Ⅳ. 研究成果の代表的論文

Down症候群のTransient Abnormal Myelopoiesis（TAM）について

菊　地　　陽

埼玉県立小児医療センター血液・腫瘍科

Transient Abnormal Myelopoiesis in Down's Syndrome

Akira KIKUCHI

Division of Hematology/Oncology, Saitama Children's Medical Center

Abstract　A total of 73 patients of transient abnormal myelopoiesis (TAM) in Down's syndrome diagnosed at 212 Japanese pediatric hematology units between 2003 and 2005 were reported. There were 33 boys and 40 girls. Median values of gestational age and birth weight were 37 weeks 3 days and 2,680 g, respectively. Congenital anomalies accompanied 48 cases and congenital heart disease was reported in 44 of 48. Two myelodysplastic syndrome and 11 acute megakaryocytic leukemia cases were reported after spontaneous resolution of TAM. GATA1 mutation was observed 9 of 10 cases examined. Fifty four cases were alive and 19 cases were dead at investigation. The causes of death were mainly due to organ failure such as hepatic failure or renal failure. Preterm gestation, WBC count more than $100\times10^9/l$ at diagnosis, maximum serum direct bilirubin level more than 5 mg/dl and presence of systemic edema were considered as poor prognostic factors. Prospective registration and standardized therapeutic intervention are needed to improve the prognosis of severe TAM cases.

要　旨　全国212の小児血液疾患診療施設に質問紙法によるアンケート調査を行って報告された，2003～2005年に本邦で発症したDown症候群のTAM 73例を報告した．内訳は男児33例，女児40例で在胎週数・出生時体重の中央値はそれぞれ37週3日，2,680 gであった．合併奇形は48例に認められ，うち先天性心疾患の合併が44例に認められた．また，TAMが自然寛解した後に骨髄異形成症候群の発症を2例に，急性巨核球性白血病の発症を11例に認めた．さらに，GATA1変異を検査が施行された10例中9例で認めた．調査時点で生存54例，死亡19例であり，死因は肝不全・腎不全等の臓器不全が多かった．予後不良因子として在胎週数37週未満，初診時白血球数10万/μl以上，直接ビリルビンの最悪値5 mg/dl以上，全身浮腫の存在などが挙げられた．前方視的登録による症例把握と標準化された推奨治療に基づく治療方針の確立が，重症TAMの予後の改善には必要である．

Key words:　TAM, Down's syndrome

I. はじめに

Down症候群（DS）の新生児の一部はtransient abnormal myelopoiesis（TAM）[1]を発症する．TAMは自然寛解する例が多いとされてきたが，その後の観察から生後4年以内にその20～30%が急性巨核球性白血病（AMKL）に進展するとされ，また近年致死的な肝線維症などを合併することが報告されてきた[2,3]．今回，日本小児血液学会会員登録施設に対し，TAMの後方視的調査を行ったので報告する．

II. 対象と方法

日本小児血液学会会員が診療を行っている全国212の小児血液疾患診療施設に調査票を送付し，2003年1月1日から2005年12月31日までに診断されたDSのTAM症例につき，質問紙法によるアンケート調査を行った．質問内容は周産期歴，DSの診断根拠，合併奇形，血液学的所見，血液生化学所見，臨床経過，治療内容，予後，死亡原因，その後の骨髄異形成症候群（MDS)/AMKLの発症などである．

別刷請求先：〒339-8551 さいたま市岩槻区馬込2100 埼玉県立小児医療センター血液・腫瘍科　菊地　陽
Reprint requests to Akira Kikuchi, Division of Hematology/Oncology, SaitamaChildren's Medical Center, 2100, Magome, Iwatsuki-ku, Saitama, 339-8551 Japan

III. 結　　果

調査票を送付した 212 施設中 150 施設から回答があり，回収率は 70.8%であった．150 施設中 110 施設では該当症例はなく，40 施設から計 73 例の報告があった．73 例の臨床所見のまとめを Table 1 に示す．73 例の内訳は男児 33 例，女児 40 例で在胎週数・出生時体重の中央値はそれぞれ 37 週 3 日，2,680 g であった．合併奇形は 48 例に認められ，うち先天性心疾患（CHD）の合併が 44 例と高頻度に認められた．また，TAM が自然寛解した後に MDS の発症を 2 例に，AMKL の発症を 11 例に認めた．また，TAM では GATA1 の変異が高率にみられることが知られているが[4]，今回の調査でも検査が行われた 10 例中 9 例で GATA1 変異を認めた．

調査時点での生存状況をみると 73 例中 54 例が生存していたが，19 例が死亡していた．全体の生存曲線を Fig. 1 に示す．死亡原因は Table 1 にあるとおりであるが，このうち CHD による 2 例と，AMKL に対する化学療法中の感染症死亡の 1 例の計 3 例を除く 16 例は，いずれも TAM に起因した早期死亡であった．

診断時の所見で生存・死亡に関連した臨床所見を以下に列挙する．

満期産/早産，体重，白血球数（WBC）10 万以上/未満，好中球%，リンパ球%，芽球%，芽球絶対数，異型リンパ球%，AST（診断時），ALT（診断時），LDH（診断時），骨髄線維化，出血症状，肝浸潤，胸水，心嚢水，全身浮腫．

これらのうち，在胎週数と白血球数でみると Table 2 に示すように，在胎 37 週未満で白血球数 10 万以上の群の生存率が極端に不良であることが明らかになった．また，直接型ビリルビン最悪値（DBmax）が 5 mg/dl 以上とそれ未満，全身浮腫の有無により層別化した生存曲線をそれぞれ Fig. 2，3 に示すが，それぞれ DBmax 5 mg/dl 以上と全身浮腫ありの群が有意に予後不良であった．TAM の重症例に対する治療介入の方法として従来 cytarabine 少量療法が報告されてきた．今回の調査でも cytarabine 少量療法が 9 例に，ステロイドの投与が 3 例に行われていたが，例数が少なく，こういった治療介入の意義についての評価は困難であった．

IV. 考　　察

今回，2003～2005 年の 3 年間に日本小児血液学会会員が診療を行っている全国 212 の小児血液疾患診療施設で診断された TAM 73 例を報告した．日本で年間におよそ 25 例が発症していたことになるが，後方視的調査であるため，その数字は正確ではない．今後，日本小児血液学会の前方視的な疾患登録事業によってより正確な発症頻度が明らかになるものと思われる．通常は自然寛解し，従来は予後良好と考えられてきた TAM であるが，調査時点での生存例は 73 例中 54 例であり，19 例は死亡していた．この 19 例のうち 16 例は TAM の病態に直接起因する早期死亡症例であり，TAM と診断された症

Table 1 Background of 73 TAM patients with DS between Jan 2003 and Dec 2005 in Japan

Gender:	Boys 33, girls 40
Gestational age:	37 weeks 3 days (27W3D–40W5D)
Birth weight:	2,680 g (1,028–4,062 g)
Accompanied anomaly:	Congenital heart disease 44, gastrointestinal 4, polydactily 2, polycytic kidney 1, rib anomaly 1
Chromosomal findings:	Simple 21 trisomy 67, 21 trisomy mosaic 2, Robertson translocation 4
GATA1 mutation:	Positive for 9 out of 10 patients examined
Later hematological malignancy:	MDS 2, AMKL 11
Survival status:	Alive 54, dead 19
Cause of death:	Hepatic failure 10, renal failure 5, DIC 3, ICH 1, MOF 1, CHD 2, Infection during chemotherapy for AMKL 1

W: weeks, D: days, DIC: disseminated intravascular coagulation, ICH: intracranial hemorrhage, MOF: multiple organ failure, CHD congenital heart disease.

Table 2 Survival rate of 73 TAM patients with DS according to gestational age and WBC count

Gestational age \ WBC count	Less than $100\times10^9/l$	$100\times10^9/l$ or more
37 weeks or more	92.1% (35/38)	50.0% (3/6)
Less than 37 weeks	85.7% (12/14)	26.7% (4/15)

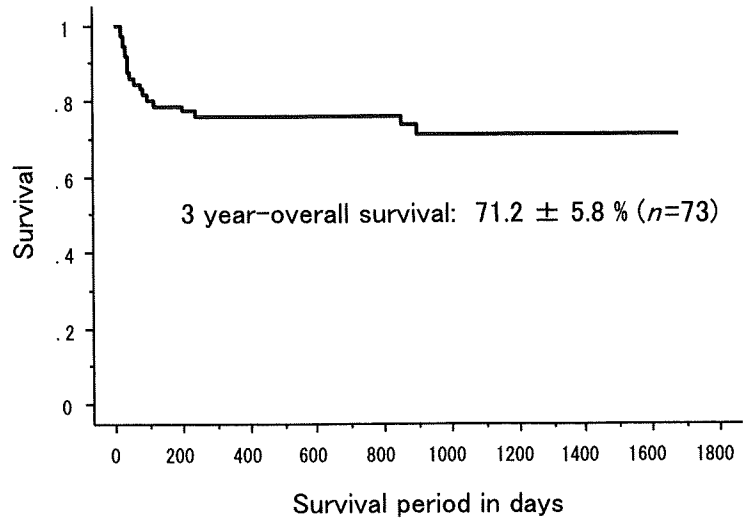

Fig. 1 Overall survival curve of 73 neonates of Down's syndrome with TAM

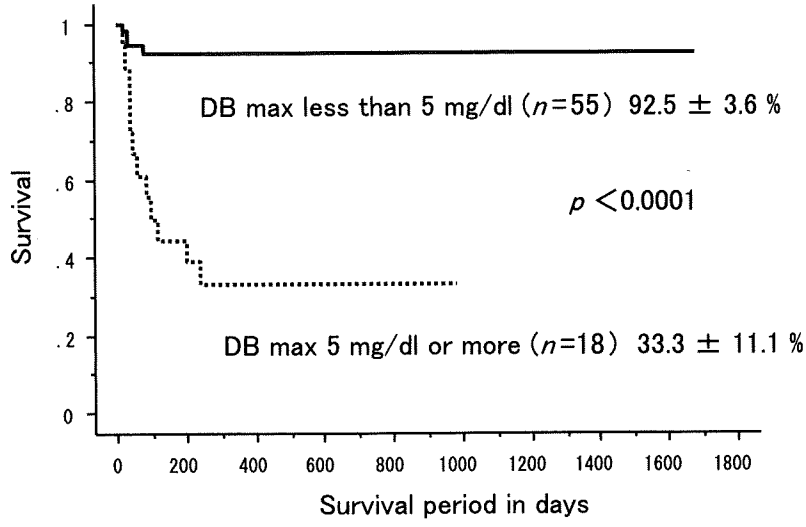

Fig. 2 Survival curves of TAM cases stratified by the maximum level of serum direct bilirubin

