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Pathology review for paediatric non-Hodgkin's lymphoma patients in Japan: a report from the Japan association of childhood leukaemia study (JACLS)

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Abstract

A central pathology review system with an immunophenotyping laboratory was established in Japan to support the clinical trial, the Japan Association of Childhood Leukaemia Study (JACLS) NHL-98, for patients with paediatric non-Hodgkin's lymphoma (NHL). Pathology samples from 155 clinically-suspected NHL cases were evaluated centrally initially using the Revised European-American Lymphoma (REAL) classification in a rapid review (within 2 weeks after surgery/biopsy) and then later at the consensus review (once a year). The samples were subsequently re-classified according to the new World Health Organisation (WHO) classification. After the pathology review, 96 (62%) patients were eligible for the study, and 58 of them (60%) had extra-nodal primaries. These NHL cases included B-cell lymphomas (precursor B-cell, 11; Burkitt, 18; diffuse large B-cell, 18; not otherwise specified, 3) and T/Natural Killer (NK)-cell lymphomas (precursor T-cell, 23; anaplastic large cell, 20; others, 3). There was excellent concordance in making the diagnoses (95/96, 99%) and typing (93/96, 97%) of NHL between the rapid and consensus reviews. Five cases, initially diagnosed as diffuse large B-cell lymphoma by the review, were re-classified as Burkitt lymphoma according to the immunocytochemical criteria by the WHO classification. A total of 59 (38%) cases were excluded from the study: they were Hodgkin lymphoma (7), leukaemias (11), reactive lymphoid hyperplasia (20), necrotizing lymphadenitis (7), no consensus diagnosis (1), insufficient materials (2), and others (11). This is the first report of the central pathology review from the paediatric NHL group study in Japan. Because various diseases, either neoplastic or reactive, mimicked NHL, clinically and histopathologically, the central pathology review system was critical and essential for patient enrollment and protocol assignment in our clinical trial. Through the two-step review system, highly reliable data were generated to support this study.
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Keywords: Non-Hodgkin's lymphoma; Japanese children; Pathology; Central review; WHO classification; Group study

1. Introduction

Historically, different systems have been applied for classifying and typing non-Hodgkin's lymphoma (NHL) cases in different countries: the Working Formulation was the most popular classification in the United States (US) [1] and the Kiel/Updated Kiel classifications were commonly used in European countries [2,3], while most of the pathologists in Japan used the Lymphoma Study Group (LSG) classification [4] that was not well accepted worldwide. This often resulted in a serious problem for haematologists/oncologists when analysing and comparing clinical data with those from other countries. In 2001, the new World Health Organisation (WHO) classification was published for international use in classifying NHL cases [5].

In 1998, before the introduction of the new WHO classification, a central pathology review system was

established to support a nationwide group study, the Japan Association of Childhood Leukemia Study (JACLS) NHL-98, for Japanese paediatric NHL patients aged between 0 and 16 years old. 95 institutions/hospitals from 24 different prefectures participated in this study, covering approximately 40% of paediatric population in Japan. The pathology review system for this study initially used the Revised European-American Lymphoma (REAL) classification [6] that was the prototype of the new WHO classification [5]. Fortunately, there are no major differences between the REAL Classification and the new WHO classification in defining the most common categories of paediatric NHL cases, such as precursor B lymphoblastic lymphoma (B-LBL), Burkitt lymphoma (BL), diffuse large B-cell lymphoma (DLBCL), precursor T lymphoblastic lymphoma (T-LBL), and anaplastic large cell lymphoma (ALCL). However, in the new WHO classification, immunostaining was mandatory for the critical distinction between atypical Burkitt/Burkitt-like variant of BL (>99% cells positive for Ki-67) and DLBCL. Accordingly, some of the cases arbitrarily diagnosed as either DLBCL or BL by the REAL classification need to be re-classified by the new WHO classification after determination of the proportion of Ki-67-positive cells [5].

After 4 years, the JACLS NHL-98 study was successfully closed in March 2002. Towards the end of the study, we had an opportunity to re-evaluate and re-classify all the NHL cases according to the new WHO classification. This report illustrates our experience of the first central pathology review system established for the paediatric NHL patients in Japan.

2. Patients and methods

Pathology material from 155 clinically-suspected NHL cases were examined through the central review system for the JACLS NHL-98 study (1 April 1998–31 March 2002). Those materials, including haematoxylin-eosin (H&E)-stained sections (172), unstained sections (1520), and snap-frozen tissues, were submitted to the Pathology Center at the Department of Pathology, Aichi Medical University, Aichi, Japan, from the participating institutions/hospitals (see Appendix). The review system was composed of two steps: i.e., rapid review and consensus review. The rapid review accepted pathology material from those cases with clinically-suspected NHL without waiting for the final pathological diagnosis from the contributing institutions, and was completed by the responsible pathologist (AN) within 2 weeks after surgery/biopsy. The rapid review diagnosis was required for patient eligibility, stratification, and protocol assignment in the study. Immunophenotyping of the proliferating cells in each case was performed at the central laboratory of the Pathology Center to sup-

port the rapid review diagnosis by using the unstained sections provided by the contributing institutions/hospitals (see the panel of primary antibodies and typical staining pattern listed in Table 1). Snap-frozen tissues were filed and kept at the Pathology Center for future investigations. The consensus review took place once every year and involved four haematopathologists (AN, SN, HN, TY) in order to ensure the reproducibility of the rapid review diagnosis and determine the final eligibility of the cases for the analysis of the study results. In selected cases, additional tests, such as detection of clonal gene rearrangements and/or chromosomal translocations, were performed to support the consensus diagnosis. Appropriate informed consent procedures were followed, and consent was obtained from patients or guardians.

All those clinically-suspected cases were evaluated histologically and placed into two major groups: i.e., NHL and other diagnoses. The tumours in the NHL group, originally evaluated by the REAL classification through this review system, were re-classified using the new WHO classification. In order to complete the process of re-classifying those NHL cases according to the criteria of the new WHO classification, additional immunostaining for Ki-67 was performed for all of the cases initially diagnosed as DLBCL and BL.

3. Results

Of the 155 cases, 95 patients were diagnosed as having NHL at the time of both the rapid and consensus reviews. One case was diagnosed as a DLBCL at the time of the rapid review, but a panel of four haematopathologists did not reach a consensus diagnosis (two voted for DLBCL and two voted for atypical lymphoid proliferation) at the time of the consensus review: a clonal B cell population was not detected by the polymerase chain reaction (PCR) method using paraffin-embedded material from this case. 57 cases were classified into the group of other diagnoses at the rapid review. 56 of them were excluded from the study after the consensus review. However, one case was diagnosed as a "fulminant T-cell proliferation in Epstein-Barr Virus (EBV) infection" at the rapid review, but was eventually included in the study with a diagnosis of "peripheral T-cell lymphoma, unspecified" after the consensus review because of an aggressive growth pattern with soft tissue infiltration and a clonal rearrangement of T-cell receptor beta chain gene by Southern hybridisation. There were an additional two cases that were also excluded from the study due to insufficient pathology material for evaluation at the time of both reviews.

In summary, 96 (62%) of 155 cases evaluated by the central pathology review system were eligible for the

Table 1a
Panel of antibodies for immunophenotyping

Antibody	Clone	Company	Antibody	Clone	Company
Anti-CD3ε	Polyclonal	DAKO	Anti-CD4	1F6	Novocastra
Anti-CD43	DF-T1	DAKO	Anti-CD8	C8/114B	DAKO
Anti-CD20	L26	DAKO	Anti-CD56	1B6	Novocastra
Anti-CD79a	JCB117	DAKO	Anti-CD45RO	UCHL-1	DAKO
Anti-TdT	Polyclonal	Supertechs	Anti-CD10	56C6	Novocastra
Anti-Ki-67	MIB-1	MBL	Anti-Bcl-2	124	DAKO
Anti-CD30	Ber-H2	DAKO	Anti-Granzyme B	GrB-7	MONOSAN
Anti-ALK-1	ALK1	DAKO	Anti-CD34	BI-3C5	DAKO
Anti-EMA	E29	DAKO	Anti-CD68	KP-1	DAKO
Anti-CD15	C3D1	DAKO	Anti-CD99 (MIC2)	12E7	DAKO

Antibodies to be tested were selected after reviewing the haematoxylin and eosin (H&E) sections from individual tumours.

