

# High p53 protein expression in therapy-related myeloid neoplasms is associated with adverse karyotype and poor outcome

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**Identification of p53-positive cells by immunohistochemistry in bone marrow from primary myelodysplastic syndrome patients correlates with the presence of *TP53* mutations and poor prognosis. Mutations in the tumor suppressor gene *TP53* are more frequent in therapy-related acute myeloid leukemia and myelodysplastic syndrome than in *de novo* disease, but the role of p53 immunohistochemistry in the therapy-related setting has not been specifically investigated. We studied p53 protein immunoreactivity in bone marrow biopsies of therapy-related myeloid neoplasms and correlated protein expression with *TP53* mutation status, clinicopathologic features and outcome. We first studied 32 patients with therapy-related acute myeloid leukemia and 63 patients with therapy-related myelodysplastic syndrome/chronic myelomonocytic leukemia from one institution and then validated our results in a separate group of 32 patients with therapy-related acute myeloid leukemia and 56 patients with therapy-related myelodysplastic syndrome from a different institution. Strong p53 immunostaining in  $\geq 1\%$  of bone marrow cells was highly predictive of a *TP53* gene mutation ( $P < 0.0001$ ) and was strongly associated with a high-risk karyotype ( $P < 0.0001$ ). The presence of  $\geq 1\%$  p53 strongly positive cells was associated with poorer overall and disease-specific survival, particularly in the subset of patients treated with stem-cell transplantation. In a multivariable Cox regression model, the presence of  $\geq 1\%$  p53 strongly expressing cells was an independent prognostic marker for overall survival in both cohorts, with hazard ratios of 3.434 (CI: 1.751–6.735,  $P < 0.0001$ ) and 3.156 (CI: 1.502–6.628,  $P = 0.002$ ). Our data indicate that p53 protein expression, evaluated in bone marrow biopsies by a widely available immunohistochemical method, prognostically stratifies patients with therapy-related myeloid neoplasms independent of other risk factors. p53 immunostaining thus represents an easily applicable method to assess risk in therapy-related acute myeloid leukemia/myelodysplastic syndrome patients.**

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Therapy-related myeloid neoplasms include acute myeloid leukemia, myelodysplastic syndrome, and less commonly myelodysplastic/myeloproliferative neoplasms such as chronic myelomonocytic leukemia. Therapy-related myeloid neoplasms occur as late complications of cytotoxic chemotherapy

and/or radiotherapy given to treat malignancies or non-malignant conditions.<sup>1,2</sup> The etiology of therapy-related myeloid neoplasms is thought to be related to the mutagenic effect of cytotoxic therapy, including alkylating agents, ionizing radiation therapy, and topoisomerase inhibitors.<sup>1</sup> Typically, therapy-related myeloid neoplasms carry complex and usually unbalanced karyotypic abnormalities, particularly loss of material on the long arm of chromosomes 5 and 7 and rearrangements of the 11q23 region involving the *MLL* gene.<sup>3</sup> The prognosis of therapy-related myeloid neoplasms is poor, likely due at least in part to the high

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proportion of cases with adverse karyotype.<sup>4,5</sup> In therapy-related myelodysplastic syndrome and oligoblastic therapy-related acute myeloid leukemia, the Revised International Prognostic Scoring System has been shown to correlate with outcome, but patients with therapy-related myelodysplastic syndrome appear to do more poorly than *de novo* myelodysplastic syndrome within each Revised International Prognostic Scoring System risk-stratum.<sup>6,7</sup> Unlike *de novo* disease, blast count is not strongly associated with outcome in therapy-related myeloid neoplasms and therapy-related myelodysplastic syndrome and therapy-related acute myeloid leukemia are considered together as one disease entity in the 2008 World Health Organization Classification.<sup>8</sup> These data suggest that additional markers are needed to stratify the prognosis of therapy-related myeloid neoplasm patients for risk-adapted therapies.

Several types of gene mutations are observed in acute myeloid leukemia and myelodysplastic syndrome, including those affecting epigenetic DNA modification and RNA splicing as well as inactivating mutations of the *TP53* tumor suppressor gene.<sup>3,9</sup> Mutational profiles confer important prognostic information that is independent of other traditional risk factors, including karyotype, blast percentage, and patient clinical characteristics in both acute myeloid leukemia and myelodysplastic syndrome.<sup>10–18</sup> The prognostic significance of mutations in therapy-related myeloid neoplasms has not been extensively characterized. Point mutations of the tumor suppressor gene *TP53* represent the most frequent single genetic abnormality in therapy-related myeloid neoplasms and are detected in 20–40% of therapy-related myeloid neoplasm patients compared with 5–20% in *de novo* acute myeloid leukemia and myelodysplastic syndrome.<sup>3,13,15,19</sup> *TP53* mutations are often associated with loss of heterozygosity of the *TP53* locus and cytogenetic defects such as  $-5/\text{del}(5q)$  and  $17p-$ .<sup>3,9,13,19–21</sup> The importance of *TP53* mutations has been validated in myelodysplastic syndrome in the non-therapy-related setting, particularly in myelodysplastic syndrome with  $\text{del}(5q)$ .<sup>21,22</sup> One recent study showed that *TP53* mutations in therapy-related myeloid neoplasms predicted an inferior clinical outcome compared with wild-type *TP53*, but the authors examined a relatively small number of cases.<sup>15</sup>

Complete assessment of *TP53* mutation requires sequencing of all exons, because mutations often occur outside of the most commonly recognized hot spots, and requires a highly sensitive assay, because the mutation may only be present in a subset of the hematopoietic cells.<sup>13,21</sup> *TP53* mutation causes overexpression of the mutated protein and several recent studies have shown that *TP53* mutations in solid tumors, lymphomas, and myelodysplastic syndrome can be accurately predicted by immunohistochemistry using widely available antibodies to

the p53 protein.<sup>21,23,24</sup> In this study, we sought to determine the prevalence of p53 protein immunoreactivity in bone marrow samples from therapy-related myeloid neoplasm patients and to evaluate its association with mutations in the *TP53* gene. We also sought to assess whether p53 immunoreactivity was associated with patient outcome in two large cohorts of therapy-related myeloid neoplasm patients with clinical follow-up.

