Leukemia-associated NF1 inactivation in patients with pediatric T-ALL and AML lacking evidence for neurofibromatosis

Leukemia-associated $NF1$ inactivation in patients with pediatric T-ALL and AML lacking evidence for neurofibromatosis

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Neurofibromatosis type 1 (NF1) is an autosomal dominant genetic disorder caused by mutations in the $NF1$ gene. Patients with NF1 have a higher risk to develop juvenile myelomonocytic leukemia (JMML) with a possible progression toward acute myeloid leukemia (AML). In an oligo array comparative genomic hybridization–based screening of 103 patients with pediatric T-cell acute lymphoblastic leukemia (T-ALL) and 71 patients with $MLL$-rearranged AML, a recurrent cryptic deletion, del(17)(q11.2), was identified in 3 patients with T-ALL and 2 patients with $MLL$-rearranged AML. This deletion has previously been described as a microdeletion of the $NF1$ region in patients with NF1. However, our patients lacked clinical NF1 symptoms. Mutation analysis in 4 of these del(17)(q11.2)-positive patients revealed that mutations in the remaining $NF1$ allele were present in 3 patients, confirming its role as a tumor-suppressor gene in cancer. In addition, $NF1$ inactivation was confirmed at the RNA expression level in 3 patients tested. Since the NF1 protein is a negative regulator of the RAS pathway (RAS-GTPase activating protein), homozygous $NF1$ inactivation represents a novel type I mutation in pediatric $MLL$-rearranged AML and T-ALL with a predicted frequency that is less than 10%. $NF1$ inactivation may provide an additional proliferative signal toward the development of leukemia. (Blood. 2008;111:4322-4328)

Introduction

Neurofibromatosis type 1 (NF1) is an autosomal genetic disorder that is clinically characterized by cafe-au-lait spots and frequent fibromatosus tumors of the skin and tumors of the central nervous system. The NF1 disorder is caused by genetic heterozygous mutations in the $NF1$ gene on chromosome 17q11.2. Most $NF1$ mutations are intragenic and have been found over the complete gene. They comprise a diversity of mutation types, where splicing mutations are intragenic and have been found over the complete $NF1$ locus. Patients with these $NF1$ microdeletions display a more severe NF1 phenotype, characterized by mental retardation, facial dysmorphism, and increased risk for developing malignant tumors, including leukemias.1,2 To this end, NF1 has also been associated with juvenile myelomonocytic leukemia (JMML), with a risk of progression toward acute myeloid leukemia (AML). These malignancies are associated with loss of the wild-type allele, either through deletions or the acquisition of point mutations. In JMML, it has also frequently been reported that the wild-type allele is replaced by the mutant allele as an effect of recombinational events leading to uniparental disomy (UPD).3,4 Previously, it was shown that biallelic inactivation of $NF1$ are found as somatic abnormalities in patients with JMML who lack clinical evidence of NF1.5,6 Somatic inactivation of $NF1$ in hematopoietic cells results in a progressive myeloproliferative disorder in mice,8 confirming that $NF1$ acts as a tumor-suppressor gene.5 The NF1 gene product, neurofibromin, is a GTPase-activating protein (GAP) that inhibits RAS signaling by hydrolysis of active RAS-GTP into inactive RAS-GDP.9,5 Therefore, $NF1$ deficiencies act as functional equivalents of activation mutations in RAS. Indeed, $NF1$ inactivation and RAS mutations have been found in a mutually exclusive manner in JMML.7 AML is a heterogeneous disease in which early treatment response and cytogenetic abnormalities are the most important prognostic factors. In AML, genetic aberrations can be classified as type I or type II mutations. One hypothesis about the development of AML is the coexistence of both type I and type II mutations that confer proliferative signals (type I mutations affecting the FLT3, C-KIT, NRAS, KRAS, or PTPN11 genes) in combination with type II differentiation-imparing mutations (such as PML-RARα, AMLETO, CBFB-MYH11, or MLL-rearrangements).10 $MLL$-rearrangements account for 8% to 20% of all cytogenetic abnormalities in pediatric AML.11,12 $HOX$ genes are the prime targets of MLL fusion products and regulate cellular differentiation in normal hematopoietic development. However, Eguchi et al point...
to another role of MLL fusion products in MLL-rearranged leukemias through the alteration of cell-cycle arrest and apoptosis.\textsuperscript{13} Most of these MLL-positive AML samples are morphologically classified as FAB-M4 and FAB-M5, and it has been suggested that MLL-rearrangements in pediatric AML are associated with a poor outcome. Interestingly, in some studies the t(9;11) subgroup has been associated with a higher sensitivity to different classes of drugs and a better prognosis.\textsuperscript{14-16} In addition, many of these patients with MLL-rearranged AML lack mutations in \textit{FLT3}, \textit{C-KIT}, \textit{NRAS}, \textit{KRAS}, and \textit{PTPN11}, indicating that the type I mutations remains to be elucidated. High-resolution genomic screening of patients with MLL-rearranged AML could provide us with further insight into novel genetic aberrations with prognostic significance or new type I mutations in MLL-rearranged AML.