Table 1b
Typical patterns of immunophenotyping for paediatric non-Hodgkin's lymphoma cases

Histological type	CD45	TdT	CD3ε	CD43	CD45RO	CD20	CD79a	CD99	CD15	CD30	EMA	ALK-1
B-LBL	+/–	+	–	–/+	–	–	+	+	–	–	–	–
BL	+	–	–	–	–	+	+	–	–	–	–	–
DLBCL	+	–	–	–	–	+	+	–	–	–/+ [a]	–/+	–
T-LBL	+/–	+	+	+	+	–	–/+	+	–	–	–	–
ALCL	–/+	–	+/–	+/–	+/–	–	–	–	–	+	+	+

B-LBL, precursor B lymphoblastic lymphoma; BL, Burkitt lymphoma; DLBCL, diffuse large B-cell lymphoma; T-LBL, precursor T lymphoblastic lymphoma; ALCL, anaplastic large cell lymphoma; +, positive; –, negative; +/–, positive in most cases; –/+, negative in most cases.
[a] Expression, extensive in anaplastic variant and variable in mediastinal primary tumour.

JACLS NHL-98 study. Among these, only two (2%) cases had discrepancies in determining their types between the rapid and consensus reviews. In those two cases (final typing from B-NHL to DLBCL and from BL to B-NHL, one case each), the changes were made after reassessment of quality/quantity of the pathology sample at the consensus review. There were no differences, except for five cases, in typing of the NHL tumours between the original review by using REAL classification and the subsequent re-classification using the new WHO classification. The five cases, all initially diagnosed as DLBCL in the review (5/23, 22%), were re-classified as BL (atypical Burkitt/Burkitt-like variant of BL) after evaluation and determination of a Ki-67 fraction of close to 100% in their tumour tissues. Those cases, as shown in Fig. 1, had ambiguous morphological features: all were positive CD10 and 4/5 were negative for Bcl-2 immunohistochemically. Two of them were reported to show a normal karyotype. During the same 4-year period, 23 cases were enrolled on the JACLS NHL-98 study without central pathology review: those cases were excluded from further analysis in the present study.

Table 2 shows the final diagnosis (typing) according to the new WHO classification and clinical information (age, gender, and primary site) for all the NHL cases in this study. These cases included B-LBL (11 cases, 11%), BL (18 cases, 19%), DLBCL (18 cases, 19%), B-cell non-Hodgkin's lymphoma, not otherwise specified (B-NHL, NOS: 3 cases, 3%, B-cell phenotype determined

by either immunostaining or flow cytometry, but further subclassification not feasible due to limited amount/quality of the samples), T-LBL (23 cases, 24%), ALCL (20 cases, 21%; including 13 cases with a T-cell phenotype and 7 cases with a null-cell phenotype), and peripheral T-cell/natural killer (NK) cell lymphoma (pT/NK-NHL: 3 cases, 3%, including hepatosplenic T-cell lymphoma, extranodal NK/T cell lymphoma, nasal type, and peripheral T-cell lymphoma, unspecified).

There were 60 males and 36 females with ages ranging between 11 months and 16 years old (median 9 years old) at diagnosis. Of these cases, 38 (40%) had primary nodal lymphoma, whereas 58 (60%) had primary extra-nodal lymphoma. Nineteen (20%) patients had bone marrow involvement (less than 25%), while no children had disease in the central nervous system (CNS) at the time of diagnosis. Those patients who had extra-medullary masses showing histology that was indistinguishable from B-LBL, T-LBL, or BL with 25% or more blasts in their bone marrow were diagnosed as having acute lymphoblastic leukaemia (ALL) and placed in the other diagnoses group (see below). As shown in Table 2, most (39/50, 78%) of the tumours with a B-cell phenotype were diagnosed in the cervical lymph node, extra-nodal head and neck region or gastro-intestinal tract. Children with BL were predominantly male (Male (M):Female (F) = 16:2), while those with B-LBL were more frequently female (M:F = 4:7). Children with T-LBL were predominantly male (M:F = 17:6), and

almost exclusively diagnosed either in the mediastinal region (15/23, 65%) or cervical lymph node (7/23, 30%). Children with ALCL were more frequently female (M:F = 8:12) and often had nodal primaries (14/20, 70%).

A total of 59 (38%) cases were excluded from the study due to other diagnoses (56 cases, Table 3), no consensus diagnosis (one case), and insufficient material (2 cases). Those cases with other diagnoses are listed in Table 3. The cases in the other diagnosis group were summarized as follows: (1) Hodgkin lymphoma was diagnosed by its characteristic histology and the presence of CD30-positive and, less frequently, CD15-positive, Reed-Sternberg cells [5,8]. However, the hallmark cells of ALCL and activated lymphocytes in reactive lymphadenopathy were also positive for CD30 [8]. (2) Like Burkitt lymphoma and B-ALL, T/B-LBL and precursor T/B-lymphoblastic leukaemias were indistinguishable cytologically [5]. All of the ALL cases in this series had more than 25% of bone marrow involvement at the time of diagnosis. In contrast, other leukaemia cases presented with an extramedullary mass, and no or few leukaemic blasts were found in the bone marrow at the time of diagnosis. (3) More than 1/3 of the cases in the other diagnoses group were classified into a category of reactive lymphoid hyperplasia (benign polyclonal lymphoproliferation). It

seemed to be a unique situation in paediatric age group to have such a large number of cases of reactive lymphoid hyperplasia as one of the differential diagnoses from NHL. (4) A case of Ewing's/peripheral Primitive Neuroectodermal Tumour (pPNET) presented us with a diagnostic difficulty, since the tumour cells, like those in many of the LBL cases, showed positive staining for CD99 (MIC2 gene product). Detection on *EWS* gene translocation and expression of neural markers confirmed the diagnosis of this particular case [9].

4. Discussion

This is the first report illustrating the central pathology review system of paediatric NHL and the experience of the JACLS NHL-98 study. Paediatric NHL cases are different from adult NHL cases: more than 90% of them are high-grade, approximately 75% present at advanced stages at diagnosis, and they often show early dissemination or leukaemic manifestation [7,10]. Tumours in paediatric NHL are frequently found in extra-nodal locations, and are difficult to diagnose, clinically as well as histopathologically [7,11,12]. Furthermore, because of striking differences in proliferative kinetics, treatment protocols for such patients should be appropriately determined based on a precise diagnosis

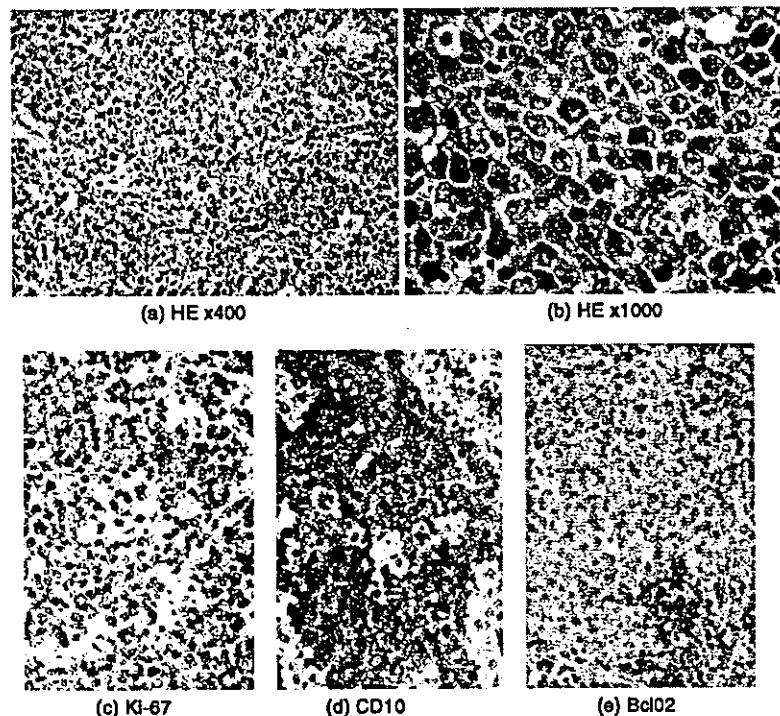(a) HE x400   (b) HE x1000

(c) Ki-67   (d) CD10   (e) Bcl02

Fig. 1. Atypical Burkitt/Burkitt-like variant of Burkitt lymphoma. (a) Diffuse proliferation of medium-sized to large lymphocytes. There are some macrophages, but atypical "starry-sky" pattern is not observed. Haematoxylin and eosin (H&E) stain. (b) Tumour cells show greater pleomorphism in nuclear size and shape than those commonly seen in classical Burkitt lymphoma. There are numerous mitotic figures. Nucleoli are more prominent. A few eosinophils are recognised. H&E stain. (c) Nearly 100% of the tumour cells are positive for Ki-67. Immunostain for Ki67. (d) Tumour cells are positive for CD10. Immunostain for CD10. (e) Tumour cells are negative for Bcl-2. Residual lymphocytes are positive for Bcl-2 (lower right). Immunostain for Bcl-2.

