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).

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

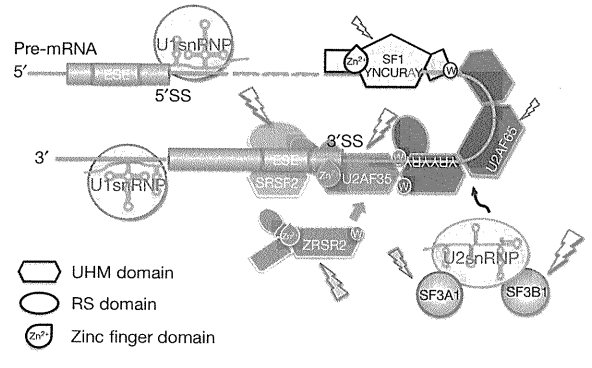

Figure 1 | Components of the splicing E/A complex mutated in myelodysplasia. RNA splicing is initiated by the recruitment of U1 snRNP to the 5′SS. SF1 and the larger subunit of the U2 auxiliary factor (U2AF), U2AF65, bind the branch point sequence (BPS) and its downstream polypyrimidine tract, respectively. 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.

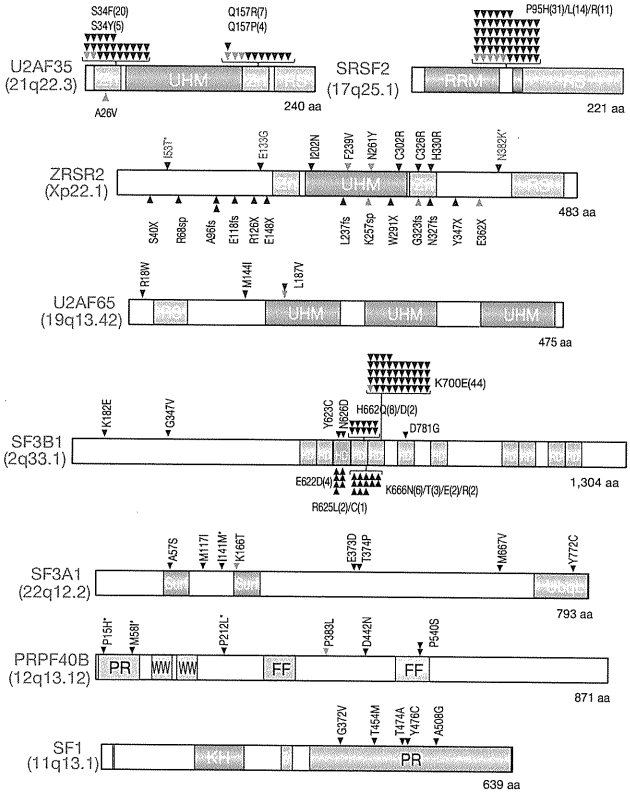

Figure 2 | Mutations of multiple components of the splicing machinery. Each mutation in the eight spliceosome components is shown with an arrowhead. Confirmed somatic mutations are discriminated by red arrows. Known domain structures are shown in coloured boxes as indicated. Mutations predicted as SNPs 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.

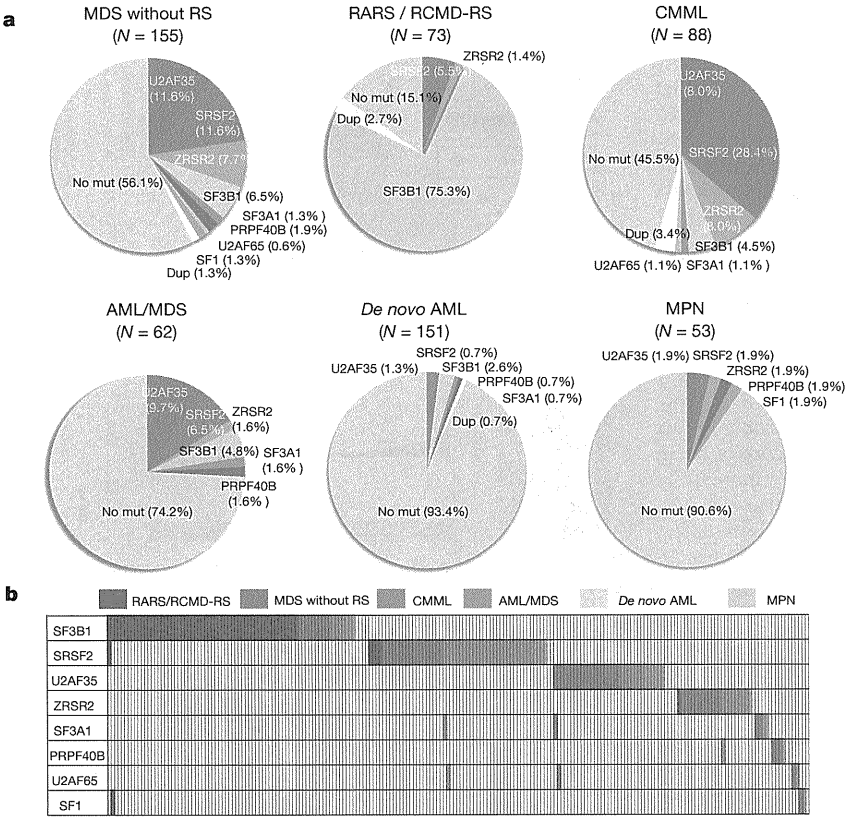

Figure 3 | Frequencies and distribution of spliceosome pathway gene mutations in myeloid neoplasms. a, Frequencies of spliceosome pathway mutations among 582 cases with various myeloid neoplasms. **b**, Distribution of mutations in eight spliceosome genes, where diagnosis of each sample is shown by indicated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

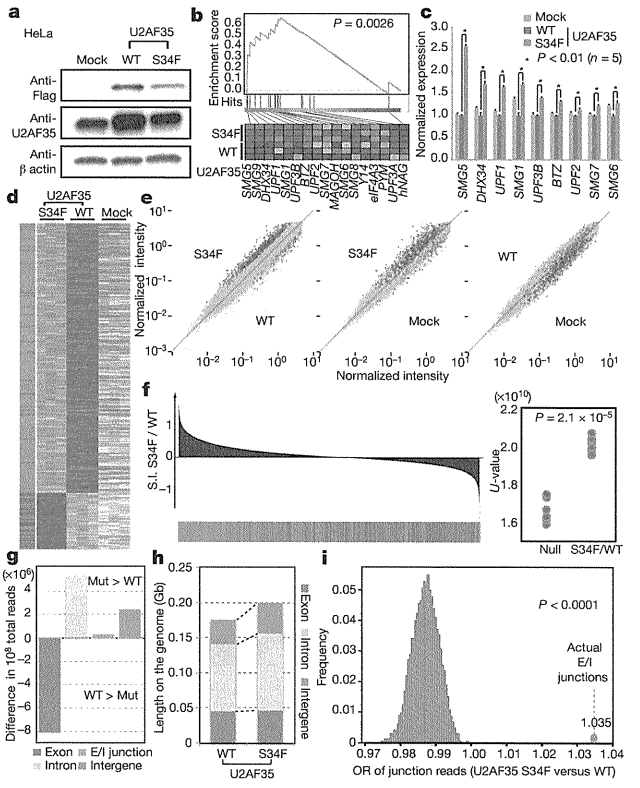

Figure 4 | Altered RNA splicing caused by a U2AF35 mutant. **a**, Western blot analyses showing expression of transduced wild-type or mutant (S34F) U2AF35 in HeLa cells used for the analyses of expression and exon microarrays. **b**, The GSEA demonstrating a significant enrichment of the set of 17 NMD 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).

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

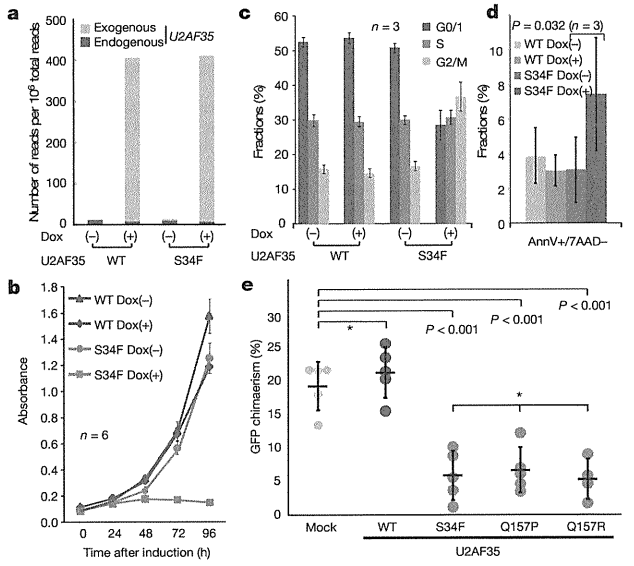

Figure 5 | Functional analysis of mutant U2AF35. a, Expression of endogenous and exogenous *U2AF35* transcripts in HeLa cells before and after induction determined by RNA sequencing. *U2AF35* transcripts were differentially enumerated for endogenous and exogenous species, which were discriminated by 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-$Kit^+Scal^+Lin^-$, $CD34^-$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

pathogenesis of MDS, where the broad spectrum of RNA species affected by impaired splicing hampers identification of responsible gene targets. Moreover, the mutated components of the splicing machinery have distinct function of their own other than direct regulation of RNA splicing, involved in elongation and DNA stability, which may be important to determine specific disease phenotypes. Clearly, more studies are required to answer these questions through understanding of the molecular basis of their oncogenic actions.

METHODS SUMMARY

Whole-exome sequencing of paired tumour/normal DNA samples from the 29 patients was performed after informed consent was obtained. SNP array-based copy number analysis was performed as previously described[17,18]. Mutation analysis of the splicing pathway genes in a set of 582 myeloid neoplasms were performed by first screening mutations in PCR-amplified pooled targets from 12 individuals, followed by validation/identification of the candidate mutations within the corresponding 12 individuals by Sanger sequencing. Flag-tagged cDNAs of the wild-type and mutant *U2AF35* were generated by *in vitro* mutagenesis, constructed into a murine stem cell virus-based retroviral vector as well as a tetracycline-inducible lentivirus-based expression vector, and used for gene transfer to $CD34^-KSL$ cells and cultured cell lines, with EGFP marking, respectively. Total RNA was extracted from wild-type or mutant *U2AF35*-transduced HeLa and TF-1 cells, and analysed on microarrays. RNA sequencing was performed according to the manufacturer's instructions (Illumina). Cell proliferation assays (MTT assays) on HeLa and TF-1 cells stably transduced with lentivirus U2AF35 constructs were performed in the presence or absence of doxycycline. For competitive reconstitution assays, $CD34^-KSL$ cells collected from C57BL/6 (B6)-Ly5.1 mice were retrovirally transduced with various *U2AF35* constructs with EGFP marking, and transplanted with competitor cells (B6-Ly5.1/5.2 F1 mouse origin) into lethally irradiated B6-Ly5.2 mice 48 h after gene transduction. Frequency of EGFP-positive cells was assessed in peripheral blood by flow cytometry 6 weeks after the transplantation (Supplementary Methods VII). The primer sets used for validation of gene mutations and qPCR of NMD gene expression are listed in Supplementary Tables 9–11. A complete description of the materials and methods is provided in the Supplementary Information. This study was approved by the ethics boards of the University of Tokyo, Munich Leukaemia Laboratory, University Hospital Mannheim, University of Tsukuba, Tokyo Metropolitan Ohtsuka Hospital and Chang Gung Memorial Hospital. Animal experiments were performed with approval of the Animal Experiment Committee of the University of Tokyo.

