OPEN

Leukemia (2018) **32**, 450–461

www.nature.com/leu

ORIGINAL ARTICLE

Low-burden *TP53* mutations in chronic phase of myeloproliferative neoplasms: association with age, hydroxyurea administration, disease type and *JAK2* mutational status

B Kubesova[1,6], S Pavlova[1,2,6], J Malcikova[1,2], J Kabathova[1], L Radova[2], N Tom[2], B Tichy[2], K Plevova[1,2], B Kantorova[1,2], K Fiedorova[2], M Slavikova[2], V Bystry[2], J Kissova[3], B Gisslinger[4], H Gisslinger[4], M Penka[3], J Mayer[1,2], R Kralovics[5], S Pospisilova[1,2] and M Doubek[1,2]

The multistep process of *TP53* mutation expansion during myeloproliferative neoplasm (MPN) transformation into acute myeloid leukemia (AML) has been documented retrospectively. It is currently unknown how common *TP53* mutations with low variant allele frequency (VAF) are, whether they are linked to hydroxyurea (HU) cytoreduction, and what disease progression risk they carry. Using ultra-deep next-generation sequencing, we examined 254 MPN patients treated with HU, interferon alpha-2a or anagrelide and 85 untreated patients. We found *TP53* mutations in 50 cases (0.2–16.3% VAF), regardless of disease subtype, driver gene status and cytoreduction. Both therapy and *TP53* mutations were strongly associated with older age. Over-time analysis showed that the mutations may be undetectable at diagnosis and slowly increase during disease course. Although three patients with *TP53* mutations progressed to *TP53*-mutated or *TP53*-wild-type AML, we did not observe a significant age-independent impact on overall survival during the follow-up. Further, we showed that complete p53 inactivation alone led to neither blast transformation nor HU resistance. Altogether, we revealed patient`s age as the strongest factor affecting low-burden *TP53* mutation incidence in MPN and found no significant age-independent association between *TP53* mutations and hydroxyurea. Mutations may persist at low levels for years without an immediate risk of progression.

Leukemia (2018) **32,** 450–461; doi:10.1038/leu.2017.230

INTRODUCTION

Leukemic transformation of Ph-negative myeloproliferative neoplasms (MPN; polycythemia vera, PV; essential thrombocythemia, ET; primary myelofibrosis, PMF) is a relatively rare but fatal event. Several intrinsic risk factors have been suggested involving MPN phenotype (PMF > PV > ET), abnormal karyotype and higher age.[1] The effect of MPN therapy has been widely discussed and alkylating agents, pipobroman and ^{32}P were shown to be leukemogenic.[1–3] Possible negative impacts of hydroxyurea (HU) remain controversial,[1,2,4–9] as summarized in.[10] Great effort has been invested into the search for genetic changes predicting and/or triggering MPN transformation to AML and MDS.[11–16]

Eliminating tumor suppressor *TP53* during myelopoiesis helps escape from control mechanisms preventing differentiation loss, aberrant self-renewal, and large genome rearrangements.[17,18] In chronic MPN phase, *TP53* gene defects have been extremely rarely detected using Sanger sequencing or cytogenetic analysis; on the contrary, they were shown to be common in post-MPN acute myeloid leukemia (AML).[19–21] This pronounced difference is indicative of *TP53* role in the transformation process. Retrospective analysis of individual cases of *TP53*-mutated post-MPN AML showed that *TP53* mutations can be traced months or even years before leukemic transformation.[11,19,22,23] The level of mutation burden was shown to remain low until complete p53 inactivation by losing the second allele (17p defects or second mutation), followed by rapid clonal expansion.[11,22]

TP53 mutations occurring at a level above detection limit of Sanger sequencing (10–20% variant allelic frequency; VAF) show negative prognostic and/or predictive impact in some types of cancer, especially in hematological malignancies.[24,25] Small *TP53*-mutated subclones below this sensitivity threshold were described to drive relapse or disease progression in many cases of chronic hematological malignancies,[26–29] but their impact is less clear in prospective setting.[30] Cytotoxic agents support a minor *TP53*-mutated subclone overgrowth.[26,31–33] Previous therapy with hydroxyurea (HU), a ribonucleotide reductase inhibitor activating p53 response via replication stress,[34,35] has been associated with *TP53*/17p defects in post-MPN AML;[4,12,22,36] however, this observation has not been confirmed in a large unbiased study.

While minor *TP53* mutations in MPN have been tracked retrospectively in individual cases and have been suggested as carrying an increased risk of leukemic transformation,[11] the occurrence of low-burden *TP53* mutations (< 5%) has not been

[1]Department of Internal Medicine—Hematology and Oncology, Faculty of Medicine, Masaryk University Brno, Czech Republic; [2]Central European Institute of Technology, Masaryk University, Brno, Czech Republic; [3]Department of Clinical Hematology, Faculty of Medicine, Masaryk University Brno, Czech Republic; [4]Department of Internal Medicine I, Division of Hematology and Blood Coagulation, Medical University of Vienna, Austria and [5]CeMM Research Center for Molecular Medicine of Austrian Academy of Sciences, Vienna, Austria. Correspondence: Dr S Pavlova, Department of Internal Medicine—Hematology and Oncology, University Hospital and Medical Faculty of Masaryk University, Cernopolni 9, Brno 61300, Czech Republic.

E-mail: pavlova.sarka@fnbrno.cz

[6]These authors contributed equally to this work.

Received 30 November 2016; revised 30 June 2017; accepted 5 July 2017; accepted article preview online 24 July 2017; advance online publication, 15 August 2017

analyzed so far. Whether therapy or other factors affect their origin and outgrowth is unknown. To map *TP53*-mutated subclones' presence in MPN patients treated with cytoreductive drugs and study their evolution over time, we used an ultra-deep next-generation sequencing (NGS) approach.

PATIENTS AND METHODS

Patients and samples

Peripheral blood (PB) samples and clinical and routine laboratory data from MPN patients were collected from Czech hospitals (University Hospital Brno and local hospitals) and Vienna General Hospital, Austria. The study was approved by the Ethical Committee of University Hospital Brno. For all samples, written informed consent approved by the Ethics Committees of the respective institutions were available in accordance with the Declaration of Helsinki. Patients were diagnosed according to the revised World Health Organization criteria.[37]

In total, 339 MPN patients were included (Supplementary Table S1). Treated patients ($N = 254$) were having or had discontinued cytoreductive therapy–HU, interferon alpha-2a (IFN) or anagrelide (ANG)—and had been diagnosed $\geqslant 4$ years (y) before sampling. To assess the effect of therapy, the treated patients were categorized as follows: (1) by administration of HU, IFN or ANG at any time during disease course (referred to as HU-yes/HU-no, IFN-yes/IFN-no, ANG-yes/ANG-no); (2) more strictly, in the HU-yes group, only patients fulfilling a criterion of HU treatment for $\geqslant 4$y were kept. This group was compared to HU-no patients. Besides these, 85 samples from patients with no cytoreductive therapy before sampling were analyzed. Retrospective and prospective samples from 31/50 patients with detected *TP53* mutations were analyzed to describe mutation development.

Ultra-deep next-generation sequencing of *TP53* amplicons

NGS analysis was performed as described previously[26] with minor modifications. Briefly, 30 ng of leukocyte or granulocyte DNA was amplified with high-fidelity Q5 Polymerase (New England Biolabs, Ipswich, MA, USA) using primers specific for the *TP53* exons 4–10. The indexed library was prepared with Nextera XT DNA Sample Preparation Kit (Illumina, San Diego, CA, USA) and sequenced using MiSeq Reagent Kit v2 (300 cycles; Illumina, San Diego, CA, USA) on a MiSeq instrument according to manufacturer recommendations. The coverage per base exceeded 5000 ($\geqslant$10 000 in 82% of exons); mean coverage reached 39535. For variant detection we used bioinformatics pipeline (Supplementary Figure S1) combining CLC Genomic Workbench version 7.5 (Qiagen, Hilden, Germany) and the deepSNV R-package.[38–42] Samples containing variants above 0.2% VAF by either approach were subjected to validation from independent sampling and/or PCR amplification (Supplementary Table S2). For over-time monitoring and validation of previously identified mutation, cutoff 0.1% was applied (minimal coverage per base $\geqslant$ 10 000).

Statistical analysis

Statistical analyses were performed within the R environment.[42] The distribution normality was tested using the Kolmogorov-Smirnov normality test. Non-parametric tests were applied because of normality violation in most clinical variables (for example, age distribution). To analyze the relationship between the variables, the Spearman correlation test, Mann–Whitney test, Kruskal–Wallis test and Fisher's exact test with simulated *P*-value (Monte-Carlo simulation) were used. Data sets were described with median and s.d. and/or range as indicated in the legends. The comparison of patients' survival was performed by log-rank test and visualized using Kaplan–Meier curves; Cox proportional hazards regression was used to model the effect of multiple predictor variables. Logistic regression models were applied to assess the significance of age and therapy in *TP53* mutational status. Age adjustment was performed by adding the age covariate into the logistic model. Finally, the age-adjusted models were compared with a model with age parameter only by anova chi-square tests. The level of statistical significance was set $P \leqslant 0.05$. All statistical tests were performed as two-sided. Plots were created with the GraphPad Prism version 5.00 for Windows (GraphPad Software, La Jolla, CA, USA).

