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Biallelic inactivation of the *NF1* tumour suppressor gene in juvenile myelomonocytic leukaemia: Genetic evidence of driver function and implications for diagnostic workup

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Summary

Juvenile myelomonocytic leukaemia (JMML) is characterized by gene variants that deregulate the RAS signalling pathway. Children with neurofibromatosis type 1 (NF-1) carry a defective *NF1* allele in the germline and are predisposed to JMML, which presumably requires somatic inactivation of the *NF1* wild-type allele. Here we examined the two-hit concept in leukaemic cells of 25 patients with JMML and NF-1. Ten patients with JMML/NF-1 exhibited a *NF1* loss-of-function variant in combination with uniparental disomy of the 17q arm. Five had *NF1* microdeletions combined with a pathogenic *NF1* variant and nine carried two compound-heterozygous *NF1* variants. We also examined 16 patients without clinical signs of NF-1 and no variation in the JMML-associated driver genes *PTPN11*, *KRAS*, *NRAS* or *CBL* (JMML-5neg) and identified eight patients with NF1 variants. Three patients had microdeletions combined with hemizygous *NF1* variants, three had compound-heterozygous *NF1* variants and two had heterozygous *NF1* variants. In addition, we found a high incidence of secondary *ASXL1* and/or *SETBP1* variants in both groups. We conclude that the clinical diagnosis of JMML/NF-1 reliably indicates a *NF1*-driven JMML subtype, and that careful *NF1* analysis should be included in the genetic workup of JMML even in the absence of clinical evidence of NF-1.

KEYWORDS

JMML, juvenile myelomonocytic leukaemia, neurofibromatosis type 1, neurofibromin, *NF1*, RAS signalling

For Affiliation refer page on 603

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INTRODUCTION

Juvenile myelomonocytic leukaemia (JMML) is an aggressive clonal myeloproliferative and myelodysplastic neoplasia in children. Malignant transformation of haematopoietic stem/progenitor cells (HSPCs) into JMML is characterized by gene variants that promote sustained activation of the RAS signal transduction pathway. Variation of the canonical RAS pathway genes *PTPN11*, *NRAS*, *KRAS*, *NF1* or *CBL* accounts for 90% of patients diagnosed with JMML.¹ The majority of patients have no known predisposing condition and the RAS pathway variants (usually in *PTPN11*, *NRAS* or *KRAS*) occur somatically in HSPCs. Patients with Noonan syndrome and a germline variant in the *PTPN11* (or, rarely, *KRAS*) gene are at increased risk of developing a JMML-like myeloproliferative disorder (MPD).² Further predisposition to JMML includes *CBL* syndrome and neurofibromatosis type 1 (NF-1).^{3,4} NF-1 is a complex multisystem developmental and tumour-predisposing disorder, which primarily affects the nervous system and skin,⁵ and has a prevalence between 1 and 10 cases per 10 000 children.^{6,7} It has been estimated that patients with NF-1 are at approximately 200-fold risk of developing JMML compared to the general population.^{4,8}

Individuals with NF-1 typically carry one defective *NF1* gene allele in the germline, with nearly half of the patients having de novo variants.⁹ *NF1* is located near the centromere on chromosome band 17q11.2 in the vicinity of several repetitive elements, increasing its susceptibility to genetic alteration. Somatic inactivation of the inherited wild-type allele in haematopoietic cells is associated with transformation to JMML.^{4,10} Biallelic *NF1* inactivation was also reported in other tumours associated with NF-1, such as neurofibroma, malignant peripheral nerve sheath tumour and pilocytic astrocytoma, and was also found in hyperproliferative lesions, such as café-au-lait macules.^{11–14} Mitotic recombination, leading to uniparental isodisomy (UPD) of the 17q chromosome arm, was found to be the most frequent mechanism behind biallelic loss of *NF1* function in JMML cells.¹⁵ Other mechanisms include somatically acquired deletions of 17q11.2 or inactivating variants on the second allele.¹⁶

Due to the presence of repeat sequences within the *NF1* locus and the existence of several *NF1* pseudogenes, it was historically difficult to obtain complete genetic analyses of the *NF1* locus. Therefore, the diagnosis of the NF-1 group in patients with JMML was usually based on clinical features, chiefly café-au-lait macules, and/or a family history of NF-1. Taking advantage of advances in next-generation sequencing (NGS) technologies and better computational methods for the detection and interpretation of variants, we now set out to clarify whether the genetic findings in leukaemic cells of JMML/NF-1 patients are consistent with the clinical assessment and the two-hit concept. Specifically, the goal was to identify more than one *NF1*-inactivating event and thus confirm the relevance of clinical NF-1 features in children with JMML/NF-1. Furthermore, we investigated the possibility

that the group of JMML patients without clinical evidence of neurofibromatosis and no abnormality in *PTPN11*, *KRAS*, *NRAS* or *CBL* (herein abbreviated as JMML-5neg) contained unrecognized cases driven by biallelic inactivation of *NF1*.