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1. Corey, S. J. *et al.* Myelodysplastic syndromes: the complexity of stem-cell diseases. *Nature Rev. Cancer* **7,** 118–129 (2007).
2. Ma, X., Does, M., Raza, A. & Mayne, S. T. Myelodysplastic syndromes: incidence and survival in the United States. *Cancer* **109,** 1536–1542 (2007).
3. Bejar, R., Levine, R. & Ebert, B. L. Unraveling the molecular pathophysiology of myelodysplastic syndromes. *J. Clin. Oncol.* **29,** 504–515 (2011).
4. Sanada, M. *et al.* Gain-of-function of mutated *C-CBL* tumour suppressor in myeloid neoplasms. *Nature* **460,** 904–908 (2009).
5. Campbell, P. J. *et al.* Identification of somatically acquired rearrangements in cancer using genome-wide massively parallel paired-end sequencing. *Nature Genet.* **40,** 722–729 (2008).
6. Chapman, M. A. *et al.* Initial genome sequencing and analysis of multiple myeloma. *Nature* **471,** 467–472 (2011).
7. Lee, W. *et al.* The mutation spectrum revealed by paired genome sequences from a lung cancer patient. *Nature* **465,** 473–477 (2010).
8. Ley, T. J. *et al.* DNA sequencing of a cytogenetically normal acute myeloid leukaemia genome. *Nature* **456,** 66–72 (2008).
9. Metzker, M. L. Sequencing technologies — the next generation. *Nature Rev. Genet.* **11,** 31–46 (2010).
10. Shendure, J. & Ji, H. Next-generation DNA sequencing. *Nature Biotechnol.* **26,** 1135–1145 (2008).
11. Shah, S. P. *et al.* Mutational evolution in a lobular breast tumour profiled at single nucleotide resolution. *Nature* **461,** 809–813 (2009).
12. Varela, I. *et al.* Exome sequencing identifies frequent mutation of the SWI/SNF complex gene *PBRM1* in renal carcinoma. *Nature* **469,** 539–542 (2011).
13. Ley, T. J. *et al. DNMT3A* mutations in acute myeloid leukemia. *N. Engl. J. Med.* **363,** 2424–2433 (2010).
14. Mardis, E. R. *et al.* Recurring mutations found by sequencing an acute myeloid leukemia genome. *N. Engl. J. Med.* **361,** 1058–1066 (2009).
15. Yan, X. J. *et al.* Exome sequencing identifies somatic mutations of DNA methyltransferase gene *DNMT3A* in acute monocytic leukemia. *Nature Genet.* **43,** 309–315 (2011).
16. Puente, X. S. *et al.* Whole-genome sequencing identifies recurrent mutations in chronic lymphocytic leukaemia. *Nature* **475,** 101–105 (2011).
17. Nannya, Y. *et al.* A robust algorithm for copy number detection using high-density oligonucleotide single nucleotide polymorphism genotyping arrays. *Cancer Res.* **65,** 6071–6079 (2005).
18. Yamamoto, G. *et al.* Highly sensitive method for genomewide detection of allelic composition in nonpaired, primary tumor specimens by use of Affymetrix single-nucleotide-polymorphism genotyping microarrays. *Am. J. Hum. Genet.* **81,** 114–126 (2007).
19. Wahl, M. C., Will, C. L. & Luhrmann, R. The spliceosome: design principles of a dynamic RNP machine. *Cell* **136,** 701–718 (2009).
20. Tronchère, H., Wang, J. & Fu, X. D. A protein related to splicing factor $U2AF^{35}$ that interacts with $U2AF^{65}$ and SR proteins in splicing of pre-mRNA. *Nature* **388,** 397–400 (1997).
21. Bevilacqua, L. *et al.* A population-specific *HTR2B* stop codon predisposes to severe impulsivity. *Nature* **468,** 1061–1066 (2010).
22. Calvo, S. E. *et al.* High-throughput, pooled sequencing identifies mutations in *NUBPL* and *FOXRED1* in human complex I deficiency. *Nature Genet.* **42,** 851–858 (2010).
23. Haase, D. *et al.* New insights into the prognostic impact of the karyotype in MDS and correlation with subtypes: evidence from a core dataset of 2124 patients. *Blood* **110,** 4385–4395 (2007).
24. Xiao, R. *et al.* Splicing regulator SC35 is essential for genomic stability and cell proliferation during mammalian organogenesis. *Mol. Cell. Biol.* **27,** 5393–5402 (2007).
25. Morin, R. D. *et al.* Somatic mutations altering EZH2 (Tyr641) in follicular and diffuse large B-cell lymphomas of germinal-center origin. *Nature Genet.* **42,** 181–185 (2010).
26. Subramanian, A. *et al.* Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc. Natl Acad. Sci. USA* **102,** 15545–15550 (2005).
27. Maquat, L. E. Nonsense-mediated mRNA decay: splicing, translation and mRNP dynamics. *Nature Rev. Mol. Cell Biol.* **5,** 89–99 (2004).
28. Ema, H. *et al.* Adult mouse hematopoietic stem cells: purification and single-cell assays. *Nature Protocols* **1,** 2979–2987 (2007).
29. Chen, M. & Manley, J. L. Mechanisms of alternative splicing regulation: insights from molecular and genomics approaches. *Nature Rev. Mol. Cell Biol.* **10,** 741–754 (2009).
30. Ni, J. Z. *et al.* Ultraconserved elements are associated with homeostatic control of splicing regulators by alternative splicing and nonsense-mediated decay. *Genes Dev.* **21,** 708–718 (2007).
31. He, H. *et al.* Mutations in U4atac snRNA, a component of the minor spliceosome, in the developmental disorder MOPD I. *Science* **332,** 238–240 (2011).
32. Edery, P. *et al.* Association of TALS developmental disorder with defect in minor splicing component *U4atac* snRNA. *Science* **332,** 240–243 (2011).
33. David, C. J. & Manley, J. L. Alternative pre-mRNA splicing regulation in cancer: pathways and programs unhinged. *Genes Dev.* **24,** 2343–2364 (2010).
34. Pajares, M. J. *et al.* Alternative splicing: an emerging topic in molecular and clinical oncology. *Lancet Oncol.* **8,** 349–357 (2007).
35. Shen, H., Zheng, X., Luecke, S. & Green, M. R. The U2AF35-related protein Urp contacts the 3′ splice site to promote U12-type intron splicing and the second step of U2-type intron splicing. *Genes Dev.* **24,** 2389–2394 (2010).
36. Barlow, J. L. *et al.* A p53-dependent mechanism underlies macrocytic anemia in a mouse model of human 5q− syndrome. *Nature Med.* **16,** 59–66 (2010).
37. Ebert, B. L. *et al.* Identification of *RPS14* as a $5q^-$ syndrome gene by RNA interference screen. *Nature* **451,** 335–339 (2008).

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Author Contributions Y.Sh., Y.Sa., A.S.-O., Y.N., M.N., G.C., R.K. and S.Miyano were committed to bioinformatics analyses of resequencing data. M.Sa., A.S.-O. and Y.Sa. performed microarray experiments and their analyses. R.Y., T.Y., M.O., M.Sa., A.K., M.Sh. and H.N. were involved in the functional analyses of U2AF35 mutants. N.O., M.S.-Y., K.I., H.M., W.-K.H., F.N., D.N., T.H., C.H., S.Miyawaki, S.C., H.P.K. and L.-Y.S. collected specimens and were also involved in planning the project. K.Y., Y.N., Y.Su., A.S.-O. and S.S. processed and analysed genetic materials, library preparation and sequencing. K.Y., M.Sa., Y.Sh., A.S.-O., Y. Sa. and S.O. generated figures and tables. S.O. led the entire project and wrote the manuscript. All authors participated in the discussion and interpretation of the data and the results.

Author Information Sequence data have been deposited in the DDBJ repository under accession number DRA000433. Microarray data have been deposited in the GEO database under accession numbers GSE31174 (for SNP arrays), GSE31171 (for exon arrays) and GSE31172 (for expression arrays). Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to S.O. (sogawa-tky@umin.ac.jp).

DNMT3A mutations are rare in childhood acute myeloid leukaemia, myelodysplastic syndromes and juvenile myelomonocytic leukaemia

Acute myeloid leukaemia (AML) is a complex disease caused by mutations and deregulated gene expression, leading to increased proliferation and decreased differentiation of haematopoietic progenitor cells. Contemporary treatments have resulted in 5-year event-free survival rates of almost 60% for paediatric AML (Pui *et al*, 2011).

Recently, a whole genome sequencing study of AML uncovered recurrent mutations of an epigenetic regulator, the *DNA methyltransferase 3A* (*DNMT3A*) gene, in approximately 20% of adult AML patients (Ley *et al*, 2010; Yamashita *et al*, 2010; Yan *et al*, 2011). In these studies, *DNMT3A* mutations were frequently associated with *FLT3*-internal tandem duplication (ITD), *nucleophosmin 1* (*NPM1*) and *isocitrate dehydrogenase 1* (*IDH1*) mutations (Ley *et al*, 2010; Yan *et al*, 2011). *DNMT3A* mutations were also found in adult myelodysplastic syndromes (MDS) (8%, 12/150) (Walter *et al*, 2011), AML secondary to myeloproliferative neoplasms (MPNs) (14%, 5/35), myelofibrosis (15%, 3/20) and polycythaemia vera (7%, 2/30) (Stegelmann *et al*, 2011).

DNMT3A is involved in epigenetic regulation of genes by enzymatic de novo addition of methyl groups to the cytosine residue of CpG dinucleotides. *DNMT3A* mutations were significantly enriched with a cytogenetic profile associated with intermediate risk, including a normal cytogenetic profile, as well as the M4 and M5 subtypes, according to the French-American-British (FAB) classification system (Ley *et al*, 2010; Yan *et al*, 2011). In AML patients with a normal karyotype and *FLT3*-ITD, patients with *DNMT3A* gene mutations showed a worse prognosis than those without *DNMT3A* gene mutations (Ley *et al*, 2010; Yan *et al*, 2011); however, the frequency and clinical impact of *DNMT3A* gene mutations in paediatric AML and myeloproliferative neoplasms (MPN) remain uncertain. We searched for *DNMT3A* gene mutations in 149 AMLs who were treated on the Japanese Childhood AML Cooperative protocol, AML 99 (range: 0–15 years old, M0: 5, M1: 23, M2: 44, M3: 13, M4: 22, M5: 21, M6: 1, M7: 17, unclassified: three patients), 40 juvenile myelomonocytic leukaemias (JMMLs; range: 2 months to 8 years), 24 myelodysplastic syndromes (MDSs) and 20 paediatric therapy-related leukaemia/MDSs (t-Leuk/MDSs, range: 1–17 years). *FLT3*-ITD and *NPM1* gene alterations have been reported in these 149 AML patients (Shimada *et al*, 2007, 2008).

Total RNA extracted from the bone marrow or peripheral blood samples at diagnosis was reverse transcribed to cDNA with a cDNA Synthesis Kit (Amersham Bioscience, Tokyo, Japan). *DNMT3A* mutations were thus far reported to be almost exclusively involved in exons 16-23 (especially codon R882 in exon 23) (Ley *et al*, 2010; Yamashita *et al*, 2010; Stegelmann *et al*, 2011; Walter *et al*, 2011; Yan *et al*, 2011); thus, we confined our analysis to these exons. cDNA was amplified using the following primers: *DNMT3A* cDNA 15F, 5′-CAGGTGCTTTTGCGTGGAGTGT-3′ and 19R, 5′-ATGC AGGAGGCGGTAGAACTCA-3′, 17F, 5′-AAGATCATGTAC-GTCGGGGA-3′ and 22R, 5′-CTTTGCCCTGCTTTA TG-GAG-3′ and 20F, 5′-CCCTGTGATGATTGATGCCA-3′ and 23R, 5′-GTATTTCCGCCTCTGTG-GTT-3′ for AML samples. For JMML, MDS and t-Leuk/MDS, we confined our analysis to exon 23, including the hotspot of codon R882, of the *DNMT3A* gene using the following primers: *DNMT3A* DNA 23F, 5′-AGAACTAAGCAGGGCC-TCAGAGGA-3′ and 23R, 5′-GTATTTCCGCCTCTGTGGTT-3′. Subsequently, direct sequencing was performed on a DNA sequencer (ABI 310; Applied Biosystems, Foster City, CA, USA) using a BigDye terminator cycle sequencing kit (Applied Biosystems). 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.

