

CLINICAL FEATURES AND GENETIC ANALYSIS OF SIX PATIENTS WITH WISKOTT-ALDRICH SYNDROME REPORTING TWO NOVEL MUTATIONS: EXPERIENCE OF ERCIYES UNIVERSITY, KAYSERI, TURKEY

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Summary: *Clinical features and genetic analysis of six patients with Wiskott-Aldrich syndrome reporting two novel mutations: experience of Erciyes University, Kayseri, Turkey:*

Aim: The Wiskott-Aldrich syndrome (WAS) is an X-linked immunodeficiency characterized by micro-thrombocytopenia, eczema, and recurrent infections. We aimed to share our experience with six children with WAS, including two patients with two novel mutations.

Material and Method: We present phenotypical and laboratory description of six patients with WAS. The initial clinical presentation, biochemical and radiological features, molecular diagnosis together with long-term follow-up data are provided.

Results: The patients showed increased serum levels of IgE; otherwise the serum levels of IgM were decreased. The percentages of CD3+ T cells were decreased or within lower limit. Four patients underwent molecular genetics analysis and Western blot studies; two of them showed unpublished mutations: a hemizygous splice site mutation in intron 8 (c.778-2A>T), and a hemizygous deletion in exon10 of the WASP gene (c.1017delT; p.S339fsX444) were detected. Western blot studies confirmed the reduced WAS protein expression in peripheral mononuclear blood cells in four studied patients.

Conclusions: The major characteristics of patients were thrombocytopenia with decreased mean platelet volume and bleeding. All patients had been previously misdiagnosed as idiopathic thrombocytopenic purpura, demonstrating the importance of a careful differential diagnosis, and intense evaluation.

Key-words: Clinical features – Molecular genetics – Wiskott Aldrich syndrome.

INTRODUCTION

Wiskott-Aldrich syndrome (WAS) is an X-linked recessive immune deficiency disease (OMIM#301000) characterized by thrombocytopenia with small platelets, eczema and recurrent infections, susceptibility to autoimmune diseases, and malignancies (24, 27). The WAS gene is located on the short arm of the X chromosome in region Xp11.22-23 and consists of 12 exons containing a total of 1,823 base pairs (13).

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The *WAS* gene encodes the WAS protein (WASP) a 502-amino acid protein expressed selectively in hematopoietic stem cell-derived lineages and involved in cell signaling and cytoskeleton reorganization, thereby affecting the functions of T, B, NK- cell, granulocytes, dendritic cells and platelets (38). Loss of function mutations in the *WAS* gene, causes X-linked thrombocytopenia (XLT), or classical WAS; whereas gain-of function mutations leading to constitutive active WASP causes X-linked congenital neutropenia (4, 5, 10). XLT is characterized by congenital micro-thrombocytopenia with an increased bleeding tendency. XLT is sometimes associated with transient eczema. The prevalence of XLT equals that of WAS. The incidence of WAS/XLT, estimated to be 10 in 1 million live births (36). In the present study, we analyzed the clinical, laboratory features of six patients with WAS, in whom two of these patients have novel mutations.

METHODS

This was a retrospective study that was conducted in the Department of Pediatric Immunology and Hematology Oncology of Erciyes University (Kayseri, Turkey). Erciyes University Children Hospital is a tertiary hospital at Central Anatolia, Kayseri, Turkey. This hospital is a referral center, serving to a population of approximately 3 million along with the surrounding cities (31). The study was carried out after obtaining a written informed consent from all parents of the subjects. The Institutional Ethical Board of Erciyes University approved the protocol. We present demographic characteristics such as age, gender and age at onset, clinical features, and physical examination findings (Table I). In addition, the hematological and immunological laboratory data and molecular genetics features of our patients with WAS are reported (Tables II and III).

HEMATOLOGIC AND IMMUNOLOGIC ASSESSMENT

The routine blood tests including platelet count and mean platelet volume (MPV) were performed by complete blood analyzer Siemens Acvia 2120i. Serum immunoglobulin (Ig) levels were determined by nephelometry. Serum Ig levels were evaluated according to age-matched reference values in healthy Turkish children (1, 6). Analysis of peripheral lymphocyte subsets were performed by using five-color flow-cytometer (Cytomics FC 500 flow cytometer (Beckman Coulter). Monoclonal antibodies including CD45 FITC, CD3 FITC, CD4 PE, CD8 PC5, CD19 FITC, CD16-56 PE (Beckmann Coulter) were used.

