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Original Article

Prognostic Impact of Immunohistochemical p53 Expression in Bone Marrow Biopsy in Higher Risk MDS: a Pilot Study

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Abstract. ***Background and objectives:*** **Mutations of the TP53 gene have an unfavorable prognosis in Myelodysplastic Syndromes (MDS). The product of the *TP53* gene is the p53 protein. Most of the *TP53* mutations entail the accumulation of the protein in the nucleus of tumor cells. The immunohistochemical (IHC) staining for p53 can be a surrogate suggesting a mutational status and, if overexpressed, seems to be of prognostic value by itself. The best prognostic cut-off value of overexpression is controversial. The aim of this pilot study is to investigate the correct value from a homogenous group of patients with higher IPSS-R risk MDS.**
Methods: **In sixty consecutive patients diagnosed with MDS and categorized as "intermediate," "high" and "very high" IPSS-risk, the bone marrow biopsies performed at diagnosis were retrospectively re-examined for IHC p53 expression. The result of p53 expression was subsequently related to survival.**
Results: **A worse overall survival was observed both in patients whose IHC p53 expression was ≥5% and ≥ 10% compared to patients with a p53 expression below 5% (p= 0.0063) or 10% (p=0.0038) respectively.**
Conclusions: **The ICH p53 expression in bone marrow biopsy in higher risk MDS was confirmed to have prognostic value. These results indicate more than 10% expression as the best cut off value.**

Keywords: MDS; Prognosis; P53 expression.

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Introduction. Myelodysplastic syndromes (MDS) are a heterogeneous group of diseases characterized by ineffective hematopoiesis and risk of acute myeloid leukemia progression. The prognosis in terms of overall survival (OS) and risk of progression is now estimated by the Revised International Prognostic Scoring System (IPSS-R),[1] developed from the previous historical system called IPSS,[2] based on the following features: cytopenias, the percentage of bone marrow blasts and cytogenetic aberrations. According to IPSS-R, five prognostic categories can be distinguished with a median OS from 5.4 years (very low risk) to 0.7 years (very high risk). The biological impact on progression and survival was recently further pointed out by studies

on recurrent gene mutation in MDS. For example, the presence of at least one among ASXL1, RUNX1, TP53, EZH2 or ETV6 somatic point mutations was shown to be enough to worsen the IPSS prognostic category.[3] On the other hand, mutations of SF3B1 (a gene encoding a core component of the RNA splicing machinery) have been associated with a favorable prognostic impact in MDS with ring sideroblasts.[4]

The TP53 is a gene mapped of the locus p13.1, on chromosome 17. It is mutated in 5-10% of cases of de novo MDS[5] and about 30% of therapy-related neoplasms. In general, this mutation is mainly observed in high-risk MDS,[6] but it is particularly frequent both in patients with isolated Del(5q) and those with complex karyotype associated with -5/5q-.

Its presence is linked with an unfavorable prognosis and with a reduced OS, regardless of prognostic or cytogenetic category. It has been shown that the cases of complex karyotype without TP53 mutations have better survival compared to those with mutations, at least in the transplant setting.[7] In addition, the presence of TP53 mutations also increases the risk of progression to leukemia in MDS patients with isolated Del(5q) and leads to poor survival in patients with normal karyotype.[3,8,9]

The product of the TP53 gene is the p53 protein, a tumor suppressor factor. When p53 is activated, it has multiple antineoplastic functions in a relationship with several transducers, including cellular growth arrest, apoptosis, DNA repairs, and angiogenesis. Most of the TP53 mutations lead to the stabilization and the accumulation of the protein in the nucleus of the tumor cells.[10] So, the immunohistochemical (IHC) staining for p53 could be a surrogate suggesting a mutation status. In other cases, a non-sense mutation may result in a truncated, and unstable protein or the injury of both alleles can lead to a complete loss of p53 production, with the absence of staining.[11] As a matter of fact, TP53 mutations are associated with the overexpression of p53 protein in 75% of cases.[5] The p53 overexpression has never been observed in cases of wild type TP53, and that was also confirmed by another study that reported 60% sensitivity and 100% specificity of IHC for p53 overexpression in detecting TP53 mutations.[12]

