Mutation analysis of therapy-related myeloid neoplasms

Takahiro Nishiyama[a], Yuichi Ishikawa[a], Naomi Kawashima[a], Akimi Akashi[a], Yoshiya Adachi[a], Hikaru Hattori[a,b], Yoko Ushijima[a], and Hitoshi Kiyoi[a,*]

[a] *Department of Hematology and Oncology, Nagoya University Graduate School of Medicine, Nagoya, Japan*

[b] *Department of Medical Technique, Nagoya University Hospital, Nagoya, Japan*

Running title: Mutation analysis of *t*-MN

***Corresponding author:** Hitoshi Kiyoi
Department of Hematology and Oncology, Nagoya University Graduate School of Medicine, Tsurumai-cho 65, Showa-ku, Nagoya 466-8550, Japan
Phone: +81-52-744-2136
Fax: +81-52-744-2157
E-mail: kiyoi@med.nagoya-u.ac.jp

Abstract

We analyzed the genetic mutation status of 13 patients with therapy-related myeloid neoplasms (*t*-MN). Consistent with previous reports, *t*-MN cells preferentially acquired mutations in *TP53* and epigenetic modifying genes, instead of mutations in tyrosine kinase and spliceosome genes. Furthermore, we compared the mutation status of three *t*-MN cells with each of the initial lymphoid malignant cells, and identified common mutations among *t*-MN and the initial malignant cells in two patients. In a patient who developed chronic myelomonocytic leukemia (CMML) after follicular lymphoma (FL), *TET2* mutation was identified in both CMML and FL cells. Notably, the *TET2* mutation was also identified in peripheral blood cells in the disease-free period with the same allelic frequency as CMML and FL cells, but not in a germ-line control, indicating that the *TET2* mutation occurred somatically in the initiating clone for both malignant cells. On the other hand, a germ-line *MYB* mutation was identified in a patient who developed myelodysplastic syndromes (MDS) after FL. These results suggest that germ-line deposition and clonal

hematopoiesis are closely associated with *t*-MN susceptibility; however, further analysis is necessary to clarify the mechanism required to provide the initiating clone with lineage commitment and clonal expansion.

Keywords: myeloid neoplasms; therapy-related; genetic alteration; founding mutation

Introduction

Therapy-related myeloid neoplasms (*t*-MN) are defined as acute myeloid leukemia (AML) or myelodysplastic syndromes (MDS) developing after exposure to cytotoxic agents or radiation therapy for unrelated malignancies, and classified into a subgroup of AML in the WHO classification [1]. Therapy-related AML (*t*-AML) was identified in about 7% of large-scale population-based AML registries, whereas the risk of *t*-MN is reportedly increasing, possibly because of the change in therapeutic strategies for malignant diseases [2-5]. Although *t*-MN after treatment with topoisomerase II inhibitors, such as etoposide, develops with a short latency, *t*-MN after treatment with alkylating agents or radiotherapy has a long latency. The former is closely associated with *KMT2A* gene rearrangement, but the latter is with unbalanced aberrations of chromosomes 5 and 7 and complex karyotypes [6]. It has been reported that several mechanisms are involved in the development of *t*-MN: induction of oncogenes and/or genetic instability, and the proliferation of a pre-existing clone

[7]. Recent advances in genetic analysis have revealed that *TP53* mutation is the most frequently identified in *t*-MN [8-10]. Mutations in epigenetic modifying genes, such as *TET2*, *DNMT3A IDH1/2* and *ASXL1*, and *RUNX1* are also frequently identified in *t*-MN [10]. Selection and proliferation of the pre-existing clone were observed in several *t*-MN patients. In those cases, a *TP53*-mutated clone was frequently identified in the peripheral blood (PB) before the onset of *t*-MN. Furthermore, it has been reported that clonal hematopoiesis exists in healthy individuals, and it may be a risk factor for the development of *t*-MN [11-14]. In those clones, *DNMT3A, TET2,* and *ASXL1* mutations are frequently involved, but the presence of *TP53* mutation reportedly increases the risk of *t*-MN development. Therefore, comparable genetic analysis between *t*-MN and the initial tumor cells is important to clarify the mechanism of clonal expansion during *t*-MN development.

In this study, we analyzed the mutation status in 13 *t*-MN patients, and conducted exome analysis in paired samples of three patients with *t*-MN developing after other lymphoid malignancies.

