Prognostic impact of genetic characterization in the GIMEMA LAM99P multicenter study for newly diagnosed acute myeloid leukemia

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ABSTRACT

Background
Recent advances in genetic characterization of acute myeloid leukemia indicate that combined cytogenetic and molecular analyses provide better definition of prognostic groups. The aim of this study was to verify this prospectively in a large group of patients.

Design and Methods
Genetic characterization was prospectively carried out in 397 patients with acute myeloid leukemia (median age, 46 years) receiving uniform treatment according to the LAM99P protocol of the Italian GIMEMA group. The impact of genetic markers on response to therapy and outcome was assessed by univariate and multivariate analyses.

Results
For induction response, conventional karyotyping identified three groups with complete remission rates of 92%, 67% and 39% (p<0.0001). Complete remission rates in *NPM1* mutated (*NPM1*+) and wild-type (*NPM1*-) groups were 76% and 60%, respectively, for the whole population and 81% and 61% in the group with normal karyotype (p<0.001 and p=0.026, respectively). Multivariate analysis indicated that low risk karyotype and *NPM1*+ were independent factors favorably affecting complete remission. Multivariate analysis of overall and disease-free survival among 269 patients who achieved complete remission showed a significant impact of karyotype on both estimates and of *FLT3* status on disease free-survival (*FLT3*-ITD *vs*. *FLT3* wild-type, p=0.0001). *NPM1* status did not significantly influence disease free-survival in either the whole population or in the patients with a normal karyotype in this series, probably due to the low number of cases analyzed.

Conclusions
These results reiterate the prognostic relevance of combining cytogenetic and mutational analysis in the diagnostic work up of patients with acute myeloid leukemia.

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Introduction

Acquired genetic lesions in acute myeloid leukemia (AML) are being increasingly recognized as relevant markers whose identification improves diagnostic refinement, classification and prognostic assessment in this heterogeneous disease. In fact, discrete AML entities requiring specific therapeutic approaches and/or showing different responses to therapy and outcome are better identified based on the detection of these alterations.[1-6] As a consequence, genetic characterization of all AML patients at presentation is nowadays regarded as mandatory to determine treatment choices and should always integrate first level diagnostic studies based on morphology, cytochemistry and immunophenotype.[7]

The genetic alterations in AML include chromosome abnormalities detectable at the karyotypic level i.e. translocations and numerical abnormalities, as well as subtle gene alterations that are identified by molecular techniques such as small duplications/insertions and point mutations.[5,6] Among the latter group, *FLT3* and *NPM1* aberrations have been reported as the most frequent genetic lesions, are consistently associated with normal karyotype, and show apparently opposite prognostic significance, *FLT3* mutations being correlated with poor outcome[8-12] and *NPM1* mutations being associated with a more favorable response to therapy.[13-19] Other aberrations, which have not yet been well defined at the gene level (e.g. numerical abnormalities such as –7, –5, +8, and others) are detectable by karyotypic or fluorescence *in situ* hybridization (FISH) analysis only, and are equally important in the clinic because of their association with specific entities (e.g. therapy-related AML) and with unfavorable outcomes.[5,6]

Based on the above considerations, modern genetic characterization of AML should combine conventional karyotyping and molecular methods – FISH, reverse transcriptase polymerase chain reaction (RT-PCR), sequencing – with the aim of analyzing all major types of clinically relevant alterations. Besides detecting submicroscopic alterations, the routine use of RT-PCR for the analysis of chromosome translocations[20] may unravel cryptic rearrangements and provide invaluable information in the case of failed karyotyping. While such an approach might be carried out routinely in experienced centers, due to logistic and standardization problems it might be more difficult in the context of large multi-institutional clinical trials.

To evaluate the prognostic relevance of an integrated genetic characterization of AML, in 1999 the Italian co-operative group GIMEMA started a clinical trial that included standard induction and consolidation therapy in all cases. Sample centralization, cell banking and standardized cytogenetic and molecular tests were planned to maximize methodological homogeneity and to establish the prognostic role of major genetic lesions in a uniform clinical context.

We report here the results of this study.

