Mitotic recombination and compound-heterozygous mutations are predominant *NF1*-inactivating mechanisms in children with juvenile myelomonocytic leukemia and neurofibromatosis type 1

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ABSTRACT

Children with neurofibromatosis type 1 (NF-1), being constitutionally deficient for one allele of the *NF1* gene, are at greatly increased risk of juvenile myelomonocytic leukemia (JMML). *NF1* is a negative regulator of RAS pathway activity, which has a central role in JMML. To further clarify the role of biallelic *NF1* gene inactivation in the pathogenesis of JMML, we investigated the somatic *NF1* lesion in 10 samples from children with JMML/NF-1. We report that two-thirds of somatic events involved loss of heterozygosity (LOH) at the *NF1* locus, predominantly caused by segmental uniparental disomy of large parts of chromosome arm 17q. One-third of leukemias showed compound-heterozygous *NF1*-inactivating mutations. A minority of cases exhibited somatic interstitial deletions. The findings reinforce the emerging role of somatic mitotic recombination as a leukemogenic mechanism. In addition, they support the concept that biallelic *NF1* inactivation in hematopoietic progenitor cells is required for transformation to JMML in children with NF-1.

Key words: mitotic recombination, NF1-inactivating mechnisms, juvenile myelomonocytic leukemia.

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Introduction

Juvenile myelomonocytic leukemia (JMML) is a malignant hematopoietic stem cell disorder that affects children at a median age of two years and is characterized by clonal hyperproliferation of monocytes and granulocytes without differentiation arrest.[1] Defining features include an absolute monocyte count of greater than 1,000/μL, circulating granulocyte precursors, less than 20% blasts in the bone marrow, and the absence of a *BCR-ABL1* fusion gene. On the molecular level, deregulation of the RAS signal transduction pathway is central to the disordered hematopoiesis in JMML.[2] Eleven percent of children with JMML have constitutional neurofibromatosis type 1 (NF-1). NF-1 patients carry in the germline one intact and one deficient allele of the *NF1* tumor suppressor gene, which is a negative regulator of RAS pathway activity.[3] The constitutional *NF1* haploinsufficiency present in patients with NF-1 appears to have no developmental consequences, as individuals with NF-1 are usually born without major birth defects. However, children with NF-1 are at a 300-fold increased risk of JMML and other myeloid malignancies. This suggests that monoallelic loss of functional *NF1* is a tumor predisposition and that a second hit to the remaining *NF1* allele in somatic cells gives rise to the formation of neoplasms. In agreement with this model, clonal inactivation of the wild-type *NF1* allele was demonstrated in leukemic cells of children with JMML and NF-1.[4] We recently described somatic loss of heterozygosity (LOH) of *NF1* in leukemic cells of 4 out of 5 children with JMML and NF-1.[5] In these cases the LOH was not restricted to a small segment surrounding the *NF1* locus on chromosome 17q11.2, but involved almost the entire 17q arm. Moreover, the 17q LOH was not the product of a simple deletion; instead, the genomic material carrying the wild-type *NF1* allele was replaced by a second copy of the *NF1*-mutant 17q arm, resulting in segmental uniparental disomy (UPD). This indicated that mitotic recombination, an otherwise rare genetic event, was a recurrent underlying mechanism, consistent with a report by others.[6] We have now expanded upon the earlier study and investigated the *NF1*-inactivating event in 10 additional cases of JMML and NF-1. The results confirm UPD as a common finding in JMML/NF-1. In cases without UPD, compound-heterozygous *NF1* mutations were frequent. The study provides data on the nature of somatic *NF1* lesions in JMML and NF-1 and supports the concept that biallelic inactivation of *NF1* function is required for full leukemic transformation.

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The online version of this article has a supplementary appendix.

