SUPPLEMENTARY APPENDIX

p53 protein expression independently predicts outcome in patients with lower-risk myelodysplastic syndromes with del(5q)

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Methods

Patients

Formalin-fixed paraffin-embedded (FFPE) BM trephines from patients enrolled in the phase III, randomized, double-blind, placebo-controlled MDS-004 trial were retrieved.[5] The MDS-004 trial assessed the efficacy and safety of lenalidomide in 205 RBC transfusion-dependent patients with IPSS-defined Low- or Int-1-risk del(5q) MDS. The inclusion criteria and treatment schedule were as previously described;[5] a biopsy specimen was recommended, but not mandatory. Patients were enrolled from 37 sites between July 8, 2005 and June 26, 2007. The present study was conducted under the ethics committee consent for the MDS-004 trial. The original ethical permit did not include any type of sequencing; therefore, *TP53* deep-sequencing analysis was only possible in a subset of patients who were still alive and provided consent to an ethical permit obtained after the MDS-004 trial had completed. In Sweden, gene-sequencing analysis in MDS patients was performed under a separate national ethical permit, which was used for pyrosequencing analysis of laser-microdissected BM cells.

BM morphology and IHC

A total of 131 BM trephines from 85 of the 205 patients (41%; IHC study cohort) obtained at baseline and follow-up were assessed in a blinded fashion. The lack of biopsies mainly reflected the country of origin, as explained in Supplementary Table S3. Serial BM biopsies were available in 25% (21 of 85) of patients. The biopsies were stained with H&E, Giemsa, and Gordon and Sweet's silver stain for routine histological examination. BM cellularity and fibrosis were assessed following the European consensus guidelines.[26] IHC was performed according to the manufacturers' guidelines, including: p53-DO7 (Novocastra; Leica Biosystems, Wetzlar, Germany); p53-DO1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA); p21 (Calbiochem, San Diego, CA, USA); CD34 (Novocastra); CD117, glycophorin A, myeloperoxidase, and hemoglobin (all Dako, Glostrup, Denmark), using the automated Bond™ (A. Menarini Diagnostics S.r.l., Firenze, Italy) and Ventana BenchMark XT systems (Ventana Medical Systems, Inc., Tucson, AZ, USA).

Assessment of p53 IHC by manual and automated image analysis

The percentage and intensity of p53 nuclear staining was assessed based on a total count of 1000 BM hematopoietic cells (lymphocytes and lymphoid aggregates excluded) at high magnification (40×/60× objectives). p53 protein expression was graded as: 0 (negative); 1+ (weakly positive); 2+ (moderately positive); 3+ (strongly positive) (Figure 1). The entire BM trephine section was also assessed using a Modified Quick Score;[27] a score of ≥3 was used to define p53-positive staining as previously described.[28] All BM samples were assessed blindly for the percentage of p53-DO1 strongly positive (3+) cells by

three independent hematopathologists from different institutions and countries. A computerized automated imaging system (3DHISTECH Ltd slide scanner, Budapest, Hungary) was used to measure p53 and the staining intensity was graded as in the manual counting. Sections from colon cancer tissue were used as positive controls. BM biopsies from 50 patients with secondary, non-MDS related cytopenia were included as negative controls for internal validation of the p53 staining.

TP53 ***mutation analysis by deep sequencing***

Deep-sequencing mutation analysis was performed in 9 of 85 (11%) patients using DNA from the FFPE baseline biopsy and, if available, a follow-up sample. From each FFPE-block, three 15 µm sections were collected and sent to an external laboratory for analysis (Covance Genomics Laboratory, Seattle, WA, USA). DNA was isolated using the QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany), and sequencing libraries were made using Illumina's TruSeq library construction kit (Illumina, Inc., San Diego, CA, USA) and quantified using KAPA qPCR reagents (Kapa Biosystems, Inc., Woburn, MA, USA) per manufacturer's instructions. Regions coding for 11 exons of the *TP53* gene were amplified (21 amplicons of ~150 bp each) and sequenced using Illumina MiSeq (Illumina, Inc.). Sequence reads were aligned against a hg19 reference genome with an average sequencing coverage of >2500×.

Laser-assisted microdissection and pyrosequencing

In 3 patients with known *TP53* mutations, laser-microdissection of p53-immunolabeled cells from serial BM samples was performed to study the relationship between protein expression and *TP53* mutation (Leica LMD7000; Leica Microsystems, Wetzlar, Germany). Briefly, 4 µm BM sections were placed on microscopic slides coated with laser pressure-catapulting membranes. The slides were dried and incubated at 56°C for 8 h, and stained with the p53-DO1 antibody. Cells with strong (3+), moderate (2+), and negative p53-staining were microdissected and collected in separate tubes (>1000 nucleated cells/tube) for DNA extraction (Arcturus® PicoPure® DNA Extraction Kit; Applied Biosystems, Carlsbad, CA, USA). The samples were incubated for 24 h at 65°C, centrifuged for 1 min at 4000 × *g*, heated to 95°C for 10 min, cooled to room temperature, and directly after used for polymerase chain reaction (PCR) using the HotStar Taq DNA Polymerase kit (Qiagen) following standard procedures. PCR temperature program was 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec, 54°C for 20 sec, and 72°C for 30 sec. Mutation specific assays were designed using PyroMark Assay Design software (Qiagen). Oligonucleotides and pyrosequencing information are presented in Supplementary Table S1. The pyrosequencing reactions were performed on a Pyromark Q24 (Qiagen) instrument using reagents and standard procedures provided by the manufacturer.

Statistical analysis

Demographic and baseline characteristics of the IHC cohort (n=85) and patients in the MDS-004 trial without IHC data (n=120) were compared using the two-sided *t*-test for continuous variables and Fisher's exact test for categorical variables. The Fisher's exact test and the Kaplan-Meier product limit methods were used to compare response rates and time-to-event endpoints. The Kaplan-Meier curves are provided along with two-sided log-rank based *P*-values. Competing risk approach was used to analyze time to progression without AML. Two-sided *P*-values based on Robert Gray's method were reported (Gray RJ. Ann Stat. 1988;16:1141-54). A *P*-value of <0.05 was considered to be statistically significant. Pearson correlation was used for the comparison of the manual and automated measurements, and the two p53 monoclonal antibodies (DO7 and DO1). Cohen's kappa test was used to measure inter-observer agreement with regard to the 1% threshold for defining strong p53-positive staining. Cox proportional hazard modeling was used to evaluate multiple risk factors for AML progression and OS. The risk factors with a *P*-value <0.2 from the univariate model were included as covariates in the multivariate model which evaluated the effects of all risk factors simultaneously. The final model only included risk factors with a *P*-value of <0.1, applying backward elimination variable selection method.