Single-nucleotide polymorphism arrays

Genome-wide analysis was performed on CytoScan HD arrays (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's instructions. CEL files were analyzed using the Chromosome Analysis Suite software, v3.1.0.15 (Affymetrix, Santa Clara, CA, USA) and annotated using NetAffx 33.1 annotation data set.

For details on methods see Supplementary Material.

RESULTS

Ultra-deep NGS analysis of *TP53* gene in treated MPN patients

To screen for *TP53* mutations and assess the effect of therapy, we analyzed 254 chronic-phase MPN patients using ultra-deep NGS. All patients were treated with one or more cytoreductive drugs (hydroxyurea, HU; interferon alpha-2a, IFN; anagrelide, ANG) and diagnosed $\geqslant 4$y before sampling (4.2–29.5; median 9.2y; Supplementary Table S1). *TP53* mutations were identified in 41 patients (41/254; 16.1%; Table 1) with VAF for the most abundant variant ranging between 0.2 and 11.6%. In a pronounced proportion of patients, more than one mutation was present (11/41; 26.8%). Colony-forming assay[43,44] performed in 3 patients confirmed the presence of *TP53*-mutated subclones within *JAK2* or *CALR*-mutated populations (Supplementary Figure S2). To verify the mutations' presence in myeloid lineage in patients examined from leukocyte DNA, the granulocyte sample was analyzed where available. No evident discrepancy was found (Supplementary Table S3).

TP53 mutations in MPN are strongly associated with higher age

TP53 mutations were found in all disease subtypes, regardless of driver gene status and even if no HU had been administered (Figure 1; Table 2). Further, we performed thorough analysis of relationships between *TP53* mutational status and disease parameters (Supplementary Table S4). The comparison of patients carrying *TP53* mutation (*TP53*-mut) to patients without *TP53* mutation (*TP53*-wt) revealed a highly significant association between *TP53* mutations and higher age ($P = 5.54 \times 10^{-5}$; median age at sampling 69.3 for *TP53*-mut and 62.4 for *TP53*-wt; Figure 2a). *TP53* mutations were less frequent in patients who obtained ANG during disease course (9/104, 8.7% in ANG-no vs 32/150, 21.3% in ANG-yes; $P = 0.0087$). Patients receiving HU at sampling carried the *TP53* mutation more frequently (HU at sampling, 28/125, 22.4% vs without HU at sampling, 13/128, 10.2%; $P = 0.0205$) but associating the *TP53* mutation with HU administration anytime during disease course did not reach significance (31/164; 18.9% in HU-yes vs 10/90; 11.1% in HU-no). As expected, the age at sampling was significantly associated to multiple therapy parameters, partially due to the frequent use of HU in older patients (Figure 2b, Supplementary Figure S3).

As the patient cohort was compiled of several hospitals' contributions, which may have introduced bias, we limited the analysis to University Hospital Brno patients ($N = 169$; $N_{TP53\text{-mut}} = 22$), which lead to similar results (data not shown).

Some of the patients received HU for a short time period and were switched to other therapy or vice versa. Thus, to further disclose the relationship between *TP53* mutations, HU, and age, we eliminated these patients from the analysis, using more stringent criteria to categorize patient therapy (Figure 2c; Supplementary Table S5): (1) patients who had obtained HU for at least 48 months ($N = 122$) and (2) patients treated with IFN and/or ANG only (HU-no; $N = 90$). Also in this subset, patient age was the most significant predictor of *TP53* mutation (Supplementary Figure S4A; *TP53*-mut 69.5y vs *TP53*-wt 63.4y; $P = 0.0009$) and *TP53* mutations were more frequent in patients in the HU subgroup (HU $\geqslant$ 4y, 28/122, 22.9% vs HU-no, 10/90, 11.1%; $P = 0.03$). In parallel, the therapy category was strongly associated to age (Supplementary Figure S4B). To eliminate the influence of age, we applied a logistic regression model with the age adjustment (Supplementary Table S6); using this approach, the *TP53* mutation frequency was not found to be significantly different in patients treated with various cytoreductive drugs. This

Table 1. Clinical and laboratory data of patients treated with cytoreductive drugs and carrying *TP53* mutations

		Disease type and course					*Therapy*						*Mutations*		*Follow-up*	
Therapy group	*Sample*	*Driver mutation* [a]	*Sex*	*Disease phenotype*	*Age at sampling*	*Time from dg to study enrollment (mo)*	*Total therapy length (mo)*	*Therapy at sampling*	*HU therapy (mo)*	*IFN therapy (mo)*	*ANG therapy (mo)*	*Busulfan or chemo/radio therapy*	*Most abundant mutation (VAF %)*	*TP53 mutations (VAF %)*	*Follow-up from study enrollment (mo)*	*Outcome*
no HU	MP315	JAK2	M	PV	62	109	89	I	0	0	0	no	0.8	p.Y126D 0.79	39	Alive
	048C	JAK2	M	PV	73	145	96	I	0	96	0	no	0.8	p.E285K 0.82	41	Alive
	JAK1716	JAK2	F	PV	63	111	110	I	0	110	0	no	0.2	p.C238S 0.20	83	Alive
	MP189	CALR +JAK2	F	ET	67	138	112	A	0	0	112	no	2.9	p.P58A 2.90	51	Alive
	MP247	CALR	F	PMF	56	122	119	A	0	5	115	no	0.3	p.Y220C 0.26	48	Alive
	MP155	CALR	F	PMF	61	122	121	A	0	0	121	no	0.2	p.V143E 0.21	52	Alive
	227A	CALR	M	PMF	72	154	123	I	0	123	0	CH	0.3	p.A138V 0.26	10	Dead
	65A	CALR	F	ET	70	158	152	IA	0	152	11	no	1.7	c.376-2A>G 1.71; p.R273H 0.2	121	Alive
	186A	JAK2	F	ET	80	180	175	I	0	175	0	no	8.3	c.454dupC 8.27	37	Dead-AML[b]
	221A	CALR	M	ET	69	180	179	I	0	179	0	no	2.2	p.I162S 2.24	118	Alive
HU < 4y	MP15	JAK2	M	PV	66	84	84	I	17	77	0	no	4.4	p.Y220C 4.4	62	Alive
	MP160	JAK2	M	ET	69	61	18	H	18	0	0	no	0.2	p.P142R 0.23	52	Alive
	MP68	JAK2	F	MPN	70	58	56	H	47	0	9	no	6.9	p.R248Q 6.90; p.A159V 6.40; p.P151R 1.73; p.I195S 0.24; p.R273H 0.23	44	Dead-AML[c]
HU ⩾ 4y	MP326	JAK2	F	PV	58	80	48	H	48	0	0	no	0.6	p.Y220C 0.55	39	Alive
	MP345	JAK2	M	PV	68	50	48	H	48	0	0	no	1.1	p.S215R 1.10	38	Alive
	MP302	JAK2	F	PV	71	231	65	H	56	0	0	B	0.4	p.G199E 0.44	29	Alive
	MP319	JAK2	M	PV	61	62	57	HA	57	0	32	no	0.4	p.R273H 0.37	28	Alive
	MP168	JAK2	F	PV	68	86	57	H	57	0	0	no	1.0	p.H179R 0.95	49	Alive
	MP153	JAK2	M	PV	63	62	61	HA	60	0	61	no	0.2	p.R213G 0.23	51	Alive
	MP96	JAK2	F	PV	55	64	62	H	62	0	0	no	0.5	p.I195T 0.51; p.R282W 0.20	54	Alive
	MP327	JAK2	F	ET	73	125	64	H	64	0	0	no	0.5	p.R273H 0.47	39	Alive
	MP317	CALR	M	PMF	81	65	65	H	65	0	0	no	0.8	p.V216M 0.83; p.H179R 0.25	17	Dead
	MP63	JAK2	F	ET	68	79	69	H	69	0	0	no	0.2	p.M246V 0.20	53	Alive
	MP5	JAK2	M	PV	68	70	70	H	70	0	0	no	0.3	p.I195T 0.26	64	Alive
	MP10	JAK2	M	PMF	79	77	76	H	76	0	0	no	10.5	p.G245S 10.50; p.R158H 0.54; p.H168R 0.29; p.F134L 0.23; p.Y234H 0.21	46	Dead-expansion[d]
	MP307	MPL	F	PV	82	103	76	H	76	0	0	no	0.2	c.572_574del 0.21	40	Alive
	MP369	JAK2	F	PV	73	84	81	H	81	0	0	no	1.4	p.T170M 1.39	35	Alive
	MP246	JAK2	F	ET	71	108	103	A	82	0	30	no	0.2	p.D259H 0.2; p.G245S 0.2	48	Alive
	MP314	JAK2	F	PV	69	233	92	H	86	0	0	B	1.3	p.C242Y 1.26; p.G245D 0.35	39	Alive
	MP8	JAK2	M	PV	82	92	90	H	90	0	0	no	0.2	p.R175H 0.24	49	Dead

