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Unique ethnic features of *DDX41* mutations in patients with idiopathic cytopenia of undetermined significance, myelodysplastic syndrome, or acute myeloid leukemia

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ABSTRACT

DDX41 mutations are associated with hematologic malignancies including myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML), but the incidence in idiopathic cytopenia of undetermined significance (ICUS) is unknown. We investigated the incidence, genetic characteristics, and clinical features of *DDX41* mutations in Korean patients with ICUS, MDS, or AML. We performed targeted deep sequencing of 61 genes including *DDX41* in 457 patients with ICUS (n=75), MDS (n=210), or AML (n=172). Germline *DDX41* mutations with causality were identified in 28 (6.1%) patients, of whom 27 (96.4%) had somatic mutations in the other position of *DDX41*. Germline origins of the *DDX41* mutations were confirmed in all of the 11 patients in whom germline-based testing was performed. Of the germline *DDX41* mutations, p.V152G (n=10) was most common, followed by p.Y259C (n=8), p.A500fs (n=6), and p.E7* (n=3). Compared with non-mutated patients, patients with a *DDX41* mutation were more frequently male, older, had a normal karyotype, low leukocyte count, and hypocellular marrow at diagnosis. Three of the four ICUS patients with germline *DDX41* mutations progressed to MDS. The incidence of *DDX41* mutations in Korean patients was high and there was a distinct mutation pattern, in that p.V152G was a unique germline variant. ICUS harboring germline *DDX41* mutations may be regarded as a hereditary myeloid neoplasm. Germline *DDX41* mutations are not uncommon and should be explored when treating patients with myeloid malignancies.

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Introduction

Inherited hematologic malignancies have been established in well-defined hereditary syndromes, which exhibit a particular phenotype, often present in childhood, or have a strong family history.[1] There is also an increasing awareness of additional autosomal dominant genetic aberrations with predisposition to myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML), which are primarily sporadic diseases and typically present in older adults. The recently updated World Health Organization (WHO) classification defined myeloid neoplasms with germline predisposition[2] and categorized familial myeloid neoplasms into three groups: those with an absence of pre-existing disorder or organ dysfunction (e.g., *CEBPA* or *DDX41* mutations), those with a pre-existing platelet disorder (e.g., *RUNX1* mutations), and those with dysfunction of other organs (e.g., *GATA2* mutations). *DDX41* mutations have recently joined the growing list of genetic alterations in familial myeloid malignancies.[3,4] MDS or AML with germline *DDX41* mutations usually occurs in the sixth decade of life or beyond, whereas

most other cases of cancers with germline predisposing mutations typically develop in adolescence or early adulthood.[5-11]

The *DDX41* gene is located at 5q35.3 and encodes a DEAD-box RNA helicase, which is involved in pre-mRNA splicing, RNA processing, and ribosome biogenesis.[12] Several mechanisms have been proposed to explain the contributions of *DDX41* mutations to the development of hematologic malignancies. *DDX41* mutations can: (i) cause aberrant mRNA splicing leading to exon retention or exon skipping, (ii) disrupt the STING-interferon pathway; and (iii) induce aberrant pre-rRNA trimming and ribosome biogenesis.[3] *DDX41* mutations include both germline and somatic mutations, with the latter being found in over half of the patients with germline mutations in the other allele of *DDX41*.[5] In recent studies, germline *DDX41* variants were found in 2.4% of 1,385 patients with MDS or AML,[11] and germline or somatic *DDX41* variants were found in 3.4% of 1,002 patients with myeloid neoplasms.[10]

Following advances in genetic testing, clinical next-generation sequencing (NGS)-based leukemia panels are being increasingly used to identify somatic mutations to facilitate the diagnosis, improve prognostication, and select optimal treatment strategies in patients with hematologic malignancies. Some variants found in the panels can also be germline mutations in genes associated with hereditary hematopoietic malignancies.[13-15] The myeloid leukemia panel used at our institute includes the *DDX41* gene, and the frequencies of *DDX41* mutations in Korean patients with MDS or AML appeared to be higher than the previously reported incidences. Importantly, *DDX41* mutations have not been evaluated in patients with idiopathic cytopenia of undetermined significance (ICUS), which is a known precursor lesion of MDS. In this study, we investigated the incidence, genetic characteristics, and clinical features of *DDX41* mutations in Korean patients with ICUS, MDS, or AML.

Methods

Patients

We included patients with ICUS, MDS, or AML whose bone marrow samples were collected between 2009 and 2019 at Asan Medical Center (Seoul, Korea). Patients with ICUS or lower-risk MDS were either prospectively enrolled (since January 2018) or retrospectively analyzed, while those with higher-risk MDS or AML were retrospectively analyzed. All patients in the study cohort were unrelated individuals, not including an index case and his or her family members. Diagnoses of MDS and AML were based on the WHO 2016 Classification.[2] ICUS was defined by the proposed criteria of the 2007 Consensus Group:[16] cytopenia in one or more of cell lineages for ≥6 months (hemoglobin <11 g/dL, neutrophils <1.5x10^9/L, and platelets <100x10^9/L) while excluding other causes of cytopenia such as a history of pelvic irradiation or cytotoxic chemotherapy, splenomegaly, heart failure or liver cirrhosis with portal hypertension, active viral infections, and a history of blood or bone marrow diseases. Clonal cytopenia of undetermined significance was defined as ICUS with myeloid neoplasm-related somatic mutations with a variant allele frequency ≥ 2%, or clonal karyotypic abnormalities. Myeloid neoplasm-related somatic mutations were based on those specified in the updated National Comprehensive Cancer Network guideline for MDS.[17]

The Institutional Review Board of Asan Medical Center approved the protocols of this study (2018-0042 and 2018-0048 [for prospective and retrospective analysis of patients with lower-risk MDS or ICUS], 2019-0794 [for sequencing the *DDX41* gene in *DDX41*-mutated patients and their family members], and 2020-0131 [for retrospective analysis of data from patients with higher-risk MDS or AML]), which was carried out in accordance with the 2008 Declaration of Helsinki.

