a   Mock   RUNX1dC

■ multiple foci
□ foci(<4)

1h

γH2AX | Hoechst

Merge

1h

% of foci positive cells

100
80
60
40
20
0

Mock   RUNX1dC

b

6h

6h

p=0.0112

% of foci positive cells

70
60
50
40
30
20
10
0

Mock   RUNX1dC

c

Number of colonies

p=0.0125

100
80
60
40
20
0

Mock   RUNX1dC

% of colonies

p=0.0251

120
100
80
60
40
20
0

■ 0 J/m²
□ 2 400 J/m²

Mock   RUNX1dC

Figure 2 RUNX1dC-transduced cells attenuate the DDR response to double-strand breaks in LSK cells. (a) Accumulations of γH2AX foci in Mock- and RUNX1dC-transduced LSK cells 1 h after γ-ray treatment (left panel). Hoechst refers to Hoechst 33342. The scale bar (10 μm) applies to all images. The percentage of γH2AX foci-positive cells in Mock- and RUNX1dC-transduced LSK cells are shown (right panel). Results are the average of three experiments. Open square indicates % of γH2AX foci-positive cells (with fewer than four foci per cell), and closed square indicates % of multiple foci-positive cells (four or more foci per cell). (b) Accumulations of γH2AX foci in Mock- and RUNX1dC-transduced LSK cells 6 h after γ-ray treatment (left panel). The percentage of γH2AX foci-positive cells in Mock- and RUNX1dC-transduced LSK cells 6 h after γ-ray treatment are shown (right panel). (c) Colony-forming ability of Mock- and RUNX1dC-transduced LSK cells after UV-B treatment. Each bar shows absolute colony numbers (left panel) or the % of colonies relative to those of non-treated cells (right panel). Data are means ± s.e.m. of triplicate experiments.

lymphoma patients, who did not have BM involvement of the disease, as controls. All of the MDS/AML patients were diagnosed at Hiroshima University Hospital and its affiliated hospitals between 2000 and 2005. Diagnosis was made based on morphologic and immunophenotypic studies according to the French-American-British classification.[18] Clinical characteristics of MDS/AML patients are shown in Supplementary Table 2.[19] This project was approved by the Institutional Review Board at Hiroshima University, and BM samples were taken after obtaining informed consent. Probes and primers used in the quantitative PCR assay are listed in the Supplementary Methods.

Statistical analysis
All values are expressed as means ± s.e.m. We performed statistical analysis using Student's t-test. Values were considered statistically significant at $P < 0.05$.

Results

RUNX1dC induces DNA-damage accumulation in LSK cells
DNA-damage accumulation and its repair pathways have important roles in tumor development.[13] Thus, we first

evaluated DNA-damage accumulation in a C-terminal truncated RUNX1 mutant-transduced LSK cells. In this study, we utilized the RUNX1dC, which lacks 225 amino acids covering both transactivating and transrepressing domains of WT RUNX1 (Figure 1a). This mutant was originally identified in a patient with MDS, and we and others previously showed it functions as a dominant-negative mutant over WT RUNX1 (RUNX1b) by inhibiting its DNA-binding activity.[20,21] RUNX1dC resembles in size an isoform of RUNX1, RUNX1a, (Figure 1a), which also has an inhibitory effect against RUNX1b.[22] Two days after gene transduction, we evaluated accumulations of DSBs using an antibody recognizing the phosphorylated histone variant H2AX at serine 139 (γH2AX).[23] As shown in Figures 1b and c, more DSBs were detected in RUNX1dC-transduced LSK cells than in Mock-transduced LSK cells (42% foci-positive cells in RUNX1dC-transduced cells vs 15% foci-positive cells in Mock-transduced cells, $P = 0.0006$).

RUNX1dC transduction results in the attenuation of the DDR response to DSBs in LSK cells
We next examined the DDR response in RUNX1dC-transduced LSK cells after exposure to environmental stressors. γH2AX foci, which indicate the immediate chromatin modification in

response to the formation of DSBs, reached a peak 30–60 min after irradiation (Figure 2a). Thereafter, the number of foci decreased with a half-life of several hours due to the DNA-repair response to DSBs.[24] Therefore, sustained accumulation of γH2AX foci indicates attenuation of the DDR response to DSBs. A total of 6 h after γ-ray treatment, RUNX1dC-transduced cells showed significantly higher accumulation of γH2AX foci than Mock-transduced LSK cells ($P = 0.0112$; Figure 2b). Moreover, although the percentage of multiple foci-positive cells carrying more severe accumulations of DSBs was only 37.6% among γH2AX-positive cells in Mock transduction, most (93.5%) γH2AX-positive cells had multiple foci in RUNX1dC-transduced LSK cells (Figure 2b right panel). In addition, we analyzed the colony-forming ability of Mock- or RUNX1dC-transduced LSK cells after treatment with UV-B. In this assay, the DDR response of tested cells can be measured by relative colony numbers because only cells that recovered from DNA damage can make colonies.[25] At steady state, there was no significant difference in colony-forming ability between Mock- and RUNX1dC-transduced LSK cells (Figure 2c left panel). On the other hand, analyses of relative colony numbers revealed RUNX1dC-transduced LSK cells showed significantly lower colony-forming ability than Mock-transduced LSK cells after UV-B exposure (32.2% in RUNX1dC-transduced LSK vs 48.8% in Mock-transduced LSK, $P = 0.0251$; Figure 2c right panel).