## Materials and methods

### Patient Population and Samples

The cases in the test set were identified by a search of the pathology database of Massachusetts General Hospital for consecutive cases of therapy-related myeloid neoplasms diagnosed between 1/1/2001 and 9/30/2013 in which paraffin blocks from the original diagnostic biopsy were available. A total of 95 therapy-related myeloid neoplasms (60 therapy-related myelodysplastic syndrome, 3 therapy-related chronic myelomonocytic leukemia, and 32 therapy-related acute myeloid leukemia) were found. Original diagnostic bone marrow biopsies from 88 patients with therapy-related myeloid neoplasms (56 therapy-related myelodysplastic syndrome and 32 therapy-related acute myeloid leukemia) diagnosed at the University of Texas MD Anderson Cancer Center between 1/1/2007 and 8/31/2010 comprised the validation set. All patients in both cohorts had a documented history of a benign or malignant condition for which they received radiotherapy alone, chemotherapy alone, or combined chemoradiotherapy prior to the diagnosis of therapy-related myeloid neoplasm. Clinical information and follow-up data were obtained from the medical record. Cytogenetic analysis was performed with trypsin-Giemsa banding techniques on bone marrow cells from aspirates obtained from the diagnostic bone marrow sample and karyotype abnormalities were described according to the International System for Human Cytogenetic Nomenclature. The myelodysplastic syndrome and chronic myelomonocytic leukemia karyotypes were stratified by the Comprehensive Cytogenetic Scoring System of the Revised International Prognostic Scoring System<sup>6,25</sup> and the acute myeloid leukemia karyotypes were stratified by the United Kingdom Medical Research Council system.<sup>26</sup> For analyses in which therapy-related myelodysplastic syndrome and therapy-related acute myeloid leukemia were considered together, high-risk karyotype was defined for therapy-related myelodysplastic syndrome/chronic myelomonocytic leukemia as Revised International Prognostic Scoring System high or very high risk and for therapy-related acute myeloid leukemia as United Kingdom Medical Research Council adverse risk. Therapies administered were recorded as supportive care (transfusion

support and anti-infectives), low-intensity therapies (low-dose cytotoxic therapies, including hypomethylating agents), induction chemotherapy, or allogeneic stem-cell transplantation. This study was approved by the Institutional Review Boards at both Massachusetts General Hospital and the University of Texas MD Anderson Cancer Center.

### Immunohistochemistry for p53

Bone marrow biopsies from Massachusetts General Hospital were fixed in B+ fixative (BBC Biochemical, Seattle, WA, USA) and decalcified in RapidCal Immuno (BBC Biochemical) for 30 min at room temperature prior to routine tissue processing and paraffin-embedding. Bone marrow biopsies from the University of Texas MD Anderson Cancer Center were fixed in neutral-buffered formalin and decalcified in 10% formic acid for 3 h at 50 °C in a microwave oven. Paraffin-embedded tissues sections (3 microns) were stained with p53 monoclonal antibody DO-7 (PA0057 Ready-to-use, Leica Biosystems, Newcastle, UK) in a Leica Bond-III immunostainer according to the manufacturer's instructions. The DO-7 antibody recognizes both wild-type and mutant p53 protein.

### p53 Immunohistochemistry Scoring

The percentage of cells with strong nuclear p53 staining was counted at  $\times 400$  magnification on immunostained bone marrow sections by a hematopathologist (RPH) who was blinded to the clinical and outcome information of the cases. Only hematopoietic cells with strong (3+) nuclear staining were counted. At least 300 cells (median 610 in test set and 576 in validation set) were counted except in cases with  $>15\%$  p53 3+ cells present throughout the biopsy and in one paucicellular case in which no p53 3+ cells were identified in all 241 mononuclear cells available. On the basis of prior studies,<sup>22</sup> a cutoff of  $\geq 1\%$  p53 3+ cells was used to define positive p53 staining (p53 immunohistochemistry-positive).

### TP53 Sequencing

Mutation analyses of the *TP53* gene was performed in 71 patients using DNA from archived fixed and frozen cell pellets from the original diagnostic bone marrow sample, using the clinically validated Snapshot-NGS-V1 assay to detect single nucleotide variants and insertions/deletions. Briefly, DNA was isolated using the QIAmp (Qiagen, Hilden, Germany). Sequencing libraries were made using an anchored multiplex PCR assay and quantified using KAPA qPCR reagents (Kapa Biosystems Inc., Woburn, MA, USA) per the manufacturer's instructions. All 11 exons of *TP53* were amplified (23

amplicons of approximately 100bp each) and sequenced using Illumina MiSeq (Illumina, Inc., San Diego, CA, USA). Sequence reads were aligned against a hg19 reference genome with an average sequencing coverage of 300x.

### Statistical Analysis

Fisher's exact test, Sperman rank-order correlation coefficient, and Mann-Whitney test were used to evaluate differences between clinicopathologic features and p53 protein expression. Overall survival was defined as time from diagnosis to death from any cause. Disease-specific survival was defined as time from diagnosis to death from myelodysplastic syndrome or acute myeloid leukemia, with patients censored if death was due to other causes (such as complications of another non-myeloid neoplasm). The method of Kaplan-Meier and the log-rank test were used to compare overall survival and disease-specific survival between groups. Independent variables predicting survival were evaluated in a multivariable model using Cox Regression analyses. All two-sided *P* values of  $<0.05$  were considered statistically significant. Prism 4.0 and XLSTAT were used for data analyses.

## Results

### Clinical, Pathologic, and Genetic Features of Therapy-Related Myeloid Neoplasm Patient Groups

The characteristics of test set and validation set are shown in Table 1. Compared with the test set population, the validation set patients were younger ( $P=0.03$ ), included fewer patients treated with radiotherapy only ( $P=0.0001$ ), and were treated less often with supportive care only ( $P=0.03$ ). The World Health Organization disease subtypes were similar between the test and validation sets. Although the myelodysplastic syndrome patients in the validation set included a higher proportion of patients with higher-risk karyotypes ( $P=0.001$ ), there was no significant difference in the overall Revised International Prognostic Scoring System risk scores between the test and validation set myelodysplastic syndrome patients (Table 1). The primary diseases for which patients in the test and validation sets received cytotoxic therapy are listed in Supplementary Table 1.

