Biological and clinical significance of the FLT3 transcript level in acute myeloid leukemia

Kazutaka Ozeki, Hitoshi Kiyoi, Yuka Hirose, Masanori Iwai, Manabu Ninomiya, Yoshihisa Kodera, Shuichi Miyawaki, Kazutaka Kuriyama, Chihiro Shimazaki, Hideki Akiyama, Miki Nishimura, Toshiko Motoji, Katsuji Shinagawa, Akihiro Takeshita, Ryuzo Ueda, Ryuzo Ohno, Nobuhiko Emi and Tomoki Naoe

From the Department of Infectious Diseases and the Department of Hematology, Nagoya University School of Medicine, Nagoya; the Department of Medicine, Japanese Red Cross Nagoya First Hospital, Nagoya; the Department of Medicine, Saiseikai Maebashi Hospital, Maebashi; the Research and Development Center for Higher Education, Nagasaki University, Nagasaki; the Department of Hematology, Kyoto Prefectural University of Medicine, Kyoto; the Department of Hematology, Tokyo Metropolitan Komagome Hospital, Tokyo; the Second Department of Internal medicine, Chiba University School of Medicine, Chiba; the Department of Hematology, Tokyo Women's Medical University, Tokyo; the Second Department of Internal Medicine, Okayama University School of Medicine, Okayama; the Department of Medicine III, Hamamatsu University School of Medicine, Hamamatsu; the Department of Internal Medicine and Molecular Science, Nagoya City University School of Medicine, Nagoya; and the Aichi Cancer Center, Nagoya, Japan.

Running title: Expression of the FLT3 transcript in AML

Scientific Section Heading: Neoplasia

Address correspondence to: Hitoshi Kiyoi, Department of Infectious Diseases, Nagoya University School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8560, Japan.
Phone: +81-52-744-2955
FAX: +81-52-744-2801
E-mail: kiyoi@med.nagoya-u.ac.jp

Abstract word count: 193

Total text word count: 4,566
Abstract

Although FLT3 mutations are essentially found in myeloid-lineage leukemia cells, a high level of FLT3 expression was recently observed in MLL gene-rearranged acute lymphoblastic leukemia without FLT3 mutations. Here, we analyzed the biological and clinical significance of the FLT3 transcript level in comparison with several gene alterations in 181 de novo acute myeloid leukemia (AML) cases. The mean expression level in AML was higher than that in normal mononuclear cells, while the range varied widely. A high level of FLT3 is related to internal tandem duplication of the FLT3 gene (FLT3/ITD), the mutations within the activation loop of FLT3 (FLT3/D835Mt) and tandem duplication of the MLL gene (MLL-TD), but not to p53 or N-RAS gene mutations. Furthermore, a high expression level in AML cases with FLT3 mutations was not related to MLL-TD. Overexpressed FLT3 revealed auto-phosphorylation, and had the same sensitivity to the FLT3 inhibitor as FLT3/ITD. Overexpression of FLT3 (over 200,000 copies/µgRNA) was an unfavorable prognostic factor for overall survival in 91 AML cases without FLT3/ITD. These results indicated that FLT3 overexpression may distinguish a novel disease entity in AML without FLT3 mutations and serve as a therapeutic target for FLT3 inhibitors.
Introduction

The prevalence and significance of several genetic abnormalities in patients with acute myeloid leukemia (AML) have been reported. The most powerful prognostic factor in AML has been the karyotype of the leukemia cells.\(^1\) Three cytogenetic risk groups (favorable, intermediate and poor) are widely accepted, but there is a practical limitation to the definition of cytogenetic risk, especially in patients falling in the intermediate group. Additional prognostic factors are therefore required. It has been reported that abnormalities in the \textit{RAS} and \textit{p53} genes as well as the \textit{FLT3} gene are implicated in the pathogenesis of AML.\(^2\text{-}^7\) Mutations in \textit{FLT3}, \textit{RAS} and \textit{p53} have been found in approximately 30\%, 20\% and 5\%-10\% of adult AML cases, respectively, indicating that mutations in these three genes are the most frequent genetic alterations in AML.

We and several groups have demonstrated that \textit{FLT3} mutations are a strong prognostic factor in AML.\(^8\text{-}^{18}\) To date, several large scale analyses have revealed that \textit{FLT3} mutations are essentially found in myeloid-lineage leukemia cells.\(^{16,19,20}\) However, \textit{FLT3} mutations within an activation-loop were found in 5 of 30 ALL cases with mixed-lineage leukemia (\textit{MLL}) gene-rearranged acute lymphoblastic leukemia (ALL).\(^{21}\) It is notable that FLT3 was highly expressed in \textit{MLL} gene-rearranged ALL, leading to the constitutive activation of wild type FLT3 kinase, and that primary ALL cells and an ALL cell line SEMK2-M1, which strongly expressed FLT3 but did not carry FLT3 mutations, had the same sensitivity to a potent FLT3 inhibitor as leukemia cells and a cell line with FLT3 mutations.\(^{21,22}\)
FLT3 is preferentially expressed on hematopoietic stem cells as well as in the brain, placenta and liver.\textsuperscript{23,24} The ligand to FLT3 (FL) is expressed as a membrane-bound or soluble form by bone marrow stroma cells, and stimulates the stem cells alone or in cooperation with other cytokines.\textsuperscript{25-32} FL-FLT3 interaction, therefore, plays an important role in the survival, proliferation and differentiation of stem cells. In FLT3-expressing leukemia cells, FL stimulation enhances proliferation and reduces apoptosis.