Supplementary Table S1. Pyrosequencing assays applied in allele quantification experiments.

p53 Mutation	Forward	Reverse	Sequencing primer	Sequence to analyze
Y163C	TCCTTCCTCTTCCTACAGTACTCC	Biotin-CCGTCATGTGCTGTGACTG	CGCGCCATGGCCATC	TRCAAGCAGTCACAGCA
L265P	GGGTGGTTGGGAGTAGATGG	Biotin-TCCCCTTTCTTGCGGAGAT	TATCCTGAGTAGTGGTAATC	TACYGGGACGGAACA
C275F	GGGTGGTTGGGAGTAGATGG	Biotin-TCCCCTTTCTTGCGGAGAT	TTGAGGTGCGTGTTT	KTGCCTGTCCTGGGAG

Supplementary Table S2. Clinical and morphological characteristics of the IHC study cohort.

	Clinical data					BM histology		IHC		
Patient no.	**Randomization group**	**IPSS**	**Cytogenetics**	**WHO group**	**BM blasts (%)**	**Cellularity (%)**	**BM fibrosis**	**CD34+ (%)**	**CD34+ clusters**	**p53+++ (%)**
1	LEN 5 mg	Int-1	Isolated del(5q)	RCMD, RCMD-RS	0	80	Grade 1	3	No	0.0
2	Placebo	Int-1	Isolated del(5q)	RCMD, RCMD-RS	NA	40	No	1	No	0.0
3	LEN 10 mg	Low	Isolated del(5q)	RCMD, RCMD-RS	4	50	No	2	No	0.0
4	LEN 10 mg	Low	Isolated del(5q)	RCMD, RCMD-RS	4	60	No	2	No	0.0
5	LEN 10 mg	Low	Isolated del(5q)	RCMD, RCMD-RS	2	40	No	2	No	0.0
6	LEN 10 mg	Low	Isolated del(5q)	RCMD, RCMD-RS	3	40	Grade 1	7	Yes	0.0
7	Placebo	Low	Isolated del(5q)	RCMD, RCMD-RS	2	40	No	0	No	0.0
8	LEN 5 mg	NA	Isolated del(5q)	NA	NA	60	Grade 1	6	No	0.5
9	LEN 10 mg	Int-1	Isolated del(5q)	RCMD, RCMD-RS	3	20	No	0	No	1.8
10	LEN 5 mg	Int-1	del(5q) + 1	RAEB-1	8	30	No	7	No	0.6
11	LEN 10 mg	Int-1	Isolated del(5q)	RA, RARS, 5q−	4	30	No	4	No	0.0
12	Placebo	Low	Isolated del(5q)	RA, RARS, 5q−	2	40	Grade 1	1	No	1.6
13	Placebo	Low	Isolated del(5q)	RCMD, RCMD-RS	4	40	No	3	No	0.6
14	LEN 5 mg	Int-1	Isolated del(5q)	RAEB-1	9	100	Grade 1	7	Yes	1.8
15	Placebo	Low	Isolated del(5q)	RCMD, RCMD-RS	3	40	No	0	No	0.3
16	LEN 10 mg	Int-1	Isolated del(5q)	RAEB-1	8	50	No	4	Yes	0.0
17	LEN 10 mg	Int-1	del(5q) + ≥2	RCMD, RCMD-RS	2	30	No	0	No	0.0
18	LEN 5 mg	Int-1	del(5q) + 1	RCMD, RCMD-RS	0	10	No	0	No	0.2
19	LEN 10 mg	Low	Isolated del(5q)	RCMD, RCMD-RS	1	70	No	2	No	0.0

	Clinical data					BM histology		IHC		
Patient no.	**Randomization group**	**IPSS**	**Cytogenetics**	**WHO group**	**BM blasts (%)**	**Cellularity (%)**	**BM fibrosis**	**CD34+ (%)**	**CD34+ clusters**	**p53+++ (%)**
20	LEN 10 mg	Int-1	Isolated del(5q)	RCMD, RCMD-RS	4	60	No	3	No	0.4
21	LEN 5 mg	Int-1	Isolated del(5q)	RAEB-1	8	70	Grade 1	10	Yes	1.4
22	LEN 10 mg	Low	Isolated del(5q)	RCMD, RCMD-RS	3	80	No	3	No	0.0
23	LEN 10 mg	NA	Isolated del(5q)	RA, RARS, 5q−	NA	60	No	7	No	0.0
24	Placebo	Low	Isolated del(5q)	RCMD, RCMD-RS	3	50	No	3	No	0.0
25	Placebo	NA	Isolated del(5q)	5q−	NA	30	No	2	No	0.0
26	LEN 10 mg	Low	Isolated del(5q)	RCMD, RCMD-RS	4	40	Grade 1	3	No	0.6
27	Placebo	Int-1	del(5q) + 1	RCMD, RCMD-RS	0	40	Grade 1	2	No	0.0
28	Placebo*	Int-2	del(5q) + ≥2	RAEB-1	9	100	Grade 2	3	No	0.0
29	LEN 5 mg	NA	Isolated del(5q)	NA	NA	60	Grade 1	5	No	0.0
30	Placebo	Int-1	Isolated del(5q)	RCMD, RCMD-RS	2	40	Grade 1	3	No	0.2
31	LEN 10 mg	NA	Isolated del(5q)	NA	NA	30	No	2	No	0.0
32	LEN 10 mg	NA	del(5q) + ≥2	NA	NA	70	Grade 1	0	No	0.0
33	Placebo*	NA	del(5q) + 1	NA	NA	30	No	0	No	0.0
34	Placebo	Low	Isolated del(5q)	RCMD, RCMD-RS	4	60	No	3	No	0.0
35	LEN 5 mg	Low	Isolated del(5q)	RCMD, RCMD-RS	1	60	No	2	No	3.2
36	Placebo	Int-1	del(5q) + 1	RCMD, RCMD-RS	1	50	No	4	No	1.4
37	LEN 5 mg	NA	Isolated del(5q)	RA, RARS, 5q−	2	50	No	4	No	3.0
38	LEN 10 mg	Int-1	del(5q) + ≥2	RAEB-1	NA	90	Grade 1	9	Yes	3.4
39	LEN 5 mg	Low	Isolated del(5q)	RA, RARS, 5q−	NA	50	No	2	No	0.0
40	LEN 5 mg	Int-1	Isolated del(5q)	RCMD, RCMD-RS	1	80	No	3	No	0.2

