Fms-like Tyrosine Kinase (FLT) 3 and FLT3 Internal Tandem Duplication in Different Types of Adult Leukemia: Analysis of 147 Patients

Aim To assess the expression level of fms-like tyrosine kinase 3 (FLT3), the incidence of FLT3/internal tandem duplications (ITD) mutation, and prognostic value of FLT3 changes in different types of adult leukemia.

Methods Bone marrow mononuclear cells were isolated from 147 adult patients with leukemia. Reverse transcriptase polymerase chain reaction (PCR) was used to screen FLT3/ITD mutation and quantitative PCR was performed to evaluate the expression of the FLT3 transcript. Flow cytometry was used for detection of FLT3 receptor protein expression on bone marrow mononuclear cells. Pearson correlation analysis was performed to estimate the significance of FLT3.

Results FLT3 expression was higher in acute myeloid leukemia and B-acute lymphoid leukemia than in T-acute lymphoid leukemia (P = 0.006, P = 0.001) and chronic myelogenous leukemia (P < 0.001). In chronic myelogenous leukemia, FLT3 expression in blast transformation phase was higher than in acceleration phase (P = 0.023). Surface expression of FLT3 protein was correlated with high percentage of bone marrow blasts and with FLT3 mRNA expression (r = 0.366, P < 0.001) in acute leukemia. FLT3/ITDs in the juxtamembrane domain were found in 25% of patients with acute myeloid leukemia and 7% of patients with acute lymphoid leukemia. FLT3/ITD positive sequences had 36, 42, and 57 nucleotides. FLT3/ITD mutation was associated with a higher white blood cell count, higher marrow blast percentage, and elevated serum lactate dehydrogenase (P = 0.045, P = 0.014, P < 0.001, respectively) and not associated with a higher FLT3 mRNA and FLT3 protein expression, and lower complete remission (P = 0.091, P = 0.060, P = 0.270, respectively).

Conclusion FLT3 expression levels differed in different types of adult leukemia. Overexpression of FLT3 and presence of a positive FLT3/ITD mutation in acute leukemia were associated with unfavorable clinical characteristics and poor prognosis.
The fms-like tyrosine kinase 3 (FLT3) gene belongs to the class III receptor tyrosine kinases and is predominantly expressed on hematopoietic progenitor cells in the bone marrow, thymus, and lymph nodes (1). An abnormality in the FLT3 gene is implicated in the pathogenesis of acute myeloid leukemia (2-4). Approximately 25% of patients with adult acute myeloid leukemia harbor internal tandem duplications (ITD) within the juxtamembrane domain of the FLT3 gene (5,6). FLT3/ITDs cause structural changes in the juxtamembrane and this disrupts the autoinhibitory conformation of the receptor (7) by promoting constitutive activation of both receptor (8-10) and downstream effectors, which all leads to a bad prognosis (11,12). In the last decade, FLT3/ITD mutations have been reported in 13%-32% of adult patients with acute myeloid leukemia and in a small number of patients with acute lymphoid leukemia (13-16). Patients with this abnormality have increased incidence of leukocytosis and decreased overall survival in comparison with patients without this abnormality. These findings indicate that FLT3/ITD not only play an important role in the pathogenesis mechanism of leukemia but also have a prognostic value.

A previous study has demonstrated that high levels of FLT3 were expressed in leukemia and lymphoma cell lines including pre-B, myeloid, and monocytic cell lines (17). Also, several studies have shown that high levels of FLT3 were expressed in 70%-100% of patients with acute myeloid leukemia and B-cell acute lymphoid leukemia and in about 30% of patients with T-cell acute lymphoid leukemia (18,19). Likewise, a small number of chronic myelogenous leukemia blast crisis and chronic lymphocytic leukemia cells has been shown to express FLT3 (18,20). These data indicate that FLT3 expression may play a role in the survival or proliferation of leukemic blasts. Using Western blotting, Carow et al (18) found no FLT3 expression in the normal bone marrow, but identified FLT3 protein in 14 of 14 B-cell acute lymphoid leukemia cases, 36 of 41 acute myeloid leukemia cases, and 1 of 4 T-cell acute lymphoid leukemia cases. Though FLT3 expression in leukemia and its clinical significance have been widely investigated, little is known about FLT3 expression level and its clinical significance in Chinese patients with adult leukemia. Most of the studies have used Western blot as FLT3 protein assay, whereas flow cytometry on intact leukemic cell surface has been rarely used. We used flow cytometry on cell surface to investigate the expression of FLT3 receptor and quantitative polymerase chain reaction (PCR) to investigate FLT3 mRNA expression, as well as performed identification of FLT3/ITDs in different types of adult leukemia.

Patients and methods

The study included 120 patients with newly diagnosed acute leukemia – 60 with acute myeloid leukemia, 30 with B-cell acute lymphoid leukemia, 30 with T-cell acute lymphoid leukemia; 27 with chronic myeloid leukemia; and 30 controls (Table 1). Diagnosis was based on May-Grunwald-Giemsa-stained bone marrow smears and cytochemistry performed according to the French-American-British (FAB) group criteria (21). Leukocyte differentiation antigens were analyzed by immunofluorescent method for some cases. Complete remission was defined as normocellular bone marrow containing less than 5% blasts and showing evidence of normal maturation of other bone marrow elements. Patients and controls provided informed consent to use their samples for this study.

mRNA expression analysis

Patients and controls’ bone marrow mononuclear cells were separated on a Ficoll-Hypaque
The fms-like tyrosine kinase 3 (FLT3) gene belongs to the class III receptor tyrosine kinases and is predominantly expressed on hematopoietic progenitor cells in the bone marrow, thymus, and lymph nodes (1). An abnormality in the FLT3 gene is implicated in the pathogenesis of acute myeloid leukemia (2-4). Approximately 25% of patients with adult acute myeloid leukemia harbor internal tandem duplications (ITD) within the juxtamembrane domain of the FLT3 gene (5,6). FLT3/ITDs cause structural changes in the juxtamembrane and this disrupts the autoinhibitory conformation of the receptor (7) by promoting constitutive activation of both receptor (8-10) and downstream effectors, which all leads to a bad prognosis (11,12). In the last decade, FLT3/ITDs mutations have been reported in 13%-32% of adult patients with acute myeloid leukemia and in a small number of patients with acute lymphoid leukemia (13-16). Patients with this abnormality have increased incidence of leukocytosis and decreased overall survival in comparison with patients without this abnormality. These findings indicate that FLT3/ITDs not only play an important role in the pathogenesis mechanism of leukemia but also have a prognostic value.

A previous study has demonstrated that high levels of FLT3 were expressed in leukemia and lymphoma cell lines including pre-B, myeloid, and monocytic cell lines (17). Also, several studies have shown that high levels of FLT3 were expressed in 70%-100% of patients with acute myeloid leukemia and B-acute lymphoid leukemia and in about 30% of patients with T-acute lymphoid leukemia (18,19). Likewise, a small number of chronic myelogenous leukemia blast crisis and chronic lymphocytic leukemia cells has been shown to express FLT3 (18,20). These data indicate that FLT3 expression may play a role in the survival or proliferation of leukemic blasts. Using Western blotting, Carow et al (18) found no FLT3 expression in the normal bone marrow, but identified FLT3 protein in 14 of 14 B-cell acute lymphoid leukemia cases, 36 of 41 acute myeloid leukemia cases, and 1 of 4 T-cell acute lymphoid leukemia cases. Though FLT3 expression in leukemia and its clinical significance have been widely investigated, little is known about FLT3 expression level and its clinical significance in Chinese patients with adult leukemia. Most of the studies have used Western blot assay as FLT3 protein assay, whereas flow cytometry on intact leukemic cell surface has been rarely used. We used flow cytometry on cell surface to investigate the expression of FLT3 receptor and quantitative polymerase chain reaction (PCR) to investigate FLT3 mRNA expression, as well as performed identification of FLT3/ITDs in different types of adult leukemia.

Patients and methods

The study included 120 patients with newly diagnosed acute leukemia – 60 with acute myeloid leukemia, 30 with B-acute lymphoid leukemia, 30 with T-acute lymphoid leukemia; 27 with chronic myeloid leukemia; and 30 controls (Table 1). Diagnosis was based on May-Grunwald-Giemsa-stained bone marrow smears and cytochemistry performed according to the French-American-British (FAB) group criteria (21). Leukocyte differentiation antigens were analyzed by immunofluorescent method for some cases. Complete remission was defined as normocellular bone marrow containing less than 5% blasts and showing evidence of normal maturation of other bone marrow elements. Patients and controls provided informed consent to use their samples for this study.

mRNA expression analysis

Patients and controls’ bone marrow mononuclear cells were separated on a Ficoll-Hypaque...
we did, probably because they used direct conjugated antibody. As to the mechanism of FLT3 protein overexpression, they suggested an autocrine stimulatory mechanism of FLT3 receptor-ligand (FL). FLT3-FL loop, which is expressed in all cell lines, plays an important role in the pathobiology of leukemia. Combined with various cytokines, FL has synergistic or additive mitogenic effect, which leads to significant anti-apoptotic effects on primary acute myeloid leukemia cells (27). FLT3 proteins on the cell surface were internalized when exogenous FL stimulation was administered (3). Our results showed that an increased FLT3 protein levels in bone marrow mononuclear cells were related to the number of blast cells in the bone marrow, suggesting that FLT3 protein assay may be a useful biomarker for making leukemia prognosis.

In our cohort, the frequency of FLT3/ITD mutation was 25% in acute myeloid leukemia and 7% in acute lymphoid leukemia, which is consistent with findings of a Japanese study (23%) and Kottaridis et al (27%) (14,15). Our initial findings showed that Chinese patients with leukemia shared the same gene alteration as patients from different genetic backgrounds, suggesting a common mechanism for the pathogenesis of acute leukemia. We found a significant association between the presence of the FLT3/ITD mutation, a higher white blood cell count, a higher marrow blast percentage, and elevated serum LDH. This suggests that FLT3/ITD is an independent prognostic factor in acute leukemia. Other studies have also reported that high FLT3 expression levels were associated with an unfavorable prognosis (26,28).

In our study, the expression of FLT3 transcript in patients with tandem duplication was increased, but the increase was not significant. Ozeki et al (26) also reported the association between FLT3/ITD mutation and expression of FLT. The explanation for such an association is that expression levels of FLT3/ITD depend on the co-expression of the wild type and the mutant alleles. It has been detected that FLT3 expression levels were related to the relative proportions of wild type and mutant FLT3 (25). Future analysis of FLT3 expression levels in patients with FLT3/ITD mutation should examine the relative fragments of wild type and mutant-FLT3.

To further assess the prognostic significance of FLT3/ITD mutation, we analyzed whether FLT3/ITD mutation influenced the complete remission rate of leukemia. The results showed that patients with FLT3/ITD had a little lower complete remission rate than patients without FLT3/ITD mutation, but the difference was not significant, which is in accordance with other studies (14,15). Lack of effect of FLT3/ITD mutation on the complete remission rate might be explained by a lack of effect of FLT3/ITD mutation on chemosensitivity of leukemic cells at diagnosis. Though it did not affect complete remission rate, FLT3 mutation has been found to predict relapse rate and overall survival (14,29). In fact, the presence of FLT3/ITD mutation has been suggested to have a major impact on long-term outcome (15).

Recently, Stirewalt et al (30) has reported that the size of FLT3/ITD has prognostic significance in patients with acute myeloid leukemia. They showed that increased ITD size was associated with decreased overall survival and relapse free survival. We sequenced three PCR products of patients with acute leukemia with FLT3/ITD mutation, detecting the insertion sizes of 36 bp, 42 bp, and 57 bp. Insertion nucleotides >40 bp were regarded as large; two patients in our group had large size ITD mutation. The patient with 42 bp insertion died three months after two courses of chemotherapy. The patient with 57 bp insertion died 5 months after diagnosis. The third patient, with a secondary acute monocytic type of leuke-
Molecular prognostic markers for adult acute myeloid leukemia with normal cytogenetics

Tara K Gregory¹, David Wald², Yichu Chen¹, Johanna M Vermaat², Yin Xiong¹ and William Tse*¹

Address: ¹Division of Medical Oncology, University of Colorado Cancer Center, University of Colorado School of Medicine, Aurora, Colorado, USA and ²Department of Medicine, Case Western Reserve University, Cleveland, Ohio, USA

Email: Tara K Gregory - tara.gregory@ucdenver.edu; David Wald - david.wald@case.edu; Yichu Chen - chapman.c@tom.com; Johanna M Vermaat - j.m.van.antwerpen@umail.leidenuniv.nl; Yin Xiong - yin.xiong@ucdenver.edu; William Tse* - william.tse@ucdenver.edu

* Corresponding author

Abstract

Acute myeloid leukemia (AML) is a heterogenous disorder that results from a block in the differentiation of hematopoietic progenitor cells along with uncontrolled proliferation. In approximately 60% of cases, specific recurrent chromosomal aberrations can be identified by modern cytogenetic techniques. This cytogenetic information is the single most important tool to classify patients at their initial diagnosis into three prognostic categories: favorable, intermediate, and poor risk. Currently, favorable risk AML patients are usually treated with contemporary chemotherapy while poor risk AML patients receive allogeneic stem cell transplantation if suitable stem cell donors exist. The largest subgroup of AML patients (~40%) have no identifiable cytogenetic abnormalities and are classified as intermediate risk. The optimal therapeutic strategies for these patients are still largely unclear. Recently, it is becoming increasingly evident that it is possible to identify a subgroup of poorer risk patients among those with normal cytogenic AML (NC-AML). Molecular risk stratification for NC-AML patients may be possible due to mutations of NPM1, FLT3, MLL, and CEBPα as well as alterations in expression levels of BAALC, MN1, ERG, and AF1q. Further prospective studies are needed to confirm if poorer risk NC-AML patients have improved clinical outcomes after more aggressive therapy.

Introduction

Acute Myeloid Leukemia (AML) is a broad range of disorders that are all characterized by an arrest of maturation along with uncontrollable proliferation of hematopoietic progenitor cells. The French-American-British classification is still widely used in clinical setting that groups AML into 8 subgroups (M0-M7) based on its degree of differentiation and morphology. Due to the heterogenous nature of AML even within specific FAB subtypes, there is a highly variable prognosis among AML patients. The overall 5-year survival rate for AML is still less than 50% in adults and significantly lower in the elderly [1]. The median survival in patients over the age of 65 is less than one year and only 20% of these patients survive two years [2]. Treatment for all subtypes of AML, except the M3 subtype, involves combination chemotherapy and a possible hematopoietic stem cell transplant as part of consolidation therapy. Acute Promyelocytic Leukemia (APL, M3 subtype) is treated with a combination of the differentiation-inducing agent all-trans retinoic acid and chemotherapy resulting in the presumed cure of 75–85% of patients [3]. In general, the prognosis of patients with AML is cur-
with normal karyotype into prognostically different subgroups [7]. Further, due to their frequency and stability, NPM1 mutations may become a new tool for monitoring minimal residual disease in AML-patients with a normal karyotype [9].

