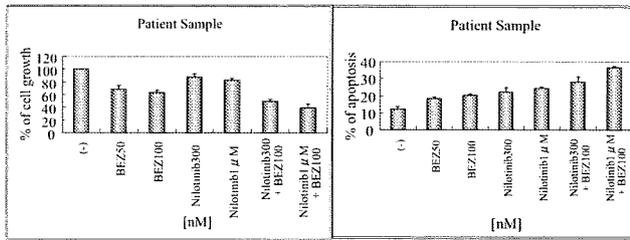
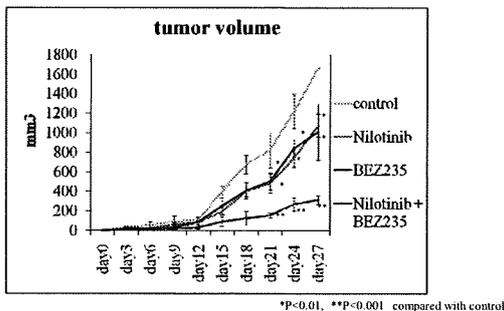


T315I変異陽性細胞に対する BEZ235、nilotinibの併用効果



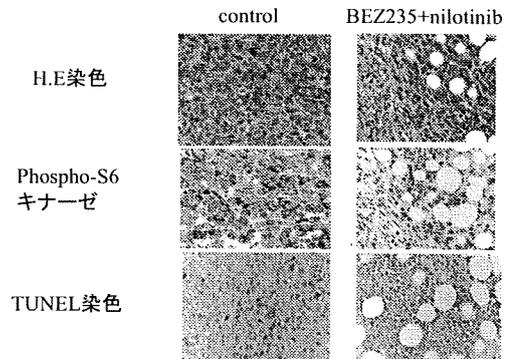
BEZ235 + nilotinibの併用効果を検体収集より得られた白血病細胞を用いて解析した。BEZ235 + nilotinibの併用によりT315I BCR-ABL発現細胞においても、細胞増殖抑制、アポトーシス誘導の増加が確認された。

BCR-ABL陽性細胞に対する BEZ235、nilotinibによるマウスモデルでの効果



Random mutagenesis BCR-ABLを感染させたBaF3細胞株をヌードマウス皮下に移植後、nilotinib単独群、BEZ235単独群、nilotinob + BEZ235併用群にて皮下腫瘍形成能について解析した。Nilotinob + BEZ235併用群では有意な腫瘍形成能の抑制が確認された。

BCR-ABL変異細胞に対する BEZ235、nilotinibによるin vivoでの効果



さらに、腫瘍組織をH. E. 染色、Phospho-S6キナーゼ免疫染色、TUNNELにて解析したところ、nilotinob + BEZ235併用群ではTUNNEL陽性細胞の増加、Phospho-S6キナーゼの抑制、アポトーシス誘導細胞の増加が確認された。今後は分子標的薬の併用効果をj確認する上で、臨床検体を用いてのin vivo studyを含めた詳細な解析が必要と考えられる。

(3) Dasatinib及びAuroraキナーゼ阻害剤併用効果の解析：

MK-0457はBaF3 WT-p185BCR-ABL細胞及びBaF3 T315I BCR-ABL細胞に対し、100nMにて細胞増殖抑制効果を示した。一方、dasatinibはBaF3 WT-p185BCR-ABL細胞に対し10nMにて細胞増殖抑制を示すのに対し、BaF3 T315IBCR-ABL細胞に対しては100nMにても増殖抑制効果は見られなかった。

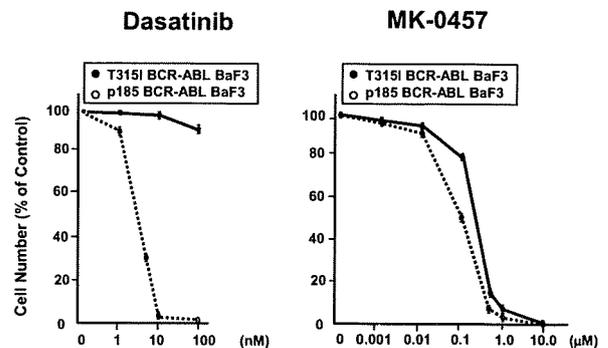


図1. MK-0457による細胞増殖抑制効果

MK-0457とdasatinib併用によるアポトーシス誘導効果をBaF3 T315IBCR-ABL細胞を用いて解析した。MK-0457はdasatinibとの併用によりアポトーシス誘導効果の増加が確認された。

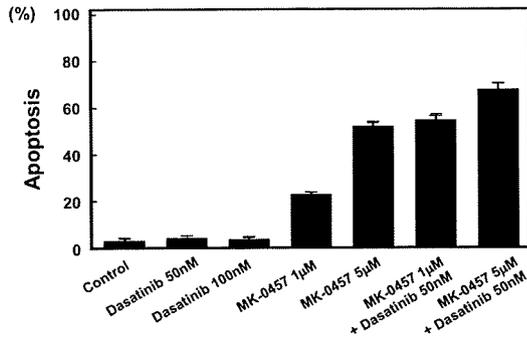
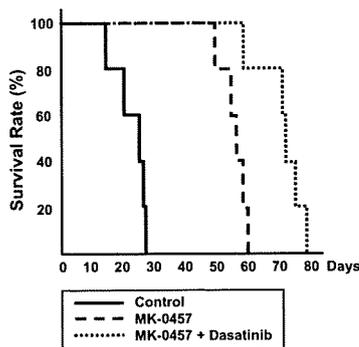