	Clinical data					BM histology		IHC		
Patient no.	**Randomization group**	**IPSS**	**Cytogenetics**	**WHO group**	**BM blasts (%)**	**Cellularity (%)**	**BM fibrosis**	**CD34+ (%)**	**CD34+ clusters**	**p53+++ (%)**
41	LEN 5 mg	Int-1	del(5q) + 1	RCMD, RCMD-RS	2	40	No	5	No	0.2
42	Placebo	Int-1	del(5q) + 1	RCMD, RCMD-RS	3	80	No	2	No	0.0
43	LEN 10 mg	NA	del(5q) + 1	NA	NA	90	Grade 2	8	Yes	1.6
44	LEN 5 mg	Int-1	del(5q) + ≥2	RCMD, RCMD-RS	4	60	No	2	No	9.6
45	Placebo	Int-1	Isolated del(5q)	RAEB-1	NA	40	No	2	No	0.0
46	LEN 5 mg	Int-2	del(5q) + ≥2	RCMD, RCMD-RS	2	90	Grade 1	9	No	7.2
47	LEN 5 mg	Int-1	Isolated del(5q)	RCMD, RCMD-RS	2	60	Grade 1	3	No	1.8
48	LEN 10 mg	Int-1	del(5q) + 1	RCMD, RCMD-RS	3	50	Grade 1	3	No	0.0
49	LEN 10 mg	Int-1	del(5q) + ≥2	RCMD, RCMD-RS	4	70	No	12	Yes	0.8
50	LEN 5 mg	NA	del(5q) + 1	NA	NA	100	Grade 2	2	No	1.2
51	LEN 10 mg	Int-1	Isolated del(5q)	RAEB-2	10	40	No	7	No	2.4
52	LEN 10 mg	Low	Isolated del(5q)	RCMD, RCMD-RS	2	80	No	3	No	0.0
53	Placebo	Low	Isolated del(5q)	RCMD, RCMD-RS	4	50	No	5	No	4.6
54	LEN 10 mg	NA	Isolated del(5q)	NA	NA	60	No	4	No	0.0
55	LEN 5 mg	NA	del(5q) + 1	NA	NA	40	Grade 1	2	No	0.0
56	LEN 10 mg	NA	NA	NA	NA	50	Grade 1	3	No	0.9
57	LEN 5 mg	Int-1	del(5q) + ≥2	RCMD, RCMD-RS	4	30	Grade 1	7	No	7.2
58	Placebo*	Int-1	Isolated del(5q)	RAEB-1	7	40	Grade 1	4	No	1.6
59	LEN 10 mg	Int-1	del(5q) + 1	RCMD, RCMD-RS	1	100	No	4	Yes	0.2
60	LEN 5 mg	Int-1	Isolated del(5q)	RAEB-1	9	40	No	6	No	5.2
61	Placebo	Low	Isolated del(5q)	RCMD, RCMD-RS	3	40	No	0	No	1.2

	Clinical data					BM histology		IHC		
Patient no.	**Randomization group**	**IPSS**	**Cytogenetics**	**WHO group**	**BM blasts (%)**	**Cellularity (%)**	**BM fibrosis**	**CD34+ (%)**	**CD34+ clusters**	**p53+++ (%)**
62	LEN 5 mg	Low	Isolated del(5q)	RCMD, RCMD-RS	3	40	No	3	No	1.5
63	Placebo	Int-1	del(5q) + 1	RCMD, RCMD-RS	3	60	No	2	No	1.0
64	Placebo	Int-1	Isolated del(5q)	RCMD, RCMD-RS	2	40	No	5	Yes	6.8
65	LEN 5 mg	NA	Isolated del(5q)	NA	NA	80	Grade 1	4	No	0.2
66	LEN 10 mg	NA	Isolated del(5q)	NA	NA	20	No	0	No	0.0
67	Placebo	NA	Isolated del(5q)	NA	NA	20	No	2	No	1.0
68	LEN 10 mg	Int-1	Isolated del(5q)	RCMD, RCMD-RS	2	50	Grade 1	7	No	1.2
69	LEN 10 mg	Low	Isolated del(5q)	RCMD, RCMD-RS	2	80	No	3	No	2.2
70	LEN 5 mg	Low	Isolated del(5q)	RCMD, RCMD-RS	2	50	No	3	No	4.2
71	LEN 5 mg	Int-1	del(5q) + 1	RCMD, RCMD-RS	2	100	Grade 1	0	No	0.0
72	Placebo	Low	Isolated del(5q)	RCMD, RCMD-RS	4	50	No	6	No	0.0
73	Placebo	Int-1	del(5q) + 1	RCMD, RCMD-RS	3	50	No	0	No	0.0
74	LEN 10 mg	Int-1	del(5q) + ≥2	RCMD, RCMD-RS	1	40	No	0	No	4.8
75	Placebo	Low	Isolated del(5q)	RCMD, RCMD-RS	1	40	No	0	No	0.0
76	LEN 5 mg	Low	Isolated del(5q)	RCMD, RCMD-RS	2	80	NA	0	No	2.0
77	LEN 5 mg	Int-1	Isolated del(5q)	RA, RARS, 5q−	3	40	No	4	No	0.0
78	LEN 10 mg	NA	del(5q) + 1	RCMD, RCMD-RS	NA	80	Grade 3	0	No	0.0
79	LEN 10 mg	Low	Isolated del(5q)	RCMD, RCMD-RS	1	30	Grade 1	1	No	0.0
80	LEN 10 mg	NA	Isolated del(5q)	NA	NA	20	No	2	No	2.2
81	LEN 10 mg	Int-1	del(5q) + 1	RCMD, RCMD-RS	2	40	Grade 2	0	No	0.0
82	LEN 5 mg	Int-1	Isolated del(5q)	RCMD, RCMD-RS	3	60	Grade 1	3	No	0.8

	Clinical data					BM histology		IHC		
Patient no.	**Randomization group**	**IPSS**	**Cytogenetics**	**WHO group**	**BM blasts (%)**	**Cellularity (%)**	**BM fibrosis**	**CD34+ (%)**	**CD34+ clusters**	**p53+++ (%)**
83	Placebo	Int-1	del(5q) + 1	RAEB-1	NA	100	Grade 3	3	No	2.4
84	LEN 10 mg	NA	Isolated del(5q)	NA	NA	60	No	2	No	0.0
85	LEN 5 mg	Int-1	del(5q) + 1	RCMD, RCMD-RS	4	50	No	0	No	0.0

Legend Supplementary Table S2. IPSS-risk category and percentage of BM blasts based on central review in the MDS-004 clinical trial. The percentage of p53-DO1 relates to the presence of strong (3+) p53-positive cells by manual assessment based on a total count of 1000 BM hematopoietic cells (lymphocytes and lymphoid aggregates excluded) at high magnification (40×/60× objectives). CD34-positive cells were quantified in ≥20 randomly selected fields (40× objectives), and the number of CD34-positive cells expressed as the percentage of the total number of BM hematopoietic cells. CD34-positive clusters were defined as a group of ≥3 CD34-positive cells. BM fibrosis and cellularity were assessed semi-quantitatively following the European consensus guidelines.[26]

*3 patients who were randomized to placebo did not crossover to LEN at 16 weeks.

BM: bone marrow; IHC: immunohistochemistry; Int: intermediate; IPSS: International Prognostic Scoring System; LEN, lenalidomide; NA: not applicable; RA: refractory anemia; RAEB: RA with excess blasts; RARS: RA with ring sideroblasts; RCMD: refractory cytopenia with multilineage dysplasia; RCMD-RS: RCMD with ring sideroblasts; WHO: World Health Organization.

Supplementary Table S3. Demographic and clinical baseline characteristics of the IHC study cohort and the MDS-004 study patients without biopsy.

