Gene Alterations Involving the CRLF2-JAK Pathway and Recurrent Gene Deletions in Down Syndrome-Associated Acute Lymphoblastic Leukemia in Japan

（本邦のダウン症候群関連急性リンパ性白血病における CRLF2-JAK 経路の遺伝子異常および白血病関連遺伝子の欠失）

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INTRODUCTION

Down syndrome (DS) shows a significantly increased incidence of both acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL) in childhood, and a complex relationship to leukemogenesis (Nižetić et al., 2012). Patients with DS and ALL have a lower incidence of favorable cytogenetic abnormalities (e.g., t(12;21) and high hyperdiploidy; Forestier et al., 2008; Maloney et al., 2010) and poorer treatment outcomes than those with sporadic ALL (non-DS patients; Buitenkamp et al., 2014). DS-ALL patients also experience increased morbidity and mortality in association with chemotherapy (Buitenkamp et al., 2014). Thus, more effective and less toxic treatments, such as targeted therapies, are needed for these patients.

The cytokine receptor-like factor 2 (*CRLF2*) gene encodes a protein that plays a role in cellular proliferation via activation of the JAK-STAT signaling pathway. Overexpression of *CRLF2* is observed in approximately 50-60% of DS-ALL patients in Western countries (Mullighan et al., 2009; Russell et al., 2009; Hertzberg et al., 2010). In most of these patients, *CRLF2* overexpression is associated with interstitial deletions of the pseudoautosomal regions (PAR1-IDs) of the sex chromosomes, resulting in the *P2RY8-CRLF2* fusion. Less frequently, translocations between the immunoglobulin heavy chain locus (*IGH*) and PAR1 that result in the *IGH-CRLF2* rearrangement are associated with *CRLF2* overexpression. *JAK2* mutations and *CRLF2* overexpression are thought to act cooperatively in leukemogenesis, and activating *JAK2* mutations are observed in approximately 20% of DS-ALL patients (Bercovich et al., 2008; Gaikwad et al., 2009; Kearney et al., 2009). Mutations in

JAK1, *CRLF2*, and interleukin-7 receptor-α (*IL7R*) are found in less than 10% of DS-ALL patients (Hertzberg et al., 2010; Blink et al., 2011; Shochat et al., 2011). Although the CRLF2-JAK signaling pathway is considered a potential therapeutic target, the incidence rates of these genetic alterations have not been established for Asian patients with DS-ALL.

In contrast to the mechanisms discussed above, the frequencies of gene deletions involved in the pathogenesis of B-cell precursor ALL (BCP-ALL) in DS-ALL patients are similar to those in non-DS ALL patients (Hertzberg et al., 2010; Loudin et al., 2011). *IKZF1* deletions have been detected in approximately 20-30% of DS-ALL patients, and are associated with a poor outcome (Buitenkamp et al., 2012; Patrick et al., 2014). The initial report of frequent *BTG1* deletions in older male DS-ALL patients (29%) was not confirmed in a subsequent study using a larger cohort (Lundin et al., 2012;Buitenkamp et al., 2013), and *EBF1* deletions have rarely been found in DS-ALL cases (Kearney et al., 2009; Hertzberg et al., 2010; Ensor et al., 2011; Loudin et al., 2011; Buitenkamp et al., 2012). The frequencies and clinical implications of these gene deletions in Asian DS-ALL patients also remain to be determined. Therefore, the goal of this study was to evaluate genetic alterations and their associated clinical effects in 38 DS-ALL patients of Japanese descent.

MATERIALS AND METHODS

Patients and Samples

This study was approved by the Ethics Committee of Hirosaki University Graduate School of

Medicine, and all clinical samples were obtained with parental informed consent in accordance with the Declaration of Helsinki. Diagnostic bone marrow samples were obtained from 28 DS-ALL patients < 20 years of age from 14 pediatric and/or hematology departments throughout Japan. These samples included frozen bone marrow cells (n = 17) and genomic DNA extracted from bone marrow specimens (n = 11). All patients were treated with curative intent according to various protocols between 1987 and 2011. Additionally, frozen bone marrow cells of 10 patients who were treated with the Japan Association of Childhood Leukemia Study (JACLS) ALL-97 or ALL-02 protocol between 1997 and 2007 were obtained from the JACLS investigators. The protocols were approved by the institutional review board of each participating institute, and written informed consent was obtained from the parents of all patients. The bone marrow samples of 25 unselected non-DS ALL patients who were treated at Hirosaki University Hospital between 2002 and 2010 served as controls for the *CRLF2* expression study. Genomic DNA was extracted from bone marrow cells with the QIAamp DNA Blood Mini Kit (QIAGEN, Venlo, Netherlands), and total RNA was extracted with the RNeasy Plus Mini Kit (QIAGEN). Complementary DNA (cDNA) was prepared using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA).

Clinical data of the first 28 patients were collected by a standardized questionnaire and included sex, age at diagnosis, initial white blood cell (WBC) count, cytogenetic and molecular genetic abnormalities, treatment protocol, and outcome. Data on the additional 10 patients were provided by JACLS.

P2RY8-CRLF2 Amplification and Mutation Analysis

Genomic PCR and reverse-transcription polymerase chain reaction (RT-PCR) for the *P2RY8-CRLF2* fusion were performed using previously described primers (Mullighan et al., 2009; Harvey et al., 2010; Morak et al., 2012). All PCR products were directly sequenced in both directions using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster city, CA) on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

Genomic PCR and/or RT-PCR were performed for exons 12-18 of *JAK1*, exons 13-19 of *JAK2*, exons 11-15 of *JAK3*, exons 1-6 of *CRLF2*, and exons 5 and 6 of *IL7R* using previously described primers (Bercovich et al., 2008; Sato et al., 2008; Hertzberg et al., 2010; Shochat et al., 2011). All PCR products were directly sequenced in both directions.

