なし

3. その他

なし

厚生労働科学研究費補助金 〔難治性疾患等克服研究事業（難治性疾患克服研究事業)〕
分担研究報告書

「RAS 関連自己免疫性リンパ球増殖症候群様疾患(RALD)の実態調査および病態病因解析に関する研究」

—既存の遺伝子変異を有さない典型的自己免疫性リンパ増殖症候群の一例—

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研究要旨：自己免疫リンパ増殖症候群（autoimmune lymphoproliferative syndrome : ALPS）は、Fasを介するアポトーシス経路の異常により、リンパ球のホメオスタシスに異常をきたす疾患である。FAS遺伝子の生殖細胞変異を伴うALPS-FAS（60-70%）、体細胞変異を伴うALPS-sFAS（10%）、FASLG遺伝子の変異によるALPS-FASLG（<1%）、CASP10遺伝子の変異によるALPS-CASP10（2-3%）に分類されるが、遺伝子変異の特定できないもの（ALPS-U）も20-30%存在する。今回典型的ALPSでありながら、既存の遺伝子変異が同定されず、ALPS-Uと考えられる一例を経験した。これに対し全エクソン解析を行ったところNRAS遺伝子の変異が認められ、RALDであることが明らかとなった。全エクソン解析がALPSもしくはALPS類縁疾患の責任遺伝子解明に有用であることが明らかとなった。

A. 研究目的

自己免疫リンパ増殖症候群（autoimmune lymphoproliferative syndrome : ALPS）は、Fasを介するアポトーシス経路の異常により、リンパ球のホメオスタシスに異常をきたす疾患である。6か月以上続く慢性非悪性非感染性リンパ増殖症および末梢血におけるCD4-CD8-TCR $\alpha\beta$+T 細胞（double negative T cells : DNT 細胞）の増加によって臨床診断される。原因遺伝子のほとんどは *FAS* 遺伝子の生殖細胞または体細胞の変異であるが、一部 FASLG ならびに CASP10 変異によるものもある。今回典型的 ALPS でありながら、FAS, FASLG, CASP10 変異が同定されない一例を経験したので報告する。

B. 研究方法

症例は 2 歳女児である。5 か月時に発熱、汎血球減少、肝機能障害を主訴に Y 大学病院に入院となった。肝脾腫、凝固線溶系の異常を認め、ステロイドならびにシクロスポリン A の投与にて速やかに解熱し、プレドニゾロン内服にて退院となった。プレドニゾロンの減量に伴い、血小板低下を認め、肝脾腫は持続し、高 IgG 血症にも気づかれ、ALPS 疑いで当大学に検査依頼があった。

患者家族から文書による同意を得て、ヘパリン加静脈血を採取した。単核球に分離後、DNT を含むリンパ球サブセットを行い、残りの単核球から活性化リンパ球を樹立後した。抗 Fas 抗体で刺激し、Annexin V 染色にてリンパ球のアポトーシスを評価した。またバッフィーコートから DNA を抽出し、*FAS*, *FASLG*, *CAPS10* 遺伝子解析を行った。さらに単核球から磁気ビーズ法にて濃縮し

た DNT 細胞から DNA を抽出し、*FAS* 遺伝子解析を行った。また責任遺伝子同定のため、全エクソン解析を行った。

（倫理面への配慮）

本研究はヒト検体を用いて解析を行うものであり、検体量および採取時の苦痛には十分な配慮を行った。遺伝子解析については各種指針を遵守して、患者個人情報の保護について十分な配慮を行った。

C. 研究結果

リンパ球サブセットにて DNT 細胞は 6.5%（正常は 1.5%以下）と増加し、肝脾腫、血球減少などの臨床症状と考え併せ、ALPS と診断した。しかし活性化 T 細胞におけるアポトーシスは健常者と同程度に観察され、ALPS-FAS は否定的であった。*FAS* はもちろんのこと *FASLG*, *CAPS10* 遺伝子解析を行ったが、いずれも変異は同定されなかった。そこで FAS 体細胞変異による ALPS-sFAS の可能性を考え、純化した DNT 細胞における FAS 遺伝子解析を行ったが、変異は同定できなかった。現在、全エクソン解析による網羅的遺伝子解析の結果、患児は NRAS 遺伝子の変異を持っていることが明らかとなった。

なお患児は現在プレドニゾロン 3mg, ミコフェノール酸モフェチル 250mg 内服にてコントロールされている。

D. 考察

非悪性リンパ増殖症、免疫抑制剤に依存する自己免疫性血球減少、高 IgG 血症ならびに DNT 細胞の増加から典型的 ALPS と診断される。わが国においては約 20 例の ALPS-FAS と 1 例の ALPS-sFAS が同定されているが、ALPS-FASLG ならびに ALPS-CASP10 の報告はこれまでない。自験例ではアポトーシスの異常は認められず、ALPS-FASLG, ALPS-CASP10、ALPS-sFAS の可能性が考えらえたが、いずれも否定的であった。そこで ALPS-U に分類されるが、何らかの遺伝的背景を有する可能性があるため、患者家族から同意を得て、本人ならびに両親の全エクソンシークエンスを行った。また ALPS の遺伝子解析においては体細胞変異も少なからず存在するため、従来のサンガー法によるシークエンスよりも次世代シークエンサーを利用したアンプリコンシークエンスの方が適していると思われる。本患者では全エクソン解析が行われ、NRAS 遺伝子の変異が同定された。全エクソン解析が ALPS もしくは ALPS 類縁疾患の責任遺伝子解明に有用であることが明らかとなった。これまで RALD では DNT 細胞の上昇は認められないとされているが、自験例では DNT 細胞の上昇が認められ、DNT 細胞だけで ALPS との鑑別ができないことが明らかとなった。

