NEOPLASIA

Prognostic Implication of *FLT3* and N-*RAS* Gene Mutations in Acute Myeloid Leukemia

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Internal tandem duplication of the *FLT3* gene and point mutations of the N-*RAS* gene are the most frequent somatic mutations causing aberrant signal-transduction in acute myeloid leukemia (AML). However, their prognostic importance is unclear. In this study, their prognostic significance was analyzed in 201 newly diagnosed patients with de novo AML except acute promyelocytic leukemia. Three patients had mutations in both genes, 43 had only the *FLT3* gene mutation, 25 had only the N-*RAS* gene mutation, and 130 had neither. These mutations seemed to occur independently. Both mutations were related to high peripheral white blood cell counts, and the *FLT3* gene mutation was infrequently observed in the French-American-British (FAB)-M2 type. AML cases with wild *FLT3*/mutant N-*RAS* had a lower complete remission (CR) rate than those with wild *FLT3*/wild N-*RAS*, whereas the presence of mutant *FLT3* did not affect the CR rate. Univariate analysis showed that unfavorable prognostic factors for overall survival were age 60 years or older ($P = .0002$), cytogenetic data ($P = .002$), FAB types other than M2 ($P = .002$), leukocytosis over 100 ± 10^9/L ($P = .003$), and the *FLT3* gene mutation ($P = .004$). However, the N-*RAS* gene mutation was only a marginal prognostic factor ($P = .06$). For the subjects under 60 years old, multivariate analysis showed that the *FLT3* gene mutation was the strongest prognostic factor ($P = .008$) for overall survival. The *FLT3* gene mutation, whose presence is detectable only by genomic polymerase chain reaction amplification and gel electrophoresis, might serve as an important molecular marker to predict the prognosis of patients with AML.

OUR UNDERSTANDING of the pathophysiology of acute myeloid leukemia (AML) has rapidly advanced over the past two decades. Cytogenetic studies have clarified the molecular mechanism of leukemogenesis. Chromosomal translocations, which are found in half of all AML cases and correlate with the French-American-British (FAB) types, target and deregulate the gene-coding transcriptional factors that are important to hematopoiesis.[1-3] Nonrandom chromosomal loss or deletion suggests that anti-oncogenes are also involved in AML.[1,2] Mutations and/or deletion of the *P53* gene were observed in AML,[4] although the incidence of these events is far lower than that in solid tumors.[5] Transfection studies using NIH/3T3 cells showed that activated *RAS* genes are associated with the pathogenesis of AML.[6] *RAS* gene mutations, the majority of which involve the N-*RAS* gene, are found in up to 30% of AML cases.[7-9] These genetic alterations have been molecularly detectable and used for diagnosis, detection of minimal residual disease, and prediction of prognosis. For routine assessment, however, the following factors are required: clinical incidence and significance, time- and cost-saving measures, and specificity and sensitivity of the examination.

Recently an internal tandem duplication of the juxtamembrane (JM) domain-coding sequence of the *FLT3* gene was found in 20% of AML.[10,11] FLT3 is a class III receptor tyrosine kinase (RTK), along with KIT, FMS, and PDGFR.[12,13] Since FLT3 preferentially expressed on hematopoietic stem cells and its ligand (FL) on bone marrow stroma cells,[13-15] FLT3-FL interaction plays an important role in primitive hematopoiesis. Furthermore, most clinical samples from AML express functional FLT3, and the FLT3-FL interaction might also be associated with the proliferation of leukemia cells.[16] The duplicated sequences of the mutant *FLT3* consisted of exon 11, but sometimes involved intron 11 and exon 12.[10,11] Although their location and length varied from sample to sample, the portion of tandem duplication was always readable in frame. Actually the transcripts with a long JM domain did not disrupt the downstream regions. The mutant FLT3 was ligand-independently phosphorylated when expressed in Cos 7 cells,[17] indicating dominant positive mutation. Interleukin-3 (IL-3)–dependent myeloid progenitor cell lines, FDC/P1 and 32D, exhibited IL-3–independent growth when transfected with mutant *FLT3* (H. Kiyoi, T. Naoe, unpublished data, 1998). The *FLT3* gene mutation was found in all types of the FAB classification and in 3% of myelodysplastic syndrome, but

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never in chronic myeloid leukemia or in lymphoid malignancies.[11] In acute promyelocytic leukemia (FAB-M3), the presence of the *FLT3* gene mutation was related to high peripheral white blood cell (WBC) counts, high peripheral leukemia cell counts, and high lactate dehydrogenase (LDH) level.[18] These findings suggest that the *FLT3* gene mutation plays an important role in leukemia progression rather than initiation.

Since the detection of the *FLT3* gene mutation requires only polymerase chain reaction (PCR) amplification using genomic DNA followed by gel electrophoresis, we studied whether it could be used as a standard molecular marker for the prognosis of AML. Here we analyzed the prognostic significance of the *FLT3* gene mutation together with the N-*RAS* gene mutations in a large number of patients with AML.

