the second largest report so far and the largest in Japan. Our aim in this retrospective study is to elucidate problems of allogeneic HSCT for this rare disorder and to devise methods to overcome these problems throughout our experience.

To our knowledge, 21 XHIM cases for which HSCT was performed have been reported in thirteen papers [12–24]. Of these, nine patients received marrow from their HLA-matched siblings, seven from HLA-matched unrelated donors, one from HLA one antigen-mismatched sibling, three from HLA one antigen-mismatched unrelated donors, and one from HLA one antigen-mismatched umbilical cord blood. T cells were depleted in seven cases whose donor was unrelated. Conditioning regimens consisted of busulphan and cyclophosphamide with or without anti-thymocyte globulin (ATG) in 14 patients, and two of these 14 also received anti-LFA-1 antibody and anti-CD2 antibody. Other myeloablative regimens, such as total body irradiation and cyclophosphamide, were used in three patients. Nonmyeloablative conditioning regimens consisted of fludarabine and melphalan with or without ATG or anti-lymphocyte globulin and were used in four cases with high-risk features, such as severe pre-transplant liver complications or sclerosing cholangitis. Although these preliminary reports are relatively successful, Khawaja et al. reported four failures out of eight transplanted patients, and that risk and death were associated with older age, pre-transplant liver damage, and cryptosporidial infection [20].

In our series, a successful outcome was achieved in five of the seven cases whether the stem cell source was an HLA-matched sibling or HLA-matched unrelated donor. In the previous reports, T-cell depletion or CD34-positive selection was often performed in order to prevent GVHD when unrelated donor marrow was selected [18,20]. However, we did not use either method for our patients because they are sometimes associated with a risk of graft rejection and EBV-associated post-transplant lymphoproliferative disease (PTLD) when transplanting the immunodeficiency patients [11]. As GVHD in our series were relatively well controlled except for one case (Patient 2), we believe that HLA-matched unrelated donor marrow transplant can be safely performed without T-cell depletion or CD34-positive selection. Among our successful five cases, the conditioning regimen, consisting of busulphan and cyclophosphamide, was well tolerated, and full engraftment and normal CD40L expression were established. Full immunological reconstitution has been achieved for all the patients with observation times of more than 3 years post-transplantation. Elevated serum levels of sCD40L were observed in all of these patients, which may indicate an immune reconstitution process in transplanted XHIM patients. Although *P. carinii* pneumonitis recurred in two cases, there were no other significant post-transplant infections. Other transplantation-related complications, such as acute GVHD, chronic GVHD, and thrombotic microangiopathy, were also well tolerated.

However, HSCT is not without risk, and two patients died. As described before, one patient suffered from pre-transplant cryptosporidial infection-related complications with poor performance state, which was a very high risk for transplantation procedure. Another patient also suffered from pre-transplant complications involving the lung, but the failure was due to severe post-transplantation infection complicated with secondary graft failure and grade 4 acute GVHD. It has been observed that older patients with primary immunodeficiency disorders tend to be at higher risk for HSCT [11,20]. The reason for this is that they may have more severe pre-transplant infections and may have serious damage in critical organs such as lung and liver because of recurrent or persistent infections. In our series, the younger patients tended to produce a good outcome as in previous reports, while the older patients also had a successful course when their status before HSCT was well managed. As for pre-transplantation infections in our series, only one patient had cryptosporidial infection and five out of seven patients had previous episodes of *P. carinii* pneumonitis. As mentioned in previous reports, cryptosporidial infection seemed to correlate with a poor outcome, but *P. carinii* infection did not [20]. Although both two patients with poor outcome had *P. carinii* pneumonitis previously, *P. carinii* infection itself was well managed after transplantation. The other two patients with previous episode of *P. carinii* pneumonitis suffered from its reactivation after HSCT, but they were successfully treated by administration of sulfamethoxazole-trimethoprim. Although the number of patients in this series is small and caution is needed before drawing conclusions, it is likely that severe pre-transplant complications, especially liver and biliary complications associated with cryptosporidial infection, correlates with a higher risk with HSCT for XHIM patients.

CONCLUSIONS

Allogeneic HSCT using a conventional conditioning regimen of busulphan and cyclophosphamide is curative and feasible for the XHIM patients without severe cryptosporidial infection and its related complications, irrespective of age. If available, transplantation from either an HLA-matched sibling or an HLA-matched unrelated donor can be performed safely. Umbilical cord blood may be an alternative for those without an HLA-matched donor [22]. GVHD prophylaxis with cyclosporine and short-term methotrexate is feasible,

although one should be aware of thrombotic microangiopathy, one of the complications induced by cyclosporine. It is also very important to manage pretransplant infection, such as *P. carinii* and *C. parvum*. *P. carinii* pneumonitis may recur in those with previous infection after HSCT, but it is controllable with early detection and treatment with co-trimoxazole. In contrast, cryptosporidial infection is generally difficult to manage, although azithromycin and paromomycin may be effective. The mainstay will be prophylaxis by avoiding intake of non-boiled water. For those with high-risk status, nonmyeloablative conditioning regimens may reduce the risks of HSCT. In fact, successful cases have been reported recently [24]. For those with severe liver complications, a combination of liver transplantation and subsequent HSCT may be a choice, as described in a previous successful report [23].

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Brief report

Female agammaglobulinemia due to the Bruton tyrosine kinase deficiency caused by extremely skewed X-chromosome inactivation

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We analyzed the cause of agammaglobulinemia in a girl whose father had been diagnosed as having X-linked agammaglobulinemia (XLA). Flow cytometric analysis revealed the lack of peripheral B cells with the block of B-cell differentiation in the stages between pro-B cells and pre-B cells in the bone marrow, and the defect of the Bruton tyrosine kinase (BTK) expression on monocytes. We found a *BTK* gene mutation in the first single base pair of intron 11 in her father and heterozygous mutation in the patient at the site. Sequence analysis of abnormally smaller-sized polymerase chain reaction (PCR) products of cDNA confirmed splicing abnormalities due to the mutation. Maternally derived X chromosome was exclusively inactivated in peripheral blood and oral mucosal cells. This is the first report of female XLA caused by heterozygous *BTK* gene abnormality and extreme nonrandom inactivation of X chromosome on which normal *BTK* gene is located. (Blood. 2004;103:185-187)

Introduction

A 10-month-old Japanese girl was admitted to our hospital because of frequent respiratory infections and otitis media during the last 2 months. She was the second child of nonconsanguineous parents. Her father was diagnosed as having X-linked agammaglobulinemia (XLA) at the age of 3 years by recurrent infections, extremely low levels of serum immunoglobulins, and the lack of peripheral B cells. Her mother, elder brother, and maternal and paternal grandmothers were healthy. Serum immunoglobulin G (IgG), IgA, and IgM concentrations were 0.06 g/L (6 mg/dL), 0.01 g/L (1 mg/dL), and 0.05 g/L (5 mg/dL), respectively. Informed consent was obtained, and the study was done in accordance with the ethical standards of the responsible committee on human experimentation (Regional Committee of Ethics for Human Research at the Faculty of Medicine, Kyushu University).

Study design

Three-color flow cytometric analysis was performed for identification of lymphocyte subsets and B-cell precursor cells in bone marrow.[1]

Each exon of Bruton tyrosine kinase (*BTK*) gene was amplified by polymerase chain reaction (PCR) as previously described.[2] PCR primer pair for intron 11 boundary of *BTK* was 5′-TAAAAGCAATGAGGCTGTAG-3′ and 5′-AAGTGGGACGGGCACAGCAT-3′. Direct sequencing of PCR products was performed and analyzed by an ABI 310 DNA sequencer (Applied Biosystems, Tokyo, Japan).

