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ORIGINAL ARTICLE

Flow cytometric analysis of de novo acute lymphoblastic leukemia in childhood: report from the Japanese Pediatric Leukemia/Lymphoma Study Group

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Abstract Although the antigen expression patterns of childhood acute lymphoblastic leukemia (ALL) are well known, little attention has been given to standardizing the diagnostic and classification criteria. We retrospectively analyzed the flow cytometric data from a large study of antigen expression in 1,774 children with newly diagnosed ALL in JPLSG. T- and B-lineage ALL accounted for 13 and 87% of childhood ALL cases, respectively. Cytoplasmic CD3 and CD7 antigens were positive in all T-ALL cases. More than 80% of T-ALL cases expressed CD2, CD5 and TdT. In B-lineage ALL, the frequencies of early pre-B, pre-B, transitional pre-B and B-ALL were 81, 15.5, 0.6 and 2.9%, respectively. More than 90% of early pre-B ALL cases expressed CD19, CD79a, CD22, CD10 and TdT. CD34 was expressed in three-fourths of early pre-B ALL cases. The frequencies of TdT and CD34 expression were lower in pre-B ALL than in early pre-B ALL. B-ALL showed less frequent expression of CD22, CD10, CD34 and TdT than other B-lineage ALL cases. Expression of CD13 and CD33, aberrant myeloid antigens, was significantly more frequently associated with B-lineage ALL than with T-ALL. Based on this retrospective study of antigen expression in 1,774 de novo childhood ALL cases in JPLSG, we propose standardized clinical guidelines for the immunophenotypic criteria for diagnosis and classification of pediatric ALL.

Keywords Acute lymphoblastic leukemia · Childhood · Flow cytometry · Immunophenotype

1 Introduction

Flow cytometric immunophenotyping of childhood acute lymphoblastic leukemia (ALL) plays an important role not

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only in the diagnosis and classification of B and T cell lineages, but also in predicting the outcome [1–8].

Childhood ALL is a heterogeneous group of diseases. Therefore, leukemic cells from patients with ALL express a variety of differentiation antigens that are also found on normal lymphocyte precursors at discrete stages of maturation. With the development of monoclonal antibodies specific for relatively lineage-restricted or hematopoietic cell antigens, it has been possible to demonstrate considerable phenotypic heterogeneity in the vast majority of ALL cases by using panels of those antibodies [1, 2, 9–12].

The immunophenotypic patterns of acute leukemia, especially ALL, are well known, and classification into major immunologic categories is also accepted [1, 2, 9–12]. However, little attention has been given to standardizing the criteria for concluding which antigens are present on childhood leukemic cells, especially in Japan.

Herein, we report for the first time the results of a large, retrospective study of antigen expression in 1,774 children, older than 1 year and younger than 19 years of age, with newly diagnosed ALL, who had been enrolled between 1997 and 2007 at hospitals affiliated to the Japanese Pediatric Leukemia/Lymphoma Study Group (JPLSG). Based on these results, we have formulated guidelines for use of immunologic markers and proper interpretation of the results. It should be noted that this study did not investigate possible associations of antigen expression with the clinical, hematological and biological features or their prognostic importance, because the present study included patients for whom a complete set of these information and the immunophenotypic characteristics based on flow cytometry were not available due to several limiting factors associated with the registration system.

2 Methods

2.1 Patient samples

This is a retrospective analysis of 1,774 pediatric patients with newly diagnosed and untreated ALL. It excluded acute undifferentiated leukemia and true mixed-lineage leukemia, defined as co-expression of golden markers of two different lineages, e.g., MPO^+ and $CD79a^+$, or MPO^+ and $CD3^+$ [10]. The analyzed patients had been enrolled between 1997 and 2007 at hospitals affiliated to the Japan Association of Childhood Leukemia Study (JACLS), the Tokyo Children's Cancer Study Group (TCCSG) and the Japanese Children's Cancer and Leukemia Study Group (JCCLSG). These three study groups, combined with the Kyushu Yamaguchi Children's Cancer Study Group (KY-CCSG), constitute the Japanese Pediatric Leukemia/Lymphoma Study Group (JPLSG). All patients were diagnosed with ALL according to the French–American–British (FAB) morphology, enzyme cytochemical analysis and immunologic phenotype based on flow cytometric analysis. Samples obtained from bone marrow or peripheral blood of patients were immediately transported in sodium heparin tubes overnight to the central reference flow cytometry laboratories of the JPLSG. Informed consent for reference laboratory studies was obtained using forms approved by the local institutional review boards.