PATIENTS AND METHODS

Patients and treatments. Two hundred one newly diagnosed patients with AML except for M3, who were treated with three protocols of the Japan Adult Leukemia Study Group, and whose leukemia cells were preserved with informed consent at initial diagnosis, were eligible for this study. Twenty-eight, 40, and 133 patients were treated by the AML-87,[19] AML-89,[20] and AML-92[21] protocols, respectively. AML was diagnosed according to the FAB classification, which was evaluated by the central review committee.

In the AML-87 study,[19] the induction therapy consisted of daily behenoyl cytarabine (BHAC) 200 mg/m^2, daily 6-mercaptopurine (6-MP) 70 mg/m^2, daily prednisolone 40 mg/m^2, and daunorubicin (DNR) 40 mg/m^2 on days 1 to 3, and if necessary on days 7, 8, and 11. The therapy was continued for 10 to 12 days until the bone marrow became severely hypoplastic with less than 5% blasts. In the AML-89 study,[20] patients were randomized to receive induction therapy that included BHAC (200 mg/m^2 by 3-hour infusion) or cytarabine (AraC, 80 mg/m^2 by continuous infusion). BHAC or AraC, and 6-MP 70 mg/m^2 were administered for 10 to 12 days, and DNR 40 mg/m^2 was administered on days 1 to 4, and if necessary, on days 10 to 12 in addition to the above schedule for AML-87. In the AML-92 study,[21] patients were randomized to receive BHAC-DM similar to the AML-87 protocol with or without etoposide (ETP, 100 mg/m^2 for 5 days). After achieving complete remission (CR), three courses of consolidation chemotherapy and six courses of intensification chemotherapy were administered. Patients 60 years or older received about two thirds of the dosage of each drug throughout the study period.

CR was determined when there were less than 5% blasts in normo-cellular bone marrow with normal levels of peripheral neutrophil and platelet counts. Overall survival (OS) was calculated from the first day of therapy to death. Disease-free survival (DFS) for patients who had achieved CR was measured from the date of CR to relapse or death. Patients who underwent bone marrow transplantation (BMT) were censored at the date of BMT.

Analysis of the internal tandem duplication of the FLT3 *gene.* High molecular weight DNA was extracted from AML cells as previously described.[9] Because previous studies showed that the location of internal tandem duplication of the *FLT3* gene was restricted to exons 11 and 12,[11,18] genomic PCR amplification was performed using the primers 11F, 5′-GCAATTTAGGTATGAAAGCCAGC, and 12R, 5′-CTTTCAGCATTTTGACGGCAACC-3′. The PCR mixture contained 500 ng of genomic DNA, 50 pmol of 11F and 12R primers, 0.2 mmol/L of each deoxynucleotide triphosphate, 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.001% gelatin (wt/vol), 50 mmol/L tetramethylammonium chloride and 2.5 U of *Taq* polymerase (Amplitaq; Perkin Elmer, Norwalk, CT). Denaturing, annealing, and extension steps were performed at 94°C for 30 seconds, 56°C for 1 minute and 72°C for 2 minutes, respectively, for 35 cycles on a GeneAmp PCR system 9600 (Perkin Elmer) including an initial 3 minutes denaturation step at 94°C and a final extension step at 72°C for 10 minutes. The amplified product was cut out from an agarose gel, purified with a Qiaex gel extraction kit (Qiagen Inc, Chatsworth, CA), and cloned into the pMOS*Blue* T-vector (Amersham, Buckinghamshire, UK) according to the manufacturer's recommendation. Ten recombinant colonies were chosen and cultured in LB medium. Plasmid DNA was prepared using a QIAprep spin plasmid miniprep kit (Qiagen Inc), and both strands were sequenced using fluorescein-conjugated −21M13 and T7 primers on a DNA sequencer (377; Applied Biosystems, Foster City, CA).

N-RAS *gene amplification and dot-blot hybridization.* To amplify the sequences spanning codons 12 and 13, and codon 61, the oligonucleotide primers were prepared as follows; 5′primer for codons 12, 13 (named NA12): 5′-GACTGAGTACAAACTGGTGG-3′, 3′primer for codons 12, 13 (NB12): 5′-CTCTATGGTGGGATCATATT-3′, 5′primer of codon 61 (NA61): 5′-GGTGAAACCTGTTTGTTGGA-3′, and 3′primer of codon 61 (NB61): 5′-ATACACAGAGGAAGCCTTCG-3′. The PCR was performed as described previously.[9] Genomic DNA was subjected to 35 cycles of PCR amplification (denaturation for 60 seconds at 92°C, annealing for 60 seconds at 55°C, and elongation for 60 seconds at 72°C). The efficiency of amplification was evaluated by agarose gel electrophoresis, and only the reactions resulting in the appropriately sized band were further analyzed. Dot-blot and oligonucleotide probe hybridization were performed as previously described.[9] Briefly, either 100 ng DNA amplified by PCR was transferred to nylon filter membranes (Hybond-N+; Amersham) with a 96-well filtration manifold. Blotted DNA was crosslinked by UV illumination and the membranes were hybridized with [γ-^{32}P] labeled oligonucleotide probes. The oligonucleotide panel included probes specific for the wild-type allele and all possible amino acid substitutions at codons 12, 13, and 61 of the N-*RAS* gene. The prehybridization, hybridization, and washing of membranes were performed under standard conditions.[9] The membranes were exposed to Kodak XAR5 films (Eastman Kodak, Rochester, NY) at −70°C using intensifying screens.

