新生児希有疾患（病態）前方視的サーベイランス事業
趣旨，実施概略ならびに登録状況

新生児希有疾患サーベイランス委員会
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■趣　旨：

前もって定められた新生児領域における希有な疾患（病態）を対象とし，公募の上決定された本学会会員がモニター役となり（サーベイランスモニター員），共通のプロトコールで前方視的にデータを集積し，資料（血液検体）などを当該担当委員へ送付し，それらの疾患（病態）の予防対策の確立など医療改善の全国的な体制を企図する。

■実施概略：

1 「サーベイランス委員会」は，公募の上，採択された各疾患（病態）に対して，「当該疾患サーベイランス担当者」を指名して具体的な調査企画の立案を促し，「サーベイランス委員会」での検討を経て，実施する。
2 公募の上，採択された「サーベイランスモニター員」は，対象疾患（病態）を経験した時点で，あらかじめ送付された調査用紙を用いて，「サーベイランス委員会」へ直ちにFAXまたはE-mailにて報告する。
3 対象疾患（病態）を経験しない「サーベイランスモニター員」は，おおよそ年3回送付されてくる新しい調査用紙に，経験なしと報告する。
4 「サーベイランス委員会」より情報を得た「当該疾患サーベイランス担当者」は，「サーベイランスモニター員」へ直接連絡の上，具体的なデータの収集，資料（血液検体）の送付の依頼などを行う。
5 「当該疾患サーベイランス担当者」は，所定の期間が終了した時点で，調査報告書を「サーベイランス委員会」へ提出する。
6 「サーベイランス委員会」は，「当該疾患サーベイランス担当者」からの報告とともに，計画全体の報告を理事会へ提出する。
7 理事会は，全会員へ報告を行う。

■登録状況：

平成22年1月15日現在，156施設にご登録いただいております。「サーベイランス委員会」では，引き続いて「サーベイランスモニター員」の新規登録の申請を受け付けております。本誌に挟み込みの申請用紙をご利用ください。

■進行状況：

平成22年1月15日現在，該当症例有り9件（新生児単純ヘルペス感染症7件，Paternal Uniparental Disomy 14が2件）ありました。

新生児希有疾患（病態）前方視的サーベイランス事業
対象疾患の追加ならびにモニター員の継続公募について

サーベイランス委員会では，当事業にご賛同いただけるモニター員を引き続き募集しております。本誌に挟み込んであります公募用紙に必要事項を記入の上，お申し込みください。なお，一施設から複数ご参加の意思をいただきました折りには，委員会にて調整させていただきますことをお断り申し上げます。

すでに登録されているサーベイランスモニター員におかれましては，下記調査対象期間中，下記当該疾患を経験された毎に，本誌に挟み込んであります報告書をコピーの上，速やかに委員会宛 FAX にてご連絡ください（E-mail も可）。なお，該当疾患を経験されなかった場合にも，「該当疾患なし」とご報告ください。

現行対象疾患，予定調査期間ならびに登録対象症例：

1　新生児単純ヘルペス感染症

サーベイランス担当者：伊藤　進（香川大学医学部小児科）

予定調査期間：2007 年 7 月 1 日～ 2010 年 6 月 30 日（3 年間）

対象の選定基準，除外基準：特になし

2　Paternal Uniparental Disomy 14（父性片親ダイソミー 14 番：pUPD 14）

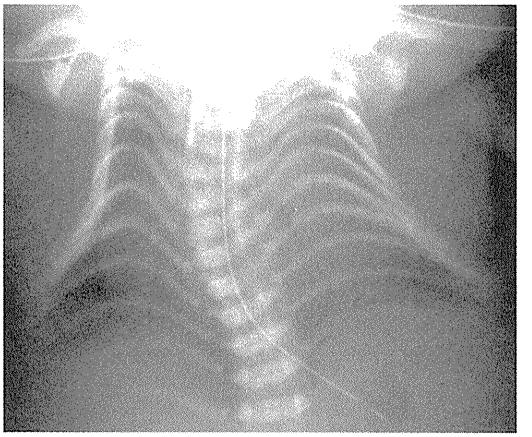
サーベイランス担当者：柴崎　淳（神奈川県立こども医療センター新生児科）

予定調査期間：2007 年 7 月 1 日～ 2010 年 6 月 30 日（3 年間）

対象の選定基準，除外基準：

1. 選定基準
   1）羊水過多
   2）特徴的な胸部レントゲン写真（ベル型胸郭・波打った肋骨 /coat hanger appearance）
   3）臍帯ヘルニアまたは腹直筋離開

以上の 3 項の全てを満たす症例。遺伝子診断が確定診断だが，臨床診断症例も該当症例とする。遺伝子診断は国立成育医療センターで施行可能。遺伝子診断の同意書も同センター作成のものがある。



2. 除外基準

その他の染色体異常，先天性筋強直性ジストロフィー

3　ダウン症候群に合併した一過性骨髄増殖症（TAM）＊新規

サーベイランス担当者：田村正徳（埼玉医科大学総合医療センター小児科）

予定調査期間：2010 年 3 月 1 日～ 2013 年 3 月 1 日（3 年間）

対象の選定基準，除外基準：

1. 選定基準
   ・ダウン症候群ないしはモザイクダウン症候群において，日齢 90 以内に末梢血中に芽球が出現した症例。ただし，体細胞は正常核型でも，芽球にトリソミー 21 または *GATA1* 遺伝子の変異を認める症例も対象とする。
   ・診断日は，生後初めて末梢血中に芽球の存在を確認した検体の採取日とする。
2. 除外基準

乳児白血病と診断された症例

Brief report

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

Takashi Yokoyama,[1] Tsutomu Toki,[2] Yoshihiro Aoki,[2] Rika Kanezaki,[2] Myoung-ja Park,[3] Yohei Kanno,[1] Tomoko Takahara,[1] Yukari Yamazaki,[1] Etsuro Ito,[2] Yasuhide Hayashi,[3] and Takuro Nakamura[1]