MATERIALS AND METHODS

The study cohort consisted of 156 patients diagnosed with JMML who were registered in the European Working Group of MDS in Childhood (EWOG-MDS) studies EWOG-MDS98 and EWOG-MDS2006 (NCT00047268 and NCT00662090; www.clinicaltrials.gov). Patients with Noonan syndrome-associated MPD or CBL syndrome were excluded by prior Sanger sequencing of *PTPN11*, *KRAS*, *NRAS* and *CBL* in hair follicles, buccal epithelium or skin fibroblasts. Parents or legal guardians of all patients provided informed consent to the scientific use of patient materials in accordance with the Declaration of Helsinki. The collection and storage of patient materials was approved by the institutional review board of each participating centre. DNA from bone marrow or peripheral blood cells collected at the time of diagnosis was used for targeted NGS. The multigene panel consisted of coding regions of canonical driver genes (*PTPN11* [NM_002834], *KRAS* [NM_004985], *NRAS* [NM_002524], *CBL* [NM_005188, exons 7–10] and *NF1* [NM_001042492]) and genes previously reported to be associated with JMML as secondary lesions (*ASXL1* [NM_015338, exons 11–12], *JAK2* [NM_004972.3, exon 14], *JAK3* [NM_000215.3, exons 11–13,15,17,19], *RRAS* [NM_006270], *RRAS2* [NM_012250], *RAC2* [NM_002872], *RUNX1* [NM_001754], *SETBP1* [NM_015559, exon 4] and *SH2B3* [NM_005475, exons 2–7]). The targeted NGS libraries were prepared using NEBNext Ultra II kits (New England Biolabs), and samples were sequenced on a MiSeq 2000 sequencer (Illumina) with 150 bp paired-end reads. CytoScan HD arrays (Affymetrix) were applied to detect segmental deletions or copy number-neutral loss of heterozygosity (LOH).

Our in-house bioinformatics pipeline was applied for the detection of sequence variants. The raw paired-end reads were trimmed using Trimmomatic (v0.30). Raw fastq files were processed with options 2:30:10, HEADCROP:3 TRAILING:10 MINLEN:25.¹⁷ The processed reads were mapped to the human genome (version hg19) using the BWA aligner (v 0.7.17) with mem mode.¹⁸ The aligned reads were further processed using Picard and GATK tools,¹⁹ converted into the mpileup format using Samtools v1.9 and subjected to variant discovery using VarScan v2.3.9.^{20,21} We set the VAF cut-off as 5%, minimum coverage as 20 and *p*-value as 0.05. Subsequently, the variants were further filtered (--min-ref-basequal 28 --min-var-basequal 28 --min-ref-readpos 0.01 --min-ref-dist3 0.01 --min-var-readpos 0.01 --min-var-dist3 0.01) using VarScan2. The identified variants were annotated using Annovar, SnpEff and InterVar tools.^{22–24} We adhered to ACMG guidelines for annotating the variants.²⁵ Variants were sequentially checked for their presence in the population databases gnomAD, ExAC,

esp6500siv2 and 1000 Genomes. Variants with a population allele frequency above 0.1% were filtered out. Novel variants were manually checked against the alignment files to identify technical artefacts. *NF1* is located near the centromere of 17q, is surrounded by repetitive elements and has several pseudogenes. We have identified and eliminated such off-target regions in our bioinformatics workflow to avoid the detection of spurious variants. Single-nucleotide variants and indels identified by our targeted NGS analysis and not previously listed in the ClinVar database were verified by Sanger sequencing.

RESULTS

Spectrum of *NF1* gene alterations in patients with JMML

We examined the driver gene profile of 156 children with JMML by targeted NGS with a panel covering the full coding sequence of the *NF1*, *PTPN11*, *KRAS*, *NRAS*, *RRAS* and *RRAS2* genes and the hotspot region (exons 7–10) of the *CBL* gene. Twenty-five of the 156 patients (16%) were clinically assigned to JMML/NF-1, based on the presence of ≥ 6 café-au-lait macules, or any number of café-au-lait macules plus positive family history. One hundred fifteen patients (74% of the 156 cases) exhibited somatic variants in the *PTPN11*, *KRAS* or *NRAS* genes and were negative for clinical features of NF-1. Sixteen children (10%) were JMML-5neg; that is, negative for clinical NF-1 and without genetic alteration of *PTPN11*, *KRAS*, *NRAS* or *CBL* in leukaemic cells. One of the 16 JMML-5neg patients carried a variant in *RRAS2*. Combining the JMML/NF-1 and JMML-5neg groups, the cohort for detailed genetic workup of *NF1* thus consisted of 41 patients. These included 26 males and 15 females with a median age of 3.0 years at diagnosis (range, 0.2–7.8 years).

The karyotype of leukaemic cells was normal in 31 cases, 5 cases had monosomy 7 and 5 carried other chromosomal abnormalities.