Design and Methods

Bone marrow or peripheral blood samples were collected in the context of European Working Group on MDS in Childhood (EWOG-MDS) studies MDS98 and MDS2006, with informed consent from guardians and approval from institutional review committees at each participating center. For short tandem repeat (STR) analysis, each locus was PCR-amplified using a fluorescently labeled forward primer (Sigma-Proligo, The Woodlands, TX, USA). Primer information for UniSTS markers can be found at http://www.ncbi.nlm.nih.gov/genome/sts. Heterozygote frequencies of markers are derived from www.gdb.org or http://genecards.weizmann.ac.il/geneloc-bin/marker_cards. Microsatellite length polymorphisms were analyzed by capillary electrophoresis (CEQ2000XL, Beckman Coulter, High Wycombe, UK). Array-based comparative genomic hybridization (CGH) was performed with the 244A Human Genome microarray kit (Agilent Technologies, Santa Clara, CA, USA), a 60-mer oligonucleotide-based microarray with median probe spacing of approximately 8.9 kilobases. The array was prepared according to the Agilent protocol. For analysis of scanned array images, default CGH settings of Feature Extraction software 9.1.1.1 (Agilent) were applied. CGH Analytics software v3.27 was used for DNA copy number analysis. The threshold of the ADM-2 aberration detection algorithm was set to 4.5. For multiplex ligation-dependent probe amplification (MLPA), the neurofibromatosis probe kits P081 and P082 (MRC-Holland BV, Amsterdam, The Netherlands) were used according to the manufacturer's instructions. For *NF1* mutation analysis, the primers used for genomic PCR amplification of *NF1* exons were based on DNA accession number NM000267.1. Amplicons were sequenced directly on an automated sequencer (MegaBace 1000, GE Healthcare, Freiburg, Germany).

Results

We tested 10 samples of leukemia cell DNA from children with JMML and NF-1 for the presence of segmental homozygosity on chromosome arm 17q, using 15 STR sequences distributed along 17q. The markers were chosen according to independent segregation and high heterozygote frequencies in the general population (ranging from 57–84%; compatible with STR haplotype distributions observed among 10 patients and 10 controls and with Hapmap data on http://www.hapmap.org). Six STR markers (D17S1841..D17S1800) were selected because of close proximity to, or position within, the *NF1* locus (*Online Supplementary Figure S1A*). The remaining 9 markers served to cover the 17q chromosome arm from D17S925 at 17q11.2 to D17S784 at 17q25.3. Patients' clinical and hematologic characteristics are shown in Table 1.

The analysis identified 2 cases (D419 and D561) where heterozygosity was lost on a large segment (>50 Mb) of chromosome arm 17q in JMML cells from children with NF-1 (*Online Supplementary Figure S1A*). Cytogenetically, the JMML cells of D419 had a complex aberrant karyotype and those of D561 had a normal karyotype; no structural or numerical aberration of chromosome 17 was seen in either case. To explore this further, we subjected both samples to MLPA, which confirmed normal genomic copy number at the NF1 locus (*data not shown*). Together, these results indicate the presence of somatic 17q UPD in D419 and D561.

In 3 other samples (D378, D566, D341), a smaller segment, which involved the *NF1*-surrounding STRs, was homozygous (*Online Supplementary Figure S1A*). We applied array-based CGH in these cases for genomic copy number analysis of the *NF1* region. No copy number irregularity was detected in D378 (*data not shown*), but 10 consecutive STR markers surrounding the *NF1* gene were homozygous. Based on heterozygote frequencies of these markers in the general population, the probability for constitutional homozygosity over the whole region is calculated to be less than 10^{-4} (squared frequencies of alleles were multiplied considering that all 10 consecutive markers do not show complete linkage disequilibrium). Therefore, the findings in D378 are indicative of interstitial UPD caused by double mitotic recombination. In samples D341 and D566, CGH demonstrated interstitial heterozygous deletions involving the *NF1* locus (*Online Supplementary Figure S1B*). In both cases the breakpoints corresponded to the segment of putative LOH defined by STR analysis.

In 5 samples (CZ051, D530, SC049, SC087, D252), the markers in close proximity to *NF1* retained heterozygosity for the selected loci (*Online Supplementary Figure S1A*). To address the two alternative possibilities of extremely focal LOH at *NF1* or compound-heterozygous *NF1* inactivation, we applied MLPA for exon-level copy number analysis, and genomic sequencing for *NF1* mutational analysis. Compound-heterozygous inactivating mutations were detected in samples CZ051, D530, SC049 and SC087 (Table 2). Consistent with this, MLPA indicated normal genomic copy number for NF1 exons 1–49 in all 4 samples (*data not shown*). By contrast, a homozygous *NF1* mutation (c.5242C>T) was found in D252. This was the only case with non-hematopoietic material (buccal epithelial cells) available. The c.5242 nucleotide was wild-type in buccal cells, indicating that the mutation found in blood cells was acquired. We assume that the constitutional *NF1* lesion in D252 is a focal intragenic deletion which does not extend to the neighboring heterozygous STRs D17S1849 and D17S1166. However, attempts to demonstrate the deletion using MLPA were unsuccessful (*data not shown*).

