Table 4 continued

S. no.	District	Institutions
120	Kinki	Osaka General Medical Center
121	Kinki	Nakano Children's Hospital
122	Kinki	Kishiwada City Hospital
123	Kinki	Japanese Red Cross Kyoto Daiichi Hospital
124	Kinki	Kyoto-Katsura Hospital
125	Kinki	Kyoto University Hospital
126	Kinki	Kyoto City Hospital
127	Kinki	National Hospital Organization Maizuru Medical Center
128	Kinki	University Hospital, Kyoto Prefectural University of Medicine
129	Kinki	Takashima General Hospital
130	Kinki	Shiga University of Medical Science Hospital
131	Kinki	Shiga Medical Center for Children
132	Kinki	Otsu Red Cross Hospital
133	Kinki	Tenri Hospital
134	Kinki	Nara Medical University Hospital
135	Kinki	Kobe University Hospital
136	Kinki	Kobe City Medical Center General Hospital
137	Kinki	Japanese Red Cross Society Himeji Hospital
138	Kinki	Akashi Municipal Hospital
139	Kinki	Hyogo Prefectural Kobe Children's Hospital
140	Kinki	Hyogo College of Medicine Hospital
141	Kinki	Nishi-Kobe Medical Center
142	Kinki	Japanese Red Cross Society Wakayama Medical Center
143	Kinki	Wakayama Medical University Hospital
144	Chugoku and Shikoku	Ehime Prefectural Central Hospital
145	Chugoku and Shikoku	Ehime University Hospital
146	Chugoku and Shikoku	National Hospital Organization Okayama Medical Center
147	Chugoku and Shikoku	Okayama University Hospital
148	Chugoku and Shikoku	Okayama Saiseikai General Hospital
149	Chugoku and Shikoku	Kawasaki Medical School Hospital
150	Chugoku and Shikoku	Kurashiki Central Hospital
151	Chugoku and Shikoku	National Hospital Organization Kagawa Children's Hospital
152	Chugoku and Shikoku	Kagawa University Hospital
153	Chugoku and Shikoku	National Hospital Organization Kochi Medical Center
154	Chugoku and Shikoku	Japanese Red Cross Kochi Hospital
155	Chugoku and Shikoku	Kochi Medical School Hospital
156	Chugoku and Shikoku	Shimane University Hospital
157	Chugoku and Shikoku	Shimane Prefectural Central Hospital
158	Chugoku and Shikoku	Tokushima University Hospital
159	Chugoku and Shikoku	Tottori University Hospital
160	Chugoku and Shikoku	Tottori Prefectural Chuou Hospital
161	Chugoku and Shikoku	Hiroshima University Hospital
162	Chugoku and Shikoku	Hiroshima Red Cross Hospital and Atomic-bomb Survivors Hospital
163	Chugoku and Shikoku	Yamaguchi University Hospital
164	Chugoku and Shikoku	Tokushima Red Cross Hospital
165	Chugoku and Shikoku	Matsue Red Cross Hospital
166	Kyushu and Okinawa	National Hospital Organization Beppu Medical Center
167	Kyushu and Okinawa	Oita Prefectural Hospital

Table 4 continued

S. no.	District	Institutions
168	Kyushu and Okinawa	Oita University Hospital
169	Kyushu and Okinawa	Hospital, University of the Ryukyus
170	Kyushu and Okinawa	Okinawa Prefectural Nanbu Medical Center and Children's Medical Center
171	Kyushu and Okinawa	Kagoshima City Hospital
172	Kyushu and Okinawa	Kagoshima University Medical And Dental Hospital
173	Kyushu and Okinawa	National Hospital Organization Kumamoto Medical Center
174	Kyushu and Okinawa	Kumamoto university hospital
175	Kyushu and Okinawa	Japanese Red Cross Kumamoto Hospital
176	Kyushu and Okinawa	Saga University Hospital
177	Kyushu and Okinawa	Nagasaki University Hospital
178	Kyushu and Okinawa	Kitakyushu Municipal Medical Center
179	Kyushu and Okinawa	Center for Pediatric Emergency Medicine Kitakyushu Municipal Yahata Hospital
180	Kyushu and Okinawa	Kurume University Hospital
181	Kyushu and Okinawa	University Hospital of Occupational and Environmental Health
182	Kyushu and Okinawa	Kyushu University Hospital
183	Kyushu and Okinawa	National Hospital Organization Kyushu Cancer Center
184	Kyushu and Okinawa	Fukuoka University Hospital
185	Kyushu and Okinawa	University of Miyazaki Hospital
186	Kyushu and Okinawa	Sasebo Municipal General Hospital
187	Kyushu and Okinawa	General Hospital Hamanomachi

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Brief Report

MYELOID NEOPLASIA

Naturally occurring oncogenic GATA1 mutants with internal deletions in transient abnormal myelopoiesis in Down syndrome

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Key Points

- Naturally occurring oncogenic GATA1 mutants with internal deletions contribute to transient abnormal myelopoiesis in Down syndrome.

Children with Down syndrome have an increased incidence of transient abnormal **myelopoiesis (TAM) and acute megakaryoblastic leukemia. The majority of these cases harbor somatic mutations in the *GATA1* gene, which results in the loss of full-length GATA1. Only a truncated isoform of GATA1 that lacks the *N*-terminal 83 amino acids (GATA1-S) remains. We found through genetic studies of 106 patients with TAM that internally deleted GATA1 proteins (GATA1-IDs) lacking amino acid residues 77-119 or 74-88 (created by splicing mutations) contributed to the genesis of TAM in 6 patients. Analyses of GATA1-deficient embryonic megakaryocytic progenitors revealed that the GATA1 function in growth restriction was disrupted in GATA1-IDs. In contrast, GATA1-S promoted megakaryocyte proliferation more profoundly than that induced by GATA1 deficiency. These results indicate that the internally deleted regions play important roles in megakaryocyte proliferation and that perturbation of this mechanism is involved in the pathogenesis of TAM. (*Blood.* 2013;121(16):3181-3184)**

Introduction

Children with Down syndrome (DS) are known to have a high risk of developing transient abnormal myelopoiesis (TAM) and subsequent acute megakaryoblastic leukemia (DS-AMKL).[1-4] Blast cells in the majority of patients with TAM and DS-AMKL have mutations in the second exon of the *GATA1* gene.[5,6] The mutations turn off the production of full-length GATA1. Instead, *N*-terminally truncated GATA1 protein (GATA1-S) was translated from the second methionine at codon 84, which is identical to the truncated GATA1 isoform found in the healthy human.[7] In contrast, only a few patients with AMKL have been reported to harbor 21-disomy blasts with the GATA1 mutation.[8,9] Therefore, GATA1-S is believed to be a prerequisite for the pathogenesis of TAM and DS-AMKL in children with DS, and unrestricted proliferation of megakaryocytic progenitors in DS-AMKL is thought to be provoked by a mechanism involving GATA1-S. However, the molecular mechanism of how GATA1-S contributes to the genesis of TAM and DS-AMKL remains elusive.

GATA1 regulates the proliferation of immature megakaryocytic progenitors. Indeed, active proliferation of immature megakaryocytic progenitors derived from GATA1-deficient mouse embryos is restricted by introduction of wild-type GATA1, but not by GATA-S.[10] GATA1-deficient mice rescued with transgenic expression of GATA1-S (or GATA1-ΔNT) are found to exhibit hyper-megakaryopoiesis in a limited embryonic and postnatal period, resembling the phenotype in human TAM cases.[11] In contrast, another report indicates that targeting mice expressing GATA1 protein with a deletion of 64 *N*-terminal amino acids, but retaining the 65th to 83rd amino acid residues intact, has demonstrated that the embryos display a transient megakaryocytic phenotype only during the early embryonic stage, not in the late-embryonic and postnatal stages.[12] We surmise that this difference simply may be a result of missing the region corresponding to the 65th to 83rd amino acids.

Here, we have identified novel GATA1 mutants with internal deletions (IDs) of either amino acid residues 77-119 or 74-88 (GATA1-IDs) in 6 patients. We found that the GATA1-IDs lost their activity in the regulation of megakaryocyte growth. These results demonstrate that disruption of ID regions is implicated in the pathogenesis of TAM.

Study design

This study was approved by the Ethics Committee of the Hirosaki University Graduate School of Medicine. All animal experiments

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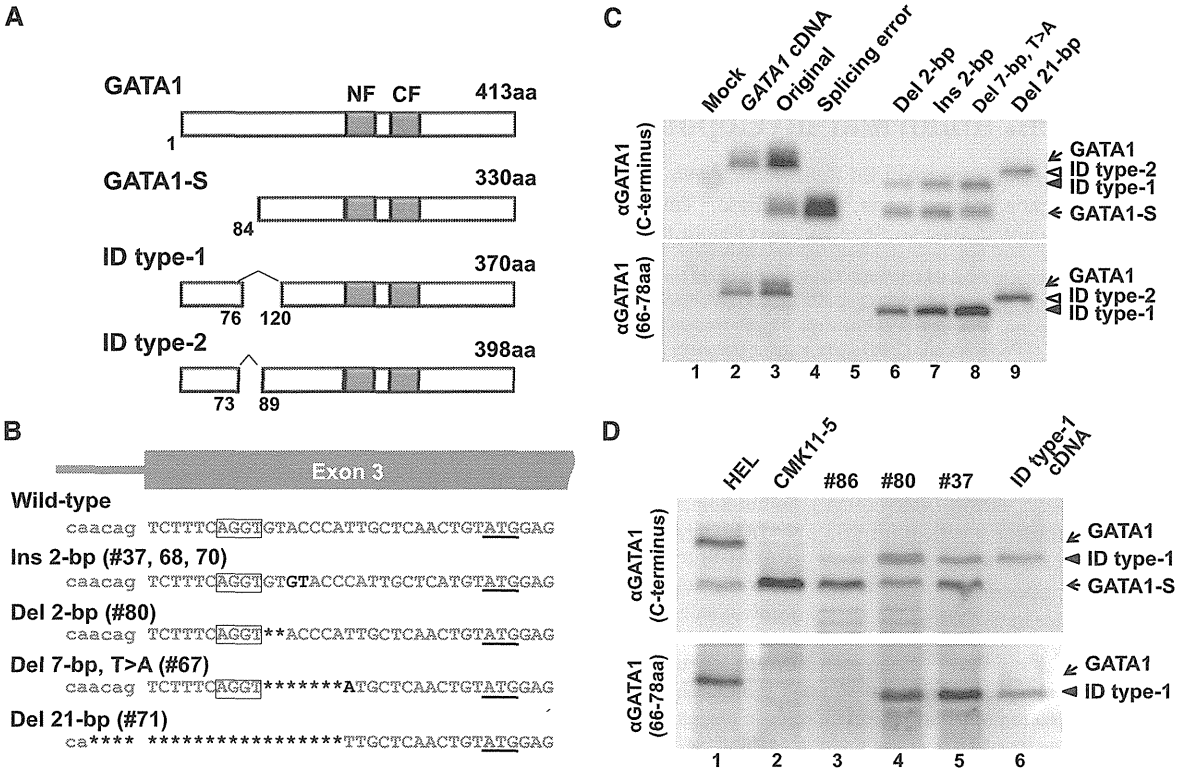

