MYELOID NEOPLASIA

Cooperativity of *RUNX1* and *CSF3R* mutations in severe congenital neutropenia: a unique pathway in myeloid leukemogenesis

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Key Points

- CN/AML patients have a high frequency of CSF3R and RUNX1 mutations.
- CSF3R and RUNX1 mutations induce elevated proliferation of CD34$^+$ cells.

Severe congenital neutropenia (CN) is a preleukemic bone marrow failure syndrome with a 20% risk of evolving into leukemia or myelodysplastic syndrome (MDS). Patterns of acquisition of leukemia-associated mutations were investigated using next-generation deep-sequencing in 31 CN patients who developed leukemia or MDS. Twenty (64.5%) of the 31 patients had mutations in *RUNX1*. A majority of patients with *RUNX1* mutations (80.5%) also had acquired *CSF3R* mutations. In contrast to their high frequency in CN patients who developed leukemia or MDS, *RUNX1* mutations were found in only 9 of 307 (2.9%) patients with de novo pediatric acute myeloid leukemia. A sequential analysis at stages prior to overt leukemia revealed *RUNX1* mutations to be late events in leukemic transformation. Single-cell analyses in 2 patients showed that *RUNX1* and *CSF3R* mutations were present in the same malignant clone. Functional studies demonstrated elevated granulocyte colony-stimulating factor (G-CSF)–induced proliferation with diminished myeloid differentiation of hematopoietic CD34$^+$ cells coexpressing mutated forms of RUNX1 and CSF3R. The high frequency of cooperating *RUNX1* and *CSF3R* mutations in CN patients suggests a novel molecular pathway of leukemogenesis: mutations in the hematopoietic cytokine receptor (G-CSFR) in combination with the second mutations in the downstream hematopoietic transcription fator (RUNX1). The detection of both *RUNX1* and *CSF3R* mutations could be used as a marker for identifying CN patients with a high risk of progressing to leukemia or MDS. (*Blood*. 2014;123(14):2229-2237)

Introduction

Congenital neutropenia (CN) is a heterogeneous bone marrow failure syndrome characterized by severe neutropenia (blood neutrophil counts $<0.5 \times 10^9$/l) and maturation arrest of myelopoiesis at the level of the promyelocytes/myelocytes.[1] Autosomal-dominant and sporadic CN cases are predominantly attributable to mutations in *ELANE*, the gene encoding neutrophil elastase.[2] Several other genetic mutations, including those in *HAX1* (HCLS1-associated protein X-1), *G6PC3* (glucose 6 phosphatase, catalytic, 3), *GFI1* (growth factor independent 1 transcription repressor), and *WAS* (Wiskott-Aldrich syndrome gene), have been described in patients with CN.[3-6] The majority of CN patients benefit from treatment with granulocyte colony-stimulating factor (G-CSF).[7] Common pathological mechanisms for the maturation arrest of myeloid development in these patients include the lack of myeloid-specific transcription factors such as LEF-1 (lymphoid enhancer-binding factor 1) and C/EBPα (CCAAT/enhancer binding protein α), and defective G-CSF signaling.[8]

CN is a preleukemic syndrome with a cumulative incidence of leukemia of >20% after 20 years.[9] Approximately 70% to 80% of CN patients who develop acute myeloid leukemia (AML) or myelodysplastic syndrome (MDS) acquire heterozygous G-CSF receptor (CSF3R) mutations, independent of the genetic subtype, suggesting that these mutations are involved in leukemogenesis.[10-12] This pattern is distinct from de novo childhood AML in which CSFR3 mutations are very rare. Current evidence indicates that CSF3R gene mutations are not sufficient for leukemic transformation. As Welch et al[13] reported, in many cases of myeloid leukemias, only 1 or 2 cooperating mutations are needed to generate the malignant

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founding clone. In our study, our hypothesis is that the initial driver mutations are the CSF3R mutations, and cell clones harboring CSF3R mutations have a growth advantage and acquire additional cooperating mutations (in the majority of patients RUNX1 mutations) that contribute to AML initiation and disease progression.

Point mutations in *RUNX1* (runt-related transcription factor 1) have been described in de novo AML or AML secondary to MDS, radiation exposure, or chemotherapy at frequencies of 6% to 33%.[14-18] High incidence of *RUNX1* mutations has been associated with monosomy 7, trisomy 21, or trisomy 13.[17,19,20] Most of the *RUNX1* mutations are acquired heterozygous point mutations, predominantly located in the Runt homology/DNA binding (RHD) or transactivation (TAD) domains. They are associated with poor prognosis in AML.[14-17,19-21] *RUNX1* germline mutations, with or without acquired *RUNX1* mutations, have also been described in a familial platelet disorder with a predisposition to AML.[22,23]

The goal of the present study was to identify the steps of leukemia progression in CN by analyzing genomic profiles of patients during the course of the disease using next-generation DNA deep-sequencing. Specifically, we sought to determine if CN patients acquired leukemia-associated mutations in the course of the development of myeloid leukemia and to assess the clinical significance of these findings.

Materials and methods

Patients

Thirty-one patients with CN who developed leukemia or MDS were analyzed. All patients had suffered from neutropenia and recurrent infections from birth and were subsequently started on treatment with G-CSF. They developed MDS or leukemia at 2 to 38 years of age. The mutation subtypes were as follows: *ELANE* (n = 18), *HAX1* (n = 6), *WAS* (n = 4), *ELANE* plus *GFI1* (n = 2), and *G6PT/SLC37A4* (n = 1). Two patients were negative for *ELANE*, *HAX1*, *G6PC3*, *GFI1*, and *WAS* mutations. We collected bone marrow or blood samples in association with annual follow-up as recommended by the Severe Chronic Neutropenia International Registry and French Severe Chronic Neutropenia Registry.[24] This study was performed with the informed consent of all subjects obtained through the respective institutional review boards and was approved by the Hannover Medical School Institutional Review Board. This study was conducted in accordance with the Declaration of Helsinki.