### Results of p53 Immunostaining in the Test Set

Strong p53-expressing cells (3+) were observed in 67/95 (71%) of the biopsies, ranging from 0.1 to 57.5% of all nucleated bone marrow hematopoietic cells. Using a  $\geq 1\%$  cutoff of p53 3+ cells (defined as p53 immunohistochemistry-positive), 40/95 (42%) of the samples, including 10/32 therapy-related acute myeloid leukemia (31%) and 30/63

**Table 1** Clinicopathologic features of therapy-related myeloid neoplasm patients from test and validation sets

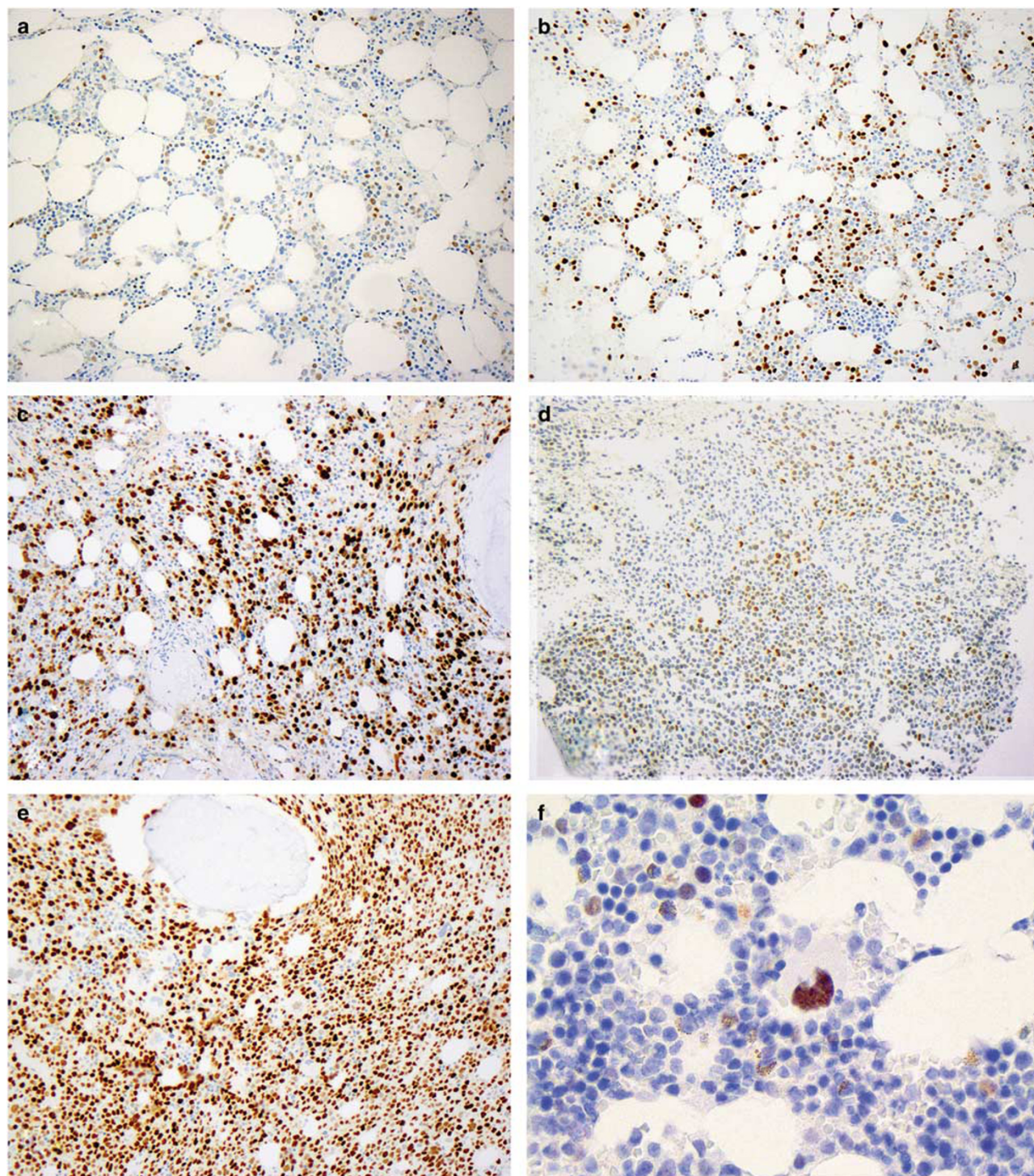
Feature	Test set (n = 95)	Validation set (n = 88)	Comparison of test and validation sets, P-value
Age, years, median (range)	69 (14–88)	65 (18–87)	0.03
Males:Females	48:47	49:39	0.55
<i>WHO Diagnoses</i>			0.29
RARS	2 (2%)	1 (1%)	
RCUD	2 (2%)	5 (6%)	
RCMD	27 (28%)	22 (25%)	
MDS del(5q)	1 (1%)	1 (1%)	
RAEB1	15 (16%)	10 (11%)	
RAEB2	9 (9%)	16 (18%)	
MDS-U	4 (4%)	1 (1%)	
CMML	3 (3%)	0	
AML	32 (35%)	32 (36%)	
<i>Prior therapy</i>			0.001
Radiation only	18 (19%)	3 (3%)	
Chemotherapy only	40 (42%)	55 (63%)	
Both	37 (39%)	30 (34%)	
Latency between first cytotoxic exposure and t-MN, median (range)	48.5 m (6–660 m)	NA	NA
<i>IPSS-R karyotype risk group (MDS/CMML only)</i>			0.001
Very low	2 (3%)	0	
Low	18 (29%)	2 (4%)	
Intermediate	5 (8%)	12 (21%)	
High	9 (14%)	12 (21%)	
Very high	26 (41%)	28 (50%)	
Not obtained	3 (5%)	2 (4%)	
<i>IPSS-R risk group (MDS/CMML only)</i>			0.14
Very low	5 (8%)	0	
Low	11 (17%)	9 (16%)	
Intermediate	13 (21%)	9 (16%)	
High	13 (21%)	17 (30%)	
Very high	15 (24%)	19 (34%)	
Not available	6 (10%)	2 (4%)	
<i>UKMRC risk group (AML only)</i>			0.99
Good	2 (6%)	2 (6%)	
Intermediate	14 (44%)	15 (47%)	
Adverse	14 (44%)	15 (47%)	
Not obtained	2 (6%)	0 (0%)	
High-risk karyotype	50/90 (56%)	56/86 (65%)	0.22
<i>Most intensive treatment administered</i>			0.03
Supportive care	34 (36%)	17 (19%)	
Low-intensity	22 (23%)	28 (32%)	
Induction	14 (15%)	23 (26%)	
Stem cell transplant	25 (26%)	20 (23%)	

AML, acute myeloid leukemia; CMML, chronic myelomonocytic leukemia; IPSS-R, Revised International Prognostic Scoring System; MDS del(5q), MDS with isolated del(5q) abnormality; MDS, myelodysplastic syndrome; MDS-U, MDS, unclassified; NA, not available; RAEB1, refractory anemia with excess blasts-1; RAEB2, refractory anemia with excess blasts-2; RARS, refractory anemia with ring sideroblasts; RCMD, refractory cytopenia with multilineage dysplasia; RCUD, refractory cytopenia with unilineage dysplasia; UKMRC, United Kingdom Medical Research Council; WHO, World Health Organization.

