Screening for NUP98 rearrangements in hematopoietic malignancies by fluorescence in situ hybridization

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Background and Objectives. The aim of this study was to determine the incidence of rearrangements of NUP98 (the gene coding for nucleoporin 98kDa protein) in childhood acute myeloid leukemia (AML) and selected patients with 11p13-15 rearrangements. This aim was achieved using a fluorescence in situ hybridization (FISH) assay that allows the detection of NUP98 aberrations independently of the partner gene involved.

Design and Methods. Screening of 59 consecutive patients enrolled in the Austrian AML-BFM93 clinical trial was performed by dual-color FISH. In addition, 14 selected cases with various hematologic malignancies and 11p13-15 aberrations were analyzed. NUP98-positive cases were further investigated by fusion gene-specific FISH and reverse transcription polymerase chain reaction assays.

Results. Among the 59 AML patients, one NUP98-NSD1 positive case (1.7%) was detected. Among the 14 selected patients, five new NUP98 positive cases were determined. Two cases showed an inv(11)(p15q22)/NUP98-DDX10 fusion, one each displayed a t(5;11)(q35;p15)/NUP98-NSD1 and a t(11;20)(p15;q12)/NUP98-TOP1 fusion, and one case with a putative new fusion partner gene at 3p24 was identified. Interpretation and Conclusions. The observed frequency of 1.7% confirmed the low incidence of NUP98 rearrangements in childhood AML. The low occurrence of NUP98 rearrangements in selected samples with 11p13-15 alterations suggests the existence of variable chromosomal breakpoints and affected genes in this region. The identification of a new NUP98 fusion partner region confirms the evident promiscuity of NUP98. Thus, analysis of NUP98 aberrations by FISH seems to be the method of choice for determining the presence of these genetic lesions in unselected patients, and to confirm the involvement of NUP98 in cases with 11p15 aberrations.

Key words: NUP98 rearrangements, FISH screening, NSD1, DDX10, TOP1.

Haematologica 2005; 90:746-752
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transcription factors containing a homeobox domain, which is a highly conserved helix-turn-helix motif. They provide an evolutionary conserved elementary regulatory mechanism for major differentiation programs from the embryo to the adult, and are also involved in hematopoietic processes. Most of the NUP98 fusion partner genes belong to the class-I-HOX group and only two, PRRX1/PMX1 and PRRX2, are members of the class-II or orphan HOX genes. The non-HOX fusion partner genes are either transcription factors or factors indirectly contributing to transcriptional control. The unifying feature of these NUP98 fusion partners is a significant probability of a coiled-coil conformation being adopted. Intriguingly, all NUP98 fusions reported so far retain the FG repeat motifs at the N-terminus and these are fused to the homeobox domain of the HOX partner genes or to the regions that encompass the predicted coiled-coil domain of the non-HOX partner genes. So far, most studies regarding NUP98 rearrangements have been performed either on selected patient samples, thus providing limited information about the true incidence of these rearrangements or were focused on the detection of specific NUP98 rearrangements. In this study, a NUP98 FISH assay that allows the detection of all NUP98 rearrangements, regardless of the NUP98 fusion partner gene involved was used to screen unselected consecutive childhood AML cases. In addition, 14 patients with various cytogenetically determined 11p13-15 rearrangements were analyzed. All cases that were positive for a NUP98 rearrangement were further evaluated to identify the specific affected partner gene.

Design and Methods

Patients

Out of 67 patients registered in the Austrian AML-BFM93 (Acute Myeloid Leukemia - Berlin-Frankfurt-Münster) study, 59 patients were analyzed by FISH for NUP98 rearrangements. These childhood AML and transient myeloproliferative disorder patients had been previously analyzed using a multiplex reverse transcription polymerase chain reaction (RT-PCR) approach that, however, did not cover NUP98 fusions. In addition, 14 childhood and adult patients with various 11p13-15 rearrangements (Table 1), who were referred to the cytogenetic laboratory of the Children's Cancer Research Institute from different hospitals for cytogenetic analysis, were screened for NUP98 rearrangements.