Pediatric AML samples were obtained from the Dutch Childhood Oncology Group (The Hague, The Netherlands), the AML-BFM Study Group (Hannover, Germany), as well as from the University Hospital in Prague (Czech Republic) and the St. Louis Hospital (Paris, France). Clinical and centrally reviewed cytogenetic and other cell-biological characteristics were made available by these cooperative groups/hospitals. All recurrent cytogenetic groups known in pediatric AML were represented among the 307 de novo pediatric AML patients (Table 2).

Mutation screening

Mutation analyses of the known leukemia-associated genes (*RUNX1*, *NPM1*, *FLT3-ITD*, *FLT3-TKD*, *CEBPA*, *NRAS*, *KRAS*, *CBL*, *TET2*, *IDH1*, *IDH2*, *DNMT3A*, *SUZ12*, *EP300*, and *CSF3R*) were performed using a sensitive next-generation amplicon deep-sequencing assay (454 Life Sciences, Branford, CT) or a SeqCap EZ library (Roche Nimblegen, Madison, WI) followed by sequencing on the Hisequation 2000 sequencing system (Illumina, San Diego, CA). Minimum coverage was at least 467-fold, and sequences were confirmed by ABI Sanger sequencing when supporting reads of mutated alleles were in excess of 20%. For sequential analyses of the time-course of occurrence of *RUNX1* and *CSF3R* mutations, mutated regions of *RUNX1* and *CSF3R* genes were amplified using polymerase chain reaction (PCR), and PCR products were sequenced using a SOLiD 5500XL ligation-based sequencing system or the SeqCap EZ library (Roche Nimblegen) followed by sequencing on the Hisequation 2000 system (Illumina). In the 302 de novo AML-samples, the protein coding sequence of the *RUNX1* gene (exon 3-8) (*RUNX1*-002, NM_001754.4) was PCR-amplified using specific primers and subsequently sequenced in forward and reverse direction. Purified PCR products were directly sequenced from both strands. Primer sequences are available upon request. The sequence data were analyzed using CLC Workbench, version 3.5.1 (CLC Bio, Aarhus, Denmark). In case of a suspected mutation, the fragment was re-amplified and sequenced in both directions.

All *RUNX1* mutated samples were also screened for *CSF3R* and *ELANE* mutations. Briefly, if cDNA was available, CSF3R cDNA was amplified and sequenced in forward and reverse direction. When only genomic DNA was available, *CSF3R* was amplified and sequenced to detect mutations and deletions around 2 previously reported *CSF3R* hotspot mutations. For *ELANE*, the protein coding exons (exons 1-5) were amplified and subsequently sequenced in forward and reverse direction on genomic DNA. Primer sequences are available upon request.

Results

High frequency of *RUNX1* gene mutations in CN patients who developed leukemia

We included 31 CN patients who developed MDS or leukemia in this study (Table 1) using materials from all of the patients available to us though our international collaborations. Twenty-one patients developed leukemia and 10 patients MDS. Of the leukemic patients, 16 patients had AML, 3 had AML after MDS, 1 had biphenotypic leukemia, and one had acute lymphoblastic leukemia (ALL). Of these 31 patients, 20 (64.5%) had heterozygous *RUNX1* mutations; these included missense mutations (n = 16), nonsense mutations (n = 5), frameshift mutations (n = 4), and mutations in the splice-acceptor site of intron 4 (n = 2) (Figure 1; Table 1). The most frequently affected position was Arg139, resulting in p.Arg139Gly, p.Arg139X (n = 2), and p.Arg139ProfsX47 (n = 4). Additionally, p.Arg64Pro, p.Arg80Ser, p.Lys83Gln, and Arg174 (p.Arg174X and p.Arg174Leu) mutations were identified, each in 2 patients. Of the 27 *RUNX1* mutations, 18 were localized within the RHD, 4 within or proximal to the TAD and 2 in splice-sites.

Simultaneous occurrence of 2 distinct heterozygous *RUNX1* mutations

In 8 patients, we detected 2 distinct heterozygous *RUNX1* mutations. In one patient, 2 insertion mutations were found at the splice-acceptor site of intron 4, predicted to affect the splicing of exons 3 and 4, which encode the RHD of *RUNX1*. In 3 patients, 2 mutations were present solely in the RHD or were present in both RHD and TAD (1 each). In 4 patients, 1 of 2 mutations was localized to the RHD. In 2 patients, we could perform allele-specific analysis of *RUNX1* mutations. Patient 10 incurred deletions leading to frame shifts on both alleles (p.Phe13TrpfsX14 and p.Arg139ProfsX47). In patient 14, 2 single missense substitutions were detected on the same allele; the first was inherited from the mother (p.Met240Ile) and is located 2 amino acids upstream of the TAD, and the second acquired mutation (p.Arg139Gly) is in the RHD of RUNX1 (supplemental Figure 1, available on the *Blood* Web site).

