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著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ
長船健二 山中伸弥	人工多能性幹(iPS)細胞の樹立とその臨床応用		最新内科学	西村書店	日本		印刷中
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Ⅳ. 研究成果の刊行物・別冊

PEDIATRICS INTERNATIONAL Official Journal of the Japan Pediatric Society

Successful treatment of pediatric immune thrombocytopenic purpura associated with ulcerative colitis

Michinori Funato, Toshiyuki Fukao, Hideo Sasai, Tomohiro Hori, Daisuke Terazawa, Kazuo Kubota, Michio Ozeki, Kenji Orii, Hideo Kaneko and Naomi Kondo

Department of Pediatrics, Graduate School of Medicine, Gifu University, Gifu, Japan

Key words hematochezia, immune thrombocytopenic purpura, iron deficiency anemia, medical therapy, ulcerative colitis.

A large number of extraintestinal manifestations of chronic inflammatory bowel disease (IBD) has been reported.[1] They are seen in approximately 25% of patients with chronic IBD, with a significantly higher proportion in patients with Crohn's disease.[1] Several hematological abnormalities, often autoimmune hemolytic anemia, have also been described in association with chronic IBD.[2] Immune thrombocytopenic purpura (ITP), also referred to as idiopathic thrombocytopenic purpura, has been sporadically reported in individuals with chronic IBD, mostly ulcerative colitis (UC). ITP is a destructive thrombocytopenia caused by autoantibodies against platelet glycoproteins (GP), and UC is a chronic IBD of unknown etiology.[3] It is not yet conclusive whether these diseases are involved in each other's pathogenesis, though the treatment of UC is known to be enough to treat ITP.[4]

Herein, we describe a 12-year-old girl with ITP associated with UC who also had anemia, hematochezia, and purpura. Her anemia was considered to be caused by intestinal hemorrhaging from the UC, and purpura was thought to be caused by ITP. She was treated with prednisolone (PSL) and 5-aminosalicylic acid (5-ASA), and the diseases now remain under control.

Case report

A 12-year-old girl was referred to us with the chief complaints of nausea and paleness for some months. Her family history was unremarkable. She had a history of mild atopic dermatitis from the age of 1, but took no medication. On admission, she showed pale and multiple purpuras in the lower extremities and had gross hematochezia, but no abdominal pain, fever nor diarrhea was observed; nor were hepatomegaly, splenomegaly or tenderness in the abdomen observed. The laboratory findings are shown in Table 1. Severe anemia, moderate thrombocytopenia, and iron deficiency were noted. Systemic computed tomography was unremarkable. Disseminated intravascular coagulation (DIC) was ruled out by the presence of normal prothrombin time and normal levels of fibrinogen and fibrin degeneration products. Platelet-associated IgG (PAIgG) rose to 39.4 ng/10^7 cells (normal value 9.0–25.0 ng/10^7 cells), and bone marrow examination revealed normocellular marrow with increased megakaryocyte numbers. We initially diagnosed her with ITP and iron deficiency anemia caused by gastrointestinal hemorrhaging, and so she was treated with sodium ferrous citrate. Her clinical course is summarized in Figure 1. Sigmoidoscopy was performed for differential diagnosis of hematochezia and revealed very friable, edematous mucosa with erosion and bleeding upon air insufflation, but no obvious findings of IBD were seen in the rectum. The stool culture of samples obtained in this examination was negative for pathogens, nor was cytomegalovirus DNA evident. In addition, to detect autoantibodies for ITP, serological analysis was performed by enzyme-linked immunosorbent assay. However, this also showed no evidence of autoantibodies against platelet GPIIb/IIIa, GPIb/ al, GPIb/IX, GPIV, or the anti-HLA Class I antibody (data not shown).

Five weeks later, the patient's hemoglobin recovered to 12.5 g/dL, but the platelet count had fallen to 7000 /mm^3. PSL (60 mg/day) treatment for ITP was then initiated on the basis of clinical evidence, and a rapid increase of the platelet count to 344 000/mm^3 was observed. However, her platelet count decreased again when PSL was tapered 2 weeks later, and again her platelet count increased with an escalating dose of PSL and decreased with a dose reduction of PSL. The gross hematochezia also continued.

Repeated endoscopic examination with biopsies was performed for refractory thrombocytopenia and gross hematochezia. Colonoscopy revealed endoscopic and microscopic evidence of scattered ulcerative colitis with cryptitis, crypt abscesses, and inflammatory polyps (Fig. 2). An upper gastrointestinal study with small intestine follow-through was normal. The symptoms, laboratory data, and colonoscopic findings indicated a diagnosis of UC using the Japanese criteria.[5] Finally, we diagnosed her as having ITP with UC, and 5-ASA (50 mg/kg/day) was initiated in addition to the PSL as further treatment for UC. Two months after starting 5-ASA, the hematochezia improved and her platelet count also became stable. The patient's UC and ITP now remain under control with tapered PSL.

Discussion

We have described a rare case of ITP associated with UC in a 12-year-old girl. The diagnosis of our patient was difficult due to her atypical condition with regard to anemia, hematochezia, and purpura. In addition, the hemorrhagic tendency made it more difficult to diagnose her without a detailed examination. Hence, we initially excluded secondary thrombocytopenia, including the adverse effects of drugs, viral infection, and DIC. We finally confirmed that she had ITP on the basis of her clinical course and

Correspondence: Michinori Funato, MD, PhD, Department of Pediatrics, Graduate School of Medicine, Gifu University, 1-1 Yanagido, Gifu 501-1194, Japan. Email: mfunato@mac.com

Received 10 March 2010; revised 19 September 2010, accepted: 2 November 2010.
doi: 10.1111/j.1442-200X.2010.03308.x