Although FLT3 is expressed on the surface of a high proportion of AML cells as well as B-lineage ALL cells,\textsuperscript{33-37} little is known about the clinical significance of the FLT3 expression level in acute leukemia. In this study, we analyzed the expression level of the FLT3 transcript quantitatively in comparison with several gene alterations in 181 \textit{de novo} AML cases. Since the prevalence of the \textit{MLL} gene rearrangement is lower in adult \textit{de novo} AML than in therapy-related AML and in infant or childhood acute leukemia, the present cohort did not contain cases with \textit{MLL} gene translocation. Chromosomal translocations involving the \textit{MLL} gene result in the production of a chimeric protein in which MLL is fused to more than 30 different fusion partners.\textsuperscript{38,39} These MLL-fusion products have been thought to contribute to leukemogenesis by conferring gain of function or interfering with normal MLL function dominant negatively.\textsuperscript{40-42} On the other hand, tandem duplication of the \textit{MLL} gene (MLL-TD) was reported in AML with normal karyotypes as well as with trisomy 11.\textsuperscript{43-47} The duplicated region of the \textit{MLL} gene essentially spans exons 3-9 or exons 3-11, and MLL-TD also results in a fusion product. Although the biological mechanism remains unclear, the prognostic implication of MLL-TD has been demonstrated by the analysis of 387 AML cases,\textsuperscript{45} suggesting the involvement of MLL-TD
in the pathogenesis of leukemia. Furthermore, coduplication of the *MLL* and *FLT3* genes was recurrently found in pediatric AML cases. Therefore, we also analyzed the prevalence of MLL-TD, and demonstrated the effects of MLL-TD on the prevalence of the *FLT3* gene mutations and the FLT3 expression, and the prognostic significance of the FLT3 expression level in AML.
Materials and Methods

Patients and samples

The diagnosis of AML was based on the morphology, histopathology, expression of leukocyte differentiation antigens and/or the French-American-British (FAB) classification. The study population included 181 newly diagnosed de novo AML patients consisting of 7 M0, 31 M1, 52 M2, 39 M3, 29 M4, 9 M5, 6 M6 and 8 M7 FAB types. Bone marrow (BM) samples from patients with AML were subjected to Ficoll-Hypaque (Pharmacia LKB, Uppsala, Sweden) density gradient centrifugation. All samples were confirmed to contain over 90% leukemia cells, then cryopreserved in liquid nitrogen before use. For the normal control, each of five BM and cord blood (CB) mononuclear cells (MNCs) was used. We obtained informed consent from all patients and volunteers to use their samples in this study. CB was collected after full-term deliveries with informed consent approved by the Review Board of Tokai Cord Blood Bank. CD34+ cells from CB were separated from MNCs using Dynabeads M-450 conjugated with an anti CD34 monoclonal antibody and DETACHaBEAD (Dynal, Oslo, Norway) according to the manufacturer's instructions. Each separated aliquot was confirmed to contain over 95% CD34+ cells by flow cytometry (data not shown).

Cytogenetic G-banding analysis was performed with standard methods. In this study, cytogenetic risk groups were determined as follows: a favorable risk group was defined by t(8; 21) or inv(16); a poor risk group by t(9; 22), del(5) or del(7); and an
intermediate risk group by normal or other karyotypes and karyotype-unknown.

**Screening for mutations of the FLT3, N-RAS and p53 genes and MLL-TD**

High-molecular weight DNA was extracted from the samples by the standard method. FLT3 gene mutations of the ITD (FLT3/ITD) and activation loop (FLT3/D835Mt), N-RAS gene mutations of codons 12, 13 and 61 and p53 gene mutations of exons 5-8 were examined as previously reported and were confirmed by the sequencing procedure.8,16,49-51 MLL-TD was examined by RT-PCR with the primer pairs 6.1 (5’-GTCCAGAGCAGAGCAACAG-3’), and E3AS (5’-ACACAGATGGATCTGAGAGG-3’), and 6.1 and 4.2R (5’-GGAGCAAGAGGTTCAGCATC-3’) according to published conditions.45,52 Amplified products were cut from the gel, purified with a QIAquick gel extraction kit (Qiagen Inc), and directly sequenced on a DNA sequencer (310; Applied Biosystems) using a BigDye terminator cycle sequencing kit (Applied Biosystems).

**Quantitation of FLT3 transcript expression**

Total RNA was extracted from the samples using a QIAamp RNA Blood Mini Kit (Qiagen Inc, Chatsworth, CA). cDNA was synthesized from each RNA using a random primer and Moloney murine leukemia virus reverse transcriptase (Super-Script II; Gibco BRL, Gaithersburg, ND) according to the manufacturer's recommendations. The expression
level of the FLT3 transcript was quantitated using a real-time fluorescence detection method on an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA). The primer and probe sequences for real-time PCR of FLT3 were: sense primer, 5’-TTTCACAGGACTTGGACAGAGATTT-3’, antisense primer, 5’-GAGTCCGGGTGTATCTGAACCTTCT-3’, and TaqMan probe, 5’-FAM-TCCAAAATCCAGCATGCCTGGTTCAAG-TAMRA-3’. The housekeeping gene, GAPDH, served as a control for cDNA quality. Relative gene expression levels were calculated using standard curves and adjusted based on the expression level of the GAPDH gene. Each gene expression level was analyzed in triplicate and the mean was subjected to analysis. Full-length human wild-type (Wt)-FLT3 cDNA cloned into the pCDHF3 vector, kindly provided by Dr. Oliver Rosnet (INSERM, France), was used as the standard. The copy number of the plasmid was calculated from the DNA concentration and the molecular weight of the plasmid. The copy number of FLT3 in each sample was calculated by comparing the Ct values of samples with that of the standard.

