

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

Leonie Saft,<sup>1,2</sup> Mohsen Karimi,<sup>1</sup> Mehran Ghaderi,<sup>3</sup> András Matolcsy,<sup>4</sup> Ghulam J. Mufti,<sup>5</sup> Austin Kulasekararaj,<sup>5</sup> Gudrun Göhring,<sup>6</sup> Aristoteles Giagounidis,<sup>7</sup> Dominik Selleslag,<sup>8</sup> Petra Muus,<sup>9</sup> Guillermo Sanz,<sup>10</sup> Moshe Mittelman,<sup>11</sup> David Bowen,<sup>12</sup> Anna Porwit,<sup>13</sup> Tommy Fu,<sup>14</sup> Jay Backstrom,<sup>14</sup> Pierre Fenaux,<sup>15</sup> Kyle J. MacBeth,<sup>14</sup> and Eva Hellström-Lindberg<sup>1</sup>

<sup>1</sup>Department of Medicine, Karolinska Institute, Karolinska University Hospital Huddinge, Sweden; <sup>2</sup>Department of Pathology, Karolinska University Hospital, Stockholm, Sweden; <sup>3</sup>Department of Oncology and Pathology, Karolinska Institute, Stockholm, Sweden; <sup>4</sup>Department of Pathology and Experimental Cancer Research, Semmelweis University, Budapest, Hungary; <sup>5</sup>King's College Hospital, London, UK; <sup>6</sup>Institute for Cell and Molecular Pathology, Medical University Hannover, Germany; <sup>7</sup>Marien Hospital Düsseldorf, Germany; <sup>8</sup>AZ ST-Jan Brugge AV, Brugge, Belgium; <sup>9</sup>Radboud University Nijmegen Medical Centre, Nijmegen, the Netherlands; <sup>10</sup>Hospital Universitaria La Fe, Valencia, Spain; <sup>11</sup>Tel Aviv Sourasky Medical Center, Sackler Faculty of Medicine, Tel Aviv, Israel; <sup>12</sup>St James's Institute of Oncology, Leeds, UK; <sup>13</sup>Department of Laboratory Medicine and Pathobiology, Toronto General Hospital, Ontario, Canada; <sup>14</sup>Celgene Corporation, Summit, NJ, USA; and <sup>15</sup>Service d'Hématologie Séniors, Hôpital St Louis, Université Paris 7, France

## ABSTRACT

Del(5q) myelodysplastic syndromes defined by the International Prognostic Scoring System as low- or intermediate-1-risk (lower-risk) are considered to have an indolent course; however, recent data have identified a subgroup of these patients with more aggressive disease and poorer outcomes. Using deep sequencing technology, we previously demonstrated that 18% of patients with lower-risk del(5q) myelodysplastic syndromes carry *TP53* mutated subclones rendering them at higher risk of progression. In this study, bone marrow biopsies from 85 patients treated with lenalidomide in the MDS-004 clinical trial were retrospectively assessed for p53 expression by immunohistochemistry in association with outcome. Strong p53 expression in  $\geq 1\%$  of bone marrow progenitor cells, observed in 35% (30 of 85) of patients, was significantly associated with higher acute myeloid leukemia risk ( $P=0.0006$ ), shorter overall survival ( $P=0.0175$ ), and a lower cytogenetic response rate ( $P=0.009$ ), but not with achievement or duration of 26-week transfusion independence response. In a multivariate analysis, p53-positive immunohistochemistry was the strongest independent predictor of transformation to acute myeloid leukemia ( $P=0.0035$ ). Pyrosequencing analysis of laser-microdissected cells with strong p53 expression confirmed the *TP53* mutation, whereas cells with moderate expression predominantly had wild-type p53. This study validates p53 immunohistochemistry as a strong and clinically useful predictive tool in patients with lower-risk del(5q) myelodysplastic syndromes. This study was based on data from the MDS 004 trial (*clinicaltrials.gov* identifier: NCT00179621).

## Introduction

Patients with myelodysplastic syndromes (MDS) with a deletion of 5q [del(5q)] defined by the International Prognostic Scoring System<sup>1</sup> (IPSS) as low- or intermediate-1-risk (lower-risk) are considered to have a favorable prognosis compared with that of patients with other types of MDS.<sup>2,3</sup> However, it is now recognized that overall survival and progression to acute myeloid leukemia (AML) vary greatly among patients with lower-risk del(5q) MDS. In a recent study of 381 untreated patients with lower-risk del(5q) MDS, the 2-year and 5-year overall survival rates were 86% and 61%, respectively, and the cumulative risks of AML were 4.7% and 14.7%, respectively.<sup>4</sup> Transfusion dependency and a bone marrow (BM) blast count of  $>5\%$  at diagnosis were significantly associated with increased risk of AML. Interestingly, neither IPSS subgroup (low- or intermediate-1-risk) nor the presence of one additional chromosomal abnormality within the intermediate-1-risk subgroup had an impact

on outcome. In the MDS-004 phase III clinical trial (*clinicaltrials.gov* identifier: NCT00179621), which included 205 red blood cell transfusion-dependent patients with IPSS-defined low- or intermediate-1-risk del(5q) MDS, the 3-year overall survival and AML-risk rates were 56% and 25%, respectively.<sup>5</sup> Failure to achieve treatment response was associated with adverse outcomes, in line with previous reports.<sup>6</sup> These studies indicate that the prognosis of patients with lower-risk del(5q) MDS is less favorable than previously considered, and that factors other than IPSS classification have an impact on patients' outcomes.<sup>7,9</sup>

Molecular mutations, including *TP53* mutations, are emerging as independent prognostic factors.<sup>8</sup> Using deep-sequencing technology, 18% of patients with lower-risk del(5q) MDS were found to have *TP53* mutated subclones which rendered them at higher risk of progression.<sup>10</sup> The negative prognostic impact of *TP53* mutations has been found in several other studies, especially in high-risk and therapy-related MDS.<sup>8,11-21</sup> Strong nuclear staining of the p53 protein by immunohisto-

©2014 Ferrata Storti Foundation. This is an open-access paper. doi:10.3324/haematol.2013.098103

The online version of this article has a Supplementary Appendix.

Manuscript received on September 27, 2013. Manuscript accepted on March 24, 2014.

Correspondence: eva.hellstrom-lindberg@ki.se

chemistry (IHC) has been used as a surrogate marker for *TP53* gene mutations in hematologic and other malignancies, including large B-cell lymphoma.<sup>22-24</sup> Recently, it was demonstrated that p53 nuclear expression correlated with hemizygous *TP53* mutation and outcomes in relapsed myeloma patients treated with lenalidomide.<sup>25</sup>

AML following MDS has a dismal prognosis, which makes prediction essential, especially in patients who potentially could be cured by stem cell transplantation. In order to develop a clinically useful prognostic tool, we measured p53 protein expression by IHC in a cohort of 85 patients with lower-risk del(5q) MDS from the MDS-004 clinical trial, and compared outcomes and responses to lenalidomide treatment in patients with respect to strong nuclear p53 expression. We demonstrate the independent prognostic value of p53 IHC in this population of patients, and show that strong nuclear staining reflects underlying *TP53* mutations. Our findings underscore the importance of including molecular markers such as *TP53* mutations in risk-assessment for del(5q) MDS patients.

