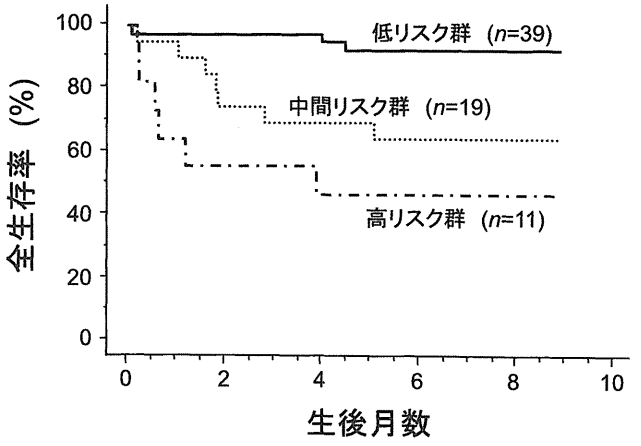

図1　東海地区におけるTAM 70例の全生存率
低リスク群（WBC＜100,000/μl かつ 満期産（在胎週数＞＝38 wks), n＝39）は，中間リスク群（WBC＜100,000/μl かつ早期産（在胎週数＜37 wks) or WBC＞＝100,000/μl かつ満期産, n＝19），高リスク群（WBC＞＝100,000/μl かつ早期産, n＝11）において，有意に全生存率が高い（p＝0.001）.

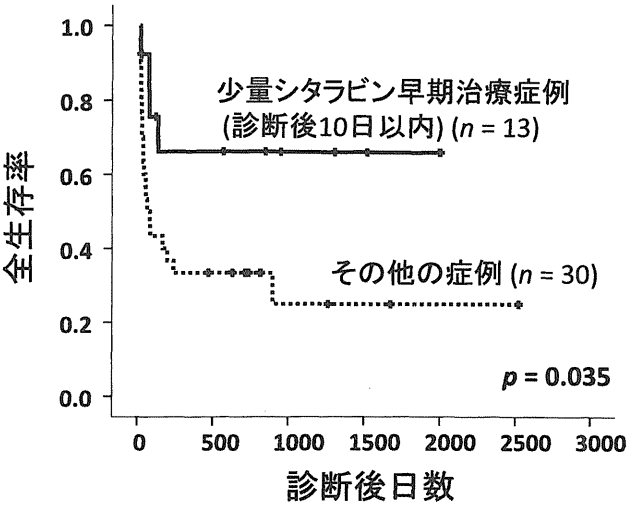

図2　少量シタラビン療法の有効性
WBC＞＝100,000/μl の TAM 患者において，診断後早期（10日以内）の少量シタラビン療法を受けた症例は有意に1年全生存率が良好であった（66.1±13.9% vs. 33.3±8.6%; p＝0.035）.

が高かったにもかかわらず，生存率は差を認めなかった（5年全生存率，78%±8% vs. 85%±3%, p＝0.44）．しかし，予後不良因子（白血球数高値，早産児，腹水，出血傾向，自然寛解なし）のいずれかを有する患者（n＝44）で累積早期死亡率の比較をすると，有意に治療群で成績が良好であり（72%±11%（n＝18）vs. 24%±9%（n＝26), p＝0.001），重症例に対する少量シタラビン療法の有効性が示された．

日本小児血液学会MDS委員会により，2003～2008年診断例に対する全国アンケート調査が行われ，153例の後方視的解析が行われている．うち28例（18%）に少量シタラビン療法が施行され，その好中球減少期間の中央値（範囲）は0日間（0～14日間）であったが，11日間連続投与を受けた1例が感染症により死亡した．白血球数10万以上の症例に限定したサブグループ解析の結果，診断後10日以内に治療開始された症例の予後は有意に予後良好であった（1年全生存率 66%±13.9%（n＝13）vs. 33.3%±8.6%（n＝30); p=0.035）[19].

IV. 提唱治療

1. 治療対象

図1のリスク分類において，低リスク群（在胎38週以上かつ WBC 100,000/μl 未満）の症例は，無治療で高い1年生存率が得られており，まずは無治療で経過観察をしてよいものと考えられる．日本小児血液学会MDS委員会の解析により，WBC 100,000/μl 以上の症例において早期の少量シタラビン療法が改善しているというデータから，治療対象として提唱する．一方，WBCが低値の症例であっても注意深く経過観察を行い，直接ビリルビンの上昇，胸水・腹水・心のう水貯留，全身浮腫など，予後不良と関連する臨床症状が出現した場合には治療対象として提唱する．

2. 少量シタラビン提唱投与量および投与方法

過去に有用であると報告された治療法として，少量シタラビン（商品名：キロサイド）療法がある[7,9]．投与量は，報告によりさまざまであるが，対象が新生児であるため体重（/kg）換算の計算式を用いたほうが簡便であると考えられる．投与目的は，芽球の根絶を目指すのではなく，腫瘍量を減弱させることで臓器障害を緩和することにある．投与期間を調節することにより，過度の骨髄抑制が起こらないように注意するべきである．

投与時修正週数34週未満の児に対しては，臓器の未熟性を考慮し，やや少ない提唱投与量とした．なお，抗がん剤投与に十分な経験のある医師の指導のもとで実施されることが望ましい．投与方法・対象について，図3にまとめた．

〈修正週数35週以上〉

シタラビン　1 mg/kg/回．適量の生理食塩水に溶解し，1日1回1時間静注投与（1～7日間）

〈修正週数35週未満〉

シタラビン　0.5 mg/kg/回．適量の生理食塩水に溶解し，1日1回1時間静注投与（1～7日間）

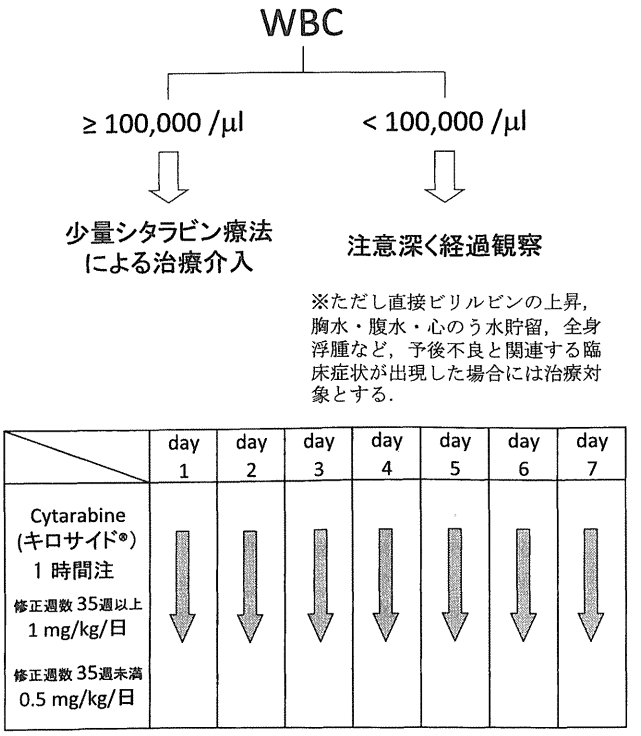

図3 TAMに対する提唱治療の概略

注意事項：WBC 20,000/μl以下となったら投与をいったん中止する．再度芽球の増加傾向がみられれば，投与を再開する．

3. 支持療法

有用性を認めるエビデンスはないが，これまでに高サイトカイン血症に対するステロイド療法，交換輸血による腫瘍量の減弱，強度の出血傾向に対するfresh frozen plasma（FFP）の大量投与などが実地臨床で試みられている．

1）ステロイド療法

プレドニゾロン（PSL）1 mg/kg/day（1〜7日間）以下漸減中止．高サイトカイン血症の存在が考えられるときに使用する．

2）交換輸血

白血球数が100,000/μl以上かつ呼吸障害等のhyperviscosity syndromeによる症状が疑われるときに施行する．敗血症や高ビリルビン血症における交換輸血の方法に準じて行う．すなわち，循環血液量の2倍（180〜200 ml/kg）の合成血（濃厚赤血球液とFFP）を使用し，末梢動脈から瀉血，末梢静脈から輸血を100 ml/kg/hの速度で同時に行う．

実施中は適宜血液ガス・末梢血血算・電解質を確認する．とくに血小板数低下と低Ca血症等に注意し，適宜補正を行う．

3）FFP補充療法

FFP 10〜15 ml/kg．フィブリノーゲン値が100 mg/dlを維持するように，適宜補充を行う．

V. おわりに

TAMは臓器障害のために早期死亡する症例が一部存在し，もはや予後良好疾患として扱うことができない．最近の国内外の多施設共同研究により，早期死亡のリスク因子および少量シタラビン療法の有用性が確認されてきており，今後適切なリスク分類に基づく早期治療介入により生命予後が改善することが期待される．なお，2011年3月よりJPLSG TAM委員会により開始された前方視的観察研究に登録することにより（http://www.jplsg.jp/index.htm），*GATA1*変異解析を含んだ中央診断およびフローサイトメトリー法による微少残存病変の評価が可能である．積極的な症例の登録をお願いしたい．

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Brief report

Identification of *TRIB1* R107L gain-of-function mutation in human acute megakaryocytic leukemia

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***Trib1* has been identified as a myeloid oncogene in a murine leukemia model. Here we identified a *TRIB1* somatic mutation in a human case of Down syndrome–related acute megakaryocytic leukemia. The mutation was observed at well-conserved arginine 107 residue in the pseudokinase domain. This R107L mutation remained in leukocytes of the remission stage in which *GATA1* mutation disappeared, suggesting the *TRIB1* mutation is an earlier genetic event in leukemogenesis. The bone marrow transfer experiment showed that acute myeloid leukemia development was accelerated by transducing murine bone marrow cells with the R107L mutant in which enhancement of ERK phosphorylation and C/EBPα degradation by Trib1 expression was even greater than in those expressing wild-type. These results suggest that TRIB1 may be a novel important oncogene for Down syndrome–related acute megakaryocytic leukemia. (*Blood*. 2012; 119(11):2608-2611)**

Introduction

The Down syndrome (DS) patients are predisposed to developing myeloid leukemia, and those patients frequently exhibit *GATA1* mutations.[1] However, it is proposed that the *GATA1* mutation is important for transient leukemia in DS but not sufficient for full-blown leukemia, suggesting that additional genetic alterations are needed.[1] Therefore, it is important to search the subsequent genetic changes for DS-related leukemia (ML-DS) to predict malignant transformation and prognosis of the patients.

