Brief report

The *FLT3*ITD mRNA level has a high prognostic impact in *NPM1* mutated, but not in *NPM1* unmutated, AML with a normal karyotype

Friederike Schneider,[1] Eva Hoster,[1,2] Michael Unterhalt,[1] Stephanie Schneider,[1] Annika Dufour,[1] Tobias Benthaus,[1] Gudrun Mellert,[1] Evelyn Zellmeier,[1] Purvi M. Kakadia,[1] Stefan K. Bohlander,[1,3] Michaela Feuring-Buske,[4] Christian Buske,[5] Jan Braess,[1] Achim Heinecke,[6] Maria C. Sauerland,[6] Wolfgang E. Berdel,[7] Thomas Büchner,[7] Bernhard J. Wörmann,[8] Wolfgang Hiddemann,[1,3] and Karsten Spiekermann[1,3]

[1]Laboratory for Leukemia Diagnostics, Department of Medicine III, University Hospital Munich, Munich, Germany; [2]Institute of Medical Statistics, Biometry, and Epidemiology, University of Munich, Munich, Germany; [3]Clinical Cooperative Group Acute Leukemias, Helmholtz Center Munich, Munich, Germany; [4]Department of Internal Medicine III, University of Ulm, Ulm, Germany; [5]Comprehensive Cancer Center Ulm, Institute of Experimental Tumor Research, Ulm, Germany; [6]Institute of Biostatistics and Clinical Research, University of Muenster, Muenster, Germany; [7]Department of Medicine, Hematology and Oncology, University of Muenster, Muenster, Germany; and [8]German Society of Hematology and Oncology, Berlin, Germany

The impact of a *FLT3*-internal tandem duplication (*FLT3*ITD) on prognosis of patients with acute myeloid leukemia (AML) is dependent on the ratio of mutated to wild-type allele. In 648 normal karyotype (NK) AML patients, we found a significant independent effect of the quantitative *FLT3*ITD mRNA level—measured as (*FLT3*ITD/wt*FLT3*)/(*FLT3*ITD/wt*FLT3* + 1)—on outcome. Moreover, this effect was clearly seen in 329 patients with a mutated *NPM1* gene (*NPM1*$^+$), but not in 319 patients without a *NPM1* mutation (wt*NPM1*). In a multivariate Cox regression model, the quantitative *FLT3*ITD mRNA level showed an independent prognostic impact on overall survival (OS) and relapse-free survival (RFS) only in the *NPM1*$^+$ subgroup (OS: hazard ratio, 5.9; [95% confidence interval [CI]: 3.1-11.2]; RFS: hazard ratio, 7.5 [95% CI: 3.4-16.5]). The *FLT3*ITD mRNA level contributes to relapse risk stratification and might help to guide postremission therapy in *NPM1*-mutated AML. (*Blood*. 2012;119(19):4383-4386)

Introduction

The prognosis of normal karyotype–acute myeloid leukemia (NK-AML) is influenced by the presence of gene mutations. *NPM1* has been shown to be the most common single mutated gene in NK-AML occurring with a frequency of ~ 50%. Combinations of *NPM1* mutations with *FLT3*-internal tandem duplication (*FLT3*ITD) have been described in ~ 20% of patients with NK-AML.[1,2] The positive prognostic impact of the *NPM1*$^+$ on outcome is mainly evident in patients lacking a *FLT3*ITD. Approximately 60% of patients carrying the *NPM1*$^+$/*FLT3*–wild-type genotype survive > 10 years.[3,4] The *NPM1*$^+$ NK-AML has been classified as an own entity of favorable prognosis in the revised World Health Organization and European LeukemiaNet classifications.[5,6] Since 2001, there have been reports that not only the presence of a *FLT3*ITD per se, but also the *FLT3*ITD/*FLT3*–wild-type (wt*FLT3*) ratio is essential for prognosis.[7,8] The aim of our work was to assess the influence of the *FTL3*ITD mRNA level according to the mutation status of *NPM1*.

Methods

Patients

Our analyses were based on patients with NK-AML treated within the AML Cooperative Group 99 study.[9] Patients were randomly assigned for induction therapy with either TAD (thioguanine, conventional-dose AraC, daunorubicin) followed by HAM (high-dose AraC, mitoxantrone) or 2 courses of HAM. As consolidation therapy in first complete remission (CR), allogeneic transplantation from an unrelated donor was recommended for high-risk patients < 60 years whereas all other patients received treatment with TAD and maintenance therapy.[9]

End points

Overall survival (OS) was calculated from randomization to death from any cause or to the latest follow-up. Relapse-free survival (RFS) was determined from the first day of CR until relapse or death in CR.

