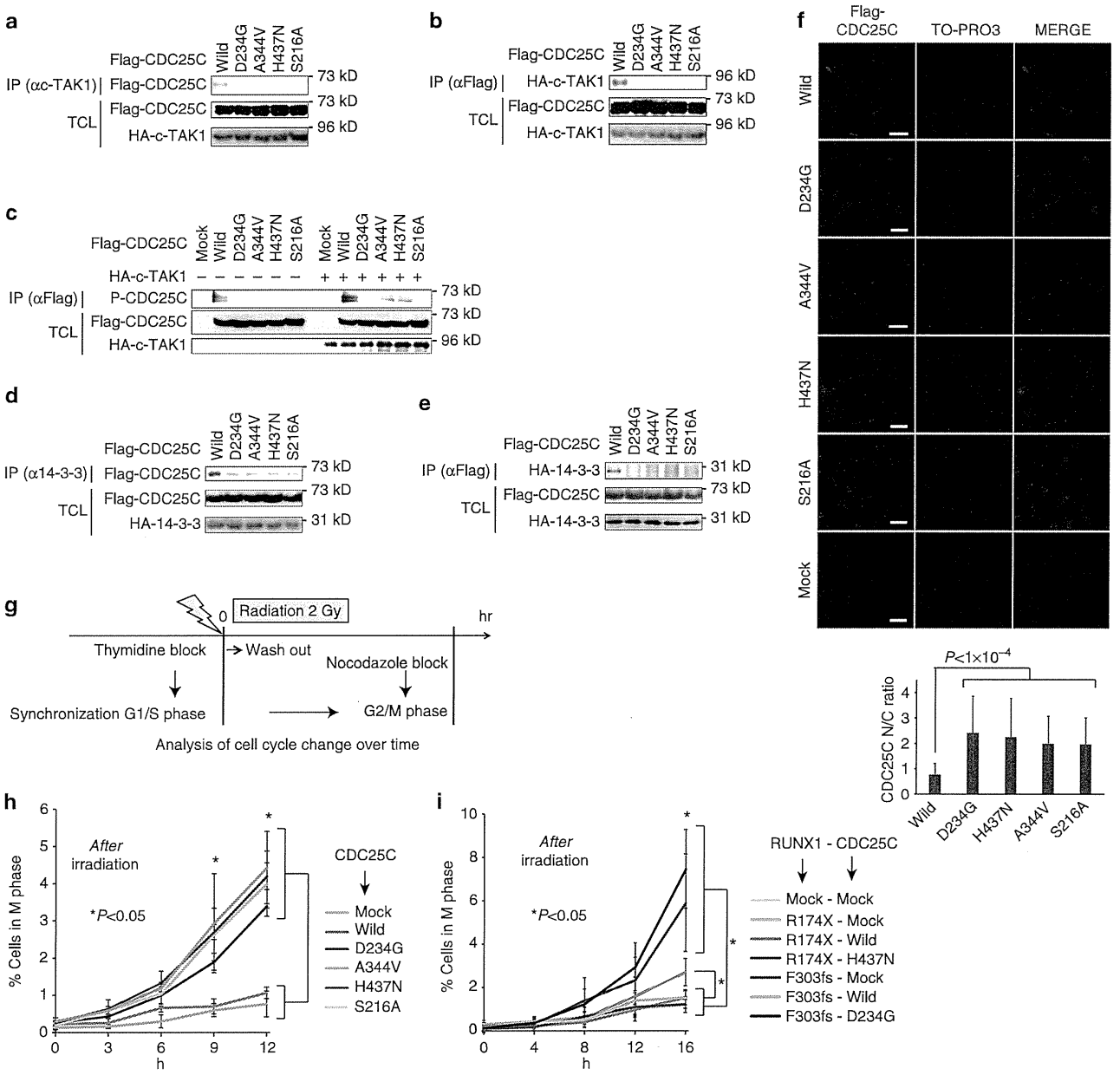

Figure 4 | Mutated CDC25C enhances mitotic entry. (**a**) HEK293T cells were transiently transfected with constructs encoding Flag-tagged CDC25C wild type or mutants, as indicated, and cell lysates were immunoprecipitated with anti-c-TAK1 antibody. Binding capacity of CDC25C was evaluated by western blotting. IP, immunoprecipitation; TCL, total cell lysate. (**b**) Reciprocal immunoprecipitation of **a** using anti-Flag (CDC25C) antibody for immunoprecipitation. (**c**) Left half; cell lysates were immunoprecipitated with anti-Flag antibody. Phosphorylation levels of CDC25C were assessed by phosphorylated-Ser216-specific anti-CDC25C antibody. Right half; the same experiment was performed with cell lysates from HEK293T cells transfected with constructs encoding Flag-tagged CDC25C wild type or mutants and HA-tagged c-TAK1. (**d**) Mutated CDC25C showed reduced capacity for binding to 14-3-3. Cell lysates were immunoprecipitated with anti-14-3-3 antibody and binding capacity of CDC25C was evaluated. (**e**) Reciprocal immunoprecipitation of **d** using anti-Flag (CDC25C) antibody for immunoprecipitation. (**f**) Localization of CDC25C or its mutants was visualized by immunofluorescence. Anti-Flag antibody and Alexa Fluor 555 antibody was used for visualization of CDC25C. N/C ratio of each cell was calculated as detailed in Supplementary Methods and Supplementary Fig. 10. The mean and s.d. of the N/C ratio is presented. Statistical significance of difference was determined by unpaired Student's *t*-test ($n>30$ for each). Scale bar, 10 μm. (**g**) Schematic description of the method used for evaluation of mitotic entry. (**h**) Mitotic entry of CDC25C-mutated cells. Percentage of mutated CDC25C-transduced cells in the M phase was compared with that of wild-type CDC25C-transduced cells. *P* values were calculated using Student's *t*-test and the differences between groups, as indicated, were all statistically significant ($^*P<0.05$) at 10 and 12 h after irradiation ($n=3$). The average and s.d. is presented. (**i**) Mutated RUNX1 and CDC25C were co-expressed in Ba/F3 cells, as indicated, and mitosis entry of these cells was evaluated. The differences between groups, as indicated, were all statistically significant ($^*P<0.05$ at 16 h after washout of thymidine ($n=3$). *P* values were determined using the Student's *t*-test. The average and s.d. is presented.

a germline *RUNX1* mutation. In addition, as Turowski and colleagues reported that CDC25C was involved in S phase entry in addition to mitotic entry[23], release from thymidine-induced G1/S block may be affected by some unknown machinery mediated by mutated CDC25Cs, which might affect the results when we observed G2/M phase fraction of these cells. It is not clear why *CDC25C* mutations are repetitively documented in FPD/AML, but not in sporadic MDS or AML cases. One possibility is that in the presence of a *RUNX1* mutation, as an initial event, an extended period is required before an additional *CDC25C* mutation is acquired. This proposal is supported by the clinical observation that ~40% of patients with FPD/AML develop leukaemia in their 30s[5]; however, the mutational status in *CDC25C* in the reported cohort was unknown.

One of the important problems in the research of FPD/AML is that definitive diagnostic criteria have not been established yet. For this purpose, more extensive studies are required for accumulating clinical characterization, genetic information and functional examination as to whether a *RUNX1* variant in families with thrombocytopenia and/or haematological malignancy is causal[24]. We clarified tentative diagnostic criteria for FPD/AML, which was used in this study (in Methods). Regarding the three missense variants in our study (p.Ser140Asn in pedigree 54, p.Gly172Glu in pedigree 57 and p.Leu445Pro in pedigree 32), Ser140 and Gly172 have been reported to be mutated in sporadic AML and/or MDS cases[25,26]. In addition, induced pluripotent stem cells from a FPD/AML pedigree with p.Gly172Glu recapitulate the phenotype of FPD/AML after hematopoietic differentiation[27]. Ser140 has been also shown to be important for RUNX1 conformation, and a mutation of this site affects hydrogen bonds and results in functional loss[28,29]. Furthermore, all the three missense variants have not been reported in the following SNP database: SNP database (dbSNP) (http://www.ncbi.nlm.nih.gov/projects/SNP), the 1000 Genomes Project (http://www.1000genomes.org), HGVB (http://www.genome.med.kyoto-u.ac.jp/SnpDB/index.html). They were also predicted as 'damaging' by Polyphen-2 (http://genetics.bwh.harvard.edu/pph2/), SIFT (http://sift.jcvi.org/) and PROVEAN (http://provean.jcvi.org/index.php). Therefore, we regarded the pedigrees with these *RUNX1* variants as having FPD/AML in this study. However, regarding pedigree 32 with p.Leu445Pro, we could not completely exclude the possibility of incidental co-occurrence of a possible non-causal *RUNX1* germline variant and hairy cell leukaemia, although co-occurrence of them is supposed to be rare. In addition, we should bear in mind the somatic as well as germline LOH of *RUNX1*, which contributes to thrombocytopenia and/or leukemogenesis in FPD/AML.

In conclusion, our results indicate that FPD/AML-associated leukaemic transformation is due to stepwise acquisition of mutations and clonal selection, which is initiated by a *CDC25C* mutation in the pre-leukaemic phase, and is further driven by mutations in other genes including *GATA2* (Supplementary Fig. 14). The identification of *CDC25C* as the target gene responsible for the leukaemic transformation will facilitate diagnosis and monitoring of individuals with FPD/AML, who are at an increased risk of developing life-threatening haematological malignancy.

Methods

Subjects. Studies involving human subjects were done in accordance with the ethical guidelines for biomedical research involving human subjects, which was developed by the Ministry of Health, Labour and Welfare, Japan; the Ministry of Education, Culture, Sports, Science, and Technology, Japan; and the Ministry of Economy, Trade, and Industry, Japan, and enforced on 29 March 2001. This study was approved by ethical committee of the University of Tokyo and each participating institution. Written informed consent was obtained from all patients whose samples were collected after the guideline was enforced. All animal experiments were approved by the University of Tokyo Ethics Committee for Animal Experiments. The clinical data, peripheral blood sample and buccal mucosa of the patients whose pedigree contained two or more individuals with thrombocytopenia and/or any haematological malignancies were collected from participating institutions. Platelet threshold depended on each institution's judge and any haematological malignancies were allowed. The diagnoses were self-reported. When all the following four criteria were fulfilled, the patient was considered as having FPD/AML in this study: (1) the pedigree has two or more individuals with thrombocytopenia and/or any haematological malignancies; (2) a germline *RUNX1* variant, including missense, nonsense, frameshift, insertion and deletion, is confirmed by Sanger sequencing and a synchronized quantitative-PCR method in at least one family member; (3) the *RUNX1* variant has not been reported in public dbSNP; (4) no germline mutations were detected in the following 16 genes: *GATA2*, *GATA1*, *CEBPA*, *MPL*, *MYH9*, *MYL9*, *GP1BA*, *GP9*, *MASTL*, *HOXA11*, *CBL*, *DIDO1*, *TERT*, *ANKRD26*, *GFI1B* and *SRP72*. Regarding the last criterion, 16 genes were selected because they have been reported to be responsible for familial thrombocytopenia and/or haematological malignancies.

Whole-exome sequencing. Genomic DNA was extracted from samples using the QIAamp DNA Mini kit (Qiagen). Exome capture was performed. Enriched exome fragments were subjected to sequencing using HiSeq2000 (Illumina). We removed any potential somatic mutations that were observed in dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP) or in the 1000 Genomes Project (http://www.1000genomes.org) data. All candidate single-nucleotide variations and indels, which were predicted to be deleterious by the Polyphen-2 algorithm, were validated by deep sequencing and Sanger sequencing. Genomic DNA samples from the buccal mucosa of the two patients (subject 20 and subject 21) were used as references. All candidate somatic mutations were validated by Sanger sequence and deep sequencing using primers listed in Supplementary Tables 3 and 4.

Deep sequencing. Using genomic DNA of the patients as template, each targeted region was PCR amplified with specific primers (Supplementary Table 4). The amplification products from an individual sample were combined and purified with the AMpure XP Kit (Beckman Coulter) and library preparation was carried out using the Ion Xpress Fragment Library Kit (Life Technologies) according to the manufacturer's instructions. The Agilent 2100 Bioanalyzer (Agilent Technologies) and the associated High Sensitivity DNA kit (Agilent Technologies) were used to determine quality and concentration of the libraries. The amount of the library required for template preparation was calculated using the template dilution factor calculation described in the protocol. Emulsion PCR and enrichment steps were carried out using the Ion OneTouch 200 Template Kit v2 DL (Life Technologies). Sequencing was undertaken using Ion Torrent PGM and Ion 318 chips Kit v2 (Life Technologies). The Ion PGM 200 Sequencing Kit (Life Technologies) was used for sequencing reactions, following the recommended protocol. The presence of *CDC25C* and *GATA2* mutations was also validated by a subclone strategy for DNA sequence analysis.