Trib1 has been identified as a myeloid oncogene that cooperates with *Hoxa9* and *Meis1* in murine acute myeloid leukemia (AML).[2] As a member of the tribbles family of proteins, TRIB1 interacts with MEK1 and enhances ERK phosphorylation.[2,3] Moreover, TRIB1 promotes degradation of C/EBP family transcription factors, including C/EBPα, an important tumor suppressor for AML, and we observed that degradation of C/EBPα by Trib1 is mediated by its interaction with MEK1.[4] Thus, TRIB1 plays an important role in the development of AML by modulating both the RAS/MAPK pathway and C/EBPα function together with Trib2 that has also been identified as a myeloid-transforming gene.[5] Potential involvement of *TRIB1* in human leukemia has been reported in cases of AML with 8q34 amplification in which both *c-MYC* and *TRIB1* are included in the amplicon.[6] The enhancing effect of TRIB1 on the MAPK signaling suggests that TRIB1 alterations may be related to AML cases, which do not show any mutations in the pathway members, such as FLT3, c-Kit, or Ras. In this report, we identified a novel somatic mutation of *TRIB1* in a case of human acute megakaryocytic leukemia developed in DS (DS-AMKL). Retrovirus-mediated gene transfer followed by bone marrow transfer indicated that the mutation enhanced leukemogenic activity and MAPK phosphorylation by TRIB1.

Methods

Patients

TRIB1 mutations have been investigated in 12 cases of transient leukemia (TL), 5 of DS-AMKL, and 4 cell lines of DS-AML. Peripheral blood leukocytes of TL and bone marrow cells of DS-AMKL were used as sources for the molecular analysis. This study was approved by the Ethics Committee of Hirosaki University Graduate School of Medicine, and all clinical samples were obtained with informed consent from the parents of all patients, in accordance with the Declaration of Helsinki.

Patient 84 showed trisomy 21 and extensive leukocytosis at birth. Hematologic findings revealed the white blood cell count to be 148×10^9/L, including 87% myeloblasts, a hemoglobin level of 19.4 g/dL, and a platelet count of 259×10^9/L. Patent ductus arteriosus and atrial septal defect have been pointed out. Based on the hematologic data and the chromosomal abnormality, the patient was diagnosed as DS-related TL. The hematologic abnormality was then improved, but 8 months later 3% of 6.9×10^9/L white blood cells became myeloblasts (Figure 1A). A karyotype analysis of bone marrow cells revealed 48, XY,+8,+21 in 3 of 20 cells. In addition, *GATA1* mutation was detected at nt 113 from A to G, resulting in loss of the first methionine.[7] He was diagnosed as AMKL at this time, and his disease was in remission by subsequent chemotherapy.

PCR and sequencing

The entire coding region of human *TRIB1* cDNA of patients' samples was amplified using Taq polymerase (Promega) and specific primer pairs (the sequences of the primers are available on request). The genomic DNA samples of patient 84 were also analyzed. The sequence analysis of *GATA1* was performed as described previously.[7] After checking the PCR products by agarose gel electrophoresis, the products were purified and directly sequenced.

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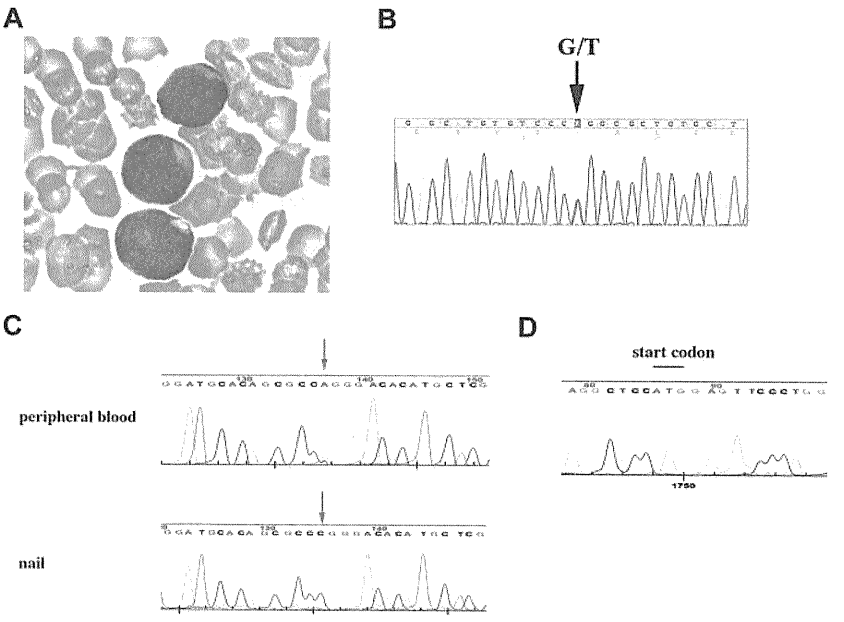

Figure 1. *TRIB1* R107L mutation identified in DS-related leukemias. (A) Giemsa staining of the case 84 peripheral blood smear diagnosed as AMKL. The image was acquired using a BX40 microscope equipped with a 100×/1.30 NA oil objective (Olympus) and a C-4040 digital camera (Olympus). (B) Fluorescent dye sequencing chromatographs of *TRIB1* genotyping by direct sequencing of the case 84 using a cDNA sample as a template. The vertical arrow indicates mixed G and T signals at codon 107. (C) Fluorescent dye sequencing chromatographs of *TRIB1* of peripheral blood leukocytes (top) or nail (bottom) in the same case at the complete remission stage. The red arrows indicate that the mutation remains in leukocytes but not in nail. The reverse strand sequences are shown. (D) *GATA1* sequence. The start codon that was mutated in AMKL[7] is normal in the peripheral blood leukocytes at the remission stage.

Retroviral infection of murine bone marrow cells and bone marrow transfer

Bone marrow cells were prepared from 8-week-old female C57Bl/6J mice 5 days after injection of 150 mg/kg body weight of 5-fluorouracil (Kyowa Hakko Kogyo). Retroviral infection of bone marrow cells and bone marrow transfer experiments were performed as described.[2] Transduction efficiencies evaluated by flow cytometric techniques were comparable between wild-type (WT; 5.3%) and R107L (3.4%). Animals were housed, observed daily, and handled in accordance with the guidelines of the animal care committee at Japanese Foundation for Cancer Research. All the diseased mice were subjected to autopsy and analyzed morphologically, and the blood was examined by flow cytometric techniques. The mice were diagnosed as positive for AML according to the classification of the Bethesda proposal.[8] The survival rate of each group was evaluated using the Kaplan-Meier method, and differences between survival curves were compared using the log-rank test.

Immunoblotting

Immunoblotting was performed using cell lysates in RIPA buffer as described.[4] Anti-p44/42 ERK (Cell Signaling Technologies), anti–phospho-p44/42 ERK (Cell Signaling Technologies), anti-C/EBPα (Santa Cruz Biotechnology), anti-FLAG (Sigma-Aldrich), and anti-GAPDH (Hy Test Ltd) antibodies were used.

Results and discussion

The important role of TRIB1 on the MAPK signaling suggests that *TRIB1* alterations may occur in some AML cases, which do not show overlapping mutations in the pathway members, such as FLT3, KIT, or RAS. Therefore, we tried to search mutations of *TRIB1* in cases of ML-DS and TL in which such mutations are infrequent.[9] In a case of DS-AMKL (case 84), a nucleotide change from guanine to thymine has been identified at 902 that results in amino acid alteration from arginine 107 (R107) to leucine (Figure 1B). The sequence changes were confirmed by subcloning the PCR product into the TA-type plasmid vector (data not shown). The nucleotide change was not observed in the DNA sample derived from the nail of the same patient at all (Figure 1C), indicating that this change is a somatic mutation. Interestingly, the mutation was retained in the peripheral blood sample in the complete remission stage in which the GATA1 mutation completely disappeared (Figure 1C-D). These results indicate that the *TRIB1* mutation precedes the onset of TL and the *GATA1* mutation, and suggest that *TRIB1* mutation occurred at the hematopoietic stem cell level and that the clone retaining the *TRIB1* mutation survived after chemotherapy. In case 84, there was no mutation for *FLT3* exons 14, 15, and 20, *PTPN11* exons 3 and 13, *KRAS* exons 2, 3, and 5, and *KIT* exons 8, 11, and 17 by the high-resolution melt analysis (data not shown).

An additional mutation was found in a case of TL (case 109) at the nucleotides 805 and 806 from GC to AT, which results in amino acid conversion from alanine (A75) to isoleucine (supplemental Figure 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). *TRIB1* expression in DS-related and DS-unrelated leukemias was examined by real-time quantitative RT-PCR (supplemental Figure 2).

R107 is located within a psuedokinase domain of TRIB1 that is considered as a functionally core domain of TRIB family proteins.[10] Sequence comparison among 3 TRIB family proteins as well as tribbles homologs in other organisms revealed that the R107 is well conserved in mammalian TRIB1 and TRIB2,[10] suggesting that this arginine residue is evolutionary conserved and may be related to an important function. On the other hand, A75 is located outside of the pseudokinase domain, not conserved between human and mouse, or other tribbles homologs. Moreover, the N-terminal domain containing A75 is dispensable for the leukemogenic activity of Trib1.[4] Therefore, we tried to investigate whether the R107L mutation could affect the leukemogenic activity of *TRIB1*.