Cytogenetic and FISH analysis

Cytogenetic analysis was performed on GTG-banded metaphases and karyotypes are described according to the International System for Human Cytogenetic Nomenclature (ISCN, 1995). For the detection of NUP98 rearrangements PAC 1175K1 (comprising exons 10-20 of NUP98) was combined with the 11p-subtelomere-specific clone, PAC 908H22 (kindly provided by Lyndal Kearney, Medical Research Council, UK). NUP98-DDX10 fusions were detected using a pool of 3 different DDX10-specific clones: RP11-35N19 (AP003384), RP11-700F9 (AP003387), and RP11-2519 (AP003027), in combination with PAC 1173K1. Depending on the resolution, in case of a NUP98-DDX10 fusion this probe mixture resulted in one or two fusion signals. NUP98-NSD1 rearrangements were detected with the NUP98 probe and the NSD1-specific RP11-265K23 (AC110005) clone, which encompasses exons 2-23 of the gene. In addition, the MLL-specific probes PAC 217A21 and PAC 167K15 (kindly provided by Ed Schuuring, Leiden University Medical Center, The Netherlands) and clones specific for ATM (RP11-241D13, AP001925), LMO1 (11p15, proximal to NUP98; RP11-379P15, AC091013), and WT1 (11p13; RP1-74J1, AL049692.13 Research Genetics, Invitrogen) were used. All BAC clones derived from the RP11 library and PAC 1175K1 were obtained from Mariano Rocchi, Resources for Molecular Cyto genetics, University of Bari, Italy. FISH was performed as previously described. In brief, probes were differentially labeled by nick translation, either with biotin-16-dUTP or digoxigenin-11-dUTP (Roche Diagnostics). Immunodetection was performed using Cy3-conjugated mouse anti-biotin (Jackson Immuno Research), Fluorescein isothiocyanate (FITC)-conjugated sheep anti-digoxigenin, and rabbit anti-sheep FITC (Roche Diagnostics) antibodies. Samples were evaluated using an Axioplan fluorescence microscope (Zeiss) equipped with the appropriate filter sets for FITC, Cy3, and DAPI and images were taken with a CCD camera (Photometrix) using the IPLab software (Vysis).

RT-PCR analysis

Total RNA was isolated from methanol/acetic acid fixed cells as previously described. RNA was reverse transcribed using random hexamers and 200 units Moloney-murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen) at 42°C for 60 min. The NUP98-DDX10-specific RT-PCR was carried out using sense primer NUP98-F2 (5’-TGTTTGGAACCAACCAACCTAAG-3’), localized in exon 12 and antisense primer DDX10-R1 (5’-TGCTCTTCTTCCTTCAGATGCGCTT-3’), localized in exon 7. RT-PCR specific for the NUP98-NSD1 fusion transcripts was performed using sense primer NUP98-F1 (5’-AGCCACTTTTGGCTTGAG-3’), located in exon 12 and antisense primer NSD1-R1 (5’-TGCACTCTGCTCTTGACCTTCC-3’), located in exon 5. The NUP98-TOP1-specific RT-PCR was carried out using

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sense primer \(\text{NUP98-F3} \ (5’-\text{GACAGCCACCTTT-GGGCTTTG-3’})\) (exon 12) and antisense primer \(\text{TOP1-R1} \ (5’-\text{CCTCGCTTTGCGGCTACG-3’})\) (exon 8). All RT-PCR reactions were carried out using Hot Start Taq (Qiagen). The cycling conditions were: initial activation 95°C for 14 min; denaturation at 95°C for 30 sec, annealing at 63-64°C for 30 sec, elongation at 72°C for 30-45 sec carried out for 35-45 cycles; final extension at 72°C for 7 min. RT-PCR products were sequenced by MWG-Biotech (Germany). The exon nomenclature of all genes followed that described at http://www.ensembl.org/.

## Results

### Detection of NUP98 rearrangements

To determine the presence of NUP98 rearrangements, independently of the partner gene involved, FISH was performed using the NUP98-specific PAC 1173K1 which splits if a rearrangement is present. In order to avoid misinterpretation of the NUP98 signal pattern due to chromosome 11 or 11p trisomies, the NUP98-specific clone was combined with an 11p-subtelomere-specific clone. This probe combination

