Expression levels of genes implicated in the working mechanism of lenalidomide predict treatment response in lower risk myelodysplastic syndrome patients

Myelodysplastic syndromes (MDS) are a heterogeneous group of clonal bone marrow malignancies characterized by ineffective hematopoiesis, morphological dysplasia, peripheral cytopenias, and risk of evolution to acute myeloid leukemia (AML).[1] Patients with a very low to intermediate risk according to the Revised International Prognostic Scoring System (IPSS-R) or very low to moderate low risk in the Molecular IPSS (IPSS-M)[2] have a low risk of progression to AML.[2,3] In these patients, treatment of anemia remains challenging and therapeutic options are limited.[3-5] Although erythropoiesis-stimulating agents (ESA) are the first line of treatment, this is insufficient in the majority of patients.[3] For subgroups of MDS patients, specific treatment can be beneficial, for example, lenalidomide in patients with an isolated chromosomal 5q deletion (del(5q)) and luspatercept in MDS with ringsideroblasts.[3-5] del(5q)-MDS is found to be sensitive to lenalidomide, with an erythroid response (hematologic improvement-erythroid [HI-E]) of 60-80% and a cytogenetic remission in more than 50% of patients.[1,4] Also in non-del(5q)-MDS-patients, lenalidomide was shown to initiate HI-E, although at a much lower response rate (±25-30%).[1,4,6-8] We have previously shown that high percentages of bone marrow lymphocytes and progenitor B cells, a low number of mutations, absence of ring sideroblasts, and the absence of *SF3B1* mutations were predictive for a response to lenalidomide in low and intermediate-risk MDS patients treated within the HOVON89 clinical trial.[1] That study was approved and registered at www.trialregister.nl; NTR1825 (former ID); NL1715 (recent ID); EudraCT 2008-002195-10; METC: 2009/50 NL25632.029.08, and was conducted according to the principles of the Declaration of Helsinki. Here, we assessed the expression levels of genes which are associated with the working mechanism of lenalidomide, and looked for correlations with regard to response to lenalidomide.

Lenalidomide binds to the E3-ubiquitin ligase cereblon (CRBN), altering its substrate specificity (Figure 1). As a result, various proteins become targets for degradation by the proteasome. Amongst the *de novo* targets are transcription factors Ikaros (IKZF1) and Aiolos (IKZF3), postulated to play a role in the response to lenalidomide in multiple myeloma, and casein kinase1A1 (CSNK1A1), which was shown

