リダイゼーション後の数値化とマッピングは Feature Extraction 、 Genomic Workbench CGH Module を用いた。

3. 遺伝子発現解析：
骨肉腫細胞株あるいは凍結腫瘍組織から調製した total RNA 1μg を出発材料に逆転写酵素を用いて cDNA を調製し定量 RT-PCR 解析を行った。標準化コントロールとして GAPDH 遺伝子を用いた。

（倫理面への配慮）
本研究で行った臨床検体を用いた実験は、関連法規ならびに指針を遵守し、倫理審査委員会ならびに実施機関長の承認を得た上で、検体提供者の人権の擁護、個人情報保護に細心の注意を払って実施した。

C．研究結果

1. 骨肉腫のゲノムコピー数異常解析と予後：
骨肉腫のゲノム異常は、高度な複雑性、heterogeneity、gain （増加）が loss （欠失）に比べ圧倒的に多いことが特徴であるが、骨肉腫の予後因子として有用なゲノムコピー数異常プロファイルはこれまで明らかにされていない。また、過去の報告では加療後に採取された腫瘍組織を用いた解析結果がほとんどであり、本来の腫瘍がもつゲノムプロファイルを反映しているとはいえない。そこで本研究では、千葉県がんセンターにて治療を受け、同センターがんバイオバンクに保存された小児骨肉腫症例 68 例のうち、臨床病理学的背景がほぼ均一な 22 例（予後良好例 14 例：2 年以上無病生存、予後不良例 8 例:転帰死亡）の生検試料を用いたアレイ CGH 解析を行い、そのゲノム異常プロファイルから予後と相関する領域の抽出を行った。まず、22 例全体を通して、1p、5p、6p、8q、17p、17q、19p、19q、21q など多くの染色体領域の増加と 6q、8p、9p、10p、10q、13q、17p などの欠失が高頻度に見られ、そのパターンは欧米人症例 20 例の過去の報告とほぼ共通であった。染色体短腕長腕単位での増加／欠失数や染色体内 breakpoint 数の比較では骨肉腫予後良好群、予後不良群間での頻度の有意差は認めなかった。さらに染色体領域毎にこれら両群間のゲノムコピー数異常プロファイルの比較を行ったところ、1q、3p、6p、9q、12q、15q の一部の連続した領域で予後に関して有意差が見られた（ $p<0.05$ ）。特に 1q22 の増加は予後良好群に、また、12q13 の増加は予後不良群に高頻度に見られた。

次に骨肉腫における化学療法感受性のマーカーの探索のため、本年度はさらに治療奏効性とゲノムコピー数異常プロファイルとの比較を行った。標準的な骨肉腫 Neoadjuvant 化学療法プロトコールにおいては、MTX、ADM、CDDP 3 剤併用による術前化学療法の後、奏効性の評価が効果不十分であった症例に対して術後に IFO を加えた治療を行う。そこで、術前治療にて良好な反応を示した症例群①、術後 IFO 追加により良好な反応を示した症例群②、効果不十分であった群③の 3 群に分け、それぞれに特徴的なプロファイルを抽出したところ、5p 増加、9q 欠失または 12q 増幅がそれぞれ症例群①、症例群③に強く相関する可能性が示された。

D．考察

これまでに治療後に採取された骨肉腫組織のゲノムコピー数異常プロファイルの報告は数例なされているものの、治療前の骨肉腫生検組織を用いた解析は国内外でもまとまった検体数の報告はなされていない。その一因として骨肉腫全体の年間発生例数が約 200 例と少なく、また、術前治療が標準であることから研究用の試料はほとんどが治療後のサンプルとなっていることによる。本研究では、長期にわたるバイオバンクへの研究用試料の保管と質の高い臨床情報の収集が可能であったことから実施が可能であった。別途並行して遺伝子発現解析用の試料も調製済みであり、今回抽出されたゲノム領域の候補遺伝子の遺伝子発現解析によるマーカーの絞り込みと検証が進行中である。特に興味深いことに、別のグループにより 6p に座位する RUNX2 遺伝子の発現レベルが骨肉腫の不良な予後と強い相関を示すことが報告されており、今回この遺伝子座がゲノム領域としても抽出されてきたことはマーカーのよい候補となりうると考えられる。

