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Brief report

Leukemic transformation in mice expressing a *NUP98-HOXD13* transgene is accompanied by spontaneous mutations in *Nras*, *Kras*, and *Cbl*

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The *NUP98-HOXD13* (*NHD13*) fusion gene occurs in patients with myelodysplastic syndrome (MDS) and acute nonlymphocytic leukemia (ANLL). We reported that transgenic mice expressing *NHD13* develop MDS, and that more than half of these mice eventually progress to acute leukemia. The latency period suggests a requirement for at least 1 complementary event before leukemic transformation. We conducted a candidate gene search for complementary events focused on genes that are frequently mutated in human myeloid leukemia. We investigated 22 ANLL samples and found a high frequency of *Nras* and *Kras* mutations, an absence of *Npm1*, *p53*, *Runx1*, *Kit* and *Flt3* mutations, and a single *Cbl* mutation. Our findings support a working hypothesis that predicts that ANLL cases have one mutation which inhibits differentiation, and a complementary mutation which enhances proliferation or inhibit apoptosis. In addition, we provide the first evidence for spontaneous collaborating mutations in a genetically engineered mouse model of ANLL. (Blood. 2008;112: 2017-2019)

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Introduction

We previously reported a transgenic mouse model for myelodysplastic syndrome (MDS), in which *NUP98-HOXD13* (*NHD13*) mice develop MDS at an early age, and progress to acute leukemia between 4 and 14 months of age.[1] This latency period is likely due to a requirement for additional genetic events before leukemic transformation. Many studies have investigated the nature of such secondary events through experimental induction of complementary events, such as retroviral insertional mutagenesis[2-4] or ENU-induced mutagenesis.[5,6] To our knowledge, no study has investigated the nature of complementary mutations that occur spontaneously. Therefore, we evaluated *NHD13* ANLL samples for the presence of mutations commonly seen in patients with ANLL.

Methods

DNA and RNA isolation

All animal experiments were conducted with the approval of the NIH Intramural Animal Care and Use Committee. Peripheral blood complete blood counts were obtained, bone marrow was harvested for cytospins, and paraffin-embedded spleen and liver were stained with hematoxylin and eosin. Routine immunohistochemical stains included F4/80, CD3, B220, and myeloperoxidase (MPO), and ANLL diagnosis was based on the Bethesda proposals for hematopoietic neoplasms in mice.[7,8] Effaced spleen tissue from *NHD13* mice with acute leukemia was snap frozen on dry ice. DNA and RNA were prepared by standard techniques.

RT-PCR and PCR

Reverse transcription (RT) was performed using Superscript II (Invitrogen, Carlsbad, CA). Genomic- and RT–polymerase chain reaction (PCR) were performed using either Supermix (Invitrogen) or Taq DNA Polymerase (Invitrogen). Primers, thermal cycling profiles, and regions amplified are listed in Tables S1 and S2 (available on the *Blood* website; see the Supplemental Materials link at the top of the online article). PCR products were purified using Qiagen (Valencia, CA) protocols, and were directly sequenced (Retrogen, San Diego, CA). Sequence chromatograms were manually inspected to detect mutations (Figure S1).

Reference sequences (NCBI accession numbers[9]) used were as follows: c-Cbl: NM_007619.2; Flt3: NM_010229.2; Kit: NM_021099.2; Kras: NM_021284.4; Nras: NM_010937.2; Npm1: NM_002520.5; Runx1: NM_001111021.1; p53: NM_011640.1.

Transfection

Wild-type and mutant *Nras* cDNAs were generated by RT-PCR using the Expand Long Template PCR System (Roche, Basel, Switzerland) and primers described in Table S1. Products were cloned into an *EF1a* expression vector and plasmids containing either wild-type or mutant *Nras* were used for transfection. Cells were transfected with Lipofectamine 2000 (Invitrogen) and selected with 500 μg/mL G418 (Invitrogen) before withdrawal of IL-3.