Analysis of X-chromosome inactivation was performed as previously described.[3] Briefly, an aliquot of DNA was digested with methylation-sensitive enzyme *Hpa*II (Takara Shuzo, Otsu, Japan) and amplified by PCR at exon 1 of androgen receptor gene with specific primers.[3] PCR products were run on a GeneGel Excel 12.5 24 Kit (Amersham Pharmacia Biotech, Uppsala, Sweden) and separated by electrophoresis on GenePhor (Amersham Pharmacia Biotech). Gels were silver-stained with Hoefer Automated Gel Stainer (Amersham Pharmacia Biotech).

We searched microsatellite markers in NT_011642 that contains *XIST* gene. The fluorescence-labeled PCR products were loaded into ABI 3100 DNA Analyzer equipped with Genescan software (Applied Biosystems) for genotyping. The marker alleles were visualized using Genotyper software (Applied Biosystems). Among 108 CA repeats that we found, 7 microsatellite markers showed polymorphism. PCR primers for the microsatellite marker no. 7 were GGAATAGCAAGGGCAGTAAAGC and TTCACTCCCTCCTGGTATGTAC.

Results and discussion

Results

Immunologic characteristics of the patients. Flow cytometric analysis revealed the lack of B cells in peripheral blood (data not shown) and the defect of BTK expression on monocytes of the patient as well as her father (Figure 1A; data not shown). Her mother and paternal grandmother expressed normal levels of BTK without showing bimodal or mosaic pattern of BTK expression (data not shown).[4] B-cell differentiation was blocked in the stages between pro- and pre-B cells in bone marrow of the patient (Figure 1B).[5] We found a mutation in the first single base pair of intron 11 (G > A) of *BTK* gene in her father and heterozygous mutation in the patient at the same site (Figure 1D; data not shown). The cDNA amplification between exon 6 and exon 13 of *BTK* showed

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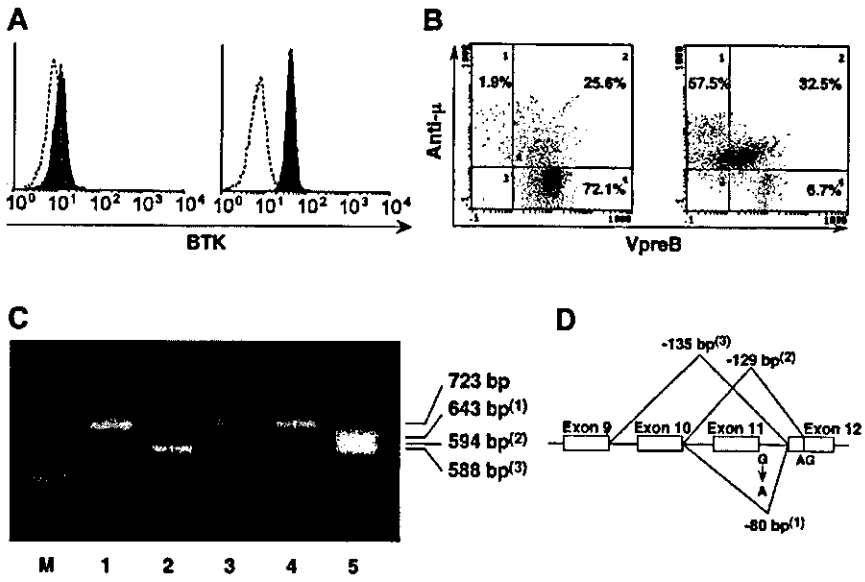

Figure 1. Defect of BTK and block of B-cell differentiation. (A) Intracellular staining of BTK was performed and BTK expression was analyzed in peripheral blood CD14$^+$ cells of the girl with agammaglobulinemia (left) and a healthy control (right). (B) The μ chain and VpreB were stained intracellularly. Analysis gate was set on CD19$^+$ and/or VpreB-positive cells. In contrast to the normal B-cell development in the bone marrow of a healthy control (right), maturational block of B-cell precursors occurred and neither VpreB- μ + (low) nor VpreB- μ + (high) cells were observed in the patient (left). (C) The cDNA amplification was performed by PCR between exon 6 and exon 13 of *BTK* in the family members. Abnormally smaller-sized PCR products (1-3) were observed in the patient (lane 2) and her father (lane 5), although her paternal grandmother (lane 1), her mother (lane 3), and her brother (lane 4) expressed BTK mRNA with normal size. The primer pair was 5'-ATGCTATGGGCTGCCAAATT-3' and 5'-GGTCCTTTGGATCAATTTCC-3', and the expected size of the normal PCR product is 723 bp. M indicates marker. (D) The abnormally smaller-sized PCR products (1-3) in panel C were sequenced and found to be the result of splicing abnormality caused by the mutation of intron 11.

abnormally smaller-sized products in the patient and her father (Figure 1C). Sequence analysis of the PCR products revealed splicing abnormality due to the mutation of intron 11 (Figure 1D). No other genetic abnormality was observed in the patient by sequencing all the coding regions of *BTK*. High-resolution X-chromosome analysis of peripheral blood cells revealed no structural abnormality (data not shown).

Nonrandom X-chromosome inactivation of the patient. As shown in Figure 2, maternal X chromosome was exclusively inactivated in mononuclear cells (Figure 2A) and oral mucosal cells (Figure 2B). On the other hand, random X-chromosome inactivation was confirmed in her mother and paternal grandmother by the presence of 2 types of CAG repeats, demonstrated by sequencing, after *Hpa*II treatment in the androgen receptor exon 1 (with 2 CAG repeat difference in her mother and 1 CAG repeat difference in her paternal grandmother; data not shown). Altogether, the patient was diagnosed as having XLA associated with a defect of BTK caused by heterozygous abnormality of the *BTK* gene and nonrandom X inactivation of maternally derived X chromosome in which normal *BTK* gene is located, which was a novel alteration without evidence of inheritance.

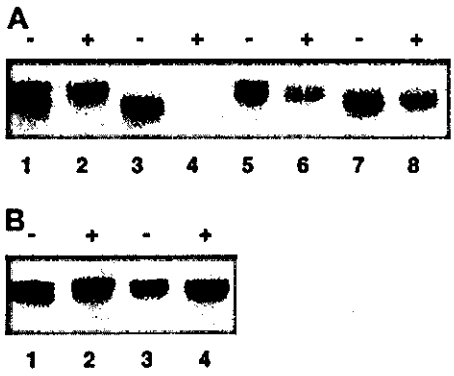

Figure 2. Nonrandom X-chromosome inactivation of the patient. DNA was extracted from peripheral blood (A) and oral mucosal cells (B) of the family members. Exon 1 of the androgen receptor, which contains CAG repeat, was amplified as described in "Study design" with (+) or without (−) the digestion by methylation-sensitive *Hpa*II before amplification. (A) Lane 1 and 2 indicate the patient; lane 3 and 4, her father; lane 5 and 6, her mother; and lane 7 and 8, her paternal grandmother. (B) Lane 1 and 2 indicate the patient; and lane 3 and 4, her mother.

Discussion

X chromosome is usually inactivated randomly in all somatic cells in early embryogenesis.[6] Skewed preferential inactivation of nonmutated X chromosome can lead to the development of X-linked recessive disorders in females.[7-11]

On the other hand, Wengler et al[12] reported that in female carriers of Wiskott-Aldrich syndrome (WAS), almost complete nonrandom inactivation of X chromosome with *WASP* gene mutation was observed in hematopoietic cells. Selective advantage of X chromosome without the mutation would inhibit the development of the disease in female heterozygotes of X-linked recessive disorders.[11] On the other hand, Ariga et al[13] reported that mutant *WASP* message was detected in peripheral blood cells in female carriers of WAS. In addition, WAS and X-linked thrombocytopenia of female heterozygotes are reported even without complete inactivation of nonmutated X chromosome.[14,15] Therefore, it is likely that WAS can develop in heterozygotes because the selective advantage of X chromosome without the mutation does not appear to be completely dominant.

