with minimal adverse effects; confirmatory results from further prospective studies are needed before optimal strategies for transplantation in PID patients can be fully determined.

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Evaluation of Risk Factors for Invasive Fungal Infection After Allogeneic Stem Cell Transplantation in Pediatric Patients

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Summary: Invasive fungal infections (IFIs) are a significant cause of morbidity and mortality after stem cell transplantation (SCT). The incidence, outcome, and risk factors for IFI after allogeneic SCT were analyzed in 149 pediatric patients treated at Hokkaido University hospital from 1988 to 2006. The cumulative incidence of IFI after allogeneic SCT was 8.1%; this comprised cases of proven, probable, and possible IFI at rates of 0.7%, 4.0%, and 3.4%, respectively. Only 1 patient complicated with IFI in the 100 days after SCT, excluding cases with rejection. Antifungal drugs were effective in 3 of the 12 patients with IFI, but the other 9 patients died because of IFI and relapse of original diseases. Nonrelapse mortality was markedly higher for patients with IFI than for those without IFI (60.0% vs. 20.0%, $P = 0.0204$). Univariate analysis showed that age at transplant, chronic graft-versus-host disease (GVHD), and a corticosteroid dose > 2 mg/kg or 60 mg/d for 10 days or longer were possible risk factors for IFI. Of these factors, chronic GVHD was the only factor associated with IFI in a multivariate analysis. Treatment of IFI is very difficult and, therefore, prevention of this condition is important, especially upon occurrence of chronic GVHD.

Key Words: invasive fungal infection, stem cell transplantation, GVHD

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Stem cell transplantation (SCT) is an effective therapeutic procedure in patients with hematologic disorders, malignant disorders, immunodeficiencies, and metabolic disorders. Recent methodological improvements in SCT have led to selection of this approach for patients with a relatively poor general status; however, invasive fungal infection (IFI) after SCT has been a problem for several decades and IFI is now a significant cause of morbidity and mortality after SCT.[1] Diagnosis of IFI is difficult and frequently the presence of fungal infection is not discovered until autopsy.[2] Despite advances in nonculture-based procedures, such as the introduction of the galactomannan antigen enzyme-linked immunosorbent assay and polymerase chain reaction tests for fungal DNA, timely diagnosis of IFI in SCT patients is still very difficult.[3,4]

Aspergillus spp. are the most common causative organism for IFI, with the incidence of invasive aspergillosis after SCT reported to be 6.3% to 15.1% in adult patients.[5,6] Reported risk factors for invasive aspergillosis include neutropenia, transplantation without laminar air flow equipment, transplantation from a matched unrelated donor, presence of graft-versus-host disease (GVHD), use of corticosteroids, older age, and underlying diseases.[5,6] The incidence of IFI among pediatric patients undergoing SCT has been less well studied. Several retrospective pediatric reviews have reported incidence rates of documented IFI ranging from 6% to 13%, with overall mortality rates of 4% to 17%.[7–9] Here, we report the incidence, outcome, and risk factors for IFI after SCT in pediatric patients treated at Hokkaido University hospital between 1988 and 2006.

PATIENTS AND METHODS

Patients

A total of 149 pediatric patients with different hematologic malignancies, metabolic abnormalities, and immunodeficiency received allogeneic SCT in our hospital between February 1988 and August 2006 (Table 1). Of these patients, 94 were boys and 55 were girls. Age of patients at transplantation was 3 months to 21 years (median: 8 y). Forty-four patients had acute lymphoblastic leukemia: 20 were in their first period of complete remission (CR), 16 were in a second CR, and 8 were in a third or later CR period. Twenty-nine patients had acute myelogenous leukemia: 14 were in their first CR, 11 were in their second CR, and 4 were in a third or later CR. Seventy-six patients had other diseases: 25 with aplastic anemia, 8 with myelodysplastic syndrome, 7 with neuroblastoma, 6 with juvenile myelomonocytic leukemia, 6 with non-Hodgkin lymphoma, 6 with chronic

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TABLE 1. Clinical Characteristics of 149 Pediatric Allogeneic SCT Patients

Sex	
Male	94
Female	55
Age	3 mo-21 y (median 8 y)
Disease	
Acute lymphoblastic leukemia	44
Acute myelogenous leukemia	29
Aplastic anemia	25
Myelodysplastic syndrome	8
Neuroblastoma	7
Juvenile myelomonocytic leukemia	6
Non-Hodgkin lymphoma	6
Chronic myelogenous leukemia	6
Wiskott Aldrich syndrome	5
Kostmann syndrome	3
Severe combined immunodeficiency	3
Hunter syndrome	1
Rhabdomyosarcoma	1
X linked hyper-IgM syndrome	1
Hurler-Scheie syndrome	1
Hurler syndrome	1
Chronic active Epstein-Barr virus infection	1
Natural killer/T leukemia	1
Origin of stem cell	
BM	108
PB	1
CB	40
Donor	
HLA-matched sibling	58
HLA 1 locus mismatched sibling	3
HLA 3 loci mismatched sibling	1
HLA-matched or mismatched parents	10
HLA-matched unrelated donor	47
HLA 1 locus mismatched unrelated donor	28
HLA 2 loci mismatched unrelated donor	2
Conditioning regimen	
BU contained	56
Melphalan contained	39
CY contained	101
ATG contained	40
TBI contained	77
GVHD prophylaxis	
CsA + MTX	68
CsA	3
CsA + mPSL	36
Tacrolims + MTX	29
MTX	10
mPSL	1
Tacrolims + mPSL	1
None	1
Year at transplantation	
1988-1990	5
1991-1995	18
1996-2000	51
2001-2006	75

ATG indicates antithymocyte globulin; BM, bone marrow; BU, busulfan; CB, cord blood; CsA, cyclosporine; CY, cyclophosphamide; mPSL, methylprednisolone; PB, peripheral blood; TBI, total body irradiation.

myelogenous leukemia, 5 with Wiskott-Aldrich syndrome, 3 with Kostmann syndrome, 3 with severe combined immunodeficiency, and 1 each with Hunter syndrome, rhabdomyosarcoma, X-linked hyper-IgM syndrome, Hurler-Scheie syndrome, Hurler syndrome, chronic active Epstein-Barr virus infection, and natural killer/T leukemia.

Treatment Protocols

The origin of the stem cells was as follows: 108 patients received bone marrow transplantation, 1 patient underwent peripheral blood stem cell transplantation, and 40 patients received cord blood stem cell transplantation. The transplantation donors were human leukocyte antigen (HLA)-matched siblings in 58 cases, a single-locus mismatched sibling in 3 cases, a 3-loci mismatched sibling in 1 case, HLA-matched or mismatched parents in 10 cases, HLA-matched unrelated donors in 47 cases, HLA single-locus mismatched unrelated donors in 28 cases, and 2-loci mismatched unrelated donors in 2 cases. The conditioning regimen contained busulfan for 56 patients, melphalan for 39 patients, cyclophosphamide for 101 patients, and antithymocyte globulin for 40 patients. In addition, total body irradiation was used as part of the conditioning regimen for 77 patients. Prophylaxis for GVHD was performed with cyclosporin A (CsA) and short-term methotrexate (MTX) for 68 patients, with CsA alone for 3 patients, with CsA and methyl prednisolone for 36 patients, with tacrolimus and short-term MTX for 29 patients, with MTX alone for 10 patients, with methyl prednisolone alone for 1 patient, and with tacrolimus and methyl prednisolone for 1 patient. One patient received no prophylactic therapy. Data were analyzed as of April 1, 2007.