No *DNMT3A* mutations were detected in any AML patients in our study. Recently, *DNMT3A* mutations have been reported in paediatric AML patients (Ho *et al*, 2011; Thol *et al*, 2011). Only two patients were identified (both 15 years old). Combined with these and our data, the frequency of *DNMT3A* mutations is extremely rare (2/524, 0·4%) in childhood AML. Furthermore, we did not identify *DNMT3A* mutations in MDS, JMML or paediatric t-Leuk/MDS. These findings were not compatible with those of adult MDS and MPN, suggesting that the frequency of *DNMT3A* gene mutations depends on age.

On the other hand, we found *FLT3*-ITD in 20 (13%) of 149 AML patients; however, no *NPM1* mutations were found (Shimada *et al*, 2007, 2008). Nine AML patients with *FLT3*-ITD were found to lack *DNMT3A* mutation. *DNMT3A* mutations have been correlated with *FLT3*-ITD and *NPM1* in adult AML, but not in paediatric AML. Although patients with *DNMT3A* mutations have been associated with FAB-M4, M5, especially *MLL*-negative M5, no mutations in these paediatric M4/M5 patients were found in this study. *DNMT3A* mutations have not been detected in any adult AML with favourable cytogenetics, including *t*(8;21) and inv(16) (Ley *et al*, 2010; Yan *et al*, 2011). Higher frequencies of *t*(8;21) and inv(16) in

paediatric than in adult AML patients may be associated with rare *DNMT3A* mutations in paediatric AML. These data suggest that the pathology of paediatric AML may be different from that of adult AML. We concluded that *DNMT3A* mutations, as well as *NPM1* mutations, may be infrequent in paediatric AML and MDS patients, especially those <15 years old.

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Authorship

TT and YH designed the study. AS, MS, SA, AT, KH and MT provided critical reagents and samples. NS and MP performed the experiments. RH, IT and HA 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 conflicts of interest.

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References

Ho, P.A., Kutny, M.A., Alonzo, T.A., Gerbing, R.B., Joaquin, J., Raimondi, S.C., Gamis, A.S. & Meshinchi, S. (2011) Leukemic mutations in the methylation-associated genes DNMT3A and IDH2 are rare events in pediatric AML: a report from the Children's Oncology Group. *Pediatric Blood & Cancer*, **57**, 204–209.

Ley, T.J., Ding, L., Walter, M.J., McLellan, M.D., Lamprecht, T., Larson, D.E., Kandoth, C., Payton, J.E., Baty, J., Welch, J., Harris, C.C., Lichti, C.F., Townsend, R.R., Fulton, R.S., Dooling, D.J., Koboldt, D.C., Schmidt, H., Zhang, Q., Osborne, J.R., Lin, L., O'Laughlin, M., McMichael, J.F., Delehaunty, K.D., McGrath, S.D., Fulton, L.A., Magrini, V.J., Vickery, T.L., Hundal, J., Cook, L.L., Conyers, J.J., Swift, G.W., Reed, J.P., Alldredge, P.A., Wylie, T., Walker, J., Kalicki, J., Watson, M.A., Heath, S., Shannon, W.D., Varghese, N., Nagarajan, R., Westervelt, P., Tomasson, M.H., Link, D.C., Graubert, T.A., DiPersio, J.F., Mardis, E.R. & Wilson, R.K. (2010) DNMT3A mutations in acute myeloid leukemia. *New England Journal of Medicine*, **363**, 2424–2433.

Pui, C.H., Carroll, W.L., Meshinchi, S. & Arceci, R.J. (2011) Biology, risk stratification, and therapy of pediatric acute leukemias: an update. *Journal of Clinical Oncology*, **29**, 551–565.

Shimada, A., Taki, T., Kubota, C., Tawa, A., Horibe, K., Tsuchida, M., Hanada, R., Tsukimoto, I. & Hayashi, Y. (2007) No nucleophosmin mutations in pediatric acute myeloid leukemia with normal karyotype: a study of the Japanese Childhood AML Cooperative Study Group. *Leukemia*, **21**, 1307.

Shimada, A., Taki, T., Tabuchi, K., Taketani, T., Hanada, R., Tawa, A., Tsuchida, M., Horibe, K., Tsukimoto, I. & Hayashi, Y. (2008) Tandem duplications of MLL and FLT3 are correlated with poor prognoses in pediatric acute myeloid leukemia: a study of the Japanese childhood AML Cooperative Study Group. *Pediatric Blood & Cancer*, **50**, 264–269.

Stegelmann, F., Bullinger, L., Schlenk, R.F., Paschka, P., Griesshammer, M., Blersch, C., Kuhn, S., Schauer, S., Döhner, H. & Döhner, K. (2011) DNMT3A mutations in myeloproliferative neoplasms. *Leukemia*, **25**, 1217–1219.

Thol, F., Heuser, M., Damm, F., Klusmann, J.H., Reinhardt, K. & Reinhardt, D. (2011) DNMT3A mutations are rare in childhood acute myeloid leukemia. *Haematologica*, **96**, 1238–1240.

Walter, M.J., Ding, L., Shen, D., Shao, J., Grillot, M., McLellan, M., Fulton, R., Schmidt, H., Kalicki-Veizer, J., O'Laughlin, M., Kandoth, C., Baty, J., Westervelt, P., Dipersio, J.F., Mardis, E.R., Wilson, R.K., Ley, T.J. & Graubert, T.A. (2011) Recurrent DNMT3A mutations in patients with myelodysplastic syndromes. *Leukemia*, **25**, 1153–1158.

Yamashita, Y., Yuan, J., Suetake, I., Suzuki, H., Ishikawa, Y., Choi, Y.L., Ueno, T., Soda, M., Hamada, T., Haruta, H., Takada, S., Miyazaki, Y., Kiyoi, H., Ito, E., Naoe, T., Tomonaga, M., Toyota, M., Tajima, S., Iwama, A. & Mano, H. (2010) Array-based genomic resequencing of human leukemia. *Oncogene*, **29**, 3723–3731.

Yan, X.J., Xu, J., Gu, Z.H., Pan, C.M., Lu, G., Shen, Y., Shi, J.Y., Zhu, Y.M., Tang, L., Zhang, X.W., Liang, W.X., Mi, J.Q., Song, H.D., Li, K.Q., Chen, Z. & Chen, S.J. (2011) Exome sequencing identifies somatic mutations of DNA methyltransferase gene DNMT3A in acute monocytic leukemia. *Nature Genetics*, **43**, 309–315.

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Letter to the Editor

CD7-positive acute myelomonocytic leukemia with trisomy 21 as a sole acquired chromosomal abnormality in two adolescents

1. Introduction

Trisomy 21 is one of the most common acquired chromosomal abnormalities in myeloid malignancies, but is rarely found as a sole anomaly. It has been reported that only 0.3% of adult patients with acute myeloblastic leukemia (AML) or myelodysplastic syndrome have trisomy 21 as a sole acquired abnormality [1]. Although several reports indicate that this karyotypic abnormality is associated with the French–American–British (FAB) AML subtypes M2, M4 and M5 [2,3] and with a poor prognosis [3,4], its clinical and prognostic implications have not been fully evaluated. Some investigators have focused on the association between this chromosomal abnormality and CD7 expression, and 4 adult patients with CD7-positive AML have been reported so far [5–8]. Although one report includes 2 adolescent AML cases with trisomy 21 as a sole acquired abnormality [3], there have been no reports on pediatric CD7-positive AML patients with this anomaly. Here, we report 2 unique adolescent cases of CD7-positive acute myelomonocytic leukemia with trisomy 21 as a minor clone and as a sole acquired chromosomal abnormality.

2. Case reports

2.1. Case 1

A 15-year-old boy was admitted to our hospital in November 2006 because of fever and gingival swelling and bleeding. Findings on physical examination included petechiae over the body and hepatomegaly (5 cm below the costal margin). Examination of the peripheral blood revealed the following: hemoglobin 8.3 g/dL, platelets 15×10^9/L, and leukocytes 227×10^9/L with 40% blasts and 8% monocytes (18.2×10^9/L). The bone marrow was hypercellular with 96% blasts, which were positive for myeloperoxidase but negative for non-specific esterase staining. Immunophenotypic analysis by flow cytometry showed that the blasts were positive for CD7, CD13, CD33, CD34, and HLA-DR expression and negative for lymphoid markers. Chromosome analysis of bone marrow cells revealed 47,XY,+21[2]/46,XY[38]. The patient had no clinical findings of Down syndrome. Interphase fluorescence in situ hybridization (FISH) analysis using a specific probe for the *RUNX1* gene labeled with Spectrum-Green plus a set of 3 probes for 21q22 (D21S259, D21S341 and D21S342) labeled with Spectrum-Orange, showed that 26 out of 1000 bone marrow cells had 3 green and 3 red signals (2.6%). Serum and urine lysozyme levels were 43.4 μg/mL (reference range, 5.0–10.2 μg/mL) and 8.6 μg/mL (undetectable in normal subjects), respectively. Although morphological analysis of bone marrow cells was suggestive of a diagnosis of AML-M2 based on the FAB classification, the final diagnosis was AML-M4 because of the increased number of peripheral blood monocytes and elevated serum and urine lysozyme levels [9]. The patient was treated with the AML-99 protocol [10] and achieved complete remission after the first course of chemotherapy. Chromosome analysis of the bone marrow cells showed a normal karyotype, 46,XY in all 20 cells analyzed. He received 5 additional courses of consolidation chemotherapy, which was finished in June 2007. Trisomy 21 was never found on chromosome analysis performed after each course of chemotherapy. He has remained in continuous complete remission for 4 years after diagnosis.

2.2. Case 2

A 14-year-old girl was referred to our hospital in April 2007 because of fever, nasal bleeding, and gingival swelling and bleeding. On admission, petechiae were observed over the body, and the liver and spleen were palpable 8 cm and 4 cm below the costal margin, respectively. Examination of the peripheral blood revealed the following: hemoglobin 5.1 g/dl, platelets 4×10^9/L, and leukocytes 73.1×10^9/L with 56% blasts and 15% monocytes (11.0×10^9/L). The bone marrow was hypercellular with 61% blasts, which were positive for myeloperoxidase but negative for non-specific esterase staining. Surface marker analysis showed that the blasts expressed CD7, CD13, CD33, CD34, and HLA-DR. Cytogenetic analysis of bone marrow cells revealed 47,XX,+21[2]/46,XX[18]. The patient did not manifest characteristics associated with Down syndrome. Interphase FISH analysis using a probe for the *RUNX1* gene showed that 41 out of 1000 bone marrow cells had trisomy 21 (4.1%). The serum lysozyme level was elevated at 43.1 μg/mL, but lysozyme was undetectable in urine. A diagnosis of AML-M2 was suggested by the morphological findings of the bone marrow cells, but peripheral blood monocytosis and an elevated serum lysozyme level led to a final diagnosis of AML-M4. The patient underwent induction chemotherapy using the AML-99 protocol and achieved complete remission after a single course of chemotherapy. Cytogenetic analysis of bone marrow cells showed a normal female karyotype in all 20 metaphases analyzed. She was subsequently treated with 5 courses of consolidation chemotherapy and completed therapy in November 2007. Trisomy 21 was never detected on chromosome analysis performed after each course of chemotherapy. She has remained in continuous complete remission for 3 years after diagnosis.