Among the 41 JMML cases subjected to in-depth *NF1* molecular genetic analysis, we found 46 sequence alterations in 33 patients, consisting of 18 nonsense variants, 16 indels, 5 splice site variants, 5 missense variants and 2 in-frame deletions (Figure 1; Table S1). The variants were distributed across the entire *NF1* sequence without obvious clusters or hot spots. p.I679Dfs*21, a single-nucleotide duplication in the homopolymer region (c. 2033dup), was identified in four patients. Two nonsense variants (p.R1362* and p.R1534*) were found in two patients each. All other variants occurred only once. Among 40 unique variants, 33 were predicted to cause premature truncation of the protein, in line with loss of neurofibromin function, the key mechanism of disease development. We also detected five missense variants in the cohort. These variants are not reported in the healthy population (gnomAD, v.2.1.1), affect amino acids with evolutionary conservation or 5' splice sites of *NF1* exons (Figure S1) and were in part reported in NF-1.^{26–28} All missense variants were predicted to impair protein function by several computational tools (Table S2).

Two cases exhibited in-frame *NF1* deletions entailing the loss of five and two amino acids respectively (p.A208_V212del and p.N2387_F2388del). Applying ACMG criteria,²⁵ 37/40 variants were pathogenic or likely pathogenic while 3/40 were scored as variants of unknown significance (Table S1). However, their non-random occurrence in concomitance with a pathogenic *NF1* lesion supports their pathogenic role.

Additional *NF1* alterations were detected by targeted NGS analysis in patients belonging to the JMML subgroups *PTPN11* (9/76 cases, 12%) and *NRAS* (3/23 cases, 13%) (Table S3). No concomitant *NF1* alterations were found in 16 patients of the *KRAS* subgroup, consistent with a previous

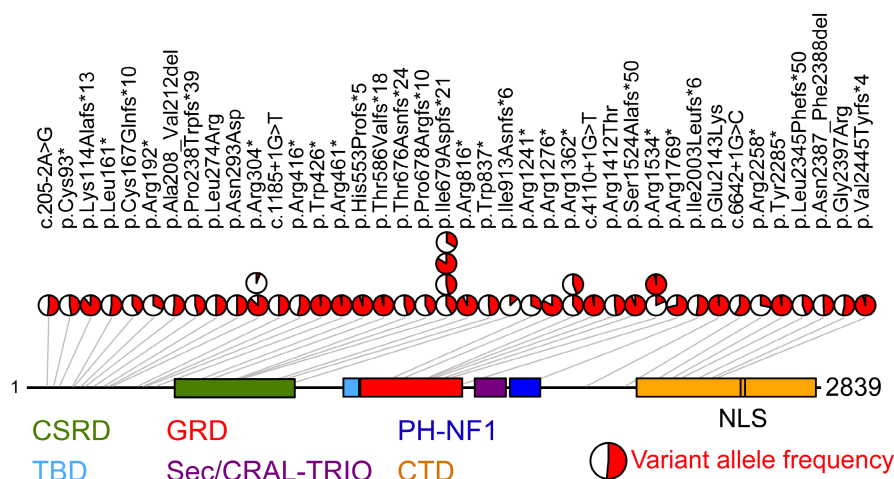


FIGURE 1 Spectrum of *NF1* gene variants in JMML. Schematic representation of the functional domains of neurofibromin (NP_001035957) and the distribution of variants identified in 33 patients. Pie charts symbolize the allele frequencies of the variants. Amino acid positions are indicated at the top. Functional domains are colour-coded. CSRD, cysteine- and serine-rich domain; CTD, C-terminal domain; GRD, GAP-related domain; NLS, nuclear localization signal; PH, pleckstrin homology domain; Sec/CRAL-TRIO, Sec14 homology/CARL-TRIO domain; TBD, tubulin-binding domain.

TABLE 1 NF1 gene variants, secondary gene variants and clinical characteristics in 25 children with JMML/NF-1 and 16 children with JMML-5neg.