Table 1 Laboratory data on admission

Peripheral Blood		Blood chemistry		Immunological tests	
White blood cells	10300/mm^3	TP	7.7 g/dl	IgG	2041 mg/dL
Neutrophils	53.0%	Alb	4.0 g/dl	IgA	237 mg/dL
Lymphocytes	33.0%	T-Bil	0.6 mg/dl	IgM	88 mg/dL
Monocytes	6.0%	AST	22 IU/l	C3	117 mg/dL
Eosinophils	5.0%	ALT	8 IU/l	C4	12 mg/dL
Basophils	0.5%	LDH	260 IU/l	CH50	32.6 U/mL
Red blood cells	389 × 10^4/mm^3	ALP	243 IU/l	CRP	0.05 mg/dL
Hematocrit	19.6%	CPK	71 IU/l	Ferritin	1.6 ng/mL
Hemoglobin	5.1 g/dL	AMY	83 IU/l		
Platelets	3.8 × 10^4/mm^3	UA	2.9 mg/dl	Urinalysis	
Reticulocytes	1.62%	BUN	7.8 mg/dl	Blood	(−)
		Cr	0.32 mg/dl	Protein	(−)
Coagulation tests		Na	139 mEq/l	Glucose	(−)
PT%	90%	K	4.0 mEq/l		
APTT	23.9 s	Cl	106 mEq/l		
Fbg	263 mg/dL	Ca	8.8 mg/dl		
FDP	<2.5 μg/mL	Fe	11 μg/dl		
D-dimer	1.9 μg/mL	UIBC	358 μg/dl		

laboratory findings, and had UC according to the Japanese criteria for diagnosis.[5]

The association of ITP with UC is rare, but has been recognized recently. Edwards and Truelove originally described three adult patients with both ITP and UC among 624 adult patients with UC, although no details for these three patients were available.[6] Mizuta *et al.* also reviewed 17 Japanese patients with ITP and UC, and strongly suggested that the association of ITP with UC is not coincidental and that ITP is secondary to UC in adults.[7] In children, the incidence of ITP associated with UC is rare, as it is in adults. Previous case reports have rarely described pediatric cases of ITP with UC, and Higuchi *et al.* reviewed only 4 pediatric cases of ITP with UC.[8]

Currently, whether UC has a causal role in the pathogenesis of ITP remains unclear. When ITP is associated with UC it usually occurs during or after the onset of colitis. Rarely, it can precede colitis.[9] Zlatanic *et al.* have suggested that antigenic mimicry plays a role in this association of UC and ITP.[9] That is, platelet membrane antigens may have peptide sequences similar to those of some bacterial GP, and antibodies may be produced when the bacterial antigens come in contact with colonic mucosa, which may cross-react with platelet GP, namely immune-mediated thrombocytopenia.[9] We examined several autoantibodies for immune-mediated thrombocytopenia, but mildly elevated PAIgG that is not specific to ITP was the only thing identified. In general, the specific autoantibodies that are most commonly identified in patients with ITP are platelet GP IIb/IIIa and Ib/IX, but these autoantibodies are not detectable in up to 50% of patients with ITP.[3] In our patient, the specific autoantibodies that cause ITP were not detected, and so further investigation related to other

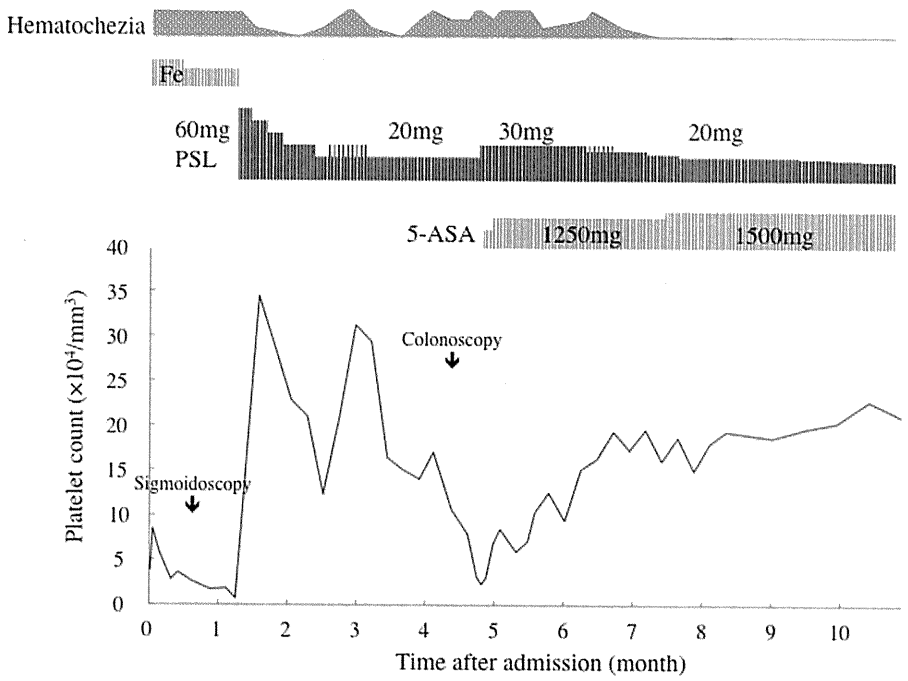

Fig. 1 Clinical course. Fe, sodium ferrous citrate; PSL, prednisolone; 5-ASA, 5-aminosalicylic acid.

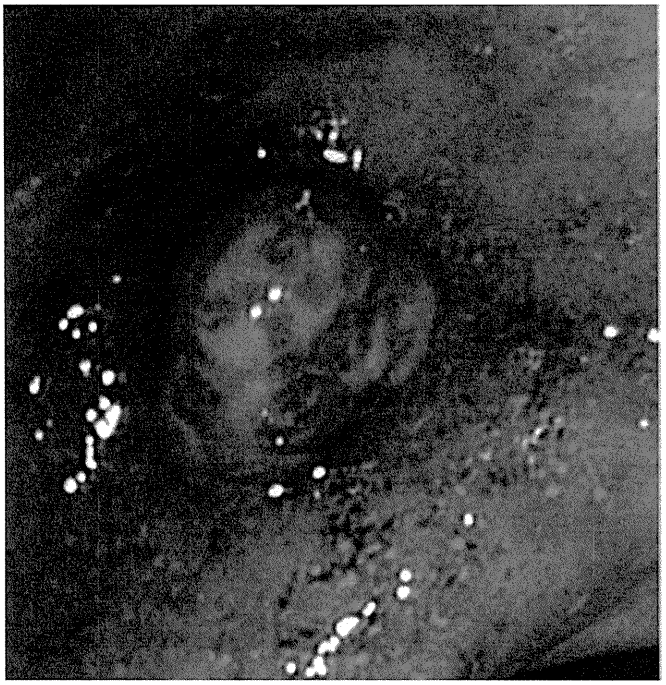

Fig. 2 Second endoscopic examination showed friable, edematous mucosa, erosions, and inflammatory polyps in the ascending colon.

specific autoantibodies against platelet GP, and which also cross-react with bacterial GP, is needed for rapid and accurate diagnosis of ITP with UC.