Figure 1. GATA1 mutant proteins with internal deletions. (A) A schema of mutant GATA1 proteins observed in patients with TAM. The amino acid sequence of GATA1-ID proteins was deduced from the sequence of *GATA1* cDNA obtained from patients with TAM. Dark boxes indicate *N*-finger (NF) and C-finger (CF) domains. ID indicates internal deletion. (B) Somatic mutations of the *GATA1* gene found in ID type 1 and type 2 patients. Missing, inserted, or substituted nucleotides are highlighted with dark color. A second translation initiation codon located in the third exon is underlined. The AGGT sequence functioning as an alternative splice donor site in mutant *GATA1* genes of ID type 1 patients is circled. Note that a mutant *GATA1* gene found in TAM patient 71f (ID type 2) lost a splice acceptor site in exon 3 because of the 21-nucleotide deletion. (C) Expression of GATA1 proteins in cells transfected with minigenes using anti-GATA1 antibodies recognizing the C terminus (upper) and residues between the 66th and 78th amino acids (lower) of the GATA1 protein. GATA1-ID proteins are recognized by the antibody against amino acid residues 66-78 of GATA1, whereas GATA1-S is not (lanes 6-9). Cells transfected with mock pcDNA3.1 (lane 1), pcDNA3.1-*GATA1* cDNA (lane 2), original minigene (lane 3), and *GATA1* minigene harboring a splicing error mutant in the 3′ boundary of intron 1[13] (lane 4) are used as positive and negative controls for GATA1 and GATA1-S, respectively. (D) GATA1 ID type 1 protein and GATA1-S are detected in the TAM blast cells from patients 80 (lane 4) and 37 (lane 5), whereas only GATA1-S is expressed in the blast cells from patient 86 harboring a conventional type of *GATA1* gene mutation in TAM cases (lane 3). Note that relatively abundant GATA1-S is recognized in patient 37 because of the intermixing of genetically distinct clone of cells expressing only GATA1-S (supplemental Table 1). Human erythroleukemia cells (HEL, lane 1) were used as a control for GATA1 and GATA1-S. DS-AMKL cells (CMK11-5, lane 2) and BHK-21 cells transfected with cDNA encoding GATA1 ID type 1 protein (lane 6) were used as controls for GATA1-S and GATA1 ID type 1, respectively.

were approved by the Institutional Animal Experiment Committee of Tohoku University. All clinical samples were obtained with informed consent from the parents of all patients with TAM in accordance with the Declaration of Helsinki. Additional information can be found in the supplemental text on the *Blood* website.

Results and discussion

Between 2003 and 2010, we screened *GATA1* mutations by direct sequencing, using cDNAs prepared from TAM blasts provided by 106 patients with DS on request from referring hospitals. Acquired *GATA1* mutations were detected in 99 (93.4%) patients (supplemental Table 1). The majority of the mutations resulted in the GATA1-S mutant protein, which lacks the entire *N*-terminal transactivation domain. Importantly, we found new mutations harboring IDs of 43 and 15 amino acids in 5 patients (patients 37, 67, 68, 70, and 80) and in 1 patient (patient 71), respectively. We refer to these mutants as GATA1-ID type 1 and GATA1-ID type 2, respectively (Figure 1A). Clinical features in patients with TAM who have GATA1-ID mutations were shown in supplemental Table 2. All of these patients showed high white blood cell counts in the peripheral blood, which is known to be a risk factor for early death.[13]

We determined the genomic DNA sequences of these cases. As shown in Figure 1B, the mutations in GATA1-ID type 1 were located in a site immediately 3′ of the consensus motif for a splice donor site AGGT[14] (Ins 2-bp in patients 37, 68, and 70; Del 2-bp in patient 80; and Del 7-bp T>A in patient 67), whereas 21 bp containing a splice acceptor site in front of exon 3 was deleted in GATA1-ID type 2 (Del 21-bp). To verify the transcripts achieved through the putative splice donor site created by mutations in GATA1-ID type 1, we introduced identified mutations into *GATA1* minigene expression vectors[13] and transduced them into hamster fibroblast cell line BHK-21. We found 3 variant transcripts in the cases of GATA1-ID type 1 mutations (supplementary Figure 1A-B): a full-length transcript with deletion or insertion of nucleotides [Ex-2 (+) (PTC)], a short transcript lacking exon 2 by alternative splice variant skipping of exon 2 for GATA1-S [Ex-2 (−)], and an aberrant transcript in which 129 nucleotides were spliced out from exon 3 (Del 129-bp). In contrast, 2 disparate transcripts with deletions of 45 or 137 nucleotides were created by

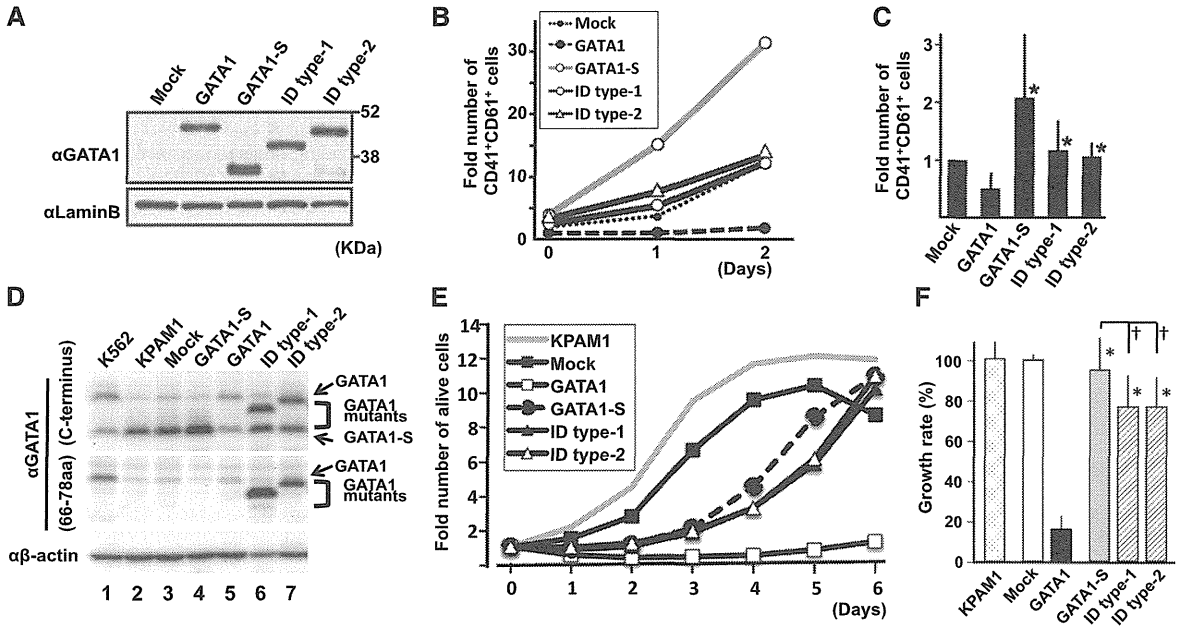

Figure 2. GATA1 ID proteins showed restricted antiproliferative activity. (A) Expression of GATA1 and GATA1 mutant proteins in cultured megakaryocytes at day 0, using an antibody against the C terminus of GATA1. The amount of protein loaded was quantified using an anti-Lamin B antibody on the same membrane. (B) Time-course change in the number of $CD41^+CD61^+$ cells. The value in the mock case at day 0 is set to 1. The result is representative of 4 independent experiments. (C) Comparison of the number of $CD41^+CD61^+$ cells at day 2. The value in the mock case is set to 1 in every experiment. The mean values and standard deviations from 4 independent experiments are presented. Asterisks indicate a significant difference compared with wild-type GATA1 ($P < .05$). (D) Immunoblot analysis of ectopic expression of GATA1 proteins in KPAM1 cells using anti-GATA1 antibodies against C terminus (upper) and residues between amino acids 66 and 78 (middle). The loading volume was quantified using anti-β-actin antibody (lower). (E) Growth curves of KPAM1 cells after ectopic expression of GATA1 proteins. Average values obtained from 6 wells are shown. The value at day zero is set to 1 for each. The growth curve of the original KPAM1 cells was analyzed as a control. Representative data from 3 independent experiments are shown. (F) Relative growth rate of KPAM1 cells at 5 days after ectopic expression of GATA1 mutant proteins. The average value of growth rate in the mock case is set to 100% in every experiment. The mean values and standard deviations from 18 wells obtained in 3 independent experiments (6 wells in each) are presented. Asterisks and daggers indicate significant differences compared with wild-type GATA1 and GATA-S, respectively ($P < .01$).

mutation in GATA1-ID type 2, using alternative acceptor sites in exon 3.

