

Analysis of clinical and molecular characteristics of Wiskott–Aldrich syndrome in 24 patients from 23 unrelated Chinese families

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The clinical data of 24 children with Wiskott–Aldrich syndrome (WAS) from 23 unrelated Chinese families were reviewed in the present study. WAS protein (WASP) expression in peripheral blood mononuclear cells was examined by flow cytometry (FCM); WASP gene was amplified by PCR and directly sequenced to analyze mutations in the WASP gene in patients and their female relatives. FCM analysis of 21 patients showed that 18 cases were WASP-negative, and three had partially WASP expression. WASP gene analysis revealed mutations in 23 patients, including five missense mutations, four nonsense mutations, four deletion mutations, three insertion mutations, six splice site mutations, and one complex mutation, among which, 20 unique mutations were detected, including seven novel mutations (168 C > A, 747–748del T, 793–797del C, 1185 ins C, Dup 1251–1267, 1277 insA and 1266 C > G; 1267–1269del C). Five WAS children underwent stem cell transplantation. After 2 months of transplantation, WASP expression was restored to normal in all five patients whereas one patient died of cytomegalovirus-induced interstitial lung disease. WASP gene analysis can make a definite diagnosis of WAS and identify mutation carriers, beneficial for timely treatment and genetic counseling for children with WAS.

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Wiskott–Aldrich syndrome (WAS) is a rare X-linked recessive immunodeficiency disorder characterized by thrombocytopenia and small-sized platelets, eczema, recurrent infections, susceptibility to autoimmune diseases, and malignancies (1, 2). If children with WAS do not receive stem cell transplantation, 70% cannot survive beyond the age of 14. The gene responsible for this syndrome, WAS protein (WASP) gene, consists of 12 exons containing 1823 base pairs (3). WASP encodes a 502-amino acid protein that is expressed selectively in hematopoietic stem cell-derived lineages and is involved in intracellular signal transduction and cytoskel-

etal reorganization, thereby playing an important role in hematopoietic cell differentiation, migration, cell signal transduction, immunologic synapse formation, and lymphocyte apoptosis (4–6).

To date, more than 300 types of WASP gene mutations have been reported. WASP gene mutations show great variation and are manifested by a wide spectrum of clinical phenotypes. Besides, the exact correlation between genotype and phenotype has not been fully characterized. WAS is prone to be misdiagnosed, and therefore treatment is often delayed. There are few studies available on WAS cases in China, a populous and developing country. Thus, in this study, we

analyzed the clinical, immunologic, and molecular characteristics of 24 children with WAS from 23 unrelated Chinese families, in an attempt to provide information for improving the diagnosis and treatment of WAS in China.

Subjects and methods

Subjects and clinical phenotypes

Totally, 24 male children from 23 unrelated Chinese families from April 2007 till July 2009 were included in this study. These children aged from 2 months to 10 yr and 9 months were from 13 provinces. Using previously published criteria (7, 8), the severity of WAS was estimated and expressed as a score of 1–5. 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 X-linked thrombocytopenia (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.

WASP gene mutation analysis

After informed consent was obtained to sequence DNA and immunologic analysis, 2 ml of fresh whole blood was collected from each case. Genomic DNA was extracted from whole blood using the QIAamp DNA Blood mini kit (Qiagen-GmbH, Hilden, Germany). RNA was isolated from peripheral blood mononuclear cells (PBMCs) using Total RNA Purification kit (Genemega, San Diego, CA, USA) and submitted to reverse transcriptase-polymerase chain reaction (RT-PCR) using the RT-PCR kit (Takara, Otsu, Japan) according to the manufacturers' instructions.

WASP cDNA was amplified by PCR in two overlapping fragments using the previously reported primers (9). Purified genomic DNA samples were amplified with primer pairs designed to span each exon and exon/intron junction (10). The PCR reaction mixture (25 µl each) contained 1.25 U Taq polymerase, 0.5 µM 5' and 3' primers, 250 µM dNTP, and 10 µM Tris-HCl. PCR amplification was performed at an initial denaturation step at 94°C for 1 min, followed by 35 cycles consisting of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min, and a final extension step at 72°C for 10 min.

PCR products were separated by 2% agarose gel electrophoresis and subjected to extraction with QIAquick PCR Purification kit (Qiagen, Hilden Germany) and directly sequenced using the same primers used in the PCR on an automated ABI PRISM 3100 Genetic Analyzer (PE Applied Biosystems Foster city, California USA). Detected mutations were confirmed by sequencing in the opposite direction. PCR amplification and sequencing were performed at mutation regions on DNA in their related female family members when WASP gene mutations were detected in children with WAS.

WASP expression detected by Flow cytometry (FCM)

PBMCs were isolated by Ficoll-Hypaque density gradient centrifugation and washed twice with phosphate-buffered saline (PBS). Cells were fixed by 0.5 ml of Cytofix/Cytoperm solution (BD Pharmingen, New jersey, USA) at room temperature for 20 min. Then, cells were washed twice with Perm/Wash solution (BD Pharmingen New jersey, USA), 0.25 g/l of anti-human WASP monoclonal antibody (BD Pharmingen New jersey, USA) was added to cells and incubated at room temperature for 30 min. After washing with Perm/Wash solution, 1:10 dilution of FITC-conjugated goat anti-mouse IgG2a (Southern Biotech, Birmingham, Alabama USA) was added to cells and incubated at room temperature for 30 min. WASP expression in PBMCs was detected by a FCM (FACS Calibur; Becton Dickinson New jersey, USA).