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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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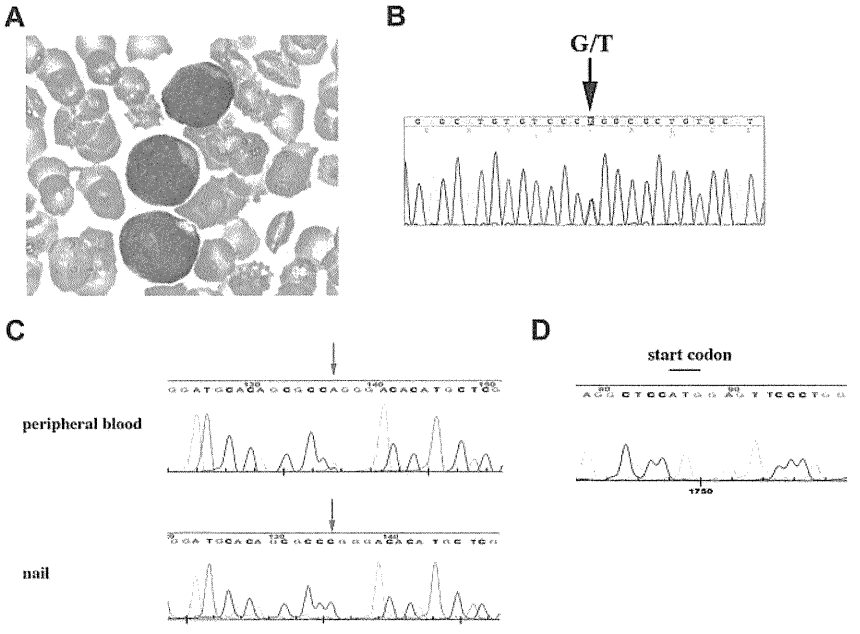

Figure 1. *TRIB1* R107L mutation identified in DS-related leukemias. (A) Giemsa staining of the case 84 peripheral blood smear diagnosed as AMKL. The image was acquired using a BX40 microscope equipped with a 100×/1.30 NA oil objective (Olympus) and a C-4040 digital camera (Olympus). (B) Fluorescent 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

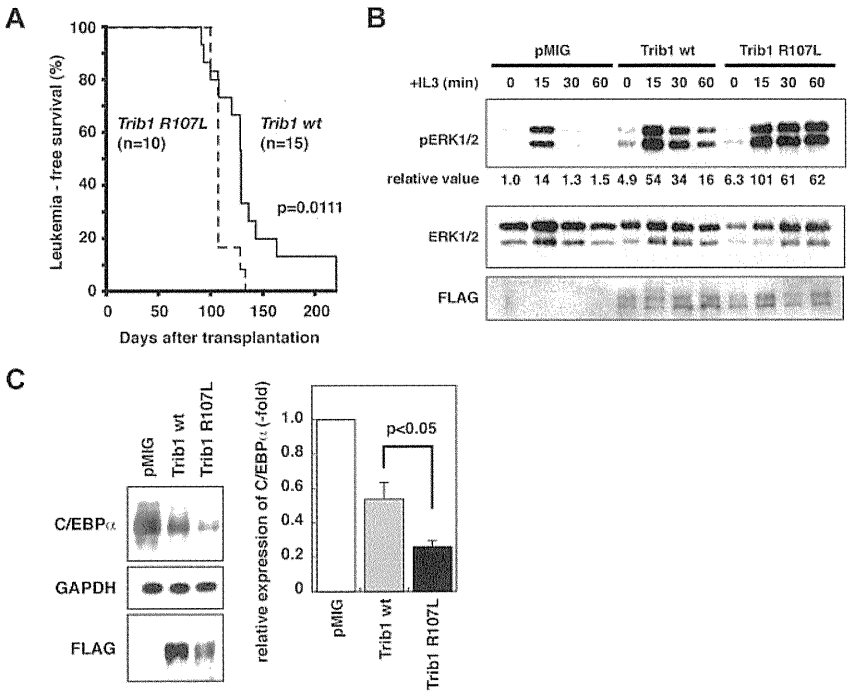

Figure 2. AML development by bone marrow transfer using *Trib1* WT and R107L. (A) Kaplan-Meier survival curves are shown. The *P* value was calculated with the log-rank test. (B) Immunoblot analysis of *Trib1* WT AML (Mac-1 56.2%, Gr-1 52.5%, $CD34^{lo}$, c-kit^{-}, Sca-1^{-}) and R107L AML (Mac-1 41.4%, 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.

Acknowledgments

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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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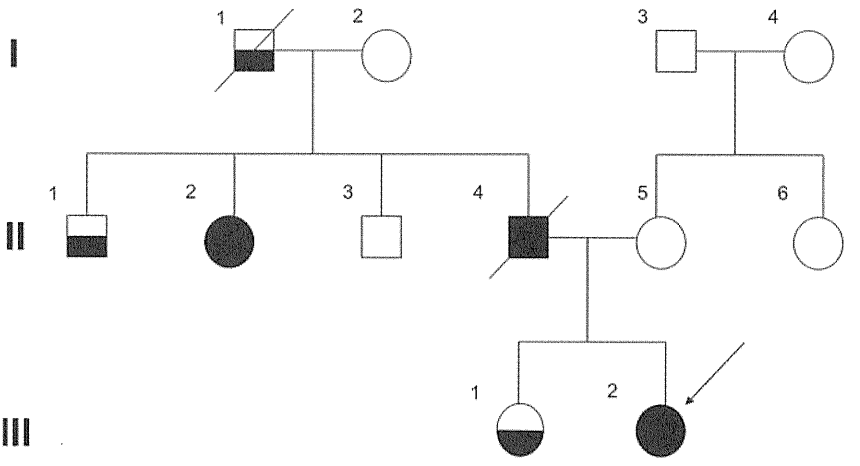

Figure 1. The family pedigree. Squares indicate males and circles indicate females. Open symbols represent unaffected persons, half-filled symbols represent persons affected by thrombocytopenia, and closed symbols represent persons affected by FPD who developed MDS/AML. The proband (III-2) is indicated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

