Characterization and Clinical Implications of p53 Dysfunction in Patients With Myelodysplastic Syndromes

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ABSTRACT

PURPOSE Tumor Protein 53 (p53) expressed from gene *TP53* is a seminal tumor suppressor. We aimed to characterize mutational and nonmutational mechanisms of p53 dysfunction in myelodysplastic syndromes (MDS) and to investigate their clinical effect.

PATIENTS AND METHODS We analyzed a cohort of 6,204 patients with MDS and subsets of patients with available information on RNA sequencing of tumor cells (n = 109), high-dimensional phenotype of immune cells (n = 77), and multiomics analysis (RNA sequencing and proteomics) on single cells (n = 15). An independent validation was performed on 914 patients.

RESULTS Biallelic *TP53* inactivation was a powerful driver of disease progression and identified high-risk patients, regardless of variant allele frequency. Monoallelic and biallelic inactivation represent disease stages occurring as a multihit process in MDS with *TP53* mutations, thus potentially refining the optimal timing of therapeutic interventions in these patients. We identified a subset of MDS (5%) characterized by *TP53* wild-type and hyperexpression of abnormal p53 protein in bone marrow progenitors that exhibit dismal outcome. These patients presented upstream p53 signaling aberrations in Pi3K cascade; RAS, WNT, and NF-KB pathways; and *MDM2* gene amplification, together with a downstream dysregulation of p53 targets. MDS with p53 dysfunction displayed a distinct immune dysregulation involving myeloid-derived inflammation and impaired antigen presentation, which may be a driver of their poor prognosis and provide the groundwork for innovative immunotherapies.

CONCLUSION The identification of nonmutational p53 dysfunction in MDS may lay the foundation for a mechanistic classification of myeloid neoplasms, moving beyond a purely molecular stratification. The recognition of patients with p53 dysfunction is relevant to provide correct disease-risk assessment and interventions, as well as to refine the design of clinical trials

ACCOMPANYING CONTENT

Appendix

Data Supplement

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INTRODUCTION

Tumor Protein 53 (p53) expressed from gene *TP53* located on the short arm of chromosome 17 is one of the seminal tumor suppressors.[1-3] p53 dysfunction occurs through a multihit process, typically involving a point mutation in one allele and loss of the other wild-type (wt) allele (biallelic inactivation). *TP53*-mutated tumors are associated with poor response to treatment and unfavorable prognosis.[1-3]

Myelodysplastic syndromes/neoplasms (MDS) are characterized by cytopenia and risk of progression to AML.[4] *TP53* mutations and/or 17p deletions occur in 5%-10% of newly diagnosed patients and 30%-40% of therapy-related

CONTEXT

Key Objective

Are *TP53* mutations and 17p deletions the sole drivers of p53 dysfunction in myelodysplastic syndromes (MDS)?

Knowledge Generated

Nonmutational p53 dysfunction identifies MDS with poor outcomes that are not efficiently captured by conventional prognostic scores. These patients typically display (1) abnormal p53 protein expression in bone marrow cells by immunohistochemistry; (2) upstream p53 signaling aberrations (including PI3K, RAS, WNT, NF-kB pathways and *MDM2* gene amplification) together with dysregulation of p53 target genes; and (3) distinct immunosuppressive profile. Recognizing MDS with p53 dysfunction is crucial for accurate risk assessment and intervention strategies, and for refining the design of clinical trials.

Relevance *(C. Craddock)*

Identification of p53 dysfunction in MDS, which may be generated independent of TP53 mutations, is crucial for accurate risk assessment and intervention strategies, and for refining the design of clinical trials.*

*Relevance section written by *JCO* Associate Editor Charles Craddock, MD.

cases.[5-7] Biallelic *TP53* inactivation defines a clinical entity with adverse prognosis.[8-11]

Our view of p53 dysfunction in human cancers has been modified by the discovery of mechanisms underlying nonmutational p53 inactivation.[12-14] The majority of *TP53* mutations occur as missense changes in the coding region[1,7,8] and have been associated with increased nuclear protein expression by immunohistochemistry in tumor cells.[1,2] Higher levels of detectable p53 result from a longer half-life of the mutated protein, arising from conformational changes. Conversely, wt p53 protein does not accumulate to amounts detectable by immunohistochemical techniques.[13,15] The finding that no mutation is detected in a proportion of patients with high p53 levels in tumor cells suggests that the nuclear stabilization of p53 could also depend on other factors, such as MDM2/4 overexpression; p14ARF inactivation; abnormalities in RAS, WNT, and NF-KB pathways; and others.[12-15]

In this study, we hypothesized that nonmutational factors could contribute to p53 dysfunction in MDS. We aimed to (1) elucidate the clinical and biological landscape of p53 dysfunction in these patients, (2) refine clinical decision-making process, and (3) promote rationalizing of innovative therapeutic approaches.

PATIENTS AND METHODS

Study design and detailed methods were reported in the Data Supplement, Appendix S1 (page 2-13 and Data Supplement, Tables S1-S3, online only).

Study Population

The study was conducted by GenoMed4All, Synthema, and Synthia consortiums, with the support of EuroBloodNET and International Consortium for MDS (icMDS). Informed consent was obtained from each participant. The Humanitas Ethics Committee approved the study (ClinicalTrials.gov identifier: NCT04889729).

We analyzed different patient populations: Cohort 1 included 6,204 retrospectively collected MDS, along with subsets of patients for whom RNA sequencing of tumor cells (n = 109) and high-dimensional immune cell phenotyping (n = 77) were available at diagnosis; Cohort 2 consisted of 914 patients with MDS and AML with clinical, genomic, and transcriptomic annotation at diagnosis, including prospective Humanitas population (n = 383) together with BeatAML (n = 362) and The Cancer Genome Atlas (TCGA; n = 169) repositories; Cohort 3 included 15 MDS with bone marrow samples collected at diagnosis and at the time of disease progression to AML for single-cell analysis.

Genomic Screening

Mutation screening was performed at diagnosis. In selected cases that were *TP53*wt, ultra-deep sequencing coupled with copy number variation (CNV) evaluation was implemented to detect subclonal *TP53* mutations or 17p microdeletions. Cytogenetic analysis was performed by Q-banding. Fluorescent in situ hybridization (FISH) analysis was used to assess *MDM2* genomic amplification.

Immunohistochemistry

At diagnosis, bone marrow cells were evaluated for p53 and MDM2 immunohistochemical expression on trephine biopsies. Nuclear p53 expression was scored according to previously published methods.[16,17] MDM2 expression was scored as percentage of positive cells.

RNA Sequencing

At diagnosis, RNA was extracted from CD34+ bone marrow progenitors, purified by MACS MicroBead kit (Miltenyi Biotech, Bologna, Italy). RNA was isolated through the RNeasy Micro kit (Qiagen, Hilden, Germany) and quality control was performed with the Agilent 2200 TapeStation system (Agilent, Santa Clara, CA). Libraries were prepared using the SMART-Seq v4 Ultra Low Input RNA Kit (Takara, San José, CA). All samples were sequenced on an Illumina Novaseq 6000 (Illumina, San Diego, CA); data analysis was performed by a standard pipeline.