Variable, n (%)	**IHC study cohort (n=85)**	**Cohort without biopsies* (n=120)**	***P***
Mean age, years (range)	67.0 (36.0-86.0)	67.6 (39.0-86.0)	0.671
Age distribution			0.252
≤65 years	38 (44.7)	44 (36.7)	
>65 years	47 (55.3)	76 (63.3)	
Sex			0.320
Male	17 (20.0)	32 (26.7)	
Female	68 (80.0)	88 (73.3)	
Mean duration of MDS, years (range)	3.4 (0.2-29.2)	3.9 (0.2-17.1)	0.331
Randomization treatment group			0.424
Lenalidomide 5 mg	27 (31.7)	42 (35.0)	
Lenalidomide 10 mg	33 (38.8)	36 (30.0)	
Placebo	25 (29.4)	42 (35.0)	
IPSS categories (central review)			0.231
Low-risk	25 (29.4)	45 (37.5)	
Int-1-risk	38 (44.7)	36 (30.0)	
Int-2-risk	3 (3.5)	7 (5.8)	
High-risk	0	1 (0.8)	
Missing	19 (22.4)	31 (25.8)	
IPSS categories (primary investigator)			0.426
Low-risk	35 (41.2)	59 (49.2)	
Int-1-risk	49 (57.6)	58 (48.3)	
Int-2-risk	1 (1.1)	3 (2.5)	
High-risk	0	0	
FAB classification (central review)			0.681
RA	46 (54.1)	61 (50.8)	
RARS	9 (10.6)	15 (12.5)	
RAEB	10 (11.8)	12 (10.0)	
RAEB-T	0	1 (0.8)	

Variable, n (%)	**IHC study cohort (n=85)**	**Cohort without biopsies* (n=120)**	***P***
CMML	0	3 (2.5)	
CML	1 (1.2)	0	
Other or missing	4 (4.7)	3 (2.5)	
Inadequate specimen	15 (17.6)	25 (20.8)	
WPSS group			0.580
Very low/Low	5 (5.9)	6 (5.0)	
Intermediate	32 (37.6)	52 (43.3)	
High	28 (32.9)	30 (25.0)	
Very high	2 (2.4)	1 (0.8)	
Missing	18 (21.2)	31 (25.8)	
5q− chromosomal abnormality			0.100
Yes	82 (96.5)	109 (90.8)	
No	2 (2.4)	2 (1.7)	
Missing†	1 (1.1)	9 (7.5)	
Baseline chromosomal abnormality			0.170
Isolated del(5q)	54 (63.5)	81 (67.5)	
del(5q) + 1 additional	19 (22.4)	19 (15.8)	
del(5q) + ≥2 additional	9 (10.6)	8 (6.7)	
Missing	3 (3.5)	12 (10.0)	
Transfusion burden, units/8 weeks	**n=64**	**n=81**	0.125
Mean (range)	5.7 (2.0-12.0)	6.4 (1.0-25.0)	
Prior erythropoietin treatment			0.887
Yes	44 (51.8)	64 (53.3)	
No	41 (48.2)	56 (46.7)	
WHO 2008 classification			0.648
RA, RARS, 5q−	6 (7.1)	10 (8.3)	
RCMD, RCMD-RS	51 (60.0)	70 (58.3)	
RAEB-1	10 (11.8)	9 (7.5)	
RAEB-2	1 (1.2)	5 (4.2)	
Missing	17 (20.0)	26 (21.7)	

Variable, n (%)	**IHC study cohort (n=85)**	**Cohort without biopsies* (n=120)**	***P***
Bone marrow blast group			0.931
<5%	54 (63.5)	73 (60.8)	
≥5%	8 (9.4)	13 (10.8)	
Missing	23 (27.1)	34 (28.3)	
26-Week transfusion independence			0.636
Yes	29 (34.1)	37 (30.8)	
No	56 (65.9)	83 (69.2)	
Cytogenetic response	**n=56**	**n=77**	0.128
Yes	21 (37.5)	19 (24.7)	
No	35 (62.5)	58 (75.3)	
AML progression	22 (25.9)	44(36.7)	0.129
Median overall survival, years (range)	3.7 (2.9-4.4)	3.5 (2.9-4.6)	0.933

*A biopsy specimen at inclusion was recommended, but not mandatory. The cohort of 120 patients without IHC included all patients enrolled in France (n=71), 18 of 25 patients enrolled in the United Kingdom, and 14 patients from other sites (n=103). In addition, 12 patients had inadequate bone marrow samples at baseline and 5 patients withdrew their consent.

†Patients with missing or failed chromosome analysis were included based on FISH-analysis.

AML: acute myeloid leukemia; CML: chronic myeloid leukemia; CMML: chronic myelomonocytic leukemia; FAB: French-American-British; FISH: fluorescence in situ hybridization; *IHC: immunohistochemistry; Int: intermediate; IPSS: International Prognostic Scoring System; MDS: myelodysplastic syndromes; RA: refractory anemia; RAEB: RA with excess blasts; RAEB-T: RAEB in transformation; RARS: RA with ring sideroblasts; RCMD: refractory cytopenia with multilineage dysplasia; RCMD-RS: RCMD with ring sideroblasts; WHO: World Health Organization; WPSS: WHO-based Prognostic Scoring System.*

Supplementary Table S4. Results from *TP53* mutation analysis.

Patient no.*	ΔTime (months)†	p53+++ IHC (%)	Chromosome position	dbSNP135	Exon	Position cDNA	Allele frequency (%)	WT-codon	Mutant codon	AA change	Mutation type
1	0	0.0	7579472	rs1042522	4	215	50	CCC	CGC	P72R	Missense
	3	1.0	7579472	rs1042522	4	215	53	CCC	CGC	P72R	Missense
	6	0.0	7579472	rs1042522	4	215	54	CCC	CGC	P72R	Missense
11	0	0.0	7579472	rs1042522	4	215	39	CCC	CGC	P72R	Missense
	3	0.0	7579472	rs1042522	4	215	52	CCC	CGC	P72R	Missense
	6	0.6	7579472	rs1042522	4	215	54	CCC	CGC	P72R	Missense
18	0	0.0	7577067	-	8	871	34	AAG	TAG	K291X	Nonsense
	3	0.2	7579472	rs1042522	4	215	100	CCC	CGC	P72R	Missense
41	0	0.2	7578231	-	6	618	49	TTG	TTA	L206L	Silent
	0		7579472	rs1042522	4	215	52	CCC	CGC	P72R	Missense
43	0	1.6	7579472	rs1042522	4	215	100	CCC	CGC	P72R	Missense
66	0	0.0	7579472	rs1042522	4	215	100	CCC	CGC	P72R	Missense
68	0	1.2	7579472	rs1042522	4	215	100	CCC	CGC	P72R	Missense
34	0	1	NA	NA	NA	NA	3	TGT	TTT	C275F	Missense
	12	1	NA	NA	NA	NA	4	TGT	TTT	C275F	Missense
	31	0	7579472	rs1042522	4	215	100	CCC	CGC	P72R	Missense
			7578210	rs1800372	6	639	45	CGA	CGG	R213R	Silent
	51	3	NA	NA	NA	NA	25	TGT	TTT	C275F	Missense
	57	5	NA	NA	NA	NA	32	TGT	TTT	C275F	Missense
	61	6	NA	NA	NA	NA	45	TGT	TTT	C275F	Missense