Patients and Methods

Patients and samples

The diagnosis of AML and MDS was based on the morphology, histopathology, expression of leukocyte differentiation antigens, and revised 2016 WHO classification [1]. Bone marrow (BM) samples from patients with *t*-AML or *t*-MDS were subjected to Ficoll-Hypaque density gradient centrifugation. For the germ-line control, buccal swabs or PB mononuclear cells (MNCs) in the disease-free period of each patient were collected. We obtained informed consent from all patients to use their samples for banking and molecular analysis, and approval was obtained from the ethics committee of the Nagoya University School of Medicine.

Cytogenetic and mutation analyses

Cytogenetic G-banding analysis was performed using standard methods. We also examined 11 chimeric gene transcripts (Major *BCR-ABL1*, Minor *BCR-ABL1*, *PML-RARA*, *RUNX1-RUNX1T1*, *CBFB-MYH11*, *DEK-NUP214*, *NUP98-HOXA9*, *MLLT1-KMT2A*, *MLLT2-KMT2A, MLLT3-KMT2A,* and *MLLT4-KMT2A*) by reverse transcriptase-mediated quantitative PCR (RQ-PCR), as previously reported [15].

High-molecular-weight DNA was extracted from the BM samples and buccal swabs using the QIAamp DNA Blood Mini Kit or QIAamp DNA investigator Kit (QIAGEN, Hilden, Germany). Target sequencing of 54 genes, which are frequently identified in the presence of myeloid malignancies, was performed using TruSight Myeloid Sequencing Panel according to the manufacturer's instructions (Illumina, San Diego, CA) (Supplementary Table 2). Additionally, we analyzed *DDX41* and *ANKRD26* mutations using TruSight Custom Amplicon Sequencing Panel (Illumina).

For exome analysis, we employed the TruSight One Sequencing Panel (Illumina), which targeted the coding region of 4,813 genes associated with the

clinical phenotype. The samples were prepared according to the manufacturer's instructions, followed by analysis with the MiSeq sequencer (Illumina). For mutation calling, Variant Studio software (Illumina) was used and targeting regions with a sequencing depth over 20 were considered suitable for analysis, and all mutations were confirmed by the Sanger sequencing method.

Results

Patient characteristics

The 13 *t*-MN patients consisted of nine with MDS, two with AML, and two with chronic myelomonocytic leukemia (CMML) (Table 1). Six of the 13 *t*-MN patients received chemotherapy for hematopoietic neoplasms: four with malignant lymphoma (ML) and two with multiple myeloma (MM). The other seven patients had solid tumors including germ cell tumor, bladder tumor, esophageal tumor, ovarian tumor, myxofibrosarcoma and liposarcoma.

Eight patients received alkylating agents, three platinum agents, one a topoisomerase inhibitor, and one alkylating agents and a topoisomerase inhibitor. Latencies of *t*-MN from the last administration of chemotherapy for the original tumor varied from one to 84 months, and one patient developed MDS during the treatment for malignant lymphoma. Although two of the 13 *t*-MN cells showed a normal karyotype by the G-banding analysis, 11 showed

abnormal karyotypes: eight showed a complex karyotype and each one t(15;17)(q22;q21), del(13)(q12q22), and t(11;19)(8q23;p13.1) and i(17)(q10). (Table 1 and Supplementary Table 2).

Mutation status

We first analyzed mutations in 54 genes as well as *DDX41* and *ANKRD26* by the target sequencing. *TP53* and *TET2* mutations were the most frequently identified, in six patients each, and two patients had both mutations. In addition, *DNMT3A, EZH2, IDH2, GATA2, NOTCH1, PTPN11,* and *ZRSR2* mutations were identified in the *t*-MN patients analyzed. However, mutations in frequently identified *de novo* AML and MDS, such as *FLT3, NPM1,* and spliceosome-related genes, were not observed in *t*-MN (Table 2). No mutation in *DDX41* or *ANKRD26* genes was identified in any patients.

Next, we compared the mutation status between *t*-MN and each original tumor by exome sequencing in three patients treated for lymphoid malignancies.

The UPN-5 patient developed *t*-MDS 30 months after the last chemotherapy for MM. In the BM cells 48 months after the diagnosis of *t*-MDS, mutations in 28 genes including *TP53* and *TET2* genes were identified. In the MM cells at diagnosis, mutations in 24 genes including *SF3B4* and *DNMT3A* genes were identified; however, no common mutations associated with MDS were identified (Figure 1 and Supplementary Table 3).