Scanning electron microscopy (SEM)

Fresh whole blood was collected from five patients with WAS, and PBMCs were isolated by Ficoll-Hypaque density gradient centrifugation and washed twice with PBS. PBMCs were examined by SEM. PBMCs from normal individuals were also examined by SEM as control.

Results

Clinical characteristics and scores

Twenty-four children with WAS met the clinical diagnostic criteria of WAS. They were characterized by bleeding, eczema, recurrent infections, thrombocytopenia, and small-sized platelets. The age of onset ranged from birth to 5 months. Eighteen patients had an onset within 1 month after birth, and two patients even developed symptoms at birth. Bleeding was the first manifestation of WAS in 17 cases, bloody stool in 13

Table 1. Analysis of clinical characteristics of 24 children with Wiskott–Aldrich syndrome

Case no.	Age at onset	Age at diagnosis	First manifestation	Bleeding	Eczema	Infection	Platelet count ($10^9/l$)	Platelet volume	HSCT	Score	Family history
1	20 days	2 months	Bloody stool	+	+	+	33–55	–	2009	4	–
2	10 days	14 months	Bloody stool	+	+	+	4–60	~7.8	2008	5	–
3	20 days	2 months	Bloody stool	+	+	+	24–46	~7.1		4	+
4	4 days	11 months	Bloody stool	+	+	+	38–49	–		4	–
5	2 days	4 yr	Petechiae	+	+	+	10–40	–		4	+
6*	2 months	10 yr	Bloody stool	+	+	+	30–47	–		4	+
7*	3 months	9 yr	Bloody stool	+	+	+	40–53	–		3	+
8	20 days	16 months	Bloody stool	+	+	+	11–30	–		4	–
9	Birth	8 months	Petechiae	+	+	+	1–41	–	2008	5	+
10	55 days	14 months	Diarrhea	+	+	+	10–69	–		3	–
11	1 month	2 yr	Blood stool	+	+	+	6–55	~4.1		4	+
12	3 days	4 months	Petechiae	+	+	+	0.4–3	–		4	+
13	1 month	21 months	Perianal abscess	+	+	+	10–39	–		4	+
14	1 month	4 months	Bloody stool	+	+	+	50	–		3	–
15	1 month	10 months	Bloody stool	+	+	+	18–27	~6.5		3	+
16	15 days	5 months	Diarrhea	+	+	+	17–74	–		4	–
17	Birth	15 months	Petechiae	+	+	+	49–55	–	2009	3	–
18	5 months	12 months	Bloody stool	+	+	+	70	6.9		5	+
19	13 days	3 yr	Bloody stool	+	+	+	40–80	–		3	–
20	2 months	18 months	Eczema	+	+	+	51	7.4		4	–
21	15 days	4 yr	Eczema	+	+	+	12–74	~8.2		3	–
22	40 days	3 yr	Diarrhea	+	+	+	11–76	–		3	–
23	1 days	11 months	Septicemia	+	+	+	5–56	~6.5	2007	4	+
24	15 days	5 months	Bloody stool	+	+	+	21–42	~6.9		4	–

–, undetectable /negative; RB, retinoblastoma; AIHA, autoimmune hemolytic anemia; HSCT, hematopoietic stem cell transplantation.

*Case 6 and case 7 were sibling brothers.

cases and skin petechiae in four cases. Infection was the first symptom of WAS in five cases, of whom there were three cases of infectious diarrhea, one case of neonatal sepsis, and one case of perianal abscess. Eczema was the first symptom in two cases. Of the 24 children with WAS showing recurrent infections, diarrhea was the most common form of infection (14 cases), followed by respiratory tract infection (11 cases), suppurative otitis externa (six cases), skin infection (five cases), perianal abscess (two cases), urinary tract infection (two cases), and purulent meningitis (one case). The scope and severity of eczema varied greatly from sporadic presence in the head and face to dense distribution on the whole body and skin folds. The clinical characteristics and scores of these patients with WAS were present in Table 1. Of the 24 children with WAS, three cases suffered from autoimmune hemolytic anemia (AIHA) or bilateral retinoblastoma scored 5, 13 cases scored 4, and the remaining 8 cases scored 3. Eleven patients had family history of bleeding tendency or recurrent infections in their related male family members.

Laboratory tests

Routine blood tests. In these children with WAS, platelet counts ranged between 1 and $80 \times 10^9/l$.

Platelet volume values were smaller than healthy subjects (normal platelet volume values in healthy children in our hospital ranged from 9.4 to 12.5 fl). The platelet volume value was undetectable in 15 cases and ranged between 4.1 and 8.2 fl in nine cases.