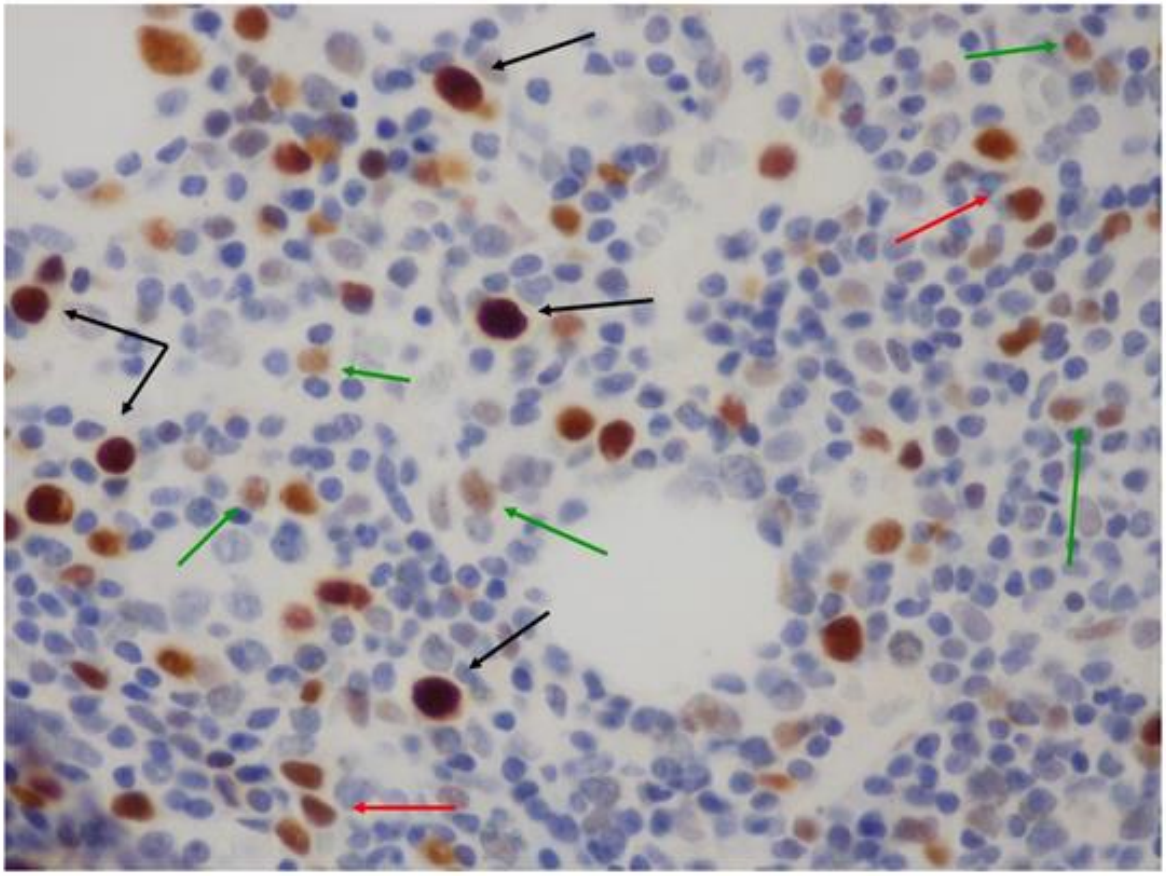
The best way to detect p53 mutations is certainly by molecular biology techniques. Otherwise, considering the low diffusion and the high cost of these procedures, the evaluation of the expression of the p53 protein as an alternative method may be considered helpful.