therapy-related myelodysplastic syndrome/chronic myelomonocytic leukemia (48%), were p53 immunohistochemistry-positive. A detailed distribution of the percentage of p53 immunohistochemistry 3+ cell percentages in the test set is shown in Supplementary Table 2. The median percentage of p53 3+ cells among the p53 immunohistochemistry-positive cases was 16.9% (range 1.1–57.5%). The cells staining for p53 were mostly early erythroid forms, blasts, and early myeloid cells that lacked

nuclear segmentation (Figure 1). Variable staining was also seen in megakaryocytes. Overall, 3+ megakaryocyte staining was present in 16/89 (18%) cases with evaluable megakaryocytes and in 15 of these 16 cases (94%),  $\geq 1\%$  p53 3+ cells were also present. In one case with 3+ megakaryocyte staining, only 0.5% of the total cells showed 3+ p53 staining.

In order to evaluate the reproducibility of p53 scoring, 40 cases (including all 18 cases with 0.5–



**Figure 1** Representative images of p53 immunostaining in bone marrow biopsies from patients with therapy-related myeloid neoplasms. (a) Case of therapy-related myelodysplastic syndrome (refractory anemia with excess blasts-1) with very rare p53 3+ cells (0.1%). (b) Case of therapy-related myelodysplastic syndrome (refractory anemia with excess blasts-2) with frequent p53 3+ cells (10.2%). (c) Case of therapy-related myelodysplastic syndrome (refractory cytopenia with multilineage dysplasia, 2% blasts) with numerous p53 3+ cells (41.3%). (d) Case of therapy-related acute myeloid leukemia with frequent p53 1+ and 2+ cells, but with only rare p53 3+ cells (0.1%). (e) Case of therapy-related acute myeloid leukemia with numerous p53 3+ cells (31.9%). (f) Case of p53 immunohistochemistry-negative therapy-related myelodysplastic syndrome (refractory cytopenia with multilineage dysplasia) with only 0.5% p53 3+ cells and with rare p53 3+ megakaryocytes.

5% p53 3+ cells) were evaluated independently by two additional observers (VN and AC). With respect to scoring cases as p53 immunohistochemistry-

positive ( $\geq 1\%$ ) or negative ( $< 1\%$ ), all three observers agreed on 37/40 cases (93%). For the three discrepant cases, the scores were 0.6-1.0-1.4,

0.0-1.1-1.1, and 0.8-1.1-2.3%. The Spearman  $r$  correlation coefficients of p53 3+ percentages between the three observers were 0.95, 0.87, and 0.86 ( $P < 0.0001$  for all comparisons) and the kappa coefficients between the three observer pairs with respect to p53 immunohistochemistry-positive vs p53 immunohistochemistry-negative were 0.95, 0.90, and 0.85.

Comparison of features between p53 immunohistochemistry-positive and p53 immunohistochemistry-negative cases in the test set is shown in Table 2. There was no association between p53 immunohistochemistry status and patient age, hemoglobin level, bone marrow cellularity, peripheral blood blast percentage, or diagnosis as therapy-related myelodysplastic syndrome/chronic myelomonocytic leukemia vs therapy-related acute myeloid leukemia. p53 immunohistochemistry-positive status was borderline associated with a lower white blood count, lower platelet count, and lower bone marrow blast percentage. Positivity for p53 was seen less often in patients with prior exposure to radiation therapy only compared with chemotherapy only or combined modality therapy ( $P = 0.02$ ), but there was no difference in latency to develop therapy-related myeloid neoplasms between p53 immunohistochemistry-positive and p53 immunohistochemistry-negative cases (Table 2). p53 immunohistochemistry positivity was also strongly associated with higher karyotype risk in the entire cohort ( $P < 0.0001$ ) and in therapy-related myelodysplastic syndrome/chronic myelomonocytic leukemia ( $P < 0.0001$ ), but was only borderline associated with higher United Kingdom Medical Research Council karyotype risk in therapy-related acute myeloid leukemia ( $P = 0.10$ ). There was a strong association between p53 immunohistochemistry positivity and the presence of  $-5/\text{del}(5q)$  and  $-7/\text{del}(7q)$  (both  $P < 0.0001$ ), but there was no significant association with the presence of 17p abnormalities. No cases with 11q23 abnormality showed p53 immunohistochemistry positivity, compared with 13% of the p53 immunohistochemistry-negative cases ( $P = 0.04$ ). p53 immunohistochemistry positivity was also associated with higher overall Revised International Prognostic Scoring System risk grouping in the therapy-related myelodysplastic syndrome/chronic myelomonocytic leukemia patients ( $P < 0.0001$ ). Regarding morphologic features, p53 immunohistochemistry positivity was associated with the presence of marked ( $>50\%$  of lineage) erythroid dysplasia ( $P = 0.015$ ) and was borderline associated with marked megakaryocytic dysplasia ( $P = 0.057$ ), but was not associated with myeloid lineage dysplasia.

#### Association of p53 Immunohistochemistry Results with TP53 Mutations

Sequencing of the *TP53* gene was informative in 65/71 (92%) of the tested cases. The sequencing results

and correlation with p53 immunohistochemistry results are shown in Supplementary Table 3. Among the p53 immunohistochemistry-positive cases, 29/31 (94%) had a *TP53* mutation identified, including 23 with single, 5 with two, and 1 with three missense mutations; 2 cases additionally had *TP53* insertions/deletions. In both of the p53 immunohistochemistry-positive cases with no *TP53* mutation identified, the p53 immunohistochemistry was only borderline positive (1.1% for both). Conversely, 4/34 (12%) p53 immunohistochemistry-negative cases had a *TP53* mutation identified. Two of these discrepant cases involved nonsense mutations creating a stop codon (c.310C>T/p.Gln104\* and c.586C>T/p.Arg196\*), resulting in a truncated protein that would likely not be detected by immunohistochemistry. The other two cases had single missense mutations (c.529C>T/p.Pro177Ser and c.393C>A/p.Asn131Lys) with allele burdens of 29% and 30%, respectively. Overall, p53 immunohistochemistry positivity was strongly associated with the presence of *TP53* mutation ( $P < 0.0001$ ). There was no correlation between the percentage of p53 3+ cells and the estimated mutant allele burden by sequence analysis (Spearman  $r$  correlation coefficient = 0.045,  $P = 0.81$ ).