Table 1. Clinical and cytogenetic characteristics as well as types of mutations of CN patients who progressed to leukemia or MDS

Patient number	AML subtype	Karyotype	Inherited mutations	Acquired *RUNX1* mutations*	Acquired *CSF3R* mutations†	Acquired AML-associated mutations
6	AML M5	45,XX,-7	*ELANE* (L152P)	R80S	Q726X	
7	MDS/ AML M1	46,XY-7, +21	*ELANE* (S126L)	R135K	Q726P	
14	AML M1	45,XY,-7[9];46,XY[11] (2010) 47,XY +21[13];46,XY[2] (2011)	*ELANE* (C151Y)	R139G M240I	Q718X	
15	AML M1	t(p1;q3)	*ELANE* (C151Y)	R139X	Q731X	
16	AML M4	46,XY	*ELANE* (G214R)	R174X	Q720X	
18	AML FAB NA	46, XY, t(9;11)	*ELANE* (N113K)	R64P	Q718X	
22	AML M1	46,XY	*ELANE* (IVS4+1G>T)	K83Q	Q718X	
30	pre-B ALL	48,XX,del(5)(q21q34),þ21, þ 22(16)/46,XX[8]	*ELANE* (G185R)	A160T S114X	Q702X	
31	RAEBT/AML FAB NA	47,XY, +21 [14] /46, XY [4]	*ELANE* (G174R)	D171N	Q718X, Q726X	*SUZ12, EP300*‡
21	AML M2	47,XX +mar[8], 47, idem, del(10)(q32)	*ELANE* (G214R) *GFI1*	R174X L294QfsX6	Q739X	
4	AML M0	45,XX,-7[12];46,XX[11]	*WAS* (S478I)	Intron 4, c.415_427dup6 Intron 4, c.421_427dup7	Q707L	*SUZ12 (*S154X) *EP300* (R2263X)
20	MDS RAEB	46,XX,add(2)(q37),add(7)(q22)	*WAS*	Q370X	Y729X	*CBL* (splice site c.1096-1G>C (Intron 7)
26	AML FAB NA	45,XY,-7	*WAS* (L270P)	R80S	Y729X	*CREBBP* (I2329M)
10	MDS RAEB	45,XY −7 [10], 46XY [5]	*HAX1* (V44X)	F13TrpfsX14 R139ProfsX47	Q726P	*FLT3-ITD*
19	MDS	46,XX	*HAX1* (V44X)	L29S, R64P	Y729X	
12	AML M2	47,XX,+21	*GPT1*	K83Q	Q720X	
25	MDS RAEB-2	46,XX,dup(21)(q22.1q22.3)[19]	*Neg*	S114P Y380_G394delinsC	Q726X	
11	AML/B-ALL	46,XY,add(21q)	*ELANE* (A57V)	R174L	neg	
13	AML FAB NA	46,XX	*ELANE* (A79VfsX9)	R139X V137D	neg	*FLT3-ITD*
17	MDS	46,XY	*HAX1* (V44X)	I22K	neg	*EP300* (C369F)
1	AML M2/M4	47,XY,+ 8	*ELANE* (D230MfsX1)	Neg	Q716X, Q726X	
9	AML M2	46,XX, del 7q [9], 46XX [1]	*ELANE* (S126X)	Neg	Q731X	
24	AML M5	5Q-deletion, a translocation of chr. I and 21	*ELANE* (Y228X)	Neg	Y729X	
27	MDS/AML FAB NA	47,XY, −7, +21, +21 [9]/46, XY [5]	*ELANE* (L92P)	Neg	Q718X	
5	MDS RAEB-T	46.XY	*HAX1* (V44X)	Neg	Q726X	
3	MDS RAEB	45,XX,-7,del(18)(q22) [11/45],idem,der (6)t(3,6)(q13;p24) [2]/ 45,XX,-7, del(13)(q13q33) [2]	*WAS* (I331M)	Neg	Q716X	*NRAS*§

NA, not available; pre-B ALL, pre-B acute lymphoblastic leukemia.

*For all patients except patient 17 amino acid positions according to the *RUNX1* transcript variant Q01196 (www.uniprot.org) was used; for patient 17 amino acid positions according to the *RUNX1* transcript variant Q2TAM6 (www.uniprot.org) was used.

†Amino acid positions were assigned as has been reported by Dong et al[10] or by UniProtKB Q99062 minus 23 amino acids of signal peptide.

‡See Beekman et al.[25]

§N-RAS mutations were measured according Nakao M. et al.[26]

Association between *RUNX1* mutations, clinical characteristics, and cytogenetics in CN patients who developed leukemia or MDS

No correlation was seen between age at progression to leukemia and *RUNX1* mutations (13.8 years in *RUNX1* mutated vs 10.2 years in *RUNX1* wild-type, (WT) groups). No gender correlation was found; 10 male and 10 female patients acquired *RUNX1* mutations, as opposed to 5 males and 6 females without *RUNX1* mutations. Among 20 patients carrying mutated *RUNX1*, one developed AML FAB M0, 4 developed M1, 2 developed M2, 1 developed M4, and 1 developed M5. A cytogenetic analysis revealed that *RUNX1* mutations were associated with monosomsy 7 in 6 patients (30%), with trisomy 21 in 6 patients (30%), and with monosomy 7/trisomy 21 in 2 patients (Table 1).

Distribution of *RUNX1* mutations based on CN-specific germline mutations

Of the 20 patients with *RUNX1* mutations, 19 were positive for known CN-causing mutations: *ELANE* (n = 12 patients, 66%), *WAS* (n = 3, 75%), *HAX1* (n = 3, 50%), both *ELANE* and *GFI1* (n = 1), and *G6PT/SLC37A4* (n = 1). Conversely, 5 of 17 patients harboring *ELANE* mutations, 3 of 6 patients with *HAX1* mutations, and 1 of 4 patients with *WAS* mutations tested negative for *RUNX1* mutations (Table 1; Figure 2A).