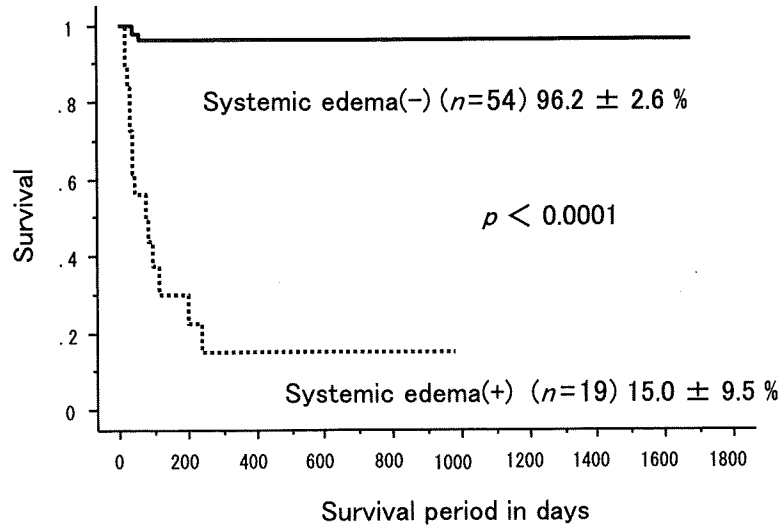

Fig. 3 Survival curves of TAM cases stratified by presence of systemic edema

例のおよそ2割が早期死亡していることがわかった．死亡原因は腫瘍細胞の浸潤による腫瘍死ではなく，肝不全・腎不全などの臓器不全が主であった．剖検例では肝の著明な線維化が報告されており，TAM の芽球から放出されるさまざまなサイトカインによる臓器障害の存在が推測されている[5]．死亡に関連した診断時の因子は調査項目の中からいくつか明らかとなったが，とくに在胎週数と診断時の WBC が重要と考えられ，在胎 37 週未満で WBC 10 万以上の症例の予後がきわめて不良であった．また他の予後因子として，DBmax 5 mg/dl 以上と全身浮腫ありの群が有意に予後不良であることが明らかとなった．これらの予後因子は従来の欧米からの報告でも同様に指摘されており[2,3]，これらの因子は DS の TAM における共通の予後因子となるものと考えられた．最近，村松らが東海地区の TAM 症例の集計を報告したが[6]，彼らが報告した症例群の病像は，死亡率・死亡原因・予後因子などが今回の結果ときわめて酷似していた．ごく一部の症例の重なりはあるものの，まったく独立して行った調査であり，今回の調査で得られた結果は DS の TAM 症例にほぼ普遍的に認められる所見と考えられた．

このようにして共通に認められた予後不良因子を背景として，治療介入をどのように行っていくのかということが今後の重要な課題である．Klusmann ら[3]は重症例に対する cytarabine 少量療法による治療介入の有用性を報告している．しかし，彼らの報告では投与対象症例の適格条件があいまいで cytarabine の投与量・投与期間に大きな幅があり，投与するタイミングについてもほとんど記載されておらず，治療レジメンとして統一したものが用いられたのではないように思われる．

今回のわれわれの調査では，在胎 37 週未満で WBC 10 万以上の症例の予後がきわめて不良であり，当面積極的に治療介入すべきなのはこの群に属する症例であると考えられた．また，DBmax が 5 mg/dl 以上であること，全身浮腫の存在することが有意に予後不良に関連するということが示された．ただ DBmax というのは最悪値であるため，この数値を示す状態まで待って治療介入するのでは時期を逸してしまうことになるので，その前に早めのタイミングでの治療開始を考慮すべきである．全身浮腫の発現機序としては，芽球から放出されるサイトカインによる血管透過性の亢進，腎不全の進行による体液貯留などが考えられるが，病態の進行に伴う終末像をみている可能性もあり，全身状態をみながら，腔水症の兆候が現れたら速やかに治療介入を考慮する必要があると考えられた．現時点で有効である可能性が示されている治療法は cytarabine 少量療法だけであるので，当面使用するのはこの治療ということになるが，具体的な至適投与量，至適投与期間についてはまだ検討の余地がある．投与対象が薬物の代謝能力が未成熟である早期産児が多くなることや，cytarabine 少量療法により骨髄の無形成が生じ，結果としてそれが死亡につながった報告例もあるため，治療介入の具体的方法に関しては慎重な検討が必要である．

従来は予後良好とされている TAM の重症例に遭遇した場合，臨床現場ではどういった症例にどのような時期にどのような治療介入をしてゆけばよいのかということが大変重要な問題になってくる．前方視的な登録による症例把握を行い，標準化された推奨治療に基づく治療を行うことが，重症 TAM の予後の改善のためには重要な課題であると考えられた．

引 用 文 献

1) Nagao T, Lampkin BC, Hug G: A neonate with Down's syndrome and transient abnormal myelopoiesis: Serial blood and bone marrow studies. Blood **36**: 443–447, 1970
2) Massey GV, Zipursky A, Chang MN, et al: A prospective study of the natural history of transient leukemia (TL) in neonates with Down syndrome (DS): Children's Oncology Group (COG) study POG-9481. Blood **107**: 4606–4613, 2006
3) Klusmann JH, Creutzig U, Zimmermann M, et al: Treatment and prognostic impact of transient leukemia in neonates with Down syndrome. Blood **111**: 2991–2998, 2008
4) 伊藤悦朗：Down 症候群の白血病発症の分子機構. 日小血会誌 **18**: 117–126, 2004
5) Hattori H, Matsuzaki A, Suminoe A, et al: High expression of platelet-derived growth factor and transforming growth factor-beta 1 in blast cells from Down syndrome suffering from transient myeloproliferative disorder and organ fibrosis. Br J Haematol **115**: 472–475, 2001
6) Muramatsu H, Kato K, Watanabe N, et al: Risk factors for early death in neonates with Down syndrome and transient leukaemia. Br J Haematol **142**: 610–615, 2008

ELSEVIER

Cancer Genetics and Cytogenetics 188 (2009) 99–102

CANCER GENETICS AND CYTOGENETICS

Short communication

Transient abnormal myelopoiesis in a Down syndrome newborn followed by acute myeloid leukemia: identification of the same chromosomal abnormality in both stages

Toshiyuki Kitoh[a,b,*], Tomohiko Taki[c], Yasuhide Hayashi[d], Kenji Nakamura[e], Tamotsu Irino[b,f], Mitsuhiko Osaka[f]

[a]*Department of Hematology/Oncology, Shiga Medical Center for Children, 5-7-30 Moriyama, Moriyama 524-0022, Moriyama, Japan*
[b]*Department of Laboratory Medicine, Shiga Medical Center for Children, Moriyama, Japan*
[c]*Department of Molecular Laboratory Medicine, Kyoto Prefectural University of Medicine Graduate School of Medical Science, Kyoto, Japan*
[d]*Gunma Children's Medical Center, Gunma, Japan*
[e]*Department of Pediatrics, Otsu Red Cross Hospital, Otsu, Japan*
[f]*Division of Cancer Research, Shiga Medical Center Research Institute. Moriyama, Japan*

Received 18 June 2008; received in revised form 31 July 2008; accepted 13 August 2008

Abstract A transient abnormal myelopoiesis was observed in a newborn with Down syndrome. Cytogenetic study revealed multiple oligoclonal abnormalities: 47,XY,inv(6)(p23q21),+21c[3]/47,XY, der(7)t(1;7)(q25;p15),+21c[1]/47,XY,del(13)(q?),+21c[1]/47,XY,+21c[15]. Ten months after the patient achieved remission, the transient abnormal myelopoiesis evolved to an acute megakaryoblastic leukemia. Cytogenetic study revealed only a single clonal abnormality, 47,XY,der(7) t(1;7)(q25;p15),+21c, identical to one of the structural changes seen at birth. Sequence analysis of the *GATA1* gene revealed a deletion–insertion mutation within the exon 2 introducing a stop codon after Arg 64. It may be that the der(7)t(1;7)(q25;p15) abnormality played some selective role in the development of acute megakaryoblastic leukemia in this patient. To our knowledge, the present case is unique in demonstrating a subclone with der(7)t(1;7)(q25;p15) evolving to acute leukemia.

1. Introduction

Approximately 10% of patients with Down syndrome are born with transient abnormal myelopoiesis (TAM) [1,2]. Of these cases, ~20% recur as acute megakaryoblastic leukemia (AMKL) [3]. Acquired mutations in *GATA1* in the leukemic blasts are detected in virtually all of these cases [4–7], but the second hit for the full expression of AMKL is still a matter of discussion [6,7]. GATA1 is a transcription factor that regulates megakaryocytic differentiation, and the mutations observed are considered to cause accumulation of poorly differentiated megakaryocytic precursors [4]. Strikingly, *GATA1* is located on chromosome X; therefore, genetic interaction of *GATA1* with one or more genes on other chromosomes presumably contributes to the development of AMKL in Down syndrome.

Acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) in Down syndrome often demonstrate chromosomal abnormalities in addition to the constitutional trisomy 21 [8–12]. These include both numerical and structural abnormalities, mostly complete or partial trisomy of a specific chromosome; reciprocal translocations are relatively rare. The role of these chromosomal translocations in developing leukemia in Down syndrome is also unknown. Here, we report the case of a Down syndrome patient who developed AMKL showing der(7)t(1;7)(q25;p15) following TAM at birth.

2. Case report

This Down syndrome patient was a boy born at 39 weeks gestational age, weighing 3,235 g. Thrombocytopenia was revealed after birth. At 5 days after birth, he was diagnosed as having TAM. Initial white blood cell count was 13,200/μL with 7% blasts, hemoglobin was 15.0 g/dL, and platelet count was 52,000/μL. Chromosomal analysis of bone marrow cells at diagnosis revealed the

* Corresponding author. Tel.: +81-77-582-6200; fax: +81-77-582-6304. (T. Kitoh).
E-mail address: tkitoh-mccs@umin.ac.jp (T. Kitoh).

0165-4608/09/$ – see front matter
doi:10.1016/j.cancergencyto.2008.08.011

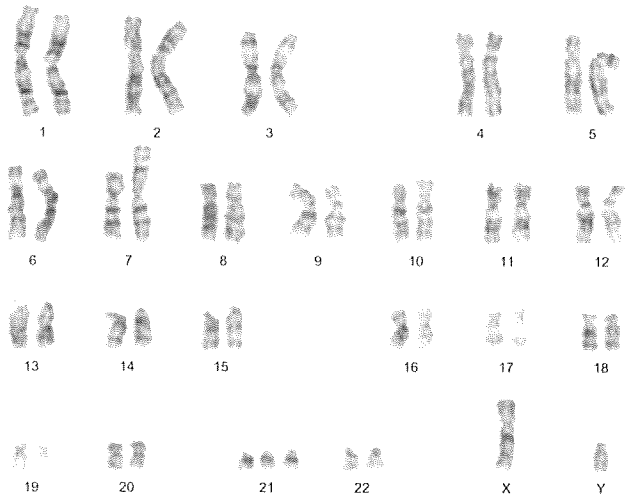

Fig. 1. Karyogram of the blast in leukemic phase showing 47,XY,der(7)t(1;7)(q25;p15),+21c.

karyotype 47,XY,inv(6)(p23q21),+21c[3]/47,XY,der(7)t(1;7)(q25;p15),+21c[1]/47,XY,del(13)(q?),+21c[1]/47,XY,+21c[15]/. Although he was not treated, his white blood cell count gradually decreased within 2 months, and at 7 months after birth his platelet count spontaneously recovered to within the normal range (although he suffered from thrombocytopenia due to an unknown viral infection). At 10 months after birth, the blasts increased suddenly.

Persistent thrombocytopenia was noted after platelet transfusions twice weekly. A bone marrow aspirate showed excessive myelofibrosis. Immunophenotyping of peripheral blasts showed $CD7^+$, $CD13^+$, $CD33^+$, $CD38^+$, $CD41^+$, $CD42b^+$, $CD56^+$, $CD157^+$, and $HLA\text{-}DR^+$. Chromosome analysis showed a der(7)t(1;7)(q25;p15) abnormality, in addition to the constitutional trisomy 21 (Fig. 1). Spectral karyotyping further confirmed the presence of the t(1;7) translocation, expressed as 47,XY,der(7)t(1;7)(q25;p15),+21c (Fig. 2), resulting in partial trisomy of 1q.

We analyzed the *GATA1* mutation in the peripheral blood sample, after written informed consent was obtained from his parents. Genomic DNA was extracted, and then polymerase chain reaction (PCR) was performed. Subcloning and nucleotide sequencing of PCR products were performed as described previously [6]. Sequence analysis of *GATA1* gene revealed a deletion–insertion mutation within exon 2, introducing premature stop codon after Arg 64 (Fig. 3).