Single-cell sequencing and genome amplification. Single cells were separated from the bone marrow of subject 20 at AML phase using FACSAria II (BD biosciences) (Supplementary Fig. 15a). Each cell was deposited into individual wells of a 96-well plate. Single cells were lysed and whole genome from single cell was amplified using GenomePlex Single Cell Whole-Genome Amplification Kit (Sigma-Aldrich). Mutation status of each gene was analysed by direct sequencing with specific primers (Supplementary Table 5). To improve the sensitivity of this procedure, we used multiple primer sets for detecting a single-nucleotide variation. We estimated the false-negative rate of this procedure based on the ratio of RUNX1 mutation, which is supposed to be observed in all of the cells. The false-negative rate was estimated to be 35% (22 cells out of 63 cells, Supplementary Table 2), which is consistent with the manufacturer's bulletin reporting the allelic dropout of 30%. In light of these results, we regard those cells with at least one gene mutation in a mutational group (coloured in red, orange, green, blue or purple) as being positive for gene mutations of the corresponding group. To assess whether mutations in *LPP*, *FAM22G*, *COL9A1* and *GATA2* and mutations in *AGAP4*, *RP1L1*, *DTX2* and *CHEK2* were mutually exclusive, we performed a statistical analysis as follows. First of all, we determine a matrix **A** that virtually represents the mutational status of eight genes (1: *LPP*, 2: *FAM22G*, 3: *COL9A1*, 4: *GATA2*, 5: *AGAP4*, 6: *RP1L1*, 7: *DTX2* and 8: *CHEK2*) of 57 cells. Concretely, **A** is defined as follows:

$$\mathbf{A}=\begin{pmatrix} a_{1,1} & \cdots & a_{8,1} \\ \vdots & \ddots & \vdots \\ a_{1,57} & \cdots & a_{8,57} \end{pmatrix} \quad a_{i,j}=\begin{cases} 0: \text{if gene i of cell j is wildtype} \\ 1: \text{if gene i of cell j is mutated} \end{cases} \qquad (1)$$

On the other hand, a matrix **R** indicates data from the actual experimental results of mutational analysis as shown in Fig. 2c. Elements of **R** is provided in

Supplementary Table 2.

$$\mathbf{R}=\begin{pmatrix} r_{1,1} & \cdots & r_{8,1} \\ \vdots & \ddots & \vdots \\ r_{1,57} & \cdots & r_{8,57} \end{pmatrix} \quad r_{i,j}=\begin{cases} 0: \text{if gene i of cell j is wild type} \\ 1: \text{if gene i of cell j is mutated} \\ 2: \text{if mutational status of gene i of cell j is undetermined} \end{cases} \tag{2}$$

Then we assumed two hypotheses: H_0 and H_1.

H_0: the mutational status of genes $1\sim4$ and genes $5\sim8$ is independent. Each matrix elements of **A** are randomly assigned 0 or 1 (at ratio of 1:1) independently of each other.

H_1: mutations in genes $1\sim4$ and genes $5\sim8$ are mutually exclusive, and cells $1\sim40$ harbour mutations of genes $1\sim4$, while cells $41\sim57$ harbour mutations of genes $5\sim8$. In mathematical representation,

$$a_{i,j}=\begin{cases} 0: (5\le i\le 8 \text{ and } 1\le j\le 40) \text{ and } (1\le i\le 4 \text{ and } 41\le j\le 57) \\ 0 \text{ or } 1 \text{ randomely}: (1\le i\le 4 \text{ and } 1\le j\le 40) \text{ and } (5\le i\le 8 \text{ and } 41\le j\le 57) \end{cases} \tag{3}$$

We assumed matrices $\mathbf{A}_0$ and $\mathbf{A}_1$ that represent virtually generated mutational status under the hypotheses H_0 and H_1, and calculate the probability of substantializing **R** for given $\mathbf{A}_0$ and $\mathbf{A}_1$.

$P_0(\mathbf{R}/\mathbf{A}_0)$ and $P_1(\mathbf{R}/\mathbf{A}_1)$ can be calculated for given matrices $\mathbf{A}_0$ and $\mathbf{A}_1$ under the condition as follows:

Probability that we cannot determine whether a cell has mutation in gene X when the cell does not actually have a mutation; 28% (based on our data shown in Supplementary Table 2).

Probability that we judge that a cell has a mutation in gene X when the cell does not actually have a mutation; 5% (because it is very unlikely to happen).

Probability that we can judge correctly that a cell does not have a mutation in gene X when the cell does not actually have a mutation; 67% ($100-28-5=67\%$).

Probability that we cannot determine whether a cell has mutation in gene X when the cell actually has a mutation; 28% (based on our data shown in Supplementary Table 2).

Probability that we judge that a cell has a mutation in gene X when the cell actually has a mutation; 35% (the estimated false-negative rate based on the ratio of *RUNX1* mutation).

Probability that we can judge correctly that a cell has a mutation in gene X when the cell actually has a mutation; 37% ($100-28-35=37\%$).

Put it simply, $\mathbf{P}_0$ represents the probability that one can get the mutational profile R when a cell harbours mutations independently of each other, while $\mathbf{P}_1$ indicates the probability that R is realized under the condition where mutations in gene groups $1\sim4$ and $5\sim8$ are exclusive. Because $\mathbf{A}_0$ and $\mathbf{A}_1$ that meet the hypotheses H_0 and H_1 can be generated innumerably, we conducted a computational simulation to acquire the distribution of P_0 and P_1 by generating $\mathbf{A}_0$ and $\mathbf{A}_1$ 100,000 times. For visibility, horizontal axis is converted to $-\ln(P)$.

Synchronized quantitative-PCR. These experiments were performed mostly as described previously[6]. Briefly, genomic DNA was denatured 95 °C for 5 min and iced immediately. Using the LightCycler 480 Instrument II (Roche), thermal cycling was performed with denatured genomic DNA, forward and reverse primers (Supplementary Table 6), THUNDERBIRD SYBR qPCR mix (TOYOBO). Threshold cycle scores were determined as the average of triplicate samples. We designed 27 primers for *RUNX1* and 3 reproducible primers (that is, primer RUNX-9, RUNX-19 and RUNX-20) were chosen by preparatory experiments. RPL5-2 and PRS7-1 primers, which were authorized previously[6], were also utilized as controls. In addition, genomic DNA extracted from the bone marrow sample of a MDS patient with a chromosome 21 deletion was also examined with the same primers as a control of *RUNX1* locus copy-number loss. Crossing points (Cps) of designed primers were examined by quantitative PCR. *RUNX1* locus copy-number relative to RPL5-2 was calculated using Cps of RUNX-9 and RPL5-2, with RPL5-2 values set at 2. Similar results were obtained when Cps of RUNX-19, RUNX-20 or RPS7-1 values were used.

LOH detection with SNP sequencing. To examine the existence of uniparental disomy, we designed four specific primers to detect nine SNPs in *RUNX1*, which are frequently seen (>40%) (Supplementary Table 7). Direct sequencing was performed with the primers, and heterogeneity of SNPs was examined.

Chemicals and immunological reagents. Thymidine and nocodazole were purchased from Sigma-Aldrich. Anti-CDC25C, anti-phospho-CDC25C (Ser216) and anti-beta-actin antibodies were purchased from Cell Signaling Technology. Anti-HA monoclonal antibody was purchased from MBL. Rabbit anti-Flag monoclonal antibody was purchased from Sigma-Aldrich. Anti-HA was purchased from Roche. Mouse anti-phospho-histone H2AX (Ser139) antibody and Alexa Fluor 488 mouse anti-phospho-H3 (Ser10) antibody were purchased from Merck Millipore. Alexa Fluor 488 rabbit anti-mouse immunoglobulin (Ig)G, Alexa Fluor 488 goat anti-rabbit IgG and Alexa Fluor 555 goat anti-rabbit IgG were purchased from Invitrogen. TO-PRO3 was purchased from Molecular Probes. Rabbit anti-14-3-3 Sigma antibody was purchased from Bethyl laboratories. Sheep anti-c-TAK1 antibody was purchased from Exalpha Biologicals. Anti-sheep IgG-HRP was purchased from RSD. Nonviable cell exclusion was performed by 7-AAD Viability Staining Solution (BioLegend).

Subclone strategy and direct sequencing. Using genomic DNA of the patients as template, each targeted region was amplified by PCR with specific primers (Supplementary Table 4). PCR products were purified with illustra ExoStar (GE Healthcare) and subcloned into *Eco*RV site of pBluescript II KS(−) (Stratagene). Ligated plasmids were transformed into *E. coli* strain XL1-Blue by 45 s heat shock at 42 °C. Positive transformants were incubated on LB plates containing 100 μg ml^{-1} ampicillin supplemented with X-gal (Sigma-Aldrich) and isopropyl β-D-1-thiogalactopyranoside (Sigma-Aldrich). For colony PCR, a portion of a white colony was directly added to a PCR mixture as the DNA template. Insert region was amplified by PCR procedure with T3 and T7 universal primers, purified with illustra ExoStar (GE Healthcare Life Sciences), and sequenced by the Sanger method with T3 and T7 primers using BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) and ABI Prism 310 Genetic Analyzer (Life Technologies).

Immunoprecipitation and western blotting. These experiments were performed as described previously[30]. Briefly, HEK293T cells were transiently transfected with mammalian expression plasmids encoding Flag-tagged CDC25C and its mutants, HA-tagged 14-3-3 or c-TAK1. All plasmids were sequence verified. After 48 h, cell lysates were collected and incubated with an antibody (anti-HA antibody (1:200, 3 h), anti-Flag antibody (1:200, 3 h), anti-c-TAK1 antibody (1:150, 3 h) and anti 14-3-3 antibody (1:150, 3 h)). After incubation, the cell lysates were incubated with protein G-Sepharose (GE Healthcare) for 1 h. The precipitates were stringently washed with high salt-containing wash buffer and analysed by western blotting. Anti-Flag (HRP-conjugated, Sigma-Aldrich), anti-HA (MBL), anti-HA (HRP-conjugated, Roche), anti-CDC25C (Cell Signaling Technology), anti-phospho-CDC25C (Ser216) (Cell Signaling Technology), anti-c-TAK1 antibody (Exalpha Biologicals) or anti-14-3-3 antibody (Bethyl laboratories) antibodies and Immunostar LD (Wako) was used for detection. Original gel images of western blot analysis are shown in Supplementary Fig. 16.

Cell cycle synchronization and analysis for mitosis entry. After transduction of wild-type CDC25C or its mutated forms to murine lymphoid cell line Ba/F3 cells (RIKEN BioResource Center), double-thymidine block was performed to obtain cell cycle synchronization at G1/S phase. In brief, 2 mM of thymidine was added to the medium. After 16 h, cells were washed and released from the first thymidine for 8 h. A second block was initiated by adding 2 mM of thymidine, and cells were maintained for 16 h. Then thymidine was washed out and the cells were incubated with 1 mM nocodazole with or without 2 Gy of irradiation (Supplementary Fig. 10a). Ba/F3 cells were fixed over time with 75% ethanol in phosphate-buffered saline (PBS) at 4 °C overnight and permeabilized with 2% Triton-X at 4 °C for 15 min. The cells were stained with anti-phospho-H3 (Ser10) Alexa Fluor 488 conjugated antibody (dilution, 1:200) in PBS with 2% fetal calf serum at 4 °C for 30 min and then treated with 5% propidium iodide and 1% RNase in PBS at room temperature (RT) for 30 min. Cell cycle was analysed using a BD LSR II Flow cytometer (BD biosciences) (Supplementary Fig. 15b). To assess the cooperation of *CDC25C* and *RUNX1* mutation, wild-type or mutant (D234G, H437N) pMXs-neo-Flag-CDC25C and mutant (F303fsX566, R174X) pGCDNsam-IRES-KusabiraOrange-Flag-RUNX1 were retrovirally transduced into Ba/F3 cells.

Immunofluorescent microscopic analysis. These experiments were performed as described previously[30]. Briefly, Ba/F3 cells were fixed, permeabilized and blocked. Staining for phosphorylated histone H2AX was performed with anti-phospho-histone H2AX (Ser139) antibody (dilution, 1:500; Merck Millipore) at RT for 3 h. After washing with PBS three times and with 1% bovine serum albumin in PBS, the cells were treated with Alexa Fluor 488 rabbit anti-mouse IgG (dilution, 1:500; Invitrogen) and TO-PRO3 (dilution, 1:1,000; Molecular Probes) for 1 h. The proteins were visualized using FV10i (Olympus) or BZ-9000 (Keyence). The percentage of γH2AX foci-positive cells was determined by examining 100 cells per sample. Three independent experiments were performed. To evaluate the localization of CDC25C, Ba/F3 cells were treated with 2 mM thymidine for 12 h and stained. Staining was underwent with anti-Flag antibody or anti-CDC25C antibody at RT for 3 h. After washing, the cells were treated with Alexa Fluor 488 or 555 antibody and TO-PRO3 for 1 h. The mean intensity of CDC25C in the nucleus and cytoplasm of each cell was measured within a region of interest placed within the nucleus and cytoplasm (Supplementary Fig. 10). Similarly, the background intensity was quantified within the region of interest placed outside the cells. All the measurements were performed using the Fluoview FV10i software or ImageJ. The background-subtracted intensity ratio of the nucleus to cytoplasm was calculated in >30 cells in each specimen.

Retrovirus production. The procedures were performed as described previously[30]. Briefly, Plat-E packaging cells were transiently transfected with each retroviral construct using the calcium phosphate precipitation method, and supernatant

containing retrovirus was collected 48 h after transfection and used for infection after it was centrifuged overnight at 10,000 r.p.m.

Statistical analysis. To compare data between groups, unpaired Student's *t*-test was used when equal variance were met by the *F*-test. When unequal variances were detected, the Welch *t*-test was used. Differences were considered statistically significant at a P value of <0.05.

