The FLT3ITD mRNA level has a high prognostic impact in $NPM1$ mutated, but not in $NPM1$ unmutated AML with a normal karyotype

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Running title: Impact of FLT3ITD mutant level in $NPM1+$ AML

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Abstract

The impact of a FLT3-internal tandem duplication (FLT3ITD) on prognosis of patients with acute myeloid leukemia (AML) is dependent on the ratio of mutated to wildtype allele. In 648 normal karyotype (NK) AML patients we found a significant independent effect of the quantitative FLT3ITD mRNA level - measured as (FLT3ITD/wtFLT3) / (FLT3ITD/wtFLT3 + 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 (wtNPM1). In a multivariate Cox regression model the quantitative FLT3ITD 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% CI: 3.1-11.2]; RFS: Hazard Ratio 7.5 [95% CI: 3.4-16.5]). The FLT3ITD mRNA level contributes to relapse risk stratification and might help to guide post-remission therapy in NPM1-mutated AML.

Introduction

The prognosis of 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 about 50%. Combinations of NPM1 mutations with FLT3ITD have been described in about 20% of patients with NK-AML1,2. The positive prognostic impact of the NPM1+ on outcome is mainly evident in patients lacking a FLT3ITD. About 60% of patients carrying the NPM1+/FLT3-wildtype genotype survive more than 10 years3,4. The NPM1+ NK-AML has been classified as an own entity of favorable prognosis in the revised WHO and ELN classifications5,6. Since 2001 there have been reports that not only the presence of a FLT3ITD per se, but that the FLT3ITD/FLT3-wildtype (wtFLT3) ratio is essential for prognosis7,8. The aim of our work was to assess the influence of the FLT3ITD mRNA level according to the mutation status of NPM1.

Patients and methods

Patients: Our analyses were based on patients with NK-AML treated within the AMLCG99 study9. Patients were randomly assigned for induction therapy with either TAD (thioguanine, conventional-dose AraC, daunorubicin) followed by HAM (high-dose AraC, mitoxantrone) or two 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 therapy9.

Endpoints: 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, FLT3ITD, FLT3TKD, MLL-PTD and CEBPA were performed according to standard protocols previously described\textsuperscript{10-12}. FLT3 mRNA RT-PCR and PCR were performed according to standard protocols\textsuperscript{13}. Labelled PCR products were electrophoresed on ABI 3100 according to protocol (Applied Biosystems). The data were collected and analyzed with Genescan and Genotyper software (Applied Biosystems). The ratio of FLT3ITD mRNA to wtFLT3 mRNA was calculated as previously published\textsuperscript{8,14}. The amount of FLT3ITD mRNA in relation to the entire FLT3 transcript signal was defined as: quantitative “FLT3ITD mRNA level” = (FLT3ITD/wtFLT3) / (FLT3ITD/wtFLT3 + 1).

**Statistical analyses:** Univariate Cox regression for OS was first performed in the complete cohort to evaluate the prognostic value of the quantitative FLT3ITD mRNA level, independently of NPM1. For visualization of significant effects, we grouped patients according to the FLT3ITD 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 FLT3ITD and wtFLT3, 0.50, indicating a heterozygous mutation, and 1.00, indicating complete wildtype 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 FLT3ITD 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 wtNPM1 patients. A significance level of 5\% was used.

**Results and Discussion**

Analyses were performed in 648 of 802 patients treated within the AMLCG99 trial (supplementary Figure 1). 119/648 patients 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 427/648 (66\%) patients in CR, median RFS was 18.0 months. In 173/648 FLT3ITD-mutated patients median FLT3ITD-level was 0.42 (0.02-1.00). Patient characteristics are summarized in supplementary Tables 1 and 2.

**Impact of FLT3ITD mutation level on OS and definition of thresholds**

Univariate Cox regression showed a significant impact of the FLT3ITD mRNA level
on OS (Hazard Ratio of 1.12 for a FLT3ITD mutation level increased by 0.10, 95% CI 1.08 – 1.17, p<0.0001). Grouping patients using the pre-specified threshold values, median OS for FLT3ITD mRNA level 0.00 (n=471/648; 73%), 0.01-0.24 (n=31/648; 5%), 0.25-0.49 (n=91/648; 14%), 0.50-0.74 (n=38/648; 6%), and 0.75-1.00 (n=17/648; 3%) were 26, 24, 12, 8, and 8 months. The threshold level of 1.00 was excluded because only 7 patients had a complete wildtype loss. Due to the low patient number, FLT3ITD-positive patients with a level below 0.25 were combined to those with a level between 0.25-0.50 into a low-level (0.01-0.49) FLT3ITD group. Similarly, patients with a positive FLT3ITD mRNA level ≥0.50 were combined to one high-level (0.50-1.00) group. Finally only the biological meaningful cutpoints 0.00 and 0.50 were retained. Median OS in FLT3ITD negative (73%), low-level (19%) and high-level FLT3ITD (8%) were 26.2, 15.6, and 7.8 months, respectively (p<0.001).