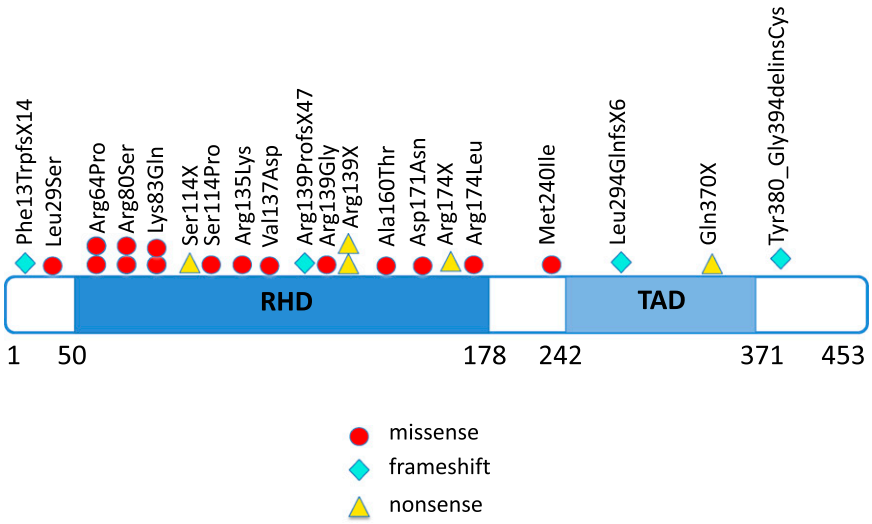

Figure 1. Localization of *RUNX1* mutations in CN patients who developed leukemia or MDS. The position of *RUNX1* mutations found in CN patients, with affected amino acids numbered. Amino acid positions correspond to the *RUNX1* transcript variant Q01196 (www.uniprot.org). Locations of the functionally important RHD and TAD are shown. Each symbol represents 1 patient.

High frequency of cooperating *RUNX1* and *CSF3R* mutations in CN patients who developed leukemia or MDS

To evaluate possible cooperating effects of "leukemogenic" gene mutations in combination with *RUNX1* mutations, we performed targeted deep-sequencing of candidate genes known to be mutated in de novo AML, namely *NPM1*, *FLT3*, *CEBPA*, *NRAS*, *KRAS*, *CBL*, *TET2*, *IDH1*, *IDH2*, *DNMT3A*, *SUZ12*, *EP300*, and *CSF3R*. In 19 of 20 patients, we detected cooperating *RUNX1* mutations. Intriguingly, 17 of 20 patients (80.5%) exhibited both *RUNX1* and *CSF3R* mutations (Figure 2B), 3 had both a *RUNX1* and an *EP300* mutation, 2 had a *RUNX1* mutation and *FLT3*, and 1 had a *RUNX1* and a *CBL* mutation. Six patients with acquired *CSF3R* mutations had WT *RUNX1*. One patient with unaffected *RUNX1*, *ELANE*, or *CSF3R* had an activating *NRAS* mutation in codon 61. This may represent a unique type of CN/MDS and could suggest that *NRAS* and *CSF3R*, or *RUNX1*, mutations are mutually exclusive (Table 1). No mutations were found in *CEBPA*, *DNMT3A*, *IDH1*, *IDH2*, *NPM1*, or *TET2*. All *CSF3R* mutations were C-terminal truncated mutations, which are localized between amino acids 680 and 780.

Intriguingly, all 6 patients who were negative for *RUNX1* and *CSF3R* mutations developed MDS only, whereas in the group of patients with cooperative *RUNX1* and *CSF3R* mutations, 11 developed AML and only 6 MDS/AML.

Molecular karyotyping by array-CGH (comparative genomic hybridization)

To elucidate the underlying molecular mechanisms of cancer susceptibility and progression in secondary acute leukemia or MDS, we conducted a comprehensive genome-wide characterization of genomic aberrations in the transformed cells from several patients with inherited bone marrow syndromes. When available, samples at different time points of disease progression were analyzed. Thirty-one CN patients were analyzed. A summary of the genomic aberrations identified by array-CGH is given in supplemental Table 1. Large genomic alterations, namely, monosomy 7/-7q, +21q, or +3q, were associated with leukemia. Apart from common copy number variants, such as *UGT2B*, *GSTT1*, and *HEATR4*, no microdeletions or microduplications were detected in primary or secondary diseases.

RUNX1 mutations in de novo pediatric AML

To evaluate whether de novo pediatric AML also has high frequency of *RUNX1* mutations, we sequenced *RUNX1* in de novo pediatric AML samples. We detected *RUNX1* mutations in 9 of 307 (2.9%) pediatric patients with de novo AML (Table 2): one deletion, 4 insertions, and 4 missense mutations (single-nucleotide substitutions) for which single-nucleotide polymorphisms have not been described. We found 6 N-terminal *RUNX1* mutations, 1 C-terminal

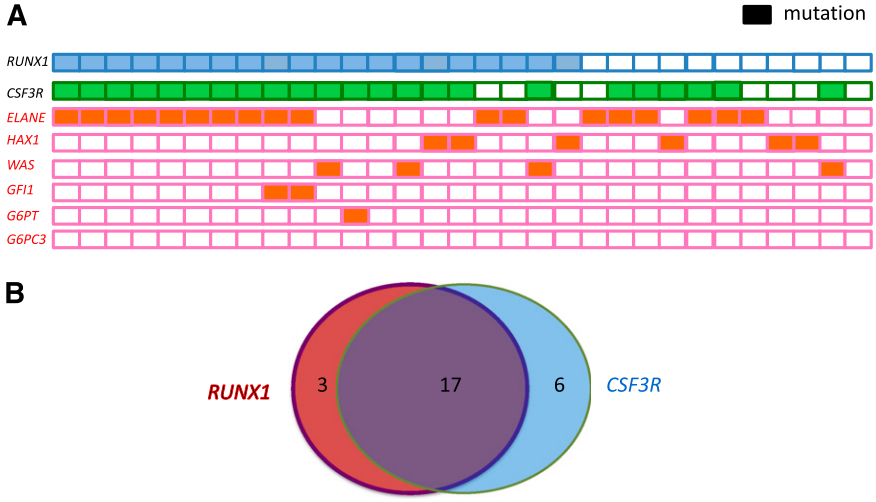

Figure 2. Frequency and distribution of acquired *RUNX1* and *CSF3R* mutations and inherited *ELANE*, *WAS*, *GFI1*, *GPT*, and *HAX1* mutations within a cohort of CN patients who developed leukemia or MDS. (A) Each patient with a *RUNX1* mutation is represented by a blue rectangle, and each patient with a *CSF3R* mutation is represented by a green rectangle. Patients with inherited mutations are represented by orange rectangles. Open rectangles correspond to patients without mutations. (B) Venn diagram illustrating the relationship among *RUNX1* and *CSF3R* mutations in CN patients who developed leukemia (n = 31). Diameters of each circle are roughly proportional to the number of mutations.