High-Dimensional Flow Cytometry

The phenotype of T, NK, and myeloid cells was investigated on bone marrow samples at diagnosis by multicolor flow cytometry according to standard recommendations.[18] Samples were acquired by BD FACSymphony A5 flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Data were analyzed with FlowJo and PhenoGraph software.[19]

Multiomics Single-Cell Analysis

Multiomics single-cell analysis of bone marrow cells was done in paired samples collected at diagnosis and AML evolution. We used CITE-Seq technology, which enables integrated transcriptomic and proteomic analysis by combining RNA sequencing with quantitative and qualitative surface protein profiling using oligonucleotide-conjugated antibodies.[20]

Statistical Analyses

Survival curves were estimated by Kaplan-Meier method and compared by log-rank test. Clinical response to and relapse after treatment were assessed according to standardized criteria.[21] Multivariable analyses were performed by Cox regression. Hierarchical clustering was applied to define patients' groups with distinct genomic profiles.[22]

RESULTS

Prevalence and Prognostic Significance of TP53 Mutations and Allelic Status

We analyzed 6,204 patients with MDS from Cohort 1. The prevalence of *TP53* mutations was 10.1%, including 270 and 356 patients with mono- and biallelic inactivation as defined per WHO 2022 criteria (Data Supplement, Table S4 and Data Supplement, Fig S1A).[9]

We analyzed the prognostic effect of mono- and biallelic *TP53* inactivation. Biallelic patients had poorer prognosis and reduced survival after treatment with hypomethylating agents and allogeneic transplantation (HSCT) in comparison with *TP53*wt patients and patients with monoallelic inactivation ($P < .001$). Monoallelic patients showed a reduced survival and increased risk of AML evolution compared with *TP53*wt patients, whereas the clinical outcome was better than that of patients with biallelic *TP53* inactivation ($P < .001$, Fig 1). The prognostic effect of mono- and biallelic *TP53* inactivation was confirmed in multivariable analyses including age, sex and Molecular International Prognostic Scoring System (IPSS-M) features as covariate and was irrespective of the mutation variant allele frequency (VAF; Data Supplement, File S1).

To assess changes in *TP53* allelic status during disease progression, we analyzed paired bone marrow samples from 42 patients collected at diagnosis and AML evolution. Among 23 patients with monoallelic *TP53* inactivation at diagnosis, 18 progressed to a biallelic status at AML evolution. In 19 patients with biallelic *TP53* inactivation, AML progression was associated with an increased *TP53* mutation VAF and acquisition of additional chromosomal abnormalities, whereas additional gene mutations were detected in only a few cases (Data Supplement, File S1).

These data confirm that biallelic inactivation of *TP53* is a potent driver of disease progression (irrespective of VAF) and suggest that mono- and biallelic inactivation are different stages occurring by a multihit process during the natural history of the disease.

Clinical Evidence Supporting the Investigation of Nonmutational p53 Dysfunction in MDS

We performed unsupervised clustering of Cohort 1 (6,204 patients) and identified 19 clusters with distinct genomic profiles. Patients with biallelic *TP53* inactivation were clustered into a distinct group (cluster 12), whereas most patients with monoallelic inactivation were classified separately (Data Supplement, Fig S2). Most relevant features for cluster 12 assignment were biallelic *TP53* and complex karyotype. We observed that a proportion of cluster 12 patients had *TP53*wt (n = 198) and showed the same dismal outcome as cases with biallelic *TP53* inactivation. Ultra-deep sequencing and CNV confirmed the absence of *TP53* genomic lesions in 24 of 25 studied participants. On the other hand, 87% of *TP53*wt patients had immunohistochemical p53 nuclear positivity in bone marrow cells, consistent with protein dysfunction.

We investigated the outcome of MDS with *TP53*wt and hyperexpression of abnormal p53 protein in a subset of Cohort 1 in which immunohistochemistry was systematically assessed (n = 2,500, Table 1). In MDS with *TP53*wt, 90 (4.1%) had hyperexpression of p53 protein in bone marrow progenitors (median 4%, 3%-81%). The most frequently identified mutated genes were *TET2* (11.1%), ASXL*1* (11.1%) and *RUNX1* (12.2%). MDS with *TP53*wt and p53 hyperexpression present comparable outcomes with respect to patients with biallelic *TP53* inactivation (Fig 1). The prognostic effect of p53 hyperexpression was confirmed in multivariable analyses including age, sex, and IPSS-M as covariate (Data Supplement, File S2).

We then investigated patterns of disease progression in paired samples collected at diagnosis and at the time of AML

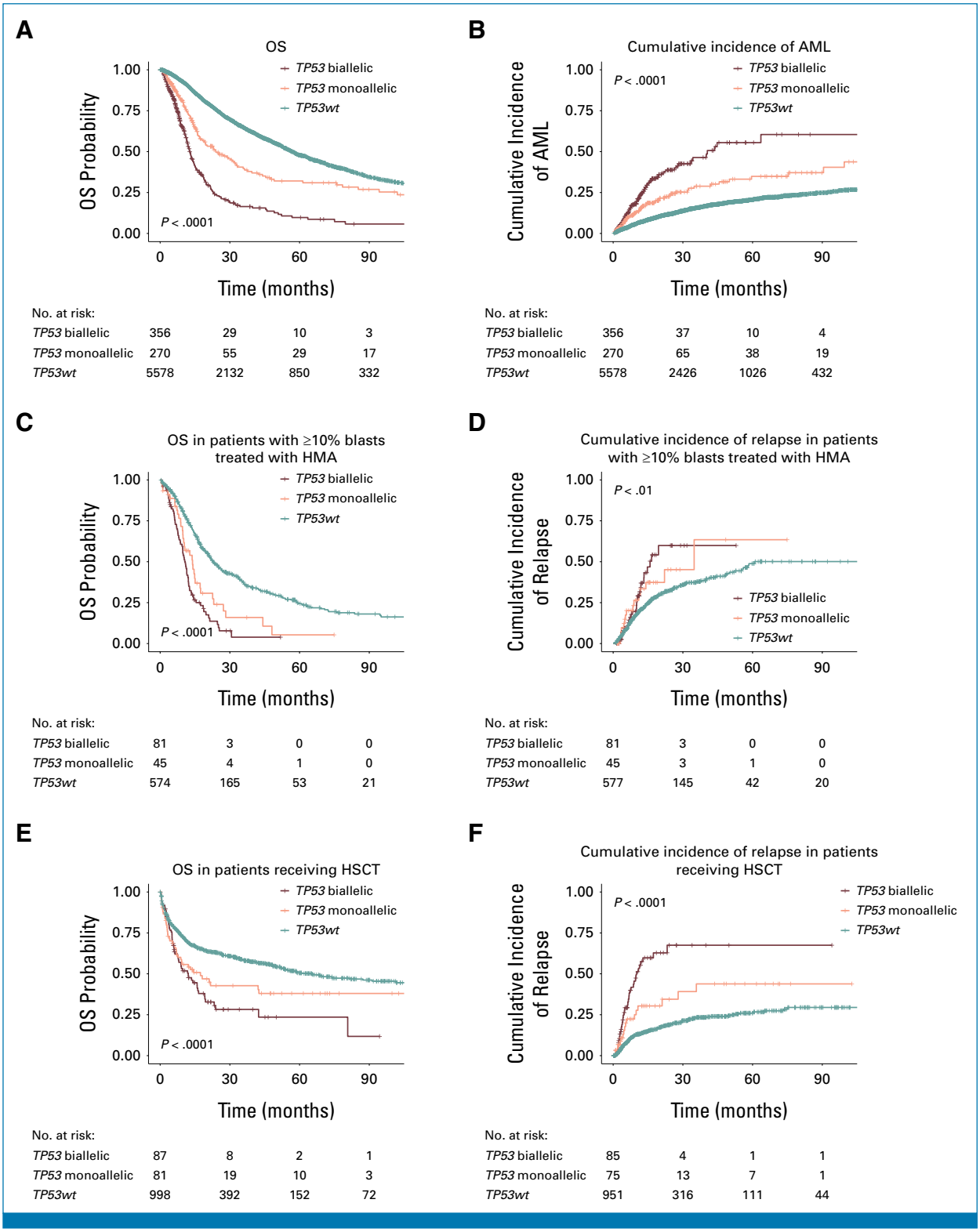

FIG 1. Clinical outcome of the study population stratified by *TP53* status (wt, mono- and biallelic *TP53* inactivation, plot A-F) and by p53 expression in bone marrow progenitors (plot G-M). HMA, hypomethylating agents; HSCT, allogenic stem cell transplantation; OS, overall survival; wt, wild-type. (continued on following page)