The IHC p53 overexpression has been evaluated as a prognostic factor in itself and considered as a low cost, easy diagnostic tool for assessing the presence of TP53 mutation, especially in low-risk Del(5q) MDS.[9,13,14] Saft et al.[14] examined p53 expression in 85 Del(5q) patients enrolled in the MDS-004 trial.[15] They also quantified the intensity of expression that identified the positivity of the marker, using a scale in which "0" was negative; "1+" weakly positive; "2+" moderately positive and "3+" strongly positive. Only the cells with a strong p53 staining (3+) were regarded as positive for the analysis. They noted that a p53 expression higher than 1% (found in 30/85 cases), was associated with higher acute myeloid leukemia (AML) evolution risk, with shorter OS and with a lower cytogenetic response rate to lenalidomide treatment. However, the correct optimal p53 positivity cut-off value is still controversial. In fact, according to the report by Jädersten et al.,[9] there is a correlation between TP53 mutation and the presence of over 2% bone marrow progenitors with strong p53 staining. Apart from Del(5q) setting, McGraw[12] indicated that the best p53 cut-off value for specificity and sensitivity to predict TP53 mutations in MDS and secondary AML is 0.5%. Iwasaki et al.[6] stated that in MDS and AML patients p53 was likely mutated when more than 5% of cells were positively stained. In a de novo MDS cohort of patients with moderate to severe reticulin fibrosis, higher levels of TP53 expression (≥10% of the cells) were associated with higher BM blast counts, poor risk karyotype, TP53 mutations and, above all, with shorter OS.[16] In another report, overall survival was significantly lower in cases with a p53 expression in more than 50% of the cells.[17]

According to the abovementioned works, the correlation of p53 expression with survival seems to be confirmed. But it is evident that there is great heterogeneity of the cut-off value above which it has to be considered as an unfavorable prognostic parameter. In particular, few homogeneous data are available on the possible prognostic impact of p53 IHC expression and overall on the cut-off levels in patients with higher risk MDS. Hence the aim of this pilot study to investigate the IHC p53 expression with OS in BM biopsies from patients with "intermediate," "high" and "very high" R-IPSS risk MDS.

Material and Methods. We performed a retrospective analysis considering a cohort of higher risk ("intermediate," "high" and "very high" risk according to IPSS-R) MDS patients. Since survival was the main endpoint of the study, we selected, from our records, patients with at least three years follow up (or who died before three years from diagnosis) and with an available bone marrow biopsy performed at the time of diagnosis. No data about TP53 mutation were available, so TP53 could not be considered. The purpose was to verify if p53 expression maintains a prognostic value in itself in higher MDS patients and investigate the best cut-off value. A cohort of 60 patients was considered. We extracted from the archives all the bone marrow samples performed at the diagnosis; IHC p53 expression was performed and evaluated by two independent pathologists (L.B.; M.T.). They evaluated p53 positivity in all hemopoietic mononucleated cells (megakaryocytes and mature granulocyte were excluded

from the count). Staining was quantified, as in the work of Saft and colleagues,[14] in an intensity scale as follows: "0" if negative; "1+" if weakly positive; "2+" if moderately positive; "3+" if strongly positive (**figure 1**). Only cells with strong p53 staining (3+) were considered as positive for the analysis. The two pathologists worked blindly and independently. The few cases (less than 5%) without full concordance were jointly reviewed and a shared conclusion was obtained for each of them. Fibrosis was also evaluated according to the European clinicopathological criteria[18] which define "MF0" the normal bone marrow fibrosis, "MF1" a slight reticulin fibrosis, "MF2" an advanced reticulin and initial collagen fibrosis and "MF3" an advanced collagen fibrosis. The number of medullar blasts for each patient was reconsidered too and reviewed on the cytological staining performed at diagnosis by A.M and M.R. The result of p53 expression was subsequently related to survival. Survival was considered globally, regardless of treatment. In fact, since a monocentric study, the best possible treatment was chosen homogeneously, according to established criteria based on the expertise of the center. In particular, transplanted patients were not censored at the time of the transplant. Lastly, we assessed a possible correlation between p53 expression and the presence of fibrosis, the amount of BM blasts and the cytogenetic risk according to the IPSS-R classification. The statistical evaluations were carried out with logistic analysis. Influence of p53 expression – as a continuous variable – on survival was analyzed by Cox proportional hazard regression, verifying the assumptions by Schoenfeld residuals. A ROC analysis was performed as a tool to evaluate the possible cut-off values suitable to dichotomize the p53 continuous variable and to individuate an optimum on the basis of their positive predictive value (PPV), negative predictive value (NPV), sensitivity and specificity, which were evaluated together with the respective 95% confidence interval (95%CI). Survivorships after dichotomizations were estimated with the Kaplan-Meier product limit method, followed by the log-rank test. The search for possible association between categorical variables was carried out by the Fisher exact test.