Various treatments have been used for the combination of UC and ITP. Short courses with steroids have frequently been able to induce remission of both diseases. However, to control ITP or to maintain platelet count, high doses of steroids and immunosuppressants including cyclosporine, intravenous immunoglobulin or splenectomy have often been required. A few reports have previously demonstrated that ITP with UC which were refractory to medical treatment and splenectomy responded to colectomy.[4,10] Recently, infliximab has also emerged as an effective treatment for immune-mediated extraintestinal manifestations often refractory to other interventions.[11] Our patient came under control with 5-ASA, which is known to suppress the production of numerous proinflammatory mediators from the colonic lumen, in addition to low-dose steroids. This may suggest that 5-ASA has a potential role in the treatment of ITP, if ITP is associated with UC.

In conclusion, we have reported a case in which consolidation of UC treatment likely results in the resolution of ITP. This case is thought to be important for considering a treatment for the cure of both diseases (UC and ITP) in children.

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Computed tomography findings of the liver in a neonate with Herpes simplex virus-associated hemophagocytic lymphohistiocytosis

Yoshiro Wada,[1,2] Masahiko Kai,[1] Hitomi Tanaka,[1] Naomasa Shimizu,[1] Masataka Shimatani[1] and Toshio Oshima[1]

[1]*Department of Pediatrics, Bell Land General Hospital, Higashiyama, Nakaku, Sakai City and* [2]*Department of Pediatrics, Rinku General Medical Center, Rinkuouraikita, Izumisano City, Osaka, Japan*

Key words computed tomography, hemophagocytic lymphohistiocytosis, Herpes simplex virus, hypoattenuation.

Correspondence: Yoshiro Wada, MD, Department of Pediatrics, Rinku General Medical Center, 2-23 Rinkuouraikita, Izumisano City, Osaka 598-8577, Japan. Email: y-wada@rgmc.izumisano.osaka.jp

Received 11 June 2009; revised 18 October 2010; accepted 12 January 2011.
doi: 10.1111/j.1442-200X.2011.03421.x

Neonatal hemophagocytic lymphohistiocytosis (HLH) is a rare and severe disease with high mortality and morbidity.[1] Neonatal HLH has been reported to be induced by Herpes simplex virus (HSV) infection.[2,3] When progressive liver dysfunction is induced by disseminated HSV infection, it is often difficult to detect HLH

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Pediatric acute lymphoblastic leukemia mimicking Henoch–Schönlein purpura

Michinori Funato, Hideo Kaneko, Kazuo Kubota, Michio Ozeki, Kaori Kanda, Kenji Orii, Zenichiro Kato, Toshiyuki Fukao and Naomi Kondo

Department of Pediatrics, Graduate School of Medicine, Gifu University, Gifu, Japan

Key words acute lymphoblastic leukemia, D-dimer, differential diagnosis, Factor XIII, Henoch–Schönlein purpura.

Henoch–Schönlein purpura (HSP) is a systemic vasculitis of unknown cause. The main clinical features include cutaneous purpura, arthritis, gastrointestinal symptoms, and nephritis, and the diagnosis is clinically established based on the presence of those clinical features. In adults, malignant diseases are sometimes associated with HSP, and a careful search for the underlying disease is needed.[1] However, pediatric cases of HSP associated with malignancy are extremely rare, even though more than 90% of patients with HSP are less than 10 years of age.[1,2] Herein, we describe a boy with acute lymphoblastic leukemia (ALL) that mimics the clinical course of HSP.

Case report

A 3-year-old boy was presented to us with bilateral knee pain and numerous purpuras. He had been suffering from acute pharyngitis prior to the onset of those symptoms. Physical examination on admission showed numerous flat or palpable purpuras that were typical for HSP, in his lower abdomen and bilateral lower extremities (Fig. 1a). No lymphadenopathy and hepatosplenomegaly were evident. His blood pressure was 96/54 mmHg. The laboratory findings (which are shown in Table 1) revealed the white blood cell count of 6160/mm^3 with 58.6% neutrophils and 37.7% lymphocytes, a hemoglobin concentration of 11.2 g/dl, and a platelet count of 355 000/mm^3. No immature cells were observed in the peripheral blood smear. Coagulation tests revealed an increase in the D-dimer level at 7.7 μg/ml and a decrease in coagulation factor XIII (FXIII) activity to 56% of the normal value. We thought he had HSP and he stayed in bed. The bilateral knee pain and numerous purpuras disappeared at 1 week after admission, but subsequently he had a recurrent fever of unknown origin, and increased D-dimer and decreased FXIII activity continued. His course of D-dimer and FXIII activity are summarized in Figure 2. One and a half months later he had pancytopenia, namely, blood examination of the white blood cell count was 2680/mm^3 with no blasts, a hemoglobin concentration of 10.4 g/dl, and a platelet count of 133 000/mm^3. The bone marrow was aspirated, showing 51.4% blast cells with mild cytological atypia and obvious nuclear body (Fig. 1b). Flow cytometric immunophenotyping in these cells was positive for CD10, CD19 and HLA-DR, and Southern blotting showed clonal immunoglobulin gene rearrangements (data not shown). In addition, cytogenetic analysis revealed the normal karyotype. These results confirmed his diagnosis of precursor B-ALL (FAB L1). Then, he had multiagent-chemotherapy, and achieved complete remission 1 month later. He is now undergoing treatment in the maintenance phase.

Discussion

The final step of the coagulation process is the formation of the crosslinked fibrin. This is activated by coagulation FXIII, and these products split by the major fibrinolytic protease plasmin are D-dimer.[3] Increased D-dimer and decreased FXIII activity are observed in children during the acute phase of HSP, and are useful

Correspondence: Michinori Funato, MD, PhD, Department of Pediatrics, Graduate School of Medicine, Gifu University, 1-1 Yanagido, Gifu 501-1194, Japan. Email: mfunato@mac.com
Received 1 August 2010; revised 14 December 2010; accepted 12 January 2011.
doi: 10.1111/j.1442-200X.2011.03445.x

