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Absence of the Wild-Type Allele Predicts Poor Prognosis in Adult *de Novo* Acute Myeloid Leukemia with Normal Cytogenetics and the Internal Tandem Duplication of *FLT3*: A Cancer and Leukemia Group B Study[1]

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ABSTRACT

The *FLT3* gene is mutated by an internal tandem duplication (ITD) in 20–25% of adults with acute myeloid leukemia (AML). We studied 82 adults <60 years of age with primary AML and normal cytogenetics, who received uniform high-dose therapy and found *FLT3* ITD in 23 (28%) patients. When the 23 *FLT3* ITD+ cases were compared with the 59 cases with wild-type (WT) *FLT3*, disease-free survival (DFS) was inferior ($P = 0.03$), yet overall survival (OS) was not different ($P = 0.14$). However, 8 (35%) of 23 *FLT3* ITD/+ cases also lacked a *FLT3* WT allele ($FLT3^{ITD-R}$) as determined by PCR and loss of heterozygosity. Thus, three genotypic groups were identified: normal $FLT3^{WT/WT}$, heterozygous $FLT3^{ITD/WT}$, and hemizygous $FLT3^{ITD/-}$. DFS and OS were significantly inferior for patients with $FLT3^{ITD/-}$ ($P = 0.0017$ and $P = 0.0014$, respectively). Although DFS and OS for $FLT3^{WT/WT}$ and $FLT3^{ITD/WT}$ groups did not differ ($P = 0.32$ and $P = 0.98$, respectively), OS of the $FLT3^{ITD/-}$ group was worse than the $FLT3^{WT/WT}$ ($P = 0.0005$) and $FLT3^{ITD/WT}$ ($P = 0.008$) groups. We propose a model in which $FLT3^{ITD/-}$ represents a dominant positive, gain-of-function mutation providing AML cells with a greater growth advantage compared with cells having the $FLT3^{WT/WT}$ or $FLT3^{ITD/WT}$ genotypes. In conclusion, we have identified the $FLT3^{ITD/-}$ genotype as an adverse prognostic factor in *de novo* AML with normal cytogenetics. A poor prognosis of the relatively young $FLT3^{ITD/-}$ adults (median age, 37 years), despite treatment with current dose-intensive regimens, suggests that new treatment modalities, such as therapy with a FLT3 tyrosine kinase inhibitor, are clearly needed for this group of patients.

INTRODUCTION

FLT3, the gene of which is located at chromosome 13q12, is a member of the class III RTKs[3] that includes FMS, platelet-derived growth factor receptor, and KIT (1–3). These RTKs are classified based on the presence of five immunoglobulin-like domains, a JM domain and two TK domains that are separated by a kinase insert domain. The *FLT3* RTK domains are activated by an allosteric dimerization process through binding with its natural ligand termed the FL. FLT3 is normally expressed on the surface of early bone marrow hematopoietic progenitor cells and has been demonstrated to have an important role in the survival and/or differentiation of multipotent stem cells (1, 2, 4). Furthermore, AML cells have been shown to express FLT3, and exogenous FL can enhance their survival and proliferative responsiveness (5). Hence, alterations in FLT3 signaling through either aberrant FL expression or through gain-of-function mutations in the *FLT3* gene itself could potentially contribute to leukemogenesis.

In the initial report, length mutations in the *FLT3* gene were detected that resulted from a partial ITD in 5 of 22 (22.7%) adults with AML (6). Additional studies (6–9) have confirmed this finding and have determined that the duplication involves the JM domain-coding sequence in a head-to-tail fashion that is always in-frame. In experimental systems, the *FLT3* ITD leads to ligand-independent *FLT3* dimerization and constitutive activation of the TK domains via autophosphorylation (10, 11). This in turn appears to constitutively activate signal transducers and activators of transcription 5 and mitogen-activated protein kinase and introduces autonomous cell growth in cytokine-dependent cell lines (11–14).

Two recent studies (8, 15) have reported that the presence of the *FLT3* ITD is associated with statistically significant worse clinical outcomes compared with patients not harboring this defect. In the latter study, the *FLT3* ITD occurred in AML at a frequency of 22% in 81 cases studied and was associated with reduced DFS for cases with *de novo* AML or intermediate-risk cytogenetics ($P = 0.004$ and $P = 0.008$, respectively; Ref. 8). However, patients were treated with a variety of treatment regimens. A recent pediatric AML study (9) was performed on patients who were treated under one protocol. A modest but significant difference in OS was noted ($P = 0.02$) between *FLT3* ITD cases and *FLT3* WT pediatric cases, with the *FLT3* ITD cases showing a poorer prognosis (9). In all of the reports to date, the analysis of the *FLT3* ITD mutation has consisted of its presence or absence by genomic or RT-PCR and sequence of its in-frame fusion. Hence, the mechanism by which this mutation may contribute to AML remains uncertain.

In the current study, we examined diagnostic bone marrow samples for the *FLT3* ITD in 82 adults <60 years of age with *de novo* AML and normal cytogenetics who were treated on a single dose-intensive treatment protocol within the CALGB. We identified three distinct genotypes in our molecular analysis: normal $FLT3^{WT/WT}$, heterozygous $FLT3^{ITD/WT}$, and hemizygous $FLT3^{ITD/-}$, *i.e.*, *FLT3* ITD-positive that also lacked the *FLT3* WT allele. Our analysis indicates that the $FLT3^{ITD/-}$ patients have a significantly shortened survival when compared with $FLT3^{WT/WT}$ and/or $FLT3^{ITD/WT}$ patients. Therefore, the data are consistent with a *FLT3* mutation and loss of WT allele resulting in a dominant-positive gain-of-function effect that is responsible for this phenotype.

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[3] The abbreviations used are: RTK, receptor tyrosine kinase; JM, juxtamembrane; FL, *FLT3* ligand; AML, acute myeloid leukemia; ITD, internal tandem duplication; DFS, disease-free survival; OS, overall survival; WT, wild-type; RT-PCR, reverse transcription-PCR; CALGB, Cancer and Leukemia Group B; PCST, peripheral blood stem cell transplant; LOH, loss of heterozygosity; CR, complete remission; FAB, French-American-British.

