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Nucleophosmin analysis in acute myeloid leukemia from a single center: value of combining immunohistochemistry with molecular mutation analysis

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Running title: Immunohistochemical and molecular analysis of NPM1 mutations

Key words
Acute myeloid leukemia; NPM1 mutation; nucleophosmin; immunohistochemical staining; molecular analysis; gene expression.

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Abstract

Mutations of nucleophosmin 1 are frequently found in acute myeloid leukemia and lead to aberrant cytoplasmic accumulation of nucleophosmin protein. Immunohistochemical staining is therefore recommended as technique of choice in front-line screening. In this study, we assessed the sensitivity and specificity of immunohistochemistry on formalin fixed bone marrow biopsies compared with gold standard molecular analysis to predict nucleophosmin 1 mutation status in 119 acute myeloid leukemia patients. Discrepant cases were further characterized by gene expression analyses and fluorescence in situ hybridization. A large overlap between both methods was observed. Nevertheless, nine patients demonstrated discordant results at initial screening. Five cases demonstrated nuclear staining of nucleophosmin 1 by immunohistochemistry, but an nucleophosmin 1 mutation by molecular analysis. In three cases this could be contributed to technical issues and in two cases minor subpopulations of myeloblasts had been discovered initially. All tested cases exhibited the characteristic nucleophosmin-mutated gene expression pattern. Four cases had cytoplasmic nucleophosmin 1 staining and an nucleophosmin-mutated gene expression pattern without a detectable nucleophosmin 1 mutation. In two of these cases we found the chromosomal translocation t(3;5)(q25;q35) encoding the NPM-MLF1 fusion protein. In the other discrepant cases the aberrant cytoplasmic nucleophosmin staining and gene expression could not be explained. In total six patients (5%) had true discordant results between immunohistochemistry and molecular analysis. We conclude that cytoplasmic nucleophosmin localization is not always caused by a conventional nucleophosmin 1 mutation and that in the screening for nucleophosmin 1 abnormalities, most information will be obtained by combining immunohistochemistry with molecular analysis.
Introduction

Mutations in the nucleophosmin gene (NPM1) are found in 30% of patients with acute myeloid leukaemia (AML) and lead to aberrant accumulation of nucleophosmin protein in the cytoplasm. Distinctive biological and clinical features can be observed in NPM1 mutated AML, including a unique gene expression profile, a distinct microRNA signature, low expression of CD34 in more than 95% of cases, increased incidence of FLT3 internal tandem duplications (ITD) in about 40% of cases, and a good response to induction chemotherapy. NPM1 mutated AML was included as a provisional entity in the 2008 World Health Organization classification of myeloid neoplasms. Currently more than 40 different mutations in the NPM1 gene have been identified. All these variants lead to common changes at the C-terminus of the gene and cause aberrant dislocation of the NPM1 protein into the cytoplasm of the AML blast cells. This feature of NPM1 mutated AML can be used for diagnostic purposes. Immunohistochemical staining of NPM1 on bone marrow biopsies has indeed been described to be fully predictive of NPM1 mutations by two independent research groups. The low costs and relative simplicity are important advantages of immunohistochemistry in diagnostic screening, especially if more sophisticated molecular techniques are not available. On the other hand, compared to molecular analyses, immunohistochemistry is more prone to inter-observer variability and variability due to technical issues. Indeed, in the study of Konoplev et al., immunohistochemical staining was not completely predictive for NPM1 mutations. Still, immunohistochemical staining is recommended as technique of choice in simple front-line screening, with a reported sensitivity and specificity of 100% on B5 fixed and EDTA decalcified bone marrow biopsies, and for diagnosis of AML patients presenting with “dry tap” or myeloid sarcoma.

In this study, we assessed the sensitivity and specificity of immunohistochemistry on formalin fixed bone marrow biopsies compared with gold standard molecular analysis to predict NPM1 mutation status in a large cohort of AML patients from our institution. The cases that were found to have a discrepancy between the two methods were extensively evaluated.
Methods

Patients
Bone marrow biopsies for immunohistochemical analysis and either peripheral blood or bone marrow aspirates for RNA isolation were obtained from untreated patients diagnosed with AML after achieving informed consent. The study protocol was approved by the Medical Ethical Committee of the University Medical Center Groningen.

