AML with mutated NPM1 carrying a normal or aberrant karyotype show overlapping biological, pathological, immunophenotypic, and prognostic features

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Running title: NPM1-mutated AML is not influenced by karyotype

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Abstract

AML with mutated *NPM1* usually carries normal karyotype (NK) but it may harbor chromosomal aberrations whose significance remains unclear. We addressed this question in 631 AML patients with mutated/cytoplasmic *NPM1*. An abnormal karyotype (AK) was present in 93/631 cases (14.7%), the most frequent abnormalities being +8, +4, -Y, del(9q), +21. Chromosome aberrations in *NPM1*-mutated AML were similar to, but occurred less frequently than additional chromosome changes found in other AML with recurrent cytogenetic abnormalities according to WHO classification. Four of the 31 *NPM1*-mutated AML patients karyotyped at different time points had NK at diagnosis but AK at relapse: del(9q) (n=2), t(2;11) (n=1), inv(12) (n=1). *NPM1*-mutated AML with NK or AK showed overlapping morphological, immunophenotypic (CD34-negativity) and gene expression profile (downregulation of CD34 and upregulation of *HOX* genes). No difference in survival was observed among *NPM1*-mutated AML patients independently of whether they carried a normal or abnormal karyotype, the *NPM1*-mutated/*FLT3*-ITD negative cases showing the better prognosis. Findings in our patients point to chromosomal aberrations as secondary events, reinforce the concept that *NPM1* mutation is a founder genetic lesion and indicate that *NPM1*-mutated AML should be clinically handled as one entity, irrespective of the karyotype.
INTRODUCTION

Acute myeloid leukemia (AML) carrying an NPM1 gene mutation causing aberrant cytoplasmic expression of nucleophosmin\(^1\) (NPMc+ AML) accounts for about one-third of adult AML. This large leukemia subgroup usually carries a normal karyotype (NK)\(^1\) and shows distinctive biological, pathological, and clinical features\(^2\), such as mutual exclusion of recurrent genetic abnormalities\(^3\), high frequency of FLT3-ITD mutations\(^1\), frequent FAB M4 or M5 morphology\(^2\), multilineage involvement\(^4\), distinctive gene expression signature\(^5\) and micro-RNA profile\(^6\,7\). Moreover, AML with mutated NPM1 is characterized by good response to induction chemotherapy\(^1\) and favourable prognosis (in the absence of a concomitant FLT3-ITD mutation)\(^8\,13\). Therefore, AML with mutated NPM1 is now included as a provisional entity in the 4\(^{th}\) Edition of the World Health Organization (WHO) classification of tumors of hematopoietic and lymphoid tissues\(^14\).

In our original study describing AML with cytoplasmic/mutated NPM1\(^1\), we found that about 85% of patients carried a normal karyotype (NK), the remaining cohort harboring chromosomal aberrations. However, the type, frequency, biological and clinical significance of these aberrations still remained poorly understood. Although the patients with abnormal karyotype (AK) represent only a minority of all AML with mutated NPM1, their definition with respect to biological, pathological and clinical terms is very important. Indeed, during preparation of the WHO-2008 classification, one of the points that was raised for designating AML with mutated NPM1 as provisional rather than a definitive entity was that the biological, clinical and prognostic significance of chromosomal aberrations in AML with mutated NPM1 was still under debate. This is due to the fact that most studies on NPM1 mutations in AML mainly focused on patients with NK and, in the few studies reporting on NPM1-mutated patients carrying other chromosomal aberrations\(^10\,11\), the
frequency of this association and its biological and clinical significance was not deeply investigated.

To address more in depth this issue, we analyzed the frequency and type of chromosomal aberrations in 631 AML patients with cytoplasmic/mutated NPM1 and compared the results with the frequency and type of additional aberrations occurring in other AML with recurrent genetic abnormalities according to the WHO-2008 classification\textsuperscript{14}, i.e. AML with t(8;21)(q22;q22), inv(16)(p13q22)/t(16;16)(p13;q22), t(15;17)(q22;q12) and t(9;11) or other 11q23-abnormalities leading to an MLL-rearrangement. Other important goals of the study were: i) to investigate variations in the karyotype of AML with mutated \textit{NPM1} during the course of the disease; and ii) to compare the biological, pathological, clinical and prognostic features of \textit{NPM1}-mutated AML carrying chromosomal aberrations with those of typical cases of \textit{NPM1}-mutated AML harboring a normal karyotype.

