Clinical and molecular correlates of somatic and germline *DDX41* variants in patients and families with myeloid neoplasms

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Abstract

The diagnosis of germline predisposition to myeloid neoplasms (MN) secondary to *DDX41* variants is currently hindered by the long latency period, variable family histories and the frequent occurrence of *DDX41* variants of uncertain significance (VUS). We reviewed 4,524 consecutive patients who underwent targeted sequencing for suspected or known MN and analyzed the clinical impact and relevance of $DDX41_{VUS}$ in comparison to $DDX41_{path}$ variants. Among 107 patients (44 [0.9%] $DDX41_{path}$ and 63 $DDX41_{VUS}$ [1.4%; 11 patients with both $DDX41_{path}$ and $DDX41_{VUS}$]), we identified 17 unique $DDX41_{path}$ and 45 $DDX41_{VUS}$ variants: 24 (23%) and 77 (72%) patients had proven and presumed germline *DDX41* variants, respectively. The median age was similar between $DDX41_{path}$ and $DDX41_{VUS}$ (66 *vs.* 62 years; *P*=0.41). The median variant allele frequency (VAF) (47% *vs.* 48%; *P*=0.62), frequency of somatic myeloid co-mutations (34% *vs* 25%; *P*= 0.28), cytogenetic abnormalities (16% *vs.* 12%; *P*=>0.99) and family history of hematological malignancies (20% *vs.* 33%; *P*=0.59) were comparable between the two groups. Time to treatment in months (1.53 *vs.* 0.3; *P*=0.16) and proportion of patients progressing to acute myeloid leukemia (14% *vs.* 11%; *P*=0.68), were similar. The median overall survival in patients with high-risk myelodysplastic syndrome/acute myloid leukemia was 63.4 and 55.7 months in the context of $DDX41_{path}$ and $DDX41_{VUS}$, respectively (*P*=0.93). Comparable molecular profiles and clinical outcomes among $DDX41_{path}$ and $DDX41_{VUS}$ patients highlights the need for a comprehensive *DDX41* variant interrogation/classification system, to improve surveillance and management strategies in patients and families with germline *DDX41* predisposition syndromes.

Introduction

Germline pathogenic variants that predispose to familial cancers have been reported in several genes.[1] In 2016 the World Health Organization recognized hematological malignancies associated with germline predisposition syndrome as a distinct sub-group with prognostic implications.[2] DEAD/H-box helicase 41 gene (*DDX41*) is located on chromosome 5q35, is thought to be a tumor suppressor gene involved in the splicing of pre-mRNA and processing of ribosomal RNA. In experimental knockouts models, defects in *DDX41* have been shown to contribute to the development of myeloid neoplasms (MN).[3-6] Unlike traditional germline predisposition syndromes, *DDX41*-associated germline predisposition syndrome has a late age of onset, commonly presents with myelodysplastic syndromes (MDS) or acute myeloid leukemia (AML), and is associated with variable family histories of MN.[7] The pro-leukemogenic properties of *DDX41* pathogenic variants is also supported by lack of concurrent cytogenetic or molecular abnormalities that are usually seen in MDS/AML.[8] Several studies including ours have demonstrated favorable prognostic outcomes associated with *DDX41* mutations in MDS/AML.[8-10] In spite of readily available genomic sequencing to assist with the diagnosis of MN, the diagnosis of *DDX41* mutated MN is hindered by a long latency, variable family history and the frequent occur-

rences of variants of uncertain significance (VUS), given inherent difficulties to characterize the *DDX41* gene/protein function. The accurate recognition of germline and somatic *DDX41* variants is vital for prognostication and the management of *DDX41* variant-associated MDS and AML, including allogeneic stem cell transplant donor selection. In this large study we report the mutational profile of 107 patients with *DDX41* variants (pathogenic and VUS) and their clinical outcomes. We also discuss challenges in interrogating and performing functional analysis for *DDX41* VUS.

Methods

We reviewed 4,524 consecutive patients who underwent next-generation sequencing (NGS; MC OncoHeme 42-gene panel) assessments between January 2018 and May 2022 for suspected or known myeloid disorders. The study was conducted at the Mayo Clinic and approved by the Mayo Clinic Institutional Review Board (IRB) (Figure 1). The clinical information was stored in a de-identified database. Germline testing in patients and families with suspected or known germline predisposition syndrome were conducted under IRB approved protocol (Mayo Clinic IRB# 16-004173; *clinicaltrials gov. Identifier: NCT02958462*). We identified 44 (0.9%) patients with *DDX41* likely pathogenic/pathogenic ($DDX41_{path}$) variants and 63 (1.3%) patients with *DDX41* VUS ($DDX41_{VUS}$) according to variant classification criteria from the American College of Medical Genetics/ the Association of Medical Genetics/ the Association for Molecular Pathology (ACMG/AMP) guidelines.[11] We evaluated baseline characteristics, mutational profiles, and clinical outcomes of patients with $DDX41_{path}$ and $DDX41_{VUS}$ variants (Figure 1).

For the purpose of this paper, we operationally defined *DDX41* variants with a variant allele frequency (VAF) of ≥40% to be presumed germline, as previously reported.[10,12] *DDX41* variants with positive germline testing on DNA derived from skin fibroblasts or hair follicles were defined as confirmed germline. Patients with *DDX41* variants who had negative germline testing defined as confirmed somatic. In the absence of direct germline testing, variants with VAF <40% were defined as presumed somatic (Figure 1). We defined clonal cytopenia(s) of undermined significance (CCUS) as unexplained cytopenia(s) associated with known somatic pathogenic variants (VAF ≥2%), with bone marrow dysplasia <10% and bone marrow blast% <5%. Patients having cytopenia(s) with an isolated $DDX41_{VUS}$, without any somatic pathogenic variants, were classified as "cytopenia associated with $DDX41_{VUS}$ alone", as the pathogenicity of $DDX41_{VUS}$ remain to be elucidated.[13,14] Responses to therapy were assessed according to the International Working Group (IWG) MDS response criteria.[15]

Clinical NGS testing was performed on DNA extracted from fresh bone marrow aspirates (102/110 [93%]) or peripheral blood samples (8/110 [7%]). The Mayo Clinic NGS panel included 42 genes (*Online Supplementary Appendix*)

