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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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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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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bjh research paper

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

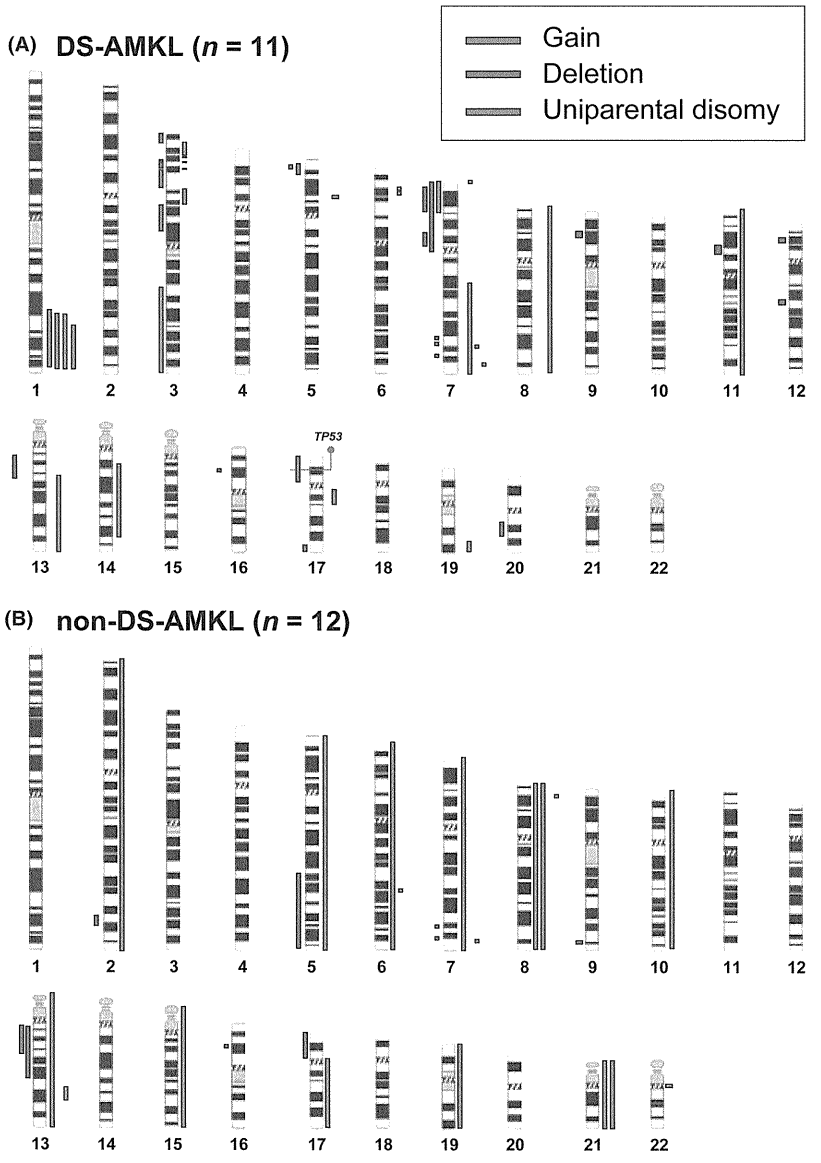

Fig 1. Single nucleotide polymorphism array-based karyotyping of AMKL. Genomic distribution and type of lesion identified by single nucleotide polymorphism array analysis in patients with DS-AMKL (n = 11) (A) and with non-DS-AMKL (n = 12) (B). Green bars represent gain, red bars indicate deletion, and 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.

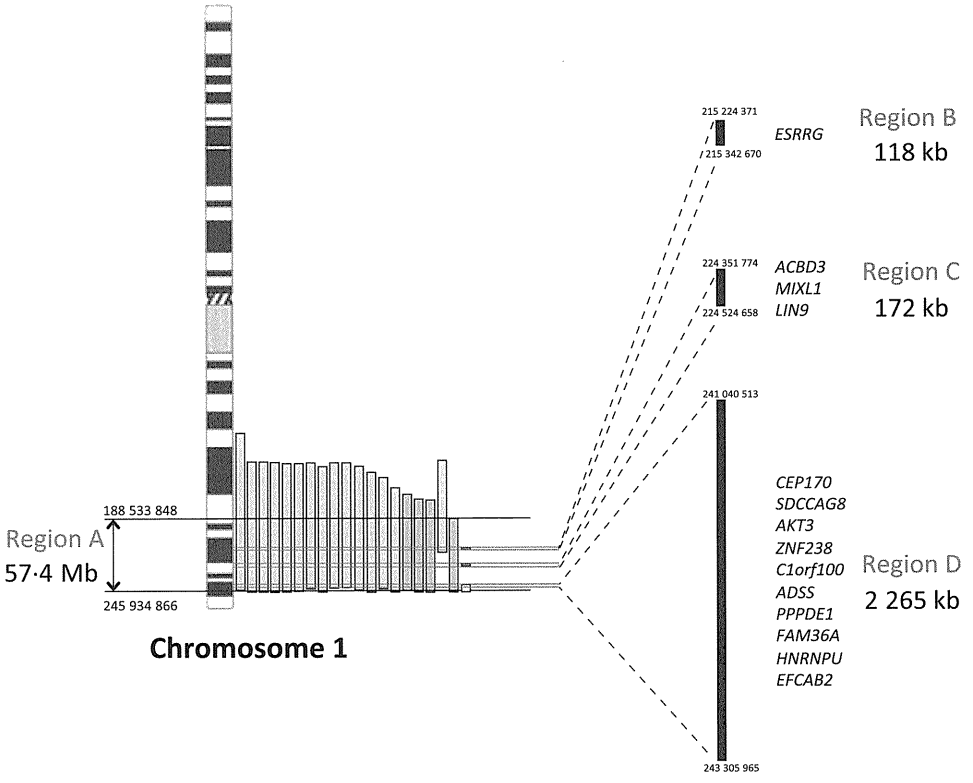

Fig 2. Summary of 1q gain lesions within the common region found in the DS-AMKL cohort: detected by single nucleotide polymorphism array (SNP-A) karyotyping in 22 of 798 patients with haematological disease. Analysis of the SNP-A karyotyping data of 798 patients with various haematological diseases identified 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.

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