To examine whether the GATA1-ID proteins were produced from the mutant alleles, we performed immunoblotting analysis with 2 distinct antibodies recognizing the C terminus and amino acids 66-78, respectively. We detected GATA1-ID type 1 protein in addition to GATA1-S in the cells transfected with the minigenes harboring Ins 2-bp, Del 2-bp, or Del 7-bp T>A mutations, whereas only GATA1-ID type 2 protein was expressed on transfection of the minigene with a Del 21-bp mutation (Figure 1C). Consistent with the minigene results, a significant amount of GATA1-ID type 1 protein and GATA1-S had accumulated in patients 80 and 37, whereas only GATA1-S was detected in the TAM blasts of patient 86, who had only a short transcript skipping exon 2 because of a point mutation in the exon 2–intron 2 boundary (Figure 1D). Thus, splicing errors were occurred in GATA1-ID type 1 and type 2 patients, leading to the production of GATA1-ID proteins.

We next examined how GATA1-ID proteins affect the proliferation of embryonic megakaryocytic progenitors. We retrovirally transduced GATA1-S and GATA1-ID mutants into lineage-negative cells derived from megakaryocyte-specific *Gata1*-deficient ($Gata1^{\Delta neo\Delta HS}$) embryos[15] and induced differentiation toward the megakaryocytic lineage. The number of $CD41^+CD61^+$ megakaryocytes was significantly higher in cases transduced with GATA1-ID proteins than with wild-type GATA1, despite almost equivalent expression levels of GATA1 proteins (Figure 2A-C). GATA1-S-transduced cells unexpectedly acquired a hyperproliferative potential compared with mock cells, probably because of an unknown function that resides in the GATA1 *N*-terminal region (Figure 2B-C).

We next analyzed cell proliferation using the DS-AMKL cell line KPAM1, in which GATA1-S was predominantly expressed with a very low level of full-length GATA1 (Figure 2D).[16] On transduction with full-length GATA1 retrovirus, proliferation of KPAM1 cells was markedly reduced. In contrast, GATA1-ID type 1 and type 2 moderately restricted the proliferation of KPAM1 cells, but the restriction activity was significantly stronger than that of GATA1-S (Figure 2E-F). These results thus demonstrate that the ID regions indeed contribute to the regulation of AMKL cell proliferation.

Our newly identified GATA1-ID mutants have highlighted a much narrower set of sequences responsible for the pathogenesis of TAM than has previously been suggested by the loss of the *N*-terminal sequence, as in GATA1-S. The missing region identified by the GATA1-ID proteins contains a consensus motif (LxCxE, amino acids 81-85) essential for the interaction with pRb,[17] which is also lost in GATA1-S. Interaction with hypophosphorylated pRb-E2F complex has been reported to be important for GATA1 to support the normal proliferation and differentiation of erythroid progenitors.[17] Consistent with this notion, GATA1-S failed to repress E2F activation, which was followed by activation of mTOR signaling in the GATA1-S fetal megakaryocytes and DS-AMKL cells.[18] Because the protein levels of cyclin D1 and $p27^{Kip}$ are reciprocally regulated by the

mTOR pathway, and thereby cause pRb to be phosphorylated,[19] cell-cycle progression in response to the mTOR pathway may be potentiated by the enfeebled function of LxCxE motif of GATA1-S. Thus, we are one step closer to a molecular understanding of GATA1-related leukemias.

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Authorship

Contribution: T.T., R.K., E.K., H. Kaneko, R.W., and K.T. contributed to the experiments; T.T., M.S., R.S., M.Y., and E.I. contributed to the study design, funding, project conception, and manuscript writing; and H. Kanegane, M.M., M.E., T.M., S.A., and Y.H. contributed to the clinical sample collection and phenotype analyses.

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nature genetics

Exome sequencing identifies secondary mutations of *SETBP1* and *JAK3* in juvenile myelomonocytic leukemia

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Juvenile myelomonocytic leukemia (JMML) is an intractable pediatric leukemia with poor prognosis[1] whose molecular pathogenesis is poorly understood, except for somatic or germline mutations of RAS pathway genes, including *PTPN11*, *NF1*, *NRAS*, *KRAS* and *CBL*, in the majority of cases[2–4]. To obtain a complete registry of gene mutations in JMML, whole-exome sequencing was performed for paired tumor-normal DNA from 13 individuals with JMML (cases), which was followed by deep sequencing of 8 target genes in 92 tumor samples. JMML was characterized by a paucity of gene mutations (0.85 non-silent mutations per sample) with somatic or germline RAS pathway involvement in 82 cases (89%). The *SETBP1* and *JAK3* genes were among common targets for secondary mutations. Mutations in the latter were often subclonal and may be involved in the progression rather than the initiation of leukemia, and these mutations associated with poor clinical outcome. Our findings provide new insights into the pathogenesis and progression of JMML.

JMML is a rare myelodysplastic/myeloproliferative neoplasm unique to childhood, characterized by excessive proliferation of myelomonocytic cells and hypersensitivity to granulocyte-macrophage colony-stimulating factor[1]. A cardinal genetic feature of JMML is frequent somatic and/or germline mutation of RAS pathway genes, such as *NF1*, *NRAS*, *KRAS*, *PTPN11* and *CBL*, which are mutated in more than 70% of JMML cases in a mutually exclusive manner[2–4]. However, it is still open to question whether RAS pathway mutations are sufficient for the development of JMML or if secondary mutations have a role in the development and progression of this cancer. To address these issues and to better define the molecular pathogenesis of JMML, we performed whole-exome sequencing of paired tumor-normal DNA from 13 cases (**Supplementary Table 1**). We obtained mean coverage in exome sequencing of 137× for tumor samples and 143× for normal samples (**Supplementary Fig. 1**). A Monte-Carlo simulation indicated that the study detected 88% of the existing somatic mutations (Online Methods and **Supplementary Fig. 2**).

Sanger sequencing of 25 candidate non-silent somatic nucleotide alterations confirmed 1 nonsense and 10 missense mutations (**Table 1** and **Supplementary Fig. 3**), with the low true positive rate consistent with the very low numbers of somatic mutations in JMML. Of the 11 somatic mutations, 6 involved known RAS pathway genes. In addition, non-overlapping RAS pathway mutations (6 somatic and 6 germline) were confirmed in 11 of the 13 discovery cases (86%; **Table 1**). For the remaining two cases that lacked documented RAS pathway mutations, we intensively searched for possible germline mutations that could be relevant to the development of JMML. In total, 179 and 167 candidate germline mutations were detected in subjects 77 and 92, respectively, but these mutations did not affect known RAS pathway genes or other cancer-related genes, including the ones registered in the pathway databases (Online Methods). A frameshift deletion in *KMT2D* (also known as *MLL2*; encoding p.Val1670fs) was found in subject 92, who had been diagnosed as having Noonan syndrome on the basis of typical features such as hypertelorism, webbed neck and congenital heart disease (**Supplementary Fig. 3**) but lacked the distinctive facial appearance of Kabuki syndrome, which was shown to be caused by germline *KMT2D* mutations[5].

Five of the 11 somatic mutations were non–RAS pathway mutations, involving *SETBP1* (3 p.Asp868Asn alterations), *JAK3* (1 p.Arg657Gln alteration) and *SH3BP1* (1 p.Ser277Leu alteration), which had not been reported in JMML cases. SETBP1 was originally isolated as a 170-kDa nuclear protein that interacts with SET, a small protein inhibitor of the putative tumor suppressors PP2A and NM23-H1 (ref. 6). Several lines of recent evidence suggest that SETBP1 has a role in leukemogenesis (**Supplementary Fig. 4**)[7–11]. *SETBP1* participates in

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Table 1 List of gene mutations identified by whole-exome sequencing

	RAS pathway mutations								Other somatic mutations			
	Somatic				Germline							
Subject number	Gene	Change at DNA level	Change at protein level	VAF[a]	Gene	Change at DNA level	Change at protein level	VAF[a]	Gene	Change at DNA level	Change at protein level	VAF[a]
11[b]	*NF1*	c.4537C>T	p.Arg1513*	40.1/24.2	*NF1*	c.5927delG	p.Trp1976fs	44.0/47.1	*SETBP1*	c.2602G>A	p.Asp868Asn	32.6/27.0
63	*KRAS*	c.38G>A	p.Gly13Asp	44.3/0.0	–	–	–	–	–	–	–	–
72	*PTPN11*	c.172A>T	p.Asn58Tyr	48.2/5.7	–	–	–	–	*SETBP1*	c.2602G>A	p.Asp868Asn	45.9/2.5
									JAK3	c.1970G>A	p.Arg657Gln	30.5/2.2
									SH3BP1	c.830C>T	p.Ser277Leu	47.8/5.1
77	–	–	–	–	–	–	–	–	*SETBP1*	c.2602G>A	p.Asp868Asn	33.4/2.1
78	*NRAS*	c.35G>C	p.Gly12Ala	45.5/9.5	–	–	–	–	–	–	–	–
82	–	–	–	–	*CBL*	c.1217del22	p.Thr406fs	34.7/38.9	–	–	–	–
83	–	–	–	–	*NF1*	c.4970A>G	p.Tyr1657Cys	50.0/51.0	–	–	–	–
84	–	–	–	–	*CBL*	c.1096–110del643	p.Glu366_Phe488del	NA/NA	–	–	–	–
85	*PTPN11*	c.226G>A	p.Glu76Lys	47.5/4.4	–	–	–	–	–	–	–	–
86	*KRAS*	c.38G>A	p.Gly13Asp	38.9/3.1	–	–	–	–	–	–	–	–
89[c]	–	–	–	–	*PTPN11*	c.1502T>G	p.Ser502Ala	50.0/49.9	–	–	–	–
91[c]	–	–	–	–	*PTPN11*	c.218C>T	p.Thr73Ile	49.0/48.0	–	–	–	–
92[c]	–	–	–	–	–	–	–	–	–	–	–	–

NA, not available.
[a]Variant allele frequency (VAF) in tumor/reference samples, where the reference was CD3+ T cells, except for subject 63, for whom umbilical cord was used as the reference. [b]Substantial contamination of tumor cell components in the CD3+ T cell reference. [c]Noonan syndrome–associated myeloproliferative disorder.

