to evaluate another data set of 83 cases of Ph-like ALL (Roberts *et al*, 2012), and found that four cases of *EBF1/PDGFRB* translocation also overexpressed *PDGFRB* (Fig 2D). It is noteworthy that Erben *et al* (2010) reported that screening of *PDGFRA or PDGFRB* translocation is facilitated by quantification of *PDGFRA or PDGFRB* expression in eosinophilia-associated myeloproliferative neoplasms (Erben *et al*, 2010). More importantly, *PDGFRB* overexpression was found in patients with diverse *PDGFRB* translocations irrespective of the fusion partner, i.e. *ETV6-PDGFRB, CCDC6-PDGFRB, GIT2-PDGFRB, MYO18A-PDGFRB and SART3-PDGFRB.* Indeed, precise detection of *PDGFRB* translocations is necessary for not only diagnostic purposes but also for providing tailored therapy, such as tyrosine kinase inhibitor (TKI) treatment (Roberts *et al*, 2012). However, the *PDGFRB* translocation is mostly cryptic and it is difficult to detect with conventional cytogenetic studies, as was shown in our case. We speculate that this might be one of the leading causes of the previous poor recognition of *PDGFRB* translocation in paediatric B-ALL. In view of the limited conventional diagnostic procedures for the detection of *PDGFRB* translocation, we speculate that the quantification of the *PDGFRB* mRNA expression level should be utilized as a simple screening test prior to performing or planning microarray-based comparative genomic hybridization or FISH analysis of the *PDGFRB* locus in specific subtypes of B-ALL, especially in the Ph-like ALL subgroup.

In summary, we have identified *ATF7IP* as a novel fusion partner in *PDGFRB* translocation in a paediatric case of B-ALL. Given the potential suitability of TKI treatment in this particular type of B-ALL (Roberts *et al*, 2012; Lengline *et al*, 2013; Weston *et al*, 2013), detection of cryptic *PDGFRB* translocation is important, especially for patients who would benefit from TKI treatment at the correct clinical phases. Thus, we ascertained the future prospective of measuring *PDGFRB* expression levels as a simple screening test to detect cryptic *PDGFRB* translocation especially in the Ph-like ALL subgroup.

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Author contributions

K.K. analysed results and wrote the manuscript; K.M., M.M., Y.K., H.T., K.I. analysed results; K.O. performed FISH analyses; H.I., N.K., K.I., K.Y., H.S., K.H., K.M. provided informatics support; T.Y., N.K., A.O. designed the research.

Competing interests

The authors have no competing interests.

References

Erben, P., Gosenca, D., Muller, M.C., Reinhard, J., Score, J., Del Valle, F., Walz, C., Mix, J., Metzgeroth, G., Ernst, T., Haferlach, C., Cross, N.C., Hochhaus, A. & Reiter, A. (2010) Screening for diverse PDGFRA or PDGFRB fusion genes is facilitated by generic quantitative reverse transcriptase polymerase chain reaction analysis. *Haematologica*, **95**, 738–744.

Fujita, N., Watanabe, S., Ichimura, T., Ohkuma, Y., Chiba, T., Saya, H. & Nakao, M. (2003) MCAF mediates MBD1-dependent transcriptional repression. *Molecular and Cellular Biology*, **23**, 2834–2843.

Jamil, A., Theil, K.S., Kahwash, S., Ruymann, F.B. & Klopfenstein, K.J. (2000) TEL/AML-1 fusion gene. its frequency and prognostic significance in childhood acute lymphoblastic leukemia. *Cancer Genetics and Cytogenetics*, **122**, 73–78.

Lengline, E., Beldjord, K., Dombret, H., Soulier, J., Boissel, N. & Clappier, E. (2013) Successful tyrosine kinase inhibitor therapy in a refractory B-cell precursor acute lymphoblastic leukemia with EBF1-PDGFRB fusion. *Haematologica*, **98**, e146–e148.

Liu, L., Ishihara, K., Ichimura, T., Fujita, N., Hino, S., Tomita, S., Watanabe, S., Saitoh, N., Ito, T. & Nakao, M. (2009) MCAF1/AM is involved in Sp1-mediated maintenance of cancer-associated telomerase activity. *The Journal of Biological Chemistry*, **284**, 5165–5174.

Lupas, A., Van Dyke, M. & Stock, J. (1991) Predicting coiled coils from protein sequences. *Science*, **252**, 162–1164.

Manabe, A., Ohara, A., Hasegawa, D., Koh, K., Saito, T., Kiyokawa, N., Kikuchi, A., Takahashi, H., Ikuta, K., Hayashi, Y., Hanada, R. & Tokyo Children's Cancer Study Group (2008) Significance of the complete clearance of peripheral blasts after 7 days of prednisolone treatment in children with acute lymphoblastic leukemia: the Tokyo Children's Cancer Study Group Study L99-15. *Haematologica*, **93**, 1155–1160.

McPherson, A., Hormozdiari, F., Zayed, A., Giuliany, R., Ha, G., Sun, M.G., Griffith, M., Heravi Moussavi, A., Senz, J., Melnyk, N., Pacheco, M., Marra, M.A., Hirst, M., Nielsen, T.O., Sahinalp, S.C., Huntsman, D. & Shah, S.P. (2011) deFuse: an algorithm for gene fusion discovery in tumor RNA-Seq data. *PLOS Computational Biology*, **7**, e1001138.

Roberts, K.G., Morin, R.D., Zhang, J., Hirst, M., Zhao, Y., Su, X., Chen, S.C., Payne-Turner, D., Churchman, M.L., Harvey, R.C., Chen, X., Kasap, C., Yan, C., Becksfort, J., Finney, R.P., Teachey, D.T., Maude, S.L., Tse, K., Moore, R., Jones, S., Mungall, K., Birol, I., Edmonson, M.N., Hu, Y., Buetow, K.E., Chen, I.M., Carroll, W.L., Wei, L., Ma, J., Kleppe, M., Levine, R.L., Garcia-Manero, G., Larsen, E., Shah, N.P., Devidas, M., Reaman, G., Smith, M., Paugh, S.W., Evans, W.E., Grupp, S.A., Jeha, S., Pui, C.H., Gerhard, D.S., Downing, J.R., Willman, C.L., Loh, M., Hunger, S.P., Marra, M.A. & Mullighan, C.G. (2012) Genetic alterations activating kinase and cytokine receptor signaling in high-risk acute lymphoblastic leukemia. *Cancer Cell*, **22**, 153–166.

Ross, T.S. & Gilliland, D.G. (1999) Transforming properties of the Huntingtin interacting protein 1/platelet-derived growth factor beta receptor fusion protein. *The Journal of Biological Chemistry*, **274**, 22328–22336.

Subramanian, A., Kuehn, H., Gould, J., Tamayo, P. & Mesirov, J.P. (2007) GSEA-P: a desktop application for gene set enrichment analysis. *Bioinformatics*, **23**, 3251–3253.

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Tefferi, A. & Vardiman, J.W. (2008) Classification and diagnosis of myeloproliferative neoplasms: the 2008 World Health Organization criteria and point-of-care diagnostic algorithms. *Leukemia*, **22**, 14–22.