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Acknowledgements

This work was supported in part by grants-in-aid from the Ministry of Health, Labor and Welfare of Japan (H23-Nanchi-Ippan-104; M. Kurokawa) and KAKENHI (24659457; M. Kurokawa). We thank R. Lewis (University of Nebraska Medical Center) and T. Kitamura (Institute of Medical Science, The University of Tokyo) for providing essential materials; T. Koike (Nagaoka Red Cross Hospital), K. Nara (Ootemachi Hospital), K. Suzuki (Japanese Red Cross Medical Center), H. Harada (Fujigaoka Hospital), Y. Morita (Kinki University), M. Matsuda (PL Hospital), H. Kashiwagi (Osaka University), T. Kiguchi (Chugoku Central Hospital), T. Masunari (Chugoku Central Hospital), K. Yamamoto (Yokohama City Minato Red Cross Hospital), T. Takahashi (Mitsui Memorial Hospital) and T. Takaku (Juntendo University) for providing patient samples; M. Kuramitsu (National Institutte of Infectious Diseases) for providing kind support of synchronized quantitative PCR; and K. Tanaka and Y. Shimamura for their technical assistance.

Author contributions

A.Y., T.T., M.I. and M. Kurokawa analysed genetic materials and performed functional studies. A.T., H.I., M.N., Y.N. and S.A. were involved in sequencing and/or functional studies. M. Kawazu, T.U. and H.M. took part in whole-exome sequencing, deep sequencing and bioinformatics analyses of the data. A.Y., T.T., M.I., H.H., K.U., Y.H., E.I., K.K. and H.N. collected specimens. A.Y. and T.T. generated figures and tables. M. Kurokawa designed and led the entire project. A.Y., T.T. and M. Kurokawa wrote the manuscript. All authors participated in the discussion and interpretation of the data.

Additional information

Accession codes: Sequence data for FPD/AML patients has been deposited in GenBank/EMBL/DDBJ sequence read archive (SRA) under the accession code SRP043031

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: The authors declare no competing financial interests.

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How to cite this article: Yoshimi, A. *et al.* Recurrent *CDC25C* mutations drive malignant transformation in FPD/AML. *Nat. Commun.* 5:4770 doi: 10.1038/ncomms5770 (2014).

12 腫瘍

2．組織球症

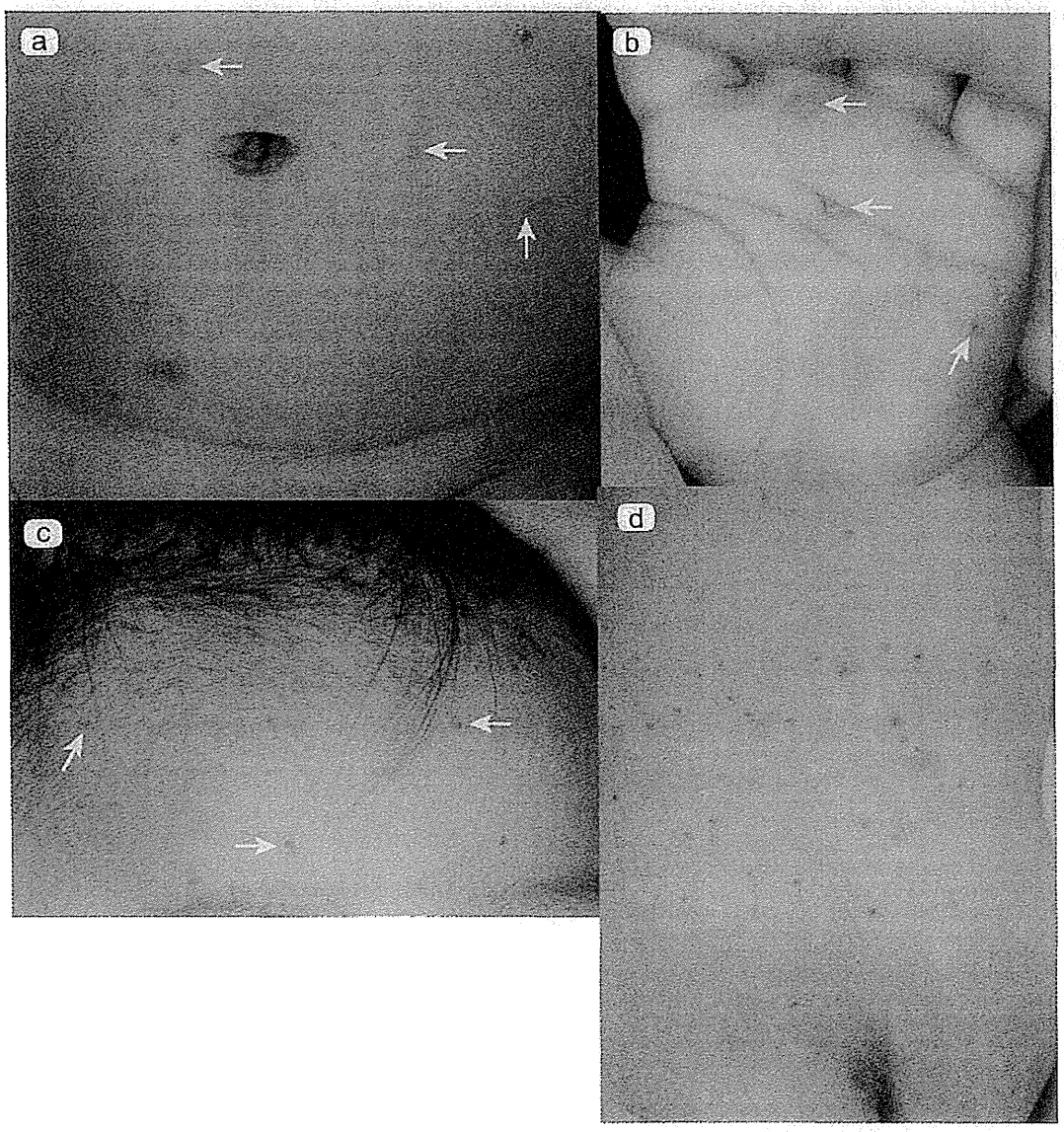

図1 ランゲルハンス細胞組織球症（LCH）の女児の臨床像

生後3週間より体幹，前額，手掌等に水疱出現。生後約3カ月時に当科（大阪大学皮膚科）受診（a～d）。ステロイド外用にても軽快せず，前額部の水疱はむしろ悪化（c）。体幹部は一部白斑も混じる（d）。生後約4カ月より両耳下腺部のリンパ節腫脹，脾腫出現。5カ月より熱発，貧血出現したためビンブラスチン・プレドニゾロンにて加療。1歳6カ月で骨髄移植。以後経過良好である。

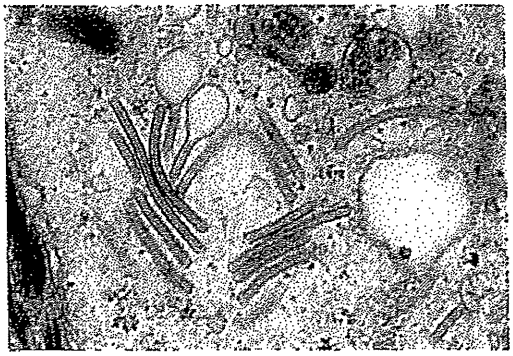

図2　LCH の男児皮膚より生検した組織の電子顕微鏡像
胞体内にラケット状の Birbeck 顆粒を認める。

1．組織球症とは

ランゲルハンス細胞などの樹状細胞やマクロファージがモノクローナルに増殖する疾患。皮膚，骨(頭蓋骨，長管骨等)，リンパ節，肺，肝，脾などの造血系，口腔粘膜，胃腸粘膜，胸腺，甲状腺，膵臓，腎臓，下垂体，眼窩，中枢神経系などさまざまな臓器に浸潤する。以下のように分類[1]。

① ランゲルハンス細胞組織球症 (Langerhans cell histiocytosis：LCH)
　Ⅰ：LCH-SS：単系統 (好酸球性肉芽腫症：多巣性骨病変)
　Ⅱ：LCH-MS：多系統で重要臓器(-) (Hand-Schüller-Christian 病：2～5 歳で発症し骨病変，眼球突出，尿崩症を 3 徴候)
　Ⅲ：LCH-MS(RO)：多系統で重要臓器 (肝，肺，脾，骨髄) を含む (Letterer-Siwe 病：2 歳未満で発症し多臓器に及ぶ)
② non-LCH：マクロファージの増殖による (若年性黄色腫，Rosai Dorfman 病，Erdheim-Chester 病)
③ 悪性組織球症

2．診断のポイント (以下 LCH について述べる)

初発症状は皮膚が多い。丘疹，水疱，びらん，結節，紫斑，ときに白斑など多彩な皮疹がさまざまな部位に出現するが 図1，非特異的な症状であるため早期診断が困難である[2]。皮膚生検で診断する。免疫学的染色にて CD1a(+)，CD207(langerin)(+)。電子顕微鏡にて胞体内にラケット状の Birbeck 顆粒を認める 図2。

3．検査

超音波検査 (肝脾腫，リンパ節腫大)，血液検査 (貧血，血小板減少，バソプレシン濃度低下)，X-P (頭蓋骨，長管骨)，MRI，骨髄穿刺，呼吸機能[3]。

4．治療

限局性の皮膚病変に対しては局所ステロイド外用を行う。LCH-MS 例に限り以前はビンブラスチン・プレドニゾロンが用いられていたが，最近では LCH の原因遺伝子の一つは *BRAF* と考えられており，これに対して海外で vemurafenib (2014 年 12 月現在国内未承認) 等による治療も試みられている[4]。骨髄移植が行われることもある[5]。

(村上有香子)

Pediatr Blood Cancer 2014;61:1215–1221

Improved Treatment Results of Children With B-Cell Non-Hodgkin Lymphoma: A Report From the Japanese Pediatric Leukemia/Lymphoma Study Group B-NHL03 Study

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Background. Previous Japanese studies of childhood B-cell non-Hodgkin lymphoma (B-NHL) have shown a favorable outcome, though the study size was too small to effectively assess the efficacy and safety of treatment for childhood B-NHL. ***Procedure.*** We performed a nation-wide prospective B-NHL03 study to assess the efficacy and safety of short-pulse intensive chemotherapy for children with B-NHL. They were stratified into four treatment groups according to disease stage, tumor resectability and bone marrow/CNS involvement: Group 1 with all resected stage I/II, Group 2 with non-resected stage I/II, Group 3 with stage III & CNS-negative stage IV, and Group 4 with CNS-positive stage IV & Burkitt leukemia. Treatment duration was 2 courses for Group 1, 4 courses for Group 2, and 6 courses for Groups 3 and 4, respectively. CNS irradiation was omitted in all patients. ***Results.*** The follow-up time ranged from 0.8 to 88 months, with a median of being 45 months. For 321 patients analyzed in this study, overall survival and event-free survival (EFS) at 4 years was 92.7% and 87.4%, respectively. The 4-year EFS according to treatment group were 94% for Group 1 (n = 17), 98% for Group 2 (n = 103), 84% for Group 3 (n = 111), and 78% for Group 4 (n = 90). There was no significant difference in outcome by histology. Therapy-related death occurred in three patients in remission. ***Conclusions.*** Our nationwide large-scale study resulted in a cure rate above 90% with <1% toxic death in childhood B-NHL. Pediatr Blood Cancer 2014;61:1215–1221.

Key words: B-NHL03; childhood; JPLSG; non-Hodgkin lymphoma

INTRODUCTION

Childhood B-cell non-Hodgkin Lymphoma (B-NHL) consists mainly of two histological subtypes, namely Burkitt lymphoma (BL), which includes Burkitt leukemia (B-ALL), and diffuse large B-cell lymphoma (DLBCL). The cure rate of childhood BL has been markedly improved over the past 30 years, and long-term event-free survival (EFS) of patients has reached to approximately 90%. This is largely due to prospective studies of European and North American groups that developed a short intensive chemotherapy regimen, including a high-dose methotrexate (HDMTX), an intermediate dose of cyclophosphamide (CPA), and anthracyclines [1–6]. Although DLBCL is a distinct disease entity from BL, the treatment is the same as that for patients with Burkitt histology, and excellent outcome has been reported [1–6]. Previously most clinical experiences of childhood B-NHL were reported by European and North American study groups, and there were few data on Japanese or Asian patients with B-NHL. In the1990s, we conducted group-wide trials for childhood B-NHL [7–10]: Horibe et al. showed a 4-year EFS with 70% for 57 patients (BL 31, B-ALL 17, DLBCL 9) [8], Kikuchi et al. showed a 6-year EFS with 82% for 91 patients (BL 45, B-ALL 9, DLBCL 26, others 11) [10], and Tsurusawa et al. showed a 7-year EFS with 93% for 30 patients with DLBCL [9]. In addition, Lee et al. has recently shown a 5-year EFS with 95% for 61 patients (BL 46, DLBCL 15) [11]. However, the treatment duration of these studies was relatively long and the number of patients was small compared to the European and North American studies [1–6].

Here, we report on the results of the nation-wide large prospective study for children with B-NHL. The primary object was to evaluate the efficacy and safety of short-pulse intensive chemotherapy regimen designed by the Japanese Pediatric Leukemia/Lymphoma Study Group (JPLSG).

DOI 10.1002/pbc.24975
Published online 13 February 2014 in Wiley Online Library (wileyonlinelibrary.com).

PATIENTS AND METHODS

Study Design and Diagnostic Criteria

The B-NHL03 study was a prospective nonrandomized trial that investigated the efficacy and safety of short-pulse intensive chemotherapy in childhood B-NHL. The chief aim was to improve the outcomes of patients enrolled in the B-NHL03 study to the level of those of European and North American studies.

Additional Supporting Information may be found in the online version of this article.

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Grant sponsor: Ministry of Health, Labor and Welfare of Japan; Grant number: H14, H15, H16, H17, H20, H23

Conflict of interest: Nothing to declare.

