Original Article

Title

Subclinical Minute *FLT3*-ITD Clone Can be Detected in Clinically *FLT3*-ITD-Negative Acute Myeloid Leukemia at Diagnosis

Authors

Shota Yokoyama[1], Masahiro Onozawa[1], Shota Yoshida[1], Naoki Miyashita[1], Hiroyuki Kimura[1], Shogo Takahashi[1], Toshihiro Matsukawa[1], Hideki Goto[1,2], Shinichi Fujisawa[2], Kosuke Miki[3], Daisuke Hidaka[4], Junichi Hashiguchi[5], Kentaro Wakasa[6], Makoto Ibata[7], Yukari Takeda[8], Akio Shigematsu[9], Katsuya Fujimoto[10], Yutaka Tsutsumi[11], Akio Mori[12], Toshimichi Ishihara[13], Yasutaka Kakinoki[14], Takeshi Kondo[12], Daigo Hashimoto[1] and Takanori Teshima[1,2]

[1]Department of Hematology, Hokkaido University Faculty of Medicine, Sapporo, Japan
[2]Division of Laboratory and Transfusion Medicine, Hokkaido University Hospital, Sapporo, Japan
[3]Department of Hematology, Teine Keijinkai Hospital, Sapporo, Japan
[4]Department of Hematology, Sapporo Hokuyu Hospital, Sapporo, Japan
[5]Department of Hematology, Kitami Red Cross Hospital, Kitami, Japan
[6]Division of Hematology, Obihiro-Kosei General Hospital, Obihiro, Japan
[7]Department of Hematology, Sapporo-Kosei General Hospital, Sapporo, Japan
[8]Department of Hematology, Tonan Hospital, Sapporo, Japan
[9]Department of Hematology, Kushiro Rosai Hospital, Kushiro, Japan
[10]Department of Hematology, National Hospital Organization Hokkaido Cancer Center, Sapporo, Japan
[11]Department of Hematology, Hakodate Municipal Hospital, Hakodate, Japan
[12]Blood Disorders Center, Aiiku Hospital, Sapporo, Japan
[13]Department of Hematology, Kin-ikyo Chuo Hospital, Sapporo, Japan
[14]Department of Hematology, Asahikawa City Hospital, Asahikawa, Japan
[15]Aiiku Hospital, Sapporo, Japan

Running short title: minute *FLT3*-ITD contributes emergence of FLT3-ITD at relapse

Keywords: minute *FLT3*-ITD, acute myeloid leukemia (AML), relapse, clonal diversity, next-generation sequencing (NGS), Hokkaido Leukemia Net (HLN)

Abstract word count: 200 words
Text word count: 2431 words
The number of tables: 2
The number of figures: 4
Supplemental materials: 0 tables, 2 figure

Corresponding author
Masahiro Onozawa, MD, PhD
Department of Hematology, Hokkaido University Faculty of Medicine, Graduate School of Medicine, Sapporo, Japan
Kita 15, Nishi 7, Kita-Ku, Sapporo, JAPAN 0608638
Phone: +81-11-706-7214
Fax: +81-11-706-7823

Abstract

Recent advances in next-generation sequencing (NGS) have enabled the detection of subclinical minute *FLT3*-ITD. We selected 74 newly diagnosed cytogenetically normal acute myeloid leukemia (AML) samples in which *FLT3*-ITD was not detected by gel electrophoresis. We sequenced them using NGS and found minute *FLT3*-ITDs in 19 cases. We compared cases with clinically relevant *FLT3*-ITD (n=37), cases with minute *FLT3*-ITD (n=19), and cases without detectable *FLT3*-ITD (n=55). Molecular characteristics (location, length) of minute *FLT3*-ITD were similar to those of clinically relevant *FLT3*-ITD. Survival of cases with minute *FLT3*-ITD was similar to that of cases without detectable *FLT3*-ITD, whereas the relapse rate within 1 year after onset was significantly higher in cases with minute *FLT3*-ITD. We followed 18 relapsed samples of cases with clinically *FLT3*-ITD-negative at diagnosis. Two of 3 cases with minute *FLT3*-ITD relapsed with progression to clinically relevant *FLT3*-ITD. Two of 15 cases in which *FLT3*-ITD was not detected by NGS relapsed with the emergence of minute *FLT3*-ITD and one of them showed progression to clinically relevant *FLT3*-ITD at the second relapse. We revealed the clonal dynamics of subclinical minute *FLT3*-ITD in clinically *FLT3*-ITD-negative AML. Minute *FLT3*-ITD at the initial AML can expand to become a dominant clone at relapse.

Introduction

The FMS-like tyrosine kinase 3 internal tandem duplication (*FLT3*-ITD) is the most common recurrent mutation in acute myeloid leukemia (AML) [1,2]. *FLT3*-ITDs are predominately located in exons 14 and 15 and affect the juxtamembrane domain of the membranous tyrosine kinase receptor [3-5]. An oligoclonal *FLT3*-ITD clone has been detected in a single patient [6,7]. Some cases acquired *FLT3*-ITD in relapsed samples despite *FLT3*-ITD being absent in initial AML samples [8-11]. Clinically, *FLT3*-ITD was detected by agarose gel or capillary electrophoresis of the polymerase chain reaction (PCR) product [9,12]. The detection sensitivity of these methods is about 1-5% variant allele frequency (VAF) [12]. The PCR amplicon sequence determined by using next-generation sequencing (NGS) enabled detection of subclinical minute *FLT3*-ITD clones [13-15]. For the cases with emerging *FLT3*-ITD on relapse, retrospective NGS assay of initial AML revealed the existence of a small subclone with *FLT3*-ITD in several cases [11,16]. However, the prevalence of subclinical minute *FLT3*-ITD in clinically *FLT3*-ITD-negative cases was not clarified. We analyzed clinically *FLT3*-ITD-negative samples by NGS to determine the prevalence of subclinical minute *FLT3*-ITD in cases with cytogenetically normal (CN)-AML, because *FLT3*-ITD was most frequent in CN-AML compared to other karyotypes [17]. We followed relapsed samples from the cohort and determined the dynamics of minute *FLT3*-ITDs.

Patients and Methods

Patients

Hokkaido Leukemia Net (HLN) is a prospective cohort study collecting acute leukemia samples from affiliated hospitals in Hokkaido, the northernmost island of Japan [17,18]. For detection of *FLT3*-ITD, genomic DNA from diagnostic samples was amplified by PCR using primer set of *FLT3*-ITD For: 5'-GCAATTTAGGTATGAAAGCCAGC-3' and *FLT3*-ITD Rev: 5'-CTTTCAGCATTTTGACGGCAACC-3' and subjected to 3% agarose gel electrophoresis (100 V, 40 min). *FLT3*-ITD alleric ratio (AR) was determined by standard capillary electrophoresis (CE) for evaluating prognostic stratification by European LeukemiaNet (ELN) 2017 [19]. The results for *FLT3*-ITD were timely returned to the doctors in charge. In this study, we focused on cytogenetically normal (CN) AML without *FLT3*-ITD diagnosed by gel electrophoresis. We

retrospectively analyzed the samples by NGS to detect subclinical minute *FLT3*-ITD. This study was conducted in compliance with the ethical principles based on the Declaration of Helsinki and was approved by the institutional review board of Hokkaido University Hospital (No. 015-0344). Written consent for participation in the study was obtained from all of the patients.