The Fms-like tyrosine kinase 3 Gene (FLT3)

FLT3 is a tyrosine kinase that is primarily expressed on hematopoietic progenitor cells and functions in the proliferation and differentiation of these cells. FLT3 is the most commonly mutated gene in AML with the mutation occurring in approximately 30–40% of AML patients [19]. The most common mutation consists of an internal tandem duplication (FLT3-ITD) in the juxtamembrane domain of the FLT3 gene. FLT3-ITD results in a constitutively active FLT3 protein that promotes Stat 5 phosphorylation. The net consequence of FLT3/Stat5 constitutive activation is uncontrolled hematopoietic cell proliferation [20]. AML patients who carry the FLT3-ITD mutation appear to have poorer clinical outcomes. Adult patients usually have a higher prevalence of FLT3-ITD than pediatric AML patients. This observation may partially explain why adult AML has a poorer clinical outcome than pediatric AML. Clinically, AML patients with FLT3-ITD tend to

<table>
<thead>
<tr>
<th>Study</th>
<th>Number of NPM1 mutants/total cases studied</th>
<th>Treatment</th>
<th>Demographics of those patients with NPM1 mutations</th>
<th>+ NPM1 mutant assessment of risk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Verhaak, et al [6]</td>
<td>95/275 (34.5%)</td>
<td>Dutch Belgian Hematology Oncology Cooperative Group (HOVON) protocols</td>
<td>- Median age 47 yo - 60% of those with FLT3 ITD - decreased in those age &lt; 35 yo - 42% of those with WBC &gt;20 K</td>
<td>HR EFS 1.96 DFS 2.0 OS 2.13</td>
</tr>
<tr>
<td>Döhner, et al [14]</td>
<td>145/300 (48.3%)</td>
<td>AML Study Group (AMLSG) AML HD93 AML HD98-A</td>
<td>- Increased in M4/M5 - extramedullary LAD - Female predominance - Decreased CD34 antigen expression - Increased LDH - Associated with FLT3 ITD - WBC &gt;20 K - Increased bone marrow blast counts</td>
<td>Odds ratio (OR) after induction CR 2.81</td>
</tr>
<tr>
<td>Schnittger, et al [15]</td>
<td>212/401 (52.9%)</td>
<td>German AMLCG99 study</td>
<td>- Associated with FLT3 ITD - Without FLT3, OS and EFS increased - Female predominance</td>
<td>Relative risk (RR) EFS 0.527</td>
</tr>
<tr>
<td>Theide, et al [16]</td>
<td>408/1485 (27.5%)</td>
<td>Deutsche Studieninitiative Leukämie (DSIL) AML 96 protocol</td>
<td>- High bone marrow blasts - Female predominance - WBC &gt;20 K - Association with FLT3-ITD mutations</td>
<td>OR OS 0.76 DFS 0.66</td>
</tr>
<tr>
<td>Boissel, et al [17]</td>
<td>50/106 (47%)</td>
<td>French Leukemia French Association (ALFA) ALFA90 ALFA9802</td>
<td>- Increased in FAB M4/M5 - 25% with FLT3-ITD - Decreased CEBPA - WBC &gt;20 K</td>
<td>No difference in CR or long term outcomes</td>
</tr>
<tr>
<td>Suzuki, et al [18]</td>
<td>64/257 (24.9%)</td>
<td>Japan Adult Leukemia Study Group protocols</td>
<td>- Associated with FLT3-ITD - NPM1 mutant unfavorable factor for relapse OR 2.106 - NPM1 wild type unfavorable for CR OR 4.908 -NPM1 mutant with FLT3-ITD favorable for CR OR 20.8</td>
<td></td>
</tr>
</tbody>
</table>
Analysis of FLT3 internal tandem duplication and D835 mutations in Chinese acute leukemia patients

Lihong Wang a,b,1, Dong Lin a,b, Xinwei Zhang a,b, Sen Chen a,b, Min Wang a, Jianxiang Wang a,b,∗

a State Key Laboratory of Experimental Hematology, Institute of Hematology and Blood Diseases Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, 288 Nanjing Road, Tianjin 300020, PR China
b Department of Clinical Hematology, Institute of Hematology and Blood Diseases Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, 288 Nanjing Road, Tianjin 300020, PR China

Received 5 February 2005; received in revised form 13 May 2005; accepted 14 May 2005
Available online 5 July 2005

Abstract

Genomic aberrations of Fms-like tyrosine kinase 3 (FLT3), including internal tandem duplication (ITD) and point mutations, have been demonstrated in 25–30% of adults acute myeloid leukemia (AML) and are markers of poor prognosis. FLT3/ITD and D835 mutations were analyzed in 194 Chinese patients with acute leukemia and myelodysplastic syndromes (MDS) by polymerase chain reaction (PCR). FLT3/ITDs and D835 mutations were found in 25.9 and 6.3% of 143 AML patients, respectively. Two patients showed both ITD and point mutations. Among the FAB subtypes of AML, the rate of FLT3 aberration was significantly higher in M3 and M5. However, neither aberrations was found in 25 patients with acute lymphoblastic leukemia (ALL), 2 acute hybrid leukemia, 17 MDS and 7 chronic myeloid leukemia in blast crisis (CML-BC). FLT3/ITD was associated to leukocytosis and lower complete remission (CR) rate, and was more prevalent in patients with normal karyotype. In contrast, D835 mutation was not associated with leukocytosis or low CR rate. Our results confirm that FLT3 activating mutations also occur in a significant percentage in Chinese AML patients. FLT3/ITD+ patients treated with standard induction regimen could achieve lower complete remission rates compared with patients not harboring this defect. Early detection of FLT3 mutations and an intensification of induction therapy might thus be useful for this group of patients to overcome the poor prognosis.

Keywords: Acute leukemia; FLT3; Mutation

1. Introduction

Fms-like tyrosine kinase 3 (FLT3), a new member of the receptor tyrosine kinase (RTK) III subfamily, was originally identified by its expression in hematopoietic stem/progenitor cells, and its importance in normal lymphohematopoietic stem cell function is now well illustrated [1]. Upon binding to the extracellular domain of FLT3, FLT3 ligand (FL) induces conformational changes and stabilizes receptor dimerization. The receptor dimerization brings the kinase domains into close proximity, and subsequent autophosphorylation of tyrosine residues further increases kinase activities. Activated RTKs then phosphorylate and/or bind multiple signal molecules leading to cell proliferation, differentiation or survival. Hence, alteration to the structure and/or expression of RTKs can result in tumorigenesis. Furthermore, acute myeloid leukemia (AML) cells have been shown to express FLT3, and exogenous FL can enhance their survival and proliferative response [2]. Therefore, alterations in FLT3 signaling through either aberrant FL expression or through gain-of-function mutations in the FLT3 gene itself could potentially contribute to leukaemogenesis. Two types of FLT3 activating mutations have been reported in leukemia patients: internal tandem duplication (ITD) and D835Y mutation. Both mutations can result in constitutive FLT3 activation [3].
Analysis of FLT3 internal tandem duplication and D835 mutations in Chinese acute leukemia patients

Lihong Wang a,b,1, Dong Lin a,b, Xinwei Zhang a,b, Sen Chen a,b, Min Wang a, Jianxiang Wang a,b,∗

a State Key Laboratory of Experimental Hematology, Institute of Hematology and Blood Diseases Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, 288 Nanjing Road, Tianjin 300020, PR China
b Department of Clinical Hematology, Institute of Hematology and Blood Diseases Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, 288 Nanjing Road, Tianjin 300020, PR China

Received 5 February 2005; received in revised form 13 May 2005; accepted 14 May 2005
Available online 5 July 2005

Abstract

Genomic aberrations of Fms-like tyrosine kinase 3 (FLT3), including internal tandem duplication (ITD) and point mutations, have been demonstrated in 25–30% of adults acute myeloid leukemia (AML) and are markers of poor prognosis. FLT3/ITD and D835 mutations were analyzed in 194 Chinese patients with acute leukemia and myelodysplastic syndromes (MDS) by polymerase chain reaction (PCR). FLT3/ITDs and D835 mutations were found in 25.9 and 6.3% of 143 AML patients, respectively. Two patients showed both ITD and point mutations. Among the FAB subtypes of AML, the rate of FLT3 aberration was significantly higher in M3 and M5. However, neither aberrations was found in 25 patients with acute lymphoblastic leukemia (ALL), 2 acute hybrid leukemia, 17 MDS and 7 chronic myeloid leukemia in blast crisis (CML-BC). FLT3/ITD was associated to leukocytosis and lower complete remission (CR) rate, and was more prevalent in patients with normal karyotype. In contrast, D835 mutation was not associated with leukocytosis or low CR rate. Our results confirm that FLT3 activating mutations also occur in a significant percentage in Chinese AML patients. FLT3/ITD+ patients treated with standard induction regimen could achieve lower complete remission rates compared with patients not harboring this defect. Early detection of FLT3 mutations and an intensification of induction therapy might thus be useful for this group of patients to overcome the poor prognosis.

© 2005 Elsevier Ltd. All rights reserved.

Keywords: Acute leukemia; FLT3; Mutation

1. Introduction

Fms-like tyrosine kinase 3 (FLT3), a new member of the receptor tyrosine kinase (RTK) III subfamily, was originally identified by its expression in hematopoietic stem/progenitor cells, and its importance in normal lymphohematopoietic stem cell function is now well illustrated [1]. Upon binding to the extracellular domain of FLT3, FLT3 ligand (FL) induces conformational changes and stabilizes receptor dimerization. The receptor dimerization brings the kinase domains into close proximity, and subsequent autophosphorylation of tyrosine residues further increases kinase activities. Activated RTKs then phosphorylate and/or bind multiple signal molecules leading to cell proliferation, differentiation or survival. Hence, alteration to the structure and/or expression of RTKs can result in tumorigenesis. Furthermore, acute myeloid leukemia (AML) cells have been shown to express FLT3, and exogenous FL can enhance their survival and proliferative response [2]. Therefore, 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. Two types of FLT3 activating mutations have been reported in leukemia patients: internal tandem duplication (ITD) and D835Y mutation. Both mutations can result in constitutive FLT3 activation [3].
There have been several reports, describing activating mutations of the FLT3 gene. In this study, we analyzed the prevalence of the two types of FLT3 activating mutations in 194 Chinese acute leukemia patients. Our findings suggest that FLT3/ITD and D835 mutation also occurs in a significant percentage of Chinese adult AML patients.

2. Materials and methods

2.1. Patient samples

Fresh bone marrow samples from 194 patients with newly diagnosed acute leukemia or myelodysplastic syndromes (MDS) admitted to our hospital between February 2000 and February 2003 were analyzed. All patients were diagnosed according to bone marrow morphology, cytochemistry and immunophenotype. Among the 194 patients, 143 were de novo AML, 2 hybrid acute leukemia (HAL), 17 MDS, 7 chronic myeloid leukemia in blast crisis (CML-BC) and 25 acute lymphoblastic leukemia (ALL). The peripheral blood samples from five healthy adult volunteers were used as control. For preservation of samples and genetic analysis, all patients gave informed consent that was approved by Ethics Committee of our institution according to Declaration of Helsinki.

2.2. Nucleic acid isolation

Genomic DNA was extracted with phenol–chloroform–isoamyl alcohol procedure from 5 ml fresh bone marrow or peripheral blood cells after Ficoll separation of mononuclear leukocytes. The concentration of DNA was determined by the ratio of absorbance at 260 and 280 and then was diluted to 1 µg/µl with water.

2.3. Detection of FLT3/ITD by DNA PCR

Polymerase chain reaction (PCR) was performed using exon 14 and 15 specific primers 14F, 5'-GAAT-TTAGGTATGAAAGCCAGC-3' and 15R, 5'-CGTTACAGGATTGGACG-3', to cover the whole JM and the first part of the TK-1 domain where most of the reported ITDs were located [4]. Amplification was performed in a 50 µl reaction volume with 1 µl DNA (1 µg/µl), 10 pmol each primer, 10 mmol dNTP, 2.5 U Ex-Taq DNA polymerase (Takara, Japan) in the buffer (10 mmol/l Tris–HCl (pH 8.3), 50 mmol/l KCl and 1.5 mmol/l MgCl2). Denaturing, annealing, and extension steps were all performed at 95 ºC for 30 s, 60 ºC for 30 s, 72 ºC for 30 s, respectively, for 30 cycles. There was an initial 5 min denaturation step at 95 ºC and a final extension step at 72 ºC for 10 min. Amplification products were analyzed on 3% agarose gels stained with ethidium bromide (Fig. 1a). The prevalence of ITD allele on the DNA level was heterogeneous, ranging from faint mutant bands in some patients to predominant mutants in others.

To detect D835 mutations, we amplified exon 20 by PCR, and then digested it with the EcoRV endonuclease. The amplified products of wild-type may be digested by EcoRV, which yielded 68 and 46 bp fragments. The D835 mutation showed an additional undigested band (114 bp) (Fig. 2).

Alterations in the FLT3 gene were detected in 44 patients among 143 AML cases analyzed. FLT3/ITDs were found in 37 (25.9%) (p = 0.001) of 143 AML patients and D835 mutations in 9 (6.3%) which was significantly lower than that of ITD mutation (p < 0.001). Two patients showed both ITD and D835Y point mutations. However, neither ITD nor D835 mutation was found in 25 ALL, 2 HAL, 17 MDS and 7 CML-BP.

2.4. Detection of D835Y mutation

PCR for detection of D835 mutation was performed by using primers 20F 5'-CGGCCAGGAAGCTGCTTG-3' and 20R 5'-GCAGCTTCACATTGCCCTCC-3'. D835 and B36 amino acids are encoded by GA TAGC, which is the recognition sequence for EcoRV [5]. Therefore, mutants can be detected by the loss of this enzyme restriction site. PCR was set up as mentioned above and 40 cycles of 94 ºC for 1 min, 55 ºC for 40 s and 72 ºC for 30 s. Purified PCR product by Quiaex (Qiagen) was digested with 5 U of EcoRV (Takara) at 37 ºC for 3 h. The digested products were separated on a 3% agarose gel, and undigested PCR product indicated the presence of a mutant. One of the mutants was confirmed by sequencing.