E. 結論

臨床症状と DNT 細胞増加から ALPS と診断したが、既存の遺伝子変異はなく、ALPS-U と考えられる一例を経験した。自験例に対し全エクソン解析を行うことにより NRAS 遺伝子の変異を同定することができ、RALD の診断が行えた。全エクソン解析が ALPS もしくは ALPS 類縁疾患の責任遺伝子解明に有用であることが明らかとなった。

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G. 知的財産権の出願・登録状況

(予定を含む)

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

Ⅳ．研究成果に関する刊行の一覧表

研究成果の刊行に関する一覧表

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森尾友宏	原発性免疫不全症	福井次矢, 黒川清	ハリソン内科学第4版(原著第18版)	メディカル・サイエンス・インターナショナル	東京	2013	2329-2339

Ⅴ. 別刷

Pediatr Blood Cancer 2013;60:836–841

Wiskott–Aldrich Syndrome Presenting With a Clinical Picture Mimicking Juvenile Myelomonocytic Leukaemia

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Background. Wiskott–Aldrich syndrome (WAS) is a rare X-linked immunodeficiency caused by defects of the WAS protein (*WASP*) gene. Patients with WAS typically demonstrate micro-thrombocytopenia. ***Procedures.*** The report describes seven male infants with WAS that initially presented with leukocytosis, monocytosis, and myeloid and erythroid precursors in the peripheral blood (PB) and dysplasia in the bone marrow (BM), which was initially indistinguishable from juvenile myelomonocytic leukaemia (JMML). ***Results.*** The median age of affected patients was 1 month (range, 1–4 months). Splenomegaly was absent in four of these patients, which was unusual for JMML. A mutation analysis of genes in the RAS-signalling pathway did not support a diagnosis of JMML. Non-haematological features, such as eczema (n = 7) and bloody stools (n = 6), ultimately led to the diagnosis of WAS at a median age of 4 months (range, 3–8 months), which was confirmed by absent (n = 6) or reduced (n = 1) WASP expression in lymphocytes by flow cytometry (FCM) and a *WASP* gene mutation. Interestingly, mean platelet volume (MPV) was normal in three of five patients and six of seven patients demonstrated occasional giant platelets, which was not compatible with WAS. ***Conclusions.*** These data suggest that WAS should be considered in male infants presenting with JMML-like features if no molecular markers of JMML can be detected. Pediatr Blood Cancer 2013;60:836–841.

Key words: children; juvenile myelomonocytic leukaemia; Wiskott–Aldrich syndrome

INTRODUCTION

Wiskott–Aldrich syndrome (WAS) is a rare X-linked recessive disorder, characterized by micro-thrombocytopenia, eczematous skin disease, and recurrent infections. The incidence of WAS is 1–10 in 1 million male new-borns. Affected patients have a predisposition to autoimmune diseases and lymphoid malignancies [1,2]. The responsible gene is *WASP*, which encodes the 502 amino acid WASP protein [3]. WASP is expressed selectively in hematopoietic cells and is involved in cell signalling and cytoskeleton reorganization [3]. Specific types of defects in WASP are often but not invariably associated with the severity of disease and clinical phenotype. Lack of WASP expression causes the most severe phenotype (i.e., classic WAS), whereas inactivating *WASP* missense mutations allow residual protein expression and can cause less severe X-linked thrombocytopenia (XLT) [4,5]. Gain-of-function mutations generate X-linked neutropenia (XLN) [6,7].

Juvenile myelomonocytic leukaemia (JMML) is a rare disease in children that occurs with an estimated incidence of 1–2 cases per million [8]. JMML has characteristics of both myelodysplastic syndrome (MDS) and myeloproliferative disorders (MPD) and is categorized in the MDS/MPD category in the World Health Organization (WHO) classification [9–11]. Clinical and haematological manifestations of JMML include hepatosplenomegaly, skin rash, lymphadenopathy, leukoerythroblastosis, monocytosis, and thrombocytopenia. Recent studies show that deregulated activation of the RAS/MAPK signalling pathway plays a central role in the pathogenesis of JMML. Gene mutations in either the *RAS*, *PTPN11*, *NF1*, or *CBL* genes involved in this pathway are detected in about 80% of JMML patients [12–18].