Mutational and cytogenetic analysis

For rhe NGS assay, we prepared the sequencing libraries from genomic DNA using customized probes (Integrated DNA Technologies, Inc., Coralville, IA, USA) to capture and enrich the entire coding regions of 61 target genes (HEMEaccuTest DNA Target Enrichment kit; NGeneBio, Seoul, Korea) (*Online Supplementary Table S1*). We carried out sequencing on the MiSeqDx (Illumina, San Diego, CA, USA) with 2×150 bp, paired-end reads according to the manufacturer's instructions. Initial read mapping was carried out against the human reference genome (hg19/GRCh37). We subsequently analyzed the sequencing data for variant calling using commercial software (CLC Genomics Workbench; QIAGEN Bioinformatics, Redwood City, CA, USA). We retained the potentially pathogenic variants by filtering out common polymorphisms (minor allele frequency in the population ≥1%) and sequencing/mapping errors, and by filtering in the known oncogenic variants based on the available population or cancer mutation-specific databases. We set the minimum cutoff of variant allele frequency at 2.0% for reporting. We performed the cytogenetic analysis using conventional G-banding techniques based on the analysis of 20 or more metaphase cells.

Germline variant confirmation and determination of causality

Variants with allele frequencies between 40% and 60% were considered to be probable germline mutations. We performed germline-based testing in 11 of 34 patients with probable germline *DDX41* mutations using sorted blood T cells. This strategy of using sorted T cells was based on recent work confirming that T cells yield sufficient DNA and high rates of somatic variant calls in MDS. It was suggested that, given the challenge of obtaining skin biopsies, T cells would be preferential germline tissues for MDS genomic studies.[18] Peripheral blood mononuclear cells were harvested by standard Ficoll (GE Healthcare, Sweden) density gradient centrifugation, and T cells were isolated using the Pan T Cell Isolation Kit, human (MACS Miltenyi Biotec, Auburn, CA, USA) according to the manufacturer's instructions. The isolated T cells were analyzed with CD3-FITC using a FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, USA), and genomic DNA was purified by the QIAamp DNA mini kit (Qiagen, QIAGEN GmbH, Germany). The pathogenicity of probable germline *DDX41* mutation was determined according to the guideline from the American College of Medical Genetics and Genomics (ACMG).[19] The concurrence of a somatic *DDX41* mutation was considered as strong evidence for causality. Thus, we classified germline *DDX41* variants as "causal" if they were either pathogenic (or likely pathogenic) by the ACMG guideline or accompanied by a somatic *DDX41* mutation regardless of the ACMG interpretation.

Statistical analysis

Categorical variables were compared using the χ^2 test or Fisher exact test, and continuous variables were compared using the Mann-Whitney U-test or the Student *t*-test, as appropriate. Survival was calculated by the Kaplan-Meier method and the resulting survival curves were compared using the log-rank test

Table 1. Patients' characteristics at diagnosis.

Characteristic	Total (n = 457)	ICUS (n = 75)	MDS (n = 210)	AML (n = 172)
Sex, n(%)				
Male	272 (59.5)	37 (49.3)	134 (63.8)	101 (58.7)
Female	185 (40.5)	38 (50.7)	76 (36.2)	71 (41.3)
Median age (range), years	59 (16-89)	54 (19-89)	61 (18-87)	57 (16-81)
ICUS, n(%)				
CCUS*		42 (56.0)		
Non-CCUS		33 (44.0)		
WHO classification				
MDS, n(%)				
MDS with SLD/RS-SLD			41 (19.5)	
MDS with MLD/RS-MLD			73 (34.8)	
MDS with EB-1			45 (21.4)	
MDS with EB-2			18 (8.6)	
MDS, unclassifiable			26 (12.4)	
MDS with isolated del(5q)			2 (1.0)	
Unknown			2 (1.0)	
AML, n(%)				
AML with RGA				84 (48.8)
AML with MRC				40 (23.3)
Therapy-related				7 (4.1)
AML, NOS				40 (23.3)
MPAL				1 (0.6)
Risk stratification, n(%)				
MDS				
IPSS-R score ≤ 3.5			75 (35.7)	
IPSS-R score > 3.5			135 (64.3)	
AML#				
Favorable				63 (36.6)
Intermediate				35 (20.3)
Adverse				73 (42.4)
Unknown				1 (0.6)
Karyotype, n(%)				
Normal	230 (50.3)	69 (92.0)	92 (43.8)	69 (40.1)
Abnormal	227 (49.7)	6 (8.0)	118 (56.2)	103 (59.9)