Table 1. (Continued)

		Disease type and course					*Therapy*						*Mutations*		*Follow-up*	
Therapy group	*Sample*	*Driver mutation* [a]	*Sex*	*Disease phenotype*	*Age at sampling*	*Time from dg to study enrollment (mo)*	*Total therapy length (mo)*	*Therapy at sampling*	*HU therapy (mo)*	*IFN therapy (mo)*	*ANG therapy (mo)*	*Busulfan or chemo/radio therapy*	*Most abundant mutation (VAF %)*	*TP53 mutations (VAF %)*	*Follow-up from study enrollment (mo)*	*Outcome*
	MP356	JAK2	F	ET	69	109	91	H	91	0	0	no	1.6	p.R248Q 1.64	37	Alive
	MP230	JAK2	M	MPN	70	96	96	H	96	0	0	no	0.5	p.R248Q 0.50; c.512_514dup 0.22; p.V274G 0.20	22	Dead
	MP289	JAK2	F	PV	61	265	140	no	98	42	0	no	3.3	p.R213* 3.29; p.I251T 0.93; p.Y234H 0.67	39	Alive
	MP273	JAK2	F	ET	75	111	111	H	111	0	0	no	0.3	p.C176S 0.31; p.S240G 0.22	47	Alive
	MP229	JAK2	F	PMF	85	112	112	H	112	0	0	no	1.0	c.376-1G>A 0.95	49	Alive
	MP363	JAK2	M	ET	87	117	112	H	112	0	0	no	0.3	p.R248Q 0.29	37	Alive
	MP7	JAK2	F	PV	69	133	130	H	121	9	0	no	0.5	p.I255T 0.46	64	Alive
	MP329	JAK2	F	ET	73	162	142	H	142	0	0	no	2.6	p.R248W 2.61	27	Alive
	MP250	JAK2	F	PV	74	152	152	H	152	0	0	no	0.4	p.W91* 0.36	11	Dead
	MP2	CALR	F	ET	68	195	186	HA	169	11	110	no	11.6	p.E286K 11.60; p.R248Q 0.45	67	Alive
	MP324	JAK2	M	PV	72	255	255	H	255	0	0	no	0.3	p.T253A 0.26	15	Dead

Abbreviations: A/ANG, anagrelide; B, busulphan; CH, chemotherapy; ET, essencial thrombocythemia; F, female; H/HU, hydroxyurea; I/IFN, interferon alpha; M, male; mo, months; PMF, primary myelofibrosis; PV, polycythemia vera; R, radiotherapy; y, years. [a]All three driver genes (*JAK2* V617F+exon 12, *CALR* exon 9 and *MPL* exon 10) were sequenced in all patients with *TP53* mutation. [b]Sample from leukemic transformation not available. [c]sAML *JAK2*-wt/*TP53*-wt. [d]Clonal expansion without leukemic transformation.

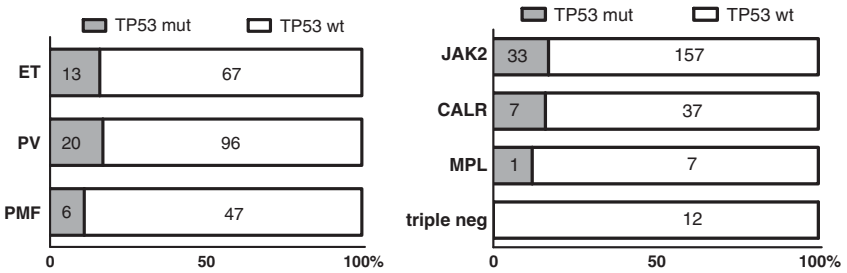

Figure 1. Disease type and driver gene mutation status stratified according to *TP53* mutation presence in patients treated with cytoreductive drugs (NS; Fisher exact test). Driver gene mutations examined in order of *JAK2* > *CALR* > *MPL*.

Table 2. Clinical characteristics of patients according to *TP53* mutational status

	Treated (N = *254*)					*Untreated* (N = *85*)				
	TP53 wt	*%*	*TP53 mut*	*%*	P	*TP53 wt*	*%*	*TP53 mut*	*%*	P
N	213	100	41	100		76	100	9	100	
Sex (male)	91	43	16	39	NS	36	47	5	56	NS
Age at study enrollment (median, range, s.d.)	62 (25–90, 13.2)		69 (55–87, 7.4)		0.0002	70 (19–89, 13.8)		70 (66–82, 5.4)		NS
Time from diagnosis (mo; median, range, s.d.)	109 (50–354, 54.9)		111 (50–265, 54.5)		NS	0 (0–255, 52.9)		4 (0–53, 17.2)		NS
Disease subtype										
ET	67	31	13	32	NS	21	28	1	11	NS
PV	96	45	20	49		20	26	2	22	
PMF	47	22	6	15		30	39	6	67	
unclassified MPN	3	1	2	5		5	7	0	0	
JAK2-mutated	157	74	33	80	NS	54	72	8	89	NS
JAK2-wt	56	26	8	20		22	29	1	11	
CALR-mut	37	17	7	17		13	17	1	11	
MPL-mut	7	3	1	2		4	5	0	0	
Triple negative	12	6	0	0		3	4	0	0	
Therapetutic history										
Total therapy length (mo; median, range, s.d.)	87 (24–265, 44.7)		92 (18–255, 45.4)		NS	0		0		
HU yes	133	62	31	76	NS	0	0	0	0	
Length of HU in HU yes (mo; median, range, s.d.)	65 (2–265, 53.9)		76 (17–255, 46.1)		0.0343	0		0		
HU at study enrollment	97	46	28	68	0.0120	0	0	0	0	
HU ⩾ 48 months	94	44	28	68	0.0060	0	0	0	0	
ANG yes	95	45	9	22	0.0392	0	0	0	0	
IFN yes	72	34	12	29	NS	0	0	0	0	
Busulfan/chemo-/radiotherapy	9	4	3	7	NS	2	3	1	11	NS

Abbreviations: %, percentage of patients with given parameter in *TP53*-wt or *TP53*-mut group; mo, months; driver gene status considered in order *JAK2* > *CALR* > *MPL*; PMF, primary myelofibrosis; PV, polycythemia vera; ET, essential thrombocythemia; post-PV MF was grouped to PV, no post-PV patient carried TP53 mutation. *MPL* status was unknown in one untreated *JAK2*-wt/*CALR*-wt/*TP53*-wt patient

is in agreement with the observation that in patients over 65y, the difference in proportion of *TP53* mutation between HU ⩾ 4y and HU-no therapy groups was much lower (23/75, 30.6% vs 6/30, 20.0%; n.s.) even though the HU-no group was significantly younger ($P = 0.026$; Supplementary Figure S5).

To further explore whether minor *TP53* mutations occur independently of the therapy, we examined a set of 85 patients with no cytoreductive treatment (Supplementary Table S1) and found *TP53* mutations in 9 of them (10.6%; Supplementary Table S7, Supplementary Figure S6).

TP53 mutations in HU-treated and other patients

We did not see any difference in mutation spectra between patients treated with HU and the others (Supplementary Figure S7). Neither the VAF of the most abundant variant nor the cumulative size of the mutated population significantly differed between the therapy groups (Figure 3). Nevertheless, we observed a trend towards the presence of more than one mutation (⩾ 0.2% VAF) in HU-treated patients (10/31) compared to patients treated with non-HU drugs (1/10) and untreated patients (2/9) (n.s.). The mutations showed typical distribution, the vast majority of them were located within the DNA-binding domain and they clustered within characteristic hot-spot sites (Supplementary Figure S8, Supplementary Table S8). All but one patient carried mutations which have been described as non-functional or, rarely, partially functional. The exception was the mutation p.P58A (MP189; VAF 2.9%) which displays no significant loss of transactivation activity.[45,46] The mutation remained stable in all three samplings (7.7y). We have not excluded the mutation