Micro-thrombocytopenia is the key haematological finding in patients with WAS. However, myelopoiesis and erythropoiesis are usually not affected, despite the fact that WASP is expressed in various hematopoietic cells [19]. The present report describes seven cases of male infants with classical WAS who demonstrated haematological abnormalities mimicking JMML. Importantly, patients can present with JMML-like features before the full clinical manifestations of WAS become apparent. Moreover, nor-

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mal mean platelet volume (MPV) and the presence of the giant platelets complicated the diagnostic evaluation in some of our patients.

PATIENTS AND METHODS

Patients

In 2007, we described a case of a male patient (patient #1) with WAS who demonstrated JMML-like clinical features [20]. Briefly, thrombocytopenia was detected shortly after birth. He suffered from bloody diarrhoea from the age of 9 days. At the age of 42 days, leukocytosis with myeloid/erythroid precursors and monocytosis was detected. Bone marrow (BM) aspirates showed hypercellularity with significant predominance of myelopoiesis and dysplastic features. The morphological features were compatible with JMML. Subsequently, the white blood cell (WBC) count increased to 52.0×10^9/L with the appearance of peripheral blasts (3%) and persistent fever. Intravenous administration of various antibiotics had no effect on fever and leukocytosis. Oral 6-mercaptopurine (6-MP) was administered, which resulted in disappearance of leukocytosis. Positive results of cytomegalovirus (CMV)-IgM/IgG and a low level pp65 CMV-antigen (Ag) cells were transitionally noted without CMV-related symptoms. Intravenous administration of ganciclovir (GCV) led to the elimination of CMV-Ag but not to any improvement of JMML-like features. At the age of 7 months, mild atopic dermatitis-like eczema was recognized, which finally led to the clinical and molecular diagnosis of WAS.

The MDS committee of the Japanese Society of Paediatric Hematology/Oncology (JSPHO) study coordinating center of the European Working Group of MDS in Childhood (EWOG-MDS) perform the morphological review of peripheral blood (PB) and BM smears and laboratory examinations for the diagnosis of JMML in Japan and Germany, respectively. By January 2011, WAS was diagnosed in six Japanese males (including patient #1) and one German male who were initially referred with a suspected diagnosis of JMML. Patient #4 was recently reported [21]. Approval for the study was obtained from the institutional review board of Nagoya University, Nagoya, Japan, and University of Freiburg, Freiburg, Germany. Informed consent was provided by parents according to the Declaration of Helsinki.

Diagnostic Tests for Wiskott–Aldrich Syndrome

Intracellular WASP expression in lymphocytes was analysed by flow cytometry (FCM) by the standard method described previously [4,22]. DNA purification and sequencing of genomic DNA, RNA isolation, reverse transcription-polymerase chain reaction, and sequencing of cDNA for the mutational analysis of *WASP* gene was performed as reported previously [23].

Diagnostic Tests for Juvenile Myelomonocytic Leukemia

Mutational screening for *PTPN11*, *NRAS*, and *KRAS* genes was performed in six patients, as previously reported [24–27]. In patients #6 and #7, the c-CBL gene, which has been recently found in about 10% of JMML patients, was also screened as described previously [16,18]. None of the patients had clinical signs of neurofibromatosis type 1 (NF1). *In vitro* colony assay for granulocyte–macrophage colony stimulating factor (GM-CSF) hypersensitivity assay was performed as a supportive diagnostic tool for JMML as previously reported [28,29].

RESULTS

Clinical Characteristics and Laboratory Findings

The clinical characteristics of these patients are summarized in Table I. Thrombocytopenia and bloody diarrhoea were observed soon after birth in all patients except for patient #6. JMML-like clinical manifestations occurred within the first few months of life. Eczema developed between 0 and 3 months after birth in all patients. Splenomegaly was seen in three of seven patients and massive splenomegaly was present in two patients. At the presentation of JMML-like features, episodes of recurrent infections, which suggest an immunodeficiency, were not observed in any patients. However, in three patients, recurrent bacterial, or viral infections (cases #5, #6, and #7) were documented during the clinical course.

The laboratory findings at the presentation of JMML-like disease are summarized in Table II. The WBC count was increased in all patients except for in patient #7. Monocytosis and myeloid/erythroid precursors were seen in PB in all patients. All patients had anaemia. The MPV before platelet transfusions ranged between 6.9 and 7.9 fl (normal, 7.2–11.7 fl) in the five patients that were evaluated. Hb F levels were normal in three patients examined. The platelet morphology demonstrated anisocytosis in all patients. Occasional giant platelets, which are defined as platelets bigger than red cells, were observed in six patients. These features were unusual for WAS. Full BM with significant predominance of myelopoiesis and a marked left shift of the myeloid lineage was seen in all patients. The number of megakaryocytes was normal or increased. Dysplasia in megakaryopoiesis, myelopoiesis, and erythropoiesis was observed in seven, four, and four patients, respectively. The common dysplasia in the megakaryopoiesis included hypolobulations of nuclei and small megakaryocytes with single or double round nuclei. In the myelopoiesis, nuclear abnormalities such as double nuclei, ring nuclei, or pseudo-Pelger–Huet anomaly nuclei were often seen. The dysplasia of erythropoiesis was mild, if observed, and included nuclear lobulation and double nuclei. The karyotype was normal in all patients. The serum levels of immunoglobulin were variable (Table II). Evaluation of T cell function revealed normal responses to phytohemagglutinin and concanavalin A in the four patients that were examined. The numbers of peripheral T and B cells and the CD4/8 ratio were normal in four patients. Patient #7 demonstrated B-lymphocytopenia and an elevated CD4/8 ratio.