FLT3-ITD NGS assay

Genomic DNA was purified using a QIAamp DNA Mini Kit (QIAGEN, Hulsterweg, Netherlands). Genomic DNA was PCR-amplified using high-fidelity Taq polymerase (PrimerSTAR GXL DNA Polymerase, TAKARA BIO INC, Shiga, Japan) with forward and reverse primers [forward primer: 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-GCAATTTAGGTATGAAAGCCAGC-3', reverse primer: 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-CTTTCGCATTTTGACGGCAACC-3', each consisting of the adapter sequence following the *FLT3* locus-specific sequence (underline) targeting *FLT3* exons 14 and 15)]. PCR products were gel-purified using a QIAquick Gel Purification Kit (QIAGEN). Purified PCR products were deep-sequenced at Research Institute for Microbial Disease, Osaka University (Osaka, Japan) using a Miseq sequencer (Illumina, San Diego, CA) with 250 bp paired-end reads. To establish sensitivity that is sufficient for the detection of 1 mutated allele in 10,000 alleles, a minimum input of 100 ng genomic DNA (30,000 alleles /15,000 cells equivalent) was tested. Each sample was sequenced at a more than 20,000 paired-end read depth. Paired-end sequencing data were sorted by read length. The reference sequence was amplified as a 329-bp read. We manually extracted elongation events equal to or more than 3 bp. A BLAT search was performed at the University of California Santa Cruz genome browser to verify genomic alterations (https://genome.ucsc.edu/cgi-bin/hgBlat). The specific location of the inserted sequence of the duplicated sequence was verified with or without several non-templated-nucleotide filler sequences between duplicated sequences. The length, origin, and VAF of each event were analyzed. Using an *FLT3*-ITD-positive AML cell line (MOLM-14), the limit of quantitative detection of minute *FLT3*-ITD was 0.0067% (Figure S1). On the other hand, none of the samples from healthy individuals (N=3) showed FLT3-ITD > 0.0067% by NGS assay. We defined "minute *FLT3*-ITD" as one that was

not detected by gel electrophoresis but was detected by NGS with a VAF of more than 0.0067%.

Statistical analysis

Overall survival (OS) was calculated as the time from the date of diagnosis to death or last follow-up; the probability of OS was estimated using the Kaplan–Meier method, and differences between patient groups were analyzed using the log-rank test. Cumulative incidence of relapse (CIR) was computed according to the method described by Gray's test. The Mann–Whitney *U* test was used to compare data between groups. P-values < 0.05 were considered statistically significant. All statistical analyses were performed using EZR [20].

Results

A total of 111 CN-AML cases were registered in HLN from January 2017 to December 2019. Thirty-seven cases were *FLT3*-ITD-positive by gel electrophoresis (cases with clinically relevant *FLT3*-ITD: cr*FLT3*-ITD). The remaining 74 clinically *FLT3*-ITD-negative cases were analyzed by the NGS assay to determine whether they had subclinical minute *FLT3*-ITD clones. Surprisingly, 28 independent minute *FLT3*-ITDs were identified in 19 cases (minute *FLT3*-ITD: m*FLT3*-ITD) and they accounted for 25.7% of the clinically *FLT3*-ITD-negative cases. We added an analysis of capillary electrophoresis for all samples with *FLT3*-ITD detected by NGS. CE detected *FLT3*-ITD in all cases with clinically relevant *FLT3*-ITD. On the other hand, CE could not detect cases with minute *FLT3*-ITD except for 1 case with a borderline *FLT3*-ITD allelic ratio (AR) of 0.066. We divided the patients into 3 groups (cases with cr*FLT3*-ITD, cases with m*FLT3*-ITD, and cases without detectable *FLT3*-ITD). The characteristics of each patient group are shown in Table 1. The percentage of blast cells, white blood cell count, *WT1* expression and percentage of cases with *NPM1* mutation were higher in cases with cr*FLT3*-ITD and cases with m*FLT3*-ITD compared to cases without detectable *FLT3*-ITD (Table1). The number of patients treated with intensive chemotherapy was larger in the cr*FLT3*-ITD group. The percentage of patients who received allogeneic stem cell transplantation was larger in the cr*FLT3*-ITD group, but the difference was not significant.

To clarify the characteristics of m*FLT3*-ITD, we analyzed 19 of 37 cr*FLT3*-ITD samples by NGS as a control. The characteristics of m*FLT3*-ITDs were compared to those of cr*FLT3*-ITD with the highest VAF in each case (n=19) (Table 2). VAF of *FLT3*-ITD was higher in cases with cr*FLT3*-ITD (cr*FLT3*-ITD vs m*FLT3*-ITD, VAF: 2.6-45.7%, median 27.5% vs 0.0077-3.1%, median 0.16%) (P<0.0001). *FLT3*-ITDs with filler sequences were more common in cases with cr*FLT3*-ITDs than in cases with m*FLT3*-ITDs (cr*FLT3*-ITD: 52.6% vs m*FLT3*-ITD: 14.3%, P=0.0085). Other parameters such as location or length of the ITD were comparable in the cr*FLT3*-ITD and m*FLT3*-ITD groups (Figure S2). The location of the ITD showed a cluster at exon 14 of *FLT3* (Figure S2). Fifteen (78.9%) of the 19 cases with cr*FLT3*-ITD and 5 (26.3%) of the 19 cases with m*FLT3*-ITD had more than two independent m*FLT3*-ITDs (Figure 1A). Oligoclonal m*FLT3*-ITDs were also detected in cases with cr*FLT3*-ITD. The number of independent ITDs ranged from 1 to 5 (median number:1) per patient in cases with m*FLT3*-ITD. The number of independent ITDs was larger in cases with cr*FLT3*-ITD (1-6, median: 3) (P<0.0027) (Figure 1B). The lengths of both *FLT3*-ITDs were similar (cr*FLT3*-ITD: 18-96 bp, median 48 bp vs m*FLT3*-ITD: 18-93 bp, median 43.5 bp) (Figure 1C) and always multiples of 3 (3N) (Table 2). The findings suggested that m*FLT3*-ITD was in-frame and had been biologically selected by its functional mutation. Clones with *FLT3*-ITD always accounted for a small population of blasts in cases with m*FLT3*-ITD (Figure 1D). On the other hand, *FLT3*-ITD percentages were mostly proportional to blast percentages in cases with cr*FLT3*-ITD, whereas cr*FLT3*-ITD could also represent a fraction of blast cells in some cases with cr*FLT3*-ITD (Figure 1D).

The 5-year survival rates of cases with cr*FLT3*-ITD, cases with m*FLT3*-ITD and cases without detectable *FLT3*-ITD were 25.7%, 53.6% and 50.2%, respectively (Figure 2A). Cases with cr*FLT3*-ITD tended to have a poor prognosis. In contrast, cases with cr*FLT3*-ITD and cases with m*FLT3*-ITD had higher relapse rates at 1 year than that in cases without detectable *FLT3*-ITD (Figure 2B).

In a median follow-up period of 25 months after initial diagnosis, 33 cases relapsed among 72 cases who achieved complete remission (CR) (11 cases with cr*FLT3*-ITD, 7 cases with m*FLT3*-ITD, and 15 cases without detectable *FLT3*-ITD). We collected 23 relapse specimens from 18 of the 22 cases in the latter two groups with clinically *FLT3*-ITD-negative [first relapse

(rel1), 18; second relapse (rel2), 5]. *FLT3*-ITD became clinically relevant in 2 cases in rel1 and 2 cases in rel2 (Figure 3A, B). Regarding rel1, 2 of 3 cases in the initial m*FLT3*-ITD-positive group developed clinically relevant *FLT3*-ITD, while none of the 15 cases in the *FLT3*-ITD undetected group developed cr*FLT3*-ITD (P=0.020). In both rel1 and rel2, cases that developed cr*FLT3*-ITD were positive for m*FLT3*-ITD at the preceding time point.

Discussion

Conventionally *FLT3*-ITD was detected by agarose gel or capillary electrophoresis [7]. An oligoclonal *FLT3*-ITD was detected by a conventional assay [6,7]. Detection limits for *FLT3*-ITD by gel electrophoresis and capillary electrophoresis were reported to be 0.034−0.072 and 0.017 in *FLT3*-ITD AR, which represented 3.3-6.7% and 1.7% in VAF [12]. Recent advances in NGS have enabled the detection of subclinical minute *FLT3*-ITDs [14,16,21]. The detection limit of *FLT3*-ITD by NGS has reached 0.001-0.01%. [13,14,16,22] A sensitive method revealed more heterogeneity of *FLT3*-ITD in each patient that might be correlated with prognosis [6,23]. Detection of m*FLT3*-ITD is also useful for MRD monitoring in *FLT3*-ITD-positive cases [13-15,22,24]. The latest consensus document from the ELN MRD working party placed the *FLT3*-ITD as the level of evidence: IV, grade of recommendation: B with the level of agreement of 94% [25]. The document said the detection of *FLT3*-ITD most likely represents residual AML when detected, but is often subclonal and has a low negative predictive value and better to use in combination with additional MRD makers [25].