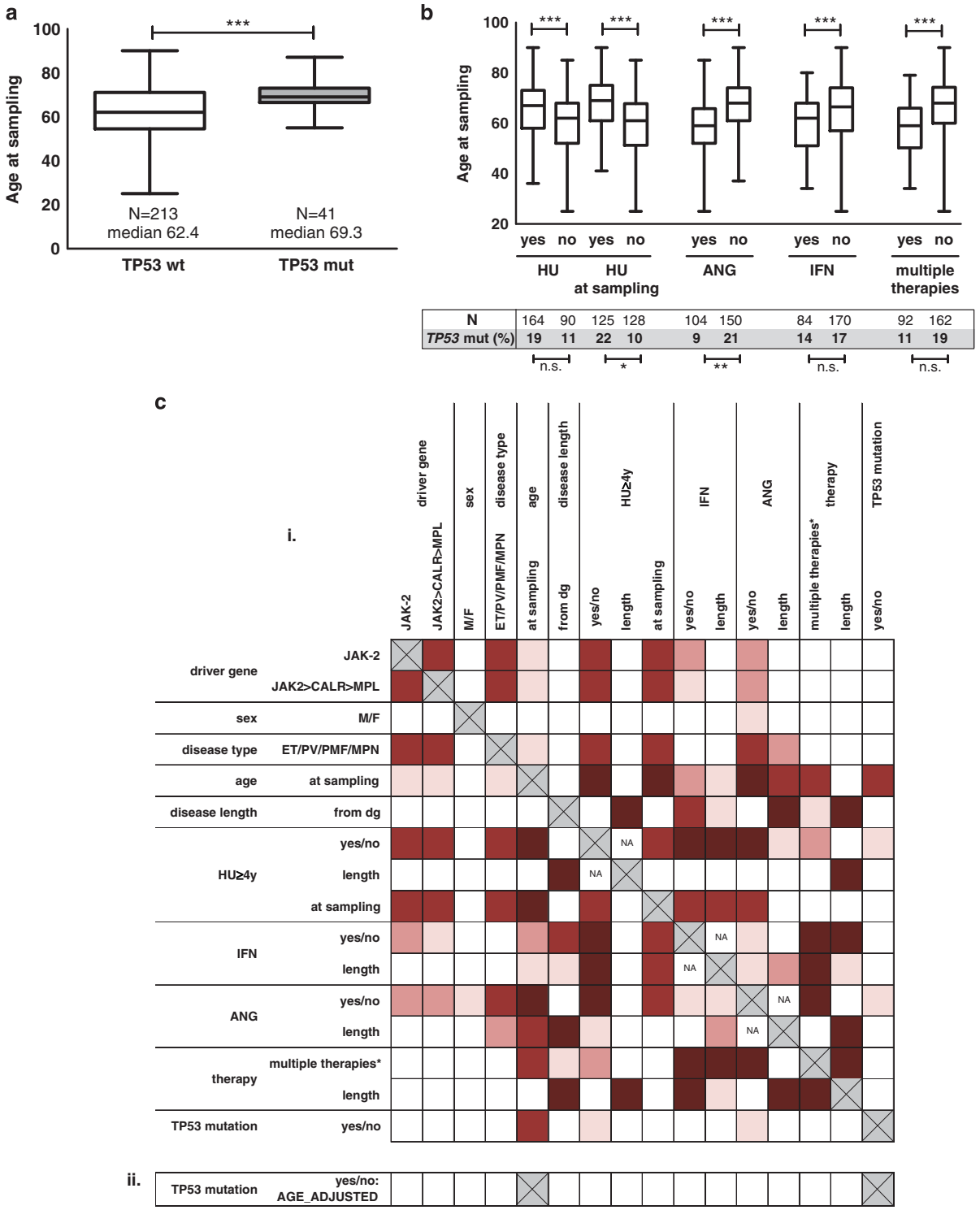

Figure 2. TP53 mutations: age and treatment in patients treated with cytoreductive drugs. (**a**) Age at sampling in *TP53*-mut and *TP53*-wt patients ($P = 5.54 \times 10^{-5}$; Kruskal–Wallis test). (**b**) Age at sampling and *TP53* mutation frequency according to therapy parameters (Kruskal–Wallis and Fisher exact test, respectively; age: HU-yes/no $P = 0.0007$; HU at sampling yes/no $P = 3.96 \times 10^{-7}$; ANG-yes/no $P = 1.32 \times 10^{-8}$; IFN-yes/no $P = 0.0006$; multiple therapies during disease course yes/no $P = 8.7 \times 10^{-8}$). Lines within boxes indicate median, box limits—25th and 75th percentiles, whiskers—minimum and maximum. (**c**) Comparison of patients treated with HU for ⩾ 4y and patients treated with IFN or/and ANG only. (i) Relationship between clinical and laboratory parameters. Red boxes: significant, scaled from $P \leqslant 0.0001$ (dark red) to $0.05 \geqslant P > 0.01$ (light red). Statistical tests used for combinations of variables: continuous—Spearman correlation test, continuous vs categorical—Kruskal–Wallis test; categorical–Fisher's exact test. (ii) Logistic regression model with the age adjustment did not reveal any age-independent significant difference. *Multiple therapies = more types of cytoreductive therapy during disease course. Length of the therapy = restricted to patients positive for given therapy type.

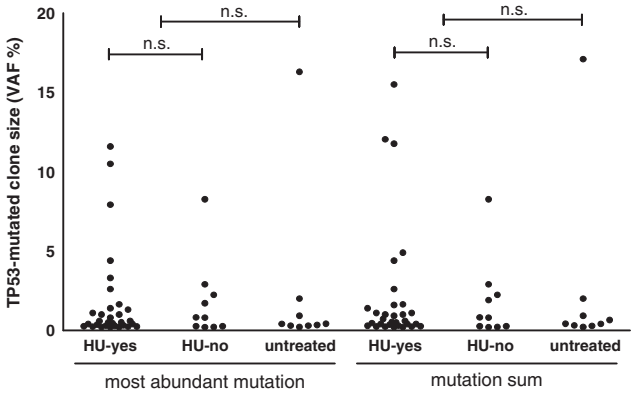

Figure 3. Mutated clone size. VAFs of most abundant mutation and VAF sums do not significantly differ between therapy groups (Mann–Whitney test).

from the analysis as we could not rule out other effects on p53 function.

TP53 mutations may escape detection if examined at diagnosis

Since the majority of mutations we describe were identified in samples taken later during the disease course, we raised the question whether the mutations can be detected earlier, or even at diagnosis. Out of 50 patients with a mutated clone, at least one retrospective sample was available in 20 (Table 2). A later identified mutation was traceable in at least one sample in 14 patients. The sample from the time of diagnosis was available in 10 patients; no *TP53* mutation was detected in 5 (50%; VAF $\geqslant$ 0.1%) of them. Correspondingly, the mutation was detectable at diagnosis in only one of two patients who later developed *TP53*^{mut} sAML (JAK22 and JAK453; Figure 4). In conclusion, despite some *TP53* mutations being found at diagnosis with VAF $\geqslant$ 10% (JAK646: 16.3%, Supplementary Table S7; 221A: 10.2%; Table 3), mutations frequently appear later in the disease course and may be undetectable ($\geqslant$ 0.1%) at diagnosis.

Monitoring patients with *TP53* mutations – dynamic behavior of mutated clones

To explore *TP53* mutation evolution in MPN prospectively, disease course was monitored and serial samples were collected. Prospective samples were available in 30 of 50 MPN patients with *TP53*^{mut}-subclones (median between study enrollment and the last serial sample 2.8y; 0.5–9.6y; Table 3). *TP53* mutations remained present in all but three serial samples (MP63, MP160 and MP307) in which mutations originally identified and confirmed in 0.2% were not detected 3.4, 2 and 1.6 years later, respectively.

TP53 mutation expanded and became predominant tumor cell population in 2/30 patients. The expansion was accompanied by the second allele inactivation in both cases. While the JAK22 patient progressed to AML carrying biallelic *TP53* inactivation (VAF 46%), the clonal biallelic expansion in patient MP10 (VAF 86%) did not result in leukemic transformation; this case is described further in detail (Figure 4; Supplementary Table S9).

When we considered all samples tested during the disease course, that is, retrospective as well as prospective samples, median interval between first and last sample was 7.1y (0.5–24.6; 31 patients). During this time, a slow mutation burden increase was the most frequent scenario (14 patients). We saw no clear association between the mutation burden changes and the VAF at study enrollment, therapy, other clinical data or mutation localization (Table 3, Supplementary Figure S8B and S10).

Impact of *TP53* mutations on overall survival or leukemic transformation

TP53 mutation with VAF $<$ 5% did not impact overall survival (OS) during the follow-up when tested either from diagnosis or study enrollment (Supplementary Figure S9). The patients carrying *TP53* mutations above 5% VAF at study enrollment had significantly shorter OS ($P = 0.0064$ OS from sampling, $P = 0.0185$ OS from diagnosis). However, the impact on OS was lost when adjusted for age (Cox proportional hazard regression model for both age and mutation: $P = 1.01 \times 10^{-10}$ for age; $P = 0.121$ for *TP53* mutation $>$ 5%). Besides, their shortened survival was not attributed to *TP53*-mut AML (Supplementary Figure S9G).

In total, AML developed in three patients with *TP53* mutation (3/50; 6%; Figure 4). Patient JAK22 (p.289fs 2%) was diagnosed with PV 4y after chemo/radiotherapy for B-cell lymphoma. The patient was treated with HU and progressed to *TP53*^{mut}-AML 5y later. On the contrary, patient MP68 (p.R248Q 6.9%) treated with HU developed *JAK2*^{wt}*TP53*^{wt}-AML outside multiple *JAK2*^{mut}*TP53*^{mut}-clones 3.5y from study enrollment (8.3y from diagnosis). Patient 186A (p.P153fs 8.27%) treated with IFN progressed to AML 2.9y after mutation detection (17.9y from diagnosis). The patient was switched to HU soon after study enrollment and an AML sample was not available, thus we cannot confirm the clonal expansion of *TP53* mutation or the effect of the therapy.