Impact of FLT3ITD mutation level on outcome according to NPM1 mutation status

In the NPM1-mutated cohort, median OS were 97.8 months in the FLT3ITD-negative, 15.6 months in the low-level (0.01-0.49) and 8.2 months in the high-level FLT3ITD (0.50-1.00) group (p<0.001, Figure 1A). Significant differences between these risk groups were evident regarding RFS (p<0.001) (Figure 1B). Median OS in wtNPM1 patients without a FLT3ITD, with a FLT3ITD-level <0.50 and ≥0.50 were not statistically different (16.8 months, 12.8 months and 6.0 months respectively, p=0.133, Figure 1A). FLT3ITD mRNA level may not impact on survival in patients with wtNPM1, although this conclusion is limited by the low statistical power due to the relatively small number of patients with a high FLT3ITD mRNA level (n=9; 1%).

In the multivariate Cox regression model with all 648 patients, the independent prognostic impact of the quantitative FLT3ITD mRNA level on outcome was detectable in NPM1+ patients (p<0.001), but not in wtNPM1 (Table 1). This was true for both age subgroups (<≥60 years, data not shown). In multiple regression in NPM1+ patients, the FLT3ITD low-level group had an adjusted hazard ratio of 1.5 (95% CI 0.96-2.3) for OS (p=0.078), and the FLT3ITD high-level group an adjusted hazard ratio of 3.1 (95% CI 1.9-5.2, p<0.001) as compared to wtFLT3 (Supplementary Table 3). Within wtNPM1 patients the FLT3ITD mRNA level did not appear as an independent prognostic factor. Similar results were observed for RFS.

Whitman et al. was the first to show that a complete loss of wtFLT3 was associated with worse outcome compared to patients without a FLT3ITD (wtFLT3/wtFLT3) or a heterozygous FLT3ITD (wtFLT3/FLT3ITD) mutation7. Thiede et al. defined the FLT3ITD/wt ratio as the relative proportion of the area under the curve (AUC) of mutant and wtFLT3 alleles (AUC-FLT3ITD/AUC-wtFLT3) in Genescan analysis. A FLT3ITD/wtFLT3 ratio above the
median of the cohort was associated with an unfavourable prognosis. 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 FLT3ITD mRNA level as the relative amount of FLT3ITD mRNA to the total FLT3 transcript, with a range from 0 (absence of mutation) to 1 (complete loss of wildtype) facilitating the estimation of the FLT3ITD mutational load. This has the advantage of direct estimation of individual prognosis according to a patient’s FLT3ITD mutant level and better comparability in different clinical studies.

The focus of our analyses was the investigation of the impact of the FLT3ITD mRNA level according to the NPM1 mutation status in NK-AML. Univariate and multivariate analyses demonstrated a distinct dose-dependent effect of the FLT3ITD mutant level on OS and RFS only in NPM1+, but not in wtNPM1 patients. In NPM1-mutated patients, multivariate analyses revealed a FLT3ITD-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 pathophysologies of heterozygous FLT3ITD mutations versus FLT3ITD mutations with a complete loss of the wildtype allele.

Our data suggest a significantly worse outcome with regard to OS and RFS for patients harbouring an NPM1 mutation and higher FLT3ITD mRNA expression compared to those NPM1-mutated patients with a low FLT3ITD mRNA expression. Thus, the FLT3ITD mRNA level might guide the decision for allogeneic transplantation in NPM1+ AML. However, such a strategy should be prospectively evaluated.