Definition of IFI

Value of C-reactive protein was measured with an automated counter twice a week, at least. When fever continued, despite administration of broad-spectrum antibiotics, or high value of C-reactive protein was detected for several times, x-rays of the chest and computed tomography of chest and abdomen were inspected. Moreover, β-D glucan and *Aspergillus* antigen (galactomannan antigen) were inspected in those cases. We identified patients who developed proven, probable, or possible IFI at any point after SCT using standardized definitions set forth by the European Organization for Research and Treatment of Cancer and the National Institute of Allergy and Infectious Diseases Mycoses Study Group.[10]

Infection Prophylaxis

Until neutrophil engraftment, which was defined as an absolute neutrophil count of $>0.5 \times 10^9$/L for 3 consecutive days, patients were housed in high-efficiency particulate air-filtered isolation rooms and provided low microbial diets. Patients routinely received acyclovir (600 mg/m^2 p.o. 4 times daily) from day −7 to +35. Anticytomegalovirus high-titered γ-globulin was infused weekly from day −6 to +90, and intravenous granulocyte colony-stimulating factor was administrated from day +5 until neutrophil engraftment. Trimethoprim-sulfamethoxazole was used before day 0 and after

engraftment for prevention of *Pneumocystis carinii* pneumonia.

From February 1988 to March 2005, oral amphotericin B 100 mg/kg/d was used from before the conditioning regimen until the date of discharge (n = 125). From April 2005 to August 2006, intravenous micafungin 1 mg/kg/d was newly introduced from the start of the conditioning regimen until neutrophil recovery followed with oral fluconazole 10 mg/kg from after neutrophil recovery until the date of discharge (n = 24).

Statistical Analysis

A *t* test or χ^2 test was used to compare patients who did and did not develop IFI. Analysis of nonrelapse mortality was performed using Kaplan-Meier method, with differences compared by log-rank test. Stepwise multivariate regression analysis was performed to explore the independent effect of variables that showed a significant effect in univariate analysis. Statistical analyses were performed using Dr SPSS II for Windows (release 11.0.1J, SPSS, Japan, Inc).

RESULTS

Incidence and Timing of IFI

The cumulative incidence of IFI after SCT was 8.1% (12/149) (Table 2), and the 12 cases comprised 1 of proven IFI (1/149: 0.7%), 6 of probable IFI (6/149: 4.0%), and 5 of possible IFI (5/149: 3.4%). IFI occurred after relapse of the original disease in 3 patients, after rejection of transplant in 3 patients, during treatment of acute GVHD in 1 patient, and during treatment of chronic GVHD in 5 patients. Only 1 patient (case 2) had a history of previous IFI. Although he had multiple liver lesions before SCT, liver lesion vanished and lung lesion was evident after SCT. Excluding cases with rejection, only 1 patient complicated with IFI during the 100 days after SCT. The median time from the date of transplant to onset of infection was 11 months for all patients with IFI and 5 months after exclusion of patients who underwent relapse. Of the 12 IFI patients, 10 had a pulmonary-specific lesion (halo sign, air-crescent sign, or cavity within an area of consolidation) and *Aspergillus* antigen was detected from blood samples in 5 cases (cases 5, 7, 8, 9, and 10). In these 5 cases, only 1 patient was diagnosed as proven IFI because *Aspergillus* hyphae were detected in lung with autopsy (case 9). *Aspergillus* was detected from sputum culture in 1 patient (case 10). One of the 10 patients with pulmonary lesions also had a brain abscess. The other 2 patients had liver-specific lesions (bull's-eye lesions) and both were judged to have probable IFI (cases 11 and 12). One of these 2 patients also had a brain abscess (case 12). In 5 patients with possible IFI, all patients were immunosuppressive state and 5 patients had lung lesion. In these 5 patients, halo sign was detected with all patients, and air-crescent sign was detected with 2 patients (cases 1 and 3). Biopsy was done with no patients because of ill condition.

Risk Factors for IFI

Risk factors for IFI after transplantation were analyzed after exclusion of 3 IFI cases after relapse. Univariate analysis showed that age at transplant, chronic GVHD, and a corticosteroid dose of > 2 mg/kg or 60 mg/d for 10 days or longer were possible risk factors for IFI (Table 3). In patients with IFI, the median age at transplantation was significantly higher than that of patients who did not develop IFI, and most patients with IFI were over 10 years old. Of these factors, the presence of chronic GVHD was the only factor associated with IFI in a multivariate analysis (Table 4).

Outcome After IFI

The administration of antifungal drugs was as follows: 9 patients used fluconazol, 6 patients used amphotericin B, 6 patients used itraconazol, 6 patients used micafungin, 2 patients used voriconazol, 2 patients used miconazol, and 1 patient 5-fluorouracil. Antifungal therapy was effective in only 3 patients with IFI after transplantation. Two of these 3 patients are still alive, but the other patient died after relapse of leukemia. Including this patient, 3 patients who developed IFI died from relapse of the original disease, and other 7 patients died after developing IFI despite receiving antifungal treatment. The nonrelapse mortality was markedly higher in patients with IFI compared with those who did not develop IFI (60.0% vs. 20.0%, $P = 0.0204$).

DISCUSSION

Our results show that the cumulative incidence of proven, probable, or possible IFI in pediatric patients undergoing allogeneic SCT was 8.1%, and the rate of proven and probable IFI were 4.7%. Other recent studies of IFI in pediatric patients undergoing SCT have indicated incidence rates ranging from 6% to 13%.[7–9] In these reports, many patients complicated with IFI within 1 year after SCT; in contrast, only 6 of our patients (4%) developed IFI within 1 year, including 3 cases with graft rejection. The incidence of IFI among our patients was lower than those in past reports, and moreover, no patients who underwent autologous SCT complicated with IFI (33 patients, data not shown). The reason of the lower rate of IFI observed in this study is not clear. However, it is of note that after April 2005 we switched to intravenous micafungin for prevention of fungal infection, after initially using oral amphotericin B for this purpose, and IFI did not occur in any patients who received micafungin, including 2 patients with graft rejection. If patients who used micafungin excluded, patients developing IFI was 9.6%, and those within 1 year was 4.8%. It becomes not different with past reports. The efficacy of micafungin has been reported to be superior to that of fluconazole in antifungal prophylaxis during the neutropenic phase after SCT.[11] It might be controversial for preventing use of micafungin, because micafungin is relatively expensive. However, the number of cases in our study is too small to judge the effect of micafungin with certainty.