Table 2
Paediatric non-Hodgkin's lymphoma cases from the JACLS NHL-98

Histological type	Age (median)	Gender	Nodal					Extra-nodal						
			Cervical LN	Axillary LN	Inguinal LN	Mesenteric LN	Other LN	Head and neck	Mediastinum	Abdomen	Gastrointestinal	Bone	Skin	Other
B-LBL (N = 11)	11 m–12 y (3 y)	M4:F7	4							1	3	1	1	1 Testis
BL (N = 18)	1 y–13 y (9 y)	M16:F2	6					5		1	5			1 Kidney
DLBCL (N = 18)	5 y–16 y (9.5 y)	M10:F8	3					8	2		3	1		1 Testis
B-NHL, NOS (N = 3)	3 y, 12 y, 14 y	M3:F0	2							1				
T-LBL (N = 23)	2 y–14 y (10 y)	M17:F6	7				1 Elbow		15					
ALCL (N = 20; T 13, Null 7)	1 y–13 y (10 y)	M8:F12	7	2	2	2	1 Lung hilar		2				2	2 Soft tissue
pT/NK-NHL (N = 3)	5 y, 6 y, 9 y	M2:F1	1					1		1				

Primary site (Nodal and Extra-Nodal), determined based on the clinical information. B-LBL, precursor B lymphoblastic lymphoma; BL, Burkitt lymphoma; DLBCL, diffuse large B-cell lymphoma; B-NHL, NOS: B-cell non-Hodgkin's lymphoma, not otherwise specified; T-LBL, precursor T lymphoblastic lymphoma; ALCL, anaplastic large cell lymphoma; pT/NK-NHL, peripheral T-cell/natural killer-cell lymphoma; m, months; y, years; M, male; F, female; LN, lymph node; JACLS, Japan Association of Childhood Leukaemia Study.

Table 3
Cases in the other diagnoses group

	Gender	Age (median)	Nodal				Extra-Nodal				
			Cervical LN	Axillary LN	Inguinal LN	Mesenteric LN	Head and neck	Mediastinum	Abdomen	Skin/soft tissue	Other
Classical Hodgkin lymphoma (N=7)											
Mixed cellularity (N=3)	M1:F2	3 y, 5 y, 7 y	3								
Nodular sclerosis (N=2)	M1:F1	11 y, 13 y	2								
Lymphocyte-rich (N=2)	M1:F1	8 y, 9 y		1							
Leukaemia (N=11)											
ALL (precursor B) (N=3)	M1:F2	6 y, 7 y, 10 y	1						1		
ALL (precursor T) (N=4)	M3:F1	6 y–11 y (7 y)	3		1						
ALL (B) (N=1)	M1:F0	13 y							1		
AML (acute monoblastic leukaemia, M5a) (N=1)	M1:F0	1 y									1 (testis)
Precursor myeloid/NK cell leukaemia (N=1)	M1:F0	1 y								1	
Mixed lineage leukaemia (Ph1-positive) (N=1)	M0:F1	1 y					1				
Reactive lymphoid hyperplasia (N=20)	M16:F4	4 m–16 y (8.5 y)	11		2	3	1		2		
Histiocytic necrotising lymphadenitis (N=7)	M5:F2	7 y–15 y (13 y)	6	1			1			1	
EBV-associated diseases (N=4)											
Infectious mononucleosis (N=2)	M1:F1	4 y, 6 y	2								
EBV-associated LPD (T-cell) (N=1)	M1:F0	10 y							1		
PTLD (DLB) (N=1)	M1:F0	12 y									1 (systemic)
HIV-related lymphadenopathy (N=1)	M0:F1	6 m				1					
Miscellaneous (N=3)											
Extramedullary plasmacytoma (N=1)	M1:F0	10 y	1								
Castleman's disease (N=2)	M1:F1	4 y, 13 y	2								
Others (N=3)											
Ewing's sarcoma/pPNET (N=1)	M0:F1	5 y								1	
Panniculitis (N=1)	M0:F1	15 y								1	
Hypertrophic thymus (N=1)	M0:F1	4 y						1			

Primary site (Nodal and Extra-Nodal), determined based on the clinical information. EBV, Epstein–Barr Virus; ALL, acute lymphoblastic leukaemia; pPNET, peripheral Primitive Neuroectodermal Tumour; PTLD, post-transplant lymphoproliferative disorder; m, months; y, years; M, male; F, female; LN, lymph node.

and classification [11,13]. Therefore, the central pathology review system, consisting of the rapid and consensus review supported by immunophenotyping results, was essential and critical to carry out our clinical trial. As described in this report, our system successfully generated the pathological data with excellent agreement rates in terms of the diagnosis and typing of the NHL cases between the rapid and consensus reviews.

All cases were initially reviewed using the REAL classification, and, subsequently, were re-classified according to the new WHO classification. There were 5 patients whose diagnoses changed from DLBCL to BL during this process of re-classification. Fortunately, we had the same treatment approaches for patients with NHL of the mature B-cell phenotype. Accordingly, the treatment protocols for these patients did not change in our clinical trial. In the classification, there still seems to be some difficulty/confusion in distinguishing between BL (atypical Burkitt/Burkitt like variant of BL) and DLBCL. For the diagnosis of BL (atypical Burkitt/ Burkitt like variant of BL), a growth fraction of nearly 100% in the tumour tissue is critical according to the WHO guideline. In addition, CD10 and Bcl-2 immunostainings are reported as useful for the distinction between BL and DLBCL [14]: in our series CD10 was positive in 15/16 BL cases and 7/12 DLBCL cases, whereas Bcl-2 was positive in 1/6 BL cases and 4/8 DLBCL cases. Cytogenetic analysis for the detection of MYC translocation was largely unsuccessful in our study.

Table 4 summarises paediatric NHL studies in the literature [15–19]. As shown in this table, paediatric NHL cases are mainly composed of only three or four different types according to the various classifications. Since these investigators used different classifications [1,3,19], it is difficult to make any direct comparisons for the incidence of NHL types between the previous studies and our current series using the new WHO classification [5] that distinguishes four major types: i.e., LBL, BL, DLBCL and ALCL. The small sample size of our study also means any conclusive statements must be cautiously made at this time. However, some or all of these types in the new WHO classification were included in the previous classifications and were given similar definitions. Recent advances in the field of haematopathology research, such as the introduction of a panel of systematic immunophenotyping with an increased number of good quality antibodies available for use on paraffin sections (Table 1), determination of a proportion of Ki-67- positive cells for the diagnosis of BL [5], and the detection of the ALK translocation for the diagnosis of ALCL [20], now enable us to recognise and distinguish these NHL types more precisely. After taking these into account, however, our study still seems to include less BL cases and more DLBCL and ALCL cases than previous reports from Western countries and Taiwan. Other investigators have reported that the distribution of adult NHL types in Japan was also different to that observed in Western countries [21]. These differ-

Table 4
Paediatric Non-Hodgkin's lymphoma studies in the literature

Author [Ref.] (country)	Study period (classification)	Number of cases[a]	Age (median)	Major histological type[b]
Wilson, JF [13] (U.S.)	1977–1980 (Rappaport)	213	≤18 years	50.2% Undifferentiated (Burkitt's and non-Burkitt's) 34.3% LBL 13.6% Large cell/histiocytic lymphoma
Murphy, SB [15] (U.S.)	1962–1986 (Working Formulation)	338	7 months–21 years (10 years)	38.3% diffuse small non-cleaved cell 28.1% LBL 26.3% DLCL
Reighter, A [16] (Germany)	1986–1990 (Updated Kiel)	261	0.6 years–17.8 years	42.5% BL 28.0% LBL 7.7% DLBCL 6.9% ALCL
Wright, D [17] (U.K.)	1990–? (Updated Kiel)	293		44.4% BL 28.7% LBL 7.5% DLBCL 15.7% ALCL
Yang, C-P [18] (Taiwan)	1992–1998 (Working Formulation)	181	2.4 months–18.3 years	42.5% BL 29.8% LBL 27.6% DLCL (including ALCL)
Present study (Japan)	1998–2002 (WHO)	96	11 months–16 years (9 years)	18.8% BL 35.4% LBL 18.8% DLBCL 20.8% ALCL

Undifferentiated: undifferentiated lymphoma; LBL: lymphoblastic lymphoma; DLCL: diffuse large cell lymphoma; BL, Burkitt's lymphoma; DLBCL, diffuse large B-cell lymphoma; ALCL, anaplastic large cell lymphoma.
[a] Non-Hodgkin's lymphoma cases only (B-ALL cases, excluded).
[b] Only major types and their percentages are listed.

ences, in both paediatric and adult NHL types, between Japanese patients and patients from other countries could be due to different ethnic as well as environment/ geographical backgrounds [21,22].