2.2 Flow cytometry

Ficoll–Hypaque-enriched blasts were stained by two-color immunofluorescence using various combinations of monoclonal antibodies, conjugated to phycoerythrin (PE) or fluorescein isothiocyanate (FITC), against the following antigens: CD1a, CD2, CD3, CD4, CD5, CD7, CD8, CD10, CD13, CD14, CD15, CD19, CD20, CD22, CD33, CD34, CD38, CD41, CD42b, CD45, CD56, CD58, CD66c, CD117, glycophorin A, HLA-DR, immunoglobulin kappa (Igκ) and lambda (Igλ) light chains, T cell receptors ($\alpha\beta$ and $\gamma\delta$) on the surface of leukemic cells and cytoplasmic Igμ chain, CD3, CD22, CD79a and myeloperoxidase antigens, as well as nuclear TdT. For detection of cytoplasmic (cCD3, cCD22, CD79a and MPO) and nuclear TdT antigens, antibodies were added after permeabilization using an Intraprep Permeabilization reagent kit (Beckman Coulter Immunotech, Miami, FL, USA). Isotypical immunoglobulins were used as negative controls. Two-color flow cytometric immunophenotyping was performed on an FACScan (Becton–Dickinson, San Jose, CA, USA) or EPICS flow cytometer (Beckman Coulter, Fullerton, CA, USA) according to the manufacturer's directions. The analysis gate was set in the forward and side light-scattering positions with lymphoid morphology. Data were recorded by an observer blinded to the patient's clinical status and diagnostic features, except for the immunophenotype. An antigen was rated as "positive" if more than 20% of the gated cells showed specific labeling above that of controls, or if a positive subpopulation was distinctively identified even in less than 20% positive cases. In principle, the criteria recommended by the European Group for the Immunological Characterization of Leukemias and others [1, 9, 10] were used for immunophenotypic classification.

2.3 Statistical analysis

Statistical analysis was performed by taking into account gender, age and the presence or absence of myeloid antigens, i.e., CD13 and CD33. Differences in the distributions of variables between groups of patients were analyzed by Mann–Whitney's U test, Kruskal–Wallis test or the χ^2 test.

3 Results

3.1 Clinical features and FAB morphology

The clinical presenting features, which include gender and age, and the FAB morphology, are summarized in Table 1. The boys-to-girls ratio of the incidence and the median age in cases of T-lineage ALL were significantly higher than in cases of B-lineage ALL ($p < 0.001$). Among patients with B-lineage ALL, these clinical characteristics were statistically more frequent in cases of mature B-ALL than in other types of B-lineage ALL ($p < 0.05$). In FAB morphology,

Table 1 Characteristics and immunophenotypic profile of 1,774 de novo cases of acute lymphoblastic leukemia

	T-ALL	B-lineage ALL		
		Early pre-B	Pre-B[a]	Mature B
Number of cases	231	1250	248	45
Frequency (%)	13.0	70.5	14.0	2.5
Clinical features				
Gender (boy/girl) (%)	74/26	55/45	51/49	74/26
Median age (range)	8 (1–16)	4 (1–18)	5 (1–15)	10 (1–15)
FAB morphology				
L1/L2/L3 (%)	72/28/0	82/17.5/0.5	84/16/0	0/0/100
T-lineage markers				
CD1a	53.7	0.3	1.5	0.0
CD2	83.5	4.1	4.0	2.2
cCD3	100	0.0	0.0	0.0
sCD3	49.3	0.0	0.0	0.0
CD4	54.8	0.8	0.0	0.0
CD5	94.2	0.5	10.1	0.0
CD7	100	3.2	6.9	2.2
CD8	68.3	1.1	0.0	0.0
TCR$\alpha\beta$	29.4	6.3	8.5	0.0
TCR$\gamma\delta$	10.9	0.0	0.0	0.0
B-lineage markers				
CD19	0.0	99.6	98.8	100
CD20	0.0	19.2	23.6	88.9
cCD22	2.9	90.1	97.3	77.8
sCD22	1.8	70.3	87.6	60.5
CD79a	21.8	99.2	100	100
cIgμ	0.0	0.0	100	88.9
sIgμ	0.0	2.1	9.0	83.3
sIgκ or λ	0.0	0.0	0.0	100
Non-lineage specific markers				
TdT	84.4	97.0	83.8	13.0
CD10	31.6	91.2	93.5	77.8
CD34	37.3	74.6	44.5	7.0
HLA-DR	16.7	99.3	94.7	97.7
Myeloid markers				
MPO	0.0	0.0	0.0	0.0
CD13	20.7	36.0	22.7	14.3
CD14	0.0	0.6	0.0	0.0
CD33	15.2	31.6	15.0	2.2
CD41	0.0	0.8	3.3	0.0
CD66c	0.5	43.5	25.9	0.0
CD117	15.6	10.1	13.4	11.5
GlyA	0.0	0.0	0.0	0.0