Green arrow: weakly positive 1+; Red arrow: moderately positive 2+; Black arrow: strongly positive 3+. Blue nuclei are negative.

Figure 1.

Table 1. patients' characteristics.

Baseline characteristics		
Patients, n	60	
Age, (years)		
Average	63.4	
Median (range)	67	(19 –82)
Gender, n (%)		
Male	37	(61.7%)
Female	23	(38.3%)
WHO 2016 subtype, n (%)		
MDS-Del5q	1	(1.7%)
MDS-MD	25	(41.6%%)
MDS-EB1	21	(35%)
MDS-EB2	13	(21.7%)
IPSS-R risk, n (%)		
"intermediate"	43	(71.7%)
"high"	9	(15%)
"very high"	8	(13.3%)
Cytogenetic risk [IPSS-R stratification], n (%)		
"very low"	1	(1.7%)
"low"	30	(50%)
"intermediate"	10	(16.7%)
"high"	12	(20%)
"very high"	7	(11.7%)
Disease Treatment, n (%)		
Lenalidomide (MDS-del5q with IPSS int-1, but IPSS-R intermediate)	1	(1.7%)
5-azacitidine (5-AZA)	13	(21.7%)
Up-front 11		
AML-like therapy previously 2		
AML-like chemotherapy only	5	(8.3%)
Allogeneic stem cell transplantation	13	(21.7%)
Up-front 4		
AML-like therapy previously 8		
AML-like therapy & 5-AZA previously 1		
BSC [including erythropoietin, transfusion and iron chelation therapy]	28	(46.7%)

MDS = myelodisplastic syndrome, AML = acute myeloid leukemia, BSC = best supportive care

Results. The median age of patients was 67 (range 19 – 82). Diagnosis, according to the WHO 2016 nomenclature, was MDS-Del5q (MDS with deletion of long arm of chromosome 5) in 1/60 cases (1.6%), MDS-MLD (MDS with multilinear dysplasia) in 25/60 cases (41.6%); MDS-EB1 (MDS with excess of blast type 1) in 21/60 cases (35%); MDS-EB2 (MDS with excess of blast type 2) in 13/60 cases (21.7%). The IPSS-R was "intermediate" in 43 cases (71.7%); "high" in 9 cases (15%) and "very high" in 8 cases (13.3%). Cytogenetic risk according to the IPSS-R stratification was "very low" in one case (1.6%); "low" in 30 cases (50%); "intermediate" in 10 cases (16.7%); "high" in 12 cases (20%); "very high" in 7 cases (11.7%) (**Table 1**). Regarding the disease treatment, 1 patient was treated with Lenalidomide (MDS-del5q with IPSS int-1, but IPSS-R intermediate), 13 patients with 5-azacitidine,2 of them also with AML-like previous therapy, 5 solely with AML-like chemotherapy and 13 patients underwent allogeneic stem cell transplantation (among them 8 with a previous AML-like therapy, 1 with both previous AML-like therapy and 5-azacitidine). In the remaining 28 cases, the best supportive care (including erythropoietin, transfusion, and iron chelation therapy) was employed (**Table 1**). The median OS considering all the patients was 41 months.