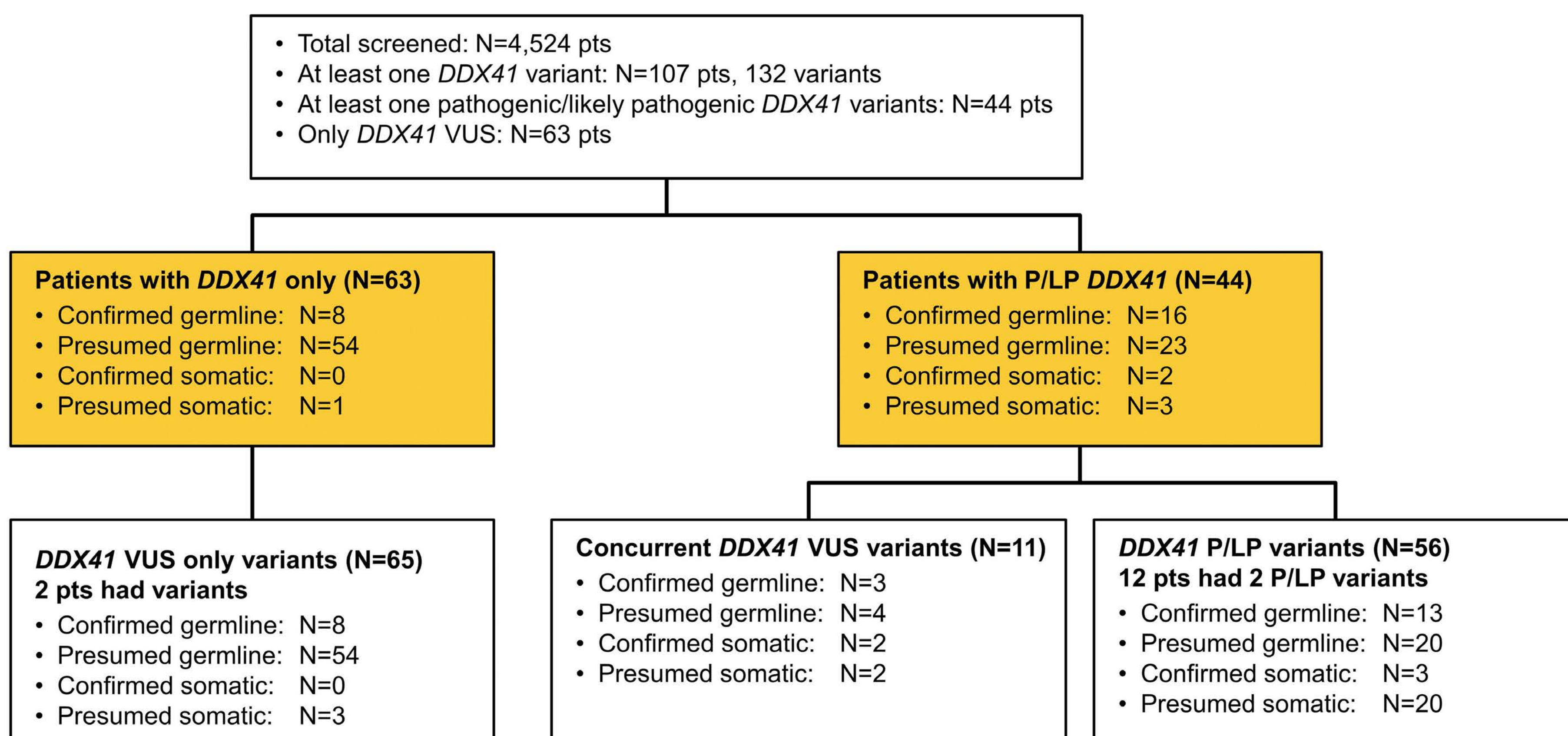

Figure 1. Flowchart of the study, illustrating germline *versus* somatic status, and proportion of patients with $DDX41_{path}$ and $DDX41_{VUS}$. In this study, 107 patients with 132 DDX41 variants are identified from 4,524 adult patients screened. The yellow boxes indicate the number of patients, and the white boxes indicate the number of variants. We identified 44 patients with pathogenic DDX41, harboring 56 pathogenic and 11 variants of uncertain significance (VUS) variants. Sixty-three DDX41 VUS only, harboring 65 VUS variants. pts: patients; path: pathogenic; P/LP: pathogenic/likely pathogenic.

and has an accuracy of >99% and reproducibility of 100% for single-base substitutions and insertion/deletion events. The panel has a variant sensitivity of >5% VAF with a minimum depth coverage of 250x. All coding regions (exons 1-17) of *DDX41* were covered by the panel. We manually reviewed the sequencing BAM files among patients with commonly occurring *DDX41* pathogenic variants (M1I and D140fs) for the presence of *DDX41* R525H somatic variants with low VAF (2-5%, below the report limit of the clinical laboratory). Germline testing was performed prospectively in our germline predisposition clinic, on DNA derived from skin fibroblasts or hair follicles as previously described.[16] We also performed in depth curation assessments of frequently occurring $DDX41_{VUS}$ to analyze allelic diversity and pathogenicity. In order to make a pathogenic prediction, based on available literature, we considered *in silico* CADD (combined annotation dependent depletion) and REVEL (rare exome variant ensemble learner) scores >25 and >0.8 as being likely pathogenic, respectively.[17,18]

Statistical analysis

Continuous variables were summarized as medians (range), while categorical variables were reported as frequencies (percentage). Unadjusted comparisons of patient characteristics and outcomes among the $DDX41_{path}$ and $DDX41_{VUS}$ variant groups were made using the Wilcoxon rank sum test (continuous variables) or Fisher's exact test (categorical variables). The Kaplan-Meier method was used to estimate overall survival (OS). The median OS was calculated from the time of diagnosis to last follow-up or death. All tests were two-sided with *P* value <0.05 considered statistically significant. We have excluded asymptomatic carriers of *DDX41* variants from survival analysis since they do not carry a diagnosis of myeloid neoplasm or cytopenia (s). At last, follow-up, none of these patients had developed cytopenia(s) or demonstrated progression to myeloid neoplasms.