TABLE 2. Clinical Characteristics and Outcomes of 12 Patients Who Developed IFI After Allogeneic SCT

	Age	Sex	Disease	SCT		Conditioning	aGVHD	cGVHD	Lesion	Interval From SCT (M)	IFI Level	Treatment	Lymphocyte Counts at Onset	Outcome
1	12	F	MDS	First CR	R-BMT	BU, CY	4	+	Lung	16	Possible	AMPH	936	Death
2	13	M	AML	Second CR	U-BMT	BU, L-PAM, ATG	0	+	Lung	24	Possible	FCZ, ITCZ	1950	Survival
3	16	M	MDS	First CR	U-BMT	BU, VP16, CY	4	+	Lung	5	Possible	FCZ, AMPH	360	Death
4	7	F	AML	Second CR	U-CBSCT	BU, L-PAM	1	+	Lung	2	Possible	FCZ, AMPH, ITCZ	3400	Death
5	13	F	ALL	Second CR	U-BMT	TBI, L-PAM	0	–	Lung	41	Probable (mold) relapse	FCZ, MCFG	200	Death
6	12	M	AML	Second CR	R-BMT	BU, L-PAM	2	+	Lung	54	Possible relapse	MCFG, VCZ	0	Death
7	3	F	AML	Second CR	U-CBSCT	Flu, BU, ATG	0	–	Lung	7	Probable (mold) rejection	FCZ, MCZ, ITCZ	0	Death
8	15	F	ALL	Second CR	U-BMT	BU, L-PAM	0	–	Lung	3.5	Probable (mold) rejection	FCZ, MCFG, AMPH, ITCZ	300	Death
9	18	M	SAA		R-BMT	Flu, CY, ATG	0	–	Lung, brain	1.5	Proven (mold) rejection	FCZ, MCFG, AMPH, 5FC	100	Death
10	17	M	AML	First CR	U-BMT	TBI, L-PAM	2	+	Lung	15	Probable (mold)	FCZ, MCFG, ITCZ, AMPH	298	Death
11	14	F	CAEBV		U-CBSCT	Flu, VP16, TBI	3	+	Liver	5	Probable	FCZ, ITCZ	42	Survival
12	13	M	NK leukemia	Second CR	U-BMT	TBI, VP16, CY	0	+	Liver, brain	16	Probable relapse	MCFG, VCZ, MCZ	30,600	Death

aGVHD indicates acute graft versus host disease; ALL, acute lymphoblastic leukemia; AML, acute myelogenous leukemia; AMPH, amphotericin B; ATG, antithymocyte globulin; BMT, bone marrow transplantation; BU, busulfan; CAEBV, chronic active Epstein-Barr virus infection; CBSCT, cord blood stem cell transplantation; cGVHD, chronic graft versus host disease; CR, complete remission; CY, cyclophosphamide; 5FC, flucytosine; FCZ, fluconazole; Flu, fludarabine; ITCZ, itraconazole; L-PAM, melphalan; MCFG, micafungin; MCZ, miconazole; MDS, myelodysplastic syndrome; R, related; SAA, severe aplastic anemia; SCT, stem cell transplantation; TBI, total body irradiation; U, unrelated; VCZ, voriconazole; VP16, etoposide.

TABLE 3. Clinical Characteristics of 146 Pediatric Allogeneic SCT Patients Excluding 3 Cases With IFI After Relapse

	Total	IFI	No IFI	*P*
Sex				
Male	92	4 (44%)	88 (64%)	0.291
Female	54	5 (56%)	49 (36%)	
Age at transplant				
Median (range)		14 (3-17)	7 (0-21)	0.00148
≥ 10 y	53	7 (78%)	46 (34%)	0.012
< 10y	93	2 (22%)	91 (66%)	
Disease				
Malignant	106	7 (78%)	99 (72%)	1.000
Nonmalignant	40	2 (22%)	38 (28%)	
Donor				
Related	71	2 (22%)	69 (50%)	0.167
Unrelated	75	7 (78%)	68 (50%)	
Source				
CB	40	3 (33%)	37 (27%)	0.706
BM, PB	106	6 (67%)	100 (73%)	
Acute GVHD				
< Grade I	116	5 (56%)	111 (81%)	0.086
≥ Grade II	30	4 (44%)	26 (19%)	
Chronic GVHD				
Yes	32	6 (67%)	26 (19%)	0.004
No	114	3 (33%)	111 (81%)	
TBI				
Yes	75	2 (22%)	73 (53%)	0.091
No	71	7 (78%)	64 (47%)	
ATG				
Yes	40	4 (44%)	36 (26%)	0.258
No	106	5 (56%)	101 (74%)	
L-PAM				
Yes	37	4 (44%)	33 (24%)	0.232
No	109	5 (56%)	104 (76%)	
Busulfan				
Yes	55	6 (67%)	49 (36%)	0.081
No	91	3 (33%)	88 (64%)	
Neutrophil recovery				
Median		22	17	0.322
≥ 29 d	18	2 (22%)	16 (12%)	0.306
< 29 d	128	7 (78%)	121 (88%)	
CMV antigenemia				
Yes	36	3 (33%)	33 (24%)	0.690
No	110	6 (67%)	104 (76%)	
Steroids				
≥ 10 d (> 2 mg/kg or 60 mg/d)	54	8 (89%)	46 (34%)	0.002
< 10 d	92	1 (11%)	91 (66%)	
Micafungin				
Yes	24	0 (0%)	24 (18%)	0.356
No	122	9 (100%)	113 (82%)	

Statistical analysis was done by χ^2 test.

ATG indicates antithymocyte globulin; BM, bone marrow; CB, cord blood; CMV, cytomegalovirus; L-PAM, melphalan; PB, peripheral blood; TBI, total body irradiation.

TABLE 4. Multivariate Analysis of Risk Factors for IFI Excluding Cases With Relapse

Factor	Relative Risk of IFI	95% CI	*P*
Chronic GVHD			
Yes	6.055	1.198-30.606	0.029
Age			
≥ 10 y	2.884	0.657-12.653	0.160
Steroids			
≥ 10 d	4.327	0.741-25.283	0.104

Risk factors for IFI in pediatric patients after SCT have been reported as abundant candidal colonization, severe acute GVHD, extensive chronic GVHD, duration of neutropenia, age > 10 years old, transplant for severe aplastic anemia or Fanconi anemia, and high-dose corticosteroid administration.[7,9] In our study, the risk factors were age > 10 years old, presence of chronic GVHD, and high-dose corticosteroid administration. Dvorak et al[9] first showed that an age of 10 years old or older was a risk factor for IFI, and suggested that this may reflect the importance of host colonization by an environmental fungus as an important step in development of invasive disease, with younger patients having had less exposure time to fungal spores in the environment. In our study, multivariate analysis revealed that chronic GVHD is the most important risk factor for IFI after SCT. However, the 3 risk factors identified in our population are closely related, because chronic GVHD more frequently occurs in older children and high-dose corticosteroids are commonly used as treatment for chronic GVHD. This suggests that preventive use of an antifungal drug, such as an azole, is important upon occurrence of chronic GVHD. However, azoles interact with drugs such as CsA and tacrolimus, which are hepatically metabolized through the cytochrome P450 3A4 pathway. Moreover, fluconazole is not effective against the *Aspergillus* spp., which often accompanies occurrence of chronic GVHD. Therefore, the selection of the antifungal drug becomes extremely important. Although itraconazole is also a member of the azole class, it is recommended for antifungal prophylaxis in high risk neutropenic patients with hematologic malignancies or for prolonged prophylaxis after allogeneic SCT, on the basis of the unambiguous evidence of efficacy and low toxicity in clinical trials and systematic reviews.[12] On the other hand, cost of antifungal drugs are high and appearance rate of side effect with antifungal drugs are relatively high. Therefore, preventive use of antifungal drug may have to be only for high-risk patients of IFI.