Molecular analyses

Mutation analyses of *NPM1*, *FLT3*ITD, *FLT3*–tyrosine kinase domain (*FLT3*TKD), *MLL*–partial tandem duplication (*MLL*-PTD), and *CEBPA* were performed according to standard protocols previously described.[10-12] *FLT3* mRNA RT-PCR and PCR were performed according to standard protocols.[13] Labeled PCR products were electrophoresed on ABI 3100 (Applied Biosystems) according to protocol. The data were collected and analyzed with Genescan and Genotyper software (Applied Biosystems). The ratio of *FLT3*ITD mRNA to wt*FLT3* mRNA was calculated as previously published.[8,14] The amount of *FLT3*ITD mRNA in relation to the entire *FLT3* transcript signal was defined as: quantitative "*FLT3*ITD mRNA level" = (*FLT3*ITD/wt*FLT3*)/(*FLT3*ITD/wt*FLT3* + 1).

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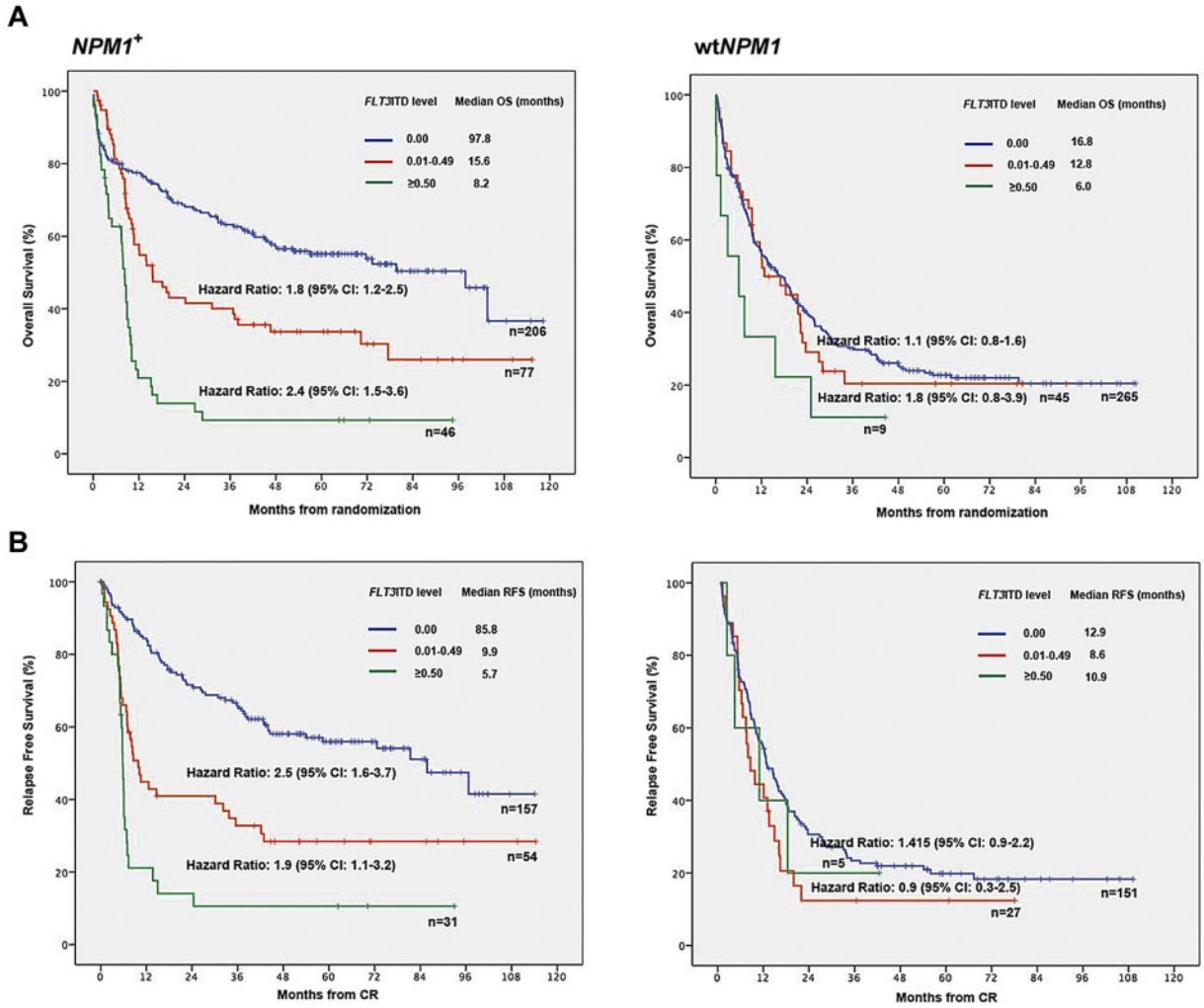

