A Leukemia-Associated CD34/CD123/CD25/CD99<sup>+</sup> Immunophenotype Identifies FLT3-Mutated Clones in Acute Myeloid Leukemia

Daniela F. Angelini<sup>1</sup>, Tiziana Ottone<sup>2,3</sup>, Gisella Guerrera<sup>1</sup>, Serena Lavorgna<sup>2,3</sup>, Michela Cittadini<sup>2,3</sup>, Francesco Buccisano<sup>2</sup>, Marco De Bardi<sup>1</sup>, Francesca Gargano<sup>1</sup>, Luca Maurillo<sup>6</sup>, Mariadomenica Divona<sup>5</sup>, Nélida I. Noguera<sup>3,4</sup>, Maria Irno Consalvo<sup>2</sup>, Giovanna Borsellino<sup>1</sup>, Giorgio Bernardi<sup>5</sup>, Sergio Amadori<sup>2</sup>, Adriano Venditti<sup>2</sup>, Luca Battistini<sup>1</sup>, and Francesco Lo-Coco<sup>2,3</sup>

Abstract

Purpose: We evaluated leukemia-associated immunophenotypes (LAIP) and their correlation with fms-like tyrosine kinase 3 (FLT3) and nucleophosmin (NPM1) gene mutational status in order to contribute a better identification of patients at highest risk of relapse in acute myeloid leukemia (AML).

Experimental Design: Bone marrow samples from 132 patients with AML were analyzed by nine-color multiparametric flow cytometry. We confirmed the presence of the mutation in diagnostic samples and in sorted cells by conventional RT-PCR and by patient-specific RQ-PCR.

Results: Within the CD34<sup>+</sup> cell fraction, we identified a discrete population expressing high levels of the IL3 receptor α-chain (CD123) and MIC-2 (CD99) in combination with the IL2 receptor α-chain (CD25). The presence of this population positively correlated with the internal tandem duplications (ITD) mutation in the FLT3 gene (r = 0.71). Receiver operating characteristics showed that, within the CD34<sup>+</sup> cell fraction a percentage of CD123/CD99/CD25<sup>+</sup> cells ≥11.7% predicted FLT3–ITD mutations with a specificity and sensitivity of >90%. CD34/CD123/CD99/CD25<sup>+</sup> clones were also detectable at presentation in 3 patients with FLT3 wild-type/NPM1<sup>+</sup> AML who relapsed with FLT3–ITD/NPM1<sup>+</sup> AML. Quantitative real-time PCR designed at relapse for each FLT3–ITD in these three cases confirmed the presence of low copy numbers of the mutation in diagnostic samples.

Conclusions: Our results suggest that the CD34/CD123/CD99<sup>+</sup> LAIP is strictly associated with FLT3–ITD–positive cells. Clin Cancer Res; 21(17); 3977–85. ©2015 AACR.

Introduction

Immunophenotyping by multiparametric flow cytometry (MFC) is a valuable and effective tool for diagnostic characterization, classification, and minimal residual disease (MRD) monitoring in acute myeloid leukemia (AML). In fact, several studies have defined surface and cytoplasmic markers that are aberrantly expressed on AML blasts at diagnosis (1–2); these combinations may identify leukemia-associated immunophenotypes (LAIP), which allow sensitive monitoring of MRD during follow-up (3–5). Compared with molecular evaluation of MRD through PCR amplification of genetic AML lesions, MFC offers the advantage of being applicable to the vast majority (i.e., >90%) of cases (6, 7).

In addition to immunophenotype, both karyotypic and molecular genetic characterization are essential parts of the diagnostic work-up in AML. Although in some cases immunophenotypic profiles have been correlated with AML genetic subsets, to date no antigenic signature has been associated with an AML genetic entity with absolute specificity. For instance, expression of CD14, CD4, CD11b, and CD64 or CD36 (markers of monocytic and granulocytic differentiation) combined with high levels of CD34 and CD117 and abnormal expression of CD2 characterizes—but is not specific for—AML inv (16) or t(16;16) (8). Similarly, acute promyelocytic leukemia (APL) with t(15;17) is characterized by very low or absent expression of CD34 and HLA-DR, and positivity for CD117/CD33; however, this antigenic profile is not completely specific for APL and some variant LAIPs have been described (9–11).

