Clinical impact of *FLT3* mutation load in acute promyelocytic leukemia with t(15;17)/*PML-RARA*

Susanne Schnittger,[1] Ulrike Bacher,[2] Claudia Haferlach,[1] Wolfgang Kern,[1] Tamara Alpermann,[1] and Torsten Haferlach[1]

[1]MLL Munich Leukemia Laboratory, Munich, Germany, and [2]Department for Stem Cell Transplantation, University of Hamburg, Germany

Manuscript received on June 17, 2011. Revised version arrived on August 11, 2011. Manuscript accepted on August 16, 2011.

Correspondence:
Susanne Schnittger, PhD, MLL Munich Leukemia Laboratory, Max-Lebsche-Platz 31, 81377 Munich, Germany.
Phone: international +49.89.99017300.
Fax: international +49.89.99017309.
E-mail: susanne.schnittger@mll.com
URL: www.mll.com

The online version of this article has a Supplementary Appendix.

ABSTRACT

Background
Combined treatment with all-trans-retinoic acid and chemotherapy is extremely efficient in patients with acute promyelocytic leukemia with t(15;17)/*PML-RARA*, but up to 15% of patients relapse.

Design and Methods
To further clarify the prognostic impact of parameters such as *FLT3* mutations, we comprehensively characterized the relation between genetic features and outcome in 147 patients (aged 19.7-86.3 years) with acute promyelocytic leukemia.

Results
Internal tandem duplications of the *FLT3* gene (*FLT3*-ITD) were detected in 47/147 (32.0%) and tyrosine kinase domain mutations (*FLT3*-TKD) in 19/147 (12.9%) patients. *FLT3*-ITD or *FLT3*-TKD mutation status did not have a significant prognostic impact, whereas *FLT3*-ITD mutation load, as defined by a mutation/wild-type ratio of less than 0.5 was associated with trends to a better 2-year overall survival rate (86.7% *versus* 72.7%; P=0.075) and 2-year event-free survival rate (84.5% *versus* 62.1%, P=0.023) compared to the survival rates of patients with a ratio of 0.5 or more. Besides the t(15;17), an additional chromosomal abnormality was detected in 57 of 147 cases and did not show a significant impact on survival. White blood cell counts of 10×10^9/L or less *versus* more than 10×10^9/L were associated with a better 2-year overall survival rate (88.3% *versus* 69.4%, respectively; P=0.015), as was male sex (P=0.040). In multivariate analysis, only higher age had a significant adverse impact.

Conclusions
Prospective trials should further investigate the clinical impact of the *FLT3*-ITD/wild-type mutation load aiming to evaluate whether this parameter might be included in risk stratification in patients with acute promyelocytic leukemia.

Key words: acute promyelocytic leukemia, *FLT3* mutations, *FLT3*-ITD/wt ratio, additional chromosomal alterations, prognosis.

Citation: Schnittger S, Bacher U, Haferlach C, Kern W, Alpermann T, and Haferlach T. Clinical impact of FLT3 *mutation load in acute promyelocytic leukemia with t(15;17)/*PML-RARA. *Haematologica 2011;96(12):1799-1807. doi:10.3324/haematol.2011.049007*

Introduction

Acute promyelocytic leukemia (APL) with the t(15;17)/*PML-RARA* is characterized by fusion of the promyelocytic leukemia (*PML*) and retinoic acid receptor alpha (*RARA*) genes. Administration of all-*trans* retinoic acid in parallel to anthracycline-based chemotherapy for induction therapy results in complete remission rates of more than 90% in newly diagnosed APL with other cases suffering mostly from early death due to hemorrhage. A 5-year overall survival rate of greater than 80% has been reported in adults.[1] Although recent population-based studies suggested a higher early death rate in patients with APL,[2-3] the high remission rates of patients who were able to complete treatment suggest that virtually all *PML-RARA*-positive APL are sensitive to all-*trans* retinoic acid and anthracycline-based chemotherapy. Still, up to 15% of patients with APL develop clinical or molecular relapses.[4-5] A high white blood cell (WBC) count (>10×10^9/L) is supposed to be the main factor associated with relapse. The Sanz score subdivides APL patients according to peripheral blood counts into three risk groups: low (WBC ≤10×10^9/L and platelet count >40×10^9/L), intermediate (WBC ≤10×10^9/L and platelet count ≤40×10^9/L), and high (WBC >10×10^9/L).[4] High-risk APL patients with a WBC count greater than 10×10^9/L were reported to achieve higher complete remission rates and better survival outcomes when cytarabine was included in the chemotherapy regimens, whereas for patients with a WBC count less than 10×10^9/L all-*trans* retinoic acid in combination with anthracyclines might be sufficient.[6-7] Lengfelder *et al.* observed no significant differences in survival outcomes and relapse incidence in 142 APL patients (who all received cytarabine within their induction and consolidation protocols) when they were separated according to a WBC threshold of 10×10^9/L.[5]

The most suitable parameters for risk stratification in APL are, therefore, still under debate. It was discussed whether patients with the French-American-British (FAB) subtype M3v might have higher rates of early death because of hemorrhagic complications when compared to patients with the classical FAB M3 morphology,[8-9] but Tallman *et al.* found that outcomes of patients with the two FAB subtypes did not differ significantly when adjustment for WBC counts or relapse risk scores was made.[10] In fact, the FAB M3v subtype has been associated with higher frequencies of *FLT3*-internal tandem duplications (ITD), which may have a negative prognostic impact.[11] *FLT3*-ITD occur in 12-38% of all APL patients and tyrosine kinase domain (TKD) mutations in 2-20%.[12] The presence of an *FLT3*-ITD was reported to worsen prognosis in APL and to be associated with higher WBC counts by several study groups,[11-12] but others found no adverse prognostic impact of this molecular marker in APL.[13-14] In fact, there were too few patients in many studies in order to be able to draw final conclusions, and it remains unclear whether *FLT3*-ITD mutation status should be incorporated into risk-adapted therapeutic algorithms for APL patients.[12,15] Other parameters, such as *FLT3*-ITD mutation level or length, and *PML-RARA* expression level have been described to be of prognostic relevance in APL.[16]

In this study, we investigated the impact of different pretreatment parameters and the influence of additional cytogenetic or molecular genetic parameters which may predict outcome in 147 adult patients with newly diagnosed APL.

Design and Methods

Patients

The study was based on 147 patients with APL at diagnosis. There were 85 males and 62 females (median age, 53.9 years; range, 19.7 – 86.3 years). One hundred and thirty-six patients had *de novo* APL and 11 had therapy-related disease (t-APL). Bone marrow and/or peripheral blood samples were sent from different hematologic centers between 8/2005-07/2010 to the MLL Munich Leukemia Laboratory for routine diagnostic purposes. Patients were selected according to availability of cytogenetic data and parallel information on molecular genetics including *PML-RARA* and *FLT3*-ITD and *FLT3*-TKD mutation status. All patients received all-*trans* retinoic acid in combination with intensive chemotherapy according to standard study protocols.[1,5,17] This cohort is completely independent of a previously published one.[11] All patients gave informed consent to the use of laboratory data for research studies. The study was approved by the Internal Review Board and adhered to the Declaration of Helsinki. Details and further characterization of the cohort are shown in *Online Supplementary Table S1*.