図2. MK-0457及びdasatinib併用によるT315IBCR-ABL変異細胞に対するアポトーシス誘導効果

さらに、in vivoにおけるMK-0457の生物活性についてBaF3 T315I BCR-ABL移植マウスを用いて解析した。①MK-0457 30mg/kg b. i. d. ip 5日間投与、3週間休薬、②MK-0457 30mg/kg b. i. d. ip + Dasatinin 10mg/kg po 5日間投与、3週間休薬、③Dasatinin 10mg/kg po 5日間投与、3週間休薬のスケジュールにて行った。コントロール群では骨髄内への白血病細胞浸潤、脾腫を伴ない移植後28日以内に全例死亡したのに対し、MK-0457投与群では60日以上生存、MK-0457 + Dasatinib投与群では70日以上生存した。

BaF3 T315I BCR-ABL



MK-0457及びdasatinib併用によりBaF3 T315I BCR-ABL細胞においてアポトーシス誘導の増加が確認された。MK-0457及びdasatinib併用投与はin vivoにおいてもBaF3 BCR-ABL移植マウス生存期間を延長させた。Imatinib耐性化症例の出現は次世代型のABLチロシンキナーゼ阻害剤の開発をもたらし、nilotinib及びdasatinibの臨床応用につながった。しかしながら、imatinib耐性の約20%を占めるT315I変異型BCR-ABLに対してはnilotinib、dasatinibともに有効性を示

さないことから、T315Iに対する対策を構築しなければならない。CML治療の今後の最大の課題はキナーゼ阻害剤を駆使してMRDを根絶させ真の治癒をめざすことであり、新規ABLキナーゼ阻害剤の開発は医療経済面においても大きな貢献になると思われる。

D. 健康危険情報
特になし。

E. 研究発表
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F. 知的財産権の出願・登録状況
(予定を含む)

1. 特許取得
該当なし。
2. 実用新案登録
該当なし。
3. その他
なし。

「染色体のセントラルレビュー」

研究分担者 滝 智彦 京都府立医科大学大学院医学研究科分子病態検査医学 講師

研究要旨

JALSG AML-97臨床試験登録症例809例の染色体レビューを行い、新規の異常を含む染色体異常の正確な頻度を明らかにした。また、染色体異常と予後との関係について検討した。その結果は、これまでの海外の臨床試験での解析結果とは必ずしも一致しないものだった。他の臨床試験の結果に頼るのではなく、独自の研究の中の症例における染色体異常と予後との関係を解析することにより、独自の予後因子を明らかにすることが重要であると思われた。今後は染色体異常だけでなく、さまざまな遺伝子異常と組み合わせての臨床像との関係の検討が必要であるが、そのための基礎となる染色体異常の正確な診断が重要である。AML-201での検討など、今後さらに症例を蓄積していくことにより日本人特有の染色体異常の臨床的な特徴を明らかにできると思われる。

A. 研究目的

急性骨髄性白血病(AML)にみられる染色体異常は、従来用いられていたFAB 分類と強く相関する。2008年に改訂されたWHO分類第4版では、さらに多くの特異的染色体異常がその分類のために採用されている。これらの染色体異常の種類と予後との関係はこれまで海外のいくつかの大規模研究によって検討され報告されてきたが、個々の染色体異常におけるリスクの評価は必ずしも一定でない。本研究では、まずJALSG AML-97臨床試験に登録された症例の染色体検査結果のセントラルレビューを行い、その結果明らかになった問題点と染色体異常の特徴について検討した。次に、その結果を基にした臨床像との関係について検討を行った。特に、染色体異常と予後との関係について検討した。JALSGのAML研究における染色体異常と予後との関係の検討によって、独自の予後因子となる染色体異常を明らかにすることを目的とした。

B. 研究方法

AML-97 登録症例 809 例について、データセンターに登録された核型データと染色体検査報告書の照合を行った。809 例のうち、最終的に 641 例について、染色体異常と臨床像との関係を検討した。

染色体レビューは、JALSG 染色体小委員会メンバー [谷脇雅史 (委員長、京都府立医科大学)、大竹茂樹 (金沢大学)、大屋敷一馬 (東京医科大学)、陣内逸郎 (埼玉医科大学)、三浦偉久男 (聖マリアンナ医科大学)、宮崎泰司 (長崎大学)、竹内 仁 (日本大学)、西田一弘 (京都府立医科大学)、滝智彦 (京都府立医科大学)] が行い、染色体異常と臨床像との関係については JALSG データセンター

[本田純久、宮崎泰司 (長崎大学)] の協力を得て行った。

C. 研究結果

1. 染色体レビューの結果

AML-97に登録された809例のうち評価可能な核型データが得られたのは650例 (80.3%)で、そのうち核型分析成功例は637例 (98.0%) であった。370例に染色体異常を認め (レビュー前381例)、正常核型は267例 (レビュー前254例) であった。染色体異常の有無についてのレビュー前後での不一致例は35例 (5.5%) であった。不一致例の中には、単純な記載ミスその他、染色体についての知識不足が原因と思われる解釈の間違いが多く含まれており、専門家によるレビューが重要であると思われた。

