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High frequency of rare structural chromosome abnormalities at relapse of cytogenetically normal acute myeloid leukemia with *FLT3* internal tandem duplication

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Abstract

FLT3 internal tandem duplication (ITD) mutations are present in acute myeloid leukemia (AML) in 30% of patients, most commonly with a normal karyotype, and are associated with short relapse-free survival. *in vitro* and *in vivo* studies of *FLT3-ITD* cell lines have demonstrated reactive oxygen species-mediated DNA double-strand breaks and associated error-prone DNA repair as a mechanism of genomic instability, and we hypothesized that genomic instability might be manifested by cytogenetic changes at relapse of *FLT3-ITD* AML. We retrospectively reviewed charts of patients with CN *FLT3-ITD* AML treated at the University of Maryland Greenebaum Cancer Center, with attention to metaphase analysis results at relapse. Cytogenetic data were available from first and, when applicable, subsequent relapses for 15 patients diagnosed with CN *FLT3-ITD* AML. Among 12 patients with documented *FLT3-ITD* at first and, when applicable, subsequent relapse, 10 had cytogenetic changes, including 9 with rare structural abnormalities. The high frequency of rare structural chromosome abnormalities at relapse in our case series supports a role of genomic instability in the genesis of relapse, and suggests that ROS-generating and DNA repair pathways might be therapeutic targets in *FLT3-ITD* AML.

Keywords

Acute myeloid leukemia; *FLT3-ITD*; relapse; genomic instability; cytogenetics; structural chromosome abnormalities

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Introduction

The receptor tyrosine kinase fms-like tyrosine kinase 3 (FLT3) is expressed on malignant blasts in 70 to 100 percent of cases of acute myeloid leukemia (AML) [1, 2] and is mutated, most commonly by internal tandem duplication (ITD), in 20 to 30 percent, with the highest frequency of *FLT3-ITD* mutations in cytogenetically normal (CN) AML cases [3–7]. *FLT3-ITD* mutations result in constitutive activation of FLT3, and although complete remission (CR) rates are similar to those of AML without *FLT3* mutations, AML with *FLT3-ITD* is associated withhigh relapse rates and short relapse-free survival (RFS) and overall survival (OS) following chemotherapy [3–8] as well as allogeneic hematopoietic stem cell transplantation (alloSCT) [9].

Regrowth of AML cells following chemotherapy has been attributed to both constitutive FLT3 signaling through signal transducer and activator of transcription (STAT) 5 [10, 11] and stimulation by FLT3 ligand, since FLT3-ITD remains responsive to FLT3 ligand despite constitutive activation [12] and FLT3 ligand levels increase following chemotherapy [13]. In addition, increased levels of reactive oxygen species (ROS), increased DNA double-strand breaks (DSBs), and error-prone DNA repair by an alternative non-homologous end-joining (ALT NHEJ) pathway have been demonstrated in *FLT3-ITD* AML cells [14, 15], and resulting genomic instability might also contribute to early relapse.

We hypothesized that if genomic instability contributes significantly to early relapse of *FLT3-ITD* AML, a high frequency of cytogenetic changes, and particularly structural cytogenetic changes, might be evident at relapse. To test this hypothesis, we retrospectively reviewed cytogenetic findings at first relapse and, when applicable, subsequent relapses of patients with CN *FLT3-ITD* AML treated at the University of Maryland Greenebaum Cancer Center (UMGCC).

Material and Methods

Charts of all patients with CN AML with and without *FLT3-ITD* mutations diagnosed and treated at UMGCC between February 2004 and December 2013 were reviewed. Patients who received remission induction chemotherapy, achieved CR, relapsed and had cytogenetic data available at relapse were selected for the study. We reviewed presenting characteristics, treatment, RFS, OS and cytogenetic changes in these patients. Relapse-free survival was calculated as time from diagnosis to first relapse, and OS from diagnosis to death. The study was approved by the University of Maryland School of Medicine Institutional Review Board. The primary focus of this review was to examine cytogenetic changes in patients with CN *FLT3-ITD*AML at diagnosis who relapsed and had *FLT3-ITD* documented at relapse (Table 1), but the frequency of cytogenetic changes in CN AML without *FLT3-ITD* at diagnosis was also determined as a control.