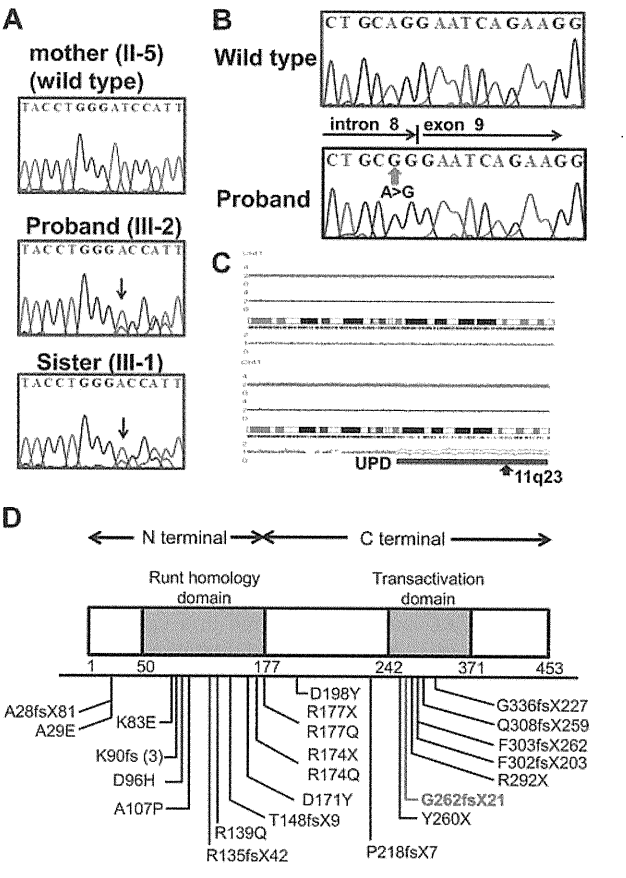

Figure 2. Mutation analysis of *RUNX1* and *CBL* genes in the pedigree. (A) Direct sequencing analysis of affected patients (III-1, III-2) and an unaffected family member (II-5) is shown. Arrow indicates a one-base deletion of adenine. (B) Mutated *CBL* is shown in the proband. (C) Identification of 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.

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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

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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

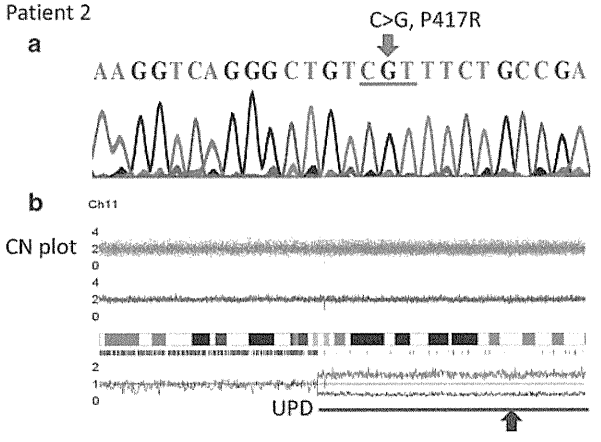

Figure 1 Identification of acquired isodisomy of 11q and *CBL* mutation in therapy-related leukemia. (**a**) Homozygous mutation of the *CBL* gene was identified in patient 2. (**b**) Copy number (CN) analysis for the gene chip output for therapy-related leukemia in patient 2. Total CNs (red plot) are 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 $BAF_{exp} = [B_1 p + B_2(1-p)]/[L_1 p + L_2(1-p)]$, 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.

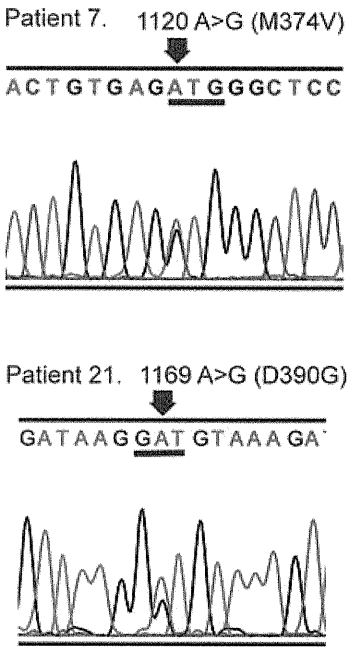

Fig 1. Identification of *CBL* mutations. Heterozygous mutations of the *CBL* gene were identified in Patients 7 and 21.

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

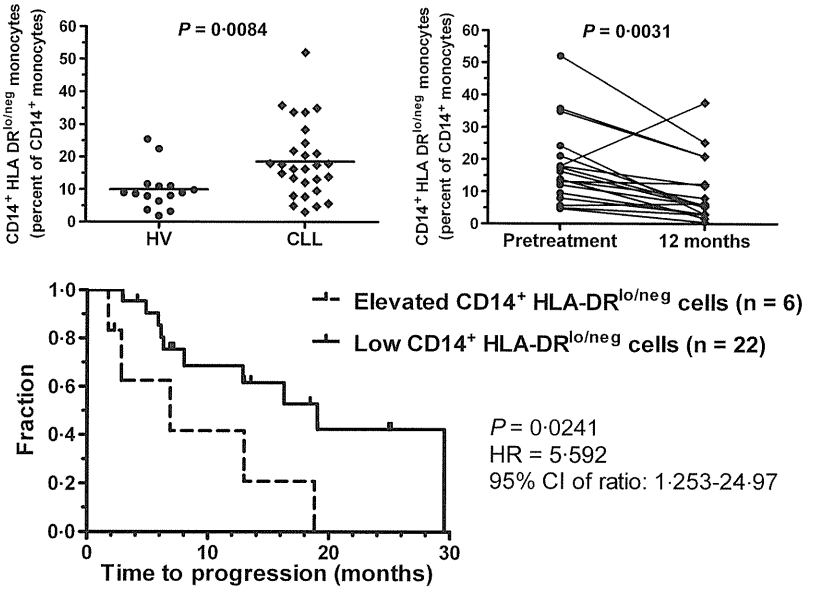

Fig 1. CD14^{+}HLA-DR$^{lo/neg}$ Monocytes in CLL. Blood from 29 CLL patients and 15 healthy volunteers were used for immune phenotyping. There was no age difference (median of 59 vs. 58 years, respectively; P = 0·2896). Patients were eligible for the clinical trial (ClinicalTrials.gov NCT00562328) if 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.