E．結論

治療前の骨肉腫臨床検体を用いた網羅的ゲノムコピー数解析から、その予後ならびに化学療法反応性に強く相関するプロファイルが抽出された。症例数をさらに広げた再現性の検証が必須であるが、バイオプシー検体のゲノムコピー数異常パターンが予後ならびに治療反応性の予測マーカーとなりうる可能性が示された。今後、臨床応用を目標にさらに検討を進める。

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G．知的財産権の出願・登録状況

（予定を含む）

1. 特許取得
   無し
2. 実用新案登録
   無し
3. その他
   無し

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平成23年度分担研究報告書

腫瘍細胞特異的遺伝子発現の経時的変化と治療の有効性についての研究

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鈴木涼子　同上

研究要旨：小児急性リンパ性白血病の治療成績向上には、治療の層別化（リスク分類）の果たしてきた役割が大きい。従来使用されてきた“発症年齢”と“白血球数”とによる層別化に加え、種々のリスク因子が検討されている。中でも治療開始初期の反応性（白血病細胞の減少速度）は、既に治療介入に使用されている。一方で、染色体の転座に伴うキメラ遺伝子は白血病細胞に特異的に発現される遺伝子である。キメラ遺伝子を標的とした検出方法を利用して治療反応性を高精度に評価できる可能性がある。これを検証することを目的として、本研究を行った。キメラ遺伝子ｍＲＮＡ定量法を用いた初期治療反応性を検討すると、現在採用しているリスク因子によって層別化された標準リスク群、高リスク群、超高危険群、及び超高危険群＋幹細胞移植群の４群それぞれにおいて、種々のキメラ遺伝子減少速度を示す症例が混在していた。現時点では登録症例を追跡中であるが、観察期間が終了した後に、再発情報・生存情報を加えて解析することによって、臨床的意義を確認できると予想される。

A. 研究目的

小児 ALL の治療成績向上には層別化治療が大きな役割を果してきた。白血病の治療効果判定として、光学顕微鏡よりも精度の高い方法が検討されている。フローサイトメトリーによる評価法や、ＡＬＬにおける免疫グロブリン遺伝子／Ｔ細胞受容体遺伝子のゲノム再構成を利用してＰＣＲで定量評価する手法が部分的に採用されている。一方で、染色体転座と関連したキメラm RNA の“定性 PCR”による評価は、予後因子としての意義が少ないという報告が複数の研究者によってなされてきた。

小児ＡＬＬにみられる転座等の染色体構造異常に起因するキメラ遺伝子由来のｍＲＮＡ定量法を用いた初期治療反応性の層別化因子としての意義を明らかにする。

B. 研究方法

東京小児がん研究グループ（ TCCSG ）登録急性リンパ性白血病（ ALL ）症例を対象として、筑波大学附属病院遺伝子検査室において発症時キメラ遺伝子スクリーニングの中央診断を行った。各医療機関（表１＝TCCSG 施設 59 ）から匿名化された骨髄または血液の送付を受け、標準的な方法によってmRNA 抽出、cDNA 作成、定量 PCR を行った。小児ＡＬＬに比較的高頻度に認められる以下のキメラ遺伝子をスクリーニングした（表２）。

- *TEL-AML1 (ETV6-AML1)*
- *E2A-PBX1*
- *Minor BCR-ABL*
- *Major BCR-ABL*
- *MLL-AF4*
- *MLL-AF9*
- *MLL-ENL*
- *SIL-TAL1*