Immunologic tests. Data on serum immunoglobulin were available in 18 patients, as listed in Table 2. Raised serum IgG (> 10 g/l) and IgA (> 1 g/l) was found in 55.6% (10/18) and 72.2% (13/18), respectively. Low serum IgM (< 0.5 g/l) was present in 16.7% (3/18) of patients, while 27.8% (5/18) had raised IgM, and serum IgE level was elevated in 72.2% (13/18) of them.

Data on lymphocyte subset were available for 15 patients, but the pattern was heterogeneous. The proportion of CD3+ T cells increased in five patients, stayed normal in four patients and decreased in four patients. Diminished CD4+ lymphocyte count was found in 10 patients, and reversed CD4/CD8 ratio was found in seven patients. Only one patient had raised natural killer (NK) cell count, eight patients had normal NK cell count, and two patients had diminished NK cell count.

Scanning electron microscopy. SEM in five patients with WAS demonstrated abnormal

Table 2. Immunologic test results of 19 children with Wiskott–Aldrich syndrome

Case no.	Immunoglobulin				Lymphocyte subclassification					
	IgG (g/l)	IgA (g/l)	IgM (g/l)	IgE (IU/ml)	CD3 (%)	CD19 (%)	CD4 (%)	CD8 (%)	CD56+16+ (%)	CD4JCD8 (%)
2	16.12	2.29	0.62	1053	67	12	25	34	14	0.74
3	3.19	0.56	0.71	56.30	46	23	38	9	29	4.22
4	12.76	1.91	0.72	391	67	9	36	26	20	1.38
5	9.88	2.94	0.93	834.3	—	—	—	—	—	—
6	9.92	3.13	0.36	110.2	—	—	—	—	—	—
8	9.65	0.63	0.51	259.7	47	46	28	20	12	1.4
9	23.53	1.32	1.41	706.1	68	7	13	46	25	0.28
10	—	—	—	—	92	5	16	73	4	0.22
11	10.35	3.68	0.66	400.5	84	6	37	44	6	0.84
12	7.33	1.149	1.03	111.4	50	9	32	15	36	2.13
13	15.86	1.31	1.45	5669	36	11	18	16	43	1.13
17	10.80	2.05	1.78	215.0	50	19	22	25	29	0.88
18	11.69	0.86	0.91	836.1	—	—	—	—	—	—
19	8.97	2.28	0.41	1070	76	2	9	64	—	0.14
20	10.60	0.87	0.71	123	38.80	—	18.90	19.7	—	0.96
21	5.88	0.37	0.59	424.3	56	5	17	38	36	0.45
22	7.11	1.24	1.35	118.6	—	—	—	—	—	—
23	11.00	1.14	0.35	169.3	68.2	26.98	19.46	38.3	—	0.51
24	15.7	1.15	2.21	449.9	64	9	23	37	22	0.62

—, not available.

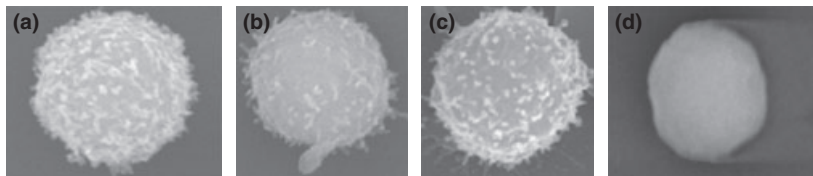


Fig. 1. Scanning electron microscopy of lymphocytes (a, b, c $\times 3.0$ K, d $\times 2.5$ K). (a) The lymphocyte from normal individuals was covered with intensive microvilli projections; (b and c) the lymphocyte from patients with Wiskott–Aldrich syndrome (WAS) was qualitatively abnormal, with short, blunted microvilli. (d) The lymphocyte from patients with WAS was devoid of microvilli.

lymphocytes, including the presence of sparse, blunted, or disrupted microvilli. Besides, microvilli were also found to be absent in some lymphocytes, also known as bald lymphocytes (Fig. 1).

WASP detection

As revealed by results of FCM present in Fig. 2, of the 21 cases detected, 15 cases had no or minimal expression of WASP in PBMCs, namely WASP-negative. Three cases (P11, P15, and P21) showed partial expression of WASP. Mothers of the 21 cases had normal levels of WASP expression.

WASP gene analysis

WASP gene detection was performed in the 24 children with WAS. Twenty unique WASP gene mutations were detected in 23 cases, including five cases of missense mutation, four cases of nonsense mutation, four cases of deletion mutation, three cases of insert mutation, six cases of

splice site mutation and one case of complex mutation. The remaining one case showed no mutation in the coding regions of the WASP gene (Table 3). Of the 20 WASP gene mutations, seven were novel mutations, including a complex mutation (168 C > A; 747–748 del T; 793–797del C; 1185 ins C; Dup 1251–1267; 1277 insA and 1266 C > G, 1267–1269del C) (Fig. 3). Hot spot mutations occurred in six cases. These mutations were distributed in seven exons (exons 1, 2, 4, 7, 8, 10, and 11) and four introns (introns 1, 3, 8, and 9) of the WASP gene. Of the five missense mutations, four were localized in the first four exons and one in exon 11. Of the seven insertion and deletion mutations, five were situated in exons 7–10. Of the six splice site mutations, three were found at the splice sites of introns 8 and 9, and the other three at the splice sites of introns 1 and 3. A variant splice site mutation occurred in intron 8 in case 21. T-A cloning was performed after purification of RT-PCR products so as to investigate the effect of WASP gene mutation on transcription. Twenty clones were selected for