Values indicate the proportion of positive cases (%)

c cytoplasmic, *s* surface

[a] Pre-B cases include transitional pre-B cases

the L3 subtype was detected in all cases of mature B-ALL and only in five cases of early pre-B ALL without *t*(8;14) or its variants. The present study did not evaluate any further possible associations of immunophenotypic characteristics with other clinical, hematological or biological features or their prognostic importance because of several limiting factors associated with the registration system.

3.2 T-lineage ALL

T-lineage ALL accounted for 13% (231/1,774) of de novo childhood ALL (Table 1). Cytoplasmic CD3 and CD7 antigens were expressed in all T-ALL cases, which we were able to analyze. More than 80% of this subset expressed CD2, CD5 and the nuclear antigen, terminal deoxynucleotidyl transferase (TdT). Surface CD1a, CD3, CD4 and CD8 were detected in 49.3–68.3% of 231 cases of T-ALL. The HLA-DR antigen was not commonly expressed, and about 30% of the T-lineage ALL cases were $CD10^+$ and/or $CD34^+$. T cell receptor (TCR) proteins were heterogeneously expressed in T-lineage ALL. About 30% of the T-lineage cases expressing surface TCR chains expressed the $\alpha\beta$ form of TCR, whereas a minority, less than 15% of the T-lineage cases, expressed TCR$\gamma\delta$ proteins. Cytoplasmic CD79a and CD22, reliable markers for B-lineage ALL, were expressed in 21.8 and 2.9% of the T-lineage ALL cases, respectively. None of the T-ALL cases expressed CD19, CD20 or immunoglobulin molecules. Myeloid-associated antigen expression analysis found that CD13 and CD33 were expressed in 20.7 and 15.2% of the T-lineage ALL cases, respectively (Fig. 1). None of the T-ALL cases in this study expressed MPO or CD14. Early T cell precursor-ALL, a poor prognosis subgroup defined by its associated distinctive immunophenotype ($CD1a^-$, $CD8^-$, CD5 weak with stem-cell/myeloid markers) [13], was found in 3.7% of de novo T-ALL cases.

3.3 Early pre-B ALL

In this study, early pre-B ALL was found in 70.5% (1,250/1,774) of our de novo ALL cases (Table 1). Almost all of the early pre-B ALL cases were positive for CD19, cytoplasmic CD79a and cytoplasmic or surface CD22, but immunoglobulins were not detected. CD20, known to be a specific marker for early pre-B ALL, was detected in just 20% of the early pre-B ALL cases. More than 90% of the early pre-B ALL cases expressed CD10, TdT and HLA-DR, which are non-lineage specific antigens for B-lineage ALL. Moreover, CD34, a progenitor cell antigen, was expressed in 74.6% of the early pre-B ALL cases. CD66c, a member of the carcinoembryonic antigen family, was detected in nearly half of the early pre-B ALL cases. CD13 and CD33 antigens were expressed in 36.0 and 31.6% of the early pre-B ALL cases, respectively (Fig. 1). It is of note that neither cytoplasmic nor surface CD3 antigens were expressed in any B-lineage ALL (early pre-B, pre-B and B cell ALL) case in this series.