3. Results

3.1. Prevalence of FLT3/ITD and D835Y mutation

The FLT3/ITD amplification yielded a higher molecular weight product on a 3% agarose gel stained with ethidium bromide (Fig. 1a). The prevalence of ITD allele on the DNA level was heterogeneous, ranging from faint mutant bands in some patients to predominant mutants in others.

3.2. Sequencing analysis

Sequence analysis of five randomly selected ITD cases revealed in-frame duplications involving exon 14. The size of the duplication length varied from 21 to 60 bp and comprised different parts of the juxtamembrane domain. Three showed simple tandem duplications, but another two cases carried tandem duplications plus insertion sequences between duplications. The origin of the insertion sequences is not clear. All duplicated fragments contained two to four tyrosine residues except for case 3. The deduced amino acid sequences are shown in Fig. 1b.
There have been several reports, describing activating mutations of the \textit{FLT3} gene. In this study, we analyzed the prevalence of the two types of FLT3 activating mutations in 194 Chinese acute leukemia patients. Our findings suggest that FLT3/ITD and D835 mutation also occurs in a significant percentage of Chinese adult AML patients.

2. Materials and methods

2.1. Patient samples

Fresh bone marrow samples from 194 patients with newly diagnosed acute leukemia or myelodysplastic syndromes (MDS) admitted to our hospital between February 2000 and February 2003 were analyzed. All patients were diagnosed according to bone marrow morphology, cytochemistry and immunophenotype. Among the 194 patients, 143 were de novo AML, 2 hybrid acute leukemia (HAL), 17 MDS, 7 chronic myeloid leukemia in blast crisis (CML-BC) and 25 acute lymphoblastic leukaemia (ALL). The peripheral blood samples from five healthy adult volunteers were used as control. For preservation of samples and genetic analysis, all samples were maintained at −80 °C until analysis. All patients gave informed consent that was approved by Ethics Committee of our institution according to Declaration of Helsinki.

2.2. Nucleic acid isolation

Genomic DNA was extracted with phenol–chloroform–isoamyl alcohol procedure from 5 ml fresh bone marrow or peripheral blood cells after Ficoll separation of mononuclear cells. The concentration of DNA was determined by the ratio of A260/A280 and then was diluted to 1 μg/μl with water.

2.3. Detection of FLT3/ITD by DNA PCR

Polymerase chain reaction (PCR) was performed using exon 14 and 15 specific primers 14F, 5′-CA TTTTGACG GCAACC-3′ and 20R, 5′-TTAGGTA TGAAAGCCAGC-3′. PCR for detection of D835 mutation was performed using primers 20F 5′-CGAGCTTC ACATTGCCC-3′ and 20R 5′-GCAGCTTC ACATTGCCC-3′. D835 and B36 amino acids are encoded by GA TA TC, which is the recognition sequence for EcoRV [5]. Therefore, mutants can be detected by the loss of this enzyme restriction site. PCR was set up as mentioned above and 40 cycles of 94 °C for 1 min, 55 °C for 40 s and 72 °C for 30 s. Purified PCR product by Qiagen (Qiagen) was digested with 5 U of EcoRV (Takara) at 37 °C for 3 h. The digested products were separated on a 3% agarose gel, and undigested PCR product indicated the presence of a mutant. One of the mutants was confirmed by sequencing.

3. Results

3.1. Prevalence of FLT3/ITD and D835Y mutation

The FLT3/ITD amplification yielded a higher molecular weight product on a 3% agarose gel stained with ethidium bromide (Fig. 1a). The prevalence of ITD allele on the DNA level was heterogeneous, ranging from faint mutant bands in some patients to predominant mutants in others.

To detect D835 mutations, we amplified exon 20 by PCR, and then digested it with the EcoRV endonuclease. The amplified products of wild-type may be digested by EcoRV, which yielded 68 and 46 bp fragments. The D835 mutation showed an additional undigested band (114 bp) (Fig. 2).

Alterations in the \textit{FLT3} gene were detected in 44 patients among 143 AML cases analyzed. FLT3/ITDs were found in 37 (25.9%) of 143 AML patients and D835 mutations in 9 (6.3%), which was significantly lower than that of ITD mutation (P < 0.001). Two patients showed both ITD and D835Y point mutations. However, neither ITD nor D835 mutation was found in 25 ALL, 2 HAL, 17 MDS and 7 CML-BP.

3.2. Sequencing analysis

Sequence analysis of five randomly selected ITD cases revealed in-frame duplications involving exon 14. The size of the duplication length varied from 21 to 60 bp and comprised different parts of the juxtamembrane domain. Three showed simple tandem duplications, but another two cases carried tandem duplications plus insertion sequences between duplications. The origin of the insertion sequences is not clear. All duplicated fragments contained two to four tyrosine residues except for case 3. The deduced amino acid sequences are shown in Fig. 1b.
the ITD was not related to gender, age or the percentage of marrow blasts. Compared with patients without FLT3/ITD, those with FLT3/ITDs had significantly higher WBC counts (median 17.7 versus 7.5, \( P = 0.01 \)).

Cytomorphology analysis of all 143 AML patients was available. FLT3/ITDs were found in M3 (37.5%) and M5 (34.8%) more frequently than in other FAB subtypes (\( P = 0.01 \)). The incidence of FLT3/ITD was ranked as follows: M3 (37.5%) > M5 (34.8%) > M4 (20.0%) > M2 (18.8%). No patient was positive for ITDs in M0, M1, M6 or M7.

Cyogenetic analyses were performed in 104 AML patients. They were divided into six groups according to cytogenetics: group 1, normal karyotype (n=36); group 2, t(15;17) (n=25); group 3, t(8;21) (n=21); group 4, inv(16) (n=6); group 5, complex karyotypes (n=7); group 6, all others (n=9). Details of the presenting features of the FLT3/ITD+ and the FLT3/ITD− groups are given in Table 2. Ordinal \( \rho \) analysis shows that the mutation is most common in the normal karyotype group (41.7%) and the t(15;17) group (40.0%). The mutation was not detected in the six patients with inv(16). Interestingly, FLT3/ITD was relatively frequent in the complex karyotype group (14.3%). The incidence of FLT3/ITD according to karyotype was ranked as follows: normal (41.7%) > t(15;17) (40.0%) > t(8;21) (19.0%) > complex karyotype (14.3%) > others (11.1%) > inv(16) (0%).

Except M3, all AML patients received standard dose chemotherapy cytarabine 100 mg/m²/12 h for 7 days, homo-harringtonine 4 mg for 7 days and/or daunorubicin 40 mg/m² for 3 days. The complete remission (CR) rates for the ITD+ or ITD− groups were 35.3% versus 68.3% (\( P = 0.01 \)) in AML patients subtype. In 34 M3 patients who received all-trans retinoic acid or arsenic trioxide as reductive therapy, the CR rates for the ITD+ or ITD− groups were 76.9% versus 100% (\( P = 0.02 \)).

3.4. Clinical significance of D835 AML patients

Among the AML patients, the incidence of D835 mutation was as follows: M2 (5/53), M3 (13/40), M5 (2/23), M6 (1/2). No patient was positive for D835 mutations in M0, M1, M4 or M7. In contrast to FLT3/ITD mutation, D835 mutations were not associated with leukocytosis or low complete remission rate.

4. Discussion

In the present report, we have demonstrated internal tandem duplications of FLT3 in 25.9% (37/143) of AML patients at diagnosis. In this population, the frequency of FLT3/ITD positivity is similar to the reported frequency of 20–35% in the European series \([6–12]\). In the same cohort of patients, point mutations of the activation loop of the second tyrosine kinase domain (D835) were identified in 6.3% (9 of 143). Two patients showed both ITD and point mutations. Taken together, constitutive activation of FLT3 was present in 20.9% (44 of 143) of these patients, which indicates that FLT3 is an important target of mutational activation in adult AML. We failed to detect FLT3 mutations in MDS, HAL, ALL and CML-BC patients.

Although the prevalence of the ITD allele on the DNA level was heterogeneous, ranging from faint mutant bands in some patients to predominant mutants in others, sequence analysis of the ITD cases revealed in-frame duplications. Most of the tyrosine residues in the JM domain were involved, indicating that ITDs might be associated with amplification of FLT3 signaling.

Our data indicate that FLT3/ITD was not equally distributed among the different FAB-types, with a significantly higher frequency in patients with FAB M3/M5. This confirms the results of other studies \([10,13]\). FLT3 is persistently expressed during monocyte differentiation, and the FLT3−/CD34− cells. Thus, constitutive activation of FLT3 might be

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Correlation of FLT3 status to cytogenetics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Karyotype</td>
<td>FLT3/ITD (%)</td>
</tr>
<tr>
<td>----------</td>
<td>---------------</td>
</tr>
<tr>
<td>Normal</td>
<td>36 (34.6)</td>
</tr>
<tr>
<td>t(15;17)</td>
<td>15 (24.0)</td>
</tr>
<tr>
<td>t(8;21)</td>
<td>21 (20.2)</td>
</tr>
<tr>
<td>inv(16)</td>
<td>6 (5.8)</td>
</tr>
<tr>
<td>Complex</td>
<td>7 (6.7)</td>
</tr>
<tr>
<td>Others</td>
<td>9 (8.7)</td>
</tr>
</tbody>
</table>

* Compared with other karyotypes except t(15;17).

* Compared with other abnormal karyotypes.

The correlation of the FLT3 status to cytogenetics
Table 1

Clinical characteristics at presentation for the patients in the FLT3/ITD- and FLT3/ITD+ groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Number</th>
<th>FLT3/ITD (total)</th>
<th>FLT3/ITD (total)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ITD percentage</td>
<td>ITD percentage</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>96</td>
<td>26 (70)</td>
<td>70 (66)</td>
<td>0.69</td>
</tr>
<tr>
<td>Female</td>
<td>47</td>
<td>11 (36)</td>
<td>36 (34)</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;15</td>
<td>10</td>
<td>1 (3)</td>
<td>9 (8)</td>
<td>0.22</td>
</tr>
<tr>
<td>15–34</td>
<td>48</td>
<td>9 (24)</td>
<td>39 (37)</td>
<td></td>
</tr>
<tr>
<td>35–54</td>
<td>62</td>
<td>21 (67)</td>
<td>41 (39)</td>
<td></td>
</tr>
<tr>
<td>&gt;55</td>
<td>23</td>
<td>6 (16)</td>
<td>17 (16)</td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>39</td>
<td>41</td>
<td>39</td>
<td>0.53</td>
</tr>
<tr>
<td>WBC (×109/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;5</td>
<td>69</td>
<td>10 (27)</td>
<td>59 (56)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>5–10</td>
<td>40</td>
<td>13 (33)</td>
<td>27 (25)</td>
<td></td>
</tr>
<tr>
<td>&gt;10</td>
<td>34</td>
<td>14 (38)</td>
<td>20 (19)</td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>9.5</td>
<td>17.7</td>
<td>7.45</td>
<td>0.01</td>
</tr>
<tr>
<td>Bone marrow blasts (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;50</td>
<td>73</td>
<td>16 (44)</td>
<td>57 (56)</td>
<td>0.19</td>
</tr>
<tr>
<td>≥50</td>
<td>38</td>
<td>13 (36)</td>
<td>25 (25)</td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>71.0</td>
<td>75.5</td>
<td>70.0</td>
<td>0.99</td>
</tr>
</tbody>
</table>

Table 2

Correlation of the FLT3 status to cytogenetics

<table>
<thead>
<tr>
<th>Karyotype</th>
<th>Total (%)</th>
<th>FLT3/ITD (%)</th>
<th>FLT3/ITD (%)</th>
<th>FLT3/ITD in karyotype group (%)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>36 (44.6)</td>
<td>15 (48.4)</td>
<td>21 (28.9)</td>
<td>41.7</td>
<td>0.04a</td>
</tr>
<tr>
<td>t(15;17)</td>
<td>25 (24.0)</td>
<td>10 (32.3)</td>
<td>15 (20.3)</td>
<td>40.0</td>
<td>0.02a</td>
</tr>
<tr>
<td>inv(16)</td>
<td>21 (20.2)</td>
<td>4 (12.9)</td>
<td>17 (25.8)</td>
<td>19.0</td>
<td>0.76a</td>
</tr>
<tr>
<td>t(8;21)</td>
<td>6 (18.2)</td>
<td>0 (0)</td>
<td>6 (18.2)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Complex</td>
<td>7 (6.7)</td>
<td>1 (3.2)</td>
<td>6 (18.2)</td>
<td>14.3</td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td>9 (8.7)</td>
<td>1 (3.2)</td>
<td>8 (11.0)</td>
<td>11.1</td>
<td></td>
</tr>
</tbody>
</table>

* Compared with other karyotypes except t(15;17).

** Compared with other abnormal karyotypes.

4. Discussion

In the present report, we have demonstrated internal tandem duplications of FLT3 in 25.9% (37/143) of AML patients at diagnosis. In this population, the frequency of FLT3/ITD positivity is similar to the reported frequency of 20–35% in the European series [6–12]. In the same cohort of patients, point mutations of the activation loop of the second tyrosine kinase domain (D835) were identified in 6.3% (9 of 143). Two patients showed both ITD and point mutations. Taken together, constitutive activation of FLT3 was present in 30.8% (44 of 143) of these patients, which indicates that FLT3 is an important target of mutational activation in adult AML. We failed to detect FLT3 mutations in MDS, HAM, ALL and CML-BC patients. Although the prevalence of the ITD allele on the DNA level was heterogeneous, ranging from faint mutant bands in some patients to predominant mutants in others, sequence analysis of the ITD cases revealed in-frame duplications. Most of the tyrosine residues in the JM domain were involved, indicating that ITDs might be associated with amplification of FLT3 signaling.