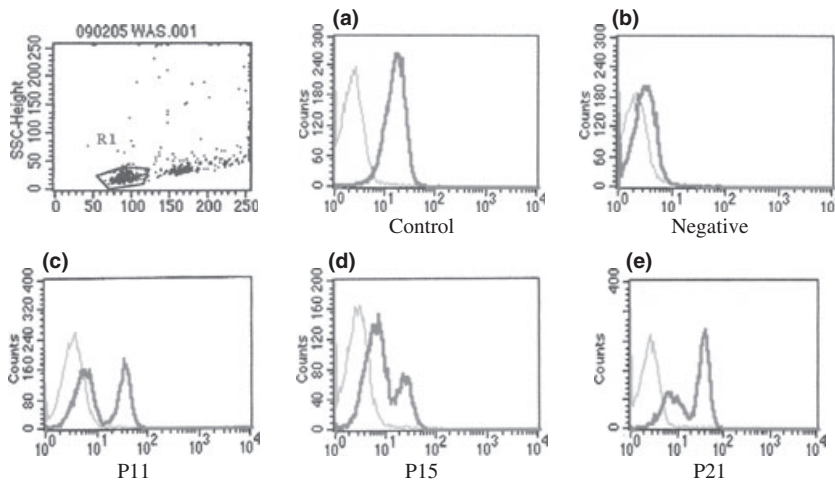


Fig. 2. Flow cytometry detection of Wiskott-Aldrich syndrome protein (WASP) expression. (a) Normal control (b) 15 cases showed no or minimal WASP, namely WASP-negative. (c, d, and e) Three cases (P11, P15, and P21) showed partial WASP expression.

Table 3. WASP mutations in 24 patients from 23 unrelated Chinese families

Patient	Score	Exon/intron	Mutation type	gDNA mutation	cDNA mutation	Effect	Carrier	WASP expression
1	4	1	Deletion	141–142del TT	ND	F36X	Mother	Absent
2	5	1	Nonsense	155 C>T	155 C>T	R41X	Mother	Absent
3	4	1	Nonsense	155 C>T	155 C>T	R41X	Mother	Absent
4	4	2	Missense	168 C>A	168 C>A	T45K	Mother	Absent
5	4	2	Missense	252 C>A	252 C>A	C73Y	Mother Sister	Absent
6	4	2	Missense	291G>A	291 G>A	R86H	Mother	Absent
7	3	2	Missense	291G>A	291 G>A	R86H	Mother	Absent
8	4	4	Deletion	470del C	470del C	Q146fsX260	Mother	Absent
9	5	7	Deletion	747–748del T	747–748del T	I238fsX260	Mother	Absent
10	3	7	Nonsense	665 C>T	665 C>T	R211X	Mother	Absent
11	4	7	Nonsense	665 C>T	665 C>T	R211X	Mother	Reduced
12	4	8	Deletion	793–797del C	ND	Q255fs260X	Mother	Absent
13	4	10	Insertion	1185 ins C	1185 ins C	P385fsX494	Mother	Absent
14	3	10	Insertion	Dup 1251–1267	ND	P413fsX450	Mother	Absent
15	3	10	Complex	1267–1269del C 1266 C>G	1267–1269del C 1266 C>G	A411fsX444 A411G	Mother	Reduced
16	4	10	Insertion	1276–1277 insA	1276–1277 insA	L415fsX494	–	ND
17	3	11	Missense	1487 G>A	ND	D485N	Mother Aunt	Minimal
18	5	Intron 1	Splice	IVS 1 +1G>T	ND	ND	Mother	Absent
19	3	Intron 1	Splice	IVS 1 +1G>A	ND	ND	Mother	Absent
20	4	Intron 3	Splice	IVS 3 +1G>A	ND	ND	–	ND
21	3	Intron 8	Splice	IVS 8 +1 to +6 del GTGA	25% exon8 del 75% normal splice	fs246X normal	Mother	Reduced
22	3	Intron 8	Splice	IVS 8 +1G>A	ND	ND	ND	ND
23	4	Intron 9	Splice	IVS 9 +2T>C	ND	ND	Mother Sister	Absent
24	4			No mutation was found in coding regions				Absent

Cases 4, 9, 12, 13, 14, 15, and 16 had novel WAS gene mutations; cases 6, 7, 10, 11, 21, and 22 had hot spot mutations in the WASP gene. Case 6 and case 7 were sibling brothers.

ND, not detected; –, no WASP gene mutation; del, deletion; ins, insertion; WASP, Wiskott-Aldrich syndrome protein.

sequencing after PCR amplification. Normal WASP gene splicing was observed in a small fraction of clones (25%), while the majority of clones (75%) showed deletion of exon 8 in WASP transcripts (Fig. 4).