The UPN-7 patient was initially diagnosed with follicular lymphoma (FL) with t(14;18), and treated with chemotherapy including high-dose chemotherapy followed by autologous peripheral blood stem cell transplantation (auto-PBSCT). Forty-eight months after the last administration of chemotherapy, the patient developed CMML. Cytogenetic G-banding analysis showed the normal karyotype. In the CMML cells, mutations in 27 genes including *TET2, ZRSR2, and EZH2* were identified. In the FL cells, 24 gene mutations were identified, and the same *TET2* mutation as noted in the CMML cells was identified (Figure 2A and Supplementary Table 4). Although the *TET2* mutation was not detected in the germ-line control obtained from the buccal swab, it was identified

in the PBSC, which were harvested for auto-PBSCT (Figure 2B). Since karyotypic analysis revealed t(14;18) in FL cells, we cloned the specific *BCL2-IgH* gene rearrangement as previously reported. We determined that the FL cells had the *BCL2-JH3* rearrangement, and prepared a FL-specific primer pair based on the rearranged sequence for PCR amplification. As shown in Figure 2C, the FL-specific *BCL2-JH3* rearrangement was detected by PCR in FL cells, but not in PBSC, CMML cells or in the germ-line control, indicating that the persistent FL cells were extremely rare after auto-PBSCT. These results collectively suggest that the *TET2* mutation somatically occurred, and that the *TET2*-mutated clone might be a common progenitor for FL and CMML.

The UPN-10 patient had a long history of chemotherapy for FL, and developed MDS one month after the last chemotherapy for FL when the patient did not achieve the complete remission (CR) of FL. In the MDS cells, mutations in 14 genes including the *TET2* gene were identified. In FL cells, mutations in 39 genes including *BCL2, CD79B,* and *EZH2* genes were identified. Of note is the fact that the *MYB* p.S404X mutation was identified in FL and MDS cells and

germ-line control from the buccal swab at almost the same variant allele frequencies: 50.3, 41.8, and 52.2%, respectively (Figure 3 and Supplementary Table 5). This indicated that the *MYB* mutation was a germ-line mutation, but we could not perform a familial study.

Discussion

In this study, we analyzed the mutation status of 13 *t*-MN patients, and demonstrated that *TP53* and epigenetic modifier genes, such as *TET2*, were frequently mutated as previously reported [8, 9]. It has been reported that a part of *t*-MN is caused by inherited cancer susceptibility. *TP53* mutation was also reported to increase the risk of cancer development as well as *t*-MN, and was sometimes observed as a germ-line deposition [10]. Although we identified *TP53* mutation in six *t*-MN patients, all were confirmed to be somatic mutations based on analysis using each germ-line control. We also examined mutations in *DDX41* and *ANKRD26*, which were included in the category of myeloid neoplasms with a germ-line predisposition in the 2016 revised WHO classification, whereas no germ-line mutations were identified in those genes. On the other hand, we identified the germ-line *MYB* mutation in the patient with *t*-MDS developing during the chemotherapy for FL. MYB is an essential transcription factor for regulating hematopoiesis, and it is critical for lymphocyte

development [16, 17]. Recurrent mutations and rearrangements of the *MYB* gene were identified in T-acute lymphoblastic leukemia (T-ALL) [18]. Furthermore, it has been reported that *MYB* is a direct target of *HOXA9* and *KMT3A* fusion genes, and the activation of MYB is involved in the development of myeloid leukemia [19, 20]. Notably, the truncating *MYB* mutation as identified in UPN-10 was reported in the TK-6 cell line, which was established from the patient with CML in T-lineage transformation [21]. The *MYB* p.S404X mutation resulted in the loss of the negative regulating domain, and the truncated MYB reportedly increased the transactivation ability. Although the *MYB* p.D402X mutation found in the TK-6 cell line was somatically acquired during disease progression, we identified the germ-line *MYB* mutation. In the UPN-10 patient, *BCL2, CD79B,* and *EZH2* mutations, which were recurrently observed and associated with the development of ML [22, 23], were identified in FL cells, and *TET2* mutation, which was closely associated with the development of myeloid leukemia, was identified in MDS cells. These results suggest that the *MYB*-mutated clone might be an initiating clone both for

lymphoid and myeloid neoplasms. Furthermore, the independent acquisition of additional mutations in lymphoid and myeloid progenitors might induce FL and MDS, respectively; however, we unfortunately could not analyze the mutation status in each hematopoietic progenitor fraction.