Figure 1. Impact of *FLT3*ITD mutation level on outcome according to *NPM1*. (A) OS in patients with *NPM1* mutation (N = 329) compared with *NPM1* wild-type (n = 319). (B) RFS in patients with *NPM1* mutation (N = 242) compared to *NPM1* wild-type (n = 183). The significant impact of *FLT3*ITD mutation level on outcome was evident in *NPM1*-mutated AML. In *NPM1*-mutated AML, the effect of the *FLT3*ITD mRNA level displayed a dose-dependency. Thus, patients with a *FLT3*ITD level ≥ 0.50 showed the worst OS and RFS compared to patients with a *FLT3*ITD level between 0.01 and 0.49 and patients without a *FLT3*ITD. Differences between the score groups were highly significant ($P \leq .001$).

Statistical analyses

Univariate Cox regression for OS was first performed in the complete cohort to evaluate the prognostic value of the quantitative *FLT3*ITD mRNA level, independent of *NPM1*. For visualization of significant effects, we grouped patients according to the *FLT3*ITD mRNA level using 5 potential threshold values. To reduce the potential bias of data-derived cutpoints, we fixed the biologically meaningful thresholds 0.00, to distinguish between *FLT3*ITD and wt*FLT3*, 0.50, indicating a heterozygous mutation, and 1.00, indicating complete wild-type loss. In addition, we investigated the values 0.25 and 0.75 as potential thresholds. Very small patient groups (≤ 5%) were combined to the next larger adjacent group.

Multiple Cox regression using the quantitative *FLT3*ITD mRNA level, together with its interaction with *NPM1* and clinical and molecular characteristics, was performed for OS and RFS. Kaplan-Meier estimation for OS and RFS and multiple Cox regression was also performed separately for *NPM1*$^+$ and wt*NPM1* patients. A significance level of 5% was used.

Results and discussion

Analyses were performed in 648 of 802 patients treated within the AMLCG99 trial (supplemental Figure 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). Patients (119 of 648) received allogeneic transplantation in first CR. Median follow-up for OS was 62.3 months. Median OS was 20.4 months with 414 events. In 427 of 648 (66%) patients in CR, median RFS was 18.0 months. In 173 of 648 *FLT3*ITD-mutated patients, the median *FLT3*ITD level was 0.42 (0.02-1.00). Patient characteristics are summarized in supplemental Tables 1 and 2.

Impact of *FLT3*ITD mutation level on OS and definition of thresholds

Univariate Cox regression showed a significant impact of the *FLT3*ITD mRNA level on OS (hazard ratio of 1.12 for a *FLT3*ITD mutation level increased by 0.10, 95% confidence interval [CI], 1.08-1.17, $P < .0001$). Grouping patients using the prespecified threshold values, median OS for *FLT3*ITD mRNA level 0.00 (n = 471 of 648; 73%), 0.01-0.24 (n = 31 of 648; 5%), 0.25-0.49 (n = 91 of 648; 14%), 0.50-0.74 (n = 38 of 648; 6%), and 0.75-1.00 (n = 17 of 648; 3%) were 26, 24, 12, 8, and 8 months. The threshold level of 1.00 was excluded because only 7 patients had a complete wild-type loss. Because of the low patient number, *FLT3*ITD-positive patients with a level below 0.25 were combined with those with a level between 0.25 and 0.50 into a low-level (0.01-0.49) *FLT3*ITD group. Similarly,