Statistical methods. The following clinical characteristics at diagnosis were analyzed: age, sex, FAB classification, peripheral WBC count, percentage of blasts in bone marrow, platelet count, serum LDH concentration, the presence of hepato-splenomegaly or extramedullary involvement, and cytogenetic findings. Analysis of frequencies was performed using the Fisher's exact test for 2×2 tables or the Pearson's χ^2 test for larger tables. Differences in median variables in age, peripheral WBC counts, platelet counts, percentage of blasts in bone marrow, and LDH were also analyzed with the Wilcoxon rank-sum test. CR rates in the two groups were compared using the Fisher's exact test. The logistic progression model was used for multivariate analysis.

Survival probabilities were estimated by the Kaplan-Meier method, and differences in the survival distributions between the mutation-positive and -negative groups were evaluated by the log-rank test. The prognostic significance of the clinical variables was assessed using the Cox proportional hazards model. These statistic analyses were performed with StatView software (Abacus Concepts Inc, Berkeley, CA) or SAS programs (SAS Institute Inc, Cary, NC). Because CR rates, OS, and DFS according to induction therapies (the AML-87, -89, and -92 studies) did not show any difference, the data of the three studies were combined and analyzed. For all analyses, the *P* values were two-tailed, and a *P* value of less than .05 was considered statistically significant.

RESULTS

A total of 201 newly diagnosed patients with AML were studied for the *FLT3* and N-*RAS* gene mutations. *FLT3* gene mutations were identified in 46 of the 201 patients. Translated into amino acids, the tandem duplications frequently involved a Y-rich stretch from codon 589 to 599 (data not shown), the same position as in previous studies.[11,18] N-*RAS* gene mutations were

detected in 28 of the 201 patients. The mutations at codons 12, 13, and 61 were observed in 12, 13, and 7 patients, respectively. Three of the 28 patients had multiple mutations at codon 12, one at codon 61, and three at codons 12 and 13. Of a total of 37 N-*RAS* gene point mutations, G to A transition was the most frequent (16/37).

In a total of 201 patients, 3 patients (1.5%) had mutations in both genes (mutant *FLT3*/mutant N-*RAS*), 43 (21.4%) had only mutant *FLT3* gene (mutant *FLT3*/wild N-*RAS*), 25 (12.4%) had only mutant N-*RAS* gene (wild *FLT3*/mutant N-*RAS*), and 130 (64.7%) had neither (wild *FLT3*/wild N-*RAS*). Clinical characteristics were analyzed comparing these four groups (Table 1). The presence of mutant *FLT3* or mutant N-*RAS* was not related to age and sex (data not shown). WBC counts in the wild *FLT3*/wild N-*RAS* group were significantly lower than in other groups ($P = .03$ *v* mutant *FLT3*/mutant N-*RAS*, $P < .0001$ *v* mutant *FLT3*/wild N-*RAS*, $P = .002$ *v* wild *FLT3*/mutant N-*RAS*) (Table 1). Serum LDH level in the wild *FLT3*/wild N-*RAS* group tended to be lower than that in the other groups. The occurrence of hepato-splenomegaly or extramedullary involvement was not significantly affected by these mutations. The incidence of the *FLT3* gene mutation according to FAB classification was ranked as follows: M4/5 (22/62) > M1 (14/48) > M2 (8/83). Additionally, the *FLT3* gene mutation was infrequently observed in the leukemia with t(8;21) ($P = .02$). The incidence of the N-*RAS* gene mutation was similarly ordered: M4/5 (11/62) > M1 (6/48) > M2 (10/83).

WBC counts were further analyzed in each FAB group. In M1 and M2, WBC counts in the wild *FLT3*/wild N-*RAS* group were lower than the mutant groups ($P = .004$ *v* mutant *FLT3*/wild N-*RAS*, $P = .09$ *v* wild *FLT3*/mutant N-*RAS*). In M2, those in the mutant *FLT3*/wild N-*RAS* group tended to be lower than other groups ($P = .2$ *v* mutant *FLT3*/wild N-*RAS*, $P = .1$ *v* wild *FLT3*/mutant N-*RAS*). In M4/5, however, there was no difference of WBC counts.

The CR rates by initial induction therapy were significantly different between the wild *FLT3*/mutant N-*RAS* group and the wild *FLT3*/wild N-*RAS* group (52.0% *v* 79.7%, $P = .005$). However, the presence of mutant *FLT3* did not affect the CR rate. Chi-squared analysis showed that FAB types other than M2, the presence of N-*RAS* gene mutation, and leukocytosis (over 100×10^9/L) were unfavorable factors for achieving CR ($P = .001$, $P = .04$, and $P = .07$, respectively). Multivariate analysis using the logistic progression model showed that FAB types other than M2 ($P = .001$) and N-*RAS* gene mutation ($P = .05$) were independent unfavorable factors for achieving CR, whereas leukocytosis was not significant.