It has been reported that *t*-MN develops from a pre-existing clone. In the present UPN-7 patient, we identified the same *TET2* mutation in FL and CMML cells. Furthermore, this mutation was also identified in the PB at the CR of FL and before developing CMML. Importantly, the variant allele frequency (VAF) of the mutated *TET2* gene was almost the same, about 50%, among FL, CMML, and PB cells, indicating that the *TET2* mutated clone clonally expanded in this patient. Age-dependent clonal hematopoiesis was reported in healthy individuals, while the VAF was usually very low [11]. Therefore, the *TET2* mutated clone might expand by acquiring other genetic alterations, which provide a growth advantage. We could not examine the presence of clonal hematopoiesis with *TET2* mutation before developing FL, nor identify another mutation in PB cells during the disease-free period; however, the present results

suggest that the proliferation of the pre-malignant clone harboring the potency for developing both lymphoid and myeloid neoplasms might be a risk factor for not only the initial but also subsequent hematological malignancies.

In the UPN-5 patient who developed MDS after MM, we could not identify common mutations among MDS and MM cells. In this case, the *DNMT3A* and *TET2* mutations, which were frequently found in age-dependent clonal hematopoiesis, were identified in the initial MM and t-MDS cells, respectively, whereas neither mutation was found in PB in the disease-free period. In the MDS cells, the VAF of *TP53* (65.8%) was almost two-times higher than that of *TET2* mutation (37.2%). However, since the karyotype analysis of MDS cells showed monosomy 17, the major MDS clone might acquire both mutations. These results collectively suggest that *TET2* and *TP53* mutations occurred during the four years of the disease-free period, and that they interacted to promote the development and progression of MDS and subsequent transformation to AML. Furthermore, since *t*-MDS cells revealed a complex karyotype, genetic instability, along with these mutations, might lead to the

development of *t*-MDS. However, we could not determine the common initiating clone, which led to the development of both MM and MDS. Further comprehensive study is necessary to clarify whether the common initiating clone with the ability to develop both lymphoid and myeloid malignancies is present, and whether another mechanism associated with cancer susceptibility exists.

In summary, we demonstrated that *t*-MN cells preferentially acquired mutations in *TP53* and epigenetic modifying genes instead of mutations in tyrosine kinase and spliceosome genes, which are frequently identified in *de novo* AML and/or MDS cells. Furthermore, we identified common mutations among *t*-MN and the initial lymphoid malignant cells in two patients: one was a somatic *TET2* mutation, and the other was a germ-line *MYB* mutation. These results indicate that a common initiating clone might be developed by both a somatic and germ-line mutation; however, further analysis is necessary to clarify the mechanism required to provide the initiating clone with lineage commitment and clonal expansion.

Authorship

T.N., Y.I. and H.K. designed the study, interpreted the data, and wrote the manuscript; T.N., Y.I., A.A., Y.A., H.H., and Y.U. performed molecular analysis and interpreted the data; T.N., Y.I., Y.A., Y.U., and H.K. collected samples and clinical data, contributed to the interpretation of the data, and critically reviewed the manuscript; and all authors approved the final version submitted for publication.

Conflict of interest

H.K. received research funding from Chugai Pharmaceutical Co. Ltd., Bristol-Myers Squibb, Kyowa Hakko Kirin Co. Ltd., Zenyaku Kogyo Co. Ltd., FUJIFILM Corporation, Nippon Boehringer Ingelheim Co. Ltd., Astellas Pharma Inc. and Celgene Corporation, consulting fees from Astellas Pharma Inc. and Daiichi Sankyo Co. Ltd., and honoraria from Bristol-Myers Squibb and Pfizer Japan Inc. The other authors have no relevant conflicts to disclose.

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Figure Legends

Figure 1. Comparison of mutational status of primary lymphoid malignancy, *t*-MN, and germ-line control cells in the UPN-5 patient.

The venn diagrams show the representative mutations in primary and secondary hematological malignancies and the germ-line control in the UPN-5 patient. Arrow heads indicate the point of sampling.

Figure 2. Comparison of mutational status of primary lymphoid malignancy, *t*-MN, and germ-line control cells in the UPN-7 patient.

A: The venn diagrams show the representative mutations in primary and secondary hematological malignancies and the germ-line control in the UPN-7 patient. Arrow heads indicate the point of sampling. B: Sequence diagrams of the *TET2* gene by the Sanger method in FL cells, PBSC, CMML cells, and the germ-line control. C: PCR amplifications of the *BCL2-JH3* region in FL cells, PBSC, CMML cells, and the germ-line control. The *BCL-JH3* rearrangement was detected only in FL cells.

Figure 3. Comparison of mutational status of primary lymphoid malignancy, *t*-MN, and germ-line control cells in the UPN-10 patient.

A: The venn diagrams show the representative mutations in primary and secondary hematological malignancies and the germ-line control in the UPN-10 patient. Arrow heads indicate the point of sampling. B: Sequence diagrams of the *MYB* gene by the Sanger method in FL and MDS cells, and the germ-line control.