Design and Methods

Patients and treatment

Between 1999 and 2003, 509 patients with FAB non-M3 AML (median age 46 years; range, 15-60) were enrolled and started induction therapy in the multicenter LAM99P study of the Italian GIMEMA group. To evaluate the prognostic impact of genetic characterization all patients received a uniform induction and consolidation protocol and diagnostic samples were sent to a central laboratory for cytogenetic and molecular studies. Therapy consisted of a pre-treatment phase with hydroxyurea (2 g/m^2 for 5 days) followed by induction with daunorubicin (50 mg/m^2 days 1, 3, and 5), cytarabine (100 mg/m^2 days 1-10) and etoposide (100 mg/m^2 days 1-5) and consolidation with cytarabine (500 mg/m^2/12 hours days 1-6) and daunorubicin (50 mg/m^2 days 4-6). After consolidation therapy, eligible patients with an identical HLA donor were planned to receive an allogeneic stem cell transplant whereas the remaining were addressed to peripheral blood autologous stem cell transplantation.

Logistics and organization of the network for centralized sample analysis and biological studies

A scheme illustrating the organization of the central sample analysis and the laboratory network for biological studies is shown in Figure 1. Anticoagulated bone marrow and peripheral blood samples were collected after informed consent at local hospitals participating in the GIMEMA LAM99P trial and sent by overnight courier to a central laboratory at the Department of Cellular Biotechnology and Hematology of *La Sapienza* University in Rome. While patients were rapidly started on cytoreductive therapy, clinicians were allowed to collect the samples during the 5 days of hydroxyurea pretreatment. This in turn facilitated the collection, shipment and delivery of samples in all cases from Monday through Thursdays thereby avoiding the week-ends. A network of six GIMEMA laboratories contributed, on a rotational basis, to carrying out the cytogenetic and RT-PCR studies. In addition to the central laboratory in Rome, the other five laboratories of the network were located at: the Department of Clinical and Biological Sciences, *S. L. Gonzaga* Hospital, Orbassano, University of Turin; the Hematology and Bone Marrow Transplantation Unit, University of Perugia; the CEINGE and Department of Biochemistry and Medical Biotechnologies, *Federico II* University of Naples; the Department of Biomedical Science, Hematology Unit, University of Ferrara; and the *Seràgnoli* Department of Hematology of the University of Bologna. The central laboratory was responsible for: i) blood sample processing to collect mononuclear cells, preparation and storage of material for genetic studies (fixed nuclei for karyotyping, dry pellets for DNA, and cells in guanidium isothiocyanate for RNA); ii) shipment of material for genetic studies to the other laboratories; and iii) participation in performing genetic characterization studies on a rotational basis. For the cytogenetic studies, fixed nuclei from heparinized vials were

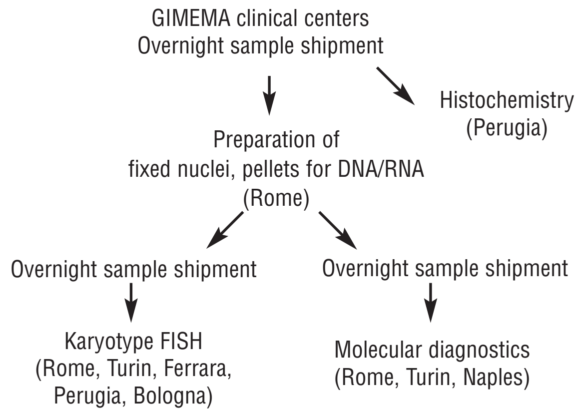

Figure 1. Schematic illustration of the national network for sample centralization, cytogenetic, molecular biology and immunohistochemistry studies on acute myeloid leukemia samples.

obtained following hypotonic lysis and fixation in sodium acetate and methanol. For molecular studies, samples anticoagulated with sodium citrate were centrifuged on a Ficoll-Hypaque gradient, mononuclear cells were washed twice in phosphate-buffered saline and aliquots were stored as dry pellets at –80°C for DNA studies and in 4M guanidium isothiocyanate at –20°C for RNA studies. Each laboratory of the network received fixed nuclei and/or cells in guanidium isothiocyanate obtained from consecutive patients during a 2-month period and carried out cytogenetic (Rome, Turin, Perugia, Ferrara, Bologna) and molecular studies (Rome, Turin, Naples) according to uniform, standardized protocols as detailed below. For immunohistochemistry, bone marrow biopsies were sent by overnight courier directly to the laboratory in Perugia for the analysis of the cellular localization of NPM1 protein, which was carried out as reported previously.[21]