The p53 expression was < 1% in 39 cases (65.0%), 1% in 5 cases (8.3%), 2% in 6 cases (10.0%), 3% in 2

cases (3.3%), 5% in 3 cases (5.0%), ≥ 10% in 5 cases (8.3%). Average MF grading was 2 (17% of patients had MF grading >2). The average number of marrow blasts was 7.3% (in 28% of patients it was ≥ 10%).

Upon univariate analysis, a significant association between the percentage of p53 expression and survival was found (p=0.013; Hazard Ratio 1.067; 95% CI: 1.014 - 1.124). The better cut-off value predicting a shorter survival was therefore investigated. Cut-off values of 1%, 2%, 3%, 5% and 10% were examined (**Table 2**). The 5% and 10% cut-off values showed a significant PPV compared to the other values in predicting the outcome (see table 2 for a synoptic comparison regarding PPV, NPV, and also sensitivity and specificity – here given as additional info – in relation to the event of death). Therefore, as shown in **figure 2**, a better OS was observed in patients whose BM p53 expression was lower than 5% or 10% compared to patients with a BM p53 expression equal or above 5% (p= 0.0063) and 10% (p=0.0038), respectively. The 10% cut-off value had the best statistical significance and therefore was considered as the best candidate to be the cut-off of reference. A different probability of outcome was not found for the lower cut-off values of 1%, 2% and 3% (p> 0.05).

Notably, considering the 8 patients with p53 expression ≥ 5%, 6 of them (75%) were treated with drugs that could potentially modify the natural course of the disease (3 with 5-azacitidine, 1 with AML-like chemotherapy, 1 with allogeneic bone marrow transplantation preceded by AML-like chemotherapy and 1 with upfront allogeneic bone marrow transplantation).

Considering p53 expression ≥ 10%, in 4 of 5 cases (80%) we administrated a therapy able to modify the natural course of the disease: two with 5-azacitidine, one with AML-like chemotherapy and one with allogeneic bone marrow transplantation preceded by AML-like chemotherapy. As a matter of fact, the best supportive care was offered only to 25% of patients with a BM p53 expression ≥ 5% and 20% of patients with a BM p53 expression ≥ 10%, compared to 46.1% (24/52) of patients with a BM p53 expression < 5%, and 45.4% (25/55) of patients with a BM p53 expression < 10%.

These observations reveal that a treatment that could potentially modify the natural course of the disease was employed to a greater extent in patients with p53 expression over 5% and to an even greater extent with p53 over 10%, apparently with no influence on the prognostic impact of high p53 expression. The low number of patients having p53 expression equal to or higher than 5 or 10% did not warrant performing a multivariate analysis to evaluate the impact of therapy on the outcome better. No association between p53 expression either with fibrosis or BM blast count was

Table 2. better cut-off value analysis. It is intended as the result of comparing survivorship of the cohort above or equal to cutpoint versus that of the cohort below the cutpoint.

cutpoint value (%)	proportion	measure (%)	95% confidence int (%)	log rank test (p-value*)
1	positive predictive value	71.4	47.8 to 88.7	0.2057
	negative predictive value	43.6	27.8 to 60.4	
	sensitivity	40.5	24.8 to 57.9	
	specificity	73.9	51.6 to 89.8	
2	positive predictive value	75.0	47.6 to 92.7	0.1207
	negative predictive value	43.2	28.3 to 59.0	
	sensitivity	32.4	18.0 to 49.8	
	specificity	82.6	61.2 to 95.0	
3	positive predictive value	70.0	34.8 to 93.3	0.3871
	negative predictive value	40.0	26.4 to 74.8	
	sensitivity	18.9	8.0 to 35.2	
	specificity	87.0	66.4 to 97.2	
5	positive predictive value	87.5	47.3 to 99.7	0.0063
	negative predictive value	42.3	28.7 to 56.8	
	sensitivity	18.9	8.0 to 35.2	
	specificity	95.7	78.1 to 99.9	
10	positive predictive value	100.0	47.8 to 100.0	0.0038
	negative predictive value	41.8	28.7 to 55.9	
	sensitivity	13.5	4.5 to 28.8	
	specificity	100.0	85.2 to 100.0	