### Table 1. Clinical characteristics, cytogenetics, FISH, and RT-PCR data of 14 patients with 11p13-15.

<table>
<thead>
<tr>
<th>No</th>
<th>Age (yr.)</th>
<th>Sex</th>
<th>Diagnosis</th>
<th>Karyotype</th>
<th>FISH</th>
<th>RT-PCR</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NUP98/11p</td>
<td>NUP98/DDX10</td>
<td>NUP98/NSD1</td>
</tr>
<tr>
<td>1</td>
<td>74,3</td>
<td>M</td>
<td>AML</td>
<td>46,Xy,inv(11)(p15q22)[20]</td>
<td>13% 1% ND ND</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>6,4</td>
<td>M</td>
<td>AML</td>
<td>46,Xy,inv(11)(p15q21)[3]/46,Xy,der(17)(p17::q11)[1]/46,Xy,der(2)(p22), der(17)(17::p17)</td>
<td>62% 65% ND ND</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>18,3</td>
<td>M</td>
<td>t-AML</td>
<td>46,Xy,inv(11)(p15q22)[20]</td>
<td>ND 80% ND ND</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>69,7</td>
<td>M</td>
<td>MDS</td>
<td>46,Xy,inv(11)(p15q23)[2]/46,Xy[18]</td>
<td>3% DDX10 deletion ND ND</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>48,6</td>
<td>F</td>
<td>AML M1</td>
<td>46,Xx,inv(11)(p15q23)[18]</td>
<td>8.7% 0% ND ND</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>56,5</td>
<td>F</td>
<td>t-AML</td>
<td>46,Xx,(11:20)(p15q12)[16]/45,XX,der(21)[4]</td>
<td>56% ND ND NUP98-TOP1</td>
<td></td>
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<tr>
<td>7</td>
<td>78,2</td>
<td>M</td>
<td>AML M0/1</td>
<td>47,X,Y,+13,+mar(16)/46,X,Y, der(X)(11:13)(p17:q11),+mar[4]</td>
<td>13% ND ND ND</td>
<td></td>
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<tr>
<td>8</td>
<td>65</td>
<td>M</td>
<td>AML M0/1</td>
<td>46,Xx,(7;11)(q22p15)[2]/46,Xy[4]</td>
<td>4.2% ND ND ND</td>
<td></td>
</tr>
<tr>
<td>9a</td>
<td>3,9</td>
<td>F</td>
<td>AML M2</td>
<td>46,Xx[17]</td>
<td>ND ND 75% NUP98-NSD1</td>
<td></td>
</tr>
<tr>
<td>9b</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>17% and 58% of the cells displayed one and two fusion signals, respectively.</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>39,1</td>
<td>F</td>
<td>AML M2</td>
<td>45,XX,del(11)(p13),del(17)(p11)[18]/45,XX,der(11)(p17::q11), -16,17, 18, +mar[4]</td>
<td>4% ND ND ND</td>
<td></td>
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<tr>
<td>11a</td>
<td>63,8</td>
<td>M</td>
<td>AML M5a</td>
<td>46,Xx,(3;11)(q24p15)[20]</td>
<td>72% ND ND ND</td>
<td></td>
</tr>
<tr>
<td>11b</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>63% ND ND ND</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>31,9</td>
<td>M</td>
<td>MDS</td>
<td>45,Xy,del(11)(p15)dup(11)(q13q25)[16]/47,Xy[6][3]</td>
<td>8% ND ND ND</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>72,8</td>
<td>F</td>
<td>AML M1</td>
<td>46,XX,der(11)(p11)(q21p15), add(11)(q22)[20]</td>
<td>13% ND ND ND</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>71</td>
<td>F</td>
<td>MDS</td>
<td>45,XX,der(11)(11;7;11)(p13::p12p15), der(11)(p17:q11)[1]/del(13q2)[12], der(17)(p17:q11)[13], p15p13, -17][15]</td>
<td>9.5% ND ND ND</td>
<td></td>
</tr>
</tbody>
</table>

Patients are grouped in inv(11) cases and in cases with other 11p13-15 aberrations. “Sample from diagnosis and “sample from relapse of the same patient.” "Relapse sample." FISH analysis using different probes specific for chromosomes 1, 5, 11, 18, the Spectra-Vysion 24-Color-Chromosome-Painting Kit (Vysis), and the NUP98/11p assay revealed a t(5;11)(q35;p15) and a t(1;18)(q32;q21). Karyotype was established after 24-Color-Chromosome-Painting and NUP98/11p FISH; the breakpoint at 11p15 is localized telomeric to NUP98. Percentages of FISH patterns suggestive for NUP98 rearrangements: NUP98/11p – 3G/2R; NUP98/DDX10 and NUP98/NSD1 – fusion signals. 13% and 52% of the cells displayed one and two fusion signals, respectively. 16.5% and 65.5% of the cells displayed one and two fusion signals, respectively. 17% and 58% of the cells displayed one and two fusion signals, respectively. AML: acute myeloid leukemia; MDS: myelodysplastic syndrome; ND: not determined; t-AML: therapy-related AML.
FISH screening for NUP98 rearrangements

resulted in 2 green and 2 red (2G/2R-NUP98/11p) signals for each probe in normal cells and, in the case of a NUP98 rearrangement, in a pattern of 3 signals for NUP98 and 2 subtelomeric signals (3G/2R). In normal peripheral blood cells the cut-off value for false positive nuclei displaying the 3G/2R pattern was determined as 7.5% (mean±3σ).