Quantitative Real-Time PCR

Quantitative real-time PCR for *CRLF2* was performed using previously described primers (Yoda et al., 2010) and iQ SYBR Green Supermix (Bio-Rad Laboratories) on the Chromo4 Real-time PCR System (Bio-Rad Laboratories). Each sample was run in triplicate. The comparative Ct method was used to quantify relative mRNA expression levels using the endogenous control gene *GAPDH*.

Copy Number Determination

Multiplex ligation-dependent probe amplification (MLPA) was performed using the SALSA MLPA probemix P335-B1 ALL-IKZF1 to detect chromosomal deletions and/or duplications associated with ALL. Reactions were performed according to the manufacturer's instructions (MRC-Holland, Amsterdam, Netherlands). Values < 0.75 and > 1.3 were considered indicative of deletions and amplifications, respectively (Schwab et al., 2010). PAR1-IDs were defined as *CSF2RA*/*IL3RA*/*P2RY8* deletions with normal or amplified *CRLF2*.

Statistical Analysis

Differences in the distribution of individual parameters among patient subsets were analyzed using the Fisher's exact test for categorized variables and the Mann-Whitney *U* test for continuous variables. Estimation of survival was performed using the Kaplan-Meier method, and differences were compared using the log-rank test. Event-free survival (EFS) was defined as the time from diagnosis to any event (induction failure, relapse, death, or second malignancy), and overall survival (OS) was defined as the time from diagnosis to death from any cause. *P*-values < 0.05 were considered statistically significant.

RESULTS

Clinical Characteristics of DS-ALL Patients

The clinical characteristics of the 38 Japanese DS-ALL patients are summarized in Table 1. Full

clinical data sets were available for 34 patients, and sex, age, and treatment protocol data were available for 35 patients. The male to female ratio was 1.1:1, and the median age at diagnosis was 6.4 years (range, 2.2-19.1 years). The median WBC count at diagnosis was 16.5 $x10^9$/l (range, 1.1-187.5 $x10^9$/l), and 47% of patients were classified as high risk according to the National Cancer Institute (NCI) risk criteria. Recurrent cytogenetic abnormalities were found in 6 patients (16%): 2 with t(12;21)(p13;q22), 2 with high hyperdiploidy, 1 with dic(9;20)(p13;q11), and 1 with t(8;14)(q11;q32). With a median follow-up of 44.0 months (range, 2-221 months), estimated 5-year EFS and OS were 65.8 ± 9.3% and 70.9 ± 9.0%, respectively.

P2RY8-CRLF2 and *JAK2* Mutations

The *P2RY8-CRLF2* fusion was identified in 11 patients (29%) by genomic PCR and/or RT-PCR (Table 1), 3 of whom had the newly discovered breakpoint 2.1 kb upstream of the *CRLF2* exon 1 (Morak et al., 2012). The *JAK2* R683G mutation was observed in 6 patients (16%; Table 1), consisting of the same single nucleotide substitution (c.2047A>G). These 6 patients also carried the *P2RY8-CRLF2* fusion. No mutations were found in *JAK1*, *JAK3*, *CRLF2*, or *IL7R* in any of the patients.

CRLF2 Expression

CRLF2 expression levels were compared among three patient groups: *P2RY8-CRLF2*-positive

DS-ALL, *P2RY8-CRLF2*-negative DS-ALL, and non-DS ALL. RNA was available from 27 DS-ALL patients, including 7 *P2RY8-CRLF2*-positive and 25 non-DS ALL patients. The median *CRLF2* expression level in the *P2RY8-CRLF2*-positive DS-ALL patients was significantly higher than that in *P2RY8-CRLF2*-negative DS-ALL ($P = 0.001$) and non-DS ALL patients ($P < 0.001$; Fig. 1). No significant difference in *CRLF2* expression levels was observed between *P2RY8-CRLF2*-negative DS-ALL and non-DS ALL patients. When a *CRLF2* level ≥10-fold the median level in non-DS ALL patients was defined as overexpression (Harvey et al., 2010), 6 of 7 *P2RY8-CRLF2*-positive and 3 of 20 *P2RY8-CRLF2*-negative DS-ALL patients exhibited *CRLF2* overexpression (9/27, 33% in total).

Copy Number Alterations of Genes Involved in BCP-ALL Development

A summary of the MLPA results is shown in Fig.2. DNA samples from 32 patients were available for analysis. PAR1-IDs were detected in 8 patients (25%), all of whom were positive for *P2RY8-CRLF2*. In one patient with *P2RY8-CRLF2*, values for *CSF2RA/IL3RA/P2RY8* were lower than that for *CRLF2*, but the result did not meet the criteria of PAR1-IDs. *EBF1* deletions were found in 5 patients (16%), significantly more in *P2RY8-CRLF2*-positive than in *P2RY8-CRLF2*-negative patients (44% vs. 4%, $P = 0.015$). Conversely, deletions of *CDKN2A/B* and *PAX5* were common in *P2RY8-CRLF2*-negative patients (48% and 39%, respectively) but rare in *P2RY8-CRLF2*-positive patients (11% each), although the differences were not statistically significant ($P = 0.103$ and 0.210, respectively). Deletions of *BTG1* and *IKZF1* were detected in 8 patients each (25%), and 4 and 3 of

these patients had *P2RY8-CRLF2*, respectively. Deletions of *ETV6* and *RB1* were found in 5 (16%) and 2 (6%) patients, respectively, all of whom were negative for *P2RY8-CRLF2*.

