博士論文

論文題目　**Investigation of a causal gene of familial myelodysplastic syndromes**

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Investigation of a causal gene of familial myelodysplastic syndromes

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Summary

Although several causal genes of familial myelodysplastic syndromes/acute myeloid leukemia (MDS/AML) have been discovered, the comprehensive mutational spectrum and the molecular pathogenesis have been poorly understood. To explore its epidemiology and novel driver genes of familial MDS/AML, I performed a Japanese nationwide survey of familial MDS/AML, and whole exome sequencing analysis with a pedigree including four MDS patients. As a result, the characteristics of Japanese familial MDS/AML pedigrees were elucidated, and twelve candidate causal genes were identified. Although I analyzed the candidate mutation status by Sanger sequencing in other familial MDS/AML pedigrees identified by the nationwide survey, no recurrent mutations was observed. Of the candidate genes, I selected *HLTF* for further functional analysis because HLTF is a chromatin remodeler, promotes error-free postreplication DNA repair, and regulates the expressions of cohesin complex-associated genes that frequently mutated in sporadic MDS/AML. Subsequently, I evaluated *HLTF* genomic mutation status in sporadic MDS samples by Sanger sequence. Importantly, a novel *HLTF* T50A mutation was identified in one out of 40 sporadic MDS samples (1/40=2.5%). I evaluated in vitro colony forming capacity using *Hltf*-knocked down 5-FU-primed murine bone marrow cells, revealing that *Hltf* silencing enhanced in vitro

replating capacity. I also found c-kit, a hematopoietic stem/ progenitor cell marker, positive cells were increased in *Hltf* shRNA-expressing colonies after the first round of colony-forming cell assay. Because HLTF has ATPase activity, and the identified mutation located near ATP-binding site of HLTF, I analyzed ATPase activity of *HLTF* wild-type and *HLTF* E259K mutant. However, no significant difference was observed. Taken together, this study could be an important first step for the understanding of familial MDS/AML.

Introduction

The myelodysplastic syndromes (MDS) are a group of clonal disorders of hematopoietic stem cells, and are defined by ineffective hematopoiesis and/or bone marrow dysplasia (1-3). More than 30,000 new cases of MDS occur in the United States every year, and approximately 30% of MDS patients eventually progress to acute myeloid leukemia (AML) (4, 5). The median overall survival of MDS patients with International Prognostic Scoring System high is around 0.4 years (6), thus, it is still challenging for physicians to cure severe MDS cases (7, 8). Whereas these novel drugs can improve clinical symptoms and quality of life of MDS patients, they have not been considered as curative options. Although allogeneic hematopoietic stem cell transplantation (HSCT) is still the only intervention to cure MDS (9, 10), very limited MDS patients are considered an indication for HSCT due to the elderly (10). Moreover, a report revealed that five-year overall survival after allogeneic HSCT was 28% in the patients with refractory anemia with excess blasts-2 (RAEB-2) (6, 11).

MDS is mostly a sporadic disease, however, familial cases have been also discovered recently. Holme et al. reported the largest cohort of 27 families with familial MDS/AML from the United Kingdom (1). Since many of familial MDS/AML cases have been recently defined, the guidelines for genetic analysis and clinical treatment are currently

based on expert opinions (12, 13). In clinical settings, clinicians are required to take a detailed family history and consult with a certified genetic counselor when necessary (14). The curative treatment for familial MDS/AML is allogeneic hematopoietic stem cell transplantation only, same as sporadic MDS (15). In this setting, using a related donor might be a problem (15).

Previous targeted sequencing research and recent comprehensive genomic mutational analysis uncovered the landscape of somatic mutations in sporadic MDS. Especially, Haferlach et al. analyzed more than 900 MDS samples by targeted sequence and revealed that approximately 90% of the MDS patients harbored at least one mutation, and that 47 genes, such as *TET2*, *SF3B1*, *ASXL1*, *SRSF2*, were frequently mutated (16). Mutated genes in MDS can be classified into epigenetic modifiers (eg, ASXL1, TET2, DNMT3A, IDH1/2, EZH2) (17-20), regulators of alternative mRNA splicing (eg, SRSF2, ZRSF2, U2AF1, SF3B1) (16), transcription factors (eg, RUNX1, TP53, GATA2) (21, 22), cytokine signaling factors (eg, NRAS, CBL) (23, 24), and cohesin complex (eg, SMC3, RAD21) (25-27). Cohesin is a protein complex which is made of four major subunits, SMC1, SMC3, RAD21, and STAG, and is related with the cohesion of sister chromatids, transcriptional control, and post-replicative DNA repair (26, 28). Recently, recurrent mutations in the cohesion-associated genes were found in a

variety of myeloid neoplasms including MDS (26). Although pathogenesis of cohesin complex in MDS was not fully understood, Smc3 was revealed to work in a dose dependent manner in malignant hematopoiesis (27). *Smc3* hetero knock-out mice showed an increase of absolute number of LSK (Lin^-, $Sca\text{-}1^+$, $c\text{-}Kit^+$) cells, especially in short-term hematopoietic stem cells (27).

As described, the genomic mutational spectrum in sporadic MDS is gradually uncovered. On the other hand, little is known about not only the causal genes but also the epidemiology of familial MDS/AML due to its rarity (1, 29). To date, few limited case reports showed the causal germline gene alterations including *GATA2* (2), *RUNX1* (29), *CEBPA* (30), *TERC* (14, 31), *TERT* (14, 32), *DIDO1* (13), *DDX41* (33), *ETV6* (34), *SRP72* (35), some of them were overlapped with sporadic MDS.

To elucidate the full picture including the epidemiology, the genomic mutational landscape, and the molecular pathogenesis, I conducted a nationwide survey and a next-generation sequencing analysis using samples obtained from familial MDS/AML patients. In this study, I identified twelve candidate genes of familial MDS/AML. Among these genes, I found a novel *HLTF* T50A mutation in a sporadic MDS sample, and loss-of-*Hltf* enhanced in vitro colony replating capacity.

Materials and methods

Subjects and ethical issues

Research on human samples was conducted in accordance with The Ethics Guidelines for Human Genome/Gene Analysis Research enforced on March 29, 2001.

This study was approved by the ethical committee of the University of Tokyo (Number; 10662, G10053), and the ethical approval was also obtained from each participating institutions or hospitals (Amagasaki Hospital, Fuchu Hospital, Hyogo Prefectural Kobe Children's Hospital, Juntendo University, Keiju Medical Center, Kochi University, Nagaoka Red Cross Hospital, National Defense Medical College, NTT Medical Center Tokyo, Oita University, and PL hospital). A written informed consent was collected from all the patients whose samples were studied. All animal experiments were approved by the University of Tokyo Ethics Committee for Animal Experiments.

Sanger sequencing

The experiment was performed as described previously (36). In using genomic DNA of the patient samples as template, each targeted region was amplified by PCR according to the specific primers as listed in Table 1. The primers were designed using Primer3 (37, 38). The PCR products were purified by illustra ExoStar (GE Healthcare). When it

was difficult to extract the sequence data, the PCR products were subcloned into EcoRV site of pBluescript II KS(-) (Stratagene). The ligated plasmids were transformed into E. coli strain of XL1-Blue or DH5alpha by heat shock for 45 seconds at 42 degrees Celsius. The positive transformants were incubated on LB plates at 37 degrees Celsius containing 100 microgram/mL ampicillin with X-gal (Sigma-Aldrich) and isopropyl beta-D-1-thiogalactopyranoside (Sigma-Aldrich). For colony PCR, a white colony was chosen, followed by adding to a PCR mixture as DNA template. The insert region was amplified by PCR using T3 and T7 universal primers, and was purified by illustra ExoStar (GE Healthcare Life Sciences). Sanger sequencing analysis was performed with T3 and T7 primers, and BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems). ABI Prism 310 Genetic Analyzer (Life Technologies) was utilized.

Table 1. The list of primers for direct sequence.

Gene	Exon	Forward	Reverse
ANKRD26	(5'UTR)	CATGGAGCACACTTGACCAC	TACTCCAGTGGCACTCAGTC
ASXL1	12	AGGTCAGATCACCCAGTCAGTT	TAGCCCATCTGTGAGTCCAACTGT
	12	AGAGGACCTGCCTTCTCTGAGAAA	TTCGATGGGATGGGTATCCAATGC
	12	ACTTGAAAACCAAGGCTCTCGT	GCAACCATCCCATCTGTCCTTGTA
	12	GGTGGACAAGGATGAGAAACCCAA	TGTCCTGTGACATAGCACGGACTT
	12	TGGATTCCAAAGAGCAGTTCTCTTC	CATGACAAAGGGCATCCCTTCCAA
	12	ACAGGAAAGCTACTGGGCATAGTC	CAAGAGTGCTCCTGCCTAAAGAGT
CBL	7	AGCAAGCACTGGCAAATTGG	GTGGAGCCCATCTCACAGTATAATTC
	8	AACCATATCACTGGACACAAGC	CCCTGACCTTCTGATTCCTGC
	9	AGGTACGGATCTAAACAGCGAC	CCTCTTTGTGCCTCAGTTTCTTCATC
CEBPA	1	GCCGGGAGAACTCTAACTCC	GCTTGGCTTCATCCTCCTC
	1	CCAAGAAGTCGGTGGACAAG	CATTTCCAAGGCACAAGGTT
DIDO1	10	AGAGTATTGCTTTCGGCTTTGTGTG	CACCATGTGGGTGCTGTCTG
	10	AGAACGTATTTCCCTGGGCCTC	ACAATAAGGCACCCTACAACTGG
DNMT3A	23	TCCTGCTGTGTGGTTAGACG	TTTTTCTCTTCTGGGTGCTGA
ETV6	3	AAGGGCTCTTGAGATGTGGA	CATCGTCATCCCTTCCTTGT
	5	TGTCTTTCCCTCTGCTCCAC	CTTCCCGATGAGAGAGGTTG
	6	ACAGGACCTCCCTCCATCTT	TGCAACTGCCTAATTGCTTG
EZH2	6	GCTTCCTTTGCCTAACACCA	AAGCAATCTGCCCACCTTAG
	7	TTCTGCTTCCCAGTGCTCTT	GGCTCATCCGCTACATTGAT
	12	CCCAAGAGGGAATTGAATGA	ACCAACAACAGCCCTTAGGA
	13	TCTTGGCTTTAACGCATTCC	TTCCAGTCAGCCTCCACTTT
	16	AGAGCACCTTGCTGAACGAT	AGCATGCAAATCCACAAACA
	17	TTGCGTTTTCTCCAGAAGGT	CACAAGAGGTGAGGTGAGCA
	18	AGGCAAACCCTGAAGAACTG	CCACTAATGCTCATGGCAAA
GATA1	5	GCCAGGGAGTGTGTGAACTG	GTCTTACCAGGCGCTTCTTG
GATA2	1	ACCTCGTGGTGGGACTTTGG	CATCCGGGAAGCAAGCAGAC
	2	TGCTGGTTCTGGGAGTCGTG	GGTACTTGACGCCGTCCTTG
	2	CACGCCACCCAAAGAAGTGTC	GGAAACCAACACTGCCACCTC
	3	ACTCCCTCCCGAGAACTTGC	TTCAAGCGGCAAAGCGTCTG
	4	AGCCCTCCTTGACTGAGCTG	ACCTCCTGAGCAGAGGCAAG
	5	TCAGCTTGACCTGCCTCTGG	GTGTCGGCCTTCGGGAAATG
GFI1B	4	GCGGGATACCGTGAAGATTA	TGCATCTCTCAGGGAAAGG
	5	TGGCCATGAGAGAAAACACA	GACTGGAGATTTGGGCATGT
GP1BA	2	CCCCTGGTTATGCAACTGTG	TGGATGCAAGGAGGAGGGCAT
GP9	1	AAGGCTGAGACCCGAGAAGG	GCTTCTGGTGGTTTGGGCTG
HOXA11	2	CAGCCCTTCTCCTCAGCTATGG	TGCATCCCTCTCTTGCACAC
IDH1	4	GTGGCACGGTCTTCAGAGA	TTCATACCTTGCTTAATGGGTGT
IDH2	4	TGAAAGATGGCGGCTGCAGT	GGGGTGAAGACCATTTTGAA
JAK2	14	GTCATGCTGAAAGTAGGAGAAAG	CTGAATAGTCCTACAGTGTTTTCAGTTTCA
KRAS	2	AAAGGTACTGGTGGAGTATTTGA	CATGAAAATGGTCAGAGAAACC
	3	CAGACTGTGTTTCTCCCTTC	TAAACCCACCTATAATGGTG
MASTL	4	TTGAACTGGCTTGAGGTTGA	CCACGTTAGCTAGGCTGGTC
MPL	1	GGAGGATGGGCTAAGGCAG	TCTTCCTGGGGCATAGGTGA
	2	CCCTTCCACATAAACATGCCT	GCAGGAAAGCTGCTGGAGT
	4	TCCAGAGGCTGAGCCATAGAC	GGTCTGGAATCCCCAAAGT
	5	GGTTGGAGGCTCTCTCAGCT	CTTTTATCTCCTCCCCATCTCC
	6	CCTATACAGTAGGGGCACACG	TGTGGCTCACTCCCATGACA
	7	GATGGGAAGCCTTGGGATTAG	GGGAACTATGTGGAAGAAT
	8	CCTTGTGCACAGAAGGACTTA	CCCCTGCGTAGTGAGGTCTG
	9	CGAAGCCCCGACGCCGGGCCA	CAGGCGCTGTGCGGCTTTGG
	10	AGGGGCGGGGCCAGAGTA	AGAGGTGACGTGCAGGAA
	11	CTGCCAATCCACTGCCATG	AGTACCAGGCAGGGTTGGTG
	12	TCCCACAGGATCTGCTTTAAT	GAGTTTAGCTCTGTCCAGGGAAC

Table 1(tbc). The list of primers for direct sequence.