The mortality rate of IFI was extremely high in our cases, despite the use of several antifungal drugs. Outcomes are also extremely poor in adult patients with IFI after allogeneic SCT, with a 1-year mortality rate of 68% to 90%.[13–15] In children, the reported mortality rate varied from 4% to 67%.[7,9,16] Although new antifungal drugs such as voriconazole and liposomal amphotericin B have been available, the prognosis for patients who develop IFI after SCT remains poor. Our data were based on retrospective study, valuable diseases, and long-term period. However, our results suggest that IFI after allogeneic SCT is associated with chronic GVHD in childhood, and because treatment of IFI is very difficult we suggest that prevention is of utmost importance, particularly in cases with chronic GVHD. Prospective study with larger scale of pediatric patients will be necessary for determination of risk factor of IFI with SCT.

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Clinical Report

Epstein–Barr Virus-Associated B-cell Lymphoma in a Patient With DNA Ligase IV (LIG4) Syndrome

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A 14-year-old Japanese girl with a progressing combined immunodeficiency had developed non-Hodgkin's diffuse large B cell lymphoma. Her molecular analysis showed a compound heterozygote of novel mutations in the *LIG4* gene, M249V substitution and a five nucleotides deletion from nucleotide position 1,270–1,274. She had also a set of characteristic clinical features of LIG4 syndrome. Mutations in the *LIG4* gene, which plays a critical role in the repair of DNA double-strand breaks, imply a correlation with malignancies and several cases with leukemia or lymphoma have already been reported. We report here on a case of LIG4 syndrome complicated with distinct EBV-associated B-cell lymphoma. © 2007 Wiley-Liss, Inc.

Key words: LIG4 syndrome; DNA ligase IV; immunodeficiency; EBV; lymphoma

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INTRODUCTION

DNA ligase IV (LIG4) syndrome (OMIM #606593) is a rare autosomal recessive disorder arising from mutations in the DNA ligase IV gene, which plays a critical role in the repair of DNA double-strand breaks by non-homologous end-joining mechanism [Riballo et al., 1999; O'Driscoll et al., 2001]. It is characterized by chromosomal instability, immunodeficiency, and developmental delay. Out of 11 previously reported patients, two cases with T cell leukemia, one case with B-cell lymphoma and one case with myelodysplasia have been reported, suggesting an increased risk for lymphoid malignancies in this disorder [Riballo et al., 1999; O'Driscoll et al., 2001; Ben-Omram et al., 2005; Buck et al., 2006; Enders et al., 2006; van der Burg et al., 2006]. In addition, a possible correlation between polymorphisms in the *LIG4* gene and a risk for malignancies such as breast cancer and multiple myeloma has been suggested [Goode et al., 2002; Roddam et al., 2002]. We present here a case of LIG4 syndrome complicated with Epstein–Barr virus (EBV)-associated large B-cell lymphoma.

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MATERIALS AND METHODS

Patient

A 14-year-old Japanese girl was admitted to Hokkaido University Hospital because of progressive gingival swelling and high fever. Her underlying clinical and laboratory features have already been precisely reported [Yamada et al., 2001]. Briefly, she had polydactyly, proportional microcephaly, short stature, and progressively decreasing serum levels of IgG and IgM, and the numbers of both peripheral B and T cells. Her skin fibroblast showed both spontaneous chromosome aberration and ionizing irradiation hypersensitivity but not mitomycin C hypersensitivity. Her clinical features including microcephaly, immunodeficiency and radiosensitivity resembled that of Nijmegen breakage syndrome (NBS) other than Bloom syndrome, Fanconi anemia, ataxia telangiectasia, and radiosensitive SCID (Artemis deficiency). However, no mutation was found in the coding lesion of *NBS1*. As well, a normal level of NBS1 protein was expressed in her fibroblast. She had been protected from severe infections by monthly intravenous immunoglobulin replacement therapy.

On admission, she had a whitely coated tumor involving her left upper gingiva and hard palate without apparent lymphadenopathy or hepatosplenomegaly. Laboratory findings were as followings; white blood cell count 1.5×10^9/L with 66% of neutrophils and 11% of lymphocytes, hemoglobin 97 g/L, platelet count 38×10^9/L, and C-reactive protein 26.3 mg/L. Flow-cytometry analysis of her peripheral blood mononuclear cells showed decreased numbers of both T ($CD3^+$, 33.1%) and B cells ($CD20^+$, 0.1%). T2-weighted magnetic resonance imaging showed a mixed intensity mass in the left maxillary sinus. The histopathological diagnosis was made as non-Hodgkin's diffuse large B cell lymphoma.

No abnormal cells were detected in either her peripheral blood or bone marrow. She was treated with the combination of reduced amounts of vincristine, cyclophosphamide, and prednisolone. Following this, erythematous desquamation of the skin, severe diarrhea, and severe persistent neutropenia developed. Finally, serious pulmonary aspergillosis developed despite anti-fungal therapy. She died of respiratory failure after four months of hospitalization. An autopsy was not performed.

Histopathology

Tissue sample from the left maxillary sinus was fixed in 10% buffered formalin, embedded in paraffin, and stained with H&E. immunostains for CD3, CD20/L26, CD79a (DAKO, Kyoto, Japan) were performed for phenotypic analysis of the proliferating lymphocytes. Detection of EBV was done by immunostains for latent membrane protein (LMP-1) and EBV-determined nuclear antigen 2 (EBNA2) (DAKO) and by in situ hybridization with an oligoprobe for EBV-encoded small non-polyadenylated RNAs (EBER) and with a sense probe (negative control) [Weiss et al., 1992].

DNA Sequencing

Genomic DNA was extracted from both peripheral blood neutrophils and skin fibroblasts. Overlapping fragments of the coding lesions of *LIG4* were amplified by polymerase chain reaction (PCR; 35 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min) with primers according to the previous report [O'Driscoll et al., 2001]. PCR products were supplied for direct sequencing reaction. Both PCR amplification and sequencing reaction were performed by GeneAmp PCR System 2400 (Perkin Elmer, Foster City, CA). Sequencing analyses were performed by ABI PRISM Gene Analyzer 310 (Perkin Elmer).

RESULTS

Histopathological Findings

Immunohistochemistry of the tumor cells demonstrated positive for both CD20 and CD79a but negative for CD3, and was histopathologically diagnosed as diffuse large B cell lymphoma (Fig. 1). Clonal immunoglobulin heavy chain gene rearrangement was noted by PCR analysis, suggesting monoclonal proliferation of the tumor cells. Although no EBV genome was demonstrated by semi-quantitative PCR analysis in her peripheral circulation, tumor cells were positive for EBV-encoded RNAs (EBERs), latent membrane protein-1, and EBV-determined nuclear antigen 2, indicating EBV-related type III latency [Okano and Gross, 2000].

Sequencing Analysis and Immunoblot Analysis

We detected novel mutations, a five nucleotide deletion from 1,270 to 1,274 and an 745A > G substitution of the *LIG4* gene, which result in a frame shift at the amino acid position 424 and M249V amino acid substitution, respectively (Fig. 2). Compound heterozygosity was confirmed by sequencing analyses of both cloned DNA from the patient and DNA from her parents. The small deletion was inherited from her mother and the point mutation was identified in her father (data not shown). Western blot analysis revealed reduced LIG4 protein levels in her fibroblasts using monoclonal antibody for LIG4 (purchased from Santa Cruz Biotechnology, Santa Cruz, CA), compared with control cells (data not shown).