Diagnostic Tests for Juvenile Myelomonocytic Leukemia

Molecular analysis of *PTPN11*, *N-RAS*, and *K-RAS* genes (n = 7) and the c-*CBL* gene (n = 2) documented no mutations in any of the examined patients. *In vitro* GM-CSF hypersensitivity was performed in all patients but patient #1 and was positive only in patient #4.

Diagnostic Tests for Wiskott–Aldrich Syndrome

FCM analysis showed absent (n = 6) or reduced (n = 1) WASP expression in the lymphocytes, which led to the confirmation of a diagnosis of WAS (Table III). Mutations of *WASP* genes

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TABLE I. Clinical Features of the Patients

Patient	1	2	3	4	5	6	7
Age at the detection of thrombocytopenia	At birth	At birth	At birth	At birth	1 month	4 months	2 months
Age at the onset of JMML like haematological features	1 month	3 months	1 month	1 month	1 month	4 months	2 months
Age at the onset of eczema	1 month	3 months	Soon after birth	3 months	1 month	3 months	2 months
Age at the onset of bloody diarrhoea	At birth	20 days	At birth	1 week	1 month	No	1 month
Hepatomegaly/splenomegaly (cm under the costal margin)	Yes (3)/no	Yes (3)/yes#	No/no	No/no	No/no	Yes (5)/yes (7.5)	Yes (6)/yes (6)
Infectious episodes before the diagnosis of WAS	CMV antigenemia	No episode	No episode	No episode	Fever of unknown origin	Otitis media	Adenovirus and Rotavirus in stool
Infectious episodes between the diagnosis of WAS and HSCT	No episode	No episode	No episode	No episode	Bacterial and RSV pneumonia Rotavirus gastroenteritis	Otitis media Anal abscess	CMV pneumonia
HSCT (age)	10 months	10 months	17 months	4 months	18 months	13 months	7 months
Donor/stem cell source	U-CBT	MSD-BMT	U-CBT	MSD-BMT	1 antigen MMUD-BMT	MUD-BMT	MUD-BMT
Survival (age at the time of the last follow-up)	Alive (6 years 5 months)	Alive (5 years 4 months)	Alive (4 years 8 months)	Alive (12 months)	Alive (1 year 9 months)	Alive (1 year 6 months)	Alive (1 year 7 months)

JMML, juvenile myelomonocytic leukaemia; WAS, Wiskott–Aldrich syndrome; RSV, respiratory syncytial virus; CMV, cytomegalovirus; # splenomegaly was noted only by ultrasound; HSCT, hematopoietic stem cell transplantation; U-CBT, unrelated cord blood transplantation; MSD-BMT, bone marrow transplantation from an HLA matched sibling donor; MUD-BMT, BMT from an HLA matched unrelated donor; MMUD-BMT, BMT from an HLA-mismatched unrelated donor.

TABLE II. Laboratory Findings Accompanying the Juvenile Myelomonocytic Leukaemia-Like Haematological Features

Patient	1	2	3	4	5	6	7
Peripheral blood							
WBC count ($\times 10^9$/L)	35.5–50.0	12.0–18.0	13.5–22.1	15.0	35.0–50.0	6.0–12.0	7.5
Monocyte count ($\times 10^9$/L)	8.9	1.0–1.5	8	2.3	1.1	1.0–1.5	1.3
Blasts (%)	3	2	2	4	2	0	1
Immature myeloid/erythroid cells	Yes/Yes	Yes/Yes	Yes/Yes	Yes/Yes	Yes/Yes	Yes/Yes	Yes/Yes
Eosinophils (%)	3	12	4	7	2	5	2
Platelet count ($\times 10^9$/L)	44	40–90	31	24	53	11	26
MPV (fl)[a]	7.0	7.4	NE	6.9	7.5	NE	7.9
Platelet anisocytosis/giant platelets	Yes/Yes	Yes/Yes	Yes/Yes	Yes/No	Yes/Yes	Yes/Yes	Yes/Yes
Hb (g/dl)	8.9	8.0	9.2	6.1	11.6	9.5	8.0
Bone marrow							
Cellularity	Full[b]	Full	Full	Full	Full	Full	Full
M/E ratio	33	4	7	5.4	11	2	2
Blasts (%)	3.5	0.5	1	0	2	3.5	2
Karyotype	46,XY	46,XY	46,XY	46, XY	46, XY	46,XY	46,XY
Immunological examination							
Age at examination (months)	8	5	2	2	10	4	2/3/5
IgG (mg/dl)	2,554	468	638	102	792	3,780	1,170/2,120/2,070
IgM (mg/dl)	156	64	37	<5	33	353	122/244/156
IgA (mg/dl)	49	52	38	39	129	124	25/45.4/58.2
IgE (mg/dl)	494	368	89	8	16	1,330 (10 months)	258/693/7,995
LBT (PHA, ConA)	Normal	Normal	NE	NE	NE	Normal	Normal
CD4/8 ratio	Normal	Normal	NE	Normal	NE	Normal	Increased (7.0/22.2/1.1)