Weston, B.W., Hayden, M.A., Roberts, K.G., Bowyer, S., Hsu, J., Fedoriw, G., Rao, K.W. & Mullighan, C.G. (2013) Tyrosine kinase inhibitor therapy induces remission in a patient with refractory EBF1-PDGFRB-positive acute lymphoblastic leukemia. *Journal of Clinical Oncology*, **31**, e413–e416.

Yeoh, E.J., Ross, M.E., Shurtleff, S.A., Williams, W.K., Patel, D., Mahfouz, R., Behm, F.G., Raimondi, S.C., Relling, M.V., Patel, A., Cheng, C., Campana, D., Wilkins, D., Zhou, X., Li, J., Liu, H., Pui, C.H., Evans, W.E., Naeve, C., Wong, L. & Downing, J.R. (2002) Classification, subtype discovery, and prediction of outcome in pediatric acute lymphoblastic leukemia by gene expression profiling. *Cancer Cell*, **1**, 133–143.

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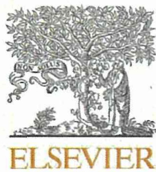

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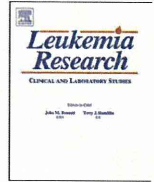

Significance of CD66c expression in childhood acute lymphoblastic leukemia

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ABSTRACT

Upon analyzing 696 childhood B-cell precursor acute lymphoblastic leukemia (BCP-ALL) cases, we identified the characteristics of CD66c expression. In addition to the confirmation of strong correlation with *BCR-ABL* positivity and hyperdiploid, we further observed that CD66c is frequently expressed in CRLF2-positive (11/15, $p < 0.01$ against chimeric gene-negative) as well as hypodiploid cases (3/4), whereas it is never expressed in *ETV6-RUNX1*, *MLL-AF4*, *MLL-AF9*, *MLL-ENL*, and *E2A-PBX1*-positive cases. Although the expression of CD66c itself is not directly linked to the prognosis, the accompanying genetic abnormalities are important prognostic factors for BCP-ALL, indicating the importance of CD66c expression in the initial diagnosis of BCP-ALL.

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1. Introduction

Although most leukemic cells retain the characteristics of their normal counterparts and exhibit commitment to any one of the hematopoietic lineages, they frequently show lineage-uncommitted antigen expression, referred to as "aberrant antigen expression" or "lineage infidelity". For example, both T-cell and B-cell precursor (BCP) acute lymphoblastic leukemia (ALL) cells commonly express aberrant myeloid lineage antigens, while acute myeloid leukemia (AML) cells often exhibit the expression of T- or B-cell lineage antigens. Several possibilities to explain this phenomenon have been postulated, whereas the precise mechanism is still unclear [1–3].

CD66c, also called CEACAM6, KOR-SA3544 antigen, and NCA 90/50, is a heavily glycosylated glycosylphosphatidylinositol (GPI)-anchored protein belonging to the carcinoembryonic antigen family, having two constant Ig-like domains and one variable Ig-like domain [4]. The expression of CD66c is observed only in granulocytes and its precursors among normal hematopoiesis [5], while it is known as the most frequently observed aberrant myeloid

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Table 1
The summary of the characteristics of patients.

	n=	Age (mean ± SD)	Range	Initial WBC/μl (mean ± SD)	Range	Gender (male:female)	NCI risk group (SR:HR)
BCR-ABL	35	8.4 ± 4.1	2–15	141,950.0 ± 202,731.2	1220–881,700	0.60:0.40	0.80:0.20
MLL-chimera	20	5.5 ± 4.8	0–15	233,237.2 ± 336,648.4	3300–1,165,400	0.45:0.55	0.80:0.20
E2A-PBX1	65	6.5 ± 4.5	1–15	39,857.4 ± 44,938.8	1730–223,300	0.52:0.48	0.94:0.06
ETV6-RUNX1	154	4.8 ± 2.8	1–15	26,426.4 ± 73,112.6	1600–788,000	0.56:0.44	1.00:0.00
Neardiploid	267	6.1 ± 4.3	1–17	31,153.1 ± 71,020.0	700–597,000	0.53:0.47	0.96:0.04
CRLF2+	15	7.7 ± 4.7	1–16	95,105.5 ± 134,773.6	4200–368,700	0.60:0.40	0.93:0.07
Hypodiploid	4	7.3 ± 5.0	2–12	10,075.0 ± 7605.4	4900–23,200	0.75:0.25	1.00:0.00
Hyperdiploid	136	4.4 ± 2.9	1–15	14,256.6 ± 27,170.5	1100–259,000	0.54:0.46	1.00:0.00
Total	696	5.6 ± 3.9		39,318.0 ± 102,794.7	700–1,165,400*	0.54:0.46	0.96:0.04
Neardiploid							
CD66c+	106	5.7 ± 3.8	1–15	24,402.5 ± 51,598.1	700–379,500	0.54:0.46	0.96:0.04
CD66c-	161	6.3 ± 4.6	1–17	35,555.7 ± 80,919.0	800–597,000	0.53:0.47	0.97:0.03
Hyperdiploid							
CD66c+	91	4.3 ± 2.4	1–12	12,339.3 ± 17,488.1	1100–116,900	0.58:0.42	1.00:0.00
CD66c-	45	4.5 ± 3.9	1–15	18,278.3 ± 40,265.9	1700–259,000	0.44:0.56	1.00:0.00

antigen in B-cell precursor acute lymphoblastic leukemia (BCP-ALL) [6]. CD66c was initially reported to be expressed highly selectively in *BCR-ABL*-positive BCP-ALL, while some *BCR-ABL*-negative cases also express this antigen [7]. Later, it was reported that CD66c was correlated strongly with *ETV6-RUNX1* and *MLL-AF4* negativity and was found at high levels in hyperdiploidy [6,8]. A number of studies to clarify the function of this molecule have been performed, and it has been reported that CD66c is involved in homo- and heterotypic adhesion [9], contributes to Ca2+-mediated signaling [10], and is involved in apoptosis induction [11]. However, the biological significance of this molecule in BCP-ALL is still not fully understood.

In an attempt to explore the significance of the expression of CD66c in BCP-ALL, we precisely characterized the properties of CD66c-positive ALL in a large cohort. In this study, we further extend previous findings and indicate that CD66c expression has a close correlation with a definite set of genetic abnormalities, although it is not limited to a specific one. The detection of CD66c at the initial diagnosis of BCP-ALL is important for the prediction of the presence and absence of certain genetic abnormalities. Although the expression of CD66c itself is not directly linked to the prognosis, the genetic abnormalities accompanying CD66c expression are important prognostic factors for BCP-ALL, and, thus, the genetic findings need to be investigated carefully with the presence of CD66c expression.