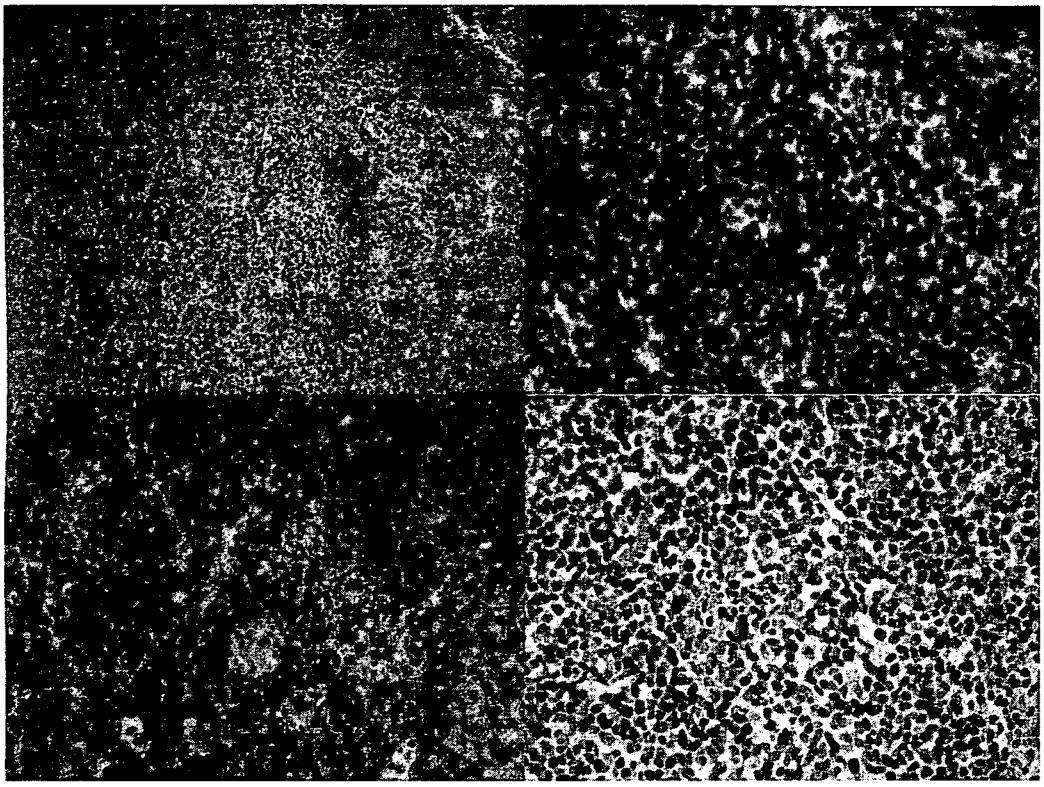

FIG. 1. Histopathology of the tumor. Tumor section was stained with hematoxylin-eosin (**A**; original × 100). Tumor cells are positive for CD79a (**B**; original × 400) and latent membrane protein-1 (**C**; original × 400) by immunohistochemistry. In situ hybridization study demonstrated EBV-encoded RNAs in the tumor cells (**D**; original × 400). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

DISCUSSION

Here we describe a case of LIG4 syndrome complicated with distinct EBV-associated B-cell lymphoma, who had many phenotypic characteristics resembling NBS. O'Driscoll et al. [2001] screened NBS-like patients who had no mutations in *NBS1* and produced normal levels of nibrin, then identified several patients with mutations in *LIG4*. We also identified two novel mutations in the *LIG4* gene, a five-nucleotides deletion and M249V substitution. The small deletion result in a frame shift and its product lacking C-terminal XRCC4 binding site could be non-functional [Grawunder et al., 1998]. Methionine at amino acid 249 is located near an ATP-binding site and is conserved among other DNA

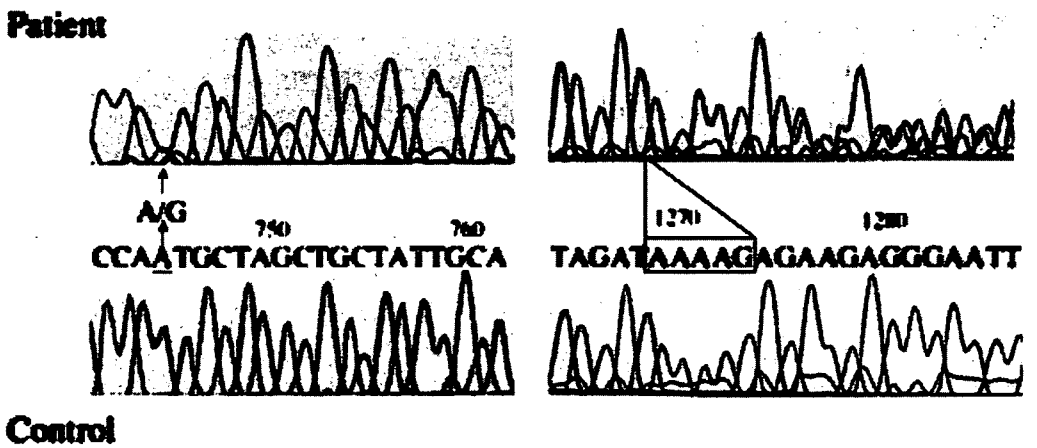

FIG. 2. Direct sequencing analysis demonstrates mutations in *LIG4*. The sequence of DNA from the patient shows heterozygote of 745A and 745G (**left upper panel**). A five nucleotides deletion from nucleotide position 1,270–1,274 (AAAAG) results in frame shift (**right upper panel**). Each mutation was detected on a different allele by sequencing analyses of cloned DNAs (data not shown). **Lower panels** show the sequence of DNA from a normal individual. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

ligases [Wel et al., 1995]. However, M249V mutant could be partially functional, because LIG4 knockout mice show embryonic lethality [Frank et al., 2000]. LIG4 plays a critical role in both DNA replication and V(D)J recombination [Riballo et al., 1999; O'Driscoll et al., 2001]. Thus its deficit might cause the death of the cells with non-functional repair or recombination during meiosis or rearrangement of both T and B cell receptors, which promotes a progressive combined immunodeficiency in LIG4 syndrome. Such a slow progression of the disease as in our case might be one of the characteristic features of LIG4 syndrome.

We detected EBV-specific RNAs and latent proteins in the tumor cells in our case, although no EBV genome was detected in her peripheral blood by PCR. EBV infection to B cells causes infectious mononucleosis in immunocompetent individuals, but promotes lymphoproliferative disorders including malignant lymphoma in immunocompromised hosts particularly with profound T cell defect [Okano and Gross, 2000]. Thus, it is possible that her deficient immunosurveillance allowed the development of EBV-associated B cell lymphoma. Otherwise, the defects in damaged-DNA repair might be responsible for the development of her malignancy as seen in other chromosome breakage syndromes such as NBS and ataxia telangiectasia. There have been two cases with T cell leukemia, one case with B-cell lymphoma and one case with myelodysplasia complicated with LIG4 syndrome to date. Collectively, LIG4 syndrome patients are likely to be at increased risk for lymphoid malignancies.