Rapid *TP53*-mutated clone expansion accompanied by cn-LOH but no other karyotype changes resulted neither in AML transformation nor HU resistance

In the *JAK2*^{mut}-PMF patient MP10, multiple *TP53* low-burden mutations were present at study enrollment. Among them, a hotspot *TP53*^{G245S} mutation grew rapidly during prospective monitoring up to 95% in granulocytes (Figure 4), reflecting the loss of heterozygosity (LOH). Since complex karyotype changes have been described as very common in AML with mutated *TP53*,[18] we analyzed the karyotype changes using CytoScan HD arrays. Only copy-neutral LOH (cn-LOH) in 17p13, including *TP53* gene, and chromosome Y loss were detected in the expanded clone. To further examine the time course of allelic changes, we analyzed myeloid progenitors. CFC assay indicated that second allele inactivation occurred intra-clonally in the clone carrying monoallelic p.G245S mutation (Supplementary Figure S2). Interestingly, despite complete p53 inactivation and clonal expansion, the patient remained clinically stable without signs of blast transformation for next 23 months, showed no signs of HU resistance and died 10.2y from diagnosis.

DISCUSSION

Previously published retrospectively analyzed cases showed that the development of *TP53*-mutated post-MPN AML is a multistep process. It likely involves mutation origin in the HSPC pool, mutated subclone propagation to level exceeding detection limit and persistence at low levels for an extended time period. Second allele inactivation was described as resulting in rapid clonal expansion and leukemic transformation.

We focused on the early phase of this process, that is, occurrence of low-burden *TP53* mutations which, in theory, may carry increased risk of leukemic transformation. Using highly sensitive and previously verified methods enabled us to detect mutations as low as 0.2%.[26] In total, we found mutations in 50 patients (14.7%). This is the first study using ultra-deep NGS to search for *TP53* mutations in MPN at a level $\leqslant$ 1%. Lundberg et al.[11] found mutations in 5/197 (2.5%) patients using NGS with sensitivity of 5%, which roughly corresponds to our data (5 patients with mutations $>$ 5% out of all 339 examined, 1.5%). As our study aimed to compare patients on HU and non-HU

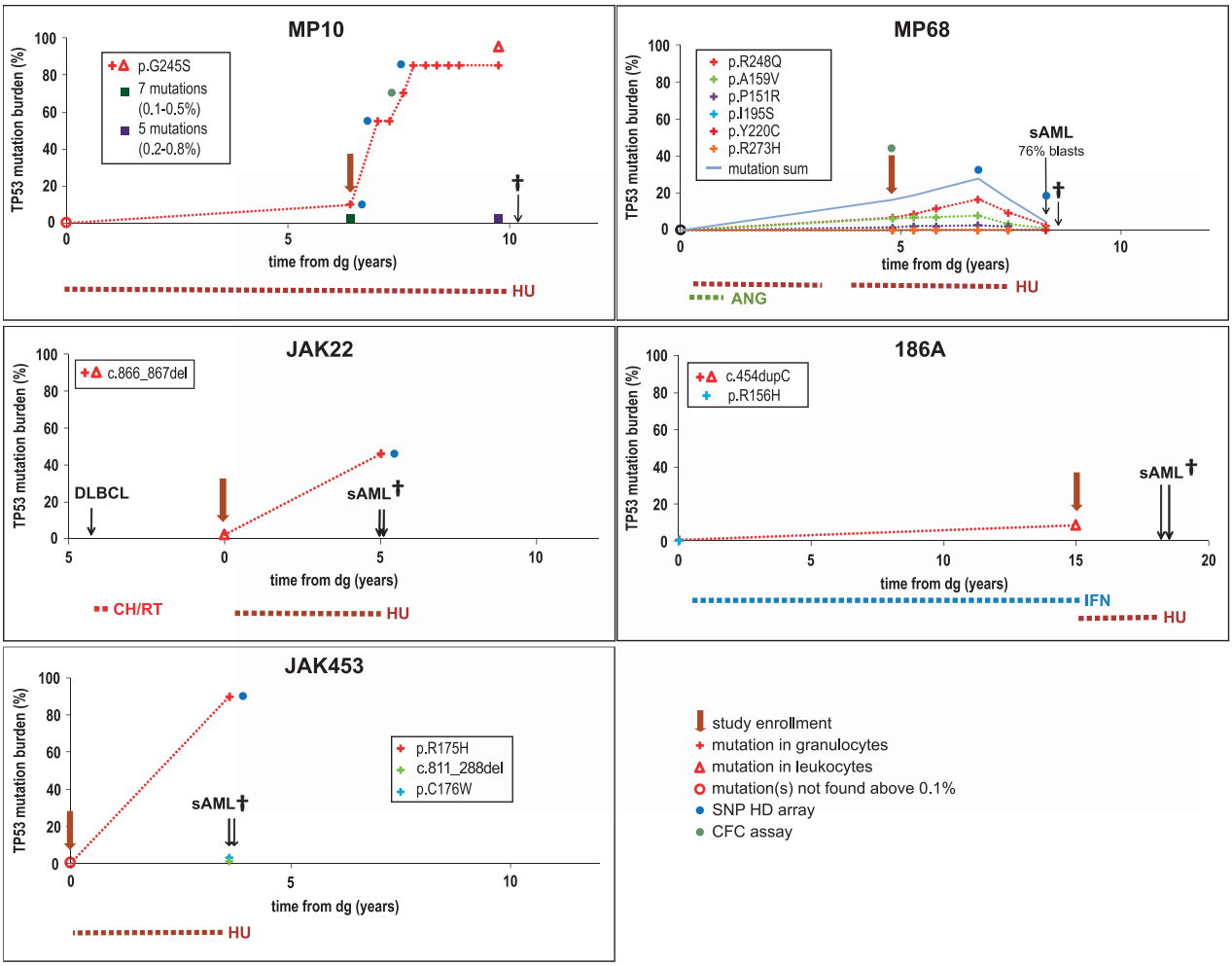

Figure 4. Selected cases of leukemic transformation or clonal expansion in patients with *TP53* mutations**.** Details on cytogenetic analysis using Single-nucleotide polymorphism (SNP) HD Array are shown in Supplementary Table S9. MP10: $TP53^{G245S}$ clonal expansion without leukemic transformation in PMF. $TP53^{G245S}$ development was monitored by Sanger sequencing. Diagnostic sample and two other samples (green and purple mark) were analyzed by NGS, for detail see Supplementary Table 1. Chromosome 17 analysis: cn-LOH(17)(p13.3p11.2) in 2nd and 3rd SNP array. MP68: $JAK2^{wt}TP53^{wt}$-AML outside multiple $JAK2^{V617F}TP53^{mut}$-clones. After transient increase of $JAK2^{V617F/wt}TP53^{R248Q}$ subclone from 8 to 16%, all $TP53^{mut}$ clones decreased accompanied by $JAK2^{V617F}$ burden drop and transformation to sAML 3.5y from study enrollment. SNP array showed no aberrations on chromosome 17. JAK22: $JAK2^{V617F}TP53^{L289fs/L289fs}$ sAML with complex karyotype changes including cn-LOH (17)(p13.3p11.2) developed from PV secondary to diffuse large B-cell lymphoma (DLBCL). Single mutation $TP53^{L289fs}$ was present at PV diagnosis (2%) and expanded in blast transformation. 186A: sAML with unknown *TP53* status developed in $JAK2^{mut}$ ET 2.9y after study enrollment when *TP53* mutation was present with VAF 8.3%. JAK453: $JAK2^{V617F}TP53^{R175H/-}$ sAML with complex karyotype changes including del (17p) developed 3.6y after study enrollment at PMF diagnosis when no *TP53* mutation and karyotype changes were found.

therapies, the frequency in the general MPN cohort was out of the scope. However, some information may be gained from our analysis of 48 consecutive newly diagnosed MPNs examined partially within the untreated cohort and partially as retrospective samples (data not shown): only one mutation $\geqslant$0.2% was found (2.1%). This observation, together with the slow increase in mutation load during disease course and strong age bias, points to the fact that *TP53* mutations are probably rare in general cohorts at diagnosis.

We detected no mutation $\geqslant$0.2% in 70 patients below 55y. In contrast, 41/179 (22.9%) patients above 65y carried *TP53* mutation. This agrees with the hypothesis that *TP53* mutations arose spontaneously and accumulate with age, as described in the elderly population without hematological abnormalities.[47] Higher age brings inherent risks of MPN transformation[1] and less vital progenitor pools in the elderly may favor cells carrying oncogenic mutations both under DNA-damaging and normal conditions.[48] On the other hand, younger patients are a subgroup with the perspective of decades living with clonally shifted hematopoiesis and using cytoreductive drugs and should be examined in detail.