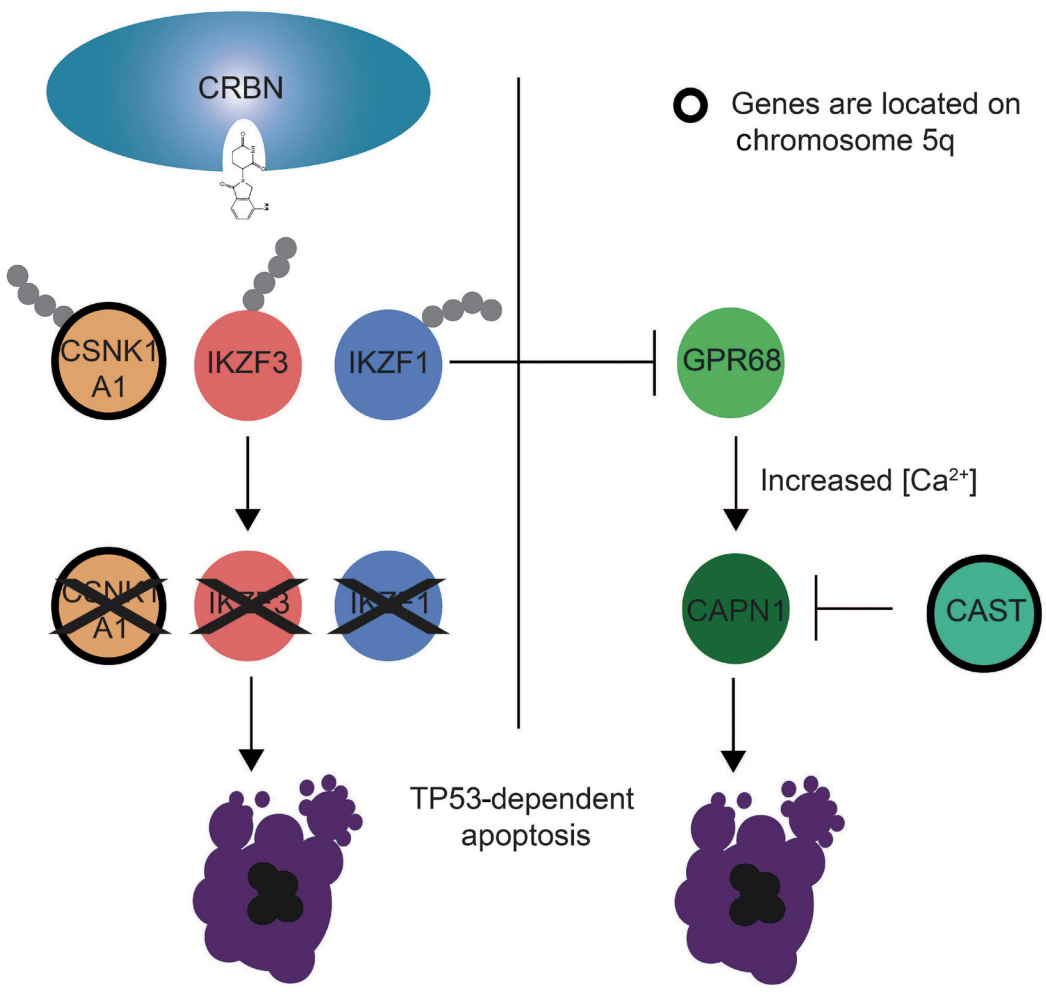

Figure 1. Proposed mechanism of lenalidomide action. Lenalidomide binds to the E3-ubiquitin ligase cereblon (CRBN) altering substrate specificity. As a result, several proteins are targeted for ubiquitination and subsequent degradation by the proteasome. These *de novo* targets include casein kinase 1A1 (CSNK1A1), Ikaros (IKZF1) and Aiolos (IKZF3) leading to TP53 activation and apoptosis. IKZF1 also has a direct inhibitory effect on G Protein-Coupled Receptor 68 (GPR68), and degradation of IKZF1 leads to increased GRP68 expression which increases cytosolic calcium levels and activation of a calcium-dependent calpain (CAPN1) which leads to apoptosis. Calpastatin (CAST) is an endogenous inhibitor of CAPN1. CK1a and CAST are both located on chromosome 5q and may, therefore, be expressed at lower levels in patients with a del(5q) which might cause enhanced vulnerability to lenalidomide.

to play a role in MDS.[9] As CSNK1A is located on chrom5q, haploinsufficiency in combination with enhanced degradation in MDS patients may have an additive effect which could explain the good response rate of lenalidomide particularly in cases with a del(5q).[10] Degradation of CSNK1A1 induces P53-dependent growth inhibition[9] and *TP53* mutations are associated with lenalidomide resistance.[11] Murine double minute 2 (MDM2) enhances degradation of P53 and increased expression of MDM2 limits P53-dependent growth inhibition.[12] Another proposed mechanism of action of lenalidomide in MDS is activation of the calcium- and calpain-dependent pathway[13] (Figure 1). *GPR68*, which encodes a G-protein-coupled-receptor implicated in calcium metabolism, is also regarded as an important modulator of sensitivity to lenalidomide. Lenalidomide was shown to induce GPR68 expression via CRBN-induced IKZF1 degradation, resulting in increased cytostolic calcium levels and activation of a calcium-dependent Calpain (CAPN1) which leads to induction of apoptosis in MDS cells. Calpastatin (CAST) is an endogenous inhibitor of CAPN1. As the *CAST* gene is located on chrom5(q15), haploinsufficiency of CAST in del(5q)-MDS may contribute to the specific sensitivity of these patients to lenalidomide.[13] We assessed the expression levels of 7 genes (*CSNK1A1, CRBN, IKZF1, IKZF3, MDM2, CAPN1, CAST*) which are associated with the working mechanism of lenalidomide in low and intermediate risk MDS patients treated within the HOVON89 clinical trial.[1] This prospective phase II randomized multicenter study (EudraCT2008-002195-10, www.trialregister.nl) included low and intermediate-1 risk MDS patients with anemia with hemoglobin (Hb) <10 g/dL or transfusion dependency. A total of 200 patients were included and, for 141 patients, RNA was available. All patients received lenalidomide (10 mg/day/day 1-21, with or without ESA/G-CSF) for a minimum of six months until loss of response or disease progression. The primary endpoint was HI-E according to the International Working Group criteria for MDS 2006.[14] The mean age was 69.9 years (range: 38-85) and the mean follow-up time was 39.2 months (range: 1.4-110.2). Of the included patients, 27 (19%) had del(5q)-MDS (according to the 2022 WHO definitions) and 109 (77%) had a non-del(5q); for 5 patients (4%), cytogenetics were unknown. In total, 57 of the 141 (40%) patients reached HI-E upon lenalidomide treatment: 85% within the del(5q)-patients and 29% within non-del(5q). There was no difference between responders and non-responders with respect to clinical characteristics such as age, bone marrow blasts, or peripheral blood cell counts (*Online Supplementary Table S1*).