Since our quantitative condition detected both Wt- and mutant-FLT3 transcripts, the results from the samples with FLT3 gene mutations revealed the total amount of both transcripts, and we then compared the relative abundance of Wt- and mutant-FLT3 transcripts by RT-PCR. In the samples with FLT3/ITD, we amplified the juxtamembrane domain using the primer pair R5 (5’-TGTCGAGCAGTACTCTAAACGT -3’) and 12R (5’-CTTCAGCATTGAGCTGGACC-3’) as previously described. Amplified products were separated on agarose gels and stained with ethidium bromide. The relative proportion of Wt- and ITD-fragments in each sample was determined by the intensities of
both fragments.

Flow cytometry

Cryopreserved AML cells were thawed, washed twice with phosphate-buffered saline and incubated with an anti-human FLT3 monoclonal antibody (SF1.340; Immunotech, Marseille, France), followed by a fluorescein isothiocyanate (FITC)-conjugated anti-mouse immunoglobulin antibody (Immunotech). The surface expression of FLT3 products was analyzed by flow cytometer (FACSCalibur; Becton Dickinson, San Jose, CA)\textsuperscript{53}. The relative expression level of FLT3 on the cell surface was adjusted to the level of the isotype control.

Western blot

Cell lysates from primary AML cells were extracted as previously described. Lysates were immunoprecipitated with a rabbit anti-human FLT3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and protein G Sepharose (Amersham Pharmacia Biotech). The precipitated samples were separated by SDS-polyacrylamide gel electrophoresis, and electroblotted onto Immobilon PVDF membranes (Millipore, Bedford, MA). Immunoblotting was performed with an anti-phosphotyrosine antibody (4G10; Upstate Biotechnology, Lake Placid, NY). The membranes were incubated with stripping buffer, then reprobed with an anti-FLT3 antibody. Signals were developed using an ECL system
To examine the total FLT3 product level, whole cell lysates from each AML cell were subjected to immunoblotting with an anti FLT3 antibody.\textsuperscript{53,54} The relative expression level of FLT3 was adjusted to the expression of Actin protein. The expression level was determined by densitometry.

**Treatment with an FLT3 inhibitor**

A potent FLT3 inhibitor, AG1296, was purchased from Calbiochem (San Diego, CA) and dissolved in dimethyl sulfoxide (DMSO) at an appropriate concentration. Primary AML cells were suspended in RPMI1640 medium (Gibco BRL) containing 10% FCS, and \(2 \times 10^4\) cells per well were seeded in 96-well culture plates with or without potent FLT3 inhibitors. Cell viability was measured using the CellTiter96 Proliferation Assay (Promega, Madison, WI) according to the manufacturer’s instructions. These procedures were performed three times independently.

To examine the effect of the FLT3 inhibitor on the phosphorylation status, leukemia cells were incubated with RPMI1640 medium containing 10% FCS and 10 \(\mu\)M AG1296 for 3 hours, then subjected to immunoblot analysis as described above.

**Analysis of clinical characteristics**

It is necessary to analyze the clinical characteristics in a well-documented cohort. Among the 181 patients analyzed, there were 39 with acute promyelocytic leukemia (APL). APL
has been considered as a separate disease entity among AML, and the introduction of all-trans retinoic acid (ATRA) has dramatically improved its clinical outcome. Since 26 patients with APL were treated with ATRA-based therapy, but the remaining patients were not included in this cohort, we excluded patients with APL from the analysis for clinical characteristics. In addition, 23 patients with AML, except for APL, were treated with independent regimens. We, therefore, analyzed the clinical characteristics of 119 patients with AML, excluding those with APL, who were treated with the AML87, AML89, and AML92 protocol of the Japan Adult Leukemia Study Group (JALSG).

**Statistical analysis**

Differences in median variables in age, peripheral WBC counts, platelet counts, and copy numbers of FLT3 were analyzed with the Mann-Whitney U-test for distribution among two groups or the Kruskal-Wallis test and the Bonferroni test for distribution among more than three groups. Analysis of the distribution between two continuous variables was performed using the Spearman rank correlation test. Analysis of frequencies was performed using Fisher's exact test for 2 x 2 tables or Pearson's $\chi^2$ test for larger tables. Survival probabilities were estimated by the Kaplan-Meyer method, and differences in the survival distributions were evaluated using the log-rank test. The prognostic significance of the clinical variables was assessed using the Cox proportional hazards model. These statistical analyses were performed with StatView-J 5.0 (Abacus Concepts Inc, Berkeley, CA). For all analyses, the $p$ values were two-tailed, and a $p$ value of less than .05 was
considered statistically significant.
Results

High-level expression of the FLT3 transcript in AML

We quantitated the expression level of the FLT3 transcript in AML cells using a real-time fluorescence detection method. As a control of normal hematopoietic cells, we also quantitated the expression level in each of five normal BM and CB MNCs. Each mean expression level was 3,709 (range, 2,352 – 9,240) and 4,736 (range, 2,285 – 7,916) copies/µgRNA in BM and CB MNCs, respectively, and there was no significant difference among them. Since FLT3 is known to be highly expressed on hematopoietic stem cells, we examined the expression level in CD34+ and CD34- cells separated from five CB. Each mean expression level was 6,643 (range, 3,814 – 8,668) and 3,712 (range, 1,169 – 3,955) copies/µgRNA, respectively. The expression level in the CD34+ cells was about 1.7 times higher than that in the CD34- cells, while it did not deviate far from the mean level of BM MNCs.