ICUS: idiopathic cytopenia of undetermined significance; CCUS: clonal cytopenia of undetermined significance; WHO: World Health Organization; MDS: myelodysplastic syndrome; SLD: single lineage dysplasia; RS: ring sideroblasts; MLD: multilineage dysplasia; EB: excess blasts; AML: acute myeloid leukemia; RGA: recurrent genetic abnormalities; MRC: myelodysplasia-related changes; NOS, not otherwise specified; MPAL, mixed phenotype acute leukemia; IPSS-R, International Prognostic Scoring System-Revised. *CCUS was defined as ICUS with myeloid neoplasm-related somatic mutations of variant allele frequency ≥2%, or clonal karyotypic abnormalities. #Risk stratification of AML according to the 2017 European LeukemiaNet risk stratification.

(univariate analysis). The Kaplan-Meier survival curves were rendered as a graph using Prism version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). In all analyses, the *P*-values were two-tailed and those <0.05 were considered statistically significant.

Results

Patients' characteristics

The clinical characteristics of the 457 included patients at diagnosis are shown in Table 1. There were 75 patients (16%) with ICUS, 210 (46%) with MDS and 172 (38% with AML. The median age at diagnosis was 59 years (range, 16-89), and 60% were men. Forty-two (56.0%) of the ICUS patients had clonal cytopenia of undetermined significance. Disease risk of the MDS patients was lower-risk in 75 (35.7%) and higher-risk in 135 (64.3%) according to the Revised International Prognostic Scoring System (IPSS-R).[20] Of the AML patients, 63 (36.6%), 35 (20.3%), and 73 (42.4%) were classified into favorable, intermediate, and adverse genetic risk categories, respectively, according to the 2017 European LeukemiaNet risk stratification.[21]

Frequency and genetic characteristics of *DDX41* mutations

We detected genetic *DDX41* mutations in 39 (8.5%) patients. Thirty-four (7.4%) patients had germline mutations, of whom 27 (79.4%) also had somatic mutations at the other position of *DDX41*. Five (1.1%) patients had somatic *DDX41* mutations only. In 28 patients, the germline *DDX41* mutations were considered causal and

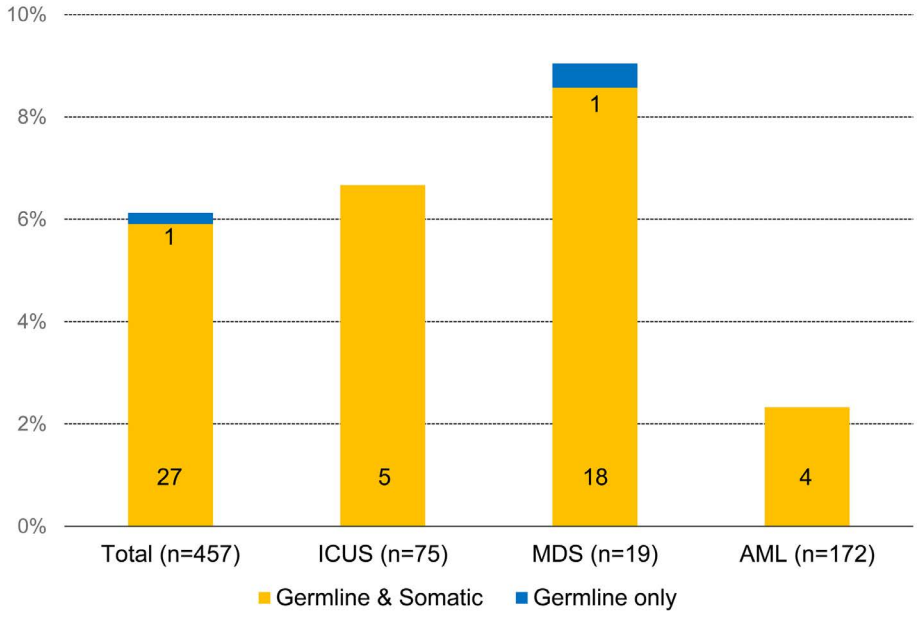


Figure 1. Frequency of *DDX41* mutations according to the type of hematologic malignancy. ICUS: idiopathic cytopenia of undetermined significance; MDS: myelodysplastic syndrome; AML: acute myeloid leukemia.

only these patients were included in further analyses. The frequency of the causal germline *DDX41* mutations was 6.1% (28 of 457); 6.7% (5 of 75) in ICUS, 9.0% (19 of 210) in MDS, and 2.3% (4 of 172) in AML (Figure 1). Detailed information on the *DDX41* variants, concurrent mutations of other genes, and karyotypes in the 28 patients are provided in *Online Supplementary Table S2*. Germline origins of the *DDX41* mutations were confirmed in all of the 11 patients who underwent germline-based testing (p.V152G in 5, p.Y259C in 3, p.A500fs in 2, and p.L328R in 1).

Of the 55 *DDX41* mutations detected in this study, 28 were germline and the other 27 appeared to be somatic. All of the somatic mutations were missense, whereas germline mutations were missense in 19 (67.9%) cases, frameshift in six (21.4%), and nonsense in three (10.7%). The majority of somatic mutations were located in the helicase C or C-terminal domain (n=18, 66.7%), whereas the majority of germline mutations were in the helicase ATP-binding or N-terminal domain (n=22, 78.6%; *P*=0.001) (Figure 2A). Of the germline *DDX41* mutations, p.V152G (n=10, 35.7%) was the most common, followed by p.Y259C (n=8, 28.6%), p.A500fs (n=6, 21.4%), p.E7* (n=3, 10.7%), and p.L328R (n=1, 3.6%).