Results and discussion

p53 is mutated in approximately 50% of all cancers.[10] In ANLL patients however, *p53* mutations are less common, occurring in approximately 10% of cases.[11] We used an RT-PCR strategy to screen this gene in a cohort of 22 mice with ANLL, and obtained a PCR product from 18 mice. Sequencing of these products detected no mutations (Table 1).

We next searched for mutations of the *Npm1* gene, which is mutated in 32% of human ANLLs,[12] in 22 ANLL samples from *NHD13* mice. These mutations cluster in exon 12 and typically occur as 4 base-pair insertions producing a frame shift. We

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Table 1. Mutations in *NHD13* acute leukemias

				Genes							
Mouse	Strain	Age, mo	Diagnosis	*Npml*	*p53*	*Runx1*	*Cbl*	*Flt3*	*Kit*	*K-ras*	*N-Ras*
63	C57BL6	11	AML with maturation	GL	GL	GL	GL	GL	GL	GL	GL
251	C57BL6	9	AML with maturation	GL	GL	ND	GL	GL	GL	GL	codon 12 GGT>GAT (G>D)
254	C57BL6	11	AML with maturation	GL	GL	GL	GL	GL	GL	GL	GL
1135	C57BL6	12	AML with maturation	GL	GL	GL	GL	GL	GL	codon 12 GGT>GAT (G>D)	GL
2434	C57BL6	13	AML with maturation	GL	GL	GL	GL	GL	GL	GL	codon 12 GGT>AGT (G>S)
2437	C57BL6	13	AML with maturation	GL	ND	GL	GL	GL	GL	GL	GL
2554	C57BL6	12	AML with maturation	GL	ND	GL	GL	GL	GL	GL	GL
1079	C57BL6	10	AML with maturation	GL	GL	GL	GL	GL	GL	GL	GL
1080	C57BL6	10	AML with maturation	GL	ND	GL	297 bp deletion	GL	GL	GL	GL
1134	C57BL6	10	AML without maturation	GL	GL	GL	GL	GL	GL	GL	GL
1155	C57BL6	10	AML without maturation	GL	GL	GL	GL	GL	GL	GL	GL
2413	C57BL6	8	AML without maturation	GL	GL	GL	GL	GL	GL	GL	GL
2417	C57BL6	9	AML without maturation	GL	GL	GL	GL	GL	GL	codon 12 GGT>GAT (G>D)	GL
2420	C57BL6	14	AML without maturation	GL	GL	GL	GL	GL	GL	GL	GL
1892	C57BL6	11	MPD-like leukemia	GL	GL	GL	GL	GL	GL	GL	codon 12 GGT>GAT (G>D)
2590	C57BL6	5	MPD-like leukemia	GL	ND	GL	GL	GL	GL	GL	GL
2679	C57BL6	6	MPD-like leukemia	GL	GL	GL	GL	GL	GL	GL	GL
1017	FVB	9	AMKL	GL	GL	GL	GL	GL	GL	codon 12 GGT>GTT (G>V)	GL
1668	FVB	10	AML with maturation	GL	GL	GL	GL	GL	GL	GL	GL
4875	FVB	15	AML with maturation	GL	GL	GL	GL	GL	GL	GL	GL
1019	FVB	14	AML with maturation	GL	GL	GL	GL	GL	GL	codon 12 GGT>GTT (G>V)	GL
1018	FVB	14	Erythroid leukemia (spleen)	GL	GL	GL	GL	GL	GL	GL	GL
1138	C57BL6	8	Biphenotypic pre-T LBL/AML	ND	ND	ND	ND	ND	ND	GL	GL
2423	C57BL6	8	Biphenotypic pre-T LBL/AML	ND	ND	ND	ND	ND	ND	GL	GL
2553	C57BL6	10	pre-T LBL	ND	ND	ND	ND	ND	ND	GL	GL
2410	C57BL6	11	pre-T LBL	ND	ND	ND	ND	ND	ND	GL	GL
1075	FVB	8	pre-T LBL	ND	ND	ND	ND	ND	ND	GL	GL
1901	FVB	10	pre-T LBL	ND	ND	ND	ND	ND	ND	GL	codon 61 CAA>AAA (Q>K)
2738	FVB	7	pre-T LBL	ND	ND	ND	ND	ND	ND	GL	GL
2918	FVB	6	pre-T LBL	ND	ND	ND	ND	ND	ND	GL	GL
8013	FVB	9	pre-T LBL	ND	ND	ND	ND	ND	ND	GL	GL
1018	FVB	14	pre-T LBL (thymus)	ND	ND	ND	ND	ND	ND	codon 12 GGT>GAT (G>D)	GL