RUNX1dC suppresses Gadd45a expression in LSK cells

As DSBs were more prominent in RUNX1dC-transduced LSK cells than in Mock-transduced LSK cells, we speculated that the defect in the DDR response gene may be a cause of enhanced DNA-damage accumulation in RUNX1dC-transduced LSK cells. Therefore, we next examined whether RUNX1dC alters expressions of DNA-damage signaling molecules. We prepared 32Dcl3 cells expressing RUNX1dC and confirmed expression by immunoblot (Supplementary Figure 1). We profiled the expression of 84 genes involved in DNA-damage signaling by RT-PCR array using this subclone. Before performing the RT-PCR array experiment, we confirmed 32D-RUNX1dC showed significantly lower colony-forming ability than 32D-neo cells after UV-B exposure, just like RUNX1dC-transduced LSK cells (at 1600 J/m²: 38.2% in 32D-RUNX1dC vs 67.2% in 32D-neo, $P < 0.0234$; Figure 3a right panel). Furthermore, we evaluated the accumulation of cyclobutane pyrimidine dimers, which are major products of DNA damage induced by UV-B, in 32D-neo and 32D-RUNX1dC cells.[26] There was no distinct difference in cyclobutane pyrimidine dimer accumulation between 32D-neo and 32D-RUNX1dC cells before UV-B treatment (Figure 3b). Although 1 h after UV-B treatment the levels of cyclobutane pyrimidine dimer accumulation were the same in 32D-neo and 32D-RUNX1dC cells, the removal of cyclobutane pyrimidine dimer was significantly impaired in 32D-RUNX1dC cells compared with 32D-neo (Figure 3b). In addition, as with RUNX1dC-transduced LSK cells, 32D-RUNX1dC cells showed sustained accumulation of γH2AX foci after γ-ray irradiation (Figure 4b). After correction using five housekeeping genes (Gusb, Hprt1, Hsp90ab1, Gapdh and Actb), we obtained four candidate genes, in which expression was repressed in 32D-RUNX1dC cells compared with 32D-neo cells with a fold-difference cutoff of <0.5 (Figure 3c and Supplementary Table 1). Among these candidate genes, the expression of Gadd45a was particularly decreased in 32D-RUNX1dC cells compared with 32D-neo cells (Figure 3c and Supplementary Figure 2a). Quantitative RT-PCR using 32D-RUNX1dC and 32D-neo cells confirmed the expression of Gadd45a was
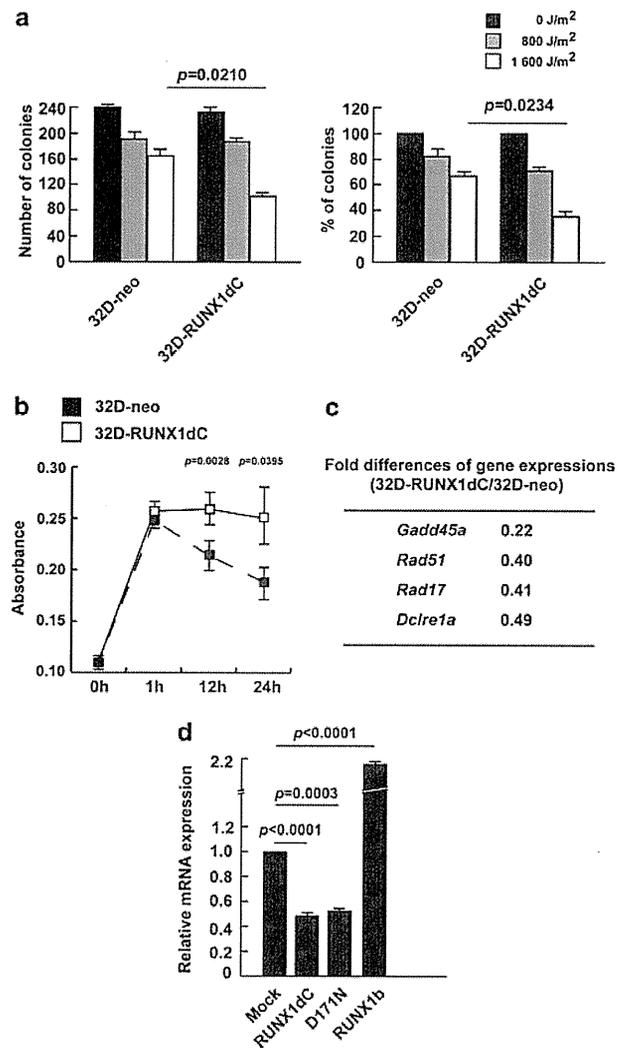

Figure 3 RUNX1dC suppresses Gadd45a expression in LSK cells. (a) Colony-forming assays for 32D-neo and 32D-RUNX1dC cells after treatment with UV-B. Test cells (1×10^3) were plated onto methylcellulose medium and cultured with IL-3. Each bar shows absolute colony numbers (left panel) or the % of colonies relative to that of non-treated cells (right panel). Data are means ± s.e.m. of triplicate experiments. (b) Determination of UV-B induced DNA damage by enzyme linked immunosorbant assay using anti-cyclobutane pyrimidine dimers antibody. Data are means ± s.e.m. of quadruplicate experiments. (c) Expression profiling of 84 genes involved in DNA-damage signaling by RT-PCR array. Fold differences of gene expressions with a cutoff of <0.5 (32D-RUNX1dC/32D-neo) are shown. (d) Relative mRNA expression levels of Gadd45a in Mock-, RUNX1dC-, D171N- and RUNX1b-transduced murine LSK cells (normalized to Gapdh). Data are means ± s.e.m. of triplicate experiments.