A trephine biopsy revealed the presence of a typical megakaryocyte proliferation and prominent fibrosis. The final diagnosis of AMKL led to the initiation of combination therapy of pirarubicin HCl (25 mg/m^2 per day for 2 days), cytosine arabinoside (100 mg/m^2 per day for 7 days), and etoposide (150 mg/m^2 per day for 3 days) [13]. Complete remission was achieved after two courses of the therapy. Continuation of the consolidation therapy was uneventful, and six cycles of the same regimen were completed. As of writing, the patient had been in continuous complete remission without marked side effects for 5 years after initiation of the therapy.

3. Discussion

Recent collaborative studies on AML in Down syndrome children have established that the most frequent cytogenetic abnormality in AML or MDS in Down syndrome is trisomy 8 or partial trisomy of 1q [2,14–17]. Because AML and MDS with Down syndrome have distinct biologic and clinical features, the identification of Down syndrome patients with a mild or normal phenotype in the AML/MDS population is of fundamental importance for clinical diagnosis and management. Partial trisomy of 1q

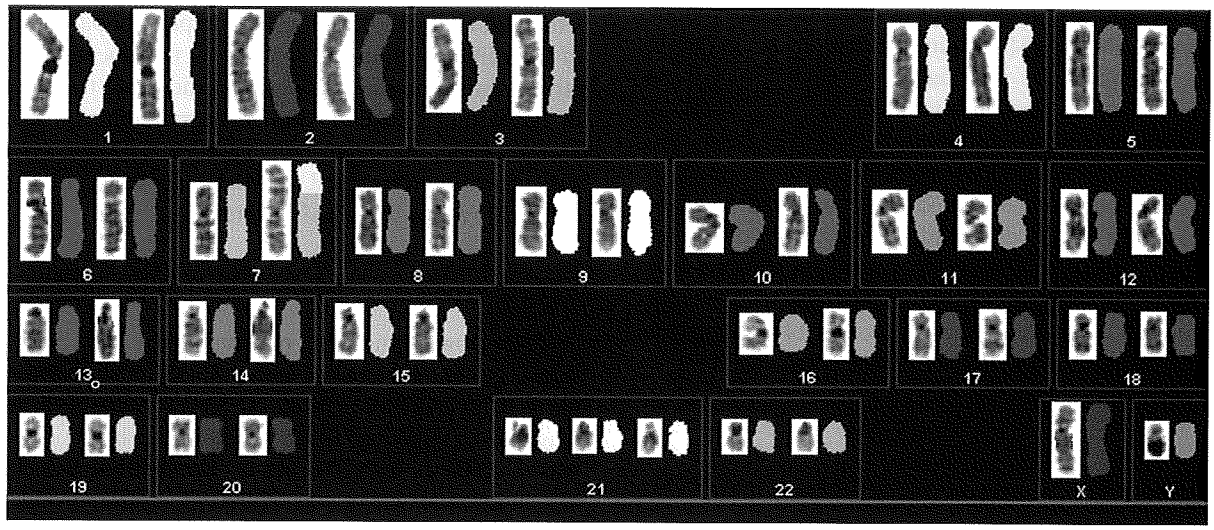

Fig. 2. Spectral karyotyping showing 47,XY,der(7)t(1;7)(q25;p15),+21c.

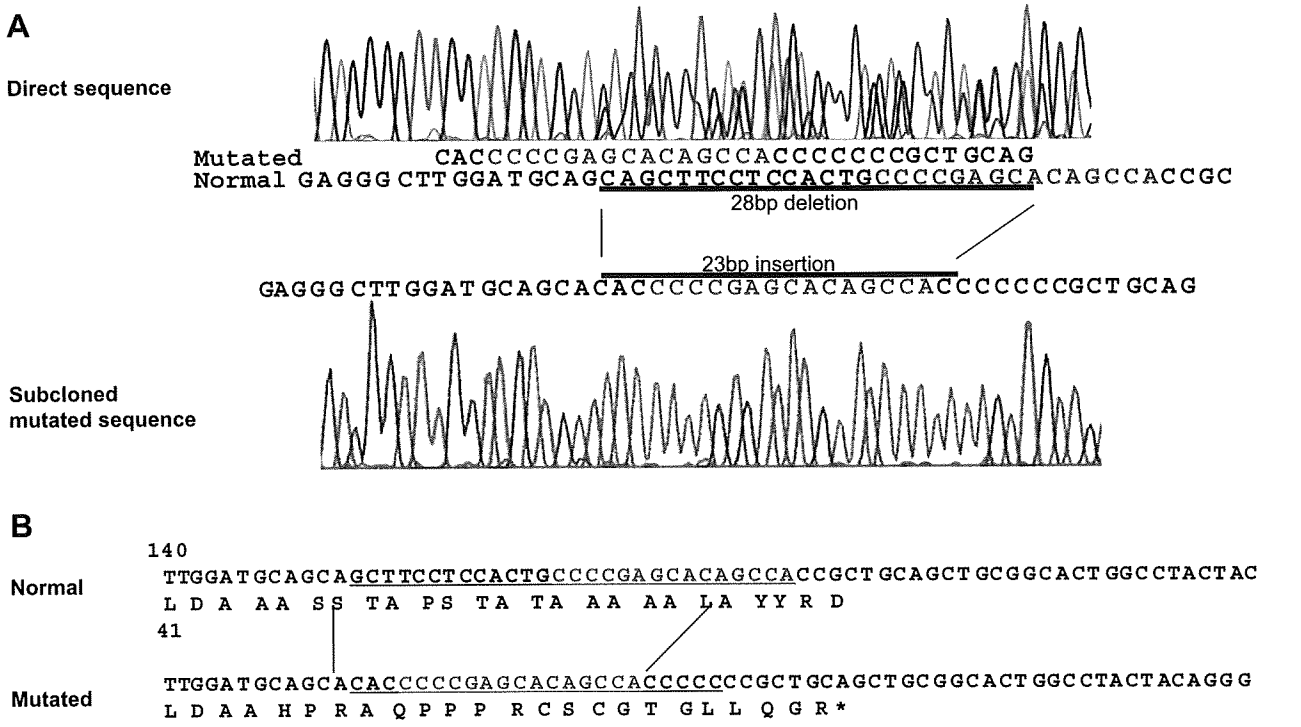

Fig. 3. Mutational analysis of the *GATA1* gene. (A) Sequence analysis was performed directly and using subcloned polymerase chain reaction product and showed a deletion–insertion mutation (28-bp deletion and 23-bp insertion) between 152 and 179 within exon 2. (B) This deletion–insertion mutation introduced a premature stop codon after Arg64. Numbers represent nucleotides from the 5′ end of exon 2.

has been reported by several authors and appears to represent a nonrandom chromosomal abnormality in patients with MDS/AML and Down syndrome [14,17]. Partial trisomy of 7q [8] or monosomy 1 [18,19] have also been reported. Unbalanced translocation t(1;7) in childhood myelodysplasia has been reported [20]. It is also possible that the t(1;7) played some role in the development of the MDS [21]. The mechanism of formation of the der(7)t(1;7) and its role in leukemogenesis are still unclear. Given that der(7)t(1;7) results in partial trisomy of 1q and partial monosomy of 7q, the increased dosage of the oncogenes located at 1q or the loss of the tumor suppressor genes located at 7q (or both factors) may be implicated in leukemogenesis of MDS and AML with der(7)t(1;7).

Cases of TAM usually have no karyotypic abnormality [1], but AMKL is associated with chromosomal abnormalities, including 8 trisomy and 19 trisomy [2]. Rare TAM cases have had chromosome abnormalities that were also observed in developing AMKL [22]. As for the *GATA1* gene, the deletion–insertion mutations within exon 2 in our patient have been reported previously in only two cases of TAM [5,7]. Reciprocal translocations are rare in TAM with Down syndrome. In the present case, a der(7)t(1;7) with partial trisomy of 1q, which is among the most frequently observed abnormalities in Down syndrome, might contribute to evolution to acute leukemia. The present report contributes insight into the mechanism of leukemic transformation from TAM in Down syndrome.

Acknowledgments

This work was supported by a grant-in-aid for cancer research from the Ministry of Health, Labor, and Welfare of Japan.

References

[1] Hayashi Y, Hanada R, Yamamoto K, Ohde S, Niitsu N, Eguchi M, Sugita K, Nakazawa S. Transient megakaryoblastic proliferation in a newborn infant with Down's syndrome. Cancer Genet Cytogenet 1987;28:373–4.

[2] Hayashi Y, Eguchi M, Sugita K, Nakazawa S, Sato T, Kojima S, Bessho F, Konishi S, Inaba T, Hanada R. Cytogenetic findings and clinical features in acute leukemia and transient myeloproliferative disorder in Down's syndrome. Blood 1988;72:15–23.

[3] Hitzler JK, Zipursky A. Origins of leukaemia in children with Down syndrome. Nat Rev Cancer 2005;5:11–20.

[4] Wechsler J, Greene M, McDevitt MA, Anastasi J, Karp JE, Le Beau MM, Crispino JD. Acquired mutations in *GATA1* in the megakaryoblastic leukemia of Down syndrome. Nat Genet 2002;32: 148–52.

[5] Rainis L, Bercovich D, Strehl S, Teigler-Schlegel A, Stark B, Trka J, Amariglio N, Biondi A, Muler I, Rechavi G, Kempski H, Haas OA, Izraeli S. Mutations in exon 2 of *GATA1* are early events in megakaryocytic malignancies associated with trisomy 21. Blood 2003;102: 981–6.

[6] Xu G, Nagano M, Kanezaki R, Toki T, Hayashi Y, Taketani T, Taki T, Mitui T, Koike K, Kato K, Imaizumi M, Sekine I, Ikeda Y, Hanada R, Sako M, Kudo K, Kojima S, Ohneda O, Yamamoto M, Ito E. Frequent mutations in the *GATA-1* gene in the transient myeloproliferative disorder of Down syndrome. Blood 2003;102:2960–8.

[7] Hitzler JK, Cheung J, Li Y, Scherer SW, Zipursky A. *GATA1* mutations in transient leukemia and acute megakaryoblastic leukemia of Down syndrome. Blood 2003;101:4301–4.

[8] Bunin N, Nowell PC, Belasco J, Shah N, Willoughby M, Farber PA, Lange B. Chromosome 7 abnormalities in children with Down syndrome and preleukemia. Cancer Genet Cytogenet 1991;54:119–26.

[9] Sawyer JR, Roloson GJ, Head DR, Becton D. Karyotype evolution in a patient with Down syndrome and acute leukemia following a congenital leukemoid reaction. Med Pediatr Oncol 1994;22:404–9.

[10] Zipursky A, Wang H, Brown EJ, Squire J. Interphase cytogenetic analysis of in vivo differentiation in the myelodysplasia of Down syndrome. Blood 1994;84:2278–82.

[11] Yamaguchi Y, Fujii H, Kazama H, Iinuma K, Shinomiya N, Aoki T. Acute myeloblastic leukemia associated with trisomy 8 and translocation 8;21 in a child with Down syndrome. Cancer Genet Cytogenet 1997;97:32–4.

[12] Duflos-Delaplace D, Lai JL, Nelken B, Genevieve F, Defachelles AS, Zandecki M. Transient leukemoid disorder in a newborn with Down syndrome followed 19 months later by an acute myeloid leukemia: demonstration of the same structural change in both instances with clonal evolution. Cancer Genet Cytogenet 1999;113:166–71.

[13] Kojima S, Sako M, Kato K, Hosoi G, Sato T, Ohara A, Koike K, Okimoto Y, Nishimura S, Akiyama Y, Yoshikawa T, Ishii E, Okamura J, Yazaki M, Hayashi Y, Eguchi M, Tsukimoto I, Ueda K. An effective chemotherapeutic regimen for acute myeloid leukemia and myelodysplastic syndrome in children with Down's syndrome. Leukemia 2000;14:786–91.