Gene	Exon	Forward	Reverse
MYH9	1	GTGATCTTGTGTGGCTGACG	CTTCTCAACCAGAGAGCCAG
	16	TTGCCCTGTCAGGTTCATAG	CCTCTGGGACTCACTGCAC
	24	ATGGCACTGAGGGCTATGTG	TGCTCACAGCTCACTAGTGC
	25	TGTCCTGCAAACTCTGCTCC	GTCCATGTCTCCAAGCCAAG
	26	GGGCTATGGGATAGATGGCTAGG	GCAGGACTGGTTTGGATTCTGTG
	30	ATAACTGGGCAGATCCCTGG	TTGCTTTGGACTCAGTGCTTG
	38-39	TCCTGGTTAGGGCTTGTTGG	TGGTGACATTCGTGCCTTGC
	40	GATGTGTGGGCTGTGCTGTG	AGGCTGTGGTGTCTGTCTGTC
MYL9	1	CTTGAATGCCAGGCCGAAGTG	CGTCTCACTACACAGGAGGTGC
	2	TCTCACACGGAGCGGTGAAG	TGGGAGATGGGTGATGTCTGTTC
	3	TGCCACGTCCTCATTCCTCAG	TGGGAACTGGGACCCTAACC
NRAS	2	GGCCGATATTAATCCGGTGT	TGGGTAAAGATGATCCGACA
	3	CAAGTGGTTATAGATGGTGAAAC	CAAATGACTTGCTATTATTGATG
PU.1	1	TCACCCAGGGCTCCTGTAGCTCA	TCGTGGGCAGGCAGGCAGGCGTCC
	2	ACTGAGCCAGGGAAGGTGAT	CTCTCTCCAGACCCCAGGA
	3	ACTATAACCTTTTCCTGCCCTGCC	AGCCTGTGTCAGCTTCCTGTGAAG
	4	GCTGTAAGCTGGCCCTTC	GGCTGCTGGGTCAGTTGG
	5	CCGGGCCCCTGTGCGTACGCAAGG	CCGGGAGCGTCCTCCCTGTGTCCG
RUNX1	3	CCCTGAACGTGTATGTTGGTCTC	AAGCTGAGACGAGTGCCTCC
	4	ATCATTGCTATTCCTCTGCAACC	ACGTTGCATGTTCCAAATCAGTC
	5	GTAACTTGTGCTGAAGGGCTGG	AGGTTGAACCCAAGGAATCTGAGAC
	6	GGCATATCTCTAGCGAGTCTATGTTGG	CAGTTGGTCTGGGAAGGTGTG
	7	ATTAAACCCTGGTACATAGGCCAC	ATGTTCTGCCAACTCCTTCATGC
	8	TCCGCAACCTCCTACTCACTTC	GCGCCGTAGTACAGGTGGTAG
	8	ACGCGCTACCACACCTACCTG	CTGACCTACAGCGAGATCCTGG
SF3B1	13-14	TGATGTGAAAGTGTAGCTTC	GGCAACATAGTAAGACCCTGT
	15-16	TGTTGGGGCATAGTTAAAACCT	TGTTAGAACCATGAAACATATCCA
SRP72	6	GAAATGGGATTTACCCAGCA	AGGAGCAGACCAGTCCTCAA
	8	CCCTAGGCAGTTCTTTGGTT	TGAAACAAAGCTGCAAATGTC
SRSF2	1	GGCCGCCACTCAGAGCTA	ACCTCACAAAGGTCCGCG
STAG2	5	GGACACCACAAAGAGGCTGT	CATCCCAAGAGTTTTCTGATGA
	6	TTTCCACATTCTTTTCAATGC	GAAGTGACTATTTGAGAGCTGCTG
	7	TATGGGGCTGTTTTCTTGTG	GCCCAGCCTAATGCTTACAA
	8	TGCATTCTAAATGAAATTGCTG	GGGGAGTGTCCTCTTAGTGGT
	9	GCAGCTGCATCTTTCTTTTG	TGGCACGGATATTCATCAGA
	10	CCCCAAAATACTGGGGAAT	TCCCTACACCACGAAATATGC
	11	AGGCCCATGCTTCATTTCTA	ATAAAGGGGAGGCTTCCAGA
	13	TTTGCAAACACTTTTCTCTTGC	AGCTGTAAACCTCCATGACG
	14	GGACGTTACTAAAAGCACCTGTTAC	CCCAGCCTACATTTCCCTTTA
	18	CACTTAACAGTGCTAATGGGCTTA	TCTGTGAGGCATTTAGGGAAA
	19	TTCCCTAAATGCCTCACAGAA	AAGCATCATTACCGCCATTC
	20	TTTCCATGGTGGTATGGTCA	ACTGCAGTAGAGGGGCTCAA
	21	TGACAAAGTTCATTTGTGGGTTT	CCCCACAACGACAACAAC
	22	TGTTAACAGTCAAGTCCAAAACAA	TGCAGTGCGTGAATAACAATC
	23	AAATGGAGACATGCCTGAGC	TGTGTGAGTTTGCTGAAAACAG
	25	GGCAGTTAGTGAGAAACCTTGG	GAAGCAGCGATCTGACTTGA
	27	TGTGACGTGTTTACATGACTAACC	GCCCAATTTCAACTGCTACC
	28	CTGCACTTTTGGTCATTTGC	TTCCAAATGAAAGGGCTAGA
	29	GCTTGGCAAAGGAAGTAGTGA	TGCCCTTAAGAATCCCAAAA
	30	TATGCCTATGCTCGCACAAC	GAACCTTAATGACAATTCAGTTGGT

Table 1(tbc). The list of primers for direct sequence.

Gene	Exon	Forward	Reverse
TERC	1	GGCCGGAAATGGAACTTTA	GCTGACAGAGCCCAACTCTT
	1	GCCTTCCACCGTTCATTCTA	TTCACGTCTCCTGCCAATTT
TERT	11	AGGAAGGCAGGAGGCTCTTTG	CAGTCACCATCAGCCTTGCAG
TET2	3	TGAACTTCCCACATTAGCTGGT	GAAACTGTAGCACCATTAGGCATT
	3	CAAAAGGCTAATGGAGAAAGACGTA	GCAGAAAAGGAATCCTTAGTGAACA
	3	GCCAGTAAACTAGCTGCAATGCTAA	TGCCTCATTACGTTTTAGATGGG
	3	GACCAATGTCAGAACACCTCAA	TTGATTTTGAATACTGATTTTCACCA
	3	TTGCAACATAAGCCTCATAAACAG	ATTGGCCTGTGCATCTGACTAT
	3	GCAACTTGCTCAGCAAAGGTACT	TGCTGCCAGACTCAAGATTTAAA
	11	GCTCTTATCTTTGCTTAATGGGTGT	TGTACATTTGGTCTAATGGTACAACTG
	11	AATGGAAACCTATCAGTGGACAAC	TATATATCTGTTGTAAGGCCCTGTGA
	11	TCTAAGCTCAGTCTACCACCCATCCATACA	TGCTCGCTGTCTGACCAGACCTCATCG
TP53	2-3	TCTCATGCTGGATCCCCACT	AGTCAGAGGACCAGGTCCTC
	4	TGCTCTTTTCACCCATCTAC	ATACGGCCAGGCATTGAAGT
	4	TGAGGACCTGGTCCTCTGAC	AGAGGAATCCCAAAGTTCCA
	5	TTCAACTCTGTCTCCTTCCT	CAGCCCTGTCGTCTCTCCAG
	6	GCCTCTGATTCCTCACTGAT	TTAACCCCTCCTCCCAGAGA
	5-6	TGTTCACTTGTGCCCTGACT	TTAACCCCTCCTCCCAGAGA
	7	CTTGCCACAGGTCTCCCCAA	AGGGGTCAGAGGCAAGCAGA
	7	AGGCGCACTGGCCTCATCTT	TGTGCAGGGTGGCAAGTGGC
	8	TTCCTTACTGCCTCTTGCTT	AGGCATAACTGCACCCTTGG
	8-9	TTGGGAGTAGATGGAGCCT	AGTGTTAGACTGGAAACTTT
	9	GACAAGAAGCGGTGGAG	CGGCATTTTGAGTGTTAGAC
	10	CAATTGTAACTTGAACCATC	GGATGAGAATGGAATCCTAT
	11	AGACCCTCTCACTCATGTGA	TGACGCACACCTATTGCAAG
U2AF35	2	GCTGCTGACATATTCCATGTG	TCTCAGACCTTCCACTGGAAGT
	6	AAAGTCTTATTAAAGCGTGGATGG	CGAACTGTGCTCAGTCACGTC
UTX	10	TTGGTTTGTTTTCTGCTTCG	TTGTAATTCAAATCTCTTAGCTGGAA
	13	GGTTTATATTCCGGTTACCCTGT	CCCCAAAATCTCTTCCCATA
	17	TTGATAACTTTAGGACTTGGGTCA	ACAAGGCAGAGAGCTGAGGA
	17	ATCCTCAGCTCTCTGCCTTG	CGGTCCAAATTTCAGCATTC
	18	GGATCCACATCCCACATCTC	TGTTTCCTAAAGGGCATCCA
	25	TTGTGACATTTTCTTCCAGTCTTAC	CGAATTACAATTCTATGCAAGGAG
ZRSR2	1	GGCTTTCCGTTTCAAGTCC	CTCCCACTCCCAGACAGTTC
	2	TCCTCAGCACCCGAACTATT	GGCGATCATTCACCAAGACT
	3	TCCTGAATTTTTGACCAAGGA	GACTGGTACTGGTTAGTAAAGGTTGA
	4	TGTGTCATTTTGCTCTCGTG	CTCACTCCAACCTCCCAAGA
	5	TGTGCGCTGTATGTGAAATG	GACCCGAAGAAGAGCATCAG
	6	TCAAAAGATCTGTGATTCAAAAGAA	TCTAAACAGGTCCAGTCCACAG
	7	ACCAGGAGCCAAGAGAGACA	CTCTCCCAAAAGGGGAACTC
	8	CCACCATGCCTGGTCTAAAG	TGTGTCCCAGCTCTCTTGTG
	9	GGGAATGTTAGCCTGGACAA	AGCGAAACTCCGTCTCAAAA
	10	CGGGGTTAATTAATAGTAGAGCTAATC	GAATATCCCTTTATATAGCAGTGGAAC
	11	GAAATGTACCTTCGGAAAAGGA	GCGGTCCCTATTTCTTCCTC
	11	TCCAGACCACTCCTACAAAAGA	TTCCAGGCTACACAGGGTTC

UTR: untranslated region

Whole exome sequencing

Genomic DNA was extracted from buccal mucosae (Bu) and peripheral blood (PB) using the QIAamp DNA Mini kit (Qiagen). After purified with AMPure XP Kit (Beckman Coulter), 560ng, 260ng, 1000ng, 330ng, 260ng, and 300ng genomic DNA in patient(Pt)1PB, Pt1Bu, Pt2PB, Pt2Bu, subject(Sb)3Bu, and Sb4Bu were fragmented to approximately 200 base pair by Covaris LE220 (Covaris). After end repairing by NEBNext End Prep (NEW ENGLAND BioLabs), adaptors were ligated. Exon capture was performed using SureSelect^{XT} Target Enrichment System Kit (Agilent Technologies) according to the manufacturer's instructions. SureSelect^{XT} Capture Library (Agilent Technologies) was version 5. The captured library was purified by AMPure XP Kit (Beckman Coulter), and the index was attached to each sample. The bridge amplification for cluster generation on Flow Cell was performed by cBot (illumina) according to the manufacturer's protocol. The pair-end sequence was performed by Hiseq 2500 (illumina), and each pair was sequenced by 104 cycles. Short read sequences were aligned to hg19, and duplicate reads were removed. SNPs from 1000 genome project (39), date base SNP 138 and 139 (40), Human Genetic Variation Database (41), Exome variant server (42), and in-house SNPs were applied to remove reported SNPs from the study.

Japanese nationwide survey of familial myelodysplastic syndromes

A questionnaire sheet was sent to 561 institutions or hospitals certified by Japanese Society of Hematology to investigate pedigrees of familial MDS/AML. The questionnaire sheet asked the number of familial MDS/AML pedigrees they had treated, and asked whether they could participate in the study (Appendix 1). Familial MDS/AML in this study was defined as the pedigree including two or more MDS/AML patients. The second questionnaire sheets were sent to 241 institutions which returned the first questionnaire sheets to obtain more detailed characteristics of the pedigrees. The following data were collected from the written questionnaires: date on diagnosis, past medical history, social history, FAB (French-American-British) and WHO (World Health Organization) classification of MDS, results of bone marrow analysis and chromosome analysis, score of International Prognostic Scoring System, clinical features, the ECOG Performance Status, treatment, and clinical outcome (Appendix 2). The clinical samples (buccal mucosae and/or peripheral blood) were obtained from four institutions.