Clones with *FLT3*-ITD mutation are unstable in the clinical course. *FLT3*-ITD-positive AML can relapse in the absence of a *FLT3*-ITD clone, while clinically *FLT3*-ITD-negative AML can relapse with a *FLT3*-ITD clone [8-11,26]. Retrospective analysis of relapsed AML cases with emerging *FLT3*-ITD revealed the existence of *FLT3*-ITD at a low VAF in the initial AML samples [11,16]. However, there has been no report on the prevalence of m*FLT3*-ITD clones in cases with clinically *FLT3*-ITD-negative AML at diagnosis. We hypothesized that the preexisting m*FLT3*-ITD could expand and contribute to a relapsed disease and we revealed the prevalence of minute *FLT3*-ITD by analyzing clinically *FLT3*-ITD-negative cases. Multiple minute *FLT3*-ITDs were detected in 31.6% (6/19) of cases with minute FLT3-ITD (Figure 2C). The fact

that there are multiple *FLT3*-ITD clones within a single case and the fact that *FLT3*-ITD is present only in a subset of blasts suggest that the *FLT3*-ITD mutation is an event that occurs in the late stages of AML pathogenesis. Furthermore, single-cell analysis clearly showed that *FLT3*-ITD occurs as a later event in the hierarchy of clonal diversity of AML [27-29].

The presence of *FLT3*-ITD in AML was known as a poor prognostic marker until the introduction of *FLT3* inhibitors [19]. Originally, the existence of *FLT3*-ITD was considered as a poor prognostic maker in the national comprehensive cancer network (NCCN) guideline 2017 [30], then *FLT3*-ITD high (AR>0.5) represented a poor prognostic maker whereas *FLT3*-ITD low (AR<0.5) did not have a prognostic impact compared to *FLT3*-ITD negative in the ELN 2017 and NCCN 2019 guideline [19,31]. The recent update further abrogated the prognostic impact of *FLT3*-ITD under upfront usage of FLT3 inhibitors [32]. Our data suggested that a minute clone with *FLT3*-ITD would not be associated with a poor prognosis because cases with m*FLT3*-ITD showed overall survival similar to that of cases without detectable *FLT3*-ITD.

It is intriguing that fillers at duplicated sites are more common in cr*FLT3*-ITDs than in m*FLT3*-ITDs (Table 2). An N-nucleotide filler at the duplicated site was speculated to be attributed to terminal deoxynucleotidyl transferase (TdT), which plays a key role in immunoglobulin rearrangement [33]. Borrow J et al. reported that a *FLT3*-ITD N-nucleotide filler had a higher G/C content (66.9%), dinucleotide composition, and length characteristics consistent with synthesis by TdT. Other than the percentages of filler sequences at the duplicated sites, we could not find any difference in the location or length of each *FLT3*-ITD between cr*FLT3*-ITDs and m*FLT3*-ITDs in terms of location or length. All m*FLT3*-ITDs were multiples of 3 bp, an in-frame duplication that seemed to be biologically selected with a gain of function. Other factors such as collaborative mutations or environmental conditions might contribute to the expansion of *FLT3*-ITD. Our findings revealed the mechanisms underlying *FLT3*-ITD emergence at relapse despite the mutation being absent in the initial sample. NGS identified m*FLT3*-ITD in one-fourth of cases with clinically *FLT3*-ITD negative AML (Figure 4). Together with cases with cr*FLT3*-ITD, almost half of the CN-AML cases had clones with *FLT3*-ITD which could increase at relapse (Figure 4). Still, we could not predict which *FLT3*-ITD clone expand or disappeared.

Cases with m*FLT3*-ITD had a relapse rate within 1 year after diagnosis that was similar to that in cases with cr*FLT3*-ITD, who were reported to have a shorter duration of remission [26]. *NPM1* mutations positively correlate with AML with *FLT3*-ITD [34]. The percentage of *NPM1* mutation was higher in cases with m*FLT3*-ITD similar to that in cases with cr*FLT3*-ITD. These similarities might be due to background conditions that contribute to the creation of *FLT3*-ITD. *NPM1* mutation, which has been considered to occur in the founder clone of AML, could lead to yielding the clone with *FLT3*-ITD as a later event [27,35].

Expansion or acquired clone with *FLT3*-ITD at relapse is also known as adaptive resistance to venetoclax-based combinations [36]. Venetoclax is a potent BCL2 inhibitor that is used for the treatment of fit and unfit AML [37,38]. Dinardo et al. reported that the expansion (n=3) or acquisition (n=2) of *FLT3*-ITD in patients with adaptive resistance to venetoclax occurred early after initial remission (within 1 to 6 months) [36]. Clonal selection by BCL2 inhibition would confer a growth advantage to the clone with *FLT3*-ITD which up-regulates MCL1 [36,39,40]. Considering these mechanisms, we speculated that cases with m*FLT3*-ITD would have a risk of relapse with emerging *FLT3*-ITD, especially if they are treated by a venetoclax-based regimen.

Conclusion

This study is the first study clarifying the clonal dynamics of minute *FLT3*-ITD in clinically *FLT3*-ITD-negative AML. One-fourth of with clinically *FLT3*-ITD-negative AML cases could have m*FLT3*-ITDs. Cases with m*FLT3*-ITD showed traits shared with cases with cr*FLT3*-ITD such as a higher percentage of blast cells, WBC count, *WT1* expression and percentage of *NPM1* mutation. Minute *FLT3*-ITD at the initial onset of AML could expand to become a dominant clone in relapsed disease. The clinical significance of an *FLT3* inhibitor for patients with m*FLT3*-ITD needs to be clarified in the future.

ACKNOWLEDGEMENTS

We thank the nursing and medical staff for their contributions to this study and their dedicated care of the patients. We thank Mr. Satoshi Oguri, Ms. Kaori Sato and Ms. Ayako Ichikawa for their dedicated support in analyzing *FLT3*-ITD.

AUTHOR CONTRIBUTIONS

Shota Yokoyama and Masahiro Onozawa designed the study, analyzed the data, and wrote the manuscript. Shota Yoshida, Naoki Miyashita, Hiroyuki Kimura, Shogo Takahashi, and Shinichi Fujisawa analyzed patients' samples. Toshihiro Matsukawa, Hideki Goto, and Takeshi Kondo provided a critique of the manuscript. Kosuke Miki, Daisuke Hidaka, Junichi Hashiguchi, Kentaro Wakasa, Makoto Ibata, Yukari Takeda, Akio Shigematsu, Katsuya Fujimoto, Yutaka Tsutsumi, Akio Mori, Toshimichi Ishihara, Yasutaka Kakinoki recruited and treated the patients. Takanori Teshima revised and approved the manuscript. All authors contributed to the final version of the manuscript and approved it for publication.

FUNDING INFORMATION

This work was supported by the Japan Society for the Promotion of Science (JSPS), Scientific Research (C) (20K08745; M.O.).

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

PATIENT CONSENT STATEMENT

Written informed consent was obtained from all individuals included in the study.

PERMISSION TO REPRODUCE MATERIAL FROM OTHER SOURCES

Excerpts from copyrighted works owned by third parties are not included in our manuscript.

CLINICAL TRIAL REGISTRATION (INCLUDING TRIAL NUMBER)

This study was part of a prospective observational study (Hokkaido Leukemia Net, UMIN000048611). It was conducted in compliance with ethical principles based on the Helsinki Declaration and was approved by the institutional review board of Hokkaido University Hospital (#015-0344).

Table 1. Characteristics of the cases categorized by *FLT3*-ITD status

Table 2. Characteristics of clinically relevant and minute *FLT3*-ITD

Figure legends

Figure 1 Comparison of *FLT3*-ITDs in clinically relevant and minute ITDs

A. Identified *FLT3*-ITDs in each case. Each circle represents independent *FLT3*-ITD. Oligoclonal *FLT3*-ITD can be detected in both cases with cr*FLT3*-ITD and cases with m*FLT3*-ITD.

