Immunophenotypic findings in acute myeloid leukemia with FLT3 internal tandem duplication

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Background and Objectives. The biological characteristics and prognostic significance of the internal tandem duplication of the FLT3 (FLT3/ITD) were investigated in a series of *de novo* acute myeloid leukemia (AML) patients. One hundred and fifty-six adult patients with AML were included in the study. FLT3/ITD was detected in 41 (26%) patients (FLT3/ITD+).

Design and Methods. The main differences observed between the groups with and without FLT3/ITD: a higher leukocyte count, a raised percentage of a normal karyotype and a more frequent M5 FAB diagnosis in the FLT3/ITD+ patients. As regards the immunophenotypic characteristics the FLT3/ITD+ group very often expressed monocytic markers (CD36 and CD11b) and less commonly immature markers (CD34 and CD117). A *promyelocytic-like* immunophenotype pattern was also detected in a minority of these patients (4/36).

Results. The FLT3/ITD+ patients had a shorter overall survival, a shorter event-free survival and a higher probability of relapse. Minimal residual disease (MRD) was investigated in the FLT3/ITD+ patients using flow cytometry. This technique had a sensitivity of 62% and a specificity of 83% in relapse prediction. Minimal residual disease analysis was hampered by the low number of patients with detectable aberrant immunophenotype.

Interpretation and Conclusions. A high frequency of changes in the phenotype and/or genotype pattern between diagnosis and relapse was detected (5/6). FLT3/ITD is a frequent molecular lesion in *de novo* adult AML and seems to be associated with monocytic differentiation, a high leukocyte count and a poor prognosis. Immunophenotype and genotype patterns observed at relapse suggest that the FLT3/ITD+ blasts may be genetically unstable and prone to clonal evolution. FLT3/ITD may not be a suitable target for minimal residual disease studies.

Key words: FLT3 internal tandem duplication, acute myeloid leukemia, immunophenotype, karyotype, minimal residual disease.

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Proliferation and differentiation of normal hematopoietic cells are regulated by a number of cell growth factors acting through specific receptors. Molecular alterations of cytokine receptors in hematologic diseases have aroused considerable interest in recent years.[1]

FLT3 is a member of the receptor tyrosine kinase (RTK) class III, which includes KIT, FMS and PDGFR.[2] Structurally, FLT3 has an extracellular region, a single transmembrane region, a juxtamembrane (JM) domain, two tyrosine kinase domains and a C-terminal domain.[3] FLT3 is preferentially expressed on hematopoietic stem cells, and its physiologic ligand (FL) on bone marrow stromal cells.[4,5] The FLT3-FL interaction plays an important role in hematopoietic development.[4-8] Earlier studies have shown that most acute myeloid leukemia (AML), acute lymphoblastic leukemias (immunological subtypes, B-ALL and T-ALL) and blast crises of chronic myeloid leukemia overexpress FLT3.[9-13] *In vitro* studies have demonstrated that FL administration produces a proliferative response in a significant proportion of cases of AML.[11-13] Activating mutations in FLT3 have frequently been found in patients with AML and these mutations have been associated with a poor prognosis.[14-21] Most FLT3 mutations are internal tandem duplications (ITDs) in the juxtamembrane domain involving exon 11, intron 11 and exon 12.[14] Subsequent studies have confirmed the presence of juxtamembrane ITDs in 20-30% of AML.[19-21] In addition, substitution mutations in the FLT3 kinase domain at Asp835 have recently been reported in approximately 7% of patients with AML.[22] FLT3-ITD mutations may also be present in occasional cases of myelodysplastic syndromes (<5%).[16,23]

In vitro studies have demonstrated that the FLT3 ligand stimulates proliferation and inhibits apoptosis in myeloid leukemic cells[10-12] and that this effect can be related to the high peripheral white blood cell counts found in patients with FLT3 mutations.[15]

Sequential studies performed in patients with FLT3 mutations showed that the FLT3 mutation may be lost in leukemic relapses whereas in some cases the mutation was acquired during progression of the disease.[23-25] Kelly *et al.*[26] developed a mouse model in which FLT3/ITD expression induced an oligoclonal myeloproliferative disorder. The mice developed an AML very similar to the human disease when FLT3/ITD was associated with PML/RARα rearrangements.[27]

The aim of this report was two-fold; i) to study the incidence and the immunophenotypic characteristics

of internal tandem duplication in the JM domain of the FLT3 gene in a consecutive series of adult AML patients and ii) to analyze, with the help of flow cytometry (FC) and polymerase chain reaction (PCR), the evolution of FLT3/ITD patients in complete remission.