#### Association of p53 Immunohistochemistry Status with Patient Outcome in the Test Set

The median follow-up of all patients was 12 months and the median follow-up of surviving patients was 43 months. The median overall survival of all patients was 12 months and the median disease-specific survival was 13 months. In univariate analysis of all patients in the test group, p53 immunohistochemistry-positive status was strongly associated with shorter overall survival (5.2 vs 20.6 months,  $P < 0.0001$ ) and disease-specific survival (5.3 vs 25.4 months,  $P < 0.0001$ ) compared with p53 immunohistochemistry-negative patients (Figures 2a and b). The unfavorable impact of p53 immunohistochemistry-positive status was seen in both therapy-related acute myeloid leukemia patients (median overall and disease-specific survival 4.2 vs 15.9 months,  $P = 0.002$ ) and therapy-related myelodysplastic syndrome/chronic myelomonocytic leukemia patients (median overall survival 5.4 vs 25.4 months,  $P < 0.0001$ , median disease-specific survival 5.5 vs 36.7 months,  $P < 0.0001$ ) and was maintained in patients with high-risk karyotypes (median overall survival 5.4 vs 19.7 months,  $P = 0.0005$ , median disease-specific survival 5.5 vs 19.7 months,  $P = 0.0006$ ) (Figure 2c). The survival of patients treated with stem-cell transplantation was also markedly influenced by p53 immunohistochemistry status (median overall survival and disease-specific survival 11.5 months for p53 immunohistochemistry-positive vs 98 months for p53 immunohistochemistry-negative patients,  $P < 0.0001$ ) (Figure 2d).

**Table 2** Comparison of clinicopathologic features of p53 immunohistochemistry-positive vs p53 immunohistochemistry-negative therapy-related myeloid neoplasm cases in test set (*n* = 95)

Feature	p53 IHC+ (n = 40)	p53 IHC- (n = 55)	Comparison of p53 IHC+ and IHC-, P-value
Age, years, median (range)	69 (25–84)	68 (14–88)	0.66
Males:Females	17:23	31:24	0.22
Latency from cytotoxic exposure, median (range)	48 m (6–300 m)	52 m (6–660 m)	0.58
<i>WHO diagnosis</i>			0.19
MDS/CMML	30 (75%)	33 (60%)	
AML	10 (25%)	22 (40%)	
<i>Prior therapy</i>			0.02
Radiation only	3 (8%)	15 (27%)	
Chemotherapy only	22 (55%)	18 (33%)	
Both	15 (38%)	22 (40%)	
White blood count, × 10 <sup>9</sup> /l, median (range)	3.1 (0.9–72.2)	4.1 (0.9–112.9)	0.05
Absolute neutrophil count, × 10 <sup>9</sup> /l, median (range)	0.68 (0.20–18.10)	1.45 (0.00–16.10)	0.44
Hemoglobin, g/dl, median (range)	9.4 (5.6–13.2)	9.5 (6.7–13.8)	0.83
Platelet count, × 10 <sup>9</sup> /l, median (range)	68 (12–355)	86 (12–382)	0.09
Peripheral blood blasts, %, median (range)	0 (0–46)	0 (0–90)	0.85
Bone marrow cellularity, %, median (range)	50 (15–100)	60 (15–95)	0.64
Bone marrow blasts, %, median (range)	6 (0–95)	8 (0–95)	0.05
<i>IPSS-R karyotype risk group (MDS/CMML only)</i>			<0.0001 (Very low/low vs very high/high)
Very low	0	2 (6%)	
Low	0	18 (55%)	
Intermediate	1 (3%)	4 (12%)	
High	2 (7%)	7 (21%)	
Very high	24 (80%)	2 (6%)	
Not obtained	3 (10%)	0	
<i>IPSS-R overall risk group (MDS/CMML only)</i>			<0.0001 (very low/low vs very high/high)
Very low	0	5 (15%)	
Low	0	11 (33%)	
Intermediate	5 (17%)	8 (24%)	
High	10 (33%)	3 (9%)	
Very high	11 (37%)	4 (12%)	
Not available	3 (10%)	2 (6%)	
<i>UKMRC risk group (AML only)</i>			0.10 (Good/intermediate vs adverse)
Good	0	2 (9%)	
Intermediate	2 (20%)	12 (55%)	
Adverse	6 (60%)	8 (36%)	
Not obtained	2 (20%)	0	
<i>Normal karyotype</i>	2/35 (6%)	24/55 (44%)	<0.0001
<i>High-risk karyotype<sup>a</sup></i>	32/35 (91%)	18/55 (33%)	<0.0001
<i>Complex karyotype<sup>b</sup></i>	32/35 (91%)	9/55 (16%)	<0.0001
– 5/del(5q)	23/35 (66%)	6/55 (11%)	<0.0001
– 7/del(7q)	22/35 (63%)	11/55 (20%)	<0.0001
11q23 abnormality	0	7/55 (13%)	0.04
17p abnormality	6/35 (17%)	5/55 (9%)	0.74
<i>Most intensive treatment administered</i>			0.38
Supportive care	18 (45%)	16 (29%)	
Low-intensity	9 (23%)	13 (24%)	
Induction	4 (10%)	10 (18%)	
Stem cell transplant	9 (23%)	16 (29%)	