In AML cells, the mean expression level of the FLT3 transcript was 20,203 copies/µgRNA. However, since each expression level varied from 0 to 2,322,706 copies/µgRNA, we compared the expression level of the FLT3 transcript with the percentage of leukemia cells in BM and found no significant association between them. In addition, since we first enriched leukemia cells from BM samples by density gradient centrifugation, we did not adjust the expression level by the percentage of leukemia cells in BM. According to the FAB classification, each mean expression level was 5,695
copies/µgRNA in M0, 53,654 in M1, 23,515 in M2, 2,546 in M3, 30,470 in M4, 21,197 in M5, 808 in M6 and 1,945 in M7, and the distribution among the FAB types was significant (p< .0001 by the Kruskal-Wallis test) (Figure 1). The Bonferroni test revealed that distributions were significantly higher in the M1, M2 and M4 FAB types than normal hematopoietic cells, but not in other FAB types.

To examine the association of the FLT3 expression level with gene alterations, we analyzed the mutations of the FLT3, N-RAS and p53 genes in 181 patients with AML. We found FLT3/ITD in 38 cases (21.0%), FLT3/D835Mt in 8 (4.4%), N-RAS mutations in 22 (12.2%) and p53 mutations in 10 (5.5%). In addition, we examined MLL-TD by RT-PCR. It has been reported that MLL-TD was detected in normal BM and PBL by nested RT-PCR, but not by single-step RT-PCR. To confirm this and our own RT-PCR conditions, we examined each of five normal BM and CB MNCs for MLL-TD by single-step RT-PCR. However, we obtained no band in these normal samples even under our conditions. We therefore used single-step RT-PCR for screening MLL-TD, and found it in 19 cases (10.5%). Direct sequencing of the amplified products revealed an exon 9/exon 3 fusion in 14 cases, exon 10/exon 3 fusion in one case, exon 11/exon 3 fusion in three cases and exon 9/exon 5 fusion in one case. It was noteworthy that seven and two of the 19 MLL-TD positive cases had FLT3/ITD and N-RAS gene mutations, respectively. In addition, two MLL-TD positive cases had both FLT3/ITD and p53 gene mutations. MLL-TD tended to be associated with FLT3/ITD (p=.07 by Fisher's exact test), but not at all with FLT3/D835Mt, N-RAS or p53 gene mutations.

Since our quantitative condition detected both Wt- and mutant-FLT3 transcripts,
we compared the relative abundance of Wt- and mutant-FLT3 transcripts in cases with FLT3 gene mutations. In this cohort, there were 38 FLT3/ITD cases. We semi-quantitatively determined the relative proportions of Wt- and mutant-FLT3 transcripts in these cases and calculated each expression level according to the proportions (Figure 2A). Furthermore, we compared the results obtained by RT-PCR with those by genomic PCR and found that the relative proportions of Wt- and mutant-FLT3 transcripts were consistent with those of Wt- and ITD-alleles (Figure 2B). Although the relative proportions of Wt- and mutant-FLT3 transcripts varied, they were not related to the total expression level of the FLT3 transcript (data not shown). In addition, the expression levels of Wt- and mutant-FLT3 transcripts were closely correlated ($p < .0001$) (Figure 2C). These results indicated that the expression levels of the FLT3 transcript in cases with FLT3 gene mutations depended not only on the mutant-transcripts levels, but also the Wt-transcript levels. We therefore subjected the total expression levels to further analyses.

A high expression level of FLT3 was related to FLT3/ITD ($p = .0020$), MLL-TD ($p = .0121$) and FLT3/D835Mt ($p = .0463$), but not to N-RAS or to p53 gene mutations (Table 1). To clarify the effects of MLL-TD and the FLT3 gene mutations on the expression level of the FLT3 transcript, we divided the 181 AML cases into five genotypes according to the presence of MLL-TD, FLT3/ITD and FLT3/D835Mt. Of these patients, seven (3.9%) had both MLL-TD and FLT3/ITD, 12 (6.6%) had only MLL-TD, 31 (17.1%) had only FLT3/ITD, eight (4.4%) had only FLT3/D835Mt, and 123 (68.0%) had neither. The FLT3 expression level in each genotype group ranged from 16,229 to 759,110 (mean,
121,219), 1,325 to 1,731,514 (mean, 69,742), 416 to 258,336 (mean, 27,490), 1,885 to 219,489 (mean, 63,756) and 0 to 2,322,706 (mean, 13,705) copies/µgRNA, respectively. There was a significant difference among these mutations ($p = .0001$ by the Kruskal-Wallis test). The Bonferroni test revealed that distributions were significantly higher in AML cases with FLT3/ITD, FLT3/D835, MLL-TD or both MLL-TD and FLT3/ITD than in those without mutations. However, there were no significant differences among cases with FLT3/ITD, FLT3/D835, MLL-TD or both MLL-TD and FLT3/ITD.

