Cereblon loss and up-regulation of c-Myc are associated with lenalidomide resistance in multiple myeloma patients

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Supplemental data

Supplementary Methods

Patients

Patients were eligible to participate in the REPEAT study if they had lenalidomide-refractory disease following at least 1 prior therapy. Lenalidomide-refractory MM was defined as progressive disease during therapy, no response (less than partial response) to prior lenalidomide-containing therapy, or progression within 60 days of discontinuation from lenalidomide-containing regimens, according to the International Myeloma Working Group criteria. The study was approved by the institutional medical ethical committee in each participating center in accordance with the declaration of Helsinki. All participants provided written informed consent. The trial was registered at www.clinicaltrials.gov as #NCT01352338.

Immunohistochemistry

Sequential dual color immunohistochemistry (IHC) assays were performed as previously described, except for the addition of Dako Protein Block (Catalog No. X0909) immediately before applying the CD138 antibody to reduce nonspecific background. Primary antibodies used were: Cereblon, Celgene custom rabbit monoclonal, CRBN65, used at 1/2000; Aiolos, Celgene custom rabbit monoclonal, Clone 9B-9-7, used at 1/400; Ikaros, Celgene custom rabbit monoclonal, Clone 36-8-5, used at 1/12000; IRF4, mouse monoclonal, Dako, Catalog No. M7259, Clone MUM1P, used at 1/7500; c-Myc, rabbit monoclonal, Abcam, Catalog No. ab32072, Clone Y69, used at 1/200; CD138, mouse monoclonal, Dako, Catalog No. M7228, Clone MI15, used at 1/1200. Mouse monoclonal IgG1 (BD Bioscience; Catalog No 550878) and rabbit monoclonal IgG (Abcam, Catalog No. Ab172730) were used.
as isotype controls at the matched concentrations as the respective primary antibodies. All slides were counterstained with hematoxylin.

Evaluation of dual color IHC slides was performed by board-certified pathologists under the light microscope. The target markers Cereblon (cytoplasmic and nuclear), Aiolos (nuclear), Ikaros (nuclear), IRF4 (nuclear), and c-Myc (nuclear) were evaluated in at least 100 CD138 (membrane) positive plasma cells in the bone marrow to generate an H-score. H-scores range from 0 to 300 and take into account frequency and intensity of staining \[H\text{-score} = (% \text{ at } 1+) \times 1 + (% \text{ at } 2+) \times 2 + (% \text{ at } 3+) \times 3\]. For Cereblon, cytoplasmic and nuclear compartments were scored separately. The sum of cytoplasmic and nuclear H-Scores accounted for the total H-Score (0-600), which was generated for each sample. This dual color IHC assay has been validated previously, showing a high specificity for a wide range of Cereblon expression levels, a low coefficient of variation for cytoplasmic and nuclear H-scores of 5% and 2% respectively, and good inter-pathologist concordance with an average \(R^2 = 0.73\).2

**Statistics**

Continuous variables were analyzed using Wilcoxon matched-pairs test or a Mann-Whitney U test. Differences in categorical variables were determined with the Fisher’s exact test for two by two tables and otherwise with the Pearson’s \(\chi^2\) test. Results are expressed as 2-tailed \(P\) values. A level of \(P < 0.05\) was considered significant. Progression-free survival (PFS) was calculated from inclusion in the REPEAT study until progression or death from any cause. Overall survival (OS) was measured from diagnosis and from inclusion in the REPEAT study until death from any cause. Prognostic factors for PFS and OS were analyzed for statistical significance using the Cox proportional hazard model. Calculations were performed in SPSS version 20.0.0 (IBM SPSS Inc., Armonk, NY, USA) and GraphPad Prism version 5.03 (GraphPad Software Inc., La Jolla, CA, USA). Correlations of various patient- and tumor-
related factors with clinical response to REP treatment were only performed for patients treated at the RDL.


Supplemental figure 1. Immunohistochemical staining of Cereblon, Ikaros, Aiolos, IRF4 and c-Myc in CD138+ bone-marrow plasma cells of MM patients.

Multiple myeloma BM core biopsies stained with dual CD138 (red membrane) and Cereblon (brown nucleus and cytoplasm - 1st column), Ikaros (brown nucleus - 2nd column), Aiolos (brown nucleus - 3rd column), IRF4 (brown nucleus - 4th column), or c-Myc (brown nucleus - 5th column). Shown are two representative patients at the time of diagnosis (upper panel) and lenalidomide-refractory disease (lower panel). Decreased Cereblon and increased c-Myc expression are noted upon development of lenalidomide resistance. Aiolos, Ikaros and IRF4 remain unchanged. H-scores range from 0 to 600 for Cereblon and from 0 to 300 for the remaining stains. Abbreviations: Len, lenalidomide.