Immunohistochemical staining for NPM1
The immunohistochemical stainings were performed on bone marrow biopsies that were fixed in 10% neutral phosphate buffered formalin (3.6% formaldehyde) for at least 12h, and decalcified in a solution containing 10% (v/v) acetic acid and 10% formalin (v/v; 3.6% formaldehyde) for 1 or 2 days. Detection of NPM1 localization was routinely performed on paraffin embedded 3 µm (and also 1-2 µm for discrepant cases) tissue sections by immunohistochemical staining using a Benchmark XT immunostainer (Ventana Medical Systems S.A., Tucson, AZ, USA). The NPM1 antigen was retrieved with TRIS EDTA buffer (pH 8.5). Endogenous peroxidase was blocked with H2O2. Slides were incubated with 1:50 diluted supernatant of the anti-NPM1 antibody (clone 376, 1G3) (kindly provided by Prof. Falini, Perugia, Italy). NPM1 was visualized using the ultraview universal DAB detection kit (Ventana). The nucleolin antigen was retrieved with a TRIS EDTA buffer (pH 9.0). The anti-nucleolin antibody (C23, Santa Cruz, Dallas, TX, USA) was used at a dilution of 1:50 and visualized using horse-radish peroxidase-labeled rabbit-anti-mouse and goat-anti-rabbit antibodies (Dako, Copenhagen, Denmark) and 3,3′-diaminobenzidine (Sigma Aldrich, St Louis, MO, USA). The NPM1 and nucleolin stainings were initially scored as being either exclusive nuclear or combined nuclear and cytoplasmic by an experienced hematopathologist (S.R., A.D. or P.M.K.).

Molecular analysis of NPM1
Molecular NPM1 mutation analysis was performed using RT-PCR of exon-12 harboring most NPM1 mutations. cDNA fragment analysis was performed with a fluorescent-labeled forward primer (CTTCCGGATGACTGACCAAGAG) and a reverse primer (CCTGGACAACATTTATCAAACG). The fragment analysis was validated by sequencing the RT-PCR product for the first 100 patient samples analyzed with fragment analysis, revealing 100% accuracy for detecting either two wild type (non-mutated NPM1) or a wild type and mutated allele (NPM1 mutated). For the cases with cytoplasmic NPM1 staining but no mutation by fragment analysis, a further analysis of exon 9 and 11 was performed by RT-
PCR using the forward primer AGCGCCAGTGAAGAAATC and the reverse primer CACGGTAGGGAAAGTTCTC and sequencing the PCR product.

qRT-PCR analyses
Total RNA was isolated using the RNeasy kit from Qiagen (Venlo, The Netherlands) according to the manufacturer’s recommendations. The presence of NUP98/NSD1 and the reciprocal NSD1/NUP98 translocations were determined by qRT-PCR. Primers sequences and cycle conditions have been described previously. Samples known to express the NUP98/NSD1 and the reciprocal NSD1/NUP98 translocations were kindly provided by dr. P.J.M. Valk (Erasmus MC, Rotterdam, The Netherlands) and used as positive controls. The sequences of the primers used for the qRT-PCR analysis of various HOX genes, MEIS1, PBX3, BAALC and MN1 and the housekeeping genes RPL27 and HPRT can be found in the supplementary material (Supplemental Table 1). All qRT-PCR analyses were performed in triplicate and the mean expression of these triplicates is indicated in the results.

Fluorescence in situ hybridization (FISH)
Cytogenetic and FISH studies were performed according to standard methods. All discrepant samples were screened for the t(3;5) NPM-MLF1 gene fusion using FISH. Both interphase nuclei and metaphases were analyzed with the TLX3 Breakapart probe (CytoCell, LPH050, Cambridge, UK) and with the artificial chromosome (BAC) (RP11-117L6 chr:170,746,923 - 170,922,033), kindly provided by Prof M. Rocchi (Department of Genetics and Microbiology, University of Bari, Italy) to confirm the presence of a break within the NPM1 gene suggestive for a translocation t(3;5).
Results

No complete concordance for immunohistochemical analysis on formalin fixed bone marrow biopsies and molecular analysis for the detection of NPM1 mutations

For a total of 119 AML patients, diagnosed between 2005 and 2010 in the University Medical Center Groningen, both immunohistochemical as well as RT-PCR analysis for NPM1 mutations was performed. Patients with a t(8;21), t(15;17), inv(16) or t(16;16)) were excluded, since NPM1 mutations were reported to be mutually exclusive with these cytogenetic abnormalities.\(^1\)\(^,\)\(^5\) Patient characteristics are provided in Table 1.