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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Novel gene targets in myelodysplasia

The list of the somatic mutations (Supplementary Table 2) included most of the known gene targets in myelodysplasia with similar mutation frequencies to those previously reported, indicating an acceptable sensitivity of the current study. The mutations of the known gene targets, however, accounted for only 12.3% of all detected mutations ($N = 33$), and the remaining 235 mutations involved previously unreported genes. Among these, recurrently mutated genes in multiple cases are candidate targets of particular interest, for which high mutation rates are expected in general populations. In fact, 8 of the 12 recurrently mutated genes were among the well-described gene targets in myelodysplasia (Supplementary Table 3). However, what immediately drew our attention were the recurrent mutations involving *U2AF35* (also known as *U2AF1*), *ZRSR2* and *SRSF2* (*SC35*), because they belong to the common pathway known as RNA splicing. Including an additional three genes mutated in single cases (*SF3A1*, *SF3B1* and *PRPF40B*), six components of the splicing machinery were mutated in 16 out of the 29 cases (55.2%) in a mutually exclusive manner (Fig. 1, Supplementary Fig. 6 and Supplementary Table 2).

Frequent mutations in splicing machinery

RNA splicing is accomplished by a well-ordered recruitment, rearrangement and/or disengagement of a set of small nuclear ribonucleoprotein (snRNP) complexes (U1, U2, and either U4/5/6 or U11/12), as well as many other protein components onto the pre-mRNAs. Notably, the mutated components of the spliceosome were all engaged in the initial steps of RNA splicing, except for PRPF40B, whose functions in RNA splicing are poorly defined. Making physical interactions with SF1 and a serine/arginine-rich (SR) protein, such as SRSF1 or SRSF2, the U2 auxiliary factor (U2AF) that consists of the U2AF65 (U2AF2)–U2AF35 heterodimer, is involved in the recognition of the 3′ splice site (3′SS) and its nearby polypyrimidine tract, which is thought to be required for the subsequent recruitment of the U2 snRNP, containing SF3A1 as well as SF3B1, to establish the splicing A complex (Fig. 1)[19]. ZRSR2 (or Urp), is another essential component of the splicing machinery. Showing a close structural similarity to U2AF35, ZRSR2 physically interacts with U2AF65, as well as SRSF1 and SRSF2, with a distinct function from its homologue, U2AF35 (ref. 20).

To confirm and extend the initial findings in the whole-exome sequencing, we studied mutations of the above six genes together with three additional spliceosome-related genes, including *U2AF65*, *SF1* and *SRSF1*, in a large series of myeloid neoplasms ($N = 582$) using a high-throughput mutation screen of pooled DNA followed by confirmation/identification of candidate mutations (refs 21 and 22 and Supplementary Methods II).

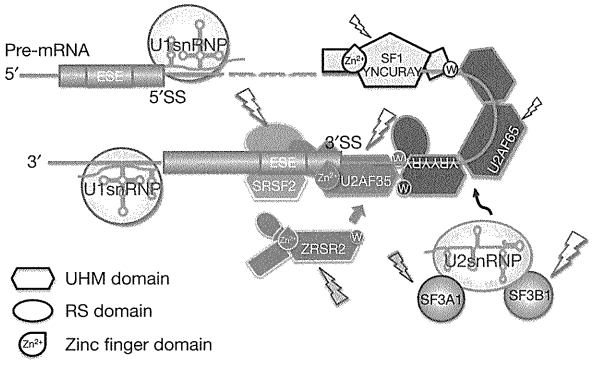

Figure 1 | Components of the splicing E/A complex mutated in myelodysplasia. RNA splicing is initiated by the recruitment of U1 snRNP to the 5′SS. SF1 and the larger subunit of the U2 auxiliary factor (U2AF), U2AF65, bind the branch point sequence (BPS) and its downstream polypyrimidine tract, respectively. The smaller subunit of U2AF (U2AF35) binds to the AG dinucleotide of the 3′SS, interacting with both U2AF65 and a SR protein, such as SRSF2, through its UHM and RS domain, comprising the earliest splicing complex (E complex). ZRSR2 also interacts with U2AF and SR proteins to perform essential functions in RNA splicing. After the recognition of the 3′SS, U2 snRNP, together with SF3A1 and SF3B1, is recruited to the 3′SS to generate the splicing complex A. The mutated components in myelodysplasia are indicated by arrows.

In total, 219 mutations were identified in 209 out of the 582 specimens of myeloid neoplasms through validating 313 provisional positive events in the pooled DNA screen (Supplementary Tables 4 and 5). The mutations among four genes, *U2AF35* ($N = 37$), *SRSF2* ($N = 56$), *ZRSR2* ($N = 23$) and *SF3B1* ($N = 79$), explained most of the mutations with much lower mutational rates for *SF3A1* ($N = 8$), *PRPF40B* ($N = 7$), *U2AF65* ($N = 4$) and *SF1* ($N = 5$) (Fig. 2). Mutations of the splicing machinery were highly specific to diseases showing myelodysplastic features, including MDS either with (84.9%) or without (43.9%) increased ring sideroblasts, chronic myelomonocytic leukaemia (CMML) (54.5%), and therapy-related AML or AML with myelodysplasia-related changes (25.8%), but were rare in *de novo* AML (6.6%) and myeloproliferative neoplasms (MPN) (9.4%) (Fig. 3a). The mutually exclusive pattern of the mutations in these splicing pathway genes was confirmed in this large case series, suggesting a common impact of these mutations on RNA splicing and the pathogenesis of myelodysplasia (Fig. 3b). The frequencies of mutations showed significant differences across disease types. Surprisingly, *SF3B1* mutations were found in the majority of the cases with MDS characterized by increased ring sideroblasts, that is, refractory anaemia with ring sideroblasts (RARS) (19/23 or 82.6%) and refractory cytopenia with multilineage dysplasia with ≥ 15% ring sideroblasts (RCMD-RS) (38/50 or 76%) with much lower mutation frequencies in other myeloid neoplasms. RARS and RCMD-RS account