Two germline variants (p.A500fs and p.E7*) were classified as pathogenic according to the ACMG guideline. The other germline variants (p.V152G, p.Y259C, and p.L328R) were classified as being of uncertain significance, but were considered causal when accompanied by somatic *DDX41* mutations (*Online Supplementary Table S3*). Notably, four mutations (p.V152G, p.Y259C, p.A500fs and p.E7*) were found at a significantly higher frequency in the study patients than in healthy Koreans, as shown by high odds ratios (38.5, 17.3, 49.6 and 26.5, respectively) (*Online Supplementary Table S4*). Two germline mutations (p.V152G and p.Y259C) were only detected in ICUS/MDS (75.0%) and not in AML (0%), whereas p.A500fs and p.E7* were detected in both ICUS/MDS and AML groups (*Online Supplementary Table S5*). Of the 27 somatic *DDX41* mutations, p.R525H (n=14, 51.9%) was the most common, followed by p.T227M (n=5, 18.5%), and the remaining eight somatic *DDX41* mutations were detected in one (3.7%) patient each. The somatic p.R525H variant was less frequently associated with the germline p.V152G variant (3 of 10) than with p.Y259C (6 of 8) or p.A500fs (4 of 6), whereas the somatic p.T227M variant tended to be more frequently associated with p.V152G (4 of 10) than with p.Y259C (1 of 8) or p.A500fs (0 of 6) (*Online Supplementary Table S6*).

Twenty-two (78.6%) of the 28 patients with mutations in *DDX41* had concurrent mutations in other genes. Genes mutated in over 10% of the patients were *PHF6* and *ASXL1* (5 patients [17.9%] each), followed by *CBL* and *NF1* (4 patients [14.3%] each), and *DNMT3A* and *TP53* (3 patients [10.7%] each) (Figure 2B; *Online Supplementary Table S2*). We observed six variants of the *PHF6* gene in five patients with *DDX41* germline mutations: p.M1T and p.R116* in one patient, and p.G248D, p.C20F, p.M1T and p.M1V in one patient each. Interestingly, *PHF6* p.M1T/V variants were detected in only three patients harboring *DDX41* germline mutations among the whole study population of 457 patients.

Clinical features and outcomes of the patients with *DDX41* mutations

There was a male predominance among the *DDX41*-mutated patients (96.4% *vs.* 57.1%; *P*<0.001), and the patients with this mutation tended to be older (median 66 *vs.* 57 years; *P*<0.001), and were more likely to have a normal karyotype (75.0% *vs.* 48.7%; *P*=0.007), lower white blood cell count (median 1.8 *vs.* 3.7×10^9/L; *P*=0.047), and lower marrow cellularity (median 30% *vs.* 60%; *P*<0.001) at diagnosis compared with the non-mutated patients (Table 2). Among patients with MDS, the *DDX41* mutations were significantly more frequent in the MDS subtypes with excess blasts (EB)-1 and EB-2, compared to other categories with bone marrow blasts <5%, although the mutation frequencies were not significantly different between patients with lower risk or higher risk according to the IPSS-R (Table 2). Of 23 MDS or AML patients with causal germline *DDX41* mutations, data regarding blood counts before diagnosis were available for 16 patients, and all 16 patients had a history of cytopenia at least 1 year prior to diagnosis.

During the median follow-up of 25.5 months, 116

Table 2. **Comparison of clinical features according to the presence of germline *DDX41* mutations.**

	DDX41 mutations (+) (n=28)	*DDX41* mutations (-) (n=429)	*P*
Sex, n(%)			
Male	27 (96.4)	245 (57.1)	< 0.001[a]
Female	1 (3.6)	184 (42.9)	
Median age (range), years	66 (41-79)	57 (16-89)	< 0.001[c]
Chromosome, n(%)			
Normal	21 (75.0)	209 (48.7)	0.007[a]
Abnormal	7 (25.0)	220 (51.3)	
WBC, $\times 10^9$/L, median (range)	1.8 (1.0-3.3)	3.7 (0.7-313.1)	0.047[c]
Hb, g/dL, median (range)	10.1 (5.2-13.2)	9.1 (2.3-16.4)	0.113[c]
Platelets, $\times 10^9$/L, median (range)	90 (13-174)	68 (3-638)	0.689[c]
BM cellularity, %, median (range)	30 (5-60)	60 (3-100)	< 0.001[c]
BM blasts, %, median (range)	6.2 (0.8-65.2)	5.2 (0-98.8)	0.016[c]
N. of mutated genes, median (range)	3 (2-6)	2 (0-12)	0.036[c]
MDS, n(%)			
MDS with SLD/MLD/del(5q)/U	7 (4.8)	138 (95.2)	0.001a[a*]
MDS with EB-1/EB-2	12 (19.0)	51 (81.0)	
Unknown	0	2 (100)	
Risk stratification, n(%)			
MDS			0.693[a]
IPSS-R ≤ 3.5 (%)	6 (31.6)	69 (36.1)	
IPSS-R > 3.5 (%)	13 (68.4)	122 (63.9)	
AML			0.215[b]
Favorable	0 (0)	63 (37.5)	
Intermediate	2 (50.0)	33 (19.6)	
Adverse	2 (50.0)	71 (42.3)	
Unknown	0 (0)	1 (0.6)	