2. AML-97登録症例の染色体の特徴

染色体レビューによって最終的に核型が決定できた症例からプロトコールの不適格症例を除いた623例についてさらに詳細な解析を行った。

染色体異常を認めたのは355例(57.0%)で、おもな染色体異常の内訳は、t(8;21) 112例 (18.0%)、inv(16) 27例 (4.3%)、11q23転座 40例 (6.4%)、-5/5q- 23例 (3.6%)、-7/7q- 31例 (5.0%)、12p- 13例 (2.1%)であった。t(8;21)の頻度は欧米での頻度に比べて高頻度であった。頻度は少ないものの特異的な染色体異常として、t(9;22) 8例 (1.3%)、11p15転座 7例 (1.1%)、t(16;21) (p11;q22) 4例 (0.6%)、t(10;11)(p12-14;q21) 4例 (0.6%)などがみられた。11p15転座は東洋人での頻度が高い可能性が示唆されていたが、本邦

での頻度が初めて明らかになり、今後欧米との結果の比較が可能になった。

3. 染色体異常と予後との関係

一般的に予後良好群に分類されるt(8;21) (112例)とinv(16) (27例)の寛解率 (89.3%と100%)、5年全生存率(OS) (64.7%と71.5%)、5年無病生存率(DFS) (47.1%と41.5%)は、従来の報告と同様に良好の傾向であった。しかし、報告によりその意義についての評価が分かれる11q23転座(40例)は、中間群とされる正常核型(268例)に比べて寛解率 (75.0% vs. 84.3%)、OS (39.5% vs. 46.7%)、DFS (29.6% vs. 38.0%)とも低い傾向であった。11q23転座は、BSHガイドラインではすべて中間群とされているが、NCCNガイドラインではt(9;11)のみが中間群、その他の11q23異常は不良群とされている。今回の解析では、t(9;11) (9例)は寛解率 (88.9%)、OS (62.5%)、DFS (50.0%)とも良好で、少ない症例数ではあるが、これらの成績はt(8;21)、inv(16)に匹敵するものであった。一方、その他の11q23異常の中ではt(6;11) (9例)の寛解率 (55.6%)、OS (0%)、DFS (0%)が他の11q23異常に比べても低い傾向だった。t(6;11)以外ではdel(11)(q23)の寛解率 (50%)、OS (33.3%)、DFS (0%)が低く、del(11)(q23)の中にはt(6;11)が多く含まれているといわれていることを反映していると考えられた。一方で、11q23転座におけるキメラ遺伝子の違いを染色体レベルで正確に区別することは困難であり、今後はRT-PCR法によるキメラ遺伝子解析を併用したさらに正確な診断が必要である。

4. 高リスク群と考えられる染色体異常の検討

高リスク群転座としては、まずこれまでのさまざまな報告で予後不良と報告されているt(9;22)(q34;q11)、11p15転座、t(10;11)(p13;q14)、t(6;9)(p23;q34)、t(16;21)(p11;q22)、8p11転座を抽出した。これらの転座によって、それぞれBCR-ABL、NUP98キメラ、CALM-AF10、DEK-CAN(NUP214)、FUS-ERG、MOZキメラが形成されると考えられる。これらを有したのは29例(4.5%)で、全体では寛解率(65.5%)、5年全生存率(18.1%)、5年無病生存率 (14.0%)とも低かった。ただし、それぞれの染色体転座ごとの症例数は2-8例といずれも少なく、個々の染色体異常が臨床的な予後不良とどの程度相関しているかの評価については今後の症例の集積が必要である。

次に、やはり多くの報告で予後不良因子とされている染色体異常の中で-5/5q-と-7/7q-について検討した。代表的な欠失型異常である-5/5q-と-7/7q-を有する症例はそれぞれ23例と31例であった。-5/5q-を有する症例は、寛解率(39.1%)、5年全生存率(0%)、5年無病生存率(0%)とも有意に低く、他の報告と同様の結果だった。一方、-7/7q-を有する症例は、寛解

率(58.1%)は有意に低かったものの5年生存率ではまったく有意差を認めなかった。-7/7q-を有する症例では、31例中27例 (87%)は他の染色体異常を合併しており、これを-7と7q-に分けて、非寛解、寛解後再発、寛解継続ごとに合併する染色体異常の比較を行った。-7の非寛解例あるいは寛解後再発例では-5/5q-、t(9;22)、3q異常などの、7q-の非寛解例あるいは寛解後再発例では-5/5q-、11p15転座、複雑核型などの、いずれも他の報告で予後不良とされている染色体異常を合併している症例が多くみられた。逆に、-7および7q-の寛解継続例ではt(8;21)、inv(16)など予後良好の染色体異常を合併していた。-7/7q-では、-7/7q-の有無よりも、-7/7q-にどのような他の染色体異常が合併するかが予後に重要な影響を与えている可能性が示唆された。-7と7q-の間で予後因子としての意義が異なるという報告もあるが、今回の解析では両者の間に差は認めなかった。同じように-7/7q-に合併する染色体異常に注目した小児および若年成人での国際共同研究の結果が最近報告されているが、-7と7q-でそれぞれに合併する染色体異常の種類が-7と7q-の間に大きく差がある点、-7または7q-単独例が高頻度である点などが我々の解析症例と大きく異なっていた。この点については症例の年齢の違いが大きく影響していると考えられるが、やはり独自の症例の解析が重要であることを改めて認識させるものであった。

このようにAML-97の結果とこれまでの海外の報告には多くの異なる点が存在する。これらの違いが民族差による遺伝的背景の違いによる可能性、プロトコルで使用している薬剤の違いによる可能性など、さまざまな要因が考えられる。現在AML-201プロトコルに登録された症例の染色体レビューを予定しており、AML-97とAML-201による症例の蓄積および比較を行うことにより、日本人特有の染色体異常の臨床的な特徴を明らかにできることが期待される。