B. Number of independent *FLT3*-ITDs per case

C. Distribution of lengths of *FLT3*-ITDs.

D. Blast percentage and highest *FLT3*-ITD percentage for each case. The dashed line represents the expected percentage of *FLT3*-ITD assuming all blasts have hemizygous *FLT3*-ITD.

Figure 2

A. Overall survival

The total cohort was analyzed. Cases with cr*FLT3*-ITD, n=37; cases with m*FLT3*-ITD, n=19; cases without detectable *FLT3*-ITD, n=55

B. Relapse rate

Patients who achieved CR were analyzed. Cases with cr*FLT3*-ITD, n=23; cases with m*FLT3*-ITD, n=15; cases without detectable *FLT3*-ITD, n=34

Figure 3

A. Dynamics of minute *FLT*3-ITD clones in relapsed cases. *FLT3*-ITD became clinically positive in 2 cases in the first relapse and in 2 cases in the second relapse. In both the first and second relapses, cases that developed clinically positive *FLT3*-ITD had been positive for m*FLT3*-ITD at the preceding time point.

B. Time course of 3 cases that acquired clinically relevant *FLT3*-ITD at relapse. Case 1, Case 2, and Case 3 correspond to each case in Figure 4A. In Case 1, the clone that had existed as m*FLT3*-ITD at initial AML became cr*FLT3*-ITD at relapse. In Case 2 and Case 3, clones that were not present as m*FLT3*-ITD became cr*FLT3*-ITD at relapse. Rel: relapse

Figure 4

Oligoclonal *FLT3*-ITD in AML.

At subclinical levels, oligoclonal minute *FLT3*-ITD exists in *FLT3* wild-type cases and may contribute to relapse with emerging cr*FLT3*-ITD.

References

1. Grimwade D, Ivey A, Huntly BJ. Molecular landscape of acute myeloid leukemia in younger adults and its clinical relevance. *Blood*. 2016;127(1):29-41.
2. Papaemmanuil E, Gerstung M, Bullinger L, et al. Genomic Classification and Prognosis in Acute Myeloid Leukemia. *N Engl J Med*. 2016;374(23):2209-2221.
3. Kiyoi H, Towatari M, Yokota S, et al. Internal tandem duplication of the FLT3 gene is a novel modality of elongation mutation which causes constitutive activation of the product. *Leukemia*. 1998;12(9):1333-1337.
4. Kayser S, Schlenk RF, Londono MC, et al. Insertion of FLT3 internal tandem duplication in the tyrosine kinase domain-1 is associated with resistance to chemotherapy and inferior outcome. *Blood*. 2009;114(12):2386-2392.
5. Schnittger S, Bacher U, Haferlach C, Alpermann T, Kern W, Haferlach T. Diversity of the juxtamembrane and TKD1 mutations (exons 13-15) in the FLT3 gene with regards to mutant load, sequence, length, localization, and correlation with biological data. *Genes Chromosomes Cancer*. 2012;51(10):910-924.
6. Kottaridis PD, Gale RE, Frew ME, et al. The presence of a FLT3 internal tandem duplication in patients with acute myeloid leukemia (AML) adds important prognostic information to cytogenetic risk group and response to the first cycle of chemotherapy: analysis of 854 patients from the United Kingdom Medical Research Council AML 10 and 12 trials. *Blood*. 2001;98(6):1752-1759.
7. Murphy KM, Levis M, Hafez MJ, et al. Detection of FLT3 internal tandem duplication and D835 mutations by a multiplex polymerase chain reaction and capillary electrophoresis assay. *J Mol Diagn*. 2003;5(2):96-102.
8. Kottaridis PD, Gale RE, Langabeer SE, Frew ME, Bowen DT, Linch DC. Studies of FLT3 mutations in paired presentation and relapse samples from patients with acute myeloid leukemia: implications for the role of FLT3 mutations in leukemogenesis, minimal residual disease detection, and possible therapy with FLT3 inhibitors. *Blood*. 2002;100(7):2393-2398.
9. Shih LY, Huang CF, Wu JH, et al. Internal tandem duplication of FLT3 in relapsed acute myeloid leukemia: a comparative analysis of bone marrow samples from 108 adult patients at diagnosis and relapse. *Blood*. 2002;100(7):2387-2392.
10. Wakita S, Yamaguchi H, Omori I, et al. Mutations of the epigenetics-modifying gene (DNMT3a, TET2, IDH1/2) at diagnosis may induce FLT3-ITD at relapse in de novo acute

myeloid leukemia. *Leukemia*. 2013;27(5):1044-1052.
11. Ottone T, Zaza S, Divona M, et al. Identification of emerging FLT3 ITD-positive clones during clinical remission and kinetics of disease relapse in acute myeloid leukaemia with mutated nucleophosmin. *Br J Haematol*. 2013;161(4):533-540.
12. Sakaguchi M, Nakajima N, Yamaguchi H, et al. The sensitivity of the FLT3-ITD detection method is an important consideration when diagnosing acute myeloid leukemia. *Leuk Res Rep*. 2020;13:100198.
13. Levis MJ, Perl AE, Altman JK, et al. A next-generation sequencing-based assay for minimal residual disease assessment in AML patients with FLT3-ITD mutations. *Blood Adv*. 2018;2(8):825-831.
14. Blatte TJ, Schmalbrock LK, Skambraks S, et al. getITD for FLT3-ITD-based MRD monitoring in AML. *Leukemia*. 2019;33(10):2535-2539.
15. Grob T, Sanders MA, Vonk CM, et al. Prognostic Value of FLT3-Internal Tandem Duplication Residual Disease in Acute Myeloid Leukemia. *J Clin Oncol*. 2022:JCO2200715.
16. Zuffa E, Franchini E, Papayannidis C, et al. Revealing very small FLT3 ITD mutated clones by ultra-deep sequencing analysis has important clinical implications in AML patients. *Oncotarget*. 2015;6(31):31284-31294.
17. Hidaka D, Onozawa M, Hashiguchi J, et al. Wilms Tumor 1 Expression at Diagnosis Correlates With Genetic Abnormalities and Polymorphism But Is Not Independently Prognostic in Acute Myelogenous Leukemia: A Hokkaido Leukemia Net Study. *Clin Lymphoma Myeloma Leuk*. 2018;18(11):e469-e479.
18. Yoshida S, Onozawa M, Miyashita N, et al. Clinical features of complex karyotype in newly diagnosed acute myeloid leukemia. *Int J Hematol*. 2022.
19. Dohner H, Estey E, Grimwade D, et al. Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel. *Blood*. 2017;129(4):424-447.
20. Kanda Y. Investigation of the freely available easy-to-use software 'EZR' for medical statistics. *Bone Marrow Transplant*. 2013;48(3):452-458.
21. Spencer DH, Abel HJ, Lockwood CM, et al. Detection of FLT3 internal tandem duplication in targeted, short-read-length, next-generation sequencing data. *J Mol Diagn*. 2013;15(1):81-93.
22. Loo S, Dillon R, Ivey A, et al. Pretransplant FLT3-ITD MRD assessed by high-sensitivity PCR-NGS determines posttransplant clinical outcome. *Blood*. 2022;140(22):2407-2411.
23. Schranz K, Hubmann M, Harin E, et al. Clonal heterogeneity of FLT3-ITD detected by high-throughput amplicon sequencing correlates with adverse prognosis in acute myeloid