It has been described that not only single accidentally arising *TP53*-mutated clones but several coexisting in parallel may be present in myeloid precursors before expansion.[49] We detected more than one mutation in one third of cases. This phenomenon, described as 'convergence',[50] occurs for example, in chronic lymphocytic leukemia (CLL)[51] and points to a selective pressure favoring the mutations in some but not all patients. In MPN, one may consider either the pressure of cytoreductive therapy, since multiple mutations tended to be more frequent in HU-treated patients, or, possibly, the effort of non-vital HPSC to survive and proliferate. The clonal competition among individual *TP53*-mutated subclones is difficult to foresee and likely depends on accompanying defects; hot-spot mutations with documented oncogenic properties may be overgrown by subclones carrying variants with lower oncogenic potential (for example, loss-of function mutations).[25,26]

Table 3. Monitoring *TP53* mutations over time

Sample ID	*Retrospective (R) /prospective (P) analysis*	*First available sample: time from dg (y)*	*Study enrollment: time from dg (years)*	*Last available sample: time from dg (years)*	*Total follow-up (years)*	*MPN therapy before study enrollment*	*Therapy after study enrollment*	*Mutation ⩾0.1% in dg sample*	*The development of most abundant mutation (%)* [a]	*Mutation development during follow-up*
186A	R	0.0	15.0	15.0	15.0	IFN	HU[b]	yes	0.4-**8.3**-NA	Increase
MP15	P/R	0.2	7.0	11.9	11.5	HU, IFN	IFN	no	0-0.8-2.1-2.6-3.8-**4.4**-10.7-11.3-11.2(W) 13.8(G)	
MP10	P/R	0.0	6.4	9.8	9.8	HU	HU	no	0-**10.1**-85.5	
JAK1716	P/R	6.0	9.3	16.2	10.2	IFN → HU	IFN → HU	NA	0-0.1-0.1-**0.2**-0.7-2.2-2-2.2	
MP326	P/R	0.0	6.7	9.1	9.1	HU	HU	no	0-**0.6**-0.5-0.5	
MP96	P/R	0.0	5.4	8.8	8.8	HU	HU	no	0-**0.5**-0.6	
MP168	P/R	2.4	7.1	10.5	8.1	HU	HU	NA	0.1-**1**-0.5-0.6	
MP319	P/R	0.0	5.2	7.5	7.5	HU, ANA	HU, ANA	yes	0.1-**0.4**-0.4-0.8	
MP369	P	0.0	0.0	7.0	7.0	none[c]	HU	NA	NA-**0.13**-1.4	
65A	P	13.2	13.2	19.6	6.5	IFN, ANA	IFN, ANA	NA	NA-**1.7**-5.4	
MP327	P/R	4.9	10.4	11.2	6.3	HU	HU	NA	0-0.1-**0.5**-0.5	
JAK22	P	0.0	0.0	5.0	5.0	none[c]	HU	yes	NA-**2.0**-46.1	
MP273	P	9.3	9.3	12.0	2.8	HU	HU	NA	NA-**0.3/0**-0.3/1.7	
MP317	P	5.5	5.5	6.0	0.5	HU	HU	NA	NA-**0.8**-1.9	
MP289	P/R	15.2	22.1	24.6	9.3	HU, IFN	none	NA	0.2/1.4-**3.3/0.7**-4.4/0.4-6.3/0.6-3.5/0.4[d]	Increase/decrease
MP68	P/R	0.0	4.8	8.3	8.3	HU, ANA	HU → none	no	0-**6.9**-8.8-10.7-11.7-16.9-9.6-2.7	
MP315	P/R	4.6	9.1	11.5	6.9	IFN	IFN	NA	0.2-**0.8**-0.4-0.2	
MP230	P	8.0	8.0	9.2	1.2	HU	HU	NA	NA-**0.5**-2.3-1.3	
MP189	P/R	6.9	11.5	14.6	7.7	ANA	HU, ANA	NA	3.9-2.8-2.5	Stable
MP155	P/R	5.7	10.2	13.0	7.3	ANA	ANA	NA	0.4-**0.2**-0.2	
MP229	P	9.4	9.4	12.4	3.0	HU	HU, ANA	NA	NA-**1**-0.5-0.6	
MP302	P	19.3	19.3	21.7	2.4	busulfan → HU	HU	NA	NA-**0.4**-0.4-0.7	
MP329	P	13.5	13.5	15.7	2.2	HU	HU, ANA	NA	NA-**2.6**-1.9-3.1/3.0	
MP324	P	21.2	21.2	22.0	0.7	HU	HU	NA	NA-0.3-0.4	
221A	P/R	0.0	15.0	24.6	24.6	IFN	IFN	yes	10.2/ND-**2.2/0.6**-0.7/2.0[d]	Decrease
MP2	P/R	12.1	16.3	21.7	9.6	HU, IFN, ANA	HU, ANA	NA	11.5-**10.6**-11.6-2.9-2.2-1.9-2.1	
MP63	P/R	1.5	6.6	10.0	8.5	HU	HU	NA	0-**0.2/0.1**-0.1/0.2-0/0[d]	
MP160	P/R	1.2	5.1	7.1	5.8	HU	HU	NA	0.5-**0.2**-0	
MP307	P	8.6	8.6	10.2	1.6	HU	HU	NA	**0.2**-0	
MP314	P/R	14.8	19.4	21.9	7.1	busulfan → HU	HU	NA	3.3/0.2-**1.3/0.4**-0.6/0.9-0.2/0.2[d]	
MP345	P/R	0.0	4.2	6.3	6.3	HU	HU	yes	0.1-**1.1**-0.9-0.5-0.6	

Abbreviations: G, granulocytes; *NA*, not available; W, leukocytes. Mutations with highest VAF are shown. "Study enrollment" describes the sampling from which the mutation was identified. For over-time monitoring and validation of previously identified mutation, cut-off 0.1% was applied (minimal coverage per base ⩾10000). VAF bellow 0.1% considered as a background (0%). Development was cathegorised as follows: increase—VAF twofold increase between first and last sample or no mutation at diagnosis; decrease—VAF decrease in VAF to half between first and last sample or no mutation at last sampling; increase/decrease—increase followed by decrease; stable—other. *y*, years; *NA*, not available; G, granulocytes; W, leukocytes. [a]Result from study enrollment is highlighted. [b]Samples not available. [c]Mutation identified at diagnosis. [d]Two mutations with the highest VAFs in distinct samplings.

The murine model and clinical observations pointed to the leukemogenic potential of *JAK2*^{V617F} overexpression in *TP53*-null background;[23,52] *JAK2*^{wt}*TP53*^{mut} AML following *JAK2*^{mut}-MPN is however not exceptional.[22] We observed no clear evidence supporting the leukemogenic potential of *TP53*^{mut} and *JAK2*^{V617F} combination. Admittedly, the *JAK2* mutation was homozygous neither in patient MP68 developing *JAK2*^{wt}-AML alongside multiple *JAK2*^{mut}*TP53*^{mut} subclones nor patient MP10 with complete *TP53* loss within the *JAK2*^{V617F} population.

TP53/17p-aberrant post-MPN AML has been repeatedly suggested - but never independently proven - to be associated with HU therapy.[4,12,22,36] Several findings support possible *TP53*^{mut}-subclone selection by HU: (1) HU blocks cell division via ribonucleotide reductase inhibition, resulting in dNTP depletion.[34] Replication stress then activates p53 and cell cycle arrest although these processes' p53 dependence is controversial and dose-dependent.[35,53,54] (2) The expansion of low-burden *TP53*^{mut}-subclones under *TP53*-triggering therapy was shown in CLL,[26–28] myelodysplastic syndrome[29] and secondary AML.[32] Further, HSPC competition triggered by low-level DNA damage in the murine model led to *TP53*$^{+/+}$ being outcompeted by *TP53*$^{+/-}$ cells via senescence-like changes.[33] Similarly, clinically relevant low- (but not high) level replication stress induced p53-dependent senescence-like arrest in fibroblasts and led to *TP53*-aberant subpopulation selection.[53] We suppose that if there was a proliferative and/or survival advantage favoring *TP53*^{mut}-subclones during HU treatment in MPN, we would have observed a pronounced difference in the abundance and incidence of low-burden *TP53* mutations after several years of therapy. Contrary to this assumption and observations from *in vitro* and *in vivo* models, we saw no significant age-independent difference between patients treated with HU and non-HU therapies; moreover, the mutations were present even if no cytoreductive therapy was given. Although long-term prospective monitoring of patients with *TP53* mutations on various therapies is necessary to fully exclude any HU impact on the second allele loss and clonal expansion, the case of patient MP10 further weakens the advantage of MPN cells with aberrant p53 during HU therapy. While the *TP53*$^{G245S/G245S}$ clone replaced *TP53*^{wt} myelopoiesis, the patient did not show any signs of HU resistance, blood counts remained unchanged and no disease progression was observed for next 2y, despite the patient being treated with a constant HU dose. The clonal competition in MPN both under and without HU treatment may differ from experimental data for several reasons: (1) chronic low-level replication stress may affect HPSC compartment differently to single dose DNA damage; (2) in highly sensitive myeloid cells, threshold for p53-dependent selection induced by low-level replication stress[55] may be shifted; (3) competition may be affected by presence of oncogenic mutations; (4) p53 activity in MPN cells may not fully correspond to that of artificially manipulated p53 in murine and cell line models. In contrast, there seems to be a difference between subclones with monoallelic and biallelic p53 defects, first increasing slowly with the latter expanding rapidly.[11]

We showed that relatively high proportion of older-age MPN patients carry low-burden *TP53* mutations. In contrast to retrospective reports, we did not observe correlation with disease progression accompanied with *TP53* mutation clonal expansion. However, our study was not designed with the primary goal to assess the prognostic impact of low-burden *TP53* mutations as we were aware that larger cohorts and long follow-up is definitely required to address this issue completely.