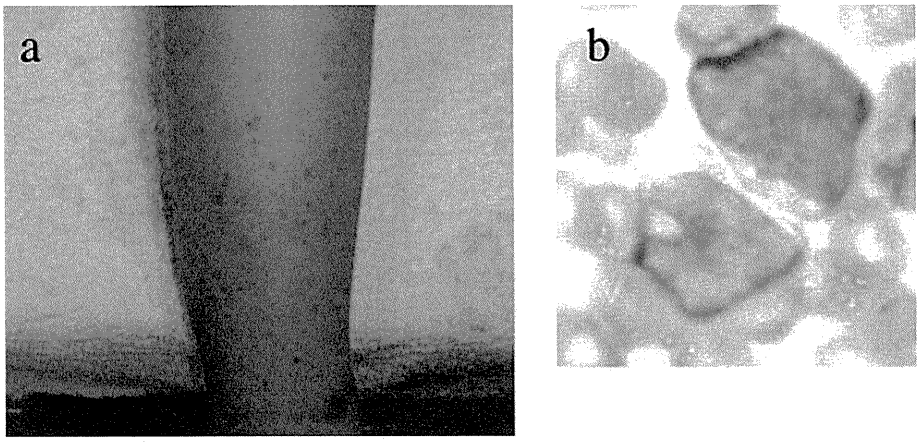

Fig. 1 (a) Numerous purpuras in the right lower extremity. (b) Bone marrow smear showing blast cells with mild cytological atypia (Wright–Giemsa, ×1000).

for early diagnosis of HSP.[4–7] These laboratory results are thought to be caused by vasculitis-induced endothelial damage, but the pathogenesis remains unclear.[4] On the other hand, a high proportion of patients with acute leukemia have coagulation abnormalities at the time of presentation, and a very similar pattern of coagulation tests in HSP is found in some cases with acute leukemia.[3] The coagulation cascade in acute leukemia is mainly known for being initiated by the transmembrane protein tissue factor. It is normally expressed on several cell types, and can be exposed to flowing blood upon endothelial damage, pathophysiological stimuli, and certain leukemic (or tumor) cell types.[3] In our patient, a few blast cells that were not grossly visible may have existed somewhere on admission and interacted with the coagulation processes both directly and indirectly. As a result, rather mild findings of ALL before the signs of overt ALL may have induced an HSP-like course with increased D-dimer and decreased FXIII activity. Tabata *et al.* reported an adult patient with T-cell lymphoma, who was initially diagnosed clinically as having HSP, and the histological finding of the skin with purpura revealed the infiltration of lymphocytes, which differed from the specific findings for HSP.[8] Unfortunately, skin biopsy could be not performed for our patient, but he also must have had different findings from the specific findings for HSP, as his clinical findings and laboratory data improved by multiagent-chemotherapy for ALL.

In conclusion, this case is thought to be important, considering the diagnosis and treatment for HSP, particularly in pediatric patients. However, the property of the blast cells that induces this phenotype needs to be investigated in further studies.

Acknowledgments

This study was supported in part by Health and Labor Sciences Research Grants of Research on Measures for Intractable Diseases from the Ministry of Health, Labor and Welfare.

Table 1 Laboratory data on admission

Peripheral blood		Blood chemistry		Immunological tests	
White blood cells	6160/mm^3	TP	7.0 g/dl	IgG	1305 mg/dl
Neutrophils	58.6%	Alb	4.2 g/dl	IgA	216 mg/dl
Lymphocytes	37.7%	T-Bil	0.5 mg/dl	IgM	81 mg/dl
Monocytes	3.2%	AST	35 IU/l	C3	159 mg/dl
Eosinophils	0.5%	ALT	9 IU/l	C4	36 mg/dl
Basophils	0.0%	LDH	386 IU/l	CRP	0.24 mg/dl
Red blood cells	420 × 10^4/mm^3	ALP	523 IU/l	Ferritin	112.9 ng/ml
Hematocrit	33.9%	CPK	49 IU/l	ASO	<40
Hemoglobin	11.2 g/dl	AMY	93 IU/l	ASK	<20
Platelets	335 × 10^4/mm^3	UA	2.5 mg/dl		
Reticulocytes	1.61%	BUN	14.8 mg/dl	Coagulation tests	
		Cr	0.23 mg/dl	PT%	96%
Urinalysis		Na	137 mEq/l	APTT	29.5 s
Blood	(−)	K	5.1 mEq/l	Fbg	408 mg/dl
Protein	(−)	Cl	104 mEq/l	D-dimer	7.7 μg/ml
Glucose	(−)	Ca	9.5 mg/dl	ATIII	111%
		Fe	78 μg/dl	XIIIfactor	56%
		UIBC	320 μg/dl		

Alb, albumin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AMY, amylase; APTT, activated partial thromboplastin time; ASK, anti-streptokinase antibody; ASO, anti-streptolysin O antibody; AST, aspartate aminotransferase; BUN, blood urea nitrogen; CPK, creatine phosphokinase; Cr, creatinine; CRP, C-reactive protein; Fbg, fibrinogen; Ig, immunoglobulin; LDH, lactate dehydrogenase; PT, prothrombin time; T-Bil, total bilirubin; TP, total protein; UA, uric acid; UIBC, unsaturated iron binding capacity.

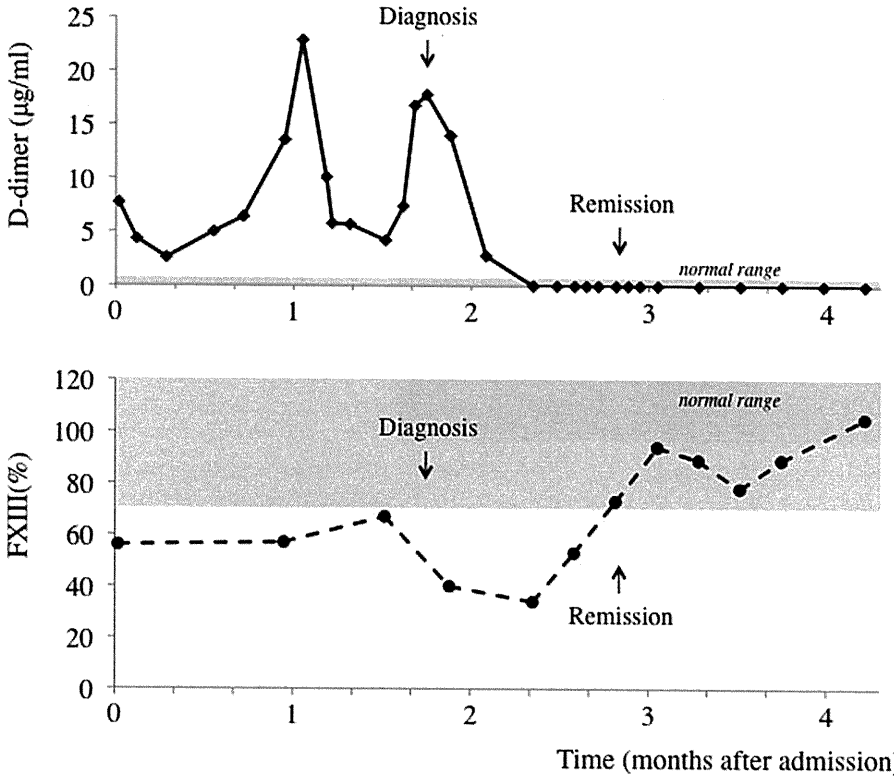

Fig. 2 Clinical course. Increased D-dimer and decrease coagulation factor XIII (FXIII) activity continued, but those data improved by multiagent-chemotherapy.