T-cell acute lymphoblastic leukemia (T-ALL) represents about 15\% of patients with pediatric ALL and is characterized by a rapid progression of disease and a 30\% relapse rate within the first 2 years after diagnosis.\textsuperscript{17} In the last decade, a large number of new

![Figure 1. NF1 microdeletions in pediatric acute leukemias.](image-url)
genomic aberrations were identified in T-ALL, including chromosomal translocations, deletions, amplifications, and mutations.\textsuperscript{18-20} All these genetic defects target different cellular processes, including the cell cycle, T-cell differentiation, proliferation, and survival. Cooperation of these genetic events initiates leukemic transformation of thymocytes.\textsuperscript{18} RAS mutations have been found in less than 5\% of patients with T-ALL, showing that proliferative hits affecting the RAS pathway remain rare.\textsuperscript{18} On the other hand, more than 50\% of the patients with T-ALL are characterized by activating mutations in the NOTCH1 pathway, including the NOTCH1 gene itself\textsuperscript{21,22} or the NOTCH1-regulating U3-ubiquitin ligase FBW7.\textsuperscript{23,24}

In this study, we used oligo array–comparative genomic hybridization (array-CGH) and identified somatic NF1 microdeletions as a cryptic genetic abnormality in patients with pediatric T-ALL and patients with MLL-rearranged AML that lack symptoms of neurofibromatosis. We present further evidence for the role of NF1 inactivation as a functional equivalent to activated RAS signaling, and suggest that this can be considered as a new type I mutation in MLL rearranged AML and a proliferative hit in T-ALL.

### Methods

#### Patients

Viably frozen diagnostic bone marrow or peripheral blood samples from 103 patients with pediatric T-ALL and 71 patients with pediatric MLL-rearranged AML were provided by the Dutch Childhood Oncology Group (DCOG), the German Cooperative Study Group for Childhood Acute Lymphoblastic Leukemia (COALL), and the Berlin-Frankfurt-Münster AML Study Group (AML-BFM-SG). Informed consent was obtained according to local law and regulations and in accordance with the Declaration of Helsinki. Leukemic cells were isolated and enriched from these samples as previously described.\textsuperscript{25} All resulting samples contained 90\% or more leukemic cells, as determined morphologically by May-Grunwald-Giemsa (Merck, Darmstadt, Germany)–stained cytospins. These leukemic cells were used for DNA and RNA extraction, and a minimum of 5\times10^6 leukemic cells were lysed in Trizol reagent (Gibco BRL/Life Technologies, Breda, The Netherlands) and stored at \(-80^\circ\text{C}.\) Genetic DNA and total cellular RNA were isolated as described before.\textsuperscript{25} From the patients with a deletion of NF1, remission and relapse material was only available for patient no. 2736.