NUP98 rearrangements in consecutive childhood AML patients

Using the NUP98/11p assay, in 58 out of 59 consecutive childhood AML cases a 3G/2R-NUP98/11p FISH pattern suggestive of a NUP98 rearrangement was observed in 1-16% of the nuclei. Probably due to the prolonged storage of the fixed bone marrow samples with ≥40% blast cells in almost all cases the hybridization was slightly less specific, and therefore these samples were considered as NUP98 negative. Moreover, taking into consideration possible 3’ deletions of NUP98, when no additional signal was observed, special attention was paid to the cytogenetics and the location of the signals on the metaphase chromosomes. Only one patient showed a 3G/2R-NUP98/11p pattern in a significant number of nuclei (37%), suggesting the presence of a NUP98-HOXA fusion. However, FISH analysis with 11p-specific probes and whole chromosome painting revealed a more complex karyotype and no involvement of 11p (data not shown).

NUP98 rearrangements in selected cases with 11p13-15 rearrangements

Based on cytogenetic data 14 cases with various 11p13-15 alterations (Table 1) were analyzed for NUP98 rearrangements. In nine samples the 3G/2R-NUP98/11p FISH pattern was observed in merely 3-13% of the nuclei. Five patients (#2, 3, 6, 9, and 11) analyzed using the NUP98/11p or fusion gene-specific FISH assays were positive for a NUP98 aberration (Table 1).

Five patients (#1-5) were karyotyped as having inv(11)(p15q21-q23) suggesting a fusion between the NUP98 and DDX10 genes. Using a NUP98- DDx10 dual-color FISH assay, two of the five patients (#2 and 3) showed NUP98-DDX10 fusion signals (Figure 2A; Table 1). Sequence analysis of the NUP98-DDX10 RT-PCR products revealed an in-frame fusion of NUP98 exon 14 with DDX10 exon 7 in both cases (Figures 2B, D). Patient #4 showed only one DDX10 and two NUP98 signals, and thus displayed a hemizygous deletion of DDX10. More detailed FISH analyses using probes specific for MLL and ATM, which is localized just 400 kb centromeric to DDX10, also revealed a hemizygous deletion of ATM, whereas MLL was not affected. In the remaining two inv(11) cases, FISH analysis revealed inver-
sion breakpoints distal to NUP98 and distal to MLL in case #1, whereas in case #5 FISH with several chromosome 11 locus-specific probes could not confirm an inversion event (data not shown).

In patient #6, whole chromosome painting confirmed the t(11;20)(p15;q12) and a NUP98 rearrangement was detected (Table 1). RT-PCR and subsequent sequencing revealed an in-frame fusion of NUP98 exon 13 and TOP1 exon 8 (Figures 2 C,E).

In patient #9, at diagnosis cytogenetics revealed a 46,XX karyotype but at relapse the presence of a del(11)(p13) suggested involvement of NUP98. FISH analysis revealed a cryptic t(5;11) (Figures 1B-C; Table 1). The presence of a NUP98-NSD1 fusion was confirmed by RT-PCR (Figure 1D) and resulted in an in-frame fusion of NUP98 exon 12 and NSD1 exon 5 (Figure 1E). Interestingly, at diagnosis and relapse metaphases displayed NUP98 signals on both derivative chromosomes including the del(11) (p13). At diagnosis both the LMO1 and the WT1 loci were retained on both chromosomes 11, whereas at relapse only LMO1 was present on the del(11)(p13) but an interstitial deletion eliminated the WT1 locus (data not shown). Patient #11 displayed a t(3;11)(p24;p15) that has not been described as a recurrent aberration. FISH analysis showed rearrangement of the NUP98 gene (Table 1). Involvement of NUP98 in a t(3;11)(p24;p15) has not been reported until now, and therefore a new NUP98 partner gene seems to be affected by this translocation.

Discussion

In this study the most comprehensive group of consecutive childhood AML patients was analyzed so far for NUP98 rearrangements. Among 59 patients, only one patient (1.7%) was positive for this specific genetic alteration confirming the overall low incidence of NUP98 rearrangements. In a previous study, 20 consecutive childhood AML cases were screened for NUP98-NSD1 fusions and one (5%) positive case was found. Also in a significant larger series of more than 200 adult AML cases a NUP98-HOXA9 fusion was observed in merely 1.5% of the cases, and in a survey of 43 adult T-ALL cases 5% were NUP98-RAP1GDS1 positive. However, due to selection bias of patient cohorts and screening for specific NUP98 fusions the overall incidence of NUP98 rearrangements remains unclear and further comprehensive studies on larger series of well-defined patient groups are needed to determine their occurrence as well as any clinical correlations.