Our results point to chromosomal aberrations occurring in AML with mutated \textit{NPM1} as secondary events, thus reinforcing the concept that \textit{NPM1} mutation is a founder genetic lesion. In addition, the finding that \textit{NPM1}-mutated AML carrying a NK or AK show overlapping pathological, immunophenotypic and prognostic features has important diagnostic and clinical implications.
MATERIALS AND METHODS

Leukemia patients

We investigated samples from 631 AML patients with cytoplasmic/mutated \( NPM1 \) for whom cytogenetic studies were available: 390 were from the Munich Leukemia Laboratory (MLL); 241 were enrolled in the GIMEMA LAM99P and GIMEMA/EORTC AML12 trials. The frequency and type of cytogenetic aberrations found in \( NPM1 \)-mutated AML were compared with those of additional chromosomal aberrations detected in 266 cases representative of the most common AML with recurrent genetic abnormalities according to the WHO-2008 classification\(^\text{14}\). The latter cases (all from the Munich Leukemia Laboratory) included: 63 AML with t(8;21), 37 AML with inv(16)/t(16;16), 83 AML with t(15;17) and 83 AML showing an 11q23/\( MLL \)-rearrangement.

For prognostic analysis, we focused on 576 AML patients: 390 from the Munich Leukemia Laboratory; 186 cases from the GIMEMA LAM99P and GIMEMA/EORTC AML12 trials who received the same treatment. Therapy in the 390 cases from Munich Leukemia Laboratory was as follows: 141 patients (36%) were treated within the AML Cooperative Group (AMLCG) trials\(^\text{15}\), 35 patients received treatment according to AMLCG protocols but were not enrolled in the AMLCG trial, and another 214 patients received other intensive AML therapy protocols.

All 186 GIMEMA/EORTC patients, both from the LAM99P and the AML12 (registration phase only) protocols, received the same treatment, consisting in a 3-drug induction cycle with Daunorubicine (DNR), Etoposide and Ara-C followed, in case of complete remission, by a single course of consolidation therapy with intermediate-dose ARA-C (500 mg/m\(^2\) every 12 hours in a 2-hour infusion on days 1-6) plus DNR, given on days 4 to 6. After consolidation, younger patients with a sibling donor were then assigned to undergo an allogeneic stem cell transplantation. Those without such a donor, as well as
older patients, were to receive an unpurged autologous stem cell transplantation. Approval for the above studies was provided by the institutional board of each participating center. Informed consent was provided by patients at each participating center according to the Declaration of Helsinki.

**Defining criteria for AML with mutated NPM1**

All 390 cases from Munich Leukemia Laboratory were defined by molecular criteria, i.e. mutational analysis of the NPM1 gene. Nucleic acid isolation from leukemic cells, cDNA synthesis and screening for NPM1 gene mutations was performed using a melting curve based LightCycler assay, as previously described\(^8\). AML samples with an aberrant melting curve underwent subsequent nucleotide sequence analysis.

All 241 AML cases from GIMEMA LAM99P and GIMEMA/EORTC AML12 trials were defined by immunohistochemical criteria, i.e. presence of aberrant cytoplasmic expression of nucleophosmin (NPMc+), which is known to be fully predictive of NPM1 mutations\(^{16,17}\). Nucleophosmin was detected by immunostaining paraffin-sections from B5-fixed/EDTA decalcified bone marrow biopsies with a specific monoclonal antibody directed against a fixative-resistant epitope of human nucleophosmin (clone 376), as previously described\(^{16}\). The antibody-antigen reaction was revealed using a highly sensitive alkaline phosphatase anti-alkaline phosphatase (APAAP) technique\(^{16}\). As expected\(^{1,16}\), all cases showed a nucleus-restricted positivity when stained with a monoclonal antibody directed against the nucleolar protein nucleolin/C23 (Santa Cruz Biotechnology, Santa Cruz, CA). In 138 of the 241 NPMc+ AML cases, material was also available for mutational analysis or Western blotting with antibodies specifically directed against NPM1 mutants. In all cases, these techniques were performed as previously described\(^{1,18}\) and confirmed the presence of NPM1 gene mutations or a mutated NPM1 protein.
Mutational analysis of the FIL3 gene

Analysis for fms-related tyrosine kinase 3 (FLT3)-internal tandem duplication (ITD) was performed in 600 cases. Point mutations in the FLT3-tyrosine kinase domain (TKD), FLT3-TKD, were assessed in 491 cases. Mutational assays were carried out as previously described19,20.

Cytogenetic studies

Cytogenetic studies were performed after short-term culture. Karyotypes, analyzed after G-banding, were described according to the International System for Human Cytogenetic nomenclature21. All NPM1-mutated patients were studied at initial diagnosis. In 31 cases from the Munich Leukemia Laboratory cytogenetic studies were available both at first presentation and at relapse.