The *FLT3-ITD* mutation was detected in bone marrow or blood (one patient) by multiplex polymerase chain reaction and capillary electrophoresis, as previously described [16].

Cytogenetic analysis consisted of analysis of at least 20 Giemsa-banded metaphases from cultures of bone marrow, or peripheral blood in one patient, at diagnosis and at first and,

when applicable, subsequent time points. Karyotypes were described according to International System for Human Cytogenetic Nomenclature (ISCN) recommendations [17].

Fluorescence *in situ* hybridization (FISH) for *BCR-ABL1* rearrangement was performed in Patient 5 (Table 1) with a *BCR/ABL1* dual fusion probe set (Rainbow Scientific, Inc, Windsor, CT), according to the manufacturer's instructions. FISH was also performed in a follow-up study in Patient 2 (Table 1) using a 1p32/1q21 probe set (Cytocell, Cambridge, UK) according to the manufacturer's instructions.

Chromosomal microarray analysis (CMA) was performed on paired DNA samples collected from Patient 9 (Table 1) at diagnosis and at relapse. Peripheral blood mononuclear cells were isolated by density gradient centrifugation. DNA extraction was performed using the Puregene Blood DNA isolation kit (Qiagen, Germantown, MD) according to the manufacturer's instructions. DNA was processed and hybridized to high-density Genome-Wide Human CytoScan HD arrays with 2.7 million markers including 750,000 single nucleotide polymorphic (SNP) and 1,900,000 non-polymorphic copy number variant (CNV) markers (Affymetrix, Santa Clara, CA), according to the manufacturer's instructions. The raw data were analyzed using the Genotyping Console software (Affymetrix, Santa Clara, CA) to reveal copy number changes with > 25 markers and 100 kb. A genomic imbalance is reported when deletions/duplications are greater than 1 Mb, unless they represent a region clearly associated with a benign copy number polymorphism in multiple independent studies. Copy number-neutral loss of heterozygosity (CNN-LOH)/acquired uniparental disomy (UPD) is reported when they are greater than 10 Mb. Chromosomal aberrations (CNN-LOH/UPD and copy number variation) were annotated based on the hg19 human genome assembly.

Results

Forty-one patients with CN AML and *FLT3-ITD* mutations were treated at UMGCC between 2004 and 2013. Twenty-six achieved CR after induction therapy and 11 were in ongoing first CR following chemotherapy or alloSCT at the time of this analysis. Cytogenetic data were available from first relapse and, when applicable, subsequent time points for all 15 patients with CN AML with *FLT3-ITD* mutations at diagnosis who relapsed. These included 12 with *FLT3-ITD* also documented at relapse (two did not have *FLT3-ITD* at relapse, and one was not studied). Among these 12 patients, 10 (83%) had cytogenetic changes at relapse. Demographic data, treatment, RFS, OS and cytogenetic and *FLT3* mutation data on these 10 patients are summarized in Table 1. Cytogenetic changes occurred at first (9 patients) or second (one patient, Patient 2 in Table 1) relapse. Of note, as is further detailed below, Patient 2 was initially noted to have an abnormal karyotype in second remission, and then relapsed shortly thereafter, with additional structural cytogenetic abnormalities. Two of the patients who developed new cytogenetic changes in first relapse (Patients 3 and 5 in Table 1) did so after initial reinduction chemotherapy. The two patients with *FLT3-ITD* mutations at diagnosis and at relapse who did not evolve new cytogenetic changes at relapse received therapy similar to that of the patients who did develop cytogenetic changes. Allelic burden data were not consistently available for the patients in our series and relapse and diagnosis allelic burden could not be compared.

Among the 12 AML patients with *FLT3-ITD* at diagnosis and at relapse, who are the focus of this report, 10 acquired cytogenetic changes at relapse. Cytogenetic changes were structural only in eight patients, numerical only in one (Patient 10 in Table 1), and both structural and numerical in one (Patient 9). Many of the structural abnormalities were rare rearrangements (Table 1). These included t(1;5)(q25;q13); dup(1)(q21q32); t(2;4)(p24;q13); der(2)t(2;?)(q21;?); del(3)(q12); t(3;14)(p21;q11.2); t(4;4)(q21;q35); t(5;13)(q31;q12); der(6)t(6;?)(p22;?); der(7)t(7;?)(p15;?); t(9;22)(q34;q11.2); inv(10)(p11.2q21.2); t(10;13)(p11.1;q11); t(X;10)(q13;q24); t(12;17)(p12;q12); and del(16)(q12). Three representative karyotypes are shown in Figure 1.