[14] Creutzig U, Ritter J, Vormoor J, Ludwig WD, Niemeyer C, Reinisch I, Stollmann-Gibbels B, Zimmermann M, Harbott J. Myelodysplasia and acute myelogenous leukemia in Down's syndrome: a report of 40 children of the AML-BFM Study Group. Leukemia 1996;10:1677–86.

[15] Kaneko Y, Rowley JD, Variakojis D, Chilcote RR, Moohr JW, Patel D. Chromosome abnormalities in Down's syndrome patients with acute leukemia. Blood 1981;58:459–66.

[16] de Alarcon PA, Patil S, Golberg J, Allen JB, Shaw S. Infants with Down's syndrome: use of cytogenetic studies and in vitro colony assay for granulocyte progenitor to distinguish acute nonlymphocytic leukemia from a transient myeloproliferative disorder. Cancer 1987;60:987–93.

[17] Litz CE, Davies S, Brunning RD, Kueck B, Parkin JL, Gajl Peczalska K, Arthur DC. Acute leukemia and the transient myeloproliferative disorder associated with Down syndrome: morphologic, immunophenotypic and cytogenetic manifestations. Leukemia 1995;9:1432–9.

[18] Blann MM, Morgan DL, Oblender M, Heinen B, Williams J, Tonk VS. Duplication of 1q in a child with Down syndrome and myelodysplastic syndrome. Cancer Genet Cytogenet 2000;119:74–6.

[19] Suarez CR, Le Beau MM, Silberman S, Fresco R, Rowley JD. Acute megakaryoblastic leukemia in Down's syndrome: report of a case and review of cytogenetic findings. Med Pediatr Oncol 1985;13:225–31.

[20] Horsman DE, Massing BG, Chan KW, Kalousek DK. Unbalanced translocation (1;7) in childhood myelodysplasia. Am J Hematol 1988;27:174–8.

[21] Hoo JJ, Szego K, Jones B. Confirmation of centromeric fusion in 7p/1q translocation associated with myelodysplastic syndrome. Cancer Genet Cytogenet 1992;64:186–8.

[22] Zipursky A. Transient leukaemia: a benign form of leukaemia in newborn infants with trisomy 21. Br J Haematol 2003;120:930–8.

Int J Hematol (2009) 89:470–481
DOI 10.1007/s12185-009-0299-0

ORIGINAL ARTICLE

Possible involvement of RasGRP4 in leukemogenesis

Naoko Watanabe-Okochi · Toshihiko Oki · Yukiko Komeno · Naoko Kato · Koichiro Yuji · Ryoichi Ono · Yuka Harada · Hironori Harada · Yasuhide Hayashi · Hideaki Nakajima · Tetsuya Nosaka · Jiro Kitaura · Toshio Kitamura

Received: 25 January 2009 / Revised: 24 February 2009 / Accepted: 8 March 2009 / Published online: 7 April 2009

Abstract It is now conceivable that leukemogenesis requires two types of mutations, class I and class II mutations. We previously established a mouse bone marrow-derived HF6, an IL-3-dependent cell line, that was immortalized by a class II mutation MLL/SEPT6 and can be fully transformed by class I mutations such as FLT3 mutants. To understand the molecular mechanism of leukemogenesis, particularly progression of myelodysplastic syndrome (MDS) to acute leukemia, we made cDNA libraries from the samples of patients and screened them by expression-cloning to detect class I mutations that render HF6 cells factor-independent. We identified RasGRP4, an activator of Ras, as a candidate for class I mutation from three of six patients (MDS/MPD = 1, MDS-RA = 1, MDS/AML = 2, CMMoL/AML = 1 and AML-M2 = 1). To investigate the potential roles of RasGRP4 in leukemogenesis, we tested its in vivo effect in a mouse bone marrow transplantation (BMT) model. C57BL/6J mice transplanted with RasGRP4-transduced primary bone marrow cells died of T cell leukemia, myeloid leukemia, or myeloid leukemia with T cell leukemia. To further examine if the combination of class I and class II mutations accelerated leukemic transformation, we performed a mouse BMT model in which both AML1 mutant (S291fsX300) and RasGRP4 were transduced into bone marrow cells. The double transduction led to early onset of T cell leukemia but not of AML in the transplanted mice when compared to transduction of RasGRP4 alone. Thus, we have identified RasGRP4 as a gene potentially involved in leukemogenesis and suggest that RasGRP4 cooperates with AML1 mutations in T cell leukemogenesis as a class I mutation.

Keywords RasGRP4 · AML1 · Class I mutation · Leukemogenesis · cDNA library

Electronic supplementary material The online version of this article (doi:10.1007/s12185-009-0299-0) contains supplementary material, which is available to authorized users.

N. Watanabe-Okochi · T. Oki · Y. Komeno · N. Kato · H. Nakajima · J. Kitaura · T. Kitamura (✉)
Division of Cellular Therapy, Advanced Clinical Research Center, The Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan
e-mail: kitamura@ims.u-tokyo.ac.jp

K. Yuji
Division of Molecular Therapy, Advanced Clinical Research Center, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan

R. Ono · T. Nosaka
Department of Microbiology, Mie University Graduate School of Medicine, Tsu-shi, Japan

Y. Harada
International Radiation Information Center, Research Institute for Radiation Biology and Medicine, Hiroshima University, Hiroshima, Japan

H. Harada
Department of Hematology and Oncology, Research Institute for Radiation Biology and Medicine, Hiroshima University, Hiroshima, Japan

Y. Hayashi
Department of Hematology/Oncology, Gunma Children's Medical Center, Shibukawa, Japan

1 Introduction

Various chromosome translocations and gene mutations were known to participate in leukemogenesis. Recently, it was recognized that multiple gene alterations are required

for leukemogenesis; coexistence of chromosomal translocations and gene mutations are frequently found in the same patient. There are some frequent combinations including c-Kit mutations and AML1/ETO [1–6], c-Kit mutations and inv(16) [1, 5–7], Ras mutations and AML1 point mutations [8, 9], FLT3-ITD and AML1 point mutations [10, 11], FLT3 mutations and PML-RARα [12–16], MLL rearrangement and FLT3-TKD [17], MLL rearrangement and Ras mutations [18], and FLT3-ITD and NPM1 mutations [19]. Interestingly, on the other hand, RAS and FLT3 mutations, which are detected in about 50% of patients with de novo AML, are negatively associated with each other [20, 21]. In mice models, while expression of PML/RARα in transgenic mice caused a nonfatal myeloproliferative syndrome, transplantation of bone marrow cells obtained from PML/RARα transgenic mice retrovirally transduced with FLT3-ITD resulted in development of an APL-like disease in a short latency [22]. Two step leukemogenesis was also suggested by an in vitro culture system of human hematopoietic cells [23]. Based on these findings, leukemia-related mutations are classified into two groups, class I and class II mutations. Class I mutations include activating mutations of tyrosine kinases and a small GTPase Ras or inactivation of apoptosis-related molecule, and these mutations induce cell proliferation or block apoptosis. On the other hand, class II mutations include dominant negative mutations of transcription factors involved in differentiation of hematopoietic cells, such as AML1/ETO, PML/RARa, or constitutively activated mutations of chromosome remodeling factors such as MLL-related fusion genes [24]. Indeed, it has been reported that a combination of class I and II mutations such as PML/RARα plus FLT3-ITD [22], AML1/ETO plus FLT3 mutation [25], AML1/EVI1 plus BCR/ABL [26], MLL/SEPT6 plus FLT3 mutation [27], K-ras plus PML/RARα [28] induced AML in a mouse BMT model, while either class I or II mutation alone led to, myeloproliferative disorders (MPD) or MDS like disease, not leukemia [22–28].

To identify class I mutations from patients with MDS/AML, MPD, or AML, we used retrovirus-mediated expression cloning; cDNA libraries from patients' samples were constructed and retrovirally transfected into an IL-3-dependent myeloid cell line, HF6, immortalized by a class II mutation MLL/SEPT6 [27]. We searched for class I mutations that abrogate IL-3 dependency of HF6 and we identified RasGRP4 as a candidate gene from three different libraries (MDS/MPD = 1, MDS/AML = 1, MDS-RA = 1). In addition, FLT3-ITD was identified in a patient with MDS/AML.

RasGRP4 belongs to a family of guanine nucleotide-exchange factors (RasGRP1-4) that positively regulate Ras and related small GTPases, and is mainly expressed in myeloid cells and mast cells [29, 30]. RasGRP4 appears to act downstream of the tyrosine kinase receptor c-Kit/CD117 [30]. RasGRP4 is located on 19q13.1 and alterations of this site have been found in several cancers (the "Cancer Chromosomes" at the NCI web site), and was previously isolated by expression cloning from cytogenetically normal AML patients using the focus-forming assay of NIH3T3 cells [29]. In the present study, we isolated RasGRP4 using expression cloning as a gene that fully transforms IL-3-dependent HF6 cells, and investigated the effect of RasGRP4 overexpression in a mouse BMT model and implicated RasGRP4 in leukemogenesis.

2 Materials and methods

2.1 Cell lines and cell culture

A mouse pro-B line Ba/F3 was maintained in RPMI1640/10% fetal bovine serum (FBS) containing 1 ng/ml recombinant mouse IL-3 (obtained from R & D systems). HF6, which had been established by introducing MLL/SEPT6 into mouse bone marrow cells, was maintained in RPMI1640/10% FBS containing 10 ng/ml mouse IL-3 as described [27].

2.2 Screening of cDNA libraries

Complementary cDNA libraries were generated from patients leukemic or MDS cells (MDS/MPD = 1, MDS/AML = 2, CMMoL/AML = 1, MDS-RA = 1, AML-M2 = 1) as described [31]. MDS or leukemic cells of these patients did not harbor recurrent chromosomal translocations involving AML1 or MLL. One patient with AML-M2 did not display t(8;21). The point mutations of AML1 were not screened. Recombinant retroviruses were generated by transient transfection using an ecotropic packaging cell line PLAT-E as described with minor modifications [32]. Bone marrow or peripheral blood samples of patients were taken under the experimental procedure approved by the ethical committees of our institute (approve no. 20-9).

We introduced each cDNA library into two IL-3-dependent cell lines Ba/F3 and HF6. After transduction with the cDNA library, the transduced cells were seeded into 96-well plates in the absence of IL-3, and factor independent clones were isolated. To identify the cDNA that confers factor independency on Ba/F3 or HF6, genomic DNA of the factor independent clones were purified and integrated cDNAs were isolated and sequenced.

2.3 Vector construction

cDNAs for human RasGRP4 were cloned from cDNA libraries of MDS/MPD patients and normal volunteers using PCR primers: 5′-GGAGCTGAGCCCTACTCTTG-3′

(forward), 5′-AGAGTCTGACGGCAGGACTC-3′ (reverse). We used pfu polymerase (Stratagene, La Jolla, CA) to amplify the coding region of human RasGRP4. We subcloned the PCR products into TOPO vector (Invitrogen, San Diego, CA). Then, the EcoRI fragment carrying RasGRP4 was inserted into the EcoRI sites of pMXs vector [32]. RasGRP4 sequences derived from patients and normal volunteers were not identical to those in the data bases as described in result section. We used an AML1 mutant, S291fsX300, identified from case number 27 among MDS/AML patients [33]. This mutant is hereafter referred to as AML1-S291fs. The AML1-S291fs was inserted upstream of the IRES-EGFP cassette of a retrovirus vector pMYs-IG [32] to generate pMYs-AML1-S291fs-IG.