Cytomorphology

Bone marrow/peripheral blood smears were available in 115 cases and were stained using the May-Grunwald Giemsa method. Cytochemistry was performed for myeloperoxidase and non-specific esterase.[18] Cases were classified as M3/hypergranular type or M3v/microgranular variant according to the FAB[19] and World Health Organization (WHO)[15] classifications.

Cytogenetics

Chromosome banding analysis and fluorescence *in situ* hybridization (FISH) were performed in all 147 cases according to standard techniques.[20]

Molecular genetics

Following extraction of mRNA and cDNA synthesis, the different *PML-RARA* fusion transcripts were detected by reverse transcription polymerase chain reaction (RT-PCR) analysis.[11] *PML-RARA* expression was quantified based on real-time PCR. Expression of *PML-RARA* was given as a ratio defined as %*PML-RARA/ABL1*.[21] Fragment analysis was used to screen and quantify the *FLT3*-ITD mutation load, and determine the length of the ITD (GeneScan, 3130 sequence detection system, ABI, Darmstadt, Germany).[22] The *FLT3*-ITD load was quantified as the ratio of the mutated allele to the wild-type allele (*FLT3*-ITD/wt ratio). Ratios of 1 or more were indicative of complete or partial loss of the wild-type allele (*FLT3*wt) in at least some of the cells. The *FLT3*-TKD were analyzed by LightCycler® (ROCHE, Mannheim, Germany) based melting curve analysis.[23]

Immunophenotyping

Immunophenotyping by multiparameter flow cytometry was done by 5-fold staining in a subgroup of 43 cases (Beckman Coulter, Krefeld, Germany).[24] For subgroup analysis cases were also investigated for CD56 and classified as CD56-positive when at least 20% of the leukemic cell population was positive by comparison with the isotype.

Statistical analysis

Overall survival and event-free survival were calculated according to the Kaplan-Meier method and compared by two-sided log rank tests. Cox regression analysis was performed for survival outcomes with different parameters as covariates. Parameters which were significant in univariate analysis were included into multi-

variate analysis. Dichotomous variables were compared between different groups using the χ^2-test and continuous variables by Student´s T-test. Spearman's rank correlation was used to analyze correlations between continuous parameters. All P values reported are two-sided. SPSS (version 14.0.1, Chicago, IL, USA) was used.

Results

Frequency of FLT3 *mutations*

FLT3 mutations were detected in 65/147 (44.2%) cases: *FLT3*-ITD in 47/147 (32.0%) and *FLT3*-TKD in 19/147 (12.9%). As one case had an *FLT3*-ITD and an *FLT3*-TKD mutation in parallel, patients with mutated *FLT3* status (either ITD or TKD) were called the "*FLT3*-mutated cohort". Patients without any *FLT3* mutation (neither ITD nor TKD) were defined as the "*FLT3*-wt cohort".

FLT3-*internal duplication mutation load*

In the 47 *FLT3*-ITD-positive APL patients the mean *FLT3*-ITD/wt ratio was 0.51 (range, 0.02 - 1.06; median, 0.54). The mean *FLT3*-ITD/wt ratio was significantly lower in APL patients than in a previous cohort of 197 *FLT3*-ITD-positive patients with normal karyotype AML[25] (0.51 *versus* 1.98, respectively; *P*<0.001). The median length of the ITD was 46 bp (range, 15-105 bp) which was in the same range as that in the normal karyotype cohort.

Biological characteristics

Sex ratio, mean age, and history of APL (*de novo* APL or t-APL) did not differ significantly between the different subgroups defined by *FLT3*-ITD or -TKD mutation status (*Online Supplementary Table S1*). The age of patients with t-APL or *de novo* APL did not different significantly (mean age, 51.5 *versus* 54.1 years, respectively). In contrast, the *FLT3*-mutated cohort had higher mean WBC counts when compared to *FLT3*-negative patients (26.8 *versus* 4.7×10^9/L, respectively; *P*<0.001). In more detail, *FLT3*-ITD-positive patients had higher mean WBC counts than either *FLT3*-ITD-negative patients (*P*<0.001) or *FLT3*-wt patients (*P*<0.001). In contrast, there were no significant differences in mean WBC counts between *FLT3*-TKD-mutated patients, *FLT3*-TKD negative patients and *FLT3*-wt cases. The mean platelet count was lower in *FLT3*-mutated patients than in *FLT3*-negative patients (30 *versus* 71×10^9/L, respectively; *P*<0.001), without significant differences between *FLT3*-ITD and *FLT3*-TKD mutated patients. Mean hemoglobin levels were lower in *FLT3*-ITD and *FLT3*-TKD patients than in the *FLT3*-wt cohort (*Online Supplementary Table S1*).

Morphological characterization

According to the FAB classification, 68 cases were classified as M3 (59.1%) and 47 as M3v (40.9%) (FAB subtypes were known for 115 cases). The proportion of M3v subtype was higher in the *FLT3*-ITD positive cohort than in the *FLT3*-ITD negative cases (25/34; 73.5% *versus* 22/81; 27.2%; *P*<0.001). Moreover, the M3v subtype was more frequent in *FLT3*-ITD-positive cases than in *FLT3*-TKD-positive cases (25/34; 73.5% *versus* 5/16; 31.3%; *P*=0.004) (*Online Supplementary Table S1*). The AML M3v patients had significantly higher mean WBC counts when compared to the M3 patients (34.5×10^9/L *versus* 4.5×10^9; *P*<0.001).

PML-RARA *transcript types*

The most frequent *PML-RARA* transcript type was bcr1 which was detected in 89 cases (60.5%), followed by bcr3 in 52 cases (35.4%). The bcr2 type was detected in 6 cases (4.1%) only. The distribution of the different bcr transcript types for *FLT3* mutations was heterogeneous (*P*<0.001): the bcr1 transcript type was correlated with *FLT3*-wt status (63/82; 76.8% in *FLT3*-wt *versus* 26/65; 40.0% in *FLT3*-mutated patients), while bcr3 was more frequent than bcr1 or bcr2 in *FLT3*-mutated patients than in *FLT3*-wt patients (38/65; 58.5% *versus* 14/82; 17.1%; *P*<0.001). *FLT3*-ITD-positive patients more frequently showed bcr3 breakpoints than did *FLT3*-TKD-positive patients (32/47; 68.1% *versus* 6/19; 31.6%; *P*=0.011) (*Online Supplementary Table S1*).