Several studies have reported correlations of aberrantly expressed markers with clinical outcome in AML. For example, CD7 and CD25 expression has been associated with poor prognosis in normal karyotype (NK) AML (12–14). The IL3 receptor-α (CD123) is overexpressed in 45% of AML patients, and this higher expression has also been associated with poor outcome (15). Finally, overexpression of CD123 has been correlated in this...
Translational Relevance

Although it is well established that FLT3 mutations confer poor prognosis in AML, the significance of minor clones harboring this alteration is unclear. Furthermore, such clones are not easily detectable through molecular assays. We show here that minor FLT3-ITD–positive subclones are clinically relevant in AML as they may emerge after therapy in patients labeled as FLT3 wild-type at presentation. Identification through multiparametric flow cytometry at diagnosis of an immunophenotypic fingerprint associated with these subclones is a novel and simplified tool with improved sensitivity to unravel these clones and allowing patient stratification and risk-adapted treatment with potential impact on outcome of the disease.

Materials and Methods

Patients

A total of 132 consecutive patients ages 19 to 85 years with newly diagnosed AML observed and treated at the Hematology Unit of the Department of Biomedicine and Prevention of the University of Tor Vergata during the period 2012 to 2014 were included in the study. The vast majority of patients ages <75 years received intensive chemotherapy according to European Organization of Research and Treatment of Cancer (EORTC)/Gruppo Italiano Malattie EMatologiche dell’Adulto (GIMEMA) protocols, whereas patients ages >75 years received supportive care or therapy with hypomethylating agents. According to the declaration of Helsinki, all patients gave informed consent for the study that was approved by the Institutional Review Board of the Policlinico Tor Vergata of Rome.

Diagnostic work-up

Routine morphologic, immunophenotypic, and genetic analyses were carried out in all cases at presentation. Conventional karyotyping was performed on bone marrow (BM) diagnostic aspirates after short-term culture and analyzed after G-banding. The description of the karyotypes was done according to the International System for Human Cytogenetic Nomenclature. As for molecular analysis, total RNA was extracted from Ficoll-Hypaque isolated BM mononuclear cells collected at diagnosis and for selected cases, during follow-up using standard procedures (25) and reverse-transcribed with random hexamers as primers. DNA was extracted using a column-based Qiagen Kit protocol (Qiagen). RNA samples extracted from sorted cells population were subjected to reverse-transcription using 200 ng of RNA with the SuperScript III One-Step RT-PCR system (Invitrogen) according to the manufacturer’s instructions. Most common AML gene fusions, FLT3 status (both ITD and TKD mutations) and NPM1 mutated (mut) or wild-type (wt) gene status were investigated using the protocols reported elsewhere (19, 26, 27). For selected patients, the FLT3-ITD data are also represented as a ratio of mut/wt cells obtained by a quantitative assay based on Genescan analysis (19). This assay compares the relative abundance of wt versus mutant FLT3 alleles.

MPFC studies and high-speed cell sorting

Immunophenotypic studies were performed on BM samples at diagnosis and, for selected cases, in samples obtained during follow-up and at disease relapse. Cells were acquired on a CyAn ADP 9 Color equipped with three lasers (Beckman Coulter, Brea, CA). Briefly, BM samples were lysed with ammonium chloride (pH 7.2) and stained with predefined optimal concentrations of 8 antibodies and a dead cell stain dye (LIVE/DEAD Fixable Aqua Dead Cell Stain Kit, Life Technologies). Each sample at diagnosis was stained with 10 distinct antibody combinations (“pre-mixes”); the markers CD45, CD34, CD117 and HLA-DR represented the “backbone” and were present in all combinations, in addition to a viability stain. The remaining 4 markers were different in each mix. The number of events acquired for each leukemic sample was determined as to have at least 500 events accumulated in the gate of CD34+ cells. Events analyzed under further back gating analysis (CD45 vs side scatter) and exclusion of doublets and dead cells. Following acquisition and analysis of the samples stained with the 10 pre-mixes, new antibody combinations were designed on the spot to include all markers expressed by the leukemic cells, thus each sample was studied specifically using custom-designed panels. Data were analyzed using FlowJo software (TreeStar Inc.).