なお、検出感度は 20 コピー／ 100ngRNA に設定した。

平成２３年４月以降平成２４年２月までに１７２症例の検体が送付され、４８症例にいずれかのキメラ遺伝子が検出された。その内訳は表３に示す。発症時スクリーニング結果を担当医に報告した。

キメラ遺伝子が検出され症例では、寛解導入療法および早期強化療法期間にかけて、該当するキメラ遺伝子の定量による微量残存白血病細胞(MRD)を追跡した。層別化とそれぞれに対する治療は、TCCSG の ALL 16 次案（表４－７）に準拠した。

MRD 追跡による治療反応生と現行の

TCCSG リスク群（標準リスク群 *SR*、高リスク群 *HR*、超高リスク群 *HEX*、同種移植群 *HEX-SCT*）との関係を解析した。

（倫理面への配慮）

臨床検体を用いた本研究の実施にあたり、関連法規を遵守し筑波大学附属病院倫理審査委員会ならび筑波大学医の倫理委員会の承認を得た。更に診療を担当する各 TCCSG 施設においても倫理委員会または研究審査委員会（IRB）の承認を得て実施した。検体提供者への人権擁護および個人情報保護に細心の注意を払った。

C. 研究結果

1． 全キメラ遺伝子定量値の推移

骨髄 MRD 追跡を行った全症例についてキメラ遺伝子定量値の推移を図１に一括表示した。治療初期反応性が最も良好な症例は、初回チェックポイントである day15 において、検出感度未満に減少していた。Day29 の時点で検出感度未満になる症例が最も多かったが、一方では、week 12 においても検出される症例もあった。

2． *BCR-ABL*

major BCR-ABL および *minor BCR-ABL* を一括して図２に示す。day15 では全例において検出された。検出感度未満まで減少した時期は、day29、day43、week12、week12 以降と非常に多様であった。BCR-ABL 陽性症例に対して HEX － SCT 型の治療戦略をとっているため、観察期間終了後に、共通の治療方針による対象集団における予後分析が可能である。

3． *MLL 再構成*

MLL-AF4、*MLL-AF9* および *MLL-ENL* を一括して図 3 に示す。検出感度未満まで減少した時期は、day15、day29、day43、week12、または week12 以降と、これも非常に多様であった。治療反応性と実際の臨床経過とを併せた評価が可能である。

4． *E2A-PBX1*

定量値の推移を図４に示す。一部の症例では、day15 までに検出感度未満に減少したが、多くは day29 に時点で陰性化を確認し、わずかであるがそれ以降まで検出された例があった。

TCCSG の層別化システムを使用して、*E2A-PBX1* 陽性例は、HR 群または HEX 群のいずれかに分類された。それぞれのリスク群ごとにキメラ遺伝子消失時期を評価した（表９）ところ、HR 群にはキメラ遺伝子消失時期が day15 の症例と day29 の症例とが含まれ、HEX 群には day29、day43、および week12 の症例が含まれた。

5． *SIL-TAL1*

定量値の推移を図５に示した。キメラ遺伝子が検出されなくなる時期は、day15 の症例と day29 の症例とがみられた。TCCSG の層別化システムによって、HEX 群と HEX-SCT 群の 2 群に分類された。HEX 群には day15 の症例と day29 の症例とが含まれたが、HEX-SCT 群には、day29 の症例のみが含まれた。

6． *TEL-AML1 (ETV6-AML1)*

定量値の推移を図６に示した。day15 までに陰性化している症例から、week12 以降まで検出され続ける症例までがあり、治療反応性は多様であった。TCCSG の層別化システムによって SR 群、HR 群、HEX 群および HEX-SCT 群に分類された。それぞれの群ごとにキメラ遺伝子陰性化確認軸時期を評価すると、SR 群と HR 群との双方に、day15、day29、または day43 にそれぞれ分布していた。