2.4 Expression of RasGRP4 in HF6

To confirm that the isolated RasGRP4 is responsible for factor-independency of HF6, the cells were infected with the retroviruses harboring pMXs-RasGRP4 derived from patients, normal volunteers or an empty vector as a control, and cultured in the absence of IL-3. To investigate the activation of the Ras pathway in the HF6 cells expressing RasGRP4, the transfected cells were lysed in lysis buffer, and lysates were subject to western blot analysis as described with minor modifications [34]. Monoclonal mouse anti-phospho-p44/42 MAPK (Thr^{202}/Tyr^{204}) antibody (Sigma) was used for phosphorylated ERK1/2.

2.5 Bone marrow transplantation

Bone marrow mononuclear cells were isolated and cultured as described [35]. The prestimulated cells were infected for 60 h with the retroviruses harboring pMXs-RasGRP4 derived from a patient with MDS/MPD, pMYs-AML1-S291fs-IG or an empty vector as a control, using six well dishes coated with RetroNectin (Takara Bio, Inc.) according to the manufacturer's recommendations. Then, 0.3–1.2 $\times$ 10^6 of infected bone marrow cells (Ly-5.1) were injected through tail vein into C57BL/6 (Ly-5.2) recipient mice (8–12 weeks of age) which had been administered a sublethal dose of 5.25 Gy total-body γ-irradiation (135Cs). Overall survival of the transplanted mice was analyzed using the Kaplan–Meier-method. All animal studies were approved by the Animal Care Committee of the Institute of Medical Science, The University of Tokyo.

2.6 Analysis of the transplanted mice

Engraftment of bone marrow cells was confirmed by measuring the percentage of Ly-5.1-positive and/or GFP positive cells in peripheral blood obtained every 1–2 months after the transplant. After the morbid mice were euthanized, their tissue samples including peripheral blood (PB), bone marrow (BM), spleen, liver, and kidney were analyzed. Circulating blood cells were counted by automatic blood cell counter KX-21 (Sysmex, Kobe, Japan). Morphology of the peripheral blood cells was evaluated by staining of air-dried smears with Hemacolor (Merck). Tissues were fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H & E). Cytospin preparations of bone marrow and spleen cells were also stained with Hemacolor. The percentage of blasts, myelocytes, neutrophils, monocytes, lymphocytes, and erythroblasts was estimated by examination of at least 200 cells. To assess whether the leukemic cells were transplantable, 2 $\times$ 10^5–1 $\times$ 10^6 total BM cells including blasts were injected into the tail veins of sublethally irradiated mice. A total of two or three recipient mice were used for each serial transplantation.

2.7 Flow cytometric analysis

Peripheral blood or single-cell suspensions of bone marrow and spleen were stained with the following phycoerythrin (PE)-conjugated monoclonal antibodies: Ly-5.1, Gr-1, CD11b, B220, CD3, CD4, CD8, CD41, c-Kit, Sca-1, CD34, and Ter-119. Then, flow cytometric analysis was performed as described [35].

2.8 RT-PCR

To confirm expression of human RasGRP4, total RNA was extracted from BM cells of transplanted mice using Trizol (Invitrogen, California, USA) and cDNA was prepared with the Superscript II RT kit (Invitrogen, California, USA) and RT-PCR was performed using a 2720 Thermal cycler (Applied Biosystems, Tokyo, Japan). The cDNA was amplified using AmpliTaq Gold (Applied Biosystems by Roche Molecular Systems, Inc., New Jersey. USA). The reaction was subject to one cycle at 95°C for 5 min, 30 cycles of PCR at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. All samples were independently analyzed at least three times. The following primer pairs were used: 5′-ACTGGCTGATGCGACACCC-3′ (forward) and 5′-GAGATGGCACTGTGACACAG-3′ (reverse) for human RasGRP4, 5′-ACCACAGTCCATGCCATCAC-3′ (forward) and 5′-TCCACCACCCTGTTGCTGTA -3′ (reverse) for GAPDH.

2.9 Quantitative RT-PCR

To examine expression levels of human RasGRP4 in patients, quantitative RT-PCR was performed. Quantitative RT-PCR was performed using a LightCycler Workflow System (Roche Diagnostics, Mannheim, Germany).

Complementary DNAs derived from bone marrow cells of leukemia or MDS patients as well as normal bone marrow cells were amplified using a SYBR Premix EX Taq (TAKARA). The reaction was subject to one cycle at 95°C for 30 s, 45 cycles of PCR at 95°C for 5 s, 55°C for 10 s, and 72°C for 10 s. All samples were independently analyzed at least three times. The primer pairs for human RasGRP4 and GAPDH were the same as described above. The samples from the patients were obtained under written consents which had been approved by the local ethical committee of each institute or hospital.

2.10 Bubble PCR

Genomic DNA was extracted from BM or spleen cells of transplanted mice and digested with EcoRI, and then the fragments were used for Bubble PCR to identify the integration sites of the retroviruses as described [35]. We confirmed inverse repeat sequence "GGGGGTCTTTCA" as a marker of junction between genomic DNA and retrovirus sequence.

3 Results

3.1 RasGRP4 induces factor-independent growth of HF6

In the screening of cDNA libraries, some wells gave rise to cell growth in the absence of IL-3 from HF6 but not from BaF3 cells. The factor-independent clones were isolated and the cDNAs integrated in the genome DNA were sequenced using PCR. FLT3-ITD was identified in one MDS/AML patient. In addition, RasGRP4 was identified from three different libraries (MDS/MPD = 1, MDS/AML = 1, and MDS-RA = 1). We introduced the isolated RasGRP4 into HF6 to confirm that RasGRP4 was responsible for autonomous growth of HF6 cells (Fig. 1a). In the sequence of RasGRP4 derived from the MDS/MPD, MDS/AML and MDS-RA patients, we found several different amino acids that compared with the sequences in two databases GenBank (accession number AF448437) and GenBank (accession number AY048119) (Table 1). Therefore, we introduced RasGRP4 derived from a patient and two Japanese normal volunteers (normal 1 and 2 in Table 1) into HF6 cells to examine if RasGRP4 from normal volunteers also gives rise to factor-independency. As a result, RasGRP4 from normal volunteers also induced factor-independent growth of HF6, indicating that overexpression of RasGRP4 by itself induced transformation of the cells, independent of some mutations in the amino acid sequence of RasGRP4. While several gene alterations were observed in the samples of patients, we focused on E468K because this change was observed only in a patient with MDS/MPD but not in the sequence derived from the two databases and two normal volunteers (Table 1). However, we did not find any functional importance of the alteration at codon 468 that changes a glutamic acid to a lysine. Moreover, SNPs of this gene are not correlated with lymphoma and leukemia (Y. Nakamura, unpublished results).

To assess the RasGRP4-mediated Ras activation, we examined phosphorylation of ERK1/2 using HF6-cells-transduced RasGRP4. Stimulation with IL-3 induced much stronger phosphorylation of ERK1/2 in the HF6 cells expressing RasGRP4 when compared with parent HF6 cells (Fig. 1b, lanes 6–8). Although we did not observe enhanced phosphorylation of ERK1/2 in the cells overexpressing RasGRP4 without IL-3 (Fig. 1b, lane 5), we assume that non-detectable enhancement of ERK1/2 was

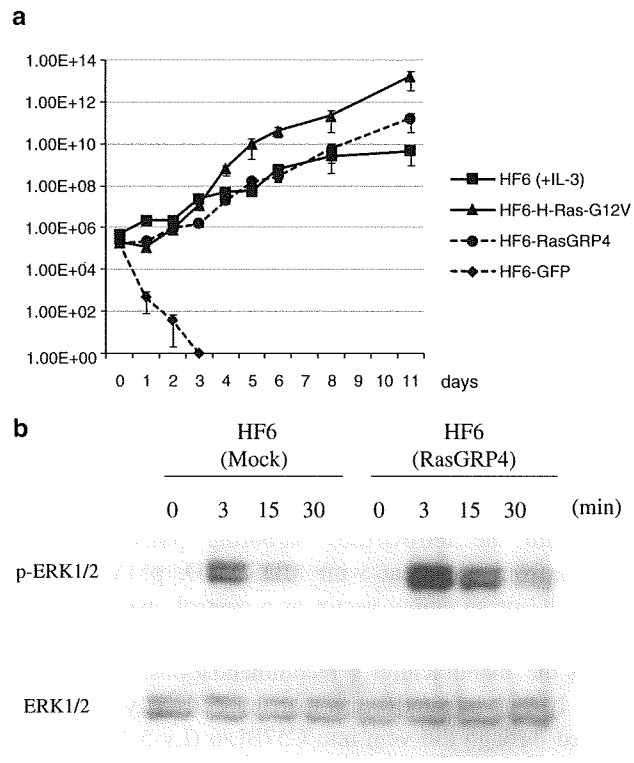

Fig. 1 RasGRP4 conferred factor independency on HF6. **a** HF6 cells expressing the H-Ras-G12V, RasGRP4 and GFP vector were deprived of IL-3, and cells were counted by trypan blue exclusion. The parental HF6 cells in the presence of IL-3 (10 ng/mL) were counted as same. **b** Stimulation with IL-3 induced strong phosphorylation of ERK1/2 in the HF6 cells expressing RasGRP4. Phosphorylation of ERK1/2 (pERK1/2) was examined in HF6 cells transfected with RasGRP4 or empty by western blot analysis using anti-phospho-p44/42 MAPK (Thr^{202}/Tyr^{204}) Ab. Loading amount was estimated by re-probing immunoblots with Abs specific for ERK1/2. The transfected HF6 cells were washed with PBS twice and cultured in RPMI1640/10% FBS without IL-3 for 4 h. Then, some of cells were collected and lysed (*lanes 1 and 5*). The remained cells were stimulated with IL-3 (100 ng/mL) for the indicated period and collected and lysed (*lanes 2–4*, and *6–8*)

Table 1 Polymorphism of RasGRP4

Position of amino acid	AF448437	AY048119	Patient 1 (MDS/MPD)	Patient 2 (MDS/AML)	Patient 3 (MDS-RA)	Normal 1	Normal 2
18	T	T	T	I	T	T	I
120	Q	L	Q	Q	Q	Q	Q
261	R	C	R	R	R	R	R
468	E	E	K	E	E	E	E
541	H	H	H	H	H	H	Y
671	L	P	P	L	L	L	L

enough to induce factor-independent growth of HF6. Only weak activation of the signaling molecule, even non-detectable in biochemical experiments, sometimes induces autonomous cell growth.