Gene expression profiling

The sample preparation assay was performed as previously reported (Affymetrix HG-U133 Plus 2.0 microarrays)22-24. Gene expression raw data were processed according to the manufacturer’s recommendations. After quality control, raw data were normalized using the robust multi-array average normalization algorithm as implemented in the R-package affy version 1.18.025. For supervised statistical analyses, samples were grouped accordingly and for each disease entity differentially expressed genes were calculated by means of t-statistics. To visualize similarity of gene expression patterns, hierarchical clustering and principal component analyses were applied. Transformed gene expression data were analyzed using GeneMaths XT Version 2.1 (Applied Maths, St-Martens-Latem, Belgium) and Partek Genomics Suite Version 6.4 (Partek Inc., St. Louis, MO).
data can be found at Gene Expression Omnibus, GEO, under accession number GSE16015.

**Statistical analysis**

Statistical analysis was performed to investigate the different distribution of quantitative variables, such as age, and qualitative variables, such as gender, CD34 expression, and FLT3 status in the two different cytogenetic groups (NK vs AK) of AML with mutated NPM1. In particular, the t-test was used to analyze mean differences and the chi-square test was applied in case of contingency tables. In case of 2x2 contingency tables, Fisher Exact Test was applied.

Prognostic analysis was performed on 576 NPM1-mutated AML patients treated as described above. Survival curves were calculated for overall survival (OS) and event-free survival (EFS) according to Kaplan-Meier and compared using the log-rank test. OS was calculated from time of diagnosis to death and EFS was calculated from time of diagnosis to death, documentation of persistent leukemia, or relapse.

For all above analyses, results were regarded as significant at a $P$ level less than 0.05 at both sides. SPSS version 14.0.1 software (Chicago, IL) was used for statistical analysis of patients from the Munich Leukemia Laboratory. Statistical analyses of patients enrolled in the GIMEMA LAM99P and GIMEMA//EORTC AML12 studies were performed using the the SAS 9.1 software (SAS Institute Inc, Cary, NC, USA).
RESULTS

Chromosomal aberrations in AML with mutated NPM1 are similar to, but occur at lower frequency than, those associated with AML carrying other recurrent genetic abnormalities

Frequency and type of chromosomal aberrations were investigated by chromosome banding analysis in 631 AML with mutated/cytoplasmic NPM1. A normal karyotype was detected in 538/631 (85.3%) and an abnormal karyotype was present in 93/631 cases (14.7%). This distribution in a large number of cases is almost identical to that we previously observed in a cohort of 166 patients with NPMc+ AML (14.5%)1.

A total of 142 chromosome abnormalities were observed in the 93 NPM1-mutated AML cases with AK (Table 1). The most frequent abnormalities were +8, +4, -Y, del(9q) and +21. Other aberrations were non-recurrent balanced rearrangements (n=10) and non-recurrent unbalanced abnormalities (n=57). In 4 cases, the definition of a complex aberrant karyotype was fulfilled (three or more clonal chromosome aberrations according to SWOG). However, these karyotypes did not show the typical pattern of chromosomal gains and losses observed in the majority of cases with complex aberrant karyotype such as loss of 5q, 7q 12p, 16q and 17p and gain of 8q, 11q and 21q (Table 1). Two of these cases were FLT3-ITD positive and 2 negative.

As expected3, none of the 93 cases of NPM1-mutated AML with abnormal karyotype harbored any of the AML-associated recurrent genetic abnormalities, as defined by the WHO-2008 classification14. No differences in the type and frequency of chromosomal aberrations were observed in AML carrying NPM1 mutation A, B, D or other rare mutation types.

Our results in a large series of leukemia patients clearly demonstrate that chromosomal aberrations are rather uncommon in AML with mutated/cytoplasmic NPM1.
We then compared the frequency and types of clonal chromosomal aberrations detected in 93/631 AML with mutated *NPM1* with those found in 266 AML with recurrent genetic abnormalities of WHO classification. The results are summarized in Table 1, and clearly indicate that the type of chromosome aberrations detected in AML with mutated *NPM1* are similar to, but occur at lower frequency than, additional chromosome changes found in AML with recurrent genetic abnormalities\textsuperscript{26-28}, according to the WHO-2008 classification\textsuperscript{14}.

**Chromosomal aberrations in AML with mutated *NPM1* are secondary events**

To assess whether chromosomal aberrations in AML with mutated *NPM1* were primary or secondary events, we applied chromosome banding at diagnosis and relapse in 31 patients with *NPM1*-mutated AML (all from Munich Leukemia Laboratory). All 31 cases showed the same *NPM1* mutation at diagnosis and relapse. Therefore, in no case a loss of the *NPM1* mutation was observed. Median time from diagnosis to relapse was 301 days (range: 71-986). Most cases (22/31; 71\%) had a normal karyotype both at diagnosis and relapse. However, 4 patients showing a normal karyotype at diagnosis were found to have an abnormal karyotype at relapse: del(9q) (n=2), t(2;11) (n=1), and inv(12) (n=1). One case with +8 at diagnosis showed +8 also at relapse. One case with +4 at diagnosis showed +4 and additional aberrations at relapse. In 1 case, clonal regression was observed (+21 -> NK). One case with an unbalanced 1;3-translocation at diagnosis showed a der(17;18)(q10;q10) at relapse; and one case with −Y at diagnosis showed a del(3p) at relapse.