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Received 4 October 2013; Accepted 16 January 2014

The diagnosis of B-NHL was based on histopathology, immunocytochemistry, and cytogenetics. All histopathological specimens were first classified by the institutional pathologist and finally each of them were reviewed by a group of seven pathologists of a central pathological review committee according to WHO classification, that is, BL or Burkitt-like lymphoma (BLL), DLBCL, mediastinal large B-cell lymphoma (MLBCL), and mature B-cell neoplasm, NOS (not otherwise specified) [12]. A mature B-cell phenotype was primarily defined as positive for C20 and/(or) CD79a and negative for CD3 and terminal deoxynucleotidyl transferase. When an immunophenotype study was not available, specific translocations t(8;14)(q24;q32), t(2;8)(p11;q24), t(8;22)(q24;q11) at cytogenetic analysis were included. CNS involvement was diagnosed by the presence of one or more of the following: any blasts with FAB L3 morphology in CSF, isolated intracerebral mass, or intra-spinal extension. The clinical stage was defined by Murphy's classification [13].

Treatments

The treatment outline is shown in Figure 1 and chemotherapy regimens are shown in Table I. They were stratified into four treatment groups according to disease stage, tumor resectability and bone marrow/CNS involvement: Group 1 with all resected stage I/II, Group 2 with non-resected stage I/II, Group 3 with stage III & CNS-negative stage IV, and Group 4 with CNS-positive stage IV & B-ALL. All groups except Group 1 received a pre-phase therapy of prednisolone (PSL), vincristine (VCR), CPA and it (intrathecal) MTX to reduce tumor volume. As shown in Figure 1, Group 1 received two courses (1A × 2), Group 2 received 4 courses (2A × 2 + 2B × 2), Group 3 received 6 courses (3A × 4 + 3B × 2), and Group 4 received 6 courses (4A1 × 2 + 4A2 × 2 + 4B × 2), respectively. No patients received prophylactic cranial irradiation. Patients with CNS involvements received HDMTX (5 g/m^2) plus an extended it regimen (14 times), but no therapeutic cranial irradiation. The schedule of HDMTX administration was identical

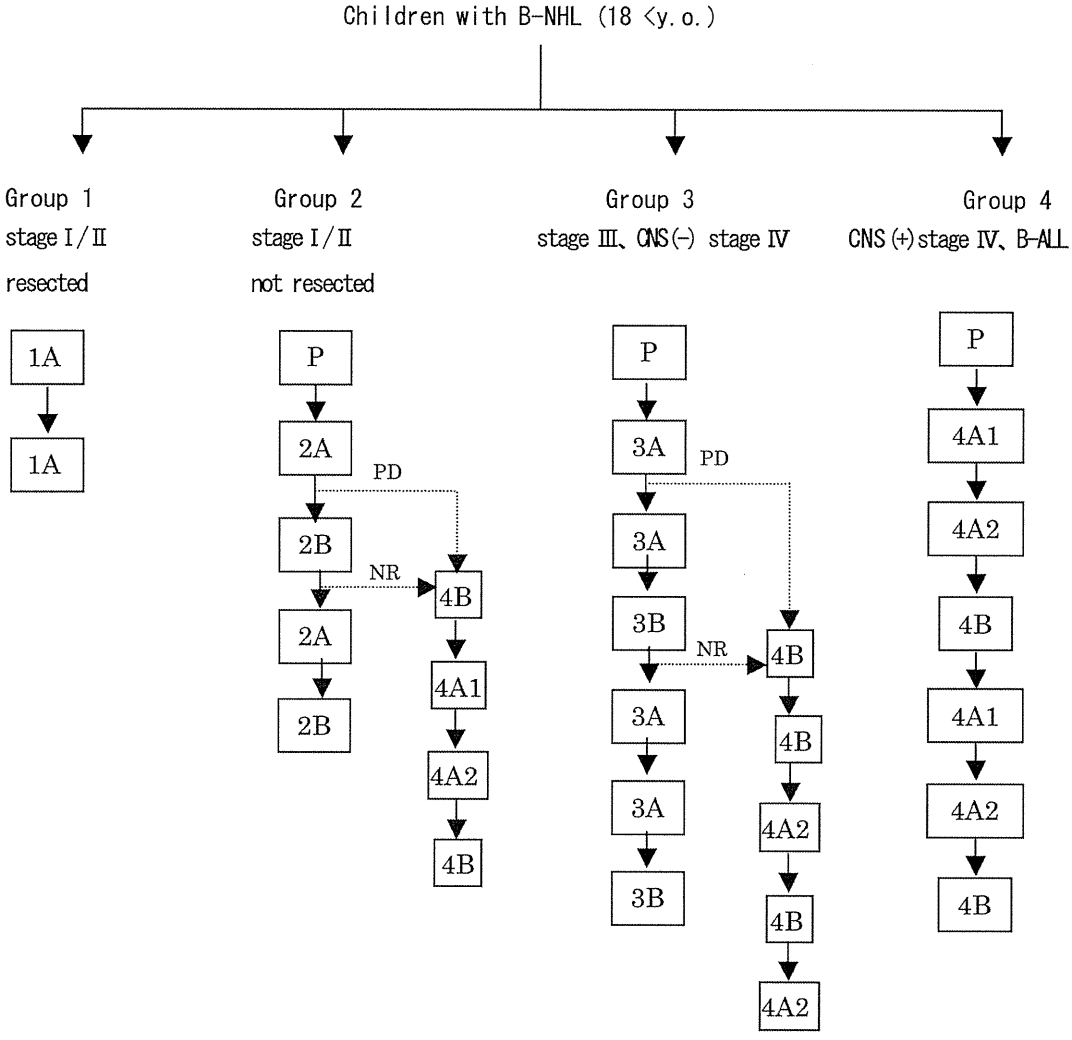

Fig. 1. Treatment framework of the B-NHL03 study. Patients were stratified into four treatment groups according to disease stage, tumor resectability, and BM/CNS involvement. All groups except Group 1 received pre-phase therapy. Group 1 received two courses of chemotherapy, Group 2 received 4 courses, Groups 3 and 4 received 6 courses, respectively. When patients in Group 2 or 3 did not achieve CR or CRu during the first 2 or 3 courses, they received salvage therapy consisting of 4B and 4A1/2 courses.

TABLE I. B-NHL03 Treatment Schedules

Regimen	Administration	Daily dose	Days
Pre-phase			
Prednisolone	Orally	30 mg and 60 mg/m^2	Days 1–3 and 4–7
Vincristine	IV	1 mg/m^2	Day 3
Cyclophosphamide	IV	150 mg/m^2	Days 4–6
Methotrexate	TIT	12 mg/m^2	Day 1, (4)[a]
Hydrocortisone	TIT	25 mg/m^2	Day 1, (4)[a]
Cytarabine	TIT	30 mg/m^2	Day (4)[a]
Regimen 1A			
Prednisolone	Orally	60 mg/m^2	Days 1–5
Methotrexate	IV	1 g/m^2	Day 1
Vincristine	IV	1.5 mg/m^2	Day 2
Cyclophosphamide	IV	250 g/m^2 × 2	Days 2–4
THP-adriamycin	IV	30 mg/m^2	Days 3, 4
Methotrexate	DIT	12 mg/m^2	Day 1
Hydrocortisone	DIT	25 mg/m^2	Day 1
Regimen 2A			
Same as 1A except for dexamethasone	Orally	10 mg/m^2	Days 1–7
Methotrexate	IV 24 hours with LV rescue	3 g/m^2	Day 1
Regimen 3A			
Same as 2A except for *t.i.t* at day 1			
Regimen 4A1			
Same as 3A except for methotrexate	IV 24 hours with LV rescue	5 g/m^2	Day 1
Methotrexate	TIT	12 mg/m^2	Day 1, (5),[a] 8
Hydrocortisone	TIT	25 mg/m^2	Day 1, (5),[a] 8
Cytarabine	TIT	30 mg/m^2	Day 1, (5),[a] 8
Regimen 4A2			
Same as 4A1 except for cyclophosphamide	IV	1 g/m^2	Days 4, 5
Regimen 2B			
Methotrexate	IV 6 hours	500 mg/m^2	Day 1
Cytarabine	cIV	150 mg/m^2	Days 1–5
Methotrexate	DIT	12 mg/m^2	Day 1
Hydrocortisone	DIT	25 mg/m^2	Day 1
Regimen 3B			
Same as 2B except for TIT at day 1, and cytarabine	cIV	150 mg/m^2	Days 1–6
Etoposide	IV	100 mg/m^2 × 2	Days 3–5
Regimen 4B			
Same as 3B except for without methotrexate, DIT at day 1 and TIT at day 8, and dexamethasone	Orally	10 mg/m^2	Days 1–7
Cytarabine	IV	2 g/m^2 × 2	Days 2–4
Etoposide	IV	150 mg/m^2	Days 2–5
Vincristine	IV	1.5 mg/m^2	Day 1

LV, leucovorin; IV, intravenous; cIV, continuous intravenous; DIT, double intrathecal; TIT, triple intrathecal. [a]For CNS positive patients.

to that of the B-NHL960 study [9]: HDMTX was administered for the first 24 hours, and 12 hours later, leucovorin (LV) 15 mg/m^2 was given orally every 6 hours, for a total of seven doses [9]. Blood MTX concentration was measured 24, 48, and 72 hours after the MTX administration. When patients showed delayed MTX clearance ($\geqq$0.2 μM after 72 hours), LV rescue was continued until MTX concentration level decreased to less than 0.2 μM.

Induction failure (IF) was defined as patients who did not achieve complete remission (CR) or unconfirmed remission (CRu) until the last evaluation time (before the second course of 2A in Group 2, before the third course of 3A in Group 3, before the second course of 4A1 in Group 4). When patients in Group 2 or 3 were evaluated to have progressive disease or no response during the first 2 or 3 courses, they received salvage therapy consisting of regimens 4B and 4A1/2. The cumulative dose of cytotoxic drugs for treatment groups was as follows: CPA 3 g/m^2, THP 120 mg/m^2 for Group 1; CPA 3.45 g/m^2, THP 120 mg/m^2 for Group2; CPA 6.45 g/m^2, THP 240 mg/m^2, VP16 0.6 g/m^2 for Group 3; CPA 7.45 g/m^2, THP 240 mg/m^2, VP16 1.2 g/m^2 for Group 4.

Statistical Analysis

Final statistical analyses were performed based on data obtained in June 2012. Overall survival (OS) was defined as the time between diagnosis and death from any causes, and EFS was defined as the time to first events defined as an occurrence of induction failure, relapse at any site, death from any causes, or second malignant neoplasm. For patients who did not experience an event, EFS was defined as the time to the last follow-up. Survival curves were prepared using the Kaplan–Meier method and standard errors (SEs) with the Greenwood formula. The significance of differences in survival outcomes was determined by means of the log-rank test.

STATA® statistical analysis software (version 11.0; StataCorp LP, College Station, TX) was used for all computations.

RESULTS

Patients

The protocol was conducted in 112 hospitals of the JPLSG after approval by each institution's review board, and written informed consent was provided by patients or legal guardians before treatment. Between November 2004 and January 2011, 346 cases of newly diagnosed B-NHL were enrolled in this study. Of these, 25 cases were excluded: 14 due to ineligible pathology, 8 for late enrollment, 2 for ineligible clinical stage, and 1 for prior chemotherapy. A total of 321 cases of four treatment groups were analyzed (Fig. 2).

Patient characteristic are shown in Table II. There were few protocol deviations: 10 patients in the Group 3/4 skipped or postponed HDMTX therapy in the A course, 5 because of retention of ascites or pleural effusion, 2 because of renal dysfunction, 2 due to septic infection, and one for stomatitis.

EFS and OS

The follow-up time ranged from 0.8 to 88 months, with a median 47 months. For the 321 patients analyzed in this study, 4-year OS was 92.7% ± 1.4% and 4-year EFS was 87.3% ± 1.8% (Fig. 3A). There was no significant difference in outcome by gender (4-year EFS, male 87.5% ± 2.2% vs. female 87.0% ± 3.8%, $P = 0.864$). The 4-year OS and EFS according to treatment subgroup were 100% and 94.1% ± 5.7% for Group 1, 100% and 98.6% ± 1.4% for Group 2, 93.6% ± 2.3% and 83.6% ± 3.5% for Group 3, and 82.1% ± 4.1% and 77.8% ± 4.4% for Group 4 (Fig. 3B). The 4-year OS and EFS according to clinical stage were 100% and 97.7% ± 2.3% for stage I, 100% and 97.8% ± 2.0% for stage II, 92.0% ± 2.9% and 82.9% ± 4.0% for stage III, 84.6% ± 5.8% and 71.8% ± 7.2% for stage IV. The 4-year OS and EFS of B-ALL were 86.2% ± 4.0% and 83.6% ± 4.3%. The 4-year EFS by histology was 86.1% ± 2.6% for BL/BLL, 87.3% ± 3.5% for DLBCL, 92.1% ± 4.3% for others, and 100% for MLBCL ($P = 0.717$) (Fig. 3C). When we analyzed the outcome of patients who had BM or CNS disease, the 4-year EFS was 83.8% ± 4.3% for patients (n = 74) with BM involvement only (BM+/CNS−), 60.0% ± 1.5%