3.2 RasGRP4 induced myeloid leukemia and T cell leukemia in mice

We further examined if overexpression of RasGRP4 induced leukemia in a mouse BMT model. We confirmed expression of human RasGRP4 in BM cells of transplanted mice by RT-PCR (Fig. 2a). Transduction of RasGRP4 (E468K) induced myeloid and/or T cell leukemia with various phenotypes, and the transplanted mice died within 2–8 months after the transplantation (Fig. 2b). For example, a mouse (ID 402) died of T cell leukemia with thymoma (weight of thymus was 1,416 mg) and hepatosplenomegaly on day 252 after the transplantation. Leukemic cells showed a CD4- and CD8-double-positive phenotype (Fig. 3). One other mouse developed a similar disease (ID 401). Unfortunately, this mouse died on day 224 before we found out. Therefore, we could only confirm hepatosplenomegaly and a giant thymoma after the death. Two mice (ID 407 and 408) died of AML with hepatosplenomegaly on days 47 and 66 after the transplantation. Severe leukocytosis, anemia and thrombocytopenia were observed in a mouse (ID 408), but severe pancytopenia was observed in the other mouse (ID 407). Leukemic cells of the mouse (ID 408) in bone marrow and thymus uniformly expressed Gr1, CD11b, and B220 on their surfaces (Fig. 3). Four of the transplanted mice (ID 403, 404, 405 and 406) developed both myeloid and T cell leukemia with hepatosplenomegaly, and in some cases, thymoma (ID 404, 405 and 406). In the mouse ID 404, both myeloid and T cell leukemia cells were observed in the bone marrow, while peripheral blood was occupied with myeloid leukemia and thymus was occupied with T cell leukemia (Figs. 3, 4). In summary, two mice died of AML after a short latency (days 47 and 66), two mice died of T cell leukemia after a long latency (days 224 and 252), and four mice died of AML and T cell leukemia (days 76, 83, 129, and 248). The

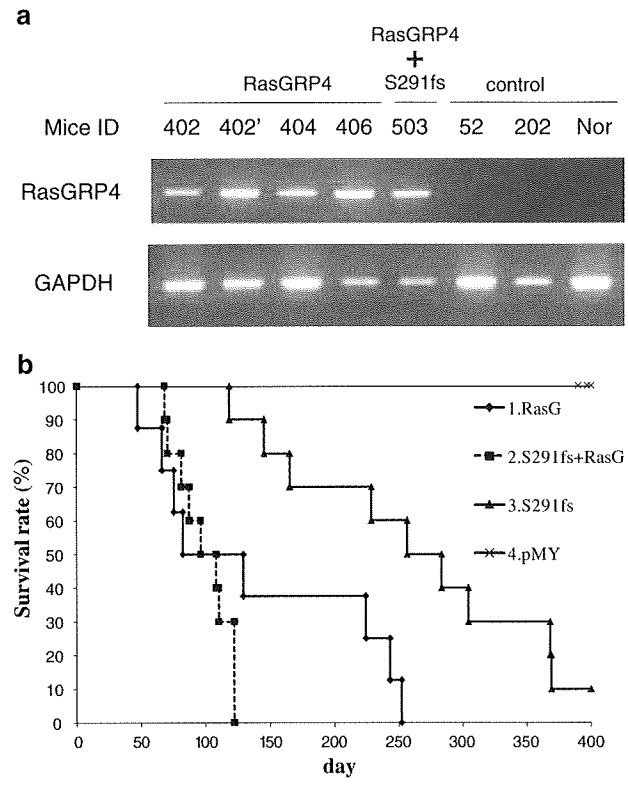

Fig. 2 Co-transduction of RasGRP4 and AML1-S291fs led to early onset of leukemia. **a** Expression of retrovirally introduced RasGRP4 in BM cells. Total RNA from BM cells of transplanted mice were extracted, and the derived cDNAs were subjected to RT-PCR. Mice IDs were shown on the top of the panel. ID 402′ is a second recipient of ID 402. Controls are AML1-S291fs (ID 52), empty vector (ID 202), and normal mouse (Nor). **b** Kaplan–Meier analysis for the survival of mice transplanted with RasGRP4, AML1-S291fs, and double-transduced BM cells. Average survival of RasGRP4 alone (139.8 days) was not significantly different when compared with double transduced mice (101.5 days) ($P = 0.223$, log rank test). Average survival of the double transduced mice (101.5 days) was significantly shorter than that of AML1-S291fs-transduced mice (263.6 days) ($P = 0.00003$, log rank test). RasGRP4 ($n = 8$), AML1-S291fs ($n = 10$), RasGRP4 + AML1-S291fs ($n = 11$), mock ($n = 16$) transduced bone marrow cells were transplanted to mice

details of individual mice are shown in Table 2 and Fig. 5. To assess whether the leukemic cells were transplantable, 2×10^5–1×10^6 total BM cells including blasts were

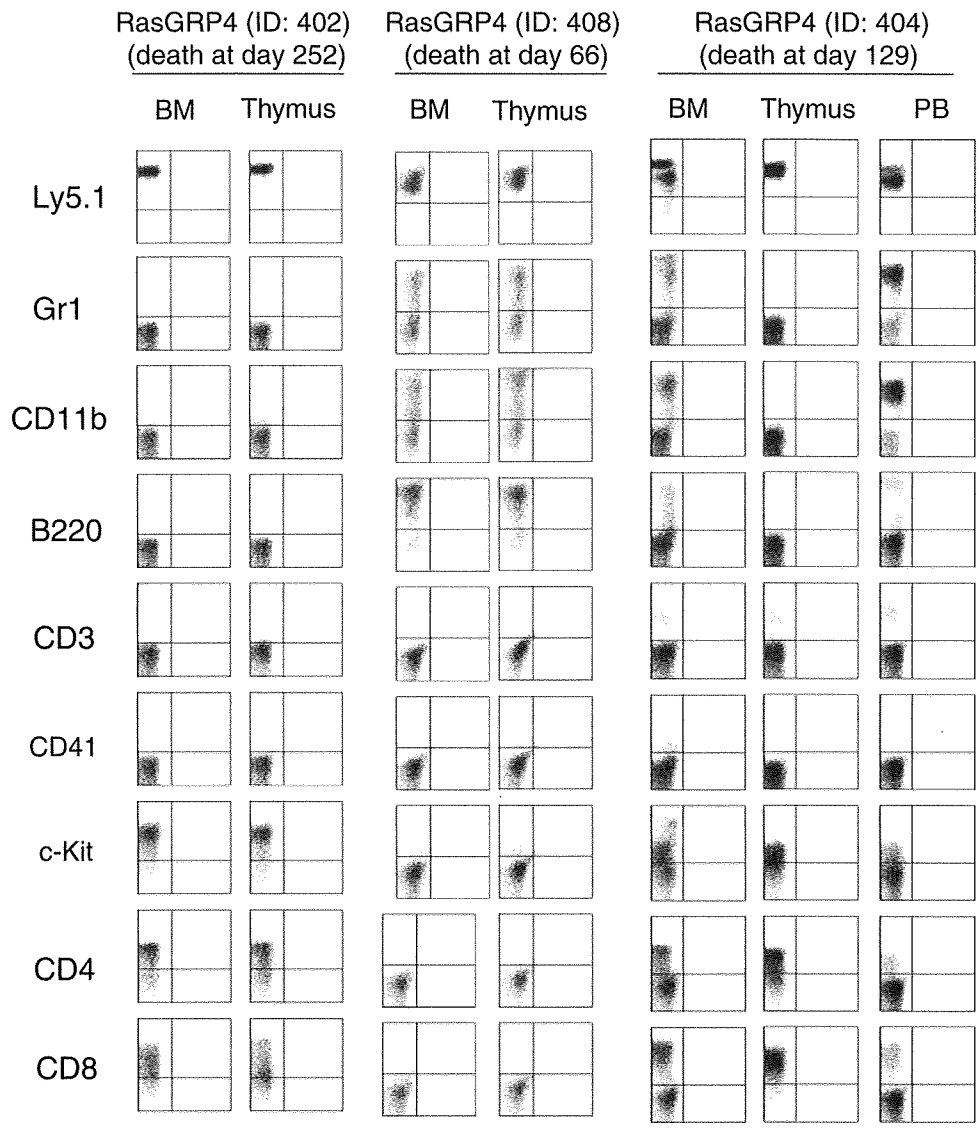

Fig. 3 RasGRP4 induced T cell leukemia and myeloid leukemia in the BMT model. The *dot plots* show Ly5.1, Gr-1, CD11b, B220, CD3, CD41, c-kit, CD4, or CD8 expression detected by corresponding PE-conjugated mAb

injected into recipient mice. We confirmed that both T cell leukemia and myeloid leukemia cells were serially transplantable although the phenotypes slightly changed after the serial transplantation (Supplemental Fig. 1).

3.3 Different integration sites were identified from T cell or myeloid leukemia cells derived from an individual mouse

To examine if the T cell and myeloid leukemia cells were derived from different clones or the same clone, we identified the integration sites in genomic DNA samples of thymus, peripheral blood or bone marrow cells. As shown in Table 3, different integration sites were identified from T cell and myeloid leukemia cells derived from an individual mouse, suggesting that T cell and myeloid leukemic cells were derived from different clones.

The integration near the Samsn1 gene was found twice in ID 405 and ID 406. These mice were transplanted on the same day. The integration site was identical among these leukemic cells indicating that leukemic cells of the two cases were derived from a single hematopoietic progenitor. This result suggests that the integration induced expansion of the transduced stem cells during the 3-day-culture period before the transplantation. Indeed, the mice with the integration at Samsn1 site developed AML with the same phenotype (CD11b positive) and similar latencies (83 and 76 days). On the other hand, different T lineage clones grew up in thymus and developed thymoma.

3.4 RasGRP4 cooperates with an AML1 mutant in leukemogenesis

RasGRP4 appears to function downstream of the tyrosine kinase receptor c-Kit/CD117 [30]. High expression of c-kit

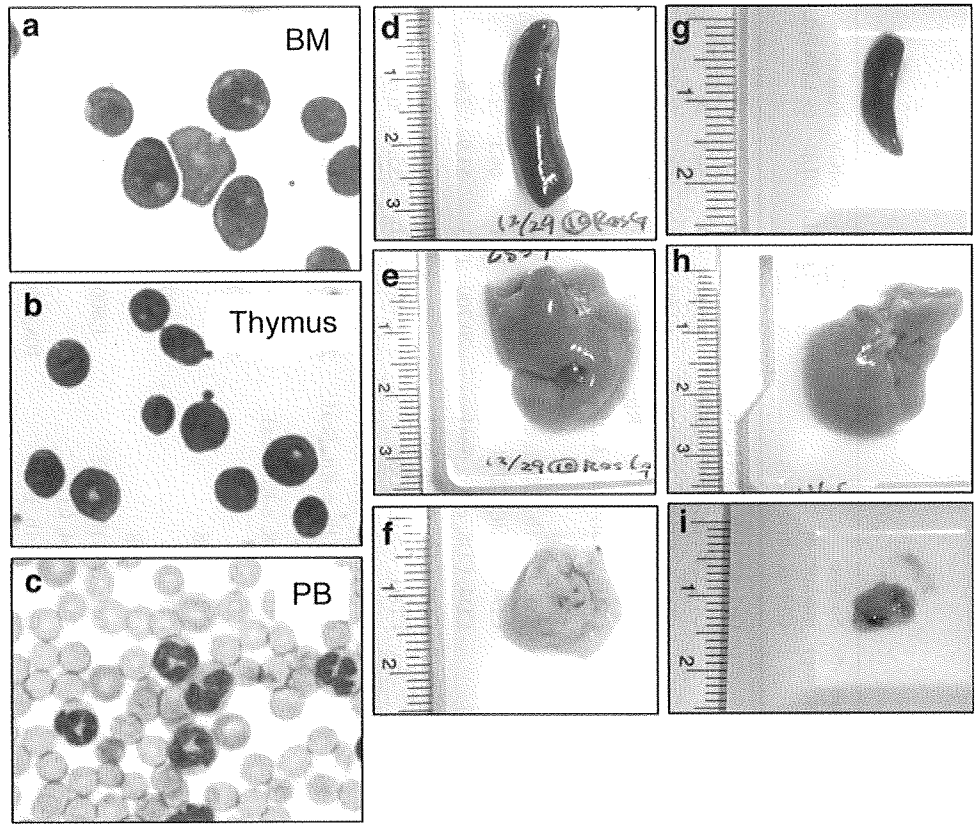

Fig. 4 RasGRP4 induced both of T cell leukemia and myeloid leukemia in the same mouse. Giemsa-stained cells derived from **a** bone marrow, **b** thymus, and **c** peripheral blood obtained from mouse ID 404. Macroscopic findings of **d** spleen, **e** liver, **f** thymus from mice ID 404; *left* **g** spleen, **h** liver, **i** thymus from normal mice; *right* are shown. Images (**a**, **b**, **c**) were obtained with a BH51 microscope and DP12 camera (Olympus, Tokyo, Japan); objective lens, UPlanFl (Olympus); ×1,000

has been found in 60–80% of AML [36] and higher expression is observed in 81.3% of patients with t(8;21) when compared with the patients with other leukemias [2]. Niimi et al. [9] reported that MDS/AML arising from AML1/RUNX1 mutations frequently involves receptor tyrosine kinase (RTK)-RAS signaling pathway activation. We have recently demonstrated that bone marrow cells transduced with AML1 mutants induced MDS-like symptoms after a long latency [35]. Therefore, we also tested if the combination of RasGRP4 and AML1-S291fs, one of the AML1 mutants, induced rapid leukemic transformation in a BMT model. As a result, co-transduction of RasGRP4 and AML1-S291fs led to early death in the transplanted mice (average 101.5 days, $n = 11$) than the expression of RasGRP4 alone (average 139.8 days, $n = 8$) (Fig. 2b). We diagnosed the double-transfected disease mice as T cell leukemia because of enlarged thymus, hepatosplenomegaly, and expansion of blast expressing CD3, CD4, and CD8 in bone marrow, peripheral blood, and thymus (Fig. 6). The onset of T cell leukemia was significantly earlier in the RasGRP4 + AML1 mutant (average 102.7 days, $n = 9$) than RasGRP4 alone (average 238 days, $n = 2$). On the other hand, onset of AML was not significantly changed between RasGRP4 + AML1 mutant (average 96 days, $n = 2$) and RasGRP4 alone (average 56.5 days, $n = 2$) transplanted mice.