PML-RARA *expression*

The mean ratio of %*PML-RARA/ABL1* expression was 25.8; the median was 18.5 and there was a wide range of 0.6 – 96.7. No significant differences of mean *PML-RARA* expression levels were observed between the different molecular subgroups defined by the different *FLT3* mutations (*Online Supplementary Table S1*). The mean *PML-RARA/ABL1* expression was higher in patients with WBC counts above 1.0×10^9/L than in those with WBC counts of 1.0×10^9/L or less (33.6 *versus* 23.4; *P*=0.031). When peripheral WBC counts and *PML-RARA/ABL1* were considered as continuous parameters by Spearman's analysis, they were significantly correlated (*P*=0.042). Mean *PML-RARA/ABL1* expression was lower in cases with bcr1 breakpoints than in patients with bcr2 and bcr3 combined (23.2 *versus* 30.3; *P*=0.059).

Additional chromosomal alterations

Additional chromosomal abnormalities (ACA) were detected in 57 patients (38.8% of the total cohort) without significant differences between the *FLT3*-mutated and the *FLT3*-wt patients. There was a trend to a higher rate of ACA in the *FLT3*-TKD-positive cohort than in the *FLT3*-TKD-negative patients (11/19; 57.9% *versus* 46/128; 35.9%; *P*=0.080), and *FLT3*-TKD-positive patients had ACA more frequently than had *FLT3*-ITD-positive patients (11/19; 57.9% *versus* 15/47; 31.9%; *P*=0.05).

Recurrent ACA (+8, 9q-, 17q-alterations) were found in 41 cases (27.9% of the total cohort; 71.9% of all ACA). The most frequent abnormality was trisomy 8 or gain of 8q (n=24); followed by 17q alterations (n=11) and 9q deletions (n=6). Infrequent and non-recurrent ACA were detected in 16 cases (10.9% of the total cohort; 28.1% of patients with ACA) (*Online Supplementary Table S1* and Table 1). In detail these abnormalities were additional translocations (n=7), insertions or deletions (n=4), a complex karyotype (n=1), other alterations (n=1) and –Y (n=3).

Chromosomal gains and losses due to ACA are depicted in *Online Supplementary Figure S1A* and breakpoints from ACA in *Online Supplementary Figure S1B* according to CyDAS.

Based on a report by Slack *et al.*, who described a significant association of the presence of ACA and the *PML-RARA* S isoform,[26] we compared the frequencies of the different bcr transcript types depending on the presence of ACA, but did not detect any significant correlations (*data not shown*).

Survival analysis

No impact of additional cytogenetic abnormalities on survival

The presence of ACA had no significant impact on survival outcomes when compared to the outcomes of patients with a sole t(15;17)/*PML-RARA* (Table 2).

Impact of FLT3 mutation status and mutation load on survival

The *FLT3*-ITD (Figure 1A,B) or *FLT3*-TKD mutation status (positive *versus* negative) had no significant impact on survival outcomes. The median overall and event-free survival of patients with *FLT3*-ITD, *FLT3*-TKD, and *FLT3*-wt also did not differ significantly (Figure 1C,D; Table 2). In contrast, when taking the mutation load expressed as *FLT3*-ITD/wt ratio into account, patients with a *FLT3*-ITD/wt ratio less than 0.5 (meaning *FLT3*-ITD-positive patients with a ratio <0.5 and *FLT3*-ITD-negative patients combined) showed better 2-year overall survival (86.7% *versus* 72.7%; *P*=0.075) (Figure 1E) and event-free survival rates (84.5% *versus* 62.1%; *P*=0.023) (Figure 1F) than those with a ratio of 0.5 or more. Results for 0.25 and 0.75 thresholds are shown in Table 2, demonstrating that the 0.25 threshold had no effect on survival. Thus, only an *FLT3*-ITD load of 0.5 or more had an adverse impact on survival in patients with *PML-RARA* positive APL.

Subsequently, we defined the influence of *FLT3* mutations on induction death, i.e. within the first 30 days following the start of therapy. The 30-day overall survival rate of *FLT3*-ITD mutation carriers and of patients with a negative mutation status did not differ significantly (85.7% *versus* 91.1%; Figure 2A). The *FLT3*-TKD mutation also had no significant impact on the 30-day overall survival rate (89.5% *versus* 88.9%; *P*=n.s.). Likewise, the presence of any of the *FLT3* mutation types did not significantly affect the 30-day overall survival rate when compared to that in *FLT3* mutation-negative patients (91.8% *versus* 86.4%; *P*=n.s.). However, using a threshold of the mutation/wild-type of 0.5, the 30-day overall survival rate was significantly better for those with a *FLT3*-ITD/wt ratio less than 0.5 compared to those with a ratio of 0.5 or above (91.7% *versus* 78.3%; *P*=0.039; Figure 2B).

No impact of immunophenotype on survival

Based on previous observations that expression of CD56 was correlated with higher relapse risk in APL,[27-28] we evaluated this parameter in 43 patients with available immunophenotypic data. CD56 expression greater than 20% was seen in 6/43 cases (14.0%) which was comparable to the frequency in the mentioned studies. In this small subcohort the 2-year overall and event-free survival rates did not differ significantly between patients with and without CD56 expression (75.0% *versus* 90.0% and 75.0 *versus* 90.9%, *P*=n.s. respectively; Table 2).

Impact of other parameters on survival

Survival data were available for 132/147 cases of the total cohort. The 2-year overall survival rate was 84.3% and the median follow-up was 767 days. Male patients had better 2-year overall survival rates compared to female patients (92.7% *versus* 78.3%; *P*=0.040) (Figure 3A, Table 2). No significant difference was found regarding 2-year event-free survival (Figure 3B, Table 2). M3 *versus* M3v FAB subtypes, history of APL (*de novo* APL *versus* t-APL), and the different *PML-RARA* fusion transcript types (bcr1-3) had no significant impact on survival outcomes.

We further separated patients according to the Sanz score,[4] based on WBC and platelet counts. Patients with WBC counts of 10×10^9/L or below had a better 2-year overall survival rate than those with WBC counts above 10×10^9/L (88.3% *versus* 69.4%; *P*=0.015) (Figure 4A) and a better 2-year event-free survival rate (85.7% *versus* 60.0%; *P*=0.006) (Figure 4B). Patients with a platelet count above 40×10^9/L had a better 2-year overall survival rate than patients with a platelet count of 40×10^9/L or below (92.7% *versus* 78.1%; *P*=0.060) (Figure 4C), and a better 2-year event-free survival rate (84.0% *versus* 76.5%; *P*=n.s.) (Figure 4D).

Table 1. Karyotypes of 13 cases from the subgroup of infrequent additional chromosomal abnormalities (the three cases with -Y as sole abnormality are not shown).