It is notable that Patient 2 acquired a dup(1)(q21q32) in 5 of 19 metaphase cells during second remission. Shortly afterwards, she developed second relapse, with additional structural abnormalities. Although the previously identified 1q duplication was observed in only one metaphase cell in the relapse sample, FISH using a 1p32/1q21 probe demonstrated the presence of this abnormality in 19% of the interphase cells. In addition to the previously identified dup(1)(q21q32), this patient acquired new DNA breaks, with a t(2;4)(p24;q13) in five cells, and a del(16)(q12) in two. Significantly, the additional changes were not seen in the metaphase with the 1q duplication, suggesting that the new aberrations were in new clones, rather than representing clonal evolution.

Patient 9 had a normal karyotype at diagnosis and complex chromosome abnormalities at relapse (179 days after diagnosis) (Figure 2A). The CMA of the diagnosis sample had three polymorphic duplications, three polymorphic deletions, and no >10 Mb CNN-LOH regions, supporting a normal copy number, without any pathogenic deletions or duplications (Fig 2B). The CMA of the relapse sample revealed the same deletions and duplications as found in the diagnosis sample, and also two additional changes acquired at relapse, including a 19.2 Mb deletion of the 11p15.1-p12 segment and a 95.7 Mb CNN-LOH of the entire 13q (Fig 2B).

Each cytogenetic change demonstrated at relapse of CN AML with *FLT3-ITD* in our series was reviewed in the Atlas of Genetics and Cytogenetics in Oncology and Hematology [18] as well as the Mitelman database [19]. Five patients (Patients 1, 2, 3, 4,and 7 in Table 1) developed inversions, translocations, deletions and a duplication with breakpoints previously undescribed in myeloid malignancies. Two patients (Patients 8and 9 in Table 1) evolved complex karyotypes with multiple structural abnormalities, including some with unspecified breakpoints.One patient (Patient 6in Table 1) developed inv(10)(p11.2q21.2), which has been reported in papillary thyroid carcinoma, but not in AML. Another patient (Patient 5 in Table 1) developed t(9;22)(q34;q11.2), with breakpoints in *BCR* and *ABL1* confirmed by FISH. The *BCR-ABL1* rearrangement is rare in AML; it has been reportedly previously in association with class II mutations including t(8;21)*/RUNX1-RUNX1T1,* inv(16)*/CBFB-MYH11* and *NPM1*, but not in association with *FLT3-ITD* [20].

During the same period, 43 patients with CN AML without *FLT3* mutations at diagnosis were treated. Thirty-five of these patients achieved CR and 21 remained in first CR at the time of this analysis. Among the 14 patients who achieved CR and subsequently relapsed, cytogenetic analysis was not performed in two and was inadequate in one. Among the 11

patients who had available cytogenetic results at relapse, five (45%) had cytogenetic changes at relapse, including four with structural changes, and six maintained a normal karyotype. The frequency of cytogenetic changes was lower than for our patients with *FLT3-ITD* at diagnosis and at relapse (p = 0.05; chi square test), albeit with very small numbers.

Discussion

Several mechanisms are thought to underlie the short RFS that characterizes AML with *FLT3-ITD*. Among these is genomic instability resulting from DNA damage due to high levels of ROS, and highly error-prone repair of DNA damage due to increased activity of an ALT NHEJ repair pathway for DNA DSBs [14, 15]. We hypothesized that genomic instability could be manifested by new structural chromosome abnormalities at relapse of CN AML with *FLT3-ITD*. To test this hypothesis, we reviewed our institution's experience with CN AML with *FLT3-ITD* mutations to look for cytogenetic evidence of genomic instability in this patient population at relapse. We found that 10 of 12 patients (83%) with CN AML with *FLT3-ITD* who also had FLT3-ITD at first and, when applicable, subsequent relapse developed new cytogenetic abnormalities; this compares to 5 of 11 patients (45%) with CN AML without *FLT3* mutation at diagnosis who developed cytogenetic changes at relapse (p=0.05). Our sample size was very small. Nevertheless, our findings support the concept of a possible role for genomic instability in the short DFS of AML patients with *FLT3-ITD*.