We next investigated the occurrence of acquired *NPM1* mutations at relapse in AML cases that, at first presentation, showed a germline *NPM1* gene and a chromosomal aberration, such as +8 or del(9q). Here, we focused on cases with +8 or del(9q), since these aberrations were commonly detected in the small subgroup of *NPM1*-mutated AML.
with AK (Table 1). Notably, all 7 cases of AML with germline \textit{NPM1} that, at the time of initial diagnosis, had presented with +8 (n=5) or del(9q) (n=2), as sole abnormality, maintained the same karyotype and did not acquire an \textit{NPM1} mutation at relapse.

Taken together, these results point to chromosomal aberrations occurring in AML with mutated \textit{NPM1} as acquired secondary events.

**AML with mutated \textit{NPM1} carrying a normal karyotype or chromosomal aberrations show a similar gene expression profile**

In order to further investigate the biological nature of the two subset of \textit{NPM1}-mutated AML (NK vs AK), gene expression microarray studies were carried out. First, restricting analysis to cases of AML with NK, a group of 42 \textit{NPM1}-unmutated (NPMc-) cases was compared against a group of 55 \textit{NPM1}-mutated cases (NPMc+). The resulting signature of the top-500 differentially expressed probe sets clearly separated the two AML groups both in a hierarchical clustering approach and a Principal Component Analysis (the gene list is provided in the Supplementary Materials). Subsequently, the analysis was expanded to include 10 additional cases of \textit{NPM1}-mutated AML carrying chromosomal aberrations: 45,X,-Y (n=4); 47,XX,+21 (n=3); 47,XX,+8 (n=2); and 46,XX,del(9)(q22q34) (n=1). Interestingly, we observed that, when using the gene list from AML with NK, the \textit{NPM1}-mutated AML cases carrying chromosomal aberrations had an expression profile similar to that of the group of \textit{NPM1}-mutated AML with NK (Figure 1).

**AML with mutated \textit{NPM1} carrying a normal karyotype or chromosomal aberrations show overlapping pathological, immunophenotypic and clinical features**

\textit{NPM1}-mutated AML carrying NK or an AK were then compared in terms of age distribution, gender, morphological appearance according to FAB/WHO, CD34 expression, \textit{FLT3}-ITD and \textit{FLT3}-TKD status.
In the full cohort of 631 *NPM1*-mutated AML patients, mean age (± standard deviation) was 54.39 (± 14.53) years in the NK group and 55.78 (± 15.78) in the AK group (p=0.39). No significant difference in age distribution of the patients emerged when they were stratified in decades (age <21, age 21-30, age 31-40, age 41-50 and age 51-60). Males were 252/538 (46.8%) in the NK group vs 47/93 (50.5%) in the AK group (p=0.57). FAB categories were available in 507 cases with a distribution in NK and AK groups not statistically significant (p=0.076).

Downregulation of CD34 is a distinguishing immunophenotypic feature of AML with cytoplasmic/mutated NPM1\(^1\). Therefore, we were interested to assess this parameter in *NPM1*-mutated AMLs with NK and AK. Material for analysis of CD34 expression by immunocytochemical labelling of cell suspensions or bone marrow tissue sections was available for 422 AML with cytoplasmic/mutated NPM1 (357 with NK; 65 with AK). Cases were regarded as negative if the percentage of CD34-positive cells was <10%. Absence of CD34 expression was found in 284/357 (79.6%) and 47/65 (72.3%) of the NK and AK group, respectively (p=0.19) (Figure 2).

Internal tandem duplication (ITD) analysis of the *FLT3* gene was available in 600 *NPM1*-mutated AML cases (515 with NK; 85 with AK). *FLT3*-ITD was detected in 190/515 (36.9%) of NK group and 23/85 (27.1%) of AK group (p=0.087). Analysis of *FLT3*-TKD mutation was available in 491 cases (422 NK; 69 AK). *FLT3*-TKD mutations were detected in 38/422 of NK group (9.0%) and 14/69 of AK group (20.3%) respectively (p=0.01) (Figure 3).