Although XLA is one of the most common primary immunodeficiency disorders affecting humoral immunity, this is the first report of a female case. As BTK is indispensable for B-cell development,[16] only progenitor cells that have inactivation of X chromosome with *BTK* mutation can develop into mature B cells. Even a small population of progenitors with inactivation of X chromosome with BTK mutation would give a chance to the development of B cells and their expansion in the periphery. Therefore, it is reasonable to suggest that the development of XLA in females with heterozygous mutation of *BTK* is more restricted than other X-linked recessive disorders including WAS.

Possible reasons for extremely unbalanced X-chromosome inactivation observed in this female patient might include a stochastic event,[11] a dysregulation of X-chromosome inactivation caused by defect of *XIST* gene[17,18] which selects the X chromosome to inactivate, and defect of *Tsix* gene,[19] which selects X chromosome to avoid accumulation of *XIST* gene mRNA in coordination with *CTCF*.[20] In this patient, no abnormality was observed in *XIST*, its promoter, or mRNA expression levels. Some polymorphisms demonstrated the *XIST* mRNA expression of both maternally and

paternally derived X chromosomes (data not shown). We further examined microsatellite markers in the NT_011642 region to analyze the possibility of deletion in a region adjacent to *XIST*. A microsatellite marker no. 7, a 180-bp distance from *XIST* gene to the telomere, showed heterozygous pattern of AC repeats, which suggests no large deletion in this region (data not shown). We therefore concluded that the nonrandom inactivation of X chromosome in this patient was caused by unknown mechanisms other than *XIST*.

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Clinical course of patients with *WASP* gene mutations

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Mutations of the Wiskott-Aldrich syndrome protein *(WASP)* gene result either in the classic Wiskott-Aldrich syndrome (WAS) or in a less severe form, X-linked thrombocytopenia (XLT). A phenotype-genotype correlation has been reported by some but not by other investigators. In this study, we characterized *WASP* gene mutations in 50 Japanese patients and analyzed the clinical phenotype and course of each. All patients with missense mutations were WASP-positive. In contrast, patients with nonsense mutations, large deletions, small deletions, and small insertions were WASP-negative. Patients with splice anomalies were either WASP-positive or WASP-negative. The clinical phenotype of each patient was correlated with the presence or absence of WASP. Lack of WASP expression was associated with susceptibility to bacterial, viral, fungal, and *Pneumocystis carinii* infections and with severe eczema, intestinal hemorrhage, death from intracranial bleeding, and malignancies. Rates for overall survival and survival without intracranial hemorrhage or other serious complications were significantly lower in WASP-negative patients. This analysis provides evidence for a strong phenotype-genotype correlation and demonstrates that WAS protein expression is a useful tool for predicting long-term prognosis for patients with WAS/XLT. Based on data presented here, hematopoietic stem cell transplantation should be considered, especially for WASP-negative patients, while the patients are young to improve prognosis. (Blood. 2004;103:456-464)

Introduction

The Wiskott-Aldrich syndrome (WAS) is a rare X-linked immunodeficiency disorder characterized by thrombocytopenia and small platelets, eczema, recurrent infections, and increased risk for autoimmunity and malignancy.[1] The gene responsible for this syndrome, *WAS protein (WASP),* consists of 12 exons containing 1823 base pairs. *WASP* encodes a 502–amino acid (aa) protein that is expressed selectively in hematopoietic stem cell–derived lineages and is involved in cell signaling and cytoskeleton reorganization.[2-5] Subsequent sequence analysis of *WASP* demonstrated that X-linked thrombocytopenia (XLT), a congenital disorder characterized by thrombocytopenia and small platelets but, in general, without the other complications of WAS, is a mild allelic variant caused by mutations of the *WASP* gene.[6,7] A correlation between clinical phenotype and genotype was reported independently by several investigators[8-10] but was not observed by others.[11] One of the difficulties in establishing a phenotype-genotype correlation for WAS/XLT has been the lack of carefully collected clinical data over time and uncertainty about a common denominator. Here we report the *WASP* gene mutations identified in 50 WAS/XLT patients from 43 unrelated Japanese families and the effects these mutations have on the expression of the WAS protein. The clinical course of each patient has been recorded longitudinally, and a correlation has been established between the clinical phenotype, the extent of the mutation, and the presence or absence of WASP in patient-derived Epstein-Barr virus (EBV)–transformed B-lymphoblastoid cell lines (B-LCLs).

Patients, materials, and methods

Patients and clinical phenotypes

Fifty affected members of 43 unrelated families with WAS or XLT were included in this analysis. Twelve patients had been described previously.[12-14] Using a simple scoring system,[7,15] the severity of WAS/XLT-associated symptoms was estimated and expressed as a score of 1 to 5. A score of 1, assigned to patients with only thrombocytopenia and small platelets, and a score of 2, assigned to patients with additional findings of mild, transient eczema or minor infections, identified XLT patients. Those with treatment-resistant eczema and recurrent infections in spite of optimal treatment received a score of 3 (mild WAS) or 4 (severe WAS). Regardless of the original score, if any patient then had autoimmune disease or malignancy, the score was changed to 5.

Approval for these studies was obtained from the Tokyo Medical and Dental University institutional review board. Informed consent was provided according to the Declaration of Helsinki.

Cell lines

B-LCLs were established by inoculating peripheral blood mononuclear cells (PBMCs) from healthy controls and WAS patients with EBV-containing supernatant, as described elsewhere.[12]

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DNA purification and sequencing of genomic DNA

Genomic DNA was extracted from granulocytes or B-LCLs using Sepa-Gene (Seikagaku kogyo, Tokyo, Japan). Purified genomic DNA samples were amplified with primer pairs designed to span each exon and exon/intron junction, and the specific causative mutation was identified by direct sequencing as described previously.[12]

RNA isolation, RT-PCR, and sequencing of cDNA

RNA isolation from B-LCLs, using the Wizard SV-total RNA isolation kit (Promega, Madison, WI), and reverse transcription–polymerase chain reaction (RT-PCR), using the RT-PCR kit (Stratagene, La Jolla, CA), were performed according to the manufacturers' instructions. *WASP* cDNA was amplified by PCR in 2 overlapping fragments using the previously reported primers.[12] PCR products were purified by agarose gel electrophoresis. PCR products were subcloned into pGEM-T Easy Vector System II (Promega). Subcloned cDNA was sequenced using the Big Dye Terminator cycle sequencing kit (Perkin-Elmer, Foster City, CA) and an ABI 310 genetic analyzer (Perkin-Elmer).

Anti-WASP antisera and Western blot analysis

Rabbit anti-WASP antibody (Ab 503) was made against a synthetic peptide (aa's 209-226 of WASP), as described previously.[9] B-LCLs from healthy control subjects and WAS patients were suspended at 1.0×10^7/mL in lysis buffer containing 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.5% aprotinin, and 10 μg/mL leupeptin at pH 7.5 and were kept on ice for 30 minutes. The protein concentration was determined in each lysate by the Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA). From each sample, 40 μg total protein was loaded onto a sodium dodecyl sulfate (SDS) polyacrylamide gel, electrophoresed, and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). After blocking with 5% nonfat milk, the membranes were incubated with anti-WASP Ab 503 at 1:5000 dilutions for 1 hour at room temperature. After washing with 0.1% Tween/phosphate-buffered saline 4 times, the membranes were incubated with alkaline phosphatase-conjugated goat antirabbit immunoglobulin (Promega) at a concentration of 1:7500 for 30 minutes at room temperature. Results were visualized by incubation with AP buffer (100 mM Tris-HCl, pH 9.5; 100 mM NaCl; and 5 mM $MgCl_2$).