The desquamative erythema, protracted diarrhea, and neutropenia during the reduced chemotherapy could be correlated with the defect in repair of DNA damage induced by the cytotoxic agents used for her treatment. Accordingly, haematopoietic stem cell transplantation that requires cytotoxic agents for myeloablation could be harmful for patients with this syndrome. Instead, anti-CD20 antibody (rituximab) or adoptive EBV-CTL therapy may be beneficial for EBV-associated lymphoma in such patients.

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原発性免疫不全症とリバージョン；「適応の破綻とその修復」のモデルとして

有賀　正*

要旨　原発性免疫不全症（primary immunodeficiency diseases；PID）は病因遺伝子の変異によって免疫機構に重大な障害が生じ、易感染性（悪性腫瘍、自己免疫疾患も発症）を示すことが特徴であり、このことは個体レベルでの適応破綻状態に該当する。さらにPIDの一部の疾患では免疫担当細胞の分化・増殖が損なわれる病態を示し、細胞レベルでの適応破綻状態をも呈している。一方、一部のPID患者において親由来の変異が *de novo* で修復、あるいは修飾された（併せてここではリバージョンと称する）細胞群をモザイク状態で保有している報告がされてきている。報告の中にはそのPID患者の臨床症状が予想以上に軽減している症例も認められた。この現象は、オリジナルの患者細胞が病因遺伝子の変異によってその機能が破綻し、その結果生体も適応の破綻としての免疫不全状態に陥っているという状況が、リバージョンによって細胞レベルでも、生体としても修復が一部でもたらされた事例と考えられる。リバージョンは自然に起こった遺伝子治療とも見なすことができ、この現象を認めた疾患では実際の遺伝子治療臨床研究を計画する際、治療効果が期待できる根拠として挙げている。本稿では我々が経験したPID患者におけるリバージョン事例を数例提示し、リバージョン発見の経緯とそのメカニズム、意義を述べる。

Spontaneous reversion of inherited mutations detected in some patients with primary immunodeficiency diseases ; a model of adaptation failure and its restoration.

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Abstract　Primary immunodeficiency diseases (PID) are characterized by their immunocompromised status, indicating one of adaptation failure diseases. Furthermore, adaptation failure in some PID is also observed in their cellular level ; immunocytes carry some defects in growth or development. Recently, somatic mosaicisms due to reversion to normal of inherited mutations have been

discovered in some PID patients. The immunocytes with reversion have growth/survival advantage over the original cells and milder clinical courses than expected, were observed in some of those patients; therefore, reversions in some patients with PID are considered restoration of the adaptation failure, and thus designating "natural gene therapy". I will show our cases with PID (cases with adenosine deaminase deficiency and Wiskott-Aldrich syndrome) who were revealed to have somatic mosaicism with *de vivo* reversion, then, the significance in the role of growth advantage in view of recent results of gene therapy will be discussed.

● Key words : primary immunodeficiency diseases, reversion of inherited mutations, Wiskott-Aldrich syndrome, adenosine deaminase deficiency, growth advantage

1. はじめに

原発性免疫不全症 (primary immunodeficiency diseases ; PID) は発症頻度が稀ではあるがその病態解析が免疫学の発展に貢献してきたこと、また人に対しての遺伝子治療臨床研究が初めて実施された対象疾患だったことなどから注目されてきた疾患群である。PIDの病因遺伝子は多岐にわたるが、共通した病態として病因遺伝子の変異によって免疫機構に重大な障害が生じ、易感染性 (悪性腫瘍、自己免疫疾患も発症) を示すのが特徴である[1]。このことから、PIDは病原体の存在する環境において個体レベルで適応が破綻している状態と考えられる。さらに一部の疾患では免疫担当細胞の分化・増殖が損なわれる病態を示し、細胞レベルでの適応破綻状態をも呈している。

一方、近年一部のPID患者において親由来の変異が*de novo*で修復、あるいは修飾された (併せてここではリバージョンと称する) 細胞群をモザイク状態で検出する報告が増えてきている[2]。報告の中にはそのPID患者の臨床症状が予想以上に軽減している症例も認められた。この現象は、オリジナルの患者細胞が病因遺伝子の変異によってその機能が破綻し、その結果生体も適応の破綻が生じて免疫不全状態に陥っているという状況が、リバージョンによって細胞レベルでも、生体としても適応の破綻が一部で修復された事例であると考えられる。リバージョンは自然に起こった遺伝子治療としても見なすことができ、この現象を認めた疾患では実際の遺伝子治療臨床研究を計画する際に治療効果が期待できる根拠として挙げている。

本稿では我々が経験したPID患者におけるリバージョン事例を数例提示し[3-5]、リバージョン発見の経緯とそのメカニズム、意義を述べることによって「適応の破綻と修復」の主旨の内容としたい。

2. リバージョンが認められたPID

これまでにリバージョンを認めたPIDをTable 1に示した。いずれの場合もリバージョンが起こった細胞はオリジナルの細胞に比べて増殖優位性（あるいは生存優位性）を示す。この性状がなければリバージョンが起こったとしてもその細胞集団を検出することは困難である。後述するが、この性状が現状の血液幹細胞を標的とした遺伝子治療の成否に重要な条件となっている。

Table 1. PID reported with reversion cases

- X-linked severe combined immunodeficiency （X-SCID）
- Adenosine deaminase （ADA） deficiency
- Omenn syndrome
- Wiskott-Aldrich syndrome （WAS）
- Leukocyte adhesion deficiency I （LAD-I）
- Bloom syndrome

Table 2. Clinical profile of two patients with ADA deficiency

Patient 1 （AT）

She frequently showed infectious respiratory symptoms since two weeks after birth, and also showed failure to thrive. She was suspected to have immunodeficiency status because of her severe lymphopenia (200/mm^3) . Screening and quantitative of ADA analysis was abnormally low, and *ADA* gene analysis revealed she had ADA deficiency. She had compound heterozygous *ADA* gene mutation with paternal Q119X and maternal R235Q.

Patient 2 （MT）

She had intractable impetigo at one month of age. She was suspected to have immunodeficiency status because of her severe lymphopenia (84/mm^3) . Screening and quantitative of ADA analysis was abnormally low, and *ADA* gene analysis revealed she had ADA deficiency. She had compound heterozygous *ADA* gene mutation with paternal del314A and maternal R235Q.