Screening for NPM1 mutations by fragment analysis revealed mutated NPM1 in 34 out of 119 patients (29%). In the subgroup of patients with a normal karyotype AML (n=68), 28 (41%) had an NPM1 mutation by fragment analysis. Screening for NPM1 dislocation by immunohistochemistry on formalin fixed bone marrow biopsies revealed cytoplasmic NPM1 in 33 out of 119 patients (28%). However, 5 cases had mutant NPM1 by fragment analysis but no characteristic cytoplasmic localization of NPM1 by initial immunohistochemistry used for diagnosis, and reversibly, 4 cases had no NPM1 mutation detected by fragment analysis but cytoplasmic localization of NPM1 by immunohistochemistry (Figure 1 and Supplementary Figure 1, Table 2).

All cases with discordant results were further analyzed. Since mutations of exon 9 and 11 of the NPM1 gene have been described to occur in rare cases,\(^1\)\(^5\)\(^,\)\(^1\)\(^6\) we performed additional sequencing analysis of exon 9, 11 and 12 for those 4 AML cases exhibiting cytoplasmic NPM1 by immunohistochemistry but no mutation by fragment analysis. This analysis did not reveal any mutations. Recently, a patient has been reported with mutated NPM1 detected by molecular analysis but predominant nuclear NPM1 by immunohistochemistry with a faint localization in the cytoplasm.\(^1\)\(^7\) It was suggested that the amino acids at position 270 and 272 could be important for proper subcellular localization of NPM1. The types of NPM1 mutations found in our discrepant cases are depicted in Supplemental Table 2. All these mutations were found in exon 12 of the NPM1 gene and did not resemble the mutation described by Planta and colleagues.

Further analysis of the 5 discrepant cases included re-cutting tissue sections at 1-2 µm and microscopic analysis at 100x magnification. In 2 discordant cases with a proven mutation, blast cells again showed exclusively nuclear staining (cases 1 and 4 in Table 2; Figure 1 and Supplementary Figure 1). In one case of AML with signs of differentiation (FAB M2) a minority of cells, probably the blasts, showed cytoplasmic staining (case 3), which had been overlooked in the original analysis of the thicker tissue sections. In an additional case with AML with multilineage dysplasia, previously classified as AML M6 (case 2), only small clusters of myeloblasts showed cytoplasmic staining. Finally the fifth discrepant case, a
patient with unclassifiable AML (dry tap due to extensive sclerosis) and very few circulating blasts, the biopsy used for the original analysis showed only nuclear staining. However, a biopsy taken one day earlier in the referring hospital and only analyzed at re-analysis, showed convincingly cytoplasmic staining in apparently more blastic cells with larger nuclei and bigger nucleoli (case 5, Supplementary Figure 1). Altogether, after re-analysis using 1-2 µm sections of the 5 discrepant cases with detectable mutations of \textit{NPM1} without cytoplasmic staining only 2 cases remained discrepant.

In the group of four discordant cases with cytoplasmic staining of \textit{NPM1} but no detectable mutation (case 6-9 in Table 2), cytoplasmic staining was very strong in one case (case 6) and fuzzy to granular in cases 8 and 9, whereas cytoplasmic staining of a very limited population of blasts was observed in case 7 (Figure 1). Immunohistochemistry for nucleolin was performed to exclude artifacts, i.e. abnormal general diffusion of nuclear proteins into the cytoplasm leading to false positive immunohistochemical results. Such an artifact was not observed (Figure 1).

To analyze the impact of the staining technique, re-analyses of all discrepant cases were performed using APAAP in the visualization of \textit{NPM1} in immunohistochemistry. Results are included as Supplemental Figure 1 and were in accordance with the results obtained by DAB visualization. After re-analysis of the 4 discrepant cases with cytoplasmic staining of \textit{NPM1} without detectable mutation all these cases remained discrepant.