3.4 Pre-B ALL

According to the general consensus [1, 10, 14, 15], pre-B ALL blasts express cytoplasmic immunoglobulin μ heavy chains, but have no detectable surface immunoglobulins in B-lineage ALL. On the other hand, lymphoblasts of transitional pre-B ALL have both cytoplasmic and surface immunoglobulin μ heavy chains, without κ or λ light chains [1, 10, 15]. Since transitional pre-B ALL cases represented only 0.5% (9/1,774) of our de novo ALL cases, we analyzed these cases together with the pre-B ALL cases. This immunophenotype accounted for 14.0% (248/1,774) of our cases of newly diagnosed childhood ALL (Table 1) and expressed CD19, cCD22 and CD79a. Surface CD20 was detected in about a quarter of these pre-B

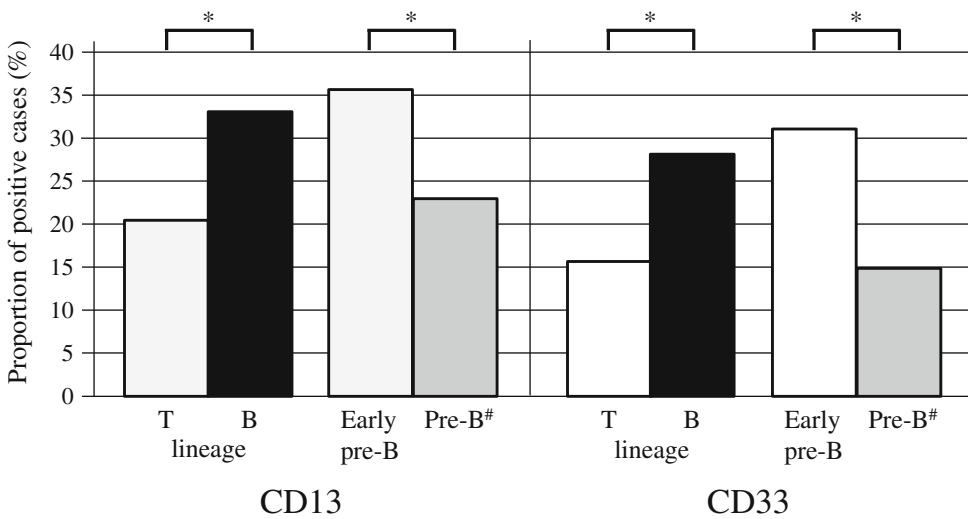

Fig. 1 Distribution of myeloid antigen (CD13 and CD33) expression. Acute lymphoblastic leukemia immunophenotypes: T-lineage ALL, B-lineage ALL, early pre-B ALL, pre-B ALL and B-ALL. *Values* indicate proportion of positive cases (%). #Pre-B cases include transitional pre-B cases. Expression was observed in all cases. *$p < 0.001$

ALL cases, and more than 90% expressed CD10 and HLA-DR. However, the frequencies of TdT and CD34 expression were 83.8 and 44.5%, respectively, which are lower than for early pre-B ALL cells. The expression frequencies of CD13 and CD33 were also lower than in the early pre-B ALL cases, at 22.7 and 15.0% ($p < 0.001$) (Fig. 1).

3.5 B cell ALL

B-ALL cells are characterized by L3 morphology, as defined in the FAB classification, and by surface membrane expression of immunoglobulin μ heavy chains (sIg) plus monotypic light chain [1, 9, 10]. In our present study, B-ALL cases accounted for 2.5% (45/1,774) of our de novo ALL cases (Table 1). The blasts of the B-ALL cases also expressed CD19, cCD79a, CD20 and HLA-DR. Both CD22 and CD10 were less frequently expressed in these cases than in other B-lineage ALL cases, including early pre-B and pre-B ALL. Although B-ALL cells are generally negative for expression of TdT and CD34, a few B-ALL cases with blasts that expressed TdT and/or CD34 have been reported [10, 16–19]. Moreover, Gluck et al. [20] diagnosed a B-ALL case that was L3 in the FAB classification with typical Burkitt's type translocation, but lacking sIg. In fact, we also identified a few cases with expression of TdT and/or CD34 and one case without sIg expression (positive for monotypic light chain) in this series. CD13 and CD33 antigens were expressed in some cases: 14.3 and 2.2%, respectively (Fig. 1).

4 Discussion

Immunophenotypic analysis of acute leukemia by flow cytometry has been used clinically as an indispensable tool for identification of the lineage association of leukemic cells and evaluation of the response to treatment [1, 2, 10–12, 21]. Recently, panels of monoclonal antibodies specific for lineage-associated antigens have been expanded. As a result, immunophenotyping of ALL has been applied to distinguish it from acute myeloid leukemia (AML) and to achieve more accurate phenotyping within ALL.