Case	Karyotype
1	45,XX,t(1;9)(p13;q22),der(2)t(2;9)(q11;p11),der(9;12)(q10;q10)t(2;9)(q11;q34),t(15;17)(q22;q12)[20]
2	46,XY,der(13)t(13;15)(q34;q11)t(13;15)(q?;q22),der(15)t(15;17)(q22;q12),der(17)(17pter->17q12::15q22->15q?::13q?->13q?::17q12->17q12::15q21->15q?::13q?->13qter).ish der(13;15)dic(13;15)(PML+,RARA+),der(15)t(15;17)(PML+,RARA+),der(17)(PML+,RARA+,PML+,RARA+[9],46,XY[9]
3	46,XX,del(1)(p22p32),t(15;17)(q22;q12)[20]
4	46,XX,der(15)t(15;17)(q26;?)ins(17;15)(q12;q22q22),r(17)ins(17;15)(?;q?q?)[19]/46,XX[1]
5	46,XX,del(15)(q22),der(17)inv(17)(p12q12)ins(17;15)(p12;q22q23).ish del(15)(q22)(PML+,RARA+),der(17)inv(17)(p12q12)ins(17;15)(p12;q22q23)(RARA+,PML+,RARA+)[16]/46,XX[4]
6	46,XY,ins(11;3)(q13;q21q26),t(15;17)(q22;q12)[12]/46,XY[3]
7	46,XX,t(4;12)(q23;q24),der(15)t(15;17)(q22;q12),der(17)t(17;19)(q12;q13),der(19)t(17;19)(q12;q13)t(15;17)(q22;q12)[19]/46,XX[1]
8	46,XY,t(15;17)(q22;q12)[3]/46,XY,t(2;20)(q35;p13),t(15;17)(q22;q12)[5]/46,XY[20]
9	46,XX,t(15;17)(q22;q12)[10]/46,XX,t(5;21)(p14;q22),t(15;17)(q22;q12)[5]/46,XX[5]
10	46,XY,der(8)t(8;17)(q11;q23),der(15)t(15;17)(q22;q21)t(8;17)(q11;q23),der(17)t(15;17)(q22;q21)[2]/45,X,-Y,der(8)t(8;17)(q11;q23),der(15)t(15;17)(q22;q21)t(8;17)(q11;q23),der(17)t(15;17)(q22;q21)[18]
11	46,XX,der(7)t(7;13)(q31;q21),t(15;17)(q22;q12)[19]/46,XX[2]
12	46,XY,der(12)t(12;15)(q24;q24),der(15)t(15;17)(q22;q12),der(17)t(15;17)(q22;q12)t(12;15)(q24;q24)[12]/46,XY[3]
13	46,XX,der(1)t(1;15)(q12;q22)t(1;17)(p12;q12),der(15)t(1;15)(q12;q22),der(17)t(1;17)(p12;q12).ish der(1)t(1;15)t(1;17)(PML+,RARA-),der(15)t(1;15)(PML+,RARA+),der(17)t(1;17)(PML-,RARA+)[20]

Uni- and multivariate analysis of survival

The following parameters were tested in univariate analysis with respect to their influence on survival outcomes: gender, WBC count (threshold: 10×10^9/L) and platelet count (threshold: 40×10^9/L) - both limits set according to the Sanz score),[4] hemoglobin level, age as a continuous variable, *de novo* APL *versus* t-APL, FAB M3 *versus* M3v subtype, *FLT3*-ITD mutant status, *FLT3*/ITD/wt ratios of 0.25 or more and of 0.5 or more, *FLT3*-TKD mutant status and the presence of ACA. A negative influence on overall survival was documented for female sex (P=0.051), higher age (P=0.001), and *FLT3*-ITD/wt ratio of 0.5 or more (P=0.084). WBC counts greater than 10×10^9/L (P=0.021) and platelet counts less than 40×10^9/L (according to the Sanz score; P=0.074) were associated with worse overall survival. In contrast, the other parameters listed above (including *FLT3*-ITD and *FLT3*-TKD mutant status or *FLT3*/ITD/wt ratio with a threshold of ≥0.25) had no significant impact on overall survival. In multivariate analysis for overall survival, significance was reached only for age as a continuous parameter (P<0.001). There was borderline significance for gender (P=0.055) and WBC counts greater than 10×10^9/L (P=0.059).

For event-free survival, significant parameters in univariate analysis were WBC counts greater than 10×10^9/L (P=0.009), age (P<0.001), and *FLT3*-ITD/wt ratio of 0.5 or more (P=0.029). In multivariate analysis for event-free survival, only age as a continuous parameter (P<0.001) was statistically significant (Table 3). We further divided patients into subgroups under 60 years old and those 60 years or more and found the same correlations as in the combined group with respect to age (*data not shown*).

Table 2. Kaplan-Meier estimates for survival outcomes according to different biological, morphological, cytogenetic, and molecular genetic parameters.

Parameter	2-years OS (%)	P	2-years EFS (%)	P
Gender				
female (n=85)	78.3	0.040	78.5	n.s.
male (n=62)	92.7		83.7	
FAB subtypes				
M3 (n=68)	86.3	n.s.	82.7	n.s.
M3v (n=47)	74.1		70.1	
APL history				
de novo (n=136)	84.6	n.s.	80.5	n.s.
therapy-related (n=11)	80.0		80.0	
WBC count (according to Sanz score)[4]				
≤10×10^9/L (n=96)	88.3	0.015	85.7	0.006
>10×10^9/L (n=31)	69.4		60.0	
Platelet count (according to Sanz score)[4]				
>40×10^9/L (n=45)	92.7	0.060	84.0	n.s.
≤40×10^9/L (n=69)	78.1		76.5	
FLT3 mutation status				
FLT3-ITD (n=46)	80.0	n.s.	77.3	n.s.
FLT3-TKD (n=18)	80.2		80.2	
FLT3 wild-type (n=82)	87.6		84.3	
FLT3-ITD				
positive (n=47)	80.5	n.s.	74.9	n.s.
negative (n=100)	86.1		83.4	
FLT3-TKD				
positive (n=19)	81.5	n.s.	74.1	n.s.
negative (n=128)	84.9		81.8	
≥1 *FLT3* mutation				
yes (n=65)	80.3	n.s.	76.3	n.s.
no (n=82)	87.6		84.3	
FLT3-ITD/wt ratio Threshold 0.25				
≥0.25 (n=36)	80.4	n.s.	72.5	n.s.
<0.25 (n=111)	85.6		83.2	
Threshold 0.5				
≥0.5 (n=26)	72.7	0.075	62.1	0.023
<0.5 (n=121)	86.7		84.5	
Threshold 0.75				
≥0.75 (n=11)	62.2	0.080	64.8	n.s.
<0.75 (n=136)	85.8		81.8	
Breakpoints				
bcr1 (n=89)	87.5	n.s.*	85.9	n.s.*
bcr2 (n=6)	83.3		66.7	
bcr3 (n=52)	78.7		73.8	
Additional chromosomal abnormalities				
presence of ACA (n=57)	86.5	n.s.	82.0	n.s.
no ACA (n=90)	82.7		79.4	
CD56 expression (defined by positivity of ≥20% of the leukemic cell population)				
positive (n=6)	75.0	n.s.	75.0	n.s.
negative (n=37)	90.0		90.9	

**for comparisons of all three different subgroups; OS: overall survival; EFS: event-free survival.*

Discussion

In current study protocols patients with APL are assigned to different therapeutic regimens (e.g. with regard to application of cytarabine) according to peripheral WBC counts,[6-7] but it remains under debate whether other parameters should be included in risk stratification in patients with this subtype of acute myeloid leukemia. We, therefore, investigated the clinical impact of *FLT3*-ITD and *FLT3*-TKD, and other parameters in 147 patients with APL at diagnosis. First, we were able to confirm the high frequency of *FLT3* mutations in APL,[12,29] as 44.2% of patients had either an ITD or TKD or both (one case). The presence of *FLT3*-ITD was associated with specific characteristics, i.e. higher WBC counts, lower platelet counts, a preponderance of the M3v subtype, and of the bcr3 *PML-RARA* fusion transcript (P<0.001 for all parameters) when compared to ITD-negative patients, confirming findings of other study groups.[29-30] *FLT3*-TKD mutated patients differed from *FLT3*-TKD negative cases by having lower platelet counts (P=0.006) and a higher frequency of ACA (P=0.080).