## Methods

### Patients

Formalin-fixed paraffin-embedded BM trephines from patients enrolled in the phase III, randomized, double-blind, placebo-controlled MDS-004 trial were retrieved.<sup>5</sup> The MDS-004 trial assessed the efficacy and safety of lenalidomide in 205 red blood cell transfusion-dependent patients with low- or intermediate-1-risk del(5q) MDS. The inclusion criteria and treatment schedule were as previously described;<sup>5</sup> a bone marrow biopsy was recommended, but not mandatory. The present study was conducted under the ethical 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 been completed. In Sweden, gene-sequencing analysis was performed under a separate national ethical permit, which was used for the pyrosequencing of laser-microdissected BM cells.

### Bone marrow morphology and immunohistochemistry

Overall, 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. Serial BM biopsies were available for 25% (21 of 85) of the patients. BM cellularity and fibrosis were assessed according to European consensus guidelines.<sup>26</sup>

The percentage and intensity of p53 staining was assessed based on a total manual count of 1000 BM hematopoietic cells (lymphocytes/lymphoid aggregates excluded) and graded as: 0 (negative); 1+ (weakly positive); 2+ (moderately positive); and 3+ (strongly positive). The entire BM trephine was also assessed using a Modified Quick Score;<sup>27</sup> a score of  $\geq 3$  was used to define p53-positive staining as previously described.<sup>28</sup> In addition, all samples were assessed blindly for the percentage of p53-DO1 strongly positive (3+) cells by three hematopathologists from institutions in different countries. A computerized imaging system was used to measure p53 and grade it as in the manual counting. BM biopsies from 50 patients with secondary cytopenias were included as negative controls for internal validation of the p53 staining.

### TP53 mutation analysis

Deep-sequencing mutation analysis was performed in nine of 85 (11%) patients using DNA from the formalin-fixed paraffin-

embedded baseline biopsy and, if available, a follow-up sample. In three patients with known *TP53* mutations, laser-microdissection of p53-immunolabeled cells was performed to study the relationship between protein expression and *TP53* mutation. Cells with strong (3+), moderate (2+), and negative p53-staining were microdissected and collected separately (>1000 nucleated cells/tube) for DNA extraction (Arcturus<sup>®</sup>PicoPure<sup>®</sup> DNA Extraction Kit; Applied Biosystems, Carlsbad, CA, USA). Oligonucleotides and pyrosequencing information are presented in *Online Supplementary Table S1*.

### 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 exact test for categorical variables. The Fisher exact test and the Kaplan-Meier methods were used to compare response rates and time-to-event endpoints. A competing risk approach was used to analyze time to progression without AML. Cox proportional hazard modeling was used to evaluate multiple risk factors for AML progression and overall survival. The final model only included risk factors with a *P*-value of <0.1, applying the backward elimination variable selection method.

## Results

### Patients

Patients in the MDS-004 trial (n=205) were randomized to lenalidomide 5 mg (n=69) or 10 mg (n=69), or placebo (n=67); the corresponding figures for the IHC cohort (n=85) were 27, 33, and 25 patients, respectively (*Online Supplementary Table S2*). Of the 25 patients randomized to placebo in the IHC cohort, 22 crossed-over to active treatment after 16 weeks; the remaining three patients discontinued placebo treatment at days 18, 26, and 30, but were followed for AML progression and survival. Baseline demographics, clinical data, IPSS risk groups, and World Health Organization subgroups did not differ between the IHC cohort and the MDS-004 trial patients without BM trephine biopsies (*Online Supplementary Table S3*). Moreover, outcomes in terms of therapeutic response, survival, and AML transformation were similar between the two groups.

### Reproducibility of the p53 immunohistochemistry staining

Results for the manual *versus* automated measurements were highly concordant with respect to the frequency of cells with weak (1+), moderate (2+), and strong (3+) p53-staining for both p53 monoclonal antibodies (Pearson correlation coefficient  $r=0.9593$ ,  $r^2=0.9203$ ;  $P<0.01$ ) (*Online Supplementary Figure S1*). In addition, the manual counts for the p53-DO1 and p53-DO7 antibodies were highly associated for different staining intensities (Pearson correlation coefficient  $r=0.9601$ ,  $r^2=0.9218$ ;  $P<0.01$ ); therefore, all subsequent statistical analyses were based on data from the manual assessment of the p53-DO1 antibody only. In addition, all BM samples were assessed by three independent investigators to evaluate inter-observer agreement with regard to the presence of cells with strong p53 expression when using the 1% threshold. Cohen's kappa test was applied and indicated perfect agreement between the three investigators (*data not shown*).



### Strong p53 protein expression in a subset of bone marrow samples

A total of 30 of 85 patients (35%) had  $\geq 1\%$  BM progenitor cells with strong (3+) p53-DO1 expression in the initial screening sample:  $\geq 1\%$  to  $<2\%$  in 14 patients; 2% to  $<5\%$  in 11 patients; and  $\geq 5\%$  in five patients. Figure 1 shows an example of p53 staining.

Detailed clinical and morphological data for the IHC study cohort are presented in *Online Supplementary Table S2*. All samples with  $\geq 1\%$  strong p53-staining by IHC were subsequently double-stained for CD34, hemoglobin, glycophorin A, and myeloperoxidase. p53 was predominantly expressed in erythroid progenitor cells, but also in the granulopoietic cell lineage and in rare cases in megakaryocytes; the latter was only seen in patients with refractory anemia with excess blasts-1. None of the samples with secondary, non-MDS related cytopenia showed strong p53 staining.

### Strong p53 expression reflects the TP53 gene mutation

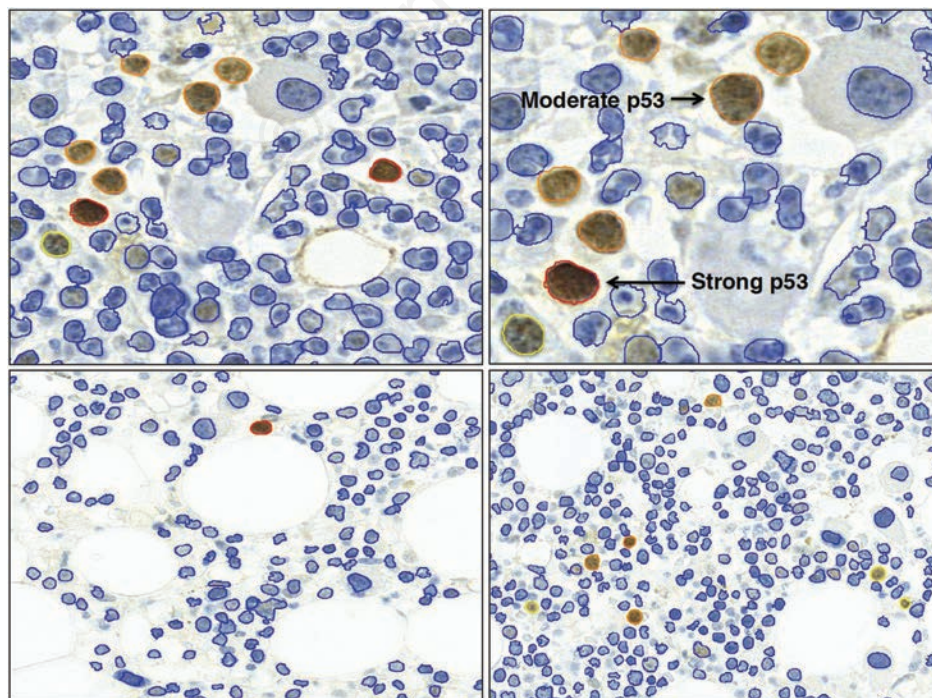
TP53 sequencing analysis was performed in a subset of nine (11%) patients in the IHC study cohort from whom additional consent was obtained (*Online Supplementary Table S4*). Mutations were detected in three patients: C275F and E294K missense mutations were associated with strong p53 expression, whereas the K291\* nonsense mutation was negative by IHC. In one patient (patient 37) the mutation was acquired at a later time point which was reflected by positive IHC, whereas previous samples stained negative. In the remaining six patients without TP53 mutations, IHC was negative in four, whereas two patients had 1.2% and 1.6% BM progenitor cells with strong p53 expression. However, it should be noted that the material used for sequencing was whole BM sections which made this method less sensitive than previous studies using archived BM smears.<sup>10</sup> In seven patients, P72R

polymorphisms were identified. These are known TP53 polymorphisms that do not represent gene mutations and were negative by IHC, as expected.