\textit{AML cases with discordant immunohistochemical and molecular results of \textit{NPM1} mutation analyses have high expression of \textit{HOX A and B} genes}

Patients with \textit{NPM1} mutated AML are considered a specific subgroup based on distinct clinical as well as biological characteristics\textsuperscript{1,4,5,18-20}. One of these features is a specific gene expression profile, characterized by strongly increased expression of \textit{HOX} genes from both the A and B cluster, \textit{MEIS1} and \textit{PBX3} and downregulation of \textit{CD34} compared to AML samples with wild-type \textit{NPM1}\textsuperscript{2,5,21}. The (re)activation of a stem cell-like \textit{HOX} gene signature in \textit{NPM1} mutated AML has been hypothesized to contribute to the leukemic transformation. We wondered whether the discordant AML cases demonstrated a specific expression profile for these genes and performed quantitative RT-PCR studies for the expression of several \textit{HOXA/HOXB} genes, \textit{MEIS1}, \textit{PBX3}, \textit{BAALC} and \textit{MN1}. The tested cases (1-4 from Table 2) with nuclear staining of \textit{NPM1} by immunohistochemistry but an \textit{NPM1} mutation by fragment analysis showed a gene expression profile as expected for \textit{NPM1} mutated AML (Figure 2), suggesting that these cases represented true false negative cases as assessed by immunohistochemistry. More interestingly, also the gene expression patterns of 3 out of 4 AML cases with cytoplasmic \textit{NPM1} by immunohistochemistry but no detectable \textit{NPM1} mutation by fragment analysis closely resembled those of \textit{NPM1} mutated AML with high
HOXA, HOXB and MEIS1 mRNA expression (Figure 2). The fourth case (case 7 in Table 2) demonstrated high expression of the HOXA genes and MEIS1, but low expression of HOXB5. Since the latter gene expression pattern is frequently observed in AML with MLL translocations22, we tested with FISH for abnormalities of chromosome 11 (q23.1) but these were not detected. Recently, Hollink et al.12 described a group of AML with NUP98/NSD1 translocations and a HOX gene expression pattern resembling the gene expression pattern of NPM1 mutated cases. We screened all discordant patients, including those with high HOXA and HOXB expression, for NUP98/NSD1 by a specific RT-PCR. This revealed no NUP98/NSD1 expression in these cases.

NPM-MLF1 gene fusion detected in two out of four discrepant cases with cytoplasmic NPM1 but no detectable NPM1 mutation

Our analysis so far identified four AML cases (cases 6-9 in Table 2) with cytoplasmic NPM1 staining by immunohistochemistry, in the absence of an NPM1 mutation detected by molecular analysis. It has been described that cytoplasmic NPM1 staining can be observed in cases without a conventional NPM1 mutation, but in the presence of the rare chromosomal translocation t(3;5)(q25;q35)23. This translocation generates the chimeric gene named NPM-myelodysplasia/myeloid leukemia factor 1 (NPM-MLF1) encoding the NPM-MLF1 fusion protein24. The mechanism explaining the cytoplasmic NPM1 dislocation in these cases remains to be clarified. We tested the discrepant cases in our study demonstrating cytoplasmic NPM1 staining, but no NPM1 mutation for the NPM-MLF1 gene translocation by FISH using the RP11-117L6 and the TLX3 Break apart probe. This analysis revealed the presence of a break within the NPM1 gene highly suggestive for a NPM-MLF1 gene translocation in 2 out of 4 discrepant cases (cases 8 and 9 in Table 2, Figure 3).
Discussion

The aim of this study was to evaluate the value of immunohistochemistry on formalin fixed bone marrow biopsies and molecular analysis for the detection of NPM1 mutations and further characterize the cases that were found to be discrepant between both techniques. We observed a high percentage of overlap between both methods of mutation detection. Nevertheless, a small subgroup of patients could be identified with discordant results (5% in the studied cohort).

To analyze the possible effects of inter-observer variability in the use of immunohistochemistry, a cohort of 50 patients, including all cases that showed discordant results for the immunohistochemistry and fragment analysis, were re-scored blindly and independently by three hematopathologists and later discussed in a panel session. All nine cases remained discrepant after this procedure (data not shown).