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Table 1. Characteristics and karyotype of *t*-MN patients

UPN	Age (years)	Primary tumor	Chemotherapy	*t*-MN	Karyotype	Interval (months)	*Genetic alterations in 54 genes*
1	24	Germ cell tumor	Topoisomerase inhibitor	MDS	complex	4	*TP53*
2	65	Bladder tumor	Platinum-based agent	MDS	complex	36	*TP53, DNMT3A, NOTCH1*
3	57	Non-Hodgkin lymphoma	Alkylating agents	CMML	complex	12	*TP53*
4	71	Esophageal tumor	Platinum-based agent	MDS	complex	6	*TP53*
5	66	Multiple Myeloma	Alkylating agents	MDS	complex	30	*TP53, TET2*
6	71	Ovarian tumor	Platinum-based agent	MDS	complex	52	*TP53, TET2*
7	58	Non-Hodgkin lymphoma	Alkylating agents	CMML	normal	48	*TET2, EZH2, ZRSR2*
8	27	Myxofibrosarcoma	Alkylating agents	AML	t(15;17)	3	*TET2*
9	66	Liposarcoma	Alkylating agents	AML	complex	6	*TET2*
10	72	Non-Hodgkin lymphoma	Alkylating agents	MDS	del(13)(q12q22)	1	*TET2*
11	76	Non-Hodgkin lymphoma	Alkylating agents	MDS	normal	84	*DNMT3A, IDH2*
12	39	Multiple Myeloma	Alkylating agents	MDS	complex	48	*PTPN11*
13	25	Germ cell tumor	Topoisomerase inhibitor, Alkylating agents	MDS	t(11;19),i(17)	4	*GATA2*

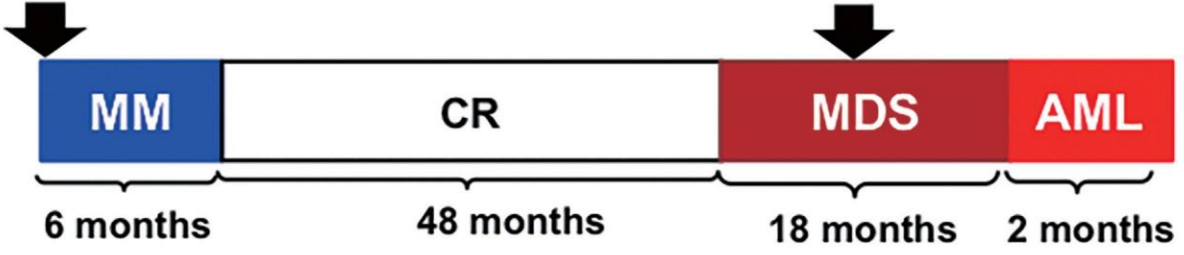
Abbreviations: MDS, myelodysplastic syndromes; CMML, chronic myelomonocytic leukemia; AML, acute myeloid leukemia.

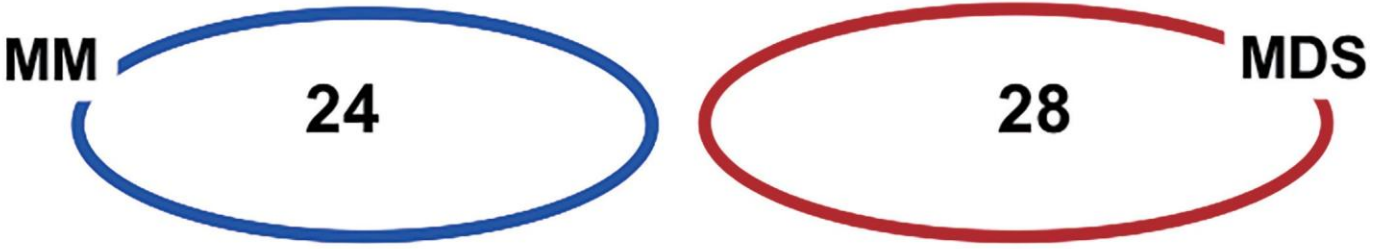
Table 2. The variant allele frequencies (VAFs) on targeted deep sequence analysis in *t*-MN patients