The clinical characteristics and laboratory data of the 2 patients are summarized in Table 1. No mutations were detected in the *RUNX1* or *GATA1* genes in DNA from the patient's bone marrow cells (data not shown).

3. Discussion

We describe the first 2 pediatric CD7-positive AML cases with trisomy 21 as a sole acquired abnormality. These cases shared several unique clinical features. First, both patients were diagnosed

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Table 1
Clinical characteristics and laboratory data of the patients.

	Case 1	Case 2
Age	15y	14y
Sex	Male	Female
WBC count ($\times 10^9$/L)	227	73.1
FAB classification	M4	M4
Non-specific esterase staining	Negative	Negative
Monocyte count ($\times 10^9$/L)	18.2	11.0
Serum lysozyme level[a] (μg/ml)	43.4	43.1
Urine lysozyme level[b] (μg/ml)	8.6	Not detected
Immunophenotype	CD7, 13, 33, 34, HLA-DR	CD7, 13, 33, 34, HLA-DR
Karyotype	47,XY,+21[2]/46,XY[38]	47,XX,+21[2]/46,XX[18]
Trisomy 21-positive cells on FISH analysis	26/1000 cells	41/1000 cells
CR after 1st course of chemotherapy	Yes	Yes
Outcome	CR, 4y after diagnosis	CR, 3y after diagnosis

WBC, white blood cell; FAB, French–American–British; FISH, fluorescence in situ hybridization; CR, complete remission.
[a] Reference range, 5.0–10.2 μg/mL.
[b] Undetectable in normal subjects.

with AML-M4 based on the FAB classification because of their peripheral blood monocytosis ($\geq 5 \times 10^9$/L) and elevated serum and/or urine lysozyme levels (>3 × normal values), although blasts in the bone marrow were negative for non-specific esterase and were morphologically classified as AML-M2 [9]. Some reports have suggested that AML with trisomy 21 as a single abnormality is associated with AML-M2, -M4 and -M5 [2,3], but monocyte counts and lysozyme levels were not discussed. Our findings indicate that evaluation of monocyte counts and serum/urine lysozyme levels are important for correct FAB classification of AML with this karyotypic abnormality.

Second, trisomy 21 was observed in only 2 out of the 40 or 20 bone marrow cells examined in Case 1 and 2, respectively, which were much lower percentages than the percentages of morphologically detected blast cells (96% and 61%, respectively). These low fractions of trisomy 21-positive cells were confirmed by interphase FISH analysis (2.6% and 4.1% in Case 1 and 2, respectively). We think that a constitutional mosaic trisomy 21 (mosaic Down syndrome) is not likely, because trisomy 21 was never found on serial cytogenetic analyses performed after remission in either patient (a total of 100 bone marrow cells were analyzed in each patient). These results clearly show that most of the blast cells had normal karyotypes and the blasts that had acquired trisomy 21 were minor clones in both patients.

Third, both patients achieved complete remission after the first course of chemotherapy and have remained in continuous complete remission for 3–4 years after diagnosis. Although several reports have indicated that this chromosomal abnormality is associated with poor prognosis [3,4], the prognostic implications have not been fully evaluated, because there are so few patients with this anomaly. To confirm that pediatric CD7-positive AML with this karyotypic abnormality has a favorable outcome, more patients need to be studied.

In conclusion, we report the first 2 pediatric cases of CD7-positive AML with trisomy 21 as a sole acquired chromosomal abnormality. The patients shared some clinical features including AML-M4 subtype, the presence of minor clones with trisomy 21, and favorable outcomes, and they might have had a distinct subtype of AML.

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References

[1] Cortes JE, Kantarjian H, O'Brien S, Keating M, Pierce S, Freireich EJ, et al. Clinical and prognostic significance of trisomy 21 in adult patients with acute myelogenous leukemia and myelodysplastic syndromes. Leukemia 1995;9:115–7.
[2] Gallego M, Solé F, Acín P, Florensa L, Sans-Sabrafèn J, Woessner S. A new case of trisomy 21 in a patient with an acute nonlymphocytic leukemia (M5b). Cancer Genet Cytogenet 1996;95:213–4.
[3] Chang Y, Wang H, Wang H, Chen Z, Jin J. Trisomy 21 in patients with acute leukemia. Am J Hematol 2008;84:193–4.
[4] Wan TSK, Au WY, Chan JCW, Chan LC, Ma SK. Trisomy 21 as the sole acquired karyotypic abnormality in acute myeloid leukemia and myelodysplastic syndrome. Leuk Res 1999;23:1079–83.
[5] Kondo H, Kobayashi A, Iwasaki H. Trisomy 21 as the sole acquired karyotypic abnormality in an adult patient with CD7-positive acute myeloid leukemia. Cancer Genet Cytogenet 2001;127:77–9.
[6] Yamamoto K, Nagata K, Hamaguchi H. A new case of CD7-positive acute myeloblastic leukemia with trisomy 21 as a sole acquired abnormality. Cancer Genet Cytogenet 2002;133:183–4.
[7] Udayakumar AM, Pathare AV, Muralitharan S, Alghzaly AA, Alkindi S, Raeburn JA. Trisomy 21 as a sole acquired abnormality in an adult Omani patient with CD7- and CD9-positive acute myeloid leukemia. Arch Med Res 2007;38:797–802.
[8] Naithani R, Singhal D, Kumar R, Raina V, Saxena R, Anand H. Sole acquired trisomy 21 in a case of CD7 and CD10 positive acute myeloid leukemia. Indian J Cancer 2008;45:132–4.
[9] Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DA, Gralnick HR, et al. Proposed revised criteria for the classification of acute myeloid leukaemia. A report of the French–American–British co-operative group. Ann Intern Med 1985;103:626–9.
[10] Tsukimoto I, Tawa A, Horibe K, Tabuchi K, Kigasawa H, Tsuchida M, et al. Risk-stratified therapy and the intensive use of cytarabine improves the outcome in childhood acute myeloid leukemia: the AML99 trial from the Japanese childhood AML cooperative study group. J Clin Oncol 2009;27:4007–13.

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Molecular lesions in childhood and adult acute megakaryoblastic leukaemia

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Summary

While acute megakaryoblastic leukaemia (AMKL) occurs in children with (DS-AMKL) and without (paediatric non-DS-AMKL) Down syndrome, it can also affect adults without DS (adult non-DS-AMKL). We have analysed these subgroups of patients (11 children with DS-AMKL, 12 children and four adults with non-DS-AMKL) for the presence of molecular lesions, including mutations and chromosomal abnormalities studied by sequencing and single nucleotide polymorphism array-based karyotyping, respectively. In children, AMKL was associated with trisomy 21 (somatic in non-DS-AMKL), while numerical aberrations of chromosome 21 were only rarely associated with adult AMKL. DS-AMKL was also associated with recurrent somatic gains of 1q (4/11 DS-AMKL patients). In contrast to trisomy 21 and gains of 1q, other additional chromosomal lesions were evenly distributed between children and adults with AMKL. A mutational screen found *GATA1* mutations in 11/12 DS-AMKL, but mutations were rare in paediatric non-DS-AMKL (1/12) and adult AMKL (0/4). *JAK3* (1/11), *JAK2* (1/11), and *TP53* mutations (1/11) were found only in patients with DS-AMKL. *ASXL1*, *IDH1/2*, *DNMT3A*, *RUNX1* and *CBL* mutations were not found in any of the patient group studied, while *NRAS* mutation was identified in two patients with paediatric non-DS-AMKL.

Keywords: acute megakaryoblastic leukaemia, children, Down syndrome, chromosome abnormality, single nucleotide polymorphism array.

Acute megakaryoblastic leukaemia (AMKL) is a heterogeneous subtype of acute myeloid leukaemia (AML) with diverse genetic and morphological characteristics. AMKL, a rare form of AML (3-14%), is more frequent in children than in adults. In Down syndrome (DS), AMKL predominates and is associated with somatic *GATA1* mutations (Wechsler *et al*, 2002; Hirose *et al*, 2003; Rainis *et al*, 2003). While most paediatric cases are *de novo* AMKL, adult AMKL is frequently observed as a secondary leukaemia after chemotherapy or leukaemic transformation of several chronic myeloproliferative neoplasms (MPNs) including chronic myeloid leukaemia (CML), polycythaemia vera (PV), essential thrombocytosis (ET), and idiopathic myelofibrosis (IMF) (Akahoshi *et al*, 1987; Radaelli *et al*, 2002; Mesa *et al*, 2005). Based on these findings, AMKL can be divided into three groups; i.e., AMKL with DS (DS-AMKL), paediatric AMKL without DS (paediatric non-DS-AMKL), and adult AMKL without DS (adult non-DS-AMKL).

Between 20% and 30% of children with DS-AMKL have a preceding history of transient abnormal myelopoiesis (TAM), in which the blasts are morphologically and phenotypically indistinguishable from those of AMKL (Zipursky *et al*, 1992). Somatic mutations of the *GATA1* gene are found in both TAM and DS-AMKL, suggesting that the acquisition of additional

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genetic alterations might be necessary for progression from TAM to AMKL (Ahmed *et al*, 2004). Although acquired mutations of *TP53, JAK3, JAK2* or *FLT3* have been found in patients with DS-AMKL, the incidence of those gene mutations was low, and *TP53* and *JAK3* gene mutations have been identified in both DS-AMKL and TAM (Malkin *et al*, 2000; Walters *et al*, 2006; De Vita *et al*, 2007; Kiyoi *et al*, 2007; Klusmann *et al*, 2007; Malinge *et al*, 2008). Mutations of *GATA1, JAK2, FLT3, KIT*, or *MPL* were also found in children with non-DS-AMKL (Malinge *et al*, 2008). *JAK3* and *TP53* mutations were reported in adult AMKL patients (Kiyoi *et al*, 2007). More recently, somatic mutations of genes, including those associated with proliferation signalling [*CBL* (Dunbar *et al*, 2008; Makishima *et al*, 2009; Sanada *et al*, 2009), *RUNX1* (Gaidzik *et al*, 2011)] and with modification of epigenetic status [*ASXL1* (Chou *et al*, 2010), *DNMT3A* (Ley *et al*, 2010)], have been found in various myeloid malignancies including AML, but not fully investigated in an AMKL cohort until now.

Recently, array-based comparative genomic hybridization (CGH-A) or single nucleotide polymorphism arrays (SNP-A) have been utilized to investigate pathogenetic lesions in haematological malignancies. Both technologies can detect microdeletions and microduplications, which are usually missed by conventional metaphase analysis. Additionally, SNP-A can detect loss of heterozygosity (LOH) due to acquired uniparental disomy (UPD), a common type of chromosomal lesion in myeloid malignancies, including AML(Gondek *et al*, 2007). Here we have analysed and compared the molecular lesions in AMKL, in particular in the three subtypes of this disease (DS-AMKL, paediatric non-DS-AMKL, and adult non-DS-AMKL).

Methods

Patients

Informed consent for sample collection from patients or their parents was obtained according to the institutional review board-approved procedures and protocols. We studied 11 patients with DS-AMKL and 16 with non-DS-AMKL (12 children and four adults) investigated at the Nagoya University Graduate School of Medicine, Nagoya, Japan and the Cleveland Clinic, Cleveland, Ohio, USA. Some of the paediatric patients (DS-AMKL-1-8, paediatric non-DS-AMKL-1-11) have been reported previously (Hama *et al*, 2008a,b). Characteristics of the patients with AMKL are summarized in Table I. The diagnosis of AMKL was based on morphology, histopathology, and the expression of megakaryocyte-specific antigens, CD41, CD42 or CD61. The diagnosis of DS was confirmed by conventional cytogenetic analysis. Bone marrow or peripheral blood samples were obtained from the patients with AMKL at the time of diagnosis. Molecular analysis of the mutational status was approved by the Ethics Committee of Nagoya University Graduate School of Medicine and Cleveland Clinic. In addition, we analysed a cohort of 642 adult and 232 paediatric patients with AML for the presence of trisomy 21 and frequency of AMKL, and a cohort of 798 various myeloid malignancies with SNP-A karyotyping data for the presence of a commonly gained region of 1q.