MATERIALS AND METHODS

Primary AML Samples. This study comprised 82 consecutive adult patients <60 years of age diagnosed with *de novo* AML and entered on CALGB treatment protocol 9621 who had both normal cytogenetics and an adequate diagnostic bone marrow sample cryopreserved and available for study. Patients' inclusion was restricted to patients whose cytogenetics were centrally reviewed before May 2000. All of the patients gave informed consent for both treatment and cryopreservation of bone marrow, blood, and buccal swabs. Patients were treated on CALGB protocol 9621, a cytogenetic risk stratification, Phase I-II study whereby patients with good risk cytogenetics [*i.e.*, inv(16) and t(8;21)] were assigned to one treatment arm and all of the other patients (normal and other cytogenetic abnormalities) were assigned to another arm. The distinct patient cohorts received induction chemotherapy with a fixed dose of infusional cytarabine and variable doses of daunorubicin and etoposide with or without concurrent administration of the multidrug resistance gene modulator PSC-833 (Valspodar; Novartis), as described previously (16). For patients with normal cytogenetics, this was followed by autologous PSCT using high-dose etoposide and cytarabine for "*in vivo* purging" and stem cell mobilization followed by a myeloablative regimen of busulfan and etoposide (17). Patients unable to receive PSCT (8 of 82 patients) were consolidated with a novel regimen consisting of the "*in vivo* purging" portion of the PSCT sequence followed by two cycles of high-dose cytarabine. After consolidation, patients received a 90-day low-dose s.c. interleukin 2 (Proleukin, Chiron Corp.) regimen interrupted with intermediate dose pulsing of s.c. interleukin-2 every 2 weeks. Growth factor support was not permissible in this study after induction and high-dose cytarabine consolidation chemotherapy.

Leukemias were classified morphologically according to the FAB Cooperative Group criteria. All of the cases were centrally reviewed. Chromosomal analysis of bone marrow was performed in institutional CALGB cytogenetics laboratories, and karyotypes were centrally reviewed biannually by an expert panel of CALGB cancer cytogeneticists as part of a prospective study of cytogenetics in acute leukemia, CALGB 8461 (18). Specimens were obtained at diagnosis and processed using unstimulated short-term (24-h, 48-h, and 72-h) cultures. G banding was typically done, although Q banding was also acceptable for inclusion in this series. The criteria used to describe a cytogenetic clone and description of karyotypes followed the recommendations of the International System for Human Cytogenetic Nomenclature (19). A minimum of 20 bone marrow metaphases/case were required to be examined for a case to be classified as having normal cytogenetics. Cells taken at diagnosis were also shipped via overnight express to the CALGB Leukemia Tissue Bank and were viably procured in liquid nitrogen after enrichment for mononuclear cells through a Ficoll gradient. Buccal swabs were often obtained from patients and snap-frozen for DNA extraction at the time of diagnosis.

Detection of a *FLT3* ITD by DNA PCR and by RT-PCR. DNA and RNA were extracted from thawed bone marrow samples by standard protocols (20). PCR and RT-PCR were carried out as described previously (6) using primers that detect all of the length mutations discovered to date for the *FLT3* gene. Amplification products after 35 PCR cycles were size fractionated through 2.5–3% agarose gels and viewed under UV illumination after ethidium bromide staining. In addition, longer range DNA PCRs were performed in the *FLT3* gene extending from exon 10 to the 3′-end of exon 12. Each standard DNA PCR product was excised from gels and purified (Qiaquick; Qiagen, Inc., Valencia, CA). After TA cloning (Invitrogen, Inc., Carlsbad, CA), a minimum of 10 clones with insert DNA were sequenced by The Sequencing and Genotyping Unit of the Ohio State University Comprehensive Cancer Center (Columbus, OH). DNA was analyzed using the basic local alignment tool, BLAST. Amino acid sequences were aligned using the MegAlign software program of DNAStar.

LOH Analysis. LOH was determined at chromosome 13 band q12 using fluorogenic primers that amplify the microsatellite markers D13S221, D13S1304, D13S1244, D13S1254, D13S1246, and D13S218 (Research Genetics, Inc., Huntsville, AL). The estimated location of these markers relative to the *FLT3* gene locus is shown in Fig. 2*B*. Markers also used that are outside this region were D13S175 at 13q11 and D13S1265 at 13q33. PCR was performed using primer pairs for each of these markers, and DNA was derived from leukemic bone marrow and buccal swab epithelial cells from the same patient. LOH analysis was performed on the sequenced PCR products using Genotyper 2.0 (The Sequencing and Genotyping Unit of the Ohio State University Comprehensive Cancer Center). Both positive and negative controls were run simultaneously.

Statistical Analyses. All of the *de novo* AML patients were classified according to one of the three genotypic groups: $FLT3^{WT/WT}$, $FLT3^{ITD/WT}$, or $FLT3^{ITD/-}$. Thereafter, clinical features at presentation were compared across the three groups. Categorical variables such as sex, FAB, and leukemic involvement of skin, gums, liver, and spleen were compared using Fisher's exact test. Continuous variables such as age, platelets, hemoglobin, and WBC count were compared using the Kruskal-Wallis test. For clinical variables reporting a significant P at the 0.05 level, pairwise comparisons were conducted to determine wherein the differences lie. Pairwise comparisons of baseline continuous variables were conducted using the Wilcoxon rank-sum test, whereas pairwise comparisons of categorical variables were conducted using Fisher's exact test. The α-level was adjusted to $\alpha = 0.05 \div 3 = 0.0167$ in assessing the significance of the pairwise comparisons.