Patient no.*	ΔTime (months)†	p53+++ IHC (%)	Chromosome position	dbSNP135	Exon	Position cDNA	Allele frequency (%)	WT-codon	Mutant codon	AA change	Mutation type
	81	5	NA	NA	NA		52	TGT	TTT	C275F	Missense
	93	15	NA	NA	NA		46	TGT	TTT	C275F	Missense
	105	11	7577114	-	8	824	23	TGT	TTT	C275F	Missense
			7579472	rs1042522	4	215	97	CCC	CGC	P72R	Missense
			7578210	rs1800372	6	639	44	CGA	CGG	R213R	Silent
	108	12	7577114	-	8	824	18	TGT	TTT	C275F	Missense
			7579472	rs1042522	4	215	100	CCC	CGC	P72R	Missense
			7578210	rs1800372	6	639	48	CGA	CGG	R213R	Silent
37	0	0	NA	NA	NA	NA	NA	NA	NA	NA	NA
	1	0	NA	NA	NA	NA	NA	NA	NA	NA	NA
	18	0	NA	NA	NA	NA	NA	NA	NA	NA	WT
	22	0	NA	NA	NA	NA	NA	NA	NA	NA	NA
	34	0	NA	NA	NA	NA	NA	NA	NA	NA	WT
	75	5	NA	NA	6	NA	10	NA	NA	N2001	Missense
	86	7	7577058	NA	8	880	10	GAG	AAG	E294K	Missense
	90	7	NA	NA	NA	NA	NA	NA	NA	NA	NA

Legend Supplementary Table S4. *TP53* mutation analysis was performed by deep-sequencing technique using DNA from FFPE BM samples from 9 patients from the IHC cohort. *TP53* mutations were identified in 3 patients (Patients 18, 34, and 37). In Patient 34, *TP53* mutations were detected at diagnosis and in serial BM samples; theses were associated with the presence of p53 by IHC with strong nuclear staining at the corresponding time points. Patient 37 was initially negative by deep-sequencing and negative for p53 by IHC, but underwent cytogenetic evolution at 75 months [(46, XX, del(5q), del(17p)] with detection of a *TP53* mutation by Sanger sequencing and deep-sequencing at 75 and 86 months from diagnosis. Patient 18 with a K291 nonsense mutation was negative for p53 by IHC as expected. In 7 patients rs1042522C→G polymorphisms

(P72R) were identified (see http://p53.iarc.fr/RefsPolymorphisms.aspx for additional information); 2 patients had 1.2% and 1.6% cells with strong p53-expression, whereas the remaining 5 patients were p53-negative.

*Patient number relates to Supplementary Table S1.

†Indicates time from randomization (patients shadowed in grey) and from initial diagnosis (Patients 34 and 37).

AA: amino acid; BM: bone marrow; FFPE: formalin-fixed paraffin-embedded; IHC: immunohistochemistry; WT: wild-type; NA, data not available

Supplementary Table S5. Comparison of baseline demographics and clinical data of the IHC cohort (N=85) by p53 IHC status.

Variable, n (%)	**p53-positive (≥1%)**	**p53-negative (<1%)**	***P***
	n=30	**n=55**	
Mean age, years (range)	67.2 (46.0-85.0)	66.9 (36.0-86.0)	0.905
Age distribution			0.649
≤65 years	12 (40.0)	26 (47.3)	
>65 years	18 (60.0)	29 (52.7)	
Sex			0.154
Male	3 (10.0)	14 (25.5)	
Female	27 (90.0)	41 (74.5)	
Mean duration of MDS, years (range)	4.2 (0.2-29.2)	2.9 (0.2-11.8)	0.158
Peripheral blood status			
Mean ANC, $\times 10^9$/L (range)	3.1 (0.5-7.0)	2.9 (0.5-20.2)	0.828
Mean WBC, $\times 10^9$/L (range)	4.9 (1.1-10.2)	5.1 (1.2-33.0)	0.847
Mean platelet count, $\times 10^9$/L (range)	312.4 (33.0-932.0)	257.2 (63.0-5223.0)	0.128
Randomization treatment group			0.167
Lenalidomide 5 mg	13 (43.3)	14 (25.4)	
Lenalidomide 10 mg	8 (26.6)	25 (45.4)	
Placebo	9 (30.0)	16 (29.0)	
IPSS categories (central review)	**n=25**	**n=41**	0.484
Low-risk	8 (32.0)	17 (41.4)	
Int-1-risk	15 (60.0)	23 (56.0)	
Int-2-risk	2 (8.0)	1 (2.4)	
IPSS categories (by primary investigator)			1.000
Low-risk	12 (40.0)	23 (41.8)	
Int-1-risk	18 (60.0)	31 (56.4)	
Int-2-risk	0	1 (1.8)	
High-risk	0	0	
Baseline chromosomal abnormality	**n=28**	**n=54**	0.265
Isolated del(5q)	18 (64.2)	36 (66.6)	
del(5q) + 1 additional	5 (17.8)	14 (25.9)	

Variable, n (%)	**p53-positive (≥1%)**	**p53-negative (<1%)**	***P***
del(5q) + ≥2 additional	5 (17.8)	4 (7.4)	
Transfusion burden, units/8 weeks	**n=22**	**n=42**	0.383
Mean (range)	6.2 (2.0-12.0)	5.5 (2.0-12.0)	
Prior Erythropoietin treatment			1.000
Yes	16 (53.3)	28 (50.9)	
No	14 (46.7)	27 (49.1)	
WHO 2008 classification			0.144
RA, RARS, 5q−	3 (10.0)	3 (6)	
RCMD, RCMD-RS	16 (53.3)	35 (64)	
RAEB-1	6 (20.0)	4 (7.3)	
RAEB-2	1 (3.3)	0	
Missing data	4 (13.3)	13 (24)	
Bone marrow blast group	**n=24**	**n=38**	0.192
<5%	19 (79.2)	35 (92.1)	
≥5%	5 (20.8)	3 (7.8)	

ANC: absolute neutrophil count; IHC: immunohistochemistry; Int: intermediate; IPSS: International Prognostic Scoring System; MDS: myelodysplastic syndromes; RA: refractory anemia; RAEB: RA with excess blasts; RARS: RA with ring sideroblasts; RCMD: refractory cytopenia with multilineage dysplasia; RCMD-RS: RCMD with ring sideroblasts; WHO: World Health Organization.

Supplementary Table S6. 26-Week RBC-TI response and cytogenetic response by p53 IHC status (N=85).