SNP-A karyotyping analysis

Mononuclear cells were isolated using Ficoll-Hypaque density gradient centrifugation and cryopreserved until use. Genomic DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Chatsworth, CA). High-density Affymetrix single nucleotide polymorphism arrays (SNP-A) (6.0) (Affymetrix, Santa Clara, CA, USA) were applied as a karyotyping platform to identify LOH, microduplication and microdeletion as previously described (Gondek *et al*, 2007, 2008).

Bioinformatic analysis

Signal intensity was analysed and SNP calls determined using Gene Chip Genotyping Analysis Software Version 4.0 (GTYPE). Genotyping console v3.0 (Affymetrix) was used for analysis of 6.0 arrays as previously described (Gondek *et al*, 2007, 2008).

We excluded germ-line encoded copy number variants (CNVs) and non-clonal areas of gene copy number-neutral LOH from further analysis by utilizing a bioanalytic algorithm based on lesions identified by SNP-A in an internal control series (N = 1003) and reported in the Database of Genomic Variants (http://projects.tcag.ca/variation/). Through calculation of their average sizes, we defined a maximal size of germ line LOH in the controls and consequently excluded all defects of this type in patient samples; according to 95% confidence interval, stretches of UPD >25·8 Mb were considered unlikely to be of germline origin. In addition, all non-clonal areas of UPD seen in controls were interstitial.

Gene mutational screening

To screen for gene mutations, genomic DNA was amplified by polymerase chain reaction (PCR); sequenced genes included *GATA1* (exons 2, 3) (Hirose *et al*, 2003), *JAK2* (exon 14), *JAK3* (exon 2–24) (Kiyoi *et al*, 2007), *TP53* (exons 5-8) (Hirose *et al*, 2003), *FLT3* (exons 11, 12, and 17) (Hirose *et al*, 2003), *NRAS* (codons 12, 13 and 61) (Hirose *et al*, 2003), *ASXL1* (exon 13) (Sugimoto *et al*, 2010), *IDH1/IDH2* (exon 2/exon 4) (Yan *et al*, 2009), *DNMT3A* (exon 23) (Ley *et al*, 2010), *RUNX1* (exons 3–8) (Kohlmann *et al*, 2010), and *CBL* (exon 8, 9) (Makishima *et al*, 2009), as previously described.

Statistical analysis

For comparison of the frequency of mutations or other clinical features between disease groups, categorical variables were analysed using the Fisher's exact test and continuous variables were tested using Mann–Whitney U test. All reported P values were two-sided, with a significance level of $\alpha = 0{\cdot}05$ used.

Table I. Patient characteristics.

Disease	Sex	Age at Dx (years)	WBC at Dx ($\times 10^9$/l)	Induction failure	Relapse	HSCT	Survival	Cause of death	Results of metaphase cytogenetics
DS-AMKL-1	M	1	6·2	–	–	–	Alive	–	47,XY,der(7)t(1;7)(q23;q36),del(20)(q11q13·1),+21c
DS-AMKL-2	M	1	4·9	+	–	UR-BMT	Dead	Heart failure	48,X,der(X)t(X;1)(q28;q25),+11,+21c
DS-AMKL-3	M	2	5·3	–	–	–	Alive	–	47,XY,del(11)(p?),+21c [18/20]/46, XY [2/20]
DS-AMKL-4	F	1	16·0	–	–	–	Alive	–	47,XX,+21c [14/20]/90,idemx2,-3,-7,-9,del(11)(q?),-18 [6/20]
DS-AMKL-5	M	1	4·4	–	–	–	Alive	–	47,XY,add(7)(q22),+add(21)(q22) [2/20]/48,idem,+add(21) [6/20]/47,idem,del(6)(q?) [2/20]
DS-AMKL-6	F	2	107·0	–	–	–	Alive	–	47,XX,t(5;12)(p15;q21),+add(7)(p11),+21c,+add(22)(q13)
DS-AMKL-7	F	2	10·7	+	+	–	Dead	Leukaemia	47,XX,del(7)(p15),+8,del(13)(q12q32),-14,der(14;21)(q10;q10) c,del(17)(p11),+21c,+mar
DS-AMKL-8	F	1	16·1	–	–	–	Alive	–	47,XX,+21c
DS-AMKL-9	F	2	6·4	–	–	–	Alive	–	47,XX,+21c
DS-AMKL-10	M	2	10·8	–	–	–	Alive	–	48,XY,+21c,+21,der(22),t(1;22)(q2?;q13)
DS-AMKL-11	M	1	3·3	–	+	UR-CBT	Dead	Relapse	47,XY,add(7)(p11·2)
Paediatric non-DS-AMKL-1	F	2	42·3	–	–	Auto	Alive	–	46,XX,del(2)(q11),del(2)(q31),der(5)t(2;5)(q11;q22),der(5)t(5;?13) (q35;?q14),-13, add(16)(p13),+mar
Paediatric non-DS-AMKL-2	F	15	2·0	–	–	Auto	Alive	–	49,XX,+5,+8,i(17)(q10),+21
Paediatric non-DS-AMKL-3	F	2	48·7	–	+	Auto	Dead	Relapse	47,XX,+add(16)(p13),+21
Paediatric non-DS-AMKL-4	F	0	37·5	+	–	–	Dead	Leukaemia	46,XX,t(1;22)(p13;q13) [5/20]
Paediatric non-DS-AMKL-5	M	1	1·0	–	–	UR-CBT	Alive	–	46,XY
Paediatric non-DS-AMKL-6	F	1	14·4	–	–	–	Alive	–	58,XX,+X,+2,+2,+6,+7,+8,+10,+13,+15,+19,+19,+22
Paediatric non-DS-AMKL-7	M	3	2·2	+	+	UR-BMT	Dead	Relapse	46,XY,t(16;21)(p11;q22) [18/20]/46,idem,add(11)(q13), del(13)(q12q14) [2/20]
Paediatric non-DS-AMKL-8	M	0	26·0	+	–	R-BMT	Alive	–	46,XY,t(2;7)(p12;p22)
Paediatric non-DS-AMKL-9	F	0	17·9	+	–	R-BMT	Alive	–	46,XX,-7,-7,del(11)(p11),+2mar,inc
Paediatric non-DS-AMKL-10	M	1	12·3	–	–	–	Alive	–	51,XY,+der(1) t(1;22)(p13;q13),t(1;22)(p13;q13),+6,+7,+10,+19 [13/20]/53, idem,+6,+8 [3/20]
Paediatric non-DS-AMKL-11	M	1	5·7	+	–	–	Dead	Leukaemia	45,XY,-11,der(11)t(3;11)(q21;p15)
Paediatric non-DS-AMKL-12	F	1	26·0	–	+	R-BMT	Dead	Relapse	47,XX,t(1;22)(p13;q13),der(2),t(1;2)(q23;q35),+19
Adult non-DS-AMKL-1	M	54	19·4	Refused chemotherapy	–	–	Dead	Leukaemia	46,XY,add(6)(p23),del(11)(q13q23),del(13)(q14q22) [4/20]/46,idem,del(7)(q22) [16/20]
Adult non-DS-AMKL-2	M	48	0·9	+	–	–	Dead	Leukaemia	46,XY,t(2;12)(q32;q24),der(3)t(3;12)(q21;q12)
Adult non-DS-AMKL-3	F	43	2·2	–	+	UR-BMT	Alive	–	46,XX,t(3;3)(q21;q26)
Adult non-DS-AMKL-4	F	60	6·1	–	–	–	Alive	–	46,XX

DS-AMKL, acute megakaryoblastic leukaemia with Down syndrome; Dx, diagnosis; WBC, white blood cell count; IF, induction failure; HSCT, haematopoietic stem cell transplantation; UR-CBT, unrelated cord blood stem cell transplantation; UR-BMT, unrelated bone marrow transplantation; R-BMT, related bone marrow transplantation.

Results

Cytogenetic and clinical characterization of AMKL patients

To enhance cytogenetic diagnosis, metaphase cytogenetics was complemented by SNP-A-based karyotyping using Affymetrix 6.0 arrays. Using conventional cytogenetics, somatic chromosomal aberrations were found in 23/27 patients. SNP-A-based karyotyping confirmed most unbalanced defects, including the germline gain of chromosome 21 in DS patients. Somatic gains, other than germline trisomy 21, were found in seven DS-AMKL patients, including recurrent duplication of 1q31q44 in 4 and 7q in 2 DS-AMKL (Fig 1A). Deletions were found in eight DS-AMKL patients, including deletion 5p and 7p in two patients each. UPD 3q and UPD 7p were found in another DS-AMKL patient. Somatic gains were found in seven paediatric non-DS-AMKL cases, including trisomy 8 in 2 and somatic trisomy 21 in two patients while deletions were present in five patients, including deletion 7q and 13q in two patients, respectively (Fig 1B). Deletions were found only in one adult non-DS-AMKL patient, including deletion 2q and 17q. An

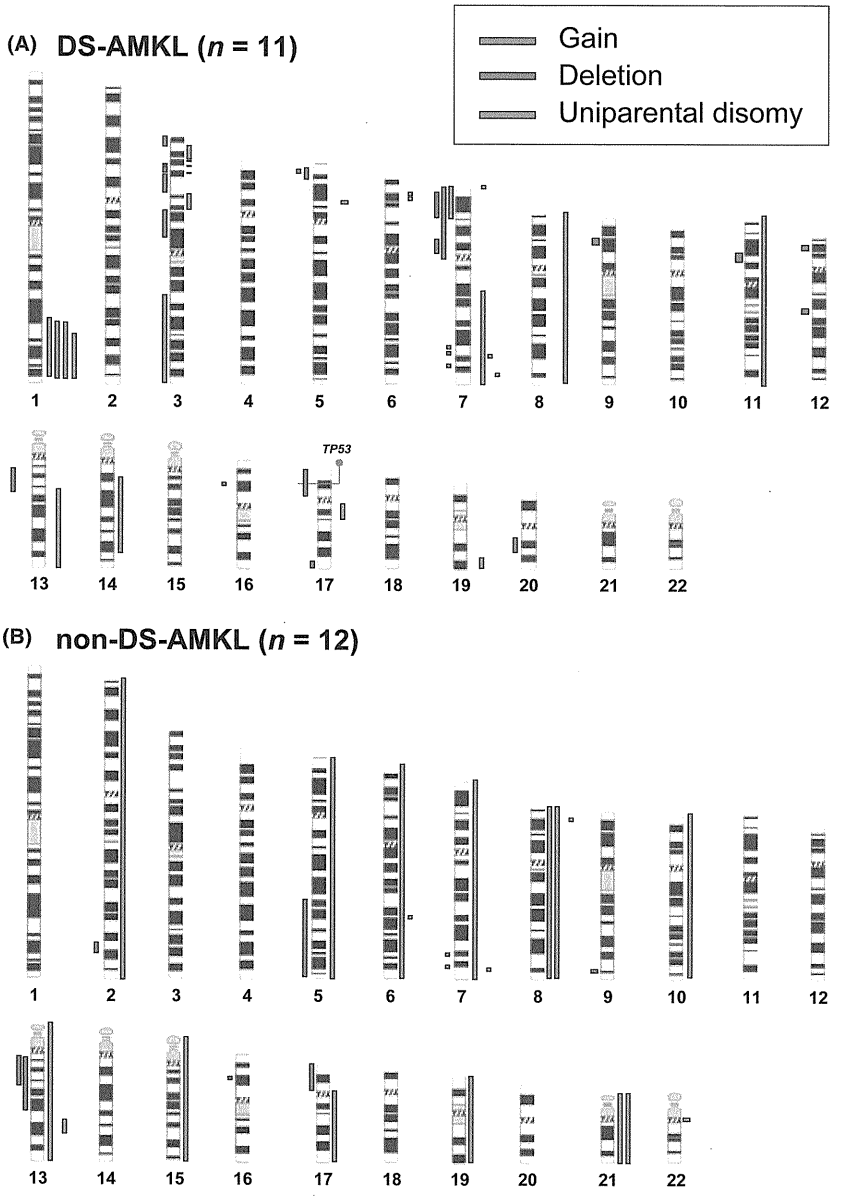

Fig 1. Single nucleotide polymorphism array-based karyotyping of AMKL. Genomic distribution and type of lesion identified by single nucleotide polymorphism array analysis in patients with DS-AMKL (n = 11) (A) and with non-DS-AMKL (n = 12) (B). Green bars represent gain, red bars indicate deletion, and blue corresponds to uniparental disomy. The fine red line pinpoints the locus of *TP53* gene mutated at that locus. Somatic gains other than trisomy 21 were found in seven DS-AMKL patients, including recurrent duplication of 1q in 4 and 7q in two patients.