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Successful treatment of very large congenital infantile fibrosarcoma

Alias Hamidah,[1] MdZin Reena,[2] A.R Abdul. Halim,[3] Sharaf Ibrahim,[3] Mariko Eguchi,[4] A Latiff Zarina,[1] Kamal N. Norazlin,[1] Rahman Jamal[1] and Hirokazu Kanegane[5]

Departments of [1]Pediatrics, [2]Pathology, and [3]Orthopedic and Traumatology, Faculty of Medicine, National University of Malaysia, Kuala Lumpur, Malaysia, [4]Department of Pediatrics, Graduate School of Medicine, Ehime University, Toon and [5]Department of Pediatrics, Graduate School of Medicine, University of Toyama, Toyama, Japan

Key words chemotherapy, congenital infantile fibrosarcoma, tumor excision.

Correspondence: Alias Hamidah, MD, Department of Pediatrics, Faculty of Medicine, National University of Malaysia, 56000 Cheras, Kuala Lumpur, Malaysia. Email: midalias@ppukm.ukm.my

Received 21 February 2010; revised 19 August 2010; accepted 12 January 2011.
doi: 10.1111/j.1442-200X.2011.03358.x

Congenital infantile fibrosarcoma (CIF) predominantly occurs in children under 24 months of age. It is important to differentiate this tumor from others such as hemangioma of infancy, lymphatic malformation, rapidly involuting congenital hemangioma, infantile fibromatosis or myofibromatosis, malignant fibrous histiocy-

JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 2011, p. 3432–3435 Vol. 49, No. 9
0095-1137/11/$12.00 doi:10.1128/JCM.00478-11

Refractory Chronic Pleurisy Caused by *Helicobacter equorum*-Like Bacterium in a Patient with X-Linked Agammaglobulinemia▽

Michinori Funato,[1]* Hideo Kaneko,[1] Kiyofumi Ohkusu,[2] Hideo Sasai,[1] Kazuo Kubota,[1] Hidenori Ohnishi,[1] Zenichiro Kato,[1,3,4] Toshiyuki Fukao,[1,5] and Naomi Kondo[1,3,4]

Department of Pediatrics[1] and Department of Microbiology,[2] Graduate School of Medicine, Gifu University, Gifu, Japan; Center for Emerging Infectious Diseases[3] and Center for Advanced Drug Research,[4] Gifu University, Gifu, Japan; Medical Information Sciences Division, United Graduate School of Drug Discovery and Medical Information Sciences, Gifu University, Gifu, Japan[5]; and Department of Clinical Research, National Hospital Organization, Nagara medical Center, Gifu, Japan[6]

Received 9 March 2011/Returned for modification 6 April 2011/Accepted 6 June 2011

We describe a 35-year-old man with X-linked agammaglobulinemia who had refractory chronic pleurisy caused by a *Helicobacter equorum*-like bacterium. Broad-range bacterial PCR targeting the 16S and 23S rRNA genes and *in situ* hybridization targeting the 16S rRNA gene of *H. equorum* confirmed the presence of this pathogen in a human for the first time.

CASE REPORT

A 35-year-old man was referred to us for a low-grade fever, fatigue, and discomfort in the right thorax. He had been diagnosed with X-linked agammaglobulinemia (XLA) during the first year of life (5). Upon diagnosis, he showed extremely low serum immunoglobulin G, A, and M levels (650 mg/liter, under 80 mg/liter, and under 60 mg/liter, respectively) and had a missense mutation, L111R (464T>G), in Bruton's tyrosine kinase (BTK) gene (Fig. 1A). His family pedigree with respect to XLA is shown in Fig. 1B. Substitution therapy with intravenous immunoglobulin was administered every 4 weeks from his childhood. During his high school years, he had acute right pleurisy and a pleural puncture, but the details are unclear. In 2006, he suffered from right pleurisy again and then was repeatedly admitted to our hospital for 2 years. No pathogens causing his chronic pleurisy have ever been detected, even though some conventional cultures of blood and sputum have been performed. However, administration of panipenem/betamipron (PAPM/BP) had been the only way to improve his chest discomfort and transiently reduce C-reactive protein (CRP) levels. Administration of other antimicrobial treatments, such as the use of macrolides, cephems, newquinolones, glycopeptides, and carbapenems other than PAPM/BP, has resulted in no improvement.

On admission in 2008, his laboratory findings showed a normal white blood cell count (7.76×10^9/liter), a high CRP level (50.3 mg/liter), and a very high endotoxin level (131 ng/liter). A chest radiograph showed a thickened right pleura and pneumonia in the right inferior lung (Fig. 2A), and a computed tomography (CT) scan of the chest also showed the thickened right pleura with calcification and an alveolar opacity in the right inferior lung (Fig. 2B). We suspected a Gram-negative bacterial infection due to the transient PAPM/BP effectiveness and the high endotoxin level or *Mycobacterium tuberculosis* complex (MTC) infection due to the CT calcification finding. However, conventional cultures of blood, urine, sputum, and feces were all negative, as they had often been in the past.

A transbronchial lung biopsy and a transcutaneous pleural biopsy were performed for definite diagnosis. Histological examination of the alveolar spaces in the right lung showed intraluminal fibrosis of distal airspaces with foamy alveolar macrophages, suggesting secondary organizing pneumonia (OP) (Fig. 2C). In addition, examination of the right pleura showed chronic inflammation (Fig. 2D). Despite these findings, conventional bacterial cultures of biopsy samples from the right pleura grown in sheep blood agar (Nissui Pharmaceutical, Tokyo, Japan) and chocolate agar (Eiken Kagaku, Tokyo, Japan) plates showed no evidence of infection, and MTC cultures grown in an egg-based solid medium (Ogawa medium) (Kyokuto Pharmaceutical, Tokyo, Japan) was also negative.