**Overexpressed Wt-FLT3 of AML cells was tyrosine phosphorylated**

To analyze the biological effects of the expression level of the FLT3 transcript, we analyzed the expression level and tyrosine-phosphorylation status of FLT3 protein in primary AML cells. Although a limited number of AML cells was available for this analysis, we selected eight primary AML samples harboring a normal karyotype: two (UPN-2 [FAB, M4] and –119 [M4] whose FLT3 expression levels were 56,635 and 19,541 copies/µgRNA, respectively) had FLT3/ITD and six (UPN-14 [M2], -78 [M2], -11 [M1], -18 [M5], -23 [M0] and -67 [M1] whose expression levels were 28,152, 29,146, 97,373, 170,237, 1,731,514 and 2,322,706 copies/µgRNA, respectively) had Wt-FLT3. Of these AML samples, UPN-23 had both MLL-TD and N-RAS mutations, but the others did not have FLT3/D835, MLL-TD, p53 or N-RAS mutations.

Although the surface expression level of FLT3 protein was not related to the FLT3 transcript level, immunoblot analysis using whole cell lysates from leukemia cells
revealed that the total amount of FLT3 protein essentially reflected the transcript level (Figures 3A and B). Of note is that FLT3 products from UPN-23 and –67, which expressed extremely high levels of FLT3 transcripts, were tyrosine phosphorylated, although those from UPN-14, –78, -11 and –18 were not (Figure 3C). To exclude the possibility that leukemia cells from UPN-23 and –67 had novel FLT3 gene mutations, we sequenced the entire coding region of FLT3 and found no mutations.

**Overexpressed FLT3 is sensitive to a potent FLT3 inhibitor**

To examine whether overexpressed FLT3 is sensitive to a FLT3 inhibitor, we analyzed the change in its phosphorylation status after treatment with a potent FLT3 inhibitor, AG1296. Leukemia cells with FLT3/ITD or overexpressing Wt-FLT3 were treated with 10 µM AG1296 for 3 hours, then subjected to Western blot analysis. All FLT3 products showed dephosphorylation at tyrosine residues by AG1296 (Figure 3C). Furthermore, we examined whether inhibition of FLT3 by AG1296 led to cytotoxicity in these leukemia cells. We assessed metabolically active cells 72 hours after the addition of AG1296 and found that leukemia cells which overexpressed Wt-FLT3 were more sensitive to treatment with AG1296 than those with FLT3/ITD (Figure 3D).

**Clinical characteristics of AML cases expressing a high level of the FLT3 transcript**

To analyze the effect of the expression level of the FLT3 transcript on the clinical
characteristics, it is necessary to determine the cut-off level of overexpression. Since this and previous studies demonstrated that FLT3 products from leukemia cells with an extremely high expression level of the transcript were constitutively tyrosine phosphorylated, it seemed logical that the transcript level, where the product was phosphorylated, was defined as overexpression. Although we could not examine the phosphorylation status of the FLT3 products from clinical samples, Western blot analysis revealed that the Wt-FLT3 product from the AML sample, whose transcript level was 170,237 copies/µgRNA, was not tyrosine phosphorylated (Figure 3C). Therefore, we tentatively determined the cut-off level of overexpression as 200,000 copies/µgRNA, and analyzed the effect of FLT3 overexpression on the prognosis of 119 patients with AML, excluding those with APL, who were treated with the JALSG protocol. In this cohort, there were 10 AML cases whose FLT3 levels were over 200,000 copies/µgRNA. Kaplan-Meier analysis for overall survival showed that overexpression of the FLT3 transcript tended to be a worse prognostic factor, though it was not statistically significant ($p = .1067$) (Figure 4A). However, since a high expression level of FLT3 transcripts was associated with FLT3/ITD, which was one of the poor prognostic factors, we analyzed the clinical impact of overexpression of the FLT3 transcript within the 91 cases without FLT3/ITD. In this group, there were five cases whose FLT3 levels were over 200,000 copies/µgRNA (Table 2). Among them, two cases, UPN-23 and –67, revealed an extremely high level of the FLT3 transcript. The FLT3 products of these two cases were confirmed to be tyrosine phosphorylated (Figure 3C), although those of the other cases could not be analyzed. Overexpression of the FLT3 transcript was not related to other
gene mutations, age, WBC count, cytogenetical findings or FAB type (Table 3). Overexpression was related to a lower CR rate, although not significantly ($p = .0686$) probably due to the small number in this cohort. Kaplan-Meier analysis for overall survival showed that the worse prognosis was the overexpression of FLT3 (Figure 4B). Univariate analysis showed that the unfavorable prognostic factors for overall survival were age 60 years or older ($p = .0006$), overexpression of the FLT3 transcript ($p = .0068$) and a high WBC count (over $100 \times 10^9$/L) ($p = .0074$) (Table 4). However, cytogenetical findings, mutations of $p53$ and $N$-$RAS$ genes and MLL-TD were not unfavorable prognostic factors. Multivariate analysis showed that overexpression of the FLT3 transcript was the strongest unfavorable factor (RR, $4.216; p = .003$), followed by age 60 years or older (RR, $2.607; p = .001$) and a high WBC count (RR, $2.101; p = .0386$) (Table 4).
Discussion

An association of high FLT3 expression was first reported for MLL-rearranged ALL. Recently, Libura et al. reported that AML with FLT3/D835Mt and the monocytic lineage AML with FLT3/ITD expressed a high level of the FLT3 transcript. In addition, they also reported that both FLT3/ITD and FLT3/D835Mt were frequently found in AML with MLL gene abnormalities, which involved DNA double strand breakage at a topoisomerase II site, and such AML cases expressed a high level of the FLT3 transcript. Since our cohort did not contain cases with MLL-rearrangements, we focused on MLL-TD as the MLL gene alteration. Seven of the 19 MLL-TD positive cases had FLT3/ITD, but none had FLT3/D835Mt. When the 181 AML cases were divided into five genotypes according to the presence of MLL-TD, FLT3/ITD and FLT3/D835Mt, there were no significant differences in FLT3 transcript level among the cases with FLT3/ITD, FLT3/D835Mt, MLL-TD or both MLL-TD and FLT3/ITD. These results indicated that the high level of FLT3 transcript in AML cases with FLT3 gene mutations was not related to MLL-TD, although we could not confirm the effects of MLL rearrangements on the prevalence of FLT3 gene mutations and the FLT3 transcript level.

