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An *Mb1-Cre*-driven oncogenic *Kras* mutation results in a mouse model of T-acute lymphoblastic leukemia/lymphoma with short latency and high penetrance

Jacob J. Junco[1], **Taylor Chen**[2], **Raushan Rashid**[1], **Maci Terrell**[1], **Vincent U. Gant Jr.**[1], **Matthew Miller**[1,3], **Rachel Rau**[1,3], **H. Daniel Lacorazza**[2], **Karen R. Rabin**[1]

[1]Texas Children's Cancer Center, Department of Pediatrics, Baylor College of Medicine (BCM), Houston TX

[2]Department of Pathology & Immunology, BCM, Houston TX

[3]Stem Cells and Regenerative Medicine Center, Departments of Pediatrics and of Molecular & Human Genetics, BCM, Houston TX

Introduction

Ras pathway-activating mutations are present in up to 44% of acute lymphoblastic leukemia (ALL) cases, and are frequently associated with chemoresistance and worse outcomes (1, 2). Activating mutations in *KRAS* make up nearly half of this group of alterations. These data underscore the importance of improving therapeutic approaches to *KRAS*-mutated ALL. However, only a few recent studies have successfully targeted forms of mutant *KRAS*, long considered to be undruggable (3). Therapies against downstream effectors of Ras signaling have yielded mixed results, likely due to the ability of Ras to compensate through multiple signaling pathways (4).

To improve effective therapies for *RAS*-mutated ALL, robust syngeneic mouse models are required. Currently available *Ras* mutation-driven mouse models of ALL have a variety of drawbacks, including variable penetrance, prolonged latency to disease onset, and/or technically challenging requirements for transduction and ablative conditioning and transplantation (5–7). Here, we report successful generation of a novel endogenous, syngeneic mouse model of *Ras*-mutated ALL. We crossed $Mb1^{Cre/+}$ mice, which express Cre in most B- and a small percentage of T-lineage cells (8), with $Kras^{LSL\text{-}G12D/+}$ mice. Resulting $Kras^{LSL\text{-}G12D/+}$. $Mb1^{Cre/+}$ mice developed T-ALL/T-lymphoblastic lymphoma (LLy), with a median latency to death of 97 days and near-complete penetrance. The disease demonstrated variable infiltration of the bone marrow, spleen, and thymus, and displayed an aberrant $CD4^{+}CD8^{+}CD44^{+}CD25^{+/-}$ immunophenotype. The disease was sensitive to standard ALL chemotherapeutic agents, and transplantable, with rapid generation of disease

Corresponding Author: Jacob J. Junco, 1102 Bates St. 760G, Houston, TX 77030, 832-824-4235, junco@bcm.edu.

Competing Interests

The authors have no conflicts of interest to disclose.

in immunocompromised recipients. This novel transgenic mouse model has numerous beneficial features for advancing the study of *KRAS*-driven T-ALL/T-LLy.

Materials and Methods (see also Supplemental Materials and Methods)

Mice

B6.C(Cg)-*Cd79a*$^{tm1(cre)Reth}$/EhobJ (*Mb1-Cre*) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). B6.129S4-*Kras*^{tm4Tyi}/J (*LSL-Kras*^{G12D}) mice were provided by Lawrence Donehower, and NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG) mice by Michele Redell. As shown in Supplemental Figure 1A, *Kras*$^{LSL\text{-}G12D/+}$ males were bred with *Mb1*$^{Cre/+}$ females (both 12-30 weeks old) to generate *Kras*$^{LSL\text{-}G12D/+}$.*Mb1*$^{Cre/+}$ (n=37) mice and control genotypes (*Kras*$^{+/+}$.*Mb1*$^{Cre/+}$. n=57, *Kras*$^{LSL\text{-}G12D/+}$.*Mb1*$^{+/+}$ n=22, and *Kras*$^{+/+}$.*Mb1*$^{+/+}$ n=20). Mice were monitored for declining health, weight loss, and CD4^{+}CD8^{+} cells in peripheral blood. Investigators were not blinded to the mouse genotypes. All animal experiments were performed with approval of the Baylor College of Medicine Institutional Animal Care and Use Committee.