Table 2. Characteristics of pediatric AML patients with *RUNX1* aberrations

	Gender	Age at dx (y)	FAB-type	Karyotype sample	Other aberrations	*RUNX1* mutation	Effect	RUNX1 protein domain	Event1	Time dx-event1 (mo)	Time dx-death (mo)	Cause of death
1	Male	5.0	M1	45,XY,-7[13]/45,idem, der(18)t(8,18)(q21; q22)[2]	—	c.179 ins GG	FS	NRDBn/ NRHn	Nonremitter	0.0	Alive	—
2	Male	2.6	M5	46,XY,t(16,21)(p11; q22)/ 46,idem,i(22) (q10)/ 46,XY	—	c.292delC	FS	RUNT	Relapse	15.8	26.3	Leukemia
3	Male	12.3	M3	46,XY[17]	*FLT3*-ITD & WT1	c.328 A>C	MS	RUNT	Nonremitter	0.0	15.1	Infection
4	Male	3.9	M1	46,XY, aberrant (pseudodiploid)[9] ISH:MLL&inv(16) notaberrant	*NRAS*	c.424_425insGG	FS	RUNT	Relapse	11.4	14.9	Leukemia
5	Male	13.8	M1	46,XY,add(8)(q22), add(16)(p13.1)[3]/ 46, XY,add(8)(q22)[13]	—	c.424_425insGGG	IF	RUNT	Relapse	2.3	7.0	Leukemia
6	Female	14.1	M4	46,XX,-7[21]	*NRAS*	c.497G>A	MS	RUNT	Relapse	25.3	43.3	Toxic, neurological syndrome
7	Female	3.9	M1	46,XX,del(5)(q31q34), del(16)(?q22)[1]/ 46, XX[4]	—	c.507_508ins11	FS	RUNT	Nonremitter	0.0	3.2	Leukemia
8	Female	15.4	M1	46,XX	*NPM1*	c.1085 C>T	MS	TAD	Early death	0.0	0.0	Leukemia
9	Male	2.1	M7	49,XY,+16,del(17) (p11),+19,+21,+21,-22[5]/ 46,XY [22]	—	c.1190 A>G	MS	TID	None	—	Alive	—

dx, diagnosis.

mutation, and 2 mutations outside the RHD and TAD. No co-occurrence of *RUNX1* mutations with *CSF3R* or *ELANE* mutations was found in these patients (Figure 3).

The median age was the same for patients with mutated and wild-type *RUNX1*. The frequency of immature phenotype (FAB M1) was higher in patients with mutated *RUNX1* (56%) than in patients with WT *RUNX1* (10%) (Fischer's exact test $P = .0019$). Interestingly, mutations were not found in patients without *MLL*-rearrangements, t,[8,21] inv,[16] t[7,12] or t,[15,17] whereas concomitant mutations in *FLT3*-ITD, *NRAS*, *NPM1*, and *WT1* were found in 5 of 9 patients (supplemental Figure 2).

RUNX1 mutations were mainly found in pediatric AML patients with an adverse prognosis. Although 4 patients received allogeneic stem cell therapy, none survived. Further, we did not find a strong gene expression signature, suggestive of specific driver alterations, in *RUNX1*-mutated pediatric AMLs (data not shown).

RUNX1 mutations are late events in the leukemic transformation of CN

To evaluate the time points and sequence of acquisition of *RUNX1* and *CSF3R* mutations, we performed a consecutive analysis of both mutations in 10 CN/AML patients. DNA from bone marrow cells collected at different time points prior to leukemia was analyzed. In 6 of 10 patients, a *CSF3R* mutation occurred prior to *RUNX1* mutations (Figure 4A and supplemental Figure 3), consistent with previous data demonstrating that acquisition of *CSF3R* mutations is an early event in the leukemic transformation in CN.[11,12] In 2 patients, both *RUNX1* and *CSF3R* mutations were detected in the earliest available DNA samples prior to leukemia/MDS. Two patients had no *CSF3R* mutations and acquired *RUNX1* mutations 2 months prior to AML or at the time point of AML progression. Interestingly, monosomy 7 or trisomy 21 appeared after acquisition of *RUNX1* mutations.

The time course of mutational events in patient 14 is shown in Figure 4A. The transient appearance of cell clones with different *CSF3R* mutations was detected as early as 8 years prior to leukemia. Three different *CSF3R* mutations (p.Q720X, p.Q726X, and p.Q731X) were detected at the age of 12 years (8 years prior to leukemia). All 3 clones fell below the detection level within 2-3 years, and one clone (p.Q731X) reappeared for a short period of time 3 years prior to leukemia. One additional clone with a p.Q718X mutation was detected 5 years prior to leukemia at the age of 15 years. Intriguingly, this clone acquired an additional *RUNX1* mutation 2 years prior to leukemia. The mutations in *RUNX1* and *CSF3R* preceded monosomy 7, which was observed nine months prior to leukemia. After acquisition of monosomy 7, treatment with G-CSF was discontinued and, intriguingly, the clone with mutated *RUNX1* and *CSF3R* was no longer detectable. Because of the life-threatening infection status of the patient, G-CSF therapy was restarted, which was rapidly followed by expansion of the cell clone with *RUNX1* and *CSF3R* mutations, an additional trisomy 21, and a bone marrow morphology that revealed overt AML.