UPN		1	2	3	4	5	6	7	8	9	10	11	12	13
t-MN		MDS	MDS	CMML	MDS	MDS	MDS	CMML	AML	AML	MDS	MDS	MDS	MDS
Karyotype		Complex	Complex	Complex	Complex	Complex	Complex	Normal	t(15;17)	Complex	del(13)	Normal	Complex	t(11;19)
VAFs (%)	*TP53*	92.1	46.7	85.8	96	65.8	73.4	-	-	-	-	-	-	-
	TET2	-	-	-	-	37.2	50.3	46.9	50.1	44.7	7.8	-	-	-
	DNMT3A	-	43.1	-	-	-	-	-	-	-	-	29.8	-	-
	EZH2	-	-	-	-	-	-	16.8	-	-	-	-	-	-
	IDH2	-	-	-	-	-	-	-	-	-	-	24.9	-	-
	GATA2	-	-	-	-	-	-	-	-	-	-	-	-	8.4
	NOTCH1	-	16.9	-	-	-	-	-	-	-	-	-	-	-
	PTPN11	-	-	-	-	-	-	-	-	-	-	-	13.9	-
	ZRSR2	-	-	-	-	-	-	83.1	-	-	-	-	-	-

Abbreviations: MDS, myelodysplastic syndromes; CMML, chronic myelomonocytic leukemia; AML, acute myeloid leukemia.