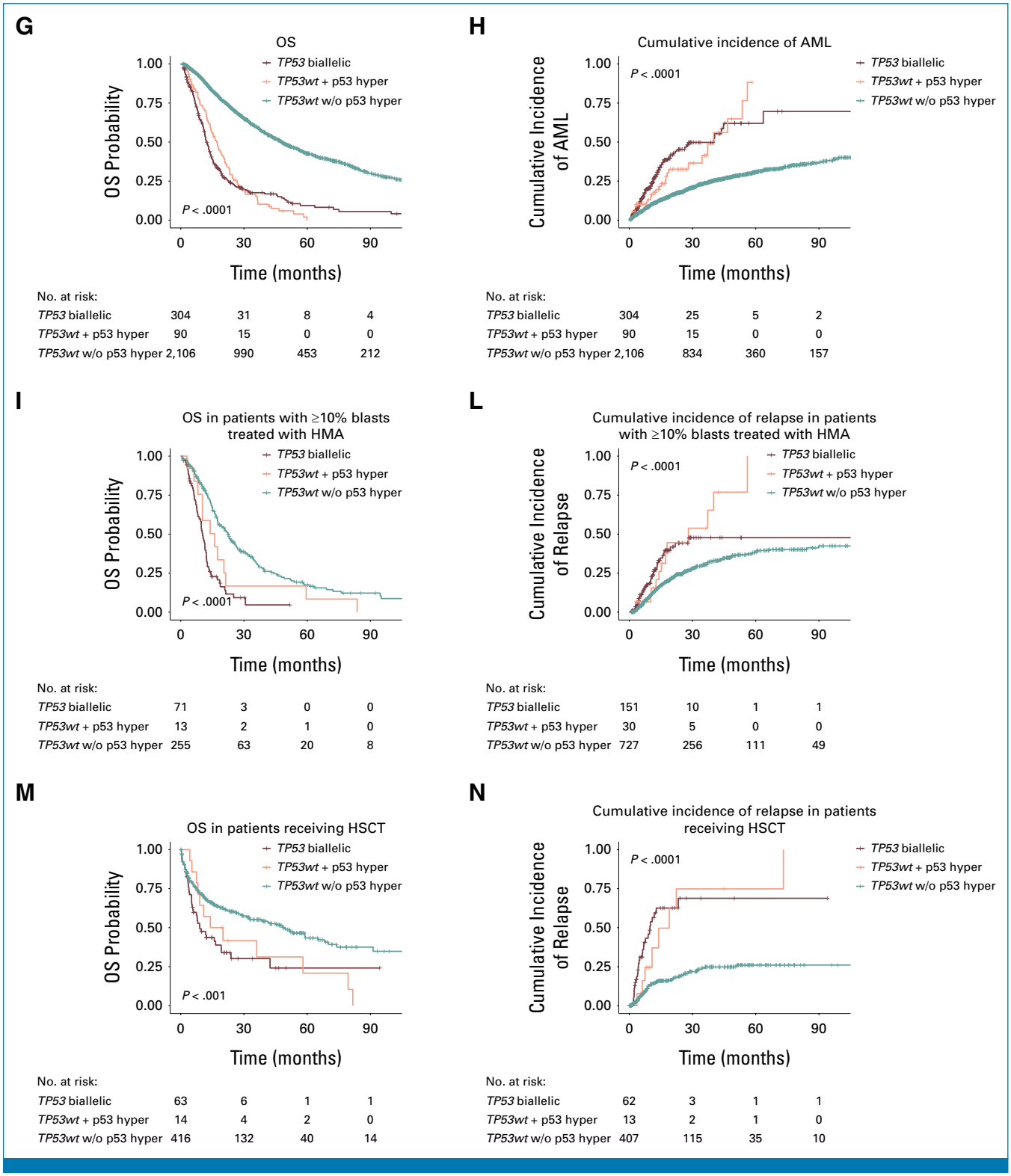

FIG 1. (Continued).

evolution from 11 patients with *TP53*wt and p53 hyperexpression: disease evolution was associated with an increased number of p53-positive cells in 10 of 11 patients (median 5%, 3%-27%), acquisition of chromosomal lesions not involving 17p deletion in nine of 11 patients, and acquisition of mutations not involving *TP53* gene in two of 11 patients (Data Supplement, File S2).

These findings suggested that MDS with *TP53wt* and p53 hyperexpression had similar dismal outcome and are

TABLE 1. Clinical Characteristics of 2,500 Patients With MDS With Available Immunohistochemical Information Stratified According to *TP53* Mutational Status and/or Abnormal p53 Hyperexpression

Cohort 1 With p53 Immunohistochemistry Available	Study Population	*TP53* Wild-Type	*TP53* Wild-Type and p53 Hyperexpression	*TP53* Biallelic Inactivation[b]	*P*[a]
No. (%)	2,500 (100)	2,106 (84.2)	90 (3.6)	304 (12.2)	
Demographic					
Male gender	1,627 (65.1)	1,375 (65.3)	60 (66.7)	192 (63.2)	.542
Age at diagnosis, years	70 (18-97)	70 (18-97)	72 (40-90)	70 (28-89)	.138
Hematologic features					
Neutrophils, $\times 10^9$/L	1.3 (0-11)	1.3 (0-11)	1.3 (0-10.2)	1.1 (0-10)	.646
Hemoglobin, g/dL	9.5 (3-16)	9.5 (3-16)	9.5 (5-15)	8.8 (4-15)	.003
Platelets, $\times 10^9$/L	102 (2-1,280)	110 (2-1,280)	87 (7-532)	67 (3-565)	.009
Bone marrow blasts, %	3 (0-19)	3 (0-19)	4 (0-19)	8 (0-19)	<.001
WHO 2016 category					
MDS 5q-	113 (4.5)	102 (4.8)	0 (0)	11 (3.6)	<.001
MDS-SLD	236 (9.4)	221 (10.5)	10 (11.1)	5 (1.6)	
MDS-MLD	684 (27.4)	612 (29.1)	24 (26.7)	48 (15.8)	
MDS-RS-SLD	173 (6.9)	167 (7.9)	1 (1.1)	5 (1.6)	
MDS-RS-MLD	205 (8.2)	178 (8.5)	8 (8.9)	19 (6.3)	
MDS-EB1	489 (19.6)	369 (17.5)	20 (22.2)	100 (32.9)	
MDS-EB2	552 (22.1)	416 (19.8)	20 (22.2)	116 (38.2)	
MDS-U	48 (1.9)	41 (1.9)	7 (7.8)	0 (0)	
WHO 2022 category					
MDS-LB-5q-	107 (4.3)	107 (5.1)	0 (0)	0 (0)	<.001
MDS-LB-*SF3B1*	301 (12)	300 (14.2)	1 (1.1)	0 (0)	
MDS-LB-RS	80 (3.2)	72 (3.4)	8 (8.9)	0 (0)	
MDS-LB	786 (31.5)	752 (35.8)	34 (37.8)	0 (0)	
MDS-IB1	324 (13)	309 (14.7)	15 (16.7)	0 (0)	
MDS-IB2	363 (14.5)	354 (16.8)	9 (10)	0 (0)	
MDS-bi*TP53*	304 (12.2)	0 (0)	0 (0)	304 (100)	
MDS with fibrosis	106 (4.2)	95 (4.5)	11 (12.2)	0 (0)	
MDS hypoplastic	91 (3.6)	83 (3.9)	8 (8.9)	0 (0)	
AML	96 (1.5)	91 (1.7)	5 (1.9)	0 (0)	
ICC 2022 category					
MDS-*SF3B1*	280 (11.2)	279 (13.2)	1 (1.1)	0 (0)	<.001
MDS-del(5q)	108 (4.3)	108 (5.1)	0 (0)	0 (0)	
MDS. NOS without dysplasia	30 (1.2)	26 (1.2)	4 (4.4)	0 (0)	
MDS. NOS with SLD	277 (11.1)	262 (12.4)	14 (15.6)	1 (0.3)	
MDS. NOS with MLD	671 (26.8)	635 (30.3)	32 (35.6)	4 (1.3)	
MDS-EB	369 (14.8)	346 (16.4)	19 (21.1)	4 (1.3)	
MDS/AML	385 (15.4)	359 (17)	20 (22.2)	6 (2)	
MDS with mutated *TP53*	222 (8.9)	39 (1.9)	0 (0)	183 (60.2)	
MDS/AML with mutated *TP53*	140 (5.6)	38 (1.8)	0 (0)	102 (33.6)	
AML	18 (0.7)	14 (0.7)	0 (0)	4 (1.3)	
IPSS-R cytogenetic risk					
Very good	100 (4)	98 (4.7)	1 (1.1)	1 (0.3)	.001
Good	1,352 (54.1)	1,327 (63)	0 (0)	25 (8.2)	
Intermediate	350 (14)	344 (16.3)	0 (0)	6 (2)	
Poor	198 (7.9)	152 (7.2)	19 (21.1)	27 (8.9)	
Very poor	500 (20)	185 (8.8)	70 (77.8)	245 (80.6)	
Chromosomal abnormalities	0 (0-11)	0 (0-10)	4 (0-8)	4 (0-11)	<.001
Complex karyotype	506 (20.2)	162 (7.7)	89 (98.9)	255 (83.9)	.001