Design and methods

Patients

Two hundred and twenty-five adult patients with *de novo* AML were enrolled sequentially in the LMA-99 protocol from the CETLAM co-operative group between November 1998 and April 2001. DNA was available from bone marrow at diagnosis from 156 of these patients. The patients were aged between 18 and 60 years old. No patient had a past history of treatment with chemotherapy, radiotherapy, or a previously diagnosed hematologic disorder. The patients were uniformly treated in accordance with the LMA-99 protocol.

The remission-induction therapy included one or two courses of idarubicin (12 mg/m^2 $\times$ 3), cytarabine (500mg/m^2/12h $\times$ 4) and etoposide (100 mg/m^2 $\times$ 3). Patients who achieved morphologic complete remission (CR) received one course of intensification therapy with cytarabine (500 mg/m^2/12h $\times$ 6) and mitoxantrone (12 mg/m^2 $\times$ 3). Finally, all the patients received consolidation treatment, which differed depending on the karyotype. The patients were classified into three risk groups: a good prognosis group including patients with t(8;21), inv 16/t(16;16); an intermediate prognosis group of patients with a normal karyotype; and a poor prognosis group of patients with karyotypes with some abnormality.[41] Patients with a good prognosis were treated with one course of high-dose cytarabine, patients with a poor prognosis received an allogeneic stem cell transplant (allo-SCT) if they had an HLA identical related donor and the remaining patients received an autologous stem cell transplant.

Morphologic studies

The diagnosis of AML and the assignment of FAB subtypes were based on standard morphologic and cytochemical criteria.[29] Morphologic CR was defined by the criteria proposed by Cheson *et al.*[30]

Cytogenetic analysis

Cytogenetic analyses were performed in 147/156 (94%) of patients. Cytogenetic G-banding analysis was performed using standard methods. The definition of a cytogenetic clone and descriptions of karyotypes followed the International System for Human Cytogenetic Nomenclature.[31]

Flow cytometry analysis

Sample preparation: in all cases immunophenotyping studies were performed at diagnosis on erythrocyte-lysed BM samples. The number of cells was quantified by microscopy and adjusted to 1$\times$10^6 in each tube. Antigen expression was analyzed using triple stainings with the following fluorochrome-conjugated (fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin-chlorophyll protein (PerCp) or phycoerythrin-cyanine 5 (PE/Cy 5) combinations of monoclonal antibodies: CD15/ CD34/HLA-DR,CD10/CD20/CD19, CD2/CD33/CD19, CD22/ CD13/CD3, CD7/CD117/CD45, CD66/CD56/ CD64, CD36/GA/CD45,CD34/CD41/CD45, CD34/ CD11b/ CD45, CD4/CD123/HLA-DR, CD14/CD135/ CD45, CD5/CD16/CD45, MPO/CD79a/CD3 and Tdt/MPO.

The monoclonal antibodies used in the study were: CD22 (4KB128 FITC), glycophorin A (JC 159 PE), CD41 (5B 12 PE), IgM (rabbit anti-human, PE), CD79a (HM57 PE) and TDT (HT-6 FITC), CD117 (104 02 PE), CD64 (10.1 PerCp), and CD66 (Kat 4c PE) from DAKO, Glostrup, Denmark; CD15 (MMA-FITC), CD34 (8G12-FITC, PE), HLA-Dr (L243 PetCp), CD10 (W8E7 FITC), CD 20 (L27 PE), CD2 (S5.2 FITC), CD33 (67.6 PE), CD7 (4H9 FITC), CD45 (2D1 PerCp), CD13 (L138 PE), CD14 (M0P9 FITC), CD3 (SK7 PerCp), CD4 (Leu 3 FITC), CD5 (Leu 1 FITC), CD56 (NCAM 16.2 PE), and CD16 (3G8 PE) purchased from Becton Dickinson, San José, California, USA (BDIS); CD19 (SJ25-C1 PE/Cy 5) and MPO (H-43-5 FITC) from Caltag Laboratories, Burlingame, USA; CD 123 (9F5 PE) from Pharmingen, San Diego, CA; CD36 (FAG-52 FITC) from Immunotech, Marseilles, France.