ID	NF1 variant	NF1 amino-acid change	VAF (%)	Mechanism of NF1 LOH	Germline analysis	Secondary gene variants	Age (years)	Café-au-lait macules	Café-au-lait macule count	Other symptoms of NF-1	Family history of NF-1	Duration of follow-up (years)	Outcome	Previous reports
NF01	c.7035_7040delinsTA c.4600C>T	p.(Leu2345Phefs*50) p.(Arg1534*)	44 18	None	Not done	None identified	3.0	Yes	6	No	No	0.6	Dead	
NF02	c.4569del	p.(Ser1524Alafs*50)	94	UPD 17q	Not done	SETBP1 c.2602G>A, p.(Asp868Asn); 44.2%	3.6	Yes	≥10	Neurofibromas	Yes	1.3	Dead	
NF03	c.5305C>T	p.(Arg1769*)	72	UPD 17q	Not done	None identified	3.6	Yes	≥10	Neurofibromas	No	17.2	Alive	Ref. 16
NF04	c.910C>T	p.(Arg304*)	6	Type 1 microdeletion	Not done	None identified	0.5	Yes	≥10	Neurofibromas	No	19.5	Alive	Ref. 16
NF05	c.6855C>A	p.(Tyr2285*)	98	UPD 17q	Not done	ASXL1 c.1934dup, p.(Gly646Trpfs*12); 36.7% JAK3 c.1970G>A, p.(Arg657Gln); 48.0%	5.2	Yes	6	Neurofibromas	No information	0.7	Dead	Ref. 16
NF06	c.1277G>A	p.Trp426*	99	UPD 17q	Not done	ASXL1 c.1934dup, p.(Gly646Trpfs*12); 28.0%	5.0	Yes	≥10	Neurofibromas; freckling	Yes	1.4	Dead	Ref. 16
NF07	c.6642+1G>C c.821T>G	p.(Glu2214asp*)? p.(Leu274Arg)	58 50	None	Not done	None identified	0.6	Yes	≥10	Neurofibromas	Yes	15.7	Alive	Ref. 16
NF08	c.1756_1759del	p.(Thr586Valfs*18)	97	UPD 17q	Not done	None identified	1.4	Yes	8	Neurofibromas	Yes	0.5	dead	Ref. 16
NF09	c.4110+1G>T	p.(Gln1370sp*)?	98	Type 1 microdeletion	Fibroblasts: Copy number loss chr17:28889920–chr17:28889920–30 501 044; absence of c.4110+1G>T	ASXL1 c.1937dup, p.(Gly649Trpfs*9); 40.0% RUNX1 c.507_508+5dup, p.(?); 10.5% RUNX1 c.1090_1103del, p.(Ile364Valfs*231); 16.0%	6.0	Yes	≥10	Neurofibromas	Yes	13.8	Alive	Ref. 16
NF10	c.1657dup	p.(His553Profs*5)	95	Atypical 2.5 Mb deletion	Not done	JAK3 c.1969C>T, p.(Arg657Trp); 6.7%	2.7	Yes	≥10	No	Yes	15.3	Alive	
NF11	c.6772C>T c.7189G>C c.2737dup	p.(Arg2258*) p.(Gly2397Arg) p.(Ile913Asnfs*6)	28 53 13	None	Not done	ASXL1 c.1934dup, p.(Gly646Trpfs*12); 9.1% ASXL1 c.2077C>T, p.(Arg693*); 8.7%	3.0	Yes	≥10	No	No	4.1	Alive	
NF12	c.574C>T	p.(Arg192*)	32	Type 1 microdeletion	Fibroblasts: Copy number loss chr17:29119495–chr17:29119495–30 228 792; absence of c.574C>T	SETBP1 c.2608G>A, p.(Gly870Ser); 5.8% SETBP1 c.2602G>A, p.(Asp868Asn); 5.2%	1.1	Yes	≥10	No	No	1.0	Dead	
NF13	c.910C>T	p.(Arg304*)	87	Type 2 microdeletion	Fibroblasts: Copy number loss chr17:29096017–chr17:29096017–30 498 238; absence of c.910C>T	CBL c.1139T>C, p.(Leu380Pro); 19.8%	3.0	Yes	≥10	No	No	7.2	Alive	

TABLE 1 (Continued)