High-speed cell sorting was performed on a MoFlo (Beckman Coulter). Cells were isolated by Ficoll-Hypaque to prevent
FLT3-ITD patient-specific quantitative analysis by RQ-PCR

For FLT3-ITD quantification, we developed patient-specific real-time assays (RQ-PCR). The strategy for FLT3-ITD patient-specific primers designing is reported elsewhere (24). On the basis of clinical outcome, molecular, and immunophenotypic results, 4 AML patients (UPN 104, 146, 213, and 241) were selected for further analysis by RQ-PCR for the patient-specific FLT3-ITD analysis using several follow-up RNA samples. UPN 104 and 146 were characterized by two FLT3-ITD clones, whereas UPN 213 and 241 by only one FLT3-ITD clone. The assays were designed to evaluate the copy number variation among different clones carrying FLT3-ITDs. The RQ-PCR for FLT3-ITD quantification was performed using patient-specific FLT3-ITD forward primers (FLT3-ITD genomic coordinates and primer sequences are available on request), a common reverse primer and probe (FLT3-ITD_forward, 5′-AGACCCCGTATGGTCGTAGC-3′, FLT3-ITD_reverse, 5′-FAM AAGGTACTAGTACGGTCTTGGAAC3′-BHQ1). The reaction mixture of 25 μL contained 1 × Master Mix (Applied Biosystems), 300 nmol/L of each primer, 200 nmol/L of Taqman probe, and 5 μL of cDNA (1/10 of RT product). Amplification conditions were: 2 minutes at 50°C, 10 minutes at 95°C followed by 60 cycles at 95°C for 15 seconds, and at 60°C for 1 minute for UPN 146, 213, and 241, and 62°C for UPN 104. A threshold value of 0.2 was used and baseline was set to 3 to 15. Patient-specific FLT3-ITDs sensitivity and specificity tests were carried out for each patient with serial water dilutions of RNA and with RNA from healthy donors.

Statistical analysis

Differences between categorical variables were evaluated by the Pearson χ² test, whereas differences between continuous variables were analyzed by an unpaired t test with 95% confidence intervals. Positive correlations between categorical and quantitative variables were analyzed by the Spearman rank correlation coefficient. Receiver operating characteristic (ROC) analysis was performed with MedCalc statistical software (Ostend Belgium). The standard error was calculated using DeLong and colleagues (28). The confidence interval for the AUC was calculated using the Binomial exact confidence interval.

Results

FLT3 and NPM1 mutational status of patients at diagnosis

Out of 132 tested patients, 36 harbored in their leukemic cells the FLT3-ITD mutations and 48 NPM1 mutations. Considering the status of both genes, 9 patients tested FLT3-ITD/NPM1-wt, 21 FLT3-ITD+/NPM1-mut, 27 FLT3-ITD-/NPM1-mut, and 75 FLT3-ITD-/NPM1-wt. Karyotypic data were available for 70% of patients. The main demographic characteristics and karyotype of patients according to NPM1/FLT3 gene status are reported in Supplementary Table S2.

MPFC analysis in total BM and in the CD34+ compartment

As shown in Supplementary Table S3, MPFC studies in the total BM revealed a statistically significant difference in the expression of CD123, CD25, CD99, CD7, and CD11b between FLT3-ITD and FLT3-ITD− patients (P = 0.02, P = 0.000006, P = 0.03, P = 0.02, P = 0.005, respectively). The fraction of CD34+ cells was significantly lower in patients with NPM1-mut, compared with patients who did not carry the mutation (Fig. 1A, top; Supplementary Table S4); accordingly, the bulk of the leukemic cell population in NPM1-mut patients was CD34+/CD117+ (Fig. 1A, bottom). In addition, CD34+ cells derived from the FLT3 and NPM1-mutated group expressed the lowest levels of CD38 (Fig. 1B).