The classification of LBL according to the new WHO guidelines deserves a brief comment. LBL cases were once classified into three subsets due to the limited number of antibodies available for immunophenotyping: approximately 2/3 of the cases were classified in the T-cell subset and the rest of the cases were classified as either B-cell or indeterminate [16,17]. By using a panel of antibodies at the central laboratory, all LBL cases included in our study were classified into either T-cell or B-cell subsets, and no cases were classified as indeterminate. Among the various immunophenotyping markers used in our series, TdT+ and $CD3_\epsilon$+ were useful for classifying a LBL in the T-cell lineage (23/34, 67.6%), and TdT+, CD20 +/−, and CD79a+ were useful to define those belonging to the B-cell lineage (11/ 34, 32.4%). In our study 7/11 (64%) of the B-LBL cases were positive for both CD20 and CD79a, and 4/11 (36%) showed positive staining for CD79a only. In our series, there was only one case whose tumour was TdT+, $CD3\epsilon$ + and CD79a+. Since the T-cell receptor beta chain gene was clonally rearranged, but the immunoglobulin heavy chain gene showed a germline configuration, this particular case was classified as being in the T-LBL subset with an aberrant CD79a expression [23]. Both T-LBL and B-LBL tumours were diagnosed in nodal (cervical lymph node) and extra-nodal locations. In our series, the extra-nodal T-LBL tumours developed exclusively in the mediastinum, while the extra-nodal B-LBL tumours (as has been reported by other investigators) were found in unusual locations, such as the bone, skin, and testis, in addition to the gastrointestinal tract [24–26]. Positive TdT staining was often helpful, and even critical, for distinguishing B-LBL cases from BL cases, especially when the tumours developed in the gastrointestinal tract. This was particularly important since the patients with B-LBL were assigned to different treatment protocols from those with BL [12,26].

The central pathology review system of our study accepted pathology materials from those cases with clinically-suspected paediatric NHL immediately after surgery/biopsy, without waiting for the final pathological diagnosis from the contributing institutions. We decided to choose this system to review a large number of suspicious cases mainly because there are considerable numbers of neoplastic and reactive lesions that mimick NHL among paediatric patients. In addition, not all of the participating institutions were fully equipped with standardised immunohistochemical and molecular techniques to perform differential diagnoses and to precisely type the NHL cases. With this system, we successfully avoided a potential failure of enrolling on the study in a timely manner some of the NHL cases

presenting diagnostic dilemma/difficulty. For example, some of ALCL cases with a feature, focally or diffusely, of either small-cell or lymphohistiocytic variant [5,27], comprising 35% (7/20) of all the ALCL cases in this series, could well have been missed because of difficulties in identifying the diagnostic hallmark cells in a background of intense inflammatory infiltrates [5,27]. Nevertheless, 59 (38%) cases were rejected from our study after the review.

In summary, we established a central pathology review system with immunophenotyping facilities for paediatric NHL cases. Pathology materials from all clinically-suspected cases were reviewed, and NHL cases were classified according to the new WHO guidelines. With this system, highly reliable pathology data were provided to support the nationwide Japanese clinical trial, the JACLS NHL-98.

Acknowledgements

Appendix

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Brief report

FLT3 mutations in the activation loop of tyrosine kinase domain are frequently found in infant ALL with *MLL* rearrangements and pediatric ALL with hyperdiploidy

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Point mutations of D835/I836 of the *FLT3* gene have been reported in adult acute myeloid leukemia (AML), but not in pediatric AML or acute lymphoblastic leukemia (ALL). *FLT3*-D835/I836 mutations were found in 6 (5.4%) of 112 children with ALL older than 1 year and in 8 (16.0%) of 50 infants with ALL. Missense mutations were found in 11 patients, 3-base pair deletions in 2 patients, and a deletion/insertion in 1 patient. Remarkably, *FLT3*-D835/I836 mutations were found in 8 (18.2%) of 44 infants with ALL with *MLL* rearrangements and in 4 (21.5%) of 19 patients with hyperdiploid ALL, but they were not found in any patients older than 1 year who had *TEL-AML1* (n = 11), *E2A-PBX1* (n = 4), or *BCR-ABL* (n = 6) fusion genes. Although infant ALL patients with mutations had poorer prognoses than did those without mutations, pediatric ALL patients with mutations who were older than 1 year had good prognoses. We also found *FLT3*-D835 mutations in 2 of 11 leukemic cell lines with *MLL* rearrangements. FLT3 was highly phosphorylated in these cell lines with *FLT3*-D835 mutations, leading to constitutive activation of downstream targets such as signal transducer and activator of transcription 5 (STAT5) without FLT3 ligand stimulation. These results suggested that *FLT3*-D835/I836 mutations are one of the second genetic events in infant ALL with *MLL* rearrangements or pediatric ALL with hyperdiploidy. (Blood. 2004;103:1085-1088)

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Introduction

Tyrosine kinases (TKs) function as the control of cellular signal transmission. Some TKs are closely associated with normal hematopoietic regulation and cell function.[1] Of TKs involved in hematopoiesis, some genes related to TKs fuse to functionally important genes, such as *ABL* or *ALK*-related fusion genes, and others have mutations, such as the *FMS* or *c-KIT* genes.[1] Constitutive phosphorylation of TK induced by these gene aberrations leads to a strong proliferative activity in hematopoietic cells, and often it is involved in the development of leukemia/lymphoma.[1,2] In these TKs, the *FLT3* gene is a receptor TK that has a transmembrane domain and plays an essential role in hematopoiesis.[2] Internal tandem duplication (ITD) of the juxtamembrane (JM) domain of the *FLT3* gene was identified in acute myeloid leukemia (AML).[3] The frequency of *FLT3*-ITDs is 17% to 27% of de novo adult AML, 5% to 17% of childhood AML, 3% to 5% of myelodysplastic syndrome (MDS), and 3% of acute lymphoblastic leukemia (ALL).[3-9] *FLT3*-ITD leads to constitutive activation and is recognized as a significant prognostic factor in adult[3-6] and pediatric[7-9] AML. FLT3-ITD activates the signal transducer and activator of transcription 5 and MAP kinase pathway.[10] In a murine bone marrow transplant model, the *FLT3*-ITD mutant induces a myeloproliferative disorder.[11]

Recently, point mutations of D835/I836 in the activation loop of the second TK domain of *FLT3* were found in adult AML, MDS, and ALL.[5,12,13] The incidence of *FLT3*-D835/I836 mutations was 7% of de novo adult AML, 3% of adult MDS, and 2.8% of ALL.[5,12,13] The D835/I836-mutant FLT3 induces the constitutive tyrosine-phosphorylation and interleukin-3 (IL-3)–independent proliferation of 32Dcl3 cells.[12] AML patients with *FLT3*-D835/I836 mutations tend to have poor prognoses,[5,12,13] suggesting that the *FLT3*-activating mutations of ITDs and D835/I836 mutations play important roles in AML. However, these *FLT3*-ITDs have rarely been found in pediatric ALL patients carrying myeloid markers and have not been associated with a poor prognosis.[8] In pediatric ALL, *FLT3*-D835/I836 mutations have not been reported previously. To clarify the relationship between the clinical features of ALL and *FLT3*-D835/I836 mutations, we analyzed *FLT3*-D835/I836 mutations in pediatric ALL, including infant ALL, and found the mutations to be relatively frequent in infant ALL with *MLL* rearrangements and pediatric ALL with hyperdiploidy.

Study design

Patient samples

We analyzed 162 patients with pediatric ALL—50 infants younger than 11 months and 112 children older than 1 year (93 B-cell precursors, 19 T-cell phenotypes)—in addition to 81 healthy donors. ALL was diagnosed in these patients according to the French-American-British (FAB) classification. Chromosomal analysis was performed using G-banding, as previously reported,[14] and karyotyping was successful in 136 (84.0%) patients. *MLL* rearrangements were examined in 50 infants with ALL with the restriction enzymes *Eco*RI and *Hind*III using Southern blotting, as previously reported.[14] DNA indexing was performed in 112 ALL patients 1 year and older.[15] A DNA index greater than 1.14 indicated hyperdiploidy. Pediatric ALL patients (age range, 1-15 years; median, 6 years) were mainly treated according to the Tokyo Children's Cancer Study Group (TCCSG) L95-14 protocol,[15] and infant ALL patients (age range, 0-11 months; median, 5 months) were mainly treated with the Japan Infant Leukemia Study MLL96 protocol.[16] Informed consent was obtained from the patients and/or the patients' parents and the healthy donors.