leukemia. *Oncotarget*. 2018;9(53):30128-30145.
24. Dillon LW, Gui G, Page KM, et al. DNA Sequencing to Detect Residual Disease in Adults With Acute Myeloid Leukemia Prior to Hematopoietic Cell Transplant. *JAMA*. 2023;329(9):745-755.
25. Heuser M, Freeman SD, Ossenkoppele GJ, et al. 2021 Update on MRD in acute myeloid leukemia: a consensus document from the European LeukemiaNet MRD Working Party. *Blood*. 2021;138(26):2753-2767.
26. Tiesmeier J, Muller-Tidow C, Westermann A, et al. Evolution of FLT3-ITD and D835 activating point mutations in relapsing acute myeloid leukemia and response to salvage therapy. *Leuk Res*. 2004;28(10):1069-1074.
27. Morita K, Wang F, Jahn K, et al. Clonal evolution of acute myeloid leukemia revealed by high-throughput single-cell genomics. *Nat Commun*. 2020;11(1):5327.
28. Shlush LI, Chapal-Ilani N, Adar R, et al. Cell lineage analysis of acute leukemia relapse uncovers the role of replication-rate heterogeneity and microsatellite instability. *Blood*. 2012;120(3):603-612.
29. Shouval R, Shlush LI, Yehudai-Resheff S, et al. Single cell analysis exposes intratumor heterogeneity and suggests that FLT3-ITD is a late event in leukemogenesis. *Exp Hematol*. 2014;42(6):457-463.
30. O'Donnell MR, Tallman MS, Abboud CN, et al. Acute Myeloid Leukemia, Version 3.2017, NCCN Clinical Practice Guidelines in Oncology. *J Natl Compr Canc Netw*. 2017;15(7):926-957.
31. Tallman MS, Wang ES, Altman JK, et al. Acute Myeloid Leukemia, Version 3.2019, NCCN Clinical Practice Guidelines in Oncology. *J Natl Compr Canc Netw*. 2019;17(6):721-749.
32. Dohner H, Wei AH, Appelbaum FR, et al. Diagnosis and management of AML in adults: 2022 recommendations from an international expert panel on behalf of the ELN. *Blood*. 2022;140(12):1345-1377.
33. Borrow J, Dyer SA, Akiki S, Griffiths MJ. Terminal deoxynucleotidyl transferase promotes acute myeloid leukemia by priming FLT3-ITD replication slippage. *Blood*. 2019;134(25):2281-2290.
34. Verhaak RG, Goudswaard CS, van Putten W, et al. Mutations in nucleophosmin (NPM1) in acute myeloid leukemia (AML): association with other gene abnormalities and previously established gene expression signatures and their favorable prognostic significance. *Blood*. 2005;106(12):3747-3754.
35. Kronke J, Bullinger L, Teleanu V, et al. Clonal evolution in relapsed NPM1-mutated acute myeloid leukemia. *Blood*. 2013;122(1):100-108.

500 36. DiNardo CD, Tiong IS, Quaglieri A, et al. Molecular patterns of response and
501 treatment failure after frontline venetoclax combinations in older patients with AML. *Blood*.
502 2020;135(11):791-803.
503 37. DiNardo CD, Jonas BA, Pullarkat V, et al. Azacitidine and Venetoclax in Previously
504 Untreated Acute Myeloid Leukemia. *N Engl J Med*. 2020;383(7):617-629.
505 38. DiNardo CD, Lachowiez CA, Takahashi K, et al. Venetoclax Combined With FLAG-
506 IDA Induction and Consolidation in Newly Diagnosed and Relapsed or Refractory Acute
507 Myeloid Leukemia. *J Clin Oncol*. 2021;39(25):2768-2778.
508 39. Yoshimoto G, Miyamoto T, Jabbarzadeh-Tabrizi S, et al. FLT3-ITD up-regulates
509 MCL-1 to promote survival of stem cells in acute myeloid leukemia via FLT3-ITD-specific
510 STAT5 activation. *Blood*. 2009;114(24):5034-5043.
511 40. Xu Y, Ye H. Progress in understanding the mechanisms of resistance to BCL-2
512 inhibitors. *Exp Hematol Oncol*. 2022;11(1):31.
513

Table 1. Characteristics of cases categorized by *FLT3*-ITD status

determined by gel electrophoresis	Clinically relevant *FLT3*-ITD	Clinically *FLT3*-ITD-Negative		
determined by NGS assay		minute *FLT3*-ITD	without detectable *FLT3*-ITD	
Number of cases	n=37	n=19	n=55	*P* value
Median age [range]	63 [32-90]	65 [21-85]	68 [26-93]	0.33
Gender: Male/Female	20/17	7/12	29/26	0.45
Specimens: BM/PB	28/9	17/2	48/7	0.28
WBC (/μL) [range]	62,165 [680-329,900]	23,100 [460-250,900]	10,700 [800-174,000]	0.0004
BM blast (%) [range]	85.0 [19.0-100.0]	83.0 [29.2-99.0]	66.0 [2.0-98.0]	0.0012
BM *WT-1* expression($\times 10^{-4}$/K562) [range]	1194.0 [23.9-7744.6]	703.0 [11.8-7244.4]	258.8 [1.0-31000.0]	0.000092
***NPM1* mutation positive (%)**	22 (59.5%)	10 (52.6%)	16 (29.1%)	0.0093
ELN2017: Favorable/Intermediate/Adverse	9/23/5	14/5/0	23/32/0	0.00041
Induction regimen				
*Intensive Chemotherapy	31 (83.8%)	13 (68.4%)	33 (60.0%)	0.051
AZA	0 (0.0%)	0 (0.0%)	6 (10.9%)	0.055
Low-dose AraC-based Chemotherapy	3 (8.1%)	5 (26.3%)	11 (20.0%)	0.13
BSC	2 (5.4%)	1 (5.3%)	5 (9.1%)	0.88
Hydroxyurea	1 (2.7%)	0 (0.0%)	0 (0.0%)	0.51
****CR rates**	23/34 (67.6%)	15/18 (83.3%)	34/50 (68.0%)	0.47
*****PIF rates**	11/31 (35.5%)	2/13 (15.4.%)	10/33 (30.3%)	0.46
Allo-HSCT	17 (45.9%)	4 (21.1%)	15 (27.3%)	0.094
Time to Allo SCT, days [range]	152 [92-225]	153.5 [104-417]	152 [92-226]	0.69
Non CR at Allo-HSCT	6/17 (35.3%)	1/4 (25.0%)	4/15 (26.7%)	0.88

Abbreviations, Allo-HSCT: allogeneic-hematopoietic stem cell transplantation, AZA: azacytidine, BM: bone marrow, BSC: best supportive care, CR: complete remission, ELN: European LeukemiaNet, NGS: next-generation sequence, PB: peripheral blood, PIF: primary induction failure, WBC: white blood cell, *WT-1*: Wilms tumor-1 gene,

*Intensive Chemotherapy consisted of anthracyclines and cytarabine.

**CR rates were calculated by excluding BSC and Hydroxyurea cases from the denominator.

***PIF rates were calculated by number of cases that achieved first CR by Intensive Chemotherapy as a denominator.

Table 2. Characteristics of *FLT3*-ITD

	clinically relevant *FLT3*-ITD (n=19)	minute *FLT3*-ITD (n=28)	*P* value
Length [range]	48 bp [18-96 bp]	43.5 bp [18-93 bp]	0.44
In-frame mutation	19 (100%)	28 (100 %)	1
VAF (%) [range]	27.5 [2.6-45.7]	0.16 [0.0077-3.1]	< 0.0001
ITD with filler (%)	10 (52.6%)	4 (14.3%)	0.0085