2. Materials and methods

2.1. Case selection

A total of 696 patients aged between 1 and 18 years (male: female; 0.54: 0.46) who had been newly diagnosed with BCP-ALL and consecutively enrolled on the Tokyo Children's Cancer Study Group (TCCSG) L16 study from December 2004 to August 2012 were included in this study. The characteristics of patients, including age, initial white blood cell (WBC) count, and NCI risk group, were summarized in Table 1. The investigations were approved by the institutional review boards of all participating institutions. Informed consent was obtained from parents or guardians, and informed assent was obtained from the patients when appropriate given their age and understanding.

Bone marrow (BM) and/or peripheral blood (PB) smears of the patients were stained by standard techniques, and the diagnosis of ALL was made according to the morphologic and cytochemical (myeloperoxidase and nonspecific esterase) criteria of the French-American-British (FAB) classification. All cases had fewer than 3% myeloperoxidase-positive, 3% Sudan black B-positive (myeloid pattern), or 20% butyrate esterase-positive (myeloid pattern) blast cells and no Auer rods. Basically, children with ALL of the mature B-cell type were not enrolled in this trial. BM aspirate or PB was immediately mixed with anti-coagulant and sent by overnight transport to the flow cytometry and fusion transcript laboratories, National Research Institute for Child Health and Development (NCH) and Univ. of Tsukuba, respectively, as part of routine pretreatment studies.

2.2. Flow cytometry

Four-color flow cytometric immunophenotyping with CD45-gating was performed on a flow cytometer (FC500, Beckman-Coulter, Brea, CA). The panel monoclonal antibodies (MoAbs) used for immunophenotyping are presented in Supplementary information. Whole blood samples were stained with various combinations of fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, PE-cyanin 5.1 (PC5)-, and PE-cyanine 7 (PC7)-conjugated MoAbs in the presence of electron-coupled dye (ECD)-conjugated CD45, following RBC-lysis treatment. For the detection of cytoplasmic (cyCD3, cyCD22, cyCD79a, cy-μ, and MPO) and nuclear TdT antigens, the cells were permeabilized with the Intraprep Permeabilization reagent kit (Beckman-Coulter). Analysis was done by collecting 10,000 gated list mode events, and selecting an appropriate blast gate for the combination of CD45 and side scatter. An antigen was considered positively expressed when at least 20% of the gated cells expressed that antigen.

DNA contents were examined by Propidium Iodide (PI)-staining. Following RBC-lysis treatment, 2.5×10^5 cells were suspended in phosphate-buffered saline (PBS) containing 0.2% of Triton X-100, 20 μg/ml of PI, and 100 ng/ml of RNase (Sigma–Aldrich, St. Louis, MO). PI fluorescence was collected through a 645-nm dichroic long-pass filter and a 620-nm band-pass filter. Upon appropriate gating, at least 10,000 events were collected and analyzed.

The detection of BCR-ABL protein by flow cytometry was performed by Cytometric Bead Array (CBA) for BCR-ABL protein (Becton Dickinson, BD, Franklin Lakes, NJ) according to manufacturer's instruction.

2.3. Detection of fusion transcripts and conventional cytogenetic analysis

The expression of 8 fusion transcripts: *MLL-AF4*, *MLL-AF9*, *MLL-ENL*, major *BCR-ABL*, minor *bcr-abl*, *ETV6-RUNX1*, *E2A-PBX*, and *SIL-TAL1*, was detected by real-time PCR using appropriate primer sets. Cytogenetic analysis was performed on bone marrow or peripheral blood specimens using standard techniques. At least 20 metaphases were examined for each case. Actual examinations were performed by Special Reference Laboratory (SRL, Tachikawa, Tokyo, Japan). In the present study, we have defined BCP-ALL cases with more than 51 chromosomes or DNA-index > 1.16 (corresponding to 51 chromosomes) as hyperdiploid (high-hyperdiploid) based on the previous reports [12,13]. Similarly, we have defined the cases with fewer than 44 chromosomes [13–15] or DNA-index < 0.95 (corresponding to 43 chromosomes) [16] as hypodiploid (near-haploid, low-hypodiploid and high-hypodiploid). The cases with 44–50 chromosomes have designated as neardiploid.

2.4. Statistical analysis

Statistical analysis was performed by means of Student's *t*-test. A *p*-value less than 0.05 was considered significant. Principal components analysis (PCA) was performed by using TriSP version2.1 developed by Yamasaki H (http://www014.upp.so-net.ne.jp/acremaker/).

3. Results

3.1. Close correlation between CD66c expression and nonrandom genetic abnormalities

We analyzed CD66c expression in 696 unselected patients' specimens with a diagnosis of BCP-ALL and available information on the presence of well-established chimeric genes, including major and minor *BCR-ABL*, *ETV6-RUNX1*, *E2A-PBX1*, *MLL-AF4*, *MLL-AF9*, and *MLL-ENL* and/or cytogenetic findings, including DNA ploidy. As shown in Table 2, CD66c was expressed in 34.9% of all BCP-ALL cases

Table 2
Expression of myeloid antigens in B-cell precursor acute lymphoblastic leukemia.

	CD66c	CD33	CD13	CD15	CD65	CD117
>20% (%)	34.91	21.73	9.20	3.44	2.46	1.62
Number	(243/696)	(151/695)	(64/696)	(23/668)	(17/692)	(11/679)
Mean (%)	23.18	13.53	6.40	3.35	2.80	1.88
SD (%)	31.26	21.85	14.11	8.57	9.59	5.08
Median (%)	4.87	2.58	1.26	0.79	0.70	0.35

and appeared to be most frequently aberrantly expressed in BCP-ALL compared to other myeloid antigens, including CD33 (21.7%), CD13 (9.2%), CD15 (3.4%), CD65 (2.5%), and CD117 (1.6%).

Consistent with previous reports, CD66c expression showed a close correlation with nonrandom genetic abnormalities and was expressed only in *BCR-ABL*-positive (91.4%, 32/35) or specific chimeric gene-negative cases (50.0%, 211/422), while none of the *ETV6-RUNX1*-positive cases expressed CD66c (Fig. 1A). In addition, not only the *MLL-AF4*-positive cases, but also *MLL-AF9* and *MLL-ENL*-positive cases were negative for CD66c. Furthermore, it is noteworthy that none of the *E2A-PBX1*-positive cases expressed CD66c.

3.2. High rate expression of CD66c in CRLF2-positive and hyperdiploid cases

Next, we further analyzed CD66c expression in BCP-ALL cases without specific chimeric genes (Fig. 1B). The chimeric gene-negative BCP-ALL cases can be subdivided into near-, hyper-, and hypodiploid based on the number of chromosomes. The abnormalities in chromosome number have been shown to have prognostic significance in BCP-ALL and hyperdiploid ALL (more than 51 chromosomes) exhibit a superior outcome [12,13], whereas hypodiploid ALL (fewer than 44 chromosomes) is characterized by extremely poor outcomes when compared with their nonhyperdiploid counterparts (44–50 chromosomes) [13–15]. As shown in Fig. 1B, hyperdiploid cases exhibited high frequency of CD66c expression (66.9%, 91/136). Interestingly, although the number of cases was small, three out of four hypodiploid cases were positive for CD66c.

