NPM1, but not FLT3-ITD mutations predict early blast cell clearance and CR rate in patients with normal karyotype AML (NK-AML) or high risk myelodysplastic syndrome (MDS)

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Running title: NPM1 mutations predict blast clearance in NK-AML
Keywords: Neoplasia, Acute Myeloid Leukemia, NPM1, FLT3-ITD, early blast cell clearance

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Abstract

Mutations in the \textit{NPM1} gene represent the most frequent genetic alterations in patients with acute myeloid leukemia (AML) and are associated with a favorable outcome. In 690 normal karyotype (NK) AML patients the complete remission rates (CR) and the percentage of patients with adequate \textit{in vivo} blast cell reduction one week after the end of the first induction cycle were significantly higher in \textit{NPM1}+ (75\% and 80\%, respectively) than in \textit{NPM1}- (57\% and 57\%, respectively) patients, but were unaffected by the \textit{FLT3-ITD} status. Multivariate analyses revealed the presence of a \textit{NPM1} mutation as an independent positive prognostic factor for the achievement of an adequate d16 blast clearance and a CR. In conclusion, \textit{NPM1}+ blast cells show a high \textit{in vivo} sensitivity towards induction chemotherapy irrespective of the \textit{FLT3-ITD} mutation status. These findings provide insight into the pathophysiology and help to understand the favorable clinical outcome of patients with \textit{NPM1}+ AML.
Introduction

In patients with a normal karyotype (NK) AML submicroscopic genetic markers are essential for risk stratification regarding response to induction therapy and survival. NPM1 is the most frequent single genetic abnormality (46%-62%) and is associated with a favorable clinical outcome\(^1\)\(^2\). The presence of a FLT3-ITD mutation found in about 30% of patients\(^3\)\(^4\) represents an unfavorable prognostic parameter for survival. The positive prognostic effect of NPM1 mutations on response to induction therapy and long-term outcome was evident only in patients lacking the FLT3-ITD mutation. In addition to FLT3-ITD and NPM1, several other genetic markers\(^5\)\(^9\) have prognostic impact in NK-AML. Kern et al.\(^10\) showed that not only pretreatment characteristics but also early response parameters e.g. early blast clearance affect outcome. In these analyses a positive prognostic impact of an adequate early blast clearance (<10% residual blasts) on complete remission (CR) rate and long-term outcome was found.

To gain further insight into the mechanisms mediating the favorable prognostic impact of NPM1 mutations we investigated effects of the NPM1 mutation combined with or without the FLT3-ITD mutation on early response parameters.

Material and Methods

This analysis was based on patients with newly diagnosed NK-AML treated within a prospective randomized multicenter trial according to the AMLCG 1999 study protocol. The details of the study have been published previously\(^11\). Cytomorphology, cytogenetic and molecular analyses of bone marrow (BM) aspirates were performed according to standard protocols\(^1\)\(^12\)\(^13\).

All pretreatment clinical (age, sex, performance status (ECOG score), AML de novo, white blood count (WBC), hemoglobin level, platelet count, lactase dehydrogenase
(LDH), BM blasts) and molecular markers (NPM1, FLT3-ITD, FLT3-TKD, CEBPA and MLL-PTD) were included in univariate and multivariate analyses. Outcome parameters CR rate and early blast clearance were dichotomous. For univariate analyses we performed cross tables and two-sided exact fisher test for categorical characteristics and univariate logistic regression for categorical and continuous parameters. In multiple logistic regression, independent prognostic factors were identified by backward elimination using the Wald statistic with significance level \( \alpha = 0.05 \). Since with regard to overall survival an interaction effect of NPM1 and FLT3-ITD is known we analyzed a potential interaction effect on CR rate or early blast cell clearance by including the interaction term together with the main parameters NPM1 and FLT3-ITD in multiple logistic regression.

**Results and Discussion**

We investigated the influence of pretherapeutic markers on early therapeutic response parameters in patients with NK-AML enrolled in the AMLCG 1999 trial. Patient characteristics are summarized in supplementary table 1.

In 690 patients the FLT3-ITD mutation status and the NPM1 mutation status could be analyzed. The NPM1 mutation was present in 51\% (N=352/690) of patients. The FLT3-ITD mutation was found in 29\% (N=200/690) of patients.