Our data indicate that FLT3/ITD was not equally distributed among the different FAB types, with a significantly higher frequency in patients with FAB M3/M5. This confirms the results of other studies [10,13]. FLT3 is persistently expressed during monocyte differentiation, and the FLT3-L is required for optimal differentiation of monocytes from CD34- cells. Thus, constitutive activation of FLT3 might be
Correlation of the FLT3 status to cytogenetics

<table>
<thead>
<tr>
<th>Karyotype category</th>
<th>FLT3/ITD (%)</th>
<th>FLT3/ITD in normal karyotype (%)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>15 (44.4)</td>
<td>15 (44.4)</td>
<td>0.04</td>
</tr>
<tr>
<td>inv(16)</td>
<td>10 (32.3)</td>
<td>15 (44.4)</td>
<td>0.02</td>
</tr>
<tr>
<td>t(8;21)</td>
<td>4 (12.9)</td>
<td>17 (52.9)</td>
<td>0.06</td>
</tr>
<tr>
<td>Others</td>
<td>0 (0)</td>
<td>6 (18.2)</td>
<td></td>
</tr>
</tbody>
</table>

Table 2

The ITD was not related to gender, age or the percentage of marrow blasts. Compared with patients without FLT3/ITD, those with FLT3/ITDs had significantly higher WBC counts (median 17.7 versus 7.5, P = 0.01).

Cytomorphology analysis of all 143 AML patients was available. FLT3/ITDs were found in M3 (37.5%) and M5 (34.8%) more frequently than in other FAB subtypes (P = 0.01). The incidence of FLT3/ITD was ranked as follows: M3 (37.5%) > M5 (34.8%) > M4 (20.0%) > M2 (18.8%). No patient was positive for ITDs in M0, M1, M6 or M7.

Cyto genetic analyses were performed in 104 AML patients. They were divided into six groups according to cytogenetics: group 1, normal karyotype; group 2, t(15;17) (n = 25); group 3, t(15;17) (n = 21); group 4, inv(16) (n = 6); group 5, complex karyotypes (n = 7); group 6, all others (n = 9). Details of the presenting features of the FLT3/ITD + and the FLT3/ITD − groups are given in Table 2. Ordinal χ² analysis shows that the mutation is most common in the normal karyotype group (41.7%) and the t(15;17) group (40.0%). The mutation was not detected in the six patients with inv(16). Interestingly, FLT3/ITD was relatively frequent in the complex karyotype group (14.3%). The incidence of FLT3/ITD according to cytogenotype was ranked as follows: normal (41.7%) > t(15;17) (40.0%) > t(8;21) (19.0%) > complex karyotype (14.3%) > others (11.1%) > inv(16) (9%).

Except M3, all AML patients received standard dose chemotherapy cytarabine 100 mg/m²/12 h for 7 days, homoharringtonine 4 mg for 7 days and/or daunorubicin 40 mg/m² for 3 days. The complete remission (CR) rates for the ITD + or ITD − groups were 35.3% versus 68.3% (P = 0.01) in AML patients subtype. In 34 M3 patients who received all-trans retinoic acid or arsenic trioxide as reductive therapy, the CR rates for the ITD + or ITD − groups were 76.9% versus 100% (P = 0.02).

3.4. Clinical significance of D835 AML patients

Among the AML patients, the incidence of D835 mutation was as follows: M2 (3/53), M3 (3/40), M5 (2/23), M6 (1/2).

No patient was positive for D835 mutation in M0, M1, M4 or M7. In contrast to FLT3/ITD mutation, D835 mutations were not associated with leukocytosis or low complete remission rate.

4. Discussion

In the present report, we have demonstrated internal tandem duplications of FLT3 in 25.9% (37/143) of AML patients at diagnosis. In this population, the frequency of FLT3/ITD positivity is similar to the reported frequency of 20–35% in the European series (6–12). In the same cohort of patients, point mutations of the activation loop of the second tyrosine kinase domain (D835) were identified in 6.3% (9 of 143). Two patients showed both ITD and point mutations. Taken together, constitutive activation of FLT3 was present in 40% (44 of 143) of these patients, which indicates that FLT3 is an important target of mutational activation in adult AML. We failed to detect FLT3 mutations in MDS, HAL, ALL and CML-BC patients.

Although the prevalence of the ITD allele on the DNA level was heterogeneous, ranging from faint mutant bands in some patients to predominant mutants in others, sequence analysis of the ITD cases revealed in-frame duplications. Most of the tyrosine residues in the JM domain were involved, indicating that ITDs might be associated with amplification of FLT3 signaling.

Our data indicate that FLT3/ITD was not equally distributed among the different FAB types, with a significantly higher frequency in patients with FAB M3/M5. This confirms the results of other studies (10,13). FLT3 is persistently expressed during monocyte differentiation, and the FLT3- L is required for optimal differentiation of monocytes from CD34+ cells. Thus, constitutive activation of FLT3 might be present in M3/M5.
groups [5,12,18,19]. Further studies including a higher number of patients are needed to make it clear.

Acknowledgement

This work was supported by National Science Fund for Distinguished Young Scholars (Grant #30025019), National Natural Science Foundation of China (Grant #30370593), Tianjin Natural Science Foundation.

Contributions. L. Wang contributed to the concept and design, analyzed and interpreted the data, collected and assembled the data, supplied statistical expertise, provided draft of the article and gave final approval of the article. D. Lin provided study materials or patients, supplied statistical expertise and gave final approval of the article. X. Zhang collected and assembled the data and gave final approval of the article. S. Chen analyzed and interpreted the data, provided technical support and gave final approval of the article. M. Lin provided study materials or patients, supplied statistical expertise and gave final approval of the article. D. Tianjin provided study materials or patients, supplied statistical expertise and gave final approval of the article. D. Tianjin Natural Science Foundation.

References


Incidence and prognostic value of FLT3 internal tandem duplication and D835 mutations in acute myeloid leukemia

Isabel Moreno, Guillermo Martin, Pascual Bolufer, Eva Barragan, Eva Rueda, Jose Roman, Pascual Fernandez, Pilar Leon, Armando Mena, Jose Cervera, Antonio Torres, Miguel A. Sanz

Background and Objectives. Cytogenetics is the most important prognostic factor in acute myeloid leukemia (AML). However, a high proportion of patients show normal or intermediate-risk karyotypes. In these patients, other determinants could help to identify those with a higher risk of relapse. Recently, internal tandem duplications (ITD) and D835 mutations in FLT3 tyrosine kinase receptor have been shown to confer a bad prognosis in AML.

Methods. We analyzed the incidence of these mutations in a total of 208 patients of different AML subsets and their prognostic relevance in non-promyelocytic de novo AML.

Results. FLT3 mutations were detected in 24% of de novo AML, 42% of acute promyelocytic leukemia (APL) and 17% of secondary AML. Four patients showed both ITD and D835 mutations. Ninety-four percent of the patients with FLT3 alterations were classified into the intermediate-risk group. There was no association between the presence of FLT3 alterations and response to induction, while the alterations were associated with a worse disease-free survival and event-free survival in both the overall and intermediate-risk patients.

Interpretation and Conclusions. Our data confirm that any of the mutations in FLT3 confer a bad prognosis in AML. Because of the high prevalence of these mutations within the intermediate-risk group, their detection could be useful to identify patients with a poor prognosis.

Key words: FLT3 mutations, acute myeloid leukemia, prognostic value, intermediate-risk cytogenetics.

Haematologica 2003;88:19-24
http://www.haematologica.org/ 2003_01/ 88019.htm
© 2003, Ferrata Storti Foundation

Although 60–80% of adult patients receiving conventional treatments for acute myeloid leukemia (AML) achieve complete remission (CR), disease relapse eventually occurs in the majority of cases. During the last few years, several efforts have been made to identify prognostic factors that may predict treatment outcomes, thereby allowing better tailoring of post-remission therapy for patients. Currently, cytogenetics is considered the strongest prognostic factor and three major risk groups (low, intermediate, and high) have been distinguished according to AML karyotype at diagnosis. However, the so-called intermediate-risk group, which represents approximately two-thirds of cases, comprises a heterogeneous group of patients with apparently normal karyotypes or patients with a variety of other aberrations whose prognostic outcome is uncertain. Therefore, better identification of prognostic indicators in this group has been of particular interest in the past few years.

Recently, mutations in the FLT3 (fms-like tyrosine kinase receptor) have been reported to be involved in leukemogenesis. This receptor belongs to the class III tyrosine kinase receptor family, and is expressed in the hematopoietic progenitor cells as well as in a high percentage of AML and acute lymphoblastic leukemias (ALL). Binding of its ligand (FL) causes dimerization and triggering of tyrosine kinase activity thus promoting stem cell proliferation.

The most frequent FLT3 mutations found in AML and myelodysplastic syndromes (MDS) are internal tandem duplications (ITD) that have been reported in 20–30% and 3–16% of cases, respectively. These mutations affect the juxtamembrane domain and can be of variable length although all cases are in-frame mutations. ITDs lead to dimerization and constitutive activation of the receptor in the absence of its ligand, and recent studies with cultured cells or animal models point to a leukemogenic potential of such alterations. 

In AML, FLT3 ITDs have been more frequently detected in patients with a normal karyotype and their presence has been associated with an adverse prognosis. More recently, several mutations affecting aspartate 835 (D835) in the tyrosine kinase domain of the receptor have been found in about 7% of AML. These mutations also seem to affect tyrosine kinase receptor activity. Several studies have suggested that D835 mutations may also confer poor prognosis in AML although their clinical significance needs to be confirmed by more analyses.

The aim of the present study was to determine the...
Incidence and prognostic value of FLT3 internal tandem duplication and D835 mutations in acute myeloid leukemia

ISABEL MORENO, GUILLERMO MARTIN, PASCUAL BOLUFER, EVA BARRAGAN, EVA RUEDA, JOSE ROMAN, PASCUAL FERNANDEZ, PILAR LEON, ARMANDO MEÑA, JOSE CERVERA, ANTONIO TORRES, MIGUEL A. SANZ

Background and Objectives. Cytogenetics is the most important prognostic factor in acute myeloid leukemia (AML). However, a high proportion of patients show normal or intermediate-risk karyotypes. In these patients, other determinants could help to identify those with a higher risk of relapse. Recently, internal tandem duplications (ITD) and D835 mutations in FLT3 tyrosine kinase receptor have been shown to confer a bad prognosis in AML.

Design and Methods. We analyzed the incidence of these mutations in a total of 208 patients of different AML subsets and their prognostic relevance in non-promyelocytic de novo AML.

Results. FLT3 mutations were detected in 24% of de novo AML, 42% of acute promyelocytic leukemia (APL) and 17% of secondary AML. Four patients showed both ITD and D835 mutations. Ninety-four per cent of the patients with FLT3 alterations were classified into the intermediate-risk group. There was no association between the presence of FLT3 alterations and response to induction while the alterations were associated with a worse disease-free survival and event-free survival in both the overall and intermediate-risk patients.

Interpretation and Conclusions. Our data confirm that any of the mutations in FLT3 confer a bad prognosis in AML. Because of the high prevalence of these mutations within the intermediate-risk group, their detection could be useful to identify patients with a poor prognosis.

Key words: FLT3 mutations, acute myeloid leukemia, prognostic value, intermediate-risk cytogenetics.

Haematologica 2003;88:19-24
http://www.haematologica.org/ 2003_01_88019.htm
© 2003, Ferrata Storti Foundation

Although 60–80% of adult patients receiving conventional treatments for acute myeloid leukemia (AML) achieve complete remission (CR), disease relapse eventually occurs in the majority of cases. During the last few years, several efforts have been made to identify prognostic factors that may predict treatment outcomes, thereby allowing better tailoring of postremission therapy for patients. Currently, cytogenetics is considered the strongest prognostic factor and three major risk groups (low, intermediate, and high) have been distinguished according to AML karyotype at diagnosis. However, the so-called intermediate risk group, which represents approximately two-thirds of cases, comprises a heterogeneous group of patients with apparently normal karyotypes or patients with a variety of other aberrations whose prognostic outcome is uncertain. Therefore, better identification of prognostic indicators in this group has been of particular interest in the past few years.

Recently, mutations in the FLT3 (fms-like tyrosine kinase receptor) have been reported to be involved in leukemogenesis. This receptor belongs to the class III tyrosine kinase receptor family, and is expressed in the hematopoietic progenitor cells as well as in a high percentage of AML and acute lymphoblastic leukemias (ALL). Binding of its ligand (FL) causes dimerization and triggering of tyrosine kinase activity thus promoting stem cell proliferation.

The most frequent FLT3 mutations found in AML and myelodysplastic syndromes (MDS) are internal tandem duplications (ITD) that have been reported in 20–30% and 3–16% of cases, respectively. These mutations affect the juxtamembrane domain and can be of variable length although all cases are in-frame mutations. ITDs lead to dimerization and constitutive activation of the receptor in the absence of its ligand, and recent studies with cultured cells or animal models point to a leuke-mogenic potential of such alterations. ITDs have been more frequently detected in patients with a normal karyotype and their presence has been associated with an adverse prognosis. More recently, several mutations affecting aspartate 835 (D835) in the tyrosine kinase domain of the receptor have been found in about 7% of AML and acute lymphoblastic leukemias (ALL). These mutations also seem to affect tyrosine kinase receptor activity. Several studies have suggested that D835 mutations may also confer poor prognosis in AML, although their clinical significance needs to be confirmed by more analyses.

The aim of the present study was to determine the...
The aim of the present study was to determine the incidence of both ITD and D835 mutations in the FLT3 gene, in a series of 208 adult patients with AML and to evaluate their potential prognostic value.

Design and Methods

Patients
Two hundred and eight patients with AML recruited from five Spanish institutions were retrospectively analyzed for the presence of FLT3 mutations. They included 166 patients with non-promyelocytic de novo AML, 19 patients newly diagnosed as having acute promyelocytic leukemia (APL) and 23 with secondary AML (sAML). Nine cases of sAML were therapy-related and 14 were secondary to MDS. The prognostic impact of FLT3 alterations was analyzed only in the group of patients with de novo AML.

Treatment
One hundred and forty-two of 166 patients were enrolled into different intensive chemotherapy trials in which induction chemotherapy consisted of a combination of anthracycline plus cytarabine, with or without etoposide. As post-remission therapy, 50 patients followed a chemotherapy program, 35 or without etoposide. As post-remission therapy, 50 patients underwent allogeneic bone marrow transplantation, and 24 patients received autologous bone marrow transplantation.

Definitions
Cytogenetic risk groups were defined as follows: high risk: −5/del(5q), −7/del(7q), abn 3q, complex aberrations (≥ 3 independent aberrations), t(9;22) and t(6;9); low risk: t(8;21) and inv(16); intermediate risk: all other karyotypic aberrations or a normal karyotype.

Complete remission (CR) and hematologic relapse were defined according to the National Cancer Institute criteria.16 Event-free survival (EFS) was defined as time from diagnosis to resistance, relapse or death from any cause. Disease-free survival (DFS) was calculated from the day CR was achieved to the time of relapse or death.