Strikingly, structural cytogenetic changes at relapse of CN AML with FLT3-ITD included a high proportion of rare translocations, inversions, and duplications. Rare changes included t(1;5)(q25;q13); dup(1)(q21q32); t(2;4)(p24;q13); der(2)t(2;?)(q21;?); del(3)(q12); t(3;14)(p21;q11.2); t(4;4)(q21;q35); t(5;13)(q31;q12); der(6)t(6;?)(p22;?); der(7)t(7;?)(p15;?); t(9;22)(q34;q11.2); inv(10)(p11.2q21.2); t(10;13)(p11.1;q11); t(X;10)(q13;q24); t(12;17)(p12;q12); and del(16)(q12). These structural abnormalities involved many DNA DSBs in various regions of multiple chromosomes, likely representing genomic instability.

The incidence of new cytogenetic abnormalities at relapse was high in our *FLT3-ITD* patients. Moreover, albeit with a very small sample size, it was higher than that reported in the literature for CN AML cases. Four previous reports addressed cytogenetic changes in CN AML at relapse without regard for *FLT3* mutation status, as these four studies antedated routine study of *FLT3* mutations [21–24]. In these four series, clonal cytogenetic changes were seen at relapse in 25, 51, 30 and 36 percent of 28, 43, 111 and 56 AML cases that were CN at diagnosis. More recently, Wang et al.[25] reviewed 17 patients with CN AML with an abnormal karyotype at relapse, including 15 characterized with regard to *FLT3* mutations at diagnosis. Eight patients had *FLT3-ITD*, and 7 had wild-type *FLT3*. New rare structural abnormalities were found at relapse in all 8 cases with *FLT3-ITD*, and also in 5 of 7 cases with wild-type *FLT3*. Finally, McCormick *et al.* [26] found that cytogenetic evolution was more frequent among *FLT3*-mutated than *FLT3*-non-mutated AML cases (10 of 14 versus 7 of 21, p= .04). In our series, new structural chromosome changes were found in 11 of 15 patients with CN *FLT3-ITD* AML at relapse, including 10 of 12 with documented *FLT3-ITD* at relapse, compared to four of 11 CN AML patients without *FLT3* mutations. Confirmation of features unique to relapse of *FLT3-ITD* AML will require a large series

with a large control group. Nevertheless, the finding of a high frequency of structural abnormalities involving various regions of multiple chromosomes supports the hypothesis that increased DNA DSBs and error-prone repair of DNA DSBs result in genomic instability that may contribute to relapse in patients with AML with *FLT3-ITD*.

Patient 9 in our series (Table 1) presented with neither cytogenetic nor chromosomal microarray alterations at diagnosis, and her relapse sample gained nine cytogenetic aberrations by routine chromosome analysis and two acquired changes by CMA. The acquired changes detected by SNP-microarray in the relapse sample are limited to a 19.2 Mb deletion in 11p and a 95.7 Mb CNN-LOH of 13q, suggesting that most of the complex abnormalities acquired in the relapse karyotype (Fig 2A) represent balanced structural changes. Although microarrays cannot reveal any balanced chromosome structural anomalies, as found by routine chromosome analysis in this patient, SNP microarrays are a useful technique to identify both cryptic chromosomal imbalances and CNN-LOH/UPD. As compared with chromosome banding analysis, SNP microarray results provided more precise breakpoints of an interstitial deletion of 11p. The two breakpoints of the 11p deletion were localized within the *PRR5L* gene on 11p12 and between the *NUCB2* and *PIK3C2A* genes on 11p15. In addition, SNP microarray analysis revealed the presence of CNN-LOH of 13q, which cannot be recognized by routine chromosome and FISH studies. CNN-LOH of 13q has been found to be the most common abnormality in AML samples at relapse, occurring in 40% of samples [27]. In most forms of cancer, LOH is generally associated with loss of a copy of a tumor suppressor gene. CNN-LOH is the occurrence of LOH in the absence of allelic loss (copy number ≥ 2). CNN-LOH has been shown to be important in cancer biology, as it can lead to tumor suppressor gene inactivation with two mutant alleles (homozygous mutation) possibly through mitotic recombination or a nondisjunction event leading to acquired isodisomy [28]. The *FLT3* gene is located at 13q. CNN-LOH of 13q may have led to a change from heterozygosity to homozygosity for *FLT3-ITD* in this patient. Homozygosity for *FLT3-ITD* has been found in AML samples at relapse [27], with homozygous mutations likely resulting in a competitive advantage and outgrowth of the clone.