Genotyping

The *Mb1-Cre* transgene was assessed with primers 29589 (5'-ACTGAGGCAGGAGGATTGG-3'), 30016 (5'-CTCTTTACCTTCCAAGCACTGA-3'), and 30017 (5'-CATTTTCGAGGGAGCTTCA-3'). *LSL-Kras*^{G12D} polymerase chain reaction (PCR) genotyping was performed with primers y116 (5'-TCCGAATTCAGTGACTACAGATG-3'), y117 (5'-CTAGCCACCATGGCTTGAGT-3'), and y118 (5'-ATGTCTTTCCCCAGCACAGT-3'). For each primer set, thermocycling conditions were: 95°C 5 min; 40 cycles of 94°C 45 sec, 57°C 30 sec, 72°C 45 sec; followed by 72°C 7 min and holding at 4°C.

Results

Kras$^{LSL\text{-}G12D/+}$.Mb1$^{Cre/+}$ mice develop T-ALL/T-LLy with high penetrance and short latency

Kras$^{LSL\text{-}G12D/+}$.*Mb1*$^{Cre/+}$ mice developed fatal T-ALL/T-LLy in nearly all cases. No mice developed B-ALL or myeloid leukemia, and none of the control genotypes developed disease. We confirmed Cre-mediated recombination of the *Kras*$^{LSL\text{-}G12D}$ allele in sorted B cells from *Kras*$^{LSL\text{-}G12D/+}$.*Mb1*$^{Cre/+}$ mice, indicating *Kras*^{G12D} was activated in B cells as intended. T-ALL/T-LLy samples from these same mice also demonstrated Cre-mediated recombination, as well as frequent loss of the wild-type *Kras* allele (Supplemental Figure 1B). *Kras*$^{LSL\text{-}G12D/+}$.*Mb1*$^{Cre/+}$ mice had significantly enlarged spleens and thymuses compared to age-matched mice of the control genotypes (Figure 1A, 1B). We performed blast immunophenotyping on 13 cases. Twelve demonstrated an aberrant CD4^{+}CD8^{+} double-positive immunophenotype, and one demonstrated CD4^{-}CD8^{+} single-positive blasts. All 13 mice had thymic involvement. Spleen involvement was present in 7 mice, with 5 of these also demonstrating blasts in the bone marrow. Three of 13 mice had >25% bone marrow involvement and thus met criteria for ALL (Figure 1C). Five cases were also stained for CD44 and CD25, markers of early T-cell differentiation. Expression of both CD44 and CD25 normally only occurs at the CD4^{-}CD8^{-} double-negative stage, but five of five cases

analyzed demonstrated aberrant $CD4^+CD8^+CD44^+CD25^{+/-}$ (Figure 1D). Hematoxylin and eosin stained spleen sections from diseased $Kras^{LSL\text{-}G12D/+}.Mb1^{Cre/+}$ mice demonstrated extensive infiltration of large blasts with immature chromatin, compared to small, mature lymphocytes with condensed chromatin in the control non-leukemic sample (Figure 1E). The median latency to death from disease was 97 days (range 75-139 days) with complete penetrance among the mice that did not die early due to non-leukemic causes (Figure 2A). Thirty-one of 37 $Kras^{LSL\text{-}G12D/+}.Mb1^{Cre/+}$ mice developed T-ALL/T-LLy. The 6 mice that did not manifest T-ALL/T-LLy were all censored at less than 84 days of age (4 had benign causes of death and 2 could not be fully evaluated). The $Kras^{G12D}$ point mutation was verified by Sanger sequencing in thymus samples from T-ALL/T-LLy mice 347, 348, and 349 (data not shown).