Variable, n (%)	**p53-positive (≥1%)**	**p53-negative (<1%)**	***P***
26-Week RBC-TI response			
Total IHC cohort	**n=30**	**n=55**	0.636
Responder	9 (30.0)	20 (36.4)	
Non-responder	21 (70.0)	35 (63.6)	
Patients randomized to lenalidomide 5 mg	**n=13**	**n=14**	0.695
Responder	4 (30.8)	6 (42.9)	
Non-responder	9 (69.2)	8 (57.1)	
Patients randomized to lenalidomide 10 mg	**n=8**	**n=25**	0.699
Responder	5 (62.5)	13 (52.0)	
Non-responder	3 (37.5)	12 (48.0)	
Cytogenetic response			
Total IHC cohort	**n=21**	**n=35**	0.009
Responder	3 (14.3)	18 (51.4)	
Non-responder	18 (85.7)	17 (48.6)	
Patients randomized to lenalidomide 5 mg	**n=8**	**n=9**	1.000
Responder	2 (25.0)	2 (22.2)	
Non-responder	6 (75.0)	7 (77.8)	
Patients randomized to lenalidomide 10 mg	**n=8**	**n=19**	0.001
Responder	1 (12.5)	16 (84.2)	
Non-responder	7 (87.5)	3 (15.8)	

IHC: immunohistochemistry; RBC-TI: red blood cell transfusion independence.

Supplementary Table S7. Assessment of p53 by IHC in serial BM samples from 21 patients (IHC cohort).

							p53 (%)				
Patient no.	**Randomization group**	**182-Day RBC-TI response**	**CyR**	**Cytogenetic progression (Δtime months)**	**Karyotype at progression**	**Time to AML (months)**	**At baseline**	**3 mos**	**6 mos**	**12 mos**	**18 mos**
5	LEN 10 mg	Yes	Minor	12	Complex [del(5q) + 3 add. aberrations]	-	Neg	NA	NA	16.8	NA
6	LEN 10 mg	Yes	None	22	Complex [del(5q) + 12 add. aberrations]	30	Neg	3.2	NA	NA	NA
8	LEN 5 mg	No	None	No	-	18	Neg	5.2	5.8	Neg*	NA
9	LEN 10 mg	Yes	Minor	6	Complex [del(5q) + 4 add. aberrations]	-	1.8	6	NA	NA	NA
12	Placebo	No	None	No	-	13	1.6	12.6	NA	NA	NA
14	LEN 5 mg	No	None	No	-	44	1.8	6.6	NA	NA	NA
17	LEN 10 mg	No	None	3	Complex [del(5q) + 5 add. aberrations]		Neg	3	NA	NA	NA
20	LEN 10 mg	Yes	Major	No	-	19	Neg	4.2	NA	NA	NA
21	LEN 5 mg	No	None	No	-	18	1.4	4.2	NA	NA	NA
13	Placebo	No	None	No	-	-	Neg	Neg	NA	NA	NA
10	LEN 5 mg	No	None	No	-	-	Neg	Neg	NA	NA	NA
3	LEN 10 mg	Yes	Major	No	-	-	Neg	Neg	Neg	Neg	1.4

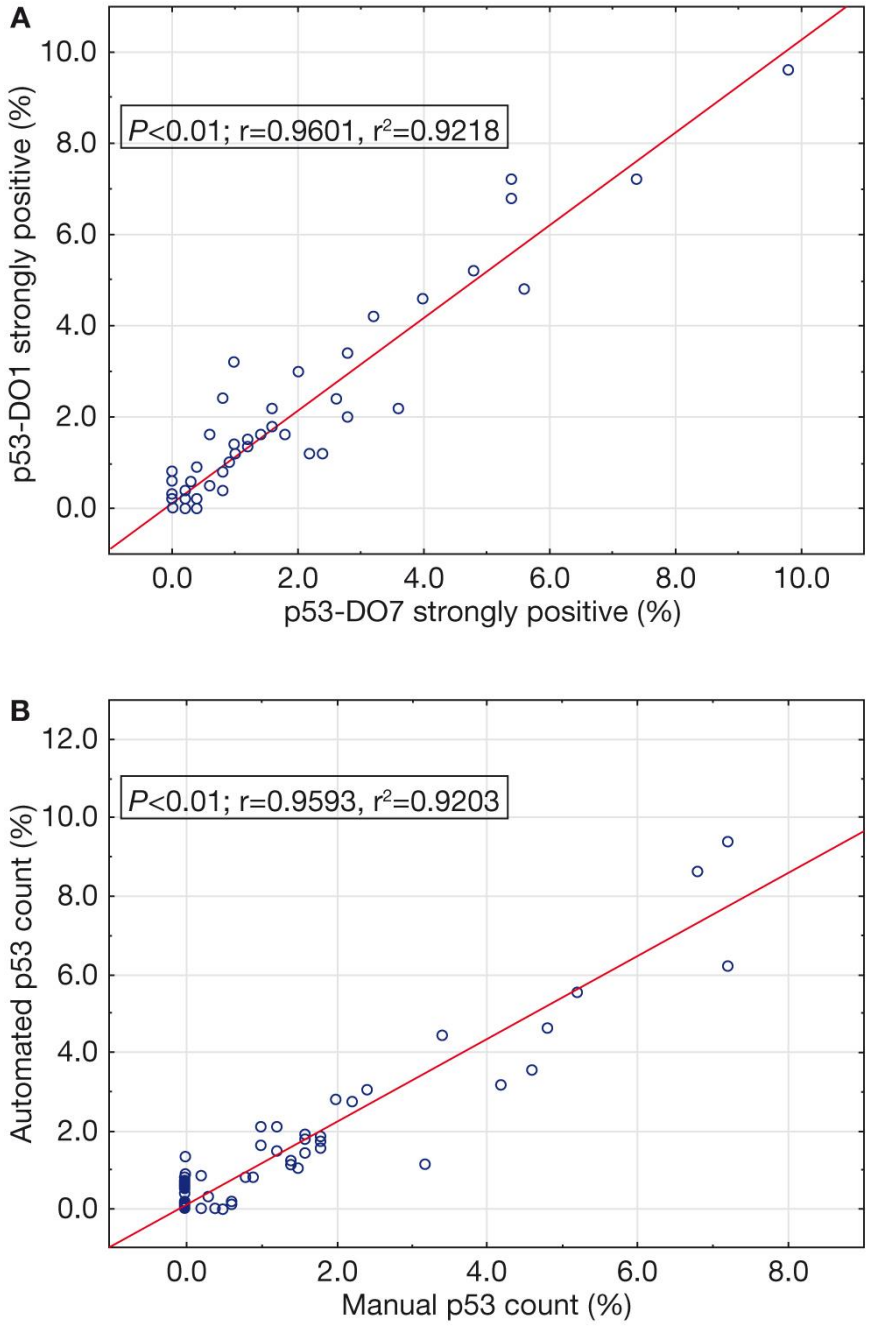
							p53 (%)				
Patient no.	**Randomization group**	**182-Day RBC-TI response**	**CyR**	**Cytogenetic progression (Δtime months)**	**Karyotype at progression**	**Time to AML (months)**	**At baseline**	**3 mos**	**6 mos**	**12 mos**	**18 mos**
15	Placebo	No	None	No	-	41	Neg	Neg	NA	NA	NA
4	LEN 10 mg	Yes	Major	No	-	-	Neg	Neg	Neg	Neg	Neg
1	LEN 5 mg	Yes	None	12	del(5q) + 1 add. aberration	-	Neg	Neg	Neg	Neg	Neg
2	Placebo	No	None	No	-	-	Neg	Neg	NA	Neg	Neg
16	LEN 10 mg	No	None	No	-	-	Neg	Neg	NA	NA	NA
18	LEN 5 mg	Yes	None	4	Complex [del(5q) + 4 add. aberrations], 2 independent clones	-	Neg	Neg	NA	NA	NA
11	LEN 10 mg	Yes	Major	No	-	-	Neg	Neg	Neg	Neg	1.6
19	LEN 10 mg	No	Major	6	Complex [del(5q) + 3 add. aberrations]	6	Neg	Neg	NA	NA	NA
7	Placebo	No	Major	No	-	-	Neg	Neg	Neg	Neg	NA