translocations that result in an aberrant fusion gene (*NUP98-SETBP1*) and overexpression of *SETBP1* in T cell acute lymphoblastic leukemia (T-ALL) and acute myeloid leukemia (AML), respectively[12,13]. *SETBP1* is one of the downstream targets induced by the Evi-1 oncoprotein[14] and, together with *EVI1* and its homolog *PRDM16* (also known as *MEL1*), was reported to be activated through retrovirus integration. SETBP1 is also known to augment the recovery of granulopoiesis after gene therapies for chronic granulomatous disease[15]. *SETBP1* overexpression is found in more than 27% of adult AML cases and is associated with poor survival[13]. The discovery of recurrent hotspot mutations of *SETBP1* provides unequivocal evidence for the leukemogenic role of deregulated SETBP1 function. Notably, the *SETBP1* mutation encoding p.Asp868Asn was identical to one of the *de novo* mutations reported to be causative in Schinzel-Giedion syndrome (SGS; MIM 269150), which is a highly recognizable congenital disease characterized by severe mental retardation, distinctive facial features and

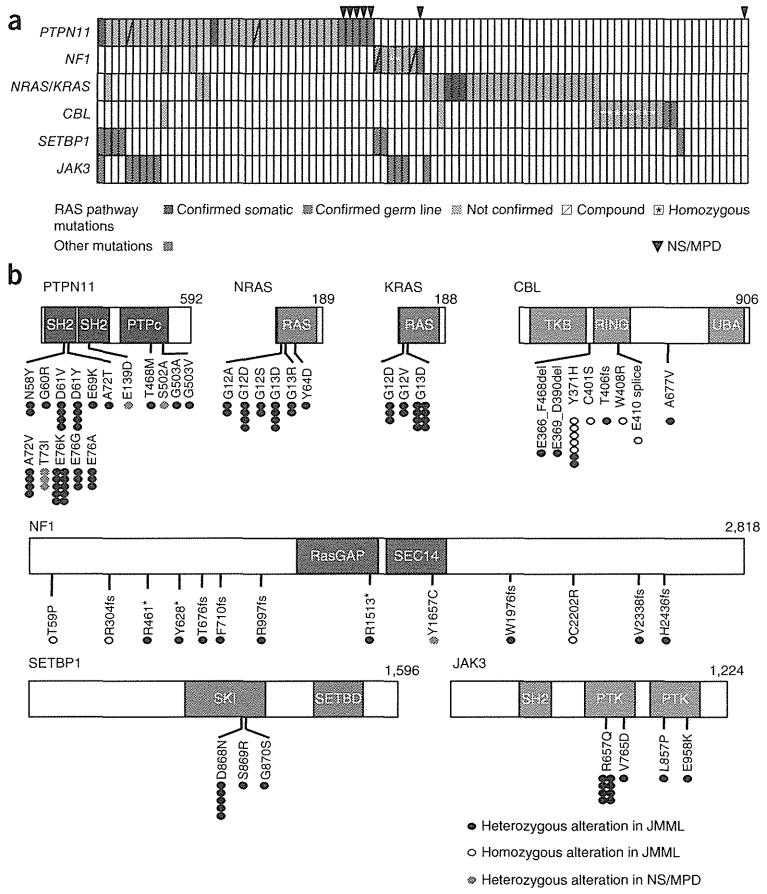

Figure 1 Mutation profiles of 92 JMML cases. (**a**) The mutation status of RAS pathway genes and 2 newly identified gene targets in a cohort of 92 JMML cases is summarized. NS/MPD, Noonan syndrome–associated myeloproliferative disorder. (**b**) The distribution of alterations is shown for each protein. SH2, Src homology 2 domain; PTPc, protein tyrosine phosphatase, catalytic domain; RAS, Ras GTPase family domain; TKB, tyrosine kinase–binding domain; RING, RING-finger domain; UBA, ubiquitin-associated domain; RasGAP, a region of similarity with the catalytic domain of the mammalian p120RasGAP protein in neurofibromin; SEC14, Sec14p-like lipid-binding domain; SKI, v-ski sarcoma viral oncogene homolog domain; SETBD, SET-binding domain; PTK, pseudokinase domain of the protein tyrosine kinases.

Table 2 Subject characteristics

Characteristic	Total cohort (n = 92)	Secondary mutations		P value
		Yes (n = 16)	No (n = 76)	
Sex (male/female)	61/31	12/4	49/27	NS
Median age at diagnosis in months (range)	19 (1–160)	38 (2–160)	13 (1–79)	<0.001
Diagnosis				
JMML	85	16	69	
NS/MPD	7	0	7	
Genetic mutations in RAS pathway				
PTPN11	39	9	30	NS
NF1	9	5	4	0.001
RAS (*NRAS* or *KRAS*)	28 (15/13)	2 (1/1)	26 (14/12)	0.08
CBL	14	0	14	0.06
Without RAS pathway mutation	10	1	9	NS
Secondary genetic mutations				
SETBP1	7	7	0	
JAK3	10	10	0	
Cytogenetics				
Normal karyotype	77	12	65	NS
Monosomy 7	8	1	7	NS
Trisomy 8	4	2	2	NS
Other abnormalities	3	1	2	NS
WBC count at diagnosis $\times 10^9$/l, median (range)	30.0 (1.0–563)	29.6 (5.6–563)	30.0 (1.0–131)	NS
Monocyte count at diagnosis $\times 10^9$/l, median (range)	4.6 (0.2–31.6)	3.1 (0.5–15.2)	4.9 (0.2–31.6)	NS
Percent HbF at diagnosis, median (range)	21 (0–68)	26 (9–55)	16 (0–68)	NS
PLT at diagnosis $\times 10^9$/l, median (range)	61.0 (1.4–483)	47.5 (1.4–175)	65.0 (5.0–483)	NS
HSCT (+/–)	56/36	16/0	40/36	
Alive/deceased	62/30	7/9	55/21	
Percent probability of 5-year overall survival (95% CI)	60 (46–71)	33 (10–59)	65 (49–77)	0.10
Percent probability of 5-year transplantation-free survival (95% CI)	15 (6–27)	0 (0–0)	18 (8–33)	0.007

JMML, juvenile myelomonocytic leukemia; NS/MPD, Noonan syndrome–associated myeloproliferative disorder; WBC, white blood cell; HbF, hemoglobin F; HSCT, hematopoietic stem cell transplantation; NS, not significant. We compared the difference between the subjects with and without secondary mutation, and P values were calculated by two-sided Fisher's exact test or Mann-Whitney U test.

multiple congenital malformations. Individuals with SGS with this mutation have a higher than normal prevalence of tumors, including of neuroepithelial neoplasia[16], although development of myeloid malignancies has not been reported so far.

To further validate our findings, we screened the entire cohort of 92 JMML cases for gene mutations in the newly identified 3 genes together with known RAS pathway targets using deep sequencing[17] (**Supplementary Fig. 5**).

RAS pathway mutations were found in 82 of 92 cases (89%) in a mutually exclusive manner, with *PTPN11* mutations predominant, followed by *NRAS*, *KRAS*, *CBL* and *NF1* mutations (**Fig. 1a** and **Table 2**). In accordance with previous reports, most of the *CBL* (8/14) and *NF1* (4/9) mutations were biallelic (**Fig. 1a,b** and **Supplementary Table 2**)[2,3,18], whereas the majority of mutations in *PTPN11*, *NRAS* and *KRAS* were heterozygous[4]. The individuals without RAS pathway mutations (n = 10) were vigorously investigated by whole-genome sequencing of tumor-normal paired samples (n = 2; **Supplementary Fig. 6**) or by whole-exome sequencing of only tumor samples (n = 8; **Supplementary Fig. 7**). As anticipated, we found no known RAS pathway mutations.

On the other hand, 18 mutations were found in *SETBP1* (n = 7) or *JAK3* (n = 11) in 16 cases (**Fig. 1a,b**, **Table 2** and **Supplementary Table 2**), with these mutations more frequent in cases with mutated *PTPN11* (and possibly *NF1*) than in cases with mutated *NRAS*, *KRAS*

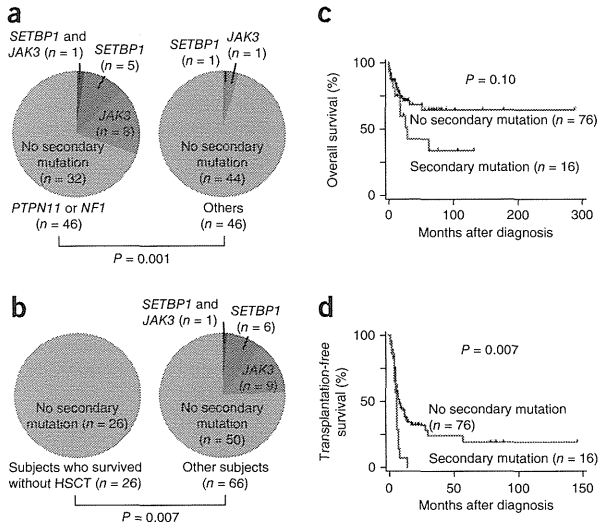

Figure 2 Clinical features of JMML cases with or without secondary mutations. (**a**,**b**) Frequency of secondary mutations in individuals with JMML depending on the type of RAS pathway mutations (left, *PTPN11* or *NF1*; right, other or no mutations) (**a**) and the status of HSCT (**b**). P values were calculated by two-sided Fisher's exact test. (**c**,**d**) The impact of secondary mutations on overall (**c**) and transplantation-free (**d**) survival is shown in Kaplan-Meier survival curves, where statistical significance was tested by log-rank test.

or *CBL* (**Fig. 2a**). Mutations in *SH3BP1*, encoding SH3 domain–binding protein 1, were not recurrent. All *SETBP1* mutations were heterozygous and occurred within the portion of the gene encoding the SKI domain, with six identical to the *de novo* recurrent mutations reported in SGS and five identical to the mutation encoding the p.Asp868Asn alteration (**Fig. 1b**). RT-PCR analysis showed that the wild-type and mutant alleles of *SETBP1* were equally expressed (**Supplementary Fig. 8**). Similarly, 8 of the 11 *JAK3* mutations in 10 cases were the well-described activating mutation (encoding a p.Arg657Gln alteration) found in various hematological malignancies, including Down syndrome–associated acute megakaryoblastic leukemia[19–23], ALL[24,25] and natural killer (NK)/T cell lymphoma[26], and the remaining 3 were also within the portions of the gene encoding the pseudokinase or kinase domain, suggestive of gain of function.