To evaluate whether *RUNX1* and *CSF3R* mutations were present in the same transformed cell clone or whether 2 different clones carried *RUNX1* and *CSF3R* mutations, we performed colony-forming assays using leukemia blasts and $CD34^+/CD33^+$ bone marrow cells isolated 7 years prior to leukemia development of one CN/AML patient. We also isolated DNA from single colonies and analyzed samples for the presence of *RUNX1* and *CSF3R* mutations by Sanger sequencing. Both *RUNX1* and *CSF3R* p.Q718X mutations were detected in 43 of 48 leukemic colonies, whereas no mutations were found in cells isolated 7 years prior leukemia (Figure 4B).

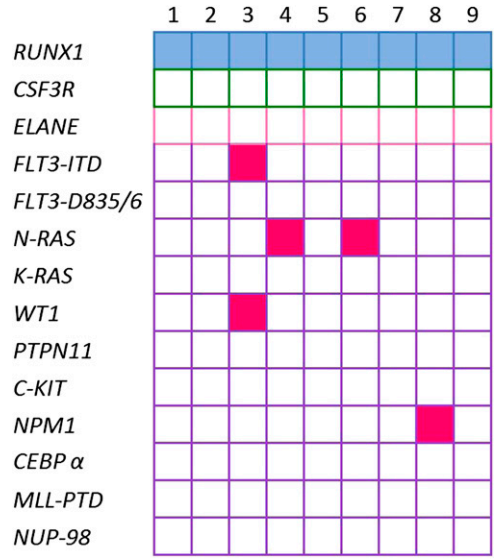

Figure 3. Mutational status of leukemia in de novo pediatric AML patients with *RUNX1* aberrations. Identified gene mutations in 9 de novo pediatric AML patients with *RUNX1* aberrations; each mutation is depicted by a blue rectangle. Patients with inherited mutations are represented by pink rectangles. Open rectangles correspond to patients without mutations.

Analysis of familial CN/AML cases

A mutation analysis of *RUNX1* was also performed in a family in which 2 siblings with *ELANE-CN* developed AML M1. The father was mosaic for the *ELANE* mutation and had a mild, asymptomatic neutropenia. One of the affected siblings had a germline *RUNX1* variation (p.Met240Ile) that was inherited from the healthy mother and which has not been previously reported as a common single-nucleotide polymorphism. A healthy sister also carried this p.Met240Ile *RUNX1* alteration. Both affected siblings had acquired distinct *RUNX1* mutations in Arg139: a p.Arg139Gly missense mutation in the brother and a p.Arg139X nonsense mutation in the sister. Both affected siblings also had *CSF3R* nonsense mutations: p.Q718X and p.Q731X in the brother and sister, respectively. None of the healthy siblings had acquired *RUNX1* or *CSF3R* mutations, or inherited *ELANE* mutations (Figure 5).

Enhanced proliferation of CD34$^+$ cells cotransduced with mutated *RUNX1* and mutated *CSF3R*

To test the effects of coexpression of *RUNX1* and *CSF3R* mutations, we cotransduced CD34$^+$ hematopoietic cells with cDNAs encoding the truncated CSF3R mutant (d715) or RUNX1 variants carrying missense mutations within the RHD (Arg135Gly or Arg139Gly). We treated transduced cells with G-CSF and evaluated proliferation and myeloid differentiation. Proliferation of cells expressing with the CSF3R mutant in combination with either RUNX1 Arg135Gly or RUNX1 Arg139Gly mutants was elevated relative to cells transduced with control vectors, the *CSF3R* mutant alone, or *RUNX1* mutants alone (Figure 6A). In parallel, we observed diminished myeloid differentiation of cells transduced with mutated *CSF3R* and *RUNX1*, as assessed by surface expression of the myeloid-specific surface markers, CD11b, CD15 and CD16 (Figure 6B; supplemental Figure 4).

Discussion

CN is a preleukemic bone marrow failure syndrome with a high risk of progression to AML or MDS.[9] Rare cases of ALL associated with CN have also been described.[27] The genetic changes involved in the evolution of CN to leukemia are still largely unknown. The frequency of acquired *CSF3R* mutations in CN/AML patients is ~80%, substantially higher than that in patients who have not yet progressed to leukemia (~20%), suggesting that *CSF3R* mutations are drivers of leukemogenesis.[10,11] Previous studies have demonstrated a clonogenic proliferative advantage of hematopoietic cells carrying a truncated *CSF3R*.[40,41] However, *CSF3R* mutations alone are not sufficient for leukemia development, and additional genetic events are required.[28-30] Moreover, the first case of leukemia in a CN patient was described in 1969, many years before G-CSF was used for the treatment of CN.[31,32] This suggests that G-CSF treatment is not causative for leukemic transformation.