Results

Among 107 patients with *DDX41* variants, we identified 17 unique $DDX41_{path}$ variants and 45 unique $DDX41_{VUS}$ (Figure 1). Eleven patients had both $DDX41_{path}$ variants and $DDX41_{VUS}$. Twelve patients had two $DDX41_{path}$ variants. Among 24 (22%) *DDX41*-mutated patients that had germline testing, 13 (54%) had $DDX41_{path}$, and 11 (46%) patients had $DDX41_{VUS}$, respectively (*Online Supplementary Table S1;* Figure 2). The previously reported *DDX41* pathogenic variants and VUS by our group are marked with (*) in the *Online Supplementary Table S1*. In addition to previously described germline and somatic variants in MN,[10,12,19,20] novel germline variants identified in the current study (i.e., not previously reported in the literature or existing genetic databases) are summarized in the *Online Supplementary Table S2*.

Germline $DDX41_{VUS}$ in patients with myeloid disorders

We identified 74 patients with $DDX41_{VUS}$, including 11 patients with both $DDX41_{path}$ and $DDX41_{VUS}$. Among patients with $DDX41_{VUS}$, frequently observed nucleotide/amino acid changes included c.773C>T; p.P258L (n=7 [9%]), c.517G>A; p.G173R (n=6 [8%]), c.465G>A; p.M155L (n=5 [7%]), c.992_994del; p. K331del (n=3 [4%]), c.1436G>A; p.R479Q (n=3 [4%]), c.1030G>T; p.D344Y (n=3 [4%]), c.1016G>A; p. R339H (n=2 [3%]), c.97T>C; p.Y33H (n=2 [3%]), c.571G>A; p.A191T (n=2 [3%]), c.616C>G; p.P206A (n=2 [3%]), c.653G>A; p.G218D (n=2 [3%]) c.740A>G; p.E247G (n=2 [3%]), c.1018T>A; p.Y340N (n=2 [3%]), c.1032C>G p.D344E (n=2 [3%]) and c.656G>A; p. R219H (n=2 [3%]). Among seven patients with the *DDX41* c.773C>T; p.P258L variant, three (43%) patients had MDS, three (43%) patients had AML and one was an asymptomatic *DDX41* carrier, identified after his family member with AML was found to have the same confirmed germline $DDX41_{VUS}$. None of these patients had concurrent somatic mutations including involvement of the second *DDX41* allele; all had VAF ≥40% (median 46.5%; range, 45-50%) and three of seven (43%) patients required treatment for their MN. The second most common $DDX41_{VUS}$ seen was *DDX41* c.517G>A; p.G173R (n=6). Of these six, four patients were diagnosed with MDS, one had clonal cytopenia of unknown significance (CCUS) with a somatic *DNMT3A* pathogenic variant (c.1233_1234insT; p. G412Wfs*9; VAF 7%) and one patient had pancytopenia. All six patients had VAF ≥40% (median 48%; range, 46-51%) and two (33%) of these six patients had concurrent somatic $DDX41_{path}$ (R525H) variants with low VAF (range, 5-7%). All six patients with this variant had a negative family history of hematological malignancies, with an indolent course only needing supportive care thus far. The *DDX41* c.465G>A; $p.M155L_{VUS}$, which has been reported previously as a germline variant,[12] was also frequent in our cohort (n=5 [7%]) and was associated with pancytopenia (n=2), AML (n=1), CCUS (n=1) and essential thrombocythemia (ET, triple negative) (n=1). None of the patients with the c.465G>A; p.M155L variant had other comutations. Other $DDX41_{VUS}$ encountered and confirmed to be germline were c.992_994del; p.Lys331del (n=3 [4%]) c.740A>G; p.E247G (n=2 [3%]), c.1016G>A; p. R339H (n=2 [3%]), c.571G>A; p.A191T (n=2 [3%]), c.1018T>A; p.Y340N (n=2 [3%]), and c.959C>T; p.T320I (n=1 [1%]). Two patients with the E247G variant had a strong family history of MDS/AML. We performed *in silico* assessments of all the $DDX41_{VUS}$ in our cohort and compared their CADD/ REVEL scores with genomic curations carried out using ClinVar and the current ACMG classification in *Online supplementary Table 2*.

Clinical demographics and mutational comparisons between $DDX41_{path}$ and $DDX41_{VUS}$

The median age in years at diagnosis of MN was comparable between $DDX41_{path}$ and $DDX41_{VUS}$ (66 *vs.* 62; *P*=0.41). Patients were predominantly male, and frequency was