(continued on following page)

TABLE 1. Clinical Characteristics of 2,500 Patients With MDS With Available Immunohistochemical Information Stratified According to *TP53* Mutational Status and/or Abnormal p53 Hyperexpression (continued)

Cohort 1 With p53 Immunohistochemistry Available	Study Population	*TP53* Wild-Type	*TP53* Wild-Type and p53 Hyperexpression	*TP53* Biallelic Inactivation[b]	*P*[a]
Mutated patients	2,029 (81.2)	1,671 (79.3)	54 (60)	304 (100)	<.001
Gene mutations	2 (0-11)	2 (0-11)	2 (1-7)	2 (1-6)	<.001
IPSS-R risk group					
Very low	330 (13.2)	322 (15.3)	0 (0)	8 (2.6)	<.001
Low	795 (31.8)	782 (37.1)	4 (4.4)	9 (3)	
Intermediate	475 (19)	437 (20.8)	12 (13.3)	26 (8.6)	
High	360 (14.4)	289 (13.7)	30 (33.4)	41 (13.5)	
Very high	540 (21.6)	276 (13.1)	44 (48.9)	220 (72.3)	
IPSS-M risk group					
Very low	119 (4.8)	119 (5.7)	0 (0)	0 (0)	<.001
Low	585 (23.4)	581 (27.6)	3 (3.3)	1 (0.3)	
Moderate low	398 (15.9)	386 (18.3)	9 (10)	3 (1)	
Moderate high	363 (14.5)	345 (16.4)	14 (15.6)	4 (1.3)	
High	453 (18.1)	404 (19.2)	28 (31.1)	21 (6.9)	
Very high	582 (23.3)	271 (12.9)	36 (40)	275 (90.5)	
Disease modifying treatment					
No	1,336 (53.4)	1,159 (55)	51 (56.7)	126 (41.4)	.011
Yes	1,164 (46.6)	947 (45)	39 (43.3)	178 (58.6)	
Hypomethylating agents	908 (36.3)	727 (34.5)	30 (33.3)	151 (49.7)	.006
Allogeneic transplantation	504 (20.2)	426 (20.2)	14 (15.6)	64 (21.1)	.25

Abbreviations: bi*TP53*, biallelic *TP53* inactivation; cnLOH, copy-neutral loss of heterozygosity; EB1/EB2, excess blasts type 1/type 2; IB, increased blasts; IPSS-M, Molecular International Prognostic Scoring System; IPSS-R, Revised International Prognostic Scoring System; LB, low blasts; MDS, myelodysplastic syndromes; MLD, multilineage dysplasia; NOS, not otherwise specified; RS-MLD, ring sideroblasts and multilineage dysplasia; RS-SLD, ring sideroblasts and single lineage dysplasia; SLD, single lineage dysplasia; U, unclassifiable.

[a]*P* values were calculated between patients with *TP53* wild-type and p53 hyperexpression and patients with *TP53* biallelic inactivation.

[b]*TP53* biallelic inactivation: Two distinct *TP53* mutations OR a single *TP53* mutation with evidence of *TP53* copy number loss or cnLOH. Patients with one *TP53* mutation not meeting criteria for biallelic inactivation were classified as *TP53* monoallelic inactivation. Patients without evidence of *TP53* mutations were classified as wild-type.

associated with similar changes at AML evolution as observed in patients with biallelic *TP53* inactivation. Therefore, we hypothesized that nonmutational factors may contribute to p53 dysfunction in MDS.

Biological Characterization of Nonmutational p53 Dysfunction in MDS

Transcriptomic Profile of CD34+ Progenitors in MDS Stratified by TP53 Allelic Status and p53 Hyperexpression

We analyzed transcriptomic profile of CD34+ cells isolated at diagnosis from 109 patients of Cohort 1 stratified as follows: (1) MDS with *TP53* biallelic inactivation, (2) MDS with *TP53*wt and hyperexpression of p53 protein, and (3) MDS without p53 dysfunction.

Unsupervised correlation plot of gene expression showed a dichotomization between patients with p53 dysfunction (defined by *TP53* biallelic mutations and/or p53 hyperexpression) and those without p53 dysfunction. Comparison by permutational multivariable analysis of variance revealed high intragroup similarity and significant intergroup differences($P < .001$, Fig 2). To understand whether a common impairment in p53 pathway exists in MDS with biallelic *TP53* inactivation and patients with *TP53*wt and p53 hyperexpression, we analyzed the expression of p53 targets signature in our cohort: p53 targets were significantly decreased in both groups compared with *TP53*wt samples ($P < .001$, Fig 2 and Data Supplement, Fig S3).

These results suggest that nonmutational mechanisms may contribute to p53 dysfunction in MDS. To further investigate transcriptomic features associated with p53 dysregulation in MDS with *TP53*wt, we conducted an analysis of reactome signatures that revealed upstream p53 signaling aberrations in Pi3K cascade, RAS processing, WNT, and noncanonical NF-kB pathway (Fig 2).

The presence of upstream p53 signaling aberrations combined with downstream dysregulation of p53 target genes in MDS with *TP53*wt and p53 hyperexpression was confirmed in an independent population of 383 patients (Cohort 2, Data Supplement, Fig S4).