At a median follow-up time of 50 months (range, 3 to 118 months), 68 of 201 patients (33.8%) were alive. The predicted OS rates at 60 months were 14.0%, 16.0%, and 44.6% in the mutant *FLT3*/wild N-*RAS*, wild *FLT3*/mutant N-*RAS*, and wild *FLT3*/wild N-*RAS* groups, respectively (Fig 1). Mutant *FLT3*/wild N-*RAS* and wild *FLT3*/mutant N-*RAS* groups had worse

Table 1. Clinical Characteristics of 201 Patients With De Novo AML Except M3

	Total (N = 201)	Mutant *FLT3*/ Mutant N-*RAS* (N = 3)	Mutant *FLT3*/ Wild N-*RAS* (N = 43)	Wild *FLT3*/ Mutant N-*RAS* (N = 25)	Wild *FLT3*/ Wild N-*RAS* (N = 130)
Age	49	60	56	45	48.5
		(41-64)	(15-77)	(16-82)	(15-85)
WBC (10^9/L)	24.7	120*	52.2‡	67.8†	19.3
		(50.2-372)	(2.1-632)	(5.3-234)	(0.9-33.7)
FAB					
M0	3	0	0	1	2
M1	48	2	12	4	30
M2	83	1	7*	9	66
M4	47	0	17	11	19
M5	15	0	5	0	10
M6	4	0	1	0	3
M7	1	0	1	0	0
Cytogenetics					
t(8;21)	28	0	2*	1	25
inv(16)	6	0	2	0	4
t(9;22)	3	0	0	1	2
del(5) or del(7)	11	0	2	2	7
Others	41	0	5	7	29
Normal	82	3	20	12	47
ND	30	0	11	2	17
Outcome					
CR	147	2	30	13†	102
Failure	51	0	13	12†	26
Unevaluable	3	1	0	0	2

Mean (minimum to maximum) values are indicated in age and WBC. Number of cases are shown in FAB, cytogenetics, and outcome.

*$.1 > P > .01$.

†$.01 > P > .001$.

‡$P < .001$ compared with wild *FLT3*/wild N-*RAS* group.

Abbreviation: ND, not determined.

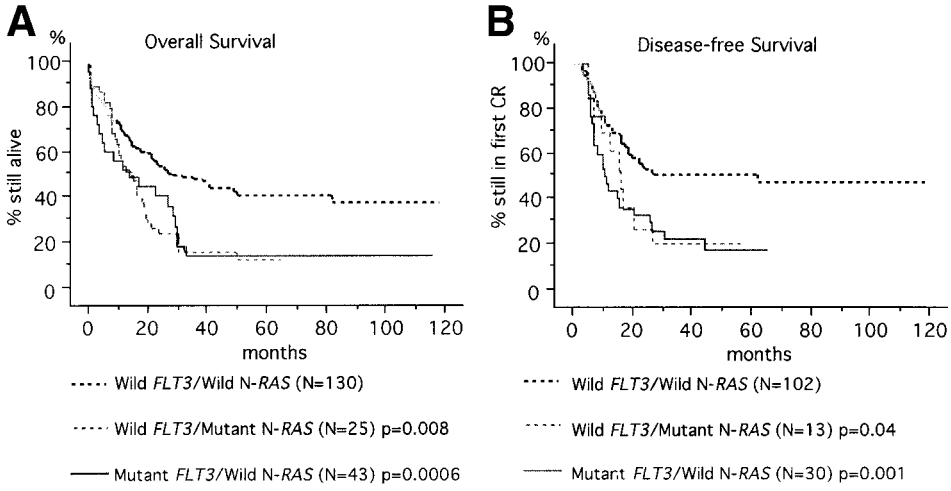

Fig 1. Kaplan-Meier curves according to the *FLT3* and N-*RAS* gene mutations. (A) OS of 198 patients. (B) DFS of 145 patients who achieved CR. Three patients with both *FLT3* and N-*RAS* gene mutations were excluded from the analysis because the number was small. Statistic difference was evaluated by the log-rank test.

prognosis than the wild *FLT3*/wild N-*RAS* group ($P = .0006$ and $P = .008$, respectively).

The prognosis of AML depends on factors such as age, initial leukocyte count, FAB classification, karyotype, immune phenotype, and response to remission-induction therapy.[19-23] Among them, cytogenetic data is thought to be the most important prognostic factor for AML.[1] Based on cytogenetic findings, the 201 patients were segregated into four groups: a good-risk group (n = 34) was defined by karyotype, t(8;21) or inv(16); a poor-risk group (n = 14) by t(9;22), 11q23 alterations, del(5) or del(7); a standard-risk group (n = 123) by normal or other karyotypes; and a karyotype-unknown group (n = 30). The predicted OS rates at 60 months were 57.1%, 33.6%, 13.3%, and 20.7% in the good-risk, standard-risk, poor-risk, and karyotype-unknown groups, respectively. In the good-risk and standard-risk groups, the *FLT3* and N-*RAS* gene mutations were associated with unfavorable prognosis (Fig 2). In the poor-risk and karyotype-unknown groups, however, there was no significant association between these mutations and prognosis.