R107L was introduced into the murine *Trib1* cDNA by site-directed mutagenesis. Both WT and R107L cDNAs were subcloned into the pMYs-IRES-GFP retroviral vector and were used for retrovirus-mediated gene transfer followed by bone marrow transfer according to the method previously described.[1] All the mice

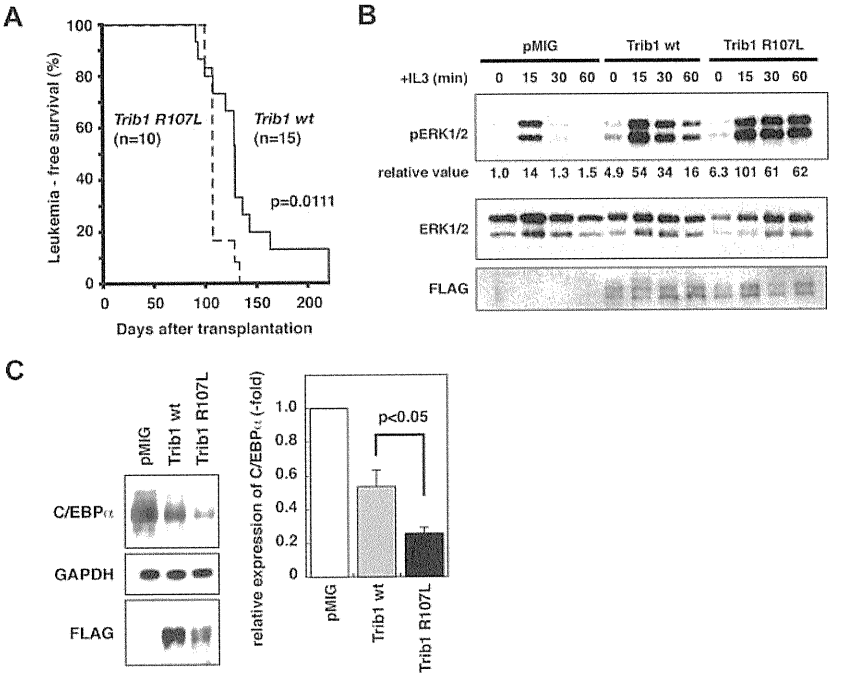

Figure 2. AML development by bone marrow transfer using *Trib1* WT and R107L. (A) Kaplan-Meier survival curves are shown. The *P* value was calculated with the log-rank test. (B) Immunoblot analysis of *Trib1* WT AML (Mac-1 56.2%, Gr-1 52.5%, $CD34^{lo}$, c-kit$^-$, Sca-1$^-$) and R107L AML (Mac-1 41.4%, Gr-1 25.2%, $Cd34^{lo}$, c-kit^{lo}, Sca-1$^-$) derived from bone marrow of recipient mice (WT #T73 and R107L #T151 in supplemental Table 1). Enhancement of ERK phosphorylation is more significant in R107L. Relative values of ERK phosphorylation were calculated by densitometric analysis. (C) Immunoblot analysis for C/EBPα of the same AML samples as in panel B. Relative expression level of C/EBPα is quantitated (right).

transplanted with bone marrow cells expressing WT (n = 15) or R107L (n = 12) developed AML (Figure 2A). The mean survival time was shorter in the recipients with R107L-expressing bone marrow cells (110 days) than those with WT (136 days; Figure 2A). The difference was significant ($P = .0111$, log-rank test). The result indicates that the R107L mutation enhances the leukemogenic activity of TRIB1. These results also suggest that *TRIB1* mutation might cooperate with *GATA1* mutation in the genesis of DS-AMKL, and that trisomy 21, *TRIB1*, and *GATA1* mutations occurred consecutively, which contributed to the multistep leukemogenic process.

We have shown that TRIB1 interacts with MEK1 and enhances phosphorylation of ERK.[2] The R107L mutant enhanced ERK phosphorylation more extensively than WT (Figure 2B) in AML cells derived from bone marrow of recipient mice, and more significant degradation of C/EBPα was induced by the R107L mutant (Figure 2C). These findings might be correlated to the enhanced leukemogenic activity of the mutant. Both R107L and WT proteins could interact with MEK1, having the binding motif in their C-termini. The residue 107 is located at subdomain II of the pseudokinase domain.[11] The mutation may affect conformation of the domain and may promote the MEK1 function on ERK, although additional studies are required to address the possibility. A recent study demonstrates that Trib1 and Trib2 failed to show ERK phosphorylation in 32D cells.[12] The different response to Trib1 between primary leukemic cells and the cell line might depend on the cellular context and/or combination of additional mutations. The AML phenotypes were somewhat varied in each case and Mac-1–positive/Gr-1–negative AMLs were more remarkable in WT than in R107L, although the difference was not statistically significant (supplemental Figures 3-4; supplemental Table 1). The current study underscores the role of TRIB1 in human leukemogenesis and the significance of the R107L mutation in its function. Further sequence analysis of tribbles family genes in a larger cohort will emphasize the importance of R107L and/or additional mutations of *TRIB1* in leukemic patients.

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Authorship

Contribution: T.Y., E.I., Y.H., and T.N. designed and performed the research and wrote the manuscript; T. Toki, Y.A., R.K., and M.-j.P. performed the research; and Y.K., T. Takahara, and Y.Y. contributed to the bone marrow transplantation analysis.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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Brief report

CBL mutation in chronic myelomonocytic leukemia secondary to familial platelet disorder with propensity to develop acute myeloid leukemia (FPD/AML)

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Familial platelet disorder with a propensity to develop acute myeloid leukemia (FPD/AML) is a rare autosomal dominant disease characterized by thrombocytopenia, abnormal platelet function, and a propensity to develop myelodysplastic syndrome (MDS) and AML. So far, > 20 affected families have been reported. Recently, a second *RUNX1* alteration has been reported; however, no additional molecular abnormalities have been found so far. We identified an acquired *CBL* mutation and 11q-acquired uniparental disomy (11q-aUPD) in a patient with chronic myelomonocytic leukemia (CMML) secondary to FPD with *RUNX1* mutation but not in the same patient during refractory cytopenia. This finding suggests that alterations of the *CBL* gene and *RUNX1* gene may cooperate in the pathogenesis of CMML in patients with FPD/AML. The presence of *CBL* mutations and 11q-aUPD was an important "second hit" that could be an indicator of leukemic transformation of MDS or AML in patients with FPD/AML. (*Blood*. 2012; 119(11):2612-2614)

Introduction

Familial platelet disorder with a propensity to develop acute myeloid leukemia (FPD/AML) is a rare autosomal dominant disease characterized by thrombocytopenia, abnormal platelet function, and a propensity to develop myelodysplastic syndrome (MDS) and AML.[1,2] Since Song et al reported haploinsufficiency of the *RUNX1/CBFA2* gene,[3] more than 20 affected families have been reported.[4-8] Notably, various types of mono-allelic mutations of the *RUNX1* gene have been found in patients with AML secondary to FPD.[3,7-9] *RUNX1*, which is a key regulator of definitive hematopoiesis and myeloid differentiation, is also commonly involved in sporadic cases of MDS and AML, by translocations in AML[10] and by point mutations in AML[11,12] and MDS.[13] Recently, a second *RUNX1* alteration has been reported[8]; however, no additional molecular abnormalities have been found so far.

In this regard, recent reports of somatic mutations of the *CBL* proto-oncogene in myeloid neoplasms are intriguing because these *CBL* mutations have been shown to result in aberrant tyrosine kinase signaling, which would also lead to the activation of RAS signaling pathways. So far, we and others have reported that *CBL* mutations occurred in a variety of myeloid neoplasms, including de novo AML,[14,15] MDS,[16,17] and myeloproliferative neoplasm,[16,17] especially in chronic myelomonocytic leukemia (CMML)[16,17] and juvenile myelomonocytic leukemia.[18] The importance of *CBL* mutations for leukemogenesis has substantially increased, which prompted us to search for possible *CBL* mutations in this pedigree.

Here, we reported that *CBL* mutation developed at the time of diagnosis of CMML, but not during refractory cytopenia, in a Japanese patient with FPD/AML harboring a *RUNX1* mutation.

Methods

RUNX1 mutation analysis

DNA and RNA were extracted from peripheral blood (PB) of the proband, her sister, and their mother after obtaining informed consent. We performed mutation analysis of the *RUNX1* gene by PCR followed by direct sequencing with the use of an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). For further confirmation of deletion mutations, the PCR products were subcloned with the use of a TOPO TA Cloning Kit (Invitrogen) and then sequenced. Mutations were screened from exons 1-8 of the *RUNX1* gene.

CBL mutation analysis

Because *CBL* mutations thus far reported almost exclusively involved exons 8-9 that encode Linker/RING finger domains, we confined our mutation analysis to these exons, which were subjected to direct sequencing. Because the frequency of 11q-acquired uniparental disomy (11q-aUPD) was reported as ~ 85%-90% in *CBL* mutations, we also analyzed the sample with Affymetrix GeneChip 250K *Nsp*I.[17-19] Genome-wide detection of copy number abnormalities or allelic imbalances was performed with CNAG/AsCNAR Version 3.0 software (http://www.genome.umin.jp), which enabled sensitive detection of copy number neutral loss of heterozygosity (or aUPD).[19] In addition, we examined mutations of the following genes in the proband as previously reported: *FLT3, KIT, RAS, JAK2, PTPN11, ASXL1, IDH1/2,* and *MPL*.[20-22] The study adhered to the principles of the Helsinki Declaration and was conducted under the regulations enacted by the Ethics Board of Gunma Children's Medical Center.