Previously, Falini and colleagues reported 100% specificity and sensitivity of immunohistochemistry to detect mutated NPM1. The superiority of their results might be explained by a difference in fixation technique, i.e. B5 fixation instead of formalin. The B5 fixation technique used for hematopoietic tissues is well known for its superior morphologic detail compared to formalin fixation. However, this technique has disadvantages, including difficulties with antigen retrieval for some antibodies (e.g. CD30, cyclin D1) and limitations to molecular studies. Moreover, the fixation can be problematic with respect to safety and disposal requirements in the laboratory, since B5 contains mercuric chloride. Therefore, currently, most pathology laboratories use purely formalin based fixation techniques, which provides a safe alternative. In view of these considerations, all biopsies of the nine initially discordant cases were re-cut for thinner tissue sections and re-analyzed at higher magnification. Further, an additional biopsy taken in the referring hospital was included for re-analysis. In three of the five cases with initially nuclear staining but proven NPM1 mutations and a gene expression pattern characteristic for NPM1-mutated AML, we observed either a minor subpopulation with cytoplasmic staining or a discordance between two subsequent biopsies (Table 2). Only in two cases, the blasts were again scored as exclusively nuclear for NPM1 staining. This suggests that fixation and histotechnical factors may indeed contribute to the generation of false negative staining results. Differences in fixation, decalcification and staining procedures leading to difficulties in microscopic evaluation have also been addressed in previously published studies. However, additional studies directly comparing different fixation techniques are necessary to assess the relevance of the fixation technique.

In addition to technical issues, characteristics of the AML blasts might also be important. We observed false negative results in an AML with myeloid maturation and one with a large
component of erythroblasts. The cytoplasmic pattern of NPM1 is generally most prominent in myeloblasts and monoblasts as well as early erythroblasts. Indeed, cytoplasmic staining may be very weak or absent in more mature cells and might be below detection levels or more easily overlooked, especially in AML cases with M5b morphology. Moreover, as is also illustrated by the results of this study, in rare cases only a very small population of blasts with cytoplasmic staining might be present.

Detection of mutant NPM1 could be improved by applying an antibody specific for mutant NPM1. Indeed, an NPM1-mutant specific antibody has been generated for the use in Western blot analysis. However, to the best of our knowledge no such antibody is available for immunohistochemistry. Importantly, none of the cases with mutated NPM1 without overt cytoplasmic staining demonstrated the frameshift mutation, leading to a truncated protein, as has been described by Pianta et al. Nevertheless, the reported truncated protein illustrates that not all identified NPM1 mutants are necessarily associated with overt cytoplasmic localization of NPM1.

The four cases with cytoplasmic NPM1 without detectable NPM1 mutation demonstrated the typical elevated expression of HOX and MEIS1 genes, which has been shown to be associated with NPM1 mutated AML, suggesting that these cases do not reflect regular NPM1 germline AML. Therefore it could be argued that immunohistochemistry should be applied to identify these rare, but interesting cases. Rare mutations in exon 9 and 11 of the NPM1 gene were excluded by sequencing. In two out of the four discrepant cases FISH analysis results were highly suggestive for the rare chromosomal translocation t(3;5)(q25;q35) encoding the NPM-MLF1 fusion protein. This finding is in line with a previous study describing aberrant cytoplasmic NPM1 localization in AML cases carrying a t(3;5). In this study AML with t(3;5) was reported to account for only 0.25% of all adult AML aged 15-60. In our complete cohort of 119 cases, t(3;5) was found in 3% of all analyzed cases. 2 out of these 4 cases demonstrated cytoplasmic NPM1. The exact breakpoints of the other two cases were not analyzed in detail. It is currently unknown how NPM-MLF1 results in cytoplasmic expression. Apparently, not only the presence of a new NES motif in NPM1 is required to cause cytoplasmic dislocation. For two out of four cases we could not find an explanation for the aberrant cytoplasmic NPM1 staining. This staining does not seem to represent an artifact as it could be reproduced and since one of both cases showed an gene expression pattern compatible with NPM1 mutated AML and also the other demonstrated high expression of HOXA genes and MEIS1 (however with low HOXB5). Thus, it is very well possible that there is a small subgroup of AML cases that present cytoplasmic NPM1 localization of (yet) unknown origin. Taken together, these data illustrate that in the screening for NPM1 abnormalities, most information will be obtained by combining immunohistochemistry with molecular analysis. Therefore, if possible, both techniques
should be performed in parallel. Nevertheless, immunohistochemistry for NPM1 could still represent a reasonable method in screening procedures for laboratories of developing countries that are not equipped for molecular studies.