significantly lower (42%) in 32D-RUNX1dC cells than in 32D-neo cells (Supplementary Figure 2b). To examine if this result was relevant to normal LSK cells, we also examined the expression of Gadd45a in RUNX1dC-transduced LSK cells. RUNX1dC-transduced LSK cells showed significantly lower expression (44%) of Gadd45a than Mock-transduced LSK cells (Figure 3d). LSK cells, which were transduced with a RUNX1 mutant harboring a point mutation in the Runt homology domain (D171N)[19,21] also showed significantly lower (53%) expression of Gadd45a compared with Mock-transduced LSK
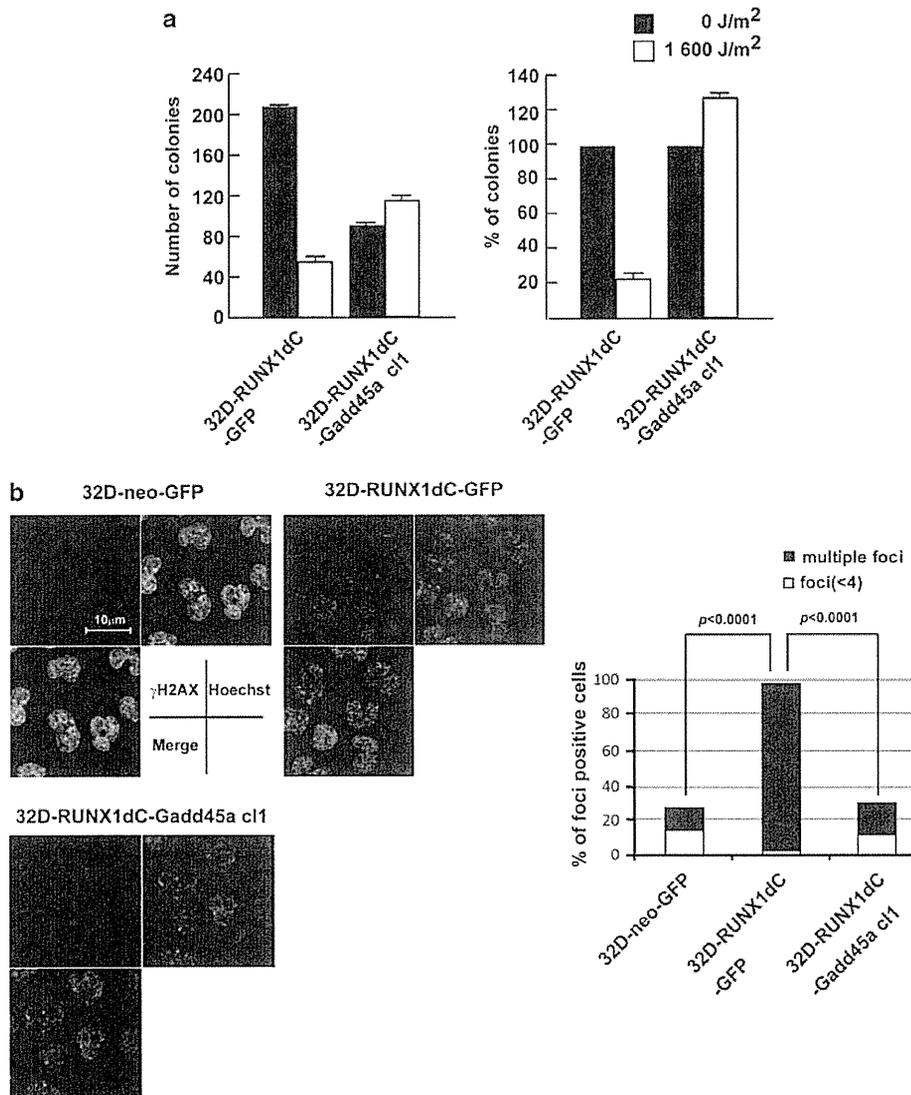
Figure 4 Exogenously expressed Gadd45a reduced γH2AX-positive foci in 32D-RUNX1dC. (a) Colony-forming ability of 32D-RUNX1dC-GFP and 32D-RUNX1dC-Gadd45a cl1 cells after UV-B treatment. Each bar shows absolute colony numbers (left panel) or the % colonies relative to those of non-treated cells (right panel). Data are means ± s.e.m. of triplicate experiments. (b) Accumulations of γH2AX foci in 32D-neo-GFP, 32D-RUNX1dC-GFP and 32D-RUNX1dC-Gadd45a cl1 cells 6 h after γ-ray treatment (left panel). The percentage of γH2AX foci-positive cells in 32D-neo-GFP, 32D-RUNX1dC-GFP and 32D-RUNX1dC-Gadd45a cl1 cells 6 h after γ-ray treatment are shown (right panel). Results are the average of three experiments. Open square indicates % of γH2AX foci-positive cells (with fewer than four foci per cell), and closed square indicates % of multiple foci-positive cells (four or more foci per cell).

cells (Figure 3d). Moreover, WT RUNX1-transduced LSK cells expressed more *Gadd45a*, an increase of 108% relative to Mock-transduced LSK cells (Figure 3d). To examine whether the impaired colony-forming ability of 32D-RUNX1dC cells attributed to the repressed expression of *Gadd45a* by RUNX1dC, we introduced the expression vector for Gadd45a into 32D-RUNX1dC cells, which was named 32D-RUNX1dC-Gadd45a (Supplementary Figure 3a). Consistent with the report from Perugini et al.,[27] Gadd45a overexpression reduced the cell proliferation ability at steady state (Figure 4a left panel). After UV-B treatment, exogenously expressed Gadd45a restored the colony-forming ability in 32D-RUNX1dC-Gadd45a cl1 cells (32D-RUNX1dC-GFP, 21.8%; 32D-RUNX1dC-Gadd45a cl1, 132%; Figure 4a right panel). Similar results were obtained from two other clones (Supplementary Figure 3b). Furthermore,

we found exogenously expressed Gadd45a reduced γH2AX-positive foci in 32D-RUNX1dC-Gadd45a cl1 cells compared with 32D-RUNX1dC-GFP cells after UV-B treatment (Figure 4b). In addition, the reduction of γH2AX-positive foci in 32D-RUNX1dC-Gadd45a cl1 cells was also observed at steady state (Supplementary Figure 3c).