Deep sequencing of the relevant mutant alleles enabled an accurate estimation of allele frequencies for individual mutations (**Supplementary Fig. 9**). *SETBP1* and *JAK3* mutations showed lower allele frequencies (but not with statistical significance for *SETBP1*) than did the corresponding RAS pathway mutations (**Supplementary Fig. 10a**), indicating that the former mutations represent secondary genetic hits that contributed to clonal evolution after the main tumor population was established (**Supplementary Fig. 10b**). Individuals with secondary mutations had shorter lengths of survival compared to those without mutations: 5-year overall survival (hazards ratio (HR) = 1.90, 95% CI = 0.87–4.19). In addition, none of the individuals with JMML who survived without hematopoietic stem cell transplantation (HSCT; $n = 26$) harbored any of the secondary mutations, and individuals with secondary mutations showed significantly inferior 5-year transplant-free survival (HR = 2.18, 95% CI = 1.18–4.02) (**Fig. 2b–d** and **Table 2**).

JMML is characterized by a paucity of gene mutations. The average number of mutations per sample (0.85; range of 0–4) was unexpectedly low compared to those reported in other human cancers (**Supplementary Fig. 11**); excluding common RAS pathway mutations, only 5 mutations were detected in 3 of the 13 discovery cases. This small number of mutations is only comparable to the figure reported for retinoblastoma (mean of 3.3 per case; range of 0–5) (ref. 27) and is in stark contrast to the abundance of gene mutations in chronic myelomonocytic leukemia (CMML) in adult cases, where the mean number of non-silent mutations was 12.4 per sample, of which 3.1 represented known driver changes (ref. 17 and K.Y., M.S., Y.S., D. Nowak, Y. Nagata *et al.*, unpublished data), underscoring the distinct pathogenesis in these two neoplasms that show indistinguishable morphology. The impact of germline events is underscored by the fact that 6 of the 13 discovery cases harbored germline RAS pathway mutations and an additional case without known RAS pathway mutations showed constitutive abnormalities similar to Noonan syndrome. Despite the central role of RAS pathway mutations, a small subset of cases had no documented RAS pathway mutations, even after whole-exome analysis in the two RAS pathway mutation–negative cases, raising the possibility that the latter cases represent a genetically distinct myeloproliferative neoplasm in childhood.

Another key finding in the current study is the discovery of secondary mutations that involve *SETBP1* and *JAK3*. Detected only in a subpopulation of leukemic cells, most of these mutations are thought to be involved in the progression rather than the establishment of JMML and were associated with poor clinical outcome. *SETBP1* is a newly identified proto-oncogene, and identical mutations in this gene have recently been reported in 15–25% of adult cases with atypical chronic myeloid leukemia (CML)[10], CMML and secondary AML[28]. Affecting one of three highly conserved amino acid positions, *SETBP1* mutations have been shown to abolish the binding of an E3 ubiquitin ligase (β-TrCP1) to SETBP1, which prevents ubiquitination and subsequent degradation, leading to gain of function through the consequent increase in SETBP1 protein amounts[10,28]. Although the precise leukemogenic mechanisms of *SETBP1* mutations are still unclear, we have shown that mutant *SETBP1* alleles confer self-renewal capability to myeloid progenitors *in vitro*, and *SETBP1* mutations in adult leukemia were associated with increases in *HOXA9* and *HOXA10* expression[28]. Recurrent *JAK3* mutations in JMML are also noteworthy. The JAK-STAT pathway is a key component of normal hematopoiesis[29]. As in other hematopoietic malignancies[20], the p.Arg657Gln alteration represents the most frequent change in JMML. This alteration confers interleukin (IL)-3 independence to Ba/F3 cells and induces STAT5 phosphorylation[20]. Targeting the JAK-STAT pathway with a pan-JAK inhibitor such as CP-690550 (ref. 30) could be a promising therapeutic possibility for patients with *JAK3*-mutated JMML.

In conclusion, our whole-exome sequencing analysis identified the spectrum of gene mutations in JMML. Together with the high frequency of RAS pathway mutations, the paucity of non–RAS pathway mutations is a prominent feature of JMML. Mutations of *SETBP1* and *JAK3* were common recurrent secondary events presumed to be involved in tumor progression and were associated with poor clinical outcomes. Our findings provide an important clue to understanding the pathogenesis of JMML that may help in the development of novel diagnostics and therapeutics for this leukemia.

URLs. Genomon, http://genomon.hgc.jp/exome/en/; BioCarta, http://www.biocarta.com/; dbSNP131, http://www.ncbi.nlm.nih.gov/projects/SNP/; RefSeq database, http://www.ncbi.nlm.nih.gov/RefSeq/.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession code. We deposited whole-genome and whole-exome sequence data in the European Genome-phenome Archive under accession EGAS00001000521.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

H.S., Y.O., H. Muramatsu, K.Y., M.T., A.K. and M.S. designed and performed the research, analyzed the data and wrote the manuscript. Y.S., K.C., H.T. and S.M. performed bioinformatics analyses of the resequencing data. X.W. and Y.X. performed Sanger sequencing. S.D., A.H., K.N., Y.T. and N.Y. collected specimens and performed the research. H. Makishima and J.P.M. designed the research and analyzed the data. S.O. and S.K. led the entire project and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Subjects. We studied 92 children (61 boys and 31 girls) with JMML, including 7 individuals with NS/MPD, who were diagnosed as having JMML in institutions throughout Japan. Written informed consent was obtained from subjects' parents before sample collection. This study was approved by the ethics committees of the Nagoya University Graduate School of Medicine and the University of Tokyo in accordance with the Declaration of Helsinki. Diagnosis with JMML was made on the basis of internationally accepted criteria[1]. Characteristics of the 92 JMML cases are summarized in **Table 2**. The median age at diagnosis was 16 months (range of 1–160 months). Karyotypic abnormalities were detected in 16 subjects, including in 8 with monosomy 7. Fifty-six of the 92 subjects (61%) received allogeneic HSCT.

Sample preparation. Genomic DNA was extracted using the QIAamp DNA Blood Mini kit and the QIAamp DNA Investigator kit (Qiagen) according to the manufacturer's instructions. The T Cell Activation/Expansion kit, human (Miltenyi Biotec) was used for the expansion of $CD3^+$ T cells from subjects' peripheral blood or bone marrow mononuclear cells[3].

Whole-exome sequencing. Exome capture from paired tumor-reference DNA was performed using SureSelect Human All Exon V3 (Agilent Technologies), covering 50 Mb of coding exons, according to the manufacturer's protocol. Enriched exome fragments were subjected to massively parallel sequencing using the HiSeq 2000 platform (Illumina). Candidate somatic mutations were detected through our in-house pipeline (Genomon) as previously described[17].

Detection of mutations from whole-exome sequencing data. Detection of candidate somatic mutations was performed according to previously described algorithms with minor modifications[17]. Briefly, the number of reads containing single-nucleotide variations (SNVs) and indels in both tumor and reference samples was determined using SAMtools[31], and the null hypothesis of equal allele frequencies in tumor and reference samples was tested using the two-tailed Fisher's exact test. A variant was adopted as a candidate somatic mutation if it had $P < 0.01$, if it was observed in bidirectional reads (in both plus and minus strands of the reference sequence) and if its allele frequency was less than 0.25 in the corresponding reference sample. For the detection of germline mutations in RAS pathway genes, SNVs and indels having allele frequencies of more than 0.25 (SNVs) and 0.10 (indels) were interrogated for 46 genes, which consisted of known JMML-related RAS pathway genes and genes registered in the pathway databases ('Ras signaling pathway' in BioCarta and 'signaling to RAS' in Reactome[32]). For variant calls in tumor samples for which the paired normal reference was not available, candidate variants in the RAS pathway were detected at an allele frequency of >0.10. Finally, the list of candidate somatic and/or germline mutations was generated by excluding synonymous SNVs and other variants registered in either dbSNP131 or an in-house SNP database constructed from 180 individual samples. All candidates were validated by Sanger sequencing as previously described.

Estimation of tumor content. The tumor content of bone marrow specimens was estimated from the allele frequency of the somatic mutations identified by deep sequencing. For homozygous mutations, as indicated by an allele frequency of >0.75, the tumor content (F_{tumor}) was calculated from the observed frequency ($F_{observed}$) of the mutation according to the following equation: $F_{tumor} = 2 \times F_{observed} - 1$. For heterozygous mutations, the tumor content was calculated by doubling the allele frequency.

Power analysis of whole-exome sequencing. The power of detecting somatic mutations at each nucleotide position in whole-exome sequencing was estimated by Monte-Carlo simulation (n = 1,000) on the basis of the observed mean depth of coverage for each exon in germline and tumor samples and the observed tumor content for each sample, which were estimated using the allele frequencies of the observed mutations. For the samples with no observed somatic mutations, the average tumor content of the informative samples was employed. Simulations were performed across a total of 192,424 exons.

Copy number analysis in whole-exome sequencing data. To detect copy number lesions at a single-exon level, the mean coverage of each exon normalized by the mean depth of coverage of the entire sample was compared with that of 12 unrelated normal DNA samples. Exons showing normalized coverage greater than 3 s.d. from the mean coverage of the reference samples were called as candidates for copy number alterations. All candidate exons of RAS pathway genes were visually inspected using the Integrative Genomics Viewer[33] and were validated by Sanger sequencing of corresponding putative breakpoint-containing fragments.

Targeted deep sequencing. Deep sequencing of the targeted genes was performed essentially as described in the 'deep sequencing of pooled target exons' section in ref. 17, except that target DNA was not pooled. Briefly, all exons of *PTPN11*, *NF1*, *KRAS*, *NRAS*, *CBL*, *SETBP1*, *JAK3* and *SH3BP1* were PCR amplified with Quick Taq HS DyeMix (TOYOBO) and the PrimeSTAR GXL DNA Polymerase kit (Takara Bio) using primers including the NotI restriction site (**Supplementary Table 3**). The PCR products from an individual sample were combined and purified with the QIAquick PCR Purification kit (Qiagen) for subsequent digestion with NotI (Fermentas). Digested PCR product was purified, concatenated with T4 DNA ligase (Takara Bio) and sonicated to generate fragments with an average size of 150 bp using Covaris. Fragments were processed for sequencing according to a modified Illumina paired-end library protocol, and sequences were read by a HiSeq 2000 instrument using a 100-bp paired-end read protocol.