The competition between an in-theory-adverse minor subclone and a major population may be more complex than one may assume from the retrospective studies. Observations on minor *TP53* mutations in CLL, another non-acute hematological malignancy, show that the mutation does not have to expand despite several specific therapy lines in some patients.[26] Further, a *TP53*-mutated subclone outgrowth occurs very rarely in patients that remain untreated, that is, strong selection pressure in the form of chemotherapy dramatically changes the *TP53*-wt vs *TP53*-mut clonal competition.[26,28] Apparently, cytoreduction regimens currently used in MPN do not create such strong pressure. More likely, other intrinsic factors (genomic instability, hematopoiesis exhaustion) lead to disease progression only in a proportion of patients carrying minor *TP53*-mutated clone.

Importantly, 3 out of 4 patients with clonal evolution (blast transformation or mut-*TP53* clonal expansion) carried *TP53* mutation(s) with VAF $\geqslant$ 5%. This indicates that *TP53* mutations increased to a certain level may be the marker of clonal instability or even a poor prognosis, as demonstrated by shorter OS in patients with mutations $\geqslant$ 5% VAF in our study which however cannot be attributed to *TP53* mutation expansion followed by leukemic transformation. Of note, we recorded 6 patients whose prospective samples were available and VAF exceeded 5% at some point, but none developed *TP53*-mutated sAML during the follow-up. Moreover, one patient with no mutations $\geqslant$ 0.1% VAF developed *TP53*-mutated sAML within 3.6y.

To conclude, we show that minor *TP53* mutations are present in a significant proportion of MPN patients and their presence is strongly associated with age. We did not see any significant age-independent association with hydroxyurea therapy, disease type or MPN driver gene mutations. We also show that even a fully expanded biallelic hotspot mutation, leading to complete loss of *TP53* transactivation activity,[45,46] does not *a priori* lead to leukemic transformation. Despite our findings do not support the assumption that there is unequivocal relation between *TP53*-mutated subclones, HU cytoreduction and leukemic transformation in MPN, larger sample sizes are warranted to definitively address this. *TP53* minor mutations in MPN undoubtedly represent a pool for further clonal evolution; however their prognostic and predictive utilization requires further investigation to identify which patients are at risk and whether any risk factors are preventable.

CONFLICT OF INTEREST

HG: AOP Orphan, Calgene, Novartis—Consultancy, Honoraria, Research Funding and Speakers Bureau; JC: Honoraria and Speakers Bureau; Baxalta-Consultancy and Honoraria. MJ: Research Funding from AOP Orphan and Novartis. KR: Research Funding from AOP Orphan, a member on an entity's Board of Directors or advisory committees in Qiagen. PM: AOP Orphan and Novartis—Consultancy, Honoraria, Research Funding and Speakers Bureau. DM: AOP Orphan, Novartis—Consultancy and Honoraria. The remaining authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We kindly thank the staff of local hospitals in Kromeriz, Znojmo, Breclav, Boskovice, Kyjov, Uherske Hradiste, Ostrava, Hodonin, Trebic, Nove Mesto na Morave for providing samples and clinical data, Hana Skabrahova for organizing blood collections, Jan Palecek for providing us methodical help and Matthew Smith for language editing. Supported by projects of Faculty of Medicine, Masaryk University MUNI/A/1106/2016 and ROZV/24/LF/2016 (SPav and JMal), by projects of MH CR 16-29447A, MEYS CR LM2015064 EATRIS, CEITEC 2020 (LQ1601) and TACR TE02000058. Also supported by Czech Leukemia Study Group for Life (CELL) and Genomics Core Facility CEITEC under MEYS CR project LM2011020.

REFERENCES

1 Finazzi G, Caruso V, Marchioli R, Capnist G, Chisesi T, Finelli C *et al.* Acute leukemia in polycythemia vera: an analysis of 1638 patients enrolled in a prospective observational study. *Blood* 2005; **105**: 2664–2670.
2 Kiladjian JJ, Chevret S, Dosquet C, Chomienne C, Rain JD. Treatment of polycythemia vera with hydroxyurea and pipobroman: final results of a randomized trial initiated in 1980. *J Clin Oncol* 2011; **29**: 3907–3913.
3 Berk PD, Goldberg JD, Silverstein MN, Weinfeld A, Donovan PB, Ellis JT *et al.* Increased incidence of acute leukemia in polycythemia vera associated with chlorambucil therapy. *N Engl J Med* 1981; **304**: 441–447.