CR required an absolute neutrophil count $\geq$1,500/μl, a platelet count $\geq$100,000/μl, no leukemic blasts in the peripheral blood, bone marrow cellularity >20% with maturation of all of the cell lines, no auer rods, <5% bone marrow blast cells, and no extramedullary leukemia, with persistence for at least 1 month. Relapse was defined as the reappearance of circulating blast cells not attributable to "overshoot" after recovery from myelosuppressive therapy or >5% blasts in the marrow not attributable to another cause or development of extramedullary leukemia. DFS was defined only for patients who achieved CR and was measured from the documented date of CR until date of relapse or death regardless of cause, censoring for patients alive in continuous CR. OS was measured from the protocol on-study date until date of death, regardless of cause of death, censoring for patients alive. Median follow-up for survival for censored patients (*i.e.*, patients still alive) was 1.7 years overall, 1.2 years for the $FLT3^{ITD/-}$ group, 2.0 years for the $FLT3^{ITD/WT}$ group, and 1.6 years for the $FLT3^{WT/WT}$ group. Kaplan-Meier curves were constructed for DFS and OS comparing the three *FLT3* genotype groups. The log-rank test was performed to determine whether there was a significant difference between the survival curves.

Finally, to adjust for potential confounding covariates, a Cox proportional hazards model was built using a backwards variable selection procedure to determine whether genotype, as an indicator variable, remained significant as a prognostic factor once other covariates adjusted the model. Firstly, univariate models for each clinical characteristic at baseline were fit. Secondly, univariate models incorporating an artificial time-dependent covariate expressed as the product of the covariate and the log of the time variable were fit to assess whether the proportional hazards assumption was met. If the proportional hazards assumption was not met for a particular variable, then the artificial time-dependent covariate was included in all of the subsequent models containing that variable. Thereafter, variables reflecting a P from the likelihood ratio test in the univariate models of <0.20 were incorporated together in a full model. Variables reporting a P of >0.05 from the corresponding Wald statistic in the full model were subsequently dropped one at a time in determining the final model. Statistical analyses were performed by the CALGB Statistical Center.

RESULTS

***FLT3* ITD Prevalence in Adult *de Novo* AML with Normal Karyotype.** A total of 82 adult patients <60 years of age with *de novo* AML and normal karyotype were evaluated for the *FLT3* ITD. An ITD within the *FLT3* gene was identified in 23 of the 82 patients (28%). Sequencing showed no significant variations in the *FLT3* length mutations compared with those described to date. However, 8 of the 23 patients with the *FLT3* ITD either lacked DNA PCR evidence of a WT *FLT3* allele or had significantly less WT *FLT3* PCR product compared with the mutant PCR product ($FLT3^{ITD/-}$; Fig. 1*A*; representative samples shown). Representative samples from $FLT3^{ITD/WT}$ and $FLT3^{ITD/-}$ patients were then subjected to RT-PCR (Fig. 1*B*). Results were completely consistent with those obtained by DNA PCR in that the WT *FLT3* transcript levels were low to absent compared with the mutant *FLT3* transcript in the $FLT3^{ITD/-}$ cases examined. To determine whether these results were indicative of a

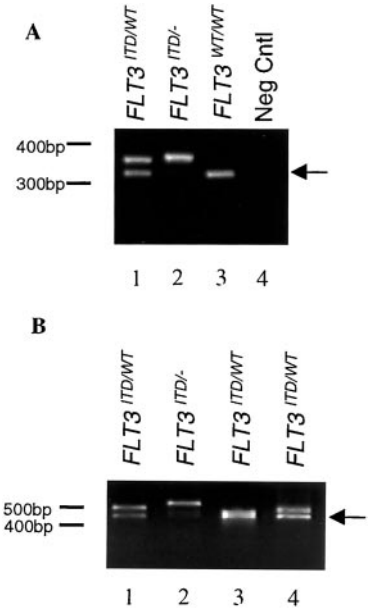


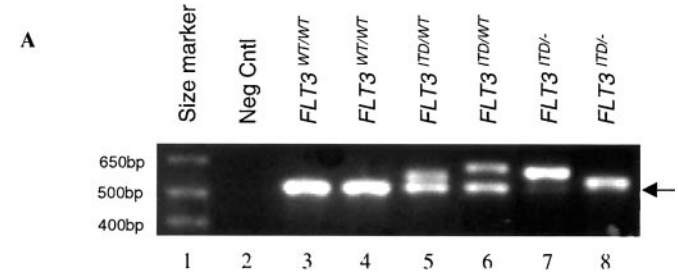
Fig. 1. PCR detection of the *FLT3* ITD and WT *FLT3* in adult *de novo* AML. *A*, DNA PCR was performed as described in "Materials and Methods." Amplification products were size fractionated through agarose gels and viewed under UV illumination after ethidium bromide staining. The WT *FLT3* genomic PCR product is indicated by the *arrow*. Representative data are shown. *B*, RT-PCR was performed as described in "Materials and Methods" on representative cases of adult *de novo* AML with a *FLT3* ITD. The WT *FLT3* RT-PCR product is indicated by the *arrow*. *Neg Cntl*, water only; $FLT3^{WT/WT}$, patient case with only the WT *FLT3* PCR product (as shown in *A*, *Lane 3*); $FLT3^{ITD/WT}$, patient cases with both a length mutation in *FLT3* exon 11 and WT *FLT3* (as shown in *A*, *Lane 1*, and *B*, *Lanes 1*, *3*, and *4*); $FLT3^{ITD/-}$, patient case with the length mutation in *FLT3* exon 11 and low (likely because of contaminating normal cells in the AML sample) levels of WT *FLT3* (as shown in *A*, *Lane 2*, and *B*, *Lane 2*).

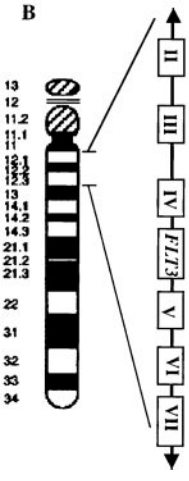
missing *FLT3* WT allele, we performed a longer range DNA PCR, extending from exon 10 of the *FLT3* gene to the end of exon 12. The results were consistent with the earlier DNA and RNA PCR results, in that there were again low to nondetectable levels of the WT PCR product in the $FLT3^{ITD/-}$ cases examined (Fig. 2*A*; representative samples shown).