D. 健康危険情報

該当無し。

E. 研究発表

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F. 知的財産権の出願・登録状況

(予定を含む)

1. 特許取得
該当無し。
2. 実用新案登録
該当無し。
3. その他
なし。

IV. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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2007年4月から2008年3月 (in press分含む)まで

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Stem cell exhaustion due to Runx1 deficiency is prevented by Evi5 activation in leukemogenesis

Bindya Jacob,^{1,2} Motomi Osato,^{1,2} Namiko Yamashita,¹ Chelsia Qiuxia Wang,¹ Ichiro Taniuchi,³ Dan R. Littman,⁴ Norio Asou,⁵ and Yoshiaki Ito^{1,2}

¹Institute of Molecular and Cell Biology, Singapore; ²Cancer Science Institute of Singapore, National University of Singapore, Singapore; ³RIKEN, Research Center for Allergy and Immunology, Yokohama, Kanagawa, Japan; ⁴Howard Hughes Medical Institute, Skirball Institute of Biomolecular Medicine, New York University, NY; and ⁵Department of Hematology, Kumamoto University School of Medicine, Kumamoto, Japan

The *RUNX1/AML1* gene is the most frequently mutated gene in human leukemia. Conditional deletion of *Runx1* in adult mice results in an increase of hematopoietic stem cells (HSCs), which serve as target cells for leukemia; however, *Runx1*^{-/-} mice do not develop spontaneous leukemia. Here we show that maintenance of *Runx1*^{-/-} HSCs is compromised, progressively resulting in HSC exhaustion. In leukemia development, the stem cell exhaustion was rescued by additional genetic changes. Retroviral insertional mutagenesis revealed *Evi5* activation as a cooperating genetic alteration and *EVI5* overexpression indeed prevented *Runx1*^{-/-} HSC exhaustion in mice. Moreover, *EVI5* was frequently overexpressed in human *RUNX1*-related leukemias. These results provide insights into the mechanism for maintenance of pre-leukemic stem cells and may provide a novel direction for therapeutic applications. (Blood. 2010;115:1610-1620)

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Introduction

The *RUNX1/AML1* gene encodes the DNA binding α subunit of heterodimeric Runt domain transcription factor, PEBP2/CBF.¹ RUNX1 and its partner protein, the non-DNA binding β subunit (PEBP2 β /CBF β), are essential for definitive hematopoiesis and are frequently targeted in human leukemia.²⁻⁴ *RUNX1* and *CBFB* are involved in chromosomal translocations, generating fusion proteins that inhibit the activity of wild-type RUNX1 in a dominant-negative manner.^{5,6} Biallelic point mutations of *RUNX1* are frequently found in the acute myeloid leukemia (AML) M0 subtype and familial platelet disorder with predisposition to AML. Monoallelic mutations are found in sporadic myelodysplastic syndrome and AML.⁷⁻¹⁰ These point mutations make the RUNX1 protein nonfunctional. Hence, loss-of-function of RUNX1 is considered to be the common underlying mechanism for RUNX1-related leukemias.

Despite the prevalence of RUNX1 loss-of-function mutations or dominant-negative fusion proteins, the *RUNX1* alteration per se does not cause leukemia. Rather, cells with loss-of-function of RUNX1 remain leukemia-prone and only with acquisition of additional hits do they become fully leukemic.¹¹⁻¹⁴ Conditional deletion of *Runx1* in adult mice results in an expansion of immunophenotypically defined hematopoietic stem cell (HSC) compartment and an accumulation of megakaryoblasts and lymphoid progenitors.¹⁵⁻¹⁷ The expansion of *Runx1*-deficient HSC/progenitor compartment is due to higher self-renewal and antiapoptotic properties and results in predisposition to leukemia.¹⁸ However, surprisingly, despite the increased number of stem cells, Gowney et al¹⁶ reported that conditional *Runx1* knockout bone marrow (BM) cells are outcompeted by simultaneously transplanted wild-type BM cells in competitive repopulation assay, indicating that *Runx1*-deficient cells are compromised in reconstituting hematopoiesis

in the recipient mice. Also, except for one group describing that *Runx1* conditional knockout mice developed lymphoma at later stages of life,¹⁷ other groups reported that leukemia/lymphoma did not develop spontaneously. The above studies indicate increased leukemia susceptibility in *Runx1*-deficient conditions, and at the same time clearly suggest that *Runx1*-deficient cells require additional genetic changes for leukemic transformation.

Retroviral insertional mutagenesis (RIM) is a powerful tool to identify oncogenes and tumor suppressor genes.¹⁹ Injection of replication-competent retrovirus into newborn mice leads to integration of virus into the host genome and activation of oncogenes or disruption of tumor suppressor genes, resulting in leukemia or lymphoma. Retrovirus usually hits multiple genes to induce leukemia or lymphoma.²⁰⁻²³ RIM on conditional *Runx1* knockout mice provides an excellent system to identify genes that cooperate with loss-of-function of *Runx1* to promote leukemogenesis. Previous RIM studies on heterozygous *Runx1* knockout mice have revealed the alterations of the *Ras* gene family and its upstream factors such as *c-Kit* and *Flt-3* as candidate "second hits" in leukemogenesis. These genes are in fact frequently mutated in human RUNX1-related leukemias.^{18,22,24}

In this study, we show that *Runx1* deficiency in HSCs leads to the phenomenon called "stem cell exhaustion" after the initial expansion. *Runx1*-deficient stem cell maintenance was compromised, probably due to defective niche interaction, resulting in decline of stem/progenitor cell numbers and decreasing contribution of these stem cells to blood cell production. We employed RIM on conditional *Runx1* knockout mice and identified overexpression of *Evi5* as an additional genetic alteration that prevents the stem cell exhaustion caused by *Runx1* deficiency. Together, these 2 genetic alterations maintain an expanded pool of aberrant