The terms NPM1-mutated and NPMc* are often used interchangeably. Our data and those of others\(^9,23\) suggest that cytoplasmic NPM1 localization is not always caused by a conventional NPM1 mutation, but can also be caused by t(3;5)(q25;q35) and possibly by other abnormalities. Moreover, mutated NPM1 does not always reveal overt cytoplasmic staining of NPM1 on formalin fixed bone marrow biopsies. Nevertheless, also when performed on formalin fixed bone marrow biopsies, immunohistochemistry has a high specificity and sensitivity to detect mutated NPM1. In view of the occasional discrepant results by immunohistochemistry compared with molecular analysis, we would recommend that the detection of NPM1 mutations for routine screening of newly diagnosed AML patients should optimally be based on both immunohistochemical and molecular analyses. Moreover, when immunohistochemical methods on formalin fixed bone marrow biopsies are used, 1-2 µm tissue sections are preferred. Further studies are needed to elucidate the relevance of different factors that might contribute to discordant results between both techniques. In addition, the clinical relevance of the discrepancies between immunohistochemical and molecular detection of NPM1 mutations will be an important issue for further research.
Authorship and disclosures
CMW performed research, analyzed data, contributed to designing experiments and wrote the manuscript. ABM, RNVS, SR, AD, EvdB performed research and analyzed data. JJS and EV analyzed data, designed experiments and contributed to writing of the manuscript. PMK and GH designed experiments, analyzed data and wrote the manuscript. The authors report no potential conflicts of interest.
Reference List


### Tables

**Table 1** Patient characteristics

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Abbreviations: yrs, years; wt, wild type; ITD, internal tandem duplication.

**Table 2** Patient characteristics of the AML cases found discrepant between immunohistochemistry and fragment analysis for the detection of NPM1 mutations.

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<th>No.</th>
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Abbreviations: FA, fragment analysis; mut, mutated; wt, wild type; IHC, immunohistochemistry; nuc, nuclear staining; cyt, cytoplasmic staining; hetero, heterogeneous staining pattern; ITD, internal tandem duplication; WBC, white blood cell count (x10⁹); FAB, French-American-British classification; WHO, World Health Organization of myeloid neoplasms; NK, normal karyotype; D, no T: dead without treatment; D, D: dead by progressive disease; D, TRM: dead by treatment related mortality; y, year.

* heterogeneous pattern with only small cluster of myeloblasts showing cytoplasmic staining;
** heterogeneous pattern with first biopsy showing nuclear and cytoplasmic staining, but a second biopsy showing only nuclear staining.
**Figure legends**

**Figure 1 Immunohistochemistry for NPM1**
Representative examples of immunohistochemical staining of AML cases are shown. (A) case with strict nuclear staining of NPM1 and wild type NPM1 by fragment analysis; (B) case with nuclear and cytoplasmic staining of NPM1 and an NPM1 mutation by fragment analysis; (C) discrepant case (case 1, Table 2) with strict nuclear staining but an NPM1 mutation found by fragment analysis; (D) discrepant case (case 2, Table 2) with a minor population of blasts with combined nuclear and cytoplasmic staining (black arrows) and many other cells with exclusively nuclear staining (orange arrows). The blasts with cytoplasmic expression had been overlooked at initial screening; (E) discrepant case (case 6, Table 2) with both nuclear and very strong cytoplasmic staining but no NPM1 mutation found by molecular analysis; (F) discrepant case (case 8, Table 2) with nuclear and cytoplasmic staining with a t(3;5)(q23;q37.3) likely involving NPM1. The inserts for cases 6 (panel E) and 8 (panel F) show the exclusively nuclear staining for the nucleolin protein as detected with the C23 antibody. All photographs were taken with a 100x oil immersion lens.

**Figure 2 Gene expression of discrepant cases**
Results of qRT-PCR of HOX genes, MEIS1, PBX3, BAALC and MN1 of AML cases found discrepant between immunohistochemistry and fragment analysis for the detection of NPM1 mutations. Relative mRNA expression of the indicated genes compared to the mean expression of the housekeeping genes RPL27 and HPRT is shown. The first eight bars from left to right in each graph represent the data from cases 1-4 and 6-9 according to Table 2.