Table II. Mutational status of pathogenic genes in DS-AMKL and non-DS-AMKL patients.

	Mutational status analysed gene										
Patient number	*GATA1*	*JAK3*	*JAK2*^{V617F}	*TP53*	*NRAS*	*ASXL1*	*FLT3*	*IDH1/2*	*DNMT3A*	*RUNX1*	*CBL*
DS-AMKL-1	■										
DS-AMKL-2	■										
DS-AMKL-3	■										
DS-AMKL-4	■										
DS-AMKL-5	■										
DS-AMKL-6	■										
DS-AMKL-7	■	■	■	■							
DS-AMKL-8	■										
DS-AMKL-9	■										
DS-AMKL-10											
DS-AMKL-11	■										
Paediatric non-DS-AMKL-1					■						
Paediatric non-DS-AMKL-2					■						
Paediatric non-DS-AMKL-3											
Paediatric non-DS-AMKL-4											
Paediatric non-DS-AMKL-5											
Paediatric non-DS-AMKL-6											
Paediatric non-DS-AMKL-7											
Paediatric non-DS-AMKL-8											
Paediatric non-DS-AMKL-9											
Paediatric non-DS-AMKL-10											
Paediatric non-DS-AMKL-11	■										
Paediatric non-DS-AMKL-12											
Adult non-DS-AMKL-1											
Adult non-DS-AMKL-2											
Adult non-DS-AMKL-3											
Adult non-DS-AMKL-4											
Total	11	1	1	1	2	0	0	0	0	0	0

Grey cells and white cells represent gene mutation and wild type, respectively.
DS, Down syndrome; AMKL, acute megakaryoblastic leukaemia.

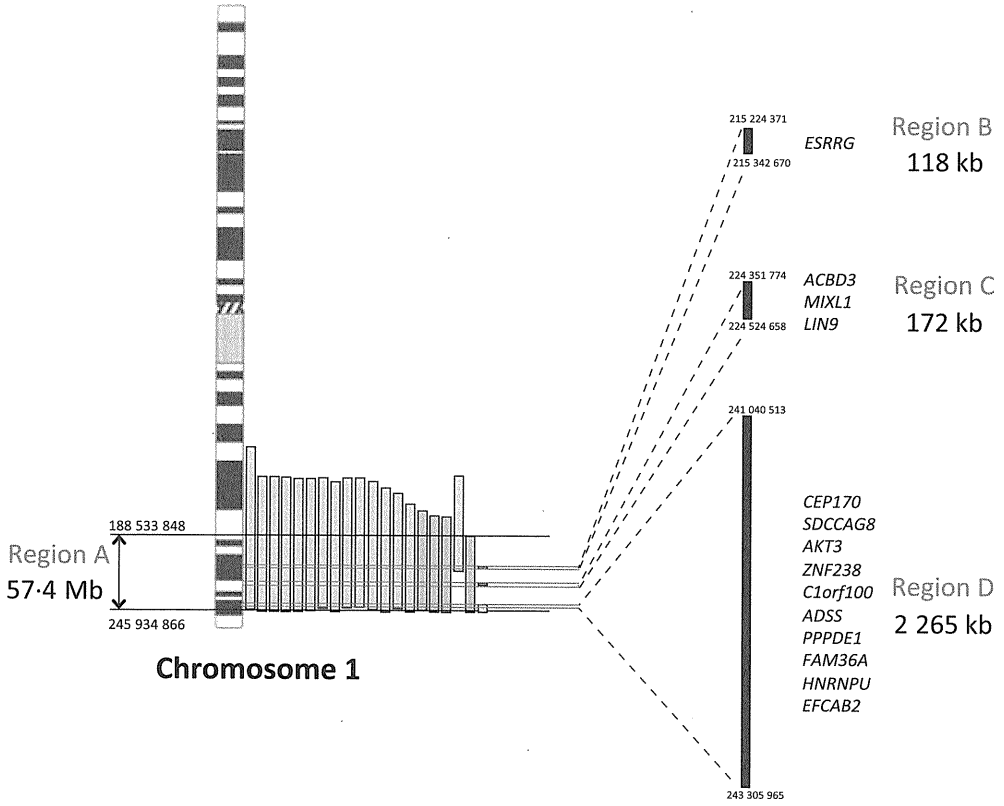

Fig 2. Summary of 1q gain lesions within the common region found in the DS-AMKL cohort: detected by single nucleotide polymorphism array (SNP-A) karyotyping in 22 of 798 patients with haematological disease. Analysis of the SNP-A karyotyping data of 798 patients with various haematological diseases identified 22 patients who harboured 1q gains that overlapped a region commonly duplicated in 4 DS-AMKL patients [Region A (57·4 Mb)]. Region B (118 kb), including only one gene (*ESRRG*) was shared by 91% (20/22) of patients. Region C (172 kb) was shared by 86% (19/22) patients, in which three genes (*ACBD3*, *MIXL1*, and *LIN9*) were located. Region D (2265kb) was shared by 86% (19/22) patients, and contained 10 genes including *AKT3*. Purple and pale blue bars indicate 1q gains found in DS-AMKL (n = 4) and other haematological diseases (n = 18), respectively. The diagnosis of 22 patients with 1q gains are as follows: DS-AMKL (n = 4), chronic myelomonocytic leukaemia (n = 5), secondary AML (n = 5), myelodysplastic syndrome (MDS) (n = 3), Fanconi anaemia (n = 2), idiopathic myelofibrosis (n = 1), chronic myeloid leukaemia (n = 1), and aplastic anaemia-derived MDS (n = 1).

overall complex karyotype (three or more abnormalities) was found in 6/11 DS-AMKL and 4/12 paediatric non-DS-AMKL.

Mutational analysis of patients with AMKL

Gene mutational status was analysed in 11 patients with DS-AMKL and 16 with non-DS-AMKL (12 children and four adults with AMKL, Table II). *GATA1* mutations were found in 10/11 (91%) with DS-AMKL, but only in one paediatric non-DS-AMKL and not found in adult patients. *NRAS* mutations (Q61R; G12S) were found in two paediatric non-DS-AMKL patients. *JAK2* (V617F), *JAK3* (Q501H), *TP53* (V157D) mutations were found in a single DS-AMKL patient (DS-AMKL-7). *ASXL1*, *IDH1/2*, *DNMT3A*, *RUNX1* and *CBL* mutations were not found in AMKL. With the exception of a hemizygous *TP53* mutation (microdeletion on 17p), all of the other mutations studied were heterozygous.

Perhaps of interest, we have identified a non-synonymous amino acid change in *ASXL1* (K888T) in a child with non-DS-AMKL. While this alteration was never reported as a SNP and not found in over 100 normal individuals from Japan, the nucleotide change was confirmed to be not of somatic origin, rather is it likely to represent a rare non-synonymous SNP found in a complete remission following successful chemotherapy.

Comparison of pathogenetic lesions among subgroups of AMKL

GATA1 mutations were more frequently found in DS-AMKL (10/11) compared to non-DS-AMKL (1/16) ($P < 0{\cdot}001$), consistent with previous reports. Additional mutations screened were only rarely found in DS-AMKL and non-DS-AMKL and thus the statistical comparisons were not warranted.

However, when the cytogenetic defects detected by SNP-A analysis were analysed, the frequency of 1q gain was significantly higher in DS-AMKL as compared to non-DS-AMKL

Table III. Comparison of somatic lesions detected by SNP-A analysis between DS-AMKL and non-DS-AMKL.

Type of somatic lesion	DS-AMKL (n = 11)	Non-DS-AMKL (n = 12)	P-value
Somatic 1q gain			
Yes	4	0	0·02
No	7	12	
Somatic gain lesion other than germline +21			
Yes	6	7	NS
No	5	5	
Somatic loss lesion			
Yes	8	5	NS
No	3	7	
Somatic UPD lesion			
Yes	1	0	NS
No	10	12	
Any somatic lesion			
Yes	8	8	NS
No	3	4	

SNP-A, single nucleotide polymorphism array; DS, Down syndrome, AMKL, acute megakaryoblastic leukaemia, UPD; uniparental disomy, NS; not significant.

[36% (4/11) vs. 0%, P = 0·02]. We also screened the SNP-A karyograms of 798 patients with myeloid malignancies (including paediatric and adult leukaemias, Fig 2) and found a commonly affected region on 1q (57·4 Mb, 188 533 848–245 934 866) in 2·8% (22/798) patients (Fig 2, region A), defined by patients with DS-AMKL. In addition to these four DS-AMKL patients, gain of 1q was also found in chronic myelomonocytic leukaemia (n = 5), secondary AML (n = 5), myelodysplastic syndrome (MDS, n = 3), Fanconi anaemia (n = 2), idiopathic myelofibrosis (n = 1), CML (n = 1), and aplastic anaemia-derived MDS (n = 1). We were also able to determine that an alternate commonly affected region on 1q (region B, 118 kb) was shared by 20/22 patients. This region was defined by a somatic microduplication in a patient with idiopathic myelofibrosis and contained only the *ESRRG* gene. Regions C (172 kb) and D (2·2 Mb) were shared by (19/22) each and harboured 3 and 10 genes, respectively (Fig 2). The frequencies of all other somatic lesions (gains, losses, UPDs) were not statistically different between patient groups (Table III).

Correlation between somatic trisomy 21 and megakaryoblastic phenotype

In addition to germline-encoded trisomy 21 of DS, somatic trisomy 21 was found in two additional patients with paediatric non-DS-AMKL. To establish whether there was an association between trisomy 21 and megakaryoblastic morphology, we analysed a cohort of paediatric AML patients without DS; AMKL was found in 23/232 of them. AMKL was found in 9/642 cases of adult AML without DS. Somatic trisomy 21 was present in 8 paediatric AML patients that showed megakaryoblastic phenotype ($P < 0·001$), while no association was found in adults (trisomy 21 was found in 21 patients, of whom none had AML with megakaryoblastic morphology; $P > 0·999$). To confirm our results, we also analysed 6009 adult AML without DS registered in the Mitelman Database of Chromosome Aberrations and Gene Fusions in Cancer (Mitelman *et al*, 2011) and found non-DS-AMKL in 130 patients. Somatic trisomy 21 was present in 264 adult AML patients, of whom only seven had non-DS-AMKL (P = 0·515, Table IV).