Legend Supplementary Table S7. Patient number relates to Supplementary Table S1. Patients shadowed in grey had an increase in p53 within 3 months of screening. Of these, 3 patients (Patients 17, 10, and 18) had del(5q) with additional chromosomal abnormalities at screening: 45,X,-Y, del(5)(q14q34)[9]/44, idem, inc[3]; 46,XX,add(5)(q23) or del(5)(q23q34)[3]/46, idem, +21[2]/46, XX [4]; 46,XX, del(5)(q14q34)[21]/47,XX,+8[4], respectively. All other patients had a del(5q) abnormality only.

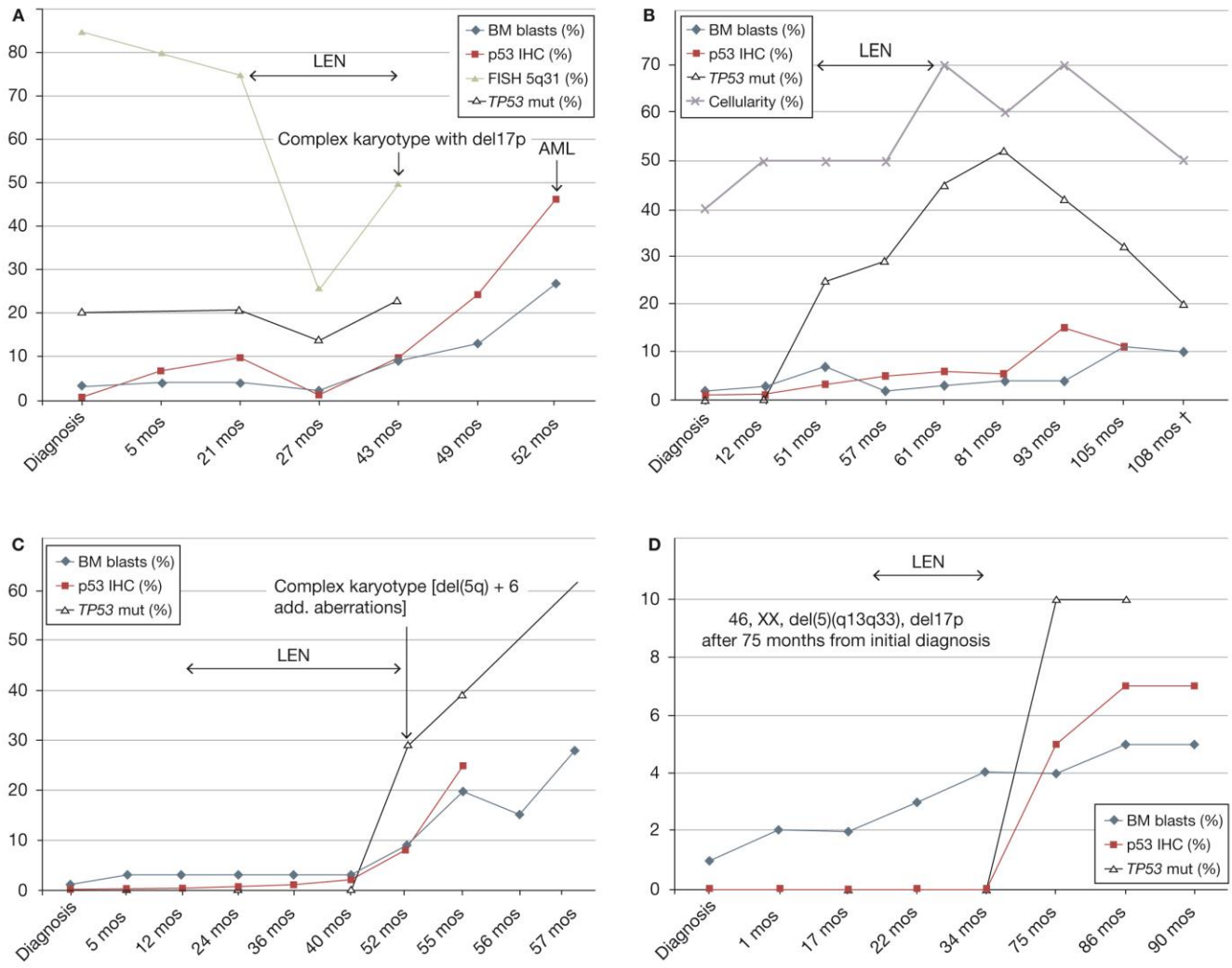
*Patient 8 stained negative for p53 in the last sample; however, BM cellularity was extremely low (10%).

AML: acute myeloid leukemia; BM: bone marrow; CyR: cytogenetic response; IHC: immunohistochemistry; LEN: lenalidomide; mos: months; NA: not applicable; Neg: negative; RBC-TI: red blood cell transfusion independence.

Supplementary Figure S1. Comparison of the two p53 monoclonal antibodies (A); and p53-DO1 by automated (Y-axis) *versus* manual (X-axis) count (B) with regard to the frequency of BM progenitor cells with strong nuclear staining.