#### Oligo array-CGH

Oligo array-CGH analysis was performed on the human genome CGH Microarray 44k-A (Agilent Technologies, Palo Alto, CA) according to the manufacturer’s protocol using a dye-swap experimental design to minimize false positive results, as previously described.\textsuperscript{25,26}

#### MLPA

Multiplex ligation-dependent probe amplification (MLPA) analysis was performed using the SALSA P081/082 MLPA assay (MRC Holland, Amsterdam, the Netherlands). SALSA P081/082 consists of 2 reaction mixes containing probes for all constitutive NF1 exons except for exons 5, 7, 17, 19A, 45, and 47. The exact localization of the MLPA probes can be downloaded from the MRC Holland website (http://www.mrc-holland.com/pages/p081_p082pag.html). The 2 reactions contain 15 and 13 control probes in other regions of the genome, respectively. The patients’ samples were analyzed with MLPA according to the manufacturer’s protocol.\textsuperscript{27,28} Data were analyzed using GeneMarker v1.5 (Softgenetics, State College, PA).

#### Mutation analysis

For the detection of NF1 mutations, DNA was subjected to 40 cycles of polymerase chain reaction (PCR) of 15 minutes at 95°C and 1 minute at

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**Table 1. Patient characteristics and truncating NF1 mutations in pediatric T-ALL and AML**

<table>
<thead>
<tr>
<th>ID</th>
<th>WBC, (10^9/\text{L})</th>
<th>Age, y</th>
<th>Sex</th>
<th>Subtype</th>
<th>Relapse or CCR, mo</th>
<th>Karyotype</th>
<th>Genotype</th>
<th>SF1 del</th>
<th>SF1 mutation analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>2736</td>
<td>13</td>
<td>10.1</td>
<td>M</td>
<td>T-ALL</td>
<td>—</td>
<td>CALMAF15</td>
<td>—</td>
<td>Yes</td>
<td>c.3734delCinsGGTTTATGGTTT</td>
</tr>
<tr>
<td>2730</td>
<td>5</td>
<td>14.0</td>
<td>F</td>
<td>T-ALL</td>
<td>—</td>
<td>CALMAF15</td>
<td>—</td>
<td>No</td>
<td>WT</td>
</tr>
<tr>
<td>167</td>
<td>—</td>
<td>17.9</td>
<td>NA</td>
<td>T-ALL</td>
<td>—</td>
<td>CALMAF15</td>
<td>—</td>
<td>Yes</td>
<td>c.3734delCinsGGTTTATGGTTT</td>
</tr>
<tr>
<td>4389</td>
<td>11</td>
<td>14.7</td>
<td>NA</td>
<td>T-ALL</td>
<td>—</td>
<td>CALMAF15</td>
<td>—</td>
<td>Yes</td>
<td>c.3734delCinsGGTTTATGGTTT</td>
</tr>
<tr>
<td>6421</td>
<td>6.5</td>
<td>19.8</td>
<td>NA</td>
<td>T-ALL</td>
<td>—</td>
<td>CALMAF15</td>
<td>—</td>
<td>Yes</td>
<td>c.3734delCinsGGTTTATGGTTT</td>
</tr>
</tbody>
</table>

In additional mutation analysis, FLT3-ITD or D835 PM, C-KIT, RAS, and PTPN11 were all WT. CCR indicates continued complete remission; —, not applicable; WT, wild-type; NA, not available; ND, not determined; PM, point mutation.
60°C, using specific primers for all NF1 exons, which are being used in NF1 diagnostics (Department of Clinical Genetics, Erasmus MC, Rotterdam, The Netherlands, A.M.W.O. manuscript in preparation; primers are available on request at a.vandenouweland@erasmusmc.nl). RAS, PTPN11, and C-KIT mutation screening was performed as described in Table S1, available on the Blood website (see the Supplemental Materials link at the top of the online article). NOTCH1 and FLT3 mutational screening were done as previously described.11,29,30 PCR products were purified by standard methods and directly sequenced from both strands. The sequence data were analyzed using Seqscape V2.5 (Applied Biosystems, Foster City, CA).