Association of Genetic Alterations with Clinical Characteristics

The distributions of sex, age at diagnosis, initial WBC count, NCI risk group, and recurrent cytogenetic abnormalities did not differ significantly between patients with and without each genetic alteration. The results for *P2RY8-CRLF2*, *BTG1*, and *IKZF1* are shown in Table 2. Of note, no *P2RY8-CRLF2*-positive patients had recurrent cytogenetic abnormalities. In patients with *BTG1* deletions, the male to female ratio was 1.0, and mean age was 4.8 years (range, 2.2-11.2). Estimated EFS and OS were not significantly different between patients with and without each genetic alteration, with the exception of inferior OS in patients with *IKZF1* deletions (41.7 ± 22.2% vs. 83.8 ± 8.9% at 5 years, $P = 0.041$; Table 2 and Fig. 3).

DISCUSSION

In this study, the frequency of *P2RY8-CRLF2* in Japanese DS-ALL patients was slightly lower (29%) than that in Western countries (~50%), a finding that was confirmed by MLPA. Initially, the frequency of *P2RY8-CRLF2* was thought to be 21% (8/38), but 3 additional patients, none of whom had available RNA samples, were positive for *P2RY8-CRLF2* by genomic PCR using primer sets that detected the newly discovered *CRLF2* breakpoints (Morak et al., 2012). These primer sets should be

included in genomic PCR analysis to avoid an underestimation of the frequency of *P2RY8-CRLF2*.

We could not investigate the *IGH-CRLF2* rearrangement because samples for fluorescence in situ hybridization analysis were not available. Instead, we studied *CRLF2* expression levels in DS-ALL and non-DS ALL patients, since *CRLF2* expression levels are higher in *IGH-CRLF2*–positive DS-ALL patients than in *P2RY8-CRLF2*–positive DS-ALL patients (Russell et al., 2009; Harvey et al., 2010). However, no significant difference in *CRLF2* expression levels was observed between *P2RY8-CRLF2*–negative DS-ALL and non–DS-ALL patients. Moreover, *CRLF2* overexpression was observed in only 3 of 20 *P2RY8-CRLF2*–negative DS-ALL patients. These results suggest that *IGH-CRLF2* is rare in our cohort. The *CRLF2* F232C mutation, which is also associated with *CRLF2* overexpression, was not found in any of the patients. Taken together, these findings suggest that the frequency of *CRLF2* rearrangements including *P2RY8-CRLF2*, *IGH-CRLF2*, and *CRLF2* mutations are lower in DS-ALL patients in Japan than in Western countries.

It is unclear why *CRLF2* rearrangements are less frequent in Japan. One study reported that *CRLF2* rearrangements were associated with Hispanic/Latino ethnicity in a high risk non-DS ALL cohort (Harvey et al., 2010), and two recent studies showed that *P2RY8-CRLF2* and *JAK2* mutations are rare in Japanese non-DS ALL patients (Asai et al., 2013; Yamashita et al., 2013). These results suggest that the frequency of these genetic alterations is dependent on ethnicity.

EBF1 deletions are very rare in DS-ALL patients in Western countries. Three studies did not find *EBF1* deletions in 10, 50, and 34 DS-ALL patients (0%; Ensor et al., 2011; Loudin et al., 2011;

Buitenkamp et al., 2012). In two earlier studies, *EBF1* deletions were only detected in 1/9 (11%; Kearney et al., 2009) and 1/15 DS-ALL patients (6.7%; Hertzberg et al., 2010). When these data are combined, the incidence of *EBF1* deletions is 2/118 (1.7%). However, a high frequency of *EBF1* deletions (5/32, 16%) were found in Japanese DS-ALL patients. Therefore, the frequency of *EBF1* deletions may also be dependent on ethnicity.

Moreover, *EBF1* deletions were significantly associated with *P2RY8-CRLF2*. *IKZF1* deletions are associated with *BCR-ABL* and other fusion genes that constitutively activate signaling pathways in *BCR-ABL*-positive ALL and *BCR-ABL*–like ALL, two pathways that are thought to contribute to leukemogenesis (Mullighan et al., 2008; Roberts et al., 2012). In the same manner, *EBF1* deletions and *P2RY8-CRLF2* may act to induce leukemogenesis in a subset of Japanese DS-ALL patients. Conversely, deletions of *CDKN2A/B* and *PAX5* were common in *P2RY8-CRLF2*–negative patients but rare in *P2RY8-CRLF2*–positive patients. It has been reported that *IKZF1* deletions are not associated with *P2RY8-CRLF2* or *JAK2* mutations in DS-ALL (Buitenkamp et al., 2012), but other associations with gene alterations remains unclear.

BTG1 deletions were detected in 8 of 32 patients (25%), with a frequency similar to that of Lundin et al. (2012) (29%) and higher than that of Buitenkamp et al. (2013) (6.9%). However, we could not confirm the previously reported associations of *BTG1* deletions with male predominance and older age (Lundin et al., 2012). *IKZF1* deletions were observed in 8 of 32 patients (25%) with a frequency similar to those in previous reports (Buitenkamp et al., 2012; Patrick et al., 2014). Although

the number of patients was limited, estimated OS was significantly shorter in patients with *IKZF1* deletions, which is also consistent with previous reports (Buitenkamp et al., 2012; Patrick et al., 2014). To determine the exact frequencies and clinical implications of these deletions in Japanese DS-ALL patients will require the evaluation of more patients.