ID	NF1 variant	NF1 amino-acid change	VAF (%)	Mechanism of NF1 LOH	Germline analysis	Secondary gene variants	Age (years)	Café-au-lait macules	Café-au-lait macule count	Other symptoms of NF-1	Family history of NF-1	Duration of follow-up (years)	Outcome	Previous reports
NF14	c.4084C>T c.279_280delinsAA	p.(Arg1362*) p.(Cys93*)	44 47	None	Not done	None identified	4.3	Yes	≥10	No	No	8.4	Alive	
NF15	c.2033dup c.1185+1G>T	p.(Ile679Aspfs*21) p.(Lys395sp*)?	41 50	None	Not done	ASXL1 c.1934dup, p.(Gly646Trpfs*12); 35.0% SETBP1 c.2572G>A, p.(Glu858Lys); 27.0%	1.8	Yes	≥10	Optic glioma	No	6.5	Alive	
NF16	c.2446C>T	p.(Arg816*)	93	UPD 17q	Fibroblasts: c.2446C>T not covered; UPD 17q absent	ASXL1 c.1934dup, p.(Gly646Trpfs*12); 38.0%	3.6	Yes	7	No	No information	5.6	Alive	
NF17	c.1381C>T	p.(Arg461*)	98	UPD 17q	Not done	None identified	2.0	Yes	≥10	No	No	2.1	Dead	
NF18	c.4235G>C c.877A>G	p.(Arg1412Thr) p.(Asn293Asp)	48 50	None	Not done	PTPN1 c.227A>C; p.(Glu76Ala); 10.7% ASXL1 c.2705dup, p.(Ser903Ilefs*3); 12.8%	3.4	Yes	≥10	No	Yes	1.9	Dead	
NF19	c.7328_7331dup	p.(Val2445Tyrfs*4)	96	UPD 17q	Not done	SETBP1 c.2608G>A, p.(Gly870Ser); 14.9%	3.0	Yes	4	No	No information	4.7	Alive	
NF20	c.7159_7164del	p.(Asn238Z Phe2388del)	49	None	Not done	CBL c.1246T>C; p.(Cys416Arg); 29.0%	0.3	Yes	6	No	No	0.8	Dead	
NF21	c.4600C>T	p.(Arg1534*)	98	UPD 17q	Not done	ASXL1 c.2077C>T, p.(Gly693*); 43.6%	3.1	Yes	≥10	Neurofibromas	Yes	13.3	Dead	
NF22	c.6427G>A	p.(Glu2143Lys)	99	UPD 17q	Not done	None identified	1.7	Yes	1	No	Yes	18.8	Alive	
NF23	c.1246C>T c.2033del	p.(Arg416*) p.(Pro678Argfs*10)	54 43	None	Not done	None identified	1.4	Yes	≥10	No	No	15.0	Alive	
NF24	c.4084C>T c.205-2A>G	p.(Arg1362*) p.(Arg69sp*)?	40 52	None	Not done	CBL c.1151G>A, p.(Cys3841Yr); 21.0% CBL c.1201_1203dup, p.(Cys401dup); 37.2%	4.2	Yes	≥10	Neurofibromas	No	1.2	Dead	Ref. 16
NF25	c.499_502del c.482T>G	p.(Cys167Glnfs*10) p.(Leu161*)	41 53	None	Not done	None identified	3.0	Yes	≥10	Neurofibromas	No	14.5	Alive	Ref. 16
NEG01	c.339dup	p.(Leu114Alafs*13)	89	Type 1 microdeletion	Buccal epithelium: c.339dup absent	None identified	2.9	No	No	No	No	2.3	Dead	
NEG02	c.711_723del	p.(Pro238Trpfs*39)	46	None	Fibroblasts: c.711_723del absent	ASXL1 c.2324T>A, p.(Leu775*); 28.6%	2.6	No	No	No	No	13.4	Alive	
NEG03	c.3721C>T	p.(Arg1241*)	32	Not assessed	Not done	ASXL1 c.1900_1922del, p.(649*); 36.0%	5.6	No	No	No	No	13.4	Alive	
NEG04	c.2033dup c.6007-5A>G	p.(Ile679Aspfs*21) p.(Leu2003Leufs*6)	46 53	None	Not done	None identified	3.0	No	No	No	No	7.7	Alive	

(Continues)

TABLE 1 (Continued)

ID	NF1 variant	NF1 amino-acid change	VAF (%)	Mechanism of NF1 LOH	Germline analysis	Secondary gene variants	Age (years)	Café-au-lait macules	Café-au-lait macule count	Other symptoms of NF-1	Family history of NF-1	Duration of follow-up (years)	Outcome	Previous reports
NEG05	c.2024dup c.2033dup	p.(Thr676Asnfs*24) p.(Ile679Aspfs*21)	45 33/16.67	None	Fibroblasts: c.2033dup and c.2024dup not covered	ASXL1 c.1934dup, p.(Gly646Trpfs*12); 32.0%	2.6	No	No	No	No	13.1	Alive	
NEG06	c.2033dup	p.(Ile679Aspfs*21)	84	Atypical 1.8 Mb deletion	Not done	ASXL1 c.1774C>T, p.(Gln592*), 43.2% SETBP1 c.2602G>A, p.(Asp868Asn); 49.2%	2.2	No	No	No	No	12.1	Alive	
NEG07	c.3826C>T	p.(Arg1276*)	83	Type 1 microdeletion	Not done	None identified	1.4	No	No	No	No	4.2	Dead	
NEG08	c.2510G>A c.622_636del	p.(Trp837*) p.(Ala208_Val212del)	48 52	None	Not done	None identified	3.4	No	No	No	No	14.1	Alive	
NEG09	No	No	No	Not assessed	Not done	ASXL1 c.1934dup, p.(Gly646Trpfs*12); 5.6% RUNX1 c.514dup, p.(Ser172Lysfs*41);5%	5.3	No	No	No	No	11.3	Alive	
NEG10	No	No	No	Not assessed	Not done	RRAS2 c.215A>T; p.(Glu72Leu);49% SETBP1 c.2572G>A; p.(Glu858Lys);46% JAK3 c.1970G>A, p.(Arg657Gln);44%	5.9	No	No	No	No	6.3	Alive	
NEG11	No	No	No	Not assessed	Not done	None identified	0.5	Yes	2	No	No	13.0	Alive	
NEG12	No	No	No	Not assessed	Not done	None identified	7.8	No	No	No	No	13.2	Alive	
NEG13	No	No	No	Not assessed	Not done	None identified	0.2	No	No	No	No	9.6	Alive	
NEG14	No	No	No	Not assessed	Not done	None identified	0.3	No	No	No	No	6.9	Dead	
NEG15	No	No	No	Not assessed	Not done	None identified	1.1	No	No	No	No	3.2	Alive	
NEG16	No	No	No	Not assessed	Not done	None identified	0.6	No	No	No	No	10.3	Alive	

Abbreviations: LOH, loss of heterozygosity; Mb, million base pairs; NF-1, neurofibromatosis type 1; UPD, uniparental disomy.

exome analysis.²⁹ The *NF1* VAFs provided no evidence of biallelic *NF1* inactivation in the JMML *PTPN11* and *NRAS* subgroups, suggesting that these changes to *NF1* were secondary events. The only exception was the case NRAS02 which harboured two pathogenic *NF1* variants. Targeted sequencing in buccal epithelial cells demonstrated that the *NRAS* variant as well as both *NF1* variants were absent from the germline. Single-cell DNA sequencing would be required to help determine whether *NF1* or *NRAS* was the initiating oncogene in this case.