Cytogenetic studies

Cytogenetic studies were carried out using either direct or 24-hour cultured preparations of bone marrow or peripheral blood cells (or both) without stimulation. GTG-banding chromosomes were classified according to the International System for Human Cytogenetic Nomenclature.[22] A successful cytogenetic analysis required the detection of two or more cells with the same structural change or chromosomal gain, three or more cells with the same chromosomal loss, or at least 20 metaphases without clonal changes. Interphase FISH analysis was routinely carried out to detect rearrangements in the *MLL* gene and to search for the following aberrations: –5, –7, +8, del(5)(q31), del(7q) and del(20)(q11). The probes and procedures for FISH studies have been reported elsewhere.[23,24]

Molecular studies

Total RNA was extracted from cells in guanidium isothiocyanate using the method of Chomczynski and Sacchi.[25] The standardized RT-PCR protocol defined by the Biomed-1 concerted action was used to detect the following fusion genes: *AML1/ETO*, *CBFβ-MYH11*, *DEK/CAN*, and *BCR/ABL*.[20] As to *FLT3* mutational analysis, internal tandem duplication (ITD) and the D835/836 mutation were investigated in all cases using RT-PCR followed by EcoRV digestion and gel visualization as described previously.[26] Denaturing high performance liquid chromatography (D-HPLC) and/or multiplex PCR followed by capillary electrophoresis was used in selected cases for the analysis of *NPM1* mutations and to confirm *FLT3* mutations detected by conventional RT-PCR. The methods used for D-HPLC and capillary electrophoresis analysis of FLT3 and *NPM1* have been reported in detail elsewhere.[27,28]

Outcome evaluation and statistical analysis

Overall survival was defined as the time from diagnosis to death or last follow-up, censoring patients alive at the last follow-up. Disease-free survival was defined, only for responding patients, as the time from achieving complete remission to either relapse or death in first complete remission or last follow-up, censoring patients alive disease-free at last follow-up. Relapse and non-relapse mortality were also analyzed as competing risks in terms of crude cumulative incidence. Differences among groups were evaluated using the χ^2 test and the Wilcoxon or Kruskal-Wallis test for categorical and continuous covariates, respectively. Complete remission rates were compared in univariate analysis by the χ^2 test and in multivariable analysis by logistic regression. Overall and disease-free survival rates were estimated using the Kaplan-Meier product limit method and compared in univariate analysis by the log-rank test and in multivariable analysis by a Cox regression model. Relapse and non-relapse mortality cumulative incidence curves were estimated using the proper non-parametric estimator and groups were compared by the Gray test. All analyses were carried out in SAS 8.02; methods for competing risks were applied using the macro CIN created by the Department of Biostatistics of St. Jude Children's Research Hospital, Memphis, USA.

Results

A total of 443 (87%) diagnostic samples from 509 patients were sent to the central laboratory for genetic studies. Conventional karyotyping on G-banded metaphases was successful in 397/443 (90%) cases. The prevalences of the main cytogenetic aberrations are shown in Table 1. No significant variations were observed in the ratio of normal to abnormal karyotypes or in the distribution of karyotypic lesions between patients whose samples were taken after receiving more (4-5 days) hydroxyurea pre-treatment and those who had received 0-1 days of hydroxyurea treatment at the time of sample collection (*data not shown*). The integration of RT-PCR analysis allowed the characterization of major translocations, including *AML1/ETO*, *CBFβ/MYH11*, *DEK/CAN* and *BCR/ABL*, in the vast majority of cases, while fewer patients were studied for *FLT3* and *NPM1* mutations because of the more recent awareness of these alterations (*NPM1* in particular) and

the lack, in many cases, of stored material for retrospective screening for these mutations. As shown in Table 1, with the exception of the higher number of cases that could be analyzed, no significant variations from karyotypic data were found when major translocations were detected by RT-PCR. As to the six cases with t(9;22), although it is not easy to distinguish chronic myeloid leukemia in blast crisis from *de novo* AML, our patients were categorized as having AML based on the following criteria: (i) no additional cytogenetic lesions at diagnosis besides the Philadelphia chromosome; (ii) absence of maturing cell elements of the granulocytic lineage reminiscent of chronic myeloid leukemia either at AML diagnosis of the leukemia or following hematopoietic reconstitution after induction chemotherapy.