Table I: Clinical phenotype

Patient no	Age of onset	Age of diagnosis	Now	First manifestation	Bleeding	Eczema	Infection	Other manifestation	Score	Family history	Present status
1	18 Month	11 Year	13 Year	Petechiae Purpura	+	+	+	Cervikal LAP Hepatosplenomegaly Brain abscess Herpetic keratitis Pigmental chorioretinal atrophy, Papilledema Bronchiectasis ANA +, ANCA+ Leukocytoclastic vasculitis Coombs test positive Aortic dilatation	5	Negative	Alive
2	6 Year	7 Year	10 Year	Petechiae	+	+	+		4	Positive	Alive
3*	3 Month	3 Month	9 month (Exitus)	Petechia Bloody stool	+	+	+	SRT CMV pneumonia	5	Positive	Died
4*	4 Month	1 Year	2 Year (Exitus)	Petechia Bloody stool	+	+	+	SRT	5	Positive	Died
5	2 Day	2 Year	4 Year	Routine follow-up		+	+	Hepatosplenomegaly	3	Negative	Alive
6	6 Month	6 Year	20 Year	Petechia		+	+	Henoch-Schönlein purpura Aortic aneurysmal dilatation ANA + Mediastinal LAP	5	Positive	Alive

SRT: severe refractory thrombocytopenia, ANA: Anti-nuclear antibodies, ANCA: Anti-neutrophil cytoplasmic antibodies, LAP: lymph adenopathy, MPV: Mean platelet volume, CMV: Cytomegalovirus, *Patient 3 and 4 are brothers.

Table II: Hematological and immunological features of the patients with Wiskott Aldrich Syndrome

Patient no	Platelet count (109/L)	MPV (7.4-10.4 fl)	IgA (mg/dl) (73-187)	IgE (iu/ml) (0-100)	IgM (mg/dl) (65-206)	IgG (mg/dl) (700-1630)	CD3 (%) (60-85)	CD4 (%) (29-59)	CD8 (%) (19-48)	CD19 (%) (11-16)	NK (%) (5-20)
1	16	7.4	115	2850	4.3	4400	40.2	36.2	29.6	10.4	7.7
2	18	6.7	434	189	41.6	1220	62.4	40.9	18.1	16.3	18.7
3*	5	6.2	220	367	29.2	2980	49	18.8	21.4	33	15.1
4*	7	5.3	183	794	132	1195	60	38.7	25	26	19.8
5	6	5.9	89	143	16.9	520	68	38	30	6.7	22.4
6	23	4.3	683	56	58	1610	48.9	35.7	15.4	24.3	21.9

*Patient 3 and 4 are brothers.

Table III: Molecular genetic studies of the patient with Wiskott Aldrich syndrome

Patient no	Exon/intron	Type of mutation	Mutation
1	Exon 2	Missense	c.G257A; p. R86H
2	Exon 10	Deletion	c. 1017delT; P.S339fsX444
3*	Exon 9	Splice-site	c.778-2A>T
4*	Not studied		
5	Exon 4	Missense	c.C371A; p. A124E
6	Not studied		

*Patient 3 and 4 are brothers.

MOLECULAR GENETIC STUDIES (PCR SEQUENCING AND PROTEIN EXPRESSION ANALYSIS)

Genomic DNA of the patients with WAS phenotype was extracted from peripheral blood cells. All exons and exon-intron boundaries of the human *WASP* gene (NM_000377) were amplified by polymerase chain reaction (PCR), and sequenced (Fig. 1). The "den Dunnen and Antonarakis" nomenclature was used for mutational description (9).

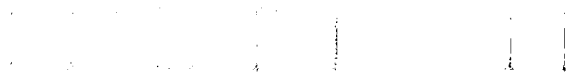


Figure 1:

Mutation of P1,2,3,5 are displayed in relation to WASp cDNA (CCDS14303) including known domains of WASp and mutational hotspots (grey shade: Ena/VASP homology 1 domain (EVH1), diagonal: GTPase binding domain (GBD), waves: proline-rich region (PPPP), longitudinal: verpoin coflin homology domains/acidic region (VCA), phosphorylation of Y291 is an important regulator of WASP activity; asterisk mark mutational hot spots.