3. 我々が経験した リバージョンを認めたPID症例

（1）Adenosine deaminase （ADA）欠損症

ADA欠損症は、常染色体劣性遺伝形式の重症複合免疫不全（SCID）であり、*ADA* 遺伝子の変異が原因である。核酸代謝酵素ADAの欠損のため毒性代謝産物の蓄積で幼弱な免疫担当細胞が死滅することによって重篤な免疫不全が生じる。根治治療としては他のタイプのSCID同様に骨髄幹細胞移植が考えられるが、本疾患特有の治療法としてはADA酵素補充療法が挙げられる。また、本疾患はヒトにおいて初めて遺伝子治療が実施された対象疾患として注目されている。我々は、2例のADA欠損症患者においてリバージョンと思われる現象を検出した[3]。2症例の簡単なプロフィールをTable 2に示す。ADAの遺伝子変異はADA症例1では父由来のQ119Xと母由来のR235Qであり、ADA症例2では父由来のdel314A（P104以降のフレームシフト）と母由来のR235Qで、両者ともにコンパウンドヘテロの変異であった。両患者の末梢血からT、B、細胞株の樹立をHerpes virus saimiri, EB virusを用いてそれぞれ試みた結果、両者からそれぞれの細胞株が樹立した。その細胞株のADA酵素活性を測定したところ、両者のB細胞株はきわめて低い値であったが、

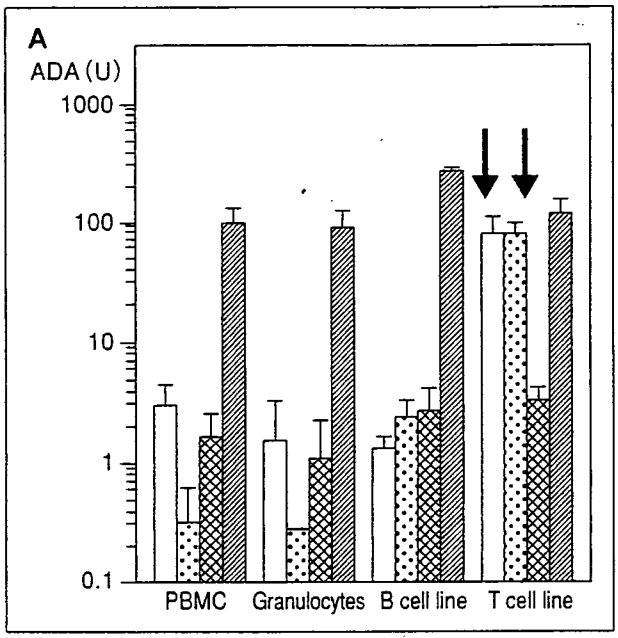

Figure 1. ADA enzyme activities (A) and ADA protein levels (B) in tne patients' cell lines

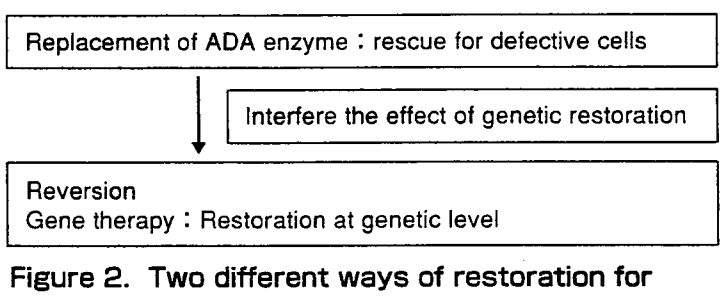

Figure 2. Two different ways of restoration for adaptation failure in ADA deficiency

予想外にもT細胞株はともに保因者レベルの高い活性を示した (Figure 1A)。ウエスタン法によるADA蛋白レベルの検査は酵素活性の結果と一致した (Figure 1B)。両症例のT細胞株が高いADA活性を示した機序の解明のため、この細胞株でのADA遺伝子の変異を再確認したところ、患者1のT細胞株では父由来の変異が正常化し、患者2では母由来の変異が正常化していた。HLA等の検索から、これらの細胞株は患者本人のものであることを確認し、T細胞受容体の解析から多クローンであることが示された。以上より、両症例の体内では片親由来の変異がリバージョンで解消された細胞群の存在が示唆された。

その後、症例2はHLA一致の同胞からの血液幹細胞移植を実施されたが、症例1ではADA酵素補充療法が開始され、その効果として末梢血T細胞の著増を認めた。症例1では酵素補充開始後何度かT細胞株の樹立を試みたが繰り返し不成功に終わり、体内でのリバージョンをもつT細胞群がオリジナルの細胞に希釈され、検出不可能になったと考えられた。ADA欠損症における適応の破綻の修復はFigure 2のような二通りが考えられる。酵素補充は酵素補充によって環境を変えることにより適応破綻している細胞を救済し、リバージョンは遺伝子レベルで破綻の修復を行っている。注目すべき点は、お互いの修復機構が干渉することで、このことは実際の遺伝子治療を実施する際に酵素補充療法の弊害 (酵素補充療法を併用した血液幹

Table 3. Clonal analysis of the WASP mutations detected in the WAS < patient 1>

	no. of clones studied	clones with A+/G-	G-	A+	no mutation
Patient	51	90.2%	9.8%	0%	0%
Mother	15	0%	40%	0%	60%
Sister	20	0%	45%	0%	55%
Control	10	0%	0%	0%	100%

細胞遺伝子治療では、遺伝子導入細胞の増殖優位性が損なわれ効果が期待できない）として再認識されている。

(2) Wiskott-Aldrich症候群 (WAS)

WASはX-連鎖遺伝形式のPIDであり、臨床的に免疫不全による易感染性、難治性湿疹、血小板減少による出血傾向を三主徴とする疾患である。自己免疫疾患や悪性腫瘍の発症も高率で長期的予後は不良である。原因分子としてアクチン調節／シグナル関連分子であるWASPが同定され、*WASP*遺伝子変異によるWASP分子の欠陥（ほとんどが欠損）が病因である。WASの診断は家族歴、臨床的特徴から疑われ、flow cytometryで細胞内のWASP分子の検索でスクリーニング診断され、最終的には遺伝子解析で行われる。根治的な治療は血液幹細胞移植が唯一であるが、遺伝子治療臨床研究の開発も進行中である。我々は3症例のWASでリバージョンを一部の細胞群に認めており、他の報告とあわせると本疾患においてはリバージョンが稀でなく起こる可能性が示唆されている。以下に、2症例での結果を紹介する。

① WAS症例1

患者はすでに死亡していて、保存されていたリンパ球ペレットから解析を行った。その結果、WASP遺伝子のエクソン10に約30塩基はなれて二つの変異を検出した。一つは一塩基Aの挿入で、他は一塩基Gの欠出であった。よく観察するとAの挿入が100%ではないことが推測され、変異を含むPCRフラグメントをクローニングして変異の状況を家族とともに解析した（Table 3）。その結果、患者ではGの欠出は100%であったがAの挿入は約90%で、挿入のない約10%のクローンを認めた。母、姉は保因者でいずれも約半数にGの欠出を認めたが、Aの挿入クローンは認めなかった。これらの結果は患者の体内で一部の細胞にAの挿入が加わり、その細胞群が増殖優位性を発揮して大多数の細胞集団となったことが示唆された。Aの挿入によってG欠出によるフレームシフトが解消され、WASP機能がある程度回復したことが背景にあると推察される。

② WAS症例2

WAS症例1での知見の後、同様の事象が他のWAS症例に起こっていないかを注意深く調べた。我々が確立したflow cytometryでWASP分子を検索する方法[6]はこの目的に極めて有用であった。WAS症例2の解析をこの方法で解析したところ大多数のWASP陰性細胞以外にWASP陽性の細胞群をリンパ球ゲートに認め（Figure 3A）、その性状を検索した。その結果、WASP陽性細胞は主にT細胞に（Figure 3B）多クローンで認め、樹立した細胞株では陽性細胞の比率と塩基配列での変異／正常の比率が一致した（Figure 3C）。WASP陽性細胞が患者本人の細胞であることはHLA等の解析から確認されたことから、母親由来の変異が生体内でリバージョンにより解消し、その細胞が増殖優位性を示した結果、このよう