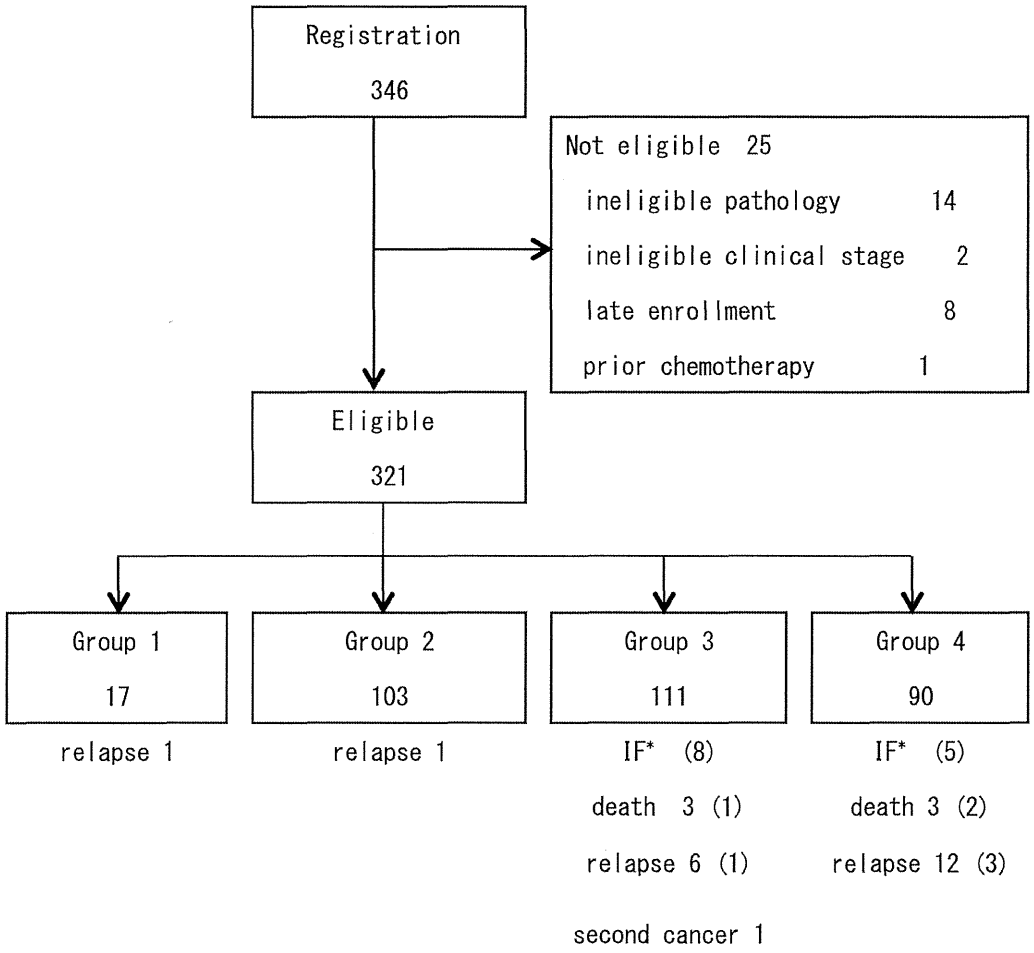

Fig. 2. Patient flow chart and events according to the treatment group. There were 40 events which consisted of each one in Group 1 and 2, 18 in Group 3, and 20 in Group 4. Number in parentheses indicates events occurred during protocol chemotherapy. Number in parenthesis indicates events occurred during protocol chemotherapy. *IF, induction failure defined as patients did not achieve complete remission or unconfirmed remission at the last evaluation time in group 3/4.

TABLE II. Patients Characteristics

Therapy groups	G1	G2	G3	G4	Total (%)
No. of patients	17	103	111	90	321
Sex					
Male	12	72	90	71	245 (76)
Female	5	31	21	19	76 (24)
Age					
0–4	2	12	18	16	48 (15)
5–9	3	45	42	39	129 (40)
10–14	8	42	42	27	119 (37)
15–	4	4	9	8	25 (8)
Histology					
BL/BLL/B-ALL	5	33	62	80	180 (56)
DLBCL	12	58	26	5	101 (31.4)
MLBCL	0	0	2	0	2 (0.6)
Others	0	12	21	5	38 (12)
Primary sites					
Thorax	5	30	7	1	43
Head & neck	5	39	12	2	58
Peripheral lymph nodes	0	3	3	0	6
Abdomen	7	29	75	11	122
Mediastinum	0	0	8	0	8
B-ALL	0	0	0	73	73
CNS	0	0	0	2	2
Other tumor site	0	2	5	0	7
Not specified	0	0	1	1	2
BM involvement	0	0	22	80	102 (32)
CNS involvement	0	0	0	38	38 (12)

BL, Burkitt lymphoma; BLL, Burkitt-like lymphoma; B-ALL, Burkitt leukemia; DLBCL, diffuse large B-cell lymphoma, MLBCL, mediastinal large.

for patients (n = 10) with CNS involvement only (BM−, CNS+), and 75.0% ± 8.2% for patients (n = 28) with BM and CNS involvements (BM+/CNS+), ($P = 0.102$) (Fig. 3D). Outcome by treatment response to initial A courses were as follows: The 4-year OS and EFS for patients who achieved CR (n = 236) or CRu (n = 54) at the last evaluation time were 95.7% ± 1.6% and 93.5% ± 1.6%, and 96.1% ± 2.7% and 86.9% ± 4.6%, respectively, while the 4-year OS and EFS for patients (n = 13) who did not achieve CR/CRu was 69.2% ± 12.8% and 15.4% ± 10.1% ($P < 0.001$), respectively.

Treatment Failure Events

Forty patients experienced an event and 25 have died (Fig. 2). The cause of death was tumor progression in 14, infection in 7, stem cell transplantation-related death in 3, and pulmonary bleeding in 1. The 40 events consisted of 13 induction failures, 6 deaths, 20 relapses, and one second cancer. Of the13 patients (6 in Group 3 and 7 in Group 4) who failed the initial treatment, 4 patients in Group 3 received salvage therapy and achieved CRu. At the time of the last analysis, 8 patients (4 in Group 3 and 4 in Group 4) were alive without tumor. Death in remission occurred in 3/321 (1%) patients: two died of infection and one died of pulmonary bleeding. The longest duration before relapse from the start of therapy was 38.9 months in DLBCL and 13.6 months in Burkitt histology. Relapse sites were 10 in local, 6 in BM, 2 in BM+CNS, one in local + CNS, and one in CNS. All CNS relapse occurred in patients with BL, but not with DLBCL. Thus, isolated CNS failure was only one among 38 patients with CNS involvement. Of the 20 relapsed patients, 11 died and 9 survived without tumor. A second cancer occurred among the patients who failed the initial treatment: a 12-year-old male with BL developed a secondary malignancy with acute myeloid leukemia (FAB M5) 17 months after the initial diagnosis.

Toxicity

Acute toxicity of treatment courses (A and B) was evaluated by the scale of NCI-CTC version 2.0., and rates of acute toxicity Grade 3 among patients in Groups 2, 3, and 4 are shown in Supplemental Table I. Anemia and neutropenia were the most frequent hematological toxicities with grade III or IV in all groups. In particular, grade IV neutropenia occurred in almost all patients (>98%) during A courses. In nonhematologic toxicity, infection was the single most frequent occurring with grade III or IV at least once in 70% of patients although the rate of grade IV infection was very small (<1%). Stomatitis and hepatotoxicity were also frequent, occurring with grade III or IV at least once in 20–35% and 24–38% of patients, respectively. The rate of renal toxicity grade III was very low. Leukoencephalopathy was reported in two patients of Group 3, and their MRI findings disappeared within 2 months without neurological symptoms. The overall incidence of renal insufficiency associated with tumor lysis syndrome was 2 out of 96 (2%) in Group 4, and these required assisted renal support with continuous hemodiafiltration.

DISCUSSION

During the last two decades, the survival outcome of children with B-NHL has been markedly improved through consecutive

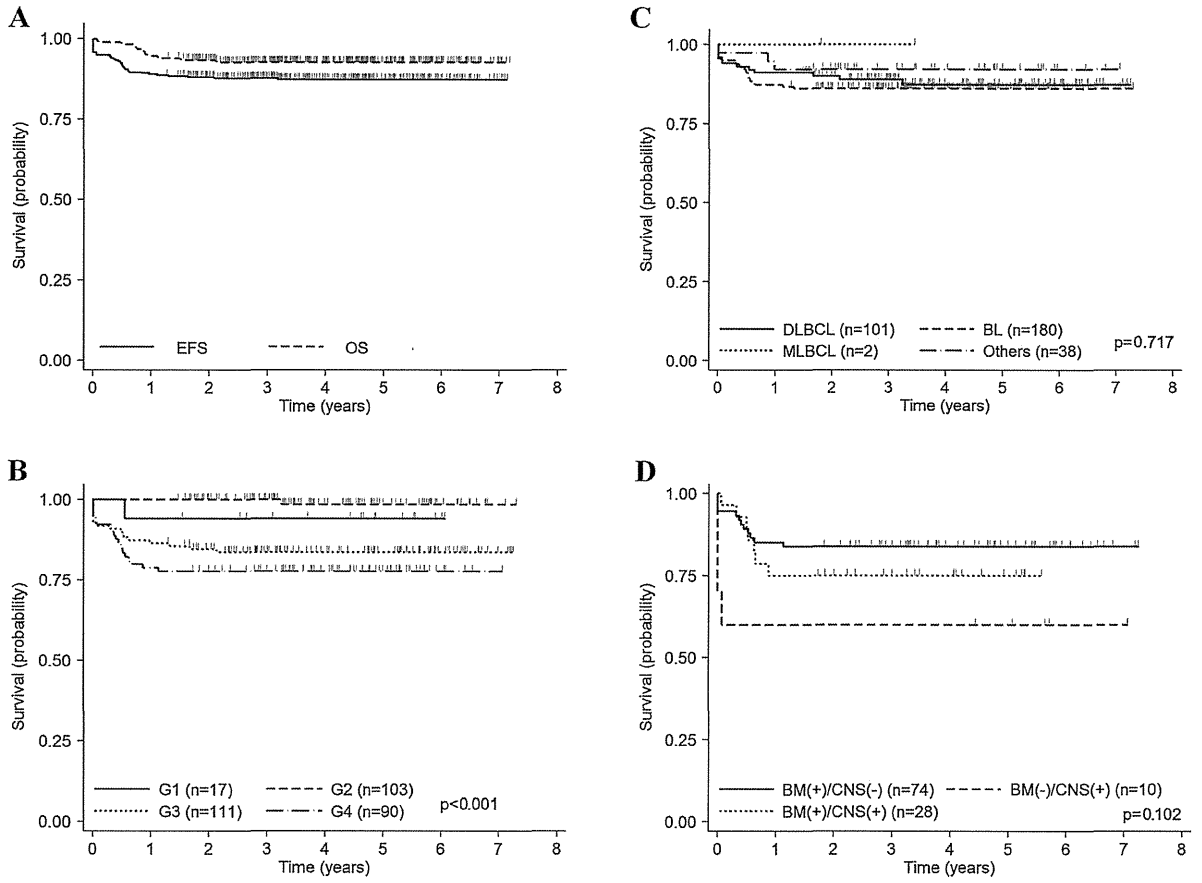

Fig. 3. Kaplan–Meier curves for OS and EFS of all patients (**A**). Kaplan–Meier curves for EFS according to treatment group (**B**), histology (**C**), and BM/CNS involvement (**D**).

clinical trials in large study groups, and the cure rate of childhood B-NHL has reached 90% [1–6]. In the present study, we showed an excellent survival outcome with 4-year OS 93% in children with B-NHL. In our study, the 4-year EFS 84% of Group 3 patients was considerably lower than the 4-year EFS 90% of intermediate risk group in the FAB/LMB96 study [5] or the 6-year EFS 88% of stage III patients in the BFM90 study [2], whereas, the 4-year EFS 78% of Group 4 patients compared favorably with the 4-year EFS 79% of high-risk group in the FAB/LMB96 study [5] and the 6-year EFS 74% of stage IV/B-ALL patients in the BFM90 study [2]. This outcome was obtained via the short-intensive chemotherapy regimen based on COPAD (CPM, VCR, PSL, and ADR) regimen plus the HDMTX of the lymphomas malin B (LMB) studies [3]. We omitted cranial irradiation for all patients, because recent studies have suggested the possibility of deleting radiotherapy in treating CNS diseases as well as CNS prophylaxis [2,3,5,9]. However, having no experience in administrating 8 g/m^2 HDMTX, we employed 5 g/m^2 HDMTX over 24 hour-infusion and not the 8 g/m^2 HDMTX over 4 hour-infusion in the LMB protocols for treating patients with CNS disease [3,5]. The treatment result for CNS disease was satisfactory, because CNS failure was only one of 38 patients with primary CNS disease in the present study.

This suggests that the 5 g/m^2 HDMTX over 24 hour-infusion is equally as effective to the CNS-positive disease as the aforementioned 8 g/m^2 HDMTX over 4 hours infusion, and reinforces the possibility that CNS irradiation could be omitted without jeopardizing the outcome of patients with CNS disease by using systemic and it MTX therapy [3,5,9].

The treatment of DLBCL as well as BL was another important focus of our study, because the incidence of DLBCL in childhood B-NHL is relatively more frequent than that of Western countries: the number of DLBCL was almost similar to that of BL (excluding B-ALL) in the present study and our recent national survey for childhood hematological malignancies has shown that the ratio of DLBCL to BL was 0.79 [14]. In our study, according to the strategy that DLBCL was treated by short-pulse chemotherapy as well as BL [15], we followed the same protocol, and achieved a favorable outcome of 4-year EFS with 87% for DLBCL which was not inferior to that of BL. This outcome can be partly explained by shared biological features, that is, that more than half of childhood DLBCL has the molecular subtypes of BL [16].