Univariate analysis showed that unfavorable prognostic factors for OS were age 60 years or older ($P = .0002$), cytogenetic data ($P = .002$), leukocytosis over 100×10^9/L ($P = .003$), the *FLT3* gene mutation ($P = .004$) (Table 2). However, the N-*RAS* gene mutation was only a marginal prognostic factor ($P = .06$). Multivariate analysis showed that age (60 years or older) was the strongest unfavorable factor (relative risk [RR], 1.9; $P = .002$), followed by cytogenetics ($P = .004$). FAB types, leukocytosis, and the *FLT3* gene mutation were less important. It might be partly associated with the poor prognosis that the dosage of chemotherapy to the patients 60 years or older was reduced. If the subjects were limited to under 60 years old, the *FLT3* gene mutation became the strongest prognostic factor

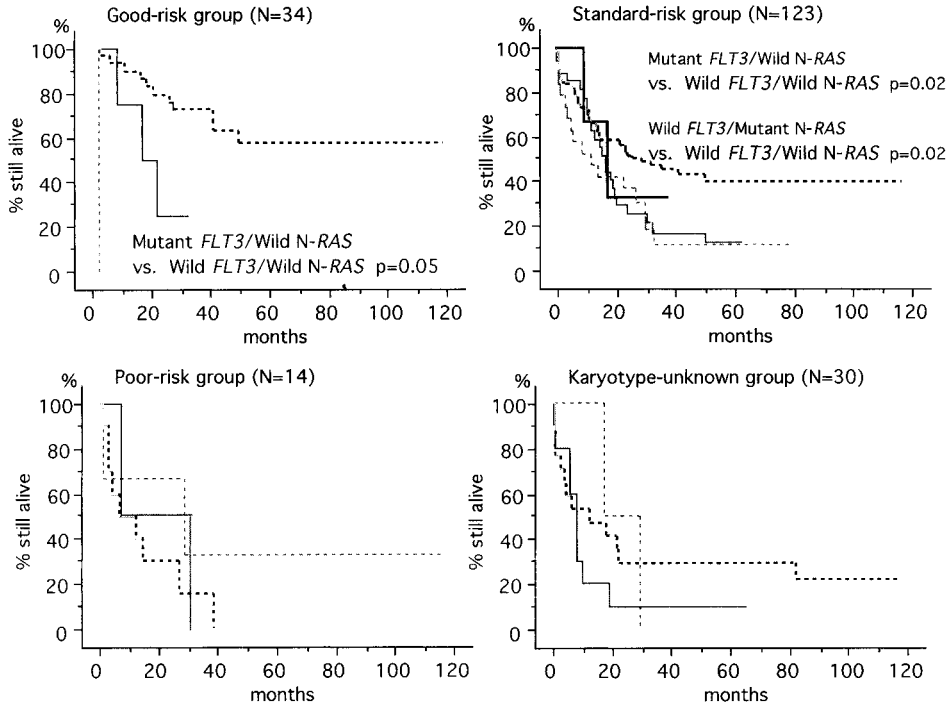

Fig 2. OS according to the *FLT3* and N-*RAS* gene mutations in each karyotype-risk group. In good-risk patients, the mutant *FLT3*/wild N-*RAS* group had worse prognosis than the wild *FLT3*/wild N-*RAS* group ($P = .05$). In standard-risk patients, the mutant *FLT3*/wild N-*RAS* group and wild *FLT3*/mutant N-*RAS* group had worse prognosis than the wild *FLT3*/wild N-*RAS* group ($P = .02$).(—), Mutant *FLT3*/mutant N-*RAS*; (—), mutant *FLT3*/wild N-*RAS*; (—·—·—), wild *FLT3*/mutant N-*RAS*; (—·—·—·—): wild *FLT3*/wild N-*RAS*.

Table 2. Unfavorable Prognostic Factors for OS in 201 Patients With De Novo AML Except M3

Prognostic Factors	Univariate	Multivariate	
	P Value	*P* Value	Relative Risk (95% CI)
Age, 60 yr or older	.0002	.002	1.9 (1.2-2.6)
Cytogenetics*	.002	.004	2.6†
FAB other than M2	.002	.2	
WBC count >100 × 10⁹/L	.003	.2	
FLT3 gene mutation	.004	.1	
N-*RAS* gene mutation	.06	.6	

*Karyotypes were segregated into four groups: a good-risk group with t(8;21) or inv(16); a poor-risk group with t(9;22), 11q23 alterations, del(5) or del(7); a standard-risk group with normal or other karyotypes; and a karyotype-unknown group.

†Comparison between the good-risk and poor-risk groups.

(RR, 2.1; $P = .008$), with a second factor being cytogenetics ($P = .07$) and N-*RAS* gene mutation (RR, 1.7; $P = .09$) (Table 3).