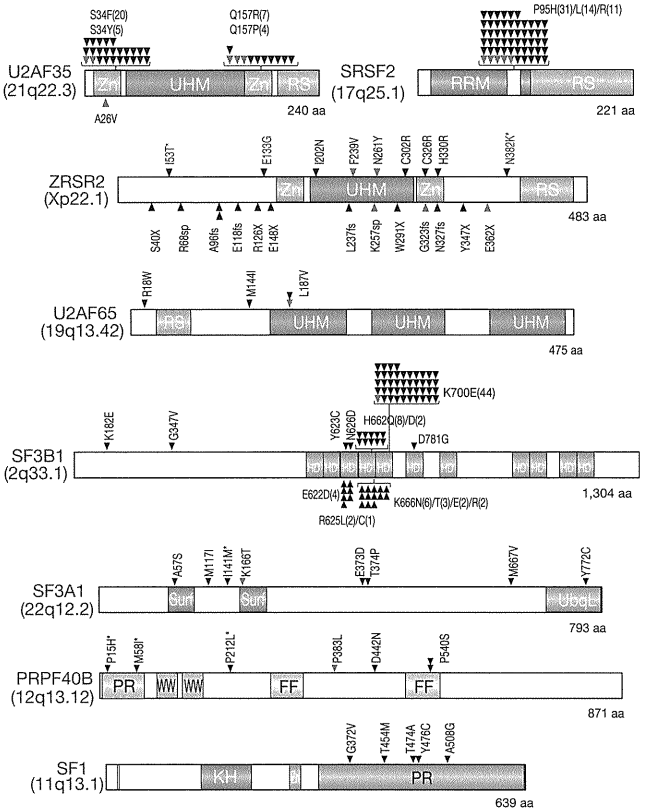

Figure 2 | Mutations of multiple components of the splicing machinery. Each mutation in the eight spliceosome components is shown with an arrowhead. Confirmed somatic mutations are discriminated by red arrows. Known domain structures are shown in coloured boxes as indicated. Mutations predicted as SNPs by MutationTaster (http://www.mutationtaster.org/) are indicated by asterisks. The number of each mutation is indicated in parenthesis. ZRSR2 mutations in females are shown in blue.

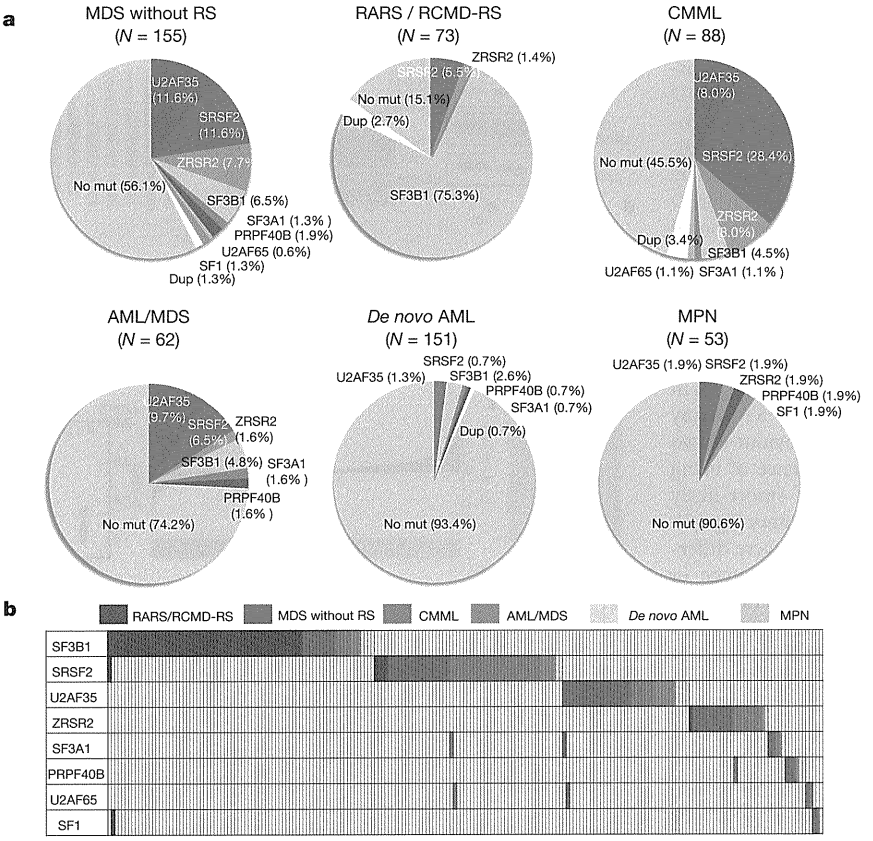

Figure 3 | Frequencies and distribution of spliceosome pathway gene mutations in myeloid neoplasms. a, Frequencies of spliceosome pathway mutations among 582 cases with various myeloid neoplasms. **b**, Distribution of mutations in eight spliceosome genes, where diagnosis of each sample is shown by indicated colours.

for 4.3% and 12.9% of MDS cases, respectively, where deregulated iron metabolism has been implicated in the development of refractory anaemia[23]. With such high mutation frequencies and specificity, the *SF3B1* mutations were thought to be almost pathognomonic to these MDS subtypes characterized by increased ring sideroblasts, and strongly implicated in the pathogenesis of MDS in these categories. Less conspicuously but significantly, *SRSF2* mutations were more frequent in CMML cases (Fig. 3 and Supplementary Table 4). Thus, although commonly involving the E/A splicing complexes, different mutations may still have different impacts on cell functions, contributing to the determination of discrete disease phenotypes. For example, studies have demonstrated that *SRSF2* was also involved in the regulation of DNA stability and that depletion of SRSF2 can lead to genomic instability[24]. Of interest in this context, regardless of disease subtypes, samples with *SRSF2* mutations were shown to have significantly more mutations of other genes compared with *U2AF35* mutations ($P = 0.001$, multiple regression analysis) (Supplementary Table 6 and Supplementary Fig. 7).