mRNA expression of 7 genes (*CSNK1A1, CRBN, IKZF1, IKZF3, MDM2, CAPN1, CAST*) implicated in the 2 proposed working mechanisms of lenalidomide[9,13] was determined in bone marrow mononuclear cells (MNC). MNC were isolated using Ficoll (GE Healthcare) density gradient isolation. cDNA was generated using M-MLV reverse transcriptase (Invitrogen). Q-PCR were performed in duplicate using 2 independent primer/probe mixes for all genes except *CSNK1A1* (Applied Biosystems) (*Online Supplementary Table S2*). The matched primer/probe sets had a Spearman correlation between 0.70-0.93 (*Online Supplementary Table S3*). Threshold cycle (CT) values were normalized using *ACTB* (β-actin) and the average of the normalized mRNA expression of both Q-PCR assays was used for the assessment of a potential effect on lenalidomide response. Survival curves and univariate analysis were calculated by the Kaplan-Meier method and compared using the log rank test. The cut-off point for mRNA expression analysis was selected by conducting univariate analysis on quartiles and median. Those that demonstrated the most distinct statistically significant differences in the HI-E log rank test were selected for reporting.

Of the genes investigated, *CSNK1A1, CAST, IKZF1, MDM2*, and *CAPN1* were significantly more highly expressed in non-del(5q) patients compared to del(5q) patients (*Online Supplementary Figure S1*). Only patients with low expression of *CAST* (lowest 25%), *MDM2* (lowest 75%), or *CAPN1* (lowest 25%) were more likely to respond to lenalidomide (1-year HI-E: 53% *vs*. 33%, *P*=0.058; 44% *vs.* 21%, *P*=0.043; 57% *vs.* 34%, *P*=0.018, respectively) (Figure 2A). This effect was retained in the non-del(5q)-group specifically; however, this was not statistically significant (1-year HI-E: 40% *vs.* 25%, *P*=0.135; *MDM2* 34% *vs.* 17%, *P*=0.125; *CAPN1* 45% *vs.* 26%, *P*=0.081) (Figure 2B). When we combined *CAST*, *MDM2*, and *CAPN1* in a response probability score (RPS), each gene receiving 1 point in case of low expression, we could stratify patients into 4 groups with response rates of 16.0% in the patients with high mRNA expression in *CAST*, *MDM2*, and *CAPN1* (0 points), and 62.2% response to lenalidomide in patients with low expression *CAST, MDM2*, and *CAPN1* (3 points). Patients with 1 or 2 points had 41.7% and 50.0% response rates, respectively (*P*=0.037) (Figure 2C). Stratification by RPS showed the same pattern in non-del(5q) patients, but this did not reach statistical significance (*P*=0.165) (Figure 2D). When incorporating RPS into a Cox regression multivariate model including the number of mutations and age, RPS was an independent factor for reaching HI-E (*P*=0.035) (Table 1A), also when taking *SF3B1* mutations into the equation (*P*=0.043) (Table 1B) .