Several factors associated with poor outcome in the high-risk group in childhood B-NHL have been reported. Cairo et al. has shown a significantly inferior outcome (4-year EFS 61% ± 6%) of the subgroup of children with combined BM and CNS involvement at diagnosis as compared with children with BM or CNS only [5]. However, our results in Group 4 showed that the outcome (4-year EFS 75% ± 8%) of this subgroup with BM+/CNS+ was not significantly inferior than that of the subgroup with BM+ (83% ± 4%) or CNS+ (60% ± 1%). Failure to initial therapy is

also known to be a strong, unfavorable prognostic factor. Past studies in LMB 89/96 have shown that non-responders to pre-phase therapy (COP regimen) suffer a significantly inferior outcome as compared with responders or incomplete responders [3,5]. In our study, an appropriate evaluation of tumor regression just after pre-phase therapy was difficult for many patients, such that we compared the outcome according to response at the final evaluation time after two or three courses of therapy. These results showed that 4-year EFS of patients who did not achieve CR/CRu was only $15\% \pm 10\%$, which was as dismal as the outcome of poor-responders to COP regimen in the FAB/LMB 96 study [5]. To rescue the poor-responders in our study, we employed salvage therapy with high-dose Ara-C and VP16 to patients who did not achieve remission after 2 or 3 courses of therapy in Group 2 or 3, as in the BFM90 or FAB96 study [2,4]. As a result, 4 of 6 patients in Group 3 received salvage therapy and survived without tumor. This response rate was similar to that of FAB96 study, in which 10 out of 16 patients who received the second phase treatment intensification after the consolidation phase were alive. Thus, our results reconfirmed the efficacy of the salvage therapy.

Management of acute toxicity by short-pulse intensive chemotherapy is essential to successfully carry out the treatment protocol for childhood B-NHL. In our study, grade IV neutropenia occurred in almost all patients, but the rate of grade IV infection was quite low. Consequently, therapy-related death was less than 1% in all patients, and 2.1% in Group 4 patients. These results show the safety and feasibility of our treatment protocol. Anthracycline cardiotoxicity and secondary malignancy by alkylating agents are serious late events in pediatric cancer treatment [17,18]. To reduce the risk of cardiotoxicity, we employed THP-adriamycin (pirarubicin) instead of ADR. Pirarubicin is a derivative of ADR with reportedly less cardiotoxicity in adults [19–24]. Recently, we have reported that no significant cardiac dysfunction was detected in long-term survivors of children with acute lymphoblastic leukemia who received THP treatment [25–27]. In the present study, there were no patients with cardiac insufficiency or cardiac myopathy during the 7-year observation period. These results suggest that late-onset cardiotoxicity induced by pirarubicin is uncommon in childhood lymphoid malignancies, at least up to the cumulative dose of 240 mg/m^2. In our study, there was one male with a second cancer with acute myeloid leukemia, although the correlation between his second cancer and the protocol treatment is uncertain because he was resistant to the pre-phase followed by arbitrary treatment.

As shown above, chemotherapy-related toxicity of our protocol treatment was within acceptable range. However, a 6-course treatment for Group 3 seemed to be more intensive as compared with a 4-course treatment for intermediate risk group in the FAB96 study [4]. In order to reduce the total dose of cytotoxic drugs without impairing the survival outcome, new approaches including targeted monoclonal antibody therapy in combination with chemotherapy [28,29], are needed for children with an advanced or resistant disease in coming studies.

In conclusion, our nationwide study resulted in a cure rate above 90% with <1% toxic death in childhood B-NHL.

ACKNOWLEDGMENTS

We would like to thank all of the patients who enrolled in the B-NHL03 study and their families. This study was supported by Grants for Clinical Cancer Research from the Ministry of Health, Labor and Welfare of Japan; H14-Koka(Gan)-031, H15-Koka(Gan)-024, H16-GanRinsho-004, H17-GanRinsho-004, H20-GanRinsho-Ippan-017, H23-GanRinsho-Ippan-014.

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Leukemia (2015) **29**, 290–296

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ORIGINAL ARTICLE

Early use of allogeneic hematopoietic stem cell transplantation for infants with *MLL* gene-rearrangement-positive acute lymphoblastic leukemia

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Sixty-two infants with *MLL* gene-rearrangement-positive acute lymphoblastic leukemia (MLL-r ALL) were treated with the MLL03 protocol of the Japanese Pediatric Leukemia/Lymphoma Study Group: short-course intensive chemotherapy followed by early allogeneic hematopoietic stem cell transplantation (HSCT) within 4 months of the initial induction. The 4-year event-free survival and overall survival rates were 43.2% (95% confidence interval (CI) = 30.7–55.1%) and 67.2% (53.8–77.4%), respectively. A univariate analysis showed younger age (<90 days at diagnosis), central nervous system disease and poor response to initial prednisolone therapy significantly associated with poor prognosis ($P<0.05$). In a multivariate analysis, younger age at diagnosis tended to be associated with poor outcome (hazard ratio = 1.969; 95% CI = 0.903–4.291; $P=0.088$). Although the strategy of early use of HSCT effectively prevented early relapse and was feasible for infants with MLL-r ALL, the fact that substantial number of patients still relapsed even though transplanted in their first remission indicates the limited efficacy of allogeneic HSCT for infants with MLL-r ALL. Considering the risk of severe late effects, indications for HSCT should be restricted to specific subgroups with poor risk factors. An alternative approach incorporating molecular-targeted drugs should be established.

Leukemia (2015) **29,** 290–296; doi:10.1038/leu.2014.172

INTRODUCTION

Acute lymphoblastic leukemia (ALL) in infants younger than 1-year old accounts for 2.5–5% of childhood ALL and carries clinical and biological features distinct from those of ALL in older children.[1] Patients have a high frequency (up to 80%) of 11q23 translocations/*MLL* gene rearrangements (MLL-r) and a highly distinctive gene expression profile, and majority of infants with MLL-r ALL are characterized by a high white blood cell count and marked hepatosplenomegaly at presentation, and by a pro-B-cell phenotype of their leukemic cells, which lack CD10 expression.[2] The prognoses of these patients are very poor, as illustrated by recent published long-term event-free survival (EFS) and overall survival (OS) rates of 42–54% and 45–61%, respectively.[3–10] In a large international study, Interfant-99, the presence of MLL-r, a very high white blood cell count, age <6 months and a poor response to prednisolone prophase were associated with inferior outcomes. Notably, the 4-year EFS of MLL-r patients was only 36.9%, which is much poorer than the 74.1% EFS of *MLL*-germline patients.[3]

Between 1995 and 2001, we conducted two consecutive Japanese nationwide studies of infant ALL, designated MLL96 and MLL98, in which we stratified patients according to their *MLL* gene configurations; all MLL-r ALL infants were assigned intensive chemotherapy followed by allogeneic hematopoietic stem cell transplantation (HSCT) at their first remission.[4] Because of the high rate of early relapse before the time for HSCT was reached, which is frequently observed in infants with ALL, the overall outcomes of these MLL-r patients were far from satisfactory. However, an encouraging result in our study for 3-year posttransplantation EFS (64.4%) in patients receiving HSCT at their first remission prompted us to speculate whether an intervention with more-effective chemotherapy and HSCT in an earlier phase could prevent early relapse and produce a better outcome.[11] Therefore, we planned the MLL03 study and analyzed the outcomes of infants with MLL-r ALL.

MATERIALS AND METHODS

Patients

Between February 2004 and January 2009, 92 consecutive infants younger than 1 year with suspected newly diagnosed ALL from 126 centers and hospitals in Japan were assessed for their eligibility for MLL03. This study included more than 90% of the same patients as the national study of the Japanese Pediatric Leukemia/Lymphoma Study Group (JPLSG). Patients with germline *MLL* gene, mature B-cell ALL, Down syndrome or congenital ALL cases with gestational age of less than 37 weeks were excluded according to the eligibility criteria of the study (Supplementary Table 1). The diagnosis of ALL was established based on bone-marrow morphology (or peripheral

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Received 19 March 2014; revised 14 May 2014; accepted 28 May 2014; accepted article preview online 3 June 2014; advance online publication, 24 June 2014

blood morphology, if bone-marrow aspiration resulted in a dry tap), cytochemical staining and immunophenotyping, which were confirmed with a central review system. The leukemic cell karyotypes were determined with a cytogenetic analysis using a G-banding technique. The *MLL* gene configuration of each patient was determined with a Southern blotting analysis, as described previously,[12] and the chimeric genes *MLL–AF4*, *MLL–AF9*, *MLL–AF6* and *MLL–ENL* were also examined with real-time PCR. With these strategies, 30 patients were excluded because they did not meet eligible criteria of the study as follows (Supplementary Table 1): *MLL*-germline ALL ($n=20$), mature B-cell ALL ($n=1$), congenital ALL cases with immature gestational age ($n=3$), death before diagnostic confirmation of ALL ($n=1$), unable to start protocol therapy within 7 days after the registration ($n=1$), refusal to participate by the guardian ($n=2$), the patient was registered before the study approval by the institutional review board ($n=1$) and the patient was transferred to a non-JPLSG member hospital ($n=1$; Figure 1). Ultimately, 62 patients with MLL-r ALL were eligible and were enrolled in the protocol study. Written informed consent was obtained from the guardians of the patients according to the Declaration of Helsinki, and institutional review board approval was obtained for all aspects of the study.

Treatment

The details of the therapeutic regimen used in the MLL03 study are described in Table 1. The patients were non-randomly assigned to commence 7-day prednisolone monotherapy. The prednisolone response and the leukemic status of the central nervous system (CNS) were evaluated on day 8, and a prednisolone good responder was defined as an infant with a peripheral blood blast count of less than 1000/μl and a poor responder as an infant with ⩾1000/μl. CNS involvement was defined as >5/μl mononuclear cells with a leukemic morphology. As most other study groups evaluate CNS status on day 1 before any treatment is given, CNS status in the current study might be influenced by the prednisolone prophase. In addition, day 8 evaluation of prednisolone prophase in this study is unique compared with other studies that usually assess peripheral blood blasts after 1 week of prednisolone concurrent with single intrathecal injection of chemotherapy.

The induction phase consisted of dexamethasone, vincristine, doxorubicin, cyclophosphamide and triple intrathecal chemotherapy with methotrexate, cytarabine (Ara-C) and hydrocortisone, followed by an intermediate dose of Ara-C and etoposide (VP-16). Based on the *in vitro* drug sensitivity data presented by Pieters *et al.*[13] showing that the lymphoblasts of infant ALL show high sensitivity to Ara-C, each of the two consolidation courses were intensified with high-dose Ara-C to prevent early relapse. However, L-asparaginase was not included throughout the therapy because of its low sensitivity in infants. All the patients received two initial courses (*induction* and *consolidation-1*) and their remission status was evaluated after each course. Complete remission (CR) was defined by testing bone marrow with less than 5% leukemic cells, regeneration of hematopoiesis and no evidence of leukemia cells elsewhere after either the *induction* or *consolidation-1* course.

Because the main objective of the MLL03 study was to evaluate the efficacy and safety of allogeneic HSCT in the early phase of the disease (within 4 months of the initial induction), all the patients with continuous CR were prescribed the following HSCT after *consolidation-2*. The donors were restricted to two types: human leukocyte antigen ⩾4/6 serologically matched unrelated cord blood or human leukocyte antigen ⩾5/6 matched related donor. The conditioning was a nonirradiation myeloablative regimen with busulfan (BU), VP-16 and cyclophosphamide. An oral formulation of BU was used until October 2006, when the intravenous formulation became available in Japan. Regardless of the drug formulation, the dose of BU was determined according to individual pharmacokinetic tests, with a targeted average steady-state concentration of 600–900 ng/ml.[14] The prophylaxis for graft-versus-host disease was either cyclosporine or tacrolimus combined with short-term methotrexate.

Statistical analyses

All the analyses were performed by the intention-to-treat approach; all the 62 eligible patients were fully analyzed even for the cases that dropped out

Table 1. Treatment for infant ALL with a rearranged *MLL* gene in MLL03 study

Phase and drug	*Delivery, duration*	*Dosage*	*Dose schedule*
PSL prophase			
PSL	IV	60 mg/m^2	Days 1–7
Induction			
DEX	IV	10 mg/m^2	Days 8–21
VCR	IV	0.05 mg/kg	Days 8, 15
CPA	IV,2 h	1 200 mg/m^2	Day 9
DXR	IV, 1 h	25 mg/m^2	Days 10, 12
TIT		age-adjusted[a]	Days 8, 22[b]
VP-16	IV, 2 h	100 mg/m^2	Days 22–25
Ara-C	IV, 4 h	500 mg/m^2	Days 22–25
Consolidation-1			
MIT	IV, 1 h	10 mg/m^2	Day 1
VP-16	IV, 2 h	100 mg/m^2	Days 1–5
Ara-C	IV, 4 h	3000 mg/m^2	Days 1–5
TIT		Age-adjusted[a]	Days 1, 8
Consolidation-2			
VCR	IV	0.05 mg/kg	Day 1
MTX	IV, 12 h	3 000 mg/m^2	Day 1
Leucovorin	IV	15 mg/m^2	36 hr after start of MTX, 7 times
Ara-C	IV, 3 h	3000 mg/m^2 × 2	Days 4, 5
TIT		Age-adjusted[b]	Days 1, 8
Conditioning regimen for hematopoietic stem cell transplantation			
BU	PO/IV	Adjusted based on PK results	Days −8, −7, −6, −5
VP-16	IV, 12 h	60 mg/kg	Day −4
CPA	IV, 2 h	60 mg/kg	Days −3, −2

Abbreviations: ALL, acute lymphoblastic leukemia; Ara-C, cytarabine; BU, busulfan; CPA, cyclophosphamide; DEX, dexamethasone; DXR, doxorubicin; IV, intravenously; MIT, mitoxantrone; MTX, methotrexate; PO, orally; PK, pharmacokinetics; PSL, prednisolone; TIT, triple intrathecal therapy; VCR, vincristine; VP-16, etoposide. The dose of each drug except VCR, PSL, and DEX were reduced by one-third in patients younger than 60 days and by one fourth in those 61–120 days of age. [a]Doses were adjusted according to the patient's age at administration as follows: 90 days old or younger, MTX 3 mg, hydrocortisone (HDC) 10 mg, Ara-C 6 mg; younger than 1 year old, MTX 6 mg, HDC 10 mg, Ara-C 15 mg; 1 year and older, MTX 8 mg, HDC 15 mg, Ara-C 20 mg. [b]Additional TITs on days 15 and 29 for patients with CNS disease.