Results and discussion

The proband (III-2), who was the second child of nonconsanguineous parents, underwent an 8-year follow-up of mild to moderate

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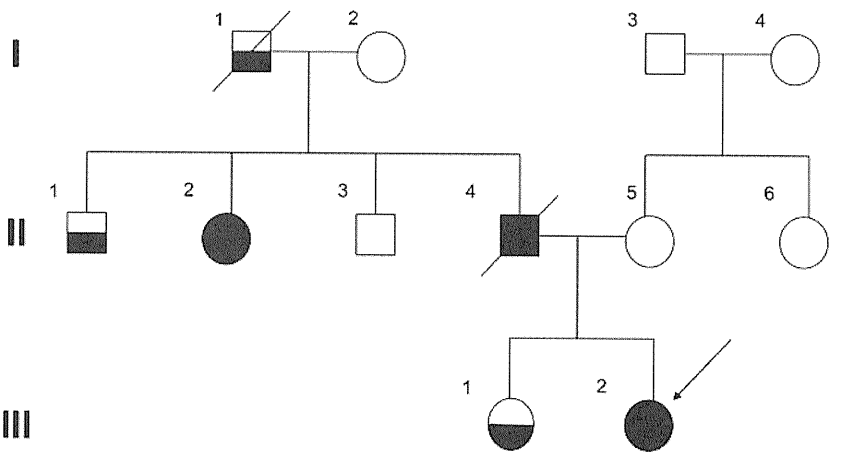

Figure 1. The family pedigree. Squares indicate males and circles indicate females. Open symbols represent unaffected persons, half-filled symbols represent persons affected by thrombocytopenia, and closed symbols represent persons affected by FPD who developed MDS/AML. The proband (III-2) is indicated by an arrow.

thrombocytopenia (50-80 $\times$ $10^3/\mu L$), and at that age of 10 years, her condition was diagnosed as refractory cytopenia. Cytogenetic analysis found a normal karyotype, and FISH showed neither monosomy 7 nor trisomy 8. The proband had been closely observed without any therapy for 2 years and 9 months because she did not require transfusion and her disease remained stable; however, at the age of 12 years, leukocytosis and monocytosis developed and she became dependent on platelet transfusions. Finally, the disease evolved to CMML, and allogeneic bone marrow (BM) transplantation from an unrelated donor was performed. During the entire course, the number of blast cells in PB was constantly $< 2\%$, and no additional symptoms were observed, such as hepatosplenomegaly. Her elder sister (III-1) was also followed for 10 years with mild thrombocytopenia; however, the morphologic findings of PB or BM were not compatible with myeloproliferative neoplasms.[17] Because her platelet count has been gradually decreasing, allogeneic BM transplantation is being considered. Although her father (II-4) developed MDS at the age of 41 and died 2 years later, her paternal aunt (II-2) developed MDS at the age of 49 and has remained in complete remission for 11 years after successful allogeneic cord blood transplantation. Her paternal grandfather (I-1) and uncle (II-1) also had a history of thrombocytopenia (Figure 1). Direct sequencing analysis of *RUNX1* found a one-base deletion of adenine at position 2364 within exon 7, resulting in a frameshift mutation that corresponded to AML1b transcript in the proband and her sister (Figure 2A). This resulted in a frameshift after amino acid change G262GfsX21. This mutation was not detected in their mother. All these data suggested that her paternal grandfather (I-1), uncle (II-1), aunt (II-2), and her father (II-4) were considered to have FPD/AML, carrying the same *RUNX1* mutation.

Although no *CBL* mutations were found in the proband sample of refractory cytopenia before development of CMML, homozygous mutation of the *CBL*, which was located in the splice acceptor site of intron 8 (Figure 2B), was identified in the proband sample in the CMML. We also found 11q-aUPD (Figure 2C) in the proband sample, confirming a strong association of *CBL* mutations with 11q-aUPD, as previously described[16-18]; however, no mutations of any other genes, including *FLT3*, *KIT*, *RAS*, *JAK2*, *PTPN11*, *ASXL1*, *IDH1/2*, and *MPL*, were found and no additional somatic *RUNX1* alterations. No *CBL* mutations were found in her sister's sample at this time.

Inherited *RUNX1* mutations were clustered in the N-terminal region in exons 3-5, which affect the runt homology domain. Mutations in the C-terminal region, detected in the present pedigree, have been reported less frequently so far and are considered to affect the transactivation domain (Figure 2D).

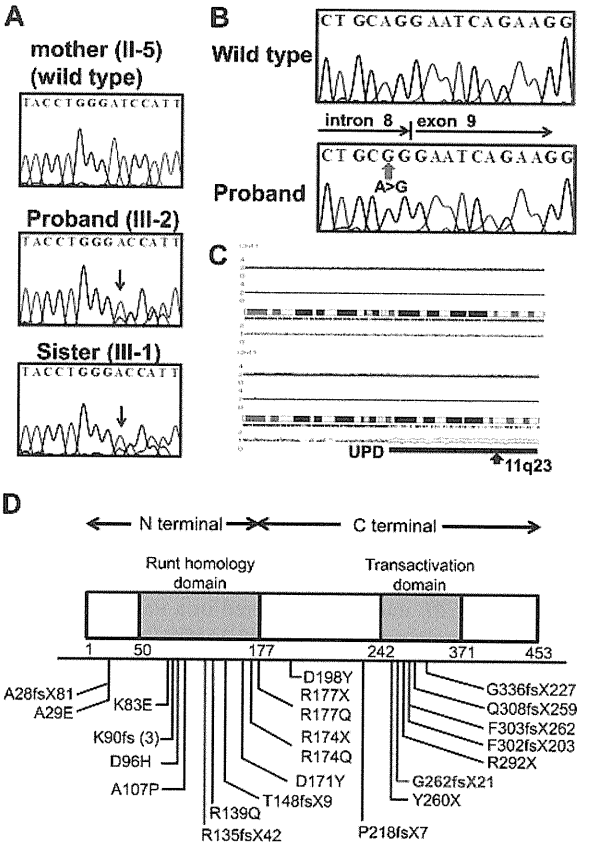

Figure 2. Mutation analysis of *RUNX1* and *CBL* genes in the pedigree. (A) Direct sequencing analysis of affected patients (III-1, III-2) and an unaffected family member (II-5) is shown. Arrow indicates a one-base deletion of adenine. (B) Mutated *CBL* is shown in the proband. (C) Identification of acquired uniparental disomy of 11q in the proband. Total copy number (tCN; red plot) is shown above the cytoband, and the results of allele-specific copy number analysis with anonymous references (AsCNAR) plots are shown below the cytoband. Larger allele is presented by a red line, and the smaller allele is presented by a blue line. Allele-specific analysis showed 11q-aUPD (blue line), which contained the *CBL* region (arrow). (D) Schematic representation of wild-type and mutated *RUNX1*. The affected *RUNX1* is truncated at the C terminus of the transactivation domain (TAD). Part of TAD is lacking in this proband (red line).

It has been postulated that disruption of the *RUNX1* gene is not sufficient to cause AML, as previously reported with monoallelic and biallelic inactivation of *Runx1* in mice[23,24] and in mice carrying the knocked-in *Runx1-Eto* chimeric gene. These data indicate that a second-hit mutation in addition to the dysfunction of *RUNX1* is required for the development of AML. Minelli et al postulated that the mutations seen in FPD cases have a mutation effect that induces additional genetic abnormalities and promotes progression to hematologic malignancies.[25]

Marked associations between chromosome translocation and gene mutations have been reported: *KIT* mutation in core binding leukemia, t(8;21)/*AML1-ETO* and inv(16)(p13q22)/*CBFβ-MYH11*, *FLT3*-ITD in leukemia with t(15;17)/*PML-RARα*, or with t(6;9)/*DEK-CAN*. We consider that it is important to find an association to administer clinically relevant treatment. In addition to the germline *RUNX1* mutation, we identified an acquired *CBL* mutation in the proband and assumed it to be a second hit mutation by which FPD evolved into CMML. To our knowledge, this is the first patient with FPD/AML in whom *CBL* mutation has developed. This finding suggests that alterations of the *CBL* gene and *RUNX1* could cooperate in the pathogenesis of CMML or AML in patients with FPD/AML. The presence of 11q-aUPD provided evidence that loss of the wild-type copy of *CBL* with duplication of the mutant copy was an important second hit that could be an indicator of leukemic transformation in patients with FPD/AML.

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Authorship

Contribution: Y.H. and C.O. designed the study; A.M., C.O., and D.H. provided critical reagents and samples; N.S., M.P., A.S.-O., and C.M. performed the experiments; H.A. and S.O. supervised the work; N.S. and M.P. analyzed the results; N.S. and D.H. constructed the figures; N.S. and Y.H. wrote the paper; and all the authors critically reviewed and revised the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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of departure from Hardy–Weinberg equilibrium ($P=0.06$), because the variant G allele is significantly more prevalent among Whites than non-Whites with the allele frequency of 0.24 versus 0.073, respectively ($P=0.0003$). Still, the observed associations retained significance in analyses restricted only to Whites. The G allele was associated with better EFS and OS in univariate analyses ($P=0.0173$ and 0.035, respectively, data not shown) and in multivariable analyses ($P=0.023$ and 0.005, respectively, data not shown).

We also observed that the variant A allele of FKBP5 SNP rs7755289 (T>A; intron 8) was significantly associated with worse EFS ($P=0.014$, hazard ratio = 3.193, 95% CI = 1.258–8.104, Figure 1c) and OS ($P=0.0036$, hazard ratio = 4.846, 95% CI = 1.68–14, Figure 1d). In addition, A allele was associated with increased day 22 MRD ($P=0.017$), increased cumulative incidence of relapse ($P=0.045$, hazard ratio = 3.4, 95% CI = 1.03–11.22) and an increased cumulative incidence of treatment-related mortality ($P=0.012$, hazard ratio = 5.57, 95% CI = 1.44–21.47). However, as this SNP occurred with the allele frequency of only ~0.2, the low sample size restricted us from performing further analysis. Although the above mentioned SNPs were the most interesting SNPs, we also observed association of SNP rs16878591 ($P=0.011$) with day 22 MRD levels and SNPs within LD block-2 with *in vitro* ara-C LC_{50} values ($P=0.03$; Table 1).