Direct immunofluorescence was performed by first incubating 1$\times$10^6 cells with the specific monoclonal antibody for 15 minutes in the dark at room temperature. An isotype-matched negative control (BDIS) was used in all cases to assess background fluorescence intensity. Cells were lysed (FACS Lysis solution, BDIS) for 5 minutes and centrifuged at 250 g for 5 minutes. The cells were washed twice with phosphate buffered saline (PBS) before being resuspended in PBS and examined. The immunologic criteria for lineage assignment followed the EGIL recommendations.[32]

Data acquisition and analysis. Measurements were performed on a FACScalibur flow cytometer (BDIS). The Cellquest (BD) software program (BDIS) was used for data acquisition. At least 10,000 events/tube were measured. The PAINT-A-GATE PRO software program (BDIS) was employed for subsequent data analysis. Thresholds for positivity were based on isotype negative controls. Analytical gates were set on desired viable cells based on forward light scatter and side light scatter combined with the exclusion of the normal cells using a CD45 tube. The positivity threshold was 20% for all markers except for cytoplasmic or intranuclear

antigens for which a 10% threshold was used.

MRD analysis. The MRD study was performed in BM samples from patients in CR after induction and intensification therapy and after SCT. The strategy for MRD detection included two criteria based on previously published data:[33] i) detection exceeding 1×10^{-3} cells coexpressing an aberrant phenotype and 2) an abnormal myeloid/lymphoid CD34$^+$ ratio:[34] a live gate was performed on a SSC/CD34$^-$FITC dot plot and only the information on the CD34$^+$ cells was stored for further analysis. Lymphoid precursors showed CD34$^+$CD33$^-$CD19$^+$ and low FSC/SSC and myeloid precursors showed CD34$^+$CD33$^+$CD19$^-$ and high FCS/SSC. The myeloid/lymphoid CD34$^+$ ratio was considered normal when the ratio was <10. In the light of our experience and that of earlier reports [33,35] three main types of aberrant phenotypes were considered: i) cross-lineage antigen expression; ii) asynchronous antigen expression and iii) antigen overexpression. Cells displaying leukemia-associated phenotypes and the myeloid/lymphoid CD34$^+$ ratio were evaluated using a two-step acquisition procedure. Analysis was performed on gated cells in accordance with previously defined methods.[35] The existence of two or more blast cell populations was established on the basis of distinct antigen expression.

Analysis of the internal tandem duplications of the FLT3 gene

High molecular weight DNA was extracted from AML cells using the salting out method. Earlier studies showed that the location of the internal tandem duplication of the FLT3 gene is restricted to exons 11 and 12.[14] Genomic PCR amplification was performed in the 150 AML samples using the primers 11F, 5′-CAATTTAGGTAT-GAAAGCC-3′; 11R, 5′-CAAACTCTAAATTTTCTCT-3′; 12F, 5′-TGTCTTTGC AGGAAGGTTAC-3′ and 12R, 5′-GTACCT TTCAGCATT TTTGAC-3′. Dilution experiments were employed to establish the sensitivity of the PCR method.

MLL studies

Genomic DNA was analyzed for MLL rearrangements using Southern blot, RT-PCR and long-range genomic PCR as previously described.[36]

Statistical methods

Complete remission (CR) and relapse were defined according to the criteria of the National Cancer Institute-sponsored workshop on AML.[30] CR duration was measured from the date of attainment of CR to the date of relapse; all patients were censored using the date of the last documented CR. Overall survival (OS) was measured from the date of entry into the treatment protocol until the date of death and event-free survival (EFS) for patients who achieved CR was measured from the date of CR to relapse or death. Overall survival, EFS and the probability of relapse were plotted by the Kaplan-Meier method; differences between curves were analyzed by the long-rank test.

Differences in age and peripheral white cell counts were analyzed with the Wilcoxon rank-sum test and the analysis of the frequencies was performed using Fisher′s test or the χ^2 test. Immunophenotypic groups, defined by expression vs lack of expression of antigens, were compared using the χ^2 test.

Results

Clinical characteristics and outcome

Internal tandem duplication of the JM domain of the FLT3 was detected in 41 out of 156 patients (38%). In two cases the normal allele was absent and in 4 additional cases the band of the normal allele was of very faint intensity. This indicates that the mutated allele was predominant in 6 out 41 patients. Details of the clinical characteristics of the FLT3/ITD$^+$ and FLT3/ITD$^-$ patients are given in Table 1.

Cytogenetic analysis was performed in 40 of the 41 FLT3/ITD$^+$ patients (98%). It is noteworthy that FLT3/ITD was present significantly more frequently in the normal karyotype subgroup ($p = 0.03$). Two patients with FLT3/ITD also had MLL self-fusions. These patients were diagnosed as having M5 FAB leukemias with a normal karyotype.