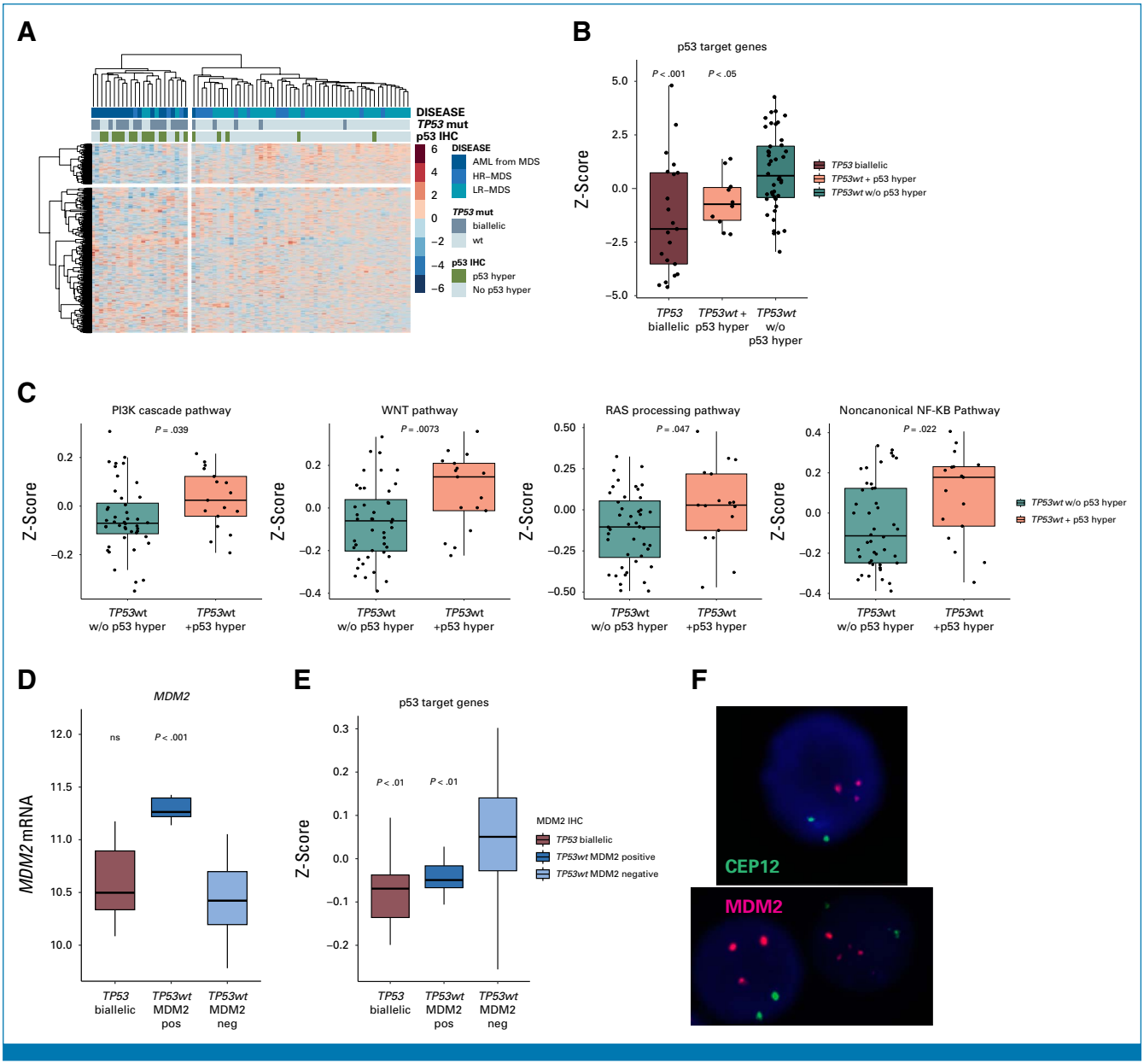

FIG 2. Landscape of transcriptomic regulation in patients with MDS (n = 109) according to *TP53* mutational status and/or p53 hyperexpression. (A) Heat map depicting unsupervised clustering using top 2,000 most variable genes identified by RNA sequencing. (B, C) Box plots of RNA-sequencing z-scores of p53 targets signature and reactome signatures in patients stratified by p53 dysfunction. (D) Violin plot of *MDM2* gene expression in patients with MDS. (E) Box plots of RNA-sequencing z-scores of p53 targets signature in patients stratified by MDM2 expression and *TP53* mutations. (F) FISH showing amplification of *MDM2* gene on the long arm of chromosome 12 (12q15). Representative images of cells from *MDM2* wild-type patient (upper panel) and cells from a patient with *MDM2* gene amplification (CEP12 probe for the centromere of chromosome 12 was used as control). FISH, fluorescent in situ hybridization; MDS, myelodysplastic syndromes.

In addition, among *TP53*wt MDS, we identified patients with highly increased RNA expression of negative p53 regulator *MDM2* (10/109 patients), associated with dysregulation of p53 targets comparable with MDS with *TP53* biallelic inactivation (Fig 2). We confirmed MDM2 upregulation in these patients also performing immunohistochemistry and by providing evidence of *MDM2* gene amplification by FISH analysis (3/10 patients; Fig 2).

Characterization of the Immunological Bone Marrow Microenvironment in MDS Stratified According to p53 Dysfunction

To dissect the biological consequences of p53 dysfunction in MDS, additional differential gene expression analysis was performed in CD34+ progenitors stratified by p53 dysfunction (n = 109, Cohort 1). In these analyses, MDS with