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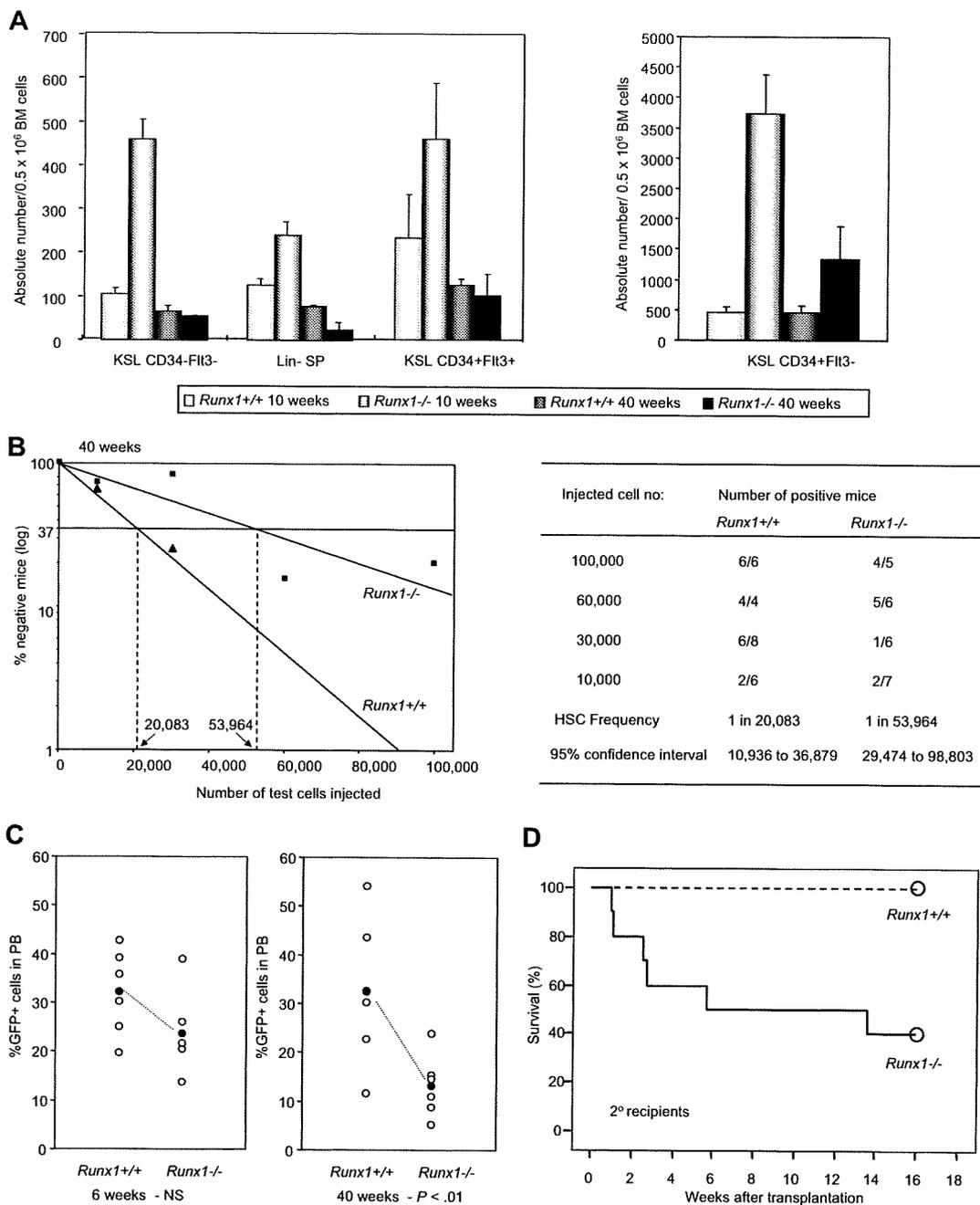


Figure 1. *Runx1*^{-/-} status leads to stem cell exhaustion. (A) Absolute number of KSL CD34⁺Fli3⁻ cells, Lin⁻ SP cells, KSL CD34⁺Fli3⁺ cells and KSL CD34⁺Fli3⁻ cells per 0.5 million BM cells from *Runx1*^{+/+} and *Runx1*^{-/-} mice of 2 distinct ages (10 and 40 weeks old). Each group comprises 3 to 4 mice. (B) Limiting dilution analysis using varying numbers of BM cells from 40-week-old CD45.2⁺ *Runx1*^{+/+} (▲), or *Runx1*^{-/-} (■) mice. Mice were considered negative when the percent chimerism was less than 1%. Left panel: estimated frequencies of the repopulating cells are indicated as vertical dashed lines (1 repopulating cell per indicated numbers of BM cells) for each genotype. Right panel: for each indicated number of transplanted cells from CD45.2⁺ *Runx1*^{+/+} or *Runx1*^{-/-} mice, the proportion of mice that are positive for test CD45.2⁺ cells is given as (number of positive mice)/(number of analyzed mice). Frequencies of HSCs were calculated using Poisson statistics. (C) GFP chimerism in PB of recipients of *Runx1*^{+/+} (n = 6) and *Runx1*^{-/-} (n = 6) cells at 6 and 40 weeks after transplantation. Each open circle represents data from an individual mouse and each closed circle is the average of a cohort. Statistical difference using unpaired Student *t* test is given at the bottom. NS indicates not significant. (D) Kaplan-Meier survival curves of secondary recipients of mock MIG vector-transfected *Runx1*^{+/+} (dashed line; n = 10) and *Runx1*^{-/-} (solid line; n = 10) BM cells. Circles represent end point of analysis.

stem/progenitor cells, which may act as targets for further oncogenic hits.