Patients with known NPM1 and FLT3-ITD mutation status were divided into four groups: NPM1+/FLT3-ITD- (N=211/690, 31\%), NPM1+/FLT3-ITD+ (N=141/690, 20\%), NPM1-/FLT3-ITD- (N=279/690, 40\%) and NPM1-/FLT3-ITD+ (N=59/690, 9\%). In 598 patients with known NPM1/FLT3-ITD mutation status information about the early blast cell clearance was available.
**NPM1, but not FLT3-ITD mutations predict early response parameters**

Of 690 patients 66% achieved a complete remission (CR), 10% had persistent leukemia (PL), 15% died of early death (ED) and 9% remained hypoplastic. 75% of patients carrying the *NPM1* mutation and 57% of *NPM1* negative patients achieved a CR (p<0.001). CR rates did not differ significantly between the *FLT3-ITD*+ (68%) and the *FLT3-ITD*- (65%) group (p=0.480). Response to induction therapy differed according to the combination of the *NPM1* and *FLT3-ITD* mutations. In *NPM1* mutated AML the CR rates were 77% (*NPM1+/FLT3-ITD*) and 71% (*NPM1+/FLT3-ITD*+). Significantly lower CR rates of 56% (*NPM1-/FLT3-ITD*) and 58% (*NPM1-/FLT3-ITD*+) were found in *NPM1* unmutated AML (p<0.001).

The amount of residual leukemic blasts in the BM measured one week after the end of induction therapy (i.e. on day 12 in the HAM regimen, on day 16 in the TAD regimen) can be used as an early independent prognostic parameter of response to therapy and is referred to as early blast cell clearance. In our data set the early blast clearance did not differ significantly between patients treated with either TAD or HAM as first induction course (p=0.661). 409 of 598 patients (68%) showed less than 10% of BM blasts one week after the end of induction chemotherapy.

80% of *NPM1*+ patients (80%: *NPM1+/FLT3-ITD*; 79%: *NPM1+/FLT3-ITD*+) but only 57% of patients without the *NPM1* mutation (58%: *NPM1-/FLT3-ITD*; 52%: *NPM1-/FLT3-ITD*+) showed a residual blast cell percentage of less than 10%. Thus the *NPM1* mutation was associated with a significantly higher blast clearance (p<0.001) compared to the *NPM1* wildtype (WT) genotype.

71% of *FLT3-ITD* positive and 67% of *FLT3-ITD* negative patients showed a blast reduction to less than 10%. Thus the presence of the *FLT3-ITD* had no influence (p=0.498) on the blast cell clearance (table 1).
Similar results were seen in younger (< 60 years) and older patients (≥ 60 years) (supplementary table 2).

The NPM1 mutation has independent prognostic impact on the CR rate and early blast cell clearance in univariate and multivariate analyses

The CR rate was analyzed in a model of the nine clinical and five molecular independent prognostic factors mentioned above. Parameters with prognostic impact on the CR rate in univariate analyses included the presence of the NPM1 mutation (OR: 2.31; 95% CI: 1.67-3.19) and de novo AML (OR: 2.14; 1.44-3.17), as well as the presence of the MLL-PTD mutation (OR: 0.46; 0.26-0.82), age (OR: 0.78; 0.69-0.88) and WBC (OR: 0.75; 0.60-0.95). In multivariate analysis the parameter with independent favorable impact on CR rate was the presence of the NPM1 mutation (OR: 2.81; 1.84-4.29) whereas a high WBC (OR: 0.53; 0.39-0.72) and higher age (OR: 0.81; 0.69-0.94) were significantly associated with a lower CR rate.

Regarding the effect on early blast clearance in univariate analyses the presence of the NPM1 mutation (OR: 2.92; 2.03-4.20) and a high LDH level (OR: 1.97; 1.11-3.50) were strong positive indicators whereas the MLL-PTD mutation (OR: 0.46; 0.24-0.89) negatively influenced blast clearance. Parameters not showing a significant effect included the hemoglobin level, WBC, platelets, BM blasts, age, sex, performance status, AML de novo and mutations of FLT3 and CEBPA. In multivariate analysis independent predictive variables were the NPM1 status (OR: 3.12; 2.01-4.83) and BM blasts at diagnosis (OR: 0.90; 0.82-0.98) (table 2).