Notch1 mutations are common in other mouse models of T-ALL/T-LLy (5) and human T-ALL (2). We performed Sanger sequencing of hotspot regions, namely *Notch1* exons 26, 27, and 34, in 9 $Kras^{LSL\text{-}G12D/+}.Mb1^{Cre/+}$ T-ALL/T-LLy samples. Six samples demonstrated exon 34 mutations resulting in frameshifts in the PEST domain of *Notch1* (Supplemental Figure 1C).

$Kras^{LSL\text{-}G12D/+}.Mb1^{Cre/+}$ leukemic blasts are sensitive to standard chemotherapy agents and cause aggressive disease in secondary recipients

Primary $Kras^{LSL\text{-}G12D/+}.Mb1^{Cre/+}$ blasts (T-ALL from mouse 347 spleen, and T-LLy from mouse 349 thymus) were transplanted into NSG mice. Recipients all developed aggressive and lethal disease, with enlarged spleens and average latency of 10 and 14 days, respectively (Figure 2B). Blasts from the secondary recipients demonstrated the same aberrant immunophenotypes as the primary blasts (primary immunophenotypes shown in Figure 1D).

Cells isolated post-mortem from the enlarged thymuses of 3 diseased $Kras^{LSL\text{-}G12D/+}.Mb1^{Cre/+}$ mice (285, 302, and 314) were incubated with the standard T-ALL/T-LLy chemotherapy agents vincristine, doxorubicin, and dexamethasone. All three cases demonstrated chemosensitivity, with low-nanomolar IC_{50} values (Figure 2C, 2D).

Discussion

$Kras^{LSL\text{-}G12D/+}.Mb1^{Cre/+}$ mice develop a highly penetrant, short latency T-ALL/T-LLy, with an aberrant immunophenotype ($CD4^+CD8^+CD44^+CD25^{+/-}$) similar to those observed in other *Kras* mutation-driven T-ALL mouse models (5, 7). Expression of the early T cell marker CD44 in our model may be useful for future studies since CD44 expression has been reported to be associated with risk of relapse in primary patient T-ALL samples (9). The aberrant combination of early (CD44 and CD25) and late (CD4 and CD8) T cell markers prevents categorizing as one of the conventional molecular genetic subtypes of T-ALL arrested at a specific T cell differentiation stage (10). Alternative T-ALL classification schemes based instead on mutations contributing to activation of particular cell signaling pathways, classify T-ALL with *RAS* and/or *PTEN* mutations as high-risk (11). Our mouse model of T-ALL/T-LLy presented here has similarities to this high-risk T-ALL subtype,

and may provide a useful preclinical system to study specific pathogenesis and potential therapies.

Most transgenic mouse models driven by *Mb1-Cre* yield B-ALL or B-cell lymphoma, and a single study of *Mb1-Cre* driven *LMO2* expression resulted in T-ALL in less than 20% of the mice (12). Thus, the exclusive development of T-lineage disease in our $Kras^{LSL\text{-}G12D/+}.Mb1^{Cre/+}$ model was unexpected. Presumably, since we observed Cre-mediated recombination in both B cells and T-ALL/T-LLy samples, Cre may be expressed in an early lymphoid progenitor cell preceding B/T lineage specification. Hobeika et al (8) showed *Mb1-Cre* mice demonstrate recombination in most B lineage cells and a small percentage of $Thy1^+$ T cells in the thymus and spleen. Another study found *Mb1-Cre*-mediated recombination in all pro-B cells and 10% of $Ly6D^+$ common lymphoid progenitors (CLPs), but no recombination in lymphoid-primed multipotent progenitors or $Ly6D^-$ CLPs, generally considered the last stage of maturation which contributes to both B and T cell populations (13). However, the $Ly6D^+$ CLP stage has been shown to also generate T cells of the DN3 stage and beyond (14). We hypothesize that *Mb1-Cre*-mediated recombination occurred at the $Ly6D^+$ CLP stage in our mice, leading to $Kras^{G12D}$ overexpression in these cells and expansion of a descendant population with a derangement in T cell maturation.