Statistics

Probabilities of outcomes were calculated using the Kaplan-Meier product limit estimate. Univariate comparisons of survival were made using the log-rank test. Comparisons of the data between WASP-positive and -negative groups were made using the Student *t* test and the Mann-Whitney *U* test.

Results

WASP mutations and protein expression by B-LCL from WAS/XLT patients

Thirty-three different mutations of the *WASP* gene were identified in 43 unrelated families studied (Tables 1 and 2). To investigate the relationship between clinical features and WASP protein expression, we performed Western blot analysis of B-LCL lysates using the polyclonal anti-WASP Ab 503. Blots for selected WAS/XLT patients are shown in Figure 1.

All 16 patients with missense mutations expressed normal-sized WASP (Figure 1A). Four patients from 3 unrelated families (patients 1, 2, 8, and 13) who had normal amounts of WASP had isolated thrombocytopenia and received a score of 1. The other 12 patients with missense mutations had a reduced amount of normal-sized WASP; 7 had a clinical score of 1 or 2, 1 had a score of 3, and 4 had autoimmune renal disease (score 5A).

WASP was not detected in the 3 unrelated patients with nonsense mutations in exons 1 and 2 (patients 17, 18, 19), but it was present as a truncated protein in the 2 brothers with nonsense mutations in exon 10 (patients 20, 21).

WASP could not be detected in cell extracts from any of the 9 unrelated patients with deletions within the *WASP* gene or from the patient with deletion of the entire *WASP* gene (Figure 1B). All patients with deletions had a classical WAS phenotype (scores 3 to 5, except for a patient with a score of 2) (Table 2). The only patient with a nucleotide insertion did not express WASP.

B-LCL extracts from 11 of 18 patients with splice-site mutations showed reduced amounts of normal-sized WASP protein (Figure 1A). It is of interest that normally spliced products and abnormally spliced products were detected in cDNA from these patients (Table 1). B-LCL extracts from the other 7 patients with splice-site mutations lacked WASP protein expression (Figure 1B).

WASP expression correlates with clinical characteristics

To correlate clinical phenotype with genotype, we divided the patients into 2 groups according to the expression of full-length WASP protein. WASP-positive patients were defined as expressing normal or reduced amounts of full-length WASP protein, as detected by Western blot analysis. WASP-negative patients were defined as having either no detectable WASP protein or C-terminal–truncated WASP protein. The entire medical history of each patient was reviewed and retrospectively analyzed and scored at the time of mutation analysis.

Table 3 summarizes the clinical phenotypes and the WAS/XLT scores of the patients. Twenty-seven patients were defined as WASP-positive and 23 patients as WASP-negative. Of 27 WASP-positive patients, 21 patients (78%) had no episodes of recurrent or life-threatening infections. On the other hand, 21 (91%) of 23 WASP-negative patients had a history of recurrent or life-threatening infections. The age distribution of 6 WASP-positive patients who had score of 3 or more (mean age, 7.7 years; range, 1-24 years) was even younger than that of 21 WASP-positive patients who had score 1 or 2 (mean age, 13.2 years; range, 2-61 years).

Twenty-six (96%) of 27 WASP-positive patients were alive at the time of this analysis compared with only 17 (74%) of 23 WASP-negative patients. Five patients (including an 8-year-old WASP-positive patient with a score of 4) died because of intracranial hemorrhage (ICH), 1 patient died of JC virus encephalitis after bone marrow transplantation (BMT), and 1 patient died of EBV-associated B-cell lymphoma. Of those alive, the WASP-positive patients were significantly older than the WASP-negative patients (mean ages, 12.1 years and 6.1 years, respectively; $P < .0001$). Mean age at death of the WASP-negative patients was 6.8 years (range, 1-16 years). Overall survival for WASP-positive and WASP-negative WAS/XLT patients is shown in Figure 2A. The 10-year probability of survival was 92.3% (range, 84.9%-100%) for WASP-positive patients and 76.0% (range, 65.3%-86.7%) for WASP-negative patients. More strikingly, the 20-year probability of survival was 92.3% (range, 84.9%-100%) for WASP-positive patients and 0% for WASP-negative patients ($P = .004$). Thus, WASP expression is closely linked to the overall outcome of the disease and has a strong prognostic value for patients with *WASP* gene mutations. ICH-free survival is shown in Figure 2B. The 10-year probability of ICH-free survival was 81.8% (range, 71.9%-91.7%) for WASP-positive and 70.6% (range, 59.2%-82.0%) for WASP-negative patients. The 30-year probability of

Table 1. Results of mutation analysis of *WASP* gene

Patient	Genomic DNA mutation	Exon or intron	cDNA mutation (predicted protein change)	Score	Western blot
Missense mutation: 13 families, 16 patients					
1*, 2*	52G > A	Exon 1	52G > A (Met6Ile)	1, 1	Positive
3	105C > T	Exon 1	105C > T (Ser24Phe)	5A	Positive
4, 5	168C > T	Exon 2	168C > T (Thr45Met)	1, 2	Positive
6	168C > T	Exon 2	168C > T (Thr45Met)	5A	Positive
7*	168C > T	Exon 2	168C > T (Thr45Met)	2	Positive
8	201C > T	Exon 2	201C > T (Ala56Val)	1	Positive
9, 10	257G > A	Exon 2	257G > A (Val75Met)	2, 1	Positive
11	290C > T	Exon 2	290C > T (Arg86Cys)	2	Positive
12	290C > T	Exon 2	290C > T (Arg86Cys)	5A	Positive
13	290C > T	Exon 2	290C > T (Arg86Cys)	1	Positive
14	291G > A	Exon 2	291G > A (Arg86His)	2	Positive
15*	291G > A	Exon 2	291G > A (Arg86His)	3	Positive
16	291G > A	Exon 2	291G > A (Arg86His)	5A	Positive
Nonsense mutation: 4 families, 5 patients					
17	71C > T	Exon 1	71C > T (Arg13Stop)	3	Negative
18*	131C > T	Exon 1	131C > T (Gln33Stop)	4	Negative
19*	196C > A	Exon 2	196C > A (Tyr54Stop)	3	Negative
20, 21	995C > T	Exon 10	995C > T (Arg321Stop)	3, 4	Positive, truncated
Deletion: 7 families, 7 patients					
22*	41-45delG	Exon 1	41-45delG (frameshift, stop aa 44)	4	Negative
23	140-142delTT	Exon 1	140-142delTT (frameshift, stop aa 36)	2	Negative
24	260-261delA	Exon 2	260-261delA (frameshift, stop aa 126)	5A/M	Negative
25	283-287del 5 nt	Exon 2	283-287del 5 nt (frameshift, stop aa 119)	4	Negative
26	406-409delG	Exon 4	406-409delG (frameshift, stop aa 126)	4	Negative
27*	899delC	Exon 9	899delC (frameshift, stop aa 307)	3	Negative
28	1178-1182delC	Exon 10	1178-1182delC (frameshift, stop aa 444)	3	Negative
Large deletion: 3 families, 3 patients					
29*	del 1872 nt (2932-4803 nt§)	Exons 3-7	del exons 3-7 (308-768) (frameshift, stop aa 105)	5A	Negative
30	del 1558 nt (3991-5548 nt§)	Exon 7	del exon 7 (594-768) (frameshift, stop aa 202)	5A/M	Negative
31	del total *WASP* gene	Exons 1-12	del total *WASP* gene	3	Negative
Insertion: 1 family, 1 patient					
32	62-64insC	Exon 1	62-64insC (frameshift, stop aa 37)	5M	Negative
Splice anomaly: 15 families, 18 patients					
33	T > G, + 2	Intron 1	ins intron 1 (297 nt) (stop aa 113)	3	Negative
34	G > A, −1	Intron 3	ins intron 3 (100 nt) (frameshift, stop aa 201) del 28 nt of exon 4 (395-422) (frameshift, stop aa 251)	5M	Negative
35	G > A, + 1	Intron 3	del exon 3 (308-394) (del aa's 92-120)‖	3	Positive, normal size
36	del 8 nt, + 1 to + 8	Intron 4	ins 31 nt of intron 4 (frameshift, stop aa 178)‖ del exon 4 (395-497) (frameshift, stop aa 251)	1	Positive, normal size
37	539G > T	Exon 5	del exon 5 (498-539) (del aa's 155-169) 539G > T (Asn169Stop)	2	Positive, small MW
38,† 39, 40	G > A, + 5	Intron 6	ins 38 nt of intron 6 (frameshift stop aa 190)‖	5A, 1, 4	Positive, normal size
41‡	G > A, + 5	Intron 6	ins 38 nt of intron 6 (frameshift stop aa 190)‖	2	Positive, normal size
42*	del G, − 1	Intron 7	del exon 8 (769-811) (frameshift, stop aa 246)	5A	Negative
43	G > C, + 1	Intron 8	del exon 8 (769-811) (frameshift, stop aa 246)	5A	Negative
44	del 4 nt, + 1 to + 4	Intron 8	ND	4	Negative
45	del 4 nt, + 1 to + 4	Intron 8	ND	5M	Negative
46	del 4 nt, + 1 to + 4	Intron 8	del exon 8 (769-811) (frameshift, stop aa 246) del exon 8, 9 (769-830) (frameshift, stop aa 267) ins intron 9 (frameshift, stop aa 327)	4	Negative
47	No mutation in coding region	—	ins intron 9 (frameshift, stop aa 327)‖ del exon 10 (966-1372) (frameshift, stop aa 357)	1	Positive, normal size
48	No mutation in coding region	—	ins intron 11 (118 bp) (frameshift, stop aa 520)‖	3	Positive, normal size
49, 50	No mutation in coding region	—	ins intron 11 (118 bp) (frameshift, stop aa 520)‖	5A, 5A	Positive, normal size