Single-cell laser-microdissection was performed in serial BM samples from three patients to assess the relationship between the degree of p53 protein expression and mutation. Of these, patient 34 was also part of the IHC cohort (Figure 2). The mutational allelic burden in microdissected cells with strong (3+) p53-staining was around 45%, indicating that 90% of the cells carried the mutation. In contrast, cells from the same sample with moderate (2+) p53-staining predominantly had wild-type TP53 (allelic burden around 16%). *Online Supplementary Figure S2A-C* shows serial data from these three patients, including the correlation between the percentage of strong (3+) p53-staining cells and the allelic burden by pyrosequencing analysis. *Online Supplementary Figure S2D* shows data for patient 37, obtained by deep-sequencing using DNA from whole BM sections.

### Strong p53 expression predicts shorter overall survival and higher risk of developing acute myeloid leukemia

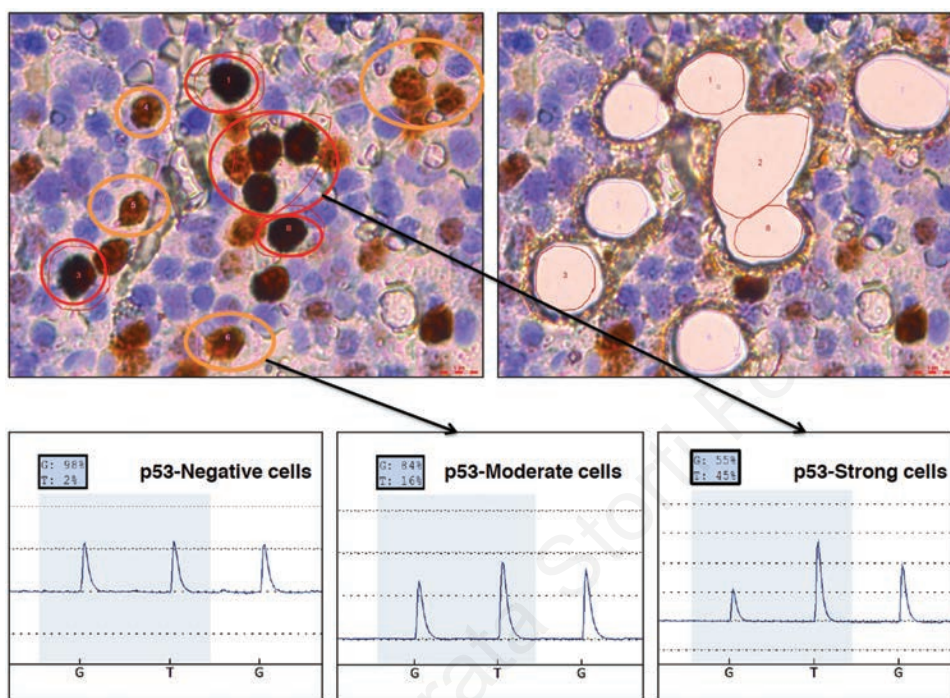
The impact of strong p53-staining on outcome was separately assessed for the presence of any strong p53-staining cells versus none, for  $\geq 1\%$  and  $\geq 2\%$  cutoffs, and for the whole IHC cohort by  $<1\%$  (n=55),  $\geq 1\%$  to  $<2\%$  (n=14), and  $\geq 2\%$  (n=16). As the  $\geq 1\%$  to  $<2\%$  and  $\geq 2\%$  curves for both overall survival and progression to AML were similarly associated with worse outcome (overlapping) and well separated from the  $<1\%$  curve (*Online Supplementary Figures S3 and S4*), the 1% cutoff was used for all subsequent analyses, with  $\geq 1\%$  defined as “p53-positive” and  $<1\%$  as “p53-negative”. Baseline demographics, clinical data, and World Health Organization subgroups did not differ significantly between patients with  $\geq 1\%$  (n=30) and  $<1\%$  (n=55) strong p53-staining (*Online Supplementary Table S5*).



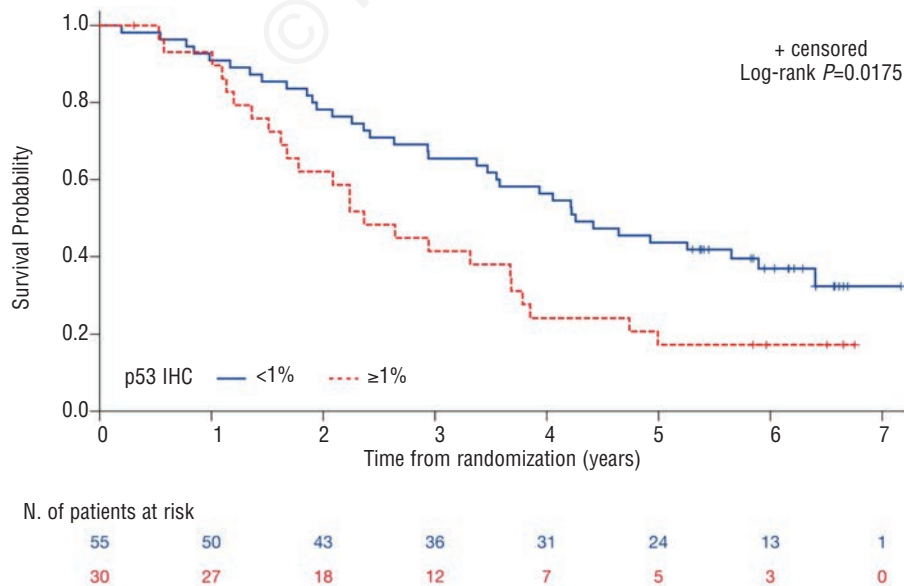
**Figure 1.** The p53-DO1 stain shows cells with moderate (2+) and strong (3+) nuclear staining (top). The lower left panel shows a BM sample with only scattered strong p53-positive staining (red circle) around the 1% level by both manual and automated image assessment. The lower right panel shows a BM sample with the presence of scattered weak (1+; yellow circle) and moderate (2+; orange circle) p53-positive staining cells, but no cells with strong (3+) nuclear staining.