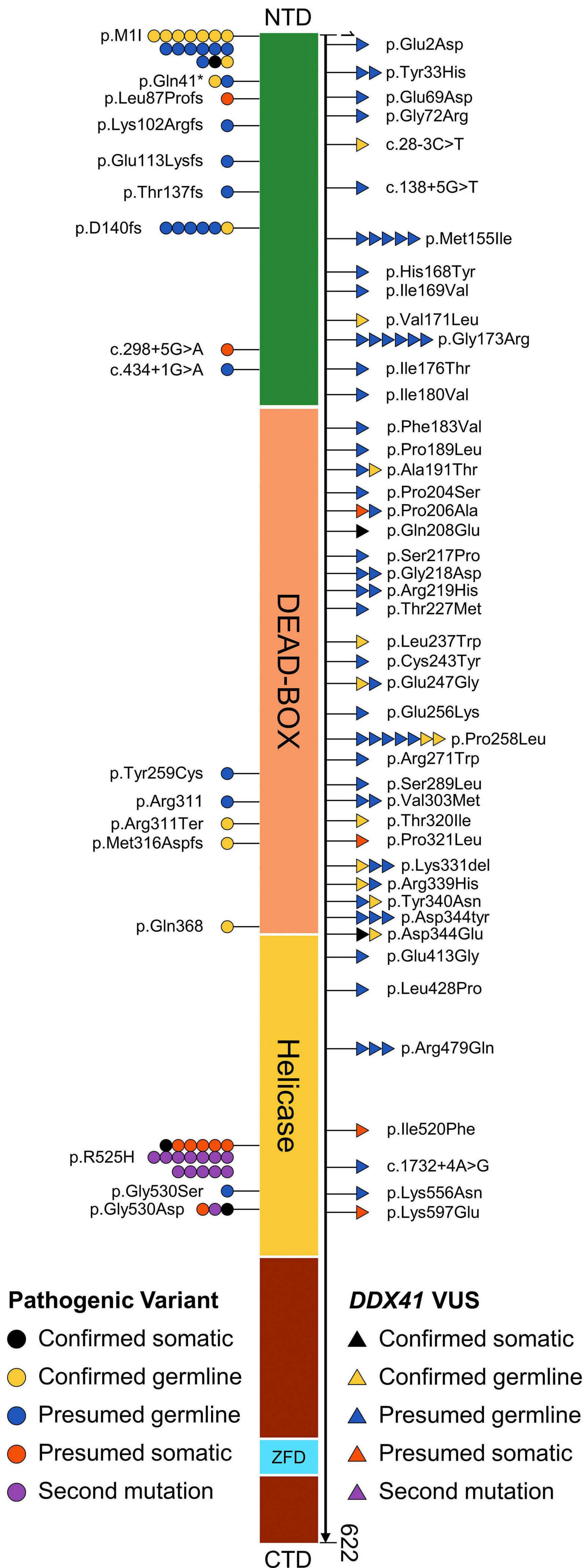

Figure 2. Distribution of *DDX41* variants detected, positioned on the DDX41 protein and its functional domains with representation of germline and somatic status. NTD: N-terminal domain; ZFD: zinc finger domain; CTD: C-terminal domain; VUS: variants of uncertain significance.

comparable between $DDX41_{path}$ and $DDX41_{VUS}$ (66% *vs.* 59%; *P*=0.64). A higher proportion of patients in the $DDX41_{path}$ group had MDS compared to $DDX41_{VUS}$ group, respectively (66% *vs.* 28%; *P*=<0.001). The proportion of patients with AML (20% *vs.* 24%; *P*=0.65), MPN (2% *vs.* 5%; *P*=0.54), CCUS (5% *vs.* 3%; *P*=0.64) or asymptomatic *DDX41* variant carrier states (9% *vs.* 6%; *P*=0.78) were comparable between both groups. The median *DDX41* VAF (47% *vs.* 48%; *P*=0.62), frequency of somatic myeloid co-mutations (34% *vs.* 25%; *P*=0.28), cytogenetic (CG) abnormalities (16% *vs.* 12%; *P*=>0.99) and family history of hematological malignancies (22% *vs.* 33%; *P*=0.59) were also comparable between the two groups (Table 1). The most frequently occurring somatic myeloid co-mutations in *DDX41path* and $DDX41_{VUS}$ groups were *DNMT3A* (15% *vs.* 5%; *P*=0.09), *ASXL1* (9% *vs.* 5%; *P*=0.45), *JAK2* (4% *vs.* 5%; *P*=>0.99) and *EZH2* (4% *vs.* 3%; *P*=>0.99), respectively (Table 1). Time to treatment in months (1.53 *vs.* 0.3; *P*=0.16) and proportion of patients progressing to AML (14% *vs.* 11%; *P*=0.68), were not statistically significantly different between $DDX41_{path}$ and $DDX41_{VUS}$, respectively. Higher proportion of $DDX41_{path}$ patients required treatment compared to $DDX41_{VUS}$ patients (73% *vs.* 48%; *P*=0.02).

We did an additional subset analysis to look for differences in clinical characteristics and outcomes between $DDX41_{path}$ and $DDX41_{VUS}$ patients with one or more concurrent somatic mutations (Figure 3). We did not find statistically significant differences in age (*P*=>0.99), sex (*P*=>0.99), hemoglobin (g/dL) (*P*=0.36), white blood cell count (WBC) (x10^9/L) (*P*=0.36), platelet count (x10^9/L) (*P*=0.06), bone marrow blast percentage (*P*=0.14), cytogenetic abnormalities (*P*=>0.99), proportion of patients progressing to AML (*P*=0.31), time on observation (*P*=0.20) and median OS (*P*=0.69); as outlined in *Online Supplementary Table S3*. We did a subset analysis and assessed for risk of AML progression among truncating and non-truncating *DDX41* variants. Among nine patients who progressed to AML, two (22%) and seven (88%) had truncating and non-truncating *DDX41* variants (*P*=0.22), respectively.

Asymptomatic individuals with germline *DDX41* variants

In our cohort, we identified eight asymptomatic individuals with germline *DDX41* variants: four patients from three affected families. Among these four patients, three have $DDX41_{path}$ (c.3G>A: p. M1I) variants and one has $DDX41_{VUS}$ (c.773C>T: p.258L). Individuals with the *DDX41* c.3G>A: p. M1I variant had a family history of advanced MDS and AML. The patient with $DDX41_{vus}$ c.773C>T: p.258L,