D 考察

予後不良因子とされる *BCR-ABL* または MLL 関連キメラ遺伝子の MRD 陰性化までの期間は、他のキメラ遺伝子に比べて明らかに長い傾向が確認された。同時に、これら予後不良とされる中にも比較的早期に MRD が陰性化するものがあり、将来は治療軽減（SCT 非実施等）の候補となる可能性がある。一方で、*TEL-AML1* および *E2A-PBX1* では、多くの症例において MRD 陰性化確認が day29 の時点であったが、一部の症例では day15 までに陰性化していた。登録症例追跡期間の終了時点で、このグループがそれ以外の *TEL-AML1* 陽性例と比較して再発率が低いか、または生存率が高い等の結果が得られれば、治療軽減の対象候補となることが推測される。一方で、陰性化が day43 以降の反応不良例が存在する。これらは逆に治療強化の対象候補となる可能性がある。

E．結論

同一リスク群であっても、骨髄中のキメラ遺伝子発現量による初期治療反応性のバラツキが確認された。

登録症例の再発状況等、追跡結果が確定した段階での再検討を計画している。

F. 研究発表

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G. 知的財産権の出願・登録状況

（予定を含む）

1. 特許取得
   なし。
2. 実用新案登録
   なし。
3. その他
   なし

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相模原協同病院小児科

表１　TCCSG 参加施設（H23 年度 59 施設）

キメラ遺伝子	対応する染色体異常（転座）	
minor BCR-ABL	Ph+	t (9;22) (q34;q11.2)
major BCR-ABL		
MLL-AF4	11q23	t (4;11) (q21;q23)
MLL-AF9		t (9;11) (p22;q23)
MLL-ENL		t (11;19) (q23;p13)
E2A-PBX1		t (1;19) (q23;p13.3)
SIL-TAL1		t (1;14) (p32;q11)
TEL-AML1		t (12;21) (p13;q22)

表２　発症時スクリーニング対象のキメラ遺伝子

	H22	H23 ＊	合計
総症例数	198	172	370
解析不能	5	9	14
キメラ遺伝子検出症例	50	48	98
TEL-AML1	22	29	51
E2A-PBX1	11	6	17
minor BCR-ABL	9	5	14
major BCR-ABL	1	0	1
MLL-AF4	3	1	4
MLL-AF9	1	2	3
MLL-ENL	1	2	3
SIL-TAL1	2	3	5

＊：Ｈ２３は２月末までの集計
表３　発症時（初発時）解析結果

末梢血白血球数	年　齢		
	1～6歳	7～9歳	10　歳～
＜20、000	SR	HR	HR
20,000 ～ 50,000	HR		
50,000 ～100,000			HEX
≧100,000	HEX		

全例にPSL単独先行投与7日間。
Day 8に末梢血芽球数を確認。

表4　TCCSG初期リスク分類

非T－ALL

初期リスク	Day 8 PB blast (/μL)	
	0～99	≧1,000
SR	SR	HEX
HR	HR	
HEX	HEX	SCT

T－ALL

初期リスク	Day 8 PB blast (/μL)	
	0～99	≧1,000
すべて	HEX	SCT

表5　Day 8 リスク分類

・寛解導入療法A
　PSL×5w, VCR×5, L-Asp×9, THP×2, TIT×2
・寛解導入療法B
　PSL×5w, VCR×5, L-Asp×9, DNR×4, CPA×2,TIT×2
・早期強化療法A
　6MP×21日, CA×15, CPA×1, DIT×2
・早期強化療法B
　DEX×5日, HD-MTX, CPA×5, HD-CA×2, L-Asp×1, TIT×1

表6　寛解導入療法レジメン

SR	寛解導入A＋早期強化A
HR	寛解導入B＋早期強化A
HEX	寛解導入B＋早期強化B
SCT	

表7　寛解導入療法の概要

	チェックポイント			
リスク群(N)	**day15**	**day 29**	**day 43**	**week12**
HR （20）	**2**	18	**0**	**0**
HEX （6）	**0**	4	**1**	**1**
SCT （0）	**0**	**0**	**0**	**0**