In our study, we examined the expression of CRLF2 using specific monoclonal antibody retrospectively and prospectively, and found 15 CRLF2-positive cases in the neardiploid cases (2.2% in our total cohort). As shown in Fig. 1B, CRLF2-positive cases exhibited a significantly high frequency of CD66c-expression and 73.3% (11/15) were CD66c-positive. No significant difference was observed between hyperdiploid and CRLF2-positive cases in CD66c-expression. In contrast, the remaining neardiploid cases exhibited less frequent CD66c-expression (39.7%, 106/267).

3.3. Correlation between CD66c expression and that of other myeloid antigens and CD21/CD27 expression

It was reported that the expression of myeloid antigens tended to be mutually exclusive with CD66c [6]. Therefore, we next examined the correlation between the expression of CD66c and other myeloid antigens. As presented above, BCP-ALL cases possessing specific chimeric genes except *BCR-ABL* never express CD66c. Since it was also reported that *ETV6-RUNX1*-positive ALL frequently expressed CD33 and CD13 [17], ALLs expressing these two antigens should be enriched in CD66c-negative/neardiploid cases. Therefore, we compared *BCR-ABL*-positive and chimeric gene-negative cases by excluding BCP-ALL cases possessing other specific chimeric genes from this analysis.

As shown in Fig. 2A and B, the expression of CD33 and CD13 was concentrated in *BCR-ABL*-positive and neardiploid cases. As described above, the vast majority of BCR-ABL-positive cases expressed CD66c and they exhibited a higher frequency of both CD33 (37.5%, 12/32) and CD13 (18.8%, 6/32) expression compared to CD66c-positive cases with neardiploid and hyperdiploid states. In contrast, although we excluded *ETV6-RUNX1*-positive cases from the analysis, neardiploid/CD66c-negative cases still exhibited a significantly higher expression of CD33 (23.6%, 38/161) compared to neardiploid/CD66c-positive (17.9%, 19/106) and hyperdiploid/CD66c-negative (4.6%, 2/44) cases. In CRLF2-positive/CD66c-positive cases, frequent expression of CD33 (36.4%, 4/11) but not CD13 was observed. Since positivity for CD15 and CD65 was low in BCP-ALL, with the exception of *MLL*-related chimeric gene-positive cases [18], no significant differences in the expression of these antigens depending on CD66c expression were observed (data not shown).

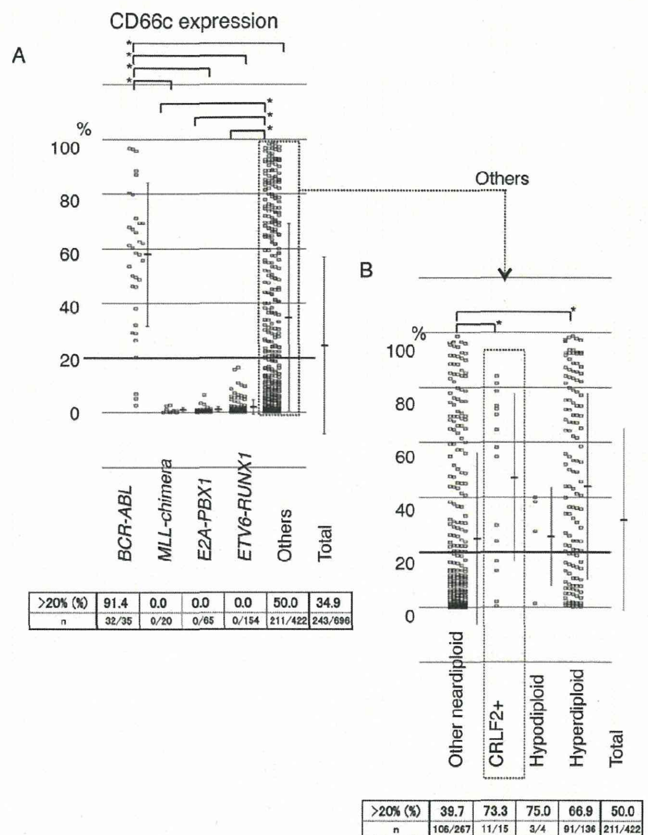

Fig. 1. Correlation between percentage CD66c positivity and acute lymphoblastic leukemia (ALL) genotype categories. (A) CD66c positivity (percentage) of B-cell precursor ALL ($n = 696$) was plotted on a scattergram categorized by the presence of well-known chimeric genes. Percentage of CD66c-positive cases (more than 20% expression in blasts) in each genotype group is listed below. (B) CD66c positivity (percentage) of B-cell precursor ALL without chimeric genes listed above ($n = 422$) was further subclassified based on the DNA-ploidy and CRLF2 expression and presented as in (A). *$p < 0.01$, using Student's t-test.