MPD-like leukemia indicates myeloproliferative disease-like leukemia; AMKL, acute megakaryocytic leukemia; GL, germline; and ND, not done.

amplified and sequenced exon 12 of the *Npm1* gene; no mutations were detected (Table 1). *Runx1* encodes a hematopoietic transcription factor mutated in 11% of ANLL patients.[12] We used RT-PCR to screen the *NHD13* samples, and obtained a PCR product from 21 mice. No mutations were detected (Table 1).

Flt3 and *Kit* encode receptor tyrosine kinases,[13] that are mutated at frequencies of 24% and 5% respectively in unselected ANLL cases,[12] and in 13% and 2% of ANLL cases that evolved from MDS.[14] We screened the juxtamembrane and kinase domain regions, where mutations are known to occur (*Flt3* exons 14, 15, and 20 and *Kit* exons 8 and 17), and found no *Flt3* or *Kit* mutations (Table 1). The "2-class" model for myeloid leukemia predicts that one class I (proliferation or survival promoting) and one class II (differentiation blocking) event are required for leukemic transformation.[15] This model would predict that *NHD13* expression and activating mutations of either *Flt3* or *Kit* would collaborate, and, given the frequent mutations of these genes in ANLL cases, we expected to find mutations of these genes among our mouse cohort. It may be that these genes are not prone to mutations in mice, or, alternatively, they do not complement the *NHD13* transgene.

We next investigated the *Nras* and *Kras* genes, which encode signaling molecules downstream of receptor tyrosine kinases.[16] These genes are mutated in 16% and 4% of ANLL cases, respectively[12]; the mutations are single nucleotide missense mutations in codons 12, 13, or 61 and result in a constitutively active RAS protein.[16] We amplified and sequenced exons 2 and 3 of these genes and identified 3 (14%) *Nras* and 4 (18%) *Kras* mutations. All 7 of these *ras* mutations occurred within codon 12 and resulted in a single amino acid substitution which is predicted to encode a constitutively active NRAS or KRAS protein. Similar to findings in patients with ANLL,[17] we noted no clear correlation between leukemic subtype and the presence of *NRAS* or *KRAS* mutations. Because of reports that indicate *NRAS* or *KRAS* mutations can be found in patients with MDS (most commonly patients with refractory anemia with excess blasts),[14] we assayed 12 *NHD13* mice with MDS for *Nras* or *Kras* mutations. No *Nras* or *Kras* mutations were identified in these mice, a statistically significant difference between the MDS and ANLL groups (7/22 vs 0/12; $P = .036$ by Fisher exact test), suggesting that the *ras* mutations occurred as leukemia progression events. Finally, because *ras* mutations have also been associated with pre-T lymphoblastic leukemia/lymphoma (pre-T LBL), we studied 10 *NHD13* mice with pre-T LBL and identified one *Nras* and one *Kras* mutation (Table 1).

We previously reported the derivation of an IL-3–dependent cell line from embryonic stem cells that expressed an *NHD13* "knock-in" allele.[18] To examine the transforming potential of the *ras* mutations in the context of *NHD13* expression, we cloned a G12D mutant *Nras* cDNA into an *EF1a*-driven expression vector. Expression of the mutant *Nras* conferred IL-3–independent growth, whereas expression of the wild-type *Nras* had no effect on growth factor dependence (Figure S2). This finding supports the assertion that *ras* mutations (class I) complement the NHD13 fusion protein (class II).