In previous reports, FKBP5 expression has been shown to positively influence response to cytarabine and gemcitabine. More recently, FKBP5 has been identified as scaffolding protein that facilitates PHLPP-mediated dephosphorylation of AKT-Ser473, thus indicating that higher expression of FKBP5 might contribute to enhanced chemosensitivity.[3–5] siRNA-mediated FKBP5 knockdown increases the resistance to cytarabine and other agents as etoposide, paclitaxel and doxetaxel.[1,3–5] Thus, FKBP5 SNPs may also be associated with response to other agents used in combination with cytarabine in AML patients. In conclusion, our preliminary results suggest that the FKBP5 polymorphisms mentioned above may also be relevant for AML treatment response. These results should be confirmed with functional studies and independent clinical studies. Identification of pharmacogenetic markers of response, such as FKBP5 SNP such as rs3798346, might help in further understanding inter-patient variation in response to chemotherapy.

Conflict of interest

The authors declare no conflict of interest.

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CBL mutation in childhood therapy-related leukemia

Leukemia (2011) **25,** 1356–1358; doi:10.1038/leu.2011.75; published online 15 April 2011

Therapy-related leukemia and myelodysplastic syndrome (t-Leuk/MDS) are mainly caused by topoisomerase II inhibitors that cause acute myeloid leukemia (AML) with an 11q23 translocation or by alkylating agents that induce MDS/AML with an *AML1* mutation and monosomy 7.[1,2] Two types of t-Leuk/MDS can be distinguished, one of which has a long latency (≥5–7 years) and is seen following alkylating agents, frequently with an preleukemic phase.[1] The other has a short latency period (1–3 years), no preleukemic phase, and is strongly associated with the administration of topoisomerase II inhibitors and chromosomal abnormalities involving 11q23 translocation/*MLL* rearrangement (*MLL*-R).[2] Repair of etoposide (VP-16)-stabilized DNA topoisomerase II covalent complexes may initiate *MLL*-R observed in patients.[3]

In this regard, recent reports of somatic mutations of the *CBL* proto-oncogene in myeloid neoplasms are intriguing, because

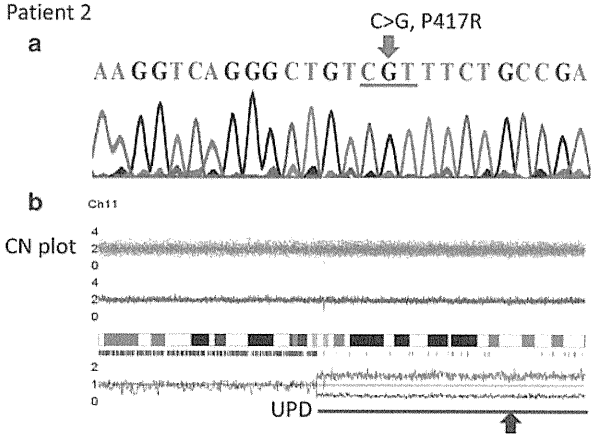

Figure 1 Identification of acquired isodisomy of 11q and *CBL* mutation in therapy-related leukemia. (**a**) Homozygous mutation of the *CBL* gene was identified in patient 2. (**b**) Copy number (CN) analysis for the gene chip output for therapy-related leukemia in patient 2. Total CNs (red plot) are shown above the cytoband, and the result of allele-specific CN analysis with anonymous references plots are shown below the cytoband. Larger allele is presented in red line, and smaller allele is presented in green line. Allele-specific analysis showed 11q-aUPD (blue line), which contained *CBL* region (black arrow).

these *CBL* mutations were shown to result in aberrant tyrosine kinase signaling, which would lead also to activation of RAS signaling pathways. We and others reported that *CBL* mutations occurred in a variety of myeloid neoplasms, including *de novo* AML,[4] MDS[4] and myeloproliferative neoplasm,[4,5] especially in chronic myelomonocytic leukemia[5] and juvenile myelomonocytic leukemia.[6] The importance of *CBL* mutations concerning about leukemogenesis is substantially increased. This prompted us to search for possible *CBL* mutations in pediatric t-Leuk/MDS.

Analysis of *CBL* gene was carried out in 20 pediatric t-Leuk/MDSs, including 15 AMLs (range: 1 year and 10 months to 17 years; 8 males and 7 females), 4 MDSs (range: 7 years to 14 years; 4 males) and 1 acute lymphoblastic leukemia (4 years and 2 months; 1 male). Median age at diagnosis was 8 years and 1 months (range: 1 year and 10 months to 17 years; 13 males and 7 females). Rearrangements of *MLL* gene were found in 17 patients (85%), including 15 of 16 who received VP-16 (Sugita *et al.*[7]), and 2 of 4 who did not receive it. An initial diagnosis was made as non-Hodgkin's lymphoma in seven patients, neuroblastoma in five, acute lymphoblastic leukemia in five, AML in two and juvenile myelomonocytic leukemia in one.

Because *CBL* mutations thus far reported almost exclusively involved exons 8–9 that encode linker/RING finger domains,[4–6] we confined our mutation analysis to these exons, in which PCR-amplified exons 8–9 were subjected to direct sequencing using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Branchburg, NJ, USA). The study adhered to the principles of the Helsinki declaration, and was conducted under the regulations enacted by the Ethics Board of Gunma Children's Medical Center.

Homozygous mutation of the *CBL* gene was identified in 1 out of the 20 t-Leuk/MDS cases (5%), which were located in the RING finger domain (P417R in patient 2). As the frequency of 11q-acquired uniparental disomy (11q-aUPD) was reported ~85–90% in *CBL* mutations,[4–6] we analyzed his sample using Affymetrix GeneChip 250K *Nsp*I array (Affymetrix, Santa Clara, CA, USA), and found the presence of 11q-aUPD, which was the sole abnormality seen by single-nucleotide polymorphism array (Figure 1), confirming a strong association of *CBL* mutations with 11q-aUPD as previously described.[4–6] Furthermore, we examined *NRAS* and *KRAS* mutations in these patients whose samples were available and found *KRAS* mutation in one patient with t-Leuk (acute monocytic leukemia having t(9;11)(p21;q23) after B-cell precursor acute lymphoblastic leukemia having 6p−, 7q+, 9q+ and 12q−).

CBL mutation was detected in MDS cells from the patient with t-MDS after malignant lymphoma. The patient was initially diagnosed as having diffuse large T-cell type malignant lymphoma, whose biopsied specimen of the buccal lymph node showed MT1(+), MB1(−) and UCHL1(+), when he was 5 years old. He subsequently was treated with chemotherapy according to T-8801 protocol including VP-16 (200 mg/m^2) given twice weekly,[7] and obtained a complete remission. However, at 7 months after diagnosis, tumor appeared in the right maxilla, and was diagnosed as the relapsed lymphoma, then, he received local irradiation (30 Gy) and chemotherapy including ifosfamide, vincristine, THP-adriamycin and L-asparaginase. At 4 months later, enlarged spleen was resected, and the infiltrated tumor cells were microscopically seen in the tumor sections. At 6 months later, 19 months after initial diagnosis, blast cells appeared in peripheral blood. His laboratory data revealed leukocytosis (14 700/μl with 18% blast cells) and an elevated serum lactate dehydrogenase level (1458 U/l). Bone marrow aspiration revealed 9.8% blasts, which were positive for cytoplasmic myeloperoxidase, suggesting MDS. Surface marker analysis showed that the leukemic blasts in the bone marrow were positive for CD33. Chromosomal analysis of bone marrow cells revealed t(5;11)(q21;q23) in 11 of 20 cells. Rearrangement of *MLL* gene of these cells was identified by Southern blotting, however, no known chimeric mRNA with *MLL*, such as *MLL-AF5q31* and *MLL-GRAF* in t(5;11)(q31;q23), could be detected. These suggested that the gene at 5q21 was a novel partner gene of *MLL*. Although another chemotherapy for AML was performed, his blast cells increased >30% blasts in bone marrow at 25 months after initial diagnosis. Therefore, he was diagnosed as having t-Leuk resembling acute monoblastic leukemia due to VP-16. He died of mycotic infection at 35 months after initial diagnosis.

No *CBL* mutations were found in his lymphoma sample at diagnosis and in tumor cells in the enlarged spleen. We also performed tissue-fluorescence *in situ* hybridization analysis with *MLL* probe on paraffin-embedded tissue sections of the tumor cells in the enlarged spleen, however, no evaluable results could be detected because of poor quality of samples. No initial samples for tissue-fluorescence *in situ* hybridization analysis could be obtained.

The 11q23 translocation/*MLL*-R in t-Leuk/MDS was considered to be induced by VP-16,[3] however, gene alterations in addition to *MLL*-R have rarely reported. Recently, *CBL* mutations were found in a variety of myeloid neoplasms.[4–6] Among 2000 samples from the patients with myeloid neoplasms, *CBL* mutations have been found in ~5% samples, including AML transformed from MDS, but not *de novo* or therapy-related acute leukemia with 11q23 translocation/*MLL*-R. To our knowledge, this is the first t-Leuk/MDS patient with 11q23 translocation/*MLL*-R and *CBL* mutation. Interestingly, a *de novo* AML case with *MLL–CBL* fusion gene has also been reported.[8] These findings suggest that alterations of *CBL* gene and 11q23 translocation/*MLL*-R may cooperate in the pathogenesis of a subtype of t-Leuk/MDS and *de novo* leukemia.

Conflict of interest

The authors declare no conflict of interest.