Finally, we performed an assessment for LOH by comparing DNA obtained from leukemic bone marrow samples with DNA obtained from buccal epithelium of three $FLT3^{ITD/-}$ cases for which buccal swab samples were available (Fig. 2*B*). In all of the three $FLT3^{ITD/-}$ patients studied, LOH was observed in the region near the *FLT3* locus. These data provide further evidence that the WT *FLT3* allele is likely lost from the non-*FLT3* ITD-containing chromosome 13 in these $FLT3^{ITD/-}$ cases. In a case where relatively low levels of WT *FLT3* DNA and transcript were detected (Fig. 1, *A–B*, *Lane 2*; and Fig. 2*A*, *Lane 7*), LOH was shown to be present (Fig. 2*B*, *Patient 1*), supporting that the observed WT *FLT3* PCR products were likely amplified from contaminating normal cellular DNA in the samples. Interestingly, one marker, D13S1246, showed retention of heterozygosity, *i.e.*, presence of two different alleles and thus two differing chromosomes, in pt 1, although the most distal marker, D13S1265, showed LOH, suggesting there are regions of microdeletions on one chromosome 13 in this patient case. Our analysis was consistent with three genotypes in this group of adult *de novo* AML with normal cytogenetics: 59 cases of AML with *FLT3* WT alleles ($FLT3^{WT/WT}$); 15 cases of AML with the *FLT3* ITD in combination with the *FLT3* WT allele ($FLT3^{ITD/WT}$); eight cases of AML with the *FLT3* ITD lacking a *FLT3* WT allele ($FLT3^{ITD/-}$).

Clinical Characteristics of AML Patients with $FLT3^{WT/WT}$, $FLT3^{ITD/WT}$, and $FLT3^{ITD/-}$ Genotypes. First, we compared the clinical characteristics at presentation between the three groups followed by relevant pairwise comparisons. As noted earlier, all of the patients in this study had a normal karyotype on diagnostic bone marrow aspirates. There were no significant differences with respect to age, sex, FAB subtypes, or other clinical characteristics such as gum hypertrophy, lymphadenopathy, splenomegaly, or hepatomegaly across the three groups (Table 1). The median WBCs increased from 16.3×10^9/liter in patients within the $FLT3^{WT/WT}$ group to 56.9×10^9/liter in the $FLT3^{ITD/WT}$ group and was highest in the $FLT3^{ITD/-}$ group (75.2×10^9/liter; $P = 0.01$). The trend of an increase in percentage of peripheral blood leukemic blasts in the three groups did not reach statistical significance ($P = 0.05$).

Evaluation of Clinical Outcome and Identification of the Prognostic Genotype. Fisher's exact test was used to determine whether there were any differences with respect to patients receiving PSC-833 or PSCT by *FLT3* genotype group. There were no differences by *FLT3* genotype group with respect to patients receiving PSC-833 ($P = 0.94$) or PSCT ($P = 0.16$) or with regards to doses of daunorubicin and VP16 among those who received PSC-833 ($P = 0.52$). The CR rate was not different among the three *FLT3* genotype groups.





Marker	Pt 1	Pt 2	Pt 3
I. D13S175 (13q11)	LOH+	Not informative	Not informative
II. D13S221	LOH+	Not informative	LOH+
III. D13S1304	LOH+	LOH+	Not informative
IV. D13S1244	LOH+	LOH+	LOH+
FLT3	**LOH+**	**LOH+**	**LOH+**
V. D13S1254	LOH+	LOH+	LOH+
VI. D13S1246	normal	LOH+	LOH+
VII. D13S218	Not informative	LOH+	Not informative
VIII. D13S1265 (13q33)	LOH+	LOH+	LOH+

Fig. 2. Long-range DNA PCR and LOH analyses confirm loss of the WT *FLT3* allele in a subset of AML cases with a *FLT3* ITD. *A*, long-range DNA PCR was performed using primers located outside the region of duplication as described in "Materials and Methods." Amplification products were size fractionated and viewed under UV light after ethidium bromide staining. The WT *FLT3* genomic PCR product is indicated by the *arrow*. *Neg Cntl*, water; $FLT3^{WT/WT}$, patient cases with only the normal *FLT3* genomic PCR product; $FLT3^{ITD/WT}$, patient cases with both a length mutation in *FLT3* exon 11 and WT *FLT3*; $FLT3^{ITD/-}$, patient cases with the length mutation in *FLT3* exon 11 and low to nondetectable levels of WT *FLT3*. Representative data are shown. *B*, six microsatellite markers (II-VII) located in the region of the *FLT3* locus on chromosome 13q12 were used to assess allele status in patients' AML and normal tissue as described in "Materials and Methods." Two markers were included: D13S175, located at 13q11, and D13S1265, located at 13q33. A schematic of banded chromosome 13 is shown on the *left*. Estimated mapping of markers and the *FLT3* locus at chromosome 13 band q12 was based on the National Center for Biotechnology Information Radiation Hybrid map, GeneMap '99, and the Ohio State University Gene Map. It should be noted that discrepancies exist in these maps to date with regards to physical locations of the markers relative to *FLT3*. Results are shown for three AML samples that were identified as $FLT3^{ITD/-}$ by genomic PCR and RT-PCR. *LOH+*, region is positive for LOH; *Not informative*, the marker was noninformative for this region; *i.e.*, alleles were identical; *normal*, heterozygosity is retained in this region on both chromosomes 13.