FLT3-ITD were found in 64/342 (19%) of patients and were significantly associated with higher white blood cell counts (p<0.001) and higher blast percentages (p=0.0007). *FLT3* D835/836 mutations were found in 21/311 (7%) of cases. NPM1 cytoplasmic dislocation was detected by immunohistochemistry in 86 of 270 (32%) cases analyzed and was significantly associated with normal karyotype (p<0.0001), *FLT3*-ITD (p=0.0002), older age (p=0.002), and higher white blood cell count (p=0.028). In 65 unselected cases, the presence of *NPM1* mutations was confirmed by D-HPLC or capillary electrophoresis. As already reported elsewhere,[21] no discrepancies were observed comparing the pattern of NPM1 histochemical staining and mutational status of the gene, whereby the nuceolar and cytoplasmic staining pattern always correlated with the absence or presence of mutations in the *NPM1* gene. The prevalences of *NPM1* and *FLT3*-ITD mutations in 111 patients with normal karyotype were calculated and are reported in Table 2.

After induction therapy, 269/397 (68%) patients achieved complete remission. As regards induction response, conventional karyotyping identified three distinct groups: a low risk group [inv(16)(p13;q22) and t(8;21)(q22;q22)], an intermediate risk group (normal karyotype and other anomalies not comprised in the high risk group) and a high risk group (t(3;3)(q21;q26), inv(3)(q21;q26), t(9;22)(q34;q11), 11(q23), chromosome 5 or 7 abnormalities, complex karyotype) with complete remission rates of 92%, 67% and 39%, respectively (p<0.0001). Complete remission rates in *NPM1*$^+$ (mutated) *vs. NPM*$^-$ (wild-type) groups were, respectively, 76% *vs.* 60% for the whole population and 81% *vs.*, 61% for patients with a normal karyotype (p<0.001 and p=0.026, respectively). Multivariate analysis indicated that low risk karyotype and *NPM1*$^+$ were independent factors favorably affecting the achievement of complete remission (odds ratios: low and high *vs.* intermediate risk karyotype groups respectively 5.68, [95% CI=2.27-14.21] and 0.46 [95%CI=0.23-0.91]; *NPM1*$^+$ *vs. NPM1*$^-$: 3.10 [95%CI=1.59-6.03]) while FLT3 status did not have a significant impact on the achievement of complete remission (Table 3). The analysis of the prognostic impact of *NPM1* and *FLT3* status on induction response was also carried out for patients with a normal karyotype. In univariate analysis, a trend (p=0.058) towards a more favorable outcome was found for *NPM1*$^+$/*FLT3*$^-$ patients, as compared to that of other subgroups. This trend, however, disappeared in the multivariate analysis.

The overall and disease-free survival rates at 24

Table 1. Results of cytogenetic and molecular characterization.

	Number	Percentage
Cytogenetics		
normal karyotype	170	42.8
inv(16)(p13;q22)	28	7.0
t(8;21)(q22;q22)	37	9.3
+8	19	4.8
t(11q23)	20	5.0
t(6;9)(p23;q34)	10	2.5
t(9;22)(q34;q11)	6	1.5
t(3;3)(q21;q26)/inv(3)(q21;q26)	6	1.5
-5/del(5q)	4	1.0
hyperdiploid	15	3.8
complex karyotype	17	4.3
other	59	14.8
Total	397	100.0
Molecular biology	*Number POS (evaluated)*	*Percentage*
CBFβ/MYH11	29 (442)	6.6
AML1/ETO	39 (443)	8.8
DEK/CAN	10(413)	2.4
BCR/ABL	6 (441)	1.4
FLT3-ITD	64 (342)	18.7
NPM+	86 (270)	31.9

Table 2. Results of ***FLT3*** (ITD only) and ***NPM1*** status in patients with a normal karyotype.