Retrovirus production

The procedures were performed as described previously (43, 44). Briefly, Platinum-E

(Plat-E) or Platinum-A (Plat-A) packaging cells were transiently transfected with retroviral constructs using polyethylenimine. The supernatant containing retrovirus was collected 48 hours after transfection and used for infection.

Plasmids

Complementary DNA (cDNA) of *HLTF* wild-type with Flag-tag was a generous gift from Professor Lajos Haracska. Plasmids encoding proteins of human wild-type Flag-tagged *HLTF* and its mutant cDNA were cloned into the bgl II/bgl II site of MSCV-neo by a PCR method. The mutant was created by KOD Plus Mutagenesis Kit (TOYOBO) according to the manufacturer's instruction. Its sequence was verified by Sanger sequencing.

shRNA interference

The procedure was performed as described previously (45). Gene-specific short hairpin RNAs (shRNAs) were constructed using RNAi designer (Clontech) (46), and cloned into pSIREN-RetroQ-ZsGreen vectors. The target sequences for shRNA were listed in Table2. A scramble shRNA was a nonfunctional construct obtained from Clontech. 5-FU primed mouse bone marrow cells were transduced with each vector and sorted

with flow cytometry (BD FACSAriaIII, BD Biosciences). A fluorescein isothiocyanate filter was used on the basis of the difference in fluorescence intensity.

Table 2. The list of target sequences for shRNA.

Gene	Sequence
mouse *Hltf*	GATTTATCAGTCTGTTAAA

In vitro colony forming assay

The experiments were performed as described previously (43, 47). One thousand sorted cells were plated for the first round, and 10,000 cells were plated from the second round into cytokine-supplemented methylcellulose medium (MethoCult M3434; STEMCELL Technologies). The colony numbers in each dish were counted every seven days.

In vitro translation of protein

The experiments were performed as described previously (48-50). PurefrexSS (GeneFrontier Corporation) was used according to the manufacturer's instructions. Template DNA was created by PCR amplification of the genes of interest. MSCV-neo-HLTF-WT, -E259K were used as the template for HLTF production. T7 promoter and ribosome binding site were attached to upstream of template DNA, and they were amplified by PCR. After purified by FastGene Gel/PCR Extraction Kit

(NIPPON Genetics), template DNA was mixed with PurefrexSS solutions as the protocols instructed, followed by incubation at 37 degrees Celsius for four hours. Changing buffer was performed using Micro Bio-Spin™ P-6 Gel Columns (BIO-RAD), and ATPase activity was evaluated.

ATPase activity analysis

The analysis of ATPase activity was performed by the QuantiChrom ATPase/GTPase Kit (BioAssay Systems) according to the manufacturer's protocols. Briefly, the malachite green reagent formed a stable dark green with phosphate, and was measured by a plate reader. After calculation of the difference of OD values between reaction and control wells, the enzyme activity was computed using the concentration of free phosphate produced from standard curve. A clear bottom 384-well plate was used. After 30 minutes reaction, the absorbance set at 620nm was measured by an absorption spectrometer (ARVO MX/Light, PerkinElmer) for 0.1 second. The formula (below) was applied for calculation of enzyme activity (EA).

EA = [phosphate concentration]*4/30 (U/L), where 1 unit of activity is the amount of enzyme that catalyzes the production of 1 micro mole of free phosphate per minute.

Quantitative real-time PCR

The experiments were performed as described previously (45). Briefly, messenger RNA was extracted by NucleoSpin RNA II kit (Clontech) and subsequently, complementary DNA was synthesized by ReverTra Ace qPCR RT Master Mix (TOYOBO) according to the manufacturer's instructions. Real-time PCR was performed by THUNDERBIRD SYBR qPCR mix (TOYOBO). The primers were designed by Universal ProbeLibrary Assay Design Center (Roche) (51). LightCycler 480 Instrument II (Roche) was operated. The results were controlled with the value of 18s ribosomal RNA. The primers for quantitative real-time PCR were listed in Table 3.

Table 3. The list of primers sequences for quantitative real-time PCR.

Gene	Forward	Reverse
mouse *Hltf*	CGCAGCTTCTCGAGTGTTCT	CCGGTCAAAGCACTGATCTT
18S	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG

Statistical analysis

Statistical significance of difference among groups was determined with a two-tailed unpaired t test. The differences were considered statistically significant when a p-value < 0.05.

Results

A pedigree with four MDS patients

A pedigree with four MDS family members was identified from our previous study (36) (Figure 1). The patient 1 was an 89 year-old female, firstly diagnosed as MDS with normal karyotype at 78 years old. Red blood cell transfusion against refractory anemia was started when she was 87. The patient 2 was a 68 year-old male, diagnosed as refractory cytopenia with multilineage dysplasia accompanied by normal karyotype at 63 years old. In order to investigate a causal gene of this pedigree, Sanger sequence was performed using the peripheral blood mononuclear cells obtained from patient 2. Although thirty-six genes previously reported as sporadic or familial MDS-related genes, such as splicing factors, epigenetic regulators, transcription factors, and kinase signaling factors (Table 4), were analyzed, no mutation were observed in their hot-spots and the coding regions. Therefore, it was hypothesized that this pedigree might have an unknown causal gene mutation of familial MDS.

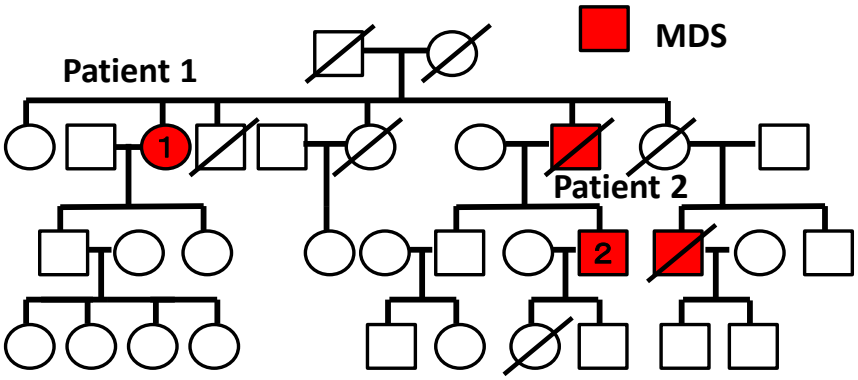


Figure 1. The index pedigree with four MDS patients (Pedigree 1).

Table 4. The list of analyzed 36 MDS-related genes.

ANKRD26	*ETV6*	*GP9*	*MASTL*	*RUNX1*	*TERT*
ASXL1	*EZH2*	*HOXA11*	*MPL*	*SF3B1*	*TET2*
CBL	*GATA1*	*IDH1*	*MYH9*	*SRP72*	*TP53*
CEBPA	*GATA2*	*IDH2*	*MYL9*	*SSF2*	*U2AF35*
DIDO-1	*GFI1b*	*JAK2*	*NRAS*	*STAG2*	*UTX*
DNMT3A	*GP1Ba*	*KRAS*	*PU.1*	*TERC*	*ZRSR2*

Whole exome sequence of the index pedigree

In order to identify the patient specific germ-line mutations of this pedigree, whole exome sequence was performed using the samples obtained from two patients and two non-MDS healthy family members as control (Figure 2A).

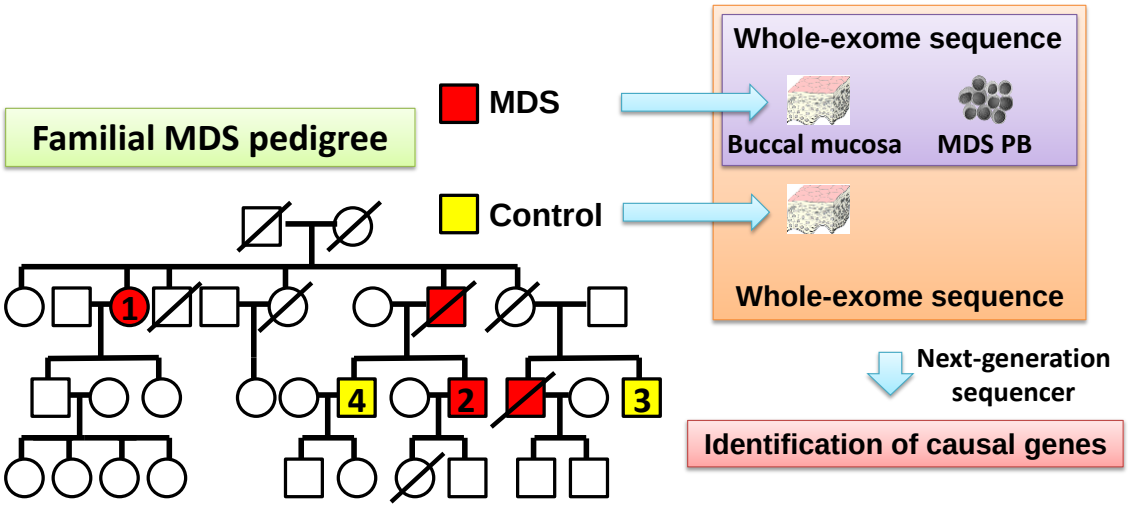


Figure 2A. Whole exome sequence strategy of the index pedigree. Both somatic and germ-line samples of MDS patients, and germ-line samples of the controls were sequenced by a next-generation sequencer.

As a result, the read numbers of germ-line samples after removing duplicate were $3.27*10^8$, $3.11*10^8$, $3.14*10^8$, and $3.30*10^8$ in Pt1, Pt2, Sb3, and Sb4, respectively. Likewise, the mean coverage of germ-line samples was 179, 170, 162, and 177 in Pt1, Pt2, Sb3, and Sb4, respectively (Table 5). The read number and mean coverage were sufficient to analyze the data of genomic mutation.

Table 5. Sample quality control and whole-exome sequence outcome.

Sample	gDNA (ng)	Raw read No.	Unique read No.	Mean coverage
Pt1 PB	560	843,820,688	527,569,654	299.004
Pt1 Bu	260	421,286,864	327,316,563	178.807
Pt2 PB	1000	812,162,088	539,764,584	305.142
Pt2 Bu	330	420,517,586	311,204,072	170.146
Control3 Bu	260	391,873,048	313,622,184	161.632
Control4 Bu	300	441,026,572	330,036,026	176.98

Bu: buccal mucosae PB: peripheral blood

In total, 16889 non-synonymous germ-line substitutions were obtained. Among them, 35 mutual single base substitutions remained after eliminating SNPs from 1000 genome project (39), in-house SNPs, data base SNP 138 and 139 (40), Human Genetic Variation Browser (41), Exome variant server (42), coverage value < 20, and variant allele frequency < 20% (Figure 2B).

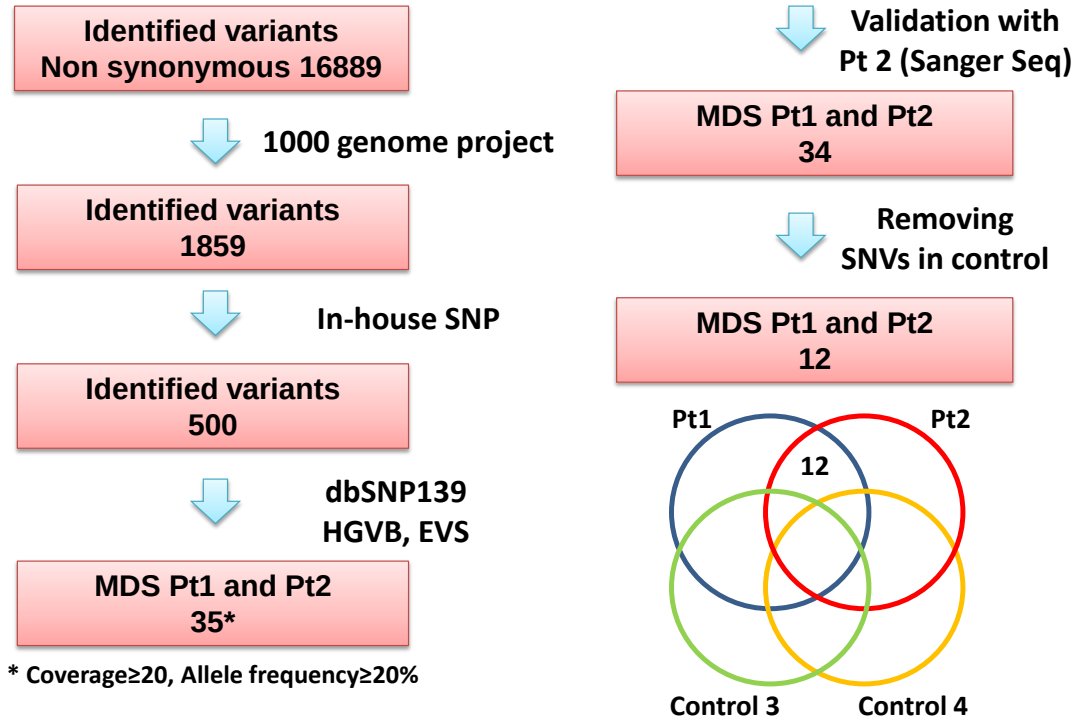


Figure 2B. The results of whole exome sequence of the index pedigree. Twelve candidate genes were obtained.