WBC: white blood cells, Hb: hemoglobin; BM, bone marrow; MDS: myelodysplastic syndrome; SLD: single lineage dysplasia; MLD: multilineage dysplasia; EB: excess blasts; IPSS-R, International Prognostic Scoring System-Revised; AML: acute myeloid leukemia. [a]by the χ^2 test; [b]by the Fisher exact test; [c]by *t*-test; *SLD/MLD/del(5q)/U *vs.* EB-1/EB-2.

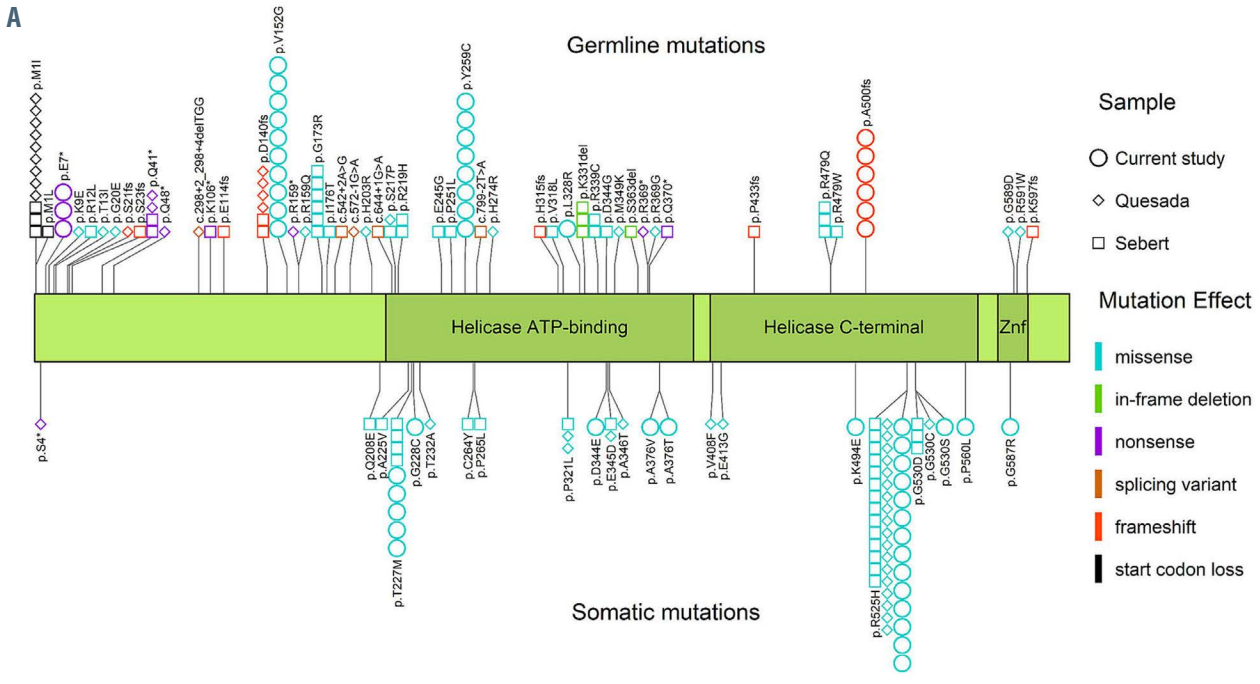
patients (7 ICUS, 55 MDS, and 54 AML) died. The 5-year overall survival rate was 60.8% in the overall population and 84.6%, 62.2%, and 38.9% in patients with ICUS, MDS, and AML, respectively. There was no significant correlation between overall survival and the presence of *DDX41* mutations in each disease category of ICUS, MDS, and AML (Figure 3) as well as in the total study population (*Online Supplementary Figure S1*).

Online Supplementary Table S7 shows the clinical course of each patient with a *DDX41* mutation. Clinical courses could be followed up in four of the five ICUS patients with probable germline *DDX41* mutations, and notably, three of these four patients showed disease progression to MDS EB-1 (n=2; 77.9 and 17.6 months after ICUS diagnosis) or MDS EB-2 with a gain of *PTPN11* mutation (n=1; 9 months after ICUS diagnosis) during the follow-up. Another ICUS patient with a germline *DDX41* mutation had a son with Hodgkin lymphoma.

Discussion

In our cohort of 457 patients with ICUS, MDS, or AML, 6.1% of the patients carried causal germline *DDX41* mutations, which is a higher incidence than those found in previous studies which ranged between 0.8% and 3.9% in patients with myeloid malignancies (mostly MDS and AML).[5,6,10,11,22] In a study comparing the clinical and genetic characteristics of *DDX41* mutations in AML and MDS patients between two ethnically distinct populations, germline *DDX41* mutations were found in 3.9% of a Japanese cohort and in 0.8% of a Caucasian cohort.[22] Therefore, there seems to be an ethnic difference in the incidence of *DDX41* mutations in patients with myeloid neoplasms between Asian and Western patients. In contrast, the clinical features of our *DDX41* -mutated patients, such as male predominance, old age at presentation,[5,6,10,11,23] hypocellular marrow,[3,4,6] leukopenia,[6] and frequent normal cytogenetics[3-6,11] were similar to those reported in other ethnic populations. The *DDX41* mutations did not show significant associations with survival outcomes.