Mutations in *WAS* lead to a reduced expression (patients #1, #2, #5), or an absent expression (patient #3) of WASP. A residual expression of a truncated protein was observed in patient #2 and patient #5 (Fig. 2).



Figure 2:

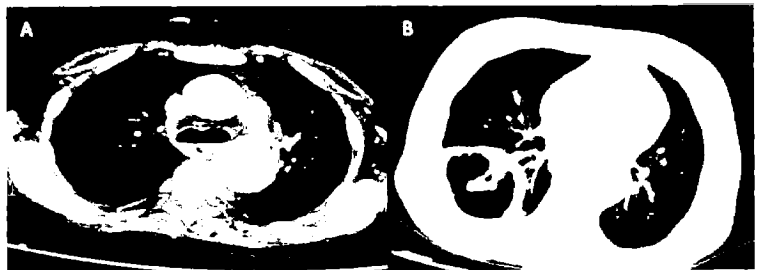
Mutations in *WAS* as described in Fig 2 lead to minimal expression (P1,2,5) or absent expression (P3) of WASP. In case of P2 and P5 a residual expression of a truncated protein is observed.

PATIENTS

PATIENT #1

This 13-year-old boy was the second child of a family with two healthy daughters. His family history revealed that his 10-year old cousin was treated for thrombocytopenia. Patient 1 was initially referred to our hospital at 18 months of age with petechiae. Previously, patient 1 had been diagnosed with ITP. At 9 years of age cervical and submandibular lymphadenopathy, as well as hepatosplenomegaly were noted. Upon admission the patient displayed eczematous and vasculitic skin lesions on his lower extremities. The platelet count was decreased to $15 - 48 \times 10^9$ cells/L. Although the mean platelet volume (MPV) was within normal limits, micro-thrombocytes were noticeable under light microscopy. Thoracic and abdominal tomography demonstrated intra-thoracic and intra-abdominal lymphadenopathy. Immunological studies showed decrease in CD3+ lymphocyte number %40.2 (%60-85). Serum levels of immunoglobulins (Ig) IgA, IgG, and IgE were increased, whereas serum level of IgM was decreased (Table II). Anti-neutrophil cytoplasmic antibody and direct Coombs tests were found to be positive. Histopathological examination of the cervical lymphadenopathy biopsy showed interfollicular plasma cell infiltrations with non-caseating granulomas. Histopathological examination of lower limb lesions revealed leukocytoclastic vasculitis. Echocardiography and MR angiography of the patient showed aortic coarctation and aortic aneurysm (Fig. 3). Thoracic computer tomography showed nodular lesions and bronchiectasis. At ophthalmological examination herpetic keratitis, pigmental chorioretinal atrophy and papilledema were detected.

Figure 3:
Thoracal axial CT image taken previously one year ago shows the normal aortic diameter and parenchyma (a). Thoracal axial CT images shows thoracal aortic aneurysm with focal rupture and surrounding hematoma (b).



He experienced skin infection due to *Staphylococcus aureus*, and herpetic keratitis due to *herpes simplex virus type 1 (HSV-1)*. Radiological and microbiological findings of the central nervous system revealed brain abscess due to *Streptococcus pneumoniae* meningitis (Fig. 4).

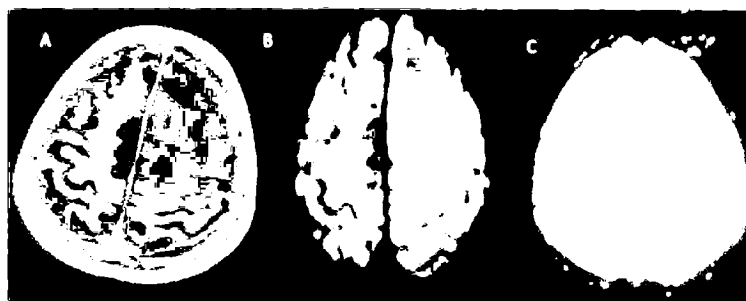


Figure 4a, b, c:
Axial brain T1 weighted
MR images after
gadolinium administration
shows contrast enhanced
multiple abscess formation.

