(**Fig. 5c,d** and **Supplementary Table 12**). The SRF pathway was reported as an essential mediator of T cell development in the thymus[24,25], although we found no clue to its functional relevance in AITL development in the literature. We did not observe enrichment of the SRF pathway in either cell type expressing Gly17Val RHOA compared to mock-transfected cells or cells expressing wild-type RHOA. These findings further support the notion that Gly17Val RHOA is a loss-of-function mutant.

The extremely high frequency and specificity of the *RHOA* mutation encoding p.Gly17Val in AITL and AITL-related PTCL cases unequivocally underscore its major role in the development of these subtypes of PTCL (**Supplementary Fig. 17**). The finding of somatic mutation of *RHOA* in lymphoma, particularly of a mutation with a loss-of-function and/or dominant-negative nature, was rather unexpected because the oncogenic potential of RHOA has been implicated in human cancers[26]. However, several lines of evidence previously suggested a tumor-suppressive role for RHOA in T-lineage cells[26,27]. Moreover, transgenic expression of C3 transferase, an inhibitor of the Rho family of proteins (RHOA, RHOB and RHOC) under the *Lck* promoter has been shown to induce thymic T cell lymphoma in mice[28]. Our observations in Jurkat cells expressing wild-type RHOA are also along these lines. Clearly, further studies are warranted to clarify the molecular pathogenesis mediated by the unique *RHOA* mutation encoding p.Gly17Val in AITL and related PTCL, and such studies might have promising implications for the development of novel diagnostics and therapeutics.

URLs. European Genome-phenome Archive, https://www.ebi.ac.uk/ega/; Genomon-exome, http://genomon.hgc.jp/exome/en/index.html; Picard, http://picard.sourceforge.net/; dbSNP131, http://www.ncbi.nlm.nih.gov/projects/SNP/; 1000 Genomes Project, http://www.1000genomes.org/; MSigDB, http://www.broadinstitute.org/gsea/msigdb.

METHODS

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

Accession codes. Genome sequence data are available at the European Genome-phenome Archive under accession EGAS00001000557.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper

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AUTHOR CONTRIBUTIONS

M.S.-Y. prepared DNA samples, sorted the tumor cells, resequenced the samples, and sorted and integrated information. T.E. analyzed the function of wild-type and mutant RHOA. K.Y. resequenced the samples and contributed to the resequencing data analyses. Y. Shiraishi, E.N., K.C., H.T. and S.M. performed bioinformatics analyses of the resequencing data. R.I. and O.N. created the model structure for mutant RHOA. Y.M., H.M., Y.K., R.N.-M., N.B.T., K.S., T.N., Y.H. and M.N. contributed to sample collection and preparation. N.T., S. Sakata, N.N. and K.T. immunostained specimens and performed pathohistological analyses. Y. Okuno and M.S. contributed to the resequencing. A.S.-O. and Yusuke Sato contributed to mRNA sequencing. K.I., Y. Ohta, J.F., S. Shimizu, T.K., Yuji Sato and T.I. collected samples. M.S.-Y., T.E., K.Y., S.O. and S.C. generated figures and tables, and wrote the manuscript. All authors participated in discussions and interpretation of the data and results.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Subjects and samples. Samples were obtained from individuals with AITL or PTCL-NOS, as well as from individuals with other mature T cell, mature B cell and myeloid neoplasms, and were used after approval was obtained from the local ethics committees at all participating institutes (**Supplementary Tables 1** and **3**). Informed consent was obtained from all living subjects. High-molecular-weight genomic DNA was extracted from archived specimens that were frozen fresh or after fixation. DNA was also extracted from paraffin-embedded, formalin-fixed samples for targeted amplicon sequencing. Constitutional DNA samples were obtained from buccal swabs, mononuclear cells from apparently tumor-free bone marrow aspirates or peripheral blood. Data on clinical outcomes were available for 71 subjects. Samples of a subcohort of PTCL-NOS cases were reviewed by four expert hematopathologists.

Within PTCL-NOS cases, a subgroup without the typical morphology of AITL but having two or more of the following immunostaining features was designated T_{FH}-like PTCL-NOS[5,9]: (i) positive staining for CD10 in tumor cells, (ii) positive staining for PD-1 in tumor cells, (iii) proliferation of CD21-positive follicular dendritic cells and (iv) the presence of EBER-positive B cells.

Sorting of the tumor cell–enriched fraction and other fractions. $CD4^+$ and $CD8^+$ T cell fractions were purified from skin tumors from subject PTCL159, and $CD4^+$ and $CD8^+$ T cell, $CD19^+$ B cell and $CD14^+$ monocyte cell fractions were purified from pleural effusion cells from subject PTCL160.

The skin tumor from subject PTCL159 was processed into single-cell suspension. Cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD4 antibody (BD Biosciences, 555346) and phycoerythrin (PE)-conjugated anti-CD8 antibody (Dako, clone DK25) and were then fractionated on a FACSAria (BD Biosciences).

Mononuclear cells (MNCs) were isolated from the pleural effusion of subject PTLC160 by Ficoll-Paque density-gradient centrifugation. MNCs were stained with FITC-conjugated anti-CD4 antibody and anti-CD14 antibody (BD Biosciences, 555397), PE-conjugated anti-CD8 antibody and PE-conjugated anti-CD19 antibody (Dako, clone HD37) and were fractionated on a FACSAria.

Whole-exome sequencing. Tumor DNA was extracted from subject biopsy samples infiltrated with lymphoma cells. DNA from either buccal mucosa, bone marrow MNCs without apparent lymphoma infiltration or peripheral blood cells was used for the paired normal control. Whole-exome capture was accomplished through the hybridization of sonicated genomic DNA to the bait cDNA library synthesized on magnetic beads (SureSelect Human All Exon 50Mb or V4 kit, Agilent Technologies). Captured targets were subjected to massively parallel sequencing using a HiSeq 2000 (Illumina) according to the standard protocol for 100-bp paired-end reads.

Detection of candidate somatic mutations was performed using our in-house pipeline for whole-exome sequencing[10] with minor modifications. Briefly, sequencing reads were first aligned to the human reference genome (hg19) using Burrows-Wheeler Aligner (BWA)[29] version 0.5.8 with default parameter settings. PCR duplicates were eliminated using Picard. The number of reads containing SNVs and indels in both tumor and germline samples was determined using SAMtools[30], and the null hypothesis of equal allele frequencies in tumor and germline samples was tested using the two-tailed Fisher's exact test. A variant was adopted as a candidate somatic mutation if it had $P < 0.01$, was observed in bidirectional reads (i.e., in both the plus and minus strands of the reference sequence) and its allele frequency was less than 0.1 in the corresponding germline sample. Finally, the list of candidate somatic mutations was generated by excluding synonymous SNVs and other variants registered in either dbSNP131, the 1000 Genomes Project or our in-house SNP database constructed from 180 individual samples. All candidates were validated by deep sequencing.

Validation of whole-exome analysis. Genomic DNA from tumors and paired normal samples was amplified using the REPLI-g mini kit (Qiagen). Regions that included candidate mutations were amplified by genomic PCR using KOD cox neo (TOYOBO) with a NotI linker attached to each primer (**Supplementary Table 13**). Products were combined, and DNA was purified using the QIAquick PCR Purification kit (Qiagen) and digested with NotI. Digested DNA was purified again, and a 1.5-μg aliquot of purified DNA was ligated with T4 DNA ligase for 5 h, sonicated into ~150-bp fragments on average using Covaris and used for the generation of sequencing libraries, according to a modified Illumina paired-end library protocol. Libraries were then subjected to deep sequencing on a MiSeq (Illumina) according to the standard protocol for 150-bp paired-end reads.

Data processing and variant calling were performed with a set of modifications to the method described in a previous publication[10]. Each read was aligned to the set of targeted sequences from PCR amplification, for which BLAT[31], instead of BWA[29], was used with the –fine option. Mapping information in the .psl format was converted to the .sam format with paired-read information using an in house–generated my_psl2sam script. The script was derived from the psl2sam.pl script distributed with SAMtools. Minor changes were applied to the original script to give the paired-end information upon conversion. Of the successfully mapped reads, the following reads were excluded from further analysis: reads that mapped to multiple sites, reads that mapped with more than four mismatched bases and reads that had more than ten soft-clipped bases. Next, the Estimation_CRME script was run to eliminate strand-specific errors and to exclude cycle-dependent errors. A strand-specific mismatch ratio was calculated for each nucleotide variant for both strands using data for those bases between 11 and 50 cycles. To calculate the frequency of each SNV, all reads were mapped to the target reference sequence using BLAT. The number of mapped reads was differentially enumerated for the dichotomic alleles, i.e., mutant and wild-type alleles. For indels, individual reads were first aligned to each of the wild-type and indel sequences and then assigned to the one with which better alignment was obtained in terms of the number of matched bases. Allele frequency was calculated by enumerating each allele according to those assignments. SNVs comprising equal to or more than 2.0% of total reads of the tumor sample rather than the germline sample at each nucleotide position, if it existed, were adopted as somatic mutations.

Targeted sequencing of the *RHOA*, *TET2*, *IDH1*, *IDH2* and *DNMT3A* genes. Targeted sequencing was performed to determine the mutation rate in a large series of PTCL samples for the *RHOA*, *TET2*, *IDH1*, *IDH2* and *DNMT3A* genes. DNA samples from 79 tumors (46 AITL and 33 PTCL-NOS) and 9 paired bone marrow or peripheral blood cell samples were analyzed, including 6 pairs of tumors and controls analyzed by whole-exome sequencing.

DNA samples were prepared as follows: 61 DNA samples were extracted from fresh frozen biopsy specimens, and 18 DNA samples were extracted from paraformaldehyde-lysine-periodate (PLP)-fixed frozen specimens (46 samples were original DNA, and 33 samples were amplified using the REPLI-g mini kit). All exons of the selected genes were captured with the SureSelect target enrichment system (Agilent Technologies), and massively parallel sequencing was then performed on a HiSeq 2000.

For each sample, all sequencing reads were aligned to hg19 using BWA version 0.5.8 with default parameters. After all duplicated reads and low-quality reads and bases were removed, allele frequencies of SNVs and indels were calculated at each genomic position by enumerating the relevant reads using SAMtools. Initially, all variants showing allele frequencies of >0.02 were extracted and annotated with ANNOVAR[32] for further consideration if they were found in >6 reads out of >10 total reads and appeared in both plus- and minus-strand reads. All synonymous variants, known SNPs in public and private databases, including dbSNP131, the 1000 Genomes Project as of 21 May 2012 and our in-house database, were removed. Candidate mutations whose allele frequencies were <5% were validated by PCR-based deep sequencing using Ion Torrent (Life Technologies).

Deep sequencing using Ion Torrent. Fragmented DNA was prepared in the same manner as described above. Libraries were then subjected to deep sequencing on Ion Torrent according to the standard protocol for 300-bp single-end reads. After excluding reads whose length was >200 bases or <50 bases to reduce sequencing errors, the allele frequency was calculated for each SNV or indel as described above.

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Hotspot sequencing to identify *RHOA* mutations encoding p.Gly17Val. Eighty DNA samples from tumors were extracted from unfixed biopsy specimens ($n = 1$), PLP-fixed frozen specimens ($n = 38$) and formalin-fixed, paraffin-embedded specimens ($n = 41$). All samples were original DNA without amplification, except for one sample amplified using the REPLI-g mini kit. Samples were subjected to genomic PCR with tagged PCR primers (**Supplementary Table 14**) and were subsequently prepared using the NEBNext DNA Library-Prep Reagent Set for Illumina (New England BioLabs). Products underwent massively parallel sequencing on a MiSeq according to the manufacturer's protocol. The SNV representing a G-to-T change comprising equal to or more than 2.0% of total reads at the c.G50 nucleotide position of the *RHOA* gene was adopted as the mutation. Methods of data analysis were the same as described above.

Antibodies. Antibodies used for protein blots or immunostaining were mouse anti-RhoA (1:1,000; Cytoskeleton, ARH03), mouse anti–β-actin (1:2,000; Sigma, A5441), mouse anti–DDDDK tag (1:10,000; MBL, M185-3), mouse anti–Myc tag (1:10,000 for WB, 1:500 for IHC; MBL, M192-3), mouse anti–GST tag (1:2,000; MBL, M071-3), rabbit anti-ECT2 (1:1,000; Millipore, 07-1364), goat anti–mouse IgG conjugated to horseradish peroxidase (HRP) (1:10,000; Dako, P0447), goat anti–rabbit IgG conjugated to HRP (1:10,000; Dako, P0448) and Alexa Fluor 647–conjugated goat anti–mouse IgG (1:1,000; Invitrogen, A-21235).