There are several noteworthy findings in our study regarding the genetic characteristics of *DDX41* mutations. First, the germline mutations were mostly N-terminal variants (78.6%), whereas somatic mutations were mostly C-terminal variants (66.7%). This finding is consistent with the observations in two recent studies.[10,11] The N-terminal region of *DDX41* has the helicase ATP binding domain,[24,25] and the structural rearrangement in the N-terminal region may change the conformation of the ATP-binding site and eventually decrease ATP-binding ability.[24] In contrast, the helicase C-terminal domain is involved in ATP hydrolysis.[24,25] Therefore, genetic alter-



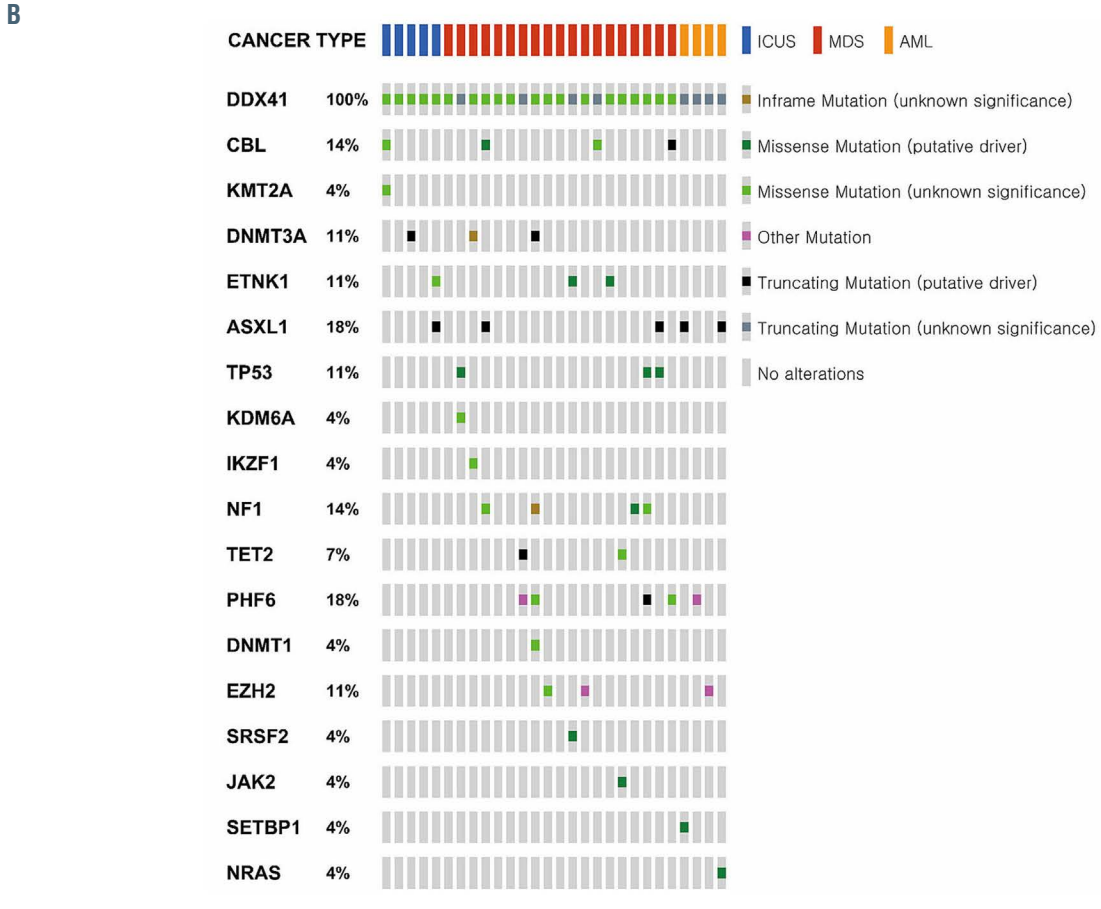


Figure 2. Distribution of *DDX41* mutations and concurrent mutations in other genes. (A) Distribution of *DDX41* mutations detected in the current study and two previous studies (Quesada *et al.*[10] and Sebert *et al.*[11]). This figure shows the differences in positional distribution (N-terminal skewed *vs.* C-terminal skewed) and mutational effects (variable *vs.* missense-dominated) between germline and somatic mutations. The protein structure of *DDX41* was based on the RefSeq accession number of NM_016222.3 and the UniProtKB entry of Q9UJV9: the 622 amino acid long protein comprises the helicase ATP-binding domain (position 212-396), the helicase C-terminal domain (position 407-567), and a zinc finger domain (position 580-597). Different colors indicate different effects of mutations: light blue, missense mutation; light green, inframe indel; purple, nonsense mutation; brown, splicing mutation; red, frameshift mutation; black, start codon loss. Different shapes represent the three studies: square, Sebert *et al.*[11] diamond, Quesada *et al.*[10] circle, current study. (B) Concurrent mutations of other genes identified in bone marrow samples from *DDX41*-mutated patients. The types of genetic alterations and diseases are presented in the legend.