3.5 RasGRP4 was overexpressed in some patients with hematological malignancies

We examined expression levels of RasGRP4 in patients with myeloid or T lineage hematological malignancies. As shown in Fig. 7, cells from some patients (T-ALL, AML-M1, MDS-RAEB, MDS-RA, CMMoL) overexpressed RasGRP4.

4 Discussions

We identified RasGRP4 from patients' cDNA libraries as a gene that renders IL-3-dependent HF6 cells factor independent when expressed at high levels via retrovirus-mediated gene transfer. Although we did not find any gain-of-function mutation of RasGRP4 in three patients from whom we identified cDNA for RasGRP4, and we detected high expression of RasGARP4 in only one out of the three patients, it is possible that overexpression or activating mutations are found in patients with malignant diseases including leukemia and MDS. Thus, RasGRP4 is a candidate gene for class I mutations. In addition to RasGRP4, we also identified FLT3-ITD from a patient with MDS/AML, thus showing the feasibility of our functional cloning strategy. The HF6 cells were immortalized by expression

Table 2 Hematological data of the transplanted mice

Gene	Mice ID	GFP (%)	Ly5.1 of BM (%)	Ly5.1 of Thymus (%)	Period from BMT (day)	Spleen weight (mg)	Liver weight (mg)	Thymus weight (mg)	Suefice markers of GFP positive cells	WBC (μL)	Hb (g/dL)	MCV (fL)	Platelet (×1,000/μL)
S291fs	51	27.4	39.8	–	368	153	1,236	–	Gr1, CD11b, CD41, cKit	4,800	6.6	60.0	27.0
S291fs	52	47.8	83.0	–	256	90	1,568	–	Gr1, CD11b, CD41, cKit	4,500	9.9	59.8	38.3
S291fs	54	29.8	45.3	–	304	338	1,812	–	CD11b, CD41, cKit, CD34	1,800	10.4	61.3	4.1
S291fs	55	75.2	83.1	–	165	166	1,574	–	Gr1, CD11b, CD41, cKit,	1,200	4.8	76.4	5.7
S291fs	56	54.9	85.2	–	118	73	1,318	–	CD41, cKit, Sca1, CD34,	2,900	4.5	72.7	4.6
S291fs	57	–	–	–	145	181	1,249	–	–	5,700	2.4		5.1
S291fs	58	72.7	56.5	–	228	225	1,582	–	Gr1, CD11b, CD41, cKit	2,100	9.9	62.7	26.7
S291fs	60	76.3		–	369	186	1,682	–	CD41, c-Kit, CD34	7,500	11.1	66.1	22.2
RasGRP4	402	–	96.5		252	318	1,975		CD4, CD8, c-Kit, Sca1	20,700	12.3	51.9	3.6
				98.9				1,416	CD4, CD8, c-Kit, Sca1				
RasGRP4	403	–	93.5		248	696	2,138		CD41, c-Kit, CD34	8,400	7.2	55.1	0.0
				85.9				92	CD3, CD4, CD8, Sca1				
RasGRP4	404	–	93.9		129	714	2,557		Gr1, CD11b, c-Kit, Sca1, CD4, CD8	282,000	11.7	56.6	3.0
				99.7				777	CD4, CD8, c-Kit, Sca1				
RasGRP4	405	–	91.3		83	1,184	3,699		CD11b	558,900	7.2	57.1	9.9
				98.3				139	CD4, CD8				
RasGRP4	406	–	94.8		76	1,348	2,678		CD11b	96,900	6.6	59.7	1.2
				99.5				609	CD4, CD8				
RasGRP4	407	–	32.0		47	670	4,795		Gr1, CD11b, c-Kit	1,800	4.5	67.6	2.7
				17.1				95	CD3, CD4, CD8				
RasGRP4	408	–	95.7		66	1,110	4,155		Gr1, CD11b, B220	108,900	5.1	104.0	3.9
				93.5				202	Gr1, CD11b, B220				
S291fs + RasGRP4	503	92.1	92.1		96	1,180	6,065		CD3, CD4, CD8	33,600	10.3	64.5	9.3
		95.0		95.0				112	CD3, CD4, CD8				
S291fs + RasGRP4	507	–	–		68	302	–		CD3, CD4, CD8				
				–				805	CD3, CD4, CD8				
S291fs + RasGRP4	513	75.8	80.7		130	544	2,784		CD3, CD8	59,500	10.7	68.8	2.2
		83.8		90.9				155	CD3, CD8				

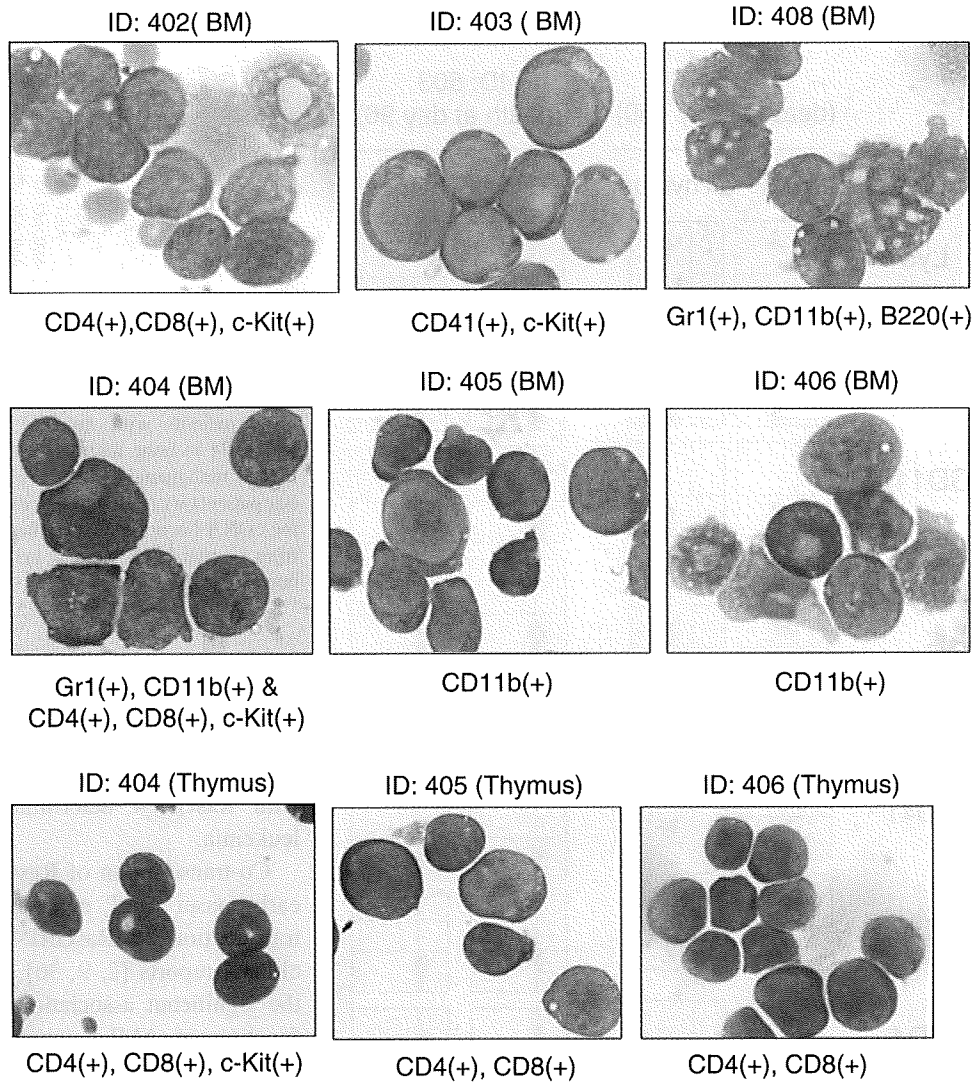

Fig. 5 Morphology of leukemic cells induced by RasGRP4. Giemsa staining photos of the leukemic cells are shown. Mice IDs were shown at *top of the panel*. Surface expression proteins were shown at bottom of the panel. Images were obtained with a BH51 microscope and DP12 camera (Olympus, Tokyo, Japan); objective lens, UPlanFl (Olympus); magnification, ×1,000

Table 3 Retroviral integration sites in the transplanted mice

Mice ID	Sample	Chr. number	Nearest gene	Gene ID	Distance to gene (start or end)	Location	Forward or reverse orientation	RTCGD hits
404	Thymus	10	Bcr	110279	Disrupt CDS	Intron 8	F	0
404	PB	15	Trio	223435	Disrupt CDS	Intron 9	F	3
405	Thymus	14	LOC100042147	100042147	12,962 bp	3′	R	0
405	BM	16	Samsn1	67742	95,998 bp	5′	R	2
406	Thymus	18	LOC100042131	100042131	Disrupt CDS	Exon 2	F	0
406	BM	16	Samsn1	67742	95,998 bp	5′	R	2

of MLL-SEPT6, and can be transformed by oncogenic Ras and Ras-related signals (manuscript in preparation). Therefore, HF6 is a suitable cell line for identification of Ras mutations as well as mutations of Ras-related signaling molecules. On the other hand, Ba/F3 cells can be transformed by STAT5 activation. In addition to these two cell lines, we have developed several other IL-3-dependent bone marrow-derived cell lines immortalized by class II mutations or related molecules (unpublished results). Because these IL-3-dependent cell lines have different signaling profiles, they would be applicable for identification of mutations in a variety of signaling molecules, providing a versatile system for functional cloning of oncogenic mutations.