**NF1 expression analysis**

NF1 expression was calculated based upon nonnormalized gene expression array data, performed on the human genome U133 Plus 2.0 array (Affymetrix, Santa Clara, CA) as previously described,31 which were available for 3 del(17)(q11.2)-positive and 7 del(17)(q11.2)-negative leukemia patients. For the NF1 probe sets, the expression was normalized to the median expression of GAPDH (6 probesets) for each patient sample. The difference in relative gene expression levels between patients with and without the del(17)(q11.2) was evaluated using the Mann-Whitney U test.

**Results**

High-resolution genomic screening of a selected subgroup of 103 patients with pediatric T-ALL and 71 patients with MLL-rearranged AML using a 44K oligo array-CGH platform led to the identification of a cryptic deletion, del(17)(q11.2). This deletion was recurrently observed in 3 patients with T-ALL and 2 patients with AML (Figure 2; Table 1). These deletions were about 1.2 Mb in size and covered the NF1 gene. For all patients, the telomeric breakpoints were situated in the pseudogene JJAZ1P (Figure 1C,D). The deletion area in these samples was equivalent to those observed in patients with NF1. Genetic and clinical patient characteristics for all del(17)(q11.2)-positive leukemia patients are summarized in Table 1. One of the 3 patients with T-ALL and at least one of the 2 patients with AML relapsed.

To further confirm the deletion breakpoints, 4 of 5 del(17)(q11.2)-positive leukemia patients and 15 del(17)(q11.2)-negative controls (7 with T-ALL and 8 with AML) were analyzed using an NF1 locus-specific MLPA assay.27,28 No residual material was available for patient no. 6421. These analyses confirmed that one copy of the NF1 locus was lost in all 4 of these patients (Figure 2; only T-ALL patient no. 2736 is shown), whereas all control patients retained both copies of the NF1 gene (only AML control no. 3339 is shown).

In order to investigate complete NF1 inactivation in our patients, we performed mutation analysis on all exons and exon/intron boundaries of the NF1 gene in the 4 del(17)(q11.2)-positive patients with leukemia, and in an additional group of 39 patients without a deletion involving chromosomal band 17q11.2 (including 21 patients with MLL-rarranged AML and 18 patients with T-ALL). Small frameshift mutations disrupting the NF1 coding region were only detected in 3 of 4 del(17)(q11.2)-positive patients (Table 1; Figure 3), leading to biallelic inactivation of NF1 in these patients. One patient with T-ALL and 2 patients with MLL-rarranged AML without a del(17)(q11.2) had a monoallelic mutation in nonfunctional domains, possibly reflecting rare polymorphisms. Furthermore, NF1 expression in the del(17)(q11.2)-positive patients with T-ALL and MLL-rarranged AML leukemias was significantly lower in 3 patients tested, as compared with 7 T-ALL and AML patient samples that are wild-type for NF1 (Figure 4).

To further verify a somatic rather than a genetic origin of NF1 inactivation, we screened relapse and remission material of T-ALL patient no. 2736, for whom material was available. At relapse, the NF1 microdeletion and NF1 mutation on the other allele were present, while in the remission sample both mutations remained undetected.