We retrospectively analyzed the flow cytometric data from a large study of antigen expression in 1,774 children with newly diagnosed ALL who were enrolled at hospitals affiliated to the Japanese Pediatric Leukemia/Lymphoma Study Group (JPLSG) between 1997 and 2007. Each central reference flow cytometry laboratory of the JPLSG made immunophenotypic diagnoses based on the criteria recommended by the European Group for the Immunological Characterization of Leukemias and others for childhood acute leukemia [1, 9, 10]. Although these criteria are actually similar to each other and standardized, they advocate some different subclasses in T- or B-lineage ALL. Additionally, ALL with myeloid antigen expression might be observed frequently in cases with mixed-lineage leukemia. However, the criteria for myeloid marker-positive childhood ALL and the clinical significance of these antigens also vary. We then formulated guidelines for the use of immunomarkers and proper interpretation of the results in childhood ALL, as summarized in Table 2.

T-lineage ALL, according to our analytical findings, is characterized by cytoplasmic or surface membrane expression of CD3 together with CD2, CD5, CD7 or CD8 (Table 2). Some of our T-ALL cells expressed CD79a or CD22 as a marker for B-lineage ALL. Although such T-ALL cases have been reported by other investigators [22, 23], none of our T-ALL cases satisfied the diagnostic criteria for B-lineage ALL described below. Recently, Campana et al. [13] reported diagnosis of early T cell precursor (ETP)-ALL, as a subgroup with a poor prognosis,

Table 2 Proposed immunophenotypic criteria for de novo cases of acute lymphoblastic leukemia

T-lineage ALL
1. $CD3^+$
2. Express CD2, CD5, CD7 or CD8
B-lineage ALL
Early pre-B ALL
Express at least two B-lineage markers (CD19, CD20, CD22 or CD79a)
Pre-B ALL[a]
1. Express at least two B-lineage markers (CD19, CD20, CD22 or CD79a)
2. Negative for surface membrane immunoglobulin κ or λ light chains
3. Express cytoplasmic and/or surface immunoglobulin μ heavy chains
B-ALL
1. Express at least two B-lineage markers (CD19, CD20, CD22 or CD79a)
2. Express surface membrane immunoglobulin κ or λ light chains
ALL with aberrant myeloid-associated antigen expression
My Ag^+ T-lineage ALL
1. $CD3^+$ and express CD2, CD5, CD7 or CD8
2. $CD79a^-$
3. MPO^- and express myeloid-associated markers (CD13, CD15, CD33 or CD65)
My Ag^+ B-lineage ALL
1. Express at least two B-lineage markers (CD19, CD20, CD22 or CD79a)
2. $CD3^-$
3. MPO^- and express myeloid-associated markers (CD13, CD15, CD33 or CD65)

[a] Pre-B ALL cases include transitional pre-B cases

characterized by absence of CD1a and CD8 expression and weak CD5 expression. At least 25% of ETP-ALL cells also express one or more of the following myeloid or stem-cell markers: CD117, CD34, HLA-DR, CD13, CD33, CD11b and CD65. Interestingly, they also pointed out that for patients with T-ALL, a diagnosis of ETP-ALL should be a stronger predictor of the outcome than is flow cytometric-based minimal residual disease [13]. We also found some ETP-ALL cases in our present study. The exact number of these immunophenotypic cases could not be indicated because not all of the myeloid or stem-cell markers reviewed above were used to diagnose our de novo ALL cases. However, six of 164 cases diagnosed using all these markers met the criteria for ETP-ALL. This frequency, 3.7%, was much less than the 12.6% reported by Campana et al. [13]. The difference in its frequency and correlation with the outcome should be ascertained in a future study.

Next, we classified B-lineage ALL into three categories, i.e., early pre-B ALL, pre-B ALL and mature B-ALL, according to the degree of B lymphoid differentiation of leukemic cells. Most cases of early pre-B ALL were positive for the common ALL antigen (CD10), CD34, HLA-DR and TdT. However, these antigens are not lineage specific. Although the immunoglobulin heavy chains are usually rearranged in these leukemic blasts, immunoglobulins were not detected. Early pre-B ALL can be conclusively defined as expression of at least two of the following four early B cell markers: CD19, CD20, CD22 and CD79a (Table 2). Pre-B ALL can be generally distinguished from transitional pre-B ALL based on their respective immunophenotypic characteristics [1, 10, 15]. However, in this study, we combined these two phenotypes as pre-B ALL, because discrimination of them might not be so important in the clinic [15, 21]. Pre-B ALL, including transitional pre-B ALL, can be defined as expression of cytoplasmic immunoglobulin μ heavy chains without κ or λ light chains and the presence of at least two of the following markers: CD19, CD20, CD22 and CD79a (Table 2). Additionally, B-ALL can be defined as expression of surface membrane immunoglobulin κ or λ light chains and at least two of the following markers: CD19, CD20, CD22 and CD79a (Table 2). Since, in rare instances, surface immunoglobulin μ heavy chains are absent in B-ALL cases, these markers are excluded from the definition of this immunophenotype [20].