Discussion

In AML, distinct molecular lesions may lead to a similar clinical phenotype; conversely, specific morphological features could be a result of recurrent lesions. For instance, AMKL is frequently seen in children with DS and is characterized by the presence of *GATA1* mutations; thus, we posed a question whether AMKL seen in children and adults share common molecular abnormalities. As expected, *GATA1* mutations were rare in non-DS-AMKL and found in only 1/11 children with this condition. To date, a few children with *GATA1* mutations in non-DS-AMKL have been reported (Rainis *et al*, 2003; Malinge *et al*, 2008). Interestingly, all of them had acquired trisomy 21 in their leukaemic cells. Our non-DS-AMKL

Table IV. Association between somatic trisomy 21 and megakaryoblastic morphology in paediatric and adult AML patients without DS.

Patient cohort	Morphology	Somatic trisomy 21		P-value
		Present	Absent	
Paediatric AMLwithout DS (N = 232) (Nagoya University)	AMKL (n = 23)	8	15	<0·001
	Other AML (n = 209)	6	203	
Adult AML without DS (N = 642) (Cleveland Clinic)	AMKL (n = 9)	0	9	>0·999
	Other AML (n = 633)	21	612	
Adult AML without DS (N = 6009) (Mitelman database)	AMKL (n = 130)	7	123	0·515
	Other AML (n = 5879)	257	5622	

patient with *GATA1* mutation did not have acquired trisomy 21 in his leukaemic cells. Recently, mutations in *JAK2*, *JAK3*, *TP53*, *FLT3*, *ASXL1*, *DNMT3A*, *IDH1*, and *IDH2* have been found in various myeloid malignancies, including AML; some of these genes could be involved in signalling along the megakaryoblastic differentiation pathway. In this series of experiments we analysed a large cohort of AMKL for the presence of distinctive or shared chromosomal and genetic defects.

In addition to genes previously reported as mutated in AMKL (*JAK2*, *JAK3*, *TP53*), we identified *NRAS* mutation in two children with non-DS-AMKL but genes recently identified in various myeloid malignancies (*ASXL1*, *DNMT3A*, *IDH1*, *IDH2*, *RUNX1*, and *CBL*) were not found to be mutated in our AMKL cohort. While having only a few cases precluded systematic analysis, it appears that adult and paediatric cases of AMKL do not display distinctive mutational patterns.

In addition to the germline trisomy 21 in DS, somatic duplications of chromosome 21 were found in two cases of paediatric non-DS-AMKL. In paediatric AML, trisomy 21 is strongly linked to megakaryoblastic AML phenotype, as further analysis of AML with this lesion revealed six additional cases of AMKL, and as result, 8/23 cases of somatic trisomy 21 were AMKL. Conversely, there is no significant association between trisomy 21 and adult AMKL in both the Cleveland Clinic cohort and Mitelman Database. It is speculated that the presence of trisomy 21 contributes to megakaryoblastic phenotype only in the paediatric population.

A distinctive feature of DS-AMKL is the presence of a gain of 1q, found in 4/11 cases and absent in all other cases of non-DS-AMKL. Gain of 1q through duplication, isochromosome formation or unbalanced translocations, is one of the most frequent acquired cytogenetic abnormalities in human neoplasia and has also been found in various haematological malignancies, including B-lineage acute lymphoblastic leukaemia (B-ALL) (Johansson *et al*, 2004), multiple myeloma (Nilsson *et al*, 2003), and non-Hodgkin lymphomas (Johansson *et al*, 1995). Expression analyses implicated several upregulated genes associated with this amplification in ALL (Davidsson *et al*, 2007). Similarly, 1q gain was also found in DS-AMKL patients (Hayashi *et al*, 1988; Silva *et al*, 2009); a recent international Berlin-Frankfurt-Munster (iBFM) study of the cytogenetic metaphase analysis reported significantly higher incidence of 1q gains in DS-AML (16%) compared to non-DS-AML (2%) (Forestier *et al*, 2008). In 2/4 patients, metaphase cytogenetics showed the presence of unbalanced translocations involving 1q but an associated gain was detected only by SNP-A. Two out of four patients showed a previously cryptic 1q gain by SNP-A but not by metaphase cytogenetics. As a result the overall frequency of this lesion was found to be higher than previously reported using only routine cytogenetics (Forestier *et al*, 2008). By comparison, 2·8% (22/798) of patients with haematological disease harboured somatic 1q gains within the regions shared by four patients with DS-AMKL. We speculate that the commonly affected region of chromosome 1q contains a putative gene involved in pathogenesis of DS-AMKL. Potential candidate genes include *AKT3* (Nakatani *et al*, 1999; Stahl *et al*, 2004) and *ESRRG* (Ijichi *et al*, 2011), both reported to be overexpressed in association with cancer development and cell proliferation.

In summary, this study comprehensively analysed the molecular lesions in AMKL using SNP-A and mutational analysis of the selected genes. High frequency of 1q gain as well as *GATA1* mutation in DS-AMKL suggests that leukemogenesis mechanisms are diverse among subgroups of AMKL.

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Authorship

A. Hama, H. Muramatsu, H. Makishima designed research, performed research, analysed data, and wrote the paper. Y.S., H.S., M.J. performed research. C.O. designed research, analysed data, and wrote the paper. Y.T., H.S., S.D., A.S., N.W., K.M., K.K. designed research. S.K. designed research and wrote the paper. J.P.M. designed research, performed research, analysed data, and wrote the paper.

Conflict of interest disclosure

The authors declare no competing financial interests.

References

Ahmed, M., Sternberg, A., Hall, G., Thomas, A., Smith, O., O'Marcaigh, A., Wynn, R., Stevens, R., Addison, M., King, D., Stewart, B., Gibson, B., Roberts, I. & Vyas, P. (2004) Natural history of GATA1 mutations in Down syndrome. *Blood*, **103,** 2480–2489.

Akahoshi, M., Oshimi, K., Mizoguchi, H., Okada, M., Enomoto, Y. & Watanabe, Y. (1987) Myeloproliferative disorders terminating in acute megakaryoblastic leukemia with chromosome 3q26 abnormality. *Cancer*, **60,** 2654–2661.

Chou, W.C., Huang, H.H., Hou, H.A., Chen, C.Y., Tang, J.L., Yao, M., Tsay, W., Ko, B.S., Wu, S.J., Huang, S.Y., Hsu, S.C., Chen, Y.C., Huang, Y.N., Chang, Y.C., Lee, F.Y., Liu, M.C., Liu, C.W., Tseng, M.H., Huang, C.F. & Tien, H.F. (2010) Distinct clinical and biological features of de novo acute myeloid leukemia with additional sex comb-like 1 (ASXL1) mutations. *Blood*, **116,** 4086–4094.

Davidsson, J., Andersson, A., Paulsson, K., Heidenblad, M., Isaksson, M., Borg, A., Heldrup, J.,

Behrendtz, M., Panagopoulos, I., Fioretos, T. & Johansson, B. (2007) Tiling resolution array comparative genomic hybridization, expression and methylation analyses of dup(1q) in Burkitt lymphomas and pediatric high hyperdiploid acute lymphoblastic leukemias reveal clustered near-centromeric breakpoints and overexpression of genes in 1q22-32.3. *Human Molecular Genetics*, **16,** 2215–2225.

De Vita, S., Mulligan, C., McElwaine, S., Dagna-Bricarelli, F., Spinelli, M., Basso, G., Nizetic, D. & Groet, J. (2007) Loss-of-function JAK3 mutations in TMD and AMKL of Down syndrome. *British Journal of Haematology*, **137,** 337–341.

Dunbar, A.J., Gondek, L.P., O'Keefe, C.L., Makishima, H., Rataul, M.S., Szpurka, H., Sekeres, M.A., Wang, X.F., McDevitt, M.A. & Maciejewski, J.P. (2008) 250K single nucleotide polymorphism array karyotyping identifies acquired uniparental disomy and homozygous mutations, including novel missense substitutions of c-Cbl, in myeloid malignancies. *Cancer Research*, **68,** 10349–10357.

Forestier, E., Izraeli, S., Beverloo, B., Haas, O., Pession, A., Michalova, K., Stark, B., Harrison, C.J., Teigler-Schlegel, A. & Johansson, B. (2008) Cytogenetic features of acute lymphoblastic and myeloid leukemias in pediatric patients with Down syndrome: an iBFM-SG study. *Blood*, **111,** 1575–1583.

Gaidzik, V.I., Bullinger, L., Schlenk, R.F., Zimmermann, A.S., Rock, J., Paschka, P., Corbacioglu, A., Krauter, J., Schlegelberger, B., Ganser, A., Spath, D., Kundgen, A., Schmidt-Wolf, I.G., Gotze, K., Nachbaur, D., Pfreundschuh, M., Horst, H.A., Dohner, H. & Dohner, K. (2011) RUNX1 mutations in acute myeloid leukemia: results from a comprehensive genetic and clinical analysis from the AML study group. *Journal of Clinical Oncology*, **29,** 1364–1372.

Gondek, L.P., Dunbar, A.J., Szpurka, H., McDevitt, M.A. & Maciejewski, J.P. (2007) SNP array karyotyping allows for the detection of uniparental disomy and cryptic chromosomal abnormalities in MDS/MPD-U and MPD. *PLoS ONE*, **2,** e1225.

Gondek, L.P., Tiu, R., O'Keefe, C.L., Sekeres, M.A., Theil, K.S. & Maciejewski, J.P. (2008) Chromosomal lesions and uniparental disomy detected by SNP arrays in MDS, MDS/MPD, and MDS-derived AML. *Blood*, **111,** 1534–1542.

Hama, A., Yagasaki, H., Takahashi, Y., Kiyoi, H., Naoe, T. & Kojima, S. (2008a) Mutations of JAK2, JAK3 and GATA1 in acute megakaryoblastic leukemia of Down syndrome. *Blood*, **111,** 2493–2494.

Hama, A., Yagasaki, H., Takahashi, Y., Nishio, N., Muramatsu, H., Yoshida, N., Tanaka, M., Hidaka, H., Watanabe, N., Yoshimi, A., Matsumoto, K., Kudo, K., Kato, K., Horibe, K. & Kojima, S. (2008b) Acute megakaryoblastic leukaemia (AMKL) in children: a comparison of AMKL with and without Down syndrome. *British Journal of Haematology*, **140,** 552–561.

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

Hirose, Y., Kudo, K., Kiyoi, H., Hayashi, Y., Naoe, T. & Kojima, S. (2003) Comprehensive analysis of gene alterations in acute megakaryoblastic leukemia of Down's syndrome. *Leukemia*, **17,** 2250–2252.

Ijichi, N., Shigekawa, T., Ikeda, K., Horie-Inoue, K., Fujimura, T., Tsuda, H., Osaki, A., Saeki, T. & Inoue, S. (2011) Estrogen-related receptor gamma modulates cell proliferation and estrogen signaling in breast cancer. *Journal of Steroid Biochemistry and Molecular Biology*, **123,** 1–7.

Johansson, B., Mertens, F. & Mitelman, F. (1995) Cytogenetic evolution patterns in non-Hodgkin's lymphoma. *Blood*, **86,** 3905–3914.

Johansson, B., Mertens, F. & Mitelman, F. (2004) Clinical and biological importance of cytogenetic abnormalities in childhood and adult acute lymphoblastic leukemia. *Annals of Medicine*, **36,** 492–503.

Kiyoi, H., Yamaji, S., Kojima, S. & Naoe, T. (2007) JAK3 mutations occur in acute megakaryoblastic leukemia both in Down syndrome children and non-Down syndrome adults. *Leukemia*, **21,** 574–576.

Klusmann, J.H., Reinhardt, D., Hasle, H., Kaspers, G.J., Creutzig, U., Hahlen, K., van den Heuvel-Eibrink, M.M. & Zwaan, C.M. (2007) Janus kinase mutations in the development of acute megakaryoblastic leukemia in children with and without Down's syndrome. *Leukemia*, **21,** 1584–1587.