Subsequently, I validated the remained 35 candidate single base mutations by Sanger sequence using the peripheral blood cells obtained from patient 2, resulting in the

exclusion of *NAT1* from the candidates (Table 6).

Table 6. The list of 34 candidate gene after validation.

Gene symbol	Amino acid change	Reference sequence No.
AGXT	R370H	NM_000030
ANK1	R256Q	NM_000037
B4GALNT2	V352L	NM_153446
BTN3A2	A334T	NM_007047
C14orf39	I55M	NM_174978
C2CD3	C1602Y	NM_015531
C9orf43	G40D	NM_152786
CYP7B1	V270I	NM_004820
DEPTOR	H242N	NM_022783
EFHB	G747V	NM_144715
ENTPD7	S413N	NM_020354
FAM160B2	L270F	NM_022749
GDAP1L1	T158M	NM_024034
HELZ2	E1684K	NM_033405
HLTF	E259K	NM_003071
INPP5J	E197X	NM_001284285
INVS	L912M	NM_014425
IRX1	A217V	NM_024337
ITPKB	R240L	NM_002221
LRRC16A	G1312V	NM_017640
MPO	R421Q	NM_000250
NAPRT1	V216I	NM_145201
NEU4	M378I	NM_080741
NID2	I342T	NM_007361
OR10D3	M118V	ENST00000318666
PLIN5	R200C	NM_001013706
PLIN5	F198L	NM_001013706
PRSS23	S157C	NM_007173
RYK	S561N	NM_002958
SCUBE1	E711K	NM_173050
SLC25A39	R300W	NM_016016
TLE6	A409V	NM_024760
TTN	A9405V	NM_003319
ZFR	E848A	NM_016107

In addition, because twenty-two of the candidate genes were also found in the control samples from healthy family members, we obtained twelve candidate gene mutations (Table 7).

Table 7. The list of twelve candidate genes and allele frequency.

Gene symbol	Amino acid change	Allele frequency(%)			
		Pt1(Bu)	Pt1(PB)	Pt2(Bu)	Pt2(PB)
ANK1	R256Q	53.4	50.0	46.3	53.1
C9orf43	G40D	48.4	47.1	47.6	52.1
CYP7B1	V270I	48.5	49.4	47.6	47.5
EFHB	G747V	49.6	46.9	54.0	45.2
ENTPD7	S413N	50.2	51.9	49.1	49.4
FAM160B2	L270F	48.7	51.1	51.3	54.2
HELZ2	E1684K	56.9	53.5	44.7	47.8
HLTF	E259K	50.8	47.0	44.2	43.1
INPP5J	E197X	43.6	52.1	52.4	47.8
ITPKB	R240L	56.8	50.9	47.6	52.1
RYK	S561N	41.0	47.8	55.4	47.7
TTN	A9405V	51.5	47.7	51.8	49.2

Bu: buccal mucosae PB: peripheral blood

As for insertions-deletions (indels), six mutual indels were identified in patient 1 and patient 2 after eliminating reported indels and in-house indels, coverage value < 20, and variant frequency < 20% (Table 8).

Table 8. The list of six indels obtained from patient 1 and patient 2.

Gene symbol	Base change (RefSeq)	Reference sequence number
CHRNA3	ctg/ctCTGg	NM_000743
FAM157B	cggcagcagcag/cgg	NM_001145249
FAM157B	cggcagcagcagcagcagcag/cgg	NM_001145249
RP11-210M15.1	----	ENST00000594607
VPS16	-/TC	NM_022575

Caverage ≥ 20, variant frequency ≥ 20%

Of these, deletion of *RP11-210M15.1* was in untranslated region and insertion of *CHRNA3* and deletion of both *FAM157B* were not validated by Sanger sequence using the peripheral blood cells from patient 2. Therefore, these indels were excluded from the analysis. Additionally, the remaining insertion of *VPS16* was detected in healthy family members. Eventually, from these analyses, I obtained 12 candidate gene mutations of familial MDS/AML.

Subsequently, I analyzed the acquired somatic mutations which were not overlapped with the germ-line mutations. After eliminating reported and in-house SNPs, eight somatic mutations were detected in patient 1 (Table 9). No acquired somatic mutation was identified in patient 2.

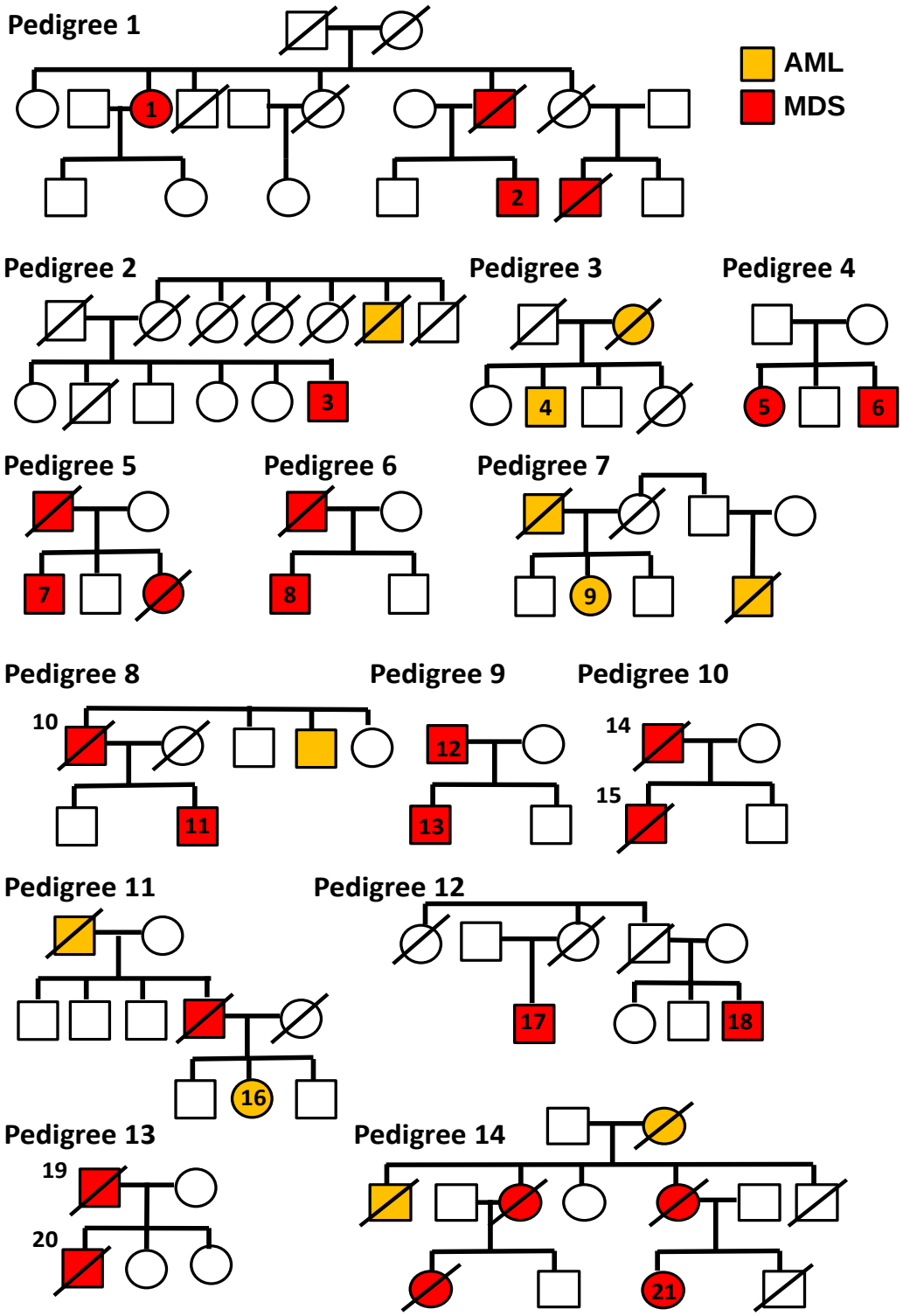
Table 9. The list of somatic mutations obtained from patient 1.

Patient	Gene symbol	Amino acid change	Reference sequence number
Patient1	*ARRDC1*	E63V	NM_152285
Patient1	*C9orf50*	*432R	NM_199350
Patient1	*DDX41*	T227M	NM_016222
Patient1	*ESM1*	N22S	NM_007036
Patient1	*GOLGA6L2*	G850R	ENST00000567107
Patient1	*IGF2R*	Y196C	NM_000876
Patient1	*OR4C13*	P77A	NM_001001955
Patient1	*PTPN1*	N162K	NM_002827

Caverage ≥ 20, variant allele frequency ≥ 20%

Japanese nationwide survey of familial myelodysplastic syndromes

To investigate the recurrence of the candidate gene mutations in familial MDS/AML, I designed a Japanese nationwide survey of familial MDS/AML, and sent a questionnaire sheet to 561 institutions or hospitals certified by Japanese Society of Hematology. Two hundred and forty-one (43.0%) institutions were answered, and 41 pedigrees of familial MDS/AML were reported in total. Subsequently, the second questionnaire sheets were sent to these institutions to obtain further characteristics of the pedigrees, and 12 out of 241 institutions (5.0%) were answered. The clinical information of 24 patients in 16 pedigrees was collected (Figure 3A). Moreover, I obtained the clinical samples of seven cases from six pedigrees (Table 10).


Pedigree 1
AML
MDS
1
2
Pedigree 2
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Pedigree 3
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Pedigree 4
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Pedigree 5
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Pedigree 6
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Pedigree 7
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Pedigree 14
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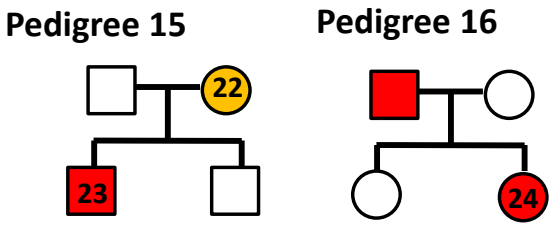


Figure 3A. The familial MDS/AML 16 pedigrees obtained from the nationwide survey. Clinical samples were obtained from patient 1 to patient 9. Whole exome sequence was performed of both patients in pedigree 1.

Table 10. The summary of the characteristics of the patients from the nationwide survey.								
Pedigree	Patient	Sex	Dx age	WHO classification	Bone marrow examination in diagnosis	Karyotype	IPSS-R and score	
1	1	female	78	MDS(N.A.)	N.A.	Normal	N.A	N.A.
	2	male	63	RCMD	N.A.	Normal	N.A.	N.A.
2	3	male	59	RAEB-2	Blasts 13.0%, Pseudo Pelger-Huet, micro MgK	Complex>3	Very high	8.5
3	4	male	71	AML-M3	APL cell 88.8%	t(15;17)	N.A.	N.A.
4	5	female	14	RA	Blast 0%, Karyorrhexis and nuclear border irregularity in erythroid lineage	Normal	Intermediate	3.5
	6	male	9	RA	Blast 0%, Multinuclear erythloblast,nuclear border irregularity in erythroid lineage	Normal	Low	2.0
5	7	male	58	RCMD	Blasts 2.7%, Pseudo Pelger-Huet, abnormal in erythroid lineage	Del(20q)	Intermediate	4.0
6	8	male	58	RAEB-2	N.A.	Normal	Intermediate	4.0
7	9	female	60	AML-M1	Blasts 56.6%	N.A.	N.A.	N.A.
8	10	male	77	MDS(N.A.)	N.A.	N.A.	N.A.	N.A.
	11	male	49	RAEB-2	Blasts 14.6%, Micro MgK, nuclear hypolobation in MgK	Normal	Intermediate	4.5
9	12	male	18	RA	Blasts 0.8%	+8	Low	3.0
	13	male	17	RCMD	N.A.	Normal	N.A.	N.A.
10	14	male	60	RAEB-1	Blasts 2.7%, Multinuclear erythloblast	-7	High	5.5
	15	male	44	RAEB-2	Blasts 10%, Nuclear border irregularity in erythroid lineage, agranularity	del(7q), -Y	Very high	7.5
11	16	female	42	AML-NOS (AML-M1)	Blasts 41.6%	N.A.	N.A.	N.A.
12	17	male	40	RA	N.A.	Normal	N.A.	N.A.
	18	male	N.A.	RAEB-2	Blasts 5.0%, Micro MgK	Complex>3	Very high	9.0
13	19	male	88	RCMD	Blasts 3.3%, Nuclear hyperlobation in erythroblast	Normal	High	5.0
	20	male	58	RAEB-2	Blasts 10.2%, Abnormality in erytyroid lineage	Normal	Intermediate	4.5
14	21	female	27	RCUD(RT)	Blast 0%, N.A.	Normal	N.A.	N.A.
15	22	female	62	AML with t(8;21)(q22;q22)	N.A.	t(8;21)(q22;q22)	N.A.	N.A.
	23	male	53	RAEB-1	Blasts 5.0%, Ring sideroblasts, agranularity, microMgK, multinucleation in MgK	Complex>3	Very high	6.5
16	24	female	36	RAEB-1	Blasts 7.2%, Multinuclear erythloblas, nuclear border irregularity in erythroid lineage, microMgK, multinucleation in MgK	Normal	Intermediate	4.5
Clinical samples were obtained from patient 1 to patient 9.								
Abbreviation Dx: diagnosis, MgK:megakaryocyte, N.A.:not applicable								

In the collected 24 patients, seven patients (7/24=29.2%) were female. The mean and the median age of the initial diagnosis were 49.6 and 58 years, respectively (Figure 3B). WHO classification ranged from refractory anemia (RA) to AML (52). RAEB-2 was the largest group (25%), followed by AML (17%), RA (17%), and RCMD (17%) (Figure 3C). Chromosomal analysis revealed that half of the patients had normal karyotypes at the initial diagnosis (Figure 3D). As for IPSS-R, “intermediate” was the largest group, followed by “very high” (17%), “high” (8%), and “low” (8%) (53) (Figure 3E). No “very low” group was observed. The characteristics of these cases were summarized in Table 10. Subsequently, twelve single base mutations were analyzed by Sanger sequencing, however, these mutations were not identified in other pedigrees.