Leukemia cell lines

FLT3-D835/I836 mutations and ITDs were examined in 44 leukemia cell lines as follows[8,17]: 20 B-precursor ALL cell lines (LC4-1, NALM-26, NALM-17, UTP-L5, REH, UTP-L10, UTP-2, NALM-20, NALM-24, BV173, OM9;22, SCMC-L10, KOCL-33, KOCL-44, KOCL-45, KOCL-58, KOCL-69, HAL-01, KOPN-41, KOPN-1), 3 B-ALL cell lines (BALM-6, DAUDI, BAL-KH), 3 T-ALL cell lines (ALL-SIL, CCRF-HSB-2, KCMC-T), 6 AML cell lines (YNH-1, KASUMI-3, KG-1, SN-1, NB4, HEL), 7 acute monocytic leukemia (AMOL) cell lines (MV4;11, THP-1, CTS, P31/FUJ, MOLM-13, KOCL-48, IMS/M1), 2 acute megakaryoblastic leukemia (AMKL) cell lines (CMS, CMY), and 3 chronic myelogenous leukemia (CML) cell lines (MOLM-1, MOLM-7, TS9;22).

RFLP-mediated PCR for the detection of *FLT3*-D835/I836 mutations and PCR for the detection of *FLT3*-ITDs

High-molecular-weight DNA or total RNA was extracted from bone marrow or peripheral blood samples from the patients using standard methods.[8,17] Total RNA (4 μg) was reverse transcribed to cDNA with a cDNA Synthesis Kit (Amersham Pharmacia Biotech, Buckinghamshire, England). *FLT3*-D835/I836 mutations were examined by restriction fragment length polymorphism (RFLP)–polymerase chain reaction (PCR) or reverse transcription-PCR (RT-PCR). The sense primer used for PCR and RT-PCR was 17F,[12] and the antisense primer for PCR or RT-PCR was FLT3-TK-R1 5'-AGTAAGCAGACTGCTGTGAG-3') or FLT3-TK-R2 5'-GTAGAAGTTAGCATCAACCGG-3'), respectively. PCR procedure has been reported previously.[8,17] Five microliters of the PCR product were digested with 5 U *Eco*RV for 1 hour at 37°C, then electrophoresed on 3% agarose gel. Undigested PCR products were directly sequenced by the fluorometric method. The *FLT3*-ITD was analyzed by PCR or RT-PCR, as previously reported.[8]

Antibodies and Western blot analysis

Monoclonal antibodies against phosphotyrosine (clone 4G10), signal transducer and activator of transcription 5 (STAT5) (clone 89), and α-tubulin (clone TU-01) were obtained from Upstate Biotechnology (Lake Placid, NY), Transduction Laboratories (Lexington, KY), and Sanbio (Uden, Netherlands), respectively. Rabbit polyclonal antibodies against FLT3 (C-20) and phospho-STAT5, which reacts to the Tyr694-phosphorylated active form of STAT5a and STAT5b, were from Santa Cruz Biotechnology (Santa Cruz, CA) and Cell Signaling Technology (Beverly, MA), respectively. Western blot analysis was performed as reported previously.[18] In brief, lysates of leukemia cells were separated, transferred, and incubated with the primary antibody, followed by incubation with horseradish peroxidase–conjugated second antibody. In the experiment for FLT3 phosphorylation, FLT3 was immunoprecipitated by anti-FLT3 antibody and protein A beads and then immunoblotted by antiphosphotyrosine antibody. The detection of bands was performed using an enhanced chemiluminescence kit (Amersham Japan, Tokyo).

Results and discussion

We detected *FLT3*-D835/I836 mutations in 6 (5.4%) of 112 children older than 1 with ALL and in 8 (16.0%) of 50 infants with ALL (Table 1). Missense mutations were found in 11 patients— Asp835Tyr in 3, Asp835His in 2, Asp835Glu in 2, and Ile836Met in 4. Deletions of 3 base pairs (Ile836del) were found in 2 patients.

Table 1. Clinical features of infant and pediatric ALL patients with *FLT3*-D835/I836 mutations

Patient	Age	Sex	WBCs × 10⁴/μL	DNA Index	Relapse	Prognosis	FLT3-D835/I836 mutations
Infants							
1*	4 mo	M	67.6	ND	+	Dead	I836M
2*	3 mo	M	14.6	ND	−	Alive	D835E
3†	3 mo	F	2.5	ND	+	Dead	D836Y
4†	6 mo	F	12.3	ND	+	Dead	D835Y
5†	6 mo	M	99.3	ND	+	Dead	D835H
6†	4 mo	M	53.7	ND	−	Dead	I836del
7*	2 mo	F	95.3	ND	+	Dead	D835Y
8*	2 mo	F	50.0	ND	−	Alive	I836del
Children							
9	3 y	M	7.4	1.19‡	−	Alive	I836M
10	2 y	F	18.5	1.15‡	−	Alive	I836M
11	4 y	F	6.9	1.2‡	−	Alive	D835H
12	2 y	F	5.6	1.19§	−	Alive	I836M
13	3 y	M	5.8	0.99‡	−	Alive	D835E
14	7 y	M	102.7	1.0§	−	Alive	9-bp del + 6-bp ins‖

MLL rearrangements were found in all infants. WBCs refers to white blood cells.

*t(4;11).

†t(11;19).

‡Normal karyotype.

§Not available.

‖Deletion (D835-S838) + insertion (A835, L836, G837).

One patient had a deletion and an insertion mutation consisting of a 4-amino acid (Asp835-Ser838) deletion in 1 allele and a 3-amino acid (Ala835, Leu836, and Gly837) insertion in another allele. All the mutations were heterozygous, and the open reading frame was conserved. The Asp835 mutation probably was an activating mutation because the Asp835Tyr, His, and Glu found in this study were constitutively tyrosine phosphorylated.[12] Ile836del has also been shown to have strong autophosphorylation,[19] suggesting an association with the proliferation of leukemic cells because these mutations were detected in adult AML and tended to reduce disease-free survival.[5,12,13]

No FLT3-ITDs were found in this study. FLT3-ITDs are rarely found in pediatric and adult ALL,[8,20] although they are frequently found in AML.[3-8] FLT3-ITDs may not be involved in growth advantage in ALL because ALL carrying FLT3-ITDs were not associated with poor prognoses.[8] Neither ITDs nor D835/I836 mutations were detected in the healthy donors in this study, as previously reported.[12,13]

FLT3-D835/I836 mutations were frequently found in infants with ALL with MLL rearrangements and children with ALL with hyperdiploidy

Of 50 infants with ALL, 44 patients had CD10⁻ early pre-B cell ALL with MLL rearrangements, 5 had CD10⁺ B-cell precursor ALL, and 1 had T-cell ALL. Interestingly, FLT3-D835/I836 mutations were found in 8 (18.2%) of 44 infants with ALL with MLL rearrangements—t(4;11)(q21;q23)/MLL-AF4 in 4 patients and t(11;19)(q23;p13)/MLL-ENL in 4 patients—but not in the remaining 6 patients without MLL rearrangements. However, this difference was statistically not significant. Six of 8 patients with the mutations died, and the overall survival rate in the patients with the mutations was lower than that in the patients with wild-type FLT3, though the difference was not significant ($P = .42$).

B-cell precursor ALL (CD10⁺, CD19⁺, CD3⁻, CD13⁻, CD33⁻) was diagnosed in 6 children with the FLT3-D835/I836 mutations. Interestingly, FLT3-D835/I836 mutations were found in 4 (21.5%) of 19 children with ALL with hyperdiploidy (all were alive). Frequencies of FLT3-D835/I836 mutations were significantly higher in pediatric ALL with hyperdiploidy than in the other patients ($P = .0074$). No mutations were found in patients with TEL-AML1 (n = 11), E2A-PBX1 (n = 4), or BCR-ABL (n = 6) fusion genes. FLT3-D835/I836 mutations were not associated with sex, age, white blood cell (WBC) or platelet counts, hepatospleno-megaly, or involvement of the central nervous system at onset in either pediatric or infant ALL (data not shown).

Of 44 leukemia cell lines with the FLT3 expression, FLT3-D835 mutations were found in 2 (4.5%) leukemia cell lines, including 1 ALL (KOCL-33 carrying t(11;19)) and 1 AMOL (KOCL-48 carrying t(4;11)) cell line, which were derived from infant leukemia. FLT3-ITDs were found in 2 (4.5%) AMOL cell lines—MV4;11 carrying t(4;11) and MOLM-13 carrying t(9;11)—which were derived from pediatric or adult AMOL patients. Interestingly, 4 (36.3%) of 11 leukemia cell lines with MLL rearrangements had FLT3-activating mutations. On the other hand, no FLT3-activating mutations were found in any B-ALL, T-ALL, AML, AMKL, or CML cell lines.