Genetic evidence of biallelic inactivation of *NF1* in JMML

Upon evaluation of targeted NGS reads, 13 of the 25 JMML/NF-1 cases exhibited a *NF1* loss-of-function variant with a variant allele frequency (VAF) in the range of 72%–99%, suggesting that these variants occurred in combination with a prior or later event causing LOH. Eight cases carried two independent pathogenic *NF1* variants, each with a VAF of almost 50%, and one case harboured three *NF1* variants with 53%, 28% and 13% VAF, suggesting two coexisting sub-clones. In the three remaining JMML/NF-1 cases, targeted sequencing identified pathogenic *NF1* variants with mono-allelic status (VAF 49%) in one sample and low VAF in the other two samples (32% and 6% respectively).

We then performed array-based single-nucleotide polymorphism (SNP) analysis to search for mechanisms of *NF1* LOH in 15/25 JMML/NF-1 cases, that is excluding those nine cases where the combined occurrence of two or more heterozygous variants already suggested biallelic inactivation without LOH and one case where additional DNA for SNP arrays was unavailable. Among 15 cases, 10 exhibited a large region of UPD that affected almost the entire chromosome 17q arm and encompassed the *NF1* locus (Table 1; Table S4), in line with previous reports.^{15,16} Based on previous literature on 17q UPD-related neoplasms in NF-1,³⁰ it is likely that the *NF1* variation was the constitutional event, and subsequent somatic acquisition of 17q UPD led to JMML in these patients. We confirmed the acquired nature of 17q UPD in one case where sufficient remaining non-haematopoietic material (fibroblasts) was available (NF16 in Table 1). However, since the combination of a high-VAF variant and UPD is only possible if the UPD comes second, it can be assumed that all UPD cases had the isodisomy as the somatic event. In 3/15 cases, SNP array analysis detected microdeletions encompassing the *NF1* locus (Table 1; Table S4). Of these, one was a recurrent type 1 deletion (i.e. encompassing 1.4 Mb and 14 protein-coding genes), one was a recurrent type 2 deletion (i.e. involving 1.2 Mb and 13 protein-coding genes but leaving the *LRRC37B* gene unaffected) and one was atypical.³¹ The remaining 2/15 cases showed *NF1* variants with low VAF, suggesting that heterozygosity of *NF1* was retained in leukaemic cells. However, SNP array analysis uncovered type 1 microdeletions in both (NF04 and NF12

in Table 1; Table S4). Non-haematopoietic material (fibroblasts) was available in three of the five microdeletion cases (NF09, NF12, NF13). The microdeletion was present but the missense variant was undetectable in all three germline samples, indicating that the sequence of constitutional microdeletion and acquired missense variant predominates in JMML/NF-1 cases without UPD. The possible combination of constitutional microdeletion and acquired 17q UPD was not detected in our cohort.

Among 16 patients in the JMML-5neg group, we identified three cases with *NF1* variants at allelic frequency near 100% (NEG01, NEG06 and NEG07 in Table 1; Table S5) and three cases with compound-heterozygous *NF1* variants (NEG04, NEG05 and NEG08), indicating biallelic loss of *NF1* in the absence of syndromic features. SNP array analysis identified one atypical and two type 1 *NF1* microdeletions in the three cases with high-VAF *NF1* variants. One case carried two frameshift single-nucleotide duplications (c.2024dup and c.2033dup) in close proximity (NEG05). It was therefore possible to determine from individual sequencing reads that the two alterations were situated in trans, substantiating the concept of compound heterozygosity. Heterozygous or low-VAF *NF1*-inactivating variants, but no second hits to *NF1*, were found in two cases (NEG02 and NEG03), providing inconclusive evidence of driver function. There was no genetic evidence of *NF1* involvement in the other eight JMML-5neg cases.

The lack of non-haematopoietic material in JMML-5neg cases with biallelic *NF1* inactivation precluded us from assessing if any of the lesions were present in the germline. We could therefore not determine genetically whether the patients were affected by constitutional neurofibromatosis with the onset of JMML before the syndrome became clinically evident. However, the median follow-up period of 12.6 years without the children developing features of NF-1 (Table 1) argues against this idea. Other possible interpretations include double somatic *NF1* hits in the haematopoietic lineage or postzygotic *NF1* mosaicism.