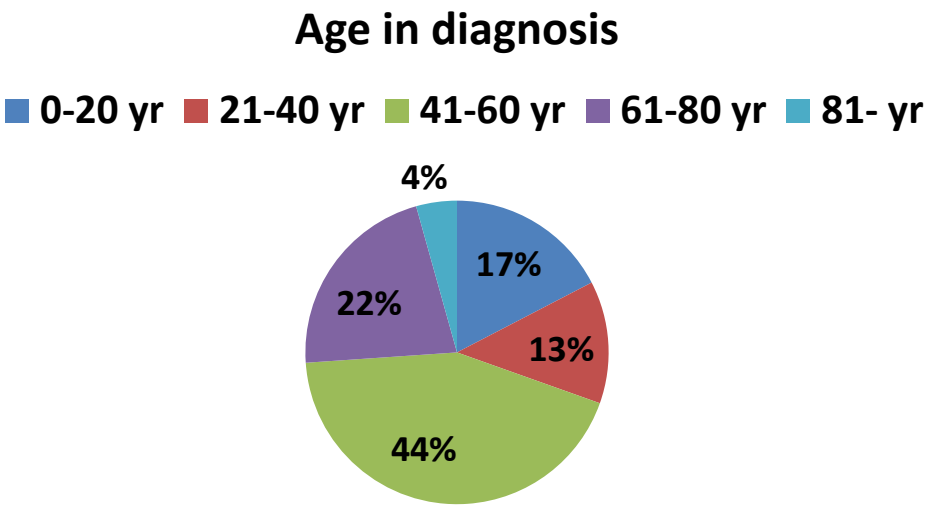


Figure 3B. Age in the initial diagnosis of familial MDS/AML.

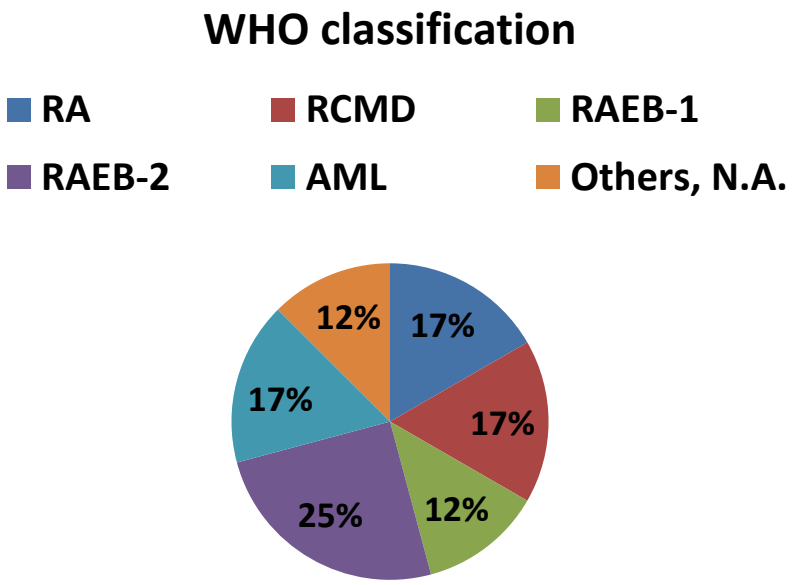


Figure 3C. WHO classification in the initial diagnosis of familial MDS/AML.

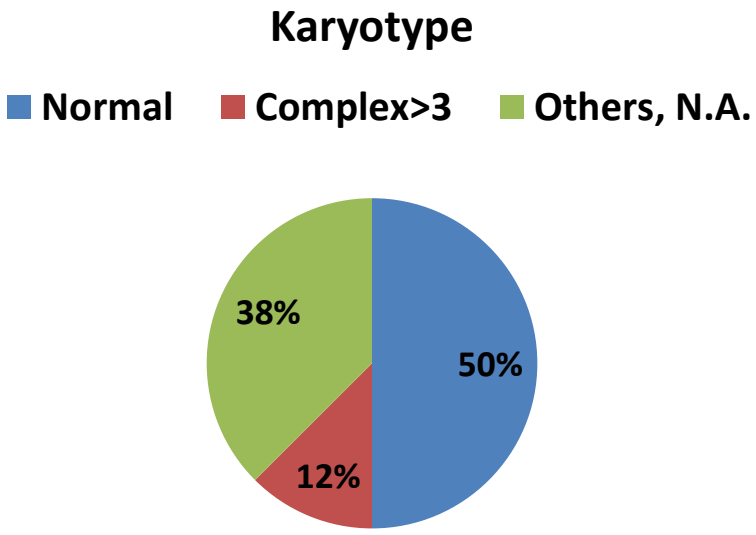


Figure 3D. Chromosome examination in the initial diagnosis of familial MDS/AML.

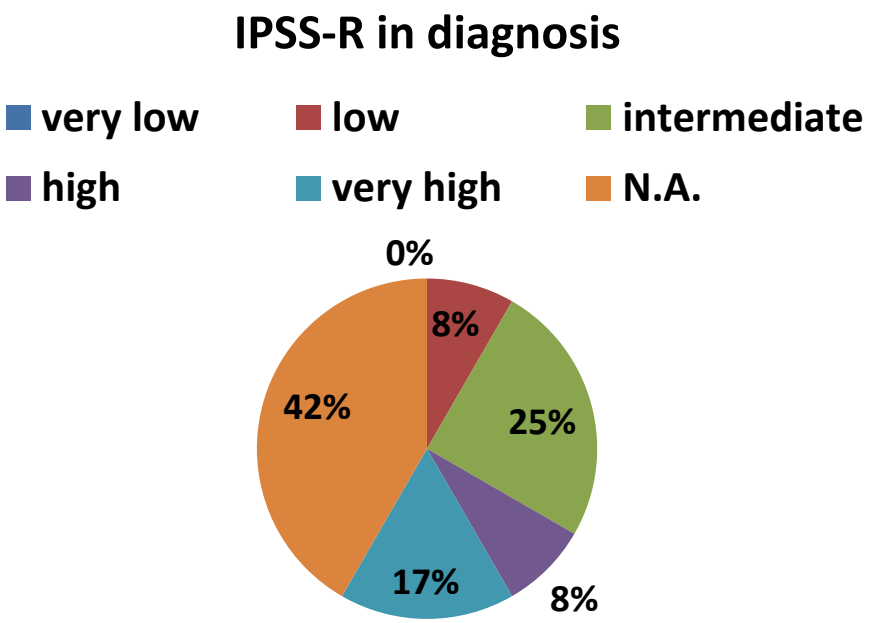


Figure 3E. IPSS-R classification in the initial diagnosis of familial MDS/AML.

***Hltf* silencing enhances in vitro colony replating capacity**

Of the twelve candidate genes (Table 7), I performed a literature survey to select a candidate gene for further functional analysis. I searched a gene with a similar function of the driver genes of sporadic MDS. Among the candidate, HLTF regulates the expressions of the cohesin complex-associated genes that frequently mutated in sporadic MDS/AML (54). Hltf null mouse brain showed more apoptotic cells and increased active caspase-3 (54). HLTF is an E3-ubiquitin ligase which supports the Lys-63 linked polyubiquitination of proliferating cell nuclear antigen (PCNA), and promotes error-free postreplication DNA repair (55). Transcript expression of main

components of the G2/M transition, such as Aurora-B, CAP-G/G2, and Histone H3.3, was decreased in Hltf null mouse brain (54). Since HLTF has an important role in chromatin remodeling, transcription, DNA damage repair, apoptosis, and cell cycle which are prominently involved in MDS pathogenesis (54, 55), I selected *HLTF* for further functional assay.

Firstly, I checked *HLTF* mutation status in sporadic MDS samples by Sanger sequence. Whole *HLTF* coding regions of 40 samples were sequenced (Table 11). As a result, a novel *HLTF* mutation (p.Thr50Ala, T50A) was identified in one sample (1/40 = 2.5%) (Figure 4A). These data suggested the possibility that *HLTF* mutation might contribute to the pathogenesis not only in familial MDS but also in sporadic MDS.

Table 11. *HLTF* Sanger sequence results of 40 sporadic MDS samples.

	exon1	exon2	exon3	exon4	exon5	exon6	exon7	exon8	exon9	exon10	exon11	exon12	exon13	exon14	exon15	exon16	exon17	exon18	exon19	exon20	exon21	exon22	exon23	exon24	exon25
MDS1	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	N.A.	O	O
MDS2	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	N.A.	O	O
MDS3	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	N.A.	O	O
MDS4	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	N.A.	O	O	O	O	O	O	N.A.	O	O
MDS5	O	O	N.A.	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
MDS6	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
MDS7	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	N.A.	O	O
MDS8	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	N.A.	O	O
MDS9	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	N.A.	O	O
MDS10	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
MDS11	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	N.A.
MDS12	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	N.A.	O	N.A.
MDS13	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	N.A.	N.A.	O	O
MDS14	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
MDS15	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
MDS16	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	N.A.	O	N.A.	O	O	O	O	O
MDS17	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	N.A.	O
MDS18	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
MDS19	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	N.A.
MDS20	O	O	N.A.	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
MDS21	O	p.T50A	N.A.	N.A.	N.A.	O	N.A.	O	N.A.	O	O	N.A.	N.A.	O	O	O	N.A.	N.A.	O	O	N.A.	O	N.A.	O	O
MDS22	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	N.A.	O	O
MDS23	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	N.A.	O	O
MDS24	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
MDS25	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	N.A.	O	O	O	O	O	N.A.	N.A.	O
MDS26	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	N.A.	O	N.A.
MDS27	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
MDS28	O	O	O	O	N.A.	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
MDS29	O	O	O	O	N.A.	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
MDS30	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	N.A.	O	O	O	N.A.	O
MDS31	N.A.	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	N.A.	O	O
MDS32	O	O	O	N.A.	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	N.A.	O	O
MDS33	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	N.A.	O	O
MDS34	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	N.A.	O	O
MDS35	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	N.A.	O	N.A.
MDS36	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	N.A.	O	O
MDS37	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	N.A.	O	O
MDS38	O	O	N.A.	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
MDS39	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	N.A.
MDS40	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	N.A.	O	O	N.A.	O	O

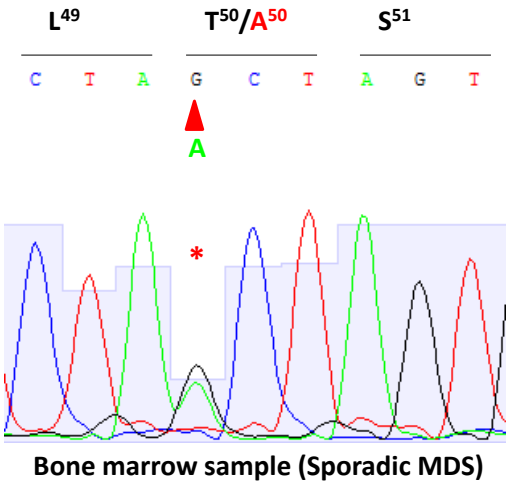
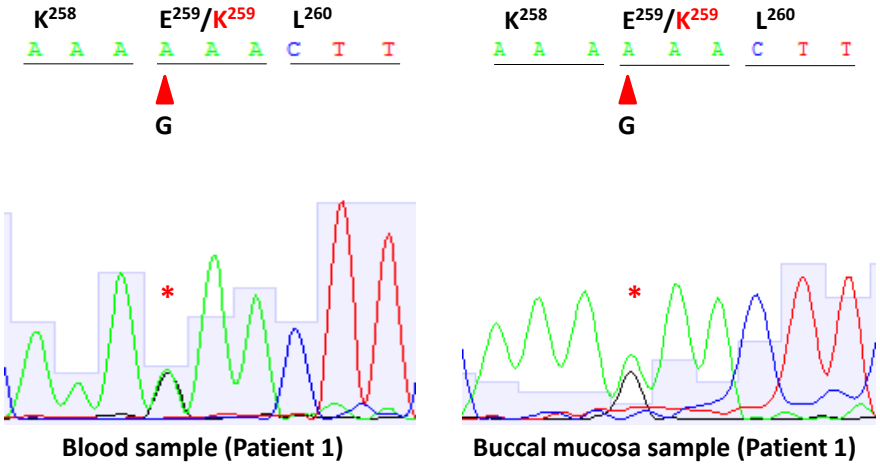


Figure 4A. *HLTF* p.Thr50Ala change in a sporadic MDS sample.