Patients' samples
DNA was obtained from the mononuclear cells of bone marrow or peripheral blood samples using the salting out procedure described by Miller et al.17 RNA was obtained by the phenol-chloroform procedure of Chomczynski and Sacchi.18 Then 0.5 µg of RNA were reverse transcribed into cDNA in a 25 µL reaction volume using the TaqMan Gold RT-PCR Kit (PE Applied Biosystems, Branchburg, New Jersey, USA).

Detection of ITD
ITDs were investigated by polymerase chain reaction (PCR) with the primers described by Nakao et al.19 Primers 11F-11R and R5-R6, which amplify the juxtamembrane domain of the receptor, were used to amplify DNA and cDNA, respectively. The amplification reaction was performed in a final volume of 50 µL with 2 µL of DNA or cDNA, 0.6 µM of each primer, 2 mM of MgCl2 and 2 U of DNA polymerase (Promega). After 2 min of initial denaturation at 95°C, 30 cycles of 45 sec at 94°C, 1 min at 58°C and 2 min at 72°C were performed. The amplified products were finally electrophoresed on a 1.5% agarose gel stained with ethidium bromide.

Detection of D835 mutations
A fragment of 114 bp, corresponding to exon 17, was amplified using the primers 17F and 17R in DNA samples.20 When analyzing cDNA, we used the reverse primer 17RC (5’–GCAGACGGGCATTGCC-CC–3’). The amplification reaction was carried out using 2 min of initial denaturation at 95°C, 35 cycles of 30 sec at 94°C, 30 sec at 55°C, and 30 sec at 72°C. The amplified product was then subjected to digestion with EcoRV restriction endonuclease (Promega). In the presence of a wild-type exon 17 the amplified fragment was digested into two fragments of 68 and 46 bp, easily distinguishable upon electrophoresis on a 2.5% agarose gel. Mutations affecting either D835 or I836 amino acids led to the detection of the undigested product of 114 bp, in addition to the two 68 and 46 bp fragments corresponding to the digestion of the wild-type allele.

In selected cases, the presence of a D835 mutation was confirmed by sequencing of the amplified products. In these cases the undigested band was purified from the agarose gel and re-amplified. The PCR product was sequenced using Big Dye Terminator cycle sequencing chemistry (Applied Biosystems). Sequences were compared with the wild-type sequence registered in Genbank [accession #XM_166272 (mRNA) and NT_033997 (DNA)].

Statistical methods
χ2 and Fisher’s exact tests were used to analyze differences in the distribution of variables among subsets of patients. For comparison, unadjusted time-to-event analyses were performed using the Kaplan-Meier estimate,21 log-rank tests and their generalizations.20–22 All survival estimates are reported plus or minus (±) 1 standard error. The median duration of follow-up of patients who remain alive was 26 months (range, 13–126 months). The patients’ follow-up was updated as of May 2002.

Computations were performed using 4F and 1L programs from the BMDP statistical library (BMDP Statistical Software Inc, Los Angeles, CA, USA).
The aim of the present study was to determine the incidence of both ITD and D835 mutations in the FLT3 gene, in a series of 208 adult patients with AML and to evaluate their potential prognostic value.

Design and Methods

Patients

Two hundred and eight patients with AML recruited from five Spanish institutions were retrospectively analyzed for the presence of FLT3 mutations. They included 166 patients with non-promyelocytic de novo AML, 19 patients newly diagnosed as having acute promyelocytic leukemia (APL) and 23 with secondary AML (sAML). Nine cases of sAML were therapy-related and 14 were secondary to MDS. The prognostic impact of FLT3 alterations was analyzed only in the group of patients with de novo AML.

Treatment

One hundred and forty-two of 166 patients were enrolled into different intensive chemotherapy trials in which induction chemotherapy consisted of a combination of anthracycline plus cytarabine, with or without etoposide. As post-remission therapy, 50 patients followed a chemotherapy program, 35 or without etoposide. As post-remission therapy, 50 patients followed a chemotherapy program, 35 patients received autologous bone marrow or peripheral blood stem cell transplantation, and 24 patients underwent allogeneic bone marrow transplantation.

Definitions

Cytogenetic risk groups were defined as follows: high risk: −5/del(5q), −7/del(7q), abn 3q, complex aberrations (≥ 3 independent aberrations), t(9;22) and t(6;9); low risk: t(8,21) and inv(16); intermediate risk: all other karyotypic aberrations or a normal karyotype.

Complete remission (CR) and hematologic relapse were defined according to the National Cancer Institute criteria.16 Event-free survival (EFS) was defined as time from diagnosis to resistance, relapse or death from any cause. Disease-free survival (DFS) was calculated from the day CR was achieved to the time of relapse or death.

Patients’ samples

DNA was obtained from the mononuclear cells of bone marrow or peripheral blood samples using the salting out procedure described by Miller et al.17 RNA was obtained by the phenol-chloroform procedure of Chomczynski and Sacchi.18 Then 0.5 µg of RNA were reverse transcribed into cDNA in a 25 µL reaction volume using the TaqMan Gold RT-PCR Kit (PE Applied Biosystems, Branchburg, New Jersey, USA).

Detection of ITD

ITDs were investigated by polymerase chain reaction (PCR) with the primers described by Nakao et al.6 Primers 11F–11R and R5–R6, which amplify the juxtamembrane domain of the receptor, were used to amplify DNA and cDNA, respectively. The amplification reaction was performed in a final volume of 50 µL with 2 µL of DNA or cDNA, 0.6 µM of each primer, 2 mM of MgCl2 and 2 U of DNA polymerase (Promega). After 2 min of initial denaturation at 95°C, 30 cycles of 45 sec at 94°C, 1 min at 58°C and 2 min at 72°C were performed. The amplified products were finally electrophoresed on a 1.5% agarose gel stained with ethidium bromide.

Detection of D835 mutations

A fragment of 114 bp, corresponding to exon 17, was amplified using the primers 17F and 17R in DNA samples.19 When analyzing cDNA, we used the reverse primer 17RC (5′-GCAGACGGGCATTGCCC-CC-3′). The amplification reaction was carried out using 2 min of initial denaturation at 95°C, 35 cycles of 30 sec at 94°C, 30 sec at 55°C, and 30 sec at 72°C. The amplified product was then subjected to digestion with EcoRV restriction endonuclease (Promega). In the presence of a wild-type exon 17 the amplified fragment was digested into two fragments of 68 and 46 bp, easily distinguishable upon electrophoresis on a 2.5% agarose gel. Mutations affecting either D835 or I836 amino acids led to the detection of the undigested product of 114 bp, in addition to the two 68 and 46 bp fragments corresponding to the digestion of the wild-type allele.

In selected cases, the presence of a D835 mutation was confirmed by sequencing of the amplified products. In these cases the undigested band was purified from the agarose gel and re-amplified. The PCR product was sequenced using Big Dye Terminator cycle sequencing chemistry (Applied Biosystems). Sequences were compared with the wild-type sequence registered in Genbank [accession #XM_166272 (mRNA) and NT_033997 (DNA)].

Statistical methods

χ2 and Fisher’s exact tests were used to analyze differences in the distribution of variables among subsets of patients. For comparison, unadjusted time-to-event analyses were performed using the Kaplan-Meier estimate,19 log-rank tests and their generalizations.20–22 All survival estimates are reported plus or minus (±) 1 standard error. The median duration of follow-up of patients who remain alive was 26 months (range, 13–126 months). The patients’ follow-up was updated as of May 2002.

Computations were performed using 4F and 1L programs from the BMDP statistical library (BMDP Statistical Software Inc, Los Angeles, CA, USA).
The aim of the present study was to determine the incidence of both ITD and D835 mutations in the FLT3 gene, in a series of 208 adult patients with AML, and to evaluate their potential prognostic value.

Design and Methods

Patients

Two hundred and eighty-two patients were enrolled into different intensive chemotherapy trials in which induction chemotherapy consisted of a combination of anthracycline plus cytarabine, with or without etoposide. As post-remission therapy, 50 patients followed a chemotherapy program, 35 patients received autologous bone marrow or peripheral blood stem cell transplantation, and 24 patients received allogeneic bone marrow transplantation.

Treatment

One hundred and forty-two of 166 patients with de novo AML, 19 patients newly diagnosed as having acute promyelocytic leukemia (APL) and 23 with secondary AML (sAML). Nine cases of sAML were therapy-related and 14 were secondary to MDS. The prognostic impact of FLT3 alterations was analyzed only in the group of patients with de novo AML.

Definitions

Cytogenetic risk groups were defined as follows: high risk: -5/del(5q), -7/del(7q), abn 3q, complex aberrations (≥ 3 independent aberrations), t(9;22) and t(6;9); low risk: t(8;21) and inv(16); intermediate risk: all other karyotypic aberrations or a normal karyotype.

Complete remission (CR) and hematologic relapse were defined according to the National Cancer Institute criteria.16 Event-free survival (EFS) was defined as time from diagnosis to resistance, relapse or death from any cause. Disease-free survival (DFS) was calculated from the day CR was achieved to the time of relapse or death.

Patients’ samples

DNA was obtained from the mononuclear cells of bone marrow or peripheral blood samples using the salting out procedure described by Miller et al.20 RNA was obtained by the phenol-chloroform procedure of Chomczynski and Sacchi.21 Then 0.5 µg of RNA were reverse transcribed into cDNA in a 25 µL reaction volume using the TaqMan Gold RT-PCR Kit (PE Applied Biosystems, Branchburg, New Jersey, USA).

Detection of ITD

ITDs were investigated by polymerase chain reaction (PCR) with the primers described by Nakao et al.6 Primers 11F-11R and R5-R6, which amplify the juxtamembrane domain of the receptor, were used to amplify DNA and cDNA, respectively. The amplification reaction was performed in a final volume of 50 µL with 2 µL of DNA or cDNA, 0.6 µM of each primer, 2 mM of MgCl₂ and 2 U of DNA polymerase (Promega). After 2 min of initial denaturation at 95°C, 30 cycles of 45 sec at 94°C, 1 min at 58°C, and 2 min at 72°C were performed. The amplified products were finally electrophoresed on a 1.5% agarose gel stained with ethidium bromide.

Detection of D835 mutations

A fragment of 114 bp, corresponding to exon 17, was amplified using the primers 17F and 17R in DNA samples.3 When analyzing cDNA, we used the reverse primer 17RC (5’–GCAGACGGGCATTGCCC–3’). The amplification reaction was carried out using 2 min of initial denaturation at 95°C, 35 cycles of 30 sec at 94°C, 30 sec at 55°C, and 30 sec at 72°C. The amplified product was then subjected to digestion with EcoRV restriction endonuclease (Promega). In the presence of a wild-type exon 17 the amplified fragment was digested into two fragments of 68 and 46 bp, easily distinguishable upon electrophoresis on a 2.5% agarose gel. Mutations affecting either D835 or I836 amino acids led to the detection of the undigested product of 114 bp, in addition to the two 68 and 46 bp fragments corresponding to the digestion of the wild-type allele.

In selected cases, the presence of a D835 mutation was confirmed by sequencing of the amplified products. In these cases the undigested band was purified from the agarose gel and re-amplified. The PCR product was sequenced using Big Dye Terminator cycle sequencing chemistry (Applied Biosystems). Sequences were compared with the wild-type sequence registered in Genbank [accession #XM_166272 (mRNA) and NT_033997 (DNA)].

Statistical methods

χ² and Fisher’s exact tests were used to analyze differences in the distribution of variables among subsets of patients. For comparison, unadjusted time-to-event analyses were performed using the Kaplan-Meier estimate,19 log-rank tests and their generalizations.20–22 All survival estimates are reported plus or minus (±) 1 standard error. The median duration of follow-up of patients who remain alive was 26 months (range, 13–126 months). The patients’ follow-up was updated as of May 2002.

Computations were performed using 4F and 1L programs from the BMDP statistical library (BMDP Statistical Software Inc, Los Angeles, CA, USA).
Results

Incidence of FLT3 mutations

Alterations in the FLT3 gene were detected in 52 patients out of the 208 cases analyzed. These aberrations included ITD in 32 cases (eight of them with more than one ITD), D835 mutations in 16 cases and both types of alteration (ITD + D835) in four patients. Forty of 166 de novo AML patients (24%) had mutations in FLT3 (24 ITD, 12 D835 and four ITD + D835), while eight of 19 APL patients (42%) had FLT3 alterations (six ITD and two D835). Finally, four of 23 sAML (17%) carried some type of mutation in the FLT3 gene (two ITD and two D835).

Sequencing of 10 samples with D835 mutation led to the identification of four different mutations (Table 1), the most frequent of which was D835Y (five cases), followed by D835H mutation (three cases). One patient carried a D835V mutation, and another the D835A mutation, which had not previously been described.

FLT3 alterations and correlation with presenting features in de novo AML patients (excluding APL)

Details of clinical characteristics at diagnosis of the 166 de novo AML patients are given in Table 2. The presence of FLT3 ITD was clearly associated with hyperleukocytosis ($p < 0.0001$), intermediate-risk cytogenetics ($p = 0.03$), and particularly with normal karyotype ($p = 0.04$). No clinical variables were associated with D835 mutations. The four patients with both ITD and D835 mutations had white blood cell counts greater than 50 $\times$ 10$^9$/L and normal karyotypes. Finally, five out of seven patients with more than one ITD alteration also had hyperleukocytosis and a normal karyotype at diagnosis.