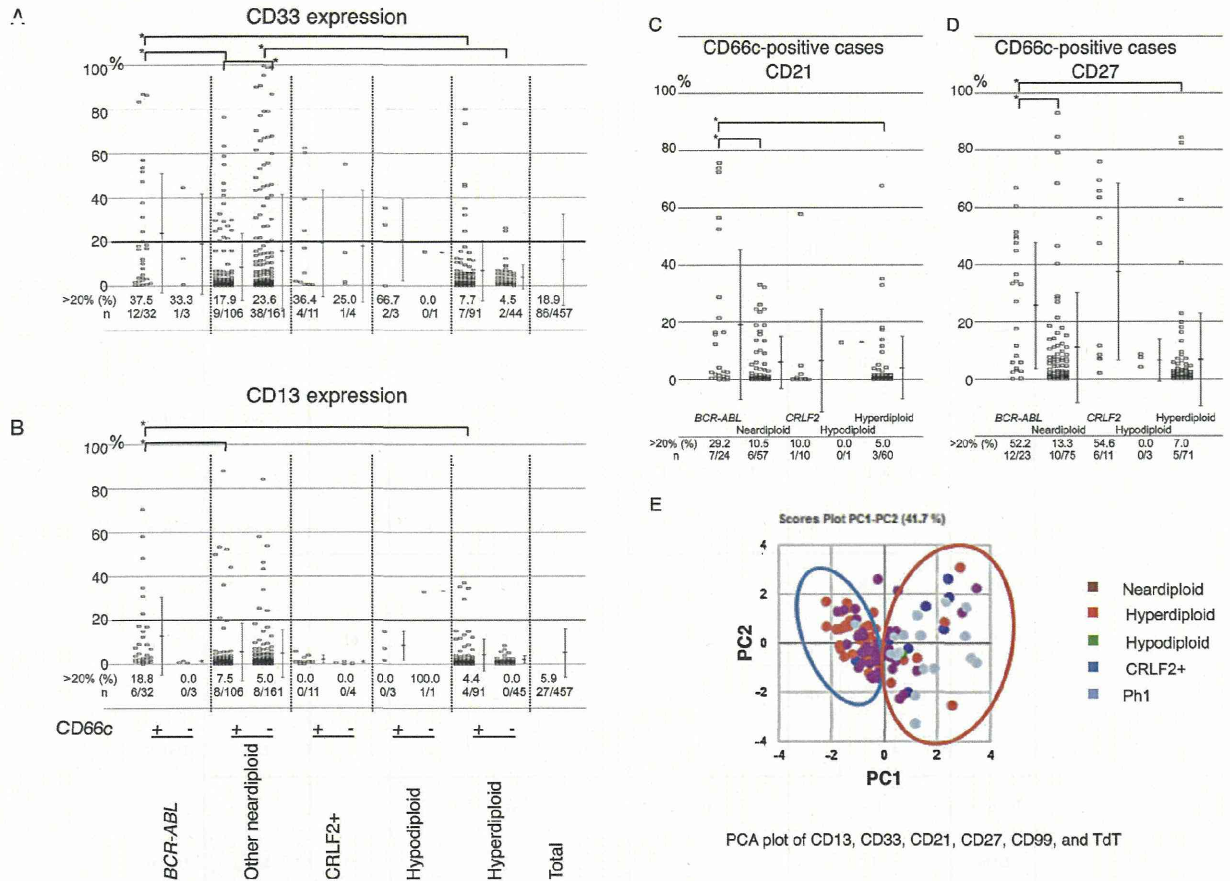

Fig. 2. Correlation between CD66c positivity and the expression of myeloid antigens, CD21 and CD27 in different genotype categories of acute lymphoblastic leukemia (ALL). The positivity (percentage) of CD33 (A) and CD13 (B) of B-cell precursor ALL was plotted on a scattergram categorized by the CD66c expression and genotype, as in Fig. 1. The positivity (percentage) of CD21 (C) and CD27 (D) of CD66c-positive B-cell precursor ALL was plotted on a scattergram categorized by the genotype as indicated in the figure. The percentage of positive cases (more than 20% expression in blasts) in each group is listed below. $^{*}p < 0.01$, using Student's t-test. (E) Principal components analysis (PCA) was performed on CD66c-positive B-cell precursor ALL cases. PCA plot of 6 antigen expression, including CD13, CD33, CD21, CD27, CD99, and TdT, is presented with two PCA axes (PC1 vs PC2).

In an attempt to explore immunophenotypic characteristics distinguishing *BCR-ABL*-positive ALLs and other CD66c-positive ALLs, we observed relatively high expression of CD21 and CD27 in *BCR-ABL*-positive ALLs. The molecule CD21 is a mature B-cell antigen and its expression in BCP-ALL is very limited [19]. The CD27 molecule is a member of the TNF receptor family and known as a marker of mature memory B cells, while some malignant and nonmalignant B precursors also express this antigen [20]. Among CD66c-positive cases, CD21 expression was revealed to be relatively high in *BCR-ABL*-positive cases (Fig. 2C), and CD27 expression was high in both *BCR-ABL*-positive and CRLF2-overexpressing cases (Fig. 2D).

To further assess the biological relevance of the expression of above antigens in CD66c-positive ALL, we performed multivariate analyses by employing PCA. As shown in Fig. 2E, PCA plot using the expression data of 6 antigens, including CD13, CD33, CD21, CD27, CD99, and TdT, could roughly separate BCR-ABL-positive and CRLF2-positive cases from remaining chimeric gene-negative cases expressing CD66c.

3.4. Correlation between risk factors and CD66c expression

We next examined the correlation between CD66c expression and risk classification in chimeric gene-negative cases. In our protocol, the patients were stratified into three risk groups, namely, standard risk (SR), intermediate risk (IR), and high risk (HR), based on presenting features (age and the leukocyte count before starting the treatment) and, then, reclassified into them three categories 7 days later according to the sensitivity to oral prednisolone monotherapy, using the cut-off counts of 1000 blasts/μL [21]. As shown in Table 3, hyperdiploidy/CD66c-positive cases were more frequently classified into SR at diagnosis, while no difference was observed at reclassification on Day 8, indicating that hyperdiploid/CD66c-positive cases tend to have favorable initial presenting features but exhibit poor response for steroid. On the other hand, neardiploid/CD66c-negative cases tended to be more frequently classified into IR and HR at the initial classification, and the tendency appeared to be more significant at reclassification on Day 8 (Table 3), indicating that neardiploid/CD66c-negative have unfavorable presenting features as well as poor steroid sensitivity. After 5-year observation, however, no significant difference in the subsequent prognosis between CD66c-positive and -negative groups was observed (data not shown).

4. Discussion

Upon precisely analyzing CD66c expression in a large cohort of childhood BCP-ALL, we further extended the previous findings, and clearly identified the characteristics of CD66c expression as follows: First, among BCP-ALL possessing well-known chimeric genes, CD66c expression is highly selective in *BCR-ABL*-positive

Table 3
Risk classification and CD66c expression.

Hyper/ CD66c+	HR (Case no.)		IR		SR			Total	HR+IR/SR (Ratio)	
	1		12		27			40	0.48	Initial
	HR-SCT	HR	HR	IR	HR	IR	SR			
	0	1	4	8	7	0	20		1.00	Day-8
Hyper/ CD66c-	HR		IR		SR					
	0		8		8			16	1.00	Initial
	HR-SCT	HR	HR	IR	HR	IR	SR			
	0	0	1	7	0	0	8		1.00	Day-8
Diploid/ CD66c+	HR		IR		SR					
	7		24		30			61	1.03	Initial
	HR-SCT	HR	HR	IR	HR	IR	SR			
	4	3	2	22	0	0	30		1.03	Day-8
Diploid/ CD66c-	HR		IR		SR					
	14		48		31			93	2.00	Initial
	HR-SCT	HR	HR	IR	HR	IR	SR			
	8	6	4	44	3	0	28		2.32	Day-8

Hyper, hyperdiploid; Diploid, neardiploid; HR, high risk; IR, intermediate risk; SR, standard risk; SCT, stem-cell transplantation; Initial, risk classification based on presenting features; Day-8, re-risk classification after 7-day oral prednisolone monotherapy.

cases, while CD66c is never expressed in cases possessing not only *ETV6-RUNX1* and *MLL-AF4*, but also *MLL-AF9*, *MLL-ENL*, and *E2A-PBX1*. Second, among BCP-ALL cases without well-known chimeric genes, CD66c expression also exhibits some selectivity that correlates with genetic abnormalities and CRLF2-positive and probably hypodiploid states, and, as in hyperdiploidy cases tend to express CD66c at a high frequency. The results were schematically summarized in Fig. 3A. Above data indicate that CD66c expression has a close correlation with definite set of genetic abnormalities, although it is not limited to a specific one.

The overexpression of *CRLF2* arises from a translocation juxtaposing *CRLF2* to the *IGH* enhancer or an interstitial deletion (*CRLF2-P2RY8*) and has been reported to be found in 4.7% to 17.5% of BCP-ALL cases as assessed by real-time PCR [22–29]. In this study, however, we found only 15 CRLF2-positive cases (2.2%) in our cohort by flow cytometry. Although the precise reason for the inconsistency in the frequency of CRLF2 overexpression between previous reports by real-time PCR and our data of flow cytometry is remaining unclear, it is possibly due to the difference of detection methods including diagnostic criteria for positive case.