	Number	Percentage
NPM1$^-$*/FLT3*$^-$	37	33.3
NPM1$^+$*/FLT3*$^-$	46	41.4
NPM1$^-$*/FLT3*$^+$	7	6.30
NPM1$^+$*/FLT3*$^+$	21	18.9

Table 3. Multivariate analysis of factors potentially influencing response to induction therapy.

Variable	*Odds ratio*	*95% CI*	*p*
Age increase by 1 year	0.98	0.96-1.00	0.032
White cell count increase by 1×10^9/mL	0.99	0.99-1.00	<0.001
Performance status = 2.3 *vs.* = 0.1	0.39	0.21-0.73	0.003
Low *vs.* intermediate risk karyotype	5.68	2.27-14.21	<0.001
High *vs.* intermediate risk karyotype	0.46	0.23-0.91	0.025
NPM1$^+$ *vs. NPM1*$^-$	3.10	1.59-6.04	<0.001

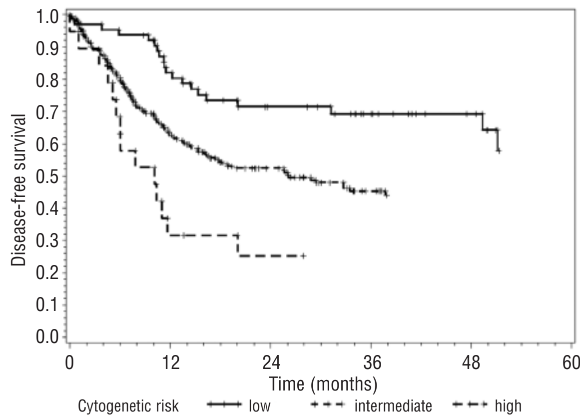

Figure 2. Disease-free survival according to karyotype. The low risk (upper curve) included patients with t(8;21) and inv(16); the high-risk group (lower curve) included patients with chromosome 5 and 7 aberrations, inv(3), t(3;3), t(9;22), 11q23 rearrangements and complex karyotypes; the intermediate risk group (middle curve) included patients with a normal karyotype or cytogenetic lesions not included in the other groups.

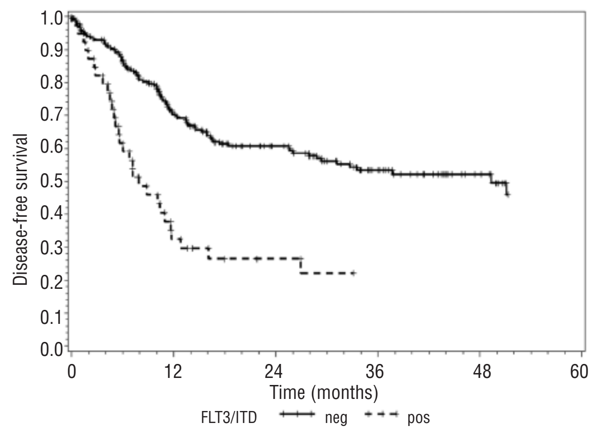

Figure 3. Disease-free survival according to the presence or absence of *FLT3*-ITD.

months for the whole series of patients were 49% (95% CI = 47-52) and 55% (95% CI = 52-59), respectively (*not shown*). The disease-free survival rates in the three karyotypically defined groups were 71% (95% CI = 64-80) in low risk group, 52% (95% CI = 49-56) in the intermediate risk group and 32% (95% CI = 26-39) in the high risk group (Figure 2). In the whole population, the disease-free survival rate according to *FLT3* status was 26% (95% CI = 23-30) and 61% (95% CI = 56-65) for patients with and without the *FLT3*-ITD, respectively (Figure 3). The disease-free survival rate according to *NPM1* status was 54% (95% CI = 50-60) for *NPM1*$^-$ patients and 49% (95% CI = 43-55) for *NPM1*$^+$ individual (Figure 4). The cumulative incidence of relapse was 36.8% (95% CI = 36.3-37.2) and 37.4% (95% CI = 36.7-38.2) for *NPM1*$^-$ and *NPM1*$^+$ patients, respectively (*data not shown*).