All of the *FLT3-ITD* AML patients in our series received standard cytarabine and anthracycline induction chemotherapy. Three patients also received the tyrosine kinase inhibitor sorafenib, and one received the histone deacetylase inhibitor vorinostat. Four patients had undergone alloSCT before relapsing with new cytogenetic changes. Nine of the 10 patients with *FLT3-ITD* AML at diagnosis and at relapse who developed new karyotype abnormalities did so in first relapse, with Patients 3 and 5 (Table 1) evolving cytogenetic changes after initial chemotherapy for AML in first relapse. Patient 2 developed cytogenetic changes while still in morphologic second remission after achieving a second CR with cytarabine and mitoxantrone therapy. She then developed additional cytogenetic changes at the time of second relapse.

The fact that the most of our patients had *FLT3-ITD* at relapse suggests that this *FLT3* mutation was a driver of relapse. Two patients with *FLT3-ITD* at diagnosis did not have *FLT3* mutations identified at relapse, and the possibility that these two patients could actually have had new therapy-related AML (t-AML) rather than relapse of their initial

AML cannot be totally excluded. However none of the cytogenetic abnormalities seen in any of our patients at relapse, including these two, were those typically seen in t-AML. Notably, they did not involve 11q23 chromosome [29] nor chromosome 7 [30] breakpoints. Additionally, time to onset was shorter than expected in t-AML [31].

It is clear that more effective treatment is needed for AML with *FLT3-ITD*. The high frequency of structural cytogenetic changes that we have demonstrated at relapse of AML with *FLT3-ITD* supports a role of genomic instability in disease progression. Laboratory data [14, 15] support the contribution of ALT-NHEJ, an error-prone pathway for repair of DNA DSBs that has been found to be upregulated in AML cells with *FLT3-ITD*. This error-prone DNA repair pathway appears to render AML cells with *FLT3-ITD* more susceptible to genetic instability at baseline. The possibility that cytotoxic chemotherapy potentiates this instability has not been evaluated in this *FLT3-ITD* AML model, but has been suggested in other tumor types [32]. The contribution of cytotoxic chemotherapy to genomic instability in *FLT3-ITD* AML should be a point of future investigation. The DNA repair protein poly(ADP)-ribose polymerase 1 (PARP1) is an essential component of the alternative repair pathway, as is DNA ligase III [15]. PARP1 inhibitors are in clinical trials [33] and DNA ligase III inhibitors are in development [34]. Down-regulation of DNA ligase III expression levels in a *FLT3-ITD* cell line using siRNA oligonucleotides decreased the frequency of DNA repair errors, supporting further investigation of DNA ligase III inhibitors as a therapeutic approach [15]. Our data may support a possible therapeutic role for these inhibitors in AML with *FLT3-ITD*.