Genetic study for carrier status was carried out in 22 families with definite genetic diagnosis, and

25 carriers of WASP gene mutations were identified in 20 families, and no WASP gene mutation was detected in the mothers of two cases (case 16's mother and case 20's mother). Case 4's mother and case 17's aunt (both were carriers of WASP gene mutation) underwent prenatal diagnosis at 20 wk of pregnancy. Their fetuses

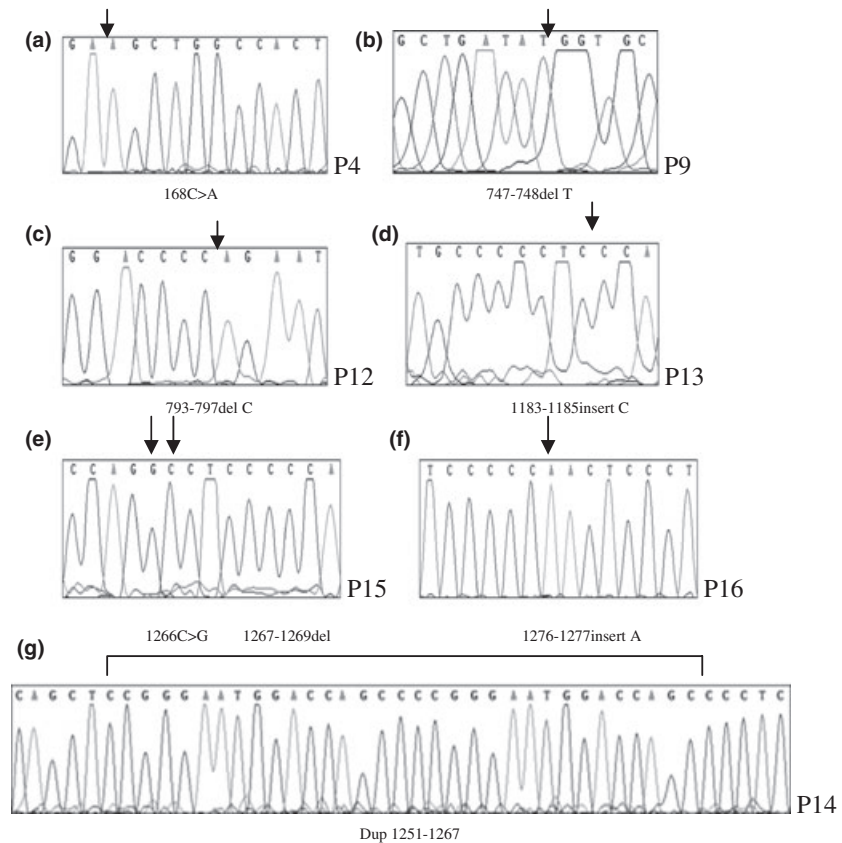


Fig. 3. Novel Wiskott–Aldrich syndrome protein (WASP) gene mutations in seven cases of WAS. (a) Transversion of C to A at position 168 was detected in exon 2 in case 4. (b) TT was deleted at position 747–748 in exon 7 in case 9. (c) A cytosine was deleted between position 793 and 797 in exon 8 in case 12. (d) A cytosine was inserted between position 1183 and 1185 in exon 10 in case 13. (e) A complex mutation in exon 10 in case 15, including a transversion of C to G at position 1266 and a deletion of cytosine between position 1267 and 1269. (f) An adenine was inserted between position 1276 and 1277 in exon 10 in case 16. (g) A duplication mutation at position 1251–1267 in exon 10 in case 14.

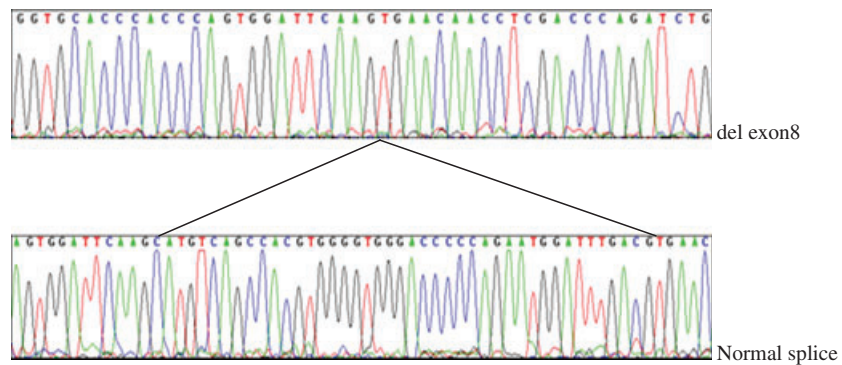


Fig. 4. T-A clone of cDNA on Wiskott–Aldrich syndrome protein (WASP) gene from case 21 with variant splice mutation of intron 8. (a) A 75% transcripts from case 21 with variant splice mutation of intron 8 had exon 8 of WASP gene deletion; (b) 25% transcripts from case 21 with variant splice mutation of intron 8 had normal splice.

were found to be female, and no WASP gene mutation was detected.