The obtained amino acid change of *HLTF* was p.Glu259Lys (E259K) in familial MDS (Figure 4B). The 259th amino acid position of HLTF is on DEXDc domain, and is close to an ATP binding site (Figure 4C) (56). DEXDc domain contains the ATP-binding region, and is involved in ATP-dependent DNA or RNA unwinding.



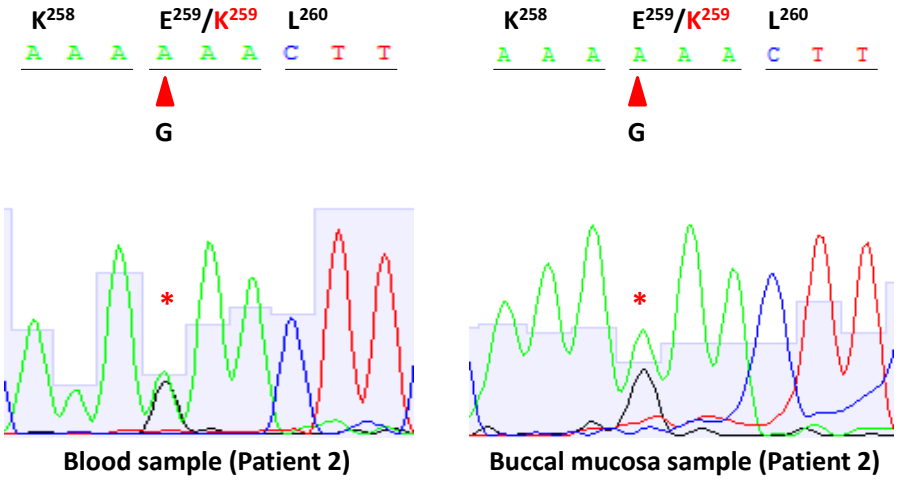


Figure 4B. *HLTF* p.Glu259Lys change in patient 1 and patient 2 by Sanger sequence.

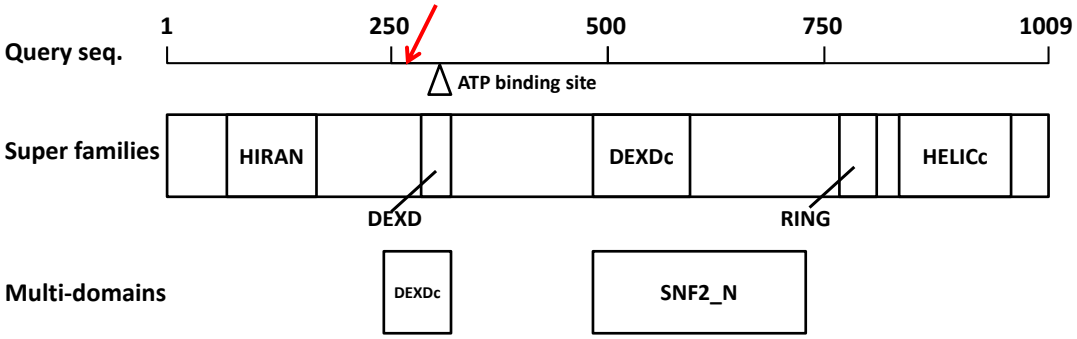


Figure 4C. Graphical summary of *HLTF*. Red arrow denotes the positon of p.Glu259Lys.

Importantly, a public dataset (GSE9476) which included the expression profile of normal hematopoietic cells from 38 healthy participants and leukemic blast cells from 26 AML patients demonstrated that *HLTF* expression was significantly lower in AML samples ($p<0.01$) (Figure 4D) (57).

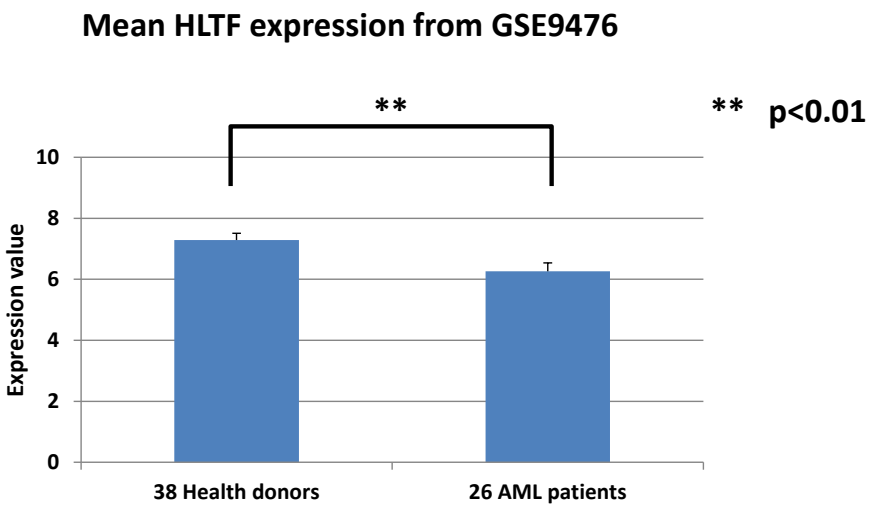


Figure 4D. Mean *HLTF* expression from GSE 9476. Error bar denotes standard error.

Subsequently, I evaluated in vitro colony-formation capacity using *Hltf*-knocked down 5-fluorouracil (5FU)-primed C57BL/6 mouse bone marrow (BM) cells. Quantitative PCR analysis of *Hltf* in BM cells showed an approximately 70% reduction of *Hltf* compared to the control shRNA vector-transduced BM cells (Figure 4E). Strikingly, *Hltf*-silenced BM cells showed enhanced in vitro colony-replating capacity (Figure 4F). While *Hltf* knockdown BM cells could be replated up to the fifth round, the control BM cells could not survive after the third plating.

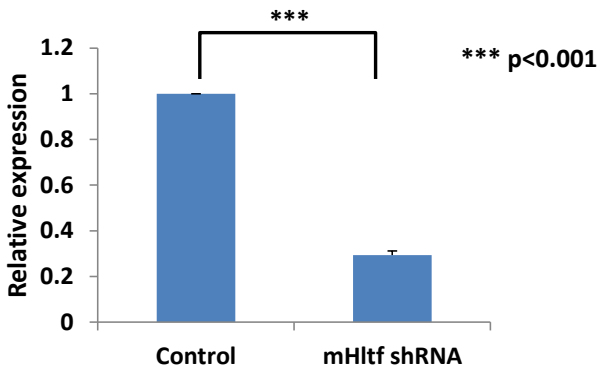


Figure 4E. Quantification of shRNA-mediated *Hltf*-knocked down efficiency by quantitative real-time PCR (n=4).

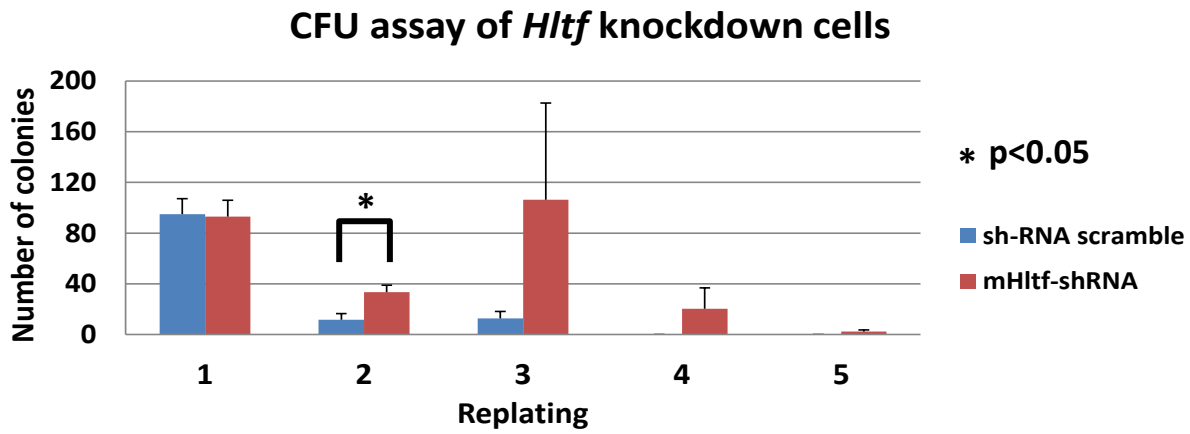


Figure 4F. Colony replating assay of control and *Hltf*-knocked down cells. Error bar denotes standard error (n=3).

Indeed, c-kit, a hematopoietic stem/ progenitor cell marker, positive cells were increased in *Hltf* shRNA-expressing colonies after the first round (Figure 4G). *Hltf* shRNA-expressing cells had morphologic characteristics of immature myeloid cells, i.e. increased nucleus-to-cytoplasm ratio, and clear nucleoli compared to control

shRNA-expressing cells (Figure 4H).

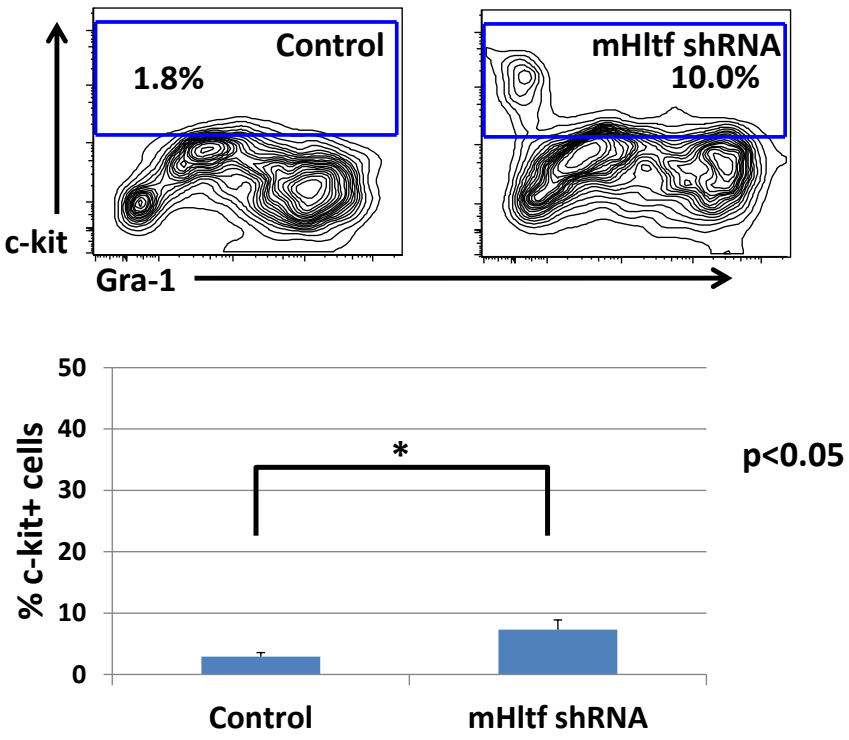


Figure 4G. (Upper) Representative image of the ratio of c-kit positive cells in control and *Hltf* shRNA-expressing colonies after the first plating. (Lower) The average of the ratio of c-kit positive cells in control and *Hltf* shRNA-expressing colonies after the first plating. Error bar denotes standard error (n=5).

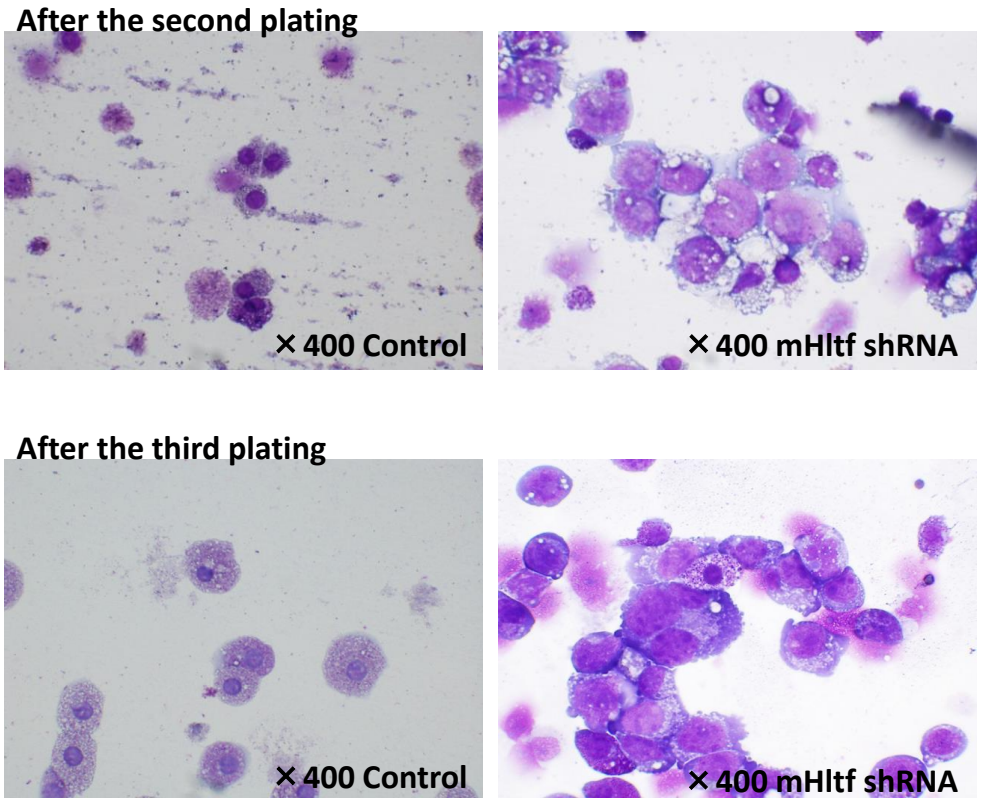


Figure 4H. Cell morphology in control colonies and *Hltf* shRNA-expressing colonies after the second plating (upper), and the third plating (lower). Cytospins of the colonies are shown.