Figure 1

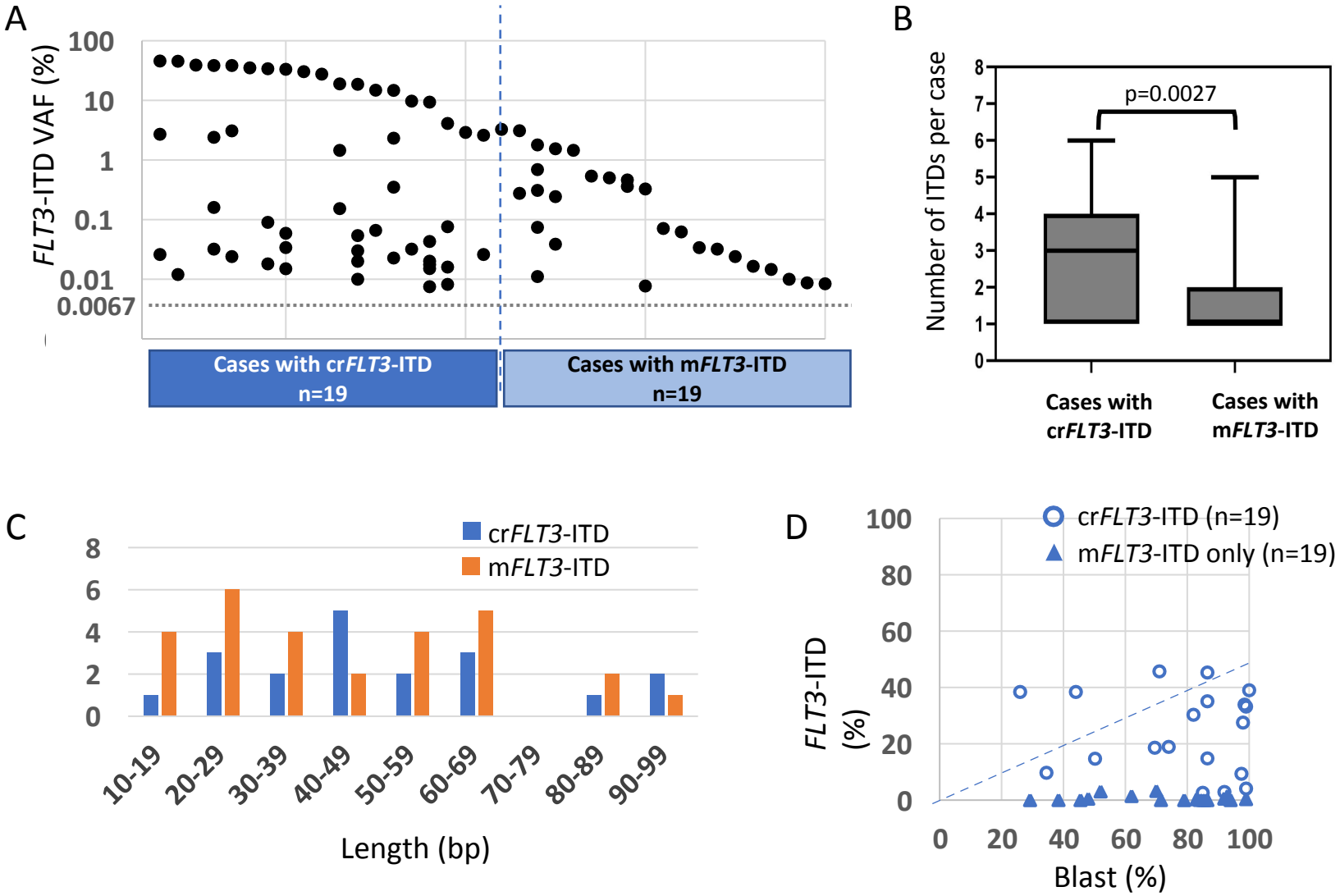

A
FLT3-ITD VAF (%)
100
10
1
0.1
0.01
0.0067
Cases with crFLT3-ITD
n=19
Cases with mFLT3-ITD
n=19
B
Number of ITDs per case
p=0.0027
Cases with crFLT3-ITD
Cases with mFLT3-ITD
C
crFLT3-ITD
mFLT3-ITD
10-19
20-29
30-39
40-49
50-59
60-69
70-79
80-89
90-99
Length (bp)
D
crFLT3-ITD (n=19)
mFLT3-ITD only (n=19)
FLT3-ITD (%)
Blast (%)

Figure 2

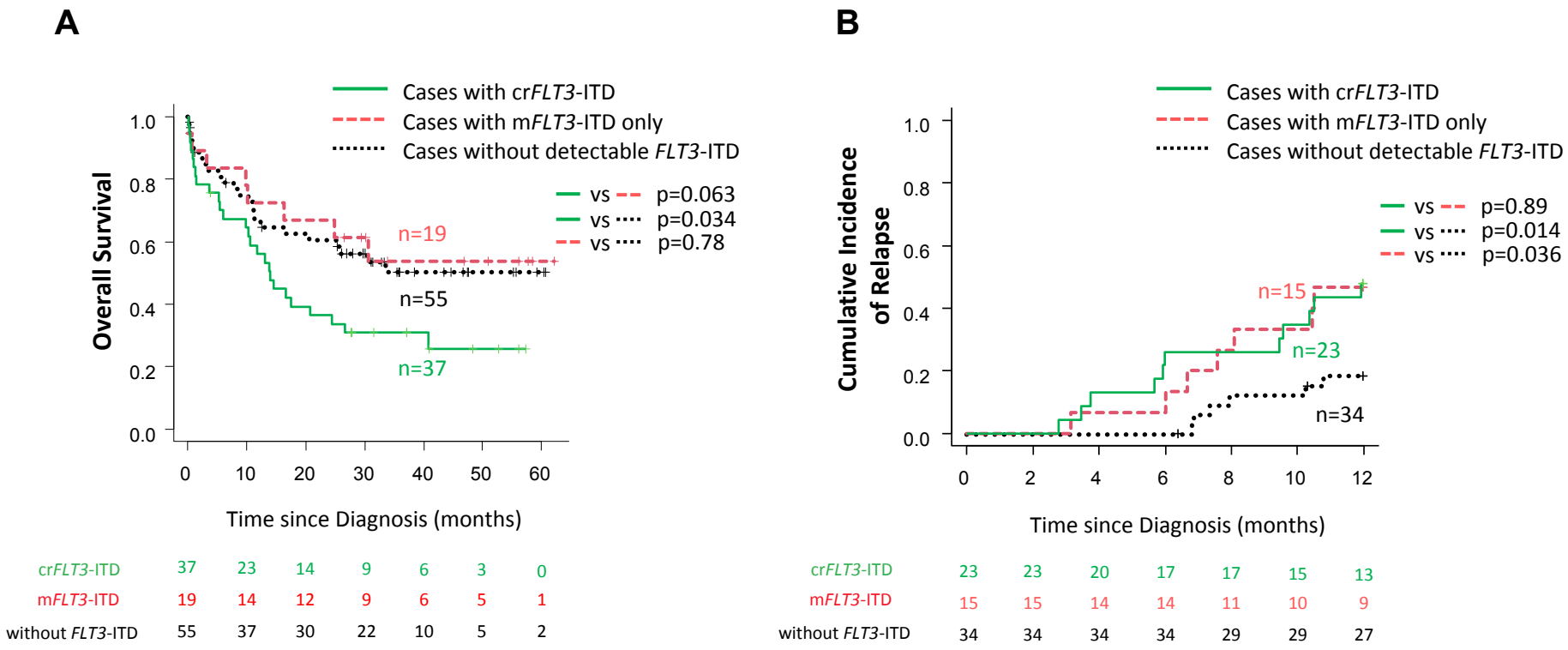

A
Cases with crFLT3-ITD
Cases with mFLT3-ITD only
Cases without detectable FLT3-ITD
vs p=0.063
vs p=0.034
vs p=0.78
Overall Survival
1.0
0.8
0.6
0.4
0.2
0.0
n=19
n=55
n=37
0
10
20
30
40
50
60
Time since Diagnosis (months)
crFLT3-ITD 37 23 14 9 6 3 0
mFLT3-ITD 19 14 12 9 6 5 1
without FLT3-ITD 55 37 30 22 10 5 2
B
Cases with crFLT3-ITD
Cases with mFLT3-ITD only
Cases without detectable FLT3-ITD
vs p=0.89
vs p=0.014
vs p=0.036
Cumulative Incidence of Relapse
1.0
0.8
0.6
0.4
0.2
0.0
n=15
n=23
n=34
0
2
4
6
8
10
12
Time since Diagnosis (months)
crFLT3-ITD 23 23 20 17 17 15 13
mFLT3-ITD 15 15 14 14 11 10 9
without FLT3-ITD 34 34 34 34 29 29 27