All the patients were treated with the same scheme, as previously described. There were no differences in the response rate between the FLT3/ITD$^+$ and the FLT/ITD$^-$ groups. Of the 31 FLT3/ITD$^+$ responders, 21 patients received a stem cell transplant (SCT) (5 patients an allogeneic SCT and 16 patients an autologous SCT), 5 patients had an early relapse and 4 patients were on intensification therapy. With a follow-up of three years FLT3/ITD$^+$ patients had a shorter OS and EFS and a higher probability of relapse (Table 1). We analyzed the outcome of the patients with normal cytogenetics. Twenty-three were FLT3/ITD$^+$ and 36 FLT/ITD-. We observed shorter survival and shorter event-free survival in the FLT3/ITD$^+$ subgroup (OS: 22$\pm$9% vs 47$\pm$9% and 27$\pm$11% vs 45$\pm$10%). Nevertheless these results were not statistically significant.

We analyzed the survival of patients who received SCT separately. Among the patients who received an autologous SCT (27 FLT3/ITD$^-$, 16 FLT3/ITD$^+$) we observed a poor survival and high probability of relapse in the FLT3/ITD$^+$ patients [OS: 50$\pm$13% FLT3/ITD$^+$ vs 79$\pm$8% FLT3/ITD- (p= 0.05); relapse: 66$\pm$12% FLT3/ITD$^+$ vs 26$\pm$9% FLT3/ITD$^-$ (p=0.002)]. In contrast, there were no statistically significant differences among the patients who received an allogeneic SCT (14 FLT3/ITD$^-$, 5 FLT3/ITD$^+$).

Table 1. Clinical characteristics of the 156 AML patients included in this series.

	FLT3 wt (n=115)	*FLT3-ITD (n=41)*	*p value*
Age, median (range) years	43 (16-60)	42 (19-60)	ns
Sex			ns
Male	64	27	
Female	51	14	
WBC, median (range)	18 (0.8-360)	84 (2-323)	0.03
FAB (%)			
M0	5 (4)	1(2.5)	ns
M1	18(16)	10(24.2)	ns
M2	35 (30.5)	2 (5)	0.01
M4	20 (17.5)	10 (24.2)	ns
M5	29 (25)	18 (44)	0.03
M6	2 (2)	0	ns
M7	0 /0	0	ns
not classified	6 (5)	0	ns
Cytogenetics (%)			
normal	36(31.5)	26(63)	0.03
t(8;21)	10(9)	0	ns
inv (16)	12(10)	1(2.5)	ns
complex	9(8)	3(7)	ns
other abnormalities	35(30.5)	6(15)	ns
Absence of mitoses	5(4)	4(10)	ns
Not performed	8(7)	1(2.5)	ns
Outcome (%)			
Complete remission	86 (75)	31 (76)	ns
Failure	16 (14)	5 (12)	ns
Death	13 (11)	4 (10)	ns
Under treatment	0	1 (2)	ns
Overall survival	41±5%	23±7%	0.002
Event-free survival	41±5%	27±8%	0.009
Probability of relapse	55±6%	67±9%	0.01

WBC: white blood count; wt: wild-type.

Immunophenotype characteristics of FLT3/ITD cases

FC studies were performed in 134 out 156 patients (86%). The immunophenotype of FLT3/ITD$^+$ cases is shown in Table 2. A consistent myelomonocytic phenotype was found in these patients. The most frequently observed pattern of antigen expression in these patients was CD34$^-$ HLA$^-$DR$^+$ CD13$^+$ CD33$^+$ CD15$^+$ CD36$^+$ CD64$^+$ CD123$^+$. Additional antigens that were frequently expressed were CD11b and CD4 in 13 and 10 cases, respectively. In contrast to the consistent pattern of committed myeloid and monocytic antigen expression, expression of early stem cell and lymphoid antigens was variable: CD34 was expressed in 42% of FLT3/ITD$^+$ cases, CD7 was expressed in 4 cases and CD2 in one case; CD3, CD5, CD10, CD20 and CD22 were always negative. Interestingly, the CD19 expression was found in 15 cases with monocytic differentiation. Anti-myeloperoxidase reactivity was demonstrated in 34 cases and nuclear TdT in only one case. Thus the antigen pattern of FLT3/ITD$^+$cases corresponded to a mature myeloid population with monocytic differentiation.

Table 2. Antigen expression in FLT3/ITD$^+$ cases.