The median overall survival was 2.4 years (interquartile range [IQR] 1.7-3.7) and 4.3 years (IQR 3.5-6.4) in p53-positive and p53-negative patients, respectively ( $P=0.0175$ ) (Figure 3). The 5 year rates to AML progression were 56.3% (IQR 33.3-79.3) and 19.6% (IQR 7.1-32.2), respectively ( $P=0.0006$ ) (Figure 4). The 5-year rates to AML progression using death without AML as a competing risk were 41.3% (IQR 23.0-58.8) and 14.5% (IQR 6.7-25.2), respectively ( $P=0.0021$ ). Similarly, p53 IHC positivity was significantly associated with worse outcomes (overall survival and AML risk) in patients randomized to lenalidomide ( $n=60$ ); 21 of 60 patients (35%) who received

lenalidomide had  $\geq 1\%$  strong p53-staining cells compared with nine of 25 patients (36%) who received placebo (Online Supplementary Table S2). For patients randomized to lenalidomide ( $n=60$ ), the median overall survival was 2.0 years (range, 1.2-5.0) and 4.1 years (range, 2.9-5.7) in p53-positive and p53-negative patients, respectively ( $P=0.0830$ ). The 5-year rates to AML progression were 51.3% (IQR 25.2-77.4) and 20.8% (IQR 5.3-36.3) for p53-positive and p53-negative patients, respectively ( $P=0.0047$ ); the 5-year rates to AML progression using death without AML as a competing risk were 39.8% (IQR 18.3-60.8) and 15.4% (IQR 6.1-28.6), respectively



**Figure 2.** Laser-microdissection of BM cells with strong (3+; red circle), moderate (2+; orange circle), and negative p53-staining collected in three separate tubes from the same formalin fixed, paraffin-embedded BM sample (patient 34, BM sample at 105 months from initial diagnosis). TP53 mutation analysis by pyrosequencing using DNA from microdissected cells. The images were taken with a Leica Laser Microscope at 60 $\times$  magnification before (left) and after (right) laser-microdissection; p53-negative microdissected cells are not shown in the upper images. Patient 34 had classical 5q- syndrome [46, XX, del(5q)] with a previously known C275F (G>T) TP53 mutation.



**Figure 3.** Overall survival by the presence of <1% (p53-negative) and  $\geq 1\%$  (p53-positive) BM progenitor cells with strong (3+) p53 staining.

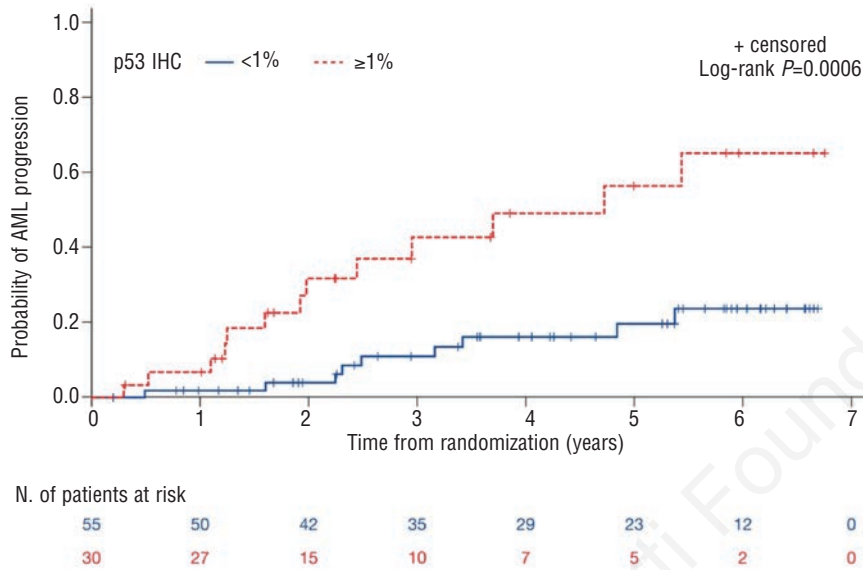


( $P=0.0140$ ) (*data not shown*). Moreover, p53 IHC positivity was associated with adverse outcome in patients with isolated del(5q) and <5% BM blast count ( $n=48$ ) (*data not shown*). The median survival after AML progression was only 0.24 years (*data not shown*).

Similar results were obtained using the p53 IHC Modified Quick Score to compare patients with a score of  $\geq 3$  versus  $< 3$  (*data not shown*). However, the  $P$ -values for the Modified Quick Score ( $P=0.04$  and  $P=0.006$ , respectively)

were higher than those for the 1% cutoff level and were not used in the final recommendation.

Using Cox proportional hazard modeling a multivariate analysis was performed to evaluate multiple risk factors for overall survival and AML progression (Table 1). p53  $\geq 1\%$  versus  $< 1\%$  was the strongest predictor of progression to AML ( $P=0.0035$ ), whereas p53 IHC and, interestingly, male sex independently predicted for overall survival (both  $P<0.001$ ).



**Figure 4.** Probability of AML progression by the presence of <1% (p53-negative) and  $\geq 1\%$  (p53-positive) BM progenitor cells with strong (3+) p53 staining.

**Table 1.** Multivariate and univariate Cox proportional regression models for AML progression and overall survival for the IHC study cohort.

Baseline characteristics	AML progression						Overall survival					
	Univariate model			Multivariate final model			Univariate model			Multivariate final model		
	HR	95% CI	P	HR	95% CI	P	HR	95% CI	P	HR	95% CI	P
Age, years	0.997	(0.961-1.034)	0.8617				1.021	(0.998-1.045)	0.0782	1.021		0.0856
Sex (male vs. female)	0.335	(0.044-2.528)	0.2887				3.482	(1.910-6.347)	<b>&lt;0.001</b>	4.088	(2.135-7.827)	<b>&lt;0.001</b>
IPSS categories (high-, int-2-, int-1-risk vs. low-risk)	1.430	(0.503-4.059)	0.5021				0.785	(0.434-1.420)	0.4236			
FAB classification (RAEB, CMML vs. RA, RARS)	1.678	(0.478-5.897)	0.4195				1.813	(0.839-3.921)	0.1304			
Duration of MDS, years	1.028	(0.944-1.120)	0.5200				0.967	(0.895-1.046)	0.4047			
Transfusion burden, units/8 weeks	1.189	(1.021-1.385)	<b>0.0259</b>	1.149	(0.985-1.341)	0.0781	1.120	(1.014-1.238)	<b>0.0254</b>			
26-week transfusion independence	0.591	(0.239-1.463)	0.2554				0.406	(0.222-0.742)	<b>0.0034</b>	0.444	(0.241-0.819)	0.0093
Bone marrow blast group (<5% vs. $\geq 5\%$ )	2.326	(0.660-8.192)	0.1889				2.198	(0.973-4.967)	0.0582			
N. of cytopenia(s) (2 or 3 vs. 1)	1.622	(0.700-3.760)	0.2593				0.806	(0.463-1.404)	0.4465			
Platelet count, $\times 10^9/L$	1.000	(0.997-1.003)	0.7500				0.998	(0.996-1.000)	0.1191	0.998	(0.997-1.000)	<b>0.0451</b>
Absolute neutrophil count, $\times 10^9/L$	0.942	(0.773-1.149)	0.5562				1.020	(0.945-1.101)	0.6150			
Hemoglobin, g/dL	0.815	(0.530-1.253)	0.3507				0.896	(0.687-1.167)	0.4139			
Erythropoietin level, mIU/mL	1.008	(0.999-1.018)	0.0763				1.007	(1.001-1.013)	0.0247			
Ferritin level, mol/L	1.000	(1.000-1.000)	0.1612				1.000	(1.000-1.000)	0.1578			
del(5q) (complex vs. isolated)	0.735	(0.287-1.878)	0.5195				1.046	(0.608-1.798)	0.8713			
WPSS group (very high, high vs. intermediate, very low/low)	1.010	(0.384-2.654)	0.9846				1.469	(0.818-2.639)	0.1976			
p53 IHC ( $\geq 1\%$ vs. $< 1\%$ )	4.017	(1.707-9.453)	<b>0.0014</b>	3.618	(1.525-8.583)	<b>0.0035</b>	1.869	(1.107-3.157)	0.0193	2.887	(1.608-5.185)	<b>&lt;0.001</b>

AML: acute myeloid leukemia; CMML: chronic myelomonocytic leukemia; FAB: French-American-British; HR: hazard ratio; 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; WPSS: World Health Organization-based Prognostic Scoring System; Statistically significant results are shown in bold.