情報が不十分な例は除いた。

表8　リスク群と骨髄 *E2A-PBX1* の消失時期

	チェックポイント			
リスク群(N)	**day15**	**day 29**	**day 43**	**week12**
HEX（4）	**1**	**3**	**0**	**0**
SCT（4）	**0**	**4**	**0**	**0**

情報が不十分な例は除いた。

表9　リスク群と骨髄 *SIL-TAL1* の消失時期

	チェックポイント			
リスク群(N)	**day15**	**day 29**	**day 43**	**≧week12**
SR（27）	**1**	**20**	**6**	**0**
HR（23）	**2**	**19**	**2**	**0**
HEX（5）	**0**	**3**	**0**	**2**
SCT（1）	**0**	**1**	**0**	**0**

情報が不十分な例は除いた。

表10　リスク群と骨髄 *TEL-AML1* の消失時期

図１　骨髄中キメラ遺伝子定量値の推移
（全キメラ遺伝子一括表示）

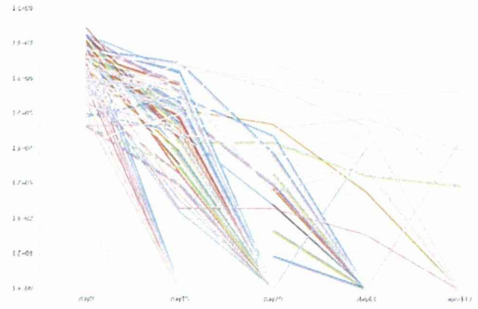
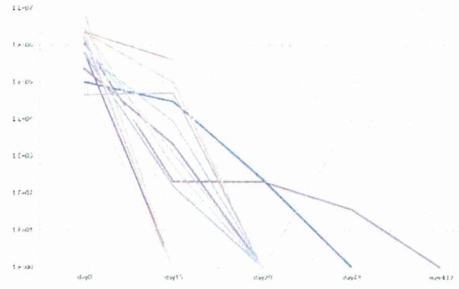


図１　*TEL·AML 1* (n=17)コピー数（/100ng RNA ）の推移

１：発症時骨髄
２：day 15
３：day 29
４：day 43 （寛解導入療法終了時）
５：早期強化療法後（week 12 相当）

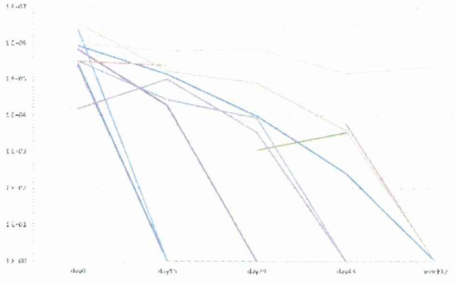
検出感度≧20 コピー／ 100ng
N=193　（H21 年以前の症例例を含む）