ations in the N-terminal region may cause greater susceptibility to protein hypofunction than those in the C-terminal region. Second, the patterns of germline *DDX41* mutations in our Korean population were distinct from those in Western populations[10,11] or even other Asian populations.[22,23] The germline *DDX41* mutations (p.V152G, p.Y259C, p.A500fs, p.E7*, and p.L328R) in our study are totally different from those reported in Western populations (p.M1I, p.D140fs, p.G173R, and Q41*). Korean and Japanese patients shared three major germline *DDX41* variants (p.Y259C, p.A500fs, p.E7*),22 but p.V152G was only found in Koreans and not in Japanese or other ethnic populations. Third, we observed the exclusive presence of *PHF6* p.M1T/V variants in three patients with probable germline *DDX41* mutations. These variants potentially cause a complete lack of protein production as a consequence of start codon loss and are causative germline mutations of the Börjeson-Forssman-Lehmann syndrome.[26-28] Thus, our findings suggest that the same genetic mutation can induce both hereditary diseases and sporadic cancer, as exemplified by mutations in *ETV6*.[29] The possible association between *PHF6* p.M1T/V variants and germline *DDX41* mutations should be investigated further.

Germline mutations that predispose an individual to MDS or AML may also contribute to the development of ICUS, but the genetic predisposition to ICUS has not been systematically investigated. In a recent study of germline *DDX41* mutations in adult patients with MDS or AML, 45.5% of patients with pathogenic germline *DDX41* mutations had a previous history of cytopenia before the diagnosis of MDS or AML, and the preexisting cytopenia might indicate the presence of ICUS in these patients.[11] We also observed similar findings. Furthermore, five (6.7%) of 75 ICUS patients had causal germline *DDX41* mutations, three of whom progressed to MDS. Our study shows that germline *DDX41* mutations are not uncommon in ICUS patients. Our findings do not indicate that the germline *DDX41* mutations contribute to the progression of ICUS to MDS, but instead do suggest that ICUS patients harboring such variants may be considered as having a hereditary myeloid neoplasm.

Our observations highlight the potential oncogenic role of germline *DDX41* mutations in the pathogenesis of ICUS/MDS in comparison with AML. First, the patients with ICUS/MDS carried germline *DDX41* mutations more frequently than did AML patients and germline missense mutations were highly enriched in ICUS/MDS rather than in AML. These findings might support the notion that less disruptive variants are associated with a milder phenotype in the disease spectrum. Second, only one (3.6%) of the 28 patients with germline *DDX41* mutations carried a mutation in the splicing factor gene. This finding is in line with previous observations that splicing factor gene mutations were largely mutually exclusive with *DDX41* mutations.[5,10] *DDX41* interacts with core splicing proteins such as SF3B, U2 complex, PRPF8 scaffold protein, and U5 complex, indicating that spliceosomal proteins are the top functional group associated with *DDX41*.[5,25] Genetic alterations of the splicing components affect the 3'-splice site recognition during pre-mRNA processing and are involved in the pathogenesis of myelodysplasia.[30] This indicates that mutant *DDX41* can have an oncogenic role in MDS via aberrant mRNA splicing with the assumption that mutations in these splicing factors have an impact on the pathways of downstream oncogenes and tumor suppressor genes.[3]

Donor-derived leukemia has been reported in several families with germline *DDX41* mutations; in all such cases, donors had the same type of germline *DDX41* variants as the respective recipients.[31,32] In our study, *DDX41* mutations (germline p.E7* and somatic p.G228C) were found in a 60-year-old man with high-risk MDS (#12). No