Secondary variants associated with JMML and *NF1* inactivation

In addition to covering canonical RAS pathway driver genes, our NGS panel also interrogated secondary gene abnormalities frequently associated with JMML (i.e. those involving *ASXL1*, *JAK2*, *JAK3*, *RUNX1*, *SH2B3* or *SETBP1*) and thus provided a picture of the variational landscape in *NF1*-driven JMML.^{29,32–34} We found at least one secondary variant in 15 of the 25 patients with clinical NF-1, and in 4 of the 8 JMML-5neg patients with *NF1* variants (Figure 2, Table 1). Among these, variants in the *ASXL1* gene were present in 12 patients. All identified variants in *ASXL1* lead to premature truncation due to a nonsense alteration, duplication of a single nucleotide or short deletion. Variants in the classic RAS pathway genes were found in four patients: The pathogenic *PTPN11* variant p.E76A

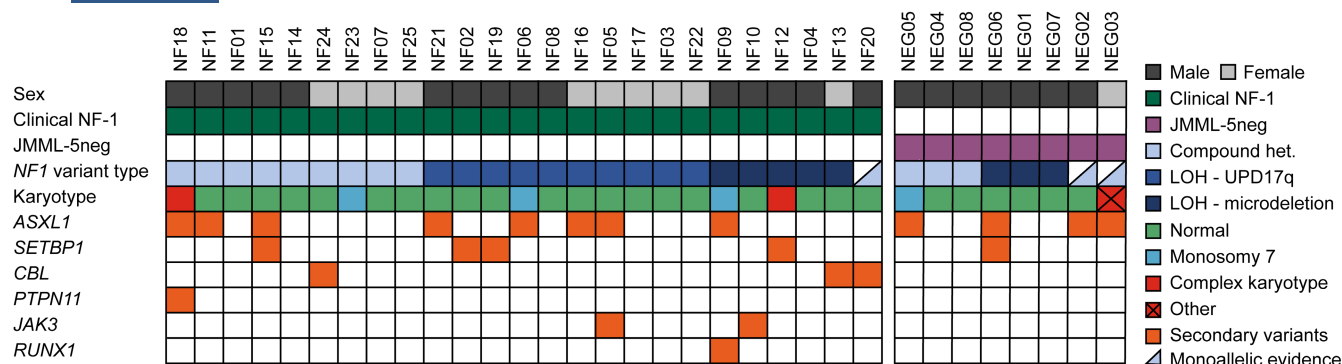


FIGURE 2 Clinical features and genetic landscape of individuals with JMML and *NF1* inactivation. The clinical and molecular characteristics of 33 JMML patients with evidence of *NF1* inactivation are displayed. Sex, clinical NF-1, JMML-5neg status, type of *NF1* variation (double heterozygosity, UPD17q, microdeletion), leukaemia karyotype (normal, monosomy 7, complex, other) and secondary variants in *ASXL1*, *SETBP1*, *CBL*, *PTPN11*, *JAK3* and *RUNX1* are indicated.

(VAF 11%) was found in one case, and the pathogenic *CBL* missense variants p.L380P, p.C384Y and p.C416R (VAFs ranging from 20% to 29%) were detected in three samples. The sample with the *CBL* p.C384Y variant additionally carried a three-nucleotide duplication in the *CBL* gene (VAF 37%), predicted to cause a non-frameshift insertion of one amino acid (p.C401dup). We identified at least one pathogenic *SETBP1* variant (p.D868N, p.G870S, or p.E858K) in five patients, with VAFs ranging from 5% to 49%. The pathogenic *JAK3* variants p.R657Q (VAF 48%) and p.R657W (VAF 7%) were found in one patient each. One sample harboured two *RUNX1* variants (VAFs 11% and 16%). There was no significant difference between JMML/NF-1 and JMML-5neg patients with regard to secondary variants in genes that are not part of the canonical RAS pathway. However, all patients with secondary variants in RAS pathway genes (1 *PTPN11* and 3 *CBL*) belonged to the JMML/NF-1 group.

DISCUSSION

The present study provides the first systematic investigation of genetic lesions that lead to biallelic loss of *NF1* gene function and thus drive the leukaemic process in a large series of 41 children with JMML. A clinical diagnosis of NF-1 was known in 25 patients, while 16 patients had no clinical features of NF-1 and were also negative for variants in the other canonical RAS pathway driver genes *PTPN11*, *KRAS*, *NRAS* and *CBL*. Although we have previously addressed the genetics of *NF1* variants or *NF1* deletions in patients with JMML and NF-1^{15,16} and *NF1* sequence analysis was part of previous whole-exome cohort studies,^{29,32,33} the additional value of the work presented here lies in a significantly increased number of cases, a more rigorous assessment of biallelic *NF1* inactivation using improved sequencing methods and high-resolution SNP arrays, and a more detailed description of the neurofibromatosis phenotype. For clarity, patients already included in previous publications are indicated in Table 1.