WBC, white blood cell; MPV, mean platelet volume; M/E myeloid-/erythroid-cells; LBT, lymphoblastic test; PHA, phytohemagglutinin; conA, concanavalin A; NE, not evaluated. [a]Normal range (7.2–11.7 fl). [b]The cellularity was high (full bone marrow), which was normal for infants.

varied between patients. In patient #1, sequencing of *WASP* cDNA identified five nucleotides (CCGGG) inserted at position c.387 in exon 4, causing a frameshift at codon 140 that gave rise to a premature stop signal at codon 262, as reported previously [20]. Patients #2 and #3 had previously known nonsense mutations in exon 1 and exon 4, which led to the absence of WASP expression and a moderate to severe clinical phenotype of WAS [4,30–32]. Patient #4 had a known deletion in intron 8, which cause a frameshift and absence of WASP expression [4,5]. Patient #5 had a known splice anomaly in intron 6, which reduced expression of WASP and led to a clinical phenotype of either XLP or WAS [4,32]. Patient #6 had known deletion in exon 1, which was associated with a classic WAS phenotype [33]. Patient #7 had a nonsense mutation in exon 1, which has not been previously described.

Clinical Course of Patients

Patient #1 received 6-MP to control leukocytosis. In other patients, the JMML-like features were stable until allogeneic hematopoietic stem cell transplantation (HSCT), which was performed at the age of 4–18 months. All patients are alive after HSCT at the time of the last follow-up (Table I). Graft failure was observed in patient #7, and a second HSCT is currently planned for this patient.

DISCUSSION

Although WASP is expressed ubiquitously in hematopoietic cells and although *in vitro* results suggest that WASP is involved in the proliferation and differentiation of all hematopoietic progenitors, overt defects are restricted to micro-thrombocytopenia and immune-dysfunction in classical WAS. We previously described a case of a male presenting with a clinical picture of JMML, in whom WAS was ultimately diagnosed (patient #1) [20]. These haematological abnormalities had not been previously reported in patients with WAS. Since then, we have encountered six additional patients with WAS who presented with similar clinical characteristics. Morphological features were not distinguishable from JMML. Moreover, normal MPV and the presence

TABLE III. Results of the Diagnostic Tests for Wiskott–Aldrich Syndrome

Patient	1	2	3	4	5	6	7
Age at examinations	8 months	4 months	4 months	3 months	8 months	4 months	3 months
WASP protein expression	Absence	Absence	Absence	Absence	Reduced	Absence	Absence
WASP mutation	Exon 4 c.387–421 ins 5nt	Exon 1 c.37C>T	Exon 4 c.424C>T	Intron 8 c.777+1_+4 delGTGA	Intron 6 c.559+5G>A	Exon1 c.31delG	Exon 1 c.C55>T
Mutation type	Insertion	Nonsense	Nonsense	Deletion	Splice anomaly	Deletion	Nonsense
Predicted protein change	Frameshift stop aa 262	R13X	Q142X	Frameshift stop aa 246	Frameshift stop aa 190	Frameshift stop aa 37	Q19X

of giant platelets in three and six patients, respectively, initially argued against a diagnosis of WAS, because micro-thrombocytes are known as a key diagnostic feature of WAS and XLP. The JMML-like features developed shortly after birth in all patients, before the full clinical picture of WAS become apparent. In our patients with JMML-like features, signs of immune defects were not present. Without recent advances in molecular diagnostic tests for WAS and JMML, it might otherwise be impossible to establish a diagnosis of WAS in these patients. Absent or reduced WASP expression by FCM–WASP and detection of *WASP* mutation ultimately led to a diagnosis of WAS. The mutations were distributed in different exons and introns, and there was no clustering. Thrombocytopenia since birth and some of the observed clinical features (e.g., atopic dermatitis-like eczema, persistent bloody stool, lack of splenomegaly) were unusual for JMML but were compatible with WAS.

The deregulated RAS signalling pathway plays a central role in the pathogenesis of JMML, and mutational analyses of *PTPN11*, *RAS*, and *c-CBL* genes located in the RAS signalling pathway have become important diagnostic tests. Mutations of one of these genes and a clinical diagnosis of NF1 can be found in more than 80% of patients with JMML. However, in up to 20% of patients without any molecular markers, a diagnosis of JMML relies on unspecific clinical and laboratory observations. We suggest that WAS should be considered within the differential diagnosis in male infants with clinical features of JMML if no mutations of the RAS signalling pathway can be detected. Importantly, clinicians should not exclude a diagnosis of WAS if the MPV is normal or if giant platelets are present. Rarely, patients with WAS can present with normal or large platelets [34,35].