Subsequently, we evaluated the prognostic impact of *FLT3*-ITD in our cohort of patients with APL. The presence of *FLT3*-ITD *per se* had no significant impact on survival outcomes, but a *FLT3*-ITD/wt ratio of 0.5 or more was prognostically adverse in univariate analysis (event-free survival, P=0.029; overall survival, P=0.084) compared to a low *FLT3*-ITD burden below 0.5% or to wild-type *FLT3* status. A high *FLT3*-ITD burden is, therefore, clinically relevant in patients with APL, and may contribute to explaining the differences in survival in patients with t(15;17)/*PML-RARA*. We were further able to confirm the 0.5 threshold for the *FLT3*-ITD/wt ratio to be prognostically relevant specifically in the induction period within the first 30 days from the start of therapy. Consistent with our results, Chillon *et al.* described an increasing *FLT3*-

ITD/wt ratio of greater than 0.66 to be related with shorter 5-year relapse-free survival in patients with APL, whereas overall survival was only weakly influenced by *FLT3*-ITD mutation status.[16] Most other studies focused on the *FLT3*-ITD mutation status in APL only and did not investigate the clinical impact of a certain *FLT3*-ITD mutation load. Gale *et al.* found higher rates of induction death in patients with mutant *FLT3*, but no significant adverse effect of *FLT3*-ITD mutated status on overall survival or relapse risk of patients with APL.[29] This was similar to the findings of Au *et al.* who described a clearly adverse impact of the *FLT3*-ITD on the achievement of remission (*P*=0.06), but failed to demonstrate a significant impact of the *FLT3*-ITD mutation status on disease-free survival in APL patients.[31] Noguera *et al.* reported a non-significant trend for worse disease-free survival or relapse risk in

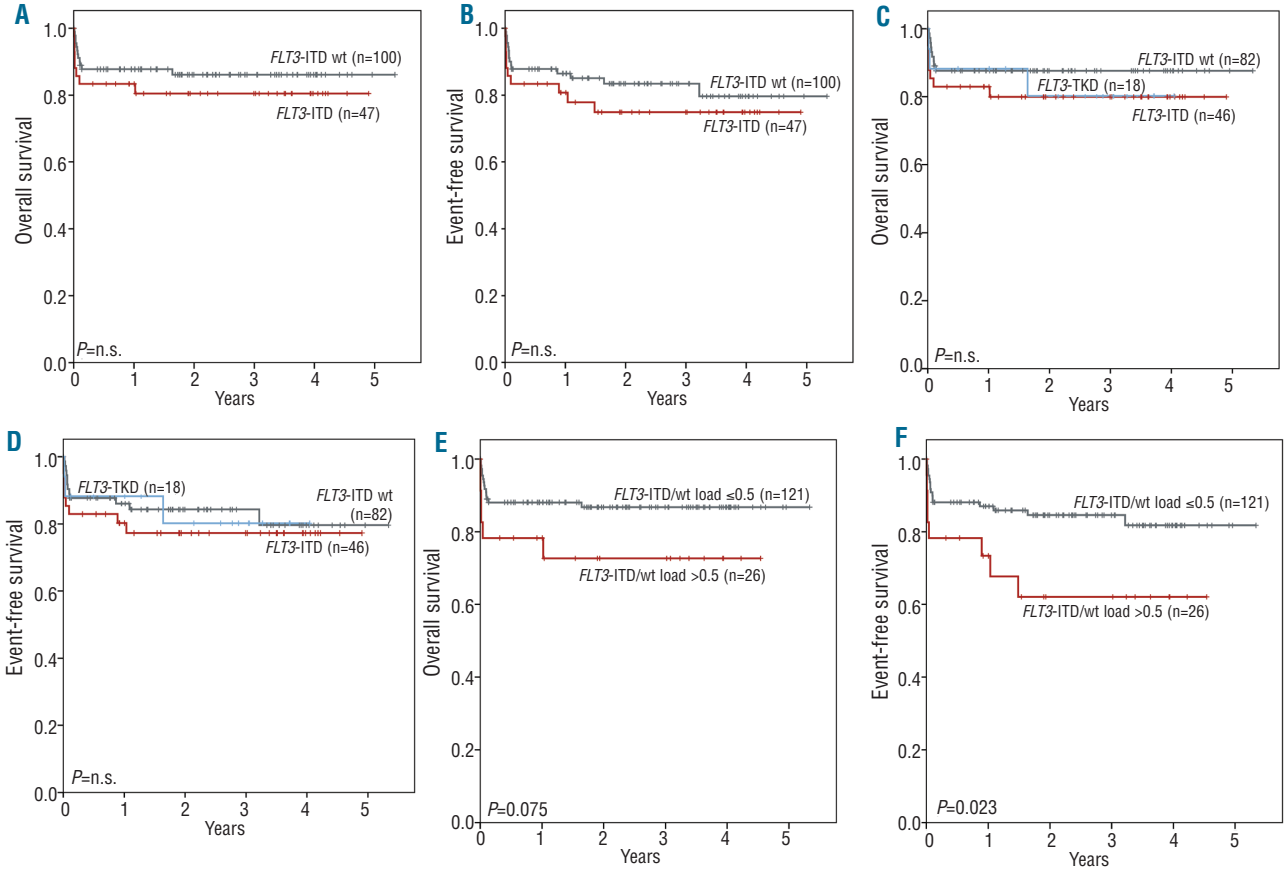

Figure 1. (A-B) Overall survival and event-free survival of *FLT3*-ITD-positive patients *versus* *FLT3*-ITD-negative patients. (C-D) Overall survival and event-free survival of patients with *FLT3*-ITD, *FLT3*-TKD, and *FLT3* wild-type. (E-F) Overall survival and event-free survival of patients with a *FLT3*-ITD/wt load ≥0.5 *versus* those with an ITD load <0.5.