Figure 1



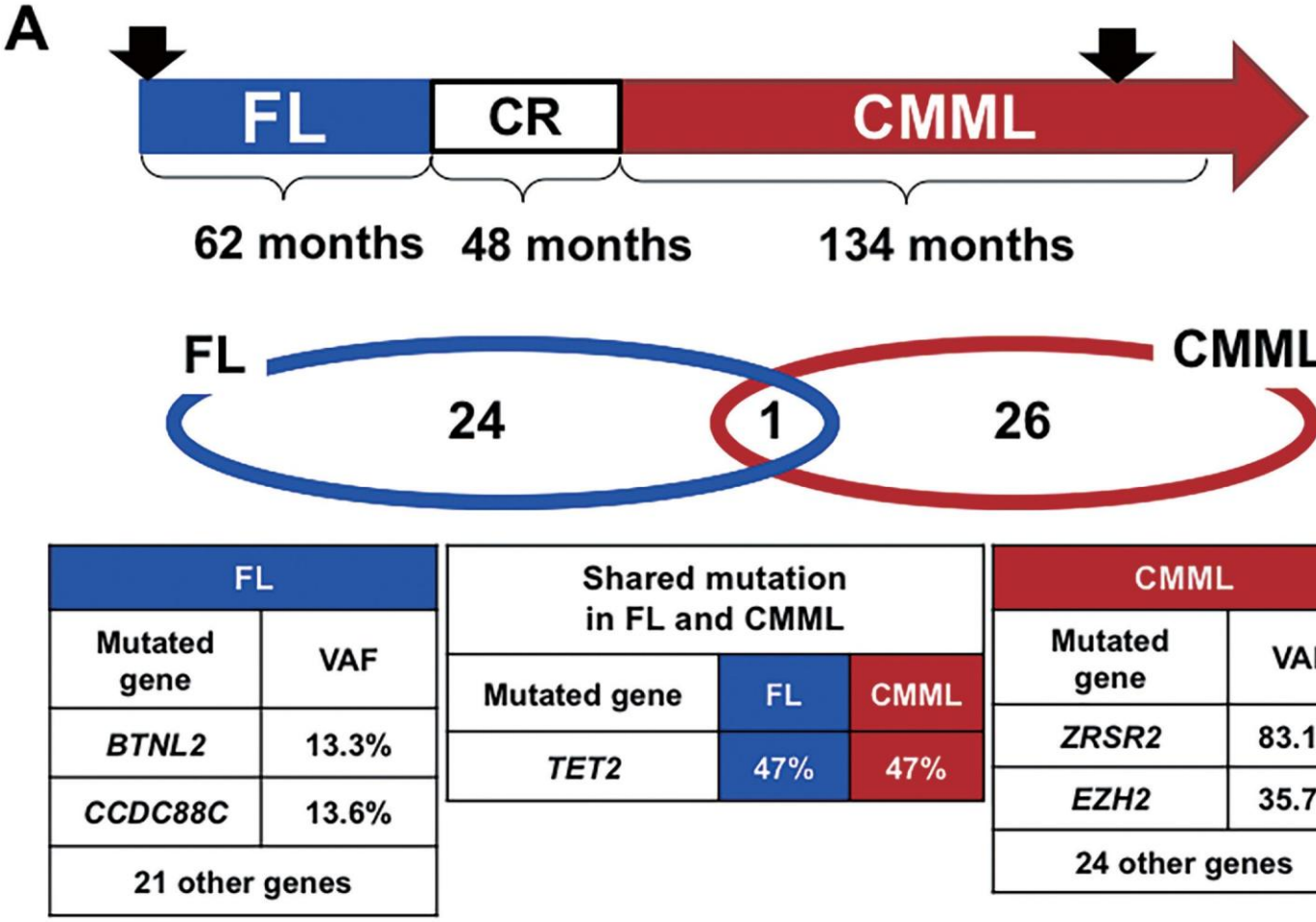


MM	
Mutated gene	VAF
SF3B4	18.1%
DNMT3A	14.5%
22 other genes	

No shared mutation

MDS	
Mutated gene	VAF
TP53	65.8%
TET2	37.2%
26 other genes	

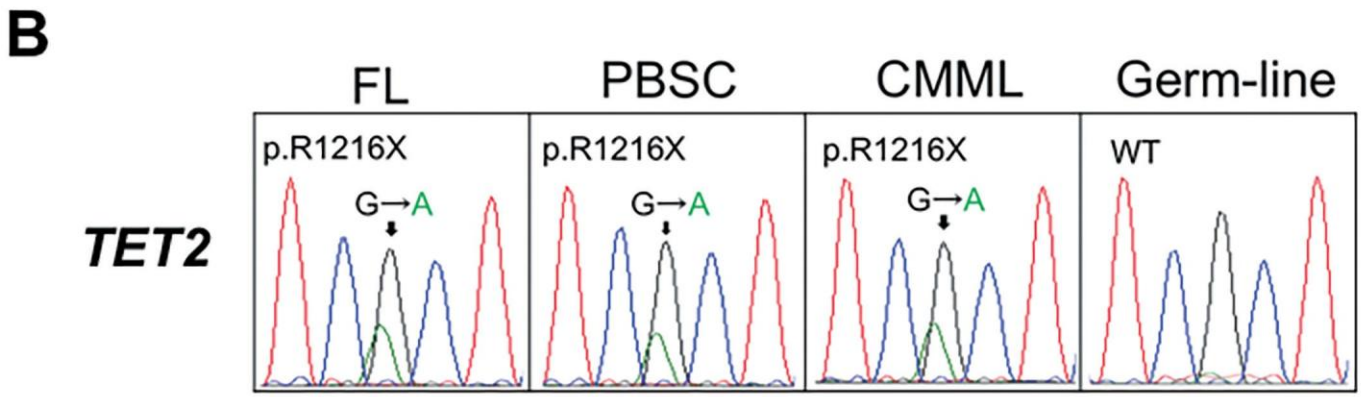
Figure 2



FL	
Mutated gene	VAF
BTNL2	13.3%
CCDC88C	13.6%
21 other genes	

Shared mutation in FL and CMML		
Mutated gene	FL	CMML
TET2	47%	47%

CMML	
Mutated gene	VAF
ZRSR2	83.1%
EZH2	35.7%
24 other genes	