### Strong p53-expression is associated with reduced cytogenetic response, but not transfusion independence

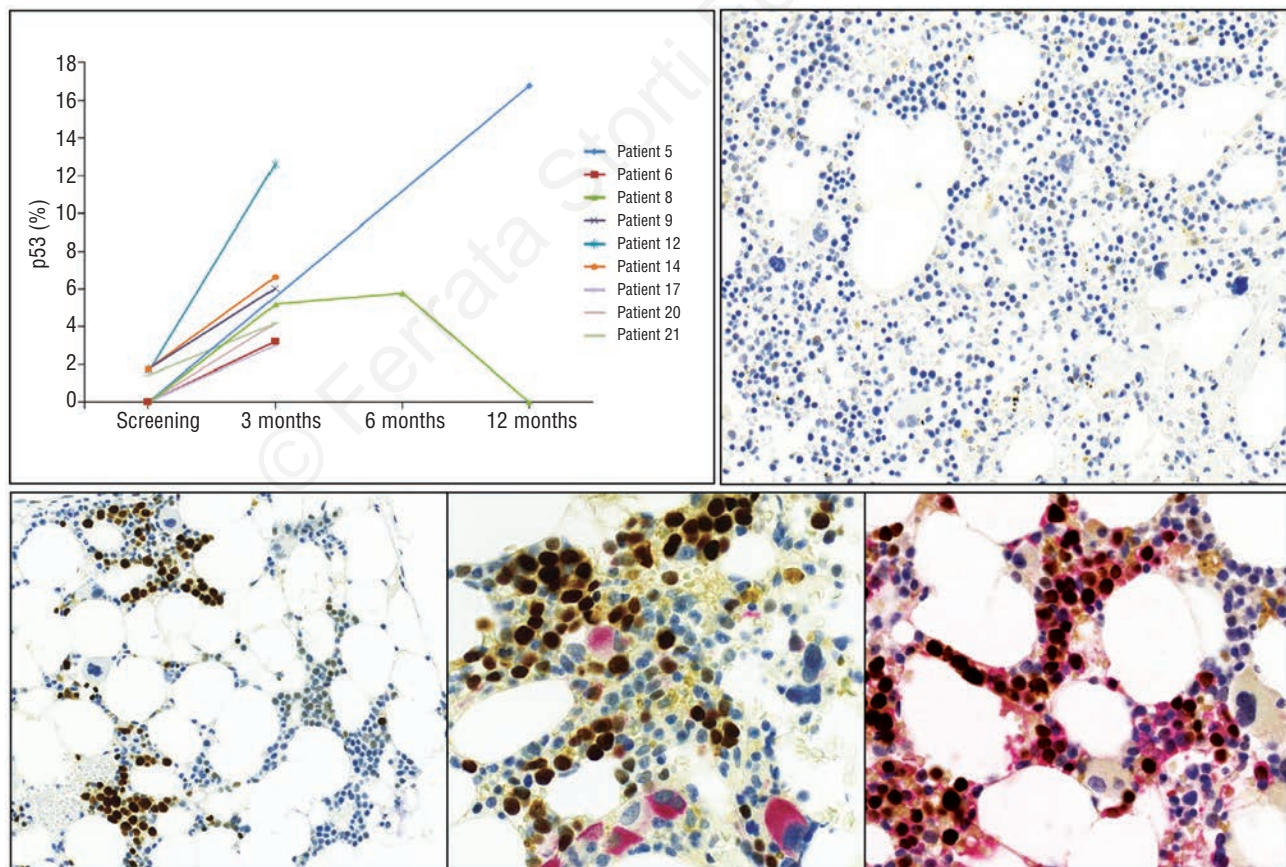
There was no difference in red blood cell-transfusion independence (RBC-TI) between p53-positive and p53-negative patients in the total cohort or in those patients randomized to receive lenalidomide. In the total cohort ( $n=85$ ),  $\geq 26$  week RBC-TI was achieved by 30.0% (9 of 30) of p53-positive and 36.4% (20 of 55) of p53-negative patients ( $P=0.636$ ) (Online Supplementary Table S6). Similarly, for patients randomized to receive lenalidomide 10 mg ( $n=33$ ), RBC-TI response was achieved in 62.5% (5 of 8) and 52.0% (13 of 25) of p53-positive and p53-negative patients, respectively ( $P=0.699$ ). In contrast, p53 IHC status was significantly associated with cytogenetic response (complete and partial response taken together): 51% (18 of 35) of p53-negative patients versus 14% (3 of 21) of p53-positive patients had a cytogenetic response ( $P=0.009$ ). As cytogenetic response rates differed for patients receiving lenalidomide 5 mg or 10 mg in the MDS-004 trial, cytogenetic response rates were also specifically evaluated for each lenalidomide dose level in the present study. For patients randomized to lenalidomide 10 mg, one of eight (12.5%) p53-positive patients had a cytogenetic response compared to 16 of 19 (84.2%)

p53-negative patients.

Therapeutic response by 26-week RBC-TI in combination with a p53-negative IHC status was associated with excellent outcome, and the median overall survival for this group was not reached at the end of the MDS-004 study period (*data not shown*). By comparison, the median overall survival was 2.3 years (IQR 1.4-3.7) and 3.9 years (IQR 2.3-4.9) for non-responding p53-positive and p53-negative patients, respectively. The 5-year cumulative incidences of AML were 71.1% (IQR 43.4-100) and 24.3% (IQR 4.5-44.2), respectively. For responding patients, the 5-year rates of AML progression were 30.0% (IQR 0.0-65.7) and 15.6% (IQR 0.0-31.8) in p53-positive and p53-negative patients, respectively. The association was also significant when using death without AML as a competing risk ( $P=0.0125$ ). Although p53 IHC status showed no association with the duration of RBC-TI ( $P=0.44$ ), the curves separated after 18 months with the response duration being shorter in the p53-positive patients (Online Supplementary Figure S5).

### Increase in p53 protein expression predicts disease progression and cytogenetic evolution

The value of p53 IHC staining during follow-up was assessed in serial BM samples from 21 patients (25%)



**Figure 5.** Increase of p53 in eight of 21 patients after 3 months, and in one patient (patient 5) after 12 months. Patients 5, 6, 9, and 17 had cytogenetic evolution with complex karyotypes at 12, 22, 6, and 3 months, respectively. The decrease in p53 in the last sample in patient 8 may reflect very low BM cellularity (10%). The histology panels show p53-D01 staining in BM samples from Patient 5 at screening (upper right) and after 12 months (lower left) at 20 $\times$ /40 $\times$  magnification. Cells with strong (3+) p53 expression were negative for CD34 (lower middle) and positive for hemoglobin (lower right). No increase in CD34 $^+$  cells was seen, but aberrant expression of CD34 in megakaryocytes was noted.