Figure 3

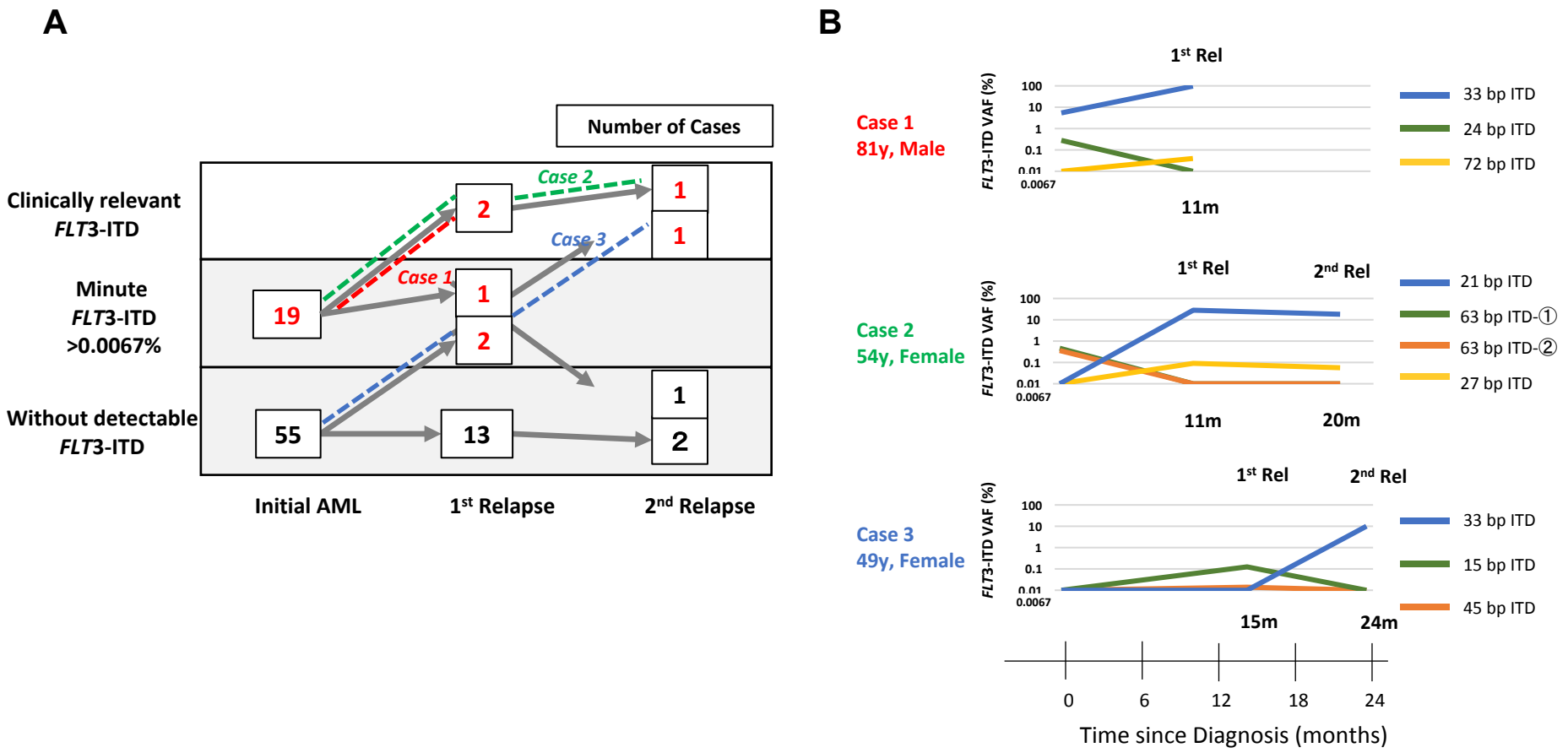

A
Number of Cases
Clinically relevant
FLT3-ITD
Minute
FLT3-ITD
>0.0067%
Without detectable
FLT3-ITD
Case 2
Case 3
Case 1
19
55
2
1
13
1
2
1
1
2
Initial AML
1st Relapse
2nd Relapse
B
Case 1
81y, Male
1st Rel
FLT3-ITD VAF (%)
100
10
1
0.1
0.01
0.0067
11m
33 bp ITD
24 bp ITD
72 bp ITD
Case 2
54y, Female
1st Rel
2nd Rel
11m
20m
21 bp ITD
63 bp ITD-①
63 bp ITD-②
27 bp ITD
Case 3
49y, Female
1st Rel
2nd Rel
15m
24m
33 bp ITD
15 bp ITD
45 bp ITD
0
6
12
18
24
Time since Diagnosis (months)

Figure 4

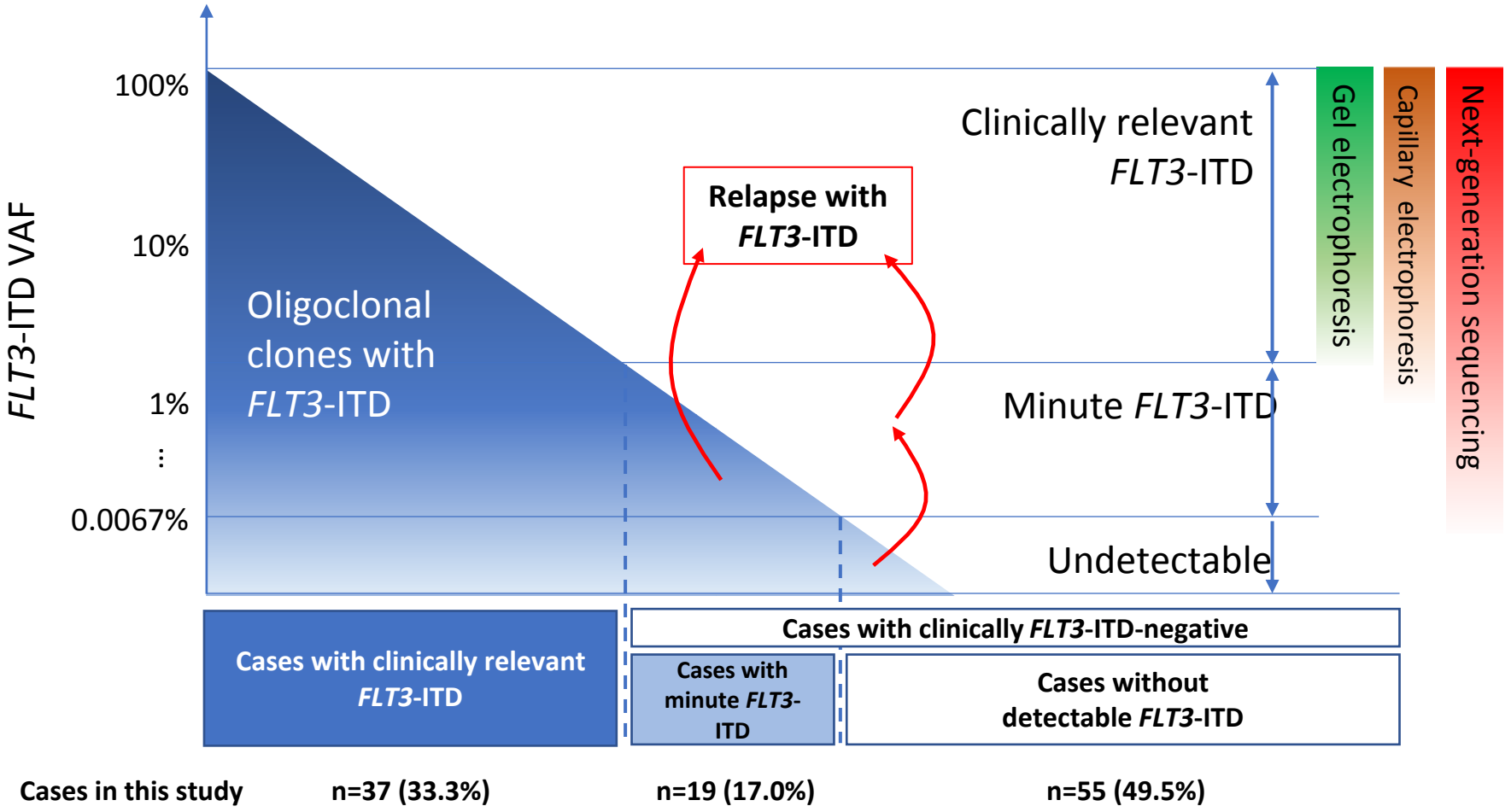
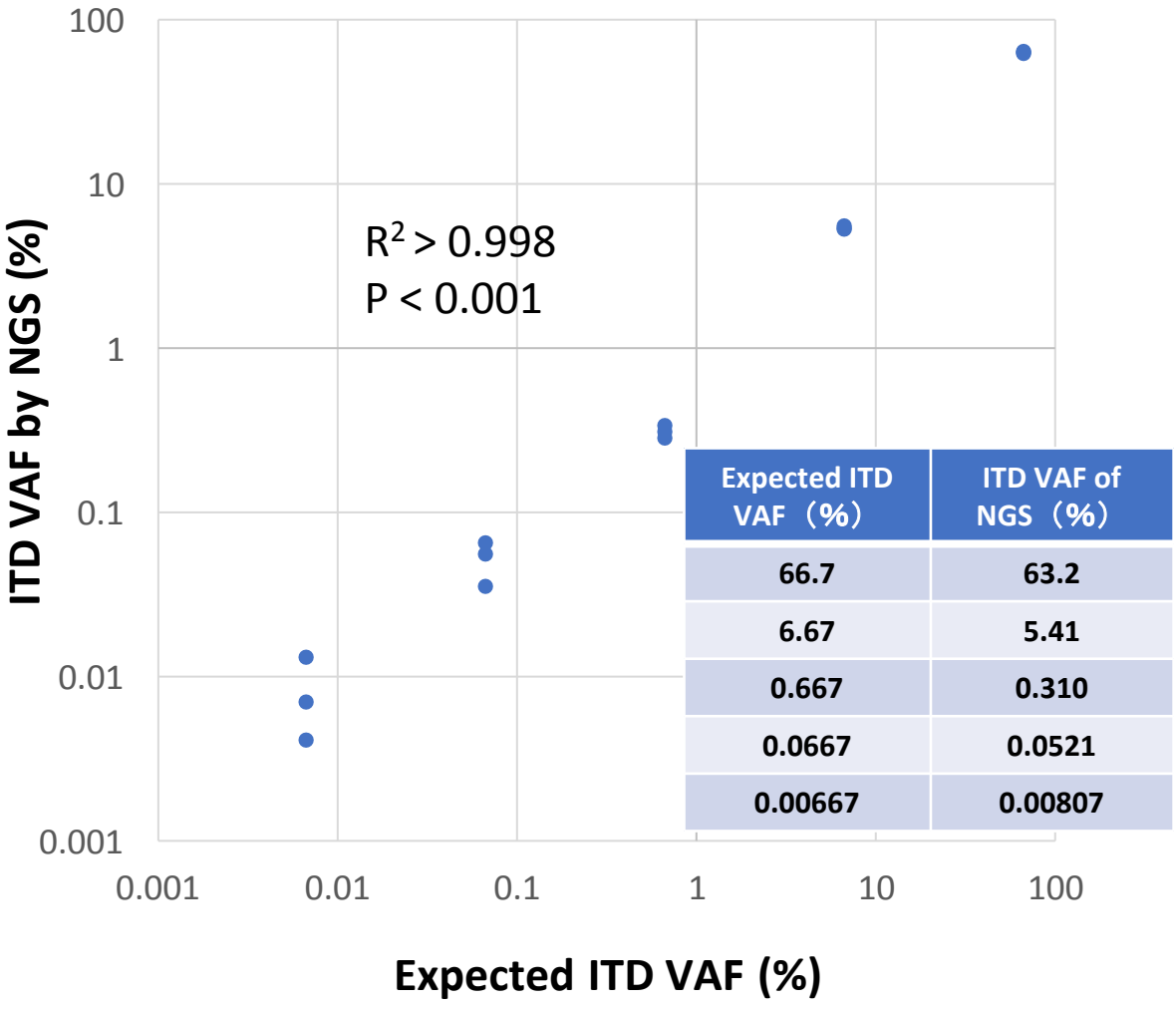