In conclusion, a relatively low frequency of *P2RY8-CRLF2*, a high frequency of *EBF1* deletions, and an association of *EBF1* deletions and *P2RY8-CRLF2* were found in Japanese DS-ALL patients. The results indicate that differences exist between the genetic profiles of DS-ALL patients in Japan and in Western countries, and that *P2RY8-CRLF2* and *EBF1* deletions may cooperate in leukemogenesis in a subset of Japanese DS-ALL patients. Further studies are needed to characterize the full spectrum of genetic alterations in Japanese DS-ALL patients and to identify a potential target for therapy.

ACKNOWLEDGMENTS

The authors would like to thank Hirokazu Kanegane, University of Toyama; Akitoshi Kinoshita, St. Marianna University School of Medicine; Kazutaka Takaki, National Hospital Organization Kumamoto Medical Center; and Satoshi Saida and Hidefumi Hiramatsu, Kyoto University for providing patient samples and data. We also thank RuNan Wang and Hisae Kudo for their technical assistance.

REFERENCES

Asai D, Imamura T, Suenobu S, Saito A, Hasegawa D, Deguchi T, Hashii Y, Matsumoto K, Kawasaki H, Hori H, Iguchi A, Kosaka Y, Kato K, Horibe K, Yumura-Yagi K, Hara J, Oda M; Japan Association of Childhood Leukemia Study (JACLS). 2013. *IKZF1* deletion is associated with a poor outcome in pediatric B-cell precursor acute lymphoblastic leukemia in Japan. Cancer Med 2: 412-419.

Bercovich D, Ganmore I, Scott LM, Wainreb G, Birger Y, Elimelech A, Shochat C, Cazzaniga G, Biondi A, Basso G, Cario G, Schrappe M, Stanulla M, Strehl S, Haas OA, Mann G, Binder V, Borkhardt A, Kempski H, Trka J, Bielorei B, Avigad S, Stark B, Smith O, Dastugue N, Bourquin JP, Tal NB, Green AR, Izraeli S. 2008. Mutations of *JAK2* in acute lymphoblastic leukaemias associated with Down's syndrome. Lancet 372:1484-1492.

Blink M, Buitenkamp TD, van den Heuvel-Eibrink MM, Danen-van Oorschot AA, de Haas V, Reinhardt D, Klusmann JH, Zimmermann M, Devidas M, Carroll AJ, Basso G, Pession A, Hasle H, Pieters R, Rabin KR, Izraeli S, Zwaan CM. 2011. Frequency and prognostic implications of *JAK 1-3* aberrations in Down syndrome acute lymphoblastic and myeloid leukemia. Leukemia 25:1365-1368.

Buitenkamp TD, Pieters R, Gallimore NE, van der Veer A, Meijerink JP, Beverloo HB, Zimmermann M, de Haas V, Richards SM, Vora AJ, Mitchell CD, Russell LJ, Schwab C, Harrison CJ, Moorman AV, van den Heuvel-Eibrink MM, den Boer ML, Zwaan CM. 2012. Outcome in children with Down's syndrome and acute lymphoblastic leukemia: role of *IKZF1* deletions and *CRLF2*

aberrations. Leukemia 26:2204-2211.

Buitenkamp TD, Pieters R, Zimmermann M, de Haas V, Richards SM, Vora AJ, Mitchell CD, Schwab C, Harrison CJ, Moorman AV, van den Heuvel-Eibrink MM, Zwaan CM. 2013. *BTG1* deletions do not predict outcome in Down syndrome acute lymphoblastic leukemia. Leukemia 27:251-252.

Buitenkamp TD, Izraeli S, Zimmermann M, Forestier E, Heerema NA, van den Heuvel-Eibrink MM, Pieters R, Korbijn CM, Silverman LB, Schmiegelow K, Liang DC, Horibe K, Arico M, Biondi A, Basso G, Rabin KR, Schrappe M, Cario G, Mann G, Morak M, Panzer-Grümayer R, Mondelaers V, Lammens T, Cavé H, Stark B, Ganmore I, Moorman AV, Vora A, Hunger SP, Pui CH, Mullighan CG, Manabe A, Escherich G, Kowalczyk JR, Whitlock JA, Zwaan CM. 2014. Acute lymphoblastic leukemia in children with Down syndrome: a retrospective analysis from the Ponte di Legno study group. Blood 123:70-77.

Ensor HM, Schwab C, Russell LJ, Richards SM, Morrison H, Masic D, Jones L, Kinsey SE, Vora AJ, Mitchell CD, Harrison CJ, Moorman AV. 2011. Demographic, clinical, and outcome features of children with acute lymphoblastic leukemia and *CRLF2* deregulation: results from the MRC ALL97 clinical trial. Blood 117:2129-2136.

Forestier E, Izraeli S, Beverloo B, Haas O, Pession A, Michalová K, Stark B, Harrison CJ, Teigler-Schlegel A, Johansson B. 2008. Cytogenetic features of acute lymphoblastic and myeloid leukemias in pediatric patients with Down syndrome: an iBFM-SG study. Blood 111:1575-1583.