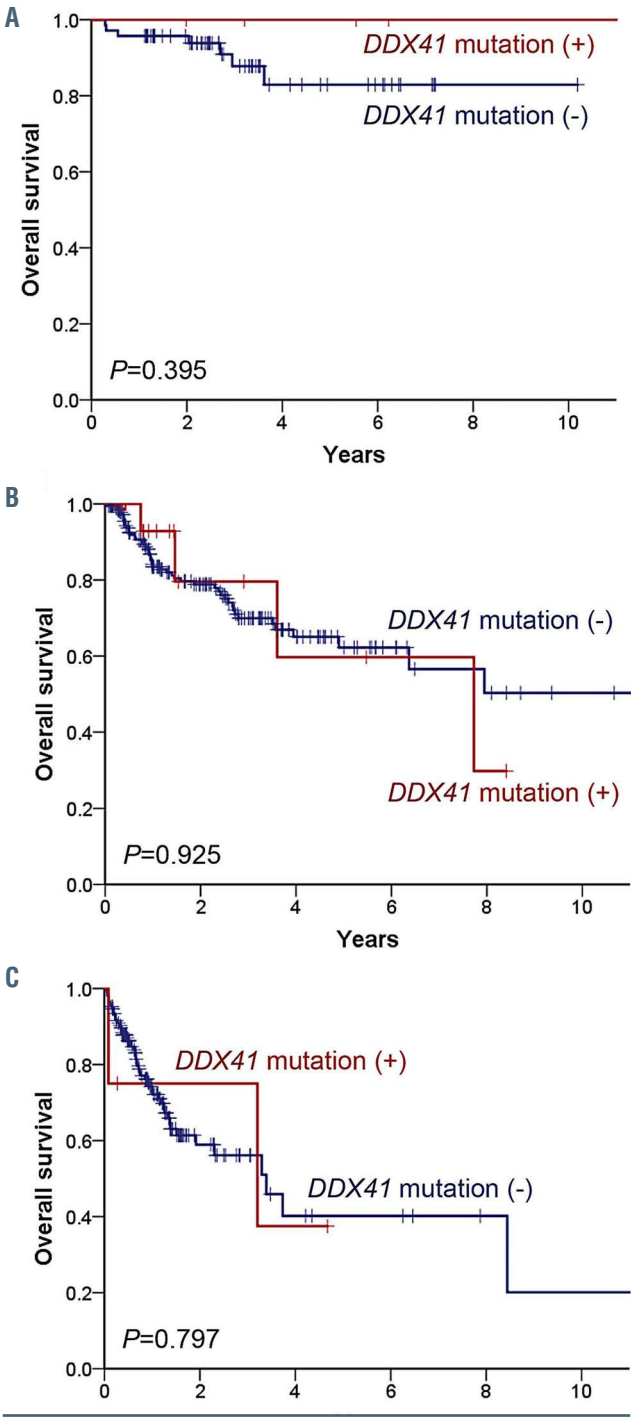


Figure 3. Overall survival of patients with different hematologic disorders according to *DDX41* mutation status. (A-C) Overall survival of patients with idiopathic cytopenia of undetermined significance (A), myelodysplastic syndrome (B) or acute myeloid leukemia (C) according to whether they had *DDX41* mutations (red) or not (blue).

HLA-matched sibling or unrelated donor was available, and his two adult offspring had the same *DDX41* mutation (p.E7*). Fortunately, the patient's HLA-haploidentical brother did not carry the *DDX41* mutation, and the patient could undergo haploidentical hematopoietic stem cell transplantation from him. Considering that the risk of malignancy in *DDX41* carriers is yet to be determined, we calculated the odds ratios of major germline *DDX41* variants detected in our study (*Online Supplementary Table S4*). Nevertheless, an extensive population-based study is needed to obtain more reliable data that may be useful in establishing genetic counseling guidelines for germline *DDX41* variants, which are currently available only for donor selection in allogeneic hematopoietic stem cell transplantation.

NGS-based targeted genotyping for somatic mutations can identify patients who are at risk of hereditary hematopoietic malignancies. In a recent study, of 25 pathogenic or likely pathogenic variants with variant allele frequency >40% in 24 patients with germline tissues available, six variants (24%) were of germline origin – three *DDX41* variants, two *GATA2* variants, and one *TP53* variant; *DDX41* had a 100% diagnostic yield for pathogenic germline variants in that study.[33] In another study, targeted NGS showed that 17 patients had putative germline *DDX41* variants with a variant allele frequency >40%, all of which were of germline origin.[11] We were also able to confirm germline origin in all of the 11 patients with probable germline *DDX41* mutations. In cases in which germline samples are not available, NGS-based leukemia panels seem to predict germline *DDX41* variants with high probability. However, it is worth mentioning that NGS-based panels may fail to detect deletions or gene rearrangements that are responsible for the predisposition syndrome.

Our study has some limitations. The number of patients included in the study was relatively small, and this might have had an impact on the analysis for clinical associations of *DDX41* mutations with clinical outcomes. Family history was not systematically collected in this study, although such information is helpful in pinpointing the pathogenicity of sequence variants. We did not perform functional studies to demonstrate that sequence variants detected in this study had a deleterious effect *in vivo*. Experimental data can be useful to support pathogenicity, particularly for missense variants of uncertain significance. We acknowledge that these limitations may hamper the precise variant classification based on the ACMG guideline. However, the concurrence of germline and somatic *DDX41* mutations was a recurrent finding across recent studies.[5,6,10,11] This also has provided another illustration that germline alterations predispose to the acquisition of somatic mutations in the same genes which act as a second hit being associated with cancer development as demonstrated by *JAK2, CEBPA,* and *RUNX1* mutations.[34,35] Therefore, in cases harboring a germline *DDX41* mutation, the acquisition of a somatic *DDX41* mutation should be considered as strong evidence for causality. Lastly, our data may not reflect the whole Korean population, although study patients included in this study come from all across the Korean peninsula.

In conclusion, our results delineate the unique ethnic features of *DDX41* mutations in Korean patients, such as higher incidence and different patterns, compared with patients from Western countries or other Asian countries. Specifically, the most common germline mutation in our cohort was p.V152G, which was not found in previous studies in other ethnicities. Our results suggest that ICUS harboring germline *DDX41* mutations may be regarded as a hereditary myeloid neoplasm. Germline *DDX41* mutations may be predicted with a high probability by using clinical NGS-based leukemia panels based on variant allele frequency levels and public databases. Germline *DDX41* mutations are not uncommon and should be explored when treating patients with myeloid malignancies.

Disclosures

No conflicts of interest to disclose.

Contributions

E-JC and Y-UC analyzed and interpreted the data; E-JC, Y-UC and J-HL contributed to the manuscript; E-HH performed experiments. All authors provided patients' data, reviewed, and approved the final manuscript.

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Data-sharing statement

For original data, please contact imeunjeee@gmail.com.

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