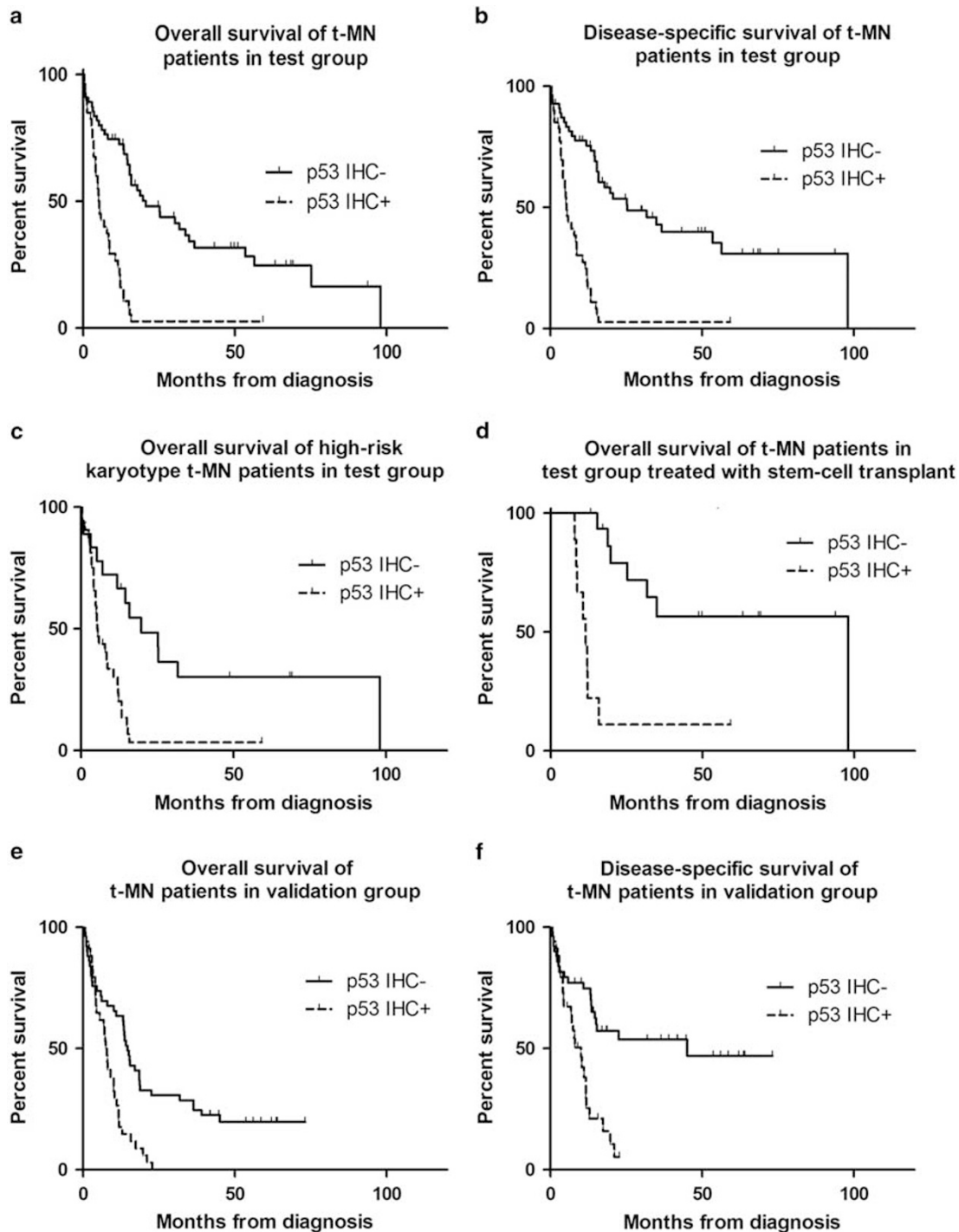
AML, acute myeloid leukemia; CMML, chronic myelomonocytic leukemia; IPSS-R, Revised International Prognostic Scoring System; MDS, myelodysplastic syndrome; p53 IHC+, p53 immunohistochemistry-positive; p53 IHC-, p53 immunohistochemistry-negative; UKMRC, United Kingdom Medical Research Council; WHO, World Health Organization.

<sup>a</sup>Defined as IPSS-R high or very high risk for MDS/CMML cases and UKMRC adverse risk for AML cases.

<sup>b</sup>Defined as three or more independent cytogenetic abnormalities.

The percentages of p53 2+ and 1+ cells were also scored, but there was no correlation between either p53 1+/2+ cells or the percentage of cells

showing any p53 staining (1–3+) with outcome (data not shown). In contrast, the presence of any (1–3+) p53 staining of megakaryocytes was



**Figure 2** Outcome of patients with therapy-related myeloid neoplasms (t-MN) in the test and validation groups according to p53 immunohistochemistry status. In the test group, overall survival (a) and disease-specific (b) survival of p53 immunohistochemistry-positive (p53 IHC+) patients ( $n=40$ ) are shorter than those of p53 immunohistochemistry-negative (p53 IHC-) patients ( $n=55$ ) ( $P<0.0001$  for both, log rank test). Among therapy-related myeloid neoplasm patients in the test group with high-risk karyotypes (c), p53 immunohistochemistry-positive patients ( $n=32$ ) have shorter overall survival than p53 immunohistochemistry-negative patients ( $n=18$ ) ( $P=0.0005$ , log rank test). Among therapy-related myeloid neoplasm patients in the test group treated with stem-cell transplantation (d), p53 immunohistochemistry-positive patients ( $n=9$ ) have shorter overall survival than p53 immunohistochemistry-negative patients ( $n=16$ ) ( $P<0.0001$ , log rank test). In the validation set, overall survival (e) and disease-specific survival (f) of p53 immunohistochemistry-positive patients ( $n=34$ ) are shorter than those of p53 immunohistochemistry-negative patients ( $n=50$ ) ( $P=0.0001$  and  $P<0.0001$ , respectively, log rank test).



**Table 3** Multivariable analysis of factors influencing overall and disease-specific survival in test set ( $n = 95$ )

Variable	HR	95% CI	P-value
<i>Overall survival</i>			
Age (per year increase)	1.002	0.984–1.020	0.81
AML (vs MDS/CMML)	1.878	1.079–3.266	0.03
High-risk karyotype <sup>a</sup>	1.874	0.968–3.627	0.06
Treatment with stem cell transplant	0.210	0.106–0.418	<0.0001
p53 immunohistochemistry positivity ( $\geq 1\%$ 3+ cells)	3.434	1.751–6.735	<0.0001
<i>Disease-specific survival</i>			
Age (per year increase)	0.982	0.982–1.019	0.97
AML (vs MDS/CMML)	2.440	1.373–4.338	0.002
High-risk karyotype <sup>a</sup>	2.395	1.196–4.795	0.014
Treatment with stem cell transplant	0.197	0.097–0.401	<0.0001
p53 immunohistochemistry positivity ( $\geq 1\%$ 3+ cells)	3.882	1.931–7.806	<0.0001

AML, acute myeloid leukemia; CMML, chronic myelomonocytic leukemia; MDS, myelodysplastic syndrome.