Since NF1 deficiency could act as a novel type I mutation, we screened all del(17)(q11.2)-positive patients with leukemia for activational mutations in RAS. Although NRAS or KRAS mutations have been described in MLL-rarranged AML and T-ALL, no somatic NRAS or KRAS mutations were found in our 5 del(17)(q11.2)-positive patients with leukemia. In addition, both patients with MLL-rarranged AML with a NF1 deletion lacked other type 1 mutations in FLT3, C-KIT, or PTPN11 in their leukemic cells. The frequency of these mutations in the 71 MLL-rarranged AML samples was low, as expected. Only 35% had one of these mutations, and all these mutations were mutually exclusive. Furthermore, the del(17)(q11.2)-positive patients with T-ALL were screened for rearrangements at the TAL1, HOX11L2, HOX11, CALM-AF10, MLL and cMYC loci or the presence of NOTCH1 mutations. One patient (no. 167) lacked rearrangements of any of the loci mentioned above, whereas a HOX11L2 translocation (no. 2780) and a CALM-AF10 fusion gene (no. 2736) were detected in 2 other patients. NOTCH1 mutations were identified in...
patients no. 2780 (heterodimerization domain; L1601P) and no. 167 (PEST domain; 2445insLL).

Discussion

Genetic events that lead to leukemogenesis by activating uncon- 
trolled cell proliferation remain to be elucidated in most patients with pediatric T-ALL and MLL-rearranged AML. We used oligo 
array-CGH to identify new abnormalities and found somatic NF1 
microdeletions as a cryptic genetic abnormality in patients lacking 
clinical symptoms of neurofibromatosis. This array-CGH study is 
currently expanded to other subtypes of leukemias. Recent single 
nucleotide polymorphism (SNP) array analysis of pediatric ALL by 
investigators from St Jude Children’s Research Hospital showed 
that this microdeletion in NF1 may be present at low frequencies in 
other types of acute leukemia as well.32

NF1 microdeletions are observed in about 5% to 20% of 
patients with NF1.7 The majority of these patients with NF1 have a 
1.4-Mb NF1 microdeletion due to interchromosomal homologous 
recombination between the low-copy repeats of the WI-12393 gene 
flanking NF1 and sequences with homology to chromosome 19 during meiosis.33 A second type of NF1 microdeletions of about 
1.2 Mb in size is due to a mitotic intrachromosomal recombination 
between the JJAZ1 and the homologous JJAZIP pseudogene.34,35

The NF1 microdeletions in our leukemia patients seemed identical 
to this 1.2 Mb microdeletion type.33 However, in contrast to 
patients with NF1 with similar NF1 deletions, our patients with 
leukemia did not meet the clinical criteria for NF1, lacking 
cafe-au-lait spots, mental retardation, and/or facial dysmor- 
phism. This suggests that the NF1 deletion in our patients is 
somatic and leukemia specific, rather than of constitutional 
genetic origin, although molecular diagnostics for NF1 was not 
performed in these patients.

Deletion of one allele of NF1 and further inactivation of the 
other NF1 allele in 3 patients through the acquisition of point 
mutations further confirms the role of NF1 as a tumor-suppressor 
gene in the pathogenesis of both pediatric MLL-rearranged AML 
and T-ALL. This point was further strengthened by the finding of 
clonal stability in one of the del(17)(q11.2) patients, where the 
deletion of NF1 on one allele and the point mutation in the other 
NF1 allele were both present at diagnosis and relapse while absent 
in the remission sample. Therefore, the NF1 abnormalities were 
somatic origin in at least patient no. 2736 and were only present in 
the leukemic cells. Similar findings have been described for 
patients with JMML,7 explaining why these patients did not have 
any clinical symptoms of neurofibromatosis.

Since NF1 deficiency leads to the activation of the RAS 
signaling pathway,9 and none of the del(17)-positive patients 
with leukemia had mutations in NRAS or KRAS, NF1 microdele- 
tions presumably provide an alternative mechanism for RAS 
activation in both MLL-rearranged myeloid and T-lymphoid

NF1 expression analysis

![NF1 expression analysis](image-url)
leukemias, thereby representing a novel type I abnormality. These patients with leukemia may potentially benefit from additional treatment with RAS inhibitors like farnesylthiosalicylic acid or downstream inhibitors.