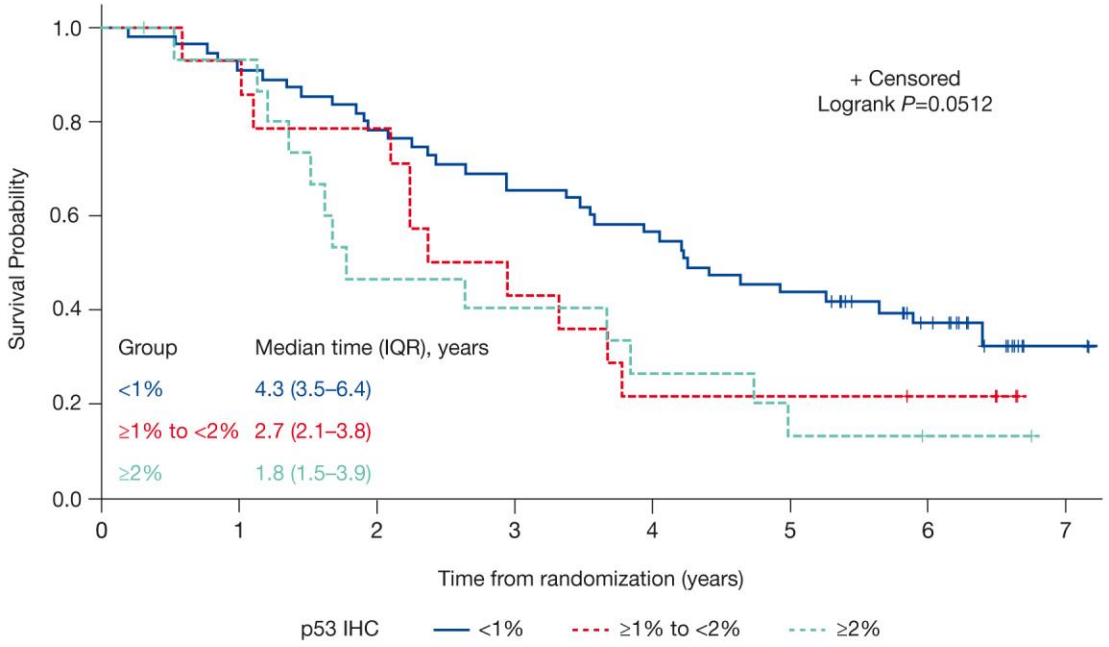


Supplementary Figure S2. Clinical course with results from serial BM assessment and *TP53* mutation analysis by deep-sequencing from whole BM sections in Patient 37 (panel D) and pyrosequencing analysis of laser-microdissected cells (panels A-C). Two patients (panels A-B) had small *TP53* mutated subclones with associated strong p53-staining already at initial diagnosis which increased as the patients progressed to high-risk MDS. The other two patients (panels C-D) acquired *TP53* mutations at 52 and 75 months, respectively, reflected by an increase in p53 by IHC.



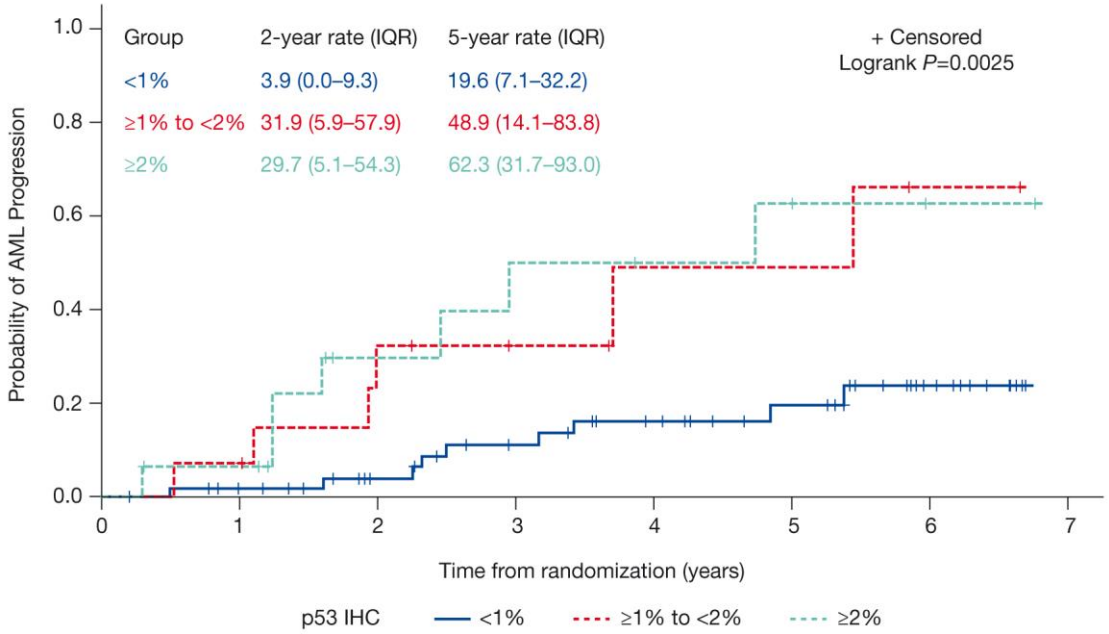
LEN: lenalidomide; mut: mutant; mos: months.

Supplementary Figure S3. OS by the presence of <1%, ≥1% to <2%, and ≥2% BM progenitor cells with strong (3+) p53 expression.



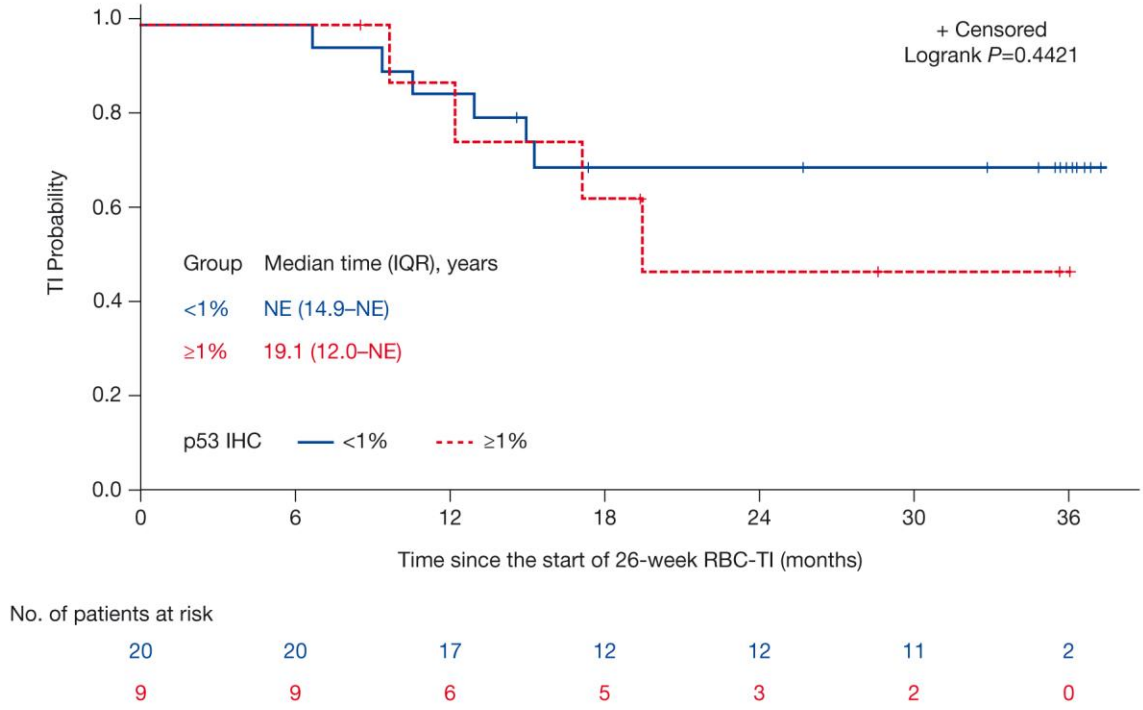
IQR: interquartile range.

Supplementary Figure S4. Probability of AML progression by <1%, ≥1% to <2%, and ≥2% BM progenitor cells with strong (3+) p53 expression.



IQR: interquartile range.

Supplementary Figure S5. Duration of RBC-TI response by p53 IHC status.



No. of patients at risk

20	20	17	12	12	11	2
9	9	6	5	3	2	0

NE: not evaluable.