Notably, with a rare exception of A26V in a single case, the mutations of U2AF35 exclusively involved two highly conserved amino acid positions (S34 or Q157) within the amino- and the carboxyl-terminal zinc finger motifs flanking the U2AF homology motif (UHM) domain. SRSF2 mutations exclusively occurred at P95 within an intervening sequence between the RNA recognition motif (RRM) and arginine/serine-rich (RS) domains (Fig. 2 and Supplementary Figs 8 and 9). Similarly, SF3B1 mutations predominantly involved K700 and, to a lesser extent, K666, H662 and E622, which are also conserved across species (Fig. 2 and Supplementary Fig. 10). The involvement of recurrent amino acid positions in these spliceosome genes strongly indicated a gain-of-function nature of these mutations, which has been a well-documented scenario in other oncogenic mutations[25]. On the other hand, the 23 mutations in *ZRSR2* (Xp22.1) were widely distributed along the entire coding region (Fig. 2). Among these, 14 mutations were nonsense or frameshift changes, or involved splicing donor/acceptor sites that caused either a premature truncation or a large structural change of the protein, leading to loss-of-function. Combined with their strong male preference for the mutation (14/14 cases), *ZRSR2* most likely acts as a tumour suppressor gene with an X-linked recessive mode of genetic action. The remaining nine *ZRSR2* mutations were missense changes and found in both males (six cases) and females (three cases), whose somatic origin was only confirmed in two cases. However, neither the dbSNP database (build131 and 132) nor the 1000 Genomes database (May 2011 snp calls) contained these missense nucleotides, suggesting that many, if not all, of these missense changes are likely to represent functional somatic changes, especially those found in males. Interrogation of these hot spots for mutations in *U2AF35* and *SRSF2* found no mutations among lymphoid neoplasms, including acute lymphoblastic leukaemia ($N = 24$) or non-Hodgkin's lymphoma ($N = 87$) (data not shown).

RNA splicing and spliceosome mutations

Because the splicing pathway mutations in myelodysplasia widely and specifically affect the major components of the splicing complexes E/A in a mutually exclusive manner, the common consequence of these mutations is logically the impaired recognition of 3′SSs that would lead to the production of aberrantly spliced mRNA species. To appreciate this and also to gain an insight into the biological/biochemical impact of these splicing mutations, we expressed the wild-type and the mutant (S34F) U2AF35 in HeLa cells using retrovirus-mediated gene transfer with enhanced green fluorescent protein (EGFP) marking (Fig. 4a and Supplementary Methods III) and examined their effects on gene expression in these cells using GeneChip Human genome U133 plus 2.0 arrays (Affymetrix), followed by gene set enrichment analysis (GSEA) (Supplementary Methods IV)[26]. Intriguingly, the GSEA disclosed a significant enrichment of the genes on the nonsense-mediated mRNA decay (NMD) pathway among the significantly upregulated genes in mutant *U2AF35*-transduced HeLa cells (Fig. 4b, Supplementary Fig. 11a and Supplementary Table 7), which was

confirmed by quantitative polymerase chain reactions (qPCR) (Fig. 4c and Supplementary Methods 5V). A similar result was also observed for the gene expression profile of an MDS-derived cell line (TF-1) transduced with the S34F mutant (Supplementary Figs 11b, c). The NMD activation by the mutant U2AF35 was suppressed significantly by the co-overexpression of the wild-type protein (Supplementary Fig. 11d), indicating that the effect of the mutant protein was likely to be mediated by inhibition of the functions of the wild-type protein. Given that the NMD pathway, known as mRNA surveillance, provides a post-transcriptional mechanism for recognizing and eliminating abnormal transcripts that prematurely terminate translation[27], the result of the GSEA analyses indicated that the mutant U2AF35 induced abnormal RNA splicing in HeLa and TF-1 cells, leading to the generation of unspliced RNA species having a premature stop codon and induction of the NMD activity.

To confirm this, we next performed whole transcriptome analysis in these cells using the GeneChip Human exon 1.0 ST Array (Affymetrix), in which we differentially tracked the behaviour of two discrete sets of probes showing different level of evidence of being exons, that is, 'Core' (authentic exons) and 'non-Core' (more likely introns) sets (Supplementary Methods IV and Supplementary Fig. 12). As shown in Fig. 4d, the Core and non-Core set probes were differentially enriched among probes showing significant difference in expression between wild-type and mutant-transduced cells (false discovery rate (FDR) = 0.01). The Core set probes were significantly enriched in those probes significantly downregulated in mutant *U2AF35*-transduced cells compared with wild-type *U2AF35*-transduced cells, whereas the non-Core set probes were enriched in those probes significantly upregulated in mutant *U2AF35*-transduced cells (Fig. 4e). The significant differential enrichment was also demonstrated, even when all probe sets were included (Fig. 4f). Moreover, the significantly differentially expressed Core set probes tended to be up- and downregulated in wild-type and mutant *U2AF35*-transduced cells compared with mock-transduced cells, respectively, and vice versa for the differentially expressed non-Core set probes (Fig. 4e). Combined, these exon array results indicated that the wild-type U2AF35 correctly promoted authentic RNA splicing, whereas the mutant U2AF35 inhibited this processes, rendering non-Core and therefore, more likely intronic sequences to remain unspliced.

The abnormal splicing in mutant *U2AF35*-transduced cells was more directly demonstrated by sequencing mRNAs extracted from HeLa cells, in which expression of the wild-type and mutant (S34F) U2AF35 were induced by doxycycline. First, after adjusting by the total number of mapped reads, the wild-type *U2AF35*-transduced cells showed an increased read counts in the exon fraction, but reduced counts in other fractions, compared with mutant *U2AF35*-transduced cells (Fig. 4g). The reads from the mutant-transduced cells were mapped to broader genomic regions compared with those from the wild-type *U2AF35*-transduced cells, which were largely explained by non-exon reads (Fig. 4h). Finally, the number of those reads that encompassed the authentic exon/intron junctions was significantly increased in mutant *U2AF35*-transduced cells compared with wild-type *U2AF35*-transduced cells (Fig. 4i and Supplementary Methods VI). These results clearly demonstrated that failure of splicing ubiquitously occurred in mutant *U2AF35*-transduced cells. A typical example of abnormal splicing in mutant-transduced cells and the list of significantly unspliced exons are shown in Supplementary Fig. 13 and Supplementary Table 8, respectively.