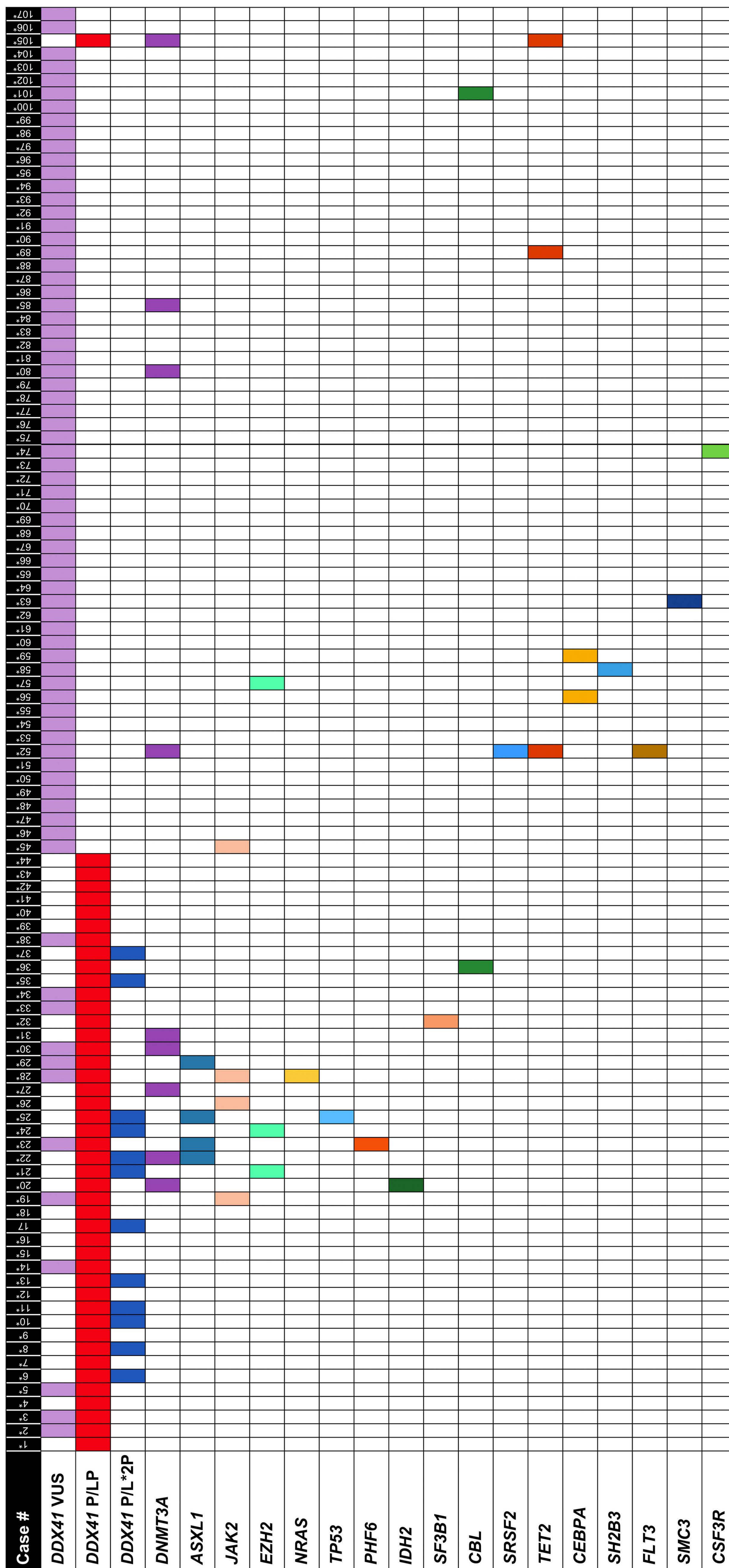

Figure 3. Integrated matrix for *DDX41*$_{path}$ and *DDX41*$_{VUS}$ per case and observed concurrent mutations. *2: second pathogenic (path) DDX41 mutation. VUS: variants of uncertain significance.

Table 1. Comparison of baseline characteristics, genomic profile, and clinical outcome between $DDX41_{path}$ and $DDX41_{VUS}$ variants.

Variable	*DDX41* pathogenic[a] N=44	*DDX41* VUS N=63	*P*
Age in years, median (range) - at diagnosis	66 (30-84)	62 (23-83)	0.41
Male sex, N (%)	29 (66)	37 (59)	0.64
BM blasts %, median (range)	12 (0.01-45)	0 (0-95)	0.005
MDS overall[b], N (%)	28 (64)	18 (28)	<0.001
MDS as per IPSS-R classification, N (%) Very low risk Low risk Intermediate risk High risk Very high risk Non-evaluable	 1 (3) 2 (6) 5 (15) 11 (33) 7 (21) 3 (9)	 0 2 (11) 5 (28) 3 (17) 0 8 (44)	<0.01
AML, N (%)	9 (20)	15 (24)	0.65
MPN, N (%)	1 (2)	3 (5)	0.54
DDX41 carrier, N (%)	4 (9)	4 (6)	0.78
CCUS, N (%)	2 (5)	2 (3)	0.64
Cytopenia associated with $DDX41_{VUS}$ alone, N (%)	-	20 (32)	<0.001
DDX41 VAF (%)	47 (5-52)	48 (14-91)	0.62
Most common *DDX41* variants	Nucleotide/amino acid change (%)		
	c.1574G>A; p. Arg525His (39)[¥]	c.773C>T; p. Pro258Leu (11)[⁋]	-
	c.3G>A; p.M1I (36)[⁋]	c.517G>A; p. Gly173Arg (9.5)[⁋]	-
	c.415_418dup; p. Asp140Glyfs*2 (14)[⁋]	c.465G>A p. Met155Ile (8)[⁋]	-
	c.1589G>A; p. p. Gly530Asp (7) [¥]	c.992_994del p. Lys331del (5)[⁋]	-
	c.931C>T; p. Arg311* (4.5)[⁋]	c.1436G>A p. Arg479Gln (5)[⁋]	-
	-	c.1030G>T. p. Asp344Tyr (5)[⁋]	-
	-	c.1016G>A; p. Arg339His (3)[⁋]	-
	-	c.656G>A; p. Arg219His (3)[⁋]	-
Co-mutated, N (%)	15 (34)	16 (25)	0.28
Abnormal cytogenetics, N (%) NI Monosomal and or complex	7 (16) 0 2 (4)	4 (12) 36 1 (3)	>0.99
Family Hx of hematological malignancies, N (%) NI	9 (20) 0	9 (33) 36	0.59
Observation in mths in no Rx group, median (range)	22.7 (0.27-49.7)	8.38 (0.17-40)	0.24
Time in mths to treatment, median (range)	1.53 (0.03-92)	0.3 (0-25.4)	0.16
Progression to AML, N (%) NI	6 (14) 0	3 (11) 36	0.68
Proportion of pts received Rx, N (%) NI	32 (73) 0	13 (48) 36	0.02
HMA based therapy, N (%) NI	22 (50) 0	5 (18.5) 36	0.004
Intensive chemotherapy*, N (%) NI	7 (16) 0	1 (4) 36	0.20
Other treatment[1], N (%) NI	3 (7) 0	7 (26) 36	0.61
Proportion of patient received allo-HCT NI	13 (32) 0	6 (24) 36	0.41

BM: bone marrow; MDS: myelodysplastic syndrome; IPSS-R: revised International Prognostic Scoring System; AML: acute myeloid leukemia; MPN: myeloproliferative neoplasms; CCUS: cytopenia of unknown significance; NI: no information; VAF: variant allele frequency; pts: patients; HMA: hypomethylating agent; Rx: treatment: Hx: history; mths: months. *Intensive chemotherapy includes 3+7, CPX-351, high dose cytarabine based treatment; [1]other treatment includes growth factors support, erythropoietin stimulating agent, hydrea, other low intensity therapy; [a]11 patients had *DDX41* pathogenic plus variants of uncertain significance (VUS); [b]total MDS patients across all risk group as per IPSS-R; [⁋]germline *DDX41* variant; [¥]somatic *DDX41* variant.

did have one affected family member with AML. Asymptomatic individuals with germline *DDX41* variants are under active surveillance at the germline predisposition syndrome clinic at Mayo Clinic (*clinicaltrial gov. Identifier: NCT02958462*).