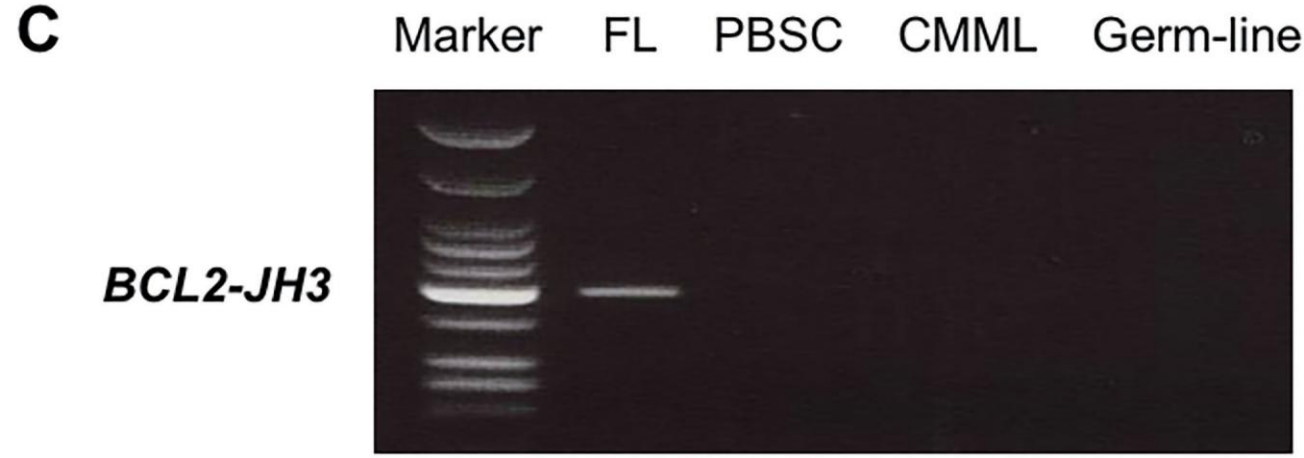
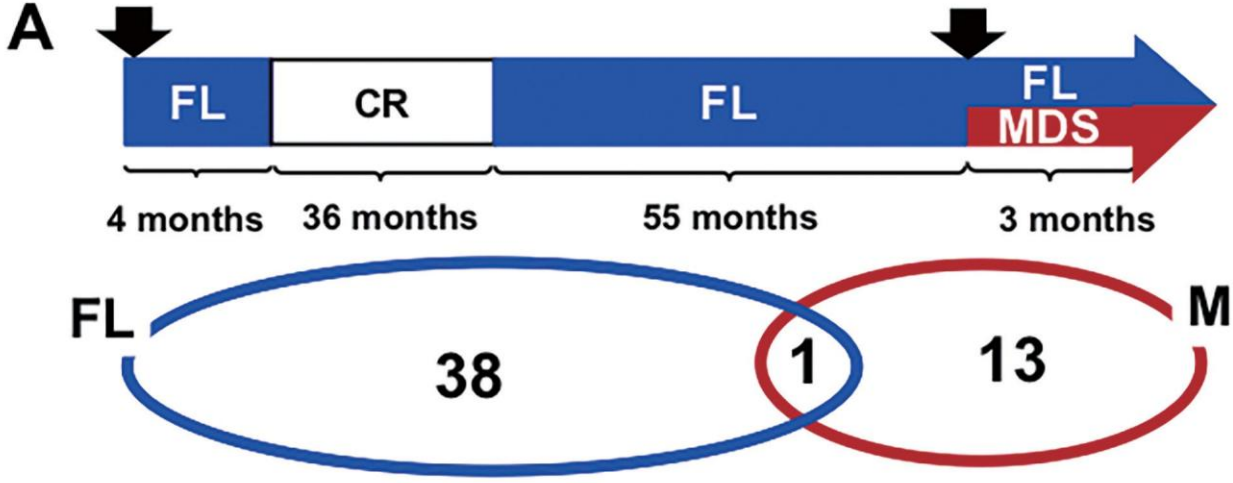


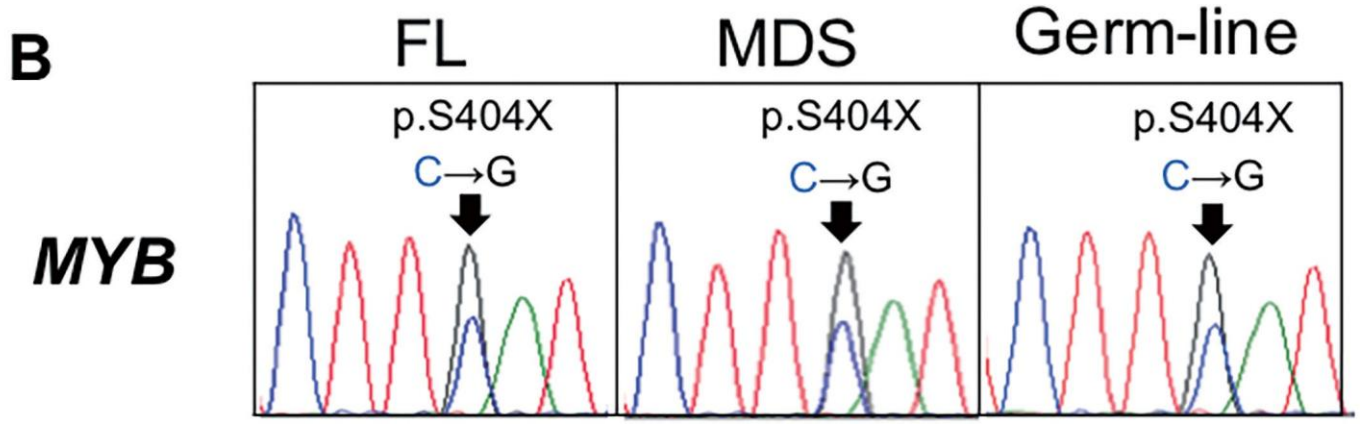
Figure 3



FL	
Mutated gene	VAF
BCL2	43.6%
CD79B	40.7%
EZH2	25%
35 other genes	

Shared mutation in FL, MDS, and germ-line control			
Mutated gene	FL	Germ-line	MDS
MYB (p.S404X)	50.3%	52.2%	41.8%

MDS	
Mutated gene	VAF
TET2	7.8%
12 other genes	



Conflict of interest disclosure

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