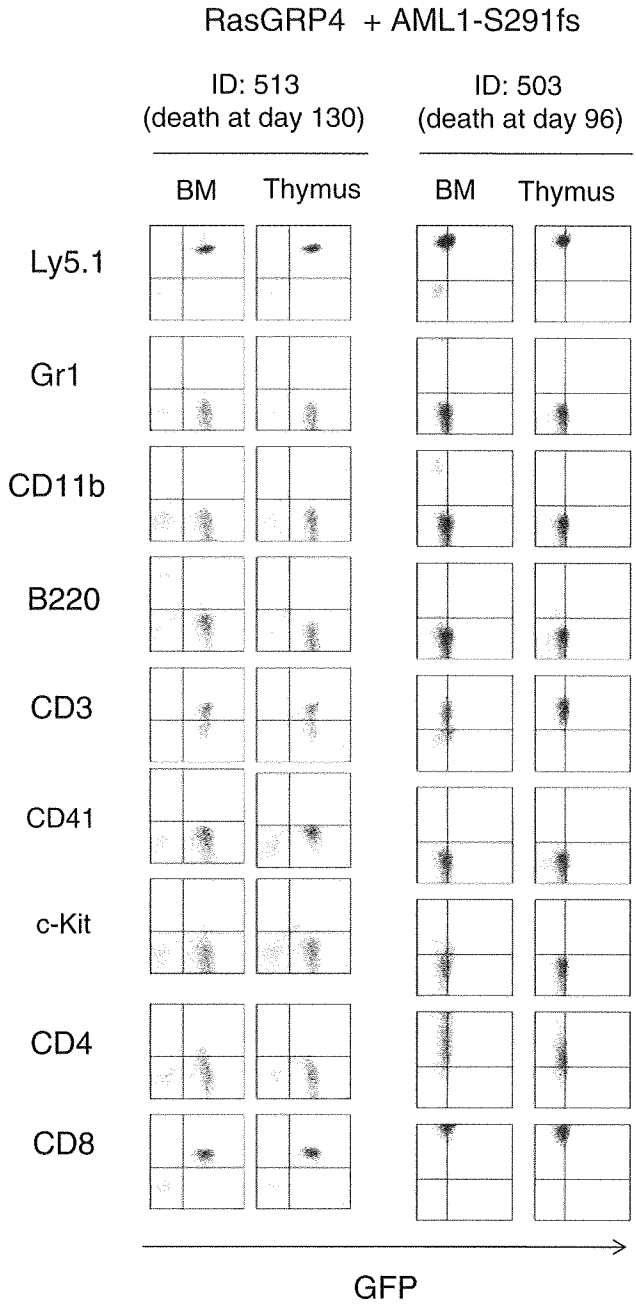

Fig. 6 RasGRP4 and AML1-S291fs induced T cell leukemia in the BMT model. The *dot plots* show Ly5.1, Gr-1, CD11b, B220, CD3, CD41, c-kit, CD4, or CD8 expression detected by corresponding PE-conjugated mAb

Overexpression of RasGRP4-induced T cell leukemia and/or myeloid leukemia in a mouse BMT model. We found that four of eight mice developed both types of leukemia and two mice died of AML after a short latency, while others died of T cell leukemia after a long latency when transplanted with RasGRP4 alone. At present, it is not clear what determines the different phenotypes of leukemia induced by RasGRP4. Although

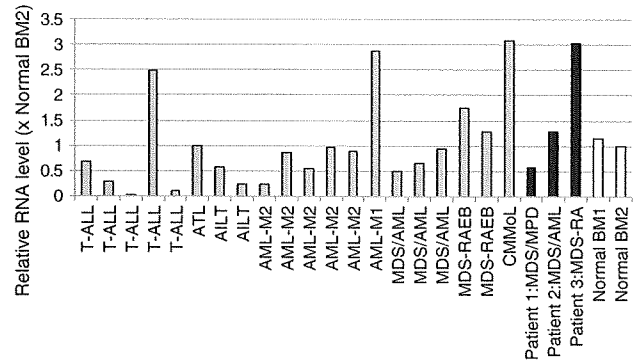

Fig. 7 RasGRP4 was overexpressed in a patient with T-ALL and some patients with myeloid malignancies. Expression levels of RasGRP4 in bone marrow cells derived from patients with hematological malignancies were evaluated by quantitative RT-PCR. *Gray bar* patients with hematological malignancies, *black bar* patients used for cDNA library and identified RasGRP4, *white bar* normal. RNAs from normal bone marrow cells served as a control (RNA level of normal BM2 = 1)

the retrovirus integration site should modify the outcome, so far we did not find any integration that could explain the differing phenotypes of leukemia. Alternatively, it is also possible that types of progenitors transduced with RasGRP4 determine the different phenotypes of leukemia.

Co-transduction of RasGRP4 and AML1-S291fs led to early onset of T cell leukemia as compared with the transduction of RasGRP4 alone. Putting together with clinical reports [2, 9, 36] and our results, we can suggest the significant association of Ras signaling pathway and function of AML1 mutation in leukemogenesis. While AML1 mutations are frequently associated with myeloid leukemia in human patients, they seemed to shorten the latency of T cell leukemia induced by forced expression of RasGRP4 in mouse BMT model. Intriguingly, while RasGRP4 induced c-Kit+/CD3−/CD4+/CD8+ T cell leukemia, combination of RasGRP4 and AML1-S291fs developed more mature T cell leukemia (c-Kit−/CD3+/CD8+/CD4− or CD4+). The reason for this difference is elusive at present. Although we need more cases of BMT mice for confirmation of this difference, AML1-S291fs may also play some roles to induce T cell differentiation in addition to its overall dominant effects on AML1 transcription. In the clinical cases, AML1-LAF4 [37] and AML1-FGA7 [38] were associated with T-ALL, although most of AML1 translocations are associated with myeloid leukemia. Because AML1 is important for transcription of TCR and silencing of CD4, it is possible that AML1-S291fs inhibited the normal ontogeny of T cells, thus accelerating leukemogenic process caused by RasGRP4 in a mice BMT model as a class II mutation that disturbs T cell ontogeny.

Reuther et al. previously isolated RasGRP4 by focus-forming assay of NIH3T3 cells from a patient with AML. This AML-derived RasGRP4 contained a point mutation at codon 620 that changes glutamic acid to lysine at the carboxyl terminus of the protein [29]. However, they found no significant difference in the ability of the AML-derived point mutated RasGRP4 (E620K) or wild-type RasGRP4 (GenBank™ accession number AF448437) in activation of Ras proteins. In our study, we found a gene alteration that induces an amino acid substitution from glutamic acid to lysine at 468 position of RasGRP4 in a patient with MDS/MPD. However, we did not detect a functional difference between RasGRP4 harboring an E468K substitution and RasGRP4 derived from normal volunteers in the ability to abrogate IL-3-dependency of HF6 cells. Moreover, SNPs of this gene are not correlated with lymphoma and leukemia (Y. Nakamura, unpublished results). These results indicate that the sequence difference simply represents a polymorphism or a neutral mutation and has no significant meaning in inducing leukemia. At present, it is not clear whether the sequence alterations in RasGRP4 gene are derived from germ line or somatic mutations.

We found overexpression of RasGRP4 in a patient with T-ALL but it is difficult to conclude the association of RasGRP4 with T-ALL. We also found overexpression of RasGRP4 in some patients with AML-M1, MDS-RAEB, MDS-RA, and CMMoL. The current results suggest that RasGRP4 plays important roles in leukemogenesis in some patients. A clinical study using a large number of patients' samples is required to fully understand the association of RasGRP4 with leukemogenesis.

In summary, we identified RasGRP4 as a candidate gene of class I mutations by our expression cloning strategy based on retrovirus-mediated gene transfer [31, 32, 39]. Although we did not find significant mutations in RasGRP4 derived from patients, overexpression of RasGRP4 confers factor independency on an IL-3 dependent cell line and induced T cell leukemia and myeloid leukemia in a mouse BMT model. Our results indicate possible involvement of RasGRP4 in leukemogenesis.

Acknowledgments We thank Fumi Shibata for technical assistance and Dr. Yusuke Nakamura for the SNPs information of RasGRP4. This work was supported by a grant-in-aid for Cancer Research supported by the Ministry of Health, Labor and Welfare and a grant from the Vehicle Racing Commemorative Foundation.

References

1. Beghini A, Peterlongo P, Ripamonti CB, Larizza L, Cairoli R, Morra E, et al. C-kit mutations in core binding factor leukemias. Blood. 2000;95:726–7.
2. Wang YY, Zhou GB, Yin T, Chen B, Shi JY, Liang WX, et al. AML1-ETO and C-KIT mutation/overexpression in t(8;21) leukemia: implication in stepwise leukemogenesis and response to Gleevec. Proc Natl Acad Sci USA. 2005;102:1104–9. doi: 10.1073/pnas.0408831102.
3. Shimada A, Taki T, Tabuchi K, Tawa A, Horibe K, Tsuchida M, et al. KIT mutations, and not FLT3 internal tandem duplication, are strongly associated with a poor prognosis in pediatric acute myeloid leukemia with t(8;21): a study of the Japanese Childhood AML Cooperative Study Group. Blood. 2006;107:1806–9. doi: 10.1182/blood-2005-08-3408.
4. Yamashita N, Osato M, Huang L, Yanagida M, Kogan SC, Iwasaki M, et al. Haploinsufficiency of Runx1/AML1 promotes myeloid features and leukemogenesis in BXH2 mice. Br J Haematol. 2005;131:495–507. doi:10.1111/j.1365-2141.2005.05793.x.
5. Care RS, Valk PJ, Goodeve AC, Abu-Duhier FM, Geertsma-Kleinekoort WM, Wilson GA, et al. Incidence and prognosis of c-KIT and FLT3 mutations in core binding factor (CBF) acute myeloid leukaemias. Br J Haematol. 2003;121:775–7. doi: 10.1046/j.1365-2141.2003.04362.x.
6. Boissel N, Leroy H, Brethon B, Philippe N, de Botton S, Auvrignon A, et al. Incidence and prognostic impact of c-Kit, FLT3, and Ras gene mutations in core binding factor acute myeloid leukemia (CBF-AML). Leukemia. 2006;20:965–70. doi: 10.1038/sj.leu.2404188.
7. Beghini A, Ripamonti CB, Cairoli R, Cazzaniga G, Colapietro P, Elice F, et al. KIT activating mutations: incidence in adult and pediatric acute myeloid leukemia, and identification of an internal tandem duplication. Haematologica. 2004;89:920–5.
8. Christiansen DH, Andersen MK, Desta F, Pedersen-Bjergaard J. Mutations of genes in the receptor tyrosine kinase (RTK)/RAS-BRAF signal transduction pathway in therapy-related myelodysplasia and acute myeloid leukemia. Leukemia. 2005;19:2232–40. doi:10.1038/sj.leu.2404009.
9. Niimi H, Harada H, Harada Y, Ding Y, Imagawa J, Inaba T, et al. Hyperactivation of the RAS signaling pathway in myelodysplastic syndrome with AML1/RUNX1 point mutations. Leukemia. 2006;20:635–44. doi:10.1038/sj.leu.2404136.
10. Matsuno N, Osato M, Yamashita N, Yanagida M, Nanri T, Fukushima T, et al. Dual mutations in the AML1 and FLT3 genes are associated with leukemogenesis in acute myeloblastic leukemia of the M0 subtype. Leukemia. 2003;17:2492–9. doi: 10.1038/sj.leu.2403160.
11. Roumier C, Eclache V, Imbert M, Davi F, MacIntyre E, Garand R, et al. M0 AML, clinical and biologic features of the disease, including AML1 gene mutations: a report of 59 cases by the Groupe Français d'Hématologie Cellulaire (GFHC) and the Groupe Français de Cytogénétique Hématologique (GFCH). Blood. 2003;101:1277–83. doi:10.1182/blood-2002-05-1474.
12. Callens C, Chevret S, Cayuela JM, Cassinat B, Raffoux E, de Botton S, et al. Prognostic implication of FLT3 and Ras gene mutations in patients with acute promyelocytic leukemia (APL): a retrospective study from the European APL Group. Leukemia. 2005;19:1153–60. doi:10.1038/sj.leu.2403790.
13. Gale RE, Hills R, Pizzey AR, Kottaridis PD, Swirsky D, Gilkes AF, et al. Relationship between FLT3 mutation status, biologic characteristics, and response to targeted therapy in acute promyelocytic leukemia. Blood. 2005;106:3768–76. doi:10.1182/blood-2005-04-1746.
14. Arrigoni P, Beretta C, Silvestri D, Rossi V, Rizzari C, Valsecchi MG, et al. FLT3 internal tandem duplication in childhood acute myeloid leukaemia: association with hyperleucocytosis in acute promyelocytic leukaemia. Br J Haematol. 2003;120:89–92. doi: 10.1046/j.1365-2141.2003.04032.x.
15. Noguera NI, Breccia M, Divona M, Diverio D, Costa V, De Santis S, et al. Alterations of the FLT3 gene in acute