Gaikwad A, Rye CL, Devidas M, Heerema NA, Carroll AJ, Izraeli S, Plon SE, Basso G, Pession A,

Rabin KR. 2009. Prevalence and clinical correlates of *JAK2* mutations in Down syndrome acute lymphoblastic leukaemia. Br J Haematol 144:930-932.

Harvey RC, Mullighan CG, Chen IM, Wharton W, Mikhail FM, Carroll AJ, Kang H, Liu W, Dobbin KK, Smith MA, Carroll WL, Devidas M, Bowman WP, Camitta BM, Reaman GH, Hunger SP, Downing JR, Willman CL. 2010. Rearrangement of *CRLF2* is associated with mutation of *JAK* kinases, alteration of *IKZF1*, Hispanic/Latino ethnicity, and a poor outcome in pediatric B-progenitor acute lymphoblastic leukemia. Blood 115:5312-5321.

Hertzberg L, Vendramini E, Ganmore I, Cazzaniga G, Schmitz M, Chalker J, Shiloh R, Iacobucci I, Shochat C, Zeligson S, Cario G, Stanulla M, Strehl S, Russell LJ, Harrison CJ, Bornhauser B, Yoda A, Rechavi G, Bercovich D, Borkhardt A, Kempski H, te Kronnie G, Bourquin JP, Domany E, Izraeli S. 2010. Down syndrome acute lymphoblastic leukemia, a highly heterogeneous disease in which aberrant expression of *CRLF2* is associated with mutated *JAK2*: a report from the International BFM Study Group. Blood 115:1006-1017.

Kearney L, Gonzalez De Castro D, Yeung J, Procter J, Horsley SW, Eguchi-Ishimae M, Bateman CM, Anderson K, Chaplin T, Young BD, Harrison CJ, Kempski H, So CW, Ford AM, Greaves M. 2009. Specific *JAK2* mutation (*JAK2*R683) and multiple gene deletions in Down syndrome acute lymphoblastic leukemia. Blood 113:646-648.

Loudin MG, Wang J, Leung HC, Gurusiddappa S, Meyer J, Condos G, Morrison D, Tsimelzon A, Devidas M, Heerema NA, Carroll AJ, Plon SE, Hunger SP, Basso G, Pession A, Bhojwani D, Carroll

WL, Rabin KR. 2011. Genomic profiling in Down syndrome acute lymphoblastic leukemia identifies histone gene deletions associated with altered methylation profiles. Leukemia 25:1555-1563.

Lundin C, Hjorth L, Behrendtz M, Nordgren A, Palmqvist L, Andersen MK, Biloglav A, Forestier E, Paulsson K, Johansson B. 2012. High frequency of *BTG1* deletions in acute lymphoblastic leukemia in children with Down syndrome. Genes Chromosomes Cancer 51:196-206.

Maloney KW, Carroll WL, Carroll AJ, Devidas M, Borowitz MJ, Martin PL, Pullen J, Whitlock JA, Willman CL, Winick NJ, Camitta BM, Hunger SP. 2010. Down syndrome childhood acute lymphoblastic leukemia has a unique spectrum of sentinel cytogenetic lesions that influences treatment outcome: a report from the Children's Oncology Group. Blood 116:1045-1050.

Morak M, Attarbaschi A, Fischer S, Nassimbeni C, Grausenburger R, Bastelberger S, Krentz S, Cario G, Kasper D, Schmitt K, Russell LJ, Pötschger U, Stanulla M, Eckert C, Mann G, Haas OA, Panzer-Grümayer R. 2012. Small sizes and indolent evolutionary dynamics challenge the potential role of *P2RY8-CRLF2*-harboring clones as main relapse-driving force in childhood ALL. Blood 120:5134-5142.

Mullighan CG, Miller CB, Radtke I, Phillips LA, Dalton J, Ma J, White D, Hughes TP, Le Beau MM, Pui CH, Relling MV, Shurtleff SA, Downing JR. 2008. *BCR-ABL1* lymphoblastic leukaemia is characterized by the deletion of Ikaros. Nature 453:110-114.

Mullighan CG, Collins-Underwood JR, Phillips LA, Loudin MG, Liu W, Zhang J, Ma J, Coustan-Smith

E, Harvey RC, Willman CL, Mikhail FM, Meyer J, Carroll AJ, Williams RT, Cheng J, Heerema NA, Basso G, Pession A, Pui CH, Raimondi SC, Hunger SP, Downing JR, Carroll WL, Rabin KR. 2009. Rearrangement of *CRLF2* in B-progenitor- and Down syndrome-associated acute lymphoblastic leukemia. Nature Genet 41:1243-1246.

Nižetić D, Groet J. 2012. Tumorigenesis in Down's syndrome: big lessons from a small chromosome. Nat Rev Cancer 12:721-732.

Patrick K, Wade R, Goulden N, Rowntree C, Hough R, Moorman AV, Mitchell CD, Vora A. 2014. Outcome of Down syndrome associated acute lymphoblastic leukaemia treated on a contemporary protocol. Br J Haematol 165:552-555.

Roberts KG, Morin RD, Zhang J, Hirst M, Zhao Y, Su X, Chen SC, Payne-Turner D, Churchman ML, Harvey RC, Chen X, Kasap C, Yan C, Becksfort J, Finney RP, Teachey DT, Maude SL, Tse K, Moore R, Jones S, Mungall K, Birol I, Edmonson MN, Hu Y, Buetow KE, Chen IM, Carroll WL, Wei L, Ma J, Kleppe M, Levine RL, Garcia-Manero G, Larsen E, Shah NP, Devidas M, Reaman G, Smith M, Paugh SW, Evans WE, Grupp SA, Jeha S, Pui CH, Gerhard DS, Downing JR, Willman CL, Loh M, Hunger SP, Marra MA, Mullighan CG. 2012. Genetic alterations activating kinase and cytokine receptor signaling in high-risk acute lymphoblastic leukemia. Cancer Cell 22:153-166.