The above findings indicate that, with the exception of a difference in the distribution of *FLT3*-TKD mutations, AML with cytoplasmic/mutated NPM1 carrying a NK or AK show overlapping morphological, immunophenotypic and clinical features.
AML with mutated *NPM1* carrying normal karyotype or chromosomal aberrations show a similar outcome

The impact of chromosomal aberrations on prognosis was assessed in a total of 576 AML patients with mutated *NPM1*, including 390 cases from the Munich Leukemia Laboratory and 186 cases from the GIMEMA LAM99P and GIMEMA/EORTC AML12 trials. In patients from the Munich Leukemia Laboratory the complete remission (CR) rate was comparable in cases with *NPM1* mutation and a normal karyotype to those with an aberrant karyotype (80.9% vs 85.7%). Overall survival and event-free survival of patients from Munich Leukemia Laboratory did not significantly differ between 328 *NPM1*-mutated AML with normal karyotype and 62 *NPM1*-mutated AML with aberrant karyotype (median OS: not reached vs not reached; % alive at 2 years 63.5% vs 59.0%,  p=0.804; median EFS: 16.0 months vs 14.0 months, p=0.849) (Figures 4A and B). OS was significantly shorter in *NPM1*-mutated cases with additional *FLT3*-ITD (n=138) as compared to *NPM1*-mutated cases without *FLT3*-ITD (n=241) (median OS: not reached vs not reached; % alive at 2 years 50.9% vs 70.5%,  p=0.015) (Supplementary Figure 1). Also EFS was significantly shorter in the *NPM1*-mutated/*FLT3*-ITD+ subgroup versus *NPM1*-mutated/*FLT3*-ITD- (11.6 months vs 17.0 months; p=0.045) (Supplementary Figure 1).

Considering only *FLT3*-ITD negative cases, no statistically significant difference emerged in OS and EFS of *NPM1*-mutated AML with NK vs AK (median OS: not reached vs 21.0 months; % alive at 2 years 74.5% vs 48.5%,  p=0.075); and median EFS was 18.1 months vs 8.6 months (p=0.176) (Figures 4C and D). Also in the subgroups of *FLT3*-ITD positive cases no difference in OS and EFS was observed between *NPM1*-mutated AML with NK or AK (median OS 21.1 months vs not reached; % alive at 2 years 47.5% vs 83.3%,  p=0.245); and median EFS was 9.5 months vs 14.9 months (p=0.354) (Supplementary Figure 2)
The same analysis was done for the 186 GIMEMA LAM99P and GIMEMA/EORTC
AML12 patients who received the same treatment (although enrolled in two consecutive
studies). The CR rate difference between \textit{NPM1}-mutated AML with normal or aberrant
karyotype was not statistically significant (79.4 vs 76.0 respectively, \( p=0.7 \)). No differences
were observed both for OS and for EFS in the whole population according to karyotype
(Figures 5A and B). Also considering separately the \textit{FLT3}-ITD negative (Figures 5C and
D) and the \textit{FLT3}-ITD positive cases (Supplementary Figure 3), again the outcome in terms
of OS and EFS was not influenced by the karyotype.
DISCUSSION

This study clearly shows that chromosomal aberrations occur infrequently in AML with mutated NPM1 (about 15% of cases), are different from the typical AML-associated recurrent genetic abnormalities (as defined by the 2008 WHO Classification)\textsuperscript{14}, and are likely represent secondary genetic events. Our results also indicate that AML with mutated NPM1 carrying a normal karyotype or concomitant chromosomal aberrations has overlapping biological, pathological, immunophenotypic and clinical features. Taken together, these findings strongly suggest that AML with mutated NPM1, irrespective of concomitant chromosome abnormalities, represents a single disease entity whose molecularly defining feature is the presence of a mutated NPM1 gene. These results have also important diagnostic and clinical implications.

A number of findings in this study point to chromosomal aberrations detected in a minority of our patients, as secondary genetic events. The type of chromosome aberrations found in NPM1-mutated AML are mostly similar to the additional chromosome aberrations detectable in AML with t(8;21), inv(16), t(15;17) or 11q23/MLL-rearrangements, which are thought to be secondary alterations\textsuperscript{26-28}. In AML with cytoplasmic/mutated NPM1, cytogenetic studies frequently showed mosaicism, i.e. the presence of cells with an abnormal karyotype as subclones within the population with a normal karyotype\textsuperscript{1}. More importantly, a proportion of NPM1-mutated AML cases carrying a normal karyotype at initial diagnosis acquired a chromosomal aberration during the course of the disease, while retaining the original NPM1 mutation. It should be added that in this study, a few NPM1-mutated AML patients with AK at diagnosis showed either clonal regression (AK to NK) or changing to a different AK at relapse, while maintaining the original NPM1-mutated gene status. Finally, we found that NPM1-mutated AML with NK or chromosomal aberrations have a very similar gene expression profile characterized by the
expected down-regulation of the CD34 and CD133 genes and overexpression of most HOX genes\textsuperscript{5,10,29}.