Table 1 *Clinical characteristics at presentation for the patients in the $FLT3^{WT/WT}$, $FLT3^{ITD/WT}$, and $FLT3^{ITD/-}$ genotype groups*

	$FLT3^{WT/WT}$ (N = 59)	$FLT3^{ITD/WT}$ (N = 15)	$FLT3^{ITD/-}$ (N = 8)	P
Age (yrs)				
Median	45	48	37	
Range	(20–59)	(20–59)	(21–57)	0.31
Gender				
Percentage male	51	33	62.5	0.37
Hemoglobin (g/dl)				
Median	9.0	10.1	7.7	
Range	(4.6–12.1)	(7.1–12.9)	(6.0–11.3)	0.09
Platelets ($\times 10^9$/l)				
Median	48.5	62	31	
Range	(5–235)	(19–154)	(25–56)	0.15
WBC ($\times 10^9$/l)				
Median	16.3	56.9	75.2	
Range	(0.9–295)	(0.9–131.6)	(19.9–261.6)	0.01
Percentage peripheral blood blasts				
Median	53	71	80	
Range	(0–97)	(12–91)	(68–90)	0.05
Percentage bone marrow blasts				
Median	62	65	79.5	
Range	(24–88)	(38–86)	(51–83)	0.28
FAB classification				
M1	21%	20%	17%	
M2	34%	27%	33%	
M4	18%	47%	50%	0.41
M5	18%	0%	0%	
M0	5%	7%	0%	
AML unclassifiable	4%	0%	0%	
Gum hypertrophy	24%	13%	12.5%	0.61
Lymphadenopathy	14%	7%	12.5%	0.87
Skin infiltrates	10%	13%	12.5%	0.86
Hepatomegaly	4%	7%	12.5%	0.33
Splenomegaly	4%	13%	12.5%	0.18
Mediastinal mass	2%	0%	0%	1.00
Central nervous system involvement	0%	0%	0%	

Table 2 *Clinical outcome for the $FLT3^{WT/WT}$, $FLT3^{ITD/WT}$, and $FLT3^{ITD/-}$ genotype groups*

	$FLT3^{WT/WT}$ (N = 59)	$FLT3^{ITD/WT}$ (N = 15)	$FLT3^{ITD/-}$ (N = 8)	P
Percentage of CR	86	79	75	0.70
DFS				
Median (months)	52[a]	24	4	
Percentage of DFS at 12 months (95% confidence interval)	71 (62, 80)	51 (35, 66)	17 (12, 22)	0.0017
Survival				
Median (months)	46[a]	46[a]	7	
Percentage alive at 12 months (95% confidence interval)	74 (65, 82)	65 (48, 81)	13 (10, 15)	0.0014

[a] Nonparametric estimate of the median is not available. Tabled value represents the median assuming the survival estimates after the last observation follows an exponential curve.

The remission induction rate was 86% for the $FLT3^{WT/WT}$ group, 79% for $FLT3^{ITD/WT}$ group, and 75% for the $FLT3^{ITD/-}$ group (P = 0.70; Table 2). Failure to achieve CR in two $FLT3^{ITD/-}$ patients resulted from primary resistant disease as it did in three $FLT3^{ITD/WT}$ patients. However, failure to achieve CR in the $FLT3^{WT/WT}$ patients was because of primary resistant disease in four patients and death during induction in four patients.

The overall analysis revealed a significant difference in the three genotype groups with respect to DFS (P = 0.0017; Fig. 3*A*). DFS was significantly shorter for the $FLT3^{ITD/-}$ group compared with the $FLT3^{WT/WT}$ group (P = 0.0008) and borderline shorter for the $FLT3^{ITD/WT}$ group (P = 0.025; Table 3). Furthermore, five of the six (83%) $FLT3^{ITD/-}$ patients who achieved CR relapsed at a median of 3 months. In contrast, 4 (36%) of the 11 $FLT3^{ITD/WT}$ group have relapsed and 10 of the 51 (20%) $FLT3^{WT/WT}$ group have relapsed at a median of 9 and 8 months, respectively. The relapse rate is significantly different among the three groups (P = 0.0026; Fisher's exact test).

OS was significantly poorer for the $FLT3^{ITD/-}$ group (P = 0.0014; Fig. 3*B*). The OS rate at 12 months was 74% for the $FLT3^{WT/WT}$ group, 65% for $FLT3^{ITD/WT}$ group, and 13% for the $FLT3^{ITD/-}$ group (Table 2). The $FLT3^{ITD/-}$ group had a much shorter OS when compared with the $FLT3^{ITD/WT}$ group (P = 0.008) and the $FLT3^{WT/WT}$ group (P = 0.0005; Table 3). There was no difference in OS between the $FLT3^{WT/WT}$ and $FLT3^{ITD/WT}$ groups (P = 0.98). Importantly, there was no difference in OS when comparing patients with and without a *FLT3* ITD (*i.e.*, $FLT3^{WT/WT}$ group *versus* combined $FLT3^{ITD/WT}$ and $FLT3^{ITD/-}$ groups; P = 0.14; Table 4). This is in contrast to several earlier reports (8, 21). The data indicate the group of patients that lack a WT *FLT3* allele in association with the *FLT3* ITD have a significantly inferior outcome in OS (*i.e.*, $FLT3^{WT/WT}$ + $FLT3^{ITD/WT}$ groups *versus* $FLT3^{ITD/-}$ group; P = 0.0003; Table 5).

In a multivariate analysis of these AML patients <60 years of age with a normal karyotype, the only significant prognostic markers for DFS (P = 0.0053) and OS (P = 0.0039) were the *FLT3* genotype