### FLT3 and STAT5 were highly phosphorylated in lymphoid and myeloid leukemic cell lines with FLT3-D835/I836 mutation

It is known that FLT3 ligand (FL) stimulation of FLT3 results in the activation of STAT5a in murine myeloid Ba/F3 cells through a Janus kinase (JAK)–independent mechanism.[21] It is also known that ITDs and D835/I836 mutations in AML induce the constitutive phosphorylation of FLT3 and the activation of its downstream targets.[2] However, it remains unknown whether the phosphoryla-tion status of FLT3 and its downstream signal transduction pathways are different between myeloid and lymphoid leukemia cells with FLT3-D835/I836 mutations. To address this point, we examined the phosphorylation of FLT3 and STAT5 on Western blot in 5 leukemia cell lines, including 2 cell lines with D835 mutations. As shown in Figure 1, lymphoid (KOCL-33, lane 1) and monocy-toid (KOCL-48, lane 2) cell lines with D835 mutations showed marked tyrosine phosphorylation of FLT3 and STAT5. Of note, the expression of FLT3 and STAT5 in KOCL-48 was lowest among the cell lines examined, but their phosphorylation status was markedly elevated. Thus, FLT3 in lymphoid and myeloid MLL rearrangement-positive leukemias with FLT3-D835/I836 mutations might be constitutively and highly phosphorylated, leading to the constitu-tive activation of downstream targets such as STAT5 without FLT3 ligand stimulation.

FLT3-D835/I836 mutations are the second genetic events in infant ALL with MLL rearrangements and ALL with hyperdiploidy

Frequent additional alterations of proliferation-related genes or tumor-suppressor genes as the second genetic events in ALL with hyperdiploidy[22,23] or MLL rearrangements have not yet been reported.[18,24] Thus, the FLT3-D835/I836 mutations found in this study are considered to be the second genetic events in ALL. As for prognoses, our results indicated that FLT3-D835/I836 mutations affected the poorer prognoses of infants with ALL and MLL rearrangements. In contrast, patients with hyperdiploid ALL and the mutations had good clinical outcomes, suggesting that the mutations may not affect the growth advantage of hyperdiploid ALL cells. Further larger prospective studies are needed. Recently, gene expression profiling by microarray showed that FLT3 expres-sion was higher in acute leukemia with MLL rearrangements[25] and in ALL with hyperdiplody.[26] These studies suggest that FLT3 high-expression or constitutive tyrosine phosphorylation possibly caused by D835/I836 mutations in ALL with MLL rearrangements or hyperdiploidy might contribute to the patho-genesis of ALL with MLL rearrangements or hyperdiploidy and that a molecularly targeted drug against activating FLT3 should be considered in the future.
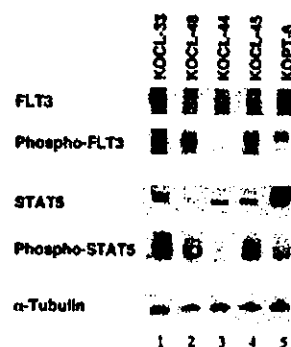
Figure 1. Western blot analysis on tyrosine phosphorylation of FLT3 and STAT5 in leukemia cell lines with or without D835 mutations. Lysates of leukemia cell lines with (lanes 1-2) or without (lanes 3-5) FLT3-D835 mutations were separated, blotted, and stained with antibodies against FLT3, phosphotyrosine (after immunopre-cipitation by anti-FLT3), STAT5, phospho-STAT5, and α-tubulin, respectively. Lanes 1, 3, and 4 were B-precursor cell lines with MLL rearrangement; lane 2 was a monocytoid cell line with MLL rearrangement; lane 5 was a T-lymphoid cell line.

Acknowledgments

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ORIGINAL ARTICLE

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L-Asparagine depletion levels and L-asparaginase activity in plasma of children with acute lymphoblastic leukemia under asparaginase treatment

Abstract *Purpose*: To determine the minimum levels of L-asparaginase (ASNase) activity necessary to maintain L-asparagine (Asn) depletion under ASNase treatment in acute lymphoblastic leukemia (ALL). *Methods*: We measured ASNase activity using an enzyme coupling method with a limit of detection of 2 U/l and examined the relationship between ASNase activity and Asn levels in blood samples from 14 children with ALL. *Results*: In all but one patient showing high ASNase antibody titers, minimum ASNase activity to maintain Asn depletion levels below the limit of detection (40 ng/ml) ranged from 6 to 180 U/l with a median value of 16 U/l. In 11 patients, the enzyme activity corresponding to minimum detectable Asn levels ranged from 2 to 32 U/l with a median value of 6.5 U/l. Patients with an ASNase activity of 2 U/l or an undetectable activity (< 2 U/l) had nearly normal Asn levels: 4140 ± 1161 ng/ml at 2 U/l and 7235 ± 3107 ng/ml at < 2 U/l (mean ± SD). Statistical analysis showed that ASNase activity in the range of 2–32 U/l was inversely correlated with Asn levels ($r = -0.803$, $P = 0.001$). *Conclusion*: These results show that Asn levels are strongly correlated with plasma ASNase activity even at low enzyme activities (< 50 U/l) and that this sensitive ASNase assay can be used to estimate plasma Asn depletion levels.

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Introduction

The antileukemic effect of L-asparaginase (ASNase), an important component of therapy for acute lymphoblastic leukemia (ALL), is believed to result from the inhibition of protein synthesis in leukemic cells that do not express a sufficient level of asparagine synthetase to synthesize asparagine (Asn) [7, 11, 13]. Since it is assumed that the pharmacologic effect of ASNase depends on the depletion of Asn from the circulating pool of amino acids, determination of the degree and duration of Asn depletion from blood is necessary to monitor the efficacy of the enzyme [12]. However, routine monitoring of Asn levels is still a laborious task in clinical practice because the accurate measurement of plasma Asn levels under ASNase treatment requires the rapid inhibition of persistent ASNase in the blood samples [3], for which a deproteinization procedure using sulfosalicylic acid (SSA) is currently employed [8]. It is desirable to estimate Asn depletion levels by measurement of the ASNase activity, but available pharmacokinetic data on ASNase treatment have not defined the minimum levels of ASNase activity required to hydrolyze Asn in vivo and ex vivo [1, 2, 5, 6, 15, 16, 20].

In this study, we measured ASNase activity using an enzyme coupling method with a lower limit of detection of 2 U/l in 14 children with ALL. Asn levels were also measured in two blood samples with or without deproteinization by SSA. The results indicate that Asn levels are strongly correlated with plasma ASNase activity even at low enzyme activities (< 50 U/l).

Materials and methods

Patients

Entered into this study were 12 children with newly diagnosed ALL and 2 with relapsed ALL. Newly diagnosed patients were treated with the Japanese Children's Cancer and Leukemia Study Group (JCCLSG) ALL-2000 protocols. The Regional Ethics Committee approved the study protocol. Verbal and written information about the study was given to the parents and written informed consent was obtained. If appropriate, informed consent was also obtained from the child. The patients were stratified to standard-risk or high-risk groups based on age and leukocyte counts at diagnosis [19]. The four-drug regimen (vincristine + prednisolone + ASNase + Adriamycin) was employed as induction therapy for the newly diagnosed patients. In this regimen, nine doses of ASNase (Kyowa Hakko, Japan) at 2000 U/m^2 were given intramuscularly three times a week starting on day 9. After remission had been achieved, the patients received intensified ASNase treatment: standard-risk patients received two weekly doses of ASNase at 2000 U/m^2 every 6 weeks for 6–18 weeks and high-risk patients received one dose of ASNase at 6000 U/m^2 weekly for 6–11 weeks. One of the patients with recurrent ALL was treated with the high-risk ALL-2000 protocol and the other patient was treated according to the ALL-REZ BFM protocol [10].