Germline *DDX41* variants in patients with myeloproliferative neoplasms

DDX41 variants in patients with MPN have not been frequently reported. We identified four (4%) patients in this cohort; two with *JAK2* V617F mutant polycythemia vera (c.138+5G>T, VAF 48% [$DDX41_{path}$], one with *JAK2* V617F mutant primary myelofibrosis (c.337del; p.E113Kfs*, VAF 48% [$DDX41_{path}$]) and one with essential thrombocythemia to: ET (triple negative) (c.465G>A; p.M155I, VAF 48% [$DDX41_{VUS}$]). One patient each with myelofibrosis and polycythemia vera, respectively, had additional somatic myeloid mutations (*DNMT3A* [c.2645G>A; p.R882H] and *IDH2* [c.419G>A; p.R140Q]); while none of them had a family history of MN and only one patient with a *CSF3R* (c.1919 C>A; p. Thr640Asn) mutant chronic neutrophilic leukemia progressed to AML.

Treatment and survival outcomes

The proportion of patients requiring treatment in the $DDX41_{path}$ and $DDX41_{VUS}$ groups was 73% (n=32) and 48% (n=13), respectively (*P*=0.02). Decisions with regards to treatment were based on the presence of worsening cytopenia(s), high-risk disease features or overt progression to AML. Fifty percent (n=22) *versus* 18% (n=5) (*P*=0.004), 16% (n=7) *versus* 4% (n=1) (*P*=0.20) and 32% (n=13) *versus* 24% (n=6) (*P*=0.41) of patients received hypomethylating agent (HMA)-based, intensive chemotherapy and allogeneic stem cell transplantation in the $DDX41_{path}$ and $DDX41_{VUS}$ groups, respectively (Table 1). Among 21 of 39 (54%) evaluable MDS patients who received treatment, six (40%), three (14.5%), and 12 (57%) had complete remission (CR), hematological improvement (HI) and no response to treatment, respectively. Among eight of 11 evaluable AML patients who received leukemia directed therapy, seven (87.5%) achieved CR and one (12.5%) was refractory to treatment. Overall survival data was available on 63 of 107 (59%) patients. The median follow-up duration was 21.2 months (range, 1.5-158.0). We compared OS outcomes among MDS and AML patients in the $DDX41_{path}$ and $DDX41_{VUS}$ groups. The median OS from date of diagnosis till last follow up or death in patients with high-risk MDS or AML was 63.4 months and 55.7 months in the $DDX41_{path}$ (n=25) and $DDX41_{VUS}$ (n=6) groups, respectively (*P*=0.93; Figure 4A). At 4 years, median OS was 65% *versus* 60% in high-risk MDS or AML with $DDX41_{path}$ $DDX41_{VUS}$, respectively. Similarly, median OS was not significantly different between patients with isolated (n=43) *versus* co-mutated (n=20) *DDX41* variants (63.43 *vs.* 158.03 months [at 4 years 78% *vs.* 59%]; *P*=0.63; Figure 4B). The median OS outcomes for patients with bone marrow (BM) blasts 10-19% (n=21) in comparison to BM blasts ≥20% (n=9) was not significantly different (136.7 *vs.* 63.4 months [at 4 years 70% *vs.* 64%]; *P*=0.90; Figure 4C). The median OS in patients with age <70 years (n=41) *versus* ≥70 years (n=22) (158 *vs.* 61.6 months [at 4 years 59% *vs.* 93%; *P*=0.93). Similarly, somatic (n=8) *versus* germline *DDX41* variants (n=55) (158 *vs.* 63.4 months [at 4 years 69% *vs.* 68%]; *P*=0.29), and normal CG (n=53) *versus* abnormal CG (n=10) (136.7 *vs.* 55.73 months [at 4 years 75% *vs.* 58%]; *P*=0.81), were not significantly different (Figure 4D-F, respectively). Of note, asymptomatic DDX41 variants carriers were excluded from the survival analysis (refer to methods section for reason). We then looked at the survival difference between *DDX41* truncating and non-truncating variants. Overall, 27 of 107 (25%) patients had truncating *DDX41* variants, among them three of 27 were $DDX41_{VUS}$ (c.138+5G>T; p?, c.1732+4 A>G; p?, c.28-3C>T; p?). The median OS was 63.4 and 96.2 months (*P*=0.44) with *DDX41* truncating and non-truncating variants, respectively.

Discussion

In this large cohort of patients who underwent NGS for a known or suspected myeloid neoplasm, we identified 17 (16%) unique $DDX41_{path}$ variants and 45 (42%) unique $DDX41_{VUS}$. Majority of the *DDX41* variants occurred in isolation (n=76/107 [71%]) without any additional somatic variant or clonal cytogenetic abnormality. Our observations validate previous reports that germline *DDX41* associated MN have a later age of onset (median age 65 years), are male predominant (61% males), usually present with indolent cytopenias (27% in $DDX41_{path}$ and 52% in $DDX41_{VUS}$ have not yet needed treatment thus far), with variable family histories of MN (26.5%).[7,10,12,21,22] Importantly, we did not find significant differences in clinical and demographic factors between patients with $DDX41_{path}$ variants and $DDX41_{VUS}$.