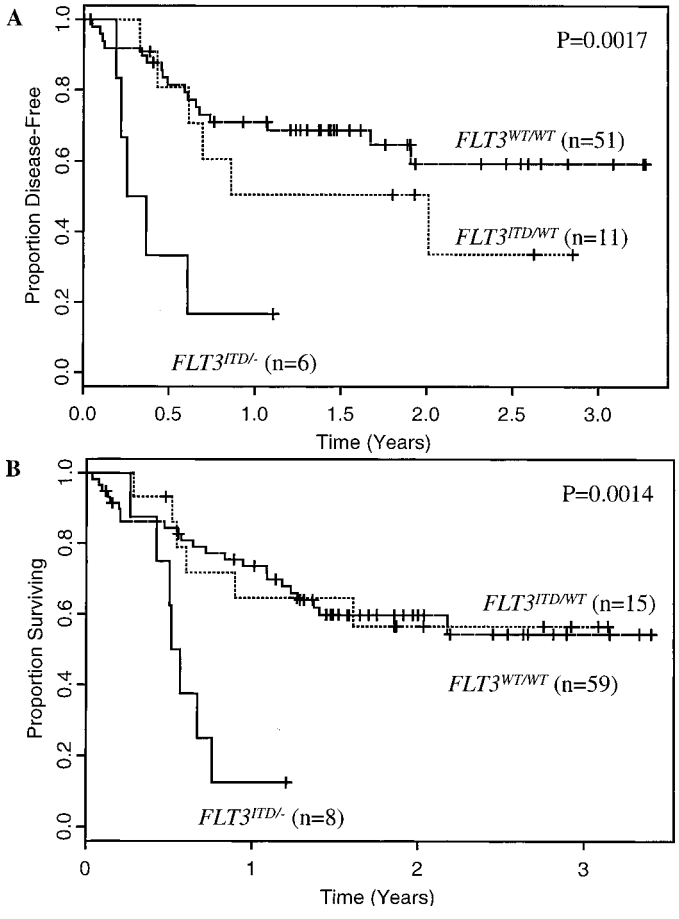


Fig. 3. AML cases with the $FLT3^{ITD/-}$ genotype have worse DFS and OS compared with AML cases with either the $FLT3^{WT/WT}$ or the $FLT3^{ITD/WT}$ genotypes. Kaplan-Meier curves were generated as described in "Materials and Methods." *Solid line*, AML patients with the $FLT3^{ITD/-}$ genotype; *dotted line*, AML patients with the $FLT3^{ITD/WT}$ genotype; *broken line*, AML patients with only a normal *FLT3* genotype, $FLT3^{WT/WT}$. DFS (*A*) and OS (*B*) were assessed as described in "Materials and Methods." Significance values (P) are indicated (also see Table 3 depicting the results of pairwise comparisons).

Table 3 *Pairwise comparisons of outcome for the three genotype groups*

Pairwise comparisons	DFS	OS
$FLT3^{WT/WT}$ *versus* $FLT3^{ITD/WT}$	0.32	0.98
$FLT3^{ITD/WT}$ *versus* $FLT3^{ITD/-}$	0.025	0.008[a]
$FLT3^{WT/WT}$ *versus* $FLT3^{ITD/-}$	0.0008[a]	0.0005[a]

[a] Significant P using the more conservative significance level of α = 0.0167 adjusted for multiple comparisons.

Table 4 *Impact on clinical outcome of the presence of the ITD mutation*

	$FLT3^{WT/WT}$ ($N = 59$)	$FLT3^{ITD/WT}$ and $FLT3^{ITD/-}$ ($N = 23$)	P
Clinical outcome			
Percentage of CR	86	77	0.33
DFS			
Median (months)	52[a]	8	0.03
Percentage of DFS at 12 months (95% confidence interval)	71 (62, 80)	39 (29, 48)	
Survival			
Median (months)	46[a]	11	0.14
Percentage alive at 12 months (95% confidence interval)	74 (65, 82)	46 (36, 55)	

[a] Nonparametric estimate of the median is not available. Tabled value represents the median assuming the survival estimates after the last observation follows an exponential curve.

indicator variables. Although there were differences among the three groups with respect to WBCs and a moderate difference with respect to percentage of blood blasts at baseline, these additional characteristics did not impact outcome nor did any other baseline characteristic. Therefore, although the multivariate model is "unadjusted" because no other potential explanatory variable was significant in the model, examination of the relative risks provides further insight into the differences in outcome between these three genotypes. The relative risk of the $FLT3^{ITD/-}$ group compared with the $FLT3^{WT/WT}$ group is 5.5 and 4.4 for DFS and OS, respectively. However, the relative risk of the $FLT3^{ITD/WT}$ group compared with the $FLT3^{WT/WT}$ group is 1.6 and 1.0 for DFS and OS, respectively. Again, we conclude that the $FLT3^{ITD/-}$ group has a poorer prognosis for DFS and OS, whereas the $FLT3^{ITD/WT}$ and $FLT3^{WT/WT}$ have a relatively similar outcome.

DISCUSSION

The *FLT3* ITD genotype has thus far been largely defined as a somatic mutation resulting in one WT *FLT3* allele and the partially duplicated *FLT3* allele, the latter of which results in constitutive activation of the RTK (6, 8–11, 13, 14, 22–25). A number of studies (8, 21) published to date have indicated that the presence of a *FLT3* ITD is a poor prognostic indicator in adult AML. The true importance of the defect has likely not been adequately defined in these patients because a variety of confounding factors that have independent prognostic value have not been considered. These include age at diagnosis, karyotype, and variation in treatment regimens. In the current report, we assessed the prognostic significance of the *FLT3* ITD in 82 adult AML patients <60 years of age with a normal karyotype at diagnosis who were treated on a single, dose-intensive protocol in a multi-institutional trial within the CALGB. Under these circumstances, we then analyzed the *FLT3* genotype in each diagnostic bone marrow sample from these patients in an attempt to define the impact of the ITD somatic mutation on clinical outcome.

We have identified at least three distinct genotypes of the *FLT3* locus: $FLT3^{WT/WT}$, $FLT3^{ITD/WT}$, and $FLT3^{ITD/-}$. The hemizygous $FLT3^{ITD/-}$ had been detected previously in one (1.1%) of 91 children with AML (8) and in four (4.9%) of 81 adult AML patients (7). However, the AML patient populations studied were cytogenetically heterogeneous and included patients with karyotypes conferring poor, intermediate, and favorable prognosis. When we restricted our analysis to adults younger than 60 with a normal karyotype, the $FLT3^{ITD/-}$ was detected in nearly 10% of all of the patients analyzed and 35% of all of the cases with a *FLT3* ITD allele. The first evidence that the $FLT3^{ITD/-}$ genotype might be associated with a distinct phenotype came from the analysis of clinical outcome, demonstrating that this subset of uniformly treated relatively young adult AML patients with normal cytogenetics had a significantly unfavorable outcome despite their otherwise "standard risk" profile.