Biological consequence of U2AF35 mutations

Finally, we examined the biological effects of compromised functions of the E/A splicing complexes. First, TF-1 and HeLa cells were transduced with lentivirus constructs expressing either the S34F U2AF35 mutant or wild-type U2AF35 under a tetracycline-inducible promoter (Fig. 5a and Supplementary Figs 14a and 15a), and cell proliferation was examined after the induction of their expression. Unexpectedly, after the induction of gene expression with doxycycline, the mutant *U2AF35*-transduced cells, but not the wild-type *U2AF35*-transduced cells, showed reduced cell proliferation (Fig. 5b and Supplementary Fig. 15b) with a marked increase in the G2/M fraction (G2/M arrest) together with enhanced apoptosis as

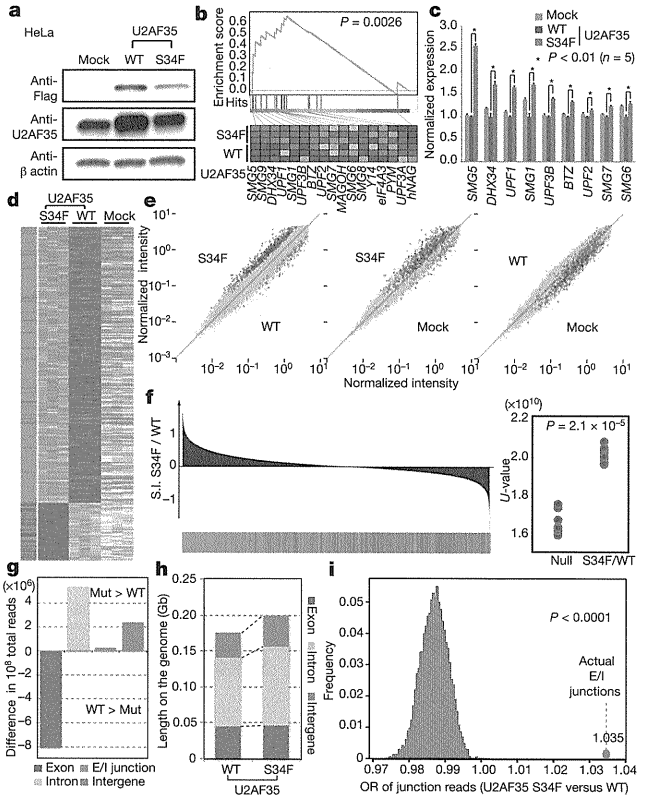

Figure 4 | Altered RNA splicing caused by a U2AF35 mutant. **a**, Western blot analyses showing expression of transduced wild-type or mutant (S34F) U2AF35 in HeLa cells used for the analyses of expression and exon microarrays. **b**, The GSEA demonstrating a significant enrichment of the set of 17 NMD pathway genes among significantly differentially expressed genes between wild-type and mutant *U2AF35*-transduced HeLa cells. The significance of the gene set was empirically determined by 1,000 gene-set permutations. **c**, The confirmation of the microarray analysis for the expression of nine genes that contributed to the core enrichment in the NMD gene set. Means ± s.e. are provided for the indicated NMD genes. *P* values were determined by the Mann–Whitney *U* test. **d**, Significantly upregulated and downregulated probe sets (FDR = 0.01) in mutant *U2AF35*-transduced cells compared with wild-type *U2AF35*-transduced cells in triplicate exon array experiments are shown in a heat map. The origin of each probe set is depicted in the left lane, where red and green bars indicate the Core and non-Core sets, respectively. **e**, Pair-wise scatter plots of the normalized intensities of entire probe sets (grey) across different experiments. The Core and non-Core set probes that were significantly differentially expressed between the wild-type and mutant *U2AF35*-transduced cells are plotted in red and green, respectively. **f**, Distribution of the Core (red) and non-Core (green) probe sets within the entire probe sets ordered by splicing index (S.I.; Supplementary Methods IV), calculated between wild-type and mutant *U2AF35*-transduced cells. In the right panel, the differential enrichment of both probe sets was confirmed by Mann–Whitney *U* test. **g**, Difference in read counts for the indicated fractions per 10^8 total reads in RNA sequencing between wild-type and mutant U2AF35-expressing HeLa cells analysis. Increased/decreased read counts in mutant U2AF35-expressing cells are plotted upward/downward, respectively. **h**, Comparison of the genome coverage by the indicated fractions in wild-type- and mutant-U2AF35-expressing cells. The genome coverage was calculated for each fraction within the 10^8 reads randomly selected from the total reads and averaged for ten independent selections. **i**, The odds ratio of the junction reads within the total mapped reads was calculated between the two experiments (red circle), which was evaluated against the 10,000 simulated values under the null hypothesis (histogram in blue).