Variant calls in targeted deep sequencing. Data processing and variant calling were performed with modifications to the protocol described in a previous publication[17]. Each read was aligned to the set of targeted sequences from PCR amplification, with BLAT[34] instead of Burrows-Wheeler Aligner (BWA)[35] used with the -fine option. Mapping information in the .psl format was converted to the .sam format with paired-read information. Of the successfully mapped reads, reads were excluded from further analysis if they mapped to multiple sites, mapped with more than four mismatched bases or had more than ten soft-clipped bases. Next, the Estimation_CRME script was run to eliminate strand-specific errors and exclude PCR-derived errors. A strand-specific mismatch ratio was calculated for each nucleotide variant for both strands using the bases from read cycles 11 to 50 on the next-generation sequencer. By excluding the top five cycles showing the highest mismatch rates, strand-specific mismatch rates were recalculated, and the smaller value between both strands was adopted as a nominal mismatch ratio for that variant. After excluding variants found in dbSNP131 or the in-house SNP database, non-silent variants having a mismatch ratio of greater than 0.05 were called as candidates, unless they had median values of the mismatch ratio at the relevant nucleotide positions in the 92 samples of greater than 0.01, as such variants were likely to be caused by systematic PCR problems. Finally, candidates with mismatch ratios of >0.15 were further validated by Sanger sequencing.

Annotation of the detected mutations. Detected mutations were annotated using ANNOVAR[36]. The positions of the mutations were based on the following RefSeq transcript sequences: NM_002834.3 for *PTPN11*, NM_000267.3 for *NF1*, NM_002524.4 for *NRAS*, NM_004985.3 for *KRAS*, NM_005188.3 for *CBL*, NM_015559.2 for *SETBP1* and NM_000215.3 for *JAK3*. The effect of the mutations on protein function was assessed by SIFT[37], PolyPhen-2 (ref. 38) and MutationTaster[39].

Whole-genome sequencing. Paired tumor-reference DNA samples were sequenced with the HiSeq 2000 platform according to the manufacturer's instructions to obtain 30× read coverage for reference samples and 40× coverage for tumor samples. Obtained FASTQ sequences were aligned to the human reference genome (hg19) using BWA[35] 0.5.8 with default parameters. Alignment of pairs of sequences, at least one of which was not mapped or was considered to have possible mapping problems (with mapping quality of less than 40, insertions or deletions, soft-clipped sequence of more than 10% of the length of the original sequence, irregular paired-read orientation or mate distance of greater than 2,000 bp), was attempted with BLAT[34] using default parameters, except for stepSize = 5 and repMatch = 2,253. Mapping statistics were calculated by counting the bases at each genomic position with SAMtools[31]. For variant calling, variant and reference bases with base quality of >30 were counted in both germline and tumor samples, and the Fisher's

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exact test was applied. Variants with P of <0.01 were called. Variants having allele frequency of >0.25 in the germline sample were excluded. Variants found in 12 unrelated germline samples with an allele frequency of >0.01 on average were also excluded owing to the high probability that they represented false positive calls. Copy number estimation was performed by calculating the averaged ratio of read depths in germline and tumor samples in 10,000-base bins. An allele-specific copy number plot was generated by measuring the allele frequency of the tumor sample at the positions in which more than 25% of the allele mismatch was observed in germline samples. For the detection of chromosomal structural variations, soft-clipped sequences that could be mapped to a unique genomic position were selected. Structural variation candidates that had more than four supporting read pairs in total and at least one read pair from each side of the breakpoint were called. Contig sequences were generated by assembling the reads within 200 bp of the breakpoint with CAP3 (ref. 40), and structural variations having the contig sequence that could be aligned to the alternate assembly of the hg19 genome with more than 93% identity were excluded as false positives. Structural variations with read depth of greater than 150 on at least one side of the breakpoint were considered to be mapped to a repeat element and were also excluded. For detection of viruses, unmapped sequences were aligned to the collection of all viral genomes in the RefSeq database using BLAT. A virus was considered to be detected if its genome was covered by mean read coverage of >1.

cDNA sequencing. Total RNA was extracted using the RNeasy Mini kit (Qiagen) and was reverse transcribed with the ThermoScript RT-PCR system (Life Technologies). Target sequences were PCR amplified with the PrimeSTAR GXL DNA Polymerase kit using the primers listed in **Supplementary Table 3** and were sequenced.

Statistical analysis. For comparison of the frequency of mutations or other clinical features between disease groups, categorical variables were analyzed using the Fisher's exact test, and continuous variables were tested using the Mann-Whitney U test. Overall survival and transplantation-free survival were estimated by the Kaplan-Meier method. Hazard ratios for survival with 95% CIs were estimated according to the Cox proportional hazards model, and difference in survival was tested by log-rank test. STATA version 12.0 (StataCorp) was used for all statistical calculations.

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To the editor:

Rabbit antithymocyte globulin and cyclosporine as first-line therapy for children with acquired aplastic anemia

Horse antithymocyte globulin (hATG) and cyclosporine have been used as standard therapy for children with acquired aplastic anemia (AA) for whom an HLA-matched family donor is unavailable. However, in 2009, hATG (lymphoglobulin; Genzyme) was withdrawn and replaced by rabbit ATG (rATG; thymoglobulin; Genzyme) in Japan. Many other countries in Europe and Asia are facing the same situation.[1] Marsh et al recently reported outcomes for 35 adult patients with AA who were treated with rATG and cyclosporine as a first-line therapy.[2] Although the hematologic response rate was 40% at 6 months, several patients subsequently achieved late responses. The best response rate was 60% compared with 67% in a matched-pair control group of 105 patients treated with hATG. The overall and transplantation-free survival rates appeared to be significantly inferior with rATG compared with hATG at 68% versus 86% ($P = .009$) and 52% versus 76% ($P = .002$), respectively. These results are comparable to those from a prospective randomized study reported by Scheinberg et al comparing hATG and rATG.[3] Both studies showed the superiority of hATG over rATG.[2,3]

We recently analyzed outcomes for 40 Japanese children (median age, 9 years; range, 1-15) with AA treated using rATG and cyclosporine. The median interval from diagnosis to treatment was 22 days (range, 1-203). The numbers of patients with very severe, severe, and nonsevere disease were 14, 10, and 16, respectively. The ATG dose was 3.5 mg/kg/day for 5 days. The median follow-up time for all patients was 22 months (range, 6-38). At 3 months, no patients had achieved a complete response (CR) and partial response (PR) was seen in only 8 patients (20.0%). At 6 months, the numbers of patients with CR and PR were 2 (5.0%) and 17 (42.5%), respectively. After 6 months, 5 patients with PR at 6 months had achieved CR and 4 patients with no response at 6 months had achieved PR, offering a total best response rate of 57.5%. Two patients relapsed at 16 and 19 months without receiving any second-line treatments. Two patients with no response received a second course of rATG at 13 and 17 months, but neither responded. Sixteen patients underwent hematopoietic stem cell transplantation (HSCT) from alternative donors (HLA-matched unrelated donors, n = 13; HLA-mismatched family donors, n = 3). Two deaths occurred after rATG therapy, but no patients died after HSCT. Causes of death were intracranial hemorrhage at 6 months and acute respiratory distress syndrome at 17 months. The overall 2-year survival rate was 93.8% and the 2-year transplantation-free survival rate was 50.3% (Figure 1).

In our previous prospective studies with hATG, the response rates after 6 months were 68% and 70%, respectively, with no increases in response rates observed after 6 months.[4,5] Our results support the notion that rATG is inferior to hATG for the treatment of AA in children. First-line HSCT from an alternative donor may be justified, considering the excellent outcomes in children who received salvage therapies using alternative donor HSCT.

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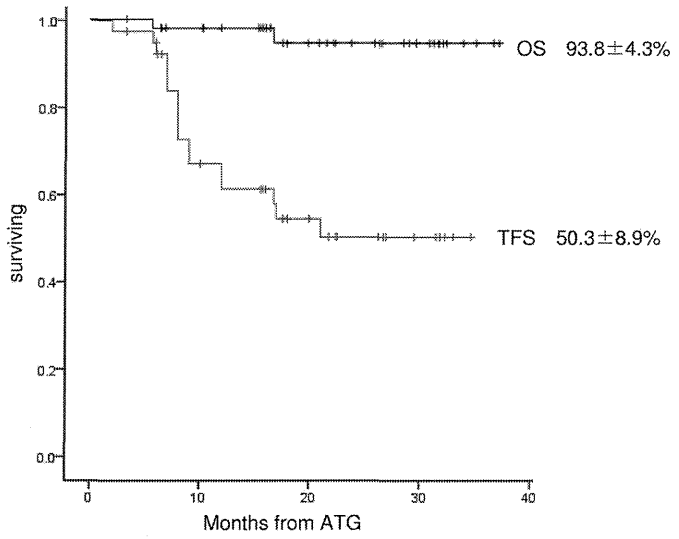

Figure 1. Kaplan-Meier estimates of overall survival (OS) and transplantation-free survival (TFS) in 40 Japanese children with AA. Survival was investigated using Kaplan-Meier methods. OS for all patients with AA after rATG and cyclosporine as first-line therapy included patients who later received HSCT for nonresponse to rATG. In the analysis of TFS for all patients treated with rATG and CSA, transplantation was considered an event.

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To the editor:

Peripheral blood stem cells versus bone marrow in pediatric unrelated donor stem cell transplantation

The relative benefits and risks of peripheral blood stem cells (PBSCs) versus bone marrow (BM) for allogeneic hematopoietic stem cell transplantation (SCT) are still a matter of highly controversial debates.[1-3] The first randomized study comparing the 2 stem cell sources in unrelated donor SCT recently documented comparable overall and event-free survival, but indicated a higher risk for chronic graft-versus-host disease (GVHD) with PBSCs.[4] Only a few pediatric patients were included in this study even though the long-term sequelae of chronic GVHD are of particular concern in this patient group.