RUNX1 transcriptionally regulates Gadd45a expression
To analyze the mechanism by which RUNX1 regulates *Gadd45a* expression, we utilized luciferase assays using a human myeloid leukemia cell line UT-7/GM. Although there was no consensus sequence for RUNX binding in the 5′-flanking region of the human *GADD45A* gene, we found two RUNX-binding sequences neighboring the p53-binding site in
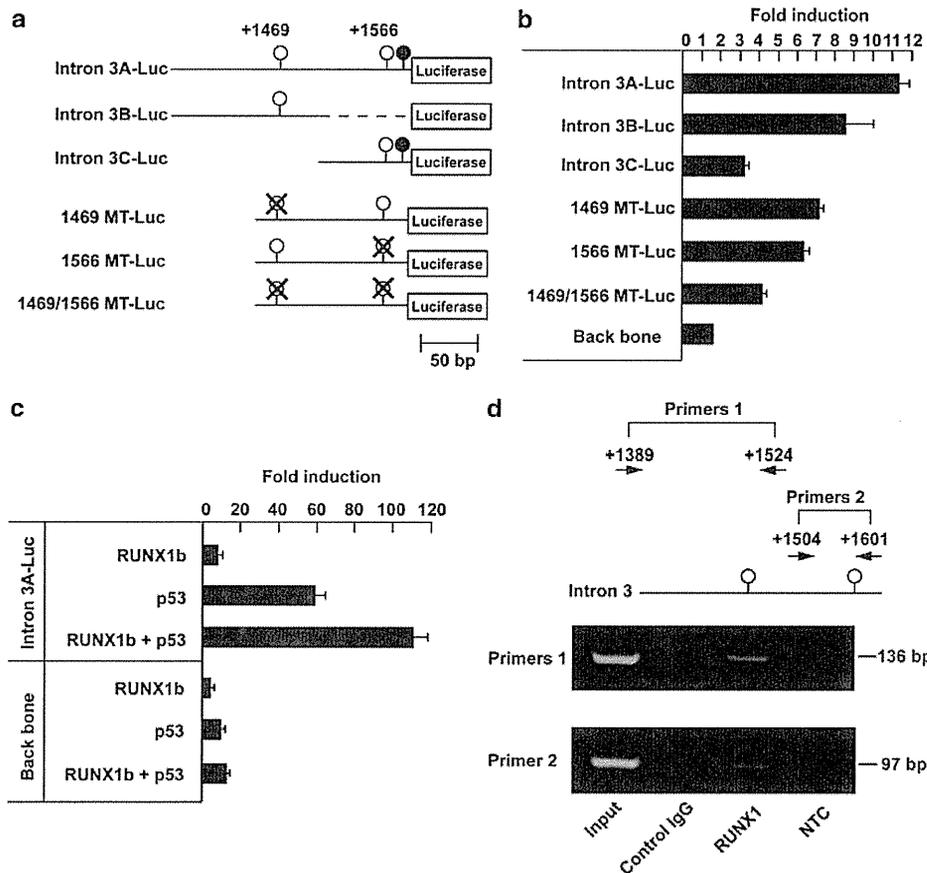
Figure 5 Analyses of transcriptional regulation of *GADD45A* by RUNX1. (a) Structures of human *GADD45A* luciferase reporter genes. Open circle indicates RUNX-binding sequences (at +1469 and +1566) and the closed circle indicates the reported p53-binding site in intron 3 of *GADD45A*. Crossed open circle indicates a mutation of the RUNX-binding sequence (TGTGGT changed into TGTTAG). (b) Reporter assays using human *GADD45A* luciferase reporter genes. Each bar indicates the fold induction of reporter activity induced by RUNX1b (relative to pRC-CMV control vector). UT-7/GM cells were transfected with 2 μg of each reporter gene (intron 3A-Luc (containing +1389–1601), intron 3B-Luc (+1389–1524), intron 3C-Luc (+1505–1601), 1469 MT-Luc, 1566 MT-Luc and 1469/1566 MT-Luc) or back bone plasmid, 0.5 μg of pRL-CMV-Rluc, 1 μg of CBFβ, and 2 μg of RUNX1b or pRC-CMV. Results shown are the means±s.e.m. of triplicate experiments. (c) Co-expression experiment of RUNX1b and p53 using the intron 3A-Luc construct. Each bar indicates the fold induction of reporter activity induced by RUNX1b and/or p53 (relative to pRC-CMV control vector). Cells were transfected with 2 μg each of RUNX1b and/or p53 with 2 μg of intron 3A-Luc or Back bone plasmid, 0.5 μg of pRL-CMV-Rluc and 1 μg of CBFβ. Results shown are the means±s.e.m. of triplicate experiments. (d) ChIP assays using the nuclear extract of UT-7/GM cells with the anti-RUNX1 Ab or control goat IgG. Two sets of closed arrows indicated primer positions in intron 3 of the *GADD45A* gene. Open circle indicates the RUNX-binding sequences and NTC means non-template control.

intron 3 of the *GADD45A* gene. Intron 3 is highly conserved between humans and rodents, and p53 transcriptionally activates *GADD45A* expression through this p53-binding site.[28,29] Therefore, we generated three types of luciferase reporter genes covering this region. Intron 3A-Luc contained the two RUNX-binding sequences (at +1469 and +1566) of the human *GADD45A* gene. Intron 3B-Luc contained only the upstream binding sequence (+1469) and intron 3C-Luc contained only the downstream binding sequence (+1566; Figure 5a). RUNX1b together with its heterodimerization partner CBFβ activated intron 3A-Luc, intron 3B-Luc and intron 3C-Luc 11.2-fold, 8.6-fold, and 3.2-fold, respectively (Figure 5b). The 1469 MT-Luc and 1566 MT-Luc constructs, which have one mutated RUNX-binding sequence (TGTGGT changed to TGTTAG), showed reduced reporter activity by RUNX1b compared with intron 3A-Luc (7.3-fold activation in 1469 MT-Luc and 6.1-fold activation in 1566 MT-Luc, Figures 5a and b). Furthermore, the 1469/1566 MT-Luc construct, which has two mutated RUNX-binding sequences, showed a 63% reduction of reporter

activity by RUNX1 compared with intron 3A-Luc (4.1-fold activation). In addition, the co-expression experiment with RUNX1b and p53 revealed these two genes synergistically activate the intron 3A-Luc construct (only p53: 59.6-fold activation and p53 with RUNX1: 108.4-fold activation; Figure 5c). We also examined the change in intron 3A-Luc activation by RUNX1b and/or p53 after γ-ray irradiation; yet, there was no difference in reporter activities between γ-ray irradiated and non-irradiated UT-7/GM cells (data not shown). Next, we examined the co-expression effects of RUNX1 mutants on *GADD45A*-reporter activation with RUNX1b. We had to transfect high amount of DNA into test cells; therefore we utilized the adherent cell line, 293T, to perform the luciferase assay.[20] Single transduction experiment of RUNX1dC revealed RUNX1dC showed reduced *GADD45A*-reporter activity compared with the transduction of RUNX1b (10-fold activation in RUNX1b and 5-fold activation in RUNX1dC). In the case of the D171N mutant, the D171N mutant did not show the significant *GADD45A*-reporter activity (3.3-fold) compared with back-bone
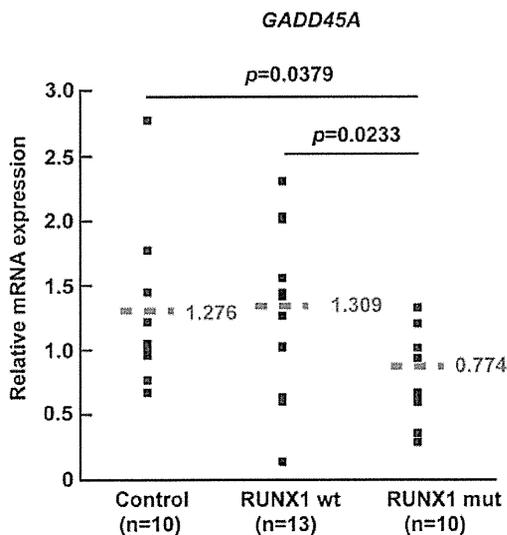
GADD45A