The analysis of prognostic factors for disease-free survival was carried out in 269 patients who achieved complete remission (median follow-up of 39 months) and multivariate analysis performed after adjusting for unfavorable factors (white cell count). As shown in Table 4, the results of multivariate analysis for disease-free survival were: low risk *vs.* intermediate risk karyotype: HR 0.53, 95% CI = 0.33-0.87, *p*=0.012; high risk *vs.* intermediate risk karyotype: HR 1.74, 95% CI = 0.99-3.05, *p*=0.054; and *FLT3*$^+$ *vs.* *FLT3*$^-$ HR=2.31, 95% CI 1.50-3.57, *p*=0.0001. *NPM1* status did not significantly influence disease-free survival in either the whole population or in the group with a normal karyotype, although a trend towards a higher disease-free survival rate (*p*=0.092) was observed in *NPM1/FLT3*$^-$ patients within the group with a normal karyotype.

Table 4. Multivariate analysis of factors potentially influencing disease-free survival.

Variable	*Hazard ratio*	*95% CI*	*p*
WBC >50×10^9/L	1.82	1.23-2.70	0.003
Low *vs.* intermediate risk karyotype	0.53	0.33-0.87	0.012
High *vs.* intermediate risk karyotype	1.74	0.99-3.05	0.054
FLT3$^+$ *vs. FLT3*$^-$	2.31	1.50-3.57	<0.001

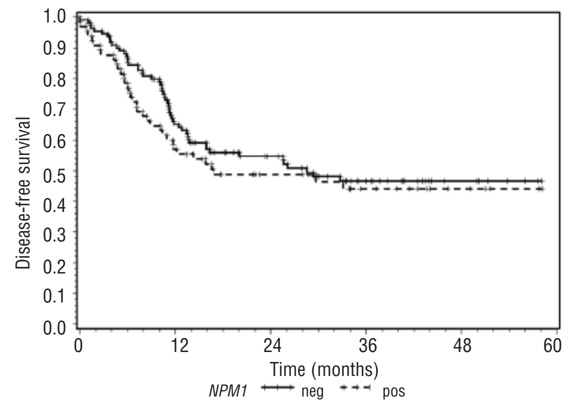

Figure 4. Disease-free survival according to *NPM1* status.

In summary, by investigating *NPM1* and *FLT3* interactions we confirmed in our series that *NPM1* status had the most important impact on complete remission (independently of *FLT3* status) whereas *FLT3* was most influential on disease-free survival (independently of *NPM1* status). The analysis of *FLT3* and *NPM1* interactions with respect to long-term outcome was, however, hampered in this study by the low number of cases in each category.

Discussion

This study highlights the clinical relevance of an integrated genetic characterization of AML in the context of large multi-institutional trials. Compared to

previous AML studies by the GIMEMA,[29] two main factors contributed to the genetic characterization, i.e. central analysis of samples and the possibility of collecting samples during the 5 days of hydroxyurea pretreatment. These factors considerably improved successful karyotyping by overcoming the problem of sampling and shipment over the week-end and by allowing cultures to be set-up on working days. Interestingly, there was no difference in the prevalence and types of karyotypic and molecular alterations between cohorts of patients whose samples were taken at day 0-1 or days 4-5 of hydroxyurea therapy (*unpublished data*).

Through the use of RT-PCR, the presence of major translocations could be searched for in virtually all samples sent for central analysis, thus enabling us to obtain relevant information on patients in whom karyotyping had failed (46/443 or 10% of cases in this study). Both the yield and quality of nucleic acids extracted from samples were good in the vast majority of patients demonstrating the feasibility and convenience of this national network for genetic diagnosis of AML. Indeed, this type of organization had already proved to be efficient in a recently reported study of the GIMEMA on adult acute lymphoblastic leukemia.[30] The lack of sufficient stored material did, however, hamper the analysis of relevant markers, including *FLT3* and *NPM1*, in a high proportion of cases of our series.