<sup>a</sup>Defined as Revised International Prognostic Scoring System high or very high risk for MDS/CMML cases and United Kingdom Medical Research Council adverse risk for AML cases.

associated with a shorter overall survival ( $P = 0.0001$ ). However, the overall p53 immunohistochemistry status ( $\geq 1\%$  3+ cells among all hematopoietic cells) had a higher hazard ratio for overall survival (HR = 5.099, 95% CI 2.906–8.944) compared with any megakaryocyte staining (HR = 2.727, 95% CI 1.635–4.547). We investigated whether cutoffs other than  $\geq 1\%$  may better define a prognostically relevant group of therapy-related myeloid neoplasms cases by p53 staining. Compared with alternative cutoffs of 0.1, 0.2, 0.3, 0.5, 0.7, 2, 5, and 10%, the cutoff of  $\geq 1\%$  p53 3+ cells provided the highest hazard ratio with respect to both overall and disease-specific survival (data not shown). With respect to correlation with *TP53* mutation status, a cutoff of  $\geq 2\%$  provided more specificity but lower sensitivity: 28/28 (100%) with  $\geq 2\%$  p53 3+ cells vs 29/31 (94%) with  $\geq 1\%$  p53 3+ cells were *TP53* mutated, while 5/37 (14%) with  $< 2\%$  p53 3+ cells vs 4/34 (12%) with  $< 1\%$  p53 3+ cells lacked *TP53* mutation. Univariate analysis excluding 12 cases with borderline p53 scores (0.6–2%) showed retained significance of p53 immunohistochemistry positivity for shorter overall and disease-specific survival ( $P < 0.0001$  for both).

In a multivariable Cox regression model including p53 immunohistochemistry status, patient age, therapy-related acute myeloid leukemia vs therapy-related myelodysplastic syndrome/chronic myelomonocytic leukemia, high-risk karyotype, and stem-cell transplantation, the p53 immunohistochemistry-positive status was a strong independent predictor of shorter overall survival (HR 3.434,  $P < 0.0001$ ) and disease-specific survival (HR 3.882,  $P < 0.0001$ ) (Table 3).

#### Association of p53 Immunohistochemistry Status with Patient Outcome in the Validation Set

p53 immunohistochemistry was performed on a separate validation set of 88 therapy-related myeloid

neoplasms diagnosed at the University of Texas MD Anderson Cancer Center, and adequate tissue for interpretation was present in 84 of the cases. p53 immunohistochemistry positivity ( $\geq 1\%$  cells with 3+ p53 staining) was observed in 34/84 (40%) of the biopsies, including 8/28 therapy-related acute myeloid leukemia (28%) and 26/56 therapy-related myelodysplastic syndrome (46%). A detailed distribution of the percentage of p53 3+ cell percentages in the validation set is shown in Supplementary Table 2. The median percentage of p53 3+ cells among the p53 immunohistochemistry-positive cases was 8.1% (range 1.2–46.7%). The median follow-up time in the validation set of all patients was 10 months and of surviving patients was 56 months. In univariate analysis of the validation set, p53 immunohistochemistry positivity was strongly associated with shorter overall survival (median 7.7 vs 14.6 months,  $P = 0.0001$ ) and disease-specific survival (median 10.1 vs 45.1 months,  $P < 0.0001$ ) (Figures 2e and f). Even after excluding the seven cases with borderline p53 3+ percentages (0.6–2.0%, comprising 8% of the entire validation cohort), p53 immunohistochemistry positivity retained its significance for both overall survival ( $P = 0.0006$ ) and disease-specific survival ( $P = 0.0004$ ).

In a multivariable Cox regression model including the same variables applied to the test set, p53 immunohistochemistry-positive status was a strong independent predictor of shorter overall survival (HR 3.156,  $P = 0.002$ ) and disease-specific survival (HR 2.467,  $P = 0.003$ ) in the validation set (Table 4).

## Discussion

Several studies have shown that *TP53* mutations in myelodysplastic syndrome predict inferior clinical outcome compared with wild-type *TP53*.<sup>4,13,15,21,22</sup> To our knowledge, the current study is the first to

**Table 4** Multivariable analysis of factors influencing overall and disease-specific survival in validation set ( $n = 84$ )

Variable	HR	95% CI	P-value
<i>Overall survival</i>			
Age (per year increase)	1.009	0.986–1.032	0.47
AML (vs MDS/CMML)	4.861	2.537–9.314	<0.0001
High-risk karyotype <sup>a</sup>	1.783	0.767–4.144	0.18
Treatment with stem cell transplant	0.413	0.184–0.929	0.03
p53 immunohistochemistry positivity ( $\geq 1\%$ 3+ cells)	3.156	1.502–6.628	0.002
<i>Disease-specific survival</i>			
Age (per year increase)	1.013	0.994–1.033	0.18
AML (vs MDS/CMML)	2.736	1.601–4.677	<0.0001
High-risk karyotype <sup>a</sup>	1.408	0.737–2.687	0.30
Treatment with stem cell transplant	0.465	0.254–0.852	0.01
p53 immunohistochemistry positivity ( $\geq 1\%$ 3+ cells)	2.467	1.364–4.461	0.003

AML, acute myeloid leukemia; CMML, chronic myelomonocytic leukemia; MDS, myelodysplastic syndrome.

<sup>a</sup>Defined as Revised International Prognostic Scoring System high or very high risk for MDS/CMML cases and United Kingdom Medical Research Council adverse risk for AML cases.

examine p53 protein expression in a large series of therapy-related myeloid neoplasms. We scored p53 by manual counting and found that, using a cutoff similar to that used in low-risk myelodysplastic syndrome ( $\geq 1\%$  3+ cells),<sup>22</sup> positivity for p53 was strongly associated with poor outcome in therapy-related myeloid neoplasms. This adverse association was independent of other factors known to influence prognosis, such as age, acute myeloid leukemia vs myelodysplastic syndrome, karyotype risk group, and treatment with stem-cell transplantation. Importantly, we validated these results in a cohort of therapy-related myeloid neoplasm patients from another institution that displayed different karyotype profiles and treatment backgrounds, likely reflecting different referral patterns and patient populations at the two institutions. The fact that we found similar results in the test and validation cohorts, with strong prognostic significance for p53 immunohistochemistry in both univariate and multivariable analyses in both cohorts, supports the utility of p53 immunohistochemistry to predict prognosis across a wide spectrum of therapy-related myeloid neoplasms in different practice settings.