Treatment and survival

In addition to use of conventional antibiotics for infection control, three cases received regular intravenous immunoglobulin (500 mg/Kg/month); 14 cases received irregular intravenous immunoglobulin whereas seven cases never received intravenous immunoglobulin. No case underwent splenectomy. Five patients underwent

hematopoietic stem cell transplantation (HSCT) and age at transplantation ranged from 2 to 3.8 yr (median, 2.6 yr). Hematopoietic stem cell source was bone marrow in one case (case 13), or cord blood in four cases (cases 1, 2, 9, and 17). Case 23 underwent human leukocyte antigen (HLA)-matched sibling bone marrow stem cell transplantation, the donor being his older sister. While two patients (cases 1 and 17) were HLA mismatched HLA at one locus (5/6), and the other two patients (cases 2 and 9) were HLA mismatched HLA at two locus (4/6). All the five

patients received a conditioning regimen consisting of busulfan (16 mg/kg total dose) and cyclophosphamide (200 mg/kg total dose) 9 days before the transplantation. Cyclosporine A was given as graft-versus-host disease (GVHD) prophylaxis following HSCT. GVHD was graded according to standard criteria. Acute grade I GVHD occurred in three patients (cases 9, 17, and 23), grade III GVHD occurred in one patient (case 2), and chronic grade IV GVHD occurred in one patient (case 1). Assessment of HSCT outcome was performed by using short tandem repeat and WASP protein staining. All the five patients exhibited normal expression of WASP, and full chimerisms were also detected 50 days after transplantation. Full chimerism was defined by the presence of 95% or more donor blood cells. Case 2 died of cytomegalovirus (CMV) infection-induced interstitial lung disease 70 days following transplantation. Case 23 had normal platelet count and lymphocyte subset within 2 yr post-HSCT. It had been reported by our group (11). The long-term effect of unrelated cord blood stem cell transplantation in cases 1, 9 and 17 should be followed up. Of the remaining 19 cases of WAS, 18 survived thus far, and one case (case 16) died of severe pulmonary infection at 6 months old.

Discussions

WAS is an X-linked recessive genetic disorder characterized by thrombocytopenia, small platelets, eczema, recurrent infections, susceptibility to autoimmune diseases, and malignancies. WAS is caused by abnormal WASP expression because of WASP gene mutations. Some cases may only show platelet abnormalities and bleeding tendency with occasional occurrence of mild eczema and infections, which is known as XLT (12). An even smaller proportion of patients with WASP gene mutations only show neutropenia, without the clinical findings characteristic for WAS/XLT, which is known as X-linked neutropenia (XLN) (13). WAS, XLT, and XLN are different phenotypes of the same disease caused by WASP gene mutations.

In the present study, we assessed the clinical data of 24 children with WAS from 23 unrelated Chinese families in 13 provinces. We found that all children had typical clinical presentations including bleeding, eczema, recurrent infections, thrombocytopenia, and reduced platelet volume. These cases had an early age of onset, with the overwhelming majority of cases having an age of onset within 1 month after birth. The first manifestation in most cases was bleeding, with

bloody stool being the most common one. The 24 patients of WAS all exhibited recurrent infections, among which diarrhea and respiratory tract infection were the most common forms; besides, suppurative otitis externa, skin infections, septicemia, and perianal abscesses were also frequently observed, consistent with previous studies (14–16). Of various forms of bleeding in the patients with WAS, bloody stool and skin petechiae accounted for an overwhelming proportion. Children with WAS are known to be susceptible to autoimmune diseases and malignancies. Previous studies have found 40% of patients with WAS would develop autoimmune disease and malignant tumors, among which lymphoma is the most common one, and its incidence increases with age. In the current study, two cases experienced AIHA, and one case suffered from bilateral retinoblastoma that finally led to blindness. Using the scoring system proposed by Ochs for WAS phenotypes, we identified three cases with a score of 5, 13 cases with a score of 4, and eight cases with a score of 3 in the 24 cases of WAS. In contrast to published case series in other ethnic groups (17–19), no mild phenotype was found in our cohort. It is uncertain whether this was a genuine characteristic feature in the Chinese population or not, but it was possible that children with XLT were under-recognized and did not receive further investigation.

The previous study found the majority of WAS cases had moderately decreased serum IgM levels, increased serum levels of IgA and IgE, and normal levels of serum IgG (17). The number of T cells decreased with age. In this study, IgA and IgE levels of most of the patients elevated, while IgG levels remained normal or elevated except decreased in one case, consistent with most previous studies. However, IgM levels decreased only in three cases. CD3⁺ T cells counts decreased in only four cases, and diminished CD4⁺ lymphocyte count was found in 10 patients. Our results revealed that cases of WAS showed various degree of immunodeficiency in humoral and cellular immunity, and therefore diagnosis of WAS should not be based on immunologic test results alone.