When focusing the analysis on CD34+ cells, FLT3-ITD patients expressed CD123, CD25, and CD99 at significantly higher levels compared with patients with FLT3-ITD−, regardless of NPM1 gene status (Supplementary Table S4; Fig. 1B). Expression of CD11b and CD7 was higher in samples with both mutations, but did not discriminate this group from the other genetic subsets. Thus, we investigated whether these three markers (CD123, CD25, and CD99) were coexpressed by the same cells. Figure 1C shows MPFC analysis of BM samples from patients with different NPM1/FLT3 gene status. Cumulative data of the percentage of CD34+ cells that stained positive for CD25, CD123, and CD99 simultaneously showed that this antigen combination is very rarely observed or barely detectable in patients with FLT3-ITD− (Fig. 1C and D). Although positivity for each marker measured separately was not sufficient to unequivocally discriminate between FLT3-ITD and FLT3-ITD− patients, the combination of all three markers simultaneously reduced the variance within the group of FLT3-ITD− patients. We then screened all patients for the presence or absence of FLT3-ITD, and we identified eight cases harboring this mutation in diagnostic BM samples, five of which were in the NPM1-wt group, whereas three carried also a mutation in NPM1. As shown in Fig. 1D (red circles), only one case presented with a significant fraction of CD34+/CD123+/CD25+/CD99− cells. It should be noted, however, that this patient also had a mutated karyotype. Interestingly, also the 2 patients without identifiable FLT3 mutations and with the LAIP had mutated karyotype and were sent to allogeneic stem cell transplantation (SCT).

Figure 1D also shows that 3 patients with FLT3-ITD and NPM1-wt presented a low percentage of CD34+/CD123+/CD25+/CD99− cells. All these 3 patients had mutated karyotype at diagnosis and underwent allogeneic SCT.

Five BM samples from healthy subjects were also analyzed for the presence of CD34+/CD123+/CD25+/CD99− cells. Figure 2 shows a representative MPFC analysis and cumulative data of CD34+ cells derived from healthy donors as compared with an FLT3-ITD+ AML patient. This combination of markers is present in healthy individuals at a rate ranging from 0.3% to 4% of CD34+ cells.

ROC curve

We found a strong positive correlation (r = 0.7136; P < 0.0001) between the percentage of CD123/CD99/CD25+ population within CD34+ cells and the presence of the FLT3-ITD mutation, as evaluated in 132 AML BM samples by conventional RT-PCR (Fig. 3A).

ROC curve analysis identified the value >11.7% of CD34+/CD123+/CD99+/CD25+ cells within the total CD34+ fraction as the best determinant for predicting the presence of FLT3-ITD mutation with a specificity and sensitivity of >90%. The area under the ROC curve (AUC) was calculated to be 0.96 with 95% confidence interval ranging from 0.91 to 0.99 and significance level of P < 0.0001 (Fig. 3B). The positive and negative predictive value calculated using 11.7% as threshold were 84.3931% and...
To note, the fraction of CD34+/CD123+/CD25+ cells within CD34+/cells is significantly higher in the group with mutations in both FLT3 and NPM1, as compared with samples that only have the mutation in FLT3 (Fig. 1D).

The ROC analysis generated using only the markers CD123 and CD25 showed similar results, but with a sensitivity and specificity lower than that calculated with also CD99 (Supplementary Fig. S1).

With respect to conventional RT-PCR, MPFC analysis proved to be superior in the ability to detect the FLT3-ITD mutation in subclones. Indeed, blasts from 3 patients (UPN 241, 213, and 104) who underwent FLT3-ITD AML relapse had been labeled as FLT3-wt by conventional RT-PCR at the time of diagnosis. These patients showed at presentation a significant number of CD123/CD99/CD25+ cells within the CD34+ population: 13%, 11.8%, and 20.7%, respectively (Supplementary Fig. S2A, red circles). Patient-specific RQ-PCR with primers designed at relapse unveiled low copy numbers of FLT3-ITD in all three cases using diagnostic samples. Supplementary Figure S2B shows that when the percentage of the CD34/CD123/CD25/CD99+ cell subset on total sample was lower than 0.1%, conventional RT-PCR failed to detect the FLT3-ITD subclone.