We also describe the occurrence of MPN in patients with *DDX41* variants. Recently, in a multicenter retrospective analysis by Li *et al.*[12] from a cohort of 176 patients ($DDX41_{path}$ [n=116], $DDX41_{VUS}$ [n=60]), 15 patients with MPN harboring *DDX41* variants were reported (11 with $DDX41_{VUS}$), with 34% of these variants being somatic in nature. *JAK2* and CALR mutations were not detected in patients with $DDX41_{path}$ and were reported in 72% of patients with $DDX41_{VUS}$. We describe four patients with MPN, including two with *JAK2* V617F mutant PV, and one each with triple-negative essential thrombocythemia and *JAK2* V617F mutant primary myelofibrosis. In this cohort, three (75%) patients had presumed germline $DDX41_{path}$ (c.337del; p. E113Kfs*14,

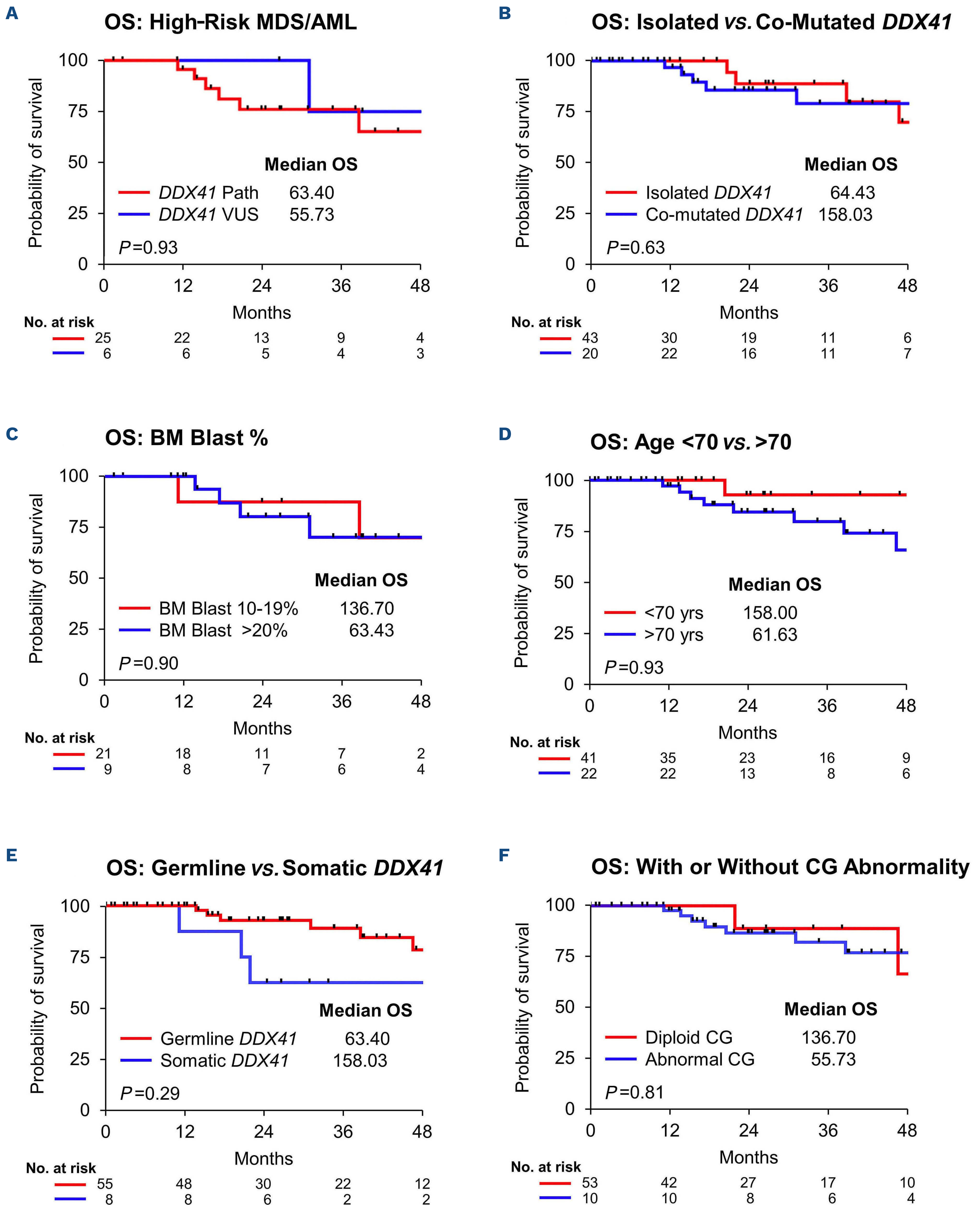

Figure 4. Kaplan Meier curves for overall survival. (A) Overall survival (OS) in high-risk myelodysplastic syndrome (MDS)/ acute myeloid leukemia (AML) patients with $DDX41_{path}$ and $DDX41_{VUS}$, (B) isolated *vs.* co-mutated *DDX41*, (C) bone marrow (BM) blast 10%-19% *vs.* ≥20% (D) age <70 years (yrs) *vs.* ≥70 yrs (E) germline *vs.* somatic *DDX41*, and (F) with or without cytogenetic abnormality. VUS: variants of uncertain significance; path: pathogenic.

c.465G>A; p.M155l and c.298+5G>A; p.?) and the remainder had presumed germline $DDX41_{VUS}$ (c.138+5G>T p?). We are not able to comment on the prognostic impact of *DDX41* mutations in MPN patients given the small sample size.

As previously reported, we did not find a significant impact of BM blast percentage (10-19% *vs.* ≥20%) on MDS/AML outcomes, in patients with *DDX41* variants, germline or somatic.[3,12,23] In contrast, *DDX41*-mutated MN with normal karyotypes had a trend towards a better OS compared to those with abnormal karyotypes, with the most common abnormal karyotype being del 20 (q11.2q13.1) (3/11 [27%]). In addition, survival outcomes for patients with DDX41-mutated MDS/AML were relatively favorable from historical cohort of high-risk MDS/AML and not significantly different between the $DDX41_{path}$ *versus* $DDX41_{VUS}$ groups. In a recent report by Makishima *et al*. on 346 patients with pathogenic/likely pathogenic germline *DDX41* variants, MDS patients with truncating *DDX41* variants had rapid progressions to AML in comparison to those with non-truncating variants, without any significant impact on survival.[24] In our cohort, 25% (n=27) of patients had truncating *DDX41* variants, among them three of 27 were $DDX41_{VUS}$ (c.138+5G>T; p?, c.1732+4 A>G; p?, c.28-3C>T; p?). One $DDX41_{VUS}$ was secondary to an inframe deletion (c.992_994del; p.Lys331del), making the adjudication as to whether or not this variant resulted in truncation of the protein challenging, hence it was excluded. We did not find significant differences in the rate of progression to AML between truncating and non-truncating *DDX41* variants. The median OS was also not significantly different between *DDX41* truncating and non-truncating variants. Further studies are needed to determine the clinical impact of truncating *DDX41* variants in MDS and AML patients.