ATPase activity of *HLTF* E259K mutant

Previously report revealed that HLTF itself has an ATPase activity (58). Since the 259th amino acid position of *HLTF* locates on near ATP binding site, I examined the ATPase activity of *HLTF* E259K compared to *HLTF* wild-type. Both *HLTF* E259K mutant and wild-type protein were synthesized by in vitro translation, and protein expressions were confirmed by Western blotting (Figure 5A). Water was used as negative control. However, in vitro ATPase activity experiment showed no significant change in

phosphate concentration between HLTF wild-type protein and HLTF E259K protein (Figure 5B). These result demonstrated that ATP-dependent DNA repair of HLTF E259K was not impaired.

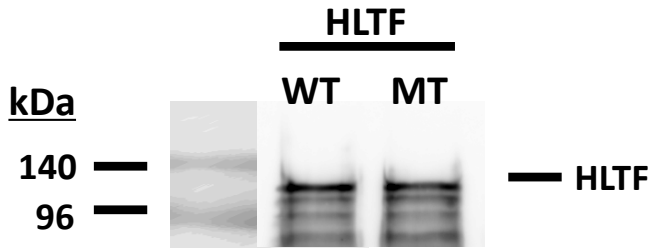


Figure 5A. *HLTF* wild-type and mutant expression by Western blotting.

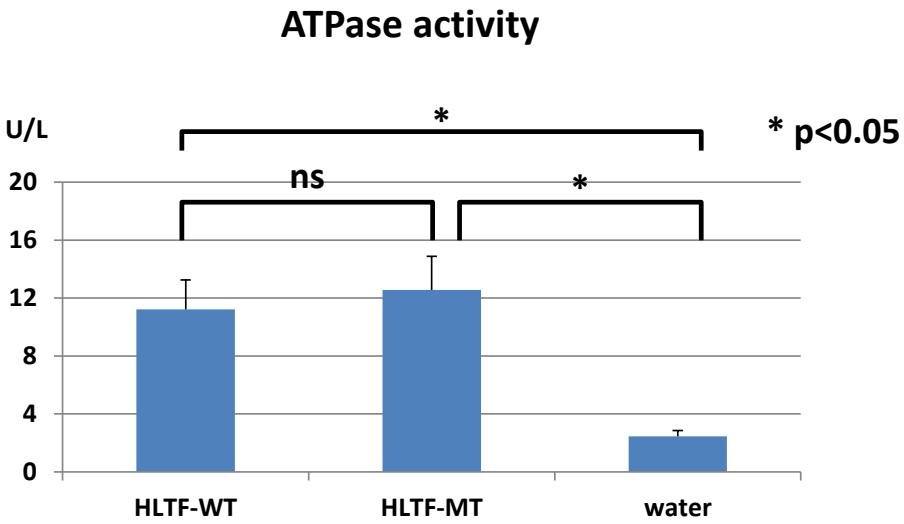


Figure 5B. ATPase activities of *HLTF* wild-type and mutant. Error bar denotes standard error (n=3).

Discussion

In this study, I elucidated the epidemiology of familial MDS/AML by the nationwide survey and identified twelve candidate genes from patient samples using whole exome sequencing. In addition, I found the novel *HLTF* T50A mutation in one sporadic MDS sample, and found the enhanced in vitro colony replating capacity of *Hltf*-silenced murine BM cells.

Holme et al. reported the cohort of 27 unrelated families with two or more MDS or AML individuals who had bone marrow failure, and analyzed 27 patients in the UK (1). The mean and the median age of the patients were 25.0 years, 23.0 years, respectively (Figure 6).

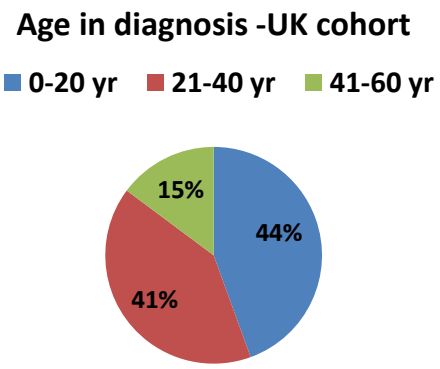


Figure 6. Age in the diagnosis of familial MDS/AML of 27 patients in the UK cohort.

Although it is reported that patients with familial MDS/AML are younger at presentation than sporadic MDS patients (29), the mean and the median age of 24

patients from our nationwide survey were 49.6 and 58 years, which were relatively older. Sporadic MDS is known as the disease of the elderly with the median age of the diagnosis ≥70 (10). Considering this issue, our results reflected the similar propensity of familial MDS/AML. In Holme's survey, 53.8% of the patients were female, which were approximately twice as many as our cohort (29.2%). While Gaitonde et al. reported the fourteenth familial MDS/AML pedigrees associated with complete or partial loss of chromosome 7 (15, 59), one same pedigree was found in our cohort (pedigree 10, 1/16=6.3%). Although Minelli et al. described that most cases of familial monosomy 7 associated MDS/AML were younger than 18 years in the first consultation (60), our patients were elder, 44 and 42 years old. Minelli et al. also reported one familial monosomy 7 associated MDS/AML patient with a trisomy 8 (60), our cohort had one patient with trisomy 8 without other karyotype abnormalities (pedigree 9, 1/16=6.3%).

Human *HLTF*, helicase-like transcription factor, is a 56.4 kb gene with 26 exons, and is located on chromosome 3 (61). HLTF is a homologue of yeast Rad5 and belongs to the SWI/SNF (switch/sucrose non-fermentable) family which leads chromatin remodeling, ubiquitin ligase, and DNA repair (55, 56, 61). When DNA is damaged in DNA replication, replication fork stalls and HLTF is recruited to be involved in template switching (61-63). HLTF promotes polyubiquitination of PCNA, a DNA polymerase

sliding clamp which leads to switch the template in order to perform error-free bypass of the lesion (55, 61, 63). HLTF also helps D-loop formation at the DNA lesion of the sister duplex so as to achieve error-free bypass (61, 63). This process is driven by an ATP-independent and/or ATP-dependent manner (61). Although *HLTF* E259K mutation was located near ATP-binding site, ATPase activity between HLTF wild-type and mutant was not altered in my assay. Therefore, according to my assay, the pathogenesis of HLTF mutant might not be related with ATP-dependent DNA repairing.

Although MacKay et al. showed that *HLTF* expression level was very low in Western blotting in one leukemia cell line (58), little is known about the relationships between HLTF and hematological malignancies (26, 61, 64, 65). HLTF is a transcription factor, an E3-ubiquitin ligase, a post-replicative DNA repair controller, and HLTF also regulates the expressions of the cohesin complex-associated genes that frequently mutated in sporadic MDS/AML (54, 55, 61, 66). These multiple MDS-related functions suggested that HLTF could be a novel causal gene of MDS pathogenesis.

In addition, *HLTF* was reported to be inactivated by promotor hypermethylation in colon and gastric cancer (67, 68). However, it is also known that *HLTF* transcript levels are more than twentyfold higher in several cancer cell lines compared to normal human tissues (61, 69). Therefore, HLTF could be considered to have propensity of both a

tumor suppressor gene and an oncogene (61).

If the promotor hypermethylation of *HLTF* was also observed in hematopoietic malignancies, DNA hypomethylating agents such as azacitidine or decitabine might be effective. In this study, 5-FU primed murine *Hltf*-knocked down cells survived longer than control cells. Considering that *HLTF* expression level was low in leukemia cell line and human leukemia samples (Figure 4D), and loss-of-*Hltf* increased replating capacity, HLTF might behave as a tumor suppressor rather than an oncogene in hematological malignancies. However, it remains a possibility that gain-of-*HLTF* E259K function may also be related with the pathogenesis of familial MDS/AML.

Importantly, I found the novel *HLTF* T50A mutation in one out of 40 samples of sporadic MDS patients (1/40=2.5%). This mutation located near HIRAN domain, which binds 3' DNA ends to regulate replication fork reversal (70). Since replication fork reversal occurs in DNA damage repairing, this mutation might impede the ability of DNA repairing. The functional assay of HLTF p.T50A remained to be performed.

In conclusion, I performed the first nationwide survey of familial MDS/AML, and elucidated the characteristics of familial MDS/AML patients in Japan. I also identified twelve candidate genes of familial MDS/AML by whole exome sequencing analysis. Of these, loss-of-*Hltf* enhanced in vitro colony replating capacity. In addition, a novel

HLTF T50A mutation was detected in a sporadic MDS sample. This study could be an important first step for the understanding of familial MDS/AML.

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Appendices

Appendix 1 -The first questionnaire sheet of the nationwide survey of familial MDS/AML.

家族性 MDS に関する全国調査　一次調査票

貴施設名＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿

貴施設住所＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿

御芳名＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿

E-mail＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿

1．貴施設において家族性 MDS・AML 症例が約何家系あったか、教えてください。

（約）　　家系

2．（上記で、該当する家系があった場合）家族性 MDS に関する調査研究にご協力いただけますか?

はい　　いいえ

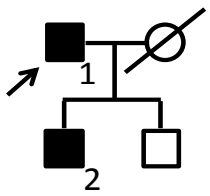
ご協力いただき、誠にありがとうございました。

Appendix 2 -The second questionnaire sheet of the nationwide survey of familial MDS/AML.

家族性MDSに関する全国調査　二次調査票

記入日	西暦　　年　　月　　日
貴施設名	
連絡御担当者	
連絡先	e−mail: Tel: FAX:

家系毎に、下記の各項目にご記入ください。

匿名化家系ID(家系毎に1から順に)	
家系図 凡例 ↗: 発端者(■●の左下) ／: 死亡者(■●に重ねて) ■: MDS/AML罹患男性 □: MDS/AML非罹患男性 ●: MDS/AML罹患女性 ○: MDS/AML非罹患女性 ー: 結婚線 ┌┐: 同胞線 番号(■●の右下): 個人番号ID (発端者を1として、2, 3, 4...)  	

個人用調査票(1個人につき1部作成してください)

個人番号(発端者を1として、2, 3, 4, ...)	
診断確定日	西暦　　年　　月　　日
診断時年齢・性別・生年月	歳　1. 男　2. 女　西暦　　年　　月生
既往歴	1. 血液疾患(　　(西暦　　年　　月頃)) (　　(西暦　　年　　月頃)) (　　(西暦　　年　　月頃)) 2. その他の腫瘍(　　(西暦　　年　　月頃)) (　　(西暦　　年　　月頃)) (　　(西暦　　年　　月頃)) (　　(西暦　　年　　月頃)) 3. 放射線治療歴　部位(　　) 線量(合計　　Gy) 4. 化学療法歴　(期間　～　内容　　) (期間　～　内容　　) (期間　～　内容　　) (期間　～　内容　　) (期間　～　内容　　) (期間　～　内容　　)
生活歴	喫煙:　1. ＿＿＿本/日×＿＿＿年間　2. 吸ったことがない 飲酒:　1. ＿＿＿合/日×＿＿＿日/(週・月) 2. 機会飲酒　3. 飲まない 化学物質曝露(　　) 放射線曝露　(　　)
診断時FAB分類(List 1から番号を選択)	
診断時WHO分類(List 2から番号を選択)	
診断時における末梢血の血算値	Hb ＿＿＿g/dl　Plt ＿＿＿×10^4/μl Neu ＿＿＿/μl (WBC ＿＿＿/μl 中 ＿＿＿%) Blasts　%
診断時骨髄穿刺所見 ※List 3から選択("その他"は具体的に)	検査日: 西暦　　年　　月　　日 Blasts: ＿＿＿% 形態異常　(　　)
診断時染色体検査所見(List 4から選択)	1. 所見: (　　) 2. 未施行
診断時IPSS-R (MDSの場合) ※List 5を参照して計算→	1. Very low　2. Low　3. Intermediate 4. High　5. Very high (Score Value:　　)
診断時臨床症状	0. なし　1. 貧血　2. 易感染性　3. 出血傾向　4. 脾腫 5. その他(　　)
診断時ECOG Performance Status (0～4)	