Patients 1 and 2 are brothers; patients 4 and 5 are cousins; patient 10 is a grandfather of patient 9; patients 20 and 21 are brothers; patient 38 is a cousin of patients 39 and 40, who are brothers; patients 49 and 50 are brothers. Score 5A or 5M is score 5 with autoimmune disease (A) or malignancy (M), respectively. ND indicates not done; —, not applicable; and MW, molecular weight.

*Reported by Itoh et al.[12]

†Reported by Kawakami et al.[13]

‡Reported by Inoue et al.[14]

§Nucleotide numbers are from GenBank accession no. AF115549.[16]

‖Normally spliced cDNA of the *WASP* gene was also detected with abnormally spliced cDNA.

Table 2. Correlation of phenotype, genotype, and WASP Western blot finding

Score	WASP	
	Positive	Negative
Missense	16	0
1, 2	11	0
3, 4	1	0
5A	4	0
Nonsense	0	5
3, 4	0	5
Deletion	0	7
1, 2	0	1
3, 4	0	5
5A/M	0	1
Large deletion	0	3
3, 4	0	1
5A	0	1
5A/M	0	1
Insertion	0	1
5M	0	1
Splice anomaly	11	7
1, 2	5	0
3, 4	3	3
5A	3	2
5M	0	2

WASP-positive numbers represent WASP detectable by Western blot. WASP-negative numbers represent WASP found absent or truncated by Western blot. All scores are clinical scores.[9,13]

ICH-free survival was 36.8% (range, 9.9%-63.5%) for WASP-positive and 0% for WASP-negative patients ($P = .048$).

WASP expression, platelet abnormalities, and hemorrhagic episodes

Patient age on first visit was similar between the 2 groups (Table 3). Most of the patients had symptoms related to thrombocytopenia. More WASP-positive patients had petechiae and more WASP-negative patients had hematorrhea as an initial symptom. By definition, WAS and XLT patients have thrombocytopenia and small platelets in common and, as a result, lifelong hemorrhagic diathesis. To determine whether the presence or absence of WASP influences bleeding tendency, we compared platelet data and hemorrhagic incidences in the 2 groups of patients (Table 4). ICH was seen in 5 of 27 WASP-positive and 6 of 23 WASP-negative patients. Of note, 4 WASP-negative patients died as a result of hemorrhage compared with only 1 WASP-positive patient. Episodes of intestinal bleeding were significantly more frequent in WASP-negative patients ($P < .0001$) (Table 4).

Platelet abnormalities (numbers, size) were the same between the 2 groups of patients. Numbers of megakaryocytes in the bone marrow were normal or increased in 11 (40%) of 28 patients tested (no difference between the 2 groups). We were unable to detect megakaryocytes in the bone marrow aspirates of 7 (46%) of 15 WASP-negative patients and 1 (8%) of 13 WASP-positive patients ($P = .07$). Most of the patients in each group had increased platelet-associated immunoglobulin G (IgG), and no significant differences were observed among them.

Table 3. WASP expression correlates with clinical characteristics

Characteristics	WASP		P
	Positive	Negative	
No. patients	27	23	—
Total patient-years observed	323	144	—
Clinical phenotype, n			
XLT phenotype (scores 1, 2, 5)	21	2	—
WAS phenotype (scores 3, 4, 5)	6	21	—
Score 5	7	8	—
XLT phenotype	5	1	—
WAS phenotype	2	7	—
5A	7	3	—
5M	0	3	—
5A/M	0	2	—
Age distribution, y			
Alive, n	26	17	—
Mean, y	12.1	6.1	< .0001
Range, y	1-61	1-15	—
Older than 15 y, n	8	1	—
Dead, n	1	6	—
Mean, y	8	6.8	—
Range, y	8	1-16	—
Older than 15 y, n	0	1	—
Age on first visit, mo			
Mean ± SD (n)	4.4 ± 5.1 (22)	2.3 ± 3.2 (22)	.19* (—)
Range	0.1-18	0.1-11	—
Initial symptoms, n			
Thrombocytopenia-related	25	20	—
No symptoms	6	4	—
Petechiae	16	3	—
Hematorrhea	2	13	—
Intracranial hemorrhage	1	0	—
Eczema	2	3	—
Recurrent infection	0	0	—

WASP-positive numbers represent WASP detectable by Western blot. WASP-negative numbers represent WASP found absent or truncated by Western blot. — indicates not applicable.
*Not significant.

WASP expression, immunologic defects, and infections

To minimize the difference in age distribution between the 2 groups, 6 adult WASP-positive patients (patients 6, 10, 12, 15, 49, 50) were excluded from the analysis of frequency of infection. WASP-negative patients were found to have significantly more episodes of infection than WASP-positive patients (0.67 episodes/patient-year and 0.14 episodes/patient-year, respectively; $P < .0001$) (Table 5). Bacterial infections (otitis media, skin abscess, pneumonia, enterocolitis, meningitis, sepsis, urinary tract infection, and others) were observed 4 times more frequently in WASP-negative patients than in WASP-positive patients (0.40 events/patient-year and 0.10 events/patient-year, respectively; $P = .003$). Viral infections were more frequent in WASP-negative patients than in WASP-positive patients (0.17 events/patient-year and 0.05 events/

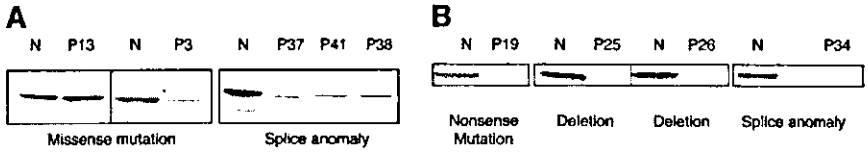

Figure 1. Western blot analysis of EBV-transformed cell lysates from WAS/XLT patients. Typical blots are shown. Patient numbers and descriptions of the mutations are shown in Table 1. (A) WASP-positive patients. (B) WASP-negative patients.