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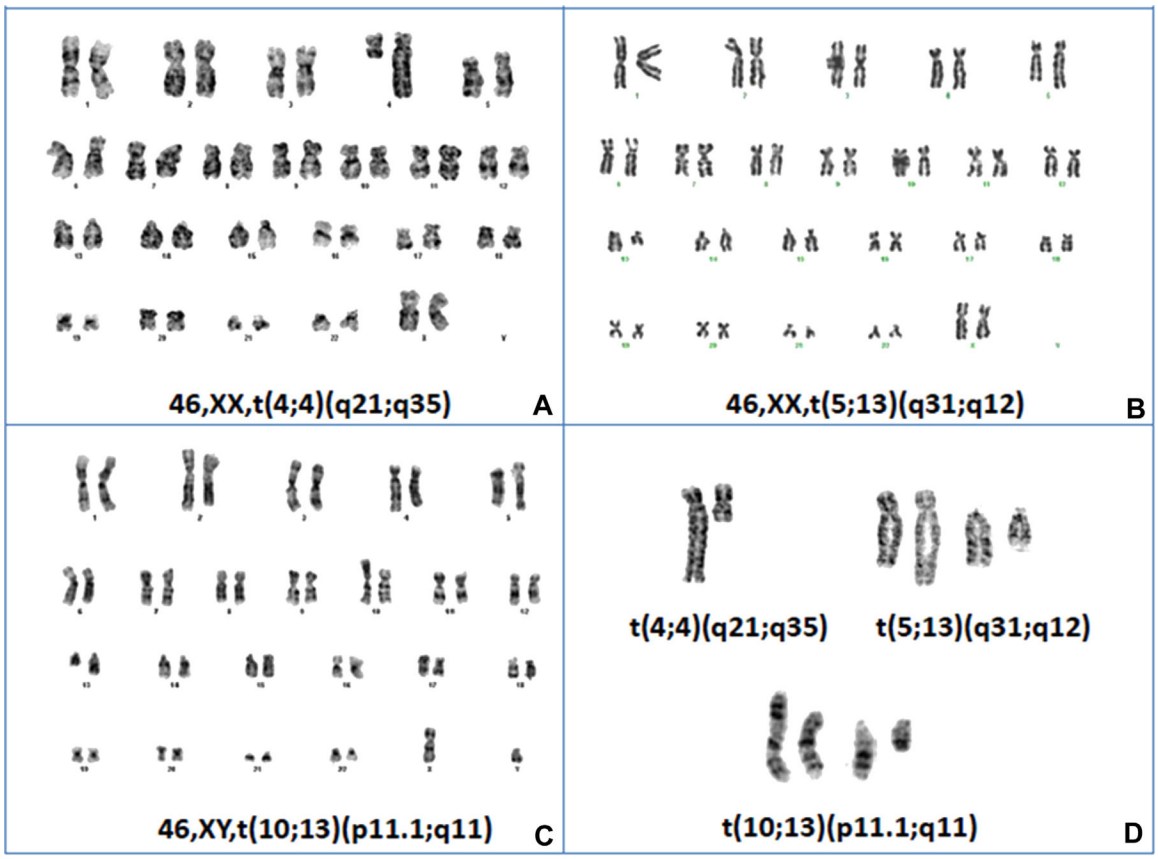
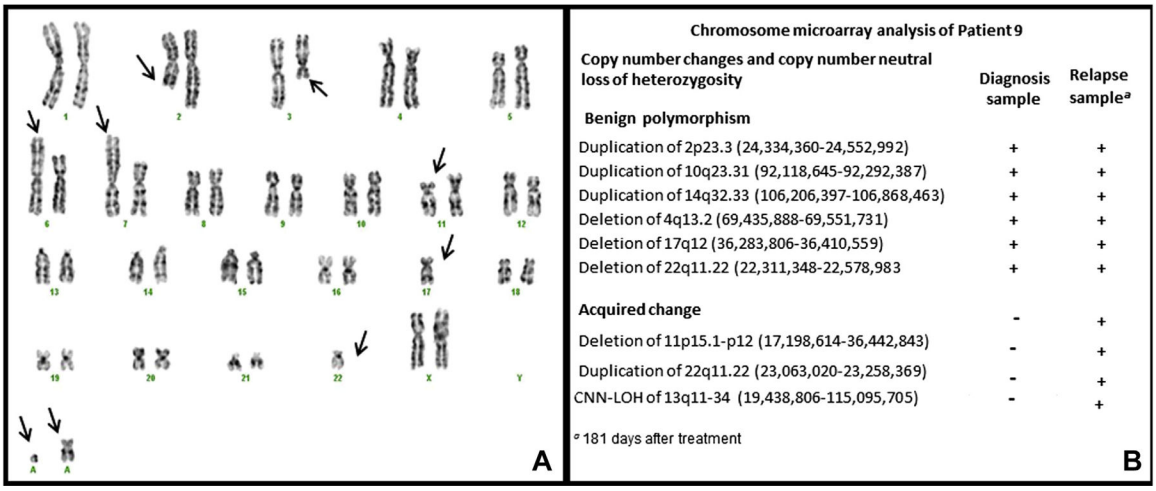


Figure 1. Representative relapse metaphase karyotypes.

Representative metaphase karyotypes from three of the FLT3-ITD patients in Table1, Patients 3, 4, and 7, labeled A, B and C, respectively. Panel D, contains enlarged views of the structural abnormalities in each of these patients.