Previous analyses revealed that most of the primary AML cells expressed FLT3 mRNA as detected by Northern blotting or the RT-PCR method, and that 60 to 70% cells expressed FLT3 protein on their surface. However, these expression levels were not quantitatively determined and the relationship between the mRNA and protein expression levels was not indicated. The MLL-rearranged ALL cell line, SEMK2-M1, was
demonstrated to undergo intrachromosomal amplification of *FLT3* by fluorescence in situ hybridization (FISH) using BAC probes spanning the *FLT3* locus, suggesting that gene amplification is a possible mechanism leading to overexpression of the transcripts.\(^{21}\)

Unfortunately, we could not examine whether *FLT3* gene amplification occurred in the AML cells harboring high levels of the FLT3 transcript, although cytogenetic analysis by G-banding revealed no alterations involving chromosome 13, where the *FLT3* gene is located (13q12), in any cases analyzed.

Although the surface expression level of FLT3 was not related to the expression level of the FLT3 transcript, the total cellular protein level essentially reflected the transcript level. It was reported that FLT3 proteins on the cell surface were internalized when exogenous FL-stimulation was administered.\(^{60}\) Furthermore, FL was preferentially expressed by AML cells and the FL/FLT3 autocrine mechanism may contribute to the anti-apoptotic effect in leukemia cells.\(^{36,60,61}\)

At present, little is known about the relationship between FL and FLT3 expression levels. However, it is possible that the overexpressed FLT3 proteins are internalized by inducing the FL expression. Alternatively, part of the overexpressed FLT3 proteins may be processed at the cell surface.

It is particularly important that the overexpressed Wt-FLT3 proteins were tyrosine phosphorylated. Previously, tyrosine phosphorylation of overexpressed ALL cells harboring the *MLL*-rearrangement has been demonstrated.\(^{21}\) In addition, three of the 27 primary AML cells without FLT3/ITD were reportedly shown to be tyrosine phosphorylated even in the absence of FL stimulation.\(^{60}\) Although these three cases were not examined for other *FLT3* gene mutations including FLT3/D835Mt and FLT3
expression levels, overexpression may be involved in auto-phosphorylation in these cases. Since it was suggested that the FL-FLT3 autocrine mechanism contributes to the anti-apoptotic effect in leukemia cells as described above, and overexpression may be related to the lower CR rate in induction chemotherapy, it seems possible that the FL expression induced by FLT3 overexpression leads to the receptor being phosphorylated by an autocrine mechanism. It was reported that the auto-phosphorylation of Wt-FLT3 induced by its overexpression was inhibited by a potent FLT3 kinase inhibitor at the same sensitivity as that of mutated FLT3. A previous study demonstrated this only in MLL-rearranged ALL cells, although our study demonstrated that it is also applicable to AML cells without MLL gene alterations. FLT3 kinase serves as an important molecular target in the treatment of leukemia, and several potent inhibitors have been subjected to clinical phase-1 and –2 trials. These inhibitors mainly target mutated FLT3 kinases, though our study suggested that the target could be extended to AML cases overexpressing FLT3 even without mutations.

Since FLT3/ITD was closely associated with high expression levels of the FLT3 transcript, high WBC counts and a poor prognosis, we examined the clinical impact of FLT3 overexpression among the 91 cases without FLT3/ITD. As shown in Table 4, overexpression of FLT3 was not related to any clinical variables with statistical significance, though it tended to be related to a high WBC count and low CR rate. Since the cut-off level of overexpression used here was tentative and there were only five patients overexpressing FLT3 in this cohort, larger scale analysis is required to clarify the clinical significance of overexpression. However, our study suggested that overexpression may
be an unfavorable prognostic factor for overall survival in AML without FLT3/ITD.

Acknowledgments

This study was performed in co-operation with JALSG. We would like to thank Ms. Manami Kira and Ms. Kyoko Aoyama for secretarial and technical assistance.
Table 1. Association of FLT3 expression with gene mutations

<table>
<thead>
<tr>
<th>Gene mutations</th>
<th>(n)</th>
<th>FLT3 transcript (copy/µgRNA)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLT3/ITD</td>
<td>38</td>
<td>30,801 (415 - 759,110)</td>
<td>.0020</td>
</tr>
<tr>
<td>MLL-TD</td>
<td>19</td>
<td>76,556 (1,324 – 1,731,514)</td>
<td>.0121</td>
</tr>
<tr>
<td>FLT3/D835</td>
<td>8</td>
<td>63,756 (1,885 – 219,489)</td>
<td>.0463</td>
</tr>
<tr>
<td>p53</td>
<td>10</td>
<td>15,888 (15 – 121,219)</td>
<td>NS</td>
</tr>
<tr>
<td>N-RAS</td>
<td>22</td>
<td>25,171 (19 – 1,731,514)</td>
<td>NS</td>
</tr>
<tr>
<td>No mutations</td>
<td>101</td>
<td>13,705 (0 – 2,322,706)</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>181</td>
<td>20,203 (0 – 2,322,706)</td>
<td></td>
</tr>
</tbody>
</table>