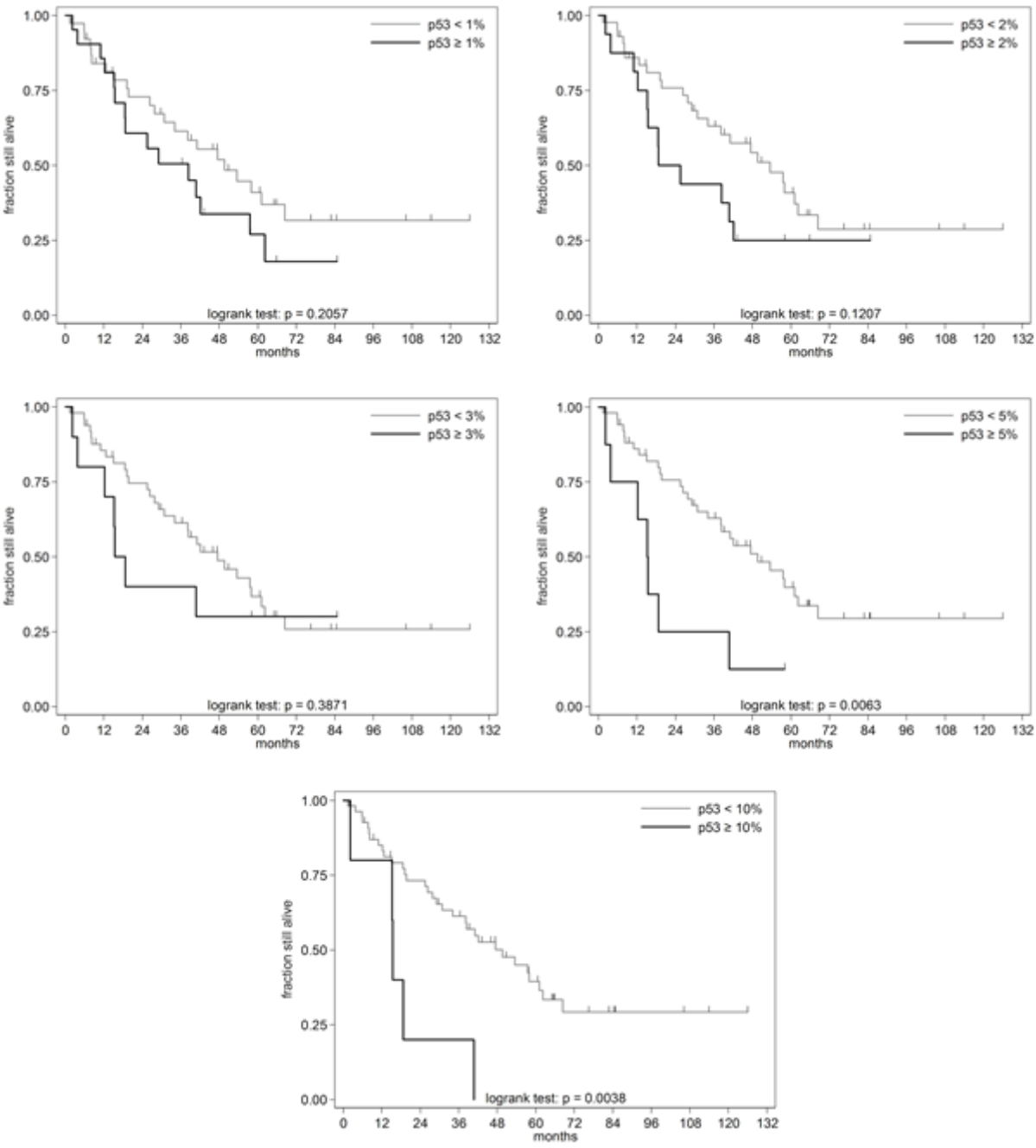


Figure 2. Overall Survival According to the p53 cutoff value.