Russell LJ, Capasso M, Vater I, Akasaka T, Bernard OA, Calasanz MJ, Chandrasekaran T, Chapiro E, Gesk S, Griffiths M, Guttery DS, Haferlach C, Harder L, Heidenreich O, Irving J, Kearney L, Nguyen-Khac F, Machado L, Minto L, Majid A, Moorman AV, Morrison H, Rand V, Strefford JC,

Schwab C, Tönnies H, Dyer MJ, Siebert R, Harrison CJ. 2009. Deregulated expression of cytokine receptor gene, *CRLF2*, is involved in lymphoid transformation in B-cell precursor acute lymphoblastic leukemia. Blood 114:2688-2698.

Sato T, Toki T, Kanezaki R, Xu G, Terui K, Kanegane H, Miura M, Adachi S, Migita M, Morinaga S, Nakano T, Endo M, Kojima S, Kiyoi H, Mano H, Ito E. 2008. Functional analysis of *JAK3* mutations in transient myeloproliferative disorder and acute megakaryoblastic leukaemia accompanying Down syndrome. Br J Haematol 141:681-688.

Schwab CJ, Jones LR, Morrison H, Ryan SL, Yigittop H, Schouten JP, Harrison CJ. 2010. Evaluation of multiplex ligation-dependent probe amplification as a method for the detection of copy number abnormalities in B-cell precursor acute lymphoblastic leukemia. Genes Chromosomes Cancer 49:1104-1113.

Shochat C, Tal N, Bandapalli OR, Palmi C, Ganmore I, te Kronnie G, Cario G, Cazzaniga G, Kulozik AE, Stanulla M, Schrappe M, Biondi A, Basso G, Bercovich D, Muckenthaler MU, Izraeli S. 2011. Gain-of-function mutations in *interleukin-7 receptor-α* (*IL7R*) in childhood acute lymphoblastic leukemias. J Exp Med 208:901-908.

Yamashita Y, Shimada A, Yamada T, Yamaji K, Hori T, Tsurusawa M, Watanabe A, Kikuta A, Asami K, Saito AM, Horibe K. 2013. *IKZF1* and *CRLF2* gene alterations correlate with poor prognosis in Japanese *BCR-ABL1*-negative high-risk B-cell precursor acute lymphoblastic leukemia. Pediatr Blood Cancer 60:1587-1592.

Yoda A, Yoda Y, Chiaretti S, Bar-Natan M, Mani K, Rodig SJ, West N, Xiao Y, Brown JR, Mitsiades C, Sattler M, Kutok JL, DeAngelo DJ, Wadleigh M, Piciocchi A, Dal Cin P, Bradner JE, Griffin JD, Anderson KC, Stone RM, Ritz J, Foà R, Aster JC, Frank DA, Weinstock DM. 2010. Functional screening identifies CRLF2 in precursor B-cell acute lymphoblastic leukemia. Proc Natl Acad Sci USA 107:252-257.

Figure legends

Figure 1. *CRLF2* expression in DS-ALL and non-DS-ALL. Open circles: DS-ALL patients with *P2RY8-CRLF2*; triangles: other patients; and horizontal bars: median *CRLF2* expression levels. NS, not significant.

Figure 2. Copy number alterations of genes involved in BCP-ALL development. The deletion frequencies are 8/32 (25%) for PAR1, 5/32 (16%) for *EBF1*, 8/32 (25%) for *BTG1*, 8/32 (25%) for *IKZF1*, 10/32 (31%) for *PAX5*, 12/32 (38%) for *CDKN2A/B*, 2/32 (6%) for *RB1*, and 5/32 (16%) for *ETV6*.

Figure 3. Clinical outcome of DS-ALL patients according to *IKZF1* deletions. (A) Event-free survival. (B) Overall survival.