The pathogenesis of JMML-like feature in these patients is unknown. There is no evidence that WASP is related to the RAS signalling pathway. The activation of this pathway does not seem to be a major cause of JMML-like features in our patients, because GM-CSF hypersensitivity was demonstrated only in one of six patients examined. Patients with WAS have an increased risk of viral infections. CMV, Epstein–Barr virus (EBV) and human herpes virus-6 (HHV-6) infections can mimic JMML in infants [36,37]. However, extensive screening failed to detect viral infections at the time, at which these patients presented with JMML-like features, except for patient #1, in whom CMV antigen was detected.

Leukocyte adhesion deficiency (LAD)-1 is a rare immunodeficiency caused by a mutation in the beta-2 integrin gene. The firm adhesion of leukocyte to the blood vessel wall is defective in LAD-1, which results in leukocytosis, mimicking JMML [38]. A defect of leukocyte adhesion due to abnormal integrin beta clustering has been described in the context of WAS [39]. A mechanism similar to that seen in LAD1 may be present in WAS with JMML-like features.

A recent report showed that WASP localizes to not only the cytoplasm but also to the nucleus and has a role in the transcriptional regulation at the chromatin level in lymphocytes [40]. Active *WASP* mutations, which cluster within the GTP-ase binding domain of WASP (L270P, S272P, and I294T), cause XLN and myelodysplasia [6,7]. Further, increased apoptosis associated with increased genomic instability in myeloid cells and lymphocytes has been described in the context of active *WASP* mutations [41,42]. Further research may identify new roles of WASP in transcriptional regulation and genomic stability in haematopoiesis, which may explain the JMML-like features, seen in WAS patients.

In conclusion, WAS should be considered in the differential diagnosis in male infants presenting with JMML-like features if no molecular markers of JMML can be demonstrated. A normal MPV and the presence of giant platelets do not exclude a diagnosis of WAS. Clinical information, such as bloody stool and eczema, may be helpful in pursuing a diagnosis of WAS in an infant with JMML like features.

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ORIGINAL ARTICLE CLINICAL IMMUNOLOGY

Clinical features and outcome of X-linked lymphoproliferative syndrome type 1 (SAP deficiency) in Japan identified by the combination of flow cytometric assay and genetic analysis

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flow cytometry; genetic analysis; hematopoietic stem cell transplantation; SLAM-associated protein; X-linked lymphoproliferative syndrome

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Abstract

Objective: X-linked lymphoproliferative syndrome (XLP) type 1 is a rare immunodeficiency, which is caused by mutations in *SH2D1A* gene. The prognosis of XLP is very poor, and hematopoietic stem cell transplantation (HSCT) is the only curative therapy. We characterized the clinical features and outcome of Japanese patients with XLP-1.

Methods: We used a combination of flow cytometric analysis and genetic analysis to identify XLP-1 and reviewed the patient characteristics and survival with HSCT.

Results: We identified 33 patients from 21 families with XLP-1 in Japan. Twenty-one of the patients (65%) who did not undergo a transplant died of the disease and complications. Twelve patients underwent HSCT, and 11 of these (92%) survived.

Conclusion: We described the clinical characteristics and outcomes of Japanese patients with XLP-1, and HSCT was the only curative therapy for XLP-1. The rapid and accurate diagnosis of XLP with the combination of flow cytometric assay and genetic analysis is important.

X-linked lymphoproliferative syndrome (XLP) is a rare inherited immunodeficiency estimated to affect approximately one in one million males, although it may be under-diagnosed (1). XLP is characterized by extreme vulnerability to Epstein–Barr virus (EBV) infection, and the major clinical phenotypes of XLP include fulminant infectious mononucleosis (FIM) or EBV-associated hemophagocytic lymphohistiocytosis (HLH) (60%), lymphoproliferative disorder (30%), and dysgammaglobulinemia (30%) (2). In addition, XLP is associated with a variety of other clinical manifestations including vasculitis, aplastic anemia, and pulmonary lymphoid granulomatosis. Patients with XLP often develop more than one phenotype over time.

The responsible gene was first identified as *SH2D1A/SLAM-associated protein (SAP)* located in the region of Xq25 (3–5). However, some of the presumed patients with

XLP do not harbor *SH2D1A* mutations, although they are clinically and even histologically similar to XLP patients with *SH2D1A* mutations. A second causative gene that encodes X-linked inhibitor of apoptosis protein (XIAP), namely *XIAP* or *BIRC4* gene, has been identified (6). Patients with XLP-2 (XIAP deficiency) sometimes present with splenomegaly and hemorrhagic colitis, but no lymphoma. The *SH2D1A* and *XIAP* genes are close together at Xq25, but the molecular pathogenesis and clinical features of these diseases seem to be distinct (7, 8).