Kohlmann, A., Grossmann, V., Klein, H.U., Schindela, S., Weiss, T., Kazak, B., Dicker, F., Schnittger, S., Dugas, M., Kern, W., Haferlach, C. & Haferlach, T. (2010) Next-generation sequencing technology reveals a characteristic pattern of molecular mutations in 72.8% of chronic myelomonocytic leukemia by detecting frequent alterations in TET2, CBL, RAS, and RUNX1. *Journal of Clinical Oncology*, **28,** 3858–3865.

Ley, T.J., Ding, L., Walter, M.J., McLellan, M.D., Lamprecht, T., Larson, D.E., Kandoth, C., Payton, J.E., Baty, J., Welch, J., Harris, C.C., Lichti, C.F., Townsend, R.R., Fulton, R.S., Dooling, D.J., Koboldt, D.C., Schmidt, H., Zhang, Q., Osborne, J.R., Lin, L., O'Laughlin, M., McMichael, J.F., Delehaunty, K.D., McGrath, S.D., Fulton, L.A., Magrini, V.J., Vickery, T.L., Hundal, J., Cook, L.L., Conyers, J.J., Swift, G.W., Reed, J.P., Alldredge, P.A., Wylie, T., Walker, J., Kalicki, J., Watson, M.A., Heath, S., Shannon, W.D., Varghese, N., Nagarajan, R., Westervelt, P., Tomasson, M.H., Link, D.C., Graubert, T.A., DiPersio, J.F., Mardis, E.R. & Wilson, R.K. (2010) DNMT3A mutations in acute myeloid leukemia. *New England Journal of Medicine*, **363,** 2424–2433.

Makishima, H., Cazzolli, H., Szpurka, H., Dunbar, A., Tiu, R., Huh, J., Muramatsu, H., O'Keefe, C., Hsi, E., Paquette, R.L., Kojima, S., List, A.F., Sekeres, M.A., McDevitt, M.A. & Maciejewski, J.P. (2009) Mutations of e3 ubiquitin ligase cbl family members constitute a novel common pathogenic lesion in myeloid malignancies. *Journal of Clinical Oncology*, **27,** 6109–6116.

Malinge, S., Ragu, C., Della-Valle, V., Pisani, D., Constantinescu, S.N., Perez, C., Villeval, J.L., Reinhardt, D., Landman-Parker, J., Michaux, L., Dastugue, N., Baruchel, A., Vainchenker, W., Bourquin, J.P., Penard-Lacronique, V. & Bernard, O.A. (2008) Activating mutations in human acute megakaryoblastic leukemia. *Blood*, **112,** 4220–4226.

Malkin, D., Brown, E.J. & Zipursky, A. (2000) The role of p53 in megakaryocyte differentiation and the megakaryocytic leukemias of Down syndrome. *Cancer Genetics and Cytogenetics*, **116,** 1–5.

Mesa, R.A., Li, C.Y., Ketterling, R.P., Schroeder, G.S., Knudson, R.A. & Tefferi, A. (2005) Leukemic transformation in myelofibrosis with myeloid metaplasia: a single-institution experience with 91 cases. *Blood*, **105,** 973–977.

Mitelman, F., Johansson, B. & Mertens, F. (eds) (2011) Mitelman database of chromosome aberrations and gene fusions in cancer (2011). http://cgap.nci.nih.gov/Chromosomes/Mitelman.

Nakatani, K., Thompson, D.A., Barthel, A., Sakaue, H., Liu, W., Weigel, R.J. & Roth, R.A. (1999) Up-regulation of Akt3 in estrogen receptor-deficient breast cancers and androgen-independent prostate cancer lines. *Journal of Biological Chemistry*, **274,** 21528–21532.

Nilsson, T., Hoglund, M., Lenhoff, S., Rylander, L., Turesson, I., Westin, J., Mitelman, F. & Johansson, B. (2003) A pooled analysis of karyotypic patterns, breakpoints and imbalances in 783 cytogenetically abnormal multiple myelomas reveals frequently involved chromosome segments as well as significant age- and sex-related differences. *British of Journal Haematology*, **120,** 960–969.

Radaelli, F., Mazza, R., Curioni, E., Ciani, A., Pomati, M. & Maiolo, A.T. (2002) Acute megakaryocytic leukemia in essential thrombocythemia: an unusual evolution? *European Journal of Haematology*, **69,** 108–111.

Rainis, L., Bercovich, D., Strehl, S., Teigler-Schlegel, A., Stark, B., Trka, J., Amariglio, N., Biondi, A., Muler, I., Rechavi, G., Kempski, H., Haas, O.A. & Izraeli, S. (2003) Mutations in exon 2 of GATA1 are early events in megakaryocytic malignancies associated with trisomy 21. *Blood*, **102,** 981–986.

Sanada, M., Suzuki, T., Shih, L.Y., Otsu, M., Kato, M., Yamazaki, S., Tamura, A., Honda, H., Sakata-Yanagimoto, M., Kumano, K., Oda, H., Yamagata, T., Takita, J., Gotoh, N., Nakazaki, K., Kawamata, N., Onodera, M., Nobuyoshi, M., Hayashi, Y., Harada, H., Kurokawa, M., Chiba, S., Mori, H., Ozawa, K., Omine, M., Hirai, H., Nakauchi, H., Koeffler, H.P. & Ogawa, S. (2009) Gain-of-function of mutated C-CBL tumour suppressor in myeloid neoplasms. *Nature*, **460,** 904–908.

Silva, M.L., do Socorro Pombo-de-Oliveira, M., Raimondi, S.C., Mkrtchyan, H., Abdelhay, E., de Figueiredo, A.F., de Souza, M.T., Garcia, D.R., de Ventura, E.M., de Sousa, A.M. & Liehr, T. (2009) Unbalanced chromosome 1 abnormalities leading to partial trisomy 1q in four infants with Down syndrome and acute megakaryocytic leukemia. *Molecular Cytogenetics*, **2,** 7.

Stahl, J.M., Sharma, A., Cheung, M., Zimmerman, M., Cheng, J.Q., Bosenberg, M.W., Kester, M., Sandirasegarane, L. & Robertson, G.P. (2004) Deregulated Akt3 activity promotes development of malignant melanoma. *Cancer Research*, **64,** 7002–7010.

Sugimoto, Y., Muramatsu, H., Makishima, H., Prince, C., Jankowska, A.M., Yoshida, N., Xu, Y., Nishio, N., Hama, A., Yagasaki, H., Takahashi, Y., Kato, K., Manabe, A., Kojima, S. & Maciejewski, J.P. (2010) Spectrum of molecular defects in juvenile myelomonocytic leukaemia includes ASXL1 mutations. *British Journal of Haematology*, **150,** 83–87.

Walters, D.K., Mercher, T., Gu, T.L., O'Hare, T., Tyner, J.W., Loriaux, M., Goss, V.L., Lee, K.A., Eide, C.A., Wong, M.J., Stoffregen, E.P., McGreevey, L., Nardone, J., Moore, S.A., Crispino, J., Boggon, T.J., Heinrich, M.C., Deininger, M.W., Polakiewicz, R.D., Gilliland, D.G. & Druker, B.J. (2006) Activating alleles of JAK3 in acute megakaryoblastic leukemia. *Cancer Cell*, **10,** 65–75.

Wechsler, J., Greene, M., McDevitt, M.A., Anastasi, J., Karp, J.E., Le Beau, M.M. & Crispino, J.D. (2002) Acquired mutations in GATA1 in the megakaryoblastic leukemia of Down syndrome. *Nature Genetics*, **32,** 148–152.

Yan, H., Parsons, D.W., Jin, G., McLendon, R., Rasheed, B.A., Yuan, W., Kos, I., Batinic-Haberle, I., Jones, S., Riggins, G.J., Friedman, H., Friedman, A., Reardon, D., Herndon, J., Kinzler, K.W., Velculescu, V.E., Vogelstein, B. & Bigner, D.D. (2009) IDH1 and IDH2 mutations in gliomas. *New England Journal of Medicine*, **360,** 765–773.

Zipursky, A., Poon, A. & Doyle, J. (1992) Leukemia in Down syndrome: a review. *Pediatric Hematology and Oncology*, **9,** 139–149.

Journal of Cellular Physiology

Neutrophil Differentiation From Human-Induced Pluripotent Stem Cells

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Induced pluripotent stem (iPS) cells are of potential value not only for regenerative medicine, but also for disease investigation. The present study describes the development of a neutrophil differentiation system from human iPS cells (hiPSCs) and the analysis of neutrophil function and differentiation. The culture system used consisted of the transfer of hiPSCs onto OP9 cells and their culture with vascular endothelial growth factor (VEGF). After 10 days, TRA 1-85^{+}CD34^{+}VEGF receptor-2 (VEGFR-2)^{high} cells were sorted and co-cultured with OP9 cells in the presence of hematopoietic cytokines for 30 days. Floating cells were collected and subjected to morphological and functional analysis. These hiPSC-derived neutrophils were similar to peripheral blood mature neutrophils in morphology, contained functional neutrophil specific granules, and were equipped with the basic functions such as phagocytosis, superoxide production, and chemotaxis. In the process of differentiation, myeloid cells appeared sequentially from immature myeloblasts to mature segmented neutrophils. Expression patterns of surface antigen, transcription factors, and granule proteins during differentiation were also similar to those of granulopoiesis in normal bone marrow. In conclusion, differentiation of mature neutrophils from hiPSCs was successfully induced in a similar process to normal granulopoiesis using an OP9 co-culture system. This system may be applied to elucidate the pathogenesis of various hematological diseases that affect neutrophils.

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Neutrophils and/or myeloid differentiation are most commonly affected in various hematological diseases including inherited bone marrow failure syndromes and neutrophil function disorders. Responsible genes have been identified in most of these syndromes or diseases, but the association between the gene mutation and the specific phenotype is not always clear. Moreover, often patients who present with a specific syndrome lack mutations in the known genes (Alter, 2007). Understanding the pathophysiology of these syndromes has been challenging despite the information provided by recent molecular findings, and in many of these syndromes, experimental models have not yet been generated.

Murine models of human congenital and acquired diseases are invaluable for disease investigation, but they provide a limited representation of human pathophysiology because they often do not faithfully mimic human diseases. The differences between murine and human physiologies make human cell culture an essential complement to research with animal models of disease.

Induced pluripotent stem (iPS) cells are reprogrammed somatic cells with embryonic stem (ES) cell-like characteristics generated by the introduction of combinations of specific transcription factors (Takahashi and Yamanaka, 2006; Meissner et al., 2007; Okita et al., 2007; Takahashi et al., 2007; Yu et al., 2007; Park et al., 2008b). Given the robustness of the approach, direct reprogramming promises to be a facile source of patient-derived cell lines. Such lines would be immediately valuable not only for regenerative medicine, but for disease investigation and drug screening as well.

The pluripotency and self-renewal potential of ES cells contributes to their value in various fields of science (Evans and Kaufman, 1981). Previous studies using normal or gene-manipulated ES cells have helped elucidate the process of normal embryogenesis and the genetic mechanisms of certain diseases (Lensch and Daley, 2006; Tulpule et al., 2010). Use of human embryos, however, faces ethical controversies that hinder the applications of human ES cells (hESCs). In addition, it is difficult to generate patient- or disease-specific ES cells, which are required for their effective application. The use of iPS cells would avoid the controversies surrounding human embryonic stem cell research.

Patient-specific iPS cells can be used for the generation of disease-corrected, patient-specific cells for cell therapy applications. Disease-specific pluripotent cells capable of differentiation into the various tissues affected in each condition can also provide new insights into disease pathophysiology by permitting analysis in a human system, under controlled conditions in vitro. Recent studies reported the generation of disease-specific iPS cell lines from patients with a variety of diseases (Park et al., 2008a; Raya et al., 2009; Agarwal et al., 2010). Therefore, disease-specific iPS cells are expected to be good models for the investigation of different diseases, and

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