Antigen	*Positive cases (%)*	*Antigen*	*Positive cases (%)*
CD34	15 (42)	CD64	22 (61)
HLADr	32 (89)	CD56	3 (8)
CD15	27 (75)	CD66	0
CD10	0	CD36	21 (58)
CD20	0	GA	0
CD19	15 (42)	CD41	0
CD33	34 (94)	CD11b	13 (36)
CD2	1 (3)	CD123	36(100)
CD13	36 (100)	CD4	10 (28)
CD3	0	CytMPO	34 (94)
CD117	27 (75)	CytCD79a	0
CD45	36 (100)	CytCD3	0
CD7	4 (11)	Tdt	1 (3)
CD14	5 (14)	Lactoferrin	0
CD5	0	Lysozyme	8 (22)

Thirteen FLT3/ITD$^+$ patients had non-M4 or M5 FAB subtypes: ten patients had M1, two patients M2 and one patient was classified as having M0. Interestingly, four of these patients (11% of the FLT3/ITD$^+$ cases) showed a characteristic phenotype pattern resembling acute promyelocytic leukemia: CD13$^+$ CD33$^+$ MPO$^+$ CD34$^-$ HLA-DR$^-$ (Figure 2). None of these cases showed expression of monocytic antigens and 2 out 4 expressed CD56. Six of the remaining FLT3/ITD$^+$ patients with non-M4 or M5 FAB subtypes showed co-expression of immature antigens with monocytic mature antigens or antigens related to monocytic differentiation. Five of these patients expressed CD64, CD56, CD19 or CD36 and two patients showed a monocytic differentiation pathway with co-expression of CD15 and CD34 in the blast cells.

The antigen expression of the 36 FLT3/ITD$^+$ patients was compared with that of 98 of the FLT3/ITD$^-$ ones. The FLT3/ITD$^+$ cases more frequently expressed some antigens related to monocytic differentiation: CD36 (p=0.004) and CD11b (p=0.03) and less frequently expressed CD34 (p=0.007) and CD117 (p=0.007). CD13, CD4 and MPO were more frequently expressed in the FLT3/ITD$^+$ cases, but we did not find statistically significant differences. As regards the lymphoid antigen expression we did not

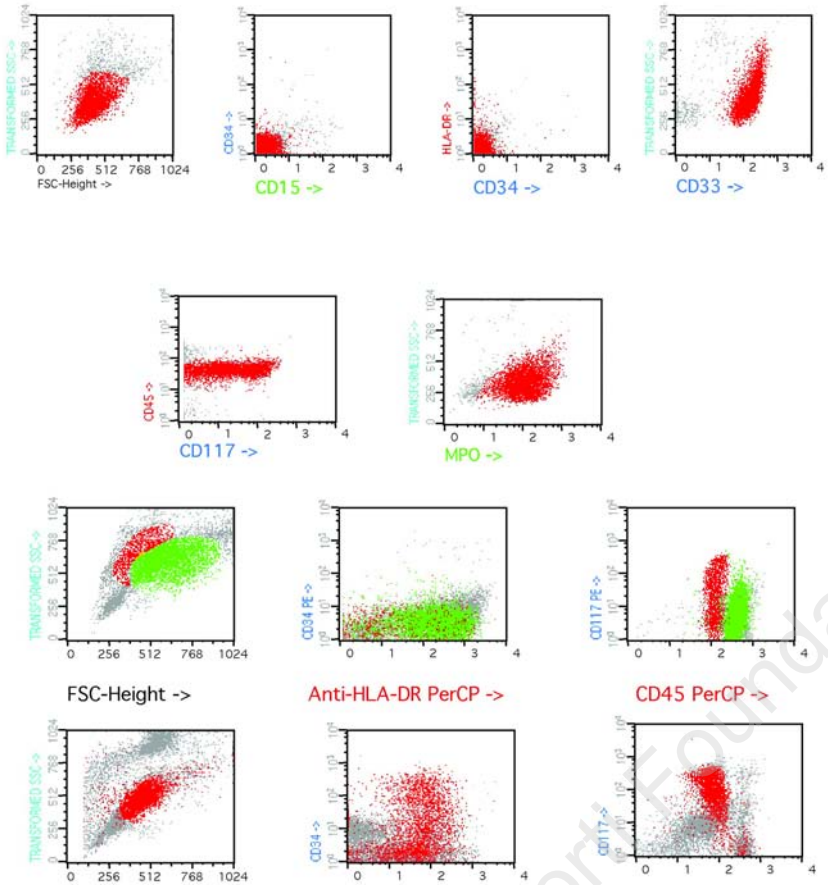

Figure 1. Top: promyelocytic-like immunophenotype in one case with FLT3-ITD. Note the CD34 and HLA-DR negativity and the marked MPO and CD33 positivity. Bottom: clonal expansion at relapse in one case of AML with FLT3-ITD. Leukemic blasts at relapse showed CD34 expression.