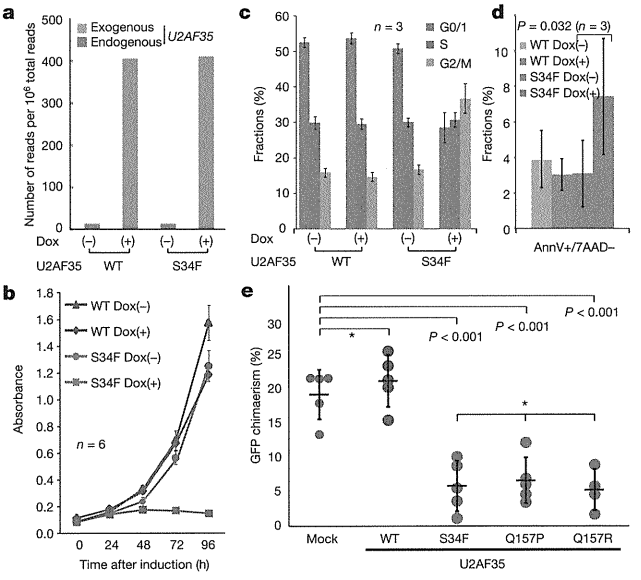

Figure 5 | Functional analysis of mutant U2AF35. a, Expression of endogenous and exogenous *U2AF35* transcripts in HeLa cells before and after induction determined by RNA sequencing. *U2AF35* transcripts were differentially enumerated for endogenous and exogenous species, which were discriminated by the Flag sequence. **b**, Cell proliferation assays of *U2AF35*-transduced HeLa cells, where cell numbers were measured using cell-counting apparatus and are plotted as mean absorbance ± s.d. **c**, The flow cytometry analysis of propidium iodide (PI)-stained HeLa cells transduced with the different U2AF35 constructs. Mean fractions ± s.d. in G0/G1, S and G2/M populations after the induction of U2AF35 expression are plotted. **d**, Fractions of the annexin V-positive (AnnV+) populations among the 7-amino-actinomycin D (7AAD)-negative population before and after the induction of U2AF35 expression are plotted as mean ± s.d. for indicated samples. The significance of difference was determined by paired *t*-test. **e**, Competitive reconstitution assays for CD34-negative KSL cells transduced with indicated U2AF35 mutants. Chimaerism in the peripheral blood 6 weeks after transplantation are plotted as mean %EGFP-positive Ly5.1 cells ± s.d., where outliers were excluded from the analysis. The significance of differences was evaluated by the Grubbs test with Bonferroni's correction for multiple testing. *not significant.

indicated by the increased sub-G1 fraction and annexin V-positive cells (Fig. 5c, d, Supplementary Fig. 14b and Supplementary Methods VI). To confirm the growth-suppressive effect of U2AF35 mutants *in vitro*, a highly purified haematopoietic stem cell population ($CD34^-c\text{-}Kit^+ScaI^+Lin^-$, $CD34^-KSL$) prepared from C57BL/6 (B6)-Ly5.1 mouse bone marrow[28] was retrovirally transduced with either the mutant (S34F, Q157P and Q157R) or wild-type *U2AF35*, or the mock constructs, each harbouring the EGFP marker gene (Supplementary Fig. 16). The ability of these transduced cells to reconstitute the haematopoietic system was tested in a competitive reconstitution assay. The transduced cells were mixed with whole bone marrow cells from B6-Ly5.1/5.2 F1 mice, transplanted into lethally irradiated B6-Ly5.2 recipients, and peripheral blood chimaerism derived from EGFP-positive cells was assessed 6 weeks after transplantation by flow cytometry. We confirmed that each recipient mouse received comparable numbers of EGFP-positive cells among the different retrovirus groups by estimating the percentage of EGFP-positive cells and overall proliferation in transduced cells by *ex vivo* tracking. Also no significant difference was observed in their homing capacity to bone marrow as assessed by transwell migration assays (Supplementary Fig. 17). As shown in Fig. 5e, the wild-type *U2AF35*-transduced cells showed a slightly higher reconstitution capacity than the mock-transduced cells. On the other hand, the recipients of the cells transduced with the various *U2AF35* mutants showed significantly lower EGFP-positive cell chimaerism than those of either the mock- or the wild-type *U2AF35*-transduced cells, indicating a compromised reconstitution capacity of the haematopoietic stem/progenitor cells expressing the U2AF35 mutants. In summary, these mutants lead to loss-of-function of U2AF35 most probably by acting in a dominant-negative fashion to the wild-type protein.

Discussion

Our whole-exome sequencing study unexpectedly unmasked a complexity of novel pathway mutations found in approximately 45% to 85% of myelodysplasia patients depending on the disease subtypes, which affected multiple but distinctive components of the splicing machinery and, as such, demonstrated the unquestionable power of massively parallel sequencing technologies in cancer research.

The RNA splicing system comprises essential cellular machinery, through which eukaryotes can achieve successful transcription and guarantee the functional diversity of their protein species using alternative splicing in the face of a limited number of genes[29]. Accordingly, the meticulous regulation of this machinery should be indispensable for the maintenance of cellular homeostasis[30], deregulation of which causes severe developmental abnormalities[31,32]. The current discovery of frequent mutations of the splicing pathway in myelodysplasia, therefore, represents another remarkable example that illustrates how cancer develops by targeting critical cellular functions. It also provides an intriguing insight into the mechanism of 'cancer specific' alternative splicing, which have long been implicated in the development of cancer, including MDS and other haematopoietic neoplasms[33,34].

In myelodysplasia, the major targets of spliceosome mutations seemed to be largely confined to the components of the E/A splicing complex, among others to *SF3B1*, *SRSF2*, *U2AF35* and *ZRSR2*, and to a lesser extent, to *SF3A1*, *SF1*, *U2AF65* and *PRPF40B*. The broad coverage of the wide spectrum of spliceosome components in our exome sequencing was likely to preclude frequent involvement of other components on this pathway (Supplementary Fig. 18). The surprising frequency and specificity of these mutations in this complex, together with the mutually exclusive manner they occurred, unequivocally indicate that the compromised function of the E/A complex is a hallmark of this unique category of myeloid neoplasms, playing a central role in the pathogenesis of myelodysplasia. The close relationship between the mutation types and unique disease subtypes also support their pivotal roles in MDS.

Given the critical functions of the E/A splicing complex on the precise 3′SS recognition, the logical consequence of these relevant mutations would be the impaired splicing involving diverse RNA species. In fact, when expressed in HeLa cells, the mutant U2AF35 induced global abnormalities of RNA splicing, leading to increased production of transcripts having unspliced intronic sequences. On the other hand, the functional link between the abnormal splicing of RNA species and the phenotype of myelodysplasia is still unclear. Mutant U2AF35 seemed to suppress cell growth/proliferation and induce apoptosis rather than confer a growth advantage or promote clonal selection. *ZRSR2* knockdown in HeLa cells has been reported to also result in reduced viability, arguing for the common consequence of these pathway mutations[35]. These observations suggested that the oncogenic actions of these splicing pathway mutations are distinct from what is expected for classical oncogenes, such as mutated kinases and signal transducers, but could be more related to cell differentiation. Of note in this regard, the commonest clinical presentation of MDS is severe cytopenia in multiple cell lineages due to ineffective haematopoiesis with increased apoptosis rather than unlimited cell proliferation[1]. In this regard, lessons may be learned from the recent findings on the pathogenesis of the 5q− syndrome, where haploinsufficiency of *RPS14* leads to increased apoptosis of erythroid progenitors, but not myeloproliferation[36,37].

A lot of issues remain to be answered, however, to establish the functional link between these splicing pathway mutations and the