Figure 6 Expression levels of *GADD45A* in MDS/AML patients. Relative expression levels of *GADD45A* in BMMNCs from controls and MDS/AML patients with WT *RUNX1* (RUNX1 wt) or C-terminal mutations (RUNX1 mut) are shown. Horizontal bar and value denotes mean *GADD45A* expressions (normalized to *GAPDH*). The patients' characteristics are shown in Supplementary Table 2.

reporter activity (2.9-fold). In addition, we found RUNX1b-induced intron 3A-Luc activity was reduced to 55% by RUNX1dC and to 37% by the D171N mutant (Supplementary Figure 4). To test whether endogenous RUNX1 binds to the *GADD45A* gene *in vivo*, we conducted ChIP assays using the nuclear extract of UT-7/GM cells. As shown in Figure 5d, the two RUNX-binding sequences were immunoprecipitated with the anti-RUNX1 antibody but not by control IgG. Together, these data indicate that endogenous RUNX1 binds to two RUNX1-binding sites in intron 3 of the *GADD45A* gene, thereby regulating its transcription.

GADD45A expression is significantly decreased in MDS/AML patients harboring *RUNX1*-C-terminal mutations compared with those with WT *RUNX1*

Finally, we evaluated *GADD45A* expression in BM mononuclear cells from 23 MDS/AML patients with or without *RUNX1* C-terminal mutations and from 10 controls.[19] We found BM mononuclear cells from MDS/AML patients harboring *RUNX1*-C-terminal mutations had significantly lower *GADD45A* expression compared with those from MDS/AML patients with WT *RUNX1* ($P = 0.0233$; Figure 6).

Discussion

We found RUNX1 transcriptionally regulates *Gadd45a*. In our experiment using LSK cells, the expression of *Gadd45a* was reduced to 44% of that of control cells by RUNX1dC (Figure 3d). This level of *Gadd45a* expression is roughly the same as that observed in *Gadd45a*^{+/-} mice, which do not develop spontaneous tumors but are susceptible to tumor development in response to mutagens because of genomic instability.[30] Like *Gadd45a*^{+/-} mice, heterozygous deletion of *RUNX1* predisposes mice to AML when accompanied by other gene alterations.[31] The similar phenotypes of *Gadd45a*^{+/-} and

RUNX1^{+/-} mice raise the possibility that both molecules have closely related roles in maintaining genome stability. As for the relationship between RUNX1 and Gadd45a, Perugini *et al.*[27] recently reported that *GADD45A* expression was significantly downregulated in RUNX1-ETO-positive AML cells compared with normal controls. Although they demonstrated ERK1/2 signaling was involved in the repression of *GADD45A* in several AML cell lines, its mechanism was not analyzed in their study. In previous papers, RUNX1-ETO was shown to decrease the expression of several genes involved in the base excision repair pathway, such as oxoguanine DNA glycosylase and polymerase epsilon, thereby DNA damage accumulated.[32,33] Gadd45a functions in the nucleotide excision repair pathway through its interaction with proliferating cell nuclear antigen;[34–36] therefore, both base excision repair and nucleotide excision repair might be impaired in hematopoietic cells carrying RUNX1 mutations. Gadd45a also functions as a stress sensor, which is mediated by a complex interplay of physical interactions with other cellular proteins that are implicated in cell-cycle regulation and the response of cells to stress.[17] Gupta *et al.*[37] reported that Gadd45a-deficient hematopoietic cells demonstrated an impaired DDR response. They found myeloid-enriched BM cells from Gadd45a- or Gadd45b-deficient mice were defective in G2/M arrest following exposure to UV and VP-16 and these cells were sensitized to genotoxic-stress-induced apoptosis.[37] Similarly, we found that RUNX1dC-transduced LSK cells showed lower colony-forming ability than Mock-transduced LSK cells after UV-B exposure. Like RUNX1dC-transduced cells, *Gadd45a* expression was reduced in D171N-transduced LSK cells compared with Mock-transduced LSK cells (Figure 3d). The D171N mutant does not have DNA-binding ability, whereas it still maintains the ability to bind to CBFβ.[5] The co-expression experiment with D171N inhibited *GADD45A* reporter activation induced by RUNX1b (Supplementary Figure 4); therefore, it is possible that D171N inhibits the function of WT RUNX1 by trapping CBFβ. Differences in the DDR response between N-terminal in-frame type mutations (such as the D171N mutant) and C-terminal truncated mutations (such as the RUNX1dC) of *RUNX1* should be explored in a future study.

Gadd45a is a well known transcriptional target of p53.[29] p53 binds to the conserved sequence within intron 3 of the *GADD45A* gene in response to ionizing radiation.[29] Here, we showed that RUNX1 activates transcription of *GADD45A* through binding to two RUNX-binding sites neighboring the p53-binding site within intron 3 (Figure 5b). RUNX1 and p53 showed synergistic effects on intron3A-Luc activation (Figure 5c); therefore, these two molecules may cooperatively regulate *GADD45A* expression. In our experiment, however, γ-ray irradiation did not influence intron 3A-Luc activation by RUNX1 and/or p53. We are now trying to examine the cooperating effect of RUNX1 and p53 on DDR response, including *GADD45A* regulation, using a more physiological setting.

MDS patients with a *RUNX1* mutation frequently present with more advanced diseases and *RUNX1* mutations are considered to be markers of a poor prognosis.[19,38,39] We speculate that a RUNX1 mutation may be a cause of additional mutation(s) through the impaired DDR response. In future studies, whole genome analysis of MDS/AML samples harboring RUNX1 mutations would clarify the role of RUNX1 mutations in the occurrence of genetic abnormalities in these patients.