Prior studies in *de novo* myelodysplastic syndrome have shown that the presence of *TP53* mutations correlates well with p53 protein expression. In a large series of patients with *de novo* myelodysplastic syndrome, 73% of patients with *TP53* mutation showed strong p53 expression by immunohistochemistry, whereas none of the patients with wild-type *TP53* showed strong p53 expression.<sup>21</sup> In that study, p53 staining was assessed based on a combination of the percentage of cells stained and staining intensity. In patients with low-risk myelodysplastic syndrome with del(5q), it was also shown that the presence of  $\geq 1\%$  p53-expressing cells was positively correlated with *TP53* mutations.<sup>13,22</sup> We also found an excellent correlation between  $\geq 1\%$  p53 3+ cells

and the presence of *TP53* mutation in the 71 cases subjected to sequencing in our study: 29/33 cases (88%) with *TP53* mutation were scored as p53 immunohistochemistry-positive. The four false-negative cases included two cases with a nonsense mutation truncating the protein, likely explaining the loss of detection by immunohistochemistry.<sup>21</sup> Interestingly, the other two cases with *TP53* mutation and negative p53 immunohistochemistry occurred in patients with non-complex karyotypes, 45,XY,-7,del(20)(q11.2) and 46,XY,del(7)(q22q31), who had relatively long survivals of 69.1 and 18.8 months, respectively. According to the database of the International Agency for Research on Cancer (<http://p53.iarc.fr/TP53GeneVariations.aspx>), both of these mutations are predicted to result in a partially functional protein, whereas in 28/29 of the *TP53* mutated cases in our series that were p53 immunohistochemistry-positive, the mutations were predicted to yield a non-functional protein. Although no definitive conclusions can be drawn from these two discrepant cases, the findings raise the possibility that missense mutations that do not increase p53 protein expression may reflect less deleterious effects on the protein and result in a disease phenotype distinct from *TP53* mutations that result in an overexpressed, non-functional protein.

Many of the cases in our series had frequent cells with weak to moderate p53 immunostaining and they appeared to have no relevance with respect to outcome or correlation with karyotypes (data not shown). Saft *et al*<sup>22</sup> showed by single-cell microdissection that cells with weak to moderate p53 staining lack *TP53* mutation. Therefore, in counting p53 immunostained cells, it is important to avoid counting moderately or weakly stained cells. Similar to the Saft *et al*<sup>22</sup> study, we found excellent interobserver concordance in manual enumeration of p53 3+ cells using a cutoff of  $\geq 1\%$ . The Saft *et al* study additionally found

concordance between manual and automated counting and between two alternative p53 antibodies (DO1 and DO7). These results suggest that p53 immunohistochemistry can represent a reliable clinical tool for risk stratification. However, we found some lack of interobserver concordance in cases with p53 immunohistochemistry scores of 1–2% and among the three cases with p53 immunohistochemistry scores of 1–2%, two cases lacked *TP53* mutation. Thus, p53 immunohistochemistry scores of 1–2% should be interpreted with caution and may be less reliable in predicting a *TP53* mutation than p53 immunohistochemistry scores of >2%. Further study is required on cases with borderline p53 immunohistochemistry scores in order to determine the mutation incidence in this subset.

The precise role of *TP53* mutations in the pathogenesis of therapy-related myeloid neoplasms is uncertain. Although *TP53* mutations are more frequent in therapy-related myelodysplastic syndrome and therapy-related acute myeloid leukemia compared with their *de novo* counterparts, it is unclear if this merely reflects the higher incidence of adverse karyotypes that are known to be strongly associated with therapy-related disease. *TP53* mutations appear to be an early and relatively stable event in myelodysplastic syndrome, as they have been shown to be present many years before disease progression<sup>13</sup> and were not seen to occur as a secondary event after the initial diagnosis in one study that evaluated for the mutation at multiple time points.<sup>21</sup> Additionally, low-level *TP53* mutations have been found in the blood and bone marrow from patients who subsequently develop therapy-related myeloid neoplasms, even prior to the cytotoxic exposure.<sup>27</sup> This has led some authors to suggest that cytotoxic therapy may select for *TP53*-mutated clones, which subsequently contribute to the complex cytogenetic features and aggressive biologic behavior of therapy-related myeloid neoplasms.<sup>27</sup> However, despite the fact that altered *TP53* is strongly associated with high-risk karyotypes, 36% of the high-risk karyotype therapy-related myeloid neoplasms in our test set were p53 immunohistochemistry-negative. We also found that p53 immunohistochemistry positivity was less frequent in patients exposed to radiation only, correlating with the observation that therapy-related myeloid neoplasms following radiation only have cytogenetic features and clinical behavior that differ from therapy-related myeloid neoplasms following cytotoxic chemotherapy.<sup>28</sup> These data suggest that the molecular mechanisms underlying the etiology of therapy-related myeloid neoplasms are diverse and are likely associated with different disease behaviors.

In our study, p53 immunohistochemistry positivity was strongly correlated with  $-5/\text{del}(5q)$  cytogenetic abnormalities and usually occurred in the context of a complex karyotype. These results are generally similar to those reported for *de novo* myelodysplastic syndrome.<sup>21,29</sup> However, we found a higher incidence of p53 immunohistochemistry

positivity in cases lacking  $-5/\text{del}(5q)$  (6/55, 11%) than the 1.5% reported in the recent series of *de novo* myelodysplastic syndrome.<sup>21</sup> Positivity for p53 immunohistochemistry occurred in two patients with normal karyotype in the test group and in two patients with isolated  $-7/\text{del}(7q)$  in the validation group (data not shown). In myelodysplastic syndrome with  $\text{del}(5q)$ , *TP53* mutation and p53 immunopositivity predicts poor response to lenalidomide<sup>13,21</sup> and none of the patients with *TP53* mutation showed a complete cytogenetic response to lenalidomide, indicating that information on the p53 status is not only important for prognostic stratification, but may also predict response to particular therapies. In our study, we found that, although the proportions of p53 immunohistochemistry-positive and p53 immunohistochemistry-negative patients treated with stem-cell transplantation were similar, p53 immunohistochemistry-positive therapy-related myeloid neoplasm patients undergoing stem-cell transplantation had a very poor outcome: eight of nine patients died because of disease relapse at a median of 5 months (range 2–10 months) after stem-cell transplantation.

In summary, we found that p53 protein expression, defined as  $\geq 1\%$  cells with strong nuclear staining in bone marrow biopsies, is frequently found in therapy-related myelodysplastic syndrome and therapy-related acute myeloid leukemia and reflects the high incidence of *TP53* missense mutations in therapy-related myeloid neoplasms. p53 immunostaining prognostically stratifies therapy-related myeloid neoplasm patients independent of other well-known risk factors and in particular identifies patients with very poor outcome following stem-cell transplantation. In combination with other markers of disease aggressiveness and patient characteristics, p53 immunohistochemistry may help guide the therapeutic approach to this aggressive group of myeloid neoplasms.

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## Disclosure/conflict of interest

The authors declare no conflict of interest.

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