Cell lines and transfection. NIH3T3 cells (American Type Culture Collection) were cultured at 37 °C in low-glucose DMEM (Sigma) supplemented with 10% heat-inactivated FCS and 1% penicillin-streptomycin. Cells were transfected with plasmids using FuGene6 transfection reagent (Promega) according to the manufacturer's protocol. Jurkat cells (European Collection of Cell Cultures) were cultured at 37 °C in RPMI-1640 (Sigma) supplemented with 10% FCS and 1% penicillin-streptomycin.

Mutagenesis and constructs. Human *RHOA* cDNA was isolated by PCR amplification from peripheral blood MNC-derived cDNA. Mutagenesis to create constructs encoding the Gly14Val, Gly17Val, Gly17del, Thr19Asn and Ala161Glu mutants was carried out with the PrimeStar Mutagenesis Basal kit (TaKaRa) according to the manufacturer's instructions. All cDNA-encoded products were tagged at their N terminus with the Flag and/or c-Myc epitope. These constructs were subcloned into the pEF-neo expression vector, the pGCDNsamIRESGFP retroviral vector and the tetracycline-inducible lentivirus-based expression vector CS-TRE-PRE-Ubc-tTA-I2G7 (ref. 33). cDNA encoding the ECT2-GFP fusion protein was kindly provided by T. Ishizaki (Oita University). An N-terminal deletion mutant (residues 414–882) of ECT2 was generated with the PrimeStar Mutagenesis Basal kit. Constructs encoding wild-type and Gly17Val RHOA were subcloned into the pGEX-2tk vector (GE Healthcare). All cDNA sequences were confirmed by Sanger sequencing.

Retrovirus production and generation of stable cell lines. For retrovirus production, each retroviral vector was transfected into 293gp packaging cells with a vesicular stomatitis virus G (VSV-G) expression plasmid[34]. Retrovirus-containing supernatant was used for the transduction of 293gpg cells to establish stable cell lines capable of producing high titers of VSV-G pseudotyped retroviral particles. To establish cell lines stably expressing wild-type or mutant RHOA, NIH3T3 cells were infected with these retroviruses. Infected cells expressing GFP were isolated using a FACSAria. The purity of sorted cell fractions consistently exceeded 95%.

Rhotekin binding assays. The amount of the GTP-bound form of the RHOA protein was measured using the RhoA Activation Assay kit (Cytoskeleton) according to the manufacturer's instructions. Briefly, cell lysate was incubated at 4 °C for 1 h with a GST fusion protein containing the RHO-binding domain of rhotekin (GST-RBD) immobilized on glutathione Sepharose beads. After washing the beads twice with lysis buffer and once with wash buffer provided by the manufacturer, we fractionated bead-bound proteins by 12% SDS-PAGE and immunoblotted with anti-RHOA and anti-Flag antibodies. Total cell lysate was also blotted with anti-RHOA and anti-Flag antibodies to assess the fractional ratios of rhotekin-bound RHOA proteins.

GEF-binding assays. GST-fused wild-type and Gly17Val RHOA proteins were prepared as previously described with minor modification[35]. Briefly, GST-fused wild-type and Gly17Val RHOA proteins were expressed in BL21 competent *Escherichia coli* cells (TaKaRa), which were lysed in lysis buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 1% Triton X-100, 1 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride) and subjected to sonication. Lysate was cleared by centrifugation at 20,000*g* for 15 min at 4 °C, incubated with Glutathione Sepharose 4B beads (GE healthcare) for 45 min at 4 °C and washed twice with lysis buffer.

NIH3T3 cells were transiently transfected with a construct expressing the N-terminal deletion mutant of ECT2 by FuGene6. After 48 h, cells were lysed in lysis buffer, cleared by centrifugation and incubated with GST-fused wild-type or Gly17Val RHOA protein bound to Sepharose beads for 2 h. Beads were washed three times with lysis buffer. Bound material was boiled with Laemmli buffer and blotted with anti-GST and anti-ECT2 antibodies.

SRF-RE reporter assays. For the measurement of activity on SRF-RE, luciferase reporter assays were performed using the pGL4.34 reporter vector (Promega), which contains an SRF-RE and a mutant form of the serum response element lacking the ternary complex factor (TCF)-binding domain. SRF-RE was designed to respond to SRF-dependent and TCF-independent signaling such as the signaling that occurs after RhoA activation[19]. NIH3T3 cells were seeded in 24-well plates and cotransfected with pGL4.34 at 40 ng/well, the expression vector pSRα containing β-galactosidase at 20 ng/well and the expression vector pEF-neo containing various *RHOA* cDNA constructs at the concentrations indicated. Luciferase activity was measured at 48 h after transfection, and values were normalized by β-galactosidase activity.

F-actin staining. NIH3T3 cells were transfected with constructs encoding wild-type or mutant RHOA on glass coverslips. After 48 h, cells were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature and permeabilized with 0.5% Triton X-100 in PBS for 10 min. After washing with PBS, cells were incubated with rhodamine phalloidin (100 nM; Cytoskeleton). For double-staining immunohistochemistry, permeabilized cells were blocked with 3% BSA and 0.1% Triton X-100 in PBS. Then, cells were incubated with mouse anti-Myc antibody (1:500 dilution) followed by Alexa Fluor 647–conjugated goat anti–mouse IgG antibody (1:1,000 dilution) and rhodamine phalloidin (100 nM). Nuclei were stained with DAPI. Images were obtained by confocal laser scanning microscopy (Leica).

Lentivirus production and generation of stable cell lines. For lentivirus production, each lentiviral vector was transfected into HEK293T cells with the psPAX2 packaging plasmid and the pMD2.G envelope plasmid. To establish cell lines inducibly expressing wild-type or Gly17Val RHOA, Jurkat cells were infected with these lentiviruses. Infected cells expressing GFP were sorted on a FACSAria. The purity of sorted cell fractions consistently exceeded 95%.

Cell proliferation assays. For cell growth assays, Jurkat cells transduced with lentiviral vectors were incubated in 96-well culture plates, and the absorbance at 450 nm was measured with Cell Counting Kit-8 (Dojindo) according to the manufacturer's instructions.

Cell cycle analysis. Cell cycle distributions were determined by 5-bromo-2′-deoxyuridine (BrdU) and aminoactinomycin D (AAD) incorporation using the APC BrdU Flow kit according to the manufacturer's protocol (BD Pharmingen). Briefly, Jurkat cells were incubated for 30 min in BrdU (10 μM). Then, cells were fixed, permeabilized, treated with DNase and stained with APC-conjugated anti-BrdU antibody and 7-AAD. Flow cytometry was performed on a FACSCalibur cytometer (BD Biosciences), and data were analyzed with FlowJo software (Tree Star).

mRNA sequencing for Jurkat and NIH3T3 cells. Jurkat cells, inducibly expressing wild-type or Gly17Val RHOA, were described above. Wild-type or Gly17Val RHOA protein expression was induced by the addition of 2 μg/ml doxycycline for 2 d ($n = 2$ for each). NIH3T3 cells were transiently transfected with pGCDNsamIRESGFP vector encoding wild-type or Gly17Val RHOA ($n = 2$ for each). After 48 h, GFP-positive cells were sorted by FACSAria.

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Total RNA was extracted by RNeasy mini kit (Qiagen) using the RNase-free DNase kit (Qiagen) to reduce contamination from genomic DNA according to the manufacturer's protocol. Libraries for sequencing were prepared using the Illumina TruSeq RNA Sample Preparation kit v2, according to the manufacturer's instructions. Briefly, poly(A)$^+$ RNA was recovered from 1 μg of total RNA using oligo(dT)-coated Sera-Mag magnetic beads. Recovered poly(A)$^+$ RNA was then chemically fragmented. RNA fragments were converted to cDNA using SuperScript II and random primers. The second strand was synthesized using RNase H and DNA polymerase I. cDNA ends were repaired using T4 DNA polymerase, T4 polynucleotide kinase and Klenow DNA polymerase. A single adenosine was added to 3′ ends using Klenow fragment (3′-to-5′ exo minus). Adaptors were attached to cDNA ends using T4 DNA ligase. Fragments were then amplified by ten cycles of PCR using Phusion DNA polymerase. Libraries were validated with an Agilent 2200 TapeStation (Agilent Technologies) and were applied to an Illumina flow cell using the Illumina Cluster Station. Sequencing was performed on a HiSeq 2000 with the paired-end 100-bp read option, according to the manufacturer's instructions.

Reads obtained from RNA sequencing were mapped to the reference transcript and genome using the Genomon-fusion pipeline. For the expression level of each gene, the fragments per kilobase of exon per million mapped reads (FPKM) value was calculated from mapped reads on the gene. GSEA was carried out using GSEA version 2.0. The top ten highest gene sets of normalized enrichment score were listed on the basis of FDR q values (<0.25). Curated gene sets (c2.kegg.version 4.0, c3.tft.version 4.0 and c5.bp.version 4.0) used in this study were obtained from MSigDB collections.

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Regular Article

LYMPHOID NEOPLASIA

Acute lymphoblastic leukemia in children with Down syndrome: a retrospective analysis from the Ponte di Legno study group

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Key Points

- Although the risk of ALL relapse is significantly higher in children with DS, good-prognosis subgroups have been identified.
- Patients with DS-ALL have higher treatment-related mortality throughout the treatment period independent of the therapeutic regimen.

Children with Down syndrome (DS) have an increased risk of B-cell precursor (BCP) acute lymphoblastic leukemia (ALL). The prognostic factors and outcome of DS-ALL patients treated in contemporary protocols are uncertain. We studied 653 DS-ALL patients enrolled in 16 international trials from 1995 to 2004. Non-DS BCP-ALL patients from the Dutch Child Oncology Group and Berlin-Frankfurt-Münster were reference cohorts. DS-ALL patients had a higher 8-year cumulative incidence of relapse (26% ± 2% vs 15% ± 1%, $P < .001$) and 2-year treatment-related mortality (TRM) (7% ± 1% vs 2.0% ± <1%, $P < .0001$) than non-DS patients, resulting in lower 8-year event-free survival (EFS) (64% ± 2% vs 81% ± 2%, $P < .0001$) and overall survival (74% ± 2% vs 89% ± 1%, $P < .0001$). Independent favorable prognostic factors include age <6 years (hazard ratio [HR] = 0.58, $P = .002$), white blood cell (WBC) count <10 × 10^9/L (HR = 0.60, $P = .005$), and *ETV6-RUNX1* (HR = 0.14, $P = .006$) for EFS and age (HR = 0.48, $P < .001$), *ETV6-RUNX1* (HR = 0.1, $P = .016$) and high hyperdiploidy (HeH) (HR = 0.29, $P = .04$) for relapse-free survival. TRM was the major cause of death in *ETV6-RUNX1* and HeH DS-ALLs. Thus, while relapse is the main contributor to poorer survival in DS-ALL, infection-associated TRM was increased in all protocol elements, unrelated to treatment phase or regimen. Future strategies to improve outcome in DS-ALL should include improved supportive care throughout therapy and reduction of therapy in newly identified good-prognosis subgroups. (*Blood*. 2014; 123(1):70-77)

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Introduction

Children with Down syndrome (DS) are predisposed to develop acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL),[1] which are characterized by unique biological features in comparison with those of non–DS-ALL.[2-4]

Children with DS-ALL have an inferior outcome compared with non-DS patients because of both higher treatment-related mortality (TRM) and a higher relapse rate.[5-9] Because attempts to decrease TRM by reducing treatment intensity may contribute to the increased risk of relapse in DS-ALL, it is important to determine whether the risk for TRM is related to a specific treatment phase or chemotherapeutic agent.[8-10] Small series suggest that DS-ALL patients have an increased risk of mucositis from methotrexate (MTX), myelosuppression from anthracyclines, and hyperglycemia from glucocorticoids.[10-16]