To determine the pathogen of this refractory pleurisy, we performed broad-range bacterial PCR and mycobacterial PCR using the pleural samples. The PCR products targeting the bacterial 16S and 23S rRNA genes revealed a 1,473-bp band and a 563-bp band, respectively (Fig. 3A). Sequencing analysis was carried out using a GenBank BLAST search (National Center for Biotechnology, Bethesda, MD). Sequence editing and phylogenetic analyses were performed with ClustalW. The sequence of a 1,473-bp fragment of 16S rRNA gene confirmed the presence of *Helicobacter equorum*-like (99.8% identical) bacterium DNA (GenBank accession no. AB571486). Moreover, the sequence of a 563-bp fragment targeting the bacterial 23S rRNA gene was 98.9% similar to that of *H. equorum* (GenBank accession no. AB571487). On the other hand, PCR amplification of the 16S rRNA gene for MTC determinations was negative.

Next, to further demonstrate that the pleural infection involved an *H. equorum*-like bacterium, we performed *in situ* hybridization

* Corresponding author. Mailing address: Department of Pediatrics, Graduate School of Medicine, Gifu University, 1-1Yanagido, Gifu 501-1194, Japan. Phone: 81-58-2306386. Fax: 81-58-2306387. E-mail: mfunato@mac.com.

▽ Published ahead of print on 15 June 2011.

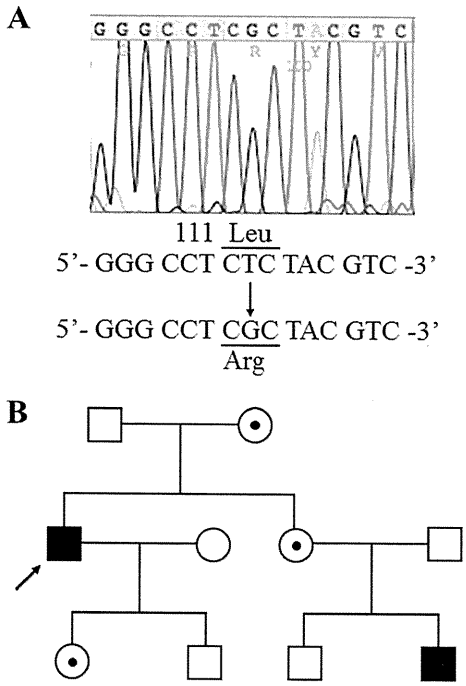

FIG. 1. Molecular analysis of the BTK gene and pedigree of our patient. (A) A missense mutation, L111R in BTK gene, identified in our patient. (B) The family tree for our patient. An arrow indicates the proband with XLA. The solid squares denote patients with XLA; circles with black dots denote mutation carriers.

using a digoxigenin-labeled single-strand RNA probe. The probe for positions 999 to 1118 in the 16S rRNA gene of *H. equorum* (GenBank accession no. AM998804) was designed as follows: 5′-UCCUCACCUUCCUCCUCCUUACGAAGGCAGUCUC CUUAGAGUGCUCAGCCAAACUGCUAGCAACUAAGG ACGAGGGUUGCGCUCGUUGCGGGACUUAACCCAAC AUCUCACGACACGAGC-3′. Positive signals in the pleural sample were confirmed with a nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP) system (Roche Diagnostics, Tokyo, Japan) (Fig. 3B) but not in the control, which included the sense probe (Fig. 3C). These results indicated that the refractory chronic pleurisy in our patient was caused by an *H. equorum*-like bacterium, which in turn caused the development of secondary OP.

We began administration of PAPM/BP at a high dose of 8 g/day and of clarithromycin orally for 2 months. Since then, the patient has had no symptoms, and tests have shown negative CRP results and an endotoxin level of less than 10 ng/liter.

Since the discovery of *Helicobacter pylori* in 1984 (7), various *Helicobacter* species have been described in a wide variety of animal hosts, and transmission to humans has been suggested (3, 10, 14). In general, *Helicobacter pylori* is associated with gastritis, peptic ulcer disease, gastric adenocarcinoma, and mucosa-associated lymphoid tissue lymphoma (3). Also, non-*H. pylori Helicobacter* species are associated with gastric, intesti-