Together, the analyses provide a picture of recurrent genetic mechanisms leading to biallelic *NF1* inactivation in JMML/NF-1 cells (Table 2). LOH of the constitutional *NF1* lesion was seen in 5 cases. The mechanism behind LOH was segmental UPD as a consequence of single mitotic recombination in 2 cases, interstitial UPD derived from double mitotic recombination in one case, and interstitial deletion in 2 cases. By contrast, no evidence of LOH at the *NF1* locus was found in 4 cases. Here, the biallelic loss of *NF1* function in leukemic cells was due to two unrelated heterozygous mutational events.

In addition to the molecular studies described above, we asked whether the different causes of *NF1* inactivation translated into specific features in the clinical or hematologic picture of JMML (Table 1). However, no correlation was evident between the genetic basis of somatic *NF1* inactivation in leukemic cells and the presentation or course of JMML in the patients studied.

Discussion

We investigated on the genomic level the mechanism that led to biallelic loss of *NF1* tumor suppressor gene function in leukemic cells from 10 children with JMML and NF-1. Together with 5 cases published previously,[5] we find evidence of mitotic recombination in hematopoietic cells in 7 of 15 children (47%). Although the number of patients in our study is too small to draw general conclusions on the frequency of each particular lesion in the JMML/NF-1 population on the whole, it appears that mitotic recombination is a predominant leukemogenic mechanism of *NF1* inactivation in JMML/NF-1. This is in accordance with reports on *NF1*-driven tumorigenesis in other tissues such as neurofibroma,[12] and with genome-wide studies indicating that partial UPD is widely found in hematologic malignancies.[13] A probable explanation for the frequent occurrence of 17q UPD in leukemias of patients with NF-1 is the existence of

Table 1. Loss-of-heterozygosity status at the *NF1* gene and clinical/hematologic characteristics in 15 patients with juvenile myelomonocytic leukemia and neurofibromatosis type 1.

Patient ID	LOH at *NF1*	Age (mo.)	Sex	Liver (cm)	Spleen (cm)	Leukocytes (10^9/L)	Monocytes (%)	Hemoglobin (g/dL)	Platelets (10^9/L)	Hemoglobin F (%)	Disease course
CZ051	no	33	M	0	3	12.1	51	11.1	207	0.5	Alive (disease-free after SCT)
D 003*	yes	4	F	4	5	103.0	17	10	160	48.6	Dead (relapse after SCT)
D 102*	yes	45	M	7	5	NA	25	10.7	103	59.5	Dead (no SCT)
D 115*	yes	3	M	3	2	39.6	22	8.4	138	NA	Dead (no SCT)
D 126*	yes	5	M	5	9	81.9	29	10	159	24.2	Alive (disease-free after SCT)
D 127*	no	66	M	6	5	26.0	29	10.1	139	5.9	Dead (relapse after SCT)
D 252	undefined	43	F	6	4	29.4	16	9.4	22	14.8	Alive (disease-free after SCT)
D 341	yes	6	M	7	6	64.6	24	9.7	225	NA	Alive (disease-free after SCT)
D 378	yes	63	F	NA	NA	27.3	9	9.6	41	NA	Dead (relapse after SCT)
D 419	yes	60	M	4	10	80.0	23	4	48	23.6	Dead (relapse after SCT)
D 530	no	7	F	4	4	79.0	29	9.4	357	NA	Alive (disease-free after SCT)
D 561	yes	17	M	4	5	48.6	30	10.1	163	25.7	Dead (transplant-related mortality)
D 566	yes	72	M	4	6	20.2	20	10	200	NA	Alive (disease-free after SCT)
SC049	no	51	F	3	3	15.7	8	9.9	15	39.5	Dead (relapse after SCT)
SC087	no	36	F	2	3	40.0	11	9.7	201	31.6	Alive (disease-free after SCT)

*LOH: loss of heterozygosity; M: male; F: female; NA: not available; SCT: stem cell transplantation. * Result of LOH analysis published previously.[5]*

Table 2. Genetic mechanisms of biallelic *NF1* inactivation, identification of *NF1* gene mutations and predicted effect on the NF1 protein in 15 patients with juvenile myelomonocytic leukemia and neurofibromatosis type 1.