DFS was further analyzed in 147 patients who achieved CR. The predicted DFS rates at 60 months were 20.0%, 23.1%, and 53.9% in the mutant *FLT3*/wild N-*RAS*, wild *FLT3*/mutant N-*RAS*, and wild *FLT3*/wild N-*RAS* groups, respectively (Fig 1). According to univariate analysis, the following pretreatment variables showed statistical significance for DFS: age ($P = .002$), cytogenetic data ($P = .004$), leukocytosis ($P = .004$), the *FLT3* gene mutation ($P = .006$) (Table 4). Multivariate analysis showed that age was the most unfavorable factor (RR, 2.0; $P = .003$), followed by cytogenetic data ($P = .001$), and leukocytosis ($P = .04$). For the subjects under 60 years old, leukocytosis was the sole important factor (RR, 2.6; $P = .01$) (Table 5).

DISCUSSION

In this study, we showed that the *FLT3* gene mutation is significantly associated with clinical feature and prognosis of AML. In our previous analysis on M3,[18] the *FLT3* gene mutation closely correlated to leukocytosis but not significantly to prognosis. The reasons for the discrepancy between M3 and the others are the sample size and that the prognosis for M3 was favorable compared with other types of AML, especially after the clinical introduction of differentiation therapy with all-*trans* retinoic acid.[24] The present study showed that the *FLT3* gene mutation was significantly associated with leukocytosis in M1 and M2 but not in M4/5. However, the *FLT3* gene mutation was associated with an unfavorable prognosis regardless of FAB type. Mutant *FLT3* might not be simply associated with cell proliferation but also with inhibition of apoptosis.[25] The relevance of mutant *FLT3* may be dependent on intracellular conditions, which are determined by cell lineage and gene alterations. Importantly, the difference of prognosis between mutant and wild *FLT3* was more remarkable in the good- and standard-risk groups than in the poor-risk and karyotype-unknown groups. Furthermore, the *FLT3* gene mutation was the strongest prognostic factor for subjects under 60 years old. Thus the *FLT3* gene mutation is a useful molecular marker to identify high-risk patients who could not be characterized by conventional criteria.

Table 3. Unfavorable Prognostic Factors for OS in the 143 Patients Younger Than 60 Years Old

Prognostic Factors	Univariate	Multivariate	
	P Value	*P* Value	Relative Risk (95% CI)
FLT3 gene mutation	.001	.008	2.1 (1.2-3.4)
WBC count >100 × 10⁹/L	.01	.2	
Cytogenetics	.03	.07	1.5*
FAB other than M2	.07	.5	
N-*RAS* gene mutation	.09	.09	

*Comparison between the good-risk and poor-risk groups.

Table 4. Unfavorable Prognostic Factors for DFS in the 147 Patients Who Achieved CR

Prognostic Factors	Univariate	Multivariate	
	P Value	*P* Value	Relative Risk (95% CI)
Age, 60 yr or older	.002	.003	2.0 (1.3-3.3)
Cytogenetics	.004	.001	4.1*
WBC count >100 × 10⁹/L	.004	.04	1.8 (1.0-3.3)
FLT3 gene mutation	.006	.2	
FAB other than M2	.1	.5	
N-*RAS* gene mutation	.2	.1	

*Comparison between the good-risk and poor-risk groups.

The prognostic significance of the N-*RAS* gene mutation is a matter of controversy. Generally *RAS* gene mutation is associated with tumor progression and was reported to be associated with poor prognosis in solid tumors and acute lymphoblastic leukemia (ALL).[26,27] In AML, however, there was no difference in survival between *RAS* mutation-positive and -negative patients.[7,8] In this study, the presence of N-*RAS* gene mutation was related to low CR rate and was marginally associated with unfavorable prognosis. One reason for the discrepancy between our data and previous reports is that we could exclude the influence of *FLT3* gene mutation in this study. When the cases with mutant N-*RAS* were compared with those with wild N-*RAS*, the prognostic difference was limited (OS, $P = .06$; DFS, $P = .2$). Because no ALL cases have mutant *FLT3*, their prognosis might be directly influenced by the presence of mutant N-*RAS*.[27]

It is particularly interesting to investigate the multiplicity of gene alterations associated with leukemia. Our results suggest that the mutations of *FLT3* and N-*RAS* genes occurred independently ($P = .07$ by the Fisher's exact test), although we could not entirely rule out the possibility that a weak adverse interaction exists between mutant *FLT3* and mutant N-*RAS*. Because both gene alterations are associated with aberrant signal-transduction, these mutations may be additively or

Table 5. Unfavorable Prognostic Factors for DFS in the 106 Patients Who Achieved CR and Were Under 60 Years Old

Prognostic Factors	Univariate	Multivariate	
	P Value	*P* Value	Relative Risk (95% CI)
WBC count >100 × 10⁹/L	.003	.01	2.6 (1.2-5.5)
FLT3 gene mutation	.02	.1	
Cytogenetics	.1	.1	
N-*RAS* gene mutation	.2	.5	
FAB other than M2	.6	.7	

synergistically associated with leukemia progression. Our serial studies indicated that no leukemia cases carry both t(9;22)/*BCR-ABL* and mutant *FLT3*.[11] Because p115CBL, which is activated by BCR-ABL,[28] is one of the downstream proteins for *FLT3*,[29] *FLT3* gene mutation may bring no growth advantage to leukemia clone with *BCR-ABL*. Aberrant signal-transduction through mutant *FLT3* should be clarified to further characterize the functional significance of mutant *FLT3*.