Lenalidomide is very effective in del(5q)-MDS to diminish transfusion dependency, but also to delay the time to transfusion dependency.[4,15] However, lenalidomide has also shown benefit in a subset of non-del(5q)-MDS patients.[4,8] We have previously identified high percentages of bone marrow lymphocytes and progenitor B cells, a low number of mutations, absence of ringsideroblasts, and *SF3B1* mutations as predictive variables for a response to lenalidomide.[1] Here we show the additional impact of mRNA expression of genes involved in the molecular mechanisms of lenalidomide response. We show that differences in expression of a variety of these genes at diagnosis correlates with HI-E. We incorporated the 3 genes (*CAST, MDM2*, and *CAPN1*) which

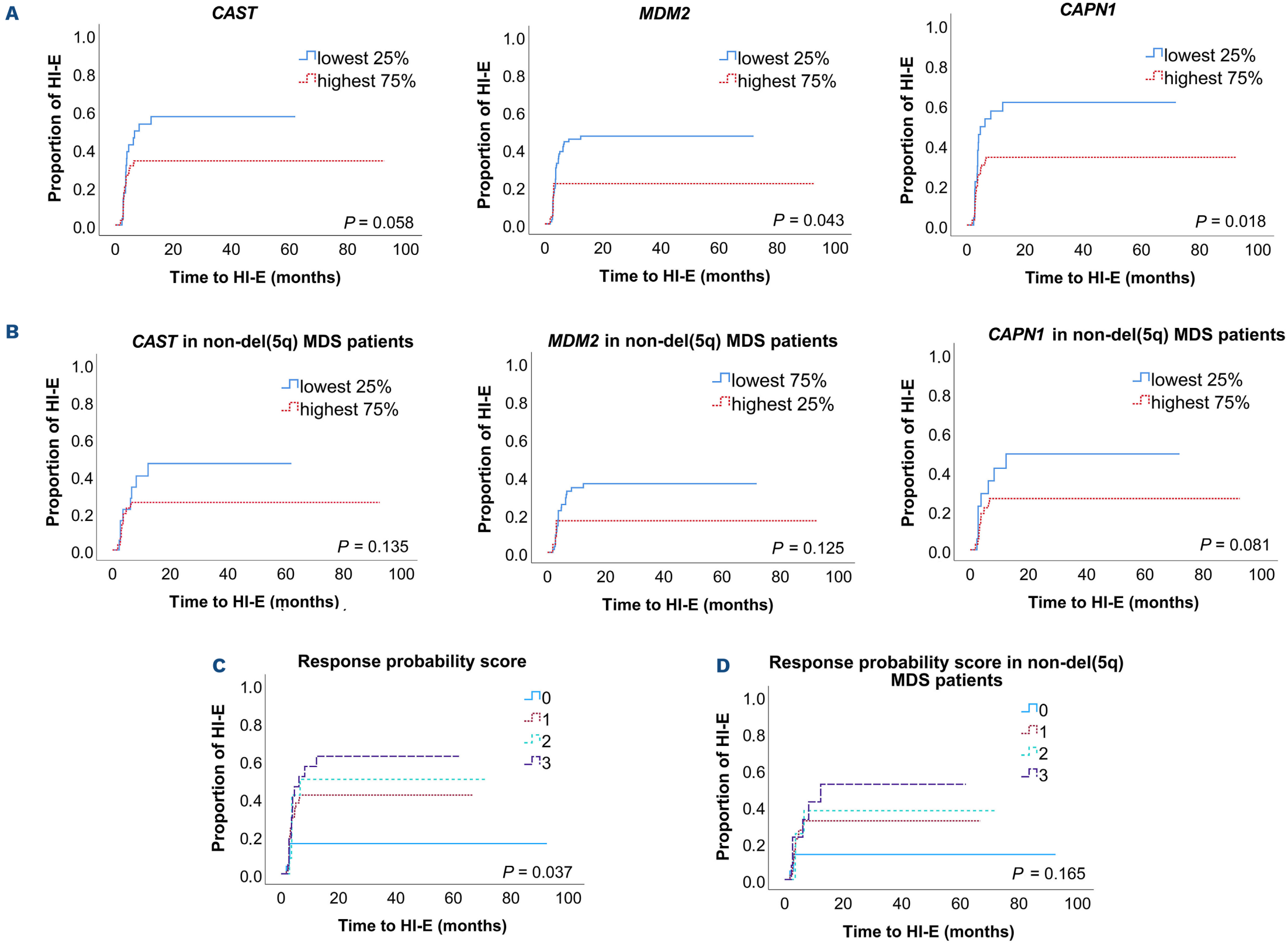

Figure 2. Hematologic improvement-erythroid divided by mRNA expression. (A) Hematologic improvement-erythroid (HI-E) divided by *CAST* mRNA expression (lowest quartile: N=30; highest 75%: N=88), *MDM2* mRNA expression (highest quartile: N=29; lowest 75%: N=88), *CAPN1* mRNA expression (lowest quartile: N=29; highest 75%: N=87). (B) HI-E in non-del(5q) myelodysplastic syndrome (MDS) patients divided by *CAST* mRNA expression (lowest quartile: N=19; highest 75%: N=74), *MDM2* mRNA expression (lowest 75%: N=67; highest 25%: N=25), *CAPN1* mRNA (lowest quartile: N=19; highest 75%: N=72). Quartiles are based on expression levels in the entire cohort. (C) HI-E divided by response probability score. 0 points: N=26; 1 point: N= 56; 2 points: N=13; 3 points: N=21. (D) HI-E divided by response probability score in non-del(5q) patients. 0 points: N=23; 1 point: N=46; 2 points: N=9; 3 points: N=13. N: number.

Table 1. Cox regression multivariate model on achieving hematologic improvement-erythroid. (A) Included variables: number of mutations, response probability score and age (>60 years). (B) Included variables: *SF3B1* mutation present, response probability score and age (>60 years).

A				
	Wald	**df**	***P***	**HR (95% CI) to achieve HI-E**
N of mutations	13.78	4	0.01	-
1	5.73	1	0.019	0.395 (0.182-0.860)
2	4.462	1	0.032	0.423 (0.193-0.927)
3	9.959	1	0.002	0.138 (0.040-0.472)
>3	1.458	1	0.227	0.455 (0.127-1.632)
Response probability score	8.598	3	0.035	-
1	5.101	1	0.024	3.435 (1.177-10.021)
2	3.973	1	0.046	3.699 (1.022-13.383)
3	8.594	1	0.003	5.592 (1.769-17.675)
Age >60 years	0.079	1	0.778	0.870 (0.329-2.298)

B				
	Wald	**df**	***P***	**HR (95% CI) to achieve HI-E**
SF3B1 mutation	2.787	1	0.095	0.575 (0.300-1.101)
Response probability score	8.152	3	0.043	-
1	4.616	1	0.032	3.276 (1.109-9.622)
2	3.804	1	0.051	3.699 (0.994-14.536)