We retrospectively compared the long-term outcome of contemporaneous unrelated donor SCT in 220 children transplanted with BM (n = 102) or PBSCs (n = 118) for hematologic malignancies and reported to the German/Austrian pediatric registry for SCT. All patients had received myeloablative conditioning followed by unmanipulated SCT from HLA-matched unrelated donors. The PBSC and BM groups were comparable with regard to patient and donor age, sex, cytomegalovirus (CMV) serostatus, disease status at transplantation, GVHD prophylaxis, growth factor use, and degree of HLA matching. The groups differed with regard to disease category with slightly more myelodysplastic syndrome patients ($P = .02$) and a higher CD34-cell dose ($P = .001$) in the PBSC group.

Neutrophil and platelet engraftment were achieved significantly faster after PBSC than BM transplantation (Figure 1A-B). In this entirely pediatric cohort, the incidence of clinically relevant grade II-IV acute GVHD (Figure 1C) did not differ. Most importantly, the incidence of chronic GVHD (PBSCs vs BM: 35% vs 33%, respectively; $P = .9$) and extensive chronic GVHD (Figure 1D) proved low and was virtually identical in the 2 groups. With a median follow-up time of 3 years, overall survival (PBSCs vs BM: 50% ± 5% vs 46% ± 6%, respectively; $P = .63$) and event-free survival (PBSCs vs BM: 45% ± 5% vs 44% ± 6%, respectively; $P = .59$) were comparable (Figure 1E-F). In multivariable analysis, taking into account all parameters with $P < .2$ in univariate analysis, the only significant independent risk factor for treatment failure was advanced disease status at the time of transplantation (relative risk = 2.4, 95% confidence interval, 1.5-3.8; $P = .001$). In contrast, stem cell source (PBSCs vs BM) had no effect (relative risk = 1.1, 95% confidence interval, 0.7-1.6; $P = .8$).

Our registry-based analysis provides evidence that in pediatric recipients of HLA-matched unrelated-donor transplantation with consistent antithymocyte globulin (ATG) use during conditioning, transplantation with PBSCs and BM results in comparable clinical outcomes without detectable differences in the risk of acute or, more importantly, chronic GVHD. Consistent with a recent study underscoring the role of ATG for the prevention of acute and chronic GVHD,[5] the use of ATG in 96% of our transplantation procedures compared with only 27% in the above-mentioned randomized study by Anasetti et al[4] might be one of the key factors responsible for the overall low and comparable incidence of

Management of adult and paediatric acute lymphoblastic leukaemia in Asia: resource-stratified guidelines from the Asian Oncology Summit 2013

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Survival for adults and children with acute lymphoblastic leukaemia has risen substantially in recent years because use of improved risk-directed treatments and supportive care has widened. In nearly all developed countries, multidisciplinary panels of leukaemia experts have formulated clinical practice guidelines in which standard treatment approaches are recommended on the basis of current evidence. However, those guidelines do not take into account resource limitations in low-income countries, including financial and technical challenges. In Asia, huge disparities in economy and infrastructure exist between countries, and even among different regions in some large countries. At a consensus session held as part of the 2013 Asian Oncology Summit in Bangkok, Thailand, a panel of experts summarised recommendations for management of adult and paediatric acute lymphoblastic leukaemia. Strategies were developed for Asian countries on the basis of available financial, skill, and logistical resources and were stratified in a four-tier system according to the resources available in a particular country or region (basic, limited, enhanced, and maximum).

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Introduction

Acute lymphoblastic leukaemia is the most common malignant disease diagnosed in patients younger than 15 years, accounting for 26% of all cancers and 78% of leukaemias in this age-group; it also represents about 20% of adult acute leukaemias.[1] The incidence of childhood acute lymphoblastic leukaemia varies substantially across geographic regions and by race and ethnic origin, partly because of familial genetic variations.[2] Environmental factors might also have a role; for example, the reduced incidence of acute lymphoblastic leukaemia in low-income countries such as Indonesia has been attributed to early exposure to infection due to social mixing of children at a young age.[3] Although a wealth of biological and epidemiological data are available for acute lymphoblastic leukaemia in North America and Europe, comparable information is not available for most countries in Asia, which do not have hospital-based or population-based registries and adequate diagnostic methods. Based on the population estimate for each of the 51 countries by the United Nations Population Division (UNPD), and assuming an age-adjusted incidence of 1·25 per 100 000 individuals per year (extrapolated from data published by the US National Cancer Institute's Surveillance, Epidemiology and End Results [SEER] programme for the population from Asia and the Pacific Islands),[1] we estimate there are at least 54 000 new cases of acute lymphoblastic leukaemia in Asia every year.

Successful management of childhood acute lymphoblastic leukaemia is one of the greatest medical achievements of the modern era, with the proportion of patients surviving for 5 years approaching and even exceeding 90% in most countries in North America and western Europe.[4] Moreover, adaptation of paediatric treatment regimens for use in adult patients has improved 5-year survival to about 50% in some clinical trials.[5] Published data in Asia are mostly available for paediatric disease, mainly from countries with high levels of resources (table 1).[6–15]

Adoption of a step-up approach to treatment of acute lymphoblastic leukaemia is desirable as Asian countries begin to grow economically. In this Review, we summarise consensus recommendations for assessment and management of acute lymphoblastic leukaemia in Asian countries, according to available resources and economic status. Because of the scarcity of published data from Asia, management guidelines for countries with few resources are based mainly on experience of using low-intensity treatment (with some modifications) in the USA and European countries during the early treatment era when supportive care was suboptimum. Strategies to overcome barriers to implementation of effective management in low-income or middle-income countries—eg, education and training, psychosocial and financial support, and establishment of strategically located oncology units or regional hospital networks—have been discussed elsewhere[16,17] and are beyond the scope of this Review.

Resource-stratified consensus recommendations

With the increasing number of diagnostic and therapeutic options available to us, and the diverse economies and infrastructures in Asian countries, clinical management guidelines for acute lymphoblastic leukaemia should not only aim to improve the proportion of patients cured and quality of life but also be sensitive to resources and cost. We have developed management guidelines and recommendations on the basis of resource availability, using the four-tier system proposed by the Breast Health Global Initiative—basic, limited, enhanced, and maximum—as a model.[18] Basic resources should support the adequate function of any health-care system. The limited category incorporates some financial means and a modest infrastructure that can be used to promote

	Year of study	Patients (n)	Age range (years)	Median WBC ($x10^9$/L)	Proportion (%) with T-cell ALL	Proportion (%) with *BCR-ABL1*-positive ALL	Event-free survival (%)	Overall survival (%)
China								
BCH-2003/CCLG-2008 (Gao et al)[6]	2003–10	1004	0–16	N/A	10·2	6·5	82·6 (SD 1·5) at 5 years for BCH-2003 82·9 (SD 2·4) at 3 years for CCLG-2008	..
TPOG-2002 (Liang et al)[7]	2002–07	788	1–18	N/A	9·7	4·4	77·4 (SD 1·7) at 5 years	83·5 (SD 1·6) at 5 years
HK 93/97 (Li et al)[8]	1997–2002	171	1–17	12·6	14	3·5	79·0 at 4 years	86·5 at 4 years
India								
Modified BFM 76/79 (Bajel et al)[9]	1985–2003	307	1–14	10	22	5·7	56 (SD 3·2) at 5 years*	59·8 (SD 2·3) at 5 years*
MCP-841 (Arya et al)[10]	1992–2002	254	1–15	N/A	31	..	51·6 (SD 3·8)	69·1 (SD 4·1)
Japan								
TCCSG L95-14 (Tsuchida et al)[11]	1995–99	597	1–15	About 10	9·7	4·0	76·8 (SD 1·8) at 5 years	84·9 (SD 1·5) at 5 years
JCCLSG ALL 2000 (Yamaji et al)[12]	2000–04	305	1–15	N/A	9·8	0	79·7 (SD 2·4) at 5 years†	89·2 (SD 1·8) at 5 years†
KYCCSG ALL-96 (Nagatoshi et al)[13]	1996–2002	201	1–15	7·3	10·4	4·9	72·1 at 7 years‡	84·8 at 7 years‡
Singapore								
Ma-Spore ALL 2003 (Yeoh et al)[14]	2002–11	556	0–18	N/A	8·8	4·0	80·6 (SD 3·5) at 6 years	88·4 (SD 3·1) at 6 years
Korea								
B-ALL (Koh et al)[15]	2004–08	98	N/A	N/A	0	N/A	..	88·8 (SD 5·3) at 3 years

ALL=acute lymphoblastic leukaemia. N/A=not available. WBC=white-blood-cell count. *30 patients were censored because they stopped treatment or were lost to follow-up. †Excluded patients with t(9;22). ‡Excluded patients with t(9;22) or t(4;11).

Table 1: **Patients' characteristics and treatment results from selected clinical trials enrolling children with ALL in Asia**

improvements in outcome. Enhanced resources not only augment outcomes but also provide therapeutic options for the patient. Although the maximum level can support all services required by contemporary management strategies for acute lymphoblastic leukaemia, some costly or less practical services might be assigned low priority.

Findings of a global study[16] showed that projected 5-year survival rates for paediatric cancer in low-income or middle-income countries were directly proportional to several health indicators, including gross domestic product per person, gross national income per person, the number of doctors and nurses, and, most importantly, annual health-care expenditure. Table 2 presents data related to child health in selected Asian countries.[19] We assigned countries to resource categories using annual total health-care expenditure per person as a guide. However, within some large Asian countries, huge disparities in economic status exist between regions. Indeed, one region might only have basic access to oncology care whereas in another the facilities and staff could be world class, enabling maximum care to be provided. Hence, Asian providers responsible for management of patients with acute lymphoblastic leukaemia should adopt the recommendations that best match their available resources.