Acquired leukemic cell genetic abnormalities have important prognostic significance in non-DS childhood ALL.[17] However, the impact of these abnormalities on treatment outcome in DS-ALL is unknown, because all published series lack a sufficient sample size to draw clear conclusions. Even the prognostic significance of well-known good prognostic factors in non–DS-ALL such as t(12;21)(p13;q22) (*ETV6-RUNX1*), high hyperdiploidy (HeH), and trisomies 4 and 10 is uncertain in DS-ALL, as is the significance of unfavorable translocations such as t(9;22)(q34;q11) (*BCR-ABL1*) and t(4;11)(q21;q23) (*MLL-AF4*).[2] Of interest, these prognostic genetic features have a lower frequency in DS-ALL.[2,7,18,19]

Recently, genetic abnormalities such as *JAK2* mutations[20] and *CRLF2* rearrangements have been identified in both DS and non–DS-ALL.[3,4,20-27] Activating *JAK2* R683 mutations were found in ~18% of DS-ALL patients.[20,24] Rearrangements of *CRLF2* occurred in ~60% of DS-ALL patients and in fewer than 10% of non–DS-ALL patients.[3,4,23] In almost all instances, *JAK2* (or rarely *JAK1* or *IL7R*) mutations were associated with *CRLF2* gene rearrangements, suggesting a model by which *CRLF2* overexpression results in JAK-STAT activation and proliferation of the leukemic clone.[3] Thus far, *CRLF2* gene rearrangements lack prognostic relevance in DS-ALL, although all series were small.[3,4,21,27]

The small size of most studies in DS-ALL patients has precluded definitive answers to the issues raised above. Hence, we undertook a large retrospective study of DS-ALL within the International ALL "Ponte di Legno" Working Group to study clinically relevant outcome parameters, the prognostic relevance of well-established and novel (cyto)genetic aberrations in ALL, and causes of treatment failure, thereby allowing a sufficient sample size to draw meaningful conclusions, despite the caveat of heterogeneity in treatment over time and between different study groups.[28]

Patients and methods

Patients

Patients eligible for this study were enrolled in various national or collaborative group clinical trials between January 1, 1995, and December 31, 2004, were ≤18 years at diagnosis and were treated with curative intent. The institutional review boards of each participating center approved treatment protocols according to the local law and guidelines. Informed consent was obtained in accordance with the Declaration of Helsinki. Participating study groups and their number of patients are detailed in supplemental Table 1 (available on the *Blood* Web site). A predefined set of data were collected, consisting of clinical data obtained at diagnosis and treatment and cytogenetic and molecular data (supplemental Table 2).

DS-ALL patients were treated according to standard ALL treatment protocols, but modifications of the standard protocol did occur. None of the protocols provided specific supportive care measures for DS-ALL children. In total, 42.3% (n = 276) DS-ALL patients received a reduced dose of chemotherapy. Most of these dose reductions (79%) were planned prior to the administration of specific courses of chemotherapy and gradually increased by observed clinical toxicity. Modifications for MTX consisted of dose reductions of high-dose MTX, varying from 10% to 75% of the maximum dose, and intensified leucovorin rescue. DS-ALL patients enrolled in EORTC 58951 protocols from September 2002 (n = 7) received 0.5 g/m^2 of MTX instead of 5 g/m^2. In addition, patients treated on protocol POG 9405 (n = 10) started with 50% of the total dose of daunorubicin, cytarabine, teniposide, histone deacetylase, and Peg-asparaginase, which was successively increased or reduced depending on toxicity. Supplemental Table 3 provides an overview of the main chemotherapeutic agents of treatment protocols used by the various study groups.

Data on either *JAK2* R683 mutations and/or *CRLF2* gene rearrangements were available from a subset of patients (n = 182) included in this study. There were no statistical differences between patients with and without available data. Some of these data have been previously reported.[20,24,25] However, several study groups contributed new unpublished data.

Non–DS-ALL reference cohort

For comparison, population-based B-cell precursor (BCP) ALL reference cohorts from the Dutch Child Oncology Group (DCOG) and the ALL Berlin-Frankfurt-Münster (BFM) study group, from exactly the same time period as the DS patients (January 1, 1995, and December 31, 2004), were added. The DCOG data set consisted of 827 non-DS BCP-ALL patients enrolled in 3 DCOG ALL treatment protocols (ALL8, ALL9, and ALL10). The BFM data set consisted of 3618 non-DS BCP-ALL patients enrolled in 2 BFM treatment protocols (BFM-95 and BFM-2000) in Germany and Austria. Details of these protocols have been reported elsewhere, except for protocol ALL10, which is ongoing.[29,30]

There were no significant differences in outcome estimates or in the distribution of cytogenetic subgroups between the DCOG and BFM data sets (data not shown) or when compared with reported data from other participating groups.[31-38] The DCOG and BFM non-DS data sets were merged for statistical analysis.

Cytogenetic analysis

Genetic abnormalities were determined by G-, Q-, or R-banded karyotyping, fluorescence in situ hybridization (FISH), or reverse-transcription polymerase chain reaction (RT-PCR). Diagnosis of rearrangements of *ETV6-RUNX1*, *BCR-ABL1*, and *MLL* were based on one or more of these techniques; diagnosis of HeH was defined by modal chromosomal number ≥52 or DNA index ≥1.13 for DS-ALL patients and ≥51 chromosomes for non-DS patients. All cytogenetic data were centrally reviewed by 2 coauthors (N.H. and E.F.). The definition and description of clonal abnormalities followed the recommendations of the International System for Human Cytogenetic Nomenclature (ISCN 2005).[39]

CRLF2 gene rearrangements were identified by genomic array, FISH, genomic PCR, Sanger sequencing, or multiplex ligation-dependent probe amplification.

Statistical analyses

Statistical analyses were conducted using SAS software (SAS-PC, version 9.1). The Kaplan-Meier method was used to estimate survival: complete remission rate (CR), event-free survival (EFS), overall survival (OS), and relapse-free survival (RFS). The survival estimates were compared using the log-rank test. The cumulative incidence of toxic death (TRM) and the cumulative incidence of relapse (CIR) were calculated by the method of

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Kalbfleisch and Prentice and compared with the use of Gray's test. CR was defined as <5% blasts in the bone marrow, with regeneration of trilineage hematopoiesis plus absence of leukemic cells in the cerebrospinal fluid or elsewhere. EFS was calculated from the date of diagnosis to the date of last follow-up or to the first event, including relapse, death in CR, failure to achieve CR (considered as event on day 0), or second malignancy. Early death was defined as any death within the first 6 weeks of treatment and was considered as an event on day 0 for statistical analysis. OS was measured from the date of diagnosis to the date of last follow-up or to the date of death from any cause. CIR included death in CR and other events as competing events.

χ^2 analysis was used to compare categorical variables, and the Fisher exact test was used for small patient numbers. The nonparametric Mann-Whitney U test was applied for continuous variables. P values $\leq$ .05 were considered as statistically significant (2-tailed testing).

For multivariate analysis, the Cox regression model was used. Continuous variables were categorized according to the National Cancer Institute (NCI) risk criteria.[40] P values $\leq$ .05 were considered as statistically significant (2-tailed testing).

Results

Patient characteristics

In total, data of 708 DS-ALL patients were collected, of which 55 were excluded because they did not meet the inclusion criteria; ie, the karyotype of 1 patient lacked constitutional trisomy 21, 39 patients were diagnosed outside the inclusion period of the study, 2 patients were not treated with curative intent, and the age of 9 patients was <18 years at diagnosis (range, 18.2-21.9 years). Furthermore, we excluded the 5 patients with T-cell ALL because this number was considered too small for meaningful statistical analysis. However, clinical and cytogenetic characteristics of these 5 T-cell ALL patients are described in supplemental Table 4. Hence, 653 patients with DS BCP-ALL were analyzed. DS-ALL patients were slightly older than non-DS patients at diagnosis (median 5.0 vs 4.7 years; P = .002) (Table 1), and DS-ALL did not occur in infants. The initial white blood cell (WBC) count of DS-ALL patients was not different compared with non-DS (median 10.2 × 10^9/L [range 0.2-459] vs 8.9 × 10^9/L [range 1.7-998], P = .14).

Genetic data

All leukemic karyotypes and FISH and RT-PCR results underwent central review; 68% (n = 444) of the DS patients had adequate genetic data (Table 1). In total, 40.3% had a cytogenetically normal (CN) karyotype (ie, only constitutional trisomy 21) compared with 6.9% of the non-DS cases (P < .001). A total of 9% of DS patients had a HeH karyotype compared with 33% of non-DS patients (P < .001). HeH DS patients were significantly older than HeH non-DS patients (median 7.2 years vs 4.2, P < .001). Trisomies of both chromosomes 4 and 10 were found in 45% of the HeH DS-ALL patients, similar to non-DS HeH patients (42.6%, P = .77).[18,41]

ETV6-RUNX1 fusion was found in 8.3% of the DS-ALL patients (compared with 25.8% in non-DS, P < .001), *BCR-ABL1* fusion in 0.7% compared with 2.4% in non-DS (P = .02), and *MLL* rearrangements in <1% compared with 1.2% in non-DS (P = .2). The previously reported t(8;14)(q11.2;q32) translocation was found in DS-ALL patients only (2%).[2,42,43]

In total, 182 patients had available data on either *JAK* and/or *CRLF2* aberrations. *JAK2* R683 mutations were found in 21% (n = 30) of the 141 DS-ALL patients with available data, of which 83% (n = 25) also had a *CRLF2* gene rearrangement. In 69% (n = 93) of the 134 DS-ALL patients with available data, *CRLF2* gene rearrangements were found, including 5.4% (n = 6) with *IGH@-CRLF2* translocations and 94.6% (n = 87) with *P2RY8-CRLF2* fusions. DS patients with *CRLF2* gene rearrangements were younger compared with DS patients with wild-type *CRLF2* (4.1 vs 7.7 years, P < .001), but no difference in diagnostic WBC was observed (14.8 vs 11.8 × 10^9/L, P = .7). This differs from non-DS patients with *CRLF2* gene rearrangements who had lower WBC counts (14.6 vs 34.6 × 10^9/L, P = .004) but did not differ in age (5.1 vs 4.7 years, P = .7) compared with wild-type patients (supplemental Table 5).

Table 1. Patient characteristics of DS-ALL patients and the DCOG non-DS BCP-ALL reference cohort

	DS-ALL	non–DS-ALL	P
Number of patients	653	4445	—
Age at diagnosis (range), y	5.0 (1.2-17.9)	4.7 (0.1-17.9)	.002
Sex			
Male	343	2431	—
Female	310	2014	.3
Median initial WBC × 10^9/L (range)	10.5 (0.2-459)	8.8 (0.2-999)	.14
Extramedullary disease			
CNS (%)	16/624* (2.5)	98/4258* (2.2)	.69
Lymph nodes (%)	134/412* (32.5)	1471/4339* (33.1)	.57
Hepatomegaly (%)	245/469* (52.2)	3156/4357* (71)	<.001
Testis (%)	1/296* (<1%)	28/4317 (<1%)	.51
Cytogenetic subgroups			
Normal karyotype	179/444* (40.3)	45/650* (6.9)	<.001
BCR-ABL1 t(9;22)	3/444* (0.7)	93/3898* (2.4)	.02
MLL (11q23)	2/444* (0.5)	36/2966* (1.2)	.15
ETV6-RUNX1 t(12;21)	37/444* (8.3)	841/3264* (25.8)	<.001
HeH$	40/444* (9)	235/708* (33)	<.001
HeH trisomy 4 and 10	18 (4.5)	100 (42.5)	<.001
HeH, other	22 (5.5)	135 (57.5)	<.001
Others	183 (41.2)	225/650* (34.6)	.03
8-y OS	74% ± 2%	89% ± 2%	<.001
8-y EFS	64% ± 2%	81% ± 2%	<.001
8-y CIR	26% ± 2%	15% ± 1%	.001
2-y TRM	7% ± 1%	2% ± <1%	<.001

CNS, central nervous system involvement at diagnosis (>5 WBC/μL; CNS-3); HeH$, DS: 52-60 chromosomes, non-DS: 51-60 chromosomes.
*Number of patients available for analysis.