Methods

Mice

The mice harboring *Runx1* allele with exon 4 flanked by loxP sites (*Runx1*^{F/+}) were generated,²⁵ backcrossed against C57BL/6 mice for

3 generations, and then intercrossed to obtain *Runx1*^{F/F} mice. They were crossed with interferon-inducible *Mx-Cre* transgenic mice,²⁶ a gift from Dr K. Rajewsky, to generate *Runx1*^{F/F}-Tg(*Mx1-Cre*) mice. For further details, see supplemental Methods (available on the *Blood* website; click on the Supplemental Materials link at the top of the online article). All mice were maintained in the Biological Resource Center (BRC), Biopolis, Singapore, and all animal experiments followed the strict guidelines set by the National Advisory Committee for Laboratory Animal Research (NACLAR) and were approved by the BRC Institutional Animal Care and Use Committee.

Retroviral insertional mutagenesis

Runx1^{Fl/Fl}-Tg(Mxl-Cre) and *Runx1^{Fl/Fl}* mice were mated, and progenies were injected with MoMuLV virus 3 days after birth and with polyinosinic-polycytidylic acid at 1 month of age. Retrovirus-injected *Runx1^{-/-}* mice and *Runx1^{+/+}* littermates were monitored by examining their health condition and by weekly checking of complete blood cell count using an automatic hematology analyzer (Celltac alpha MEK-6358; Nihon Kohden). Necropsy of diseased mice, hematology analysis, and identification of RIS using inverse polymerase chain reaction (PCR) were carried out as previously described.^{22,23}

Flow cytometric analysis

Flow cytometric analysis was performed using a fluorescence-activated cell sorter (FACS) Vantage instrument as previously described.^{18,22} Monoclonal antibodies were usually purchased from BD Biosciences (supplemental Methods).

Patient samples

Thirty-five human patients with leukemia belonging to the following categories were screened for expression level of *EVI5*: AML with t(8;21) (n = 9); inv(16) (n = 7); other AML (n = 10); chronic myeloid leukemia (CML) blast crisis (n = 6); and complete remission from AML (n = 3). Each patient gave informed consent to this study based on the tenets of the revised Helsinki protocol produced by the Institutional Committees for the Protection of Human Subjects and Analysis of the Human Genome. All studies of human samples were approved by the institutional review board of Kumamoto University Hospital.

Additional procedures

For complete information on bone marrow transplantation (BMT) procedures; plasmid construction, retroviral transduction, and in vitro cell culture assays; quantitative real-time PCR (qRT-PCR); luciferase assay; in vivo homing assay; and the BrdU incorporation assay, see the supplemental Methods.

Results

Runx1^{-/-} stem/progenitor cell population declines after the initial expansion

Runx1 knockout (*Runx1^{-/-}*) BM cells, generated by Cre-recombinase-mediated knockout of *Runx1*, show an increase in hematopoietic stem/progenitor cell fraction compared with control wild-type (*Runx1^{+/+}*) mice. However, we found that *Runx1^{-/-}* HSC expansion is followed by exhaustion, resulting in a progressive decline of stem/progenitor cell numbers. At 10 weeks of age, *Runx1^{-/-}* mice showed a significant increase in long-term HSCs (c-Kit⁺Scal⁺Lineage⁻ [KSL] CD34⁻Flt3⁻), short-term HSCs (KSL CD34⁺Flt3⁻), and multipotential progenitors (KSL CD34⁺Flt3⁺). However, at 40 weeks of age, *Runx1^{-/-}* stem/progenitor cell numbers declined significantly and were equivalent to or lesser than corresponding *Runx1^{+/+}* cell numbers (Figure 1A, supplemental Figure 1). Side population analysis of lineage-negative cells (lineage-SP) also showed a similar trend of expansion of *Runx1^{-/-}* HSCs at 10 weeks, followed by decline at 40 weeks (Figure 1A). To analyze the number of functional competitive repopulating units (CRUs) in aged (40 weeks old) *Runx1^{-/-}* and *Runx1^{+/+}* mice, we carried out limiting dilution BMT. The frequency of CRUs in BM of aged *Runx1^{-/-}* mice was 1 in 53 964, lower than the frequency, 1 in 20 083, in *Runx1^{+/+}* littermate controls (Figure 1B). These results suggest that stem cell exhaustion may occur in *Runx1^{-/-}* mice.