Authorship contributions:

F. Schneider: statistics, author, wrote the manuscript
K. Spiekermann: corresponding author, wrote the manuscript
E. Hoster, M. Unterhalt, M.C. Sauerland, A. Heinecke: statistical support
S. Schneider, A. Dufour, T. Benthaus, G. Mellert, E. Zellmeier, Purvi M. Kakadia: molecular diagnostics
S.K. Bohlander, M. Feuring-Buske, C. Buske, J. Braess, K. Spiekermann: central diagnostics
W. Hiddemann, T. Buechner, B.J. Woermann, W.E. Berdel: principal investigators of AMLCG99 study

Disclosure of Conflicts of Interest

The author indicates no potential conflict of interest.
References

The independent prognostic impact of the FLT3ITD mutation level on overall survival (OS) and relapse free survival (RFS) was evaluated using multivariate Cox regression models. The FLT3ITD mutation level was introduced as a continuous parameter into the model. Due to the known interaction between NPM1 and FLT3ITD an interaction term NPM1* FLT3ITD mutation level was included in the model. Besides the FLT3ITD mutation level, mutations of the molecular markers NPM1 (NPM1+), CEBPA (moCEBPA=monoallelic CEBPA mutation; biCEBPA=biallelic CEBPA mutation), FLT3TKD, MLL-PTD, and the clinical parameters age, sex, ECOG performance status, AML de novo, white blood count (WBC), platelet count, hemoglobin level, lactase dehydrogenase (LDH) and amount of bone marrow blasts were introduced into the model. The multivariate prognostic factors were identified using a logistic regression model with a significance level of 5%. HR=Hazard. CI= confidence interval, CL=confidence limit, P =p value; n=number.

Table 1: Multiple Cox regression models for OS and RFS

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Stratum</th>
<th>OS (n=508)</th>
<th>RFS (n=333)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HR</td>
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<tr>
<td></td>
<td></td>
<td>Lower CL</td>
<td>Upper CL</td>
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<td>wtFLT3</td>
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<td>0.2</td>
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<td>wtNPM1</td>
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<td>NPM1+</td>
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<td>3.1</td>
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<td>Interaction NPM1* FLT3ITD mutation level</td>
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<td>moCEBPA vs. wtCEBPA</td>
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<td>0.3</td>
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<td>biCEBPA vs. wtCEBPA</td>
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<td>0.1</td>
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<tr>
<td>FLT3TKD pos.vs. neg.</td>
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<td>0.9</td>
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<tr>
<td>MLL-PTD pos.vs. neg.</td>
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<tr>
<td>WBC (x 10^6/l) 10 fold</td>
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<td>1.1</td>
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<tr>
<td>Platelets (x 10^6/l) 10 fold</td>
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<td>0.7</td>
<td>0.6</td>
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<tr>
<td>Hemoglobin level (mg/dl) + 1 g/dL</td>
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<td>1.0</td>
<td>0.997</td>
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<tr>
<td>LDH (U/l) 10 fold</td>
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<td>0.8</td>
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<tr>
<td>Bone marrow blasts (%) + 10%</td>
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<td>0.997</td>
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<tr>
<td>Age (years) +10 years</td>
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<td>1.2</td>
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<td>Performance status (ECOG) 2-4 vs. 0,1</td>
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<td>1.3</td>
<td>0.996</td>
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<td>Sex female vs male</td>
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<td>De novo AML vs. non de novo</td>
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<td>0.7</td>
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Figure Legends:

Figure 1: Impact of FLT3ITD mutation level on outcome according to NPM1

(A) Overall survival (OS) in patients with NPM1 mutation (N=329) compared to NPM1 wildtype (n=319). (B) Relapse free survival (RFS) in patients with NPM1 mutation (N=242) compared to NPM1 wildtype (n=183).

The significant impact of FLT3ITD mutation level on outcome was evident in NPM1-mutated AML. In NPM1-mutated AML, the effect of the FLT3ITD mRNA level displayed a dose-dependency. Thus, patients with a FLT3ITD-level ≥ 0.50 showed the worst OS and RFS compared to patients with a FLT3ITD-level between 0.01-0.49 and patients without a FLT3ITD. Differences between the score groups were highly significant (p ≤ 0.001). CI=confidence interval.
Figures:

Figure 1:

**NPM1+**

<table>
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<th>FLT3/ITD level</th>
<th>Median OS (months)</th>
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<td>0.00</td>
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<tr>
<td>0.01-0.49</td>
<td>15.6</td>
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<td>≥0.50</td>
<td>8.2</td>
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**wtNPM1**

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<th>Median OS (months)</th>
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<tbody>
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<td>15.8</td>
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<tr>
<td>0.01-0.49</td>
<td>12.0</td>
</tr>
<tr>
<td>≥0.50</td>
<td>4.0</td>
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</table>

**1A**

**Overall Survival (%)**

- Hazard Ratio: 1.8 (95% CI: 1.2-2.8)
- Hazard Ratio: 2.4 (95% CI: 1.6-3.0)
- N=946

**1B**

**Relapse-Free Survival (%)**

- Hazard Ratio: 2.9 (95% CI: 1.6-0.7)
- Hazard Ratio: 1.9 (95% CI: 1.1-3.2)
- N=946
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