Table 1. Multiple Cox regression models for OS and RFS

			OS, n = 508				RFS, n = 333			
				95% CI				95% CI		
Parameter		Stratum	HR	Lower CL	Upper CL	*P*	HR	Lower CL	Upper CL	*P*
NPM1	pos vs neg	wt*FLT3*	0.3	0.2	0.4	< .001	0.2	0.1	0.3	< .001
*FLT3*ITD mutation level		wt*NPM1*	1.1	0.4	3.0	.789	0.6	0.2	2.2	.436
*FLT3*ITD mutation level		*NPM1*$^+$	5.9	3.1	11.2	< .001	7.5	3.4	16.5	< .001
Interaction *NPM1*FLT3*ITD mutation level			5.2	1.7	15.3	.003	12.7	3.0	55	.001
mo*CEBPA*	vs wt*CEBPA*		0.6	0.3	1.04	.067	0.5	0.2	1.1	.075
bi*CEBPA*	vs wt*CEBPA*		0.3	0.1	0.5	< .001	0.3	0.1	0.6	.001
*FLT3*TKD	pos vs neg		1.4	0.9	2.3	.149	1.0	0.5	2.2	.890
MLL-PTD	pos vs neg		0.9	0.6	1.4	.699	0.8	0.4	1.4	.377
WBC, $\times 10^6$/L	10-fold		1.4	1.1	1.8	.002	1.3	0.9	1.7	.118
Platelets, $\times 10^6$/L	10-fold		0.7	0.6	1.01	.059	0.8	0.6	1.2	.343
Hemoglobin level, mg/dL	+1 g/dL		1.0	0.99	1.005	.595	1.0	0.99	1.01	.858
LDH, U/L	10-fold		1.2	0.8	1.8	.503	1.2	0.7	2.1	.616
BM blasts, %	+10%		1.0	0.997	1.01	.243	1.0	0.998	1.01	.178
Age, y	+10 y		1.4	1.2	1.5	< .001	1.2	1.1	1.4	< .001
Performance status, ECOG	2-4 vs 0,1		1.3	0.996	1.6	.054	1.2	0.9	1.6	.310
Sex	Female vs male		0.9	0.7	1.1	.353	0.8	0.6	1.1	.243
De novo AML	vs non–de novo		0.9	0.7	1.3	.709	0.9	0.6	1.4	.781

The independent prognostic impact of the *FLT3*ITD mutation level on OS and RFS was evaluated using multivariate Cox regression models. The *FLT3*ITD mutation level was introduced as a continuous parameter into the model. Due to the known interaction between *NPM1* and *FLT3*ITD, an interaction term *NPM1*FLT3*ITD mutation level was included in the model. Besides the *FLT3*ITD mutation level, mutations of the molecular markers *NPM1* (*NPM1*$^+$), *CEBPA* (mo*CEBPA*; bi*CEBPA*), *FLT3*TKD, *MLL-PTD*, and the clinical parameters age, sex, ECOG performance status, AML de novo, WBC, platelet count, hemoglobin level, LDH, and amount of BM blasts were introduced into the model. The multivariate prognostic factors were identified using a logistic regression model with a significance level of 5%.

OS indicates overall survival; RFS, relapse-free survival; mo*CEBPA*, monoallelic *CEBPA* mutation; bi*CEBPA*, biallelic *CEBPA* mutation; TKD, tyrosine kinase domain; PTD, partial tandem duplication; ITD, internal tandem duplication; WBC, white blood count; LDH, lactate dehydrogenase; ECOG, Eastern Cooperative Oncology Group; HR, hazard ratio; CI, confidence interval; CL, confidence limit; pos, positive; neg, negative; and AML, acute myeloid leukemia.

patients with a positive *FLT3*ITD mRNA level ≥ 0.50 were combined to one high-level (0.50-1.00) group. Finally, only the biologic meaningful cutpoints 0.00 and 0.50 were retained. Median OS in *FLT3*ITD-negative (73%), low-level (19%), and high-level *FLT3*ITD (8%) were 26.2, 15.6, and 7.8 months, respectively ($P < .001$).