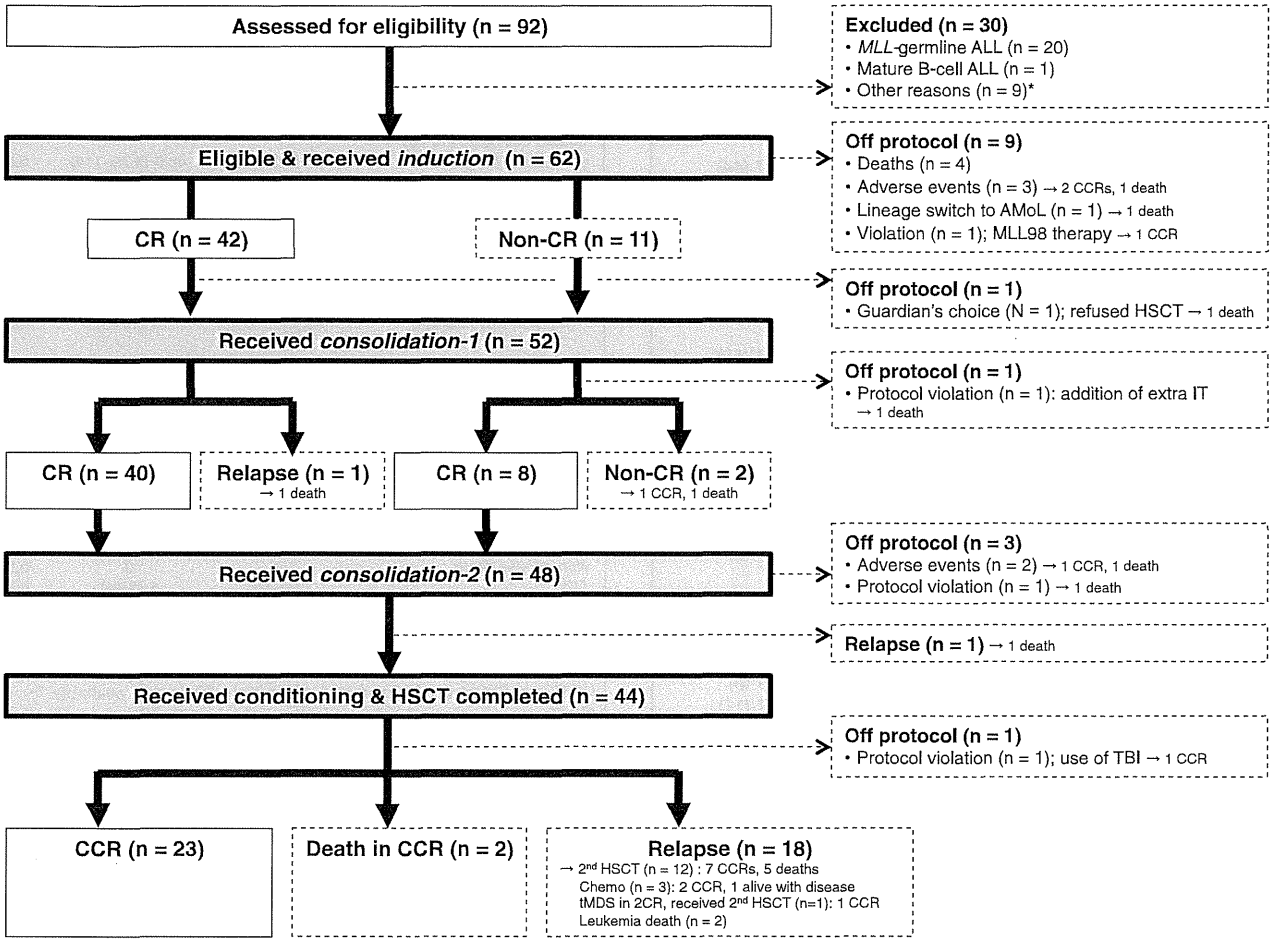

Figure 1. Patient flow chart in the MLL03 study. ALL, acute lymphoblastic leukemia; AMoL, acute monocytic leukemia; CR, complete remission; CCR, continuous CR; HSCT, hematopoietic stem cell transplantation; IT, intrathecal therapy; TBI, total-body irradiation; tMDS, therapy-related myelodysplastic syndrome; 2CR, second CR. *Reasons for exclusion of the nine patients are as follows: congenital ALL cases with immature gestational age ($n = 3$), death before diagnostic confirmation of ALL ($n = 1$), unable to start protocol therapy within 7 days after the registration ($n = 1$), refusal to participate by the guardian ($n = 2$), the patient was registered before the study approval by the institutional review board ($n = 1$) and the patient was transferred to a non-JPLSG member hospital ($n = 1$).

of the study before completing the protocol-specified therapy for various reasons (Table 1). EFS was defined as the length of time from the diagnosis of ALL to the last follow-up or first event (failure to achieve remission, relapse, secondary malignancy or death from any cause). OS was defined as the length of time from the diagnosis of ALL to death from any cause. The probabilities of EFS and OS were estimated with the Kaplan–Meier method and standard errors (s.e.) with the Greenwood formula, and were then compared with the log-rank test; 95% confidence intervals (CIs) were computed. A Cox proportional hazards regression model was used to identify the risk factors associated with the EFS rate. Variables including age at initial diagnosis (<90 days vs ⩾90 days), white blood cell count at initial diagnosis (⩾100 000/μl vs <100 000/μl), CNS disease (positive vs negative), cytogenetics (t(4;11)(q21;q23) vs others) and response to initial prednisolone monotherapy (poor vs good responders) were considered for inclusion in the model. The significant variables associated with the EFS rate were then identified. No statistical adjustment was made for the performance of multiple tests, but two-sided *P* values greater than 0.05 were interpreted with caution. All data analyses were performed with the STATA statistical software (version 11.0; StataCorp LP, College Station, TX, USA).

RESULTS

Patient characteristics

The characteristics of the 62 enrolled infants with MLL-r ALL are shown in Table 2. Notably, the proportion of younger infants aged <180 days (6 months) at diagnosis was very high (68% (41/62)) in the present report compared with those in previous reports, in which they usually constituted as much as 50%.[3–5]

Treatment outcomes

Remission induction results. The prednisolone response was evaluated in 59 out of 62 patients (95%): 43 (69%) infants were good responders and 16 (26%) were poor responders. Forty-two patients (67.7%) achieved CR after the initial induction therapy, four patients died (because of sepsis ($n = 2$), acute respiratory distress syndrome after respiratory syncytial virus infection ($n = 1$) and liver failure ($n = 1$)) and five patients dropped out of the protocol with one of the following reasons: severe adverse events ($n = 3$: heart failure or renal failure from tumor lysis syndrome and respiratory syncytial virus bronchiolitis. The patient with heart failure eventually died of leukemia progression, and the other two are alive in continuous CR.), lineage switch to acute monocytic leukemia ($n = 1$: died of leukemia) and protocol violation ($n = 1$: alive with continuous CR after HSCT following MLL98 chemotherapy; Figure1). Notably, five of these eight patients (excluding the protocol violation case) were less than 90 days of age (six were <180 days of age) at diagnosis. In addition, one patient, although achieved CR after *induction*, dropped out of the protocol because

Table 2. Characteristics of 62 MLL-r ALL infants enrolled on study MLL03

	No. of patients (%)
Sex	
Male	27 (44)
Female	35 (56)
Age, days	
<90	22 (36)
90 to <180	20 (32)
180 to <366	20 (32)
WBC count, 10^9/L	
<100	34 (55)
100 to <300	11 (18)
⩾300	17 (27)
Immunophenotype	
Pro-B	42 (68)
Pre-B	4 (6)
Common B	9 (14)
AMLL	6 (10)
AUL	1 (2)
11q23 abnormality	
t(4;11)(q21;q23) or *MLL-AF4*	31 (50)
t(9;11)(p22;q23) or *MLL-AF9*	4 (6)
t(11;19)(q23;p13) or *MLL-ENL*	3 (5)
Other 11q23 abnormalities	5 (8)
Other abnormalities	7 (11)
Normal karyotype	9 (15)
Not evaluable	3 (5)
CNS disease	
Positive	11 (18)
Negative	48 (77)
Not evaluable	3 (5)

Abbreviations: AMLL, acute mixed-lineage leukemia; AUL, acute undifferentiated leukemia; CNS, central nervous system; MLL-r ALL, *MLL* gene-rearrangement-positive acute lymphoblastic leukemia; WBC, white blood cell.

the guardian refused the HSCT strategy and withdrew the consent. As a result, total 52 patients received *consolidation-1* and 40/41 patients continued to be CR, 8 extra cases entered CR, 1 relapsed (died of leukemia), 2 failed to achieve CR (one is alive in continuous CR and the other died of leukemia progression) and 1 patient dropped out of the study because of protocol violation ($n=1$: died of leukemia after the second relapse).

Thus, the overall CR rate (CR after either *induction* or *consolidation-1*) was 80.6% (50/62).

Transplantation outcome. Total 48 patients received *consolidation-2*, and another 3 patients dropped off the study because of severe adverse events ($n=2$: one is alive in continuous CR and the other died of leukemia progression) and protocol violation ($n=1$: died of leukemia after the second relapse), and 1 patient relapsed (died of leukemia after the second relapse; Figure1). Thus, 44 patients received HSCT in their first remission (1CR), however, one case dropped out of the study because of protocol violation using total-body irradiation as a conditioning regimen. Among the 43 patients who received HSCT per protocol, 31 patients underwent unrelated cord blood transplantation and 12 patients underwent related bone-marrow transplantation. Although the median infused cell dose was higher in the related bone-marrow transplantation group and the median days to platelet engraftment was longer in the unrelated cord blood transplantation group, there were no differences between the two groups in the incidence of acute or chronic graft-versus-host disease, relapse,

Table 3. Comparison of results of HSCT by different donor sources in MLL03 study

	UCBT, n = 31	*RBMT, n = 11*[a]	P *value*
Infused cell dose, x10^7/kg			
Median (range)	10.7 (5.00–21.5)	48.0 (8.70–119)	0.01
Neutrophil engraftment			
n	31 (100%)	11 (100%)	
Median days (range)	16 (14–30)	16 (11–31)	0.65
Platelet engraftment			
n	30 (97%)	11 (100%)	
Median days (range)	40.5 (16–69)	25 (11–52)	0.04
Acute GVHD			
I–II	19	6	
III–IV	2	0	0.67
Chronic GVHD	6	1	0.75
Relapse			
Total	13	5	0.87
BM relapse	10[b]	2	
Isolated EM relapse	1	1	
Combined BM/EM relapse	2	2	
Non-relapse death	1	0	0.58
CCR	17 (54%)	6 (54%)	0.73

Abbreviations: BM, bone marrow; CCR, continuous complete remission; EM, extramedullary; GVHD, graft-versus-host disease; HSCT, hematopoietic stem cell transplantation; RBMT, related bone marrow transplantation; UCBT, unrelated cord blood transplantation. [a]Data not available for one RBMT case, because of sudden death on day +125. [b]One case developed therapy-related myelodysplastic syndrome 4 years after the second CR.

non-relapsed death or the percentage of continuous CR (Table 3). Eighteen patients relapsed after HSCT: twelve in the bone marrow, two in an extramedullary site and four in both. Among these patients, 13 underwent a second HSCT: 7 are alive in continuous CR for median follow-up of 4.4 years (range = 2.5–5.4 years) post second HSCT, 1 is alive but has developed secondary myelodysplastic syndrome and 5 died (3 of disease-related and 2 of HSCT-related causes). Of the remaining five patients, two with isolated subcutaneous relapse are alive in CR after chemotherapy with or without local irradiation, one is alive but relapsed after salvage chemotherapy and two died of disease progression.

Analysis of overall outcome. The median follow-up period of all the 62 patients was 4.0 years (range = 0–7.4 years). The 18-month EFS rate, a primary end point of this study, was 53.2% (95% CI = 40.1–64.6%). The 4-year EFS and OS rates were 43.2% (95% CI = 30.7–55.1%) and 67.2% (95% CI = 53.8–77.4%), respectively (Figure 2). The 4-year EFS rates according to the different risk factors are presented in Table 4; younger age at diagnosis (<90 days), CNS disease and poor response to initial prednisolone monotherapy were significantly associated with a poor prognosis in the univariate analysis. In the multivariate analysis, only younger age at diagnosis tended to be associated with a poorer EFS rate (hazard ratio = 1.969 (95% CI = 0.903–4.291); $P=0.088$), but the association was not statistically significant, probably because the number of infants analyzed was small. Other associations, such as with CNS disease (hazard ratio = 1.243 (95% CI = 0.421–3.655); $P=0.694$) and poor prednisolone response (hazard ratio = 1.078 (95% CI = 0.507–2.291); $P=0.875$), were also statistically insignificant.