No difference in overall survival was found between the $FLT3^{WT/WT}$ group and the $FLT3^{ITD/WT}$ group, alone or in combination with the $FLT3^{ITD/-}$ group. How can this be explained? In the absence of a WT *FLT3* allele, a myeloblast with the *FLT3* ITD mutant homodimers could continuously as well as aberrantly activate downstream signaling pathways (26), when compared to myeloblasts with either the $FLT3^{WT/WT}$ or $FLT3^{ITD/WT}$ genotype (see Fig. 4). In this regard, a single amino acid substitution in the JM domain of the related receptor, PDGFR, causes constitutive kinase activity, and changes in the JM domain of PDGFR alter downstream docking sites for proteins that normally bind to the activated WT receptor (27, 28). In the $FLT3^{ITD/WT}$ cases, presence of the WT FLT3 has the potential to diminish the effect of the FLT3 ITD, whereas signal transduction by constitutively active FLT3 ITD homodimers in the $FLT3^{ITD/-}$ cases could enhance myeloblast survival and cell cycling, priming the cell for secondary molecular events contributing to malignant transformation and chemoresistance. Indeed, seven of the eight patients with the $FLT3^{ITD/-}$ genotype and otherwise intermediate risk had either refractory AML or relapsed shortly after one or two cycles of consolidation therapy. Therefore, this report identifies a subset of relatively young adult AML patients (median age of 37) with a normal karyotype and $FLT3^{ITD/-}$ genotype that has an especially poor prognosis despite dose-intensive chemotherapy. Because this is a relatively small study, a larger study of the kind we have presented could provide additional strength to our conclusions, especially as to whether the $FLT3^{WT/WT}$ and $FLT3^{ITD/WT}$ genotypes still have relatively similar outcomes after longer follow-up data are obtained. Moreover, it will also be interesting to examine cases we have defined as $FLT3^{WT/WT}$ and $FLT3^{ITD/WT}$ for evidence of an activating point mutation (29), recently discovered in 8 of 201 (4%) *de novo* AML cases, to determine whether its presence impacts our results.

With virtually all of the *FLT3* ITDs described to date having been in-frame and experimental models demonstrating ligand-independent dimerization and constitutive autophosphorylation in the *FLT3* ITD mutations, a gain-of-function mutation is quite likely the responsible mechanism for the phenotype. Most gain-of-function mutations are associated with a dominant-negative effect where the mutant protein interferes in one way or another with the function of a normal protein arising from the WT allele on the other chromosome in a heterozygous situation. In the $FLT3^{ITD/-}$ cases, it is the constitutive activation of *FLT3* by the ITD in the absence of a WT allele, as demonstrated by LOH, that clearly predicts the poor prognosis phenotype in this subset of AML patients <60 years of age with a normal karyotype. This gain-of-function by the mutated *FLT3* ITD allele in the absence of the second WT *FLT3* allele is referred to as a dominant-positive effect.

Table 5 *Impact on clinical outcome of the absence of WT FLT3*

	$FLT3^{WT/WT}$ and $FLT3^{ITD/WT}$ ($N = 74$)	$FLT3^{ITD/-}$ ($N = 8$)	P
Clinical outcome			
Percentage of CR	85	75	0.61
DFS			
Median (months)	44[a]	4	
Percentage of DFS at 12 months (95% confidence interval)	68 (59, 76)	17 (12, 22)	0.0005
Survival			
Median (months)	47[a]	7	
Percentage alive at 12 months (95% confidence interval)	72 (64, 79)	13 (10, 15)	0.0003

[a] Nonparametric estimate of the median is not available. Tabled value represents the median assuming the survival estimates after the last observation follows an exponential curve.