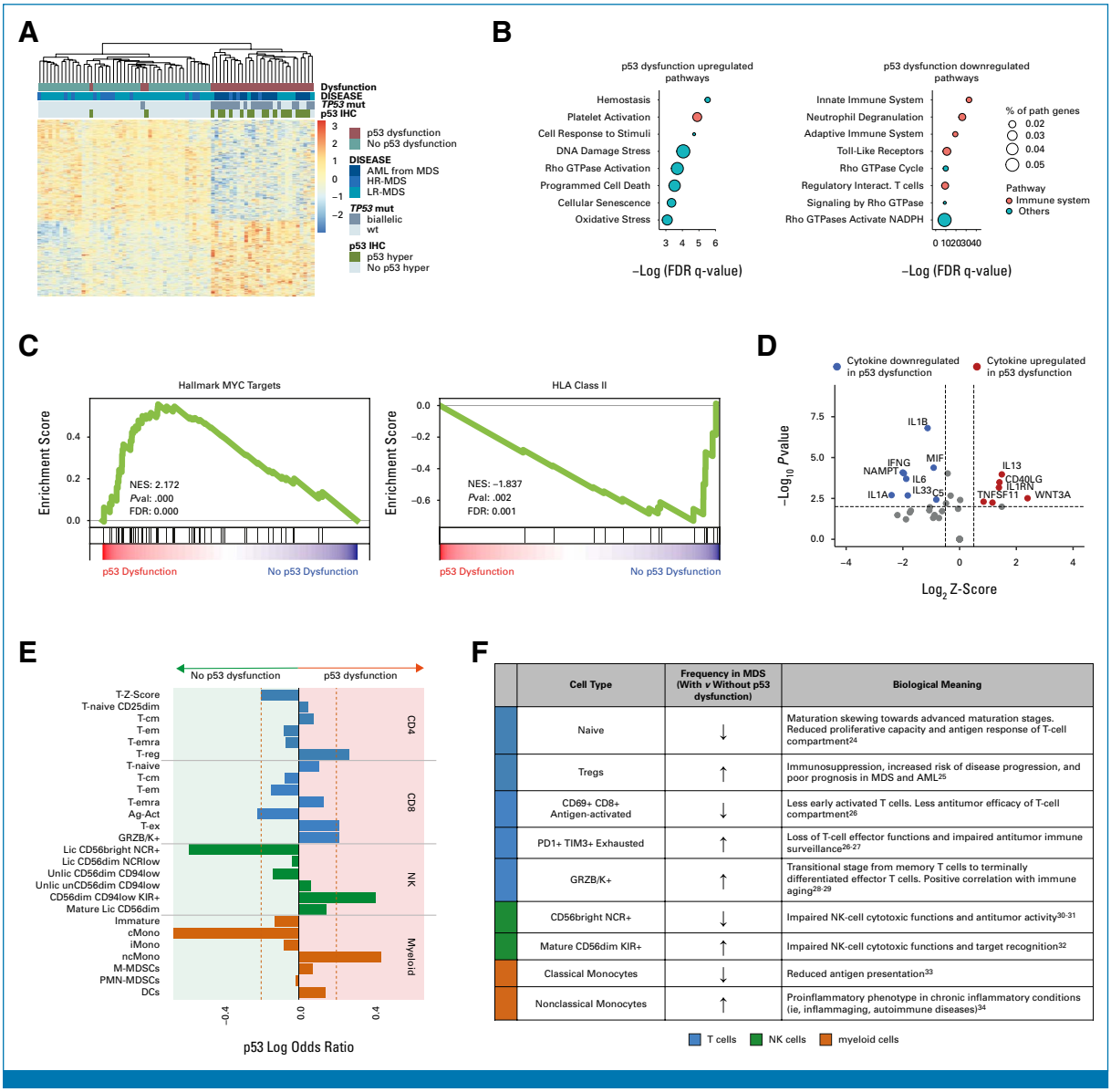

FIG 3. Characterization of the immunological bone marrow environment in MDS stratified according to p53 dysfunction. (A) Heat map of differentially expressed genes in patients stratified according to p53 dysfunction. (B) Functional enrichment analysis: the dot plot depicts the activity of reactome pathways from significant upregulated genes (on the left) or downregulated genes (on the right) in patients with versus without p53 dysfunction. (C) GSEA results of *MYC* targets gene signature (left) and HLA class II genes (right) in patients with versus without p53 dysfunction. (D) Upstream regulator analysis using Ingenuity Pathway Analysis. Volcano plot shows the significant activated and inhibited cytokines in patients with versus without p53 dysfunction. (E) Bar plot shows the variation of each immune cell type frequency in patients with versus without p53 dysfunction. The dashed line highlights the cell types with a significant variation (Bonferroni-corrected *P* value < .0001) with log(2) odds ratio >|0.2|, meaning an increase or reduction of at least 30% compared with patients with no p53 dysfunction. (F) Immunological effects of dysregulated cell types in patients with p53 dysfunction. FDR, false discovery rate; HLA, human leukocyte antigen; MDS, myelodysplastic syndromes; NES, normalized enrichment score.

TP53 biallelic inactivation and those with *TP53*wt and p53 hyperexpression were grouped on the basis of their similar transcriptomic profile and compared with MDS with TP53wt and absence of p53 hyperexpression.

Overall, we identified 467 differentially expressed genes in MDS with versus without p53 dysfunction (119 upregulated and 348 downregulated). Innate and adaptive immune system, and MHC class II antigen presentation pathways were downregulated in MDS with versus without p53 dysfunction, whereas upregulated pathways in MDS with p53 dysfunction included oxidative stress–related pathway, regulation of transcription pathway, and MYC targets. Ingenuity Pathway Analysis was done to predict which soluble factors could be enriched/depleted based on observed changes in the expression of downstream genes: in MDS with

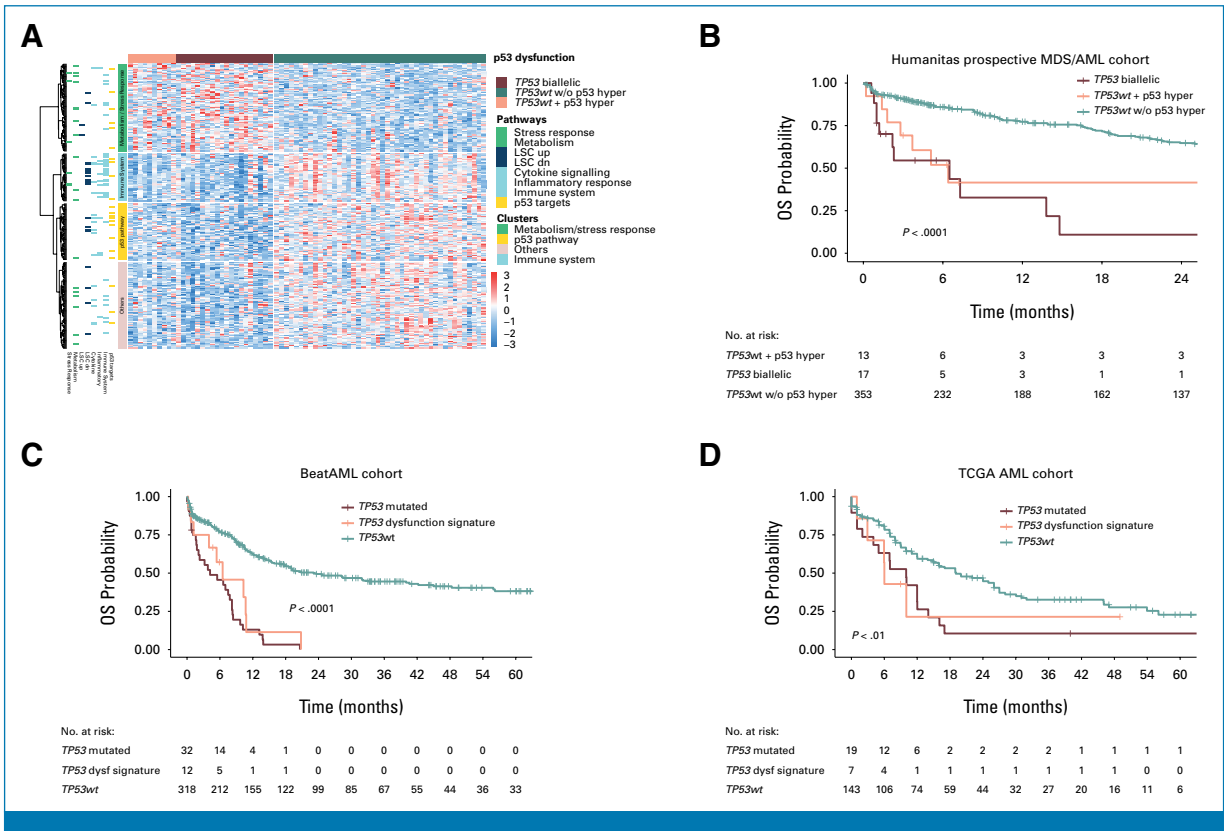

FIG 4. Identification of a transcriptomic signature associated with p53 dysfunction. (A) Heat map of gene signature identified by the feature selection procedure. Patients are stratified by p53 dysfunction. Genes are annotated based on their specific pathways, and clusters summarizing these pathways are highlighted with different colors. (B-D) Overall survival of the Humanitas prospective MDS/AML cohort (N = 383), BeatAML cohort (N = 362), and TCGA AML cohort (N = 169) stratified by *TP53* mutational status and transcriptomic signature associated with p53 dysfunction. MDS, myelodysplastic syndromes; OS, overall survival; TCGA, The Cancer Genome Atlas.

versus without p53 dysfunction there was a predicted increase of anti-inflammatory and reduction of proinflammatory cytokines (Fig 3 and Data Supplement, Fig S5).

Taken together, transcriptomic data highlighted a possible immunosuppressive phenotype of tumor cells in MDS with p53 dysfunction. We thus deepened the analysis of immune cell populations in bone marrow by high-dimensional flow cytometry. Data were available in 77 patients from Cohort 1 who underwent RNA-sequencing analyses (Data Supplement, Figs S6-S10).