found ($p > 0.05$ for both the variables). On the contrary, we observed a significant association between p53 expression and cytogenetic risk according to IPSS-R stratification. Note that seven of the eight patients with p53 expression of at least 5%, had a complex karyotype; none of them showed a 17p alteration. For any single arbitrary unitary increase in the cytogenetic risk score according to the R-IPSS stratification, the odds of a BM p53 expression > 10% rise by 1600% ($p=0.015$).

Discussion. This pilot study confirmed the unfavorable prognostic significance of BM p53 expression also in a population of intermediate, high and very high IPSS-R risk patients. These results were expected since overexpression is never observed in cases of wild type TP53, meanwhile not all TP53 mutations lead to p53 overexpression. In other terms, IHC p53 overexpression is always a sign of a molecular alteration with negative prognostic impact, even though it underestimates the real frequency of TP53 mutations.

The only cohort of patients with homogeneity regarding the prognostic risk and in which ICH p53 expression was related to survival was analyzed in the Saft work on low-risk Del(5q).[13] There are some differences in comparison to our higher risk group of patients. First of all, the prevalence of p53 overexpression was more evident in our data: we found 35% patients with p53 expression ≥ 1%, 27% cases with p53 expression ≥ 2% and 13% higher or equal to 5%. Our rate was higher than that found in the cohort

analyzed by Saft et al. (30%, 19%, and 6% respectively). Evidently, higher risk MDS are characterized by a more frequent occurrence of TP53 mutations compared with the low-risk category Del(5q). The other important difference was the cut-off value which has to be considered significant for the prognostic impact of IHC p53 overexpression. In our cohort of higher risk MDS patients, the cut-off levels were considerably higher (5-10%) than those reported by Saft et al. (1%). This discrepancy may be due to the fact that different factors from the p53 expression, could strongly affect survival in patients with higher-risk MDS. Thus, the negative prognostic value of p53 overexpression emerges only at higher levels of its expression. This hypothesis can only work if based on the assumption that a greater accumulation of the protein in tumor nucleus is linked to a TP53 mutation with a more severe impact on cellular homeostasis. In other terms, we may suppose that the higher the p53 cellular accumulation is, the higher the impairment of the protein is in its anti-neoplastic functions, especially inducing apoptosis.

In this pilot study, 10% cut-off value appears to be the best to identify a poor prognosis, according to statistical analysis, and that must be considered only a preliminary finding and needs to be confirmed in a larger series.

Another interesting issue is the association between p53 expression and the IPSS-R cytogenetic risk score. We speculate a correlation between the presence of a TP53 mutation with a severe injury of p53 function and the presence of further DNA damage. If we consider that a properly functioning p53 protein is related to different DNA repair mechanisms, this hypothesis appears appropriate from a biological point of view. However, this theory has to be confirmed in a larger group of patients too.

Overall, IHC detectable p53 cellular accumulation may be considered as an unfavorable prognostic marker in MDS; whereas, the absence of this protein in the IHC assessment is not evidence of TP53 mutation absence. In higher-risk MDS, IHC identification of p53 expression seems to be an unfavorable prognostic factor only when largely overexpressed (best cut-off value seems to be 10%), contrary to the lower risk, at least in the setting of patients with Del(5q). The IHC for p53 is a low-cost test if compared to molecular detection of TP53 mutations by PCR or NGS techniques. Furthermore, it should be routinely employed in the MDS diagnostic workup, independently from the IPSS-R risk, and used as a tool to help clinical decisions.

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