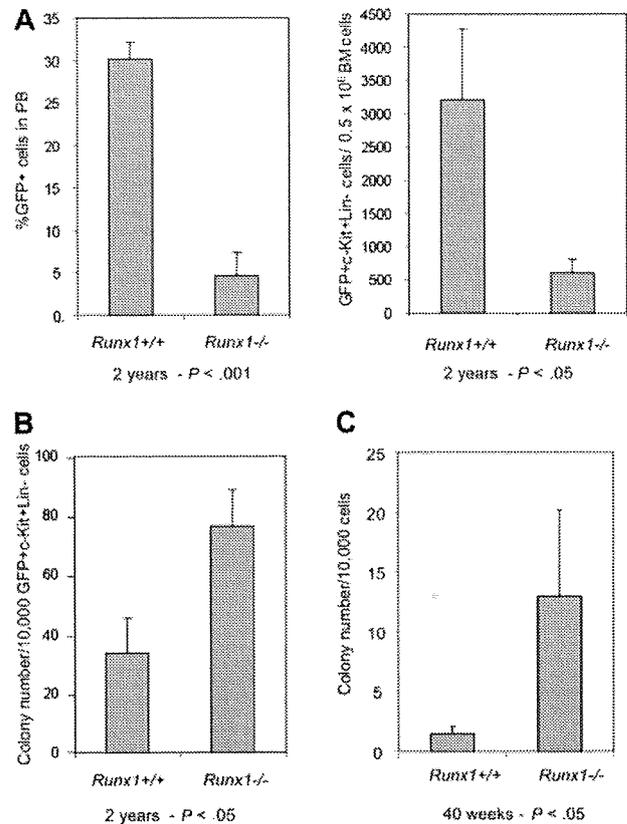


Figure 2. Aged *Runx1^{-/-}* stem/progenitor cells maintain proliferative ability. (A) Graphs showing percentage of GFP⁺ cells in PB and number of GFP⁺c-Kit⁺Lin⁻ cells in BM of recipients of *Runx1^{+/+}* (n = 3) and *Runx1^{-/-}* (n = 3) BM cells, 2 years after transplantation. (B) Colony assay of GFP⁺c-Kit⁺Lin⁻ cells from recipients of *Runx1^{+/+}* (n = 3) and *Runx1^{-/-}* (n = 3) BM cells, 2 years after transplantation. (C) Colony assay of KSL cells from 40-week-old mice, after 30 days of long-term culture on OP9 stromal cells. Statistical differences using the unpaired Student *t* test are given at the bottom.

The decline of *Runx1^{-/-}* HSCs is further observed in another BMT experiment. In the recipient mice that underwent transplantation with BM cells from *Runx1^{-/-}* and *Runx1^{+/+}* mice transfected with MIG (MSCV-IRES-GFP) retroviral vector expressing enhanced green fluorescent protein (EGFP) as a surrogate marker, contribution of donor cells to hematopoiesis was monitored periodically by the percentage of GFP⁺ cells in the peripheral blood (PB). At 6 weeks after transplantation, the GFP chimerism in PB of the recipients (n = 6) of *Runx1^{+/+}* and *Runx1^{-/-}* cells was comparable, with a mean value of 32.1% and 23.6% respectively. After 40 weeks, the mean GFP chimerism in the recipients of *Runx1^{+/+}* remained the same at 32.5%, whereas it was significantly lower (P < .01) at 13.1% in the recipients of *Runx1^{-/-}* cells (Figure 1C). By 2 years after transplantation, the GFP chimerism in PB of recipients of *Runx1^{-/-}* cells dropped even further. There was also a concomitant decrease in absolute number of immature *Runx1^{-/-}* (c-Kit⁺Lineage⁻GFP⁺) cells in the BM of the recipients, again suggesting stem cell exhaustion (Figure 2A).

To ascertain the phenomenon of *Runx1^{-/-}* stem cell exhaustion, a secondary transplantation experiment was carried out. Two to 3 primary recipients with similar GFP chimerism were killed at an average of 4 months after transplantation and BM cells were transplanted into 10 lethally irradiated (8 Gy) secondary recipients. Six of 10 recipients of *Runx1^{-/-}* cells died within 3 months of secondary transplantation due to pancytopenia arising from graft failure, while all the recipients of control *Runx1^{+/+}* cells survived