100%
10%
1%
⋮
0.0067%
FLT3-ITD VAF
Oligoclonal clones with FLT3-ITD
Relapse with FLT3-ITD
Clinically relevant FLT3-ITD
Minute FLT3-ITD
Undetectable
Gel electrophoresis
Capillary electrophoresis
Next-generation sequencing
Cases with clinically relevant FLT3-ITD
Cases with clinically FLT3-ITD-negative
Cases with minute FLT3-ITD
Cases without detectable FLT3-ITD
Cases in this study
n=37 (33.3%)
n=19 (17.0%)
n=55 (49.5%)

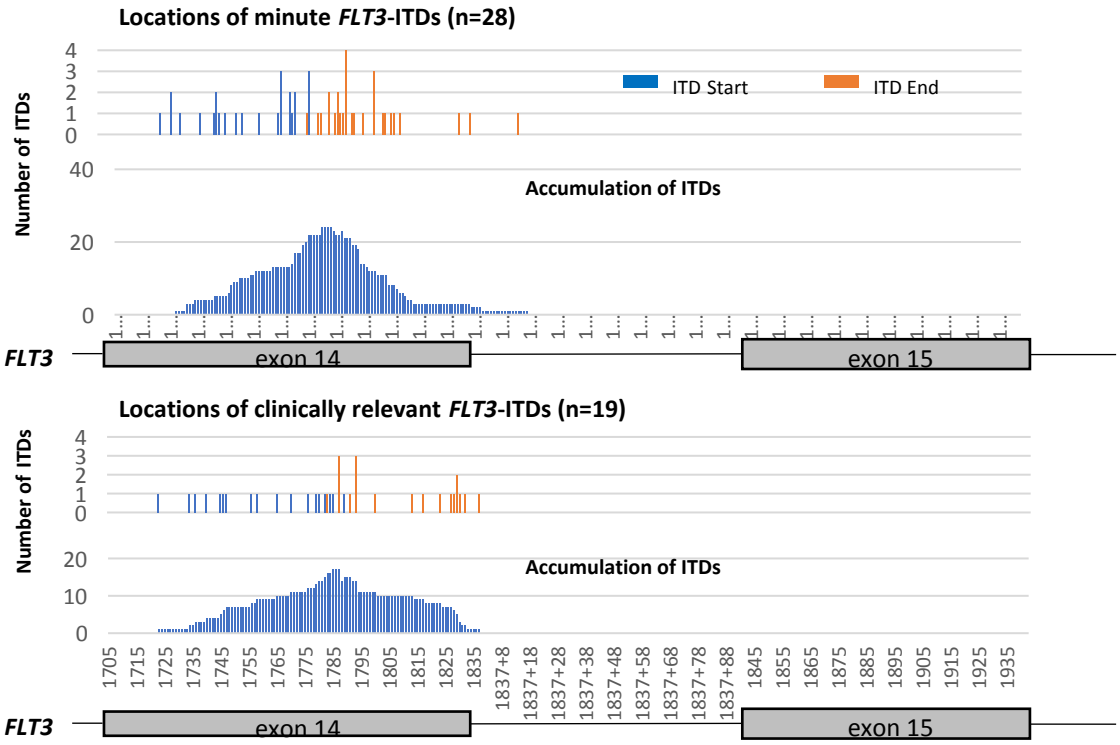
Figure S1



Expected ITD VAF（%）	ITD VAF of NGS（%）
66.7	63.2
6.67	5.41
0.667	0.310
0.0667	0.0521
0.00667	0.00807

Assessment of assay sensitivity, accuracy, and reproducibility. DNA of a *FLT3*-ITD-positive AML cell line (MOLM-14) was serially diluted in DNA of a *FLT3*-ITD-negative cell line (HL-60). Each dilution sample was analyzed in triplicate. The table shows expected ITD VAF and average ITD VAF by NGS of triplicated samples in each dilution.

Figure S2



Locations of *FLT3*-ITDs in clinically relevant and minute ITDs.
The locations of the duplicated regions of cr*FLT3*-ITD (upper panel) and minute *FLT3*-ITD (lower panel) are shown. The start and end positions of the duplicated regions of each ITD and the accumulation of duplicated regions are shown. The *FLT3* nucleotide position is shown on the horizontal axis. The numbering of intron 14 is shown as the last nucleotide of exon 14 (c.1837) plus the position of the intron (1837+N).