The vast majority of patients with XLP die in childhood; the survival rate is very poor, even with treatment (2). Hematopoietic stem cell transplantation (HSCT) is the only curative therapy for XLP (9, 10). Therefore, rapid definitive diagnosis and immediate treatment are extremely significant for better prognosis and survival of patients with XLP. We previously established the anti-SAP monoclonal antibody (mAb) and applied it to flow cytometric diagnosis of patients with XLP-1 (11). We performed a nationwide survey for XLP-1 with the flow cytometric assay and genetic analysis and identified a total of 33 patients from 21 families with XLP-1 in Japan (11–15). In this study, we elucidated the clinical and genetic characteristics of these patients. Twelve patients with XLP-1 underwent HSCT, and 11 of these (92%) survived. We also describe the outcomes of HSCT in Japan.

Materials and methods

Study subjects

The subjects in this study were largely male patients with FIM or EBV–HLH treated until the end of 2011. In addition, a few male patients with lymphoma or hypogammaglobulinemia with unknown genetic origin were suspected of having XLP. After written informed consent was obtained, 5–10 ml of venous blood was collected into heparin-containing syringes and delivered to the laboratory. Patients and families provided informed consent for genetic analyses in accordance with the 1975 Declaration of Helsinki, and the study protocol was approved by the Ethics Committee of the University of Toyama. Several patients were described in our previous reports (11–15).

Flow cytometric analysis of SAP

Flow cytometric analysis of SAP was performed as previously described (11, 12). The peripheral blood mononuclear cells (PBMC) were isolated by Ficoll–Hypaque density gradient centrifugation and immediately fixed in 1% paraformaldehyde for 30 min at room temperature and then permeabilized in 0.5% saponin for 15 min on ice. To test the expression of SAP in lymphocytes, these cells were incubated with 2 μg/ml anti-SAP mAb, termed KST-3 (rat IgG1) or irreverent rat IgG1, for 20 min on ice and further stained with a 1:1000 dilution of FITC-labeled goat anti-rat IgG antibody (Zymed, South San Francisco, CA, USA) or Alexa Fluor 488-conjugated goat anti-rat IgG antibody (Molecular Probes, Eugene, OR, USA) for 20 min on ice. To evaluate SAP expression in $CD8^+$ T and NK cells, PBMC were stained with phycoerythrin (PE)-conjugated anti-CD8 and anti-CD56 mAbs (DAKO Japan, Kyoto, Japan), respectively, before cellular fixation and permeabilization. The stained cells were analyzed using a flow cytometer (EPICS XL-MCL; Beckman Coulter KK, Tokyo, Japan).

SH2D1A mutation detection

The *SH2D1A* mutations were detected by direct sequencing as described previously (5, 14). Genomic DNA was purified from PBMC with a QIAamp Blood Kit (Qiagen, Hilden, Germany) and amplified using primers encompassing each exon–intron boundary of the *SH2D1A* genes. The sequencing reaction was carried out using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) with an automated ABI PRISM 310 DNA sequencer (Applied Biosystems).

Results

SAP expression in patients with XLP-1

Fresh blood cells were available in 19 patients with XLP-1. All the examined patients demonstrated markedly deficient SAP expression in lymphocytes, especially in $CD8^+$ T cells and NK cells (Fig. 1 and Table 1).

SH2D1A mutations

All the mutations including unpublished data are summarized with the clinical data (Table 1). There were three gross deletions (the whole gene and two exons 3 and 4), four nonsense mutations (all Arg55stop), eight missense mutations (Ala3Ser, Tyr7Cys, two His8Asp, Gly27Ser, Asp33Tyr, Ser34Gly and Gly49Val), two small deletions (584delA and 1021delAA), two small insertions (312insG and 545insA), and two splicing anomalies (416C > T and IVS2 + 1G > A). The substitution of 416C with T revealed an aberrantly spliced cDNA with deletion of the last 22 bases of exon 1, and IVS2 + 1G > A resulted in skipping of exon 2.

Clinical characteristics of Japanese patients with XLP-1

Eighteen of the 33 patients (55%) had FIM or EBV–HLH, 12 patients (36%) had hypogammaglobulinemia, seven patients (21%) had malignant lymphoma or lymphoproliferative disease, and two patients (P4.2 and P7.2) had lymphocytic vasculitis. One patient (P7.1) had aplastic anemia. Twenty-seven patients (82%) were associated with EBV infection at the disease onset. Two patients (P16.1 and P19.3) presented with non-EBV–HLH. Interestingly, malignant lymphoma and lymphocytic vasculitis in P4.2 were not associated with EBV infection, but the patient later developed EBV–HLH at the age of 14 yr and died of HLH. Two patients (P17.2 and P21.1) had encephalitis: and P17.2 developed acute disseminated encephalomyelitis caused by human