Treatment outcome according to FLT3 status in de novo non-promyelocytic AML patients

FLT3 alterations had no influence on response to induction (Table 3). The three-year Kaplan-Meier estimate of DFS for the whole series was 40±5%. FLT3 alterations were associated with a shortened DFS (ITD-positive 41±12%, D835-positive 14±12%, ITD/D835-negative 43±6%) and EFS (ITD-positive 28±9%, D835-positive 11±9%, ITD/D835-negative 43±6%), as shown in Figures 1A and 1B. In the intermediate cytogenetic group, patients with a mutated FLT3 had a shorter EFS (ITD-positive 30±10%, D835-positive 17±11%, ITD/D835-negative 36±26%) and DFS (ITD-positive 39±12%, D835-positive 22±4%, ITD/D835-negative 44±7%), as shown in Figures 2A and 2B. DFS in FLT3-positive AML patients was 17±4%, 38±20% and 47±19%
Mutations of receptor tyrosine kinases are implicated in the constitutive activation and development of human malignancy. An internal tandem duplication (ITD) of the juxtamembrane (JM) domain-coding sequence of the FLT3 gene (FLT3/ITD) is found in 20% of patients with acute myeloid leukemia (AML) and is strongly associated with leukocytosis and a poor prognosis. On the other hand, mutations of the c-KIT gene, which have been found in mast cell leukemia and AML, are clustered in 2 distinct regions, the JM domain and D816 within the activation loop. This study was designed to analyze the mutation of D835 of FLT3, which corresponds to D816 of c-KIT, in a large series of human hematologic malignancies. Several kinds of missense mutations were found in 30 of the 429 (7.0%) AML cases, 1 of the 29 (3.4%) myelodysplastic syndrome (MDS) cases, and 1 of the 36 (2.8%) acute lymphocytic leukemia patients. The D835Y mutation was most frequently found (22 of the 32 D835 mutations), followed by the D835V (5), and D835H (1), D835E (1), and D835N (1) mutations. Of note is that D835 mutations occurred independently of FLT3/ITD. An analysis in the 201 patients newly diagnosed with AML (excluding M3) revealed that, in contrast to the FLT3/ITD mutation (n = 46), D835 mutations (n = 8) were not significantly related to the leukocytosis, but tended to worsen disease-free survival. All D835-mutant FLT3 were constitutively tyrosine-phosphorylated and transformed 32D cells, suggesting these mutations were constitutively active. These results demonstrate that the FLT3 gene is the target most frequently mutated to become constitutively active in AML. (Blood. 2001;97:2434-2439)
We examined the D835 and ITD mutations of the *FLT3* gene in a total of 589 patients with hematologic malignancies (Table 1). We found several kinds of missense mutations of D835 in 30 of the 429 (7.0%) AML, 1 of the 29 (3.4%) MDS, and 1 of the 36 (2.8%) ALL patients. Among AML patients, D835 mutations were found in 7.0% (30 of 429), an incidence significantly lower than that of ITD mutation (81 of 429, 18.9%, P < .001, Fisher exact test). According to the FAB classification, D835 mutation was frequently found in the M5 type (P = .015, Pearson χ² test); 1 of 4 (25%) of M0, 2 of 63 (3.1%) of M1, 3 of 99 (3.0%) of M2, 11 of 141 (7.8%) of M3, 4 of 70 (5.7%) of M4, 9 of 40 (22.5%) of M5, 0 of 6 of M6, and 0 of 6 of M7 cases. In 3 AML patients, whose leukemia cells had the D835 mutations at the initial diagnosis, the mutations were lost at the complete remission (CR; Figure 2). Furthermore, no mutation was found in peripheral blood mononuclear cells from 30 healthy volunteers.

Sequence analysis showed that there were several kinds of D835 mutations, though all were missense. The first nucleotide G of D835 was most frequently substituted with T (22 of the 32 D835 mutations), resulting in an Asp to Tyr amino acid change (D835Y). The T substitution for the second nucleotide A of D835, resulting in an Asp to Val change (D835V), was found in 5 patients. Furthermore, D835H, D835E, and D835N mutations were each found in one patient. Of interest is that D835Y and D835E mutations were found in one AML (M3) patient, whereas cloning analysis showed that these mutations occurred in different alleles. In one MDS (RAEB in T) patient, a different mutation was found at I836. This mutation consisted of the insertion of 3 nucleotides (TTG) between D835 and I836, and A T to GA substitutions at the first and second nucleotides of I836, resulting in insertion of Leu and an Ile to Asp amino acid change (I836L+D).

Of note is that both D835 and ITD mutations were found in only one AML (M3) patient. To clarify whether these mutations occurred on the same allele, we amplified the region from JM through TK2 domains by reverse transcriptase-mediated PCR. After *Eco* RV digestion, the amplified products were separated through a polyacrylamide gel. The result showed that the product with ITD was completely digested by *Eco* RV but not the product without ITD, suggesting that these mutations occurred on different alleles (data not shown).

**Clinical characteristics and prognosis of AML patients with or without *FLT3* gene mutations**

Among the AML patients, 201 individuals excluding those with M3 who were treated with the AML87, AML89, and AML92
not express myeloid antigens, suggesting that D835 mutation might be involved in lymphoid lineage cells.

To exclude the possibility of polymorphism, we also analyzed normal individuals and the patients both at initial diagnosis and at CR. In all normal individuals, no D835 mutation was found. Furthermore, in the patients with D835 mutation at initial diagnosis, the mutation was lost at CR. These results confirmed that D835 mutations of FLT3 are somatic mutations associated with leukemia.

The incidence of D835 mutations was significantly lower than that of ITD mutations. Both mutations were mainly found in AML. D816 mutations of c-KIT, which are equivalent to D835 of FLT3, were found in many patients with mastocytosis as well as AML.16 Although most of the D816 mutations of c-KIT were an Asp to Val substitution (D816V), the major D835 mutation of FLT3 was an Asp to Tyr substitution (D835Y). Furthermore, although 3 kinds of D816 c-KIT mutations (D816V, D816Y, and D816F) have been found in patients with mastocytosis and AML, D835H, D835E, and D835N mutations of FLT3 were found in addition to D835Y and D835V mutations. However, the mutants found in this study showed constitutive activation of the receptor in accordance with mutant c-KITs, in which the Asp residue was mutated to a series of other amino acids.24

In one patient with AML (M3), 2 kinds of mutations (D835Y and D835E) were found. Cloning analysis demonstrated that these 2 mutations did not occur on the same allele. Likewise, D835 and ITD mutations were found in one patient with AML (M3), but further analyses showed that these mutations occurred on different alleles. These results suggest that continuing mutations of the FLT3 gene seem to occur in leukemia cells. However, this raises the question of whether all kinds of mutations have the same potential functions in leukemia cells. Because it remains unclear whether all mutations have the same kinase activity, and are associated with the same signal-transduction pathway, we could not entirely rule out the possibility that different kinds of mutations are additively or synergistically associated with the progression of leukemia.

Clinical characteristics were analyzed in 201 patients with newly diagnosed AML excluding the M3 cases. In contrast to ITD mutations, D835 mutations did not significantly affect any clinical variables or prognosis. However, these results do not indicate whether D835 mutations have an adverse effect on leukemia cells because such a mutation was found in only 8 patients (4%). Indeed, all 6 patients in the D835-Mt group who achieved CR relapsed within 28 months, whereas there was no significant difference from the Wt group. If D835 mutations are not considered, ITD mutations do not become a poor prognostic factor for DFS. However, if D835 mutations are considered, the ITD group had a significantly lower DFS than the Wt group (P = .023). We previously reported that age 60 years or older and cytogenetics data were the strongest unfavorable factors for DFS in a multivariate analysis in these patients.8 We, therefore, analyzed the effect of D835-Mt on DFS in the 101 patients who were under 60 years old and did not have the karyotypic abnormalities associated with poor prognosis, specifically t(9; 22), 11q23 alterations, del(5) or del(7). Among them, 19 patients had an ITD mutation, 5 had a D835 mutation, and 77 had neither. Although the D835-Mt group was too small for statistical analysis, it showed a tendency for a worse prognosis (P = .09).

It has been reported that N-RAS gene mutations were found in 10% to 20% of patients with AML and associated with several clinical variables.25,26 Previously, we also reported that N-RAS gene mutations were found in 28 of the same 201 AML patients and associated with leukocytosis.4 However, N-RAS gene mutations were found in only 3 patients with FLT3/ITD and not in patients with D835 mutations, suggesting that N-RAS and FLT3 gene mutations occur independently. Because it has been demonstrated that the MAP kinase pathway is activated by either FLT3 or RAS,10,27 the leukemia clone, in which MAP kinase is activated, seems not to acquire the other mutation. To exclude the effect of N-RAS gene mutations, we reanalyzed the relationship between D835 mutations and clinical variables in 173 patients without N-RAS mutations. However, we found no significant differences in the patients with D835 mutations. To clarify the clinical significance of the FLT3 gene mutations, a larger scale analysis is required.

Although D835 mutants were constitutively activated and caused the autonomous proliferation of 32D cells like ITD mutants, it remains to be clarified whether or not the level of kinase activity and signal-transduction pathway are the same between them. In addition, it has been demonstrated that the level of kinase activity differed with the amino acid substituted for the Asp residue (D814 in c-KIT and D802 in c-FMS) within the A-loop of murine c-KIT and c-FMS. In murine c-KIT, all mutants except D814C had an increased amount of tyrosine phosphorylation without ligand stimulation, and D814Y, D814V, D814L, D814H, and D814W mutants, especially, revealed markedly elevated kinase activity.24 In murine c-FMS, D802Q, D802R, and D802G were inactivating mutations, whereas all other mutants were active.19 According to
Detection of KIT and FLT3 Mutations in Acute Myeloid Leukemia with Different Subtypes

Farhad Zaker PhD*, Mohammad Mohammadzadeh MSc*, Mohammad Mohammadi MSc**

Background: Mutations in KIT and fms-like tyrosine kinase 3 genes lead to uncontrolled proliferation of leukemic cells with a poor prognosis. Since data concerning the incidence and associations with patients characteristics vary amongst different studies, the aim of the present study is to identify and quantify the frequency of mutations in Iranian patients suffering from acute myeloid leukemia.

Methods: Internal tandem duplication and D835 mutations in the fms-like tyrosine kinase 3 gene of acute myeloid leukemia patients were studied through polymerase chain reaction and polymerase chain reaction-RFLP analysis. Amplified products for a point mutation in D816 for KIT have also been identified through the polymerase chain reaction-RFLP technique. The mutations in exon 8 of KIT were detected by using the PCR and the Conformational Sensitive Gel Electrophoresis techniques, and amplified products have been confirmed by sequencing techniques.

Results: Internal tandem duplication and D835 mutations in the fms-like tyrosine kinase 3 gene occurred in 18% and 6% of AML patients, respectively. Frequencies of mutation were 1.4% and 4.7% in exon 8 and D816 of the KIT gene in acute myeloid leukemia patients. These results were substantially different for various subclasses of French-American-British classification.

Conclusion: This study revealed that approximately 30% of acute myeloid leukemia patients have either KIT or fms-like tyrosine kinase 3 genetic mutations. The presence of fms-like tyrosine kinase 3 was significantly associated with M3 morphology and mutations of KIT were significantly associated with M2 and M4 subtypes.

Keywords: Acute Myeloid Leukemia • CSGE • mutations • PCR

Introduction

Amongst various factors, mutations for cell differentiation and proliferation are considered to be effective factors in the development of acute myeloid leukemia (AML). fms-like tyrosine kinase 3 (FLT3) and KIT genes belong to the family of tyrosine kinase class III receptors that induce signals for cell proliferation. Mutations of these genes, however, result in autonomously leukemic cell proliferation and an unfavorable prognosis.1-3

A significant over expression of the FLT3 gene (70 – 100%) occurs in both AML and acute lymphoblastic leukemia (ALL)4-6 and point mutations in both the D835 and internal tandem duplication (ITD) of this gene occur as main mutations. These mutations lead to a steady and continual activation of the FLT3 tyrosine kinase receptor without ligand stimulation.7,8 The ITD mutation is the most frequent abnormality that occurs in 20 – 25% of AML patients and the D835 point mutation has been identified in 6 – 10% of patients.9-12 The augmented activation of KIT with SCF causes cell proliferation whereby in AML, an abnormal increase of proliferation occurs in two ways; first in AML, mutations of D816 (in exon 17) or exon 8 KIT lead to autonomous activation of KIT, and also through over expression of KIT in...
Detection of KIT and FLT3 Mutations in Acute Myeloid Leukemia with Different Subtypes

Farhad Zaker PhD*, Mohammad Mohammadzadeh MSc*, Mohammad Mohammadi MSc**

Background: Mutations in KIT and fms-like tyrosine kinase 3 genes lead to uncontrolled proliferation of leukemic cells with a poor prognosis. Since, data concerning the incidence and associations with patients characteristics vary amongst different studies, the aim of the present study is to identify and quantify the frequency of mutations in Iranian patients suffering from acute myeloid leukemia.

Methods: Internal tandem duplication and D835 mutations in the fms-like tyrosine kinase 3 gene of acute myeloid leukemia patients were studied through polymerase chain reaction and polymerase chain reaction-RFLP analysis. Amplified products for a point mutation in D816 for KIT have also been identified through the polymerase chain reaction-RFLP technique. The mutations in exon 8 of KIT were detected by using the PCR and the Conformational Sensitive Gel Electrophoresis techniques, and amplified products have been confirmed by sequencing techniques.

Results: Internal tandem duplication and D835 mutations in the fms-like tyrosine kinase 3 gene occurred in 18% and 6% of AML patients, respectively. Frequencies of mutation were 1.4% and 4.7% in exon 8 and D816 of the KIT gene in acute myeloid leukemia patients. These results were substantially different for various subclasses of French-American-British classification.

Conclusion: This study revealed that approximately 30% of acute myeloid leukemia patients have either KIT or fms-like tyrosine kinase 3 genetic mutations. The presence of fms-like tyrosine kinase 3 was significantly associated with M3 morphology and mutations of KIT were significantly associated with M2 and M4 subtypes.

Introduction

Amongst various factors, mutations for cell differentiation and proliferation are considered to be effective factors in the development of acute myeloid leukemia (AML). fms-like tyrosine kinase 3 (FLT3) and KIT genes belong to the family of tyrosine kinase class III receptors that induce signals for cell proliferation. Mutations of these genes, however, result in autonomously leukemic cell proliferation and an unfavorable prognosis.1-3

A significant over expression of the FLT3 gene (70 – 100%) occurs in both AML and acute lymphoblastic leukemia (ALL)4-6 and point mutations in both the D835 and internal tandem duplication (ITD) of this gene occur as main mutations. These mutations lead to a steady and continual activation of the FLT3 tyrosine kinase receptor without ligand stimulation.7,8 The ITD mutation is the most frequent abnormality that occurs in 20 – 25% of AML patients and the D835 point mutation has been identified in 6 – 10% of patients.9,12 The augmented activation of KIT with SCF causes cell proliferation whereby in AML, an abnormal increase of proliferation occurs in two ways; first in AML, mutations of D816 (in exon 17) or exon 8 KIT lead to autonomous activation of KIT, and also through over expression of KIT in

Archives of Iranian Medicine, Volume 13, Number 1, 2010: 21 – 25.

Keywords: Acute Myeloid Leukemia • CSGE • mutations • PCR
in the AML patient. Overall, the present study indicated that in 18% of the ITD mutations; 16% have been found in the M3 subclass and the remainder belong to both M2 and M4 subclasses. Furthermore, of the 6% with mutations in D835; 4% were located in the M3 subclass and the rest in both M2 and M5 subclasses.