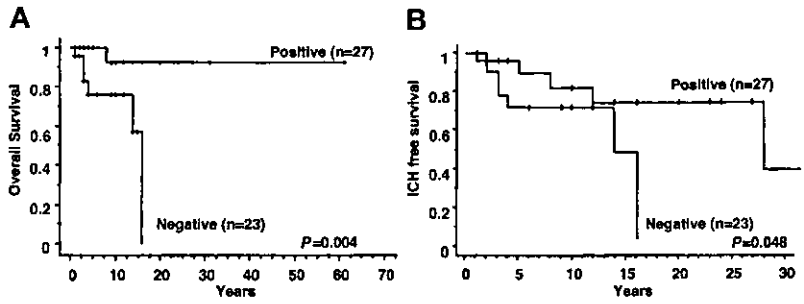

Figure 2. WASP expression and probability of survival of patients. (A) Overall survival of the patients. Positive and negative indicate the survival curves of patients found positive or negative for WASP on Western blot analysis. (B) Intracranial hemorrhage-free survival of WAS/XLT patients. Positive and negative indicate the survival curves of patients found positive or negative for WASP on Western blot analysis.

patient-year, respectively). WASP-negative (but not WASP-positive) patients were highly susceptible to herpes simplex virus (HSV) infections (13 episodes). Cytomegalovirus (CMV) encephalitis occurred in 2 WASP-negative patients and CMV hepatitis in 3 WASP-positive patients. WASP-negative patients were also reported to have extensive candidiasis (9 patients), aspergillosis (3 patients), and *Pneumocystis carinii* pneumonia (PCP) (2 patients). On the other hand, recurrent herpes zoster was only observed in WASP-positive patients (2 patients).

The bias of antimicrobial prophylaxis did not contribute to the susceptibility of infections seen in WASP-negative patients (Table 5). All WASP-positive patients except one did not receive antimicrobial prophylaxis. On the other hand, 13 (56.5%) of 23 WASP-negative patients received antimicrobial prophylaxis after clinical or molecular diagnosis. Thirteen of 14 received trimethoprim-sulfamethoxazole and amphotericin B syrup. PCP was observed before the prophylaxis started in 2 patients affected, indicating the importance of prophylaxis by trimethoprim-sulfamethoxazole. Only 1 WASP-negative patient received acyclovir to prevent recurrent herpes infections. Candidiasis (4 patients) and aspergillosis (1 patient) developed even under prophylactic treatment with amphotericin B syrup, indicating the limitation of this prophylaxis. These observations strongly support aggressive prophylactic therapy with fungal and antiviral agents (such as acyclovir) and with PCP for WASP-negative patients.

Lymphocyte subsets and in vitro lymphocyte proliferation to mitogens (phytohemagglutinin [PHA] or concanavalin A [ConA]) were normal in both groups of patients, as has been reported previously.[1] Serum IgG concentrations were normal or increased in both groups. Low serum IgM concentrations were observed in 23% of the WASP-positive and 20% of WASP-negative patients, and increased serum IgA concentrations were observed in WASP-positive (53%) and in WASP-negative (36%) patients. These immunologic data were not significantly different between the 2 groups.

Table 4. Platelet-associated data

	WASP		
	Positive	Negative	*P*
No. patients	27	23	—
Major bleeding problems			
Intracranial hemorrhage, n (%)	5 (18.5)	6 (26.1)	.52†
Events/patient-year	0.015	0.042	.06†
Age, y, mean	11.0	3.2	.10†
Age range, y	2-28	1-7	
Intestinal bleeding, n (%)	3 (11.1)	15 (65.2)	< .0001
Events/patient-year	0.01	0.13	< .0001
Death due to hemorrhage, n (%)	1 (3.7)	4 (17.4)	.11†
Platelet count on first visit, × 10^9/L			
Mean ± SD (n)	36 ± 31 (13)	37 ± 19 (14)	.94†
Range	0.6-11.6	1.0-7.0	—
Mean platelet volume, fL			
Mean ± SD (n)	6.0 ± 1.4 (18)	6.2 ± 1.7 (15)	.63†
Range	4.0-8.8	4.0-10.5	—
BM megakaryocyte*			
Mean ± SD (n)	97.6 ± 97.0 (13)	65.2 ± 108.5 (15)	.42†
Range, /μL	0-320	0-385	—
Normal or increased, n (%)	7 (54)	4 (27)	—
Decreased, n (%)	5 (38)	4 (27)	—
Undetectable, n (%)	1 (8)	7 (46)	—
PAIgG			
Mean ± SD, ng/10^7 platelets (n)	434.6 ± 979.7 (20)	272.8 ± 228.0 (14)	.55†
Range, ng/10^7 platelets	12.5-4157	2.5-789	—
No. PAIgG-positive patients	20	13	—

WASP-positive numbers represent WASP detectable by Western blot. WASP-negative numbers represent WASP found absent or truncated by Western blot. PAIgG indicates platelet-associated IgG; and —, not applicable.

*BM megakaryocyte indicates number of bone marrow megakaryocytes per microliter BM aspirate. For BM megakaryocyte subrows, normal or increased indicates more than 50/μL; decreased, 20-50/μL; and undetectable, less than 20/μL.

†Not significant.

Table 5. WASP expression and infections

	WASP		
	Positive	Negative	*P*
No. patients for analysis	21	23	—
Total patient-years observed	134	144	—
Total episodes of infections	19	96	—
Total infectious events/patient-year	0.14	0.67	< .0001
Bacterial infections, n	13	58	—
Events/patient-year	0.10	0.40	.003
Severe or recurrent viral infections, n	6	24	—
Events/patient-year	0.05	0.17	.10‖
Severe viral infections, n*	4	7	.93‖
Recurrent viral infections, n†	2	17	.01
Miscellaneous infections, n	0	14§	—
Events/patient-year	0	0.10	.001
Patients with antimicrobial prophylaxis‡	1	13	—
Patients without antimicrobial prophylaxis	26	10	—

WASP-positive numbers represent WASP detectable by Western blot. WASP-negative numbers represent WASP found absent or truncated by Western blot. — indicates not applicable.

*WASP-positive patients: CMV hepatitis (n = 3), severe varicella (n = 1). WASP-negative patients: CMV encephalomeningitis (n = 2), measles pneumonitis (n = 2), mumps meningitis (n = 2), EBV reactivation (n = 1).

†WASP-positive patients: molluscum contagiosum infections (n = 1), recurrent herpes zoster (n = 1). WASP-negative patients: HSV infections (n = 13), molluscum contagiosum infections (n = 4).

§Fungal infections: aspergillosis (n = 3), candidiasis (n = 9), and *P carinii* pneumonia (n = 2).

‡WASP-positive patients: trimethoprim-sulfamethoxazole (ST) + amphotericin B (AMPH) (syrup) + acyclovir (ACV, orally) + vancomycin (orally). WASP-negative patients: 12 ST + AMPH (syrup), 1 ST, 2 antibiotics (rifampicin, kanamycin), 1 fluconazole, 1 ACV (orally).

‖Not significant.

WASP expression and atopic diseases

The severity of eczema was estimated by the scoring system, described in "Patients, materials, and methods." As shown in Table 6, only 1 WASP-positive patient had persistent difficulty controlling eczema; 16 patients (59%) never had or had only transient and mild eczema. In contrast, severe eczema was reported in 11 WASP-negative patients; eczema was transient in 1 and absent in 1 WASP-negative patient. Asthma and food allergy, an often reported complication associated with atopic dermatitis,[17] was observed in 8 patients (16%) only and did not correlate with the expression of WASP. Very high IgE concentrations (more than 1000 IU/mL) were found in 62% of WASP-negative patients compared with 25% of WASP-positive patients ($P = .05$). IgE concentrations did not correlate with the severity of eczema.