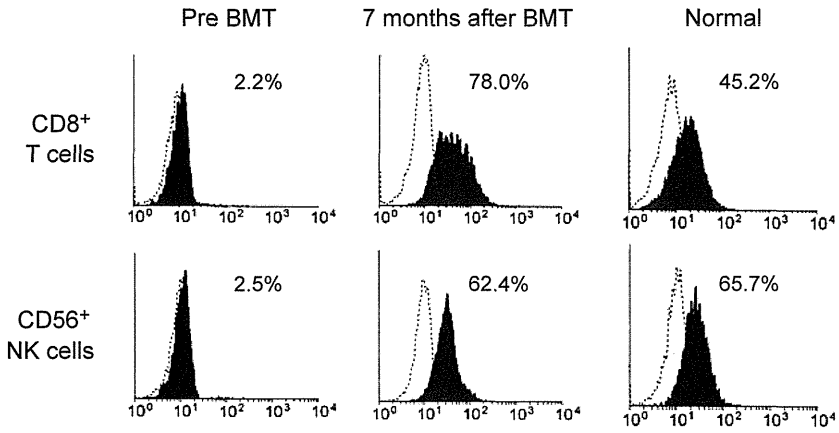

Figure 1 The SAP expression in CD8$^+$ T cells and NK cells from the patient (P16.1) and a normal adult donor. Dotted lines and shaded areas indicate staining by the control antibody and anti-SAP mAb (KST-3), respectively. A flow cytometric analysis demonstrated that deficient SAP expression in CD8$^+$ T cells and NK cells from the patient increased after he had undergone hematopoietic stem cell transplantation.

herpes virus 6 infection and P21.1 developed EBV encephalitis. Approximately 70% of the patients (23 of 33) were diagnosed by the time they were 5 yr of age, but two patients (P13.1 and P20.1) were diagnosed in adulthood. Eleven families (52%) had X-linked family histories. Ten patients (30%) presented with more than one clinical manifestation over time. Ten sibling cases were observed in this study, and seven families manifested different phenotypes. Fifteen patients (45%) were treated with intravenous immunoglobulin replacement therapy. In this study, the mortality rate was 21 of 32 patients (66%), and all the living patients were post-transplanted. Clinical characteristics of this study are summarized in comparison with those of previous study (Table 2).

Hematopoietic stem cell transplantation for patients with XLP-1

Twelve patients with XLP underwent HSCT in Japan (Table 3), and one patient (P9.2) died of *Pseudomonas* sepsis and multiple organ failure 14 days after HSCT. Two patients (P1.2 and P7.2) were transplanted from matched sibling donors, but the other patients were transplanted from matched or one-locus-mismatched unrelated donors, or mismatched familial donors. Various types of conditioning regimen were performed. Five patients (P1.2, P7.2, P9.1, P10.1, and P14.1) underwent HSCT following myeloablative conditioning, but the other patients did so following reduced intensity conditioning (RIC). Acute graft versus host disease (GVHD) was observed in 6 of 11 patients (Grade I, two patients; Grade II, three patients; Grade III, one patient). Chronic GVHD was observed in five patients, among whom 4 (P1.2, P7.2, P10.1, and P18.1) had extensive types and one (P14.1) had a limited type. Eleven patients (92%) have survived and had complete chimerism with a median follow-up of 7 yr and 9 months. A flow cytometric assay could be conducted to evaluate SAP expression in CD8$^+$ T cells and NK cells after HSCT in five patients (P7.2, P10.2, P16.1, P17.2, and P18.1). All the patients demonstrated an increase in SAP expression in CD8$^+$ T cells and NK cells after undergoing HSCT (Fig. 1).

Discussion

X-linked lymphoproliferative syndrome is a rare but life-threatening disease. A large cohort showed that most patients with XLP died by the age of 40 yr and more than 70% of the patients died before the age of 10 yr (2). Early diagnosis in non-familial cases may be difficult because XLP is heterogeneous in its clinical presentation. The ability to screen rapidly and make an accurate diagnosis of patients with XLP facilitates the initiation of life-saving treatment and preparation for HSCT. In a previous study, we generated an anti-SAP mAb, termed KST-3, which was applied to the flow cytometric evaluation of SAP deficiency (XLP-1) (11). All the patients evaluated in this study showed deficient SAP expression, although some patients with missense mutations might demonstrate normal expression of SAP, as shown in Western blotting (16).

Various types of *SH2D1A* mutation have been identified in Japan (11–15). The *SH2D1A*base (http://bioinf.uta.fi/SH2D1Abase) discloses that 133 unrelated patients were identified to have *SH2D1A* mutations. Missense and nonsense mutations appear in one-quarter each, and other types of mutation appear in half of the patients in this database. In the present study, Arg55stop mutations were most frequently found, in keeping with the *SH2D1A*base. No genotype and phenotype correlation was evident in this study, as well as in previous studies (1, 17).

Large cohort studies have shown that the major clinical phenotypes of XLP include FIM (60%), dysgammaglobulinemia (30%), and malignant lymphoma (30%) (1, 2). Aplastic anemia, lymphoid granulomatosis, and systemic vasculitis are minor clinical presentations at frequencies of approximately 3%. Although the present study included a limited number of patients with XLP-1, the distribution of the clinical manifestations seems to be similar to that in previous large studies