A search for submicroscopic (or karyotypically silent) alterations appears increasingly relevant for the clinical management of AML because of their frequency and potential to discriminate prognostic groups, particularly among the large group of patients with a normal karyotype.[5,6,13] Given the need to identify these prognostically significant subtle lesions, the use of other techniques in addition to RT-PCR, such as D-HPLC and multiplex PCR followed by capillary electrophoresis, therefore appear to be necessary for routine molecular characterization of this disease. Alongside the well-standardized strategies for detecting major AML fusion genes, more effort appears to be required to standardize technical approaches for detecting subtle gene mutations whose clinical relevance has emerged more recently. In fact, heterogeneous methods have been reported for mutational analysis of these alterations.[8–19,26-28] *NPM1* mutational status can be predicted with high fidelity by immunohistochemistry on trephine bone marrow biopsies, since the pattern of nuclear *vs.* cytoplasmic staining correlates closely with germline *vs.* mutated *NPM1* gene.[21]

Two important limitations of the present study are the lack of analysis of other subtle gene aberrations that occur with a certain incidence in AML and the low number of cases for which *NPM1* data were available in order to analyze to long-term prognostic value of this gene's mutational status in the multivariate model and its interaction with *FLT3*. Mutations with potential prognostic impact, such as those in *c-Kit*, *CEBPA*, *N-RAS*, *p53*, as well as the prevalence of *MLL*-self fusion were not investigated in this study. However, whereas most investigators would now include detection of *FLT3* and *NPM1* mutations among routine screening (also in light of their higher frequency), no consensus exists on which of the other less prevalent gene alterations should be included in a genetic diagnostic panel for AML. *CEBPA* and *c-Kit* alterations have been reported to bear prognostic relevance according to some recent studies and may carry opposite prognostic significance in the important subset of core binding factor AML.[15,16,18,31-35] In addition, mutations in *CEBPA* have been detected in a sizable proportion of patients with normal karyotype AML and correlated with a favorable outcome.[35,36] Other yet unknown lesions will likely be discovered in the near future in the fraction of normal karyotype AML still undefined at the genetic level.[36] This underscores the relevance of routinely establishing banks of samples from AML patients enrolled in large clinical trials, as recommended by an International Working Group on AML.[5]

In our series, the analysis of outcome based on karyotype confirms the main findings of large co-operative studies including those reported by the MRC[1], SWOG[3], CALGB[4] among others, as reviewed by Estey *et al.*[6] The prognostic significance of karyotype as an independent factor influencing outcome was clearly confirmed both for achievement of complete remission and disease-free survival. With regards to the analysis of factors influencing complete remission, our results on *NPM1* are in line with those reported in most previous series[14–19] and confirm that mutations in this gene confer a better response to induction therapy. The biological reasons underlying this finding are unclear. We did, however, recently observe that *NPM1* status at diagnosis correlates strongly with the rate of spontaneous apoptosis in AML as measured by the Bcl2/Bax ratio (*unpublished observations*), which may, at least in part, explain the more favorable outcome in *NPM1*$^+$ patients.

As far as regards factors influencing post-remission outcome, our results lend further support to the important role of *FLT3* gene status, whereby the presence of ITD in this gene significantly worsened prognosis in the long-term while not influencing initial response. In line with all other studies reported so far,[36-38] *FLT3* gene status overcomes the prognostic role of *NPM1* for long-term outcome, since the presence of *FLT3*-ITD mutations seems to abrogate the favorable impact of *NPM1* mutations. As mentioned above, however, the analysis of the long-term prognostic impact of *NPM1* and its interaction with *FLT3* was hampered in the present study by the low number of cases in each category (only seven patients in the *NPM1*$^-$*FLT3*$^+$ subgroup). Discrepancies with other studies as concerns correlations with outcome[14-19] may be due to relevant differences in the ages of the populations analyzed, as well as to heterogeneity of the therapeutic context.

In conclusion, this study highlights the clinical relevance of a combined multidisciplinary approach to genetic characterization of AML which clearly improves the availability of prognostic determinants in individual patients. Furthermore, the increasing number of molecular markers should, in the future, allow better assessment of response to treatment and monitoring in this disease.

Authorship and Disclosures

FL-C, SA, FM and GS conceived and designed the study; DC, DD, MM, NT, AB, NB and RLS carried out experimental studies to characterize leukemia patients, analyzed and reviewed the data and revised the manuscript; PF, SI, AP, MV were responsible at the GIMEMA data center for data management and statistical analysis. AC, FP, PGP, CM, and BF contributed significantly to plan experimental genetic studies, supervised the results of genetic characterization and critically reviewed the manuscript. All authors approved the final version of the manuscript. FL-C and GS accept direct responsibility for the manuscript. The authors reported no potential conflicts of interest.

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