(Online Supplementary Table S7). At the scheduled 12-week assessment, an increase in p53 expression from baseline was seen in eight patients (Figure 5); 12 patients remained negative and one patient had inadequate BM samples at 3 and 6 months, but adequate material at 12 months. The increase in p53-staining was associated with cytogenetic evolution (all complex karyotypes) in four patients (patients 5, 6, 9, and 17; all randomized to lenalidomide 10 mg). Overall, six of the eight p53-positive patients developed AML within 13-44 months, compared with two of 12 patients who were p53-negative ( $P < 0.01$ ). The increase in p53 by IHC at 12 weeks was also associated with shorter overall survival compared with that of patients who remained p53-negative ( $P = 0.0005$ ).

In addition, we assessed p53 IHC status and *TP53* mutational status in serial BM samples from four patients, including single-cell laser-microdissection in three patients' samples, as described previously (Online Supplementary Figure S2). Briefly, two patients (Online Supplementary Figure S2A,B) had small *TP53* mutated subclones already at initial diagnosis which, at first, remained stable, but then increased as the patients' disorder progressed to high-risk MDS. The *TP53* mutation was reflected by the presence of BM progenitors with strong p53-staining at the corresponding time points. The remaining two patients (Online Supplementary Figure S2C,D) acquired *TP53* mutations at 52 and 75 months, associated with an increase in p53 by IHC. The acquisition of *TP53* mutations was associated with rapid disease progression.

## Discussion

This study demonstrates the utility of p53 IHC as a significant and independent clinical decision-making tool in lower-risk del(5q) MDS patients treated with lenalidomide. Strong p53 expression of  $\geq 1\%$  prevalence was independently associated with a higher risk of AML transformation and poor overall survival. The results for manual versus automated p53 counting were highly concordant and the assessment of strong p53-staining by three independent hematopathologists reflected high inter-observer reproducibility. p53 IHC status was not associated with the achievement or duration of RBC-TI but did influence overall survival, while in the original report of the MDS-004 study, RBC-TI  $> 8$  weeks was associated with better overall survival.<sup>5</sup> Interestingly, p53 IHC status was associated with achievement of a cytogenetic response to lenalidomide. Complete hematologic response without disappearance of del(5q) is, therefore, insufficient to recognize patients with a low risk of progression, when considering p53 status at baseline. The dismal survival after AML transformation underscores the importance of predicting rather than treating this condition.

A strong association between p53 protein expression, *TP53* mutation, and adverse outcome has been reported in various hematologic malignancies including MDS.<sup>18,23-25,29-34</sup> *TP53* mutations occur primarily in high-risk/therapy-related MDS, MDS-derived leukemia, and in the context of complex chromosomal abnormalities including del(17p).<sup>11,12,14,15,18-20,35,36</sup> Studies also reported on the poor prognostic impact of *TP53* mutations and the association between *TP53* mutations and poor therapy response. Interestingly, a high-frequency of chromosome 5 abnormalities was found in patients with *TP53* mutations.<sup>16,35,37,38</sup>

In fact, a recent study encompassing 318 MDS patients of all IPSS categories found that *TP53* mutations were strongly correlated with aberrations of chromosome 5.<sup>28</sup> The *TP53* mutant clones persisted in patients not responding to chemotherapy. Our present findings underscore the role of the *TP53* pathway in the pathogenesis of lower-risk del(5q) MDS as well.

Our previous study,<sup>10</sup> as well as two recent investigations using conventional Sanger sequencing in patients with lower-risk del(5q) MDS,<sup>39</sup> and higher-risk MDS and AML,<sup>7</sup> found that patients with *TP53* mutations do not obtain a complete cytogenetic response to lenalidomide.

Although the 1% cutoff level put forward in this study is arbitrary, it is able to divide patients into two groups with very different outcomes. Of the 55 patients with  $< 1\%$  strong p53-staining cells, 40 patients had none and 15 patients had 0.2-0.9% cells. We do not know how many *TP53* mutated cells are needed to promote disease progression in an individual patient. Nevertheless, the outcome curves for patients with  $\geq 1\%$  p53-positive cells were identical to those using the 2% cutoff, and significantly different from those with  $< 1\%$  p53-positive cells. In fact, p53-positive IHC  $\geq 1\%$  was the strongest independent predictor of AML transformation and also an independent predictor of overall survival. Thus, even the presence of small p53-positive populations has prognostic significance. Recently, a similar effect was observed for *NRAS* mutations in patients with lower-risk MDS.<sup>40</sup>

In our previous study,<sup>10</sup> nine of ten patients with a mutation showed  $> 2\%$  strong p53-staining cells. Interestingly, the association with disease progression and AML risk was stronger for IHC compared with *TP53* mutation, an observation also made in cases of pediatric glioma.<sup>41</sup>

Failure to detect *TP53* mutations in IHC-positive samples may reflect the presence of very small subclones and dilution of hematopoietic DNA. In contrast to our previous study which used DNA from BM smears or biobanked samples,<sup>10</sup> deep-sequencing in the present study was performed on DNA from sections of biopsies, including varying amounts of hematopoietic and non-hematopoietic cells, and stromal components. The negative mutational analysis in two patients with 1-2% IHC-positive cells is, therefore, in line with previous reports. Also, by separately analyzing DNA from both bulk BM cells and microdissected cells with strong p53-staining, we demonstrated that a small fraction of cells with mutated *TP53* can exist without detection by bulk tissue sequencing (*data not shown*). This indicates greater sensitivity of the p53 IHC method for identifying patients with small *TP53* mutant clones. However, the small number of BM samples that were assessed by IHC and sequencing is a limitation in the present study and the correlation between IHC and *TP53* mutational status should be further assessed in a larger cohort.

A drawback of the IHC method is that it fails to detect nonsense mutations giving rise to a stop codon. However, such mutations occur in  $< 10\%$  of high-risk myeloid malignancies and seem to be uncommon in lower-risk del(5q) MDS. None of the *TP53* mutated patients with lower-risk del(5q) MDS in the study by Kulasekararaj *et al.*<sup>28</sup> or in our previous report<sup>10</sup> had stop codon mutations and, in the present study, only one nonsense mutation was detected by deep sequencing. Therefore, a negative IHC status, in spite of an underlying mutation, is likely to be infrequent in this population.



Strong p53-expression has occasionally been described in hematologic malignancies and non-neoplastic conditions in the absence of a *TP53* mutation, indicating alternative mechanisms of acquiring a p53-positive phenotype.<sup>12,21,33,42-44</sup> One of the major factors contributing to p53 stabilization is via expression of p14ARF.<sup>45,46</sup> Alternatively, inhibition of MDM2-mediated degradation of p53 could block the degradation of p53 protein.<sup>47,48</sup> Studies by Barlow *et al.*<sup>49</sup> and Pellagatti *et al.*<sup>50</sup> have elegantly shown that wild-type p53 activation is a key mechanism underlying the anemia of the 5q- syndrome, with moderate p53 IHC staining observed in these studies. However, in our experience, strong p53-staining is not seen in normal/reactive BM samples. Here, we microdissected cells with strong and moderate staining, and demonstrated an underlying mutation in the former, but not in the latter.

The study of sequential BM samples demonstrated that an increase in p53-positive cells may predict disease progression and cytogenetic evolution. Repeated testing may, therefore, be a way to investigate patients with borderline p53 IHC frequencies. In addition, the development of non-histological methods, including flow cytometry and immunocytochemistry, to assess p53-protein expression could be useful.