Mean (minimum to maximum) expression levels of the FLT3 transcript are indicated according to the gene mutations. Statistical analysis was performed by comparing with cases harboring no mutations. ‘No mutations’ indicates cases without FLT3/ITD, MLL-TD, FLT3/D835, p53 and N-RAS mutations. NS indicates no significance. Seven cases had both FLT3/ITD and MLL-TD, and two of them had p53 gene mutations. Two cases had both MLL-TD and N-RAS gene mutations.
Table 2. Clinical characteristics of AML overexpressing Wt-FLT3

<table>
<thead>
<tr>
<th>UPN</th>
<th>Age (year)</th>
<th>FAB</th>
<th>WBC (x 10^9/L)</th>
<th>FLT3 transcripts (copies/µgRNA)</th>
<th>Karyotype</th>
<th>Gene mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>UPN-112</td>
<td>69</td>
<td>M4</td>
<td>82.2</td>
<td>266,947</td>
<td>normal</td>
<td>none</td>
</tr>
<tr>
<td>UPN-185</td>
<td>32</td>
<td>M2</td>
<td>30.0</td>
<td>516,010</td>
<td>t(8; 21)</td>
<td>none</td>
</tr>
<tr>
<td>UPN-187</td>
<td>43</td>
<td>M2</td>
<td>24.7</td>
<td>561,052</td>
<td>normal</td>
<td>none</td>
</tr>
<tr>
<td>UPN-23</td>
<td>64</td>
<td>M0</td>
<td>197.4</td>
<td>1,731,514</td>
<td>normal</td>
<td>MLL-TD N-RAS</td>
</tr>
<tr>
<td>UPN-67</td>
<td>21</td>
<td>M1</td>
<td>56.1</td>
<td>2,322,706</td>
<td>normal</td>
<td>none</td>
</tr>
</tbody>
</table>

There were five cases whose FLT3 transcript level was over 200,000 copies/µgRNA in AML without FLT3/ITD. In particular, two cases, UPN-23 and –67, revealed an extremely high level of the FLT3 transcript. The FLT3 products of these two cases were tyrosine phosphorylated, as shown in Figure 3C.
Table 3. Relationship between overexpression of the FLT3 transcript and clinical characteristics in 91 AML cases without FLT3/ITD

<table>
<thead>
<tr>
<th></th>
<th>Total (N=91)</th>
<th>FLT3 transcripts &gt; 200,000 copies/µgRNA (N=5)</th>
<th>FLT3 transcripts ≤ 200,000 copies/µgRNA (N=86)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (year)</td>
<td>48 (15 – 85)</td>
<td>43 (21 – 69)</td>
<td>48 (15 – 85)</td>
</tr>
<tr>
<td>Age &gt; 60 years</td>
<td>27</td>
<td>2</td>
<td>25</td>
</tr>
<tr>
<td>WBC (x 10^9/L)</td>
<td>23.4</td>
<td>56.1</td>
<td>22.2</td>
</tr>
<tr>
<td>WBC &gt;100 x 10^9/L</td>
<td>14</td>
<td>1</td>
<td>13</td>
</tr>
<tr>
<td>FAB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M0</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>M1</td>
<td>19</td>
<td>1</td>
<td>18</td>
</tr>
<tr>
<td>M2</td>
<td>45</td>
<td>2</td>
<td>43</td>
</tr>
<tr>
<td>M4</td>
<td>18</td>
<td>1</td>
<td>17</td>
</tr>
<tr>
<td>M5</td>
<td>6</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>M6</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>M7</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cytogenetics</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>favorable</td>
<td>20</td>
<td>1</td>
<td>19</td>
</tr>
<tr>
<td>t(8; 21)</td>
<td>17</td>
<td>1</td>
<td>16</td>
</tr>
<tr>
<td>inv(16)</td>
<td>3</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>intermediate</td>
<td>67</td>
<td>4</td>
<td>63</td>
</tr>
<tr>
<td>poor</td>
<td>4</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>t(9; 22)</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>del(7)</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Outcome</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CR</td>
<td>71</td>
<td>2</td>
<td>69</td>
</tr>
<tr>
<td>Failure</td>
<td>20</td>
<td>3</td>
<td>17</td>
</tr>
</tbody>
</table>
OZEKI et al.
EXPRESSION OF THE FLT3 TRANSCRIPT IN AML

<table>
<thead>
<tr>
<th>Mutation Type</th>
<th>Count</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>MLL-TD mutation</td>
<td>9</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>p53 gene mutation</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>N-RAS gene mutation</td>
<td>14</td>
<td>1</td>
<td>13</td>
</tr>
<tr>
<td>FLT3/D835 mutation</td>
<td>5</td>
<td>0</td>
<td>5</td>
</tr>
</tbody>
</table>

Mean (minimum to maximum) values are indicated for age and WBC. Number of cases is shown by age (> 60 years), WBC count (>100 × 10⁹/L), FAB, cytogenetics, outcome and gene mutations. The favorable risk group was defined by t(8;21) or inv(16), the poor risk group by t(9;22) or del(7), and the intermediate risk group by normal or other karyotypes and karyotype-unknown. CR indicates complete remission.
Table 4. Unfavorable prognostic factors for overall survival in 91 AML cases without FLT3/ITD