Sample collection

Blood samples were obtained when the last dose of intensified ASNase treatment was administered in the JCCLSG protocol or when the last dose of ASNase was administered in course R1 of the BFM protocol. Blood samples for ASNase activity and Asn level measurements were collected on day 0 (just before administration of the last dose) and every 2 to 3 days for 2 weeks as part of routine laboratory testing. ASNase antibodies were also measured in samples on day 0. Samples were placed in heparinized tubes and centrifuged at –4°C. The plasma was then divided into three parts: one was deproteinized by adding an equal volume of 10% (w/v) SSA, the second was immediately frozen for Asn determination, and the third was frozen for measurements of ASNase activity and antibodies.
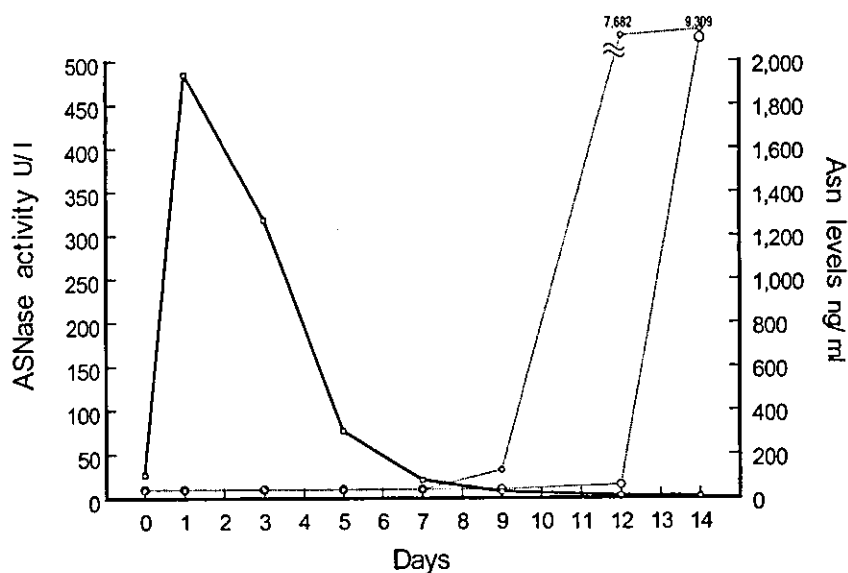
Measurement of ASNase levels

A series of enzyme reactions are triggered when ASNase catalyzes the substrate L-asparagine to produce L-aspartate in the presence of 2-oxoglutarate, NADH, and the conjugating enzymes L-glutamic oxaloacetic transaminase (GOT) and L-malate dehydrogenase (MDH). Through these reactions, NADH is oxidized and the absorbance of the reaction solution is decreased. We measured the ASNase levels using this series of reactions [4]. To the patient's plasma in a 96-well plate was added a mixed reagent solution of 2-oxoglutarate, NADH, GOT and MDH. The plate was allowed to stand at 37°C and, after the addition of L-asparagine solution to each well, placed in a plate reader and reacted at 37°C for 5 or 45 min to measure the decrease in absorbance at 340 nm. The same procedure was applied to a standard solution of known ASNase level (phosphate buffer solution containing BSA) to produce a calibration curve. To correct for the effect of L-aspartate contained in the patient's plasma, the same procedure was carried out simultaneously by adding phosphate buffer solution instead of L-asparagine solution continuously to obtain a blank correction. The determination limit of this method was 2 U/l.

Measurement of the L-asparagine level

The patient's plasma was mixed with an equal volume of SSA under ice cooling. The mixture was centrifuged and the supernatant was used as the sample solution. A given volume of the sample solution was automatically injected into an amino acid analysis system. The amino acid analysis was performed by RP-HPLC using precolumn derivation with o-phthalaldehyde and subsequent fluorescence detection according to the method of Yasui [21]. The lower determination limit of this method was 40 ng/ml.

Measurement of anti-ASNase IgG and IgE antibody titers

Anti-ASNase IgG antibody and IgE antibody titers in patients' samples were measured by the ELISA methods described by Tsukimoto et al. [18] and by Takatsuka et al. [17], respectively.

Fig. 1 Plasma ASNase activity and Asn levels after the last injection of L-asparaginase at 6000 U/m^2 in patient 11. Blood samples on day 0 were obtained just before the administration of ASNase (□ trough ASNase activity, ◇ Asn levels in plasma with SSA, ○ Asn levels in plasma without SSA)

Statistics

The correlation between Asn levels after deproteinization with SSA and plasma ASNase activities was assessed by Spearman's rank correlation test. SPSS statistical analysis software (SPSS 9.0 J) was used for all computations.

Results and discussion

The mean ($\pm$SD) baseline level of Asn in plasma obtained from 11 children with ALL before ASNase treatment was 7045 ± 1785 ng/ml. In the 14 patients

Table 1 Plasma ASNase activities and Asn levels in children with ALL treated with 2000 U/m^2 ASNase. Some Asn levels and ASNase activities after day 1 are not shown in the table because they were less important (ND not detected)

| Patient number	Day	Asn (ng/ml)		ASNase (U/l) enzyme coupling	Antibodies (U/ml)	
		Plasma	Plasma + SSA		IgG	IgE
1	0	ND	ND	82	ND	ND
	1	ND	ND	178		
	3	ND	ND	58		
	6	ND	210	9		
	8	4,830	5,600	2		
	10	5,580	5,660	<2		
2	0	ND	ND	11	ND	ND
	1	ND	ND	123		
	7	ND	ND	14		
	10	ND	1,360	4		
	14	6,430	6,510	3		
3	0	ND	ND	19	3	8
	1	ND	ND	218		
	7	ND	ND	17		
	10	ND	860	6		
	13	80	1,650	3		
	14	2,100	3,610	2		
	16	3,670	3,930	2		
4	0	6,013	6,122	<2	6	324
	1	ND	ND	12		
	3	216	2,622	2		
	5	5,851	5,871	<2		
5	0	14,930	15,750	<2	3	283
	1	ND	ND	344		
	3	ND	ND	169		
	5	ND	90	32		
	7	8,270	8,120	<2		
6	0	ND	ND	107	ND	ND
	1	ND	ND	180		
	5	ND	42	19		
	7	4,000	4,940	2		
	9	7,310	6,520	<2		
7	0	ND	ND	222	ND	ND
	3	ND	ND	163		
	7	ND	ND	15		
	10	ND	98	3		
	12	4,720	4,980	<2		
8	0	ND	ND	60	ND	ND
	1	ND	ND	246		
	5	ND	ND	17		
	7	ND	2,070	4		
	9	7,000	7,720	<2		
9	0	ND	ND	243	ND	ND
	1	ND	ND	378		
	12	ND	ND	6		

Table 2 Plasma ASNase activities and Asn levels in children treated with 6000 U/m^2 (patients 10, 11, 12 and 14) or 10,000 U/m^2 (patient 13) ASNase. Patients 13 and 14 relapsed. Some data for Asn levels and ASNase activities after day 1 are not shown in the table because they were less important (ND not detected)

Patient number	Day	Asn (ng/ml)		ASNase (U/l) enzyme coupling	Antibodies (U/ml)	
		Plasma	Plasma + SSA		IgG	IgE
10	0	ND	ND	36	2	23
	1	ND	ND	482		
	7	ND	ND	37		
	10	ND	60	10		
	12	170	1220	7		
	14	1600	2120	4		
11	0	ND	ND	27	4	61
	1	ND	ND	485		
	7	ND	ND	21		
	9	ND	129	7		
	12	61	7682	3		
	14	2107	9309	<2		
12	0	ND	ND	140	ND	ND
	1	ND	ND	933		
	2	ND	ND	964		
	11	100	ND	14		
	13	ND	1050	6		
13	0	ND	ND	6500	ND	ND
	1	ND	ND	3430		
	10	ND	ND	36		
	13	ND	ND	12		
	14	ND	ND	9		
14	0	5850	6310	<2	1360	11,200
	1	6010	6080	<2		
	16	4900	4870	<2		

studied here, Asn levels were strongly correlated with plasma ASNase activity. Figure 1 shows a representative patient (patient 11, Table 2), in whom Asn levels in the deproteinized samples were below the level of detection (<40 ng/ml) until day 7 (ASNase activity 21 U/l), rose to 129 ng/ml on day 9 (ASNase activity 7 U/l), increased dramatically to 7682 ng/ml on day 12 (ASNase activity 3 U/l) and then increased further to 9309 ng/ml on day 14 (ASNase activity <2 U/l). In this patient, Asn levels were significantly lower in untreated plasma samples than in deproteinized samples from day 9 to day 14. These results show that very small amounts of residual ASNase (2–7 U/l) in the plasma sample can hydrolyze Asn before measurement [3].