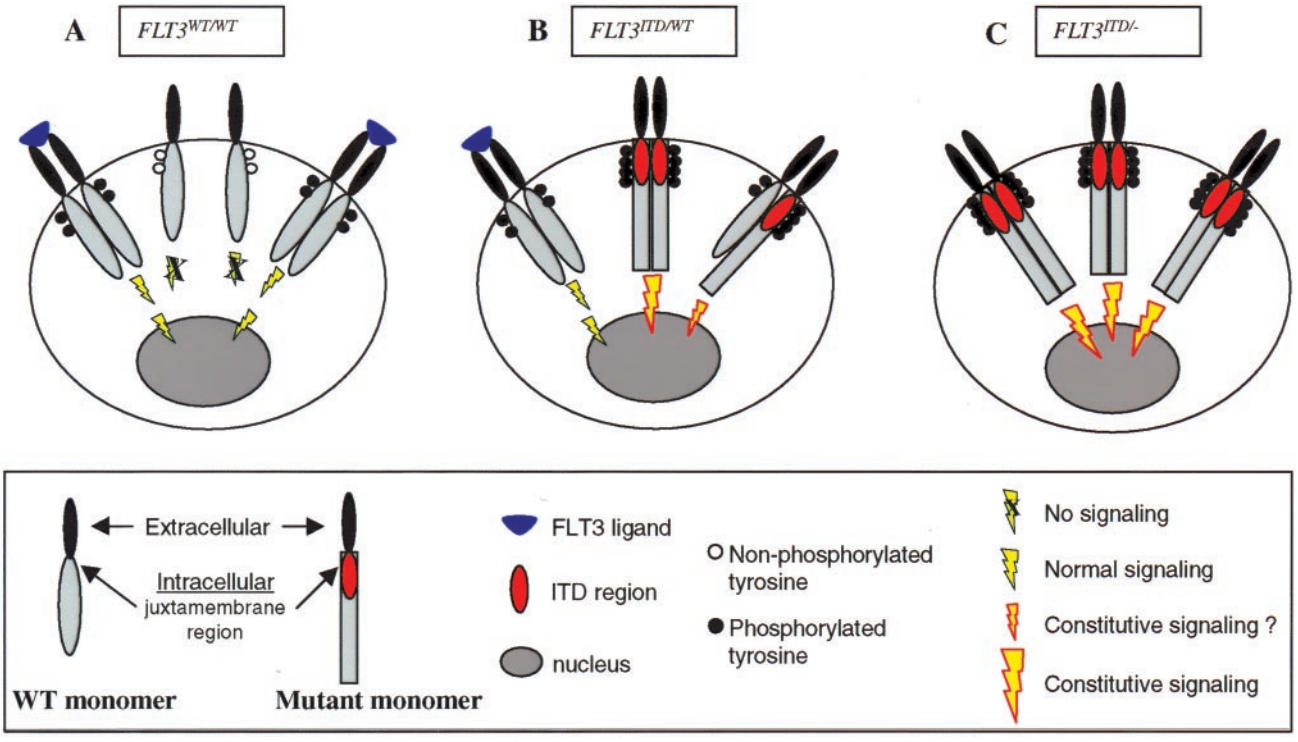


Fig. 4. A proposed model demonstrating how a greater growth advantage may be conferred by the $FLT3^{ITD/-}$ genotype compared with the $FLT3^{WT/WT}$ and $FLT3^{ITD/WT}$ genotypes. *A*, in the presence of FLT3 ligand, WT FLT3 proteins dimerize and phosphorylate tyrosines located in the intracellular JM domain, thereby activating the kinase and inducing signaling in AML cells with the $FLT3^{WT/WT}$ genotype. *B*, in AML cells with the $FLT3^{ITD/WT}$ genotype, expression of both WT and FLT3 ITD proteins may produce aberrant, intermediate signaling in the presence and/or absence of FLT3 ligand, compared with $FLT3^{WT/WT}$ and $FLT3^{ITD/-}$ cells. Potentially, signaling pathways activated by different dimers could be antagonistic, thereby having a neutralizing effect on the end point of those pathways. This diminishing effect by WT FLT3 is supported by the clinical data showing an increase in WBCs but no survival advantage for AML cases with the $FLT3^{ITD/WT}$ compared with the $FLT3^{WT/WT}$ cases (Tables 1 and 3). *C*, independent of FLT3 ligand, constitutive activation of FLT3 ITD homodimers leads to constitutive activation of downstream signaling molecules, potentially both physiological and aberrant, and occurs in AML cells that have the $FLT3^{ITD/-}$ genotype, providing the cell with a greater growth advantage compared with AML cells having the other two *FLT3* genotypes.

The *FLT3* ITD has been suggested to arise via a slippage-repair mechanism (10), but the mechanism whereby the loss of the WT *FLT3* allele is achieved is currently unknown. The presence of a normal karyotype, containing two intact copies of chromosome 13, in each patient with $FLT3^{ITD/-}$ indicates that loss of the WT *FLT3* allele is not a result of a monosomy 13 or a cytogenetically detectable deletion involving band 13q12. Results of LOH studies suggest that an interstitial, submicroscopic deletion of 13q12 may be responsible for the observed WT *FLT3* allele loss. Alternatively, the whole chromosome 13 with WT allele could be initially lost to be later replaced by a duplicated copy of the chromosome 13 containing the *FLT3* ITD allele (thus generating a $FLT3^{ITD/ITD}$ genotype). However, this is unlikely to represent a consistent mechanism of WT *FLT3* allele loss because no LOH of an informative marker D13S1246 was detected in blasts of patient 1 in Fig. 2*B*. Moreover, careful review of karyotypes of all eight patients with $FLT3^{ITD/-}$ revealed that in two of them, including patient 3 in Fig. 2*B*, the two homologous chromosomes clearly differed morphologically with regard to the heteromorphic regions in their short arms. Thus, we believe that duplication of the chromosome 13 homologue containing the *FLT3* ITD allele has likely not taken place in these three patients. On the other hand, LOH of several markers, including D13S175 mapped to 13q11 and D13S1265 mapped to 13q33, suggests a high degree of genetic instability in morphologically normal chromosomes 13 in this subset of patients with AML. We are investigating this further, along with the possibility that a subset of the $FLT3^{WT/WT}$ cases may, in actuality, have a $FLT3^{WT/-}$ genotype as well as the possibility that LOH has occurred elsewhere on chromosomes 13 near the *FLT3* locus in cases with either the $FLT3^{WT/WT}$ or $FLT3^{ITD/WT}$ genotype.

There appears to be a greater number of $FLT3^{ITD/WT}$ AML patients than $FLT3^{ITD/-}$ AML patients in all of the series reported to date, and our data show no clear adverse impact on OS or DFS in the former group compared with $FLT3^{WT/WT}$ patients. Therefore, it seems reasonable to speculate that the *FLT3* ITD somatic mutation likely occurs before the loss of the *FLT3* WT allele and that it may in some way contribute to the latter event, possibly through the induction of genomic instability. Further analysis of the *FLT3* locus by fluorescence *in situ* hybridization or LOH analysis in AML cases that lack the *FLT3* ITD might provide additional insight into this possibility.

The identification of a new subset of poor prognosis patients in AML patients without karyotypic abnormalities is important for directing early aggressive therapeutic alternatives for this otherwise "standard risk" group of patients. However, it is especially important given the nature of their molecular defect. The aberrant kinase activity that is likely maximized in the hemizygous cases would certainly suggest that therapy with a TK inhibitor specific for FLT3 should be beneficial to these unfortunate patients, the same way STI571 has been beneficial in patients with stable phase chronic myelogenous leukemia (30). Such targeted therapies are in development at this time and might need to be directed to this specific subset of AML patients to demonstrate a significant impact on the disease. It is possible, however, that the other molecular defects arising before or after this mutation will require targeting as well. Perhaps allogeneic stem cell transplantation with a suitable donor should be incorporated into the primary induction regimen for these patients.

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Absence of the Wild-Type Allele Predicts Poor Prognosis in Adult *de Novo* Acute Myeloid Leukemia with Normal Cytogenetics and the Internal Tandem Duplication of *FLT3*: A Cancer and Leukemia Group B Study

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