Dependable genetically-engineered mouse models are needed for preclinical studies of *de novo RAS*-mutated T-ALL. One widely-used technique for modeling *Ras* mutation-driven T-ALL/T-LLy involves treating $Kras^{LSL\text{-}G12D/+}.Mx1^{Cre/+}$ mice with polyinosinic:polycytidylic acid to induce Cre recombination. These mice develop myeloproliferative neoplasms, and secondary transplantation of bone marrow into lethally-irradiated mice is required for T-ALL/T-LLy development (5, 7). This approach results in variability in the type of hematologic malignancy generated and/or a prolonged latency to disease. Finally, similar to xenografting or transduction/transplantation mouse models, the recipient conditioning and transplantation of myeloproliferative cells yield mice less suitable for studies of microenvironment interactions and early stages of leukemogenesis. There is currently only one other mouse model, $Kras^{LSL\text{-}G12D/+}.Lck^{Cre/+}$, which consistently develops *Kras* mutation-driven T-ALL with minimal technical manipulation, but its latency to disease is longer (median 121 days) than for $Kras^{LSL\text{-}G12D/+}.Mb1^{Cre/+}$ mice (15).

Compared to other models of *Ras* mutation-driven T-ALL/T-LLy, the $Kras^{LSL\text{-}G12D/+}.Mb1^{Cre/+}$ model has advantages in terms of high penetrance, short latency, and lack of technical manipulations. Because the blasts demonstrate sensitivity to standard chemotherapy agents, this model may be useful for studying novel targeted therapies in combination with, or in comparison to these agents. It is also well-suited to studies of pro-leukemic interactions between blasts and the native bone marrow microenvironment during leukemogenesis. In sum, this model holds promise as a tool for gaining insights into more effective therapies for *RAS* mutation-driven leukemias and lymphomas.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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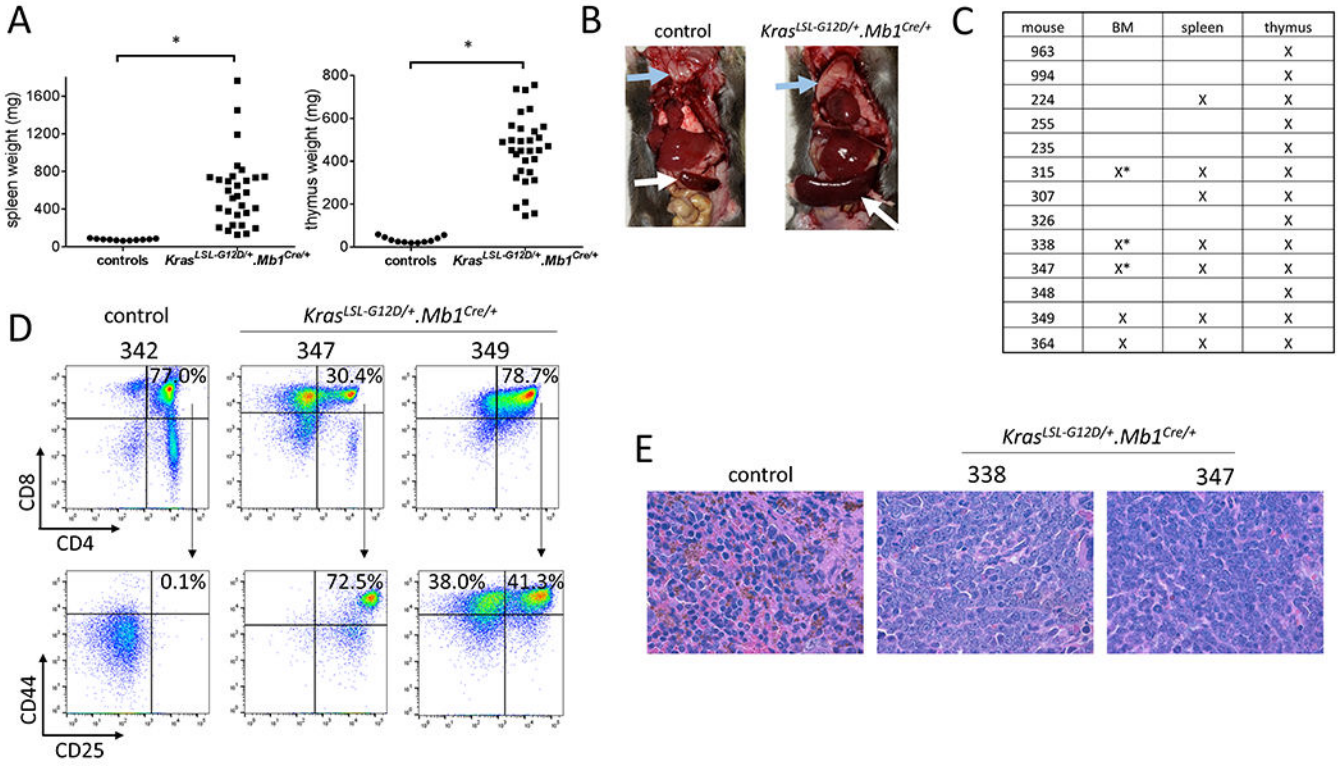
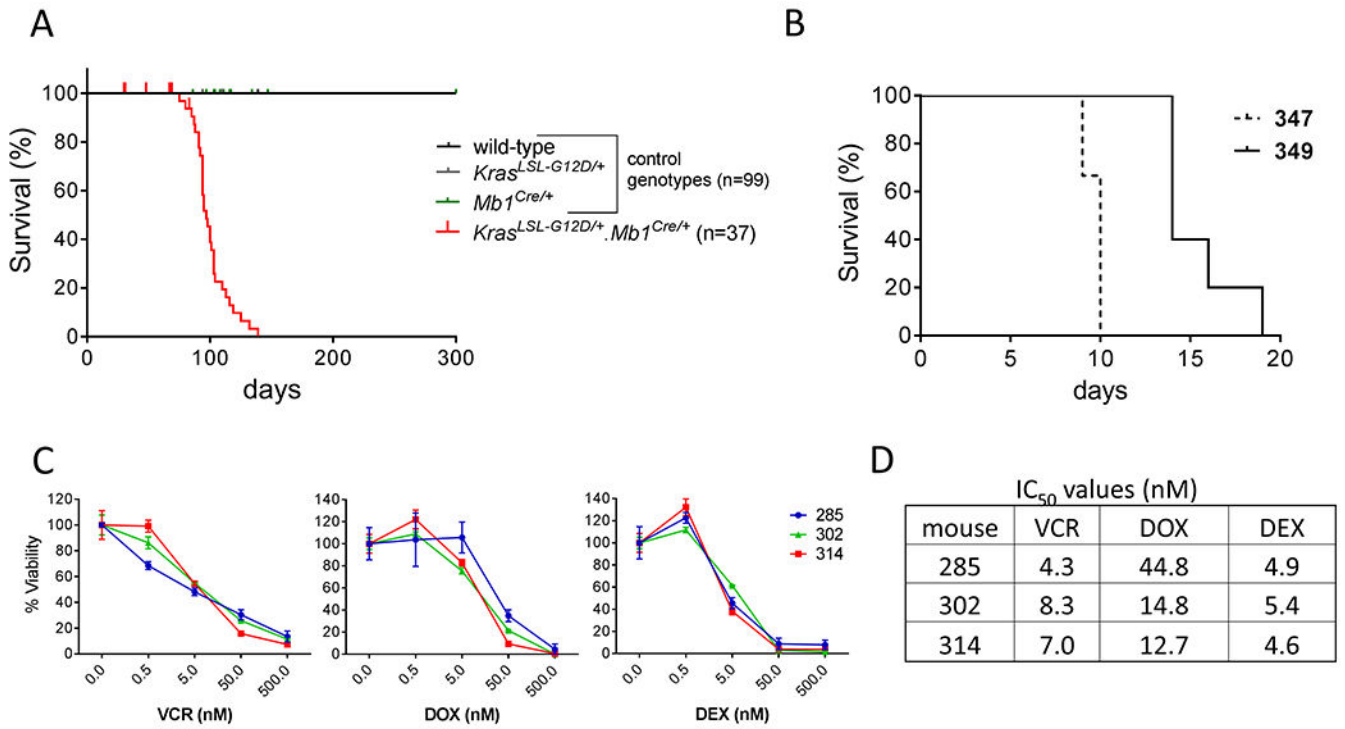