<table>
<thead>
<tr>
<th>Prognostic Factors</th>
<th>Univariate</th>
<th>Multivariate</th>
<th>Relative Risk (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age &gt; 60 yr</td>
<td>.0006</td>
<td>.0011</td>
<td>2.607 (1.464 – 4.644)</td>
</tr>
<tr>
<td>Overexpression of FLT3 &gt; 200,000 copy/µgRNA</td>
<td>.0068</td>
<td>.0030</td>
<td>4.216 (1.631 – 10.897)</td>
</tr>
<tr>
<td>WBC count &gt; 100 x 10^9/L</td>
<td>.0074</td>
<td>.0386</td>
<td>2.101 (1.040 – 4.246)</td>
</tr>
<tr>
<td>Cytogenetics</td>
<td>.0801</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p53 gene mutation</td>
<td>.0805</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MLL-TD mutation</td>
<td>.1046</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-RAS gene mutation</td>
<td>.2350</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FAB other than M2</td>
<td>.6483</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FLT3/D835 mutation</td>
<td>.8687</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
References


38. Bernard OA, Berger R. Molecular basis of 11q23 rearrangements in hematopoietic


Wormann B, Hiddemann W, Griesinger F. Screening for MLL tandem duplication in 387 unselected patients with AML identify a prognostically unfavorable subset of AML. Leukemia. 2000;14:796-804

46. Christiansen DH, Pedersen-Bjergaard J. Internal tandem duplications of the FLT3 and MLL genes are mainly observed in atypical cases of therapy-related acute myeloid leukemia with a normal karyotype and are unrelated to type of previous therapy. Leukemia. 2001;15:1848-1851


Figure 1. Expression level of the FLT3 transcript according to the FAB type.

Distribution of the expression level of the FLT3 transcript is indicated according to the FAB type. There was a significant difference among the FAB types and normal hematopoietic cells \((p < .0001\) by the Kruskal-Wallis test). The Bonferroni test revealed that distributions were significantly higher in the M1, M2 and M4 FAB types than in normal hematopoietic cells, but not other FAB types. Normal hematopoietic cells included each of five BM and CB MNCs. CD34\(^+\) and CD34\(^-\) cells were isolated from CB MNCs. Horizontal bars indicate each mean value.
Figure 2. Proportion of the Wt- and mutant-FLT3 fragments.

Representative results of RT-PCR (A) and genomic PCR (B) in seven AML samples with FLT3/ITD are shown. The proportion given for each sample denotes the relative intensity of Wt-FLT3 and FLT3/ITD fragments. Each relative proportion obtained by RT-PCR was consistent with that by genomic PCR. (C) The correlation between the expression levels of the Wt-FLT3 and FLT3/ITD transcripts in 34 AML samples with FLT3/ITD is shown. Four AML samples, which lost the Wt-FLT3 allele, were excluded from this analysis. Both expression levels were closely correlated ($p < .0001$ by the Spearman rank correlation test).
Figure 3. Biological effects of the expression level of the FLT3 transcript.

**A** Biological effects of the FLT3 transcript level were analyzed using eight AML samples harboring a normal karyotype. Two, UPN-2 (FAB, M4) and –119 (M4) had FLT3/ITD and six, UPN-14 (M2), -78 (M2), -11 (M1), -18 (M5), -23 (M0) and -67 (M1), had Wt-FLT3. Of these AML samples, UPN-23 had both MLL-TD and N-RAS mutations, but the others did not have FLT3/D835, MLL-TD, p53 or N-RAS mutations. (A) Surface expression level of FLT3 was examined by flow cytometry. There was no marked difference according to the expression level of the FLT3 transcript. (B) Immunoblot analysis revealed that the total cellular level of FLT3 protein reflected the expression level of the FLT3 transcript. Each expression level of FLT3 protein was adjusted to that of
Actin protein. Ratio indicates the relative expression level of the whole cellular protein (Protein), surface protein (Surface) and the transcript when compared to that of UPN-14.

(C) Tyrosine phosphorylation of each FLT3 protein was examined. Overexpressed Wt-FLT3 was phosphorylated as well as FLT3/ITD (upper two panels). These phosphorylations were inhibited by treatment with AG1296 for 3 hours (lower two panels).

(D) Cell viability was measured using the CellTiter96 Proliferation Assay 72 hours after the addition of AG1296 at the indicated concentration to primary AML cells. The Y-axis indicates the ratio of absorbance for AG1296-treated cells to untreated cells. Wt-average indicates the results from UPN-14 and –78 cases, ITD from UPN-2 and –119, and Wt-High from UPN-23 and –67.
Figure 4. Overall survival according to overexpression of FLT3.

Overall survival according to the overexpression of FLT3 in all AML cases (A) and in 91 AML cases without FLT3/ITD (B) are indicated. In the total cases, overexpression of the FLT3 transcript tended to indicate a worse prognosis, although not significantly. However, it was a poor prognostic factor within the cases without FLT3/ITD. Statistical difference was evaluated using the log-rank test.
Biological and clinical significance of the FLT3 transcript level in acute myeloid leukemia

Kazutaka Ozeki, Hitoshi Kiyoi, Yuka Hirose, Masanori Iwai, Manabu Ninomiya, Yoshihisa Kodera, Shuichi Miyawaki, Kazutaka Kuriyama, Chihiro Shimazaki, Hideki Akiyama, Miki Nishimura, Toshiko Motoji, Katsuji Shinagawa, Akihiro Takeshita, Ryuzo Ueda, Ryuzo Ohno, Nobuhiko Emi and Tomoki Naoe