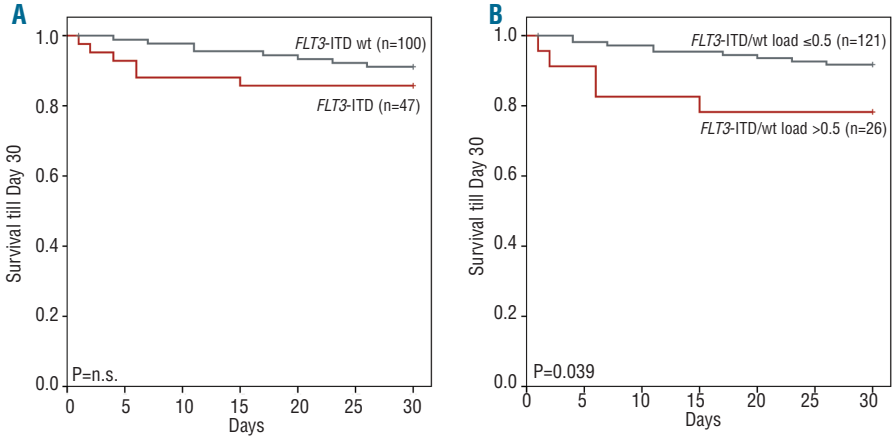

Figure 2. (A) Overall survival of the APL patients within the first 30 days after starting therapy comparing those with *FLT3*-ITD and without. (B) Comparison of patients with *FLT3*-ITD ratio ≥0.5 and below this threshold.

patients with *FLT3*-ITD-positive APL.[30] In accordance with Chillon *et al.*[16] and previous data from our group[11] we found no significant impact of the *FLT3*-TKD mutant status in our APL cohort. In contrast, Gale *et al.* described a worse overall survival, of borderline statistical significance, in APL patients with *FLT3*-TKD (*P*=0.05).[29]

The mean *FLT3*-ITD/wt ratio was significantly lower (P<0.001) in APL patients than in a cohort of 197 patients with *FLT3*-ITD-positive normal karyotype acute myeloid leukemia[25] which we had previously analyzed. It remains speculative whether this aspect could contribute to explain the weaker prognostic impact of the *FLT3*-ITD in

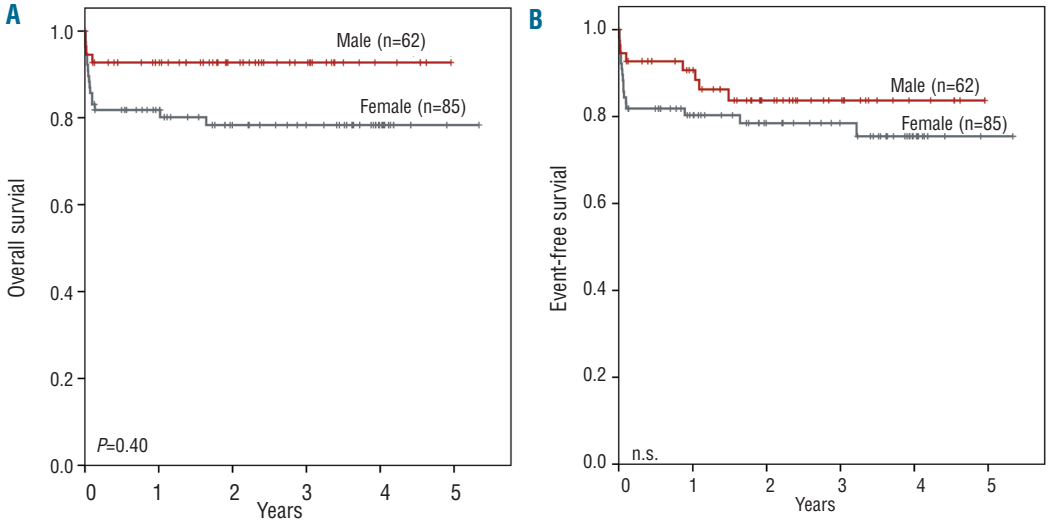

Figure 3. (A) Overall survival and (B) event-free survival of male *versus* female patients.

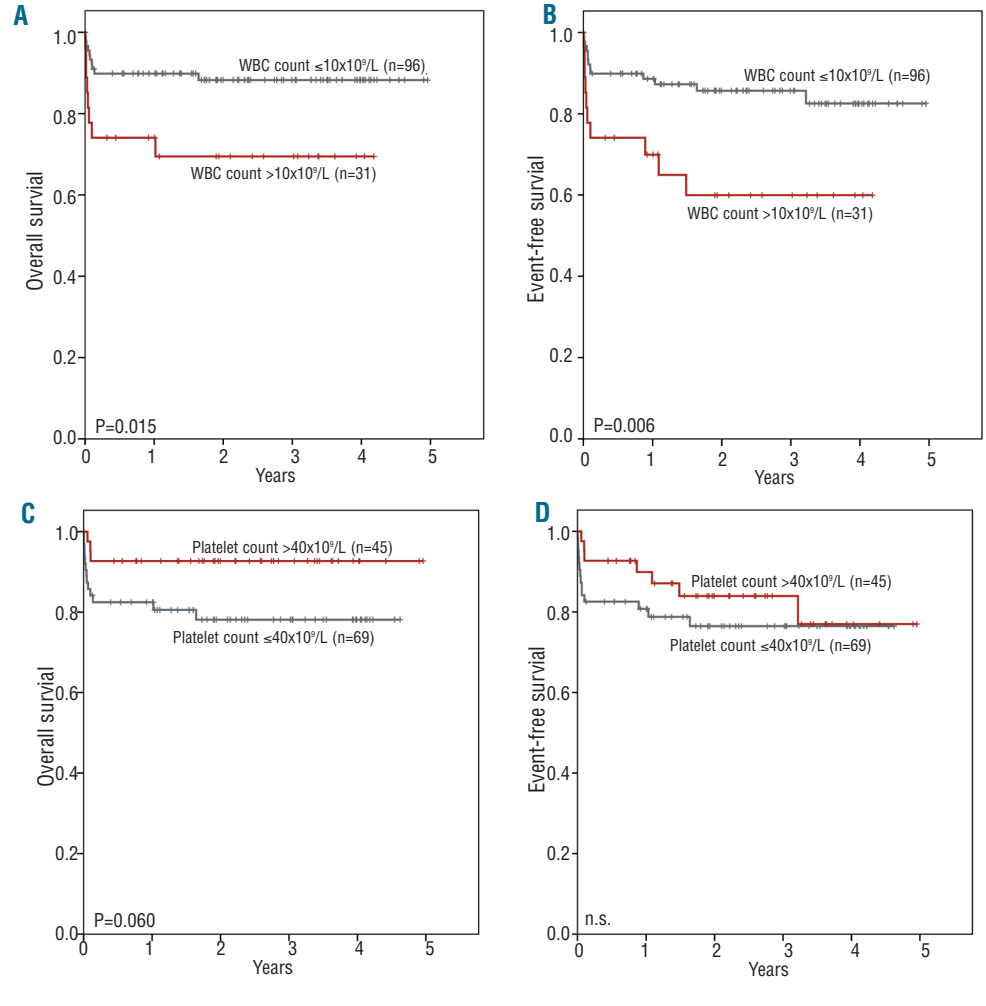

Figure 4. (A) Overall survival and (B) event-free survival according to WBC count thresholds following the Sanz score[4] (≤10x10⁹/L *versus* >10x10⁹/L). (C) Overall survival and (D) (EFS) according to platelet count thresholds following the Sanz score[4] (>40x10⁹/L *versus* ≤40x10⁹/L)

patients with APL than in those with normal karyotype acute myeloid leukemia.