Treatment outcome according to clinical data

The median follow-up time was 6.8 years for DS-ALL and 8.4 years for non-DS survivors. The CR rate was 96.7% in DS-ALL and 99% in non-DS patients (P < .001). Induction failures were more frequent in DS-ALL compared with non-DS (3.0% and 1.0%, respectively; P < .001). DS patients had a higher CIR (26% ± 2% vs 15% ± 1% at 8 years, P < .0001) and TRM (7% ± 1% vs 2% ± <1% at 2 years, P < .0001) than non-DS patients, resulting in a lower EFS (64% ± 2% vs 81% ± 2% at 8 years, P < .0001) and OS (74% ± 2% vs 89% ± 2%, P < .0001) (Figure 1). In total, 144 DS patients relapsed compared with 650 non-DS patients. The time to relapse after CR was significantly longer for DS (median 2.8 years, p25: 1.8 years, p75: 4.0 years) than for non-DS patients (median 2.4 years, p25: 1.4 years, p75: 3.5 years, P = .007). In addition, 23 DS-ALL patients relapsed after 5 years vs 33 non–DS-ALL patients (P < .001). Treatment outcome did not differ significantly between the early (1995-2000) and late (2000-2004) treatment eras for DS patients (8-year: OS 77% ± 3% vs 73% ± 3%, P = .7; CIR 26.7% ± 3% vs 31% ± 6%, P = .4).

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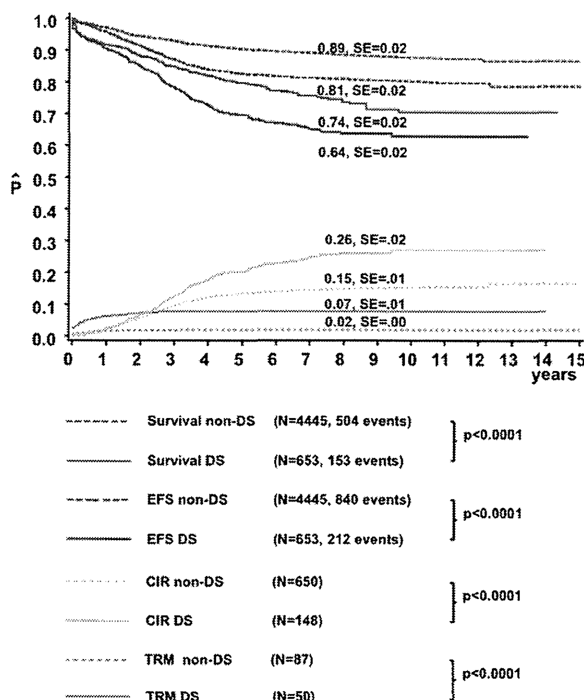

Figure 1. Treatment outcome of the DS-ALL and non–DS-ALL patients. The continuous lines represent DS-ALL patients, and the dotted lines represent non–DS-ALL patients. The red line represents OS, the blue line EFS, the green line the cumulative incidence of TRM, and the light blue line CIR. SE, standard error.

The 379 DS-ALL children <6 years fared significantly better than the 272 older children (8-year: EFS 70% ± 3% vs 54% ± 4%, $P < .0001$; OS 78% ± 2% vs 67% ± 3%, $P = .002$; CIR 21% ± 2% vs 34% ± 3%, $P \leq .001$; and 2-year cumulative incidence of TRM, 7% ± 1% vs 8% ± 2%; $P = .33$). Notably, the 126 children aged 6 to 9 years had a relatively poor outcome (8-year: EFS 51% ± 3%, OS 70% ± 5%), which was due to a very high frequency of relapse (CIR 41% ± 6%) not attributable to any known risk factor(s). Outcome declined with increasing WBC count and was best for the 319 patients with WBC $<10 \times 10^9$/L due to a low risk of TRM (8-year: 4% ± 1% vs 11% ± 2% for WBC $\geq 10 \times 10^9$/L, $P = .0003$) and relapse (8-year: 21% ± 3% vs 30% ± 3%, $P = .03$). These features thus define a favorable risk group with age <6 years and WBC $<10 \times 10^9$/L, when compared with the remaining DS patients (8-year: EFS 78% ± 3% vs 58% ± 3%, $P < .0001$; OS 87% ± 3% vs 68% ± 3%, $P < .0001$; CIR 17% ± 3% vs 30% ± 2%, $P = .003$; 2-year TRM 3% ± 1% vs 9% ± 1%, $P = .002$) (Figure 2, Table 2). These criteria predicted outcome more accurately than the classical NCI criteria (supplemental Figure 1).[40] These features remained significant after excluding patients with *ETV6-RUNX1* rearrangements or trisomies 4 and 10 from the analysis. The effect of this new Ponte di Legno (PdL) risk stratification was consistent among the larger study groups including AIEOP, BFM, CCG, POG, and the UK with a hazard ratio (HR) of 1.62 for high-risk patients from the UK and 3.79 for BFM patients. Among patients with age >6 years and WBC $>10 \times 10^9$/L, DS patients had a poorer outcome than non-DS patients (8-year: EFS 58% ± 3% vs 78% ± 1%, $P < .001$; OS 68% ± 3% vs 86% ± 1%, $P < .001$; CIR 30% ± 2% vs 17% ± 1%, $P < .001$; 2-year TRM 10% ± 1% vs 2% ± <1%, $P < .0001$). The clinical characteristics of DS-ALL patients (n = 246) classified as NCI low risk but considered high risk according to our criteria are described in supplemental Table 6.

In total, 18 (2.8%) of the DS-ALL patients received a stem cell transplantation: 3 in CR1 and 15 in CR2. Of these patients, 6 are alive in continuous CR and 12 patients died (1 graft-versus-host disease, 1 toxic noninfectious event, 1 infection, and 9 relapses).

Treatment outcome according to genetic data

The 37 DS-ALL patients with *ETV6-RUNX1* had significantly better outcome than the other DS patients: 8-year EFS 95% ± 4% vs 63% ± 3% ($P = .001$), OS 97% ± 3% vs 75% ± 2% ($P = .007$), CIR 3% ± 3% vs 26% ± 2% ($P = .004$), and 2-year TRM 3% ± 3% vs 8% ± 1%; ($P = .2$). DS-ALL patients with *ETV6-RUNX1* did not differ in outcome when compared with the 841 non-DS patients with this abnormality (8-year: EFS 95%, $P = .48$; OS 96%, $P = .91$; CIR 7%, $P = .32$; 2-year TRM 1%, $P = .19$).

The 40 HeH DS-ALL patients had a significantly lower CIR than the other DS-ALL patients (8-year: 8% ± 5% vs 26% ± 3%, $P = .02$). However, a relatively high rate of TRM (2-year: 13% ± 5% in HeH vs 7% ± 1% in non-HeH DS, $P = .2$) resulted in similar 8-year EFS (77% ± 7% vs 65% ± 3%, $P = .28$) and OS (79% ± 6% vs 76% ± 2%, $P = .88$). TRM in these HeH patients was not exclusively seen in one treatment strategy but was spread across the different treatment protocols. HeH DS-ALL patients showed lower OS when compared with the 235 HeH non-DS patients due to increased TRM (8-year: OS 79% ± 6% vs 93% ± 2%, $P = .009$; EFS 77% ± 7% vs 86% ± 2%, $P = .06$; CIR 8% ± 5% vs 11% ± 2%, $P = .7$; 2-year: TRM 13% ± 5% vs 1% ± 1%, $P < .001$).

The subgroup of HeH DS-ALL patients with trisomies 4 and 10 (n = 18) showed a trend toward better outcome when compared with all other DS-ALL patients (8-year: EFS 88% ± 8% vs 65% ± 3%, $P = .09$; OS 88% ± 8% vs 76% ± 2%, $P = .32$; CIR 0% vs 25% ± 2%, $P = .03$; 2-year: TRM 12% ± 8% vs 7% ± 1%, $P = .6$). No DS patients with these trisomies relapsed, and all events were due to toxicity. Their outcome was similar when compared with non-DS patients with trisomy 4 and 10 (8-year: EFS 90.8% ± 3%; $P = .75$, OS 92.3% ± 4%; $P = .65$, CIR 5.1% ± 2%; $P = .34$, 2-year: TRM 3.0% ± 2%, $P = .1$).

DS-ALL patients with or without *JAK2* mutations had similar treatment 8-year outcomes (EFS 57% ± 10% vs 69% ± 5%, $P = .1$; CIR 26% ± 9% vs 23% ± 5%, $P = .48$). No data were available in the reference cohort. The 93 DS-ALL patients with *CRLF2* aberrations showed no significant difference in 8-year survival compared with the 41 wild-type DS-ALL patients (EFS 62% ± 6% vs 71% ± 8%, $P = .21$; OS 73% ± 5% vs 83% ± 8%, $P = .13$; CIR 26% ± 6% vs 22% ± 8%, $P = .44$). DS-ALL patients with *CRLF2* gene rearrangements did not differ in outcome from non–DS-ALL patients with these aberrations (8-year: EFS 62% ± 6% vs 58% ± 9%, $P = .7$; OS 73% ± 5% vs 79% ± 8%, $P = .6$; CIR 26% ± 6% vs 38% ± 9%, $P = .15$). Median time to relapse for DS patients with *CRLF2* aberrations was 29 months vs 51 months in patients with wild-type *CRLF2* ($P = .11$).

Treatment-related mortality

In total, 7.7% of the DS-ALL patients died of other causes than relapsed/refractory disease compared with 2.3% in non-DS ($P < .001$). TRM occurred at all phases of therapy, including maintenance (supplemental Table 7). TRM death during induction occurred in 2.8% (n = 18) of the DS patients (13 infectious, 5 noninfectious deaths). In CR, 4.9% (n = 32) of the DS patients died of TRM (25 infectious, 7 noninfectious). The most common cause of TRM

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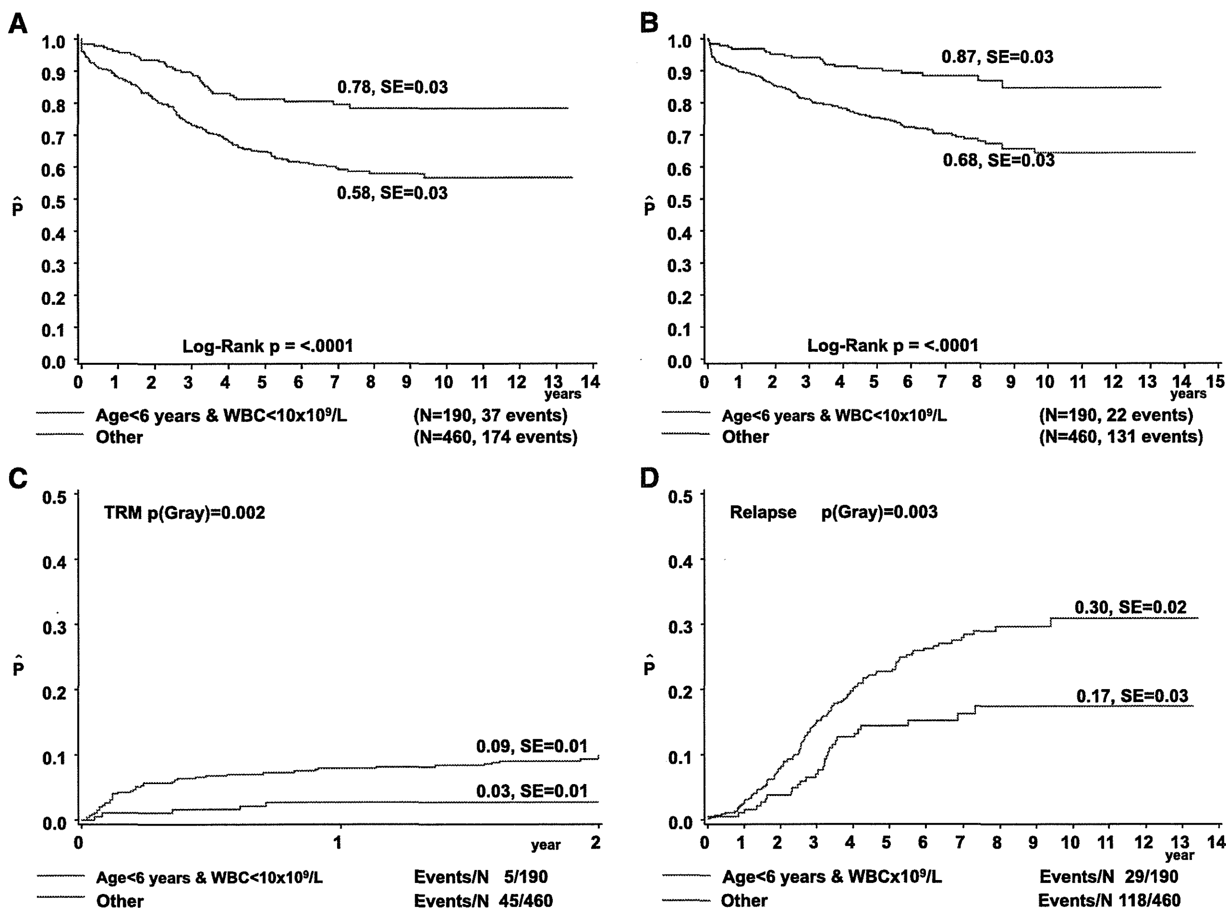

Figure 2. Treatment outcome according to age and WBC in DS-ALL. The OS (A), EFS (B), cumulative incidence of TRM (C), and CIR (D) are depicted for patients aged <6 years with WBC <10 × 10^9/L (blue line) vs all other DS-ALL patients (red line). The numbers on the curves for OS and EFS represent results at 8 years. The numbers on the curves for TRM are 2-year results (during treatment only) and those for relapse are results at 8 years. SE, standard error.

was infection, mainly respiratory and bacterial infections. Only 0.3% (n = 2) of the DS ALL patients died of second malignancies in CR1 (secondary AML and Epstein-Barr virus lymphoproliferative disease), compared with 1.3% of the non-DS patients ($P < .04$). Secondary malignancies in non-DS patients included 28 AML/myelodysplastic syndromes, 5 brain tumors, 9 other tumors, and 13 other malignancies.