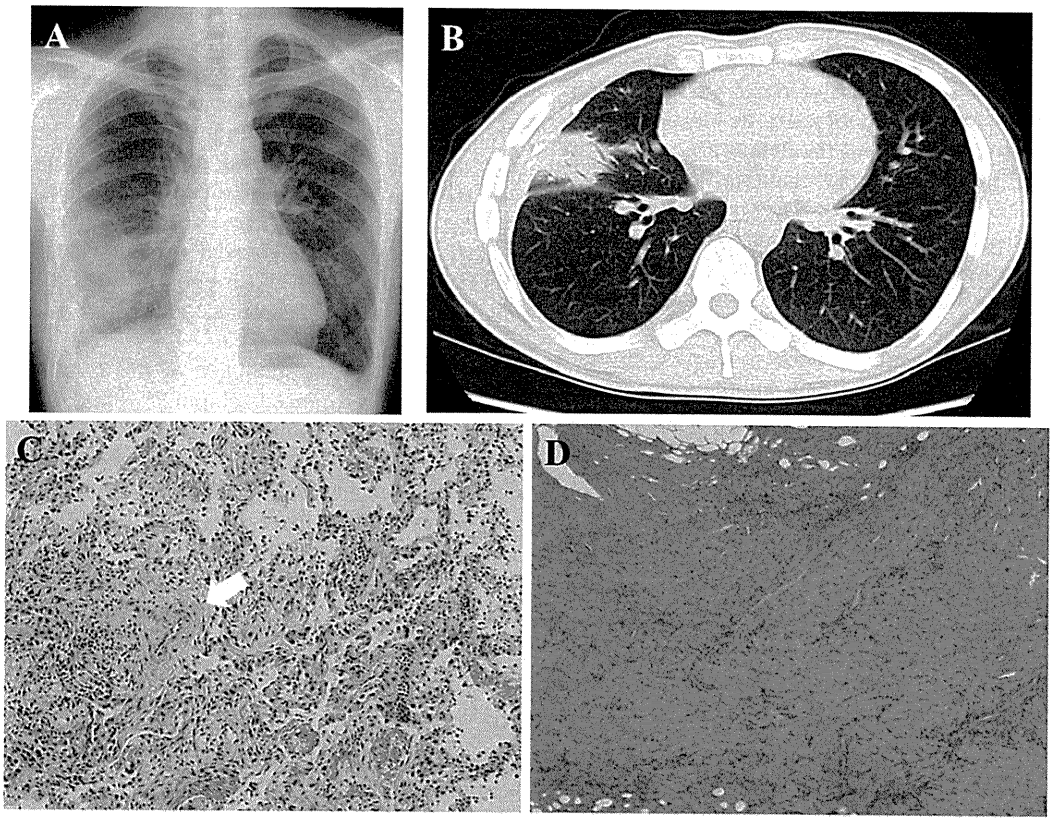

FIG. 2. Imaging and histological findings of refractory chronic pleurisy and secondary OP. (A and B) Results of a chest radiograph (A) and a chest CT scan (B) upon the latest admission of the patient to the hospital. (C) Histological finding in alveolar spaces, showing intraluminal fibrosis (arrow) (hematoxylin and eosin). (D) Histological finding in the right pleura, showing chronic inflammation (hematoxylin and eosin).

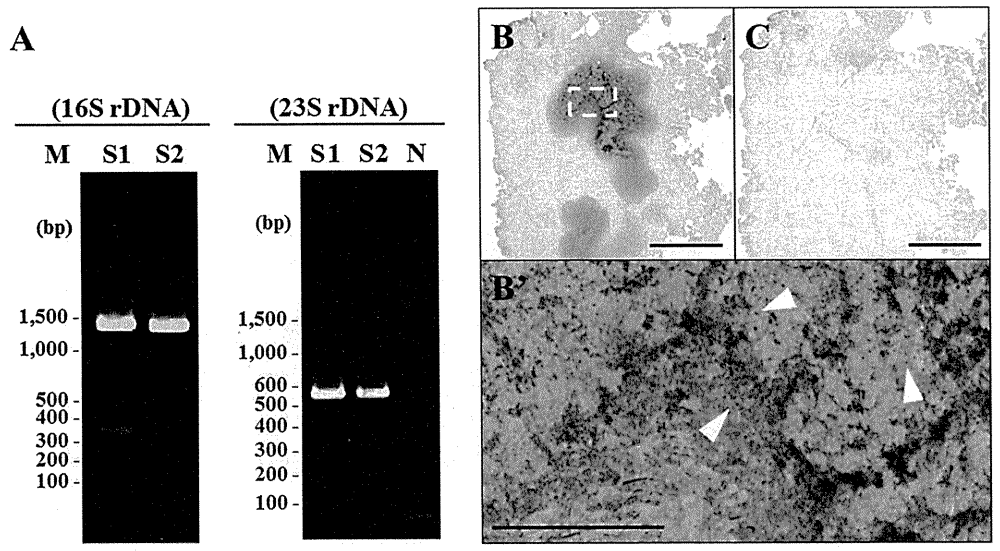

FIG. 3. Detection of *H. equorum*-like bacterium DNA in samples from the right pleura. (A) Broad-range bacterial products from a PCR targeting the 16S rRNA gene (left) and the 23S rRNA gene (right) determined using biopsy samples from the right pleura. S1 and S2 denote DNA samples from our patient. One-tenth the amount used in S1 was used in S2. N, negative control; M, marker. (B) Result of *in situ* hybridization of the pleural samples performed using the probe for the 16S rRNA gene of *H. equorum*. Scale bar, 500 μm. (B') Higher magnification of the bracketed areas shown in panel B. The signals of *H. equorum* were detected (arrowheads). Scale bar, 100 μm. (C) Negative control. Scale bar, 500 μm.

nal, and hepatobiliary diseases in humans (3, 10, 14). This understanding is attributed to molecular diagnosis based on the sequencing of bacterial 16S and 23S rRNA genes, an analytical technique that has already proved useful for various bacterial infections during antimicrobial treatment (11), for rare or unexpected pathogens (11), and particularly for difficult-to-culture bacteria such as non-*H. pylori Helicobacter* species (3). Herein, we have also described a case of refractory chronic pleurisy caused by an *H. equorum*-like bacterium that was subjected to molecular analysis.

Our patient had XLA, which is a rare genetic disorder of B-cell maturation characterized by the absence of mature B cells, very low serum levels of all immunoglobulin isotypes, and a lack of specific antibody production (6). He suffered for 2 years from right chronic pleurisy due to an unknown pathogen. We treated him with PAPM/BP on the basis of the clinical findings, but we were confused because the efficacy was transitory. Molecular diagnosis targeting bacterial 16S and 23S rRNA genes revealed that only DNA of an *H. equorum*-like bacterium that has not previously been reported to have been found in samples from humans was isolated from biopsy samples of our patient. Unfortunately, a culture for the *Helicobacter* species could not be performed for our patient because of the unexpected bacterium, but such culture is also difficult to perform in general, particularly for non-*H. pylori Helicobacter* species (3). We therefore performed *in situ* hybridization using the probe for the 16S rRNA gene of *H. equorum* and thereby confirmed that the infection had been caused by an *H. equorum*-like bacterium.

H. equorum, which is a Gram-negative, curved, and motile bacterium, was recently isolated from horse feces by molecular diagnosis (8). Additional investigation revealed that the prevalence of *H. equorum* was significantly higher in horses under veterinary care than in healthy horses, and *H. equorum* DNA has never been detected in human samples (9). To the best of our knowledge, this is the first case of infection with *H. equorum*-like bacterium in a human with XLA and in the respiratory system. So far, *Helicobacter* infections in patients with XLA have rarely been reported (2, 4, 12, 13), and none of those reported have been due to the presence of *Helicobacter* species in the respiratory system. Freeman and Holland illustrated the importance of humoral immunity in *Helicobacter* infections involving mucosal surfaces, because patients with XLA have been prone to chronic bacteremia, skin infections, and bone infections by the *Helicobacter* species (1). Our patient with XLA showed no evidence of bacteremia or other infections due to the presence of an *H. equorum*-like bacterium. In addition, the studied patient had not had any contact with horse feces, which is a possible vector of *H. equorum*, for the previous 2 years, though he had a history of right pleurisy. Finally, the source of the infection in our patient could not be identified, but we think it would be accurate to say that this infection, which exhibited abnormal humoral immunity, may have been associated with XLA.