Gain-of-function mutation of the *FLT3* gene suggests that new strategies would be applicable for the treatment of AML. Inhibitors of FLT3-pathway may selectively inhibit leukemia cell proliferation. It has been reported that inhibition of Jak-2 activity by a specific tyrosine kinase blocker blocks leukemic cell growth in vitro and induces programmed cell death in vivo.[30] In the future, the best choice of therapy may be established depending on an individual set of molecular alterations in each patient with leukemia.

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REFERENCES

1. Mrózek K, Heinonen K, de la Chapelle A, Bloomfield CD: Clinical significance of cytogenetics in acute myeloid leukemia. Semin Oncol 24:17, 1997

2. Caligiuri MA, Strout MP, Gilliland DG: Molecular biology of acute myeloid leukemia. Semin Oncol 24:32, 1997

3. Look AT: Oncogenic transcription factors in the human acute leukemias. Science 278:1059, 1997

4. Wattel E, Preudhomme C, Hecquet B, Vanrumbeke M, Quesnel B, Dervite I, Morel P, Fenaux P: p53 mutations are associated with resistance to chemotherapy and short survival in hematologic malignancies. Blood 84:3148, 1994

5. Greenblatt MS, Bennett WP, Hollstein M, Harris CC: Mutations in the *p53* tumor suppressor gene: Clues to cancer etiology and molecular pathogenesis. Cancer Res 54:4855, 1994

6. Bos JL: Ras oncogenes in human cancer: A review. Cancer Res 49:4682, 1989

7. Radich JP, Kopecky KJ, Willman CL, Weick J, Head D, Appelbaum F, Collins SJ: N-*ras* mutations in adult de novo acute myelogenous leukemia: Prevalence and clinical significance. Blood 76:801, 1990

8. Neubauer A, Dodge RK, George SL, Davey FR, Silver RT, Schiffer CA, Mayer RJ, Ball ED, Wurster-Hill D, Bloomfield CD, Liu ET: Prognostic importance of mutations in the *ras* proto-oncogenes in de novo acute myeloid leukemia. Blood 83:1603, 1994

9. Kubo K, Naoe T, Kiyoi H, Fukutani H, Kato Y, Oguri T, Yamamori S, Akatsuka Y, Kodera Y, Ohno R: Clonal analysis of multiple point mutations in the N-*ras* gene in patients with acute myeloid leukemia. Jpn J Cancer Res 84:379, 1993

10. Nakao H, Yokota S, Iwai T, Kaneko H, Horiike S, Kashima K, Sonoda Y, Fujimoto T, Misawa S: Internal tandem duplication of the flt3 gene found in acute myeloid leukemia. Leukemia 10:1911, 1996

11. Yokota S, Kiyoi H, Nakao M, Iwai T, Misawa S, Okuda T, Sonoda Y, Abe T, Kashima K, Matsuo Y, Naoe T: Internal tandem duplication of the FLT3 gene is preferentially seen in acute myeloid leukemia and myelodysplastic syndrome among various hematological malignancies. A study on a large series of patients and cell lines. Leukemia 11:1605, 1997

12. Matthews W, Jordan CT, Wiegand GW, Pardoll D, Lemischka IR: A receptor tyrosine kinase specific to hematopoietic stem and progenitor cell-enriched populations. Cell 65:1143, 1991

13. Rosnet O, Schiff C, Pébusque MJ, Marchetto S, Tonnelle C, Toiron Y, Birg F, Birnbaum D: Human FLT3/FLK2 gene: cDNA cloning and expression in hematopoietic cells. Blood 82:1110, 1993

14. Small D, Levenstein M, Kim E, Carow C, Amin S, Rockwell P, Witte L, Burrow C, Ratajczak MZ, Gewirtz AM, Civin CI: STK-1, the human homolog of Flk-2/Flt-3, is selectively expressed in CD34+ human bone marrow cells and is involved in the proliferation of early progenitor/stem cells. Proc Natl Acad Sci USA 91:459, 1994

15. Lyman SD, Jacobsen SEW: c-kit ligand and Flt3 ligand: Stem/progenitor cell factors with overlapping yet distinct activities. Blood 91:1101, 1998

16. Drexler HG: Expression of FLT3 receptor and response to FLT3 ligand by leukemic cells. Leukemia 10:588, 1996

17. Kiyoi H, Towatari M, Yokota S, Hamaguchi M, Ohno R, Saito H, Naoe T: Internal tandem duplication of the FLT3 gene is a novel modality of elongation mutation which causes constitutive activation of the product. Leukemia 12:1333, 1998