Variant curation of *DDX41* is challenging since many aspects of its protein function are incompletely understood and for which functional assays or strong heritability links are not yet described. Our molecular hematology laboratory uses standard ACMG criteria, with the help of genetic counselors for evaluating *DDX41* germline variants. We tend to be conservative with our curation approach, to not assign potential risk allele and disease causality without sufficient evidence. Thus, several variants classified as VUS in this manuscript have been reported in ClinVar with different and sometimes conflicting ACMG classification.[11] We are cautious about using ClinVar entry assertions when case counts are very limited. There are many inaccuracies in ClinVar and many entries with assertions provide little to no data for a designation of likely pathogenic variants. An example of this is the p.R339 site, which is now increasingly recognized as a recurrent event and a likely disease predisposing germline variant. It is also evident that these alterations have associations in SNP databases (e.g., gnomAD) with variable frequency among ethnic populations (overall frequency 0.003%). We feel this is a prudent approach given that data continues to emerge on the true effects of different *DDX41* variants in relation to disease outcomes and currently there remains a large gap in our understanding due to a lack of functional data. Li *et al*.[12] recently described a valuable *DDX41*-specific classification approach but included assumptions on certain ACMG criteria to enable a more dichotomous classification as causal/likely causal, *versus* uncertain. Specifically PM2 was applied in the situation that the polymorphic association of a new possible germline causal variant was less frequent in the "general population" than the most common two *DDX41* germline variants (c.465G>A; p.M1I and c.415_418dup; p.D140fs). While the basis for this approach is reasonable, it may inadvertently segregate rare polymorphic variants of limited or no significance with potential disease-associated risk variants. We also apply this criterion (PM2) in our process, but not at its full value. Similarly, this group applied PM3 (typically used in recessive disorder assessment) in a modified fashion when considering occurrence of *DDX41* somatic genetic variants. While these maneuvers are certainly logical, they are not necessarily definitive. Another recent paper by Duployez *et al*.[25] applied ACMG/AMP criteria without apparent modification, although they did consider accompanying *DDX41* somatic variants as strong evidence for a germline finding to be causal. Both papers identify many potential germline variants in patients with hematologic malignancies. In our cohort we find overlapping alterations, specifically with c.773C>T; p.P258L, c.1016G>A; p.R339H and c.992_994del; p.K331del variants, that have been described in several publications with a prevalence now exceeding that in 'control' patients with hematological malignancies. While the accumulating data supports the association of these rare alleles with disease risk, the standard ACMG classification would still render these as VUS calls. It is notable that in a more recent large international study Makishima *et al*.,[24] the c.992_994del; p.K331del variant was not identified in our reading of the paper. While this may reflect an effect of different ethnic group distribution, this finding also supports a conservative approach to curation, along with appropriate interpretive commentary in our reporting.

The pathogenicity of germline VUS associated with adult MN is difficult to determine through analysis of patient observational data alone, since these diseases often present with a complex array of co-mutations and cytogenetic abnormalities that may be epistatic to the effects of the $DDX41_{VUS}$. Thus, experimental analysis of the effect of these variants on gene function is necessary, in conjunction with analysis of available patient data to confirm pathogenicity. Secondly, the effect of VUS on the tumor suppressive activities of *DDX41* likely depends on the effect of each variant on the structure and function of the *DDX41* protein. Most VUS are missense or cause deletion of a single

amino acid and, thus their effect on tumor suppression activity is likely dependent on the role of the effected amino acid in the protein structure and function. The challenge in determining the effect of a variant on tumor suppression is that *DDX41* has multiple functions and variants may not affect all functions equally. Experimental testing of the effect of each VUS on all known functions of *DDX41* is required to resolve this question. However, this experimental analysis can be tedious because each variant must be analyzed separately. *DDX41* is particularly difficult to examine experimentally since it is an essential gene, causing *DDX41*-knockout cell lines to grow inefficiently in culture and making it difficult to engineer cells where the VUS can be studied in isolation from wild-type *DDX41*. Furthermore, the precise function(s) of *DDX41* that are responsible for its role as a tumor suppressor remain incompletely defined, adding additional complication to functional analysis of variants. Importantly, *DDX41* is an essential gene for hematopoiesis and potentially other physiological processes relying on cell proliferation and it is unlikely that inhibition of *DDX41* would provide clinical benefit as a therapeutic approach. However, since *DDX41* mutant MDS/AML is known to have favorable treatment outcomes and slower progression rates than other adult MDS/AML subtypes, understanding the effect of each *DDX41* variant on protein function would allow for further classification and risk stratification with individualized medicine approaches.[5]

In summary, we provide a comprehensive genomic landscape, including germline and somatic pathogenic variants and VUS in patients with *DDX41* variant-associated hematological disorders. We report on the likely pathogenicity of several unique $DDX41_{VUS}$ and provide a detailed analysis on their clinical course and outcomes. We show that patients with $DDX41_{path}$ and $DDX41_{VUS}$ had similar clinical characteristics and clinical outcomes, underscoring the need for better variant interrogation and classification methods.

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Contributions

TB developed the concept, cured data, and wrote and submitted the original draft. AN helped in collecting and analyzing. JMF, TL, CF, HBA, RH, DV, NG, AT, AAM and LJO contributed patients. AA, MS and MRL contributed patients and reviewed the manuscript. TC reviewed and edited the manuscript. AF reviewed genetic data and performed additional variant curation; he also reviewed the manuscript. MMP contributed patients, supervised the review, and edited the manuscript.

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Data-sharing statement

Original data can be provided on reasonable request.

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