Purified CD34+/CD123+/CD99+/CD25+ cells carry the FLT3-ITD mutation

Based on the availability of samples, we proceeded to investigate the mutational profiles of cell-sorted subfractions within the
CD34+ population. We identified two patients harboring FLT3-ITD (UPN 118 and 146) in whom MPFC had revealed 64% and 36% of CD123/CD99/CD25 + within CD34+ cells (1.7% and 1.9% of the total sample). Cells from both patients were purified by cell sorting according to CD34, CD117, CD123, CD99, CD25, CD7, and CD38 expression (Fig. 4) at diagnosis. The isolated subfractions showed an enrichment of the mutated allele within the cell population, expressing the markers we have identified as LAIP for the FLT3-ITD mutation: in both patients, the CD34+, CD117+, CD123+, CD99+, and CD7−/− fraction was highly enriched in cells carrying the mutation, with a FLT3 ratio of 21.67 in one patient and of 8.46 in the other (Fig. 4, subset 1). In patient UPN 118, we identified a second subpopulation of cells expressing high levels of CD38 and which contained a heterogeneous cell population with a FLT3 ratio similar to the whole MNC (value = 2.04). The ratio is an indirect quantification of the number of FLT3-ITD-positive and -negative cells. In particular, Thiede and colleagues (19) found that a high FLT3-ITD allelic burden confers a poor prognosis for AML patients.

Within the CD34+ cells of patient UPN 146, we could identify and isolate another two subpopulations: one containing CD117+, CD123+, CD99-/−/−, CD25−/−, CD7−/−/−, and CD38+ cells, mostly harboring FLT3 wt (subset 2), and the other (subset 3) characterized by expression of CD99 and negativity for CD38, which on the contrary was highly enriched in mutated FLT3 (ratio 7.18).

By patient-specific RQ-PCR, we could define the copy number of FLT3-ITD clones in the three phenotypic subsets, which is reported in Supplementary Table S5. These results confirm that cells carrying the FLT3-ITD mutation are contained within distinct subfractions of the CD34+ cell population and can be enriched based on the expression of surface markers.

**Figure 2.** MPFC analysis of CD34+ cells in healthy bone marrow samples. A, MPFC analysis of a bone marrow sample from a healthy donor. The sequential gating strategy shows the expression of CD123 vs. CD25 and CD123 vs. CD99 within CD34+CD117− cell subset. B, cumulative data of the percentage of CD123/CD25/CD99− within CD34+ cells in FLT3-ITD mutated bone marrow samples and in healthy donors (HD).

**Figure 3.** ROC curve analysis. A, Spearman rank correlation coefficient (r) between the percentage of CD34+CD25+CD99+ cells within the total CD34 compartment and the FLT3-ITD mutation in 132 AML samples. The coefficient was calculated giving the value 1 to the presence of FLT3-ITD and −1 to the absence of FLT3-ITD. B, the ROC curve is generated by plotting the variables mentioned above. Statistical analysis was performed by “Med Calc” software.
Sequential immunophenotypic and RQ-PCR monitoring studies through patient-specific FLT3-ITD

Four patients (UPN 241, 213, 146, and 104) were followed longitudinally through post-induction, post-consolidation therapy, and at relapse by patient-specific RQ-PCR using RNA derived from sequential BM samples (Supplementary Table S5). Following serial dilution experiments with patient-specific RQ-PCR for FLT3-ITD, the assay showed maximum reproducible sensitivity and specificity at 10^{-6} in UPN 104 and 213; and at 10^{-4} in UPN 146 and 241. The coefficient of the standard curves for the 4 samples ranged from 0.995 to 0.999 and the slope ranged from 3.418 to 4.24.