In general, NUP98 fusions appear to be associated with a poor prognosis; however, there are few comprehensive data sets available. It seems that the NUP98-NSD1 fusion is strongly associated with childhood AML and so far, including the new case described herein (case #9), eight cases have been identified. Of note, the first case of an adult with MDS harboring a NUP98-NSD1 fusion resulting from a cryptic insertion of NUP98 into the NSD1 locus was recently described. In childhood AML the
NUP98-NSD1 rearrangement may occur with a concurrent del(5q) that appears to be associated with an even poorer prognosis than those cases without the additional deletion. In support of this hypothesis, the new case (#9) presented in this study displayed no obvious deletion at 5q and at present is in complete remission almost four years after diagnosis, whereas the other patient displayed a del(5q) and died 10 months after diagnosis.

The analysis of five patients with an inv(11)(p15q22) detected two new NUP98-DDX10-positive cases. Of note, at diagnosis one of these patients (#3) presented with an AML-M3 and showed a t(15;17)(q22;q21) with a PML-RARA rearrangement. Five years later he developed a secondary leukemia with an AML-M4 phenotype and the inv(11)(p15q22)/NUP98-DDX10 fusion confirming the association of NUP98 aberrations with therapy-related diseases. Detailed FISH analysis of the inv(11) cases negative for NUP98-DDX10 suggested, in at least one case (#1), the existence of another type of inv(11) involving genes that have not been identified yet. Although in two further cases (#4 and 5) the presence of a cytogenetically suspected inv(11) could not be verified with the FISH probes applied, its presence cannot be excluded with certainty.

Patient #2 displaying a NUP98-DDX10 fusion suffered from aggressive disease and due to treatment failure died 2 months after diagnosis, whereas patient #3 has now been in remission for 6 months. The NUP98-TOPO fusion described herein was also found in a patient with secondary AML-M2 with rapid disease progression who died within a few months, again emphasizing the association of NUP98 aberrations with an adverse outcome. The same held true for the patient with the new NUP98/3p24 rearrangement who died of refractory disease 20 months after diagnosis. However, NUP98 rearrangements are rare and occur in various hematologic diseases, and thus it is difficult to reach any conclusions regarding their definite prognostic significance. Nevertheless, studies of NUP98 aberrations elucidate the potential mechanisms of leukemic transformation and will eventually identify specific subgroups of leukemia that require particular treatment approaches. So far, 17 NUP98 fusion partners have been detected, and this evident promiscuity of NUP98 makes it reasonable to suspect the existence of other yet unknown partners. Screening for NUP98 aberrations by FISH allows the detection of all rearrangements independently of the partner gene, and seems to be the method of choice for determining the overall incidence of these genetic lesions and for identifying novel fusion partners. In this regard, a further improvement might be achieved by a NUP98 dual-color split-apart FISH assay that would completely exclude lack of detection of NUP98 rearrangements associated with 3′ deletions. However, unfortunately an adequate probe set for screening studies is not available to date. Interestingly, the low frequency of NUP98 rearrangements even in selected samples with 11p13-15 alterations suggests the existence of variable chromosomal breakpoints and affected genes in recurrent translocations involving 11p15. These findings emphasize the importance of combining cytogenetics, FISH techniques and molecular analysis in order to determine the specific genes involved in 11p15 rearrangements unequivocally.

While all authors fulfill the requirements for authorship, more specifically KN, SS, MK and OAH had the major input designing the study and were involved in analyzing and interpreting of data. HHS, DL, WRS, KK, SB and MND were mostly involved in acquisition of available preserved samples and clinical data. KN wrote the first draft of the paper, which was critically revised by SS. Additionally, OAH contributed fruitfully in revision of the paper and the final version was approved by all authors. The authors declare that they have no potential conflicts of interest. The authors gratefully thank H. Haidinger, U. Horčíka, E. Lang, G. Neumann, B. Nistler, T. Pass, H. Pirc-Danosewinata, and E. Winkler for the excellent cytogenetic analysis. We thank all pediatric oncologists in Austria for contributing samples and clinical data for this study.

This work was supported by the „Österreichische Kinderkrebshilfe” and by the research program “Genome Research for Health” of the Austrian Ministry of Education, Science and Culture (GEN-AU Child, GZ 200.073-VI/2a/2002). Manuscript received January 10, 2005. Accepted April 27, 2005.

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