Here, we provide the first report of the high frequency of *RUNX1* mutations in CN/AML patients. It is important to mention that there is no other clinical entity with this high frequency of RUNX1 mutations. In contrast, we found that *RUNX1* mutations are a rare

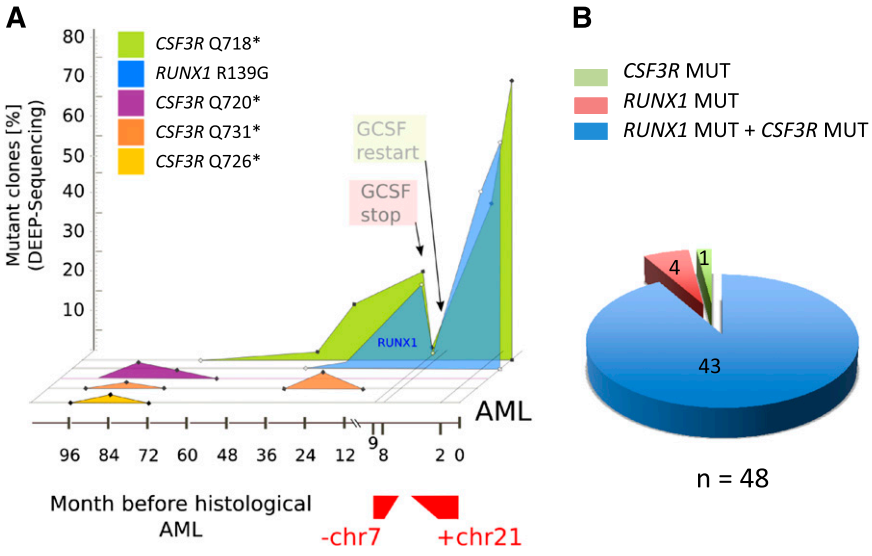

Figure 4. Cooccurrence of *RUNX1* and *CSF3R* mutations in combination with monosomy 7 and trisomy 21 in a leukemic clone of a CN/AML patient. (A) Graphic presentation of a mutational analysis of deep-sequencing data for *RUNX1* and *CSF3R* genes in CN/AML patient 14. Results of sequencing DNA samples from different time points prior to overt AML (x-axis) and the percentage of mutant clones (y-axis) are presented. (B) Diagram of the distribution of *RUNX1* and *CSF3R* mutations in DNA isolated from single clones (n = 48) of AML samples from CN patient 14.

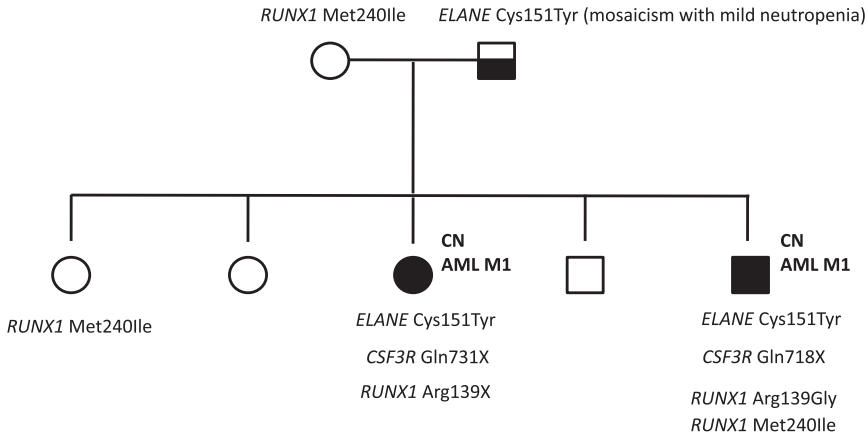

Figure 5. Mutation analysis of a CN/AML pedigree. Squares indicate males and circles indicate females. Open symbols represent unaffected persons; the half-filled square represent the father with a mosaicism for *ELANE* mutation and mild neutropenia; closed symbols represent 2 persons affected by CN, both of whom also developed AML-M1. Inherited mutations in *ELANE* (p.Cys151Tyr) and *RUNX1* (p.Met240Ile) as well as acquired *RUNX1* (p.Arg139Gly; p.Arg139*) and *CSF3R* (p.Gln718X; p.Gln731*) mutations are indicated for each affected person.

event in de novo childhood AML (frequency <3%) and are not associated with the acquisition of *CSF3R* mutations. Interestingly, *RUNX1* mutations were found in pediatric AML patients mutually exclusive of MLL-rearrangements, t(8,21), inv(16), t(7,12), or t(15,17) whereas concomitant mutations in the *FLT3*, *NRAS*, *NPM1*, and *WT1* genes were found in 5 of 9 patients. Intriguingly, patients with CN associated with glycogen storage disease type Ib, or those harboring *ELANE*, *HAX1*, *WAS*, or *GFI1* mutations who developed secondary leukemia, acquired *RUNX1* mutations. This observation demonstrates that RUNX1 is a crucial driver of leukemogenesis, independent of the underlying diverse inherited aberrations. The vast majority of *RUNX1* mutations in CN/AML and de novo AML patients impair RUNX1 function (loss of DNA binding or transactivation capacity) and would be predicted to have dominant negative effects or to generate a null allele (no protein or extremely truncated protein).[33-37]

The high frequency of combined RUNX1 and CSF3R mutations and the presence of both mutations in the same leukemic cell clones clearly suggest that these 2 mutations cooperate as drivers in leukemogenesis, Our hypothesis is that the initial driver mutations are the *CSF3R* mutations and cell clones harboring *CSF3R* mutations having a growth advantage acquire in the majority of patients *RUNX1* mutations that contribute to AML initiation and disease progression.