The combined application of NGS and SNP array analysis identified biallelic *NF1* inactivation due to gene variation plus LOH in 13 of 25 patients with JMML/NF-1 and 3 of 16 JMML patients with no clinical diagnosis of NF-1. Ten patients exhibited large regions of interstitial UPD involving almost the entire 17q chromosome arm. Six patients had *NF1* microdeletions (four recurrent and two atypical). An interesting observation is that 17q UPD was restricted to JMML/NF-1 patients but did not occur in JMML-5neg children, and that it was detected only in the context of *NF1* single-nucleotide variants or indels but not other types of *NF1* inactivation. As a possible interpretation, UPD on top of an interstitial deletion would lead to nullisomy of other genes in the region and this could protect the cells from transformation to leukaemia. We also uncovered a high proportion of *NF1* microdeletions in the cohort. These were germline events in all cases where non-haematopoietic material was available for testing. The overrepresentation of constitutional deletions in JMML/NF-1 patients compared to the general NF-1 population³⁵ may suggest that patients with microdeletions are at higher risk of JMML, consistent with the view that codeleted flanking genes are likely to act as phenotype modifiers.³¹ *SUZ12* deletion might be a potential link as inactivation of polycomb repressive complex 2 has previously been shown to be associated with JMML.²⁹

Some interesting aspects emerged from the analysis of secondary variants in *NF1*-driven JMML. Consistent with the notion that JMML/NF-1 is a subtype with aggressive presentation and poor prognosis, secondary variants were detectable at the time of diagnosis in the majority (15/25) of JMML/NF-1 patients. Co-occurrence of another RAS pathway lesion with *NF1* inactivation was noted in 4 of these 15 patients (3 *CBL*, 1 *PTPN11*). Both the clinical phenotype and the comparison of allele frequencies clearly indicated that *NF1* was the original driver in these cases whereas the changes in the other RAS pathway genes were limited to subclones. Further, the JMML/NF-1 group contained eight patients with *ASXL1* lesions and four with *SETBP1* alterations. Concerning *ASXL1* in particular, this suggests an

overrepresentation compared to patients with other subtypes of JMML.^{29,32–34}

For the management of patients, we conclude from our study that the clinical assignment to the JMML/NF-1 group is reliable and can almost always be confirmed genetically. This is remarkable since the children are often too young at the onset of JMML to display the full spectrum of NF-1-associated symptoms.^{5,36} In fact, our genetic data show that the presence of ≥ 6 café-au-lait macules (or less in the case of affected parents) in a child with JMML is already sufficient to diagnose NF-1 with a very high probability.

Our data illustrate that the two definitions of the *NF1*-driven JMML subtype recently published by expert groups have imperfections.^{37,38} The ICC definition calls for “germline *NF1* mutation and LOH of *NF1* or clinical diagnosis of NF-1”, but this does not accommodate compound-heterozygous variants in children with clinical NF-1 or double somatic *NF1* inactivation in children without NF-1 phenotype.³⁷ The WHO definition requires “biallelic pathogenic alterations in *NF1*” but this does not account for JMML cases with a clear clinical diagnosis of NF-1 where genetic *NF1* analysis is uninformative or missing.³⁸ Both definitions do not specify whether testing for LOH (e.g. by SNP array) is explicitly required or whether LOH can be inferred from a high *NF1* VAF; also, no VAF thresholds are provided for the latter alternative.

Our genetic analysis in JMML patients who lacked clinical features of NF-1 and harboured no driver alteration in *PTPN11*, *KRAS*, *NRAS* or *CBL* demonstrated the biallelic involvement of *NF1* in a relevant number of cases (38%). Since no NF-1 symptoms developed in these children even after long observation periods, we consider postzygotic mosaics or double somatic *NF1* inactivation in haematopoietic cells to be more likely than constitutional NF-1. Future studies are needed to determine whether this variety of *NF1*-driven JMML differs haematologically and clinically from JMML in constitutional NF-1. Finally, in JMML-5neg cases without *NF1* alteration, other forms of myeloproliferative neoplasms should be considered, for example, those involving alterations or rearrangements of tyrosine kinase genes.

AUTHOR CONTRIBUTIONS

Senthilkumar Ramamoorthy, Dirk Lebrecht, Denny Schanze, Ina Schanze, Ilse Wieland, Geoffroy Andrieux, Patrick Metzger, Maria Hess, Marcin W. Wlodarski, Melanie Boerries, Martin Zenker and Christian Flotho performed experiments, analysed the data and interpreted variants. Michael H. Albert, Arndt Borkhardt, Dorine Bresters, Jochen Buechner, Albert Catala, Valerie De Haas, Michael Dworzak, Miriam Erlacher, Henrik Hasle, Kirsi Jahnukainen, Franco Locatelli, Riccardo Masetti, Jan Stary, Dominik Turkiewicz, Luca Vinci, Ayami Yoshimi and Charlotte M. Niemeyer provided clinical samples, collected and interpreted clinical phenotypes, and analysed the data. Martin Zenker and Christian Flotho conceived the study.

Senthilkumar Ramamoorthy and Christian Flotho wrote the manuscript. All authors revised the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

ETHICS STATEMENT

The study was approved by Research Ethics Committee “Ethik-Kommission der Albert-Ludwigs-Universität”, University of Freiburg, Germany.

PATIENT CONSENT STATEMENT

The authors attest to the fact that written consent for participation was received from every individual whose data are included.

CLINICAL TRIAL REGISTRATION NUMBER

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