In conclusion, we demonstrated that a *RUNX1* C-terminal deletion mutant attenuates the DDR response to environmental and physiological stresses in LSK cells. As a possible explanation

for this mechanism, we found a novel role for RUNX1 in the transcriptional regulation of *Gadd45a*. The impaired Gadd45a function leads to genomic instability; therefore, Gadd45a dysfunction induced by *RUNX1* mutations can cause the additional mutation(s) required for multi-step leukemogenesis. Further studies on RUNX1/Gadd45a would be useful to understand the pathophysiology of MDS/AML patients harboring RUNX1 abnormalities and to prevent disease progression.

Conflict of interest

The authors declare no conflict of interest.

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Chromosome abnormalities in advanced stage T-cell lymphoblastic lymphoma of children and adolescents: a report from Japanese Paediatric Leukaemia/Lymphoma Study Group (JPLSG) and review of the literature

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Summary

T-cell acute lymphoblastic leukaemia (T-ALL) and T-cell lymphoblastic lymphoma (T-LBL) are combined into one category as T lymphoblastic leukaemia/lymphoma in the current World Health Organization (WHO) classification. However, there is still ongoing discussion on whether T-ALL and T-LBL are two separate entities or represent two variant phenotypes of the same disease. Cytogenetic analysis has been used to identify the molecular background of haematological malignancies. To compare the distribution of chromosomal abnormalities of T-ALL and T-LBL, large series of cytogenetic data are required, but are absent in T-LBL in contrast to the abundant data in T-ALL. Among 111 T-LBL cases in our clinical trial, we obtained complete cytogenetic data from 56 patients. The comparison between our cytogenetic findings and those from three published T-LBL studies revealed no significant difference. However, meta-analysis showed that translocations involving chromosome region 9q34 were significantly more common in T-LBL than in T-ALL. In particular, four out of the 92 T-LBL cases, but none of the 523 paediatric T-ALL cases, showed translocation t(9;17)(q34;q22–23) ($P = 0.0004$). Further studies are needed for the possible linkage between abnormal expression of genes located at 9q34 and/or 17q22–23 and the unique 'lymphoma phenotype' of T-LBL.

Keywords: T-cell lymphoma, child, non-Hodgkin lymphoma, cancer cytogenetics, leukaemia.

In children and adolescents, precursor T lymphoblastic neoplasms have been classified into two diseases: T-cell acute lymphoblastic leukaemia (T-ALL) and T-cell lymphoblastic lymphoma (T-LBL). Although the current World Health Organization (WHO) classification designates both malignancies as T lymphoblastic leukaemia/lymphoma (Borowitz & Chan, 2008), there is continuing discussion on whether T-ALL and T-LBL are two separate entities or whether they represent

two different clinical presentations of the same disease. They show overlapping clinical, pathological and immunophenotypic features. In general, the word 'lymphoma' is used if there is a bulky mass in the mediastinum or elsewhere, with less peripheral blood and bone marrow (BM) involvement. Most study groups distinguish between leukaemia and lymphoma on the basis of the extent of BM involvement: patients with <25% lymphoblasts in the BM are diagnosed with lymphoblastic lymphoma; in cases

of 25% or more BM blasts, the diagnosis is leukaemia. While this distinction may appear somewhat arbitrary, a notable observation is that T-LBL patients with large mediastinal masses frequently exhibit little, if any, evidence of tumour dissemination and BM involvement, but the molecular background for this difference is unknown.

Chromosomal analysis has been widely used as a primary step that is required to narrow down the responsible genes that define a disease entity. For instance, discovery of Ph chromosome led to the identification of the chimeric *BCR/ABL1* gene, which is responsible for and defines chronic myeloid leukaemia. Compared with T-ALL, chromosomal abnormalities in T-LBL are not well defined. Reports in the literature and current textbooks claim that the typical chromosomal aberrations reported in T-ALL can also be found in T-LBL (Borowitz & Chan, 2008). However, there are no large series of cytogenetic data on T-LBL (Burkhardt, 2010).

This study aimed to fill the gap regarding cytogenetic data in T-LBL and compare the cytogenetic findings of T-ALL and T-LBL, which may lead to identification of the molecular background behind phenotypical differences between the two disease entities.

Study patients

From November 2004 to October 2010, 154 eligible children (aged 1–18 years) with newly diagnosed advanced stage LBL (Murphy stages III and IV) (Murphy, 1980) were entered in the Japanese Paediatric Leukaemia/Lymphoma Study Group (JPLSG) ALB-NHL03 study (UMIN000002212, http://www.umin.ac.jp/ctr/index-j.htm). Patients with primary immunodeficiencies, Down syndrome and T-cell diseases as second malignancies were excluded. The ethics committee of each participating institute approved the study protocol.

Cytogenetic analysis

Cytogenetic analysis was performed on cell suspensions obtained from 31 tumour/lymph nodes, 19 pleural effusions and six bone marrow samples. The methods of chromosome preparation for cytogenetic analysis are described elsewhere (Sanger *et al*, 1987; Horsman *et al*, 2001). Karyotypes are described according to the International System for Human Cytogenetic Nomenclature (ISCN) (Shaffer & Tommerup, 2005). Only those cases with abnormal cytogenetic study results, defined as two or more cells with the same structural abnormality or the same numerical gain, three or more cells with the same numerical loss or isolated cells with disease-associated abnormalities, were eligible for inclusion in this study.

Statistical methods

Two-tailed Fisher's exact test was used to analyse the patients' characteristics and the frequency of each chromosome abnormality. Significant differences in the analysis of he frequency of

each chromosome abnormality were determined by the two-tailed Fisher's exact test with Bonferroni correction comparison. The *P* value threshold for inclusion of a new variable was chosen to be $P < 0.003$ in this analysis (0.05/17, after Bonferroni correction). A review of T-LBL and T-ALL karyotypes reported in the literature was obtained from a PubMed search and information on chromosome abnormalities and gene fusions was obtained from Mitelman Database of Chromosome Aberrations and Gene Fusions in Cancer (http://cgap.nci.nih.gov/Chromosomes/Mitelman).