mouse	BM	spleen	thymus
963			X
994			X
224		X	X
255			X
235			X
315	X*	X	X
307		X	X
326			X
338	X*	X	X
347	X*	X	X
348			X
349	X	X	X
364	X	X	X

Figure 1: *Kras*$^{LSL\text{-}G12D/+}$*.Mb1*$^{Cre/+}$ mice develop T-ALL/T-LLy.

(A) *Kras*$^{LSL\text{-}G12D/+}$*.Mb1*$^{Cre/+}$ mice had enlarged spleens and thymuses compared to age-matched control mice (Welch's t-test, *p<0.0001). **(B)** Representative images of a control and a *Kras*$^{LSL\text{-}G12D/+}$*.Mb1*$^{Cre/+}$ mouse. Blue and white arrows indicate thymus and spleen, respectively. **(C)** Disease involvement of the bone marrow, spleen, and thymus in 13 mice analyzed by flow cytometry. "X" indicates tissue involvement, and * indicates >25% bone marrow involvement, the criterion for T-ALL. **(D)** *Kras*$^{LSL\text{-}G12D/+}$*.Mb1*$^{Cre/+}$ leukemic blasts from the thymus demonstrated aberrant expression of both early and late T-cell progenitor markers. T-ALL/T-LLy mice 347 and 349 had $CD4^{+}CD8^{+}CD44^{+}CD25^{+/-}$ blasts. Thymocytes from a representative age-matched healthy control mouse (342) are shown for comparison. (E) Hematoxylin and eosin stained spleen sections of leukemic mice 338 and 347 show loss of normal splenic architecture, with diffuse infiltration by large blasts with immature chromatin, compared to a control mouse (100X magnification).



mouse	VCR	DOX	DEX
285	4.3	44.8	4.9
302	8.3	14.8	5.4
314	7.0	12.7	4.6

Figure 2: *Kras*$^{LSL-G12D/+}$.*Mb1*$^{Cre/+}$ T-ALL/T-LLy has a short latency, causes disease in secondary recipients, and blasts are sensitive to standard chemotherapy agents.

(A) *Kras*$^{LSL-G12D/+}$.*Mb1*$^{Cre/+}$ mice developed T-ALL/T-LLy and died at a median of 97 days (range 75-139 days). **(B)** NSG mice transplanted with primary *Kras*$^{LSL-G12D/+}$.*Mb1*$^{Cre/+}$ T-ALL (mouse 347) and T-LLy (mouse 349) blasts died with median latencies of 10 and 14 days, respectively. **(C)** Thymic blasts from three leukemic *Kras*$^{LSL-G12D/+}$.*Mb1*$^{Cre/+}$ mice (285, 302, 314) were incubated for 48 hours with varying concentrations of standard T-ALL/T-LLy chemotherapy agents vincristine (VCR), doxorubicin (DOX), and dexamethasone (DEX), followed by viability determination with ATP assay. Error bars for each point show standard deviation. **(D)** Each case demonstrated chemosensitivity, with IC_{50} values in the low nanomolar range.