In conclusion, we found that strong p53 protein expression by IHC is the strongest independent predictor of AML transformation, and also an independent predictor of overall survival and lower cytogenetic response in lower-

risk del(5q) MDS patients treated with lenalidomide. Furthermore, the presence of BM progenitor cells with strong nuclear p53 expression may reflect an underlying *TP53* mutation. As deep-sequencing at present is neither a validated nor a generally available method, we suggest that assessment of p53 by IHC should be integrated in the routine diagnostic work-up and follow-up of del(5q) MDS patients. Moreover, *TP53* mutational status should be included in the risk assessment of del(5q) MDS patients.

### Acknowledgments

*This work was supported by grants from Celgene Corporation. We thank Sabina Botic, Hanna Bergman, Ann Kanfeldt, and Erika Ezelius (Biomedical Laboratory Technicians at the Department of Pathology, Division of Hematopathology, Karolinska University Hospital, Stockholm, Sweden) for sectioning bone marrow samples and immunohistochemistry staining. We also thank Doris Kiri, manager of the original MDS-004 study and the clinical investigators in this trial. The authors received editorial assistance (Christian Geest, PhD) from Excerpta Medica, sponsored by Celgene Corporation. The authors are fully responsible for all content and editorial decisions regarding the manuscript.*

### Authorship and Disclosures

*Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at [www.haematologica.org](http://www.haematologica.org).*

## References

- Greenberg P, Cox C, LeBeau MM, Fenau P, Morel P, Sanz G, et al. International scoring system for evaluating prognosis in myelodysplastic syndromes. *Blood*. 1997;89(6):2079-88.
- Giagounidis AA, Geming U, Wainscoat JS, Boultonwood J, Aul C. The 5q- syndrome. *Hematology*. 2004;9(4):271-7.
- Geming U, Strupp C, Kuendgen A, Isa S, Knipp S, Hildebrandt B, et al. Prospective validation of the WHO proposals for the classification of myelodysplastic syndromes. *Haematologica*. 2006;91(12):1596-604.
- Geming U, Lauseker M, Hildebrandt B, Symeonidis A, Cermak J, Fenau P, et al. Survival, prognostic factors and rates of leukemic transformation in 381 untreated patients with MDS and del(5q): a multicenter study. *Leukemia*. 2012;26(6):1286-92.
- Fenau P, Giagounidis A, Selleslag D, Beyne-Rauzy O, Mufti G, Mittelman M, et al. A randomized phase 3 study of lenalidomide versus placebo in RBC transfusion-dependent patients with low-/intermediate-1-risk myelodysplastic syndromes with del5q. *Blood*. 2011;118(4):3765-76.
- Göhring G, Giagounidis A, Büsche G, Kreipe HH, Zimmermann M, Hellström-Lindberg E, et al. Patients with del(5q) MDS who fail to achieve sustained erythroid or cytogenetic remission after treatment with lenalidomide have an increased risk for clonal evolution and AML progression. *Ann Hematol*. 2010;89(4):365-74.
- Mallo M, Cervera J, Schanz J, Such E, García-Manero G, Luño E, et al. Impact of adjunct cytogenetic abnormalities for prognostic stratification in patients with myelodysplastic syndrome and deletion 5q. *Leukemia*. 2011;25(1):110-20.
- Bejar R, Stevenson K, Abdel-Wahab O, Galili N, Nilsson B, Garcia-Manero G, et al. Clinical effect of point mutations in myelodysplastic syndromes. *N Engl J Med*. 2011;364(26):2496-506.
- Kantarjian H, O'Brien S, Ravandi F, Borthakur G, Faderl S, Bueso-Ramos C, et al. The heterogeneous prognosis of patients with myelodysplastic syndrome and chromosome 5 abnormalities: how does it relate to the original lenalidomide experience in MDS? *Cancer*. 2009;115(22):5202-9.
- Jädersten M, Saft L, Smith A, Kulasekararaj A, Pomplun S, Göhring G, et al. TP53 mutations in low-risk myelodysplastic syndromes with del(5q) predict disease progression. *J Clin Oncol*. 2011;29(15):1971-9.
- Kaneko H, Misawa S, Horiike S, Nakai H, Kashima K. TP53 mutations emerge at early phase of myelodysplastic syndrome and are associated with complex chromosomal abnormalities. *Blood*. 1995;85(8):2189-93.
- Jonveaux P, Fenau P, Quiquandon I, Pignon JM, Lai JL, Loucheux-Lefebvre MH, et al. Mutations in the p53 gene in myelodysplastic syndromes. *Oncogene*. 1991;6(12):2243-7.
- Ludwig L, Schulz AS, Janssen JW, Grünwald K, Bartram CR. P53 mutations in myelodysplastic syndromes. *Leukemia*. 1992;6(12):1302-4.
- Padua RA, Guinn BA, Al-Sabah AI, Smith M, Taylor C, Pettersson T, et al. RAS, FMS and p53 mutations and poor clinical outcome in myelodysplasias: a 10-year follow-up. *Leukemia*. 1998;12(6):887-92.
- Kita-Sasai Y, Horiike S, Misawa S, Kaneko H, Kobayashi M, Nakao M, et al. International prognostic scoring system and TP53 mutations are independent prognostic indicators for patients with myelodysplastic syndrome. *Br J Haematol*. 2001;115(2):309-12.
- Christiansen DH, Andersen MK, Pedersen-Bjergaard J. Mutations with loss of heterozygosity of p53 are common in therapy-related myelodysplasia and acute myeloid leukemia after exposure to alkylating agents and significantly associated with deletion or loss of 5q, a complex karyotype, and a poor prognosis. *J Clin Oncol*. 2001;19(5):1405-13.
- Pedersen-Bjergaard J, Andersen MK, Andersen MT, Christiansen DH. Genetics of therapy-related myelodysplasia and acute myeloid leukemia. *Leukemia*. 2008;22(2):240-8.
- Kitagawa M, Yoshida S, Kuwata T, Tanizawa T, Kamiyama R. p53 expression in myeloid cells of myelodysplastic syndromes. Association with evolution of overt leukemia. *Am J Pathol*. 1994;145(2):338-44.
- Wattel E, Preudhomme C, Hecquet B, Vanrumbeke M, Quesnel B, Dervite I, et al. p53 mutations are associated with resistance to chemotherapy and short survival in hematologic malignancies. *Blood*. 1994;84(9):3148-57.
- Lai JL, Preudhomme C, Zandeck M, Flactif M, Vanrumbeke M, Lepelle P, et al. Myelodysplastic syndromes and acute myeloid leukemia with 17p deletion. An entity characterized by specific dysgranulopoiesis and a high incidence of P53 mutations. *Leukemia*. 1995;9(3):370-81.
- Sugimoto K, Hirano N, Toyoshima H, Chiba S, Mano H, Takaku F, et al. Mutations of the p53 gene in myelodysplastic syndrome (MDS) and MDS-derived leukemia. *Blood*. 1993;81(11):3022-6.
- Kamata H, Mitani S, Fujiwara M, Aoki N,