Both del(17)-positive patients with AML were further screened for any of the other currently known type I mutations in AML. As expected, no other type I mutations were detected, indicating that NF1 microdeletions could act as a novel type I mutation which cooperate with the MLL translocation (type II mutation) in the pathogenesis of AML.

The idea of a multistep pathogenesis in T-cell leukemia is widely accepted.18-20,37 Cooperative genetic events affect cell cycle, T-cell differentiation, proliferation, and survival. We identified a number of cooperative aberrations in the del(17)(p11.2)-positive T-ALL samples. NOTCH1 mutations, generally present in about 50% of T-ALL,21 were identified in 2 of 3 del(17)(p11.2)-positive T-ALL samples. In addition, genetic aberrations that induce a T-cell differentiation arrest were identified in patient no. 2780 (HOX11L2 translocation) and patient no. 2736 (CALM-AF10 translocation). These data further suggests that loss of NF1 can be involved in the development of T-ALL, as one of the genetic hits in multistep oncogenesis.

In this study, we identified 3 patients with a deletion of NF1 and an inactivational mutation on the remaining allele. We could not identify homozygous somatic NF1 mutations in 21 patients with MLL-rearranged AML and 18 patients with T-ALL without a microdeletion. This suggests that the frequency of biallelic inactivation, until now the only mechanism described for oncogenesis, is less than 10% in these groups.

Other mechanisms of NF1 inactivation, such as inactivation through the duplication of the mutated (UPD) NF1 allele at the expense of the remaining wild-type allele, as observed in patients with NF1 with JMML, may have been missed. Of interest, recent SNP array analysis of pediatric ALL, and JMML without underlying NF1, showed that there was no UPD involved in the NF1 region.6,32 In addition, in adult AML approximately 20% have large regions of UPD, but none of them involves the NF1 locus.38,39 Hence, UPD of the NF1 locus may be a rare event in leukemias of somatic origin compared with leukemias which originate from patients with clinical evidence of NF1. Therefore, the frequency of biallelic NF1 inactivation in pediatric MLL-rearranged AML and T-ALL as we reported here may be underestimated. Future studies should be extended by sequencing the NF1 locus, including the promoter region and the 3′ untranslated region (UTR), and look for abnormalities in NF1 protein expression.

In conclusion, we report the identification of NF1 microdeletions in patients with pediatric T-ALL and MLL-rearranged AML without clinical evidence of NF1. We confirmed NF1 inactivation by reduced NF1 expression levels and biallelic NF1 mutations in 3 of 5 patients, confirming the role of NF1 as a tumor-suppressor gene in cancer. NF1 inactivation is a novel type I mutation in MLL-rearranged AML and a new proliferative hit in T-ALL.

Authorship

Contribution: B.V.B. and P.V.V. designed and performed research and wrote the paper; G.J.L. collaborated on the MLL-AML study; E.E.W., D.R., and M.H. made this research possible by collecting patient samples and characteristics in their own study groups and providing additional information; A.M.W.O. and J.N.R.T. performed and designed NF1 analysis; and J.P.P.M., M.M.H., H.B.B., C.M.Z., and R.P. designed research and wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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References

13. Eguchi M, Eguchi-Ishimae M, Knight D, Kearney L, Slany R, Greaves M. MLL chimeric protein activates raf protein through distal elements of the raf promoter region and the 3′ untranslated region (UTR), and look for abnormalities in NF1 protein expression.

In conclusion, we report the identification of NF1 microdeletions in patients with pediatric T-ALL and MLL-rearranged AML without clinical evidence of NF1. We confirmed NF1 inactivation by reduced NF1 expression levels and biallelic NF1 mutations in 3 of 5 patients, confirming the role of NF1 as a tumor-suppressor gene in cancer. NF1 inactivation is a novel type I mutation in MLL-rearranged AML and a new proliferative hit in T-ALL.