Our patient with XLA has been treated with PAPM/BP, but we are unsure as to which antimicrobial treatment to use in a case like this. Because of the difficulty of performing culture, *in vitro* susceptibility testing has scarcely been evaluated or standardized for *H. equorum*. Moyaert et al. reported resistance to cephalotin and nalidixic acid and sensitivity to metronidazole for *H. equorum* (8). We also noted evidence of multiple drug resistance of this organism clinically, as our patient improved only after treatment with PAPM/BP; administration of many other antimicrobial treatments resulted in no improvement. Further investigation is needed, because antimicrobial treatment for *H. equorum* may be difficult.

In conclusion, we have described a case of chronic pleurisy associated with the presence of an *H. equorum*-like bacterium.

All of the clinical findings for our patient—transient PAPM/BP effectiveness, a high serum endotoxin level, and imaging-histological findings of chronic inflammation—were consistent with infections by this organism. This case illustrates both the usefulness of molecular diagnosis of infections with unknown organisms and the pathogenicity of the *H. equorum*-like bacterium in immunocompromised humans. In the future, the issues of whether *H. equorum* is associated with diseases in immunocompetent humans or not and of how patients infected with *H. equorum* are to be treated need to be investigated.

ACKNOWLEDGMENTS

We thank Yoshinobu Hirose, Yasushi Ohno, Kouyou Shirahashi, and Hisahi Iwata for contributing to the diagnosis and Hideki Hiraiwa for technical support with the *in situ* hybridization.

This work was supported by a grant from the Ministry of Health, Labor, and Welfare of Japan.

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Int J Hematol (2011) 94:185–192
DOI 10.1007/s12185-011-0900-1

ORIGINAL ARTICLE

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

Shotaro Iwamoto · Takao Deguchi · Hideaki Ohta · Nobutaka Kiyokawa · Masahito Tsurusawa · Tomomi Yamada · Kozo Takase · Junichiro Fujimoto · Ryoji Hanada · Hiroki Hori · Keizo Horibe · Yoshihiro Komada

Received: 6 November 2010 / Revised: 6 July 2011 / Accepted: 6 July 2011 / Published online: 30 July 2011

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

For the Immunological Diagnosis Committee of the Japanese Pediatric Leukemia/Lymphoma Study Group.

S. Iwamoto (✉) · T. Deguchi · H. Hori · Y. Komada
Department of Pediatrics and Developmental Science, Mie University Graduate School of Medicine, 2-174 Edobashi, Tsu, Mie 514-8507, Japan
e-mail: siwamoto@clin.medic.mie-u.ac.jp

H. Ohta
Department of Pediatrics, Osaka University Graduate School of Medicine, Suita, Osaka, Japan

N. Kiyokawa · J. Fujimoto
Department of Developmental Biology, National Research Institute for Child Health and Development, Setagaya-ku, Tokyo, Japan

M. Tsurusawa · T. Yamada
Department of Pediatrics, Aichi Medical University, Nagakute, Aichi, Japan

K. Takase
Division of Research Development, Department of Health Science Policies, Graduate School of Medicine and Dentistry, Tokyo Medical and Dental University, Bunkyo-ku, Tokyo, Japan

R. Hanada
Division of Hematology/Oncology, Saitama Children's Medical Center, Saitama, Saitama, Japan

K. Horibe
Clinical Research Center, National Hospital Organization Nagoya Medical Center, Nagoya, Aichi, Japan

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

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

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

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

2 Methods

2.1 Patient samples

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

2.2 Flow cytometry

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

2.3 Statistical analysis

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

3 Results

3.1 Clinical features and FAB morphology

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

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

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

Values indicate the proportion of positive cases (%)

c cytoplasmic, *s* surface

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

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

3.2 T-lineage ALL

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

3.3 Early pre-B ALL

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

3.4 Pre-B ALL

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

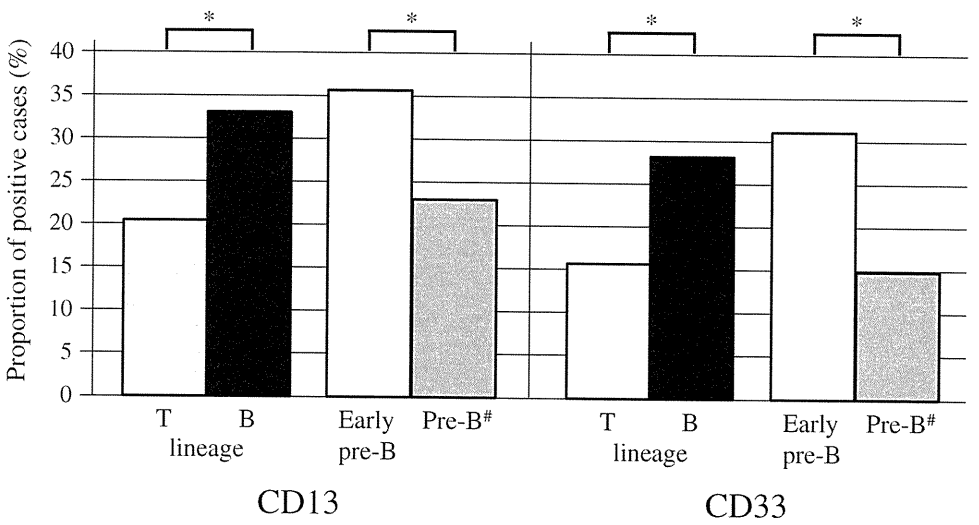

Fig. 1 Distribution of myeloid antigen (CD13 and CD33) expression. Acute lymphoblastic leukemia immunophenotypes: T-lineage ALL, B-lineage ALL, early pre-B ALL, pre-B ALL and B-ALL. *Values* indicate proportion of positive cases (%). #Pre-B cases include transitional pre-B cases. Expression was observed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

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

3.5 B cell ALL

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

4 Discussion

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

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

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

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

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

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

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

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

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

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

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

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

Acknowledgments We thank the committee members of the JPLSG for sending bone marrow and peripheral blood samples. This study was supported by a grant for Clinical Cancer Research from the Ministry of Health, Labor, and Welfare of Japan. We thank Dr. K. Nakahara, Ms. E. Ogawa and Mr. W. Hashimoto for their insightful and helpful comments.

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