最終確認日と転帰	西暦　　年　　月　　日 転帰:　1. 無病生存　2. 有病生存　3. 死亡
転帰/理由	上記2.の場合: 1. 転院　2. 通院中　3. その他(　　) 上記3.の場合: 死因(　　)
患者検体はありますか? 患者検体はこれから採取可能ですか?	0. いいえ　1. はい　(a. 骨髄　b. その他(　　)) 0. いいえ　1. はい　(a. 骨髄　b. 末梢血　c. 口腔粘膜)
治療①(初回治療; List 6から選択) ※治療①の種類が移植の場合は治療③へ	治療の種類: (　　)　開始日: 西暦　　年　月　日 1. 西暦　　年　月　日まで　2. 現在に至る
治療①開始前の末梢血血算値	1. 検査日: 西暦　　年　　月　　日 Hb＿＿＿g/dl　Plt＿＿＿×10^4/μl Neu＿＿＿/μl (WBC＿＿＿/μl 中＿＿＿%) Blasts＿＿＿% 2. 診断時と同じ
治療①開始前に要した輸血単位・頻度	1. 赤血球LR　治療①開始前の8週間に(　　)単位 2. 血小板LR　治療①開始前の8週間に(　　)単位 3. 輸血依存なし
治療①開始前の骨髄穿刺所見 ※List 3から選択("その他"は具体的に)	1. 検査日: 西暦　　年　　月　　日　Blasts:＿＿＿% 形態異常: (　　) 2. 診断時と同じ
治療①開始前の骨髄染色体検査所見	1. 所見(List 4): (　　) 2. 診断時と同じ
治療①の治療効果	MDSの場合(List 7から選択): AMLの場合(List 8から選択):
治療①後の骨髄穿刺所見 ※List 3から選択("その他"は具体的に)	1. 検査日: 西暦　　年　　月　　日　Blasts:＿＿＿% 形態異常: (　　) 2. 未施行
治療①後の染色体検査所見	1. 染色体異常消失、かつ新たな異常の出現なし 2. 染色体異常が50%以上減少 3. その他(　　) 4. 未施行
治療①を終了/中止/変更した理由(List 9)	1. 終了　2. 中止　3. 変更　理由: (　　)
治療②(初回治療; List 6から選択) ※治療②の種類が移植の場合は治療③へ	治療の種類: (　　)　開始日: 西暦　　年　月　日 1. 西暦　　年　月　日まで　2. 現在に至る

治療②開始前の末梢血血算値	1. 検査日: 西暦　　年　　月　　日 Hb ＿＿＿＿ g/dl　　Plt ＿＿＿＿ ×10^4/μl Neu ＿＿＿＿ /μl (WBC ＿＿＿＿ /μl中 ＿＿＿＿ %) Blasts　　%
治療②開始前の骨髄穿刺所見 ※List 3から選択(“その他”は具体的に)	1. 検査日: 西暦　　年　　月　　日　Blasts: ＿＿＿＿ % 形態異常: (　　) 2. 治療①後と同じ
治療②開始前の骨髄染色体検査所見 ※List 2から選択(“その他”は具体的に)	1. 所見: (　　) 2. 治療①後と同じ
治療②の治療効果	MDSの場合(List 7から選択): AMLの場合(List 8から選択):
治療②後の骨髄穿刺所見 ※List 3から選択(“その他”は具体的に)	1. 検査日: 西暦　　年　　月　　日　Blasts: ＿＿＿＿ % 形態異常: (　　) 2. 未施行
治療②後の染色体検査所見	1. 所見:　1. 染色体異常消失、かつ新たな異常の出現なし 2. 染色体異常が50%以上減少 3. その他 (　　) 2. 未施行
治療②を終了/中止/変更した理由(List 9)	1. 終了　2. 中止　3. 変更　理由: (　　)
治療③: 移植 ※HLA一致: 血清6座以上一致	移植前の状態:　1. 未治療　2. CR　3. その他(　　) 移植日:　西暦　　年　月　日 HLA:　1. 血縁者　2. HLA一致非血縁者　3. 臍帯血 4. その他(　　) 移植の効果:　1. CR維持　2. 再発 3. 死亡(死因:　　)

移植治療を2回施行した場合：2回目の移植はこちらに記載

治療③: 移植 ※HLA一致: 血清6座以上一致	移植前の状態:　1. 未治療　2. CR　3. その他(　　) 移植日:　西暦　　年　月　日 HLA:　1. 血縁者　2. HLA一致非血縁者　3. 臍帯血 4. その他(　　) 移植の効果:　1. CR維持　2. 再発 3. 死亡(死因:　　)

List 1: 診断時分類(FAB)

1. RA　2. RARS　3. RAEB　4. RAEB-t　5. CMMoL
6. MDS-overt leukemia　7. AML-M0　8. AML-M1　9. AML-M2　10. AML-M3
11. AML-M4　12. AML-M4Eo　13. AML-M5a
14. AML-M5b　15. AML-M6　16. AML-M7
17. その他(具体的に記載してください)

List 2: 診断時分類(WHO)

1. RCUD(1.1 RA; 1.2 RN; 1.3 RT)(1.1～1.3のどれかに○)
2. RARS　3. RCMD　4. RAEB-1　5. RAEB-2　6. MDS-U
7. MDS associated with isolated del(5q)
8. AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11
9. APL with t(15;17)(q24.1;q21.1); PML-RARA
10. AML with t(9;11)(p22;q23); MLLT3-MLL
11. AML with t(6;9)(p23;q34); DEK-NUP214
12. AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); RPN1-EVI1
13. AML (megakaryoblastic) with t(1;22)(p13;q13); RBM15-MKL1
14. AML with mutated NPM1
15. AML with mutated CEBPA
16. AML with MDS-related changes (AML-MRC)
17. Therapy-related AML
18. AML-NOS (M0 M1 M2 M4 M5 M6 M7)
19. Acute basophilic leukemia
20. Acute panmyelosis with myelofibrosis
21. その他(具体的に記載してください)

List 3: 形態異常

E. 赤芽球系形態異常

E1. 環状鉄芽球(RS)
E2. 核辺縁不整
E3. 核間(染色質)架橋
E4. 核崩壊像
E5. 多核赤芽球
E6. 過分葉核赤芽球
E7. 巨赤芽球様変化
E8. 赤血球系細胞質空胞化
E9. 赤血球系PAS陽性
E10. その他

G. 顆粒球系形態異常

G1. 低分葉好中球(Pelger核異常)
G2. 脱顆粒(a-Gr/hypo-Gr)
G3. 小型または大型好中球
G4. 過分葉核好中球
G5. 偽Chédiak-Higashi顆粒
G6. その他

M. 巨核球系形態異常

M1. 微小巨核球(mMgk)
M2. 非分葉核
M3. 分離多核
M4. その他

List 4: 染色体異常

0. 正常核型
1. -Y
2. del(11q)
3. del(5q)
4. del(12p)
5. del(20q)
6. double including del (5q)
7. del(7q)
8. +8
9. +19
10. i(17q)
11. any other single/double independent clones
12. -7
13. inv(3)/t(3q)/del(3q)
14. double including -7/del(7q)
15. complex (3 abnormalities)
16. complex (>3 abnormalities)

List 5: IPSS-R(Revised International Prognostic Scoring System)

	Score value						
	0	0.5	1	1.5	2	3	4
核型*	Very good		Good		Intermediate	Poor	Very Poor
骨髄芽球(%)	≤2		>2～<5		5～10	>10	
Hb (g/dl)	≥10		8～<10	<8			
Plt (万/μ l)	≥10	5～<10					
Neu (/μ l)	≥800	<800					

核型(List 2)*

Very good: 1. ～ 2.　Intermediate: 7. ～ 11.　Very poor: 16.

Good: 0., 3. ～ 6.　Poor: 12. ～ 15.

リスク群(score total)

Very low: ≤1.5　Intermediate: >3～4.5　Very high: >6

Low: >1.5～3　High: >4.5～6

List 6: 治療(複数選択の場合、中心となった治療を最初に記載してください)

0. 経過観察
1. 輸血
2. G-CSF
3. エリスロポエチン
4. ビタミンK2
5. ビタミンD3
6. ビタミンB6
7. ATG
8. シクロスポリン(CsA)
9. 副腎皮質ホルモン
10. 蛋白同化ホルモン
11. レナリドミド
12. アザシチジン(ビダーザ)
13. キロサイド皮下注
14. スタラシド内服
15. ラステット点滴
16. キロサイド点滴
17. ハイドレア内服
18. ベプシド内服
19. IDR + Ara-C
20. DNR + Ara-C
21. High-dose Ara-C
22. Ara-C + MIT
23. Ara-C + ACR
24. A-triple-V (Ara-C + VP-16 + VCR + VDS)
25. HAM (High-dose Ara-C + MIT)
26. AEM (Ara-C + VP-16 + MIT)
27. DCM (DNR + Ara-C + 6-MP)
28. GO (マイロターグ)
29. Intermediate-dose Ara-C
30. CAG (Ara-C + ACR + G-CSF)
31. WT-1ワクチン
32. weekly IDR
33. ATRA + IDR + Ara-C
34. ATRA + DNR + Ara-C
35. MTX + 6-MP
36. 三酸化砒素(ATO(トリセノックス))
37. AM80(アムノレイク)
38. その他(具体的に記載してください)

List 7: International Working Group (IWG) Response Criteria for MDS

1.～7.: Altering natural history; 4週間以上の持続を必要とする。

1. CR (complete remission)

骨髄: 芽球≤5%かつ3系統の成熟(異形成残存は許容)

末梢血: 芽球0%, Hb≥11g/dl, Plt≥10万/μl, Neu≥1000/μl

2. PR (partial remission)

骨髄: 芽球が治療前の≥50%減少するが、5%よりも多く残存

骨髄細胞密度と細胞形態の異常あり

その他はCRと同じ

3. Marrow CR

骨髄: 芽球が5%以下、かつ治療前に比べて50%以上減少

末梢血: CRの条件を満たさないが、HI(後述)があれば明記

4. Stable disease

PRに達しないが、>8週間増悪の徴候がない

5. Failure

治療中の死亡、または次のいずれかを伴うDP(後述):

・血球減少の悪化

・骨髄中の芽球増加

・治療前よりもFAB subtype増悪

6. Relapse after CR or PR: 次の3項目のうち1項目以上を満たす。

・骨髄中の芽球が治療前の比率に戻る

・顆粒球または血小板が寛解時最大値から≥50%減少

・Hbが≥1.5g/dl減少または輸血依存

7. DP (disease progression)

a) 芽球5%以下: ≥50%増加し、>5%

b) 5%<芽球≤10%; ≥50%増加し、>10%

c) 10%<芽球≤20%; ≥50%増加し、>20%

d) 20%<芽球≤30%; ≥50%増加し、>30%

上記a)～d)のいずれかに該当し、かつ 次の1項目以上を満たす:

- 顆粒球または血小板が寛解時最大値から≥50%減少
- Hbが≥2g/dl減少
- 輸血依存へ移行

8.～9.: Hematologic improvement; 8週間以上の持続を必要とする。

8. Hematological improvement

8-1. HI-E: 治療前がHb<11g/dl, 治療後1.5g/dl以上のHb上昇

治療前に比べ、8週間あたり8単位以上の赤血球輸血回数減(Hb≤9.0g/dlに対して)

8-2. HI-P: 治療前が2万/μl<Plt<10万/μlの場合、治療後30000/μl以上のPlt増加

治療前がPlt≤2万/μlの場合、≥100%増加かつ>2万/μlに増加

8-3. HI-N: 治療前Neu<1000/μl, 治療後100%以上の上昇率かつNeu>500/μlの増加量

9. Progression/Relapse after hematological improvement: 次のうち1項目以上を満たす。

・顆粒球または血小板が治療後の最大値から50%以上減少

・1.5g/dl以上のHb減少

・輸血依存

List 8: Response criteria in AML
(European LeukemiaNet(Döhner H et al., Blood 2010;115:453-74.)の基準を元に作成)

1. Complete remission (CR)
   骨髄芽球<5%; Auer小体を有する芽球なし; 髄外病変なし;
   好中球>1000/μl; 血小板>100 000/μl; 赤血球輸血依存なし
2. CR with incomplete recovery (CRi)
   好中球<1000/μlまたは血小板<100 000/μl;
   その他の上記CRの条件を全て満たす
3. Morphologic leukemia-free state
   骨髄芽球<5%; Auer小体を有する芽球なし; 髄外病変なし;
   血球回復の基準は満たさなくて良い
4. Treatment failure
   a. Resistant disease:
      CRまたはCRiに至らなかった
      初回治療が完結してから7日以上生存
      末梢血または骨髄で白血病遷延
   b. Death in aplasia:
      血球減少を伴う死亡
      初回治療が完結してから7日以上生存
      無形成～低形成骨髄(死亡する7日前以降)
      白血病遷延の所見なし
   c. Death from indeterminate cause
      治療完了前、または治療完了後7日以内に死亡、または
      治療完了後7日以上後に死亡したが末梢血芽球なし、骨髄所見なし
5. Relapse
   骨髄芽球≥5%、または末梢血中に芽球再出現、または髄外病変出現

List 9: 治療を終了・中止・変更した理由

1. ガイドライン等が定める最多コース数に達した。
2. 十分な治療効果を得ることができた。
3. 予算や保険の制約による。
4. 患者からの要求(治療拒否を含む)。
5. 十分な治療効果が得られなかった。
6. 副作用・有害事象の発生:
   6.1. 感染症
   6.2. 出血傾向
   6.3. 貧血
   6.4. 心機能異常
   6.5. 肝機能異常
   6.6. 腎機能異常
   6.7. 神経系異常
   6.8. 薬疹等
   6.9. その他(具体的に記載)
7. 併存する他の疾患の増悪(具体的に記載)

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