Most recently, a subtype of BCP-ALL including CRLF2-overexpressing cases has been called "Ph-like ALL" and identified to be sharing a transcriptional signature that significantly overlaps with a *BCR-ABL*-positive ALL and accompanied by high rates of relapse and poor overall survival [30]. Besides CRLF2-overexpressing cases, our preliminary results indicate that other Ph-like ALL cases also tend to frequently express CD66c (data not shown).

As well as CRLF2 overexpression [22–29], both *BCR-ABL*-positive [13,31] and hypodiploid patients are well known to show a poor prognosis [13–15]. In contrast, hyperdiploid BCP-ALL patients are generally accompanied by a relatively favorable therapeutic outcome [12,13]. Therefore, the expression of CD66c itself is not directly linked to the prognosis, whereas the genetic abnormalities accompanying CD66c expression are important to make a prognosis for BCP-ALL patients. Concerning the chimeric gene-negative cases, our data further indicated that the combination of CD66c expression and chromosome number abnormalities is closely related to risk classification and steroid sensitivity. Thus the genetic findings must be paid attention when CD66c expression is detected.

Since *CRLF2*-overexpressing BCP-ALLs and *BCR-ABL*-positive cases share overlaped transcriptional signature as we described above [24], the transcription of CD66c might be regulated by a common downstream factor in both pathways. Similarly, CD66c expression in hyper- and hypodiploid cases might also share the same pathway, whereas the precise mechanism that induces the aberrant expression of CD66c in BCP-ALL is unclear. In the

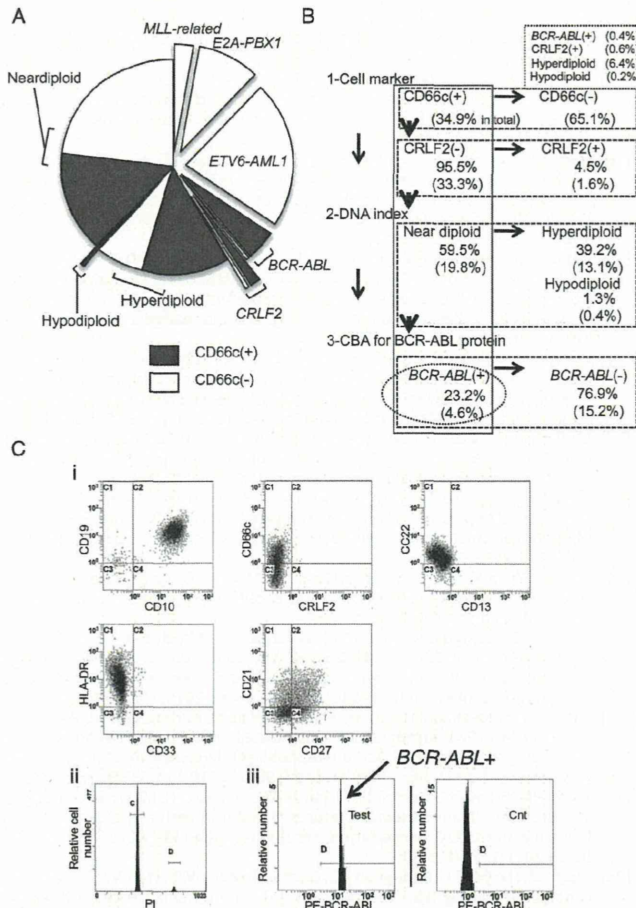

Fig. 3. Summary of CD66c expression and diagnostic flow to detect BCR-ABL-positive acute lymphoblastic leukemia (ALL) by flow cytometry. (A) Summary of CD66c expression and correlation with genetic abnormalities. (B) An initial diagnostic flow of the subclassification of BCP-ALL using flow cytometry is indicated. In the case of neardiploid B-cell precursor ALL that is CD66c-positive and CRLF2-negative, it is recommended to perform the Cytometric Bead Array (CBA) for BCR-ABL protein. Cases expressing any of the myeloid antigens, CD21, or CD27 and exhibiting extreme elevation of peripheral blood white blood cell counts are highly probable of being BCR-ABL-positive. The frequency (%) of each subclass in our study is indicated as a percentage. The number in parentheses indicates the frequency in the total cases. (C) Case diagnosed as BCR-ABL-positive ALL by flow cytometry. The leukemic cells exhibit CD19+, CD10+, HLA-DR+, CD22+, CD66c+, CRLF2-, myeloid-antigen-, CD21+, and CD27+. The DNA-index analysis revealed neardiploidy, and CBA for BCR-ABL protein was positive.

case of hyperdiploid ALLs, a significant correlation between the chromosomal location of upregulated genes and the presence of trisomies/tetrasomies was observed, and, thus, the reflection of a gene-dosage effect has been suggested [32]. On the other hand, hyperdiploid ALL is characterized by a nonrandom gain of chromosomes commonly including chromosomes X, 4, 6, 10, 14, 17, 18, and 21, but CD66c is located on chromosome 19 [33]. Considering the findings, the expression of CD66c in hyperdiploid cases should not be mediated by the gene-dosage effect.

It was reported that the expression of CD13, CD33, CD15, and CD65 tended to be mutually exclusive with CD66c [6]. Since both *ETV6-RUNX1*-positive ALL frequently expressing CD33 and CD13 [17] and ALL with *MLL*-related chimeric genes commonly expressing CD15 and CD65 [18] are highly concentrated in neardiploid/CD66c-negative cases, it is quite reasonable that the expressions of CD66c and other myeloid antigens tend to be mutually exclusive. Therefore, we excluded BCP-ALL cases possessing well-known chimeric genes lacking CD66c expression and then analyzed the correlation between CD66c expression and that of other myeloid antigens. However, upon excluding *ETV6-RUNX1*-positive cases from the analysis, neardiploid/CD66c-negative cases still exhibited a significantly higher expression of CD33. On the other hand, both *BCR-ABL*-positive and CRLF2-expressing cases exhibited frequent expression of CD33 besides CD66c. Further investigation of the underlying mechanisms that induce the aberrant expression of CD66c and other myeloid antigens should be conducted in the future.

Based on our analysis, we propose an initial diagnostic flow of the prognosis-based subclassification of BCP-ALL using flow cytometry. As presented in Fig. 3B and C, in addition to a regular diagnostic panel, the immunocytological detection of CD66c and CRLF2 in combination with PI staining should be a useful tool for the initial diagnosis of BCP-ALL. By assessing cases with PI staining, more than one-third of the patients should be diagnosed as hyperdiploid, and hypodiploid cases might be rarely detected. After the exclusion of hyper- and hypodiploid BCP-ALL cases, less than 10% of the cases will be CRLF2-positive. In the remaining cases, approximately one quarter of the cases should be *BCR-ABL*-positive ALL. As we presented in Fig. 2E by PCA, the coexpression of myeloid antigens, CD21 or CD27 with CD66c, as well as extreme elevation of peripheral blood white blood cell counts, suggests the presence of a *BCR-ABL* chimeric gene at a high probability, while the findings are not definitive. However, by utilizing the recently developed CBA for BCR-ABL protein, we can make a final diagnosis of *BCR-Abl*-positive ALL at the initial presentation of the patient without waiting for the results of RT-PCR or chromosomal analysis. Since the effectiveness of tyrosine kinase inhibitors as first-line treatment has been reported [31,34], the prompt diagnosis of *BCR-ABL*-positive ALL is important. In our pilot study on 20 patients suspected of *BCR-ABL*-positive ALL, including 5 cases subsequently confirmed as true *BCR-ABL*-positive, the results showed a complete concordance between prior CBA for BCR-ABL fusion proteins and following real-time PCR for *BCR-ABL* chimeric genes (a typical result was presented in Fig. 3C).