Chromosome microarray analysis of Patient 9

Copy number changes and copy number neutral loss of heterozygosity	Diagnosis sample	Relapse sample[a]
Benign polymorphism		
Duplication of 2p23.3 (24,334,360-24,552,992)	+	+
Duplication of 10q23.31 (92,118,645-92,292,387)	+	+
Duplication of 14q32.33 (106,206,397-106,868,463)	+	+
Deletion of 4q13.2 (69,435,888-69,551,731)	+	+
Deletion of 17q12 (36,283,806-36,410,559)	+	+
Deletion of 22q11.22 (22,311,348-22,578,983	+	+
Acquired change		
Deletion of 11p15.1-p12 (17,198,614-36,442,843)	-	+
Duplication of 22q11.22 (23,063,020-23,258,369)	-	+
CNN-LOH of 13q11-34 (19,438,806-115,095,705)	-	+
	-	+

[a] 181 days after treatment

A B

Figure 2. Chromosome and SNP microarray results of Patient 9.

A. G-band karyogram in the relapse sample. Arrows point to abnormal chromosomes including monosomy 17 and 22, rearrangements of 2q21, 3q12, 6p22 and 7p15, possible deletion of 11p, and gain of two marker chromosomes. **B.** SNP microarray profiles from the diagnosis and relapse samples.

Table 1

Patient characteristics, treatment, outcomes, and karyotype changes

Patient	Age,Sex	Diagnosis karyotype[a]	*FLT3* status at diagnosis	Therapy	RFS, OS[b]	Subsequent karyotype	AML status when new karyotype emerged	*FLT3* status when new karyotype emerged
1	53, F	46,XX	ITD	AraC, Ida: HIDAC, topotecan: sorafenib; alloSCT	163, 197	46,XX,t(1;5) (q25;q13) [5]/46,XX[15]	Relapse 1	ITD and D835
2	56, F	46,XX	ITD	AraC, DNR; HIDAC	408, 966	46,XX,t(2;4) (p24;q13) [5]/46,XX,del(16) (q12) [2]/dup(1) (q21q32) [1]/46, XX[12] nucish(1p32×2) (1q21×3) [38/200]	CR2; Relapse 2	ITD
3	74, F	46,XX	ITD	AraC, Ida, sorafenib; HIDAC	183, 394	46,XX,t(4;4) (q21;q35) [7]/46,XX[13]	Relapse 1. after chemotherapy	ITD
4	58, F	46,XX	ITD	AraC, Ida: HIDAC	783, 989	46,XX,t(5;13) (q31;q12) [20]	Relapse 1	ITD
5	64, M	46,XY	ITD	AraC, Ida: HIDAC	229, 434	46,XY,t(9;22) (q34:q11.2) [4]/46,XY[8]	Relapse 1, after chemotherapy	ITD
6	48, M	46,XY	ITD	AraC, DNR. VP16; alloSCT	227, 243	46,XY,inv(10) (p11.2q21.2)[12]/ 46,XY[8]	Relapse 1	ITD
7	64, M	46,XY	ITD	AraC, Ida, sorafenib; alloSCT	177, 726+	46,XY,t(10;13) (p11.1q11)[9]/46,XY[9]// 46,XX[2]	Relapse 1	ITD
8	53, F	46,XX	ITD	AraC, DNR. VP16; vorinostat, AraC, VP16; alloSCT	330, 435	46,X,t(X;10) (q13;q24),t(3;14) (p21;q11.2), t(12:17) (p12;q12) [15]// 46,XY[5]	Relapse 1	ITD
9	43, F	46,XX	ITD	AraC, DNR. VP16; HIDAC	179, 413	46,XX,der(2)t(2;?) (q21;?),del(3) (q12), der(6)t(6;?) (p22;?), der(7)t(7;?) (p15;?), ?del(11) (p12p15), −17,−22,−2mar(19]/ 46,XX[1]	Relapse 1	ITD
10	49, F	46,XX	ITD	AraC, Ida; HIDAC	175, 567+	49,XX,+6,+8, +13[2]/ 46,XX[18]	Relapse 1	ITD

Abbreviations: M, male; F, female; AraC, cytarabine; Ida, idarubicin; DNR, daunorubidn; VP16, etoposide; HIDAC, high dose cytarabine

[a] 20 metaphases.

[b] Days.