The frequency of ACA in our cohort (38.8%) was similar to that in previous studies.[32-33] In general we did not find that ACA had a significant impact on prognosis, which is in accordance with our previous study in 50 patients with APL.[34] Cervera *et al.* from the PETHEMA study group found that ACA were associated with higher rates of coagulopathy, lower platelet counts, and higher relapse risk scores in APL, but remission rates of patients with and without ACA were nearly the same, being 90% and 91%, respectively. In their study, no specific ACA was an independent risk factor for relapse.[33] The European APL group also did not find that ACA had a significant impact on prognosis,[32] and Slack *et al.* found no difference in overall survival between APL patients with an isolated t(15;17)/*PML-RARA* and patients with ACA.[26] Accordingly, we were not able to show a significant influence of ACA on outcome in patients with APL.

Finally, we were able to confirm the prognostic power of a WBC count threshold of 10×10^9/L, as introduced by Sanz *et al.*, to separate different risk groups.[4] Other than that, male gender showed a borderline significance for better overall survival in univariate and multivariate analyses, and age as a continuous parameter was a strong independent prognostic parameter ($P<0.001$ for overall and event-free survival in multivariate analysis) in our study. Survival outcomes of patients with *de novo* APL or t-APL did not differ significantly in our study, which was in accordance with the results of a previous study on 106 patients with t-APL by Beaumont *et al.*[35] Nevertheless, given that for all subtypes of AML, clinical outcomes of patients with therapy-related disease were found to be worse than those of patients with *de novo* acute myeloid leukemia, as recently described in a large cohort including 200 patients with therapy-related acute myeloid leukemia and 2653 with *de novo* acute myeloid leukemia by the AMLSG Study Group,[36] the potential clinical decisions in t-APL should be further studied before definite conclusions are drawn.

In conclusion, we were not able to show a significant impact of the *FLT3*-ITD mutation status *per se* on prognosis in APL, but a higher *FLT3*-ITD/wt ratio (≥0.5%) was prognostically adverse. Prospective trials should further investigate the clinical impact of the *FLT3*-ITD/wt mutation load aiming to evaluate whether this parameter might be included in risk stratification in APL.

Table 3. Results of univariate and multivariate analyses according to Cox regression analysis (* thresholds according to the Sanz score[4]).

Parameter	*P* value (hazard ratio)			
	Overall survival		Event-free survival)	
	univariate	multivariate	univariate	multivariate
Gender	0.051 (0.336)	0.055 (0.288)	n.s.	-
WBC count ≤10×10^9/L*	0.021 (2.999)	0.059 (3.417)	0.009 (2.983)	n.s.
Platelet count >40×10^9/L*	0.074 (0.319)	n.s.	n.s.	-
Hemoglobin level (g/dL)	n.s.	-	n.s.	-
Age (as a continuous parameter)	0.001 (1.4955**)	<0.001 (1.745**)	<0.001 (1.481**)	<0.001 (1.5493**)
De novo versus t-APL	n.s.	-	n.s.	-
FAB M3 *versus* M3v subtype	n.s.	-	n.s.	-
bcr subtype	n.s.	-	n.s.	-
FLT3-ITD mutant status	n.s.	-	n.s.	-
FLT3/ITD/wt ratio ≥0.25	n.s.	-	n.s.	-
FLT3/ITD/wt ratio ≥0.5	0.084 (2.325)	n.s.	0.029 (2.552)	n.s.
FLT3-TKD mutant status	n.s.	-	n.s.	-
Presence of ACA	n.s.	-	n.s.	-

** *per 10 years of increase.*

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

Financial and other disclosures provided by the authors using the ICMJE (www.icmje.org) Uniform Format for Disclosure of Competing Interests are also available at www.haematologica.org.

References

1. Sanz MA, Grimwade D, Tallman MS, Lowenberg B, Fenaux P, Estey EH, et al. Management of acute promyelocytic leukemia: recommendations from an expert panel on behalf of the European LeukemiaNet. Blood. 2009;113(9):1875-91.
2. Lehmann S, Ravn A, Carlsson L, Antunovic P, Deneberg S, Mollgard L, et al. Continuing high early death rate in acute promyelocytic leukemia: a population-based report from the Swedish Adult Acute Leukemia Registry. Leukemia. 2011;25(7):1128-34.
3. Park JH, Qiao B, Panageas KS, Schymura MJ, Jurcic JG, Rosenblat TL, et al. Early death rate in acute promyelocytic leukemia remains high despite all-trans retinoic acid. Blood. 2011;118(5):1248-54.
4. Sanz MA, Lo Coco F., Martin G, Avvisati G, Rayon C, Barbui T, et al. Definition of relapse risk and role of nonanthracycline drugs for consolidation in patients with acute promyelocytic leukemia: a joint study of the PETHEMA and GIMEMA cooperative groups. Blood. 2000;96(4): 1247-53.
5. Lengfelder E, Haferlach C, Saussele S, Haferlach T, Schultheis B, Schnittger S, et al. High dose ara-C in the treatment of newly diagnosed acute promyelocytic leukemia: long-term results of the German AMLCG. Leukemia. 2009;23(12):2248-58.
6. Ades L, Sanz MA, Chevret S, Montesinos P, Chevallier P, Raffoux E, et al. Treatment of newly diagnosed acute promyelocytic leukemia (APL): a comparison of French-Belgian-Swiss and PETHEMA results. Blood. 2008;111(3):1078-84.
7. Sanz MA, Lo-Coco F. Modern approaches to treating acute promyelocytic leukemia. J Clin Oncol. 2011;29(5):495-503.
8. Cunningham I, Gee TS, Reich LM, Kempin SJ, Naval AN, Clarkson BD. Acute promyelocytic leukemia: treatment results during a decade at Memorial Hospital. Blood. 1989;73(5):1116-22.
9. Bassan R, Battista R, Viero P, d'Emilio A, Buelli M, Montaldi A, et al. Short-term treatment for adult hypergranular and microgranular acute promyelocytic leukemia. Leukemia. 1995;9(2):238-43.
10. Tallman MS, Kim HT, Montesinos P, Appelbaum FR, de la Serna.J., Bennett JM, et al. Does microgranular variant morphology of acute promyelocytic leukemia independently predict a less favorable outcome compared with classical M3 APL? A joint study of the North American Intergroup and the PETHEMA Group. Blood. 2010; 116(25):5650-9.
11. Kuchenbauer F, Schoch C, Kern W, Hiddemann W, Haferlach T, Schnittger S.