In conclusion, CD66c expression is not always specific for *BCR-ABL-positive* ALL, whereas it is frequently associated with some genetic abnormalities, which are important for the prognosis. Although further analysis is needed to elucidate the underlying genetic characteristics as well as clinico-pathological features of CD66c-positive neardiploid BCP-ALL cases, our observations should shed light on the significance of CD66c expression in BCP-ALL.

Conflict of interest statement

The authors have no conflict of interest to declare with regards to this work.

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Contributions. NK designed the research study, performed research, analyzed data and wrote the paper. OT, KI, MM, DH, KK, HO, MK, HS, AM, TK, NT and KK performed the research. AK, JF, YH AO analyzed data. TK, AM and KS analyzed data and wrote the paper.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.leukres.2013.10.008.

References

[1] Greaves MF, Chan LC, Furley AJ, Watt SM, Molgaard HV. Lineage promiscuity in hemopoietic differentiation and leukemia. Blood 1986;67:1–11.
[2] Schmidt CA, Przybylski GK. What can we learn from leukemia as for the process of lineage commitment in hematopoiesis? Int Rev Immunol 2001;20:107–15.
[3] Béné MC, Porwit A. Acute leukemias of ambiguous lineage. Semin Diagn Pathol 2012;29:12–8.
[4] Sugita K, Mori T, Yokota S, Kuroki Ma, O-Koyama T, Inukai T, et al. The KOR-SA3544 antigen predominantly expressed on surface of Philadelphia chromosome-positive acute lymphoblastic cells is nonspecific cross-reacting antigen-50/90 (CD66c) and invariably expressed in cytoplasm of human leukemia cells. Leukemia 1999;13:779–85.
[5] Boccuni P, Di Noto R, Lo Pardo C, Villa MR, Ferrara F, Rotoli B, et al. CD66c antigen expression is myeloid restricted in normal bone marrow but is a common feature of CD10+ early-B-cell malignancies. Tissue Antigens 1998;52:1–8.
[6] Kalina T, Vaskova M, Mejstrikova E, Madzo J, Trka J, Stary J, et al. Myeloid antigens in childhood lymphoblastic leukemia: clinical data point to regulation of CD66c distinct from other myeloid antigens. BMC Cancer 2005;5:38.
[7] Mori T, Sugita K, Suzuki T, Okazaki T, Manabe A, Hosoya R, et al. A novel monoclonal antibody, KOR-SA3544 which reacts to Philadelphia chromosome-positive acute lymphoblastic leukemia cells with high sensitivity. Leukemia 1995;9:1233–9.
[8] Hrusák O, Trka J, Zuna J, Housková J, Bartůnková J, Starý J. Aberrant expression of KOR-SA3544 antigen in childhood acute lymphoblastic leukemia predicts TEL-AML1 negativity. The Pediatric Hematology Working Group in the Czech Republic. Leukemia 1998;12:1064–70.
[9] Yamanka T, Kuroki M, Matsuo Y, Matsuoka Y. Analysis of heterophilic cell adhesion mediated by CD66b and CD66c using their soluble recombinant proteins. Biochem Biophys Res Commun 1996;219:842–7.
[10] Klein ML, McGhee SA, Baranian J, Stevens L, Hefta SA. Role of non-specific cross-reacting antigen, a CD66 cluster antigen, in activation of human granulocytes. Infect Immun 1996;64:4574–9.
[11] Kanderová V, Hrusák O, Kalina T. Aberrantly expressed CEACAM6 is involved in the signaling leading to apoptosis of acute lymphoblastic leukemia cells. Exp Hematol 2010;38:653–60.
[12] Paulsson K, Johansson B. High hyperdiploid childhood acute lymphoblastic leukemia. Genes Chromosomes Cancer 2009;48:637–60.
[13] Pui CH, Mullighan CG, Evans WE, Relling MV. Pediatric acute lymphoblastic leukemia: where are we going and how do we get there? Blood 2012;120:1165–74.
[14] Harrison CJ, Moorman AV, Broadfield ZJ, Cheung KL, Harris RL, Reza Jalali G, et al. Childhood and Adult Leukaemia Working Parties. Three distinct subgroups of hypodiploidy in acute lymphoblastic leukaemia. Br J Haematol 2004;125:552–9.
[15] Nachman JB, Heerema NA, Sather H, Camitta B, Forestier E, Harrison CJ, et al. Outcome of treatment in children with hypodiploid acute lymphoblastic leukemia. Blood 2007;110:1112–5.
[16] Greipp PR, Trendle MC, Leong T, Oken MM, Kay NE, Van Ness B, et al. Is flow cytometric DNA content hypodiploidy prognostic in multiple myeloma? Leuk Lymphoma 1999;35:83–9.
[17] Baruchel A, Cayuela JM, Ballerini P, Landman-Parker J, Cezard V, Firat H, et al. The majority of myeloid-antigen-positive (My+) childhood B-cell precursor acute lymphoblastic leukaemias express TEL-AML1 fusion transcripts. Br J Haematol 1997;99:101–6.
[18] Behm FG, Smith FO, Raimondi SC, Pui CH, Bernstein ID. Human homologue of the rat chondroitin sulfate proteoglycan, NG2, detected by monoclonal antibody 7.1, identifies childhood acute lymphoblastic leukemias with t(4;11)(q21;q23) or t(11;19)(q23;p13) and MLL gene rearrangements. Blood 1996;87:1134–9.
[19] Uckun FM. Regulation of human B-cell ontogeny. Blood 1990;76:1908–23.
[20] Vaskova M, Fronkova E, Starkova J, Kalina T, Mejstrikova E, Hrusak O. CD44 and CD27 delineate B-precursor stages with different recombination status and with an uneven distribution in nonmalignant and malignant hematopoiesis. Tissue Antigens 2008;71:57–66.
[21] Manabe A, Ohara A, Hasegawa D, Koh K, Saito T, Kiyokawa N, et al. Significance of the complete clearance of peripheral blasts after 7 days of prednisolone treatment in children with acute lymphoblastic leukemia: the Tokyo Children's Cancer Study Group Study L99-15. Haematologica 2008;93:1155–60.
[22] Mullighan CG, Collins-Underwood JR, Phillips LA, Loudin MG, Liu W, Zhang J, et al. Rearrangement of CRLF2 in B-progenitor- and Down syndrome-associated acute lymphoblastic leukemia. Nat Genet 2009;41:1243–6.
[23] Russell LJ, Capasso M, Vater I, Akasaka T, Bernard OA, Calasanz MJ, et al. Deregulated expression of cytokine receptor gene, CRLF2, is involved in lymphoid transformation in B-cell precursor acute lymphoblastic leukemia. Blood 2009;114:2688–98.
[24] Yoda A, Yoda Y, Chiaretti S, Bar-Natan M, Mani K, Rodig SJ, et al. Functional screening identifies CRLF2 in precursor B-cell acute lymphoblastic leukemia. Proc Natl Acad Sci USA 2010;107:252–7.
[25] Harvey RC, Mullighan CG, Chen IM, Wharton W, Mikhail FM, Carroll AJ, et al. 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 2010;115:5312–21.
[26] Cario G, Zimmermann M, Romey R, Gesk S, Vater I, Harbott J, et al. Presence of the P2RY8-CRLF2 rearrangement is associated with a poor prognosis in non-high-risk precursor B-cell acute lymphoblastic leukemia in children treated according to the ALL-BFM 2000 protocol. Blood 2010;115:5393–7.
[27] Ensor HM, Schwab C, Russell LJ, Richards SM, Morrison H, Masic D, et al. Demographic, clinical, and outcome features of children with acute lymphoblastic leukemia and CRLF2 deregulation: results from the MRC ALL97 clinical trial. Blood 2011;117:2129–36.
[28] Chen IM, Harvey RC, Mullighan CG, Gastier-Foster J, Wharton W, Kang H, et al. Outcome modeling with CRLF2, IKZF1, JAK, and minimal residual disease in pediatric acute lymphoblastic leukemia: a Children's Oncology Group study. Blood 2012;119:3512–22.
[29] Palmi C, Vendramini E, Silvestri D, Longinotti G, Frison D, Cario G, et al. Poor prognosis for P2RY8-CRLF2 fusion but not for CRLF2 over-expression in children with intermediate risk B-cell precursor acute lymphoblastic leukemia. Leukemia 2012;26:2245–53.
[30] Roberts KG, Morin RD, Zhang J, Hirst M, Zhao Y, Su X, et al. Genetic alterations activating kinase and cytokine receptor signaling in high-risk acute lymphoblastic leukemia. Cancer Cell 2012;22:153–66.
[31] Hunger SP. Tyrosine kinase inhibitor use in pediatric Philadelphia chromosome-positive acute lymphoblastic anemia. Hematology Am Soc Hematol Educ Program 2011;2011:361–5.
[32] Andersson A, Olofsson T, Lindgren D, Nilsson B, Ritz C, Edén P, et al. Molecular signatures in childhood acute leukemia and their correlations to expression patterns in normal hematopoietic subpopulations. Proc Natl Acad Sci USA 2005;102:19069–74.
[33] Inazawa J, Abe T, Inoue K, Misawa S, Oikawa S, Nakazato H, et al. Regional assignment of nonspecific cross-reacting antigen (NCA) of the CEA gene family to chromosome 19 at band q13.2. Cytogenet Cell Genet 1989;52:28–31.
[34] Foà R, Vitale A, Vignetti M, Meloni G, Guarini A, De Propris MS, et al. GIMEMA Acute Leukemia Working Party. Dasatinib as first-line treatment for adult patients with Philadelphia chromosome-positive acute lymphoblastic leukemia. Blood 2011;118:6521–8.