18. Kiyoi H, Naoe T, Yokota S, Nakao M, Minami S, Kuriyama K, Takeshita A, Saito K, Hasegawa S, Shimodaira S, Tamura J, Shimazaki C, Matsue K, Kobayashi H, Arima N, Suzuki R, Morishita H, Saito H, Ueda R, Ohno R: Internal tandem duplication of FLT3 associated with leucocytosis in acute promyelocytic leukemia. Leukemia 11:1447, 1997

19. Ohno R, Kobayashi T, Tanimoto M, Hiraoka A, Imai K, Asou N, Tomonaga M, Tsubaki K, Takahashi I, Kodera Y, Yoshida M, Murakami H, Naoe T, Shimoyama M, Tsukada T, Takeo T, Teshima H, Onozawa Y, Fujimoto K, Kuriyama K, Horiuchi A, Kimura I, Minami S, Miura Y, Kageyama S, Tahara T, Masaoka T, Shirakawa S, Saito H: Randomized study of individualized induction therapy with or without vincristine, and of maintenance-intensification therapy between 4 or 12 courses in adult acute myeloid leukemia. AML-87 Study of the Japan Adult Leukemia Study Group. Cancer 71:3888, 1993

20. Kobayashi T, Miyawaki S, Tanimoto M, Kuriyama K, Murakami H, Yoshida M, Minami S, Minato K, Tsubaki K, Ohmoto E, Oh H, Jinnai I, Sakamaki H, Hiraoka A, Kanamaru A, Takahashi I, Saito K, Naoe T, Yamada O, Asou N, Kageyama S, Emi N, Matsuoka A, Tomonaga M, Saito H, Ueda R, Ohno R: Randomized trials between behenoyl cytarabine and cytarabine in combination induction and consolidation therapy, and with or without ubenimex after maintenance/intensification therapy in adult acute myeloid leukemia. The Japan Leukemia Study Group. J Clin Oncol 14:204, 1996

21. Miyawaki S, Tanimoto M, Kobayashi T, Minami S, Tamura J, Hatake K, Kuriyama K, Ohmoto E, Saito K, Kanamaru A, Oho H, Ohtake S, Jinnai I, Sakamaki H, Yamada O, Asou N, Takahashi M, Takeyama K, Matsuda S, Ueda R, Ohno R: Randomized study of individualized induction therapy with or without etoposide in adult acute myeloid leukemia (except for M3) (JALSG-AML92 study) and analysis of prognostic factors. Blood 90:503a, 1997 (abstr, suppl 1)

22. Bradstock K, Matthews J, Benson E, Page F, Bishop J: Prognostic value of immunophenotyping in acute myeloid leukemia. Australian Leukaemia Study Group. Blood 84:1220, 1994

23. Bishop JF: The treatment of adult acute myeloid leukemia. Semin Oncol 24:57, 1997

24. Kanamaru A, Takemoto Y, Tanimoto M, Murakami H, Asou N, Kobayashi T, Kuriyama K, Ohmoto E, Sakamaki H, Tsubaki K, Hiraoka A, Yamada O, Oh H, Saito K, Matsuda S, Minato K, Ueda T, Ohno R: All-*trans* retinoic acid for the treatment of newly diagnosed acute promyelocytic leukemia. Japan Adult Leukemia Study Group. Blood 85:1202, 1995

25. Lisovsky M, Estrov Z, Zhang X, Consoli U, Sanchez-Williams G, Snell V, Munker R, Goodacre A, Savchenko V, Andreeff M: Flt3 ligand stimulates proliferation and inhibits apoptosis of acute myeloid leukemia cells: Regulation of Bcl-2 and Bax. Blood 88:3987, 1996

26. Slebos RJ, Kibbelaar RE, Dalesio O, Kooistra A, Stam J, Meijer CJ, Wagenaar SS, Vanderschueren RG, van Zandwijk N, Mooi WJ, Bos JL, Rodenhuis S: K-*RAS* oncogene activation as a prognostic marker in adenocarcinoma of the lung. N Engl J Med 323:561, 1990

27. Lübbert M, Mirro J Jr, Miller CW, Kahan J, Issac G, Kitchingman G, Mertelsmann R, Herrmann F, McCormick F, Koeffler HP: N-*Ras* gene point mutations in childhood acute lymphocytic leukemia correlate with a poor prognosis. Blood 75:1163, 1990

28. Gordon MY, Goldman JM: Cellular and molecular mechanisms in chronic myeloid leukaemia: Biology and treatment. Br J Haematol 95:10, 1996

29. Lavagna-Sevenier C, Marchetto S, Birnbaum D, Rosnet O: FLT3 signaling in hematopoietic cells involves CBL, SHC and an unknown P115 as prominent tyrosine-phosphorylated substrates. Leukemia 12: 301, 1998

30. Meydan N, Grunberger T, Dadi H, Shahar M, Arpaia E, Lapidot Z, Leeder JS, Freedman M, Cohen A, Gazit A, Levitzki A, Roifman CM: Inhibition of acute lymphoblastic leukaemia by a Jak-2 inhibitor. Nature 379:645, 1996