図２　骨髄中 *BCR-ABL* 発現量の推移



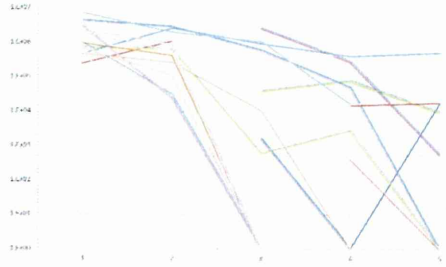
N=22 （H21 年以前の症例例を含む）

図３　骨髄中 *MLL* 関連キメラ遺伝子発現量の推移



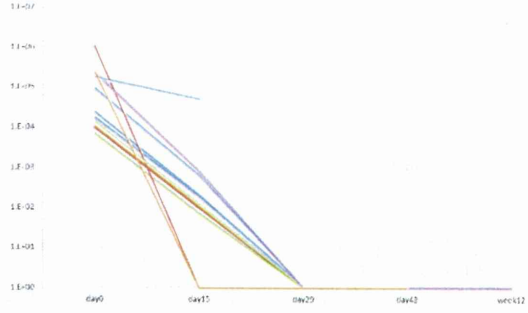
N=21 （H21 年以前の症例例を含む）

図４　骨髄中 *E2A-PBX1* 発現量の推移



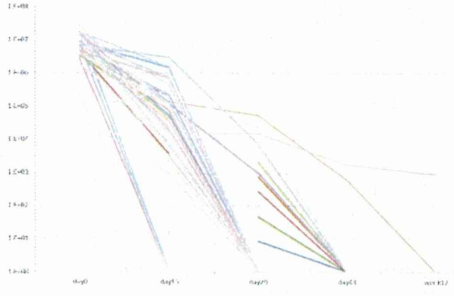
N=37 （H21 年以前の症例例を含む）

図５　骨髄中 *SIL-TAL1* 発現量の推移



N=13 （H21 年以前の症例例を含む）

図６　骨髄中 *TEL-AML1* 発現量の推移



N=100 （H21 年以前の症例例を含む）

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LETTER TO THE EDITOR

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

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Immunophenotypic analysis has become a powerful tool for the correct identification of leukemic cell lineage. Our study evaluates the diagnostic utility of flow cytometric immunophenotyping of pediatric AML. We retrospectively collected data of immunophenotype from 375 cases of de novo AML studied from 1997 to 2007 at central laboratory institutions of the Japanese Pediatric Leukemia/Lymphoma Study Group (JPLSG): Department of Pediatrics and Developmental Science, Mie University Graduate School of Medicine; Department of Pediatrics, Osaka University Graduate School of Medicine; Center for Clinical Research, National Center for Child Health and Development; and Department of Pediatrics, Aichi Medical University. The diagnosis of AML was made according to the French-American-British (FAB) classification based on morphology and enzyme cytochemical analysis as follows: M0 (acute myeloid leukemia without differentiation, $n = 11$), M1 (acute myelocytic leukemia with little differentiation, $n = 41$), M2 (acute myelocytic leukemia with differentiation, $n = 113$), M4 (acute myelomonocytic leukemia, $n = 47$), M5 (acute monocytic leukemia, $n = 54$), M6 (acute erythroleukemia, $n = 6$), and M7 (acute megakaryoblastic leukemia, $n = 61$).

Mononuclear cells of bone marrow or peripheral blood samples were stained with various combinations of fluorescein isothiocyanate (FITC)- and phycoerythrin (PE)-labeled monoclonal antibodies against the following antigens: CD4, CD7, CD13, CD14, CD15, CD19, CD33, CD34, CD36, CD41, CD42b, CD45, CD56, CD61, CD65, CD117, glycophorin A (GPA: CD235a), and HLA-DR. Cytoplasmic MPO was also detected by anti-MPO antibody after permeabilization. Two-color flow cytometric immunophenotyping was performed by collecting 10,000 ungated list mode events. An antigen was considered as

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Table 1 Immunophenotypic profile of 375 de novo cases of acute myeloid leukemia