TRM was not significantly different between DS patients treated in the CCG/POG/UK studies (3-drug induction) and those DS patients treated in AIEOP/BFM studies (4-drug induction); the rate of death during induction was 1.1% ± 1% vs 1.9% ± 1% ($P = .7$) and the 2-year cumulative rate was 7% ± 2% vs 8% ± 3% ($P = .99$). The inclusion of an anthracycline in induction (4-drug induction) had no impact on TRM.

Table 2. Contingency table representing outcome of DS patients by NCI risk group and PdL risk group criteria

	PdL risk criteria		
Classical NCI criteria	Standard risk	High risk	
Low risk	n = 187 EFS 78% ± 3% OS 87% ± 3% TRM 3% ± 1% CIR 17% ± 3%	n = 246 EFS 63% ± 4% OS 73% ± 3% TRM 7% ± 2% CIR 30% ± 4%	NCI SR n = 433
High risk	n = 0	n = 218 EFS 57% ± 4% OS 62% ± 4% TRM 12% ± 2% CIR 29% ± 3%	NCI HR n = 218
	PdL SR model n = 187	PdL HR model n = 464	Total n = 651

Classical NCI risk criteria: age 1-9 or ≥ 10 y at diagnosis and WBC < or ≥50 × 10^9/L; PdL low-risk criteria: age <6 y and WBC <10 × 10^9/L; PdL high-risk criteria: all other patients.

HR, high risk; SR, standard risk.

Multivariate analysis

Stepwise multivariate Cox regression analysis of EFS revealed age <6 years (HR = 0.58, 95% confidence interval [CI] = 0.41-0.81, $P = .002$), WBC <10 × 10^9/L (HR = 0.60, 95% CI = 0.42-0.86, $P = .005$), and *ETV6-RUNX1* (HR = 0.14, 95% CI = 0.03-0.57, $P = .006$) as independent predictors for favorable outcome. They also independently predicted OS (age HR = 0.66, $P = .04$; WBC <10 × 10^9/L HR = 0.51, $P = .003$; and *ETV6-RUNX1* HR = 0.12, $P = .04$). RFS was predicted by age, *ETV6-RUNX1*, and HeH (Table 3).

In non–DS-ALL, the classical NCI criteria are age and the initial WBC count; however, *ETV6-RUNX1* and trisomy 4 and 10 are independent predictors for favorable outcome (*ETV6-RUNX1*: HR = 0.29, 95% CI = 0.15-0.58, $P < .001$; or trisomy 4 and 10: HR = 0.37, 95% CI = 0.17-0.79, $P = .011$). NCI criteria retained

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Table 3. Multivariate analysis of the DS-ALL data set

Outcome	Variable	HR	95% CI	*P* value
	Age <6 y	0.58	0.41-0.81	.002
EFS	WBC <10 × 10⁹/L	0.60	0.42-0.86	.005
	ETV6-RUNX1	0.14	0.03-0.57	.006
	HeH	0.68	0.34-1.36	.275
	Age <6 y	0.66	0.44-0.99	.044
OS	WBC <10 × 10⁹/L	0.51	0.33-0.79	.003
	ETV6-RUNX1	0.12	0.02-0.86	.035
	HeH	1.01	0.48-2.11	.983
	Age <6 y	0.48	0.32-0.73	.000
RFS	WBC <10 × 10⁹/L	0.71	0.46-1.08	.105
	ETV6-RUNX1	0.10	0.01-0.64	.016
	HeH	0.29	0.09-0.92	.036

HeH ≥ 52 chromosomes.

their prognostic value in a Cox model with these 3 variables (HR = 1.96; 95% CI = 1.30-2.95, P = .001). In addition, multivariate analysis showed that the PdL criteria are not driven by the large group of DS-ALL patients having *CRLF2* aberrations (HR = 0.66, 95% CI = 0.33-1.33, P = .25), but more likely by age and initial WBC (HR = 2.16, 95% CI = 0.95-4.90, P = .07).

Discussion

Many study groups have reported a worse clinical outcome for DS-ALL, but almost all reports lack sufficient power to answer relevant biological questions in DS-ALL, which is the reason the PdL group undertook this retrospective review. The unprecedented size of this study cohort resolves the controversy of the frequency and clinical impact of specific (cyto)genetic aberrations in DS-ALL.[2,18] Moreover, the scale of the study enabled the identification of relatively small subgroups of DS-ALL with favorable outcomes. Analysis of 444 DS-ALL patients with known cytogenetics demonstrated that the genetic subgroups predicting favorable outcome in non–DS-ALL[2,6,7,18,41,44] also predict favorable outcome in DS-ALL. Most significant is the discovery that *ETV6-RUNX1* conferred an excellent prognosis and that HeH with trisomy of chromosomes 4 and 10 was associated with a very low CIR. Hence, these patients, comprising 12% of DS-ALL, may be eligible for future treatment reduction to reduce TRM and can be treated according to the same risk-stratified algorithms as non-DS patients in the collaborative study group protocols.

Another novel finding of this study was the identification of a clinically favorable prognostic subgroup of DS-ALL patients, characterized by age <6 years and WBC <10 × 10⁹/L. These cut points differ from those used in the classical NCI ALL risk criteria, although the biological basis for this difference is not fully understood.[40] No genetic abnormalities were identified that could explain this difference between the classical NCI and the herein-reported criteria. Remarkably, children aged between 6 and 9 years at diagnosis had a relatively poor outcome similar to high-risk ALL patients, which was due to a high frequency of relapse. This subgroup may be treated according to a medium- or high-risk arm of future collaborative study group protocols. Unraveling the genetic background of the leukemia in this subgroup will be required in order to design more rational therapy for these patients. Noteworthy, minimal residual disease (MRD) was not routinely determined during the era of this study, and it is unclear whether MRD would confirm these novel risk groups. Because MRD was proven to be a powerful tool in non–DS-ALL risk assignment,[45,46] further research is needed to validate whether an MRD-based strategy is desirable in future DS-ALL treatment protocols.

In general, we showed that DS-ALL patients have an inferior survival when compared with a representative non–DS-ALL cohort treated in the same time period, which is in agreement with previous smaller studies.[5,10,47] Despite a high rate of TRM, and different from what is often suggested, relapse remained the main cause of treatment failure in DS patients. Interestingly, the relapses tend to occur later in DS. It is unclear if this is due to the genetic makeup of DS-ALL or to decreased immune surveillance of the residual leukemia in DS patients. It cannot be ruled out that underreported treatment reduction of patients with DS-ALL contributes to the increased relapse risk.[48] This finding suggests that the currently accepted strategy of treatment reduction in DS-AML, which is characterized by a chemotherapy-sensitive phenotype,[49] is not applicable to DS-ALL.[47] The only exception may be DS-ALL patients with *ETV6-RUNX1* or HeH, in which TRM outweighed the risk of relapse, for whom a 3-drug induction and a limited reinduction might be adequate. Interestingly, and in accordance with previous results, the incidence of secondary malignancies was significantly lower in DS patients as compared with non–DS-ALL patients. This is in agreement with the reduced propensity for solid tumors in DS patients reported before.[50]

The genetic basis of the aggressive clinical behavior of DS-ALL is still unknown. A high proportion of DS-ALLs have normal karyotype (40.3% compared with 6.9% of non-DS), suggesting the presence of cytogenetically invisible molecular abnormalities. One of these abnormalities, detected in 60% of DS-ALLs, is the aberrant expression of *CRLF2*, which is often associated with *JAK-STAT* mutations. In contrast to some studies showing deleterious effects of *CRLF2* alterations in non-DS high-risk ALL,[26,51] no such association was found in this study or in several prior smaller studies of DS-ALL.[3,4,21,27] Nevertheless, a substantial proportion of DS-ALL patients carry these aberrations, thereby providing a pathway that might be targeted by inhibitors of the JAK-STAT pathway or mTOR signaling.[52]

IKZF1 mutational status was unknown in our data set. Recently, it was shown that this gene was frequently deleted in DS-ALL patients (in ~35%) and was found to be an independent predictor for dismal outcome.[27] Of note, the median age of patients with *IKZF1* aberrations in the DS-ALL study was significantly higher compared with wild-type patients (8.2 vs 4.3 years), which could be an important genetic factor underlying the biological basis for the age cutoff point of 6 years reported here as clinically significant.

Previous studies reported increased TRM in children with DS-ALL,[9] also in relapse protocols.[53] The large size of our cohort enabled the observation that the increased TRM is present throughout treatment, with about half of the deaths occurring during maintenance therapy. While doses of myelosuppressive chemotherapy are typically adjusted during maintenance therapy, to maintain an adequate neutrophil count, this phase of treatment may nevertheless lead to B-cell depletion and hypogammaglobulinemia, and hence to a higher infection rate in already immune-compromised DS patients.[54,55] To reduce TRM, we suggest improving supportive care throughout the treatment period with aggressive treatment of infections and studies analyzing the potential benefit of antibacterial and antifungal prophylaxis and/or immunoglobulin substitution. Patients should be leukocyte depleted as non-DS patients during maintenance in order to prevent relapse, but with prompt interruptions for aplasia and with more intensive surveillance than non-DS children.

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In conclusion, this large international study demonstrated that the poorer survival seen in DS-ALL is mainly due to a higher relapse rate and less so to TRM. Therefore, treatment reduction is not warranted, except for the 12% of patients with HeH or *ETV6-RUNX1* in which toxicity is the major cause of mortality. Because TRM occurs throughout therapy and is not associated with a specific chemotherapy regimen, better surveillance and improved supportive care measures throughout therapy need to be evaluated. As a result of this study, an initiative is underway to develop an international treatment protocol for children with DS-ALL.

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Authorship

Contribution: T.D.B., S.I., J.W., C.H.P., and C.M.Z. conceived of, designed, and wrote the manuscript; T.D.B., S.I., M.Z., E.F., N.A.H., M.M.v.d.H.-E., R.P., C.M.K., L.B.S., K.S., D.-C.L., K.H., M.A., A.B., G.B., K.R.R., M.S., G.C., G.M., M.M., R.P.-G., V.M., T.L., H.C., B.S., I.G., A.V.M., A.V., S.P.H., C.-H.P., C.G.M., A.M., G.E., J.R.K., J.A.W., and C.M.Z. provided all study materials or patients; T.D.B., S.I., J.W., and C.M.Z. collected and assembled data; T.D.B., S.I., M.Z., J.W., N.H., E.F., M.M.v.d.H.-E., and C.M.Z. analyzed and interpreted data; and all authors gave final approval of the manuscript.