We conducted genetic diagnosis on the levels of DNA and cDNA in the 24 children with WAS from 23 unrelated families, and we also further examined WASP expression in peripheral lymphocytes by FCM. Twenty unique mutations in 23 patients, including seven novel mutations and six hot spot mutations, were identified. The mutations consisted of six splice site mutations, five missense mutations, four nonsense

mutations, four deletion mutations, three insertion mutations, and one complex mutation. However, no mutations could be identified in one of the families, although the patient was diagnosed as having a WAS phenotype with a score of 4, and no expression of normal WASP was determined by FCM. We cannot discard that the mutation could be lie in the non-coding parts of the gene, such as the promoter, regulatory region, or introns, or this could be a case of genetic heterogeneity (20). Furthermore, a study from Hong Kong also found two typical WAS cases with no mutation in the WASP gene (15). These findings suggest that the pathogenic mechanism of WAS warrants to be further studied. Of the 23 WAS cases with a definite diagnosis, genetic study for maternal carrier status was carried out in 22 cases, 20 cases were confirmed to be heterozygous for the mutation detected in their child/children, and two cases showed no abnormal WASP gene. We speculate that some ovum of the mother may harbor a mutant WASP gene, or that the child may develop spontaneous WASP gene mutation.

Most studies have shown that missense mutations are typically located in exons 1, 2, 3, and 4 (14, 15). In the present study, of the five missense mutations, four lied in exon 2 except for one in exon 11, similar to that reported in many previous studies. The two missense mutations (R86H) in exon 2 contained mutation-prone CpG sites. Amino acid substitution at these sites might partially inhibit WASP functions (21). The C73Y substitution in a patient with severe WAS involves a cysteine residue that is conserved in WASP. Disruption of a disulfide bond can alter protein conformation and cause degradation of the misfolded protein. Although the D485N mutation in exon 11 is a missense mutation, Lemahieu et al. found that the D485N mutation could affect the 5' terminal conservative nucleotide sequence of intron 11, resulting in skipping of exon 11 and occurrence of frameshift mutation. In addition, transcript analysis revealed that some transcripts had normal splicing (22). In the present study, patients with the D485N mutation showed minimal WASP expression and had mild WAS manifestations, further confirming the above findings.

Mutations are heterogeneously distributed in the WASP gene. Except for missense mutation, which is predominantly localized at the N-terminus of the WASP gene (exons 1–4), nonsense, insertion, deletion, and splice site mutations occur preferably at the C-terminus of the WASP gene (exons 6–11). In this study, of the 18 cases with the latter four types of WASP gene

mutations, eleven cases had mutations localized at the C-terminus, consistent with the basic features of mutation distribution in the WASP gene. The most common type of mutations is splice site mutation, including one variant splice site mutation and five invariant splice site mutations scattered in four introns. Case 21 in the present study had a variant splice site mutation (IVS: intervening sequence, that is intron: 8 + 1 to +6 del GTGA). RT-PCR products of this case were sequenced after T-A cloning. We found that 25% (5/20) exhibited normal splicing, whereas 75% (15/20) had frameshift mutation attributed to deletion of exon 8, resulting in a truncated WASP of 246 amino acids. This result was in line with the milder clinical manifestations of this case. Similar patients were reported by several groups (18, 23, 24). In addition, we found another hot spot mutation in intron 8 (IVS 8 + 1 G > A). A number of previous studies have showed that the mutation results in deletion of exon 8 and frameshift, causing a premature termination at amino acid 246 in the WASP (18, 25). Mutation at the splice site of intron 9 (IVS 9 + 2 T > C) is an invariant splice site mutation. Most studies indicate that this splice site mutation leads to deletion of exon 9, clinically manifested by typical WAS. In addition to the above three splice site mutations, the other three splice site mutations in our present study occurred in the N-terminus of the WASP gene. It should be noted that case 18 had a mutation occurring in the splice site of intron 1, and this case developed bilateral retinoblastoma at 3 yr and 9 months old and finally lost his sight.

In addition to splice site mutations, our study also identified seven insertion and deletion mutations. In addition to two localized in exons 1 and 4, respectively, the other five were found in exons 7–10, suggesting that insertion or deletion mutations are preferably clustered in the carboxyl terminus of the WASP. In addition, of the seven cases, six were severe WAS cases and had a clinical score of 4, indicating that insertion or deletion mutation often causes severe WAS. In addition, nonsense mutations were found in four cases, of whom two in exon 1 and two in exon 7. The two cases with the R41X mutation in the present study had severe WAS phenotype. Moreover, one case developed AIHA at 1 yr and 2 months old. R211X is a hot spot mutation occurring in exon 7. Our present study also found the R211X mutation in two cases that were typical WAS.