Secondariness of chromosomal aberrations in NPM1-mutated AML is in line with the growing body of evidence that NPM1 mutation is a founder genetic lesion in AML, as supported by the following observations: i) cytoplasmic mutated nucleophosmin is specific for AML\textsuperscript{1,30,31} and clinically shows close association with AML of de novo origin\textsuperscript{1,32-34}; ii) NPM1 mutations are mutually exclusive of other recurrent genetic abnormalities in AML\textsuperscript{3} (with the exception of rare cases in which both NPM1 and CEPBA mutations co-exist); iii) AML with mutated NPM1 shows distinctive gene expression signatures\textsuperscript{5,10,29} and microRNA profiles\textsuperscript{5,7}; iv) all NPM1 mutations generate common changes at the C-terminus of nucleophosmin protein which appear to maximize nuclear export of NPM1 leukemic mutants\textsuperscript{1,35-37}, pointing to their cytoplasmic dislocation as the central event for leukemogenesis\textsuperscript{36,38}; v) NPM1 mutations are stable during the course of the disease\textsuperscript{39-41}, as the same type of NPM1 mutation is consistently detected at relapse in medullary and extramedullary sites. Loss of NPM1 mutation has been rarely observed in NPM1-mutated AML\textsuperscript{42}. These cases, however, could represent secondary treatment-related AML rather than relapse of the de-novo AML\textsuperscript{43}, and vi) quantitative real-time PCR shows that NPM1 mutations disappear at complete remission\textsuperscript{44,45}.

Interestingly, we found that the incidence of associated chromosomal aberrations was lower in AML with mutated NPM1 (14.7\%) than in AML with 11q23/MLL rearrangement, inv(16), t(15;17) and t(8;21) (frequency ranging from 34\% to 70\%). These findings and the fact that about 85\% of AML with mutated NPM1 carry a normal karyotype suggest that this type of leukemia may be characterized by a higher genomic stability than other leukemias listed in the group of “AML with recurrent genetic abnormalities” of the WHO classification\textsuperscript{14}, such as AML with 11q23/MLL rearrangement, inv(16), t(15;17) and t(8;21).
High genomic stability was recently observed in an NPM1-mutated/FLT3-ITD positive AML patient who, at whole-genome sequencing\textsuperscript{46}, showed no deletions or LOH, and mutations of yet unknown significance of only 8 genes (in addition to those affecting NPM1 and FLT3). The eight mutated genes were: CDH24 and PCLK (encoding protocadherin/caderin family members), GPR123 and EBI2 (encoding G-protein-coupled receptors), PTPRT (encoding a protein phosphatase), KNDC1 (encoding a potential guanine nucleotide exchange factor), SLC15A1 (encoding a peptide/drug transporter), and GRINL1B (encoding a glutamate receptor gene). Notably, when the nonsense and missense mutations affecting these 8 genes were searched in additional 187 AML cases (including 43 carrying an NPM1 mutation), none of them were detected\textsuperscript{46}. In our opinion, these results further support the concept that NPM1 is a primary genetic lesion, since it is specific for AML\textsuperscript{1,31}, is recurrent in about 30% of adult AML\textsuperscript{2}, and defines a type of leukemia with distinctive biological and clinical features\textsuperscript{2} as well as unique gene expression\textsuperscript{5,10,29} and microRNA signatures\textsuperscript{6,7}. On the contrary, the eight additional mutated genes are likely to represent secondary events that, in this particular whole-sequenced patient\textsuperscript{46}, may signify either “passenger” or cooperating genetic alterations.

One possible explanation for the high genomic stability of AML with mutated NPM1 is that the NPM1 mutant alone induces leukemic transformation. Notably, in transfected cells, NPM1 leukemic mutants decreased the activity of tumor oncosuppressor ARF\textsuperscript{47,48} and caused activation of the MYC oncogene\textsuperscript{49}. When combined with haploinsufficiency for wild-type NPM1\textsuperscript{50}, this double-edged sword could result into full potential to induce leukemic transformation. This would be also in accordance with a recently developed one-mutation mathematical model \textsuperscript{51} for NPM1-mutated AML. However, clarification of the leukemogenic role of NPM1 mutants awaits the development of appropriate animal models.
Our results have also important diagnostic and clinical implications. The finding that NPM1-mutated AML with NK or AK have overlapping biological, pathological, immunophenotypic and clinical features justifies their inclusion in the new WHO classification, as a single entity under the term of “AML with mutated NPM1”\(^{14}\).