3	8.076	1	0.004	5.251 (1.673-16.479)
Age >60 years	0.250	1	0.617	0.781 (0.296-2.059)

CI: confidence interval; df: degree of freedom; HI-E: hematologic improvement-erythroid; HR: Hazard Ratio; Wald: Wald test.

showed the most impact on response to lenalidomide in a univariate model into a response probability score (RPS). This score allows stratification of patients into 4 distinct groups with a significantly different probability of attaining HI-E. The best group was 3 times more likely to respond to lenalidomide compared to patients with the lowest score. The RPS was an independent predictive factor in a multivariate model containing previously identified factors, including *SF3B1* mutations, which we recently identified as a novel (negative) predictive factor for lenalidomide response. In non-del(5q) patients, the score still showed the same pattern, but significance was lost, probably due to the small number of patients in each score group. *TP53* mutations are correlated with poor response to lenalidomide. In our cohort, we found *TP53* mutations in 6 patients, 3 with a variant allele frequency (VAF) >5%. None of the patients with a *TP53* mutation with a VAF>5% reached HI-E. Two of these patients had a response probability score (RPS) of 1 and one patient had an RPS of 3. The patient with an RPS of 3 also had a del(5q), which could explain the high RPS. The *TP53* mutation probably contributed to the lack of response; however, the numbers are too small to make any general comments on the gene expression patterns and *TP53* mutations.

In summary, based on the proposed working mechanism of lenalidomide, we identified several markers that may help to predict response to lenalidomide in MDS patients developing an RPS. This could help to further identify those patients who are more likely to respond to lenalidomide treatment. This may broaden the use of lenalidomide in a disease where therapeutic options are limited.

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Contributions

FH, AL and JJ contributed to the conception and design of the work, and wrote the manuscript. TS acquired the laboratory data. FH, JJ, AdG, BvR, TM, TS and AL contributed to the analysis and interpretation of data. CA, CD, EC, HV, AV, DC and AL contributed to patient accrual and interpretation of the data. All authors reviewed critically and approved the final manuscript.

Data-sharing statement

Clinical data are available in our previously published data.[1] All other datasets analyzed during the current study are available from the corresponding author on reasonable request.

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