Tables 1 and 2 show plasma Asn levels, ASNase activity and antibody levels in 14 patients. In all patients except one (patient 14 with a high antibody titer), minimum ASNase activity to maintain Asn depletion levels below the limit of detection ranged from 6 to 180 U/l with a median value of 16 U/l. This finding is in accordance with data reported by other investigators [1, 2, 5, 6, 16] and strongly suggests that the recommended plasma level of 100 U/l to secure Asn depletion is not required in all patients. In 11 of these 13 patients, the enzyme activity corresponding to minimum detectable Asn levels ranged from 2 to 32 U/l with a median of 6.5 U/l. In the other two patients (patients 9 and 13), Asn depletion (<40 ng/ml) persisted for the observation

Fig. 2 Plasma ASNase activity
and Asn levels after the last
injection of ASNase in ten
children with ALL. Each
patient shows a inverse
correlation between trough
ASNase activity (*solid lines*) and
Asn levels in plasma with SSA
(*dashed lines*) (○ patient 1, □
patient 2, △ patient 3, ◇ patient
4, ▽ patient 5, ● patient 6, ■
patient 7, ▲ patient 9, ◆ patient
10, ▼ patient 11)
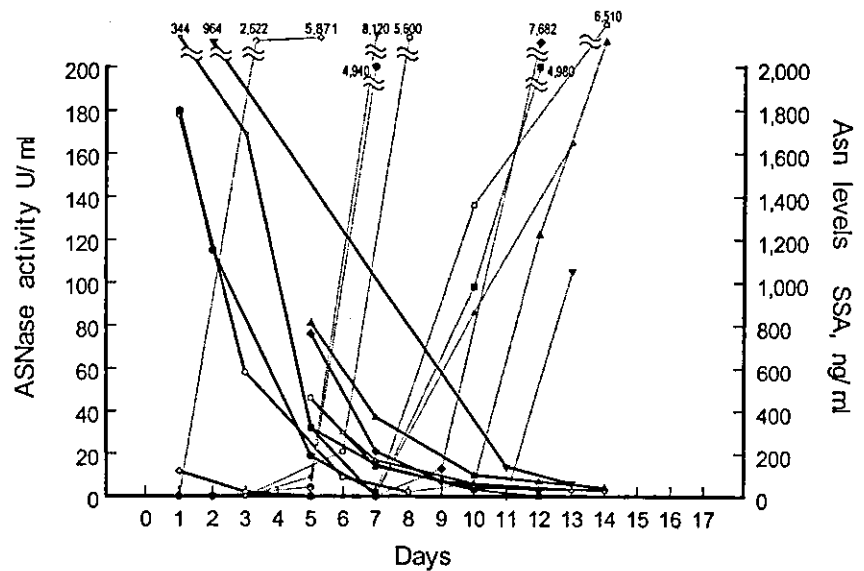
period (12 or 14 days). Patients with an ASNase activity of 2 U/l or an undetectable activity (< 2 U/l) had nearly normal Asn levels: 4140 ± 1161 ng/ml at 2 U/l and 7235 ± 3107 ng/ml at < 2 U/l (mean $\pm$ SD). The Asn levels (range 42–6510 ng/ml) after deproteinization with SSA and plasma ASNase activities (range 2–32 U/l) in 14 samples obtained from 11 patients were significantly inversely correlated by Spearman's rank correlation test ($r = -0.803$, $P = 0.001$). This inverse correlation between Asn levels and plasma ASNase activities for individual patients is shown in Fig. 2. Thus, our assay system showed that Asn levels are strongly correlated with plasma ASNase activity in the very low range of 2–32 U/l and that the detection limit is sensitive enough to estimate the asparagine depletion levels under ASNase treatment in plasma of children with ALL.

In one patient who had a relapse (patient 14) and high ASNase antibody titers, ASN activity was undetectable and Asn levels remained almost at baseline for 16 days after administration. Two other patients with high antibody levels (patients 4 and 5) also showed a rapid decline in ASNase activity and a very short duration of Asn depletion. These results suggest that "silent inactivation" by neutralizing antibodies reduces the therapeutic effect of ASNase [4, 9, 14] and that determination of antibody levels coupled with a sensitive ASNase assay is more important in monitoring the efficacy of ASNase treatment.

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Two Distinct Gene Expression Signatures in Pediatric Acute Lymphoblastic Leukemia with *MLL* Rearrangements[1]

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ABSTRACT

Acute lymphoblastic leukemia (ALL) with 11q23 translocations is usually associated with *MLL* gene rearrangement, but little is known about its leukemogenesis. We analyzed the gene expression profiles of pediatric ALL samples according to their translocations. Using oligonucleotide microarray analysis, we identified distinct expression profiles for 23 ALL samples with 11q23 translocations, including t(4;11) (*n* = 15), t(11;19) (*n* = 6), and t(5;11) (*n* = 2), compared with 9 ALL samples with other translocations, including t(12;21) (*n* = 6) and t(1;19) (*n* = 3). Gene expression scores of *FLT3*, *MeisI*, and *CD44* for samples with *MLL* rearrangements were particularly high compared with those for other ALL samples. Statistical analysis of the gene expression profiles for the 21 ALL samples with *MLL* rearrangements at diagnosis revealed two subgroups that exclusively correlated with prognosis but not with any other clinico-pathological factor. The transcription factors *CBF2* and *CDP* were highly expressed in the poor and good prognosis subgroups, respectively. In addition, their downstream target genes were differentially expressed. These findings provide new insights into the biological mechanisms of leukemogenesis and prognosis for pediatric ALL with *MLL* rearrangements.

INTRODUCTION

The prognosis of children with ALL[3] has improved remarkably over the last 2 decades (1–3). This success has been achieved by using risk-directed therapy, which was developed after the realization that pediatric ALL is a heterogeneous disease (4). However, 20–25% of ALL patients still experience a relapse. Attempts to classify pediatric ALL into therapeutically relevant risk categories have relied mainly on clinical parameters, including age and WBC count at diagnosis, as well as early response to treatment (4). Recent advances in molecular biology have identified several genes involved in chromosomal translocations of ALL, such as the *E2A-PBX1* chimeric gene in t(1;19), *ETV6/TEL-AML1* in t(12;21), *BCR-ABL* in t(9;22), and *MLL-AF4* in t(4;11) (Refs. 1–5). Patients with t(12;21)-ALL have a good prognosis while those with t(9;22)- or t(4;11)-ALL have a poor prognosis. Infant ALL with *MLL* rearrangements (*MLL*-Re-ALL), including t(4;11) and

t(11;19), is strongly associated with poor prognosis (6). Thus, cytogenetic or direct molecular genetic methods have become an essential part of the routine diagnosis and follow-up of acute leukemia patients, as well as increasing our understanding of leukemogenesis.

The *MLL* gene (also known as *ALL-1* or *HRX*), located at 11q23, encodes a protein of 3969 amino acids containing zinc fingers and AT-hook motifs and has homology with *Drosophila* trithorax protein (7–9). The *MLL* gene fuses with >30 genes on various partner chromosomes (10–12) and is highly conserved across species. Through its regulation of the *HOX* genes, *MLL* is essential for normal mammalian development and hematopoiesis. Although the function of the various *MLL* fusion genes and proteins is poorly understood, it appears that their fusion proteins disrupt the ability of wild-type *MLL* to regulate *HOX* gene expression, leading to leukemogenesis (13).

Recently, a genomic approach to cancer classification, including leukemia classification (14–17), based on gene expression monitoring using DNA microarrays, has been reported, with a distinct gene expression in pediatric T-ALL shown to be associated with a poor/good prognosis (17). *MLL*-Re-ALL has been reported to have characteristic, distinct gene expression profiles that are consistent with an early hematopoietic progenitor cell expressing selected multilineage markers and individual *HOX* genes. Clustering algorithms reveal that, based on their gene expression patterns, acute leukemia with *MLL* rearrangements can clearly be separated from conventional ALL and AML (18), suggesting that they constitute a distinct disease. Among *MLL*-Re-ALLs, infant patients have a poor prognosis. However, children > 1 years old have a relatively good prognosis (4). We used an oligonucleotide microarray to analyze the expression of >12,600 genes in leukemic cells from 31 pediatric ALL patients, including 15 with t(4;11), 6 with t(11;19), and 2 with t(5;11). We found that *MLL*-Re-ALL could be identified from the distinct expression pattern of several genes, including *FLT3*, *CD44*, *HOXA9*, and *MEIS1*. Furthermore, using the gene expression profiles, each of the t(4;11), t(11;19), or t(5;11) found in *MLL*-Re-ALL could be classified into two distinct groups, with differential prognosis, irrespective of their translocation partner chromosomes.

MATERIALS AND METHODS

Leukemia Samples. Leukemia cells from the bone marrow or peripheral blood of ALL patients were obtained with informed consent at diagnosis or relapse. In each case, the percentage of blasts was >90%. CD19 was expressed in all samples, but CD2, CD5, and CD7 were not expressed in any samples. We analyzed 32 ALL samples with chromosomal translocations, comprising 3 samples with t(1;19), 6 with t(12;21), and 23 with *MLL* rearrangements, including 15 t(4;11), 6 t(11;19), and 2 t(5;11). Samples were obtained both at diagnosis and relapse from one patient with t(4:11) and only at relapse from one *MLL*-Re-ALL sample. Therefore, the remaining 21 samples were obtained only at diagnosis. All of the translocations were subjected to karyotype analysis, fluorescence *in situ* hybridization, and/or Southern blot analyses, and *MLL* partner genes were confirmed by RT-PCR as described elsewhere (11, 19–21). The t(1;19), t(12;21), t(4;11), t(11;19), and t(5;11) samples were found