**Mutational analysis of KIT**

Of the 212 AML patients investigated in this study, three patients' specimens had mutations in exon 8. Figure 3 demonstrates lanes 7, 11, and 13 that relate to patients M4, M2, and M4, each of them had 3 different bands. The remainder show one band which represented a wild type with 386 bp. In addition, genomic sequencing confirmed insertion and deletion sequences in exon 8 in two patients' samples but the third sample was failed. These new mutations have been submitted and documented in the Gene Bank (FJ177639 and FJ189474). In one case, the fragment GACAGGCT has been deleted and TGGCA was inserted. In the other case, however, only the GACAGGGCTT fragment has been inserted without any deletion. Of all patients studied, samples from ten patients were shown to have a point mutation at D816. The AatII enzyme was capable of identifying the sequence present in the wild type of KIT and hence the 106 bp PCR products were spliced into 85 bp and 21 bp, which this enzymatic DNA analysis would not occur if a mutation was present at D816. While the 85bp band was detectable, the 21bp band ran off the gel. In Figure 4, the D816 mutation has occurred in columns 4, 5, and 8. Due to sequence alteration at the breaking point, the 106 bp nucleotides have not been digested. In general, out of 212 AML patients, 3 (1.4%) contain exon 8 mutations and 10 (4.7%) have D816 point mutations in the KIT gene. One of the M2 samples had mutations in both exon 8 and D816. These mutations were typically located within the M2 and M4 subclasses, whereas only one case of M1 had a D816 mutation.

**Discussion**

Mutations of both the KIT and FLT3 genes have a great impact on leukemia pathogenesis, most specifically on patients who suffer from AML. Mutations also lead to uncontrolled proliferation of leukemic cells. Despite the pathogenic effect of these mutations, they are not the sole cause of acute leukemia, which may require other genomic alterations related to cell differentiation. These mutations are usually associated with a poor prognosis.25-28

In this study of adults with AML, a total of 24% mutations were observed in the FLT3 gene of which 18% were ITD that mostly occurred in the M3 subclass which was characterized by the T.15-17 No positive cases of ITD mutation were observed in patient specimens within the M0, M1, and M5 subclasses. Evidence to date has suggested that PML/RARA gene fusion is not sufficient for
leukemogenesis. Other factors such as mutations of FLT3 could also confer a proliferative advantage hence halting the differentiation process.\textsuperscript{26,26,27} Various studies have reported a high occurrence of ITD in 385 of 1595 of adult patients (24\%) with AML and another study has shown that ITD mutations occur in 20 to 30\% of AML cases and D835 occurs in 7 to 10\% of patients.\textsuperscript{21,29} ITD has mostly been observed within AML M3 samples and the least occurrence within AML M2 samples.\textsuperscript{20,20} However, other studies have reported an irregular occurrence of mutations among the subclasses.\textsuperscript{25,28} In this study, most FLT3 mutations have been observed within M3 subclasses. The second most abundant FLT3 mutation occurred in D835 of this gene which has been identified within 6\% of our patient samples, but it was not detected in people with M1, M0, and M4 subclasses. The D835 substitution has been reported in 30 out of 429 cases (7\%) of AML, in 1 out of 29 cases (3\%) of MDS and in 1 out of 36 cases (3\%) of ALL.\textsuperscript{21,30}

There are a few reports about the involvement of c-KIT mutations (exon 8 and 17) in AML patients, of which these mutations are not a rare event in core binding factor (CBF) leukemia.\textsuperscript{3,25} In AML patients that were investigated in this study, the mutation of exon 8 KIT gene has occurred in 1.2\% of M2 cases, 3.7\% of M4 cases, and in 1.4\% of total cases. Most mutations and alteration in sequences were detected in the KIT gene when exon 17 in this gene was analyzed. This mutation is a point mutation in which the aspartic amino acid in the 816 (D816) locus is substituted with other amino acids, leading to loop activation.\textsuperscript{31,32} In the present work, the D816 mutation occurred in 3.7\% of the M1, 9.8\% of M2, and 1.8\% of the M4 subclasses as well as 4.7\% of total cases with AML, whereas the frequency of mutations in the previous study were 0.9\%, 3.1\%, 1.8\%, and 1.7\%, respectively.\textsuperscript{33} Therefore the frequency of the D816 mutation has been shown to be more than reported in the past. Ethnicity may strongly influence the frequency of the reported mutated gene. Out of all the studied patients, one patient with an M2 subtype had both exon 8 and D816 mutations, hence harboring 5.6\% of the KIT mutation.

**Conclusion**

In this study we demonstrated that FLT3 mutations are frequent molecular abnormalities in AML patients with an incidence of 24\%. The presence of ITD was significantly associated with M3 morphology. Mutations of c-KIT resulted in 5.6\% of AML (one patient had both mutations together) that was significantly associated with M2 and M4 subtypes. These data show that approximately one third of AML patients had mutations in the tyrosine kinase receptor. Although our data do not support its value as a prognostic factor in AML patients, further investigation is required.

**Acknowledgment**

The authors of this article gratefully appreciate the Research Council at Iran University of Medical Sciences, Tehran for their financial support. The project was conducted according to the university ethical code. We thank the Hematology Oncology Research Center of Shariati Hospital in Tehran for providing blood samples. Also, we thank Dr. S. A. Moosavi for reviewing this manuscript.

**References**


Detection of KIT and FLT3 mutations in AML with different subtypes
STUDIES OF FLT3 MUTATIONS IN PAIRED PRESENTATION AND RELAPSE SAMPLES FROM PATIENTS WITH ACUTE MYELOID LEUKEMIA: IMPLICATIONS FOR THE ROLE OF FLT3 MUTATIONS IN LEUKEMOGENESIS, MINIMAL RESIDUAL DISEASE DETECTION, AND POSSIBLE THERAPY WITH FLT3 INHIBITORS

Panagiotis D. Kottaridis, Rosemary E. Gale, Stephen E. Langabeer, Marion E. Frew, David T. Bowen, and David C. Linch

FLT3 mutations, either internal tandem duplications (ITDs) or aspartate residue 835 (D835) point mutations, are present in approximately one third of patients with acute myeloid leukemia (AML) and have been associated with an increased relapse rate. We have studied FLT3 mutations in paired presentation and relapse samples to ascertain the biology of these mutations and to evaluate whether they can be used as markers of minimal residual disease. At diagnosis, 24 patients were wild-type FLT3, and 4 acquired a FLT3 mutation at relapse (2 D835+, 2 ITD+), with a further patient acquiring an ITD at second relapse. Of 20 patients positive at diagnosis (18 ITD+, 2 D835+), 5 who were all originally ITD+ had no detectable mutation at relapse, as determined by a sensitive radioactive polymerase chain reaction. One of these patients had acquired an N-Ras mutation not detectable at presentation. Furthermore, another patient had a completely different ITD at relapse, which could not be detected in the presentation sample. These results indicate that FLT3 mutations are secondary events in leukemogenesis, are unstable, and thus should be used cautiously for the detection of minimal residual disease.

Introduction

Approximately 80% of patients with acute myeloid leukemia (AML) younger than 55 years of age achieve complete remission (CR) following intensive induction therapy.1 However, the actuarial survival at 5 years is only just over 40% because most patients relapse and die of their disease.1 Considerable effort has therefore been directed at identifying molecular markers that can be used to detect residual disease or predict relapse at an earlier stage and lead to therapeutic intervention before overt hematological relapse occurs.

Ideally, a candidate marker for minimal residual disease (MRD) should always or frequently be present in a specific disease and it should be easy to assay and stable; that is, always present at relapse. In lymphoid malignancies, clonal rearrangements of either immunoglobulin or T-cell receptor genes are suitable for such analysis.2 They occur in up to 90% of patients, can be detected using sensitive polymerase chain reaction (PCR) techniques, and reappear at relapse, although development of secondary rearrangements has been noted in some cases.3 Studies evaluating their usefulness in clinical practice as a predictor of relapse have shown that the presence and level of residual leukemia can correlate with the risk of early relapse in acute lymphoid leukemia.4 Similarly, the BCR/ABL fusion transcript is present in nearly all patients with chronic myeloid leukemia (CML), and a number of different clonal populations have been used to correlate this marker with disease status and outcome.5-8

In AML, the use of MRD has been limited by the lack of suitable specific molecular markers and the diversity of those that do exist. Although cytogenetic markers have proved to be significant prognostic indicators, fewer than 50% of patients have an identifiable cytogenetic abnormality.1 Some patients have partial or complete loss or gain of a chromosome, but such abnormalities can be identified only using relatively insensitive “whole cell” techniques, and even the more sensitive fluorescence in situ hybridization technique requires evaluation of thousands of cells.9 There are 3 molecular markers that have been used for MRD studies: PML/RARα fusion transcript arising from the t(15;17) translocation, AML1/ETO arising from t(8;21), and CBFB/MYH11 from inv(16).10-13 Quantitative assays are necessary, particularly because several groups have shown that patients who are positive for AML1/ETO using sensitive reverse transcription (RT)–PCR assays can remain in morphologic and clinical remission for many years.14,15 Nevertheless, reappearance of the marker, or persistence at a certain level after induction chemotherapy, does generally signify an increased risk of relapse.16,17

However, these 3 markers together account for only approximately one fourth of adult AML patients,1 and other markers are required to extend the potential application of MRD to more AML patients. Recent studies have demonstrated that mutations in the FLT3 gene occur in about one third of adult AML patients. In 22% to 27% of patients, there is an internal tandem duplication (ITD) in the juxtamembrane region between exons 14 and 15 (previously called exons 11 and 12).20 The extra sequence varies between 18 base pairs (bp) and 222 bp (as detected in one patient in the
Studies of FLT3 mutations in paired presentation and relapse samples from patients with acute myeloid leukemia: implications for the role of FLT3 mutations in leukemogenesis, minimal residual disease detection, and possible therapy with FLT3 inhibitors

Panagiotis D. Kottaridis, Rosemary E. Gale, Stephen E. Langabeer, Marion E. Frew, David T. Bowen, and David C. Linch

Introduction

Approximately 80% of patients with acute myeloid leukemia (AML) younger than 55 years of age achieve complete remission (CR) following intensive induction therapy. However, the actuarial survival at 5 years is only just over 40% because most patients relapse and die of their disease. Considerable effort has therefore been directed at identifying molecular markers that can be used to detect residual disease or predict relapse at an earlier stage and lead to therapeutic intervention before overt hematological relapse occurs.

Ideally, a candidate marker for minimal residual disease (MRD) should always or frequently be present in a specific disease and it should be easy to assay and stable; that is, always present at relapse. In lymphoid malignancies, clonal rearrangements of either immunoglobulin or T-cell receptor genes are suitable for such analysis. They occur in up to 90% of patients, can be detected using sensitive polymerase chain reaction (PCR) techniques, and reappear at relapse, although development of secondary rearrangements has been noted in some cases. Studies evaluating their usefulness in clinical practice as a predictor of relapse have shown that the presence and level of residual leukemia can correlate with the risk of early relapse in acute lymphoid leukemia. Similarly, the BCR/ABL fusion transcript is present in nearly all patients with chronic myeloid leukemia (CML), and a number of different approaches have been used to correlate this marker with disease status and outcome.

In AML, the use of MRD has been limited by the lack of suitable specific molecular markers and the diversity of those that do exist. Although cyogenetic markers have proved to be significant prognostic indicators, fewer than 50% of patients have an identifiable cytogenetic abnormality. Some patients have partial or complete loss or gain of a chromosome, but such abnormalities can be identified only using relatively insensitive “whole cell” techniques, and even the more sensitive fluorescence in situ hybridization technique requires evaluation of thousands of cells. There are 3 molecular markers that have been used for MRD studies: PML/RARα fusion transcript arising from the t(15;17) translocation, AML1/ETO arising from t(8;21), and CBFB/MYH11 from inv(16). Quantitative assays are necessary, particularly because several groups have shown that patients who are positive for AML1/ETO using sensitive reverse transcription (RT)–PCR assays can remain in morphologic and clinical remission for many years. Nevertheless, reappearance of the marker, or persistence at a certain level after induction chemotherapy, does generally signify an increased risk of relapse.

However, these 3 markers together account for only approximately one fourth of adult AML patients, and other markers are required to extend the potential application of MRD to more AML patients. Recent studies have demonstrated that mutations in the FLT3 gene occur in about one third of adult AML patients. In 22% to 27% of patients, there is an internal tandem duplication (ITD) in the juxtamembrane region between exons 14 and 15 (previously called exons 11 and 12). The extra sequence varies between 18 base pairs (bp) and 222 bp (as detected in one patient in the
Detection of FLT3 Internal Tandem Duplication and D835 Mutations by a Multiplex Polymerase Chain Reaction and Capillary Electrophoresis Assay


8. Murphy2003 (condensed)

Kathleen M. Murphy,* Mark Levis,†
Michael J. Hafez,* Tanya Geiger,*
Lisa C. Cooper,† B. Douglas Smith,‡
Donald Small,† and Karin D. Berg*†
From the Departments of Pathology* and Oncology‡, Johns
Hopkins University School of Medicine, Baltimore, Maryland

FLT3 is a receptor tyrosine kinase that is expressed on early hematopoietic progenitor cells and plays an important role in stem cell survival and differentiation. Two different types of functionally important FLT3 mutations have been identified. Internal tandem duplication mutations arise from duplications of the juxtamembrane portion of the gene and result in constitutive activation of the FLT3 protein. This alteration has been identified in ∼20% to 30% of patients with acute myelogenous leukemia and appears to be associated with a worse prognosis. The second type of FLT3 mutation, missense mutations at aspartic acid residue 835, occurs in ∼7.0% of acute myelogenous leukemia cases. These mutations also appear to be activating and to portend a worse prognosis. Identification of FLT3 mutations is important because it provides prognostic information and may play a pivotal role in determining appropriate treatment options. We have developed an assay to identify both internal tandem duplication and D835 FLT3 mutations in a single multiplex polymerase chain reaction. After amplification, the polymerase chain reaction products are analyzed by capillary electrophoresis for length mutations and resistance to EcoRV digestion. Here we describe the performance characteristics of the assay, assay validation, and our clinical experience using this assay to analyze 147 clinical specimens. (J Mol Diagn 2003, 5:96–102)

FMS-like tyrosine kinase 3 (FLT3 also known as STK1 and flk2) is a member of the class III receptor tyrosine kinase family that also includes PDGF-R, KIT, and FMS.1 The FLT3 protein is normally expressed on hematopoietic stem progenitor cells and appears to play an important role in stem cell survival, and the development of dendritic and natural killer cells.2,3 FLT3 is overexpressed in most cases of acute myeloid leukemia (AML).4,5 In addition, analysis of leukemic blasts from AML patients has identified two specific somatic mutations of the FLT3 gene.6,7 Identification of these mutations in AML patients provides independent prognostic information that may also prove important for treatment optimization.