- Okada S, Mori S. Mutation of the p53 tumour suppressor gene and overexpression of its protein in 62 Japanese non-Hodgkin's lymphomas. *Clin Exp Med.* 2007;7(2):39-46.
23. Chang H, Yeung J, Qi C, Xu W. Aberrant nuclear p53 protein expression detected by immunohistochemistry is associated with hemizygous P53 deletion and poor survival for multiple myeloma. *Br J Haematol.* 2007;138(3):324-9.
  24. Chang H, Jiang AM, Qi CX. Aberrant nuclear p53 expression predicts hemizygous 17p (TP53) deletion in chronic lymphocytic leukemia. *Am J Clin Pathol.* 2010;133(1):70-4.
  25. Chen MH, Qi CX, Saha MN, Chang H. p53 nuclear expression correlates with hemizygous TP53 deletion and predicts an adverse outcome for patients with relapsed/refractory multiple myeloma treated with lenalidomide. *Am J Clin Pathol.* 2012;137(2):208-12.
  26. Thiele J, Kvasnicka HM, Facchetti F, Franco V, van der Walt J, Orazi A. European consensus on grading bone marrow fibrosis and assessment of cellularity. *Haematologica.* 2005;90(8):1128-32.
  27. Allred DC, Clark GM, Elledge R, Fuqua SA, Brown RW, Chamness GC, et al. Association of p53 protein expression with tumor cell proliferation rate and clinical outcome in node-negative breast cancer. *J Natl Cancer Inst.* 1993;85(3):200-6.
  28. Kulasekararaj AG, Smith AE, Mian SA, Mohamedali AM, Krishnamurthy P, Lea NC, et al. TP53 mutations in myelodysplastic syndrome are strongly correlated with aberrations of chromosome 5, and correlate with adverse prognosis. *Br J Haematol.* 2013;160(5):660-72.
  29. Iwasaki T, Murakami M, Sugisaki C, Sobue S, Ohashi H, Asano H, et al. Characterization of myelodysplastic syndrome and aplastic anemia by immunostaining of p53 and hemoglobin F and karyotype analysis: differential diagnosis between refractory anemia and aplastic anemia. *Pathol Int.* 2008;58(6):353-60.
  30. Kurotaki H, Tsushima Y, Nagai K, Yagihashi S. Apoptosis, bcl-2 expression and p53 accumulation in myelodysplastic syndrome, myelodysplastic-syndrome-derived acute myelogenous leukemia and de novo acute myelogenous leukemia. *Acta Haematol.* 2000;102(3):115-23.
  31. Kikukawa M, Aoki N, Sakamoto Y, Mori M. Study of p53 in elderly patients with myelodysplastic syndromes by immunohistochemistry and DNA analysis. *Am J Pathol.* 1999;155(3):717-21.
  32. Schlette EJ, Admirand J, Wierda W, Abruzzo L, Lin KI, O'Brien S, et al. p53 expression by immunohistochemistry is an important determinant of survival in patients with chronic lymphocytic leukemia receiving frontline chemo-immunotherapy. *Leuk Lymphoma.* 2009;50(10):1597-605.
  33. Nakano Y, Naoe T, Kiyoi H, Kitamura K, Minami S, Miyawaki S, et al. Prognostic value of p53 gene mutations and the product expression in de novo acute myeloid leukemia. *Eur J Haematol.* 2000;65(1):23-31.
  34. Dimopoulos MA, Kastritis E, Christoulas D, Migkou M, Gavriatopoulou M, Gkatzamanidou M, et al. Treatment of patients with relapsed/refractory multiple myeloma with lenalidomide and dexamethasone with or without bortezomib: prospective evaluation of the impact of cytogenetic abnormalities and of previous therapies. *Leukemia.* 2010;24(10):1769-78.
  35. Horiike S, Kita-Sasai Y, Nakao M, Taniwaki M. Configuration of the TP53 gene as an independent prognostic parameter of myelodysplastic syndrome. *Leuk Lymphoma.* 2003;44(6):915-22.
  36. Haferlach C, Bacher U, Tiu R, Maciejewski JP, List A. Myelodysplastic syndromes with del(5q): indications and strategies for cytogenetic testing. *Cancer Genet Cytogenet.* 2008;187(2):101-11.
  37. Jasek M, Gondek LP, Bejanyan N, Tiu R, Huh J, Theil KS, et al. TP53 mutations in myeloid malignancies are either homozygous or hemizygous due to copy number-neutral loss of heterozygosity or deletion of 17p. *Leukemia.* 2010;24:216-9.
  38. Horiike S, Misawa S, Kaneko H, Sasai Y, Kobayashi M, Fujii H, et al. Distinct genetic involvement of the TP53 gene in therapy-related leukemia and myelodysplasia with chromosomal losses of Nos 5 and/or 7 and its possible relationship to replication error phenotype. *Leukemia.* 1999;13(8):1235-42.
  39. Jädersten M, Saft L, Pellagatti A, Göhring G, Wainscoat JS, Boultonwood J, et al. Clonal heterogeneity in the 5q- syndrome: p53 expressing progenitors prevail during lenalidomide treatment and expand at disease progression. *Haematologica.* 2009;94(12):1762-6.
  40. Murphy DM, Bejar R, Stevenson K, Neuberger D, Shi Y, Cubrich C, et al. NRAS mutations with low allele burden have independent prognostic significance for patients with lower risk myelodysplastic syndromes. *Leukemia.* 2013;27(10):2077-81.
  41. Pollack IF, Finkelstein SD, Woods J, Burnham J, Holmes EJ, Hamilton RL, et al. Expression of p53 and prognosis in children with malignant gliomas. *N Engl J Med.* 2002;346(6):420-7.
  42. Adamson DJ, Dawson AA, Bennett B, King DJ, Haites NE. p53 mutation in the myelodysplastic syndromes. *Br J Haematol.* 1995;89(1):61-6.
  43. Elghetany MT, Alter BP. p53 protein overexpression in bone marrow biopsies of patients with Shwachman-Diamond syndrome has a prevalence similar to that of patients with refractory anemia. *Arch Pathol Lab Med.* 2002;126(4):452-5.
  44. Seliger B, Papadileris S, Vogel D, Hess G, Brendel C, Störkel S, et al. Analysis of the p53 and MDM-2 gene in acute myeloid leukemia. *Eur J Haematol.* 1996;57(3):230-40.
  45. Vonlanthen S, Heighway J, Tschan MP, Borner MM, Altermatt HJ, Kappeler A, et al. Expression of p16INK4a/p16alpha and p19ARF/p16beta is frequently altered in non-small cell lung cancer and correlates with p53 overexpression. *Oncogene.* 1998;17(21):2779-85.
  46. Xue Q, Sano T, Kashiwabara K, Saito M, Oyama T, Nakajima T. Aberrant expression of pRb, p16, p14ARF, MDM2, p21 and p53 in stage I adenocarcinomas of the lung. *Pathol Int.* 2002;52(2):103-9.
  47. Moll UM, Petrenko O. The MDM2-p53 interaction. *Mol Cancer Res.* 2003;1(14):1001-8.
  48. Koga T, Hashimoto S, Sugio K, Yoshino I, Nakagawa K, Yonemitsu Y, et al. Heterogeneous distribution of P53 immunoreactivity in human lung adenocarcinoma correlates with MDM2 protein expression, rather than with P53 gene mutation. *Int J Cancer.* 2001;95(4):232-9.
  49. Barlow JL, Drynan LF, Hewett DR, Holmes LR, Lorenzo-Abalde S, Lane AL, et al. A p53-dependent mechanism underlies macrocytic anemia in a mouse model of human 5q- syndrome. *Nat Med.* 2010;16(1):59-66.
  50. Pellagatti A, Marafioti T, Paterson JC, Barlow JL, Drynan LF, Giagounidis A, et al. Induction of p53 and up-regulation of the p53 pathway in the human 5q- syndrome. *Blood.* 2010;115(13):2721-3.