A progressive decrease in FLT3-ITD copy number was detected in UPN 146 after induction therapy but unlike the smaller FLT3-ITD clone (ITD, 30bp), the most representative clone (ITD, 60bp) detected at the diagnosis in this case (Fig. 4B, top; Supplementary Table S5) remained unchanged after consolidation therapy. Accordingly, MPFC analysis after post-consolidation therapy detected a CD123/CD25/CD99+ cell subset comprising 20% of CD34+ cells and 0.6% of the total blast population (Fig. 5A).

UPN 104, 213, and 241 displayed a normal karyotype and had been labeled as FLT3-wt using the conventional RT-PCR; however, within the small fraction of CD34+ cells, the CD123/CD99/CD25+ population was detected by MPFC above the defined threshold (Figs. 5B, top and 6A; Supplementary Fig. S2A and S3A). The FLT3-ITD patient-specific analysis by RQ-PCR performed on all samples at relapse allowed identifying retrospectively FLT3-ITD in the diagnostic sample (Supplementary Table S5).

In the case of UPN 104, the FLT3-ITD-specific RQ-PCR showed a low expression of the two ITD clones at diagnosis, which were below the detection limit of conventional RT-PCR assays and which decreased after induction therapy. The FLT3-ITD copy number remained undetectable after the second course of consolidation therapy with Ara-C (Supplementary Table S5 and Fig. 6B). This patient relapsed during follow-up and MPFC analysis showed an expansion of the CD34/CD123/CD99/CD25+ subset, which at this time point represented over 65% of the total blast population (Fig. 6C).

The FLT3-ITD patient-specific RQ-PCR of UPN 213 and 241 showed a low expression of the ITD clone at diagnosis, which decreased after induction and post consolidation therapy. MPFC analysis had initially shown a percentage of the aberrant population very close to the threshold defined by the ROC curve (11.8% and 13% of CD34+ cells, respectively). This percentage decreased to 2% after post consolidation treatment for UPN 213 (Fig. 5B, bottom) in parallel with FLT3-ITD copy number fluctuations, but unfortunately there is no MPFC data from the relapse. However, MPFC analysis for UPN 241 showed a 96% of CD123/CD25/CD99 within CD34+ cells when the patient relapsed, reflecting the very high FLT3-ITD copy number (Supplementary Figs. S3B–S3C).

Figure 6 and Supplementary Fig. S3 also show that, both at diagnosis and relapse, the CD34/CD123/CD99/CD25+ cell subsets expressed low levels of CD38.

Discussion

Using MPFC in leukemic blasts from AML patients, we identified a CD34+ cell subset characterized by CD25/123/99 coexpression that, compared with the total mononuclear cell fraction, contained an enriched FLT3-ITD–positive population. Our established threshold of 11.7% cells staining positive for this antigenic profile allowed to predict FLT3-ITD with specificity and sensitivity >90% and enabled the identification of FLT3-mutated subclones also in cases labeled as FLT3 wt by conventional RT-PCR during diagnostic routine work-up. As reported previously by our group and by others (29–31), these minor FLT3-mutated subclones, which escape diagnostic recognition, may undergo clonal evolution and ultimately result in overt disease relapse, hence their improved identification at diagnosis through MPFC appears to be clinically relevant.
Our results suggest that the CD34+/CD25/CD123/CD99+ LAIP is strictly associated with FLT3-ITD mutations in the context of NK-NPM1–mutated AML. A total of six cases in this study showed discordant results with respect to the correlation between the established LAIP threshold and FLT3 mutational status (Fig. 1D). Of these, three patients with FLT3-ITD had CD34+/CD25+/CD123+/CD99+ cells below the ROC-assessed 11.7% threshold, and 3 patients with FLT3 wt configuration disclosed a CD34+/CD25+/CD123+/CD99 LAIP above the threshold; interestingly, all these six cases showed mutated karyotype and NPM1 wt gene configuration. Although our findings require confirmation in other independent and larger studies, we outline the clinical relevance of the precise diagnostic definition of NK-NPM1 mutated AML—a subset commonly associated with a favorable prognostic outcome (32)—for the possible concomitant presence of small subclones harboring the FLT3-ITD mutation.