WASP expression and autoimmune disorders

Autoimmune diseases and malignancies are frequent complications observed in WAS patients.[1] As shown in Table 7, autoimmune/inflammatory diseases were seen almost equally as much in WASP-positive and WASP-negative patients (26% and 22%, respectively). Five WASP-positive patients (19%) had IgA nephropathy. Age at onset was 10 to 20 years, and the disease progressed to chronic renal failure in 2 of the 5 patients at the ages of 22 and 24, respectively.

WASP expression and malignancies

During the observation period, malignancies developed in 5 WASP-negative patients (Table 7). Lymphoma developed in 2 patients and was related to EBV infection in one of them, as reported previously.[18] EBV-related lymphoma was detected at age 1, and the patient died at age 2. Myelodysplasia developed in 3 patients. None of the WASP-positive patients had a malignancy. However, we obtained positive family histories from 2 unrelated kinships in which the surviving patients were WASP-positive. Each family reported the death of an affected male relative; 1 died of malignant lymphoma at age 32 and the other of a brain tumor at age 38.

Table 6. WASP expression and atopic diseases

	WASP		
	Positive, n (%)	Negative, n (%)	*P*
Eczema (score)			.0003
None (1)	9	1	
Transient (2)	7	1	
Moderate (3)	10	10	
Severe (4)	1	11	
Asthma	2 (7.4)	2 (8.7)	—
Food allergy	2 (7.4)	2 (8.7)	—
IgE, IU/mL			.05
Less than 100	6 (30)	4 (19)	
100-1000	9 (45)	4 (19)	
More than 1000	5 (25)	13 (62)	

WASP-positive numbers represent WASP detectable by Western blot. WASP-negative numbers represent WASP found absent or truncated by Western blot. — indicates not applicable.

WASP expression and response to therapy

Hematopoietic stem cell transplantations (HSCTs) were performed in 3 of 27 WASP-positive and in 12 of 23 WASP-negative patients. Eleven patients received bone marrow from HLA-matched donors (sibling, 1; unrelated donor, 10), and 1 patient received bone marrow from an HLA-haploidentical donor. Three patients underwent reconstitution with cord blood stem cells from partially matched, unrelated donors. Mean observation time was 2.2 years (range, 0.25-8.0 years), and all but 1 patient are alive. Three patients rejected grafts, and 2

Table 7. WASP expression, autoimmune disease, and malignancies

	WASP		
	Positive	Negative	*P*
Autoimmune or inflammatory diseases			
Affected patients, n (%)	7 (26)	5 (22)	.31*
Total events	11	6	—
Events/patient-year	0.03	0.04	—
Vasculitis, n	3	1	—
Arthritis, n	1	2	—
Inflammatory bowel disease, n	0	2	—
Autoimmune hemolytic anemia, n	2	1	—
IgA nephropathy, n (%)	5 (19)	0	.03
Chronic renal failure, n	2	0	—
Age at onset, y	10, 10, 17, 18, 20	—	—
Malignant diseases			
Affected patients, n (%)	0	5 (22)	.05
Malignant lymphoma, n	0	2	—
Age at onset, y	—	1.8, 15	—
Myelodysplasia, n	0	3	—
Age at onset, y	—	0.1, 0.2, 3	—

WASP-positive numbers represent WASP detectable by Western blot. WASP-negative numbers represent WASP found absent or truncated by Western blot. — indicates not applicable.

*Not significant.

patients achieved partial grafting after the first HSCT (Table 8). Two patients achieved engraftment after second HSCT, and 1 patient achieved it after receiving donor lymphocyte transfusions. A 14-year-old WASP-negative patient died after undergoing BMT from an unrelated HLA-matched donor. Initially he achieved partial engraftment, but EBV-related posttransplantation lymphoproliferative disease developed. Treatment with donor lymphocyte transfusions resolved the lymphoproliferation. However, HSV and fungal pneumonitis then developed, and he died 200 days after BMT of JC-virus–related progressive multifocal leukoencephalopathy.

Splenectomy was performed in 10 patients (5 WASP-positive, 5 WASP-negative). Good responses (platelet counts higher than 100×10^9/L) were achieved in 5 patients. All 5 WASP-positive patients survived without ICH after splenectomy for an average of 17.0 years (range, 0.5-59 years). In contrast, 2 of the 5 WASP-negative patients died of ICH after splenectomy. After splenectomy all patients were placed on prophylactic antibiotics. In spite of this, 2 WASP-positive and 3 WASP-negative patients acquired but survived pneumococcal sepsis/meningitis.

High-dose intravenous immunoglobulin therapy was given to 24 patients (13 WASP-positive, 11 WASP-negative). No patient had a good response; therefore, it is no longer recommended for treatment of WAS-associated thrombocytopenia. Prednisolone or methylprednisolone pulse therapy was attempted in 26 patients. Sixteen patients (62%) did not respond, and 10 patients (38%) achieved transient responses to this therapy.

Table 8. WASP expression and response to therapy

	WASP		
	Positive	Negative	*P*
Stem cell transplantation, n	3	12	—
Mean age, y	12.4	12.8	.017
Age range, y	0.25-19	0.5-14	—
Alive, n	3	11	—
Dead, n	0	1	—
Splenectomy, n	5	5	—
Mean age at present, y	17.0	5.0	.017
Range, y	0-59	1.0-8.2	—
Mean age at splenectomy, y	8.8	6.0	—
Range, y	2-23	0.3-9	—
Alive, n	5	2	—
Dead, n	0	3†	—
Good response, n (%)*	2 (40)	3 (60)	—
Partial response, n (%)*	3 (60)	2 (40)	—
No response, n	0	0	—
Pneumococcal sepsis/meningitis after splenectomy, n	2	3	—
High-dose IVIG, n	13	11	—
Good response, n*	0	0	—
Transient response, n (%)	4 (31)	2 (18)	—
No response, n (%)	9 (69)	9 (82)	—
Steroids, n	15	11	—
Good response, n*	0	0	—
Transient response, n (%)	8 (53)	2 (18)	—
No response, n (%)	7 (47)	9 (82)	—

WASP-positive numbers represent WASP detectable by Western blot. WASP-negative numbers represent WASP found absent or truncated by Western blot. — indicates not applicable.

*Good response indicates a platelet count greater than 100×10^9/L; partial response, a platelet count less than 100×10^9/L.

†Two patients died of ICH, and 1 died of complications related to BMT.

Discussion

To compile this comprehensive phenotype-genotype study, we collected clinical and molecular data from a population of patients treated at major medical centers in Japan for classic WAS or XLT. Clinical data were collected over many years and addressed disease severity, complications, clinical outcomes, and extent of immunodeficiency. Each patient underwent an extensive molecular evaluation that included mutation analysis of *WASP*, both at the genomic DNA and at the transcriptional/translational levels. Thirty-three unique *WASP* mutations were observed in the 43 unrelated families enrolled in the study, demonstrating that mutations were highly diverse in Japanese patients, as had been observed in other populations.[9,10,19] In agreement with previous reports, missense and nonsense mutations were found predominantly in exons 1 and 2, and splice-site mutations were found within the C-terminal half of the gene; insertions and deletions were distributed throughout the *WASP* gene. To test the hypothesis that clinical phenotype correlates with the presence or absence of WASP, we divided the patients into 2 groups. Patients with normal or reduced amounts of full-length mutated WASP were categorized as WASP-positive patients (group 1), and those with no detectable or truncated WASP were categorized as WASP-negative patients (group 2). Group 1 consisted of 27 WASP-positive and group 2 of 23 WASP-negative subjects. As expected, patients with missense mutations expressed normal-sized WASP, often at a decreased level. Patients with nonsense mutations either lacked WASP or had truncated protein if the mutation affected exons at the C-terminal end. Patients with deletions and the only patient with an insertion did not express WASP, presumably because of frameshift and early termination of transcription. Some patients with splice-site mutations had multiple splicing products and expressed WASP, but others failed to express detectable WASP.