find any differences between the two groups. We analyzed the presence of a promyelocytic phenotype in FLT3/ITD$^-$ cases. The promyelocytic pattern (CD13$^+$CD33$^+$CD34$^-$HLA$^-$DR-MPO$^+$) was less common in the FLT3$^-$ITD$^-$ group (3%) (p =0.058). We analyzed the incidence and the phenotypic pattern of the different blast cell subpopulations in the FLT3/ITD$^+$ patients. More than one blast cell subpopulation was identified in 29 patients (81%). In 19 cases (65%) the blast cell subpopulations differed in reactivity for the CD34 antigen; in eight of these, this was the only antigenic feature that distinguished the cell subsets, whereas in the remaining patients other antigenic variations were detected. The most common immunophenotypic abnormality in the FLT3-ITD$^+$ cases was asynchronous expression of maturation-associated antigens, detected in 21 (72%) patients.

Minimal residual disease analysis

We performed a follow-up of the FLT3/ITD$^+$ patients in morphologic complete remission (CR) using flow cytometry in order to detect residual leukemic cells. A total of 48 bone marrow (BM) samples from 19 FLT3/ITD$^+$ patients in CR were analyzed by immunophenotype using the two aforementioned criteria. In nine of these patients, no aberrant immunophenotype was found and immunologic MRD analysis relied exclusively on abnormal CD34 ratios. Samples were obtained after induction and intensification therapies in all patients and in 6 of them we analyzed MRD after the SCT.

MRD was detected in 9 patients (MRD$^+$) after the induction therapy and leukemic residual cells persisted in 5 of them after intensification therapy. Eight patients showed aberrant phenotypes at diagnosis and analysis of the MRD was performed on the basis of these phenotypes. The remaining patient without an aberrant phenotype showed a persistently abnormal CD34 ratio after induction and intensification therapies and after SCT. Eight out of the 9 MRD$^+$ patients relapsed and the remaining patient died in morphologic CR 6 months after SCT with persistence of residual leukemic cells. In 10 patients MRD was persistently not detected (MRD$^-$). In contrast to the MRD$^+$ patients, only 4 out of the MRD$^-$ patients showed aberrant phenotypes at diagnosis. In these patients the MRD analysis was performed only by studying the abnormal CD34 ratio. Five of the MRD$^-$ patients relapsed and the remaining patients were in CR.

Table 3. Phenotype and genotype changes between diagnosis and relapse in FLT3/ITD$^+$ patients.

Patient	Phenotype at diagnosis		PCR genotype at diagnosis	Phenotype at relapse		PCR genotype at relapse
	Major blast cell subpopulation	Minor blast cell subpopulation		Major blast cell subpopulation	Minor blast cell subpopulation	
1	34+ 15- DR+ 19+ 33+ 117+ 13+ 64+ 36+ 11b+	34+15-DR+ 19- 33+ 117+13+ 64+ 36- 11b-	FLT3^{ITD} MLL+	34+ 19- 33+ 117+ 13+ 11b-	None	FLT3^{WT} MLL-
2	34- 15- DR- 33+ 117+ 13+ 64- 11b- 36-	none	FLT3^{ITD}	34- 15- DR- 33+ 117+ 13+64- 11b- 36-	None	FLT3^{WT}
3	34- 15- DR+ 33+ 117+ 13+ 64- 11b 14-	34+ 15- DR+ 33+ 117+ 13+ 64- 11B- 14-	FLT3^{ITD}	34+ 15- DR+ 33+ 117+ 13+ 64- 11B- 14-	34- 15- DR+ 33+ 117+ 13+ 64- 11b- 14-	FLT3^{ITD}
4	34- 15+ DR+ 33+ 117- 19+ 13+ 64+ 11b+ 14+	34- 15+ DR+ 33+ 117+ 19+13+ 64+ 11b- 14-	FLT3^{ITD}	34+ 15+ DR+ 33+ 19+ 117+13+ 64+ 11b- 14-	34- 15+ DR+ 117- 13+ 64+ 11b- 14- 19+	FLT3^{ITD}
5	34- 15+ DR+ 33+ 117+ 13+ 64+ 11b- 14-	34+ 15+ DR+ 33+ 117+ 13+ 64- 11b- 14-	FLT3^{ITD}	34+ 15+ DR+ 33+ 117+ 13+ 64- 11b- 14-	None	FLT3^{ITD}
6	34+ 15+ DR+ 19+ 33+ 117+ 13+ 64+ 36+ 11b- 14-	none	FLT3^{ITD}	34+ 15+ DR+ 19+ 33+ 117+ 13+ 64+ 36+ 11b- 14-	None	FLT3^{ITD}

DR: HLA-DR; FLT3^{ITD}: internal tandem duplication; FLT3^{WT}: wild type; MLL+: MLL gene rearranged: MLL-: MLL gene germinal.