Impact of *FLT3*ITD mutation level on outcome according to *NPM1* mutation status

In the *NPM1*-mutated cohort, median OS was 97.8 months in the *FLT3*ITD-negative, 15.6 months in the low-level (0.01-0.49), and 8.2 months in the high-level *FLT3*ITD (0.50-1.00) group ($P < .001$, Figure 1A). Significant differences between these risk groups were evident regarding RFS ($P < .001$; Figure 1B). Median OS in wt*NPM1* patients without a *FLT3*ITD, with a *FLT3*ITD level < 0.50 and ≥ 0.50 were not statistically different (16.8 months, 12.8 months, and 6.0 months, respectively, $P = .133$, Figure 1A). *FLT3*ITD mRNA level may not impact on survival in patients with wt*NPM1*, although this conclusion is limited by the low statistical power because of the relatively small number of patients with a high *FLT3*ITD mRNA level (n = 9; 1%).

In the multivariate Cox regression model with all 648 patients, the independent prognostic impact of the quantitative *FLT3*ITD mRNA level on outcome was detectable in *NPM1*$^+$ patients ($P < .001$), but not in wt*NPM1* (Table 1). This was true for both age subgroups (</≥ 60 years, data not shown). In multiple regression in *NPM1*$^+$ patients, the *FLT3*ITD low-level group had an adjusted hazard ratio of 1.5 (95% CI, 0.96-2.3) for OS ($P = .078$), and the *FLT3*ITD high-level group an adjusted hazard ratio of 3.1 (95% CI 1.9-5.2, $P < .001$) compared with wt*FLT3* (supplemental Table 3). Within wt*NPM1* patients, the *FLT3*ITD mRNA level did not appear as an independent prognostic factor. Similar results were observed for RFS.

Whitman et al were the first to show that a complete loss of wt*FLT3* was associated with worse outcome compared to patients without a *FLT3*ITD (wt*FLT3*/wt*FLT3*) or a heterozygous *FLT3*ITD (wt*FLT3*/*FLT3*ITD) mutation.[7] Thiede et al defined the *FLT3*ITD/wt ratio as the relative proportion of the area under the curve (AUC) of mutant and wt*FLT3* alleles (AUC-*FLT3*ITD/AUC-wt*FLT3*) in Genescan analysis. A *FLT3*ITD/wt*FLT3* ratio above the median of the cohort was associated with an unfavorable prognosis.[8] Median-defined risk groups have to be determined in large patient cohorts before a definite statement about individual prognosis can be made. In contrast, we defined the *FLT3*ITD mRNA level as the relative amount of *FLT3*ITD mRNA to the total *FLT3* transcript, with a range from 0 (absence of mutation) to 1 (complete loss of wild type), facilitating the estimation of the *FLT3*ITD mutational load. This has the advantage of direct estimation of individual prognosis according to a patient's *FLT3*ITD mutant level and better comparability in different clinical studies.

The focus of our analyses was the investigation of the impact of the *FLT3*ITD mRNA level according to the *NPM1* mutation status in NK-AML. Univariate and multivariate analyses demonstrated a distinct dose-dependent effect of the *FLT3*ITD mutant level on OS and RFS only in *NPM1*$^+$, but not in wt*NPM1* patients. In *NPM1*-mutated patients, multivariate analyses revealed a *FLT3*ITD level of 0.50 as cutoff between an intermediate group (26% long-term survivors) and a poor-risk group with 9% survivors in 7 years. In accordance with Whitman et al, these observations suggest different pathophysiologies of heterozygous *FLT3*ITD versus *FLT3*ITD with a complete loss of the wild-type allele.[7]

Our data suggest a significantly worse outcome with regard to OS and RFS for patients harboring an *NPM1* mutation and higher *FLT3*ITD mRNA expression compared to those *NPM1*-mutated patients with a low *FLT3*ITD mRNA expression. Thus, the *FLT3*ITD mRNA level might guide the decision for allogeneic

transplantation in *NPM1*$^+$ AML. However, such a strategy should be prospectively evaluated.

Authorship

Contribution: F.S. performed statistical analysis and wrote the manuscript; E.H., M.U., A.H., and M.C.S. provided statistical support; S.S., A.D., T. Benthaus, G.M., E.Z., and P.M.K. performed molecular diagnostics; S.K.B., M.F.-B., C.B., J.B., and K.S. performed central diagnostics; W.E.B., T. Büchner, B.J.W., and W.H. were principal investigators of AMLCG99 study; and K.S. wrote the manuscript.

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Correspondence: Karsten Spiekermann, MD, Department of Medicine III, University Hospital Grosshadern, Marchioninistr 15, 81377 Munich, Germany; e-mail: karsten.spiekermann@med.uni-muenchen.de.

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