	CD34	CD117	HLADR	MPO	CD13	CD33	CD14	CD15	CD65	GPA	CD36	CD41	CD42b	CD61	CD7	CD4	CD19	CD56	CD45
M0 (11)	72.7 (11)	90.9 (11)	63.6 (11)	45.5 (11)	54.5 (11)	90.0 (11)	0 (11)	33.3 (9)	16.7 (6)	0 (11)	9.1 (11)	9.1 (11)	9.1 (11)	ND	54.5 (11)	9.1 (11)	9.1 (11)	45.5 (11)	90.0 (10)
M1 (41)	85.4 (41)	100 (36)	73.2 (41)	100 (41)	90.2 (41)	97.6 (41)	2.6 (39)	60.7 (28)	75.0 (20)	0 (37)	18.9 (37)	10.0 (40)	0 (36)	ND	51.2 (41)	2.7 (37)	7.3 (41)	19.5 (41)	90.9 (33)
M2 (113)	83.8 (111)	94.4 (89)	89.2 (111)	96.4 (84)	91.2 (113)	92.9 (113)	7.4 (108)	55.1 (89)	33.3 (63)	0 (93)	12.0 (92)	4.5 (112)	2.2 (92)	ND	14.3 (112)	0 (95)	24.8 (113)	36.4 (110)	97.3 (74)
M3 (42)	14.3 (42)	76.3 (38)	4.8 (42)	96.9 (32)	92.9 (42)	97.6 (42)	4.8 (42)	15.6 (32)	53.8 (26)	2.8 (36)	5.6 (36)	0 (42)	10.8 (37)	ND	0 (42)	2.7 (37)	2.4 (42)	7.1 (42)	85.2 (23)
M4 (47)	53.2 (47)	76.7 (43)	78.7 (47)	94.9 (39)	87.2 (47)	93.6 (47)	29.8 (47)	80.0 (30)	80.6 (31)	2.3 (43)	51.2 (43)	10.6 (47)	4.5 (44)	ND	8.5 (47)	23.1 (39)	2.1 (47)	15.2 (46)	94.4 (36)
M5 (54)	24.1 (54)	39.6 (48)	81.5 (54)	68.6 (35)	64.8 (54)	98.1 (54)	34.6 (52)	74.5 (47)	87.1 (31)	2.3 (43)	60.5 (43)	5.6 (54)	2.1 (48)	ND	3.7 (54)	52.1 (48)	1.9 (54)	57.4 (54)	93.8 (32)
M6 (6)	50.0 (6)	66.7 (6)	50.0 (6)	80.0 (5)	100 (6)	100 (6)	0 (6)	0 (4)	25.0 (4)	66.7 (6)	83.3 (6)	0 (6)	0 (6)	ND	33.3 (6)	16.7 (6)	0 (6)	0 (6)	60.0 (5)
M7 (61)	41.1 (56)	74.5 (51)	49.1 (57)	2.8 (36)	73.7 (57)	90.0 (60)	1.9 (53)	8.9 (45)	5.7 (35)	32.0 (50)	78.0 (50)	72.4 (58)	58.5 (53)	85.7 (14)	69.6 (56)	20.0 (50)	1.7 (58)	45.6 (57)	96.8 (31)

Values indicate proportion of positive cases (%); parentheses indicate evaluable cases, *ND* not done

positive, if more than 30% of the gated cells showed specific labeling above that of controls, or if positive subpopulation was distinctively identified even in <30% positive cases.

The result is summarized in Table 1. Cytoplasmic MPO expression was found in less than half of cases with M0 (45.5%), which is consistent with other reports [1, 2]. However, M0 blasts expressed CD33 (90.0%) and CD117 (90.9%), and, less frequently, CD34 (72.7%), suggesting myeloid lineage. The low expression of CD13 as compared to CD33 in our study may reflect a more mature myeloid profile in pediatric cases [1, 3]. CD7, expressed in more than half cases, is known to be expressed in a proportion of AML-M0 and M1 cases [3–5], consistent with the fact that CD7 is expressed during early stages of normal myeloid differentiation [6]. CD56 was also expressed in nearly half of cases, but only one case co-expressed CD7 and CD56 consistent with NK/myeloid-cell precursor acute leukemic cells [7].

M1 and M2 blasts expressed CD34, CD117, HLA-DR, MPO, CD13, CD33, and HLA-DR in more than 80% of cases, and less commonly CD15 and CD65. CD7 was detected in 51.2% of M1 cases, while its expression was repressed in M2. CD19, detected in 24.8% of M2 cases, was reported to be detected in 78–81% of M2 cases with t(8;21) translocation [8, 9].