Among CD4+ T cells, in MDS with p53 dysfunction we observed increased frequency of immunosuppressive T-regs combined with a decrease in naïve cells ($P < .001$). The majority of T-regs were CD45RO+CD95+, that is, proliferative and highly suppressive.[23] Among CD8+ T cells, in MDS with p53 dysfunction we found increased frequency of exhausted PD1+TIM3+ cells and GRZB/K+ double-positive T cells combined with reduced frequency of CD69+ antigen-activated T cells ($P < .001$) and a positive correlation between *PDL1* mRNA expression in CD34+ cells and the percentage of exhausted PD1+CD8+ T lymphocytes ($P < .001$). Considering NK-cell compartment, in MDS with p53 dysfunction we observed increased percentage of educated NK cells expressing Killer Ig-like Receptors ($P < .001$) together with a reduction of less differentiated NK cells expressing the activating natural cytotoxicity receptors NKp30 and NKp46 ($P < .001$). Finally, considering myeloid cells, in MDS with p53 dysfunction we observed an increased frequency of nonclassical monocytes and a decreased frequency of classical ones ($P < .001$, Fig 3 and Data Supplement, Fig S11).[24-34]

Immunological abnormalities related to p53 dysfunction were confirmed in both patients with biallelic *TP53* inactivation and patients with *TP53*wt and p53 hyperexpression (Data Supplement, Fig S11).

Identification of a Transcriptomic Signature Consistent With p53 Dysfunction and Validation of Its Clinical Effect in Independent Populations of Myeloid Neoplasms

We aimed to validate the clinical impact of nonmutational p53 dysfunction across various patient populations with myeloid neoplasms and available RNA-sequencing data at diagnosis (Cohort 2, N = 914, Data Supplement, Tables S5-S7). Using

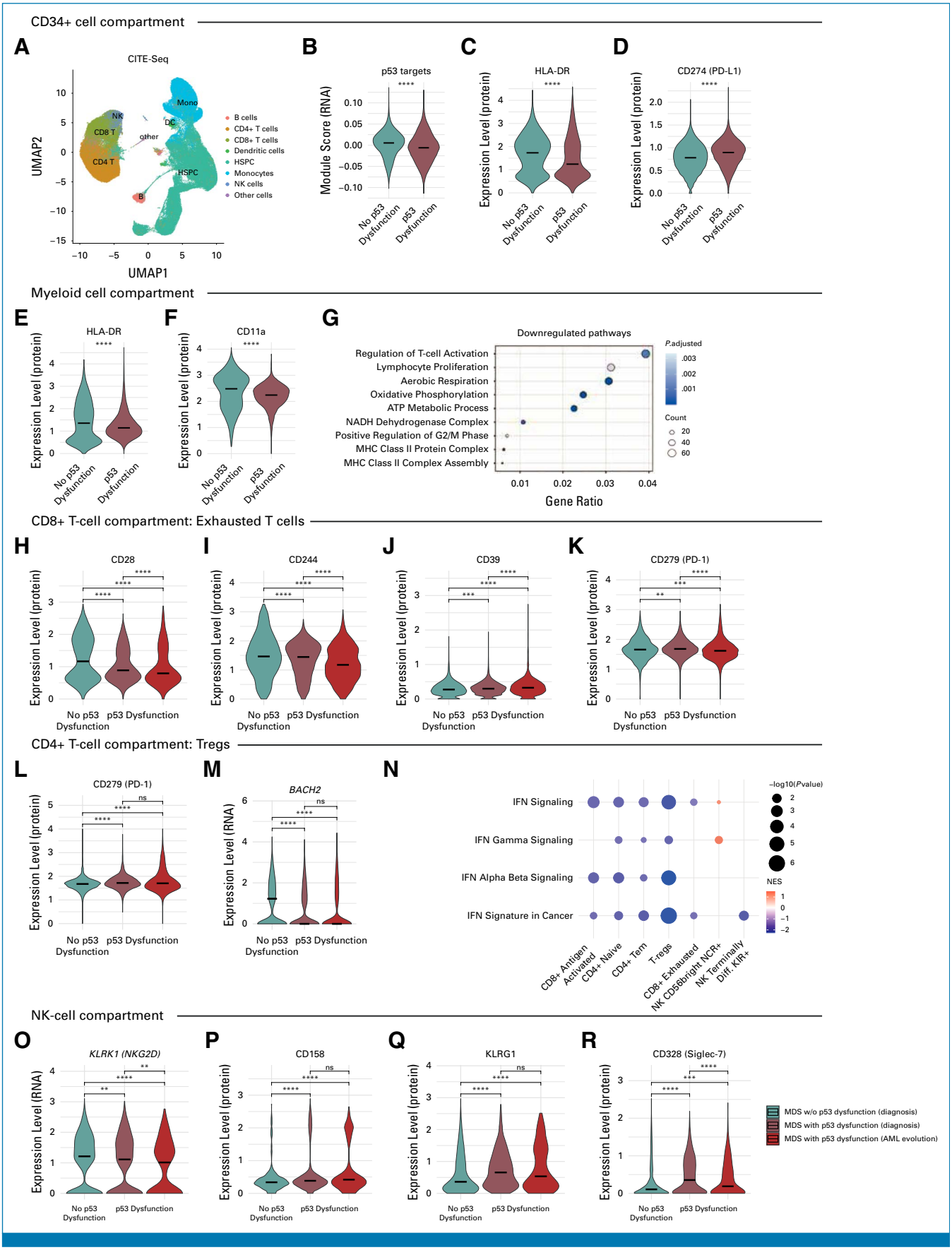

FIG 5. Single-cell multiomics longitudinal analysis of MDS with p53 dysfunction. (A) UMAP plot depicting the main cell types identified in a multiomics CITE-seq experiment, based on 27,290 cells. (B) Violin plot showing the p53 target signature (continued on following page)

FIG 5. (Continued). score in CD34+ cells. (C) Violin plot showing HLA-DR protein expression on the surface of CD34+ tumor cells. (D) Violin plot showing PD-L1 protein expression on the surface of CD34+ tumor cells. (E, F) Violin plots showing HLA-DR and CD11a protein expression on the surface of monocytes. (G) Bubble plot depicting gene enrichment analysis of GO terms for downregulated genes associated with p53 dysfunction in the monocyte compartment. (H, I) Violin plots showing protein expression levels of activation markers CD28 and CD244 on CD8+ exhausted T cells. (J, K) Violin plots showing protein expression levels of the immunosuppressive enzyme CD39 and the immune checkpoint PD-1 on CD8+ exhausted T cells. (L) Violin plot showing protein expression levels of the inhibitory molecule PD-1 on Tregs at both disease onset and AML evolution. (M) Violin plot showing expression levels of the *BACH2* in Tregs which suggest exposure to inflammation. (N) Gene set enrichment analysis of reactome IFN-related pathways in T- and NK-cell clusters. (O) Violin plot showing gene expression levels of the activation receptor *NKG2D* on NK cells. (P-R) Violin plots showing protein expression levels of the inhibitory molecules CD158, KLRG1, and Siglec-7 on NK cells in our cohort. Black dashed lines represent the median. Wilcoxon test, **<.01; ***<.001; ****<.0001. HLA, human leukocyte antigen; IFN, interferon; MDS, myelodysplastic syndromes; MHC, major histocompatibility complex; NES, normalized enrichment score.

a random-forest approach in 109 MDS from Cohort 1, we identified a transcriptomic signature of CD34+ progenitors (253 genes) consistent with p53 dysfunction (Fig 4). This signature was then applied to identify patients with p53 dysfunction in three populations, including a prospective Humanitas cohort of myeloid neoplasms (n = 383), TCGA[35] (n = 169), and BeatAML[36] (n = 362) populations.

In Humanitas prospective cohort, the transcriptomic signature correctly identified both patients with biallelic *TP53* inactivation and those with *TP53*wt and p53 hyperexpression (accuracy 96%). *TP53*wt patients with p53 hyperexpression had a comparable survival with respect to patients with *TP53* biallelic inactivation, both exhibiting worse prognosis compared with patients without p53 dysfunction ($P < .0001$, Fig 4). In both TCGA and BeatAML cohorts, patients with AML with *TP53*wt and transcriptomic signature consistent with p53 dysfunction showed survival outcomes comparable with those with *TP53* mutations, both significantly reduced with respect to patients without p53 dysfunction ($P < .0001$, Fig 4 and Data Supplement, Fig S12).