In contrast, the association between *RUNX1* and *CSF3R* mutations was not found in de novo pediatric AML, suggesting a distinct and specific mechanism in CN. Only 2 patients with a *RUNX1* mutation had no *CSF3R* mutation; conversely, 6 patients with a *CSF3R* mutation had no *RUNX1* mutations. Intriguingly, 5 patients who were negative for *CSF3R*, and *RUNX1* mutations had MDS only and no overt AML, suggesting that *CSF3R/RUNX1* mutations are rather a feature of AML and less frequent of MDS. There was no evidence from a copy number analysis that the chromosomal region 21q22.1 containing *RUNX1* was lost in these latter 6 cases, but the loss of *RUNX1* expression (eg, through DNA or histone methylation) cannot be excluded. CN patients require lifelong treatment with high therapeutic doses of G-CSF.[1] Most of the *CSF3R* mutations described in CN patients lead to a truncated CSF3R protein lacking the intracellular domain responsible for the termination of proliferative signals.[10-12,38,39] Moreover, we demonstrated the transitory appearance of clones carrying different *CSF3R* mutations in CN patients many years prior to AML (Figure 4A). These data are in line with our previously published findings showing more than one mutation in *CSF3R* in a CN patient who developed AML.[42] Our data suggest that as soon as a cell clone carrying a *CSF3R* mutation is hit by an additional mutation in *RUNX1*, the inevitable fate of this clone is transformation into leukemic cells. It has been reported that a *RUNX1* loss-of-function mutation per se is not sufficient to cause leukemia but does block myeloid differentiation.[43] Our patient data taken together with in vitro studies strongly support the hypothesis that, by markedly elevating proliferation and diminishing myeloid differentiation of hematopoietic cells, *CSF3R* and *RUNX1* mutations are the major drivers of leukemogenesis in patients carrying both mutated proteins.

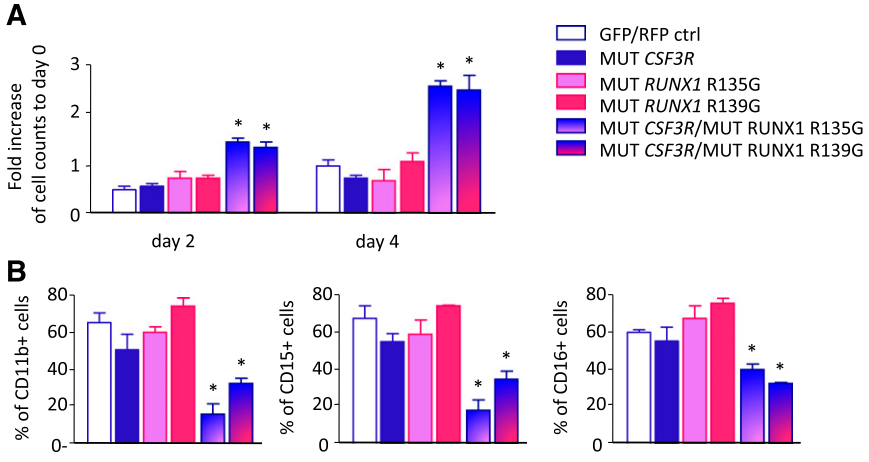

Figure 6. Enhanced proliferation and diminished myeloid differentiation of hematopoietic cells. $CD34^+$ bone marrow cells from healthy individuals were transduced with lentivirus-based green fluorescent protein (GFP)-tagged *RUNX1* mutants (p.Arg135Gly or p.Arg139Gly) or red fluorescent protein (RFP)-tagged *CSF3R* mutant (d715) alone, cotransduced with *RUNX1* and *CSF3R* mutants, or cotransduced with control GFP- and RFP-tagged lentiviral constructs. Transduced cells were treated with 10 ng/mL of G-CSF. (A) Cell number was evaluated on days 2 and 4 of culture by estimation of RFP^+/GFP^+ cells using fluorescence-activated cell sorter. (B) G-CSF–triggered myeloid differentiation was evaluated on day 8 of culture. Data represent means ± standard deviations and are derived from 2 independent experiments, each in triplicate (*$P < .05$).

The importance of *RUNX1* mutations in leukemic transformation was substantially strengthened by the analysis of a unique family with 2 siblings suffering from CN that subsequently transformed into AML. In both children, cooperating *RUNX1* and *CSF3R* mutations were detected that were not present in healthy family members. Inherited *RUNX1* mutations have been described in familial platelet disorder-AML, another inherited preleukemic bone marrow failure syndrome with a high propensity to develop into AML.[22,23] Cryptic *RUNX1* lesions (translocations, deletions, or mutations) have also been observed in Fanconi anemia patients who developed AML or MDS.[44] These data, together with our observations, suggest that *RUNX1* mutations could represent an important common event in triggering secondary leukemia in patients with bone marrow failure syndromes.

In conclusion, our findings may improve monitoring of the possible leukemic transformation in CN patients in which *CSF3R* mutations are already present. We recommend yearly bone marrow examinations. Patients with both *RUNX1* and *CSF3R* mutations appear to be at high risk for rapid evolution to leukemia and should be considered candidates for stem cell transplantation.

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Authorship

Contribution: J.S. and D.S. collected and analyzed data, supervised experiments, and wrote the manuscript; J.S. also provided financial support; J.E.K.-K., J.O., C.M.Z., D.R., and M.M.v.d.H.-E. collected and analyzed data on de novo pediatric AML patients and wrote the part of the manuscript describing de novo AML patients data; C.Z. provided patient materials; O.K. performed in vitro experiments with $CD34^+$ cells; M.K. performed DNA sample preparation, analysis of sequenced data, sequencing of single CFU clones, and allele-specific PCR; M.Ü. and S.K. analyzed deep-seq data of CSF3R; R.B., K.B., and C.S. made lentiviral constructs with WT and MUT RUNX1; S.S. and A.K. performed deep-seq of RUNX1 and other leukemia-associated mutations and wrote manuscript; M.G.V., R.H., and I.P.T. performed RUNX1 and CSF3R analysis of some CN/AML patients; I.P.T. wrote the manuscript; V.M., D.C.D., P.V., and J.D. provided some patient materials; D.C.D. wrote the manuscript; G.G. and B.S. provided some patient materials and cytogenetic analysis; M.S. wrote the manuscript; and K.W. provided patients material, supervised experiments, analyzed data, wrote the manuscript, and provided financial support.

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