According to these data, flow cytometry showed a high specificity (83%) in predicting relapse but its sensitivity was only 62%. Six FLT3/ITD$^+$ patients who relapsed were studied using flow cytometry and PCR. The sensitivity of genomic PCR used was 1/100 as determined by dilution experiments. A high frequency of changes in the phenotype and genotype was detected. As regards the immunophenotype, in all cases the changes were subclonal expansions of a more immature blast cell subpopulation present at diagnosis. Different *change-pattern* relapses were detected in these patients (Table 3): one patient relapsed with a different immunophenotype and the FLT3/ITD became negative. In the same patient an associated MLL rearrangement disappeared in Southern blot analysis of a relapse sample. Another patient showed the same phenotype but the PCR was negative for the FLT3/ITD. Three patients showed different phenotypes but the FLT3 mutation was conserved at relapse. Finally, the immunophenotype and the PCR patterns were the same at relapse in only one case.

Discussion

Internal tandem duplication of the FLT3 gene is a common molecular defect present in AML. It has been related to high leukocyte cell counts and a poor prognosis.[14-21] Nevertheless, there is controversy about its prognostic significance given that there are other confounding factors that have independent prognostic value (age, karyotype and variation in the treatment regimens).[24,36] The adverse prognostic significance of FLT3 seemed to be particularly relevant in AML patients with a normal karyotype. In this group of patients FLT3 was associated with a poor evolution when the abnormal allele was exclusively detected.[20]

In line with earlier reports, we found a high incidence of FLT3/ITD which was significantly associated with a poor outcome. In this series there were very few cases with a single abnormal allele (6/41). Our study included patients under 60 years old who were treated homogenously. Patients with M3 leukemia, which is very often associated with FLT3/ITD, were excluded from this series.

FLT3 plays an important role in regulating normal hematopoietic cell growth and differentiation.[4-8] FLT3 is activated by ligand–dependent dimerization and transphosphorylation of tyrosine residues. The mutation appears to activate the kinase domain of the receptor through constitutive dimerization.[10-12] It has been suggested that activated FLT3 might activate the JAK-STAT signaling cascade.[37,38] These effects could explain the high peripheral white cell counts found in FLT3/ITD$^+$ cases. In our study FLT3/ITD$^+$ patients had a significantly higher leukocyte count.

FLT3/ITD is preferentially associated with myelomonocytic and monocytic leukemias.[19] Thus, in our study 67% of the FLT3/ITD patients had M4 or M5 subtype leukemia. Nevertheless, 25% of the dupli-

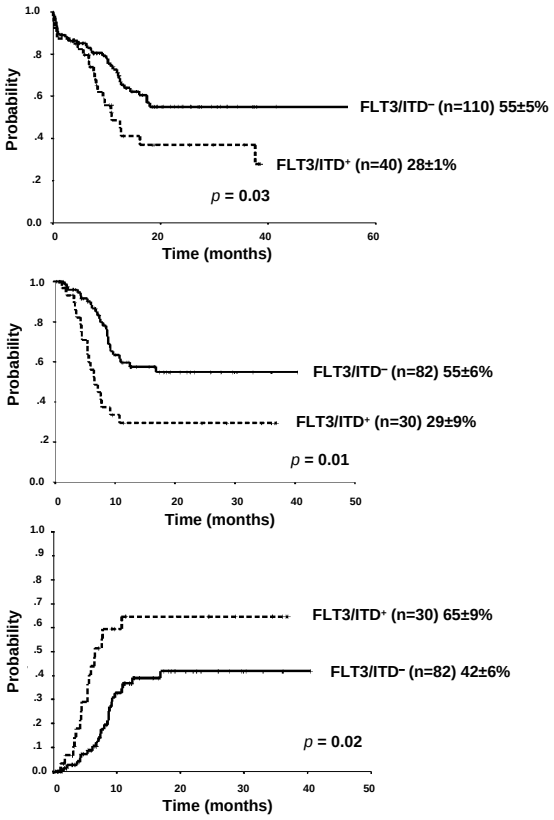

Figure 2. Overall survival (top), Event-free survival (middle) and probability of relapse (bottom) curves.

cated cases had a more immature leukemia. The immunophenotype of the blast cells of a considerable number of FLT3/ITD+ patients with M0, M1 or M2 FAB subtypes expressed monocytic markers and/or showed myelomonocytic differentiation. Recent studies have demonstrated that leukemic cells of M4 or M5 subtypes commonly respond to FL. Our phenotypic data are in accordance with these findings because FLT3/ITD was preferentially detected in cases with monocytic differentiation. A high proportion of cases showed heterogeneous blast cell populations.