Impact of FLT3 mutations and promyelocytic leukaemia-breakpoint on clinical characteristics and prognosis in acute promyelocytic leukaemia. Br J Haematol. 2005;130 (2):196-202.
12. Beitinjaneh A, Jang S, Roukoz H, Majhail NS. Prognostic significance of FLT3 internal tandem duplication and tyrosine kinase domain mutations in acute promyelocytic leukemia: a systematic review. Leuk Res. 2010;34(7):831-6.
13. Kiyoi H, Naoe T, Yokota S, Nakao M, Minami S, Kuriyama K, et al. Internal tandem duplication of FLT3 associated with leukocytosis in acute promyelocytic leukemia. Leukemia Study Group of the Ministry of Health and Welfare (Kohseisho). Leukemia. 1997;11(9):1447-52.
14. Kainz B, Heintel D, Marculescu R, Schwarzinger I, Sperr W, Le T, et al. Variable prognostic value of FLT3 internal tandem duplications in patients with de novo AML and a normal karyotype, t(15;17), t(8;21) or inv(16). Hematol J. 2002;3(6):283-9.
15. Arber B, Brunning R, Le Beau M, Falini B. Acute myeloid leukemia with recurrent genetic abnormalities. In: Swerdlow S, Campo E, Lee Harris N, Jaffe E, Pileri S, Stein H et al. WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues, 4th ed. Lyon: IARC Press, 2008:110-23.
16. Chillon MC, Santamaria C, Garcia-Sanz R, Balanzategui A, Maria ES, Alcoceba M, et al. Long FLT3 internal tandem duplications and reduced PML-RARalpha expression at diagnosis characterize a high-risk subgroup of acute promyelocytic leukemia patients. Haematologica. 2010;95(5):745-51.
17. Avvisati G, Lo-Coco F, Paoloni FP, Petti MC, Diverio D, Vignetti M, et al. AIDA 0493 protocol for newly diagnosed acute promyelocytic leukemia: very long-term results and role of maintenance. Blood. 2011;117(18):4716-25.
18. Löffler H, Raststetter J, Haferlach T. Atlas of Clinical Hematology, 6th ed. Berlin: Springer, 2004.
19. Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DA, Gralnick HR, et al. Proposed revised criteria for the classification of acute myeloid leukemia. A report of the French-American-British Cooperative Group. Ann Intern Med. 1985;103(4):620-5.
20. Schoch C, Schnittger S, Bursch S, Gerstner D, Hochhaus A, Berger U, et al. Comparison of chromosome banding analysis, interphase- and hypermetaphase-FISH, qualitative and quantitative PCR for diagnosis and for follow-up in chronic myeloid leukemia: a study on 350 cases. Leukemia. 2002;16(1):53-9.
21. Schnittger S, Weisser M, Schoch C, Hiddemann W, Haferlach T, Kern W. New score predicting for prognosis in PML-RARA+, AML1-ETO+, or CBFBMYH11+ acute myeloid leukemia based on quantification of fusion transcripts. Blood. 2003;102(8):2746-55.
22. Thiede C, Steudel C, Mohr B, Schaich M, Schäkel U, Platzbecker U, et al. Analysis of FLT3-activating mutations in 979 patients with acute myelogenous leukemia: association with FAB subtypes and identification of subgroups with poor prognosis. Blood. 2002;99(12):4326-35.
23. Bacher U, Haferlach C, Kern W, Haferlach T, Schnittger S. Prognostic relevance of FLT3-TKD mutations in AML: the combination matters-an analysis of 3082 patients. Blood. 2008;111(5):2527-37.
24. Kern W, Voskova D, Schoch C, Hiddemann W, Schnittger S, Haferlach T. Determination of relapse risk based on assessment of minimal residual disease during complete remission by multiparameter flow cytometry in unselected patients with acute myeloid leukemia. Blood. 2004;104 (10):3078-85.
25. Schnittger S, Bacher U, Kern W, Alpermann T, Haferlach C, Haferlach T. Prognostic impact of FLT3-ITD load in NPM1 mutated acute myeloid leukemia. Leukemia. 2011; 25(8):1297-304.
26. Slack JL, Arthur DC, Lawrence D, Mrozek K, Mayer RJ, Davey FR, et al. Secondary cytogenetic changes in acute promyelocytic leukemia--prognostic importance in patients treated with chemotherapy alone and association with the intron 3 breakpoint of the PML gene: a Cancer and Leukemia Group B study. J Clin Oncol. 1997;15(5):1786-95.
27. Ferrara F, Morabito F, Martino B, Specchia G, Liso V, Nobile F, et al. CD56 expression is an indicator of poor clinical outcome in patients with acute promyelocytic leukemia treated with simultaneous all-trans-retinoic acid and chemotherapy. J Clin Oncol. 2000;18(6):1295-300.
28. Montesinos P, Rayon C, Vellenga E, Brunet S, Gonzalez J, Gonzalez M, et al. Clinical significance of CD56 expression in patients with acute promyelocytic leukemia treated with all-trans retinoic acid and anthracycline-based regimens. Blood. 2011;117(6): 1799-805.
29. Gale RE, Hills R, Pizzey AR, Kottaridis PD, Swirsky D, Gilkes AF, et al. Relationship between FLT3 mutation status, biologic characteristics, and response to targeted therapy in acute promyelocytic leukemia. Blood. 2005;106(12):3768-76.
30. Noguera NI, Breccia M, Divona M, Diverio D, Costa V, De Santis S., et al. Alterations of the FLT3 gene in acute promyelocytic leukemia: association with diagnostic characteristics and analysis of clinical outcome in patients treated with the Italian AIDA protocol. Leukemia. 2002;16(11):2185-9.
31. Au WY, Fung A, Chim CS, Lie AK, Liang R, Ma ES, et al. FLT-3 aberrations in acute promyelocytic leukaemia: clinicopathological associations and prognostic impact. Br J Haematol. 2004;125(4):463-9.
32. de Botton S., Chevret S, Sanz M, Dombret H, Thomas X, Guerci A, et al. Additional chromosomal abnormalities in patients with acute promyelocytic leukaemia (APL) do not confer poor prognosis: results of APL 93 trial. Br J Haematol. 2000;111 (3):801-6.
33. Cervera J, Montesinos P, Hernandez-Rivas JM, Calasanz MJ, Aventin A, Ferro MT, et al. Additional chromosome abnormalities in patients with acute promyelocytic leukemia treated with all-trans retinoic acid and chemotherapy. Haematologica. 2010; 95(3):424-31.
34. Schoch C, Haase D, Haferlach T, Freund M, Link H, Lengfelder E, et al. Incidence and implication of additional chromosome aberrations in acute promyelocytic leukaemia with translocation t(15;17)(q22;q21): a report on 50 patients. Br J Haematol. 1996;94(3):493-500.
35. Beaumont M, Sanz M, Carli PM, Maloisel F, Thomas X, Detourmignies L, et al. Therapy-related acute promyelocytic leukemia. J Clin Oncol. 2003;21(11):2123-37.
36. Kayser S, Döhner K, Krauter J, Köhne CH, Horst HA, Held G, et al. The impact of therapy-related acute myeloid leukemia (AML) on outcome in 2853 adult patients with newly diagnosed AML. Blood. 2011; 117(7):2137-45.