Outcome by minimal residual disease (MRD). The significance of MRD was evaluated by measuring selected *MLL*-fusion transcripts in several patients using real-time quantitative PCR in an add-on study. Unfortunately, MRD could not be consistently monitored and the results were not used to guide therapy. Only 11 samples

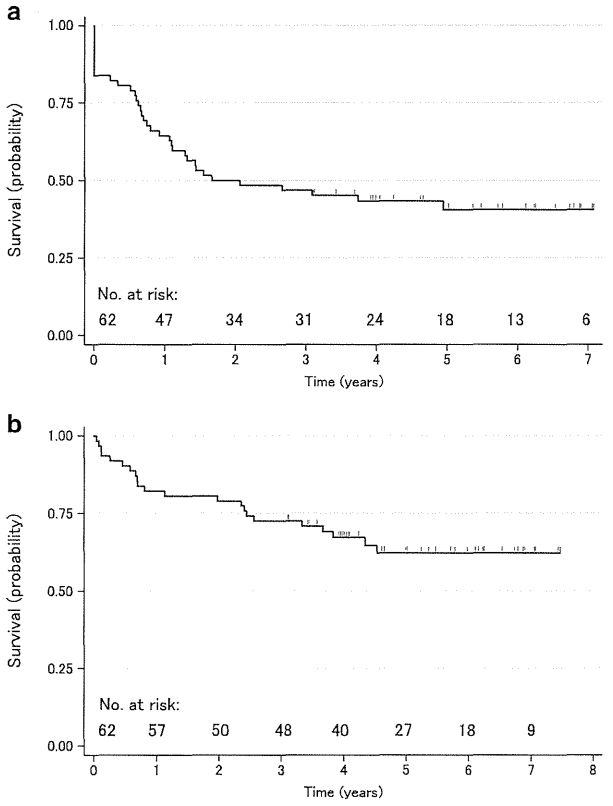

Figure 2. Outcomes of infants with ALL and *MLL*-gene rearrangements enrolled in the MLL03 study. (**a**) Event-free survival (EFS). (**b**) Overall survival.

were available at the end-of-induction time point and 10 samples at the transplantation time point because it was difficult to collect paired samples. At the end-of-induction time point, two patients were MRD positive and one eventually relapsed. The 4-year EFS rate for the nine patients negative for MRD after induction was 40.0% (95% CI = 12.2–67.0%; $P = 0.052$). At the pretransplantation time point, two patients were MRD positive and ultimately relapsed. Of the eight patients with negative MRD at HSCT, five relapsed and three cases are in continuous CR; the 4-year EFS rate was 37.5% (95% CI = 8.7–67.4%; $P = 0.081$).

Safety analysis

The grade 3 and 4 toxicities in each treatment phase, evaluated according to the second version of the National Cancer Institute Common Toxicity Criteria, are described in Table 5. Hematological toxicities and nonhematological toxicities, such as diarrhea, elevated liver transaminases and infections, were quite common throughout all treatment phases. Serious complications, such as pulmonary or neurologically related complications or hemorrhage, were predominantly observed during the *induction* phase. Notably, tumor lysis syndrome was observed in 40% of patients presumably because rasburicase, a recombinant urate oxidase, was not available in Japan during the study period.

DISCUSSION

Infant MLL-r ALL is one of the most difficult to cure of all the subtypes of childhood acute leukemia, and the EFS rates are estimated to be less than 40%, even in recently reported studies of patients treated with intensive chemotherapy with or without HSCT.[3–5] The major factor responsible for the high failure rate is the high relapse rate in the early postremission phase of treatment. In fact, more than half treatment failures occurred before HSCT in our previous MLL96 and MLL98 studies, which made it difficult to assess the true impact of allogeneic HSCT on infants with MLL-r ALL.[4] Therefore, in the present study, we intensified the pretransplantation chemotherapy with high-dose Ara-C and assigned all eligible patients to receive allogeneic HSCT in the early postremission phase, within 4 months of the initial induction therapy.

This strategy was feasible because nearly 90% (44/50) of those who achieved remission were able to undergo allogeneic HSCT in 1CR. However, the low overall CR rate, attributable to high induction toxicity, and the substantial number of patients who still relapsed after HSCT resulted in a 4-year EFS rate of 43.2%, which is no better than the previous reports including the study Interfant-99; only 12% (37/297) of the MLL-r cases in the Interfant-99 study received allo HSCT in 1CR.[3] One factor that affected the outcome of this study was an unexpectedly high proportion of younger infants, less than 180 days of age, at diagnosis. It is well known that a younger age at diagnosis is associated with a higher risk of induction toxicity and relapse, and is definitely a poor prognostic factor in MLL-r infants with ALL.[3–5,15] The 4-year EFS rate of the 42 patients <180 days old was 35.3%, whereas that of the 20 patients ⩾180 days old was 60.0%. Another potentially associated factor was the lower treatment potential of the pretransplantation chemotherapy given in this study. We completely eliminated the use of L-asparaginase because its activity against infant MLL-r ALL is low, and this strategy could have adversely affected the outcome, despite the treatment intensification with high-dose Ara-C. One should realize that *in vitro* resistance to a certain drug does not mean absolute resistance to that drug, but is a relative to that of other types of ALL. Furthermore, our strategy of minimizing the chemotherapy courses given before HSCT could have meant that they were insufficient to reduce the leukemic burden, which might have resulted in post-HSCT relapse in some cases. The correlation between MRD and treatment outcome was evaluated in a small proportion of patients in this study, and those with

Table 4. Comparison of 4-year EFS according to the risk factors in 62 MLL-r ALL in MLL03 study

	No. of patients	*4-Year EFS rate, %*	*95% CI*	P *value*
Age, days				
<90	22	22.7	8.2–41.4	0.016
⩾90	40	54.4	37.7–68.4	
<180	42	35.3	21.3–49.7	0.102
⩾180	20	60.0	35.7–77.6	
WBC count, × 10^9/l				
<100	34	46.4	29.0–62.1	0.328
⩾100	28	39.2	21.6–56.5	
CNS disease				
Positive	11	27.2	6.5–53.8	0.046
Negative	48	49.7	34.9–62.8	
Karyotype				
t(4;11)(q21;q23)	31	41.9	24.6–58.3	0.613
Others	31	47.4	28.3–64.2	
Prednisolone response				
PGR	43	53.1	37.2–66.7	0.013
PPR	16	25.0	7.7–47.1	

Abbreviations: CI, confidence interval; CNS, central nervous system; EFS, event-free survival; MLL-r ALL, *MLL* gene-rearrangement-positive acute lymphoblastic leukemia; PGR, prednisolone good responder; PPR, prednisolone poor responder; WBC, white blood cell.

Table 5. Grade 3 and 4 toxic events by different treatment phases in 62 MLL-r ALL infants in MLL03 study

Toxicities *Patients assessed*	*Induction* n = *62 (%)*	*Cons-1* n = *52 (%)*	*Cons-2* n = *48 (%)*	*Conditioning* n = *44 (%)*[a]
Blood/bone marrow				
Hemoglobin	58 (94)	44 (85	41 (85)	40 (91)
Leukocytes (total WBC)	59 (95)	52 (100)	48 (100)	44 (100)
Neutrophils/granulocytes	62 (100)	52 (100)	48 (100)	44 (100)
Platelets	59 (95)	50 (96)	46 (96)	40 (91)
Gastrointestinal				
Stomatitis/pharyngitis	7 (11)	2 (4)	0 (0)	18 (41)
Vomiting	0 (0)	2 (4)	0 (0)	4 (9)
Diarrhea	13 (21)	15 (29)	6 (13)	12 (27)
Constipation	1 (2)	0 (0)	0 (0)	1 (2)
Pancreatitis	0 (0)	0 (0)	0 (0)	0 (0)
Hepatic				
Total bilirubin	7 (11)	1 (2)	2 (4)	8 (18)
AST/ALT	27 (44)	7 (13)	26 (54)	4 (9)
Metabolic/laboratory				
Amylase	0 (0)	0 (0)	0 (0)	—
Hyperglycemia	2 (3)	0 (0)	0 (0)	—
Renal/genitourinary				
Creatinine	1 (2)	0 (0)	0 (0)	0 (0)
Proteinuria	1 (2)	0 (0)	0 (0)	
Cardiovascular				
Thrombosis/embolism	1 (2)	0 (0)	0 (0)	0 (0)
Other cardiovascular	1 (2)	0 (0)	0 (0)	
Pulmonary				
Dyspnea	10 (16)	0 (0)	0 (0)	3 (7)
Hypoxia	15 (24)	1 (2)	0 (0)	5 (11)
Infection/febrile neutropenia				
Infection	45 (73)	36 (69)	17 (35)	32 (73)
Allergy/immunology				
Allergic reaction/ hypersensitivity	0 (0)	0 (0)	0 (0)	0 (0)
Syndromes				
Tumor lysis syndrome	25 (40)	0 (0)	0 (0)	—
Thrombotic microangiopathy	—	—	—	0 (0)
Veno-occlusive disease	—	—	—	6 (14)
Dermatology/skin				
Rash/desquamation	1 (2)	0 (0)	0 (0)	5 (11)
Neurology	4 (6)	0 (0)	0 (0)	1 (2)
Hemorrhage	6 (10)	0 (0)	1 (2)	1 (2)

Abbreviations: AST, aspartate aminotransferase; ALT, alanine aminotransferase; *Cons-1, consolidation-1*; *Cons-2, consolidation-2*; MLL-r ALL, *MLL* gene-rearrangement-positive acute lymphoblastic leukemia; WBC, white blood cell. [a]One patient who discontinued the study because of protocol violation (use of total body irradiation) is included.

residual MRD at both the postinduction and pretransplantation time points tended to show a worse outcome. Moreover, the fact that nearly half the patients ultimately relapsed even though transplanted in 1CR indicates the limited efficacy of allogeneic HSCT itself for infants with MLL-r ALL.

Although the EFS rate of our study cohort was not satisfactory, their 4-year OS rate of 67.2% was relatively good. This can be explained by the low HSCT-related mortality rate and the relatively high salvage rate after relapse compared with those in our previous MLL96 and MLL98 studies.[16] Among the 43 patients who received allogeneic HSCT per protocol, only 2 (4.6%) died of non-HSCT toxicities (1 of transfusion-related lung injury and 1 with unknown sudden death), although this death rate was high (15.0%, 8/53) in our previous MLL96 and MLL98 studies.[4] Several reasons can be proposed to explain this observation. One is the introduction of an appropriate dose of BU in the conditioning regimen, based on individual pharmacokinetic studies. It is well recognized that the pharmacokinetics of BU vary widely among infants, which may lead to severe post-HSCT organ damage, including lung injury, hepatic veno-occlusive disease, etc.[14,17] Second, the minimum course of pre-HSCT chemotherapy might have reduced the potential organ damage that could have occurred if the patient were instead heavily treated with multiple courses of chemotherapy. Therefore, our strategy of including short-course chemotherapy and the early use of HSCT with individually tailored doses of BU could have reduced both the early-relapse rate and the transplantation-related deaths. The fact that 7 out of 13 cases of post-HSCT relapse were salvaged with a second HSCT may also reflect the low toxic potential of the early HSCT strategy, as mentioned above. Of course, late effects are yet to be evaluated in this study and must be observed especially carefully in these cases.

Because of the limited effectiveness of HSCT and the potential risk of late effects, alternative strategies with novel targeted therapies should be explored for infants with MLL-r ALL.[4,18,19] Recent research has demonstrated that the aberrant epigenetic status, induced by a reciprocal *MLL* translocation via the H3K79 methyltransferase DOT1L, has a central role in MLL-r leukemogenesis.[20–22] The clinical development of epigenetic modifiers, such as DNA methyltransferase inhibitors and/or histone deacetylase inhibitors, is currently in progress. A small-molecule inhibitor of DOT1L is also in clinical development. Meanwhile, HSCT should be restricted to patients at higher risk of relapse, who are likely to benefit from this treatment modality.[23] This stratification is currently being evaluated in our ongoing JPLSG MLL-10 study.

In conclusion, short-course chemotherapy and the early use of HSCT in our study was feasible for infants with MLL-r ALL. However, given the limited effects of HSCT and the potential risk of late effects, the indication for HSCT should be restricted to specific subgroups with poor risk factors, and an alternative approach incorporating molecular-targeted drugs should be established in the future.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank all JPLSG investigators and the following investigators who contributed to the central diagnosis in this study: H Nakamura and S Yamagata (Chiba University, Chiba) for the BU pharmacokinetic study; Y Komada (Mie University Graduate School of Medicine, Mie) and H Ohta (Osaka University, Osaka) for immunophenotypic diagnostics; T Taki (Kyoto Prefectural University of Medicine, Kyoto) for the molecular biological analyses; and J Fujimoto (National Center for Child Health and Development, Tokyo) for the preservation of the diagnostic specimens. This work was supported by a Grant for Clinical Cancer Research and a Grant-in-Aid for Cancer Research from the Ministry of Health, Labour and Welfare of Japan.

AUTHOR CONTRIBUTIONS

K Koh, DT, TM, MH, Y Takahashi, AO, K Kato, KS and EI (principal investigator) participated actively in the study conception and design; K Koh, DT and EI reviewed the data analysis and interpretation and were the main authors of the manuscript; AMS and TW conducted the statistical analysis; TS was responsible for the busulfan pharmacokinetic study; TD and MT were responsible for the immunophenotyping diagnostics; YH was responsible for coordinating the molecular biology analyses; K Koh, K Kato, JT and Y Takeshita recruited patients; MT, KH and SM contributed to the financial and administrative support of the study; and all authors contributed to the conduct of the trial and were involved in the review of the results and the final approval of the manuscript.

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Supplementary Information accompanies this paper on the Leukemia website (http://www.nature.com/leu)