Seven novel WASP gene mutations were identified, including one in exon 2, one in exon 7, one in exon 8, and four in exon 10. The seven novel

gene mutations include one missense mutation, two deletion mutations, three insertion mutations, and one complex mutation. 168 C>T(T45M) was a hot spot mutation (18, 23, 26). However, 168 C>A(T45K) had not been previously reported. Deletion of 747–748 T in exon 7 of the WASP gene in case 9 generated a truncated WASP of only 260 amino acids (I238fsX260), thus leading to loss of the functional GBD domain and the carboxyl terminus. A novel mutation (793–797delC, Q255fs260X) was detected in case 12 and predicted to generate a truncated WASP, consistent of no WASP expression determined by FCM. The only one complex mutation included a missense mutation at position 1266 (1266 C>G) and a deletion mutation at position 1267–1269 (1267–1269del C), resulting in a truncated WASP of only 444 amino acids (A411fsX444). Exon 10 is a proline-rich region whose reported mutations include insertion, deletion, and nonsense mutations as well as rare missense mutations. In this study, the four novel mutations in exon 10 all caused generation of truncated WASPs, resulting in loss of the functional VCA: verprolin-like central and acidic domain and seriously impairing WASP functions. Consequently, four cases with such novel mutations were typical severe cases of WAS. Complex mutation is a rare form of genetic mutations, generally including two types of missense mutations, point mutation followed immediately by deletion mutation as well as the combination of deletion and insertion mutations. Notably, previous study has shown children having WASP prematurely terminated at amino acid 444 or 493 because of frameshift mutations are predisposed to autoimmune disease or malignancies (18). Therefore, the three cases with such mutations in the present study should be closely followed.

WASP expression in the 21 cases of WAS were examined by FCM. Results demonstrated that 18 cases showed no or minimal expression of WASP, and three cases showed partial WASP expression. Of the three cases with partial expression of WASP, there was one case of variant splice site mutation in intron 8 (IVS 8 + 1 to + 6 del GTGA), one case of complex mutation (1266 C>G, 1267–1269del C), and one case of nonsense mutation in exon 7 (R211X). In the case of variant splice site mutations, a small amount of normal WASP may be generated owing to a certain degree of normal splicing as detected by T-A cloning. The complex mutation in exon 10 (1266 C>G; 1267–1269del C) was predicted to cause a frameshift and generated a WASP of only 444 amino acids (A411fsX444).

However, a small amount of normal WASP was detected by FCM. A similar mutation (1109 C>A; 1110–1113delC) occurring in an American child is also predicted to generate a truncated WASP with 444 amino acids in length. Nevertheless, weak expression of normal WASP was revealed in this case by Western blot (18). Further studies are needed to elucidate the specific mechanism. R211X is a hot spot mutation of the WASP gene. No study is currently available reporting that such mutation could lead to partial expression of normal WASP. One possible explanation is the occurrence of reverse mutation in the WAS case, leading to synthesis of normal or partially normal WASP. We found no second site reverse mutation or chimera in the coding regions or flanking splicing areas of the WASP gene. Another explanation suggests that nonsense mutations are also capable of inducing errors in mRNA splicing while generating partially normal splicing products (18, 22). Further studies are needed to determine whether the 665C>T mutation can also lead to abnormal mRNA splicing and generation of reduced amounts of normal WASP.

HSCT is currently the most effective means in curing patients with WAS. Negative WASP expression is a strong indication for administration of stem cell transplantation as early as possible. In other published series, the overall 5-yr survival rate in various donor types was 70–78.2% (27–30). The two most recent cohorts (29, 30) showed that the survival rate was up to 80% especially in patients who received transplant at younger age. We performed stem cell transplantation on five cases with definite genetic diagnosis and negative WASP expression. Hematopoietic stem cell (HSC) source was bone marrow in one case and cord blood in four cases. GVHD occurred in all the five cases, and chronic grade IV GVHD occurred in one case. As clinical GVHD has no demonstrated beneficial role with respect to durable engraftment or cure of the disease, efforts to reduce its incidence and severity are likely to further improve transplantation results. Normal expression of WASP and full chimerisms were detected in all the five cases 50 days after transplantation. One patient who received HLA-matched sibling bone marrow transplantation had complete disease remission. Platelets and WASP were shown to be normal upon multiple reexaminations within 2 yr after transplantation. Unfortunately, one case died of CMV infection-induced interstitial lung disease after transplantation. Therefore, particular attention should be given to such viral infections as CMV and Epstein–Barr virus in patients with WAS.

No sufficient information regarding the incidence of WAS in China is currently available. When estimated by the incidence of one WAS case in 100,000 live births, at least 250 of the 25 million neonates develop WAS each year. Since 1988 only 60-odd cases of WAS have been diagnosed in Mainland China, and no case of XLN or XLT has been reported. It suggests a high proportion of misdiagnosis and missed diagnosis in addition to selection bias and/or genetic variations. For patients with atypical clinical manifestations or mild symptoms, FCM detection of WASP expression together with genetic analysis of the WASP gene provides a definite diagnosis for WAS and has potential for prenatal diagnosis, which is of great significance to prenatal and postnatal care.

In conclusion, we confirm a heterogeneous distribution of WASP gene mutations in Chinese children with WAS by analysis of molecular characteristics. We also identify seven novel mutations in the WASP gene, thereby broadening the spectrum of WASP gene mutations. Currently, the correlation between genotype and phenotype in WAS cases is yet to be fully elucidated. In future, more cases need to be collected, and understandings of the medical staff on this disease, especially atypical or mild cases, should be deepened, so that more children with WASP gene mutations will receive timely diagnosis and treatment.

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