Patient ID	Mechanism of biallelic *NF1* inactivation	*NF1* mutation(s)	Effect on NF1 protein
D419	LOH (segmental UPD)	ND	
D561	LOH (segmental UPD)	ND	
D003*	LOH (segmental UPD)	*homo*: c.3861_3862delCT; p.F1287LfsX30	Truncation
D102*	LOH (segmental UPD)	*homo*: c.574C>T; p.R192X **	Truncation
D115*	LOH (segmental UPD)	*homo*: c.2066delT; p.V689GfsX58	Truncation
D126*	LOH (segmental UPD)	*homo*: c.7699C>T; p.Q2567X **	Truncation
D378	LOH (interstitial UPD)	ND	
D341	LOH (interstitial deletion)	ND	
D566	LOH (interstitial deletion)	ND	
CZ051	compound-heterozygous mutation	*het*: c.1748A>G; p.K583R *het*: c.2027delC; p.T676TfsX11 **	Alteration of kinase recognition site Truncation
D530	compound-heterozygous mutation	*het*: c.821T>G; p.L274R *het*: c.6579+1G>C (splice donor) **	Alteration of conserved domain Messenger splicing defect
SC049	compound-heterozygous mutation	*het*: c.205-2A>G (splice acceptor) *het*: c.4084C>T; p.R1362X **	Messenger splicing defect Truncation
SC087	compound-heterozygous mutation	*het*: c.482T>G; p.L161X ** *het*: c.495_498delTGTT; p.T165TfsX11 **	Truncation Truncation Truncation
D127*	compound-heterozygous mutation	*het*: c.2288_2295dupTGAGGCGC; p.R765fsX1 *het*: c.3366delT; p.D1122EfsX19	Truncation Truncation
D252	undefined	*homo*: c.5242C>T, p.R1748X **	Truncation

*LOH: loss of heterozygosity; UPD: uniparental disomy; het: heterozygous; homo: homozygous; ND: not done. * Case published previously.[5] ** NF1 mutation described in the literature.[7-11]*

repetitive sequences adjacent to *NF1*, which may be subject to a higher rate of erroneous recombination in faster dividing tissues.[14]

Compound-heterozygous mutation emerged as another recurrent *NF1*-inactivating mechanism, present in 5 of 15 cases (33%). By contrast, interstitial heterozygous deletion was seen in only 2 of 15 cases (13%). No case of interstitial homozygous deletion at the *NF1* gene locus was identified. Other authors have noted that the predominant type of somatic *NF1* lesion in NF-1 associated tumors appears to depend on the tumor entity. For example, large heterozygous deletions involving *NF1* and flanking genomic material occur in the majority of malignant peripheral nerve sheath tumors,[15] but are uncommon in dermal neurofibromas.[16] One may speculate that concomitant deletion of *NF1*-flanking genes could be involved in the development of specific tumor types. In summary, our data indicate that mitotic recombination and compound-heterozygous intragenic *NF1* mutations, but not deletions, are common somatic events in the pathogenesis of JMML in children with NF-1. However, we observed no correlation between the genetic basis of *NF1* inactivation and the clinical phenotype of the resultant leukemia.

With respect to *NF1* mutational spectrum, we detected a total of 15 different sequence alterations. Eight of these were previously reported in the literature. Five alterations correspond to nonsense mutations (all 5 described in the literature), resulting in a truncated neurofibromin protein. Six alterations are small deletions or duplications (2 described in the literature; all 6 are exonic), causing a frameshift and resulting in a truncated neurofibromin protein via premature termination codons. Two alterations affect splice donor or acceptor sites (one described in the literature), resulting in disrupted messenger RNA composition. Only 2 alterations correspond to single nucleotide exchanges not previously described in the literature (c.1748A>G and c. 821T>G). Both alterations are exonic, cause an amino acid exchange and affect evolutionally conserved domains of neurofibromin, suggesting pathogenicity. In addition, the c.1748A>G affects a well-defined cAMP-dependent protein kinase recognition site and generates a new splice acceptor site. Benign sequence variations in the vicinity of both alterations are not documented in available data bases. A schematic map of *NF1* mutations identified and the protein domain structure is provided as *Online Supplementary Figure S2*.

In each of the 15 cases analyzed here and previously,[5] we found evidence of biallelic *NF1* inactivation in leukemic cells of children with JMML and NF-1. This reinforces the long-standing concept that neurofibromatosis type 1, characterized by heterozygous germline defects of *NF1*, constitutes a tumor predisposition syndrome but somatic second events, which abolish *NF1* function completely, are required for actual tumor formation. Nevertheless, the question remains open as to whether *NF1* inactivation is by itself sufficient to drive the malignant transformation of a hematopoietic progenitor cell, or whether it is a secondary event that merely sustains the proliferation of a progenitor cell clone transformed through other mechanisms.[2,17]

Authorship and Disclosures

DS and CF designed the study. DS, LA, IP, MS and CF performed experiments and/or analyzed data. HH, JS, BS and CMN contributed research materials and patients. DS and CF wrote the paper. All authors read and approved the final version.

The authors reported no potential conflicts of interest.

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