This would account for the findings at relapse when some patients had lost the FLT3/ITD. The frequency of subclonal events found at diagnosis in our study and the high number of phenotype changes at relapse in FLT3/ITD+ patients could be a consequence of an increased genetic instability. The phenotypic and genotypic variability among FLT3/ITD+ patients observed in our study may limit the potential use of this mutation as a marker of MRD.

Our data demonstrated a consistent myeloid and monocytic immunophenotype in adult AML with FLT3/ITD, with expression of the committed stem cell and myeloid antigens HLA-DR, CD13, CD33, CD45 and MPO in almost all cases and CD64, CD15, CD117 and CD36 in more than 50% of cases. In contrast, the immature antigen, CD34, was expressed in 42% of FLT3/ITD+ cases. FLT3/ITD+ patients did not display a unique immunophenotype. These data suggest that FLT3/ITD does not represent a specific genetic lesion, such as PML/RARα or BCR/ABL, with a characteristic phenotype. FLT3/ITD could be associated with leukemic progression, especially in leukemias with mature monocytic differentiation.

FLT3 duplication has been associated with other molecular lesions such as PML/RARα and p53 mutations.[15,19,36] In our study we found co-existence of FLT3/ITD in two patients with the MLL tandem duplication and in another patient with the CBFb/MYH-11 rearrangement. Inactivation of a core binding factor associated with constitutive activation of a receptor tyrosine-kinase may be a common theme in human leukemogenesis.

Tse *et al.*[38] demonstrated that transplantation of cells with an activated FLT3 receptor into syngeneic mice caused death 3 weeks after the injection. Histopathologic analysis demonstrated a massive infiltration of mononuclear cells in the liver, spleen and bone marrow.[37] However, Kelly *et al.*,[26,27] using a murine bone marrow transplantation assay, demonstrated that FLT3/ITD induced only an oligoclonal myeloproliferative disorder characterized by splenomegaly and leukocytosis. In this study the FLT3 mutation was sufficient to induce a myeloproliferative disorder. Some authors suggest that the FLT3 duplication could be associated with leukemia progression and would thus be a marker of clonal evolution. In line with this view, sequential analyses in patients with leukemia arising from myelodysplastic syndromes revealed that FLT3 duplication was a late genetic event.[23,25]

The presence of the internal tandem duplication of the FLT3 gene identifies a subgroup of adult AML patients with a poor prognosis. The bad outcome associated with FLT3 mutations may be linked to increases in genetic instability and/or subclone development.[39] This is important for identifying patients who need aggressive or new therapeutic alternatives. Thus, the presence of a mutation that activates a tyrosine kinase receptor paves the way for the use of specific kinase inhibitors to treat this disease. The genetic variability of this molecular lesion during the progression of the disease may yield new insights into the leukemogenic process.

Appendix

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Pre-publication Report & Outcomes of Peer Review

Contributions

LM: was primarily responsible for collecting and interpreting the data of this work, she also prepared the first draft of the manuscript. JFN was in charge of conceiving the study and revised the paper. The remaining authors: MR, AA, GA, AD, MT, JJ, JPT, NV and SB are members of the CETLAM group and were responsible for collecting samples and for the biological and clinical data. They also provided comments on and suggestions for the final version.

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In the following paragraphs, Professor Béné summarizes the peer-review process and its outcomes.

What is already known on this topic

Mutations and duplication of the FLT3 gene have been identified in about one third of AML cases and reported by several groups as poor prognosis factors. Relationships between these genetic alterations and other features of the disease have been less frequently reported, and a possible link with typical immunophenotypic features, that could orient towards the search for this genetic anomaly, has not been noted.

What this study adds

This study provides extensive data on the immunophenotype of 36 AML cases with FLT/ITD. It also shows a few significant differences with cases lacking the genetic anomaly. There is no real, strongly specific feature, but this work had to be done and improves knowledge about this particular anomaly. It also confirms previous work regarding the association of FLT/ITDwith poor prognosis and interest in the detection of minimal residual disease.