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bjh research paper

Secondary cancers among children with acute lymphoblastic leukaemia treated by the Tokyo Children's Cancer Study Group protocols: a retrospective cohort study

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Summary

With improvement in survival, it is important to evaluate the impact of treatment on secondary cancers in acute lymphoblastic leukaemia (ALL) survivors. A retrospective cohort study comprising 2918 children diagnosed with ALL and enrolled on Tokyo Children's Cancer Study Group (TCCSG) protocols between 1984 and 2005 was conducted to evaluate the incidence of secondary cancers and associated factors including treatment protocol, cranial irradiation and other characteristics of the primary ALL. Thirty-seven patients developed secondary cancers, including acute myeloid leukaemia (n = 11), myelodysplastic syndrome (n = 5), non-Hodgkin lymphoma (n = 2), brain tumours (n = 13) and other solid carcinomas (n = 6) within a median follow-up duration of 9·5 years. The cumulative incidence of any secondary cancers was 1·0% (95% confidence interval (CI), 0·7–1·4%) at 10 years and 2·4% (95% CI, 1·5–3·7%) at 20 years, respectively. Standardized incidence rate ratio of secondary cancers was 9·3 (95% CI, 6·5–12·8). Multivariate analyses showed an increased risk of secondary cancers associated with the recent treatment protocol and cranial irradiation. There was no evidence of a reduction in secondary cancer incidence despite marked decreases in cranial irradiation use in the recent protocols.

Keywords: secondary cancers, acute lymphoblastic leukaemia, children, cumulative incidence, standardized incidence rate ratio.

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Intensive multidrug therapy has steadily improved the overall survival (OS) of children with acute lymphoblastic leukaemia (ALL) despite decreasing prophylactic cranial irradiation (Pui *et al*, 2009; Tsuchida *et al*, 2010). The immunosuppressive and cytotoxic therapy necessary to achieve this improvement increases the risk of subsequent late effects. One of the most serious late effects is the development of a secondary cancer.

Reports from previous studies including the Childhood Cancer Survivor Study (CCSS) and British CCSS (BCCSS) have contributed important evidence regarding the risk of subsequent primary neoplasms among survivors of childhood cancers, such as ALL.(Hawkins *et al*, 1992; Neglia *et al*, 2001; Mody *et al*, 2008; Meadows *et al*, 2009; Friedman *et al*, 2010; Reulen *et al*, 2011) However, the study populations comprising both of these large cohorts are childhood cancer patients who have survived at least 5 years following primary cancer diagnosis and the results do not account for the time at risk during the first 5 years.(Hawkins & Robison, 2006).

A few studies have described the overall risk of secondary cancers among children with ALL with the period of observation beginning from a time shortly following successful complete remission (CR). (Neglia *et al*, 1991; Nygaard *et al*, 1991; Kimball Dalton *et al*, 1998; Loning *et al*, 2000; Bhatia *et al*, 2002; Hijiya *et al*, 2007; Schmiegelow *et al*, 2009) Compared with the general population, the survivors with a history of childhood ALL have been estimated to have a 10- to 20-fold greater risk of developing a secondary cancer. In addition to genetic predisposition, previously administered chemotherapy and/or radiotherapy are considered the most important risk factors. (Loning *et al*, 2000) Based on the cohort of patients previously enrolled onto a Tokyo Children's Cancer Study Group (TCCSG) protocol since 1984, the current study is the first report from an Asian country to describe the incidence and types of secondary cancers observed among survivors of childhood ALL. We also aimed to evaluate potential risk factors for secondary cancers, particularly the influence of treatment protocol and cranial irradiation use.

Patients and methods

Study population

A total of 2,918 newly diagnosed children with ALL aged 1–15 years were entered into 5 consecutive TCCSG studies between 1984 and 2005 (L84-11, L89-12, L92-13, L95-14, and L99-15/L04-1502; Figure S1). The current analysis was primarily based on 2,807 patients who underwent a successful induction phase, achieved CR and survived for at least 2 months or more in the intention-to-treat group, including a total of 621 stem cell transplantations (SCT) had been performed for the primary ALL during the observation period of the study population (Fig 1). Details of the treatment regimens and main therapeutic results have been previously published. (Tsunematsu *et al*, 1974; Toyoda *et al*, 2000; Manabe *et al*, 2001; Igarashi *et al*, 2005; Hasegawa *et al*, 2012) Although the patients in our cohort were treated according to therapeutic protocols, we do not have detailed information regarding actual doses of additional therapeutic exposures given to the relapsed patients, which potentially could have influenced the development of secondary cancers. As a sensitivity analysis, we conducted the same analysis on 1716 patients (referred to as the per protocol group), limited to the patients who had completed all planned treatment leading to first CR (Fig 1).

The cumulative doses of the important treatment contents are listed in Table I. The cumulative anthracycline dose was converted to doxorubicin (DOX)-equivalent doses, which ranged from 0 to 415 mg/m^2. The cumulative cyclophosphamide (CPM) dose ranged from 0 to 6·8 g/m^2 and etoposide (up to 2·4 g/m^2) was administered in only some ALL high-risk regimens. The actual doses of oral drugs given to the patients, such as methotrexate and mercaptopurine (6-MP) were adjusted by white blood count (WBC) counts; therefore we evaluated maintenance duration in our analyses instead of oral antimetabolites doses. A major change over time across the TCCSG treatment protocols included a decrease in the executed proportion and dosage of prophylactic cranial radiation therapy (CRT) and intensified systemic and intrathecal chemotherapy. Prophylactic CRT was part of the treatment protocol for all patients in the L84-11 trial, whereas only 8·6% of the patients in the more recent L99-15/L04-1502 trial received CRT, which was limited to the high-risk group (Table I).

Follow-up and data collection

Follow-up of the patients were performed by the treating institution every 2 years, at which time any late effects including secondary cancer were documented into the TCCSG database. To obtain additional information on characteristics of the secondary cancer diagnosis, we distributed a survey to the treating institution to collect data on the date of diagnosis, cytological or histological characteristics including cytogenetic findings, cancer site, cumulative treatment exposures before secondary cancers, treatment contents given for secondary cancers and its outcomes. The time at risk for secondary cancers was computed from the date of ALL diagnosis to the date of secondary cancer diagnosis, date of death or date of last contact, whichever came first. The end of follow-up for the study was December 2011.

Statistical analysis

Cumulative incidence of secondary cancers over time was calculated using competing risk methods (considering any death as a competing event).(Gooley *et al*, 1999) The incidence rates of cancer in the Japanese general population (obtained from the regional cancer registry of National Cancer Centre Hospital in Japan) (Japanese National Cancer Centre Hospital, 2013) were used to calculate the number of cancers expected to occur in the patient cohort by calculating the total person-years at risk by gender and 5-year age

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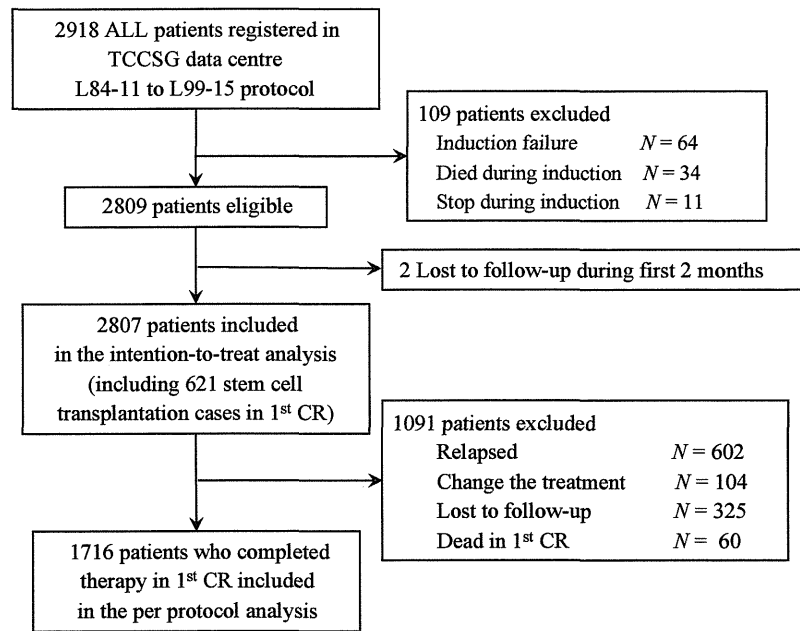

Fig 1. Flow diagram describing the criteria for patient selection. Total of 2918 newly diagnosed children with ALL aged 1–15 years entered into 5 consecutive Tokyo Children's Cancer Study Group (TCCSG) studies (L84-11, L89-12, L92-13, L95-14 and L99-15/L04-1502. The current analysis is primarily based upon 2807 patients who successfully acheived complete remission (CR) and survived at least 2 months or more as the intention-to-treat analysis. 1st CR, first complete remission.

groups and multiplying by the corresponding cancer rates observed in the general population. The standardized incidence rate ratio (SIR), defined as the ratio of the number of observed cancers divided by the number of expected cancers, was used to evaluate the difference in cancer occurrence between the ALL group and the general population. Absolute excess risk (AER) was calculated as the difference between the number of observed events and expected events divided by the number of person-years of follow-up, and was expressed as per 100 000 person-years. Survival analysis was conducted using Kaplan-Meier methods (Log-rank method for comparison) and Cox regression model for hazard ratio (HR) estimates. Variables examined in the regression model included age at ALL diagnosis, risk classification, age at last follow-up, CRT (yes or no), specific anti-cancer agents (yes or no), and duration of maintenance therapy. Treatment protocol and the anticancer agents could not be entered as co-variable factors in the same regression model due to their highly correlated nature. Thus, treatment protocol was included in the main analysis, but the same model replacing protocol with the anticancer agents was also performed to evaluate their effects. Data were analysed using the SPSS statistical software, version 20.0 (IBM Japan Ltd., Tokyo, Japan) and EZR (Saitama Medical Centre, Jichi Medical University), a graphical user interface for R. (Kanda, 2013).

Results

The OS proportions of the TCCSG ALL L84-11 to L04-1502 protocols are shown in Table I. Ninety-seven percent of the whole study population achieved CR and 602 (21·4%) of the 2,807 children among the intention-to-treat group suffered a relapse. Of the total patients, about 70% were followed until after 2008. Even with reduction in CRT use, from 100% to 8·6%, 10-year OS has increased steadily from 74% to more than 85%. The median follow-up duration after diagnosis of ALL was 9·5 years (range 0·2–27 years), with a total of 27 495 person-years of follow-up. At December 2011, a total of 37 secondary cancers had been diagnosed in our cohort, including acute myeloid leukaemia (AML, n = 11), myelodysplastic syndrome (MDS, n = 5), non-Hodgkin lymphoma (NHL, n = 2), brain tumours (n = 13) and other solid carcinomas (n = 6).

Cumulative incidence

The overall cumulative incidence of secondary cancers was 1·0% (95% confidence interval [CI], 0·7–1·4%) at 10 years, 1·4% (95%CI, 0·9–2·0%) at 15 years and 2·4% (95%CI, 1·5–3·7%) at 20 years from the diagnosis of ALL, respectively (Fig 2A). The corresponding cumulative incidence among patients remaining in first CR was 3·9% at 20 years (95% CI: 2·3%–6·1%), which was significantly higher (P < 0·001) than patients not in first CR (Fig 2B). The cumulative incidence in persons who received CRT was 2·9% at 20 years (95% CI, 1·8–4·4%), which appeared higher than the patients without CRT (P = 0·057, Fig 2C). There was no statistically significant difference in cumulative incidence by TCCSG therapeutic protocol (Fig 2D).

Clinical characteristics of secondary cancers

The clinical characteristics of the patients with secondary cancers are summarized in Table II according to type of secondary cancer. Females were predominant (75%) in secondary AML/MDS. Types of secondary cancers differed also according to the age at diagnosis of ALL; brain tumours and

Table I. Cumulative doses of selected chemotherapeutic agents and radiation of ALL trials L84-11 to L99-15 according to risk groups.