According to our results, cytogenetic status (NK vs AK) seems to have no significant impact in the prognosis of patients with NPM1-mutated AML. The most clinically relevant finding was that FLT3-ITD negative NPM1-mutated AML patients carrying a normal or aberrant karyotype showed the same relatively favourable prognosis. These results were based upon the comparison between NPM1-mutated AML patients with NK or AK group, as a whole. Thus, we cannot exclude that single sporadic chromosomal aberrations within the NPM1-mutated AK group may have an impact on prognosis. In spite of this potential limitation, our findings raise the question of whether assessment of the prognostic value of NPM1 and FLT3-ITD mutations in the framework of normal karyotype\(^8-13\) represents the most rationale way for risk-stratification of AML patients. In fact, this approach has two major limitations: i) it excludes a significant number of AML patients because of failure of cytogenetic analysis (up to 25% in large multicenter studies, including GIMEMA/EORTC); and ii) it prevents assignment of AML patients to the group with favorable genotype (NPM1 mutated/FLT3-ITD negative) if a chromosomal aberration is present. Thus, in line with the new WHO classification\(^{14}\), a patient should simply be classified as “AML with mutated NPM1” (when carrying an NPM1 mutation or aberrant cytoplasmic expression of nucleophosmin), independently of whether the karyotype is normal or not. In the absence of FLT3-ITD, this patient should then be regarded as potentially belonging to the good favourable prognostic group. This approach would also offer the opportunity to investigate in future large multicenter clinical trials whether single, rare chromosomal abnormalities may affect the prognosis of NPM1-mutated AML patients. Use of normal karyotype as initial framework for analysis of other mutations or prognostic factors is likely to be more...
important in the group of AML patients that does not carry \textit{NPM1} mutations (about 40% of all AML with NK), whose molecular nature still remains uncertain.

In conclusion, our studies further support the concept that AML with mutated \textit{NPM1} represents a distinct entity and have important diagnostic and clinical implications.

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For a complete list of GIMEMA study group participants and AMLCG participant centers, see the supplemental appendices.

\section*{Authors’ contributions}

CH studied cytogenetically the cases from Munich Leukemia Laboratory and contributed to write the paper; CM, MM, AC, NT and GRC performed the cytogenetic studies on patients from the GIMEMA/EORTC studies and contributed to write the paper; SS carried out the molecular studies on patients from Munich Leukemia Laboratory and contributed to write the paper; AK investigated the gene expression profile of cases from
Munich Leukemia Laboratory and contributed to write the paper; AS performed the statistical analyses on patients enrolled in the GIMEMA/EORTC trials; MPM characterized by immunohistochemistry/WB the samples from the GIMEMA/EORTC patients; MV and PF carried out the prognostic analysis of the GIMEMA/EORTC patients. TH carried out clinical and statistical correlations among AML patients from the Munich Leukemia Laboratory and contributed to write the paper; BF had the original idea for the study and wrote the paper.

Conflict of interest disclosure: B.F. and C.M. applied for a patent on clinical use of NPM1 mutants. All other authors declare no conflict of interest.
References


<table>
<thead>
<tr>
<th>Karyotype</th>
<th>AML</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>NPM1-mut*</td>
</tr>
<tr>
<td>(N= 631)</td>
<td>(N=63)</td>
</tr>
<tr>
<td>Additional abnormalities</td>
<td>93/631*</td>
</tr>
<tr>
<td></td>
<td>(14.7%)</td>
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<tr>
<td>-X/-Y</td>
<td>11</td>
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<tr>
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<tr>
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<tr>
<td>ider(17)(q10)t(15;17)</td>
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<tr>
<td>Other</td>
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<tr>
<td>Total</td>
<td>142*</td>
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</tbody>
</table>

*More than one abnormality was present in a subset of cases with aberrant karyotype.

*AML with mutated NPM1.

# Includes four cases fulfilling the definition of a complex aberrant karyotype but without a typical pattern of chromosomal gains and losses:

- 90,XXXX,-3,+8,+8,-10,-11,-17; FLT3-ITD- (74 days, alive).
- 49,XY,+der(5)t(5;17)(q11:?),+8,+del(13)(q12); FLT3-ITD- (30 days, alive).
- 53,XX,+4,+5,+8,+8,der(12)t(12;13)(q24;q?),+16,+18,+20; FLT3-ITD+ (86 days, dead).
- 55,XY,+X,+4,+5,+8,+10,+13,+14,+17,+18; FLT3-ITD+ (5 days, dead).
Figure 1. Gene expression profiling analysis of NPM1-mutated AML (NK vs AK).
Analysis based on the top-500 differentially expressed genes between AML cases NPM1c- (unmutated NPM1) and NPM1c+ (mutated NPM1). The gene discovery process was restricted to AML with normal karyotype, i.e. a supervised analysis was performed. A) Hierarchical clustering heatmap including also 10 NPM1 mutated AML cases with other chromosomal aberrations. The similarity of the genes was computed by Euclidean distance, and then Ward's method was used to cluster the gene expression profiles based on these measures. The normalized expression value for each probe set is coded by color (standard deviation from mean). Red cells indicate high expression and green cells indicate low expression. B) Principal Component Analysis (PCA). The AML samples were plotted in a three-dimensional space using the three principal components (PC) capturing most of the variance in the original dataset (PC1=34.0%, PC2=7.0%, PC3=5.2%). Each patient sample is represented by a single color-coded sphere.