Aberrant expression of one or more immunologic markers of another lineage might be observed in cases with mixed-lineage leukemia, which include myeloid antigen-positive ALL (B-lineage or T-lineage), lymphoid antigen-positive AML and true mixed-lineage leukemia [10]. Although our study included myeloid antigen-positive ALL, we did not find either biclonal or oligoclonal leukemias, which consist of two or more morphologically or immunophenotypically distinct leukemic cell populations. Expression of aberrant myeloid antigens (MyAgs) reportedly occurs in 5–22% of pediatric patients with de novo ALL [24–29]. We chose CD13 and CD33 as MyAgs, because they have been the most common antigens in MyAg-positive ALL. In our study, CD13 and CD33 were expressed in 31.7 and 26.5%, respectively, of de novo childhood ALL cases. Moreover, the frequency of CD13 expression was 33.3% in B-lineage ALL compared with 20.7% in T-ALL, while CD33 expression was 28.1% in B-lineage ALL versus 15.2% in T-ALL. These MyAgs were significantly more frequently associated with B-lineage ALL than with T-ALL ($p < 0.001$). In addition, the expression of these MyAgs was more frequent in early pre-B ALL cases than in pre-B ALL cases ($p < 0.001$). These incidences of MyAg expression in our study are in line with the data reported in the literature [24–29].

Recently, several notable studies investigated differences of race and ethnicity in the immunophenotypic subsets of childhood ALL [30–32]. Bhatia et al. [30] analyzed 8,762 children with de novo ALL who were categorized according to five groups: white, black, Hispanic, Asian and others. They showed that there was a significantly greater incidence of black children (25%) with T-ALL compared with Asian (19%), white (15%) and Hispanic (13%) children. In comparison, the frequency of T-ALL in our present report (the largest scale report in Japan to date), as representative data of East Asian children with ALL, was 13% of all cases, which is less than the 19% reported by Bhatia et al. [30]. This disparity cannot be readily explained. However, Kandan-Lottick et al. [32] pointed out that the reason might be that the Asian children analyzed by Bhatia et al. [30] were not Japanese, but from the Indian subcontinent and South Asia because they had been enrolled in the Children's Cancer Group Study.

In conclusion, based on the results of our large, retrospective study of antigen expression in 1,774 children with newly diagnosed ALL enrolled between 1997 and 2007, we have formulated clinically useful guidelines for flow cytometric immunophenotypic criteria for the diagnosis and classification of pediatric ALL in the JPLSG. The JPLSG was established in 2003 to create a research base for multi-center clinical trials for promotion of evidence-based medicine in pediatric hematologic malignancies. The JPLSG unifies several pediatric leukemia study groups, including the Japan Association of Childhood Leukemia Study (JACLS), the Tokyo Children's Cancer Study Group (TCCSG), the Japanese Children's Cancer and Leukemia Study Group (JCCLSG) and the Kyushu Yamaguchi Children's Cancer Study Group (KYCCSG), which had been functioning in Japan since the 1970s. The patients analyzed in this study have been treated according to different clinical protocols in each study group, and some of

them have not been clinically observed long enough. In addition, the central reference flow cytometry laboratories of the JPLSG received samples and made immunophenotypic diagnoses even during the intervals between clinical studies. Therefore, in this study we did not concern ourselves with possible associations of antigen expression with the clinical, hematological or biological features, or attempt to determine the prognostic importance of antigen expression for the decision of treatments. Nevertheless, flow cytometric data generated by extensive use of our newly proposed immunological criteria together with common diagnostic panels developed according to the present analysis may be valuable for achieving more precise characterization of the leukemic blasts in each individual patient. This information, combined with the molecular and clinical features presented in the next standard clinical protocol for childhood ALL that will be issued by the JPLSG, will also contribute to the development of personalized medicine, the so-called tailor-made therapy, for each patient.

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