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SNP array analysis of leukemic relapse samples after allogeneic hematopoietic stem cell transplantation with a sibling donor identifies meiotic recombination spots and reveals possible correlation with the breakpoints of acquired genetic aberrations

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Allogeneic hematopoietic stem cell transplantation (HSCT) with a sibling donor is commonly used for treating hematologic malignancies.[1] Although this procedure is frequently curative, a proportion of the patients eventually suffers a relapse of the original malignancy.[1] Leukemogenesis is associated with acquired genetic aberrations caused by various mechanisms including induction of double-stranded DNA breaks by DNA toposiomerase II poisons followed by non-homologous end joining, recombination between homologous sequences and illegitimate V(D)J recombination.[2] It has been hypothesized that neoplasia-associated breakpoints may correlate with the breakpoints of meiotic events, that is, some parts of the genome are more prone to both meiotic and somatic rearrangements; however, this remains controversial.[3–5]

During the last five years, numerous studies have used single-nucleotide polymorphism (SNP) array analysis to investigate genetic abnormalities in hematologic malignancies, including paired diagnostic and relapse samples.[6] To the best of our knowledge, however, the particular scenario of a relapse occurring after allogeneic HSCT with a sibling donor has not been addressed with this technique. In such cases, the bone marrow consists of a mixture of the patient-derived leukemic cells and the donor-derived normal hematopoietic cells, displaying different degrees of chimerism depending on the proportion of leukemic cells. In the present study, we have investigated hematologic malignancies that relapsed after allogeneic HSCT with a sibling donor, and we here provide examples and discuss the particular properties of these samples in terms of SNP array analysis. Furthermore, we have, for the first time, investigated whether the breakpoints of acquired leukemia-associated genetic abnormalities and meiotic recombination events are correlated in a single individual genome.

The study included six cases of relapsed hematologic malignancies after HSCT with a sibling donor, comprising one acute myeloid leukemia M0, two acute myeloid leukemia M5, two myelodysplastic syndromes and one chronic myeloid leukemia. DNA was extracted according to standard methods from bone marrow samples obtained at relapse. In addition, a dilution series of a mixture of peripheral blood samples from two unrelated healthy individuals was prepared in ratios of 1:9, 2:8, 3:7, 4:6 and 5:5. SNP array analysis was performed using the Illumina 1M-duo bead Infinium BD BeadChip platform (Illumina, San Diego, CA, USA) as previously described.[7] Expected B-allele frequency (BAF) values for each combination of genotypes in two mixed cell populations were calculated using the formula $BAF_{exp} = [B_1p + B_2(1-p)]/[L_1p + L_2(1-p)]$, where B is the number of B alleles in the respective cell population, p is the frequency of cell population 1, and L is the

CBL mutations in infant acute lymphoblastic leukaemia

Infant acute lymphoblastic leukaemia (ALL) is relatively rare, occurring in approximately 2·5–5% of cases of childhood ALL (Biondi *et al*, 2000). Infant ALLs are much more likely to present with high leucocyte counts, hepatosplenomegaly and overt central nervous system (CNS) diseases (Taki *et al*, 1996). T cell phenotype is much less common in infants, while myeloid antigen co-expression and the absence of CD10 expression are more frequent in infants than in older children with ALL. When molecular techniques [such as fluorescence *in situ* hybridization (FISH) or Southern blot analysis] are used in addition to karyotype, *MLL* gene rearrangements (*MLL*-R) are found in 70–80% of infant ALL compared with only 2–4% of older children with ALL (Taki *et al*, 1996; Biondi *et al*, 2000). Thus, infant ALL appears to be biologically distinct from the disease in older children (more than 1 year old).

In this regard, recent reports of somatic mutations of the *CBL* proto-oncogene in myeloid neoplasms are intriguing, because these *CBL* mutations were shown to result in aberrant tyrosine kinase signalling, which also leads to activation of RAS signalling pathways. So far, we and others have reported that *CBL* mutations occur in a variety of myeloid neoplasms, including *de novo* acute myeloid leukaemia (AML) (Caligiuri *et al*, 2007), myelodysplastic syndrome (MDS), and myeloproliferative neoplasm, especially in chronic myelomonocytic leukaemia (CMML) (Sanada *et al*, 2009), and juvenile myelomonocytic leukaemia (JMML) (Shiba *et al*, 2010). The importance of *CBL* mutations regarding leukaemogenesis is substantially increased. Recently, we found *CBL* mutation in therapy-related AML with *MLL*-R (Shiba *et al*, 2011). Interestingly, the *MLL-CBL* fusion gene has been reported in a *de novo* AML case (Fu *et al*, 2003), and this prompted us to search for possible *CBL* mutations in infant ALL with *MLL-R*.

Because *CBL* mutations thus far reported were almost all clustered within exons 8–9 that encode Linker/RING finger domains (Caligiuri *et al*, 2007; Sanada *et al*, 2009; Shiba *et al*, 2010), we confined our mutation analysis to these exons, in which polymerase chain reaction-amplified exons 8–9 were subjected to direct sequencing using an ABI PRISM 310 Genetic Analyser (Applied Biosystems, Branchburg, NJ, USA). The study adhered to the principles of the Helsinki Declaration, and was conducted under the regulations enacted by the Ethics Board of Gunma Children's Medical Centre.

CBL gene analysis was performed in 41 infant ALL patients in which *MLL*-R was found in 33 patients (80·5%), including 15 patients with t(4;11)(q21;q23), 4 with t(9;11)(p22;q23) and 5 with t(11;19)(q23;p13.3). Median age at diagnosis was 4·7 months (range, 0–12 months). We also performed *CBL* gene mutation analysis in 28 B cell precursor (BCP)-ALL patients (age range, 1–14 years).

Heterozygous mutations of the *CBL* gene were identified in 2 (4·9%) of 41 infant ALL patients, but not in older children with BCP-ALL. These were located in exon 8 (Fig 1). One patient was a 3-month-old female with t(4;11)(q21;q23) and the other patient was a 6-month-old male with t(11;19)(q23;p13.3). They were registered and treated on two Japanese infant leukaemia protocols, MLL96 and MLL98 respectively (Isoyama *et al*, 2002; Kosaka *et al*, 2004). Although strong association between *CBL* mutations and 11q-acquired uniparental disomy (aUPD) has been reported (Sanada *et al*, 2009), we did not perform the single nucleotide polymorphism array analysis due to lack of DNA.

MLL-R are more frequent in younger infants; up to 90% of infant ALL less than 6 months old at diagnosis have detectable *MLL*-R compared with 30–50% of infant ALL aged 6–12 months (Taki *et al*, 1996). *MLL*-R ALL has a characteristic gene expression profile that significantly differs from that of non-*MLL*-R BCP-ALL and of AML, confirming that *MLL*-R ALL is a biologically unique leukaemia subtype.

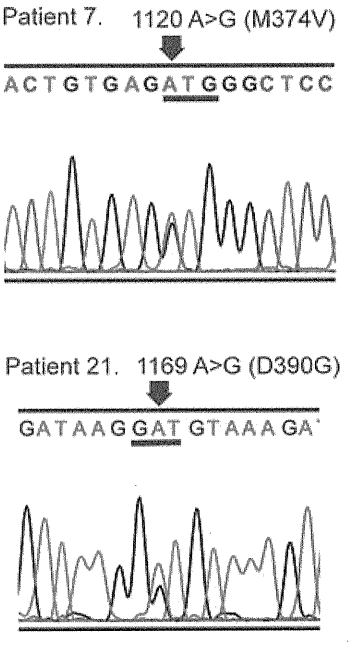

Fig 1. Identification of *CBL* mutations. Heterozygous mutations of the *CBL* gene were identified in Patients 7 and 21.

Thus, the distinctive presenting features and clinical behaviour of infant ALL appear to be primarily due to the high frequency of *MLL*-R in this age group. However, outcome data comparing infant and non-infant patients with *MLL*-R suggest that there may be other factors which impact the prognosis of infant ALL. Both of the patients with *CBL* mutations were diagnosed before 6 months of age. In our previous report, all of three cases with *CBL* mutation developed JMML before 4 months of age (Shiba *et al*, 2010). These data suggested that *CBL* mutation may have a strong association with very early onset disease. *CBL* mutations have been reported as germline mutations in JMML (Niemeyer *et al*, 2010). Unfortunately, we could not investigate whether the mutations in our cases were germline mutations or not, because somatic cells were not available.

CBL mutations have been found in approximately 5% of 2000 samples from patients with myeloid neoplasms, including AML transformed from MDS. Gene aberrations in addition to *MLL*-R have rarely been reported in infant ALL. No reports of ALL with *CBL* mutations have so far been reported, suggesting that the pathogenesis of infant ALL is different from paediatric or adult ALL. To our knowledge, this is the first report of infant ALL patients with 11q23 translocation/*MLL*-R and *CBL* mutations. The present study suggests that alterations of *CBL* gene and *MLL*-R may cooperatively play a pathogenic role in the development of infant ALL with *MLL*-R.

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Author's contributions

TT and YH designed the study. JT, MH, TK, MS and EI provided critical reagents and samples. NS and MP performed the experiments. EI, HA and SO supervised the work. NS and MP analysed the results. NS, TT, and YH wrote the paper and all the authors critically reviewed and revised it.

Conflict of interest

The authors declare no conflict of interest.

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Association of an increased frequency of CD14^{+}HLA-DR$^{lo/neg}$ monocytes with decreased time to progression in chronic lymphocytic leukaemia (CLL)

Clinically important immune dysregulation is an early feature of leukaemia/small lymphocytic lymphoma (CLL) that often precedes other clinical manifestations of this disease. Defects in the adaptive immune system in patients with CLL results in quantitative and qualitative abnormalities in antibody production, and profound changes in T and Natural Killer (NK) cell numbers, ratios, and function (Palmer *et al*, 2008; Gonzalez-Rodriguez *et al*, 2010). Monocytes and macrophages, critical for adaptive and innate immune responses, also have an important role in the function of the CLL cell microenvironment, and this relationship continues to be explored (Caligaris-Cappio, 2011). However, little is known about the effects of CLL on monocyte/macrophage physiology and whether alterations in monocytes have any clinical role in CLL. We were especially interested in the possible role of immunosuppressive CD14+ monocytes with reduced HLA-DR expression in CLL as we have observed this phenomena in glioblastoma (Gustafson *et al*, 2010), non-Hodgkin lymphoma (Lin *et al*, 2011) and prostate cancer (Vuk-Pavlovic *et al*, 2010).