Figure 2. Morphology and immunophenotype of NPMc+ AML with abnormal karyotype. The figure shows the morphological and immunohistological features of a representative example of NPM1-mutated AML with aberrant karyotype 46,XX,der(19)t(13;19)(q12;q13)/ 47,XY,+8 (Top panel). These features are identical to those observed in the typical NPM1-mutated AML cases with normal karyotype. Middle left) Diffuse marrow infiltration by leukemic cells with myelomonocytic appearance. The arrow points to a dysplastic megakaryocyte (paraffin section from bone marrow biopsy; hematoxylin-eosin; x 800). Middle right) Leukemic cells are CD34-negative. The single arrow indicates a dysplastic megakaryocyte whilst the double arrows indicate a CD34-positive vessel. Bottom left) Myelomonocytic leukemic cells and a dysplastic
megakaryocyte (arrow) show aberrant cytoplasmic expression of nucleophosmin (NPM1), indicating they belong to the same clone; cells showing nucleus-restricted expression of nucleophosmin represent normal residual hemopoietic precursors. Bottom right) Leukemic cells and a dysplastic megakaryocyte (arrow) show nucleus-restricted positivity for C23/nucleolin. Middle right and bottom panels: immunostaining of paraffin sections from bone marrow biopsy using the alkaline phosphatase monoclonal anti-alkaline phosphatase (APAAP) technique; hematoxylin counterstaining; x 800.

Figure 3. Distribution of FLT3-ITD and FLT3-TKD mutations in NPM1-mutated AML with normal karyotype and abnormal karyotype.

Figure 4. Survival curves of NPM1-mutated AML patients (NK vs AK) from the Munich Leukemia Laboratory.

A) No significant differences in overall survival between NPM1-mutated AML with normal karyotype (n=328) and NPM1-mutated cases with aberrant karyotype (n=62) are observed (p=0.804).

B) No significant differences in event free survival between NPM1-mutated AML with normal karyotype (n=328) and NPM1-mutated cases with aberrant karyotype (n=62) are observed (p=0.849).

C) No significant differences in overall survival between NPM1-mutated/FLT3-ITD-negative AML with normal karyotype (n=197) and NPM1-mutated cases with aberrant karyotype (n=44) are observed (p=0.075).

D) No significant differences in event free survival between NPM1-mutated/FLT3-ITD-negative AML with normal karyotype (n=197) and NPM1-mutated cases with aberrant karyotype (n=44) are observed (p=0.176).
Figure 5. Survival curves of *NPM1*-mutated AML patients (NK vs AK) from the GIMEMA LAM99P and GIMEMA/EORTC AML12 trials. A) No significant differences in overall survival are observed between *NPM1*-mutated AML with normal and aberrant karyotype (p=0.877); B) No significant differences in event free survival are observed between *NPM1*-mutated AML with normal and aberrant karyotype (p=0.827); C) No significant differences in overall survival are observed between *NPM1*-mutated/*FLT3*-ITD-negative AML with normal and aberrant karyotype (p=0.814); D) No significant differences in event free survival are observed between *NPM1*-mutated/*FLT3*-ITD-negative AML with normal and aberrant karyotype (p=0.970).
Figure 1

A

B

42 AML normal karyotype, $NPM1c^-$

55 AML normal karyotype, $NPM1c^+$

10 AML other aberrations, $NPM1c^+$

Normal karyotype

$NPM1c^-$

$NPM1c^+$
Figure 2
Figure 3

No. of Cases

FLT3/ITD +

FLT3/ITD -

(p=0.08)

TKD +

TKD -

(p=0.01)

NPMc+AML (NK=515)
NPMc+AML (AK=85)

NPMc+AML (NK=422)
NPMc+AML (AK=69)
Figure 4

A. For overall survival, the probability for aberrant karyotype, n=62, is shown compared to normal karyotype, n=328. The p-value is 0.804.

B. For event-free survival, the probability for aberrant karyotype, n=62, is shown compared to normal karyotype, n=328. The p-value is 0.849.

C. For FLT3-ITD negative, the probability for overall survival, normal karyotype, n=197, is shown compared to aberrant karyotype, n=44. The p-value is 0.075.

D. For FLT3-ITD negative, the probability for event-free survival, normal karyotype, n=197, is shown compared to aberrant karyotype, n=44. The p-value is 0.176.
Figure 5

A

FLT3-ITD negative

Overall Survival

normal karyotype, n=161
aberrant karyotype, n=25

p=0.877

B

FLT3-ITD negative

Event Free Survival

aberrant karyotype, n=25
normal karyotype, n=161

p=0.827

C

FLT3-ITD negative

Overall Survival

normal karyotype, n=98
aberrant karyotype, n=16

p=0.814

D

FLT3-ITD negative

Event Free Survival

aberrant karyotype, n=16
normal karyotype, n=98

p=0.970
AML with mutated NPM1 carrying a normal or aberrant karyotype show overlapping biological, pathological, immunophenotypic, and prognostic features

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