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DNA Methylation Profile Distinguishes Clear Cell Sarcoma of the Kidney from Other Pediatric Renal Tumors

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Abstract

A number of specific, distinct neoplastic entities occur in the pediatric kidney, including Wilms' tumor, clear cell sarcoma of the kidney (CCSK), congenital mesoblastic nephroma (CMN), rhabdoid tumor of the kidney (RTK), and the Ewing's sarcoma family of tumors (ESFT). By employing DNA methylation profiling using Illumina Infinium HumanMethylation27, we analyzed the epigenetic characteristics of the sarcomas including CCSK, RTK, and ESFT in comparison with those of the non-neoplastic kidney (NK), and these tumors exhibited distinct DNA methylation profiles in a tumor-type-specific manner. CCSK is the most frequently hypermethylated, but least frequently hypomethylated, at CpG sites among these sarcomas, and exhibited 490 hypermethylated and 46 hypomethylated CpG sites in compared with NK. We further validated the results by MassARRAY, and revealed that a combination of four genes was sufficient for the DNA methylation profile-based differentiation of these tumors by clustering analysis. Furthermore, *THBS1* CpG sites were found to be specifically hypermethylated in CCSK and, thus, the DNA methylation status of these *THBS1* sites alone was sufficient for the distinction of CCSK from other pediatric renal tumors, including Wilms' tumor and CMN. Moreover, combined bisulfite restriction analysis could be applied for the detection of hypermethylation of a *THBS1* CpG site. Besides the biological significance in the pathogenesis, the DNA methylation profile should be useful for the differential diagnosis of pediatric renal tumors.

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Introduction

In the pediatric population, the types of renal tumor are entirely different from those occurring in adults. It is estimated that 85% of pediatric renal malignancies comprise nephroblastoma, 5% congenital mesoblastic nephroma (CMN), 4% clear cell sarcoma of the kidney (CCSK), and 2% rhabdoid tumor of the kidney (RTK) [1], and these 4 major entities account for 96% of the total. The remaining 4% tend to occur in older children and include miscellaneous tumors, such as the Ewing's sarcoma family of tumors (ESFT). Nephroblastoma is malignant but still a relatively favorable tumor prognostically, being derived from nephrogenic blastemal cells that can show divergent differentiation. CMN is a kind of fibroblastic sarcoma of infancy and characterized by a specific chromosomal translocation, t(12;15)(p13;q25), which results in the fusion of *ETV6* and *NTRK3* genes [2]. On the other hand, CCSK is a relatively unfavorable tumor prognostically, being composed of clear mesenchymal cells with a characteristic vascular pattern [3]. RTK is a highly aggressive tumor occurring in young children, has a dismal outcome, and is characterized by pathological rhabdoid features and molecular biallelic inactivation of the *SMARCB1* (*hSNF5/INI1*) gene [4–6].

Since pediatric renal tumors are diverse neoplastic entities, as described above, and require different therapeutic strategies, rapid and accurate diagnosis is crucial for adequate treatment. However, all those tumors are composed of small-to-medium-sized, round, oval, or spindle-shaped undifferentiated or immature cells, and often deceptively mimic each other, making the diagnosis difficult [7]. In RTK and CMN, molecular markers, i.e., loss of SMARCB1 expression and *ETV6-NTRK3* fusion, respectively, are useful for an ancillary diagnosis, whereas the diagnosis of nephroblastoma and CCSK is exclusively based on histologic features. Although numerous studies have been done, immunohistochemical features or recurrent genetic changes that can reliably distinguish CCSKs from other pediatric renal tumors have not identified [3,8]. Therefore, the identification of molecular signatures that can distinguish CCSK from other renal tumors should be useful and provide diagnostic confidence and accuracy.

Alterations of DNA methylation have been well documented as an important peculiarity of cancer cells [9,10], and two patterns of