Figure 1

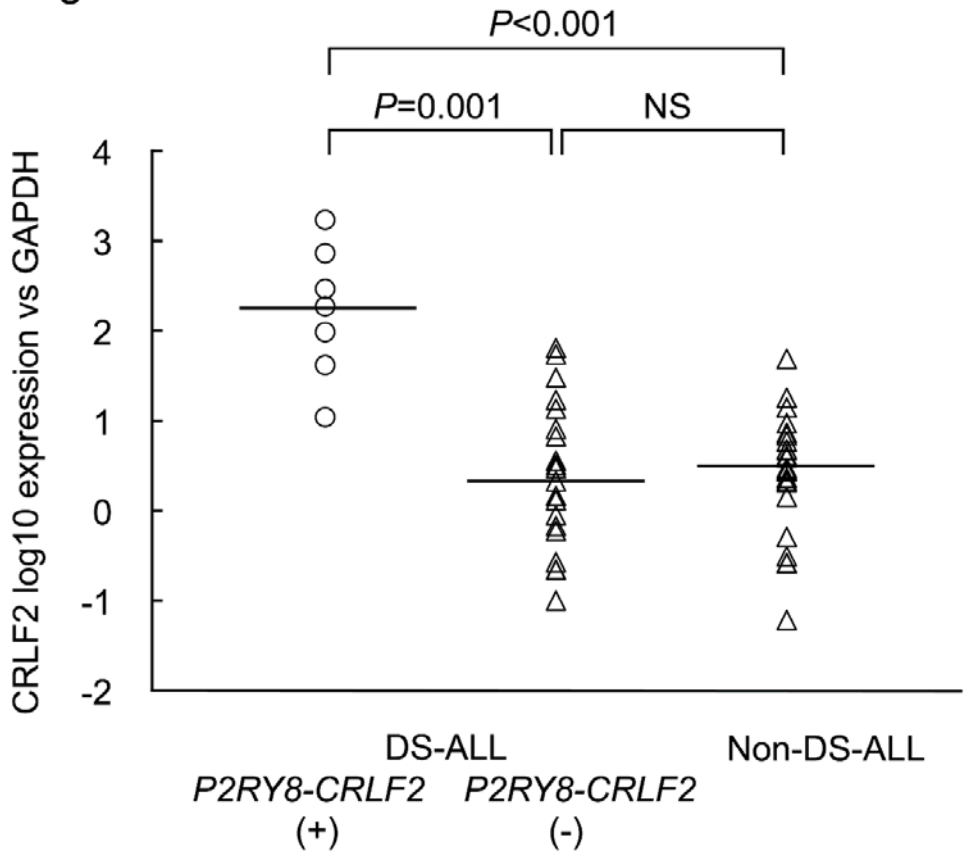
P<0.001
P=0.001
NS
CRLF2 log10 expression vs GAPDH
4
3
2
1
0
-1
-2
DS-ALL
Non-DS-ALL
P2RY8-CRLF2
(+)
P2RY8-CRLF2
(-)

Figure 2

Patient No.	*P2RY8-CRLF2*	*JAK2*	*PAR1*	*EBF1*	*BTG1*	*IKZF1*	*PAX5*	*CDKN2A/B*	*RB1*	*ETV6*
1	Positive	R683G								
2	Positive	R683G								
3	Positive	R683G								
4	Positive	R683G								
5	Positive	R683G								
6	Positive	R683G								
7	Positive	WT								
8	Positive	WT								
9	Positive	WT								
10	Positive	WT								
11	Positive	WT								
12	Negative	WT								
13	Negative	WT								
14	Negative	WT								
15	Negative	WT								
16	Negative	WT								
17	Negative	WT								
18	Negative	WT								
19	Negative	WT								
20	Negative	WT								
21	Negative	WT								
22	Negative	WT								
23	Negative	WT								
24	Negative	WT								
25	Negative	WT								
26	Negative	WT								
27	Negative	WT								
28	Negative	WT								
29	Negative	WT								
30	Negative	WT								
31	Negative	WT								
32	Negative	WT								
33	Negative	WT								
34	Negative	WT								
35	Negative	WT								
36	Negative	WT								
37	Negative	WT								
38	Negative	WT								

Deleted Not done

Figure 3

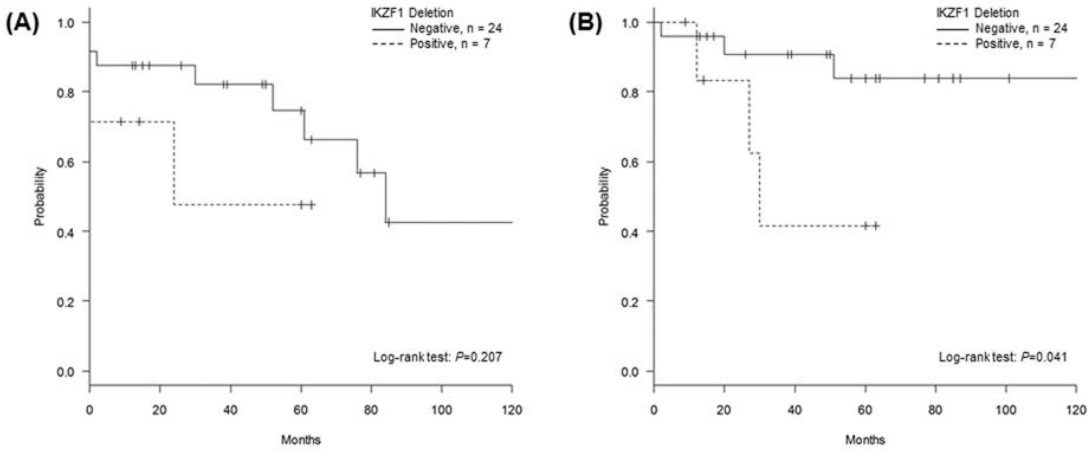
(A)
IKZF1 Deletion
Negative, n = 24
Positive, n = 7
Probability
1.0
0.8
0.6
0.4
0.2
0.0
Log-rank test: P=0.207
0
20
40
60
80
100
120
Months
(B)
IKZF1 Deletion
Negative, n = 24
Positive, n = 7
Probability
1.0
0.8
0.6
0.4
0.2
0.0
Log-rank test: P=0.041
0
20
40
60
80
100
120
Months