M3 cells expressed CD13, CD33, and MPO at high frequency, as for M1 or M2 cells. However, the frequency of CD117 expression was 76.7%, lower than for M1 or M2 cells. A striking feature is that the expression of CD34 and HLA-DR was low, at 14.3 and 4.8%, respectively. The lack of CD34 and HLA-DR was a feature of M3 blasts [4, 5, 10].

Leukemic cells of most M4 and M5 cases expressed monocyte markers, CD15 and CD65. The less common expression of CD14 has been reported by others, particularly in M5 cases [2, 5, 10]. M4 and M5 expressed CD33 at similarly high frequencies. The progenitor-associated antigens, CD34 and CD117, were seen in a lower proportion of M5 cases, which might reflect commitment to monocytic lineage. CD4 was expressed in 52.1% of M5 cases and 23.1% of M4 cases, in line with other reports [2, 10].

We observed only six M6 cases. Leukemic erythroblasts expressed CD36 and GPA in 66.7 and 83.3% of cases, respectively. Myeloid antigens (MPO, CD13, and CD33) and hematopoietic progenitor-associated markers (CD34 and CD117) were also expressed at variable frequencies. The expression of monocytic markers (CD14 and CD15) was absent, as well as megakaryocyte-associated antigens (CD41 and CD42b).

The expression frequencies of megakaryocyte-associated antigens, CD41 and CD42b in cases with M7, were

72.4 and 58.5%, respectively. All cases expressed CD41 and/or CD42b. CD36 was expressed at a high frequency, but its expression was also seen in other subtypes (M4, M5, and M6). Myeloid antigens (CD13 and CD33) were expressed in most cases, but lack of MPO expression was observed. Hematopoietic progenitor-associated antigens (CD34 and CD117) were expressed in many cases, and CD7 was expressed in 69.6% of cases.

In conclusion, each subtype of AML possesses distinguishing features of antigen expression. Some antigens appear to be associated with certain subtypes, but are not necessarily specific. Uncommon expression must be interpreted in the context of the entire immunophenotyping profile for correct identification of AML subtypes.

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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

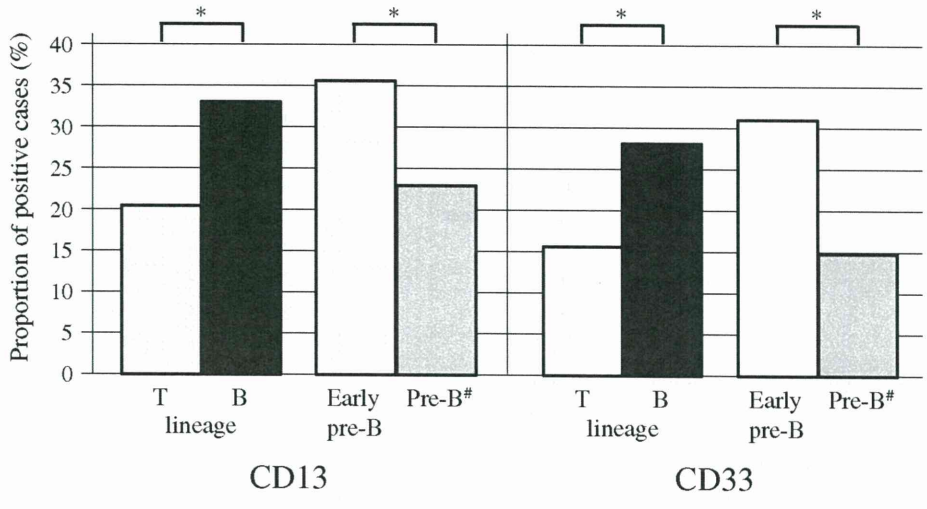

Fig. 1 Distribution of myeloid antigen (CD13 and CD33) expression. Acute lymphoblastic leukemia immunophenotypes: T-lineage ALL, B-lineage ALL, early pre-B ALL, pre-B ALL and B-ALL. *Values* indicate proportion of positive cases (%). #Pre-B cases include transitional pre-B cases. Expression was observed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