Our data show that the presence of an NPM1 mutation is associated with the initial response to chemotherapy and thus represents a marker of sensitivity towards induction chemotherapy in vivo.
Pathophysiologically this hypothesis is supported by in vitro experiments in which leukemic blasts carrying the \textit{NPM1} mutation\textsuperscript{16} showed significantly higher rates of apoptosis after treatment with chemotherapeutic agents compared to the \textit{NPM1 WT} leukemic cells\textsuperscript{17}. Abnormal activation of the transcription factor NF-kappaB has been described in leukemic blasts resistant to chemotherapy\textsuperscript{18}. Cilloni \textit{et al}.\textsuperscript{17} showed that \textit{NPM1} plays a central role in enhancing apoptotic cell death in AML by interacting with NF-kappaB. Interestingly NF-kappaB activity did not differ in \textit{NPM1+/FLT3-ITD+} and \textit{NPM1+/FLT3-ITD-} blasts\textsuperscript{17} supporting our in vivo data that the presence of \textit{NPM1} mutation is associated with a higher sensitivity towards induction chemotherapy regardless of the \textit{FLT3-ITD} mutation status.

These data allow further insight into the biology of AML and provide a rationale for the better clinical outcome of this genetic AML subgroup.
Authorship Section

F. Schneider: statistics, author, wrote the manuscript
E. Hoster, M. Unterhalt, M.C. Sauerland, A. Heinecke, S. Fritsch: statistics
S. Schneider, A. Dufour, T. Benthaus, G. Mellert, E. Zellmeier: molecular diagnostics
B.J. Woermann, W.E. Berdel: Coordinators for the inclusion of patients into the AMLCG99 study and acquisition of patient data in cooperating hospitals
S.K. Bohlander, M. Feuring-Buske, C. Buske, J. Braess, K. Spiekermann: central diagnostics
K. Spiekermann: corresponding author, wrote the manuscript
W. Hiddemann, T. Buechner: principal investigators of AMLCG99 study

There is no conflict of interest to be declared.

References

10. Kern W, Haferlach T, Schoch C, et al. Early blast clearance by remission induction therapy is a major independent prognostic factor for both achievement of complete remission...
# Tables

## Table 1 Influence of the mutation status of NPM1 or FLT3-ITD on early response parameters in all patients

<table>
<thead>
<tr>
<th>End point</th>
<th>NPM1+ (N=299)</th>
<th>NPM1- (N=299)</th>
<th>FLT3-ITD+ (N=174)</th>
<th>FLT3-ITD- (N=424)</th>
<th>p NPM1+ vs. NPM1-</th>
<th>p FLT3-ITD+ vs. FLT3-ITD-</th>
</tr>
</thead>
<tbody>
<tr>
<td>d16 blasts &lt; 10% rate, %</td>
<td>80 (238)</td>
<td>57 (171)</td>
<td>71 (123)</td>
<td>67 (286)</td>
<td>&lt; 0.001</td>
<td>0.498</td>
</tr>
<tr>
<td>No. of patients</td>
<td>238</td>
<td>171</td>
<td>123</td>
<td>286</td>
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<td></td>
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<table>
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<tr>
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<th>NPM1+ (N=352)</th>
<th>NPM1- (N=338)</th>
<th>FLT3-ITD+ (N=200)</th>
<th>FLT3-ITD- (N=490)</th>
<th>p NPM1+ vs. NPM1-</th>
<th>p FLT3-ITD+ vs. FLT3-ITD-</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR rate, %</td>
<td>75 (264)</td>
<td>57 (191)</td>
<td>68 (136)</td>
<td>65 (319)</td>
<td>&lt; 0.001</td>
<td>0.480</td>
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<tr>
<td>No. of patients</td>
<td>264</td>
<td>191</td>
<td>136</td>
<td>319</td>
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</table>