Risk Group by protocol	Patients (n)	Anthracycline (mg/m²)						CPM (mg/m²)	VP-16 (mg/m²)	IV MTX (g/m²)	Maintenance (weeks)	MTXIT	CRT (Gy)	CRT rate (%)	10-year OS (%)
		DNR	DOX	THP	ACR	MIT	Total								
L84-11	484													100	74·3 ± 2·0
SR (A/B arm)†	194	0	0	0	0	0	0	0	0	2/3·5	172	9/15	18	100	
HR (A/B arm)†	244	180	0	0	150	0	224	6800/6000	0	1/2·5	172	5/11	24	100	
HEX	48	75	100	0	0	0	162	4000	0	0	96	11	24	100	
L89-12	418													80	73·5 ± 2·2
SR (A/B arm)†	142	0	100/0	100/150	0	0	160/90	0	900	9	91	9/9	0 vs 18	44	
IR	100	0	0	210	60	0	135	3100	2400	6	91	7	18	100	
HR	146	0	0	240	60	20	210	3600	2400	6	87	6	18	100	
L92-13	347													44	77·9 ± 2·2
SR	124	0	0	150	0	20	170	0	0	6	24	8	0	0	
HR (A/B arm)†	122	0	0	100	0	20	140	1000	1200	6/0	22	10	0 vs 12/18	47	
HEX	101	0	0	100	0	40	220	1000	1200	0	16	9 (6)	18	100	
L95-14	597													44	82·0 ± 1·6
SR	231	0	0	100	0	0	60	2000	0	10·6	54	11	0	0	
HR (A/B arm)†	129	0	0	220	0	0	132	4000	0	10/1	54	8	0 vs 12/18	18	
HEX	237	100	200	220	0	0	415	4000	0	1	54	8	18	100	
L99-15/L04-1502	1007													8·6	87·6 ± 1·2‡
SR	381	100	0	0	0	0	83	2000	0	13·15	104	11	0	0	
HR (A/B arm)†	404	100	100	120	0	0	245	4000/5000	0	10	52	10/11	0	0	
HEX	242	100	0	0	0	20	163	5600	1000	6	54	17	12/18	27·4	

SR, Standard risk; IR, Intermediate risk; HR, High risk; HEX: extremely high risk; DNR, daunorubicin; DOX, doxorubicin; THP, pirarubicin; ACR, acracinomycin; MIT, mitoxantrone; Total, DOX-equivalent dose; CPM, cyclophosphamide; VP-16, etoposide; MTX, methotrexate; CRT, cranial irradiation; IT, intrathecal; OS, overall survival.

†(A/B arm): cumulative doses of A arm/B arm; Additional details of treatment regimen are provided as supplemental information.

‡4-year overall survival rate.

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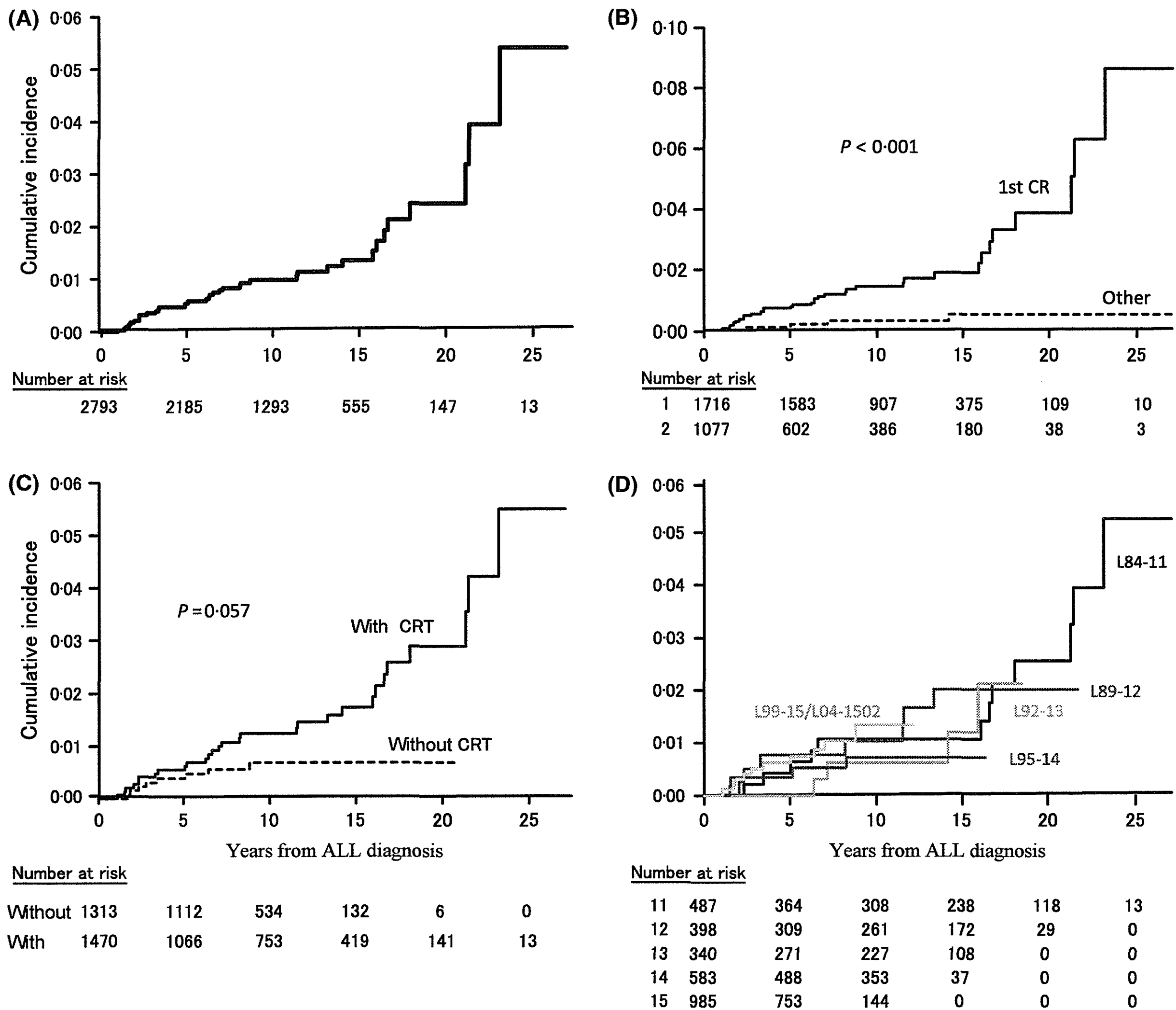

Fig 2. Cumulative incidence of secondary cancers. Shown are the cumulative incidences of secondary cancers as a function of time since primary ALL diagnosis over a maximum follow-up of 27 years. (A) Overall cumulative incidence of secondary cancer among the total patient cohort. (B) Overall cumulative incidence of patients remaining in first complete remission (1st CR) compared to others. (C) Overall cumulative incidence according to treatment with or without cranial radiation therapy (CRT). (D) Overall cumulative incidence according to treatment protocols: L84-11 (black line), L89-12 (red line), L92-13 (green line), L95-14 (purple line), and L99-15/L04-1502 (blue line). The numbers of patients at risk at a specific time point are indicated below each of the four plots.

other carcinoma tended to develop more commonly in children that were older at ALL diagnosis. There was no difference between types of secondary cancers with regard to initial WBC, immunophenotype and risk classification of the primary ALL. The median latency period from ALL diagnosis to secondary cancers was 6 years (range 1–23 years) and varied by type of secondary cancer (Fig 3). The median time to diagnosis for haematological cancers (AML, MDS and NHL) was shortest (median 3·0 years), followed by brain tumours (median 11·5 years) and other solid carcinoma (median 16·3 years). Haematological cancers developed most commonly during the first 10 years followed by brain tumours from 15 to 20 years (Fig 3A, B). The age distributions at diagnosis of secondary cancers are shown in (Fig 3C). Median age at diagnosis of secondary was earlier for haematological cancers than brain tumour (median 14 years) and other carcinomas (median 19 years). Among AML, the most common morphological type was M5 (3 had t (9;11) (p22;q23) with *MLL-MLLT3* translocation). Four MDS cases showed chromosomal abnormality. Lymphoma and solid tumours did not show any chromosomal abnormalities.

As for the characteristics related to the treatment of primary ALL, the secondary cancers with the highest proportions of patients who underwent CRT were MDS, brain tumour and other carcinoma, while the haematological cancers showed elevated cumulative anticancer drug doses. Among a total of 621 SCT that had been performed for the primary ALL during the observation period of the study population, only 3 patients

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Table II. Clinical characteristics of patients with secondary cancers.

	AML	MDS	NHL	Brain tumour	Other carcinoma
Total number of secondary cancers	11	5	2	13	6
Gender (Male:Female)	3:8	1:4	2:0	8:5	3:3
Primary ALL					
Age at diagnosis of ALL (years)	5 (1–14)	5 (2–13)	4 (2–6)	8 (2–12)	11 (3–14)
Initial WBC count ($\times 10^9$/l)	20·5 (1·9–168)	11·2 (2·9–70)	8·7 (3·4–14)	12·6 (1·9–112)	4·9 (2·1–163)
Immunophenotype (B:T:Other)	7:0:4	3:0:2	2:0:0	7:1:5	6:0:0
Risk group (SR:IR:HR)	1:8:2	1:2:2	2:0:0	1:10:2	2:4:0
Secondary cancer (SC)					
Incubation time to SC (years)	3·3 (1·6–11·6)	2·3 (1·0–6·3)	3·1 (2·8–3·4)	11·5 (2·3–23·2)	16·3 (7·2–21·4)
Diagnosis on therapy	4/11 (36%)	2/5 (40%)	1/2 (50%)	0/13 (0%)	0/6 (0%)
Age at diagnosis of SC (years)	9·0 (6·4–21·3)	11·1 (4·0–14·5)	7·5 (5·3–9·7)	18·5 (10·3–27·7)	23·9 (18·8–32·6)
Sub-classification	M4: 2, M5: 7, M7: 1, Unknown: 1	RAEB: 1, CMML: 2, Unknown: 2	Diffuse large B-cell lymphoma: 1, Burkitt lymphoma: 1	Glioma: 8, Meningioma: 3, Other: 2	Oral cancer: 2, parotid cancer: 2, breast cancer: 1, thyroid cancer: 1
Treatment for primary ALL					
Protocol (11:12:13:14:15)	1:3:0:2:5	2:1:0:1:1	0:0:0:0:2	6:3:1:1:1	3:0:2:0:1
Cranial irradiation	6/11 (55%)	5/5 (100%)	0/2 (0%)	13/13 (100%)	5/6 (83%)
Dose of cranial irradiation (Gy)	18 (0–28)	18 (18–24)	24 (18–36)	0	18 (0–24)
Anthracyclines (DOX equivalent)	230 (50–330)	72 (0–190)	112 (82–142)	120 (0–190)	47 (0–230)
Cyclophosphamide ($\times 10^3$ g)	4·0 (3·1–6·0)	4·0 (0–5·6)	1·0 (0–2·0)	4·0 (0–6·8)	1·1 (0–6·0)
Etoposide ($\times 10^3$ g)	0 (0–2·4)	0 (0–2·4)	0 (0–2·4)	0	0 (0–1·2)
Duration of maintenance (weeks)	52 (28–172)	96 (62–172)	96 (22–175)	78 (52–104)	112 (0–172)
Stem cell transplantation	0	0	0	1/13 (8%)	2/6 (33%)
Treatment for secondary cancer (SC)					
Surgery	0	0	0	9	6
Radiation	0	0	0	7	3
Chemotherapy	11	4	2	6	3
Stem cell transplantation	8	1	0	0	0
Median survival duration (years)	1·7 (0·2–4·3)	4·6 (0·9–11·1)	3·6 (0·5–6·7)	2·0 (0·1–11·3)	3·0 (0·8–10·4)
4 year survival rate (%)	24%	60%	50%	50%	83%
Standardized incidence ratio (SIR) and absolute excess risk (AER)					
No. observed/expected	16/0·64	2/0·52	13/0·36	6/2·45	
SIR (95%CI)	25 (14–41)	3·8 (0·5–14)	36 (19–62)	2·5 (0·9–5·3)	
AER/100 000 person-years	118	9·4	90	26	

ALL, acute lymphoblastic leukaemia; AML, acute myeloid leukaemia; MDS, myelodysplastic syndrome; RAEB, refractory anaemia with excess blasts; CMML, chronic myelomonocytic leukaemia NHL, Non-Hodgkin lymphoma; WBC, white blood cell; SR/IR/HR, standard/intermediate/high risk; DOX, doxorubicin; 95% CI, 95% confidence interval.
Numbers shown as median (range; minimum-maximum).

developed a secondary cancer (1 brain tumour and 2 other cancers). All 3 cases received total body irradiation-containing conditioning regimens, two of 3 developed tongue carcinoma whilst suffering from chronic graft-versus-host disease after allogeneic SCT. SCTs were common among secondary AML patients as treatment. Kaplan–Meier OS curves for the patients with secondary cancers are shown in (Fig 3D). The lowest survival probabilities were observed for patients with AML/MDS/NHL compared to patients with brain tumours and other carcinoma (P = 0·045 by log-rank test).