General management

Initial diagnosis and classification of acute lymphoblastic leukaemia should be done in specialised tertiary centres. In large countries with limited infrastructure, where travel to main centres might be difficult, diagnostic bone-marrow and blood samples can be transported to referral centres for specialised tests. Initial bone-marrow smears should be made on new glass slides and additional samples transported at room temperature within 48 h in sterile EDTA (edetic acid) or heparinised (preservative-free heparin, sodium or lithium heparin) tubes. In countries with basic and limited resources, where public diagnostic resources might be rudimentary, certified private laboratories—if available—could possibly provide the service.

Findings of retrospective studies show that adolescents treated in paediatric centres fare much better than do children of similar ages treated in adult centres.[20] Factors contributing to this observation include intensive use of non-myelosuppressive agents (asparaginase, glucocorticoids, and vincristine), early administration of intrathecal treatment, and good adherence to treatment by patients and clinicians and a better social support network and parental involvement.[20,21] Moreover, many adolescents with acute lymphoblastic leukaemia need specialist paediatric supportive care, including admission to paediatric intensive-care units and dialysis, and some children might have undue psychological stress if they are admitted to an adult ward with mainly elderly patients. Therefore, children younger than 18 years with acute lymphoblastic leukaemia are best managed in paediatric centres instead of adult centres.

Diagnostic tests

Bone-marrow aspiration done under sterile conditions in the posterior iliac region is recommended for diagnosis of acute lymphoblastic leukaemia because

the morphology of leukaemia cells in peripheral blood can differ from that in bone marrow; furthermore, as many as 20% of patients with acute lymphoblastic leukaemia do not have circulating blast cells at diagnosis.[22] Sternal aspiration is contraindicated in young children and is rarely necessary in older adolescents. Bone-marrow smears treated with either May-Grünwald-Giemsa or Wright-Giemsa stain should be examined by light microscopy (panel 1). If flow cytometry is not available, cytochemistry for myeloperoxidase and non-specific esterase should be done to exclude acute myeloid leukaemia. However, cytochemical staining is quite expensive; in countries with basic or limited resources, batched cytochemical staining once a week is acceptable. Indeed, in countries with basic resources, cytochemistry is typically the only means by which diagnosis of acute lymphoblastic leukaemia can be made. T-cell acute lymphoblastic leukaemia is suspected if an anterior superior mediastinal mass is seen on chest radiography at diagnosis.

At centres in which samples are couriered to central laboratories for cytochemical staining or flow cytometry, treatment can be started on the basis of findings in the initial peripheral-blood or bone-marrow smear, particularly if the patient has hyperleucocytosis or a mediastinal mass. In such cases, remission induction treatment should be non-intensive, starting with one or two drugs only (eg, prednisolone with or without vincristine). Although infants with acute lymphoblastic leukaemia usually present with a high leucocyte count, special care must be taken when diagnosing young children with low leucocyte counts, whose immature atypical lymphocytes can mimic lymphoblasts. In countries with basic and limited resources, delaying treatment for infants who present with low leucocyte counts until the bone-marrow examination is completed might be preferable.

	Total population (x1000)	Proportion age 0–14 years (%)	Doctors (n/10 000 population)	Nurses and midwives (n/10 000 population)	Tuberculosis prevalence (cases/100 000 population)	Proportion of underweight children among under-5s (%)	Under-5 mortality (deaths/ 1000 births)	Measles immunisation coverage among under-1s (%)	Annual government expenditure per person (US$)	Annual total health-care expenditure per person (US$)
Basic resources										
Burma	48337	25%	5	8	525	30%	62	88%	4	34
Afghanistan	32358	46%	2	5	352	33%	101	62%	5	44
Bangladesh	150494	31%	3	3	411	41%	46	94%	19	57
Pakistan	176745	35%	8	6	364	31%	72	86%	23	59
Nepal	30486	35%	2	5	238	39%	48	86%	22	66
Timor-Leste	1154	46%	1	22	643	45%	54	66%	47	84
Laos	6288	34%	3	10	130	36%	42	64%	32	97
Limited resources										
Indonesia	242326	27%	3	20	289	20%	32	89%	55	112
Cambodia	14305	31%	2	8	660	29%	43	93%	45	121
India	1241492	30%	6	13	256	44%	61	74%	39	132
Philippines	94852	35%	12	60	502	21%	25	88%	50	142
Sri Lanka	21045	25%	5	19	101	22%	12	99%	66	148
Vietnam	88792	23%	12	10	334	20%	22	98%	81	215
Mongolia	2800	28%	28	35	331	5%	31	97%	120	218
Bhutan	738	29%	<1	3	181	13%	54	95%	239	275
Enhanced resources										
Thailand	69519	20%	3	15	182	7%	12	98%	247	330
China	1347565	19%	14	14	108	3%	15	99%	203	379
Maldives	320	26%	16	45	13	18%	11	97%	281	464
Malaysia	28859	30%	9	27	107	13%	7	96%	356	641
Brunei	406	26%	14	49	91	N/A	7	94%	1230	1449
Maximum resources										
South Korea	48391	16%	20	53	151	N/A	5	98%	1193	2023
Singapore	5188	17%	18	59	44	3%	3	95%	825	2273
New Zealand	4415	20%	27	109	9	N/A	6	91%	2514	3020
Japan	126497	13%	21	41	27	N/A	3	94%	2644	3204
Australia	22606	19%	30	96	8	N/A	5	94%	2340	3441

Data are for 2012 and are taken from the United Nations Economic and Social Commission for Asia and the Pacific.[19] Data were not available for Hong Kong or Taiwan. N/A=not available.

Table 2: **Health-related statistics in selected Asian countries**

Panel 1: Recommendations for diagnostic work-up, according to resource availability

Basic resources

Children and adults

- Morphology with or without cytochemistry
- Chest radiography to detect mediastinal mass

Limited resources

Children

- Morphology and cytochemistry
- Immunophenotyping (restricted)
- DNA index
- RT-PCR of *BCR-ABL1*, *MLL-AFF1*, and *ETV6-RUNX1*

Adults

- Morphology and cytochemistry
- Immunophenotyping (restricted to exclude acute myeloid leukaemia and mixed-lineage acute leukaemia)
- RT-PCR of *BCR-ABL1*
- Cytogenetics for Philadelphia chromosome or fluorescence in-situ hybridisation of *BCR-ABL1*

Enhanced resources

Children

- Morphology
- Immunophenotyping
- DNA index
- RT-PCR of *BCR-ABL1*, *MLL-AFF1*, *ETV6-RUNX1*, and *TCF3-PBX1*
- Cytogenetics for hyperdiploidy >50 or hypodiploidy <44
- Fluorescence in-situ hybridisation of chromosomes 4, 10, and 17, and *BCR-ABL1*

Adults

- Morphology
- Immunophenotyping
- RT-PCR of *BCR-ABL1* and *MLL-AFF1*
- Cytogenetics for hyperdiploidy >50
- Fluorescence in-situ hybridisation of *BCR-ABL1*
- HLA typing

Maximum resources

Children and adults

As for enhanced, plus

- Genome-wide analysis
- Pharmacogenetics

Flow cytometry

Establishing the immunophenotype of leukaemia cells is necessary for accurate diagnosis and to initiate risk-directed treatment.[23] Without this knowledge, acute lymphoblastic leukaemia can be misdiagnosed as acute myeloid leukaemia, or vice versa, which happens in about 10% of cases;[24] moreover, in some situations, B-cell precursors (haematogones) that are regenerating normal bone marrow after a severe infection could be misdiagnosed as leukaemia. Immunophenotyping by flow cytometry based on two or three fluorochromes is widely available. For countries with limited resources, we recommend a minimum panel of markers for acute lymphoblastic leukaemia that includes: CD19 plus CD22 or cytoplasmic CD79a for B-cell acute lymphoblastic leukaemia; CD7 and cytoplasmic CD3 for T-cell acute lymphoblastic leukaemia; and myeloperoxidase to exclude acute myeloid leukaemia.[23] These analyses should be done by skilled laboratory workers. If trained technicians are not available, the flow cytometry plots can be reviewed remotely by experts via a web-based system.[24,25] In addition to CD19 and CD22, CD20 could also be measured in countries with enhanced or maximum resources, because monoclonal and bispecific antibodies against these antigens are available for high-risk or refractory cases.[5]

Cytogenetic and molecular genetic analyses

Molecular genetic abnormalities and the cytogenetic features of leukaemia cells are highly prognostic for treatment outcome. Such analyses permit precise risk assignment to avoid overtreatment or undertreatment of individual patients.[26] Detection of genetic alterations with common oncogene fusion transcripts can be done by cytogenetic techniques (eg, karyotyping and fluorescence in-situ hybridisation) and by molecular analyses (eg, reverse transcription-PCR [RT-PCR]), which are recommended for countries with limited resources or better.

When resources are limited, molecular screening for oncogene fusion transcripts is preferable to standard karyotyping. Tertiary centres can partner with academic institutions to screen for common oncogene fusion transcripts of prognostic importance (panel 1). Care must be taken in such studies to avoid cross-contamination by using replicates, positive and negative controls, and housekeeping genes (eg, *GUSB* or *ABL1*), to ensure that no mRNA is degraded. By contrast, lymphoblasts grow and band poorly in cytogenetic cultures, and every karyotype must be analysed manually by trained technicians, making delivery of timely cytogenetic results difficult in busy, poorly staffed centres. Large cooperative groups—such as the Italian and US Children's Oncology Groups—depend on a combination of molecular oncogene fusion screening, flow cytometric determination of DNA content, and fluorescence in-situ hybridisation for genetic subgrouping, in lieu of, or in addition to, cytogenetics.[27,28] Indeed, molecular and cytogenetic analyses have been done successfully in some centres in China with an enhanced level of resources.[6,29,30] Compared with US and European cohorts, study findings in Chinese paediatric patients with acute lymphoblastic leukaemia suggest that the frequency of t(9;22)(q34;q11) with the *BCR-ABL1* fusion and expression of *TLX1* (formerly *HOX11*) in T-cell leukaemia are increased, and the frequency of t(12;21)(p13;q22) with the *ETV6-RUNX1* fusion gene and hyperdiploidy greater than 50 are decreased.[29,31]

With the advent of genome-wide analysis and falling costs, large-scale sequencing studies of whole cancer and