<table>
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<tr>
<th>End point</th>
<th>NPM1+ FLT3-ITD- (N=177)</th>
<th>NPM1+ FLT3-ITD+ (N=122)</th>
<th>NPM1- FLT3-ITD- (N=247)</th>
<th>NPM1- FLT3-ITD+ (N=52)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>d16 blasts &lt; 10% rate, %</td>
<td>80 (142)</td>
<td>79 (96)</td>
<td>58 (144)</td>
<td>52 (27)</td>
<td>&lt; 0.001</td>
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<tr>
<td>No. of patients</td>
<td>142</td>
<td>96</td>
<td>144</td>
<td>27</td>
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<table>
<thead>
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<th>End point</th>
<th>NPM1+ FLT3-ITD- (N=211)</th>
<th>NPM1+ FLT3-ITD+ (N=141)</th>
<th>NPM1- FLT3-ITD- (N=279)</th>
<th>NPM1- FLT3-ITD+ (N=59)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR rate, %</td>
<td>77 (163)</td>
<td>71 (100)</td>
<td>56 (156)</td>
<td>58 (34)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>No. of patients</td>
<td>163</td>
<td>100</td>
<td>156</td>
<td>34</td>
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<thead>
<tr>
<th>End point</th>
<th>NPM1+ FLT3-ITD- (N=27)</th>
<th>NPM1+ FLT3-ITD+ (N=27)</th>
<th>NPM1- FLT3-ITD- (N=35)</th>
<th>NPM1- FLT3-ITD+ (N=7)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>ED rate, %</td>
<td>13 (28)</td>
<td>10 (14)</td>
<td>17 (46)</td>
<td>22 (13)</td>
<td>0.104</td>
</tr>
<tr>
<td>No. of patients</td>
<td>28</td>
<td>14</td>
<td>46</td>
<td>13</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>End point</th>
<th>NPM1+ FLT3-ITD- (N=35)</th>
<th>NPM1+ FLT3-ITD+ (N=35)</th>
<th>NPM1- FLT3-ITD- (N=42)</th>
<th>NPM1- FLT3-ITD+ (N=7)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>PL rate, %</td>
<td>5 (10)</td>
<td>9 (13)</td>
<td>15 (42)</td>
<td>12 (7)</td>
<td>0.003</td>
</tr>
<tr>
<td>No. of patients</td>
<td>10</td>
<td>13</td>
<td>42</td>
<td>7</td>
<td></td>
</tr>
</tbody>
</table>

| Definition of early response parameters: Complete Remission (CR): normocellular BM with < 5% blasts, > 1 500 neutrophils/µl, > 100 000 platelets/µl; Early Death (ED): death after < 7 days after completion of first induction; Persistant Leukemia (PL) ≥ 5% blasts in the BM with a leukemic phenotype (eg, Auer rods) after complete induction treatment; Hypoplasia without AML: < 1 000 neutrophils/µl, < 100 000 platelets/µl, no blasts. |

The analyses were performed in Cross tables using two-sided exact Fisher test.
Table 2 Impact of clinical and molecular parameters on early response parameters (multivariate analysis)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Comparison</th>
<th>p</th>
<th>OR</th>
<th>Lower CI</th>
<th>Upper CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPM1</td>
<td>pos vs. neg</td>
<td>&lt; 0.001</td>
<td>3.12</td>
<td>2.01</td>
<td>4.83</td>
</tr>
<tr>
<td>Initial BM blasts (%)</td>
<td>+ 10</td>
<td>0.019</td>
<td>0.90</td>
<td>0.82</td>
<td>0.98</td>
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</table>

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Comparison</th>
<th>p</th>
<th>OR</th>
<th>Lower CI</th>
<th>Upper CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPM1</td>
<td>pos vs. neg</td>
<td>&lt; 0.001</td>
<td>2.81</td>
<td>1.84</td>
<td>4.29</td>
</tr>
<tr>
<td>WBC (/µl)</td>
<td>10 fold</td>
<td>&lt; 0.001</td>
<td>0.53</td>
<td>0.39</td>
<td>0.72</td>
</tr>
<tr>
<td>Age (years)</td>
<td>+ 10</td>
<td>0.006</td>
<td>0.81</td>
<td>0.69</td>
<td>0.94</td>
</tr>
</tbody>
</table>

The candidate prognostic factors included were mutations of the molecular markers NPM1, FLT3-ITD, FLT3-TKD, MLL-PTD, CEBPA and the clinical parameters age, sex, ECOG performance status, AML de novo, WBC count, platelet count, hemoglobin level, LDH and amount of BM blasts. The analyses were performed using 514 complete cases with regard to CR rate and 448 complete cases with regard to d16 blast clearance for the candidate prognostic factors. The multivariate prognostic factors were identified using backward Wald logistic regression model with a significance level of 5%.

OR=Odds ratio for achieving a CR / early blast cell clearance. Lower CI=lower limit of the 95% confidence interval. Upper CI=upper limit of the 95% confidence interval.
NPM1, but not FLT3-ITD mutations predict early blast cell clearance and CR rate in patients with normal karyotype AML (NK-AML) or high risk myelodysplastic syndrome (MDS)

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