SIR and AER

We compared the incidence of secondary cancers in our cohort with that of the general population using the regional cancer registration database of the National Cancer Centre Hospital in Japan. As shown in Table II, the SIR was 25 (95% CI, 14–41) for AML/MDS, 3·8 (95% CI, 0·5–14) for lymphoma, 36 (95% CI, 19–62) for brain tumours and 2·5 (95% CI, 0·9–5·3) for other solid carcinoma. This represents a 9·3-fold (95% CI, 6·5–12·8) increase risk of all secondary cancers during a total of 27 658 person-years of observation. The total AER for secondary cancers was 256 per 100 000 person-years.

Risk factors for secondary cancers

The unadjusted analyses comparing patients with and without secondary cancers showed differences in age at ALL diagnosis, risk classification, CPM and CRT, while there were no statistically significant differences with respect to gender,

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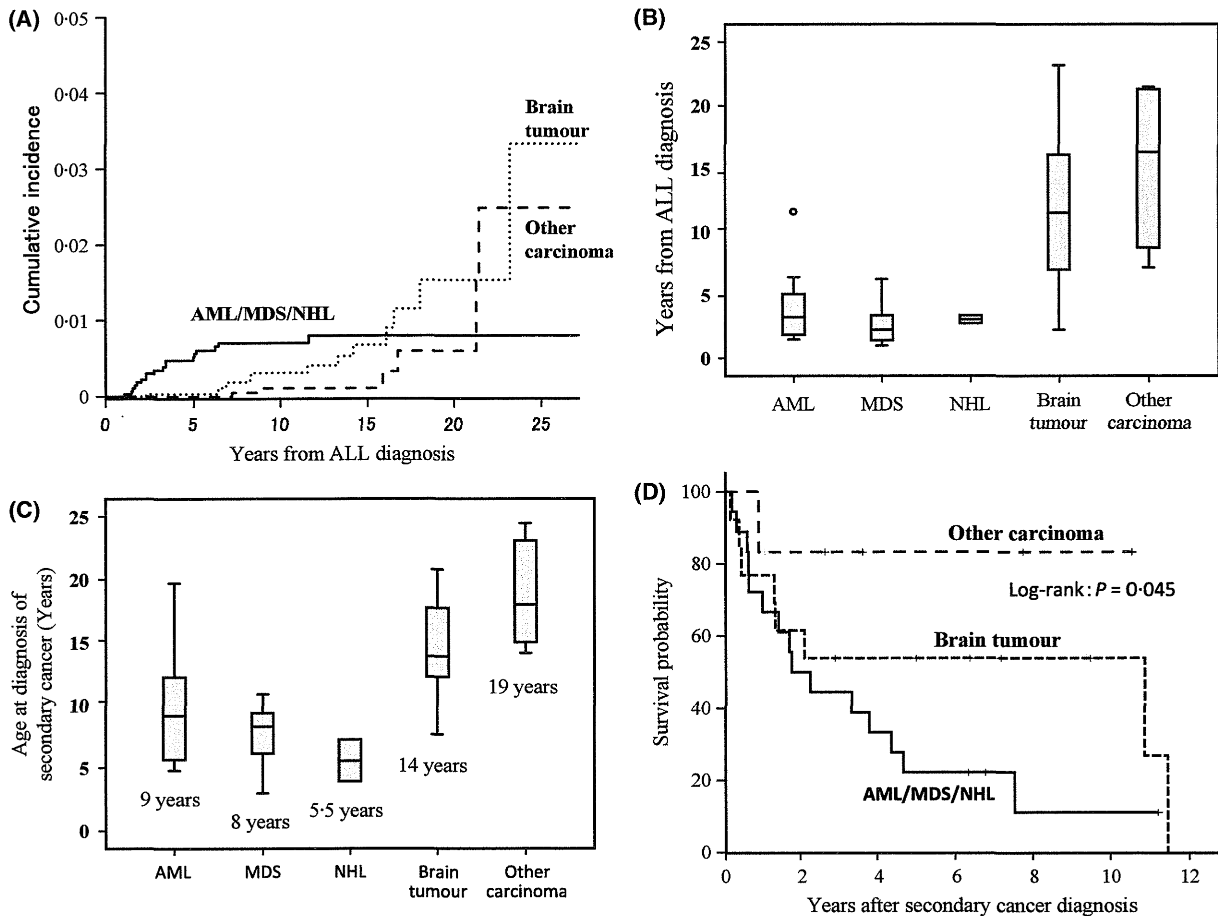

Fig 3. Clinical characteristics according to types of secondary cancer. (A) Cumulative incidence by years since ALL diagnosis of specific secondary cancers including AML/MDS/NHL (solid line), brain tumour (dotted line), and other carcinoma (dashed line). (B) The median latency period from diagnosis of ALL to development of specific secondary cancers. The median time for haematological cancers (AML, MDS and NHL) was shortest, followed by brain tumours and other solid carcinoma. (C) Age at diagnosis of secondary cancers; generally, the median age of haematological cancers was younger compared to brain tumours and other carcinomas. (D) Overall survival of secondary cancer patients are shown using Kaplan–Meier survival curves. Survival probabilities were the lowest for patients with AML/MDS/NHL. Actuarial survival at 4 years from diagnosis of secondary cancers depend on the type; AML/MDS/NHL 33%; brain tumours 54%; other carcinoma 83%. AML, acute myeloid leukaemia; MDS, myelodysplastic syndrome; NHL, Non-Hodgkin lymphoma.

initial WBC, immunophenotype, anticancer agents (with the exception of CPM) and maintenance duration of the primary ALL (Table III and Figure S2). Because protocol and anticancer drugs were highly correlated, we were unable to effectively evaluate them in the same multivariate regression analysis. Thus, results using Cox regression adjusting for covariates including treatment protocol (but not anticancer drug) (Table III) showed that CRT was associated with a 6-fold increased risk of secondary cancers compared to patients not receiving CRT (HR = 6·02, 95% CI 1·46–24·8). When CRT was categorized into 3 groups based on dose (i.e. no CRT, 18 Gy, and >24 Gy), similarly increased risks were observed for the moderate and high dose categories (data not shown). Age at ALL diagnosis >7 years (versus 3 years or younger, HR = 3·01, 95% CI 1·14–7·94) and inclusion in the more recent TCCSG L99-15/L04-1502 protocol (versus L84-11, HR = 8·15, 95% CI 1·03–64·7) were independently associated with an increased risk of secondary cancers. The same model, but replacing treatment protocol with the anticancer drugs (i.e. CPM, yes versus no; etoposide, yes versus no; high-dose methotrexate, yes versus no) showed an attenuated risk estimate for CPM (HR = 1·84, 95 CI 0·32–10·4), despite it being statistically significant in the unadjusted analysis (OR = 3·05, 95% CI 1·06–8·76).

Discussion

The risk of secondary cancers in childhood ALL survivors may be influenced by genetic predisposition, but growing evidence shows therapeutic regimen to be another major contributing factor. The risk of developing secondary cancers should be interpreted in the context of the survival

Table III. Cox-regression analysis evaluating the association between select characteristics of the primary ALL diagnosis and risk of developing a secondary cancer.

Intention to treat analysis group (*n* = 2807)	Patients with Secondary cancer	Patients without Secondary cancer	Crude HR (95%CI)	Adjusted HR (95%CI)	*P*-value
Protocol					
L84-11	12	476	Reference	Reference	
L89-12	7	392	1·00 (0·37–2·69)	1·35 (0·47–3·84)	0·576
L92-13	4	336	0·78 (0·24–2·56)	3·64 (0·45–29·1)	0·224
L95-14	4	584	0·56 (0·17–1·91)	4·47 (0·46–43·6)	0·198
L99-15/L04-1502	10	982	1·12 (0·42–3·01)	8·15 (1·03–64·7)	0·047
Risk classification					
Standard risk	7	1021	Reference	Reference	
Intermediate risk	20	956	3·42 (1·44–8·08)	2·70 (0·84–8·69)	0·096
High risk	10	771	2·67 (1·02–7·03)	1·01 (0·21–4·84)	0·992
Age at ALL diagnosis					
3 years or younger	8	986	Reference	Reference	
4–7 years	12	965	1·63 (0·67–3·98)	1·76 (0·71–4·40)	0·224
8 years or older	17	888	3·10 (1·34–7·21)	3·01 (1·14–7·94)	0·026
Gender: Male/Female	18/19	1530/1207	1·29 (0·68–2·46)	1·37 (0·71–2·62)	0·347
Attained age ≥20 years: No/Yes	20/17	2054/685	0·89 (0·42–1·90)	0·46 (0·19–1·12)	0·089
Cranial irradiation: No/Yes	8/29	1310/1445	2·57 (1·15–5·75)	6·02 (1·46–24·8)	0·013
Maintenance >1·5 years: No/Yes	15/22	1547/1209	1·16 (0·57–2·36)	3·19 (0·55–18·4)	0·194
Anticancer drugs					
Anthracycline: No/Yes	4/33	182/2574	1·32 (0·45–3·89)	N/A	N/A
Cyclophosphamide: No/Yes	4/33	448/2308	3·05 (1·06–8·76)	N/A	N/A
Etoposide: No/Yes	24/13	1910/846	1·30 (0·65–2·60)	N/A	N/A
High-dose Methotrexate: No/Yes	15/22	793/1963	0·77 (0·23–2·54)	N/A	N/A

ALL, acute lymphoblastic leukaemia; HR, hazard ratio; 95% CI, 95% confidence interval; N/A, not available.
Total number of patients may not equal 2807 for all variables due to missing data.

probability for a given treatment protocol, as low survival will result in fewer secondary cancers. Although the lifetime incidence of secondary cancers has not yet been defined, within the first 20 years of initial diagnosis of childhood ALL, previous studies conducted the U.S. and Europe have estimated it to be between 2% and 5%. To our knowledge, our study is the first conducted among an Asian population to report estimates of the cumulative incidence of secondary cancers in childhood ALL survivors. We found that the cumulative incidence of any secondary cancers in ALL survivors was 1·0% at 10 years and 2·4% at 20 years, respectively.

The previous reports on secondary cancers in childhood ALL survivors are summarized in Table IV. In 1991, the Children's Cancer Group (CCG) evaluated 9720 cases of ALL diagnosed since 1972 (Neglia *et al*, 1991) with a more recent update reported by Bhatia *et al*(2002) The CCG report showed a cumulative incidence of 1·3% at 10 years after ALL diagnosis, whereas the Berlin-Frankfurt-Munster (BFM) study (Loning *et al*, 2000) observed an overall cumulative incidence of secondary cancers at 15 years of 3·3% and 2·9% (95% CI: 1·6%–4·2%) among patients in first CR. In 1991, a Norwegian study found an overall cumulative incidence of 2·9% by 20 years after diagnosis in a group of 895 patients treated between 1958 and 1985 (Nygaard *et al*, 1991). In the St. Jude study reported by Hijiya *et al* (2007) a comparatively higher cumulative incidence of 4·2% at 15 years and 11% at 30 years was found. Our study of Japanese patients resulted in cumulative incidence and SIR estimates that are consistent with these results reported by the CCG, BFM, and Norwegian studies.

Previous reports from the CCSS and BCCSS (Mody *et al*, 2008; Reulen *et al*, 2011) calculated cumulative incidence and SIR estimates of secondary cancers within cohorts of childhood cancer patients that have survived at least 5 years. The distribution of secondary cancer types reported by those studies appeared to be different compared to ours and other prospective clinical studies (Table IV). As shown previously and in our study, most AML and MDS developed within 5 years after diagnosis of ALL. Thus, studying 5 year childhood cancer survivors probably influenced the comparatively fewer numbers of AML/MDS secondary cancers observed in the CCSS and BCCSS (Table IV).

Our results are also consistent with previous studies with respect to the median latency period by secondary cancer type (shortest for AML/MDS/NHL)(Loning *et al*, 2000; Bhatia *et al*, 2002; Hijiya *et al*, 2007) over-representation of females (Neglia *et al*, 2001; Bhatia *et al*, 2002; Meadows *et al*, 2009) in secondary AML/MDS, and CRT as a strong risk factor for secondary cancer development.(Neglia *et al*, 1991; Nygaard *et al*, 1991; Loning *et al*, 2000; Borgmann *et al*,

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