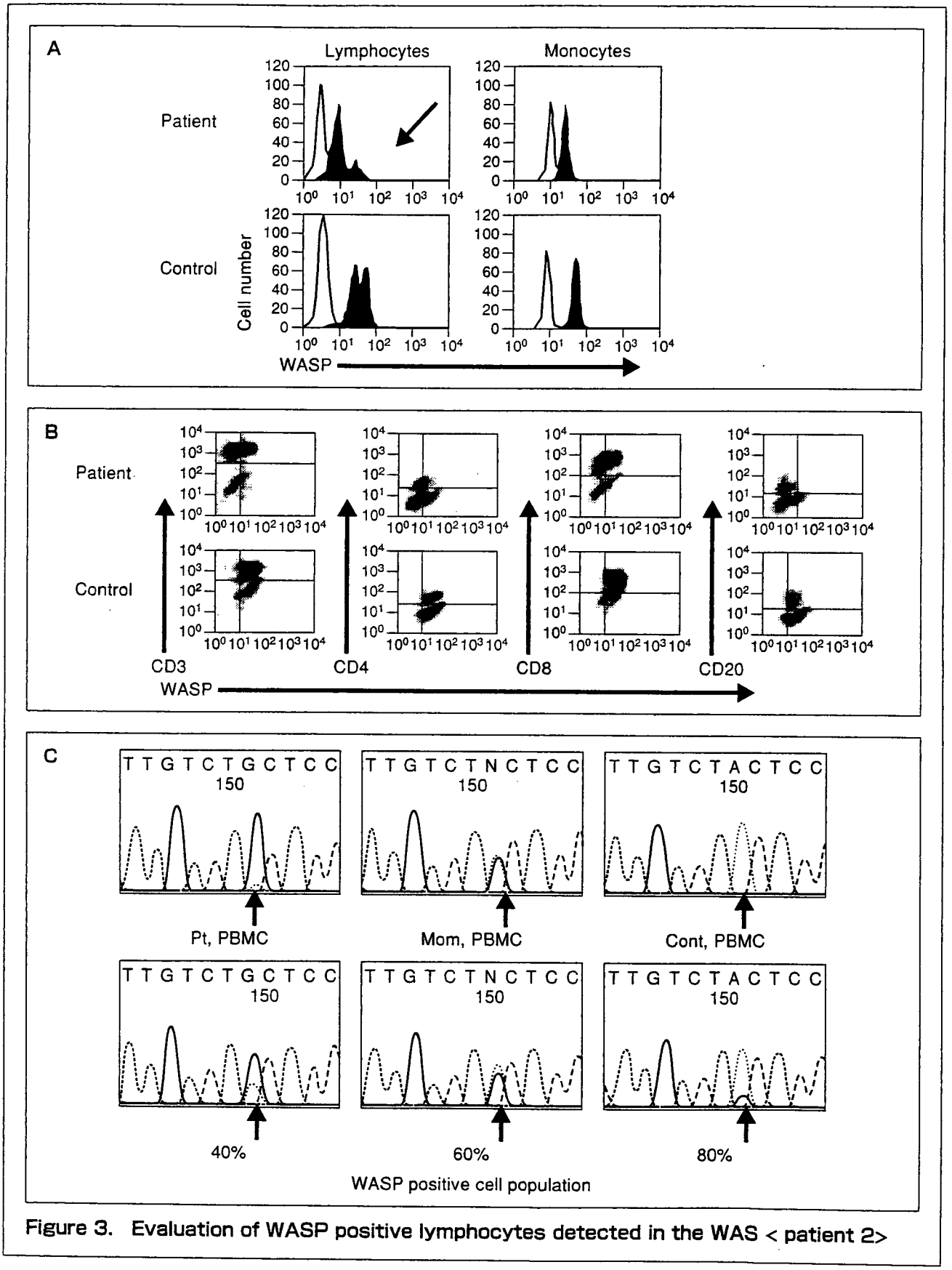

Figure 3. Evaluation of WASP positive lymphocytes detected in the WAS < patient 2>

な現象が検出されたと思われる。WASに認められるリバージョンの症例は我々の報告後に複数の報告があり稀ではないことが示唆されている。WAS症例におけるリバージョン事例の確認は、本疾患に対する血液幹細胞遺伝子治療の可能性を支持する重要な事象と考えられる。

4. 最後に

PIDは病原体が存在する環境において、個体レベルで適応が破綻している状態と考えることができる。その適応の破綻に対する修復は、PIDに対する治療を意味するが、本稿で紹介したリバージョンのような自然の修復事象も存在する。本稿ではPIDにおけるリバージョンという事象から、適応の破綻に対する異なった角度からの修復が時に干渉し合うこと、また患者に見られた稀な修復事象（リバージョン）が新たな将来的根治治療（遺伝子治療）に対して示唆を与えうることを紹介した。

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神経疾患に対する遺伝子治療の可能性
—現状と問題点—

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Ⅰ．はじめに

遺伝子治療は新しい概念の治療法としてすでに遺伝性疾患をはじめ悪性腫瘍，心血管系の疾患などの一部でその実施が開始されており，将来的には難治性感染症，自己免疫疾患，変性疾患などへの応用や，移植・再生医療の補助療法，ワクチンへの展開などが期待される治療技術である．特に現状では有効な治療法が確立していない多くの疾患に対して，その応用による新たな局面が期待されている．その意味で神経疾患は有効な治療法が確立されていない病態が多く，遺伝子治療研究が盛んに行われている領域である．本講演では，代表的な神経変性疾患に対しての遺伝子治療の可能性について現状と問題点を含めて解説したい．

Ⅱ．遺伝子治療技術の現状

現状の遺伝子治療では正常な遺伝子を標的細胞へ導入し，導入遺伝子が長期にわたって発現することによって病態を改善させることを目標としている．つまり，標的細胞に不足している分子を補うことが主な戦略であり，変異している遺伝子の修復や，病因分子の作用を抑制するような技術はまだ研究段階にとどまっている．言い換えると，遺伝子を治療するのではなく，遺伝子で治療するのが現状の遺伝子治療である．導入遺伝子の発現も本来の発現パターンを再現する技術はまだ確立しておらず，強制的に発現させるような方法が用いられているが，それでも長期にわたると体内での発現低下が問題になっている．また，標的細胞は体細胞に限られ，子孫に影響を及ぼす生殖細胞の遺伝子を改変する操作は認められていない．さらに，出生後の治療が主体であるため，発生過程の異常による構造異常に起因する疾患に対しては無力である．

Ⅲ．遺伝子治療の適応が考慮されている神経関連疾患（表1）

症状が神経系に及ぶ疾患には各種の病態があり，病態が神経系に限局したものもあるが，代謝病など全身におよぶ病態の一部としての神経疾患も存在する．これらの神経関連疾患のなかで遺伝子治療が考慮されている疾患を表1に示した．すべての疾患が適応になるわけではないが，現状の治療法では確実な治療効果の得られない多様な病態が含まれている．神経疾患に対する遺伝子治療は表2のようにこの分野特有の特徴を有しており，治療効果を得ることは必ずしも容易ではない．しかし，現状での治療ではきわめて限られた効果しか得られない疾患が多いため，遺伝子治療に対する期待が大きく，臨床応用を目指