Other authors have correlated the expression of CD25 and CD123 with FLT3-ITD mutation in AML (13). In particular, the study by Gonen and colleagues (13) showed a significant association of CD25 and CD123 with FLT3 mutation but did not find a correlation of CD25 expression with the putative stem cell markers like CD34. Our data have revealed high copy number of FLT3-ITD in the CD34+/CD123+/CD99−CD38+CD117+ population, suggesting that CD25 expression may appear at a later stage from CD34+/CD123+/CD99− subset. However, an ROC analysis performed on this phenotype (AUC = 0.89) indicates that this LAIP is not optimal for the identification of samples with FLT3-ITD.

Rollins-Raval and colleagues (33) described the association of CD123 in AML patients with both FLT3 and NPM1 mutations, in keeping with our observations. Moreover, we reported a higher CD123 expression in FLT3 mutated AML patients as compared with those with germline FLT3 (16). In the study by Ehninger and colleagues (34), the analysis of CD33 and CD123 restricted to the CD34+ compartment allowed to detect an association with FLT3 mutations in up to 73.1% of AML patients.

Our study shows that AML blasts with FLT3 mutations also coexpress CD99. The addition of CD99 to CD123 and CD25 and the restriction of the analysis to the CD34 population allowed to increase the specificity and sensitivity of the association of immunophenotypes with FLT3-ITD from 73.1%, as detected by Ehninger and colleagues (34), to more than 90%. Notably, in this study, the CD34+/CD123+/CD25+/CD99+ cell fraction was detectable in healthy marrow samples at levels <4%, that is far below the 11.7% ROC-generated threshold for the association with FLT3-ITD. CD99, also known as MIC2 or single-chain type-1 glycoprotein, is a O-glycosylated transmembrane antigen expressed on all leukocytes and is involved in the regulation of apoptosis and adhesive properties of T cells (35, 36). The expression level of this antigen in normal hemopoiesis is related to distinct maturational stages: It is high in bone marrow CD34+ cells, and then declines during the differentiation process (37). In normal BM, we detect CD99 expression on CD34+ cells, but it does not correlate with CD123 or CD25. Interestingly, CD99 expression by tumor cells has been described in Ewing Sarcoma, where this antigen is the potential target of immunotherapy (38).

Bachas and colleagues (29, 31) have studied in detail the subclones detectable within the leukemic blasts of AML patients and analyzed their role in the development of relapse. By analyzing AML cases with FLT3 mutations collected both at diagnosis and at relapse, these authors found a most significant enrichment of cells carrying the FLT3 mutation in the CD34+/CD38− compartment. Our results further improve the identification of these...
clones by a more accurate LAIP, which strengthens the association with FLT3 mutations.

As to the potential clinical use of our findings, we observe that search of this LAIP at diagnosis may complement routine genetic characterization. In particular, we recommend that samples from AML patients with normal karyotype and NPM1-mutated/FLT3 wt genotype by routine RT-PCR, which show a CD34/CD123/CD25/CD99 population above the indicated threshold be strictly monitored by both molecular and MPFC during follow-up for the possible emergence of FLT3-mutated clones.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

Conception and design: D.F. Angelini, T. Ottone, G. Borsellino, L. Battistini, F. Lo-Coco

Development of methodology: D.F. Angelini

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): G. Guerrera, S. Lavorgna, M. Cittadini, F. Buccisano, F. Gargano, M. Divona, N.I. Noguera

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D.F. Angelini, T. Ottone, F. Buccisano, M.I. Consalvo, G. Borsellino

Writing, review, and/or revision of the manuscript: D.F. Angelini, F. Buccisano, L. Maurillo, G. Borsellino, G. Bernardi, S. Amadori, A. Venditti, L. Battistini, F. Lo-Coco

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. De Bardi

Study supervision: S. Amadori, L. Battistini

Other (performed the experiments): G. Guerrera

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