Results

Patient characteristics

A total of 154 children were enrolled on JPLSG ALB-NHL03 protocols; 111 cases were T-LBL. Among 111 T-LBL cases, the study population for the current analysis included 56 patients for whom complete cytogenetic data were obtained. With respect to presenting features, patients with reviewed and accepted cytogenetic data were similar to both those without accepted cytogenetic data and the entire cohort of concurrently enrolled T-lineage LBL patients (Table S1).

Frequency of chromosomal abnormalities

Multiple chromosome abnormalities were identified in 31 patients (45%). Structural chromosome abnormalities were identified in 29 patients (52%), and numerical chromosome abnormalities were identified in 18 patients (32%). Ploidy results included pseudodiploid in 14 patients (25%), hypodiploid in three patients (5%), hyperdiploid with 47–50 chromosomes in 10 patients (18%), hyperdiploid with more than 50 chromosomes in four patients (7%) and diploid in 25 patients (45%) (Table S2).

All of the hypodiploid cases had 43–45 chromosomes; none had a near-haploid karyotype. Of the four cases with more than 50 chromosomes, two had near-tetraploid karyotypes. The frequencies of ploidy groups in this series are compared with those reported in other series of karyotyped T-LBL patients and paediatric T-ALL (Table S2). Structural chromosome abnormalities were identified in 29 patients (52%). In the current study, seven patients (13% of those with abnormal karyotypes) exhibited a rearrangement at one or more of the chromosome bands (7p15, 7q32–36 and/or 14q11–13) that are the locations of T-cell receptor chain genes. Rearrangements in the 14q11–13 region, in which the T-cell receptor α/δ chain genes are located, were present in three patients (5%) of the karyotypically abnormal cases in this series (Table S2). Structural abnormalities involving chromosome region 9q34 were identified in nine patients (16%). Translocations involving chromosome region 9q34 were identified in three patients (5%) (t(9;17)(q34;q22), t(7;9)(q34;q34) and t(2;9)(q23;q34)). In comparison between cytogenetic findings in the current data and combined data of three published reports (Burkhardt

et al, 2006; Lones et al, 2007; Uyttebroeck et al, 2007; Table S1), the frequencies of numerical and structural cytogenetic abnormalities in T-LBL and T-ALL had no significant difference (Table S2).

We compared the cytogenetic findings in the current study with the published reports from the three largest-scale studies on T-LBL (Burkhardt et al, 2006; Lones et al, 2007; Uyttebroeck et al, 2007; Table S3) and those from the two largest-scale studies on T-ALL combined (Heerema et al, 1998; Schneider et al, 2000; Table S3) (Table I). The frequencies of almost all of the cytogenetic abnormalities in T-LBL and T-ALL had no significant difference, but translocation involving chromosome region 9q34 was significantly more common in T-LBL than in T-ALL ($P = 0.0004$, Table S3) and translocation t(9;17) was also more common in T-LBL (4%, 4/92) than in T-ALL (0%, 0/523, $P = 0.0004$) (Table I).

The current study included a patient with translocation t(9;17)(q34;q22). As far as we could tell from the consulted published reports, all T-LBL patients with translocation t(9;17) presented with a mediastinal mass and without any bone marrow involvement (Kaneko et al, 1988; Shikano et al, 1992) (Table II).

Discussion

This is the largest study involving cytogenetic analysis of T-LBL and the first study to directly compare cytogenetic findings of T-LBL and T-ALL. The frequencies of almost all of the cytogenetic abnormalities in both entities were found to have no significant difference, but translocation involving chromosome region 9q34 was significantly more common in T-LBL than in T-ALL. The current study included a patient with unique translocation t(9;17)(q34;q22). Interestingly, four out of the 92 T-LBL cases, but none of the 523 paediatric T-ALL cases, showed this translocation ($P = 0.0004$) (Table I). Translocation t(9;17) has been reported in several haematological diseases, such as precursor B-cell ALL (Coyaud et al, 2010), acute myeloid leukaemia (Mrózek et al, 2001), chronic myeloid leukaemia (DeAngelo et al, 2004), chronic lymphocytic leukaemia (Michaux et al, 2005), diffuse large B-cell lymphoma (Hammond et al, 1992) and follicular lymphoma (Aamot et al, 2007), but these breakpoints, 9q34 and 17q22–23, are limited in the cases of T-LBL (http://cgap.nci.nih.gov/Chromosomes/Mitelman). These results imply a linkage between abnormal expression of genes located at 9q34 and/or 17q22–23 and the unique phenotypes of the T-LBL mentioned above.

Cytogenetic analysis has been used to identify the molecular background of haematological malignancies. To compare the distribution of chromosomal abnormalities of T-ALL and T-LBL, large series of cytogenetic data are required, but are absent in T-LBL in contrast to the abundant data in T-ALL. Three recent series of cytogenetic data on paediatric T-LBL have been published, reporting the cytogenetic findings in 13, 11 and 12 paediatric T-LBL cases (Burkhardt et al, 2006; Lones

Table I. Comparison of cytogenetic findings between T-LBL and T-ALL.

	T-LBL		T-ALL		
	n	%	n	%	P value
Total	92		523		
Normal karyotype†	36	39	219	42	0·6478
Abnormal karyotype	56	61	304	58	0·6478
Hypodiploid	4	4	20	4	0·9999
Pseudodiploid	30	33	204	39	0·2000
Hyperdiploid(47–50)	18	20	64	12	0·0328
Hyperdiploid(>50)	4	4	16	3	0·5217
Any translocation	26	28	177	34	0·3367
Any del chrome.	19	21	160	31	0·0328
Any der chrome.	4	4	58	11	0·0583
del(6q)	6	7	69	13	0·0833
Loss of 9p	10	11	44	8	0·5487
Any 14q11–13 abnormality	10	11	72	14	0·5100
Any 7q32–36 abnormality	7	8	35	7	0·8220
Any translocation including 9q34	8	9	7	1	0·0004*
t(7;10)	1	1	2	0	0·3855
t(10;11)	1	1	8	2	0·9999
t(9;17)	4	4	0	0	0·0004*

†Includes one Klinefelter syndrome, and one inv(9) without other abnormality in current report.

The P value threshold for inclusion of a new variable was chosen to be 0·003 (0·05/17, after Bonferroni correction). *P < 0·003.

T-LBL: current study (JPLSG ALB-NHL03) combined with three published reports(Burkhardt et al, 2006; Lones et al, 2007; Uyttebroeck et al, 2007).