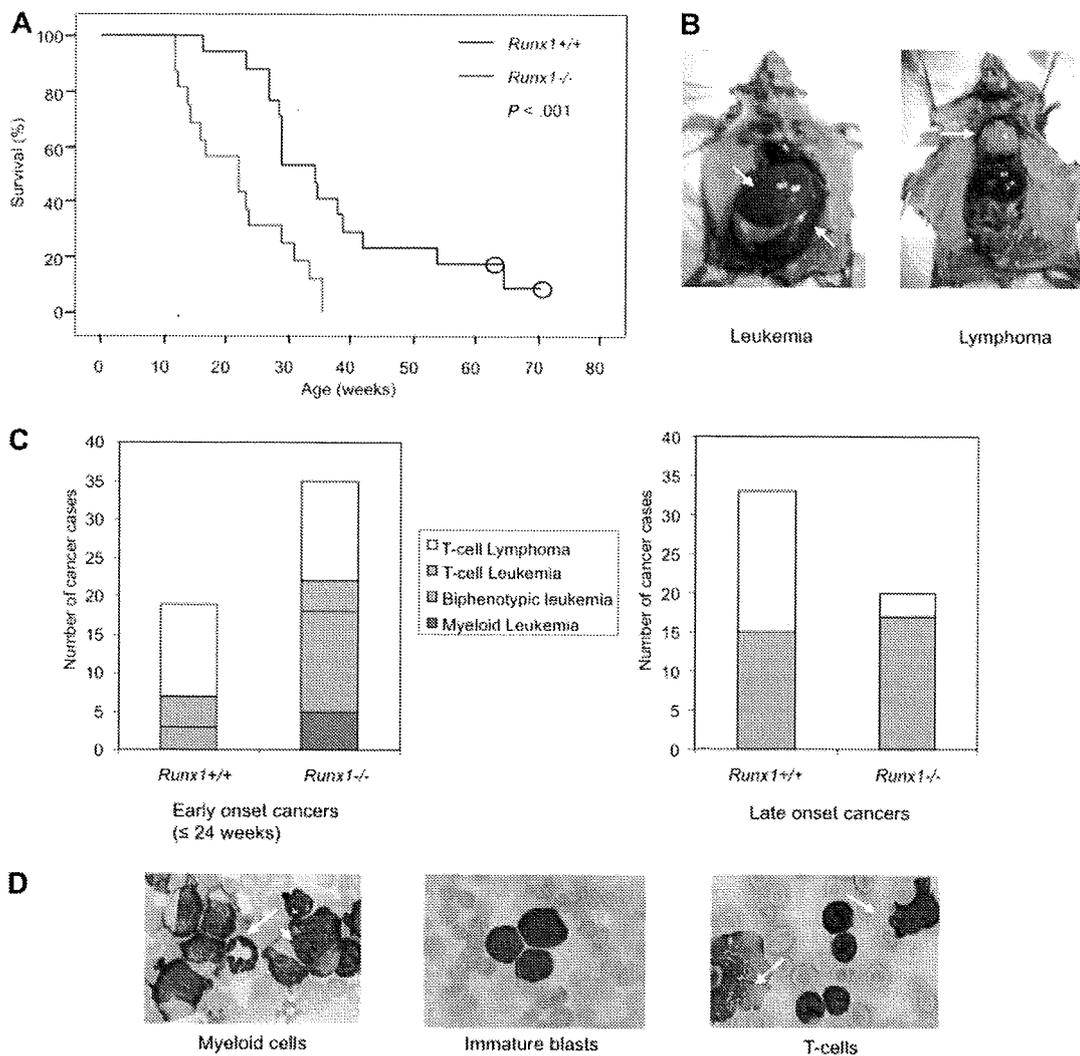


Figure 3. *Runx1*^{-/-} mice show early onset and high frequency of leukemia with myeloid features in RIM. (A) Kaplan-Meier survival curves of *Runx1*^{+/+} (blue line; $n = 17$) and *Runx1*^{-/-} (red line; $n = 16$) mice injected with MoMuLV retrovirus. Kaplan-Meier method showed significant difference between the 2 genotypes ($P < .001$, Mantel-Cox test). Open circles represent censored cases. (B) Necropsy of diseased mice; leukemic mice usually show enlarged spleen (bottom arrow) and liver (top arrow) while T-cell lymphoma mice show enlarged thymus (arrow). (C) Graphs showing frequency of different types of leukemia/lymphoma, groups 1 to 4, in early-onset (≤ 24 weeks) cancers of *Runx1*^{+/+} ($n = 19$) and *Runx1*^{-/-} ($n = 34$) mice; and frequency of leukemia or lymphoma cases in late-onset cancers of *Runx1*^{+/+} ($n = 33$) and *Runx1*^{-/-} ($n = 20$) mice. In total, $n = 52$ for *Runx1*^{+/+} mice and $n = 54$ for *Runx1*^{-/-} mice. (D) Morphology of cells from PB of representative leukemic case from group 1 showing granulocyte (arrow) and monoblast (arrowhead); group 2 showing immature blasts; and group 3 showing T cells and ghost cells (arrows) which are frequently seen in T-cell malignancy.

beyond that (Figure 1D). Taken together, the above results prove that *Runx1*^{-/-} status results in progressive stem cell exhaustion.

Surprisingly, colony assay of immature *Runx1*^{-/-} cells ($\text{GFP}^+ \text{c-Kit}^+ \text{Lineage}^-$) from BM of recipient mice showed an increased number of precursors even 2 years after transplantation, similar to the observations made soon after the conditional deletion of the *Runx1* gene¹⁸ (Figure 2B). Furthermore, long-term culture initiating cell (LTC-IC) assay of KSL cells from BM of aged 40-week-old *Runx1*^{-/-} mice showed an increased number of progenitor cells after 28 days of culture on OP9 stromal cells (Figure 2C). These results suggest that immature *Runx1*^{-/-} cells maintain their inherent properties of increased proliferation even after long periods of time. Hence, stem cell exhaustion may not be due to cell intrinsic defects of *Runx1*^{-/-} stem/progenitor cells.

***Runx1*^{-/-} status results in increased susceptibility to myeloid leukemia development**

It is conceivable that for *Runx1*^{-/-} mice to develop leukemia, *Runx1*^{-/-} stem cells would have to acquire an ability to survive long, possibly through additional genetic hits. Therefore, RIM was

employed to induce leukemia in *Runx1*^{-/-} mice and to identify genes that prevent stem cell exhaustion and aid in leukemia development. *Runx1*^{-/-} mice showed a significantly shorter latency of leukemia development than wild-type littermates (Figure 3A), thus confirming that the *Runx1*-deficient status accelerates leukemia development.

When the mice became moribund, necropsy was carried out and the disease was divided into leukemia or lymphoma cases. Leukemia cases showed elevated leukocyte counts and hepatosplenomegaly with normal thymus, whereas lymphoma cases showed normal or elevated leukocyte counts and enlarged thymus/lymph node (Figure 3B). Based on combination of leukocyte counts, necropsy, and further immunophenotype and morphologic analyses, tumors were classified into the following 4 groups: group 1, myeloid leukemia (supplemental Figure 2A); group 2, biphenotypic (myeloid and T-cell) leukemia; group 3, T-cell leukemia; and group 4, T-cell lymphoma (supplemental Tables 1-2). Most of the biphenotypic leukemia cases belonging to group 2 were considered to be mixtures of myeloid and lymphoid leukemia originating from different clones, with a certain subset of the leukemic cells