Single-Cell Multiomics Longitudinal Analysis of MDS With Versus Without p53 Dysfunction

Multiomics single-cell analysis was performed on paired samples collected at diagnosis and AML evolution from 15 patients with MDS (including five with p53 dysfunction) using CITE-seq technology.[20] We aimed to further characterize abnormalities in CD34+ and immune cell compartments through a cutting-edge methodology. Detailed information on these analyses is available in the Data Supplement (Figs S13 and S14), whereas a graphical representation of the most relevant findings is presented in Figure 5.

CITE-seq analysis of MDS with p53 dysfunction confirmed the downregulation of p53 target genes in CD34+ cells, alongside activation of PI3K, RAS, WNT, and NF-kB pathways. Regarding immune evasion, we observed downregulation of human leukocyte antigen (HLA) class I-II molecules and interferon (IFN)γ signaling together with upregulation of immune escape proteins, including CD274 (PD-L1), CD35 (CR1), CD39 (ENTPD1), CD73 (NT5E), and HLA-E, along with reduced expression of immune activation markers (CD44, CD86, CD154, $P < .001$). These alterations persisted at disease evolution.

In MDS with p53 dysfunction, mature myeloid cells (monocytes and dendritic cells [DCs]) showed higher interferon signaling and increased expression of inflammasome-related genes (*NLRP3*, *CASP1*, *IL-1β*, *TLR4*), with reduced HLA-DR, adhesion molecules(CD54, CD11a), and T-cell activation pathways($P < .001$), indicating impaired antigen presentation.

CITE-seq analysis of CD8+ T cells revealed lower expression of activating receptors (CD25, CD40, OX40) and higher levels of CD39, KLRG1, and PD-1 ($P < .001$) at diagnosis, worsening with disease evolution, reflecting progressive T-cell dysfunction. Exhausted $CD8^+$ T cells exhibited further immune exhaustion, with downregulation of activating receptors (CD28 and CD244) and upregulation of immune checkpoint molecules ($P < .001$).

T-regs were more frequent in MDS with p53 dysfunction ($P < .05$), with higher expression of *CD69* gene and CD39, PD-1 proteins ($P < .001$), along with activation of myeloid-derived inflammation pathways (S100A9, IRAK2, FOS, CIITA, S100A8) and downregulation of IFNγ-related genes ($P < .001$), suggesting an inflammatory-exposed profile. This was further supported by lower expression of the *BACH2* gene and upregulation of CD161 protein ($P < .001$).[37] All T-cell clusters exhibited significant downregulation of IFN response genes.

Finally, NK cells displayed impaired functionality at both diagnosis and disease evolution, with reduced RNA transcription of activating receptors (NKG2D, NKp30, NKp46, CD244) and increased expression of inhibitory molecules (CD158, PD-1, Siglec-7, KLRG1, TIGIT).

DISCUSSION

In this study, we have uncovered new insights into p53 dysfunction in MDS and potentially other myeloid neoplasms. These findings may affect diagnostic processes and patients' clinical management, advancing precision medicine beyond conventional disease stratification on the basis of clinical features and gene mutations.

We confirmed that biallelic inactivation of *TP53* was a powerful driver of disease progression and identified very-high-risk patients, regardless of VAF.[7-10,38] Our findings potentially refine the clinical significance of monoallelic *TP53* status, which, in a previous study, was not associated with a different prognosis compared with *TP53wt* patients.[8] Finally, we provided evidence that mono- and biallelic inactivation represent disease stages occurring as multihit process during the natural history of the disease.[39]

These observations may have practical implications for the management of patients with MDS carrying *TP53* mutations. As an example, in patients eligible for HSCT, those with monoallelic inactivation have better outcome than biallelic patients.[40-42] Therapeutic interventions in monoallelic patients could be initiated earlier than would be indicated by currently available scores.[41,42] Accordingly, 39% of patients with monoallelic *TP53* had IPSS-R score[43] ≤3.5 (Data Supplement, Table S4) and therefore not candidate to disease-modifying treatments by conventional prognostic assessment. Moreover, IPSS-M, developed in a patient population where monoallelic status was not linked to a negative prognosis, may underestimate the severity of the disease and the risk of clonal evolution in some of these patients.[44,45]

In addition, we identified a subgroup of patients (5%) characterized by *TP53*wt and abnormal p53 hyperexpression in bone marrow progenitors, who exhibited similar poor outcomes as seen in patients with biallelic *TP53* inactivation. This finding aligns with growing evidence that nonmutational factors can contribute to the p53 dysfunction in human cancers.[12-15] We identified specific upstream signaling aberrations associated with nonmutational p53 dysfunction in MDS, including dysregulation in Pi3K cascade, RAS processing pathway, WNT pathway, and the noncanonical NF-kB pathway, together with a dysregulation of p53 targets.[46-48] Additionally, we identified a small population of patients with the amplification of the negative p53 regulator *MDM2* gene.[49] Again, conventional prognostic scoring systems failed to capture reliable prognostic information in a proportion of these patients (Table 1).

We provided an independent validation of these results across different populations with myeloid neoplasms. Clinically, these patients should be managed similarly to patients with biallelic *TP53* inactivation, based on a common signature associated with p53 dysfunction. Moreover, identifying these patients could have important implications for the design of clinical trials, particularly regarding the eligibility criteria in p53-focused studies and the stratification factors of study populations.

Immunohistochemical evaluation of p53 in the bone marrow biopsy (combined with staining for MDM2 protein) may serve as a surrogate marker for p53 dysfunction and identify these patients during routine diagnostic work-up.[16,17]

The identification of *TP53*wt patients with p53 dysfunction may lay the foundation for a mechanistic classification of MDS based on multiomics data to define disease-associated biological pathways, moving beyond a pure molecular stratification (Data Supplement, Table S8).[50]

Finally, our study aimed to promote the rationale for innovative therapeutic approaches in patients with p53 dysfunction, who typically exhibit resistance to treatment.[5,8,51] Previous studies suggested that MDS with *TP53* mutations have a distinct immunosuppressive profile, which may be the primary driver of the poor prognosis in these patients.[52,53] Our findings provide new insights into the potential role of p53 dysfunction in shaping the immune microenvironment in MDS. We observed that p53 dysfunction does not trigger an IFNγ-driven expansion of T-regs through CD34+ progenitor cells, as seen in AML.[54] Instead, the inflammatory signature observed in mature myeloid cells and T-regs is indicative of a distinct immune dysregulation involving myeloid-derived inflammation and impaired antigen presentation. The downregulation of *BACH2* and subsequent enrichment of CD161+ T-regs with heightened suppressive capacity further suggest that chronic myeloid-derived inflammation may drive an immunosuppressive state, independent of IFNγ pathways.[37] These observations raise important questions about the interplay between inflammation and immune escape mechanisms in these patients. Future studies should aim to explore therapeutic strategies targeting these pathways to restore immune balance and improve patient outcomes.

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EQUAL CONTRIBUTION

M.Z., E.R. and L.L. equal contribution as first author. S.K., F.F. and M.G.D.P. equal contribution as last senior author.

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AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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DATA SHARING STATEMENT

Requests for access to data from the study should be addressed to GenoMed4All/Synthema/icMDS scientific committees (please contact Matteo G Della Porta at matteo.della_porta@hunimed.eu). All proposals requesting data access will need to specify how the data will be used, and all proposals will need the approval of the GenoMed4All/Synthema/icMDS scientific committees before data release.

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AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Characterization and Clinical Implications of p53 Dysfunction in Patients With Myelodysplastic Syndromes

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