4 Sterkers Y, Preudhomme C, Laï JL, Demory JL, Caulier MT, Wattel E *et al.* Acute myeloid leukemia and myelodysplastic syndromes following essential thrombocythemia treated with hydroxyurea: high proportion of cases with 17p deletion. *Blood* 1998; **91**: 616–622.
5 Björkholm M, Derolf AR, Hultcrantz M, Kristinsson SY, Ekstrand C, Goldin LR *et al.* Treatment-related risk factors for transformation to acute myeloid leukemia and myelodysplastic syndromes in myeloproliferative neoplasms. *J Clin Oncol* 2011; **29**: 2410–2415.
6 Tefferi A. Is hydroxyurea leukemogenic in essential thrombocythemia? *Blood* 1998; **92**: 1459–1460, author reply 1460–1451.
7 Nand S, Stock W, Godwin J, Fisher SG. Leukemogenic risk of hydroxyurea therapy in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis. *Am J Hematol* 1996; **52**: 42–46.
8 Liozon E, Brigaudeau C, Trimoreau F, Desangles F, Fermeaux V, Praloran V *et al.* Is treatment with hydroxyurea leukemogenic in patients with essential thrombocythemia? An analysis of three new cases of leukaemic transformation and review of the literature. *Hematol Cell Ther* 1997; **39**: 11–18.
9 Baz W, Najfeld V, Yotsuya M, Talwar J, Terjanian T, Forte F. Development of myelodysplastic syndrome and acute myeloid leukemia 15 years after hydroxyurea use in a patient with sickle cell anemia. *Clin Med Insights Oncol* 2012; **6**: 149–152.
10 Björkholm M, Hultcrantz M, Derolf Å. Leukemic transformation in myeloproliferative neoplasms: therapy-related or unrelated? *Best Pract Res Clin Haematol* 2014; **27**: 141–153.
11 Lundberg P, Karow A, Nienhold R, Looser R, Hao-Shen H, Nissen I *et al.* Clonal evolution and clinical correlates of somatic mutations in myeloproliferative neoplasms. *Blood* 2014; **123**: 2220–2228.
12 Thoennissen NH, Krug UO, Lee DH, Kawamata N, Iwanski GB, Lasho T *et al.* Prevalence and prognostic impact of allelic imbalances associated with leukemic transformation of Philadelphia chromosome-negative myeloproliferative neoplasms. *Blood* 2010; **115**: 2882–2890.
13 Abdel-Wahab O, Manshouri T, Patel J, Harris K, Yao J, Hedvat C *et al.* Genetic analysis of transforming events that convert chronic myeloproliferative neoplasms to leukemias. *Cancer Res* 2010; **70**: 447–452.
14 Zhang SJ, Rampal R, Manshouri T, Patel J, Mensah N, Kayserian A *et al.* Genetic analysis of patients with leukemic transformation of myeloproliferative neoplasms shows recurrent SRSF2 mutations that are associated with adverse outcome. *Blood* 2012; **119**: 4480–4485.
15 Ding Y, Harada Y, Imagawa J, Kimura A, Harada H. AML1/RUNX1 point mutation possibly promotes leukemic transformation in myeloproliferative neoplasms. *Blood* 2009; **114**: 5201–5205.
16 Green A, Beer P. Somatic mutations of IDH1 and IDH2 in the leukemic transformation of myeloproliferative neoplasms. *N Engl J Med* 2010; **362**: 369–370.
17 Zhao Z, Zuber J, Diaz-Flores E, Lintault L, Kogan SC, Shannon K *et al.* p53 loss promotes acute myeloid leukemia by enabling aberrant self-renewal. *Genes Dev* 2010; **24**: 1389–1402.
18 Haferlach C, Dicker F, Herholz H, Schnittger S, Kern W, Haferlach T. Mutations of the TP53 gene in acute myeloid leukemia are strongly associated with a complex aberrant karyotype. *Leukemia* 2008; **22**: 1539–1541.
19 Harutyunyan A, Klampfl T, Cazzola M, Kralovics R. p53 lesions in leukemic transformation. *N Engl J Med* 2011; **364**: 488–490.
20 Neri A, Fracchiolla NS, Radaelli F, Boletini A, Ribera S, Migliorini C *et al.* p53 tumour suppressor gene and RAS oncogenes: molecular analysis in the chronic and leukaemic phases of essential thrombocythaemia. *Br J Haematol* 1996; **93**: 670–673.
21 Gaidano G, Guerrasio A, Serra A, Carozzi F, Cambrin GR, Petroni D *et al.* Mutations in the P53 and RAS family genes are associated with tumor progression of BCR/ABL negative chronic myeloproliferative disorders. *Leukemia* 1993; **7**: 946–953.
22 Beer PA, Delhommeau F, LeCouédic JP, Dawson MA, Chen E, Bareford D *et al.* Two routes to leukemic transformation after a JAK2 mutation-positive myeloproliferative neoplasm. *Blood* 2010; **115**: 2891–2900.
23 Rampal R, Ahn J, Abdel-Wahab O, Nahas M, Wang K, Lipson D *et al.* Genomic and functional analysis of leukemic transformation of myeloproliferative neoplasms. *Proc Natl Acad Sci USA* 2014; **111**: E5401–5410.
24 Robles A, Harris C. Clinical outcomes and correlates of TP53 mutations and cancer. *Cold Spring Harb Perspect Biol* 2010; **2**: a001016.
25 Malcikova J, Pavlova S, Kozubik KS, Pospisilova S. TP53 mutation analysis in clinical practice: lessons from chronic lymphocytic leukemia. *Human Mutation* 2014; **35**: 663–671.
26 Malcikova J, Stano-Kozubik K, Tichy B, Kantorova B, Pavlova S, Tom N *et al.* Detailed analysis of therapy-driven clonal evolution of TP53 mutations in chronic lymphocytic leukemia. *Leukemia* 2015; **29**: 877–885.
27 Zenz T, Krober A, Scherer K, Habe S, Buhler A, Benner A *et al.* Monoallelic TP53 inactivation is associated with poor prognosis in chronic lymphocytic leukemia: results from a detailed genetic characterization with long-term follow-up. *Blood* 2008; **112**: 3322–3329.
28 Rossi D, Khiabanian H, Spina V, Ciardullo C, Bruscaggin A, Famà R *et al.* Clinical impact of small TP53 mutated subclones in chronic lymphocytic leukemia. *Blood* 2014; **123**: 2139–2147.
29 Jädersten M, Saft L, Pellagatti A, Göhring G, Wainscoat JS, Boultwood J *et al.* Clonal heterogeneity in the 5q- syndrome: p53 expressing progenitors prevail during lenalidomide treatment and expand at disease progression. *Haematologica* 2009; **94**: 1762–1766.
30 Belickova M, Vesela J, Jonasova A, Pejsova B, Votavova H, Merkerova MD *et al.* TP53 mutation variant allele frequency is a potential predictor for clinical outcome of patients with lower-risk myelodysplastic syndromes. *Oncotarget* 2016; **7**: 36266–36279.
31 Marusyk A, Porter CC, Zaberezhnyy V, DeGregori J. Irradiation selects for p53-deficient hematopoietic progenitors. *PLoS Biol* 2010; **8**: e1000324.
32 Wong TN, Ramsingh G, Young AL, Miller CA, Touma W, Welch JS *et al.* Role of TP53 mutations in the origin and evolution of therapy-related acute myeloid leukaemia. *Nature* 2015; **518**: 552–555.
33 Bondar T, Medzhitov R. p53-mediated hematopoietic stem and progenitor cell competition. *Cell Stem Cell* 2010; **6**: 309–322.
34 Krakoff IH, Brown NC, Reichard P. Inhibition of ribonucleoside diphosphate reductase by hydroxyurea. *Cancer Res* 1968; **28**: 1559–1565.
35 Nayak BK, Das GM. Stabilization of p53 and transactivation of its target genes in response to replication blockade. *Oncogene* 2002; **21**: 7226–7229.
36 Furgerson JL, Vukelja SJ, Baker WJ, O'Rourke TJ. Acute myeloid leukemia evolving from essential thrombocythemia in two patients treated with hydroxyurea. *Am J Hematol* 1996; **51**: 137–140.
37 Vardiman JW, Thiele J, Arber DA, Brunning RD, Borowitz MJ, Porwit A *et al.* The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. *Blood* 2009; **114**: 937–951.
38 van Dongen JJ, Langerak AW, Brüggemann M, Evans PA, Hummel M, Lavender FL *et al.* Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. *Leukemia* 2003; **17**: 2257–2317.
39 Vandenbroucke I, Van Marck H, Verhasselt P, Thys K, Mostmans W, Dumont S *et al.* Minor variant detection in amplicons using 454 massive parallel pyrosequencing: experiences and considerations for successful applications. *Biotechniques* 2011; **51**: 167–177.
40 Varley JM. Germline TP53 mutations and Li-Fraumeni syndrome. *Hum Mutat* 2003; **21**: 313–320.
41 Forbes SA, Beare D, Gunasekaran P, Leung K, Bindal N, Boutselakis H *et al.* COSMIC: exploring the world's knowledge of somatic mutations in human cancer. *Nucleic Acids Res* 2015; **43**(Database issue): D805–811.
42 R_Core_Team. R: A Language and Environment for Statistical Computing, 2015. Available at https://www.R-project.org.
43 Olcaydu D, Harutyunyan A, Jäger R, Berg T, Gisslinger B, Pabinger I *et al.* A common JAK2 haplotype confers susceptibility to myeloproliferative neoplasms. *Nat Genet* 2009; **41**: 450–454.
44 Klampfl T, Gisslinger H, Harutyunyan AS, Nivarthi H, Rumi E, Milosevic JD *et al.* Somatic mutations of calreticulin in myeloproliferative neoplasms. *N Engl J Med* 2013; **369**: 2379–2390.
45 Bouaoun L, Sonkin D, Ardin M, Hollstein M, Byrnes G, Zavadil J *et al.* TP53 variations in human cancers: new lessons from the IARC TP53 Database and Genomics Data. *Hum Mutat* 2016; **37**: 865–876.
46 Soussi T, Hamroun D, Hjortsberg L, Rubio-Nevado JM, Fournier JL, Béroud C. MUTTP53 2.0: a novel versatile matrix for statistical analysis of TP53 mutations in human cancer. *Hum Mutat* 2010; **31**: 1020–1025.
47 Jaiswal S, Fontanillas P, Flannick J, Manning A, Grauman PV, Mar BG *et al.* Age-related clonal hematopoiesis associated with adverse outcomes. *N Engl J Med* 2014; **371**: 2488–2498.
48 Marusyk A, DeGregori J. Declining cellular fitness with age promotes cancer initiation by selecting for adaptive oncogenic mutations. *Biochim Biophys Acta* 2008; **1785**: 1–11.
49 Beer PA, Ortmann CA, Stegelmann F, Guglielmelli P, Reilly JT, Larsen TS *et al.* Molecular mechanisms associated with leukemic transformation of MPL-mutant myeloproliferative neoplasms. *Haematologica* 2010; **95**: 2153–2156.
50 Gerlinger M, Rowan AJ, Horswell S, Larkin J, Endesfelder D, Gronroos E *et al.* Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. *N Engl J Med* 2012; **366**: 883–892.
51 Jethwa A, Hüllein J, Stolz T, Blume C, Sellner L, Jauch A *et al.* Targeted resequencing for analysis of clonal composition of recurrent gene mutations in chronic lymphocytic leukaemia. *Br J Haematol* 2013; **163**: 496–500.

52 Tsuruta-Kishino T, Koya J, Kataoka K, Narukawa K, Sumitomo Y, Kobayashi H *et al.* Loss of p53 induces leukemic transformation in a murine model of Jak2 V617F-driven polycythemia vera. *Oncogene* 2017; **36**: 3300–3311.
53 Marusyk A, Wheeler LJ, Mathews CK, DeGregori J. p53 mediates senescence-like arrest induced by chronic replicational stress. *Mol Cell Biol* 2007; **27**: 5336–5351.
54 Gottifredi V, Shieh S, Taya Y, Prives C. p53 accumulates but is functionally impaired when DNA synthesis is blocked. *Proc Natl Acad Sci USA* 2001; **98**: 1036–1041.
55 Marusyk A, DeGregori J. Replicational stress selects for p53 mutation. *Cell Cycle* 2007; **6**: 2148–2151.

Supplementary Information accompanies this paper on the Leukemia website (http://www.nature.com/leu)