations on the response to targeted cancer therapies have been frequently discussed in relation to various cancers.[57] Of interest, in a recent publication, there was evidence that low-frequency mutations in *KRAS* may cause resistance to anti-epidermal growth factor receptor therapy in colorectal cancer, even if mutant allele frequency was as low as 0.4% to 17%,[58] and similar reports were published for gefitinib resistance in lung cancer.[59,60] The authors discuss potential explanations, including amplification of the gene locus and the possibility of parallel evolution of distinct resistance mechanisms within different subclones of a tumor. Whether such explanations might hold true in rMM remains to be addressed.

Including the mutation described above, we have detected 15 *CRBN* mutations in 9 MM patients thus far by M[3]P sequencing at the Mayo Clinic, all of the patients being clinically resistant to IMiD treatment.[7,19] This includes an additional patient who was initially responsive to thalidomide and lenalidomide treatment, who acquired resistance to lenalidomide over the disease course, and who was finally unresponsive to pomalidomide treatment at the time of tumor sampling. In this patient, we detected a total of 6 mutations within *CRBN*: (p.Lys401Glu, 40% VR; p.His397Tyr, 6% VR; p.Pro352Ser, 2% VR; p.Ile314Ser, 11% VR; p.Leu308Phe, 5% VR; and p.Leu190Phe, 7% VR). Of note, all mutations but 1 were located within the thalidomide-binding domain, critical for the response to IMiDs. We had 2 prior samples available from earlier time points and, as with the 3 patients described above, we were unable to detect the mutations in *CRBN* when the patient was still sensitive to IMiD treatment.

In summary, our study supports the hypothesis that *CRBN* mutations predominantly, but not exclusively, occur in advanced MM.

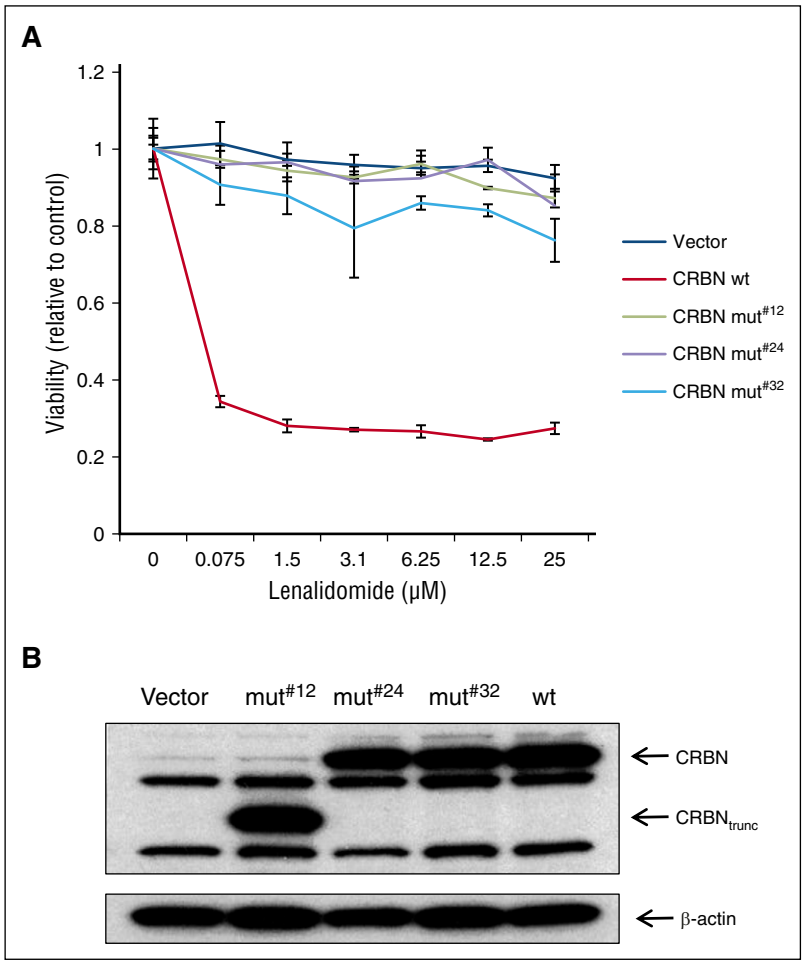

Figure 4. CRBN mutations identified in our cohort confer lenalidomide resistance in OCI-MY5 cells. OCI-MY5 cells are resistant to IMiD treatment because of low CRBN expression levels. IMiD sensitivity can be restored by CRBN wt overexpression. (A) All 3 CRBN mutants failed to resensitize the cell line; thus, the mutations conferred IMiD resistance. (B) Protein expression by western blot.

These CRBN mutations are probably evoked by clonal selection under prolonged IMiD-based therapy, and their appearance probably contributes to IMiD-resistant MM disease. Clearly, the subclonal appearance of CRBN mutations in some patients requires further investigation. In addition, our study provides evidence of an altered mutational landscape in drug-resistant MM that presents most significantly with an increased number of mutations in the MAPK pathway. Whether these MAPK mutations are associated with resistance to IMiD treatment or are, more likely, positively selected by repeated drug exposures in advanced disease, remains to be determined.

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Authorship

Contribution: A.K.S., E.B., E.K.M., K.M.K., M.S.R., and P.L.B. designed the research; A.J., C.-X.S., J.X., K.M.K., L.B., M.A., N.H.H., P.J., and Y.-X.Z. performed experiments; A.K.S., E.B., E.K.M., K.M.K., M.S.R., N.H.H., R.A.S., and S.B. analyzed data; A.K.S., E.B., E.K.M., K.M.K., and M.S.R. wrote the manuscript; and E.K.M., M.M., J.H., and H.G. recruited patients and provided clinical information.

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