Using a scoring system, most patients with missense mutations had a score of 1 or 2 (XLT phenotype). In contrast, patients who had nonsense mutations or deletions/insertions had scores of 3 to 5 (WAS phenotype). Two thirds of WASP-positive patients with splice-site mutations had mild disease with scores of 1 and 2, possibly because of the presence of small quantities of normally spliced WASP. All patients with splice-site mutations who were WASP-negative had severe disease with scores of 3 to 5.

A striking correlation between WASP expression and susceptibility to infection was observed. The number of bacterial and viral infections per patient-year was almost 4 times higher in WASP-negative patients than in WASP-positive patients. Fungal infections and *P carinii* pneumonia were only observed in WASP-negative patients. Interestingly, standard screening tests to assess immune responses were minimally affected, and no difference was observed between the 2 groups of patients. In-depth evaluation of the immune system in WAS patients has revealed reproducible defects; lymphocytes from patients with classic WAS fail to proliferate in vitro when exposed to anti-CD3 mAb.[20] If they are immunized with bacteriophage ΦX174, WAS patients have severely defective in vivo antibody responses characterized by failure for isotype switching and for developing immunologic memory.[21] In contrast, after bacteriophage immunization, patients with XLT are able to isotype switch and to develop immunologic memory though it is usually less than that of healthy controls.[22] The increased incidence of infections and the severe defect in antibody production justifies

prophylactic treatment of WAS patients with intravenous immunoglobulin infusions and, in those younger than 3 years of age, with trimethoprim-sulfamethoxazole and anti-herpes virus prophylaxis.

Platelet abnormalities are characteristic findings in patients with WASP mutations, but no differences in platelet number or size between the 2 groups were observed. Almost half the patients from each group had normal or increased numbers of megakaryocytes in the bone marrow. However, the proportion of patients with undetectable megakaryocytes was greater in WASP-negative than in WASP-positive patients (46% vs 8%, respectively). Nevertheless, as shown in Table 4, the number of serious episodes of bleeding (intracranial hemorrhage, intestinal bleeding) was higher in the WASP-negative group. One patient (3.7%) from the WASP-positive group and 4 patients (17.4%) from the WASP-negative group died as a result of hemorrhage.

As expected, the severity of eczema correlated with the expression of WASP. Only 1 of 27 WASP-positive patients had severe eczema compared with 11 of 23 WASP-negative patients. High IgE levels (more than 1000 IU/mL) were observed in most (62%) WASP-negative patients compared with only 25% in the WASP-positive group, strongly suggesting that the lack of WASP is directly or indirectly responsible for the atopic diathesis observed in WAS patients.

Whereas malignancies did not develop in any of the 27 WASP-positive patients during the observation period, malignancies were diagnosed in 5 of 23 WASP-negative patients (lymphoma, 2; myelodysplasia, 3). This observation correlates with the findings of others, who reported that the incidence of malignancies is low in XLT patients compared with classic WAS (H.D.O., unpublished observation; J.A. Winkelstein, personal communication).

In contrast, autoimmune/inflammatory diseases were equally frequent in WASP-positive and WASP-negative patients (26% and 22%, respectively). Characteristic nephropathy with IgA deposits was observed in 5 of 27 WASP-positive patients, but not in WASP-negative patients. IgA nephropathy progressed through the years and led to chronic renal failure in 2 patients. The incidence of nephropathy in patients with classic WAS was reported previously (5 of 32 patients),[23] and there are some case reports of XLT with nephropathy.[24-26] It is possible that IgA nephropathy required the generation of specific IgA antibody, which may be less likely to be produced in WASP-negative patients with defective isotype switching. In WAS patients there were some defects of glycosylation.[27] The *O*-glycan side chains in the hinge of the glomerular IgA1 were highly underglycosylated in IgA nephropathy.[28] Thus the impaired glycosylation of IgA1 molecules caused by mutated WASP might have contributed to the pathogenesis of IgA nephropathy seen in the patients.

Finally, we observed a striking difference in long-term outcomes. All but 1 of the 27 WASP-positive patients were alive at the completion of the study, compared with only 17 of 23 WASP-negative patients. Five patients (including an 8-year-old WASP-positive patient with a score of 4) died of intracranial hemorrhage, 1 patient died of JC virus encephalitis after BMT, and fatal EBV-associated B-cell lymphoma developed in 1 patient. Of those alive, the WASP-positive patients were significantly older than the WASP-negative patients (mean ages, 12.1 years and 6.1 years, respectively; $P < .001$). The 10-year probability rate of survival was higher for WASP-positive patients than for WASP-negative patients (92.3% vs 76.0%). Furthermore, when we calculated the 20-year probability rate of survival, none of the WASP-negative patients were expected to survive, whereas 92.3% of WASP-positive patients were expected to survive ($P = .004$). As shown in Figure 2B, intracranial hemorrhage-free survival was highly dependent on WASP expression. The 30-year probability of intracranial hemorrhage-free survival is 36.8% for WASP-positive and 0% for WASP-negative patients. Thus, the expression of WASP is closely linked to the overall disease outcome and has a strong prognostic value for patients with *WASP* gene mutations.

This retrospective analysis revealed that bone marrow transplantation was more frequently performed in WASP-negative patients (12 of 23) than in WASP-positive (3 of 27). Only 1 patient, a 14-year-old WASP-negative patient who underwent BMT from an unrelated HLA-matched donor, died of complications related to the procedure, which included EBV-related lymphoproliferative disease and JC-virus–associated progressive multifocal leukoencephalopathy. Both groups responded equally to splenectomy; however, 2 of the WASP-negative patients who underwent splenectomy died of intracranial hemorrhage, and sepsis/meningitis developed in 5 (2 from WASP-positive and 3 from WASP-negative group), developed in spite of antibiotic prophylaxis. Neither high-dose intravenous immunoglobulin infusions (IVIG) nor prednisone had a significant impact on thrombocytopenia in either group of patients, and neither is recommended in WAS/XLT.

This retrospective study demonstrates a strong phenotype-genotype correlation in patients with WASP mutation. Although this has been reported by others,[9,10,29] more detailed observations, such as the data related to thrombocytopenia, types of infections, incidence and course of autoimmune and malignant diseases, response to therapies, and prognosis, especially in a large number of XLT patients, gives us more informations to understand and to manage this complex disease. There is some overlap between the 2 groups, however, as has been observed by others.[11] Infants and young children may not yet have developed the final clinical phenotype; other genes may influence the severity of the clinical symptoms; some patients are exposed to more pathogenic microorganisms. Recently, reversal of the *WASP* gene mutations to normal has been observed in several WAS patients, resulting in improvement of the clinical phenotype.[30,31] The guarded long-term prognosis of patients with the classic WAS phenotype associated with the complete absence of WASP is a strong indication for early stem cell transplantation using matched related or unrelated bone marrow or cord blood stem cells because these methods were shown to have high probability of hematopoietic and immunologic reconstitution, as seen in this and other studies.[32] If there is a suitable donor, HSCT can also be a treatment of choice for WASP-positive patients with the XLT phenotype because of their equivalent or higher frequency of complications, among them ICH, autoimmune diseases, and IgA nephropathy, that lead to chronic renal failure. Prospective studies of HSCT for XLT are needed to establish the method and to assess the safety of HSCT for this patient group.

Splenectomy should be carefully indicated in WASP-negative patients if they have no suitable HSCT donor because 3 patients acquired pneumococcal sepsis/meningitis and 2 patients died because of ICH after splenectomy in our study in spite of the favorable outcomes of a previous report.[33] Indicating splenectomy for WASP-positive patients remains controversial. Further study is needed to establish the standard treatment regimen for WAS and XLT patients.

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