The first and best-studied FLT3 mutation is an internal tandem duplication (ITD) mutation. ITD mutations typically result from the duplication and tandem insertion of a portion of the juxtamembrane (JM) region (exons 11 to 12) of the FLT3 wild-type gene.6 The lengths of the duplicated segments have been reported to range in size from 6 to 180 bases and are always in frame.8,9 ITD mutations result in the constitutive autophosphorylation of the FLT3 receptor and are thus gain-of-function mutations of the FLT3 proto-oncogene.10 FLT3 ITD mutations have been reported to occur in 20 to 30% of patients with AML and have been associated with an increased relapse risk, decreased disease-free survival, decreased event-free survival, and decreased overall survival.8,9,11 In a multivariate analysis of FLT3 ITD mutations, cytogenetic risk group, presentation white blood cell count, percentage BM blasts at diagnosis, age, gender, and FAB type in 854 AML patients, the presence of a FLT3 ITD mutation was the most significant factor adversely affecting relapse risk (P < 0.0001) and disease-free survival (P < 0.0001).9 FLT3 ITD mutations are amenable to polymerase chain reaction (PCR)-based molecular diagnostic DNA testing because they are limited to a small, predictable region of the FLT3 gene.

Recently, an additional type of FLT3 mutation has been described. These alterations are missense mutations that alter the wild-type aspartic acid residue at position 835 (D835) within the activation loop of the FLT3 protein.7,12 Alteration of D835 also appears to result in constitutive activation of the FLT3 receptor and portends a worse disease-free survival in at least some studies.7 D835 mutations have been reported to occur in ∼7% of patients with AML, 3% of patients with myelodysplastic syndrome (MDS), and 3% of patients with acute lymphocytic leukemia.7 D835 and ITD mutations appear to occur independently but not exclusively of one another and the presence of concurrent D835 and ITD mutations has been reported.7 The D835 wild-type gene sequence is
Detection of FLT3 Internal Tandem Duplication and D835 Mutations by a Multiplex Polymerase Chain Reaction and Capillary Electrophoresis Assay

Journal of Molecular Diagnostics, Vol. 5, No. 2, May 2003

8. Murphy 2003 (condensed)

Kathleen M. Murphy,* Mark Levis,† Michael J. Hafez,* Tanya Geiger,* Lisa C. Cooper,* B. Douglas Smith,† Donald Small, † and Karin D. Berg* †

From the Departments of Pathology* and Oncology,† Johns Hopkins University School of Medicine, Baltimore, Maryland

FLT3 is a receptor tyrosine kinase that is expressed on early hematopoietic progenitor cells and plays an important role in stem cell survival and differentiation. Two different types of functionally important FLT3 mutations have been identified. Internal tandem duplication mutations arise from duplications of the juxtamembrane portion of the gene and result in constitutive activation of the FLT3 protein. This alteration has been identified in ~20% to 30% of patients with acute myelogenous leukemia and appears to be associated with a worse prognosis. The second type of FLT3 mutation, missense mutations at aspartic acid residue 835, occurs in ~7.0% of acute myelogenous leukemia cases. These mutations also appear to be activating and to portend a worse prognosis. Identification of FLT3 mutations is important because it provides prognostic information and may play a pivotal role in determining appropriate treatment options. We have developed an assay to identify both internal tandem duplication and D835 FLT3 mutations in a single multiplex polymerase chain reaction. After amplification, the polymerase chain reaction products are analyzed by capillary electrophoresis for length mutations and resistance to EcoRV digestion. Here we describe the performance characteristics of the assay, assay validation, and our clinical experience using this assay to analyze 147 clinical specimens. (J Mol Diagn 2003, 5:96–102)

FMS-like tyrosine kinase 3 (FLT3 also known as STK1 and flk2) is a member of the class III receptor tyrosine kinase family that also includes PDGF-R, KIT, and FMS.1 The FLT3 protein is normally expressed on hematopoietic stem progenitor cells and appears to play an important role in stem cell survival, and the development of dendritic and natural killer cells.2,3 FLT3 is overexpressed in most cases of acute myeloid leukemia (AML).4,5 In addition, analysis of leukemic blasts from AML patients has identified two specific somatic mutations of the FLT3 gene.6,7 Identification of these mutations in AML patients provides independent prognostic information that may also prove important for treatment optimization.

The first and best-studied FLT3 mutation is an internal tandem duplication (ITD) mutation. ITD mutations typically result from the duplication and tandem insertion of a portion of the juxtamembrane (JM) region (exons 11 to 12) of the FLT3 wild-type gene.6 The lengths of the duplicated segments have been reported to range in size from 6 to 180 bases and are always in frame.8,9 ITD mutations result in the constitutive autophosphorylation of the FLT3 receptor and are thus gain-of-function mutations of the FLT3 proto-oncogene.10 FLT3 ITD mutations have been reported to occur in 20 to 30% of patients with AML and have been associated with an increased relapse risk, decreased disease-free survival, decreased event-free survival, and decreased overall survival.8,9,11 In a multivariate analysis of FLT3 ITD mutations, cytogenetic risk group, presentation white blood cell count, percentage BM blasts at diagnosis, age, gender, and FAB type in 854 AML patients, the presence of a FLT3 ITD mutation was the most significant factor adversely affecting relapse risk (P < 0.0001) and disease-free survival (P < 0.0001).9 FLT3 ITD mutations are amenable to polymerase chain reaction (PCR)-based molecular diagnostic DNA testing because they are limited to a small, predictable region of the FLT3 gene.

Recently, an additional type of FLT3 mutation has been described. These alterations are missense mutations that alter the wild-type aspartic acid residue at position 835 (D835) within the activation loop of the FLT3 protein.7,12 Alteration of D835 also appears to result in constitutive activation of the FLT3 receptor and portends a worse disease-free survival in at least some studies.7 D835 mutations have been reported to occur in ~7% of patients with AML, 3% of patients with myelodysplastic syndrome (MDS), and 3% of patients with acute lymphocytic leukemia.7 D835 and ITD mutations appear to occur independently but not exclusively of one another and the presence of concurrent D835 and ITD mutations has been reported.7 The D835 wild-type gene sequence is

Accepted for publication February 3, 2003.

Address reprint requests to Kathleen M. Murphy, Ph.D., Carnegie Bldg., Room 367, 600 North Wolfe St., Baltimore, MD 21287. E-mail: kmurphy4@jhmi.edu.
FLT3 internal tandem duplication and FLT3-D835 mutation in 80 AML patients categorized into cytogenetic risk groups

Wewnętrzna tandemowa duplikacja oraz mutacja D835 genu FLT3 u 80 pacjentów z AML w trzech cytogenetycznych grupach ryzyka

Ewa Mały¹, Marta Przyborska¹, Tomasz Nowak¹, Jerzy Nowak², Danuta Januszkiewicz¹,²,³

¹ Department of Medical Diagnostics, Poznań
² Institute of Human Genetics, Polish Academy of Sciences, Poznań
³ Department of Pediatric Hematology, Oncology and Transplantology of the Medical University, Poznań

Summary

Background: Acute myeloid leukemia (AML) is a clonal disorder characterized by various genetic abnormalities and variable response to treatment. About 50% of patients with AML have no cytogenetic aberrations, presenting normal karyotype, and are categorized in the intermediate risk group. In this group detection of FLT3 mutations move a patient from the intermediate to the adverse risk group.

Material/Methods: Bone marrow from 80 AML patients was cultured to obtain chromosome slides and then karyotype. Simultaneously DNA was isolated from bone marrow and PCR reaction was conducted to test the FLT3 mutation status (ITD and D835). For statistical analysis Chi squared test was used.

Results: From the group of 80 AML patients seven were classified as a favorable risk group and FLT3/ITD was found only in one of these patients (14.28%), and FLT3/D835 in another one (14.28%). Fifteen patients showed a complex karyotype with more than three aberrations or with any aberration known as a poor prognosis. Among the adverse group FLT3/ITD was detected in three patients (20%) and D835 mutation in two other patients (13.33%). Among 58 patients with normal karyotype in GTG banding FLT3/ITD occurred in six cases (10.34%) and D835 mutation in two cases (3.45%). No significant difference was found among these three risk groups regarding presence or absence of FLT3/ITD and FLT/D835.

Discussion: Molecular characterization of mutations in several genes, such as FLT3, NPM1, MLL, CEBPA, in acute myeloid leukemia, especially in normal karyotype cases, could be another factor after cytogenetic analysis to stratify AML patients into different prognostic categories.

Key words: acute myeloid leukemia • cytogenetic • FLT3-ITD internal tandem duplication
slides a standard protocol was applied and bone marrow was cultured in vitro for 24 hours. Then slides were banded using a GTG technique and karyotyping was performed. For fluorescent in situ hybridization (FISH) with Kreatech probes (t(8;21) AML/ETO, t(15;17) PML/RARA and inv16/(16;16) CBFB slides were obtained from uncultured cells. Microscopic analysis was conducted using Zeiss Imager D1 with Metasystems software (Ikaros and Isis v 5.3.3.). Twenty GTG metaphases for karyotype and 200 interphase nuclei were analyzed for each probe in the FISH experiment.

For FLT3 analysis DNA was isolated from bone marrow cells using NucleoSpin Blood kit (Macherey-Nagel). Then polymerase chain reaction was conducted using FLT3 Mutation Assay kit (In Vivo Scribe Technologies).

For statistical analysis Chi squared test was used.

Results

From the group of 80 AML patients (43 men and 37 women; range 18–60 years) seven were classified as a favorable group risk with karyotypes presenting t(8;21) (cases 2 and 6), t(15;17) (case 1) and inv16 (cases 3, 4, 5 and 7). FLT3/ITD was found only in one of these patients (case 1) (14.28%), and FLT3/D835 in another one (case 2) (14.28%) (table 1). Fifteen patients showed a complex karyotype with more than three aberrations or with an aberration known as a poor prognosis (such as t(6;9) in case 77 or isolated tetrasomy 13 in case 78). Among the adverse group FLT3/ITD was detected in three patients (20%) and D835 mutation in two other patients (13.33%). The most numerous group was with intermediate prognosis according to normal karyotype. Among 58 patients FLT3/ITD occurred in six cases (10.34%) and D835 mutation in two cases (3.45%). The median age in the favorable risk group was 51 years, in the intermediate risk group 50 years and in the unfavorable risk group 44 years. The median age in all 80 AML patients was 49 years. FLT3/ITD was found in 10 patients (12.5%) and D835 mutation in 5 patients (6.25%). No significant difference was found among these three risk groups regarding presence or absence of FLT3/ITD and FLT/D835.

Discussion

For patients with de novo AML the best clinical approach is the classical cytogenetic analysis including FISH technique to categorize patients into specific risk groups. For AML patients with normal karyotype (NK-AML) it is particularly difficult to establish the prognosis during diagnosis. Their overall survival rate can range from 24% to 42% [8]. Further studies are needed in order to elucidate the molecular background in AML. Detection of mutations in genes such as FLT3, NPM1 or CEBPA can be valuable in providing prognostic information during diagnosis [7].

AML patients with FLT3/ITD mutation have shorter remission duration and overall survival [5]. Not only the presence of ITD affects poor prognosis, but also the size of the internal tandem duplication, which can vary from three to hundreds of nucleotides. Longer tandem duplication correlates with worse overall survival [8]. The occurrence of D835 mutation is still unclear; the data from the literature are inconsistent and further studies are required in order to establish the role of this mutation as a prognostic factor in AML [7].

Nowadays cytogenetic prognostic factors are more precise and in wide clinical use. But there is a need to improve molecular factors (the mutation status of genes such as FLT3, MLL, NPM1, CEBPA), especially in cases with normal karyotype [14]. FLT3/ITD, which is known to be a poor prognostic factor, is present in 20–30% of AML patients with different karyotype status. In the group with complex karyotype or with aberration with poor prognosis (monosomy 5 or 7) there is no difficulty in interpretation of clinical outcome. However, the group with a good cytogenetic factor such as t(15;17) with coexisting FLT3/ITD is more difficult to interpret. Patients with acute promyelocytic leukemia (APL) showed a similar frequency of FLT3/ITD and D835 mutation as the other groups within AML. A study with transgenic mice revealed that PML/RARα is necessary but not sufficient for APL to develop and additional aberrations are needed [2]. Presence or absence of FLT3/ITD in APL cases seems to have no impact on survival, but a study using quantitative techniques on FLT3/ITD ratio to wild type of FLT3 showed a significant impact on the outcome in APL. Patients with higher ITD ratios and lower PML/RARα transcript levels at diagnosis have much worse prognosis [6].

The group of AML patients with normal karyotype is the most difficult clinical problem in treatment selection. After FLT3 the most studied gene is NPM1 (nucleophosmin gene), which plays a role as a partner in many chromosomal translocations and as a nuclear chaperone has a function in genome stability, DNA duplication and transcriptional regulation [3]. The frequency of NPM1 mutations in AML patients with normal karyotype is about 50% and their presence is associated with favorable outcome (if not coexisting with FLT3/ITD) [11]. Detection of NPM1 mutations should be accessible during diagnosis of de novo AML, but unfortunately it is rare in Polish laboratories.

In our study the group of patients with normal karyotype was fairly numerous (72.5%) while in the literature NK patients represent about half of all AML cases [13]. The frequency of FLT3/ITD in all 80 AML patients (only 12.5%) was lower compared to other authors (in the literature about 20–30% of AML cases carry this tandem duplication). Small [12] indicates a different percentage frequency of FLT3/ITD, ranging from 15% to 34%. In our study only in the adverse group the frequency of FLT3/ITD was 20%. Within AML patients the frequency of D835 mutation was about 6.25% and this is consistent with other authors [5].

In recent years novel targeted therapies for FLT3/ITD patients have been developed. Mutated FLT3 has the activity of tyrosine kinase, and special FLT3 inhibitors have been created such as lestaurtinib (CEP-701) which in preclinical studies reduced blasts with mutations [4]. However, the best approach at the present time is to combine new drugs such as inhibitors for FLT3 mutation together with conventional chemotherapy.