Table 1. Clinical and Genetic Characteristics of DS-ALL Patients

Patient No.	Sex	Age (years)	WBC ($x10^9$/l)	NCI risk	Recurrent cytogenetic abnormality	EFS (months)	OS (months)	*P2RY8-CRLF2*	*JAK2*
1	F	4.5	46.5	SR	No	12+	12+	Positive	R683G
2	M	6.2	26.9	SR	No	30, relapse	87+	Positive	R683G
3	F	8.2	65.4	HR	No	38+	38+	Positive	R683G
4	F	3.4	3.7	SR	No	2, death in CR	2	Positive	R683G
5	M	2.7	27.2	SR	No	63+	63+	Positive	R683G
6	F	4.8	2.2	SR	No	9+	9+	Positive	R683G
7	M	4.4	2.1	SR	No	24, relapse	30	Positive	WT
8	M	15.0	187.5	HR	No	60+	60+	Positive	WT
9	F	4.2	1.9	SR	No	17+	17+	Positive	WT
10	NA	NA	NA	NA	NA	NA	NA	Positive	WT
11	F	15.8	2.0	HR	No	4, death in CR	4	Positive	WT
12	M	2.2	57.3	HR	No	85+	85+	Negative	WT
13	M	6.4	55.0	HR	No	50+	50+	Negative	WT
14	F	7.0	2.2	SR	No	49+	49+	Negative	WT
15	M	16.8	70.7	HR	No	0, IF	27	Negative	WT
16	M	11.4	9.0	HR	No	39+	39+	Negative	WT
17	NA	NA	NA	NA	NA	NA	NA	Negative	WT
18	M	11.2	1.1	HR	No	81+	81+	Negative	WT

19	F	8.1	4.5	SR	No	26+	26+	Negative	WT
20	F	4.5	107.5	HR	t(12;21)(p13;q22)	13+	13+	Negative	WT
21	F	4.8	108.9	HR	t(12;21)(p13;q22)	128+	128+	Negative	WT
22	M	2.8	10.9	SR	High hyperdiploidy	221+	221+	Negative	WT
23	M	6.7	10.5	SR	No	0, IF	51	Negative	WT
24	M	19.1	2.6	HR	No	60+	60+	Negative	WT
25	F	2.9	38.9	SR	No	76, relapse	128+	Negative	WT
26	M	4.8	51.2	HR	No	77+	77+	Negative	WT
27	F	9.7	44.9	SR	dic(9;20)(p13;q11)	63+	63+	Negative	WT
28	F	9.8	61.7	HR	t(8;14)(q11;q32)	52, relapse	56+	Negative	WT
29	M	7.3	13.6	SR	No	0, IF	20	Negative	WT
30	M	8.7	51.6	HR	No	0, IF	12	Negative	WT
31	F	17.2	47.3	HR	No	15+	15+	Negative	WT
32	M	13.6	13.2	HR	No	84, relapse	101+	Negative	WT
33	F	7.1	19.5	SR	No	14+	14+	Negative	WT
34	M	5.3	3.8	SR	High hyperdiploidy	61, relapse	64+	Negative	WT
35	NA	NA	NA	NA	NA	NA	NA	Negative	WT
36	F	6.4	3.9	SR	No	10, relapse	22	Negative	WT
37	M	4.8	NA	NA	NA	NA	NA	Negative	WT
38	F	6.3	3.0	SR	No	27+	27+	Negative	WT

WBC, white blood cell; NCI, National Cancer Institute; EFS, event-free survival; OS, overall survival; M, male; F, female; SR, standard risk; HR, high risk; CR, complete remission; IF, induction failure; WT, wild type; NA, not available.

Table 2. Association Between Genetic Alterations and Clinical Characteristics

	P2RY8-CRLF2		*P*-value	*BTG1*		*P*-value	*IKZF1*		*P*-value
	Positive (n = 10)	Negative (n = 25[a])		Positive (n = 8)	Negative (n = 23)		Positive (n = 7)	Negative (n = 24)	
Sex									
Male, n (%)	4 (40%)	14 (56%)	0.471	4 (50%)	13 (57%)	1.000	4 (57%)	13 (54%)	1.000
Female, n (%)	6 (60%)	11 (44%)		4 (50%)	10 (43%)		3 (43%)	11 (46%)	
Age, years									
Median (range)	4.7 (2.7-15.8)	7.0 (2.2-19.1)	0.165	4.8 (2.2-11.2)	7.1 (2.7-19.1)	0.21	8.7 (4.4-16.8)	6.3 (2.2-19.1)	0.228
WBC, $x10^9$/l									
Median (range)	15.3 (1.9-187.5)	16.5 (1.1-108.9)	0.345	48.9 (1.1-107.5)	13.6 (1.9-187.5)	0.47	44.9 (2.1-187.5)	20.2 (1.1-108.9)	0.603
NCI risk group									
SR, n (%)	7 (70%)	11 (46%)	0.270	3 (38%)	13 (57%)	1.000	4 (57%)	12 (50%)	1.000
HR, n (%)	3 (30%)	13 (54%)		5 (63%)	10 (43%)		3 (43%)	12 (50%)	
Recurrent cytogenetic abnormality									
Yes, n (%)	0 (0%)	6 (25%)	0.148	1 (13%)	5 (22%)	1.000	1 (14%)	5 (21%)	1.000
No, n (%)	10 (100%)	18 (75%)		7 (87%)	18 (78%)		6 (86%)	19 (79%)	
5y EFS									
% ± SE	48.0 ± 19.0	72.0 ± 10.2	0.394	80.0 ± 17.9	65.7 ± 11.0	0.155	47.6 ± 22.5	74.6 ± 10.3	0.207
5y OS									
% ± SE	64.0 ± 17.5	74.4 ± 10.2	0.348	100	68.6 ± 10.9	0.162	41.7 ± 22.2	83.8 ± 8.9	0.041

WBC, white blood cell; NCI, National Cancer Institute; SR, standard risk; HR, high risk; EFS, event-free survival; OS, overall survival.

[a] In one patient, data were only available for sex and age.