Cbl is a ubiquitin ligase responsible for targeting degradation of receptor tyrosine kinases. Intriguingly, *CBL* mutations that lead to

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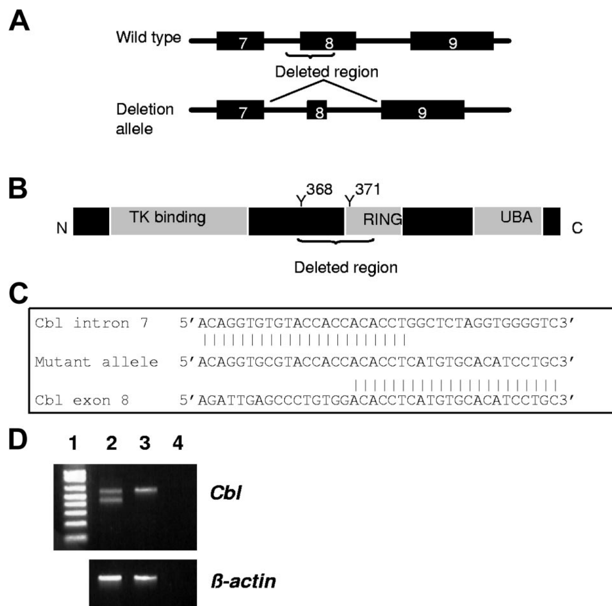

Figure 1. Cbl deletion mutant. (A) Exons 7-9 of the wild-type and mutant *Cbl* gene. Deletion of the exon 8 splice acceptor causes exon 8 to be spliced of the transcript. (B) CBL protein structure showing known functional domains. Y indicates tyrosine residue that is phosphorylated to activate CBL function. (C) Nucleotide sequence at breakpoint, demonstrating 6 base pair microhomology. (D) Expression of the mutant allele. Lane 1, 1 kb ladder, lane 2, cbl mutant, lane 3, wild-type control, lane 4, no template. Note the lower PCR product in lane 2 corresponding to the mutant allele.

FLT3 activation have recently been identified in AML patients, clustered around *CBL* exon 8.[19,20] We screened the ANLL samples for mutations of *Cbl* exon 8, and identified a deletion of 297 bp in one sample that results in loss of the exon 8 splice acceptor. RT-PCR and sequence analysis of the mutant PCR product demonstrated an aberrant *Cbl* transcript lacking exon 8 (Figure 1), which is similar to findings identified in the MOLM-13 cell line and one ANLL patient.[19] This transcript encodes a protein lacking a portion of the RING domain and 2 tyrosine residues which are critical for activation of the CBL protein.[21] The transcripts lacking exon 8 are in-frame and may produce a dominant negative protein similar to that produced by the *v-Cbl* oncogene.[19,21] The genomic breakpoint shows a 6 nucleotide microhomology region (Figure 1C) suggesting the deletion was mediated via nonhomologous end-joining.[22]

Although we cannot exclude the possibility that the NHD13 transgene alone was sufficient to initiate leukemia, the results regarding *Nras*, *Kras*, *Cbl*, *Runx1* and *Npm1* are consistent with the current "2-class" model for leukemogenesis. Of note, although *ras* mutations have been studied for decades, and there are numerous reports of mutagen-induced *ras* mutations in mice, there have been no reports of spontaneous *ras* mutations in murine leukemia. The absence of *Kit* and *Flt3* mutations was surprising in the context of the aforementioned 2-class hypothesis, and it will be of interest to learn whether these mutations were not identified in *NHD13* mice because they do not complement *NHD13*, or because they simply did not occur. The results described herein reinforce the prevailing 2-class model of leukemogenic events, and also have implications for the nature of *NHD13*-mediated leukemogenesis.

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Authorship

Contribution: C.S. designed and performed research, analyzed data, and wrote the first draft of the manuscript; L.Y.L. and S.B. designed and performed research and analyzed data; and P.D.A. designed research, analyzed data, and wrote the final draft of the manuscript.

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