To identify potential monocyte alterations by CLL in patients, we performed flow cytometric analysis of peripheral

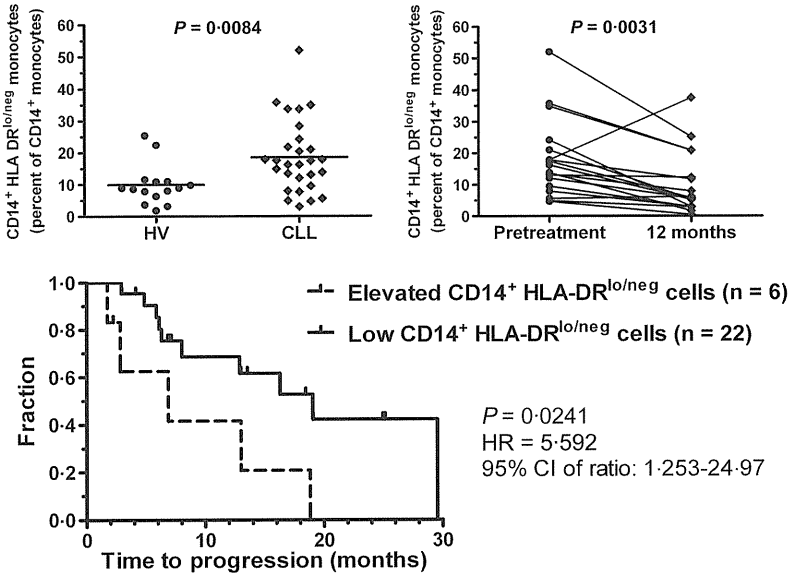

Fig 1. CD14^{+}HLA-DR$^{lo/neg}$ Monocytes in CLL. Blood from 29 CLL patients and 15 healthy volunteers were used for immune phenotyping. There was no age difference (median of 59 vs. 58 years, respectively; P = 0·2896). Patients were eligible for the clinical trial (ClinicalTrials.gov NCT00562328) if diagnosed with previously untreated high-risk CLL using standard criteria and did not meet guidelines for conventional treatment. Blood was collected before initiation of treatment and 6, 9, and 12 months after completion of treatment in patients who had a sustained response. The percentage of CD14+ cells with a loss of HLA-DR staining was determined and compared between CLL patients and healthy volunteers (HV; upper left). CLL patients with a sustained response to treatment had a decrease in the frequency of CD14^{+}HLA-DR$^{lo/neg}$ monocytes 12 months after completion of treatment compared to measurement prior to treatment (upper right). Kaplan–Meyer survival curve comparing CLL patients with elevated ratios (>2·5 standard deviations) of CD14^{+}HLA-DR$^{lo/neg}$ monocytes when compared to healthy volunteers (dashed line) or with ratios similar to those seen in healthy volunteers (solid line; bottom panel). HR, Hazard Ratio; 95% CI, 95% confidence interval.

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IDH1 and *IDH2* mutations are rare in pediatric myeloid malignancies

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Recently, recurrent somatic missense mutations in $NADP^+$-dependent isocitrate dehydrogenase gene (*IDH1*) at codon R132, as well as *IDH2* at codon R172, have been identified in low-grade gliomas/secondary glioblastoma by high-throughput sequencing.[1] Subsequent studies also revealed that acquired somatic mutations in *IDH1* frequently occurred in adult hematological malignancies, such as acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS).[2,3] More recently, Paschka *et al.*[4]reported that not only *IDH1* but also *IDH2* mutations occurred relatively frequently in adult AML, and that these mutations were associated with older age, poor prognosis, cytogenetically normal AML (CN-AML) and the genotype of mutated *NPM1* without *FLT3*-internal tandem duplication (ITD). Exon 4 of both *IDH1* and *IDH2*, which was previously identified as a hot spot for mutations in these genes, encodes three arginine residues (R100, R109 and R132 in *IDH1* and R140, R149, and R172 in *IDH2*) that are important for protein activities.[5] Tumor-derived *IDH1* and *IDH2* mutations impair the affinity of enzymes for substrates, and dominantly inhibit wild-type *IDH1* and *IDH2* activities through the formation of catalytically inactive heterodimers.[5] Ho *et al.*[6] previously reported that *IDH1* mutations are not detected in pediatric AML; however, little is known about the incidence and prognostic values of *IDH1* and *IDH2* mutations in pediatric myeloid malignancies. Here, we analyzed mutations that involve the activation sites of *IDH1* and *IDH2* (exon 4 and exon 7 in both *IDH1* and *IDH2*) using genomic DNA-polymerase chain reaction amplification/sequencing in a total of 199 samples of pediatric myeloid malignancies, including 17 AML-derived cell lines, 115 primary cases of AML, 28 primary cases of MDS, 15 primary cases of juvenile myelomonocytic leukemia (JMML), 6 chronic myeloid leukemia (CML)-derived cell lines and 18 primary cases of CML. Moreover, to assess whether *IDH1* and *IDH2* mutations overlap with known gene abnormalities, such as *FLT3*, *c-KIT* and *NPM1* mutations, mutational analyses of *FLT3*, *c-KIT* and *NPM1* were also performed in AML samples. This study was approved by the ethics committee of the University of Tokyo (Approval Number 3043).

The common *IDH2* R140Q mutation was detected in a single AML case, whereas no *IDH1* mutation including G123E, as well as no other *IDH2* mutations, such as R172K, were detected in our study (Figure 1). The *IDH2* R140Q mutation detected in the AML case was a heterozygous substitution. No *IDH1* and *IDH2* mutations were detected in the JMML, MDS or CML samples examined. As the additional activation sites of both *IDH1* and *IDH2* are located in exon 7 of these genes, direct sequencing of exon 7 of *IDH1* and *IDH2* was also performed, but no mutations were detected in our series. Six AML samples including one cell line had *c-KIT* mutations (D816V, N822K and D419fs), and 12 AML samples had *FLT3-ITD*. The *NPM1* mutation was detected in 2 of 132 AML samples. The AML case harboring the *IDH2* mutation, case 39, showed no abnormalities of *NPM1*, *c-KIT* and *FLT3*. Case 39 was a 12-year-old boy diagnosed as AML-M2 according to the French–American–British cooperative group classification system. Bone marrow blasts obtained at initial diagnosis showed t(8;21)(q22;q22). After complete remission was achieved by the ACMP (adriamycin, cytarabine, 6-mercaptopurine, prednisolone) two-step induction therapy, the patient underwent consolidation therapy every 5 weeks, but hematological relapse occurred 11 months after the initial diagnosis. He was treated with low-dose cytarabine, but died 5 months after relapse with progressive disease. To assess the genetic mechanisms involved in the pathogenesis of the disease of this case, we further performed genome-wide copy number analysis of bone marrow blasts obtained at initial diagnosis of this case, using single-nucleotide polymorphism (SNP)-genotype microarrays (Affymetrix GeneChip Mapping 250 K *Sty*I arrays,

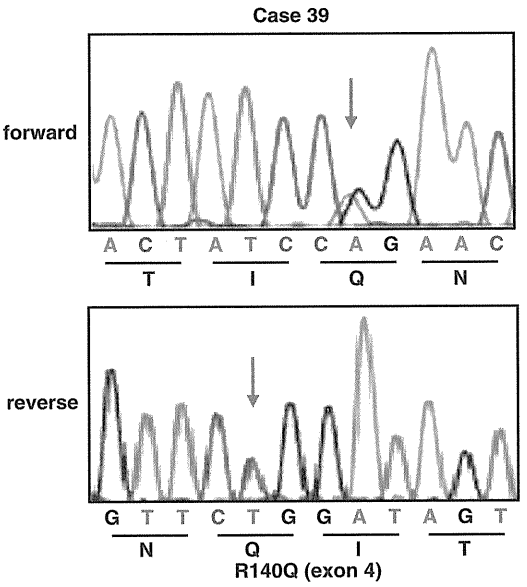

Figure 1 Sequence chromatogram of the *IDH2* mutation detected in a pediatric AML patient. A heterozygous mutation at R140 in exon 4 of *IDH2* is shown (top and bottom: forward and reverse sequencing results, respectively). Mutated nucleotides are indicated by arrows.

Affymetrix, Inc., Santa Clara, CA, USA). As shown in Figure 2, complex chromosomal abnormalities, such as heterozygous deletions at chromosomes 7q11.2, 7q34-qter, 9q13-q21.33, 9q22.33, 16q23.1-q24.3 and 17q12qter, as well as gains of 4q24.3, 17q12-qter and 22q12.3-q13.33 were detected in leukemic cells of this patient (Figure 2).