**Figure 3 FISH analysis for NPM-MLF1 translocation**
On the left, results of metaphase FISH analysis of an AML case with a split in the TLX3 gene on chromosome 5q35. Spectrum red and green TLX3 breakapart probe (Cytocell, LPH, 050). On the right, metaphase FISH analysis of an AML case with a split in the NPM1 gene on chromosome 5q35. Spectrum red NPM1 specific BAC (RP11-117L6) and spectrum green specific BAC (RP11-600N9; control on 5p15.33). The pictures show results of the FISH analysis of case 8 from Table 2.
**Supplemental tables**

**Supplemental Table 1 Primer sequences**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
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| **HOXA5** | Forward AGATCTACCCCTGGATGCGC  
Reverse CCTTCTCCAGCTCCAGGGTC |
| **HOXA6** | Forward GAGCCCGGTTTACCTTGAGTG  
Reverse TCTGTAGCGCGTGTAAGTGCTG |
| **HOXA7** | Forward ATCACTCTACCTCGTAAAACCAGAC  
Reverse ACAATAAAGAAGAAGCTTAATTTG |
| **HOXA9** | Forward ACACATGAAACCGCCATTTG  
Reverse GGAAGCCCAAGATTCAAGG |
| **HOXA10** | Forward ATGATATGGCTTTTTCCCCCAG  
Reverse TCTTTGTGTTTGTGGTGCTG |
| **HOXB5** | Forward GGCAAGCTCCGCAAATATTCCC  
Reverse GGTACGGTTGAGTGGAAACTC |
| **MEIS1** | Forward TCTGCCACCGGTATATTGC  
Reverse GAACGAGTAGATGCCGTGTC |
| **PBX3** | Forward ACAGTGATGCGCTTGAGG  
Reverse GCCGCTCTGAGGAGCTGTC |
| **BAALC** | Forward AATCCACCTGGCTACCCCTAC  
Reverse TTGGAGGGCGAGTCATCTTC |
| **MN1** | Forward GTACATGCCGCTGACAGGG  
Reverse GAGGTCGTGGGCTTCTTTC |

**Supplemental Table 2 NPM1 mutation in discrepant cases**

<table>
<thead>
<tr>
<th>No.</th>
<th>NPM1 mutation</th>
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<tbody>
<tr>
<td>1</td>
<td>Type I</td>
</tr>
<tr>
<td>2</td>
<td>Del GGAGGAA / Insertion CCCTAGCTAGG (exon 12)</td>
</tr>
<tr>
<td>3</td>
<td>Type A</td>
</tr>
<tr>
<td>4</td>
<td>Type A</td>
</tr>
<tr>
<td>5</td>
<td>ND*</td>
</tr>
</tbody>
</table>

Numbering of cases is according to Table 2. Mutation types according to Falini et al. Blood 2007, Vol 109;874-885. ND, not determined because not enough material left for sequencing analysis.
**Legend Supplemental Figure 1**

**Supplemental Figure 1 Immunohistochemistry for NPM1 of all cases**

Immunohistochemistry of all cases for NPM1 on 1-2 µm tissue sections, using an alternative staining procedure. The NPM1 antigen was retrieved with TRIS EDTA buffer (pH 8.5). Slides were incubated with 1:50 diluted supernatant of the anti-NPM1 antibody (clone 376, 1G3) (kindly provided by Prof. Falini, Perugia, Italy). NPM1 was visualized using the ultraview universal alkaline phosphatase red detection kit with fast red/naphthol (Ventana). All cases are numbered according to Table 2. The arrows indicate cytoplasmic expression.

Cases 1 and 4: only nuclear pattern. Case 2: two different areas from the same biopsy, 2a: nuclear staining, partially in probably erythroblasts, 2b: small clusters of myeloblasts with a nuclear and cytoplasmic staining. Case 3: two different areas from the same biopsy, 3a: almost exclusively nuclear staining in more mature leukemic cells, 3b: small clusters of blasts with a nuclear and cytoplasmic staining. Case 5: two subsequent biopsies (interval one day) in a patient with probably a myelomonocytic leukemia and bone marrow fibrosis resulting in a dry tap. The first biopsy (5b) shows many blasts with obvious nucleoli with a combined nuclear and cytoplasmic staining. The second biopsy taken one day later (5a) showed a less blastic appearance of the cells with only nuclear staining. Cases 6-9 show an obvious combined nuclear and cytoplasmic staining, albeit in case 7 in only a minority of the cells.