T-ALL: combined two published reports (Heerema et al, 1998; Schneider et al, 2000).

et al, 2007; Uyttebroeck et al, 2007). Thus, this study can play a role to fill the gap of cytogenetic data on T-LBL.

Translocation involving chromosome region 9q34 was found to be significantly more common in T-LBL than in T-ALL (Table I). Among genes located in the 9q34 region, SET, PKN3, ABL1, NUP214 and NOTCH1 have previously been implicated in malignancy, with SET, ABL1, NUP214 and NOTCH1 being implicated in leukemogenesis (Ellisen et al, 1991; van Vlierberghe et al, 2008; Hagemeijer & Graux, 2010).

An oncogenic SET-NUP214 fusion gene has been reported in a case of acute undifferentiated leukaemia with a reciprocal translocation t(9;9)(q34; q34) (von Lindern et al, 1992) and NK adult acute myeloid leukaemia as a result of a cryptic deletion of 9q34 (Rosati et al, 2007). van Vlierberghe et al (2008) identified the SET-NUP214 fusion gene in three patient samples out of 92 paediatric cases of T-cell leukaemia. SET-NUP214 may contribute to T-ALL pathogenesis by inhibition of T-cell maturation through the transcriptional activation of the HOXA genes (van Vlierberghe et al, 2008). However, the frequency of this mutation in T-LBL is unknown.

NOTCH1, previously termed TAN1, was discovered as a partner gene in T-ALL with a translocation t(7;9)(q34;q34.3), and was found in <1% of T-ALLs (Ellisen et al, 1991). Several

Table II. Clinical characteristics and detailed karyotype data in T-LBL patients with t(9;17).

	Age (years)	Sex	Tumour site	Stage	BM blast %	Karyotype
Kaneko et al (1988)	14	F	Mediastinum	III	0	46,XX,t(9;17)(q34;q23)
	15	M	Mediastinum	III	0	46,XY,-9,del(6)(q13q21),t(9;17)(q34;q23),+der(9)t(9;17)(q34;q23)
	10	M	Mediastinum	III	0	47,XY,+19,t(9;17)(q34;q23)
Shikano et al (1992)	14	F	Mediastinum	III	0	46,XX,t(9;17)(q34;q23)
	7	M	Mediastinum	III	0	49,XY-l,+der(l)t(l;?)(p36;?),t(9;17)(q34;q23),+14,+marl,+mar2
	5	F	Mediastinum	III	0	47,XX,t(9;17)(q34;q23),+der(17)t(9;17)(q34;q23)
Burkhardt et al (2006)	ND	ND	ND	ND	ND	46,XX,del(6)(q1?2q1?6),t(9;17)(q34;q22)
	ND	ND	ND	ND	ND	47,XX,t(9;17)(q34;q22),+20
Lones et al (2007)	8	M	Mediastinum	III	0	47,XY,t(9;17)(q3?4;q2?3),+20
Current study	7	M	Mediastinum	III	0	46,XY,t(9;17)(q34;q22)

ND, no data available.

study groups reported NOTCH1 mutations in 31–62% of T-ALL patients (Weng et al, 2004; Breit et al, 2006; van Grotel et al, 2006; Zhu et al, 2006; Malyukova et al, 2007; Asnafi et al, 2009; Gedman et al, 2009; Park et al, 2009). In contrast, only two studies reported NOTCH1 mutation analyses in T-LBL: Park et al (2009) reported NOTCH1 mutations in six out of 14 paediatric T-LBL patients (43%), and Baleydier et al (2008) reported mutations in six out of nine paediatric T-LBL (66%), with 32 adult patients with NOTCH1 mutations in 16 cases (54% in all patients) (Baleydier et al, 2008). According to these reports, the frequencies of NOTCH1 mutation were not significantly different between T-LBL and T-ALL.

ABL1 fusion genes have been identified that provide proliferation and survival advantage to lymphoblasts. NUP214-ABL1, EML1-ABL1, BCR-ABL1 and ETV6-ABL1 chimeric genes have been reported. The most frequent one in T-ALL is the NUP214-ABL1 fusion gene, which has been identified in 6% of cases, in both children and adults (Graux et al, 2009). In addition, using an oligonucleotide microarray, ABL1 overexpression was identified in 8% of cases in T-ALL (Chiaretti et al, 2007). Our review of these published reports indicated that the frequency of ABL1 mutation in T-LBL is unknown.

Raetz et al (2006) analysed the gene expression profiles of ten T-ALL BM samples and nine T-LBL samples using a microarray. They identified 133 genes for which the expression levels differed between T-LBL and T-ALL. ZNF79 (encoding zinc finger protein 79) and ABL1, both located in chromosome region 9q34, were included in these genes and showed at least twofold higher overexpression in T-LBL than that in T-ALL. Additionally, MED13 (previously termed THRAP1), which is located in 17q22-q23, also showed at least twofold higher overexpression in T-LBL than that in T-ALL (Raetz et al, 2006). Taking these findings together, it is possible that ZNF79, ABL1 or THRAP1 as well as other genes at 9q34 and 17q22–23 are involved in the 'lymphoma phenotype' such as a bulky mass in the mediastinum and minimal BM involvement. These findings need further study to determine if this linkage constitutes a unique 'lymphoma phenotype'.

Acknowledgements

Authorship

MS designed the study, prepared the data file, performed the analysis, interpreted data and wrote the manuscript. SS is a lead principal investigator for the JPLSG ALB-NHL03 study. AN contributed to pathological diagnosis. YH contributed to chromosome analysis. YO is a principal investigator contributing a patient to this study. AMS contributed to statistical analysis. KH received a research grant from the Ministry of Health, Labour and Welfare of Japan. MT is a chairperson of JPLSG. TM is a chairperson of JPLSG lymphoma committee. SS, KH, MT and TM were primarily responsible for the study design, data analysis and interpretation of the data. All authors approved the final manuscript.

Disclosure

The authors declare no competing financial interests.

Supporting Information

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

Table S1. Respective clinical characteristics with and without karyotype data in 111 T-LBL patients in the current study.

Table S2. Comparison of cytogenetic findings in T-LBL between current study and combined data of three published reports.

Table S3. Published data of cytogenetic findings in T-LBL and T-ALL.

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