To our knowledge, this is the first report to describe the *IDH2* mutation in a pediatric AML patient. In the present study, we detected the *IDH2* R140Q mutation in a single AML case out of 199 samples of pediatric myeloid malignancies, which suggests that the involvement of *IDH1* and *IDH2* mutations in the pathogenesis of pediatric AML is extremely rare compared with those in adult AML cases. Likewise, although *IDH* mutations are frequently observed in adult brain tumors, they are not observed in pediatric cases.[1] Therefore, somatically acquired *IDH1* and *IDH2* mutations may be related to an acquired neoplastic pathway exclusive to adult patients. Several groups have reported that *IDH1* and *IDH2* mutations are significantly associated with a normal karyotype in adult AML.[4,6] However, our patient with an *IDH2* mutation had t(8;21) together with complex chromosomal changes. Furthermore, a previously reported genome-wide study of pediatric AML revealed that, in contrast to our AML patients with *IDH2* mutation, pediatric *de novo* AML was characterized by a very low burden of genomic alterations.[7] These clinical and cytogenetic data suggest that pediatric AML with t(8;21) and *IDH2* mutation might be a specific subtype of AML with complex chromosomal abnormalities and poor prognosis. Thus, our result has important clinical and pathological implications regarding the role of *IDH2* mutations in the development of AML. t(8;21) is considered as a distinct AML subtype associated with characteristic morphology and a favorable prognosis.[8] Although approximately 90% of AML patients with t(8;21) achieve remission, relapse is frequent.[8] Once the disease relapses, the prognosis is poor, with an overall survival of 50% at 5 years.[8] Although the *c-KIT* mutation and *FLT3-ITD* are considered as poor prognostic factors in AML patients with t(8;21), these abnormalities occur in approximately 10% of AML patients with t(8;21).[9] Notably, *IDH1* and *IDH2* mutations constitute a poor prognostic factor in CN-AML with mutated *NPM1* without *FLT3-ITD*, which allows refined risk stratification of this AML subset.[4] Although treatment contents as well as clinical and genetic backgrounds were some of the parameters influencing the patient's outcome, our findings suggest that the *IDH2* mutation may also be related to an inferior outcome in pediatric AML patients with t(8;21) even if they lack the *c-KIT* mutation and *FLT3-ITD*. As *IDH2* mutation with t(8;21) is an extremely rare event and the prognostic values of *IDH2* mutations in AML

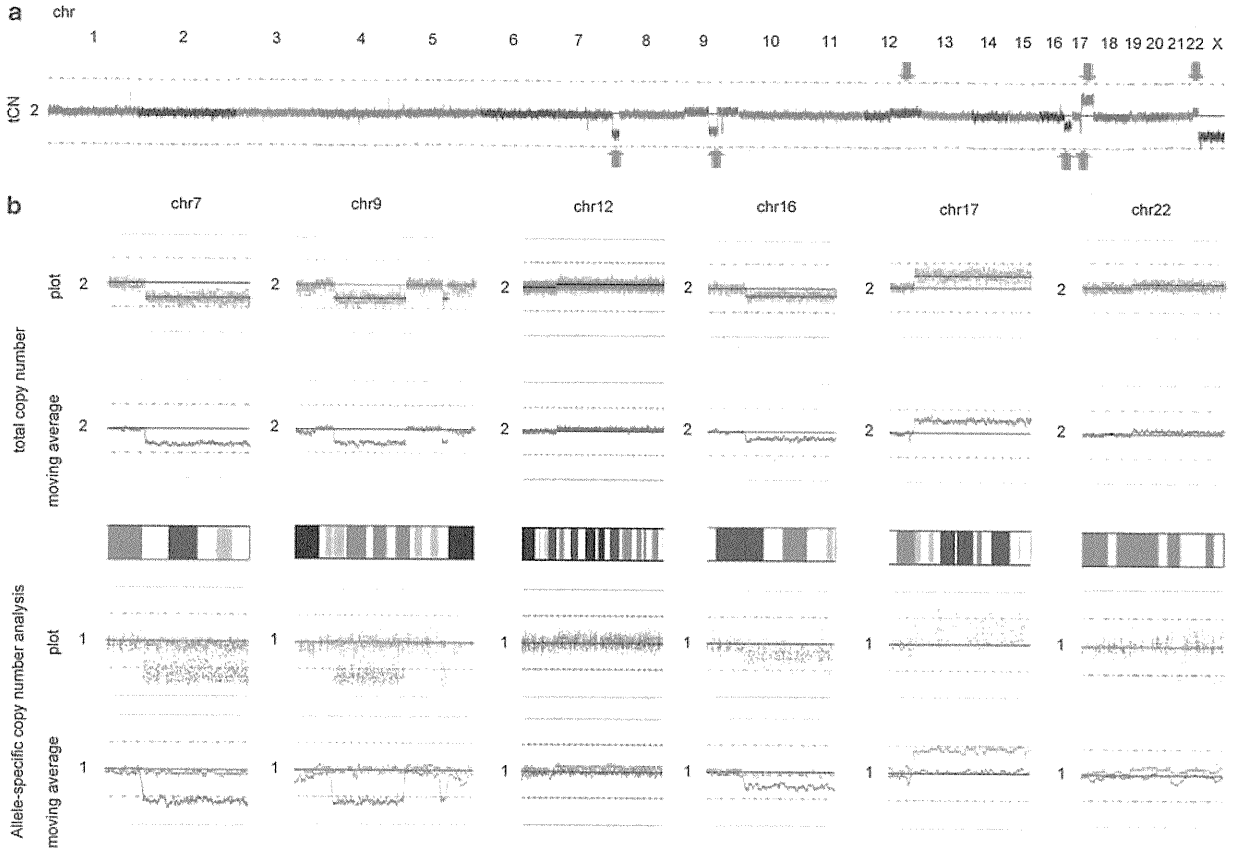

Figure 2 The result of copy number analysis using SNP-genotyping microarrays. (**a**) The moving average of the total copy number plot is presented. Each chromosome is indicated by different colors. Deletions in the regions at 7q, 9q, 16q and 17q, and gains in the region at 12q, 17q and 22q are indicated by the red arrows. (**b**) Deletions of 7q, 9q, 16q and 17q, and gains of 12q, 17q and 22q. The total copy number plot from each probe (red points) and the moving average (blue line) are shown above the cytobands. The results of the allele-specific analysis with CNAG/AsCNAR are shown below the cytobands. The larger allele is presented in red, and the smaller allele is presented in green. The numbers located at the left edge of each lane indicate a normal copy number (2 for total copy number analysis and 1 for allele-specific copy number analysis).

with t(8;21) are still unclear, further data accumulation is necessary. Although uncommon in pediatric myeloid malignancies, *IDH1* and *IDH2* mutations, particularly *IDH2* mutations, could contribute to the advanced phenotype of AML. Our findings provide additional impetus for investigating the role of *IDH1* and *IDH2* in the pathophysiology of errors of metabolism and in neoplastic disorders.

Conflict of interest

The authors declare no conflict of interest.

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ARTICLE

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Frequent pathway mutations of splicing machinery in myelodysplasia

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Myelodysplastic syndromes and related disorders (myelodysplasia) are a heterogeneous group of myeloid neoplasms showing deregulated blood cell production with evidence of myeloid dysplasia and a predisposition to acute myeloid leukaemia, whose pathogenesis is only incompletely understood. Here we report whole-exome sequencing of 29 myelodysplasia specimens, which unexpectedly revealed novel pathway mutations involving multiple components of the RNA splicing machinery, including *U2AF35*, *ZRSR2*, *SRSF2* and *SF3B1*. In a large series analysis, these splicing pathway mutations were frequent (~45 to ~85%) in, and highly specific to, myeloid neoplasms showing features of myelodysplasia. Conspicuously, most of the mutations, which occurred in a mutually exclusive manner, affected genes involved in the 3′-splice site recognition during pre-mRNA processing, inducing abnormal RNA splicing and compromised haematopoiesis. Our results provide the first evidence indicating that genetic alterations of the major splicing components could be involved in human pathogenesis, also implicating a novel therapeutic possibility for myelodysplasia.

Myelodysplastic syndromes (MDS) and related disorders (myelodysplasia) comprise a group of myeloid neoplasms characterized by deregulated, dysplastic blood cell production and a predisposition to acute myeloid leukaemia (AML)[1]. Although the prevalence of MDS has not been determined precisely, more than 10,000 people are estimated to develop myelodysplasia annually in the United States[2]. Their indolent clinical course before leukaemic transformation and ineffective haematopoiesis with evidence of myeloid dysplasia indicate a pathogenesis distinct from that involved in *de novo* AML. Currently, a number of gene mutations and cytogenetic changes have been implicated in the pathogenesis of MDS, including mutations of *RAS*, *TP53* and *RUNX1*, and more recently *ASXL1*, *c-CBL*, *DNMT3A*, *IDH1/2*, *TET2* and *EZH2* (ref. 3). Nevertheless, mutations of this set of genes do not fully explain the pathogenesis of MDS because they are also commonly found in other myeloid malignancies and roughly 20% of MDS cases have no known genetic changes (ref. 4 and unpublished data). In particular, the genetic alterations responsible for the dysplastic phenotypes and ineffective haematopoiesis of myelodysplasia are poorly understood. Meanwhile, the recent development of massively parallel sequencing technologies has provided an expanded opportunity to discover genetic changes across the entire genomes or protein-coding sequences in human cancers at a single-nucleotide level[5–10], which could be successfully applied to the genetic analysis of myelodysplasia to obtain a better understanding of its pathogenesis.

Overview of genetic alterations

In this study, we performed whole-exome sequencing of paired tumour/control DNA from 29 patients with myelodysplasia (Supplementary Table 1). Although incapable of detecting non-coding mutations and gene rearrangements, the whole-exome approach is a well-established strategy for obtaining comprehensive registries of protein-coding mutations at low cost and high performance. With a mean coverage of 133.8, 80.4% of the target sequences were analysed at more than ×20 depth on average (Supplementary Fig. 1). All the candidates for somatic mutations ($N = 497$) generated through our data analysis pipeline were subjected to validation using Sanger sequencing (Supplementary Methods I and Supplementary Fig. 2). Finally, 268 non-synonymous somatic mutations were confirmed with an overall true positive rate of 53.9% (Supplementary Fig. 3), including 206 missense, 25 nonsense, and 10 splice site mutations, and 27 frameshift-causing insertions/deletions (indels) (Supplementary Fig. 4). The mutation rate of 9.2 (0–21) per sample was significantly lower than that in solid tumours (16.2–302)[7,11,12] and multiple myeloma (32.4)[6], but was comparable to that in AML (7.3–13)[13–15] and chronic lymphocytic leukaemia (11.5)[16]. Combined with the genomic copy number profile obtained by single nucleotide polymorphism (SNP) array karyotyping, this array of somatic mutations provided a landscape of myelodysplasia genomes (Supplementary Fig. 5)[17,18].

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