The patient was treated with intravenous cephtriaxon, linezolid, metronidazol, vorikonazol and acyclovir for 30 days. The intracranial abscess was completely resolved (8). Clinical presentation and family history of patient 1 were suggestive of WAS. A Mutation in *WAS* gene was detected in exon-2 (Table III). Hematopoietic stem cell transplantation (HSCT) from his HLA identical daughter was performed in another center without any complication.

PATIENT #2

A 10-year old boy was presented with skin eruptions. He has been followed in another medical center because of eczema since 5.5 years of age. The parents were consanguineous, and his brother had thrombocytopenia. In the physical examination eczematous lesions at the cheeks, area beneath the chin, flexor aspects of joints, and petechiae were noted. Laboratory findings showed thrombocytopenia of 18×10^9 cells/L with microthrombocytes observed in the peripheral blood smears. Bone marrow aspiration showed normal numbers of megakaryocytes and a hypercellular bone marrow. The immunological evaluation showed: IgM, 41.6 mg/dL (65-206); IgA, 434 mg/dL (73-187); IgG, 1220 mg/dL (700-1630); IgE, 189 IU/mL (0-100). We identified a novel hemizygous deletion mutation in exon 10 of the *WAS* gene: c.1017delT; p.S339fsX444. In the absence of an HLA-matched family donor, allogeneic hematopoietic stem cell transplantation from full matched unrelated donor has been scheduled. Meanwhile, supportive care (platelet suspension transfusion, prophylaxis and treatment of infections) has been administered to patient #2.

PATIENT #3

Patient #3 presented with recurrent fever, cough, gastrointestinal bleeding, with eczema and mild infections, at age 3 month. One brother diagnosed with thrombocytopenia died due to cerebral hemorrhage

(patient 4). On physical examination, a petechial rash and ecchymosis were noted. Clinically, hepatosplenomegaly was noted (Liver palpated as 4 cm, while spleen as 2 cm). Hematological findings revealed micro-thrombocytopenia with platelet count of 5×10^3 cells/L. Immunological findings showed: IgG, 2,980 mg/dL; IgA, 220 mg/dL; IgE, 367 iu/mL; IgM, 174 mg/dL. Lymphocyte subgroups were normal. Cytomegalovirus positive pneumonia was diagnosed and the patient was treated with intravenous gancyclovir. Clinical presentation and family history suggest WAS. Sequencing of *WAS* gene showed a novel hemizygous mutation in the splice-site in intron 8 of the *WAS* gene: c.778-2A>t. Bone marrow transplantation was scheduled from an HLA-matched related donor. Unfortunately, the patient developed platelet refractoriness; the calculated Corrected Count Increment (CCI) for platelets 60 minutes after a platelet transfusion was found to be 1500. Intravenous immunoglobulin (IVIG), methylprednisolone, and vincristine were given but no response was detected. He died due to intra-cranial hemorrhage confirmed post mortem by CT scan.

PATIENT #4

Patient #4 presented with recurrent bloody stools at the age of 4 months. On physical examination eczema was noted. Peripheral blood analysis disclosed severe micro thrombocytopenia (platelet count of 21×10^9 /L; MPV of 5.3 fL (normal range, 7.4–10.4 fL)). Immunological evaluation, showed: IgG, 1195 mg/dL; IgA, 183 mg/dL; IgE, 794 iu/mL; IgM, 132 mg/dL. Lymphocyte subgroups were normal. Several treatment including IVIG and steroid were applied to this patient but he did not respond to treatment. Unfortunately, the patient died because of intracranial bleeding.

PATIENT #5

Patient #5 was referred to hospital for evaluation of petechial, thrombocytopenia and hepatosplenomegaly at the age of 3 month. The referring physician had suspected the diagnosis of leukemia. There is no consanguinity between parents. The family history was unremarkable for WAS. On physical examination at the age of 4 years, patient 5 showed petechia and hepatosplenomegaly. The platelet count varied from 6 to 13×10^9 cells/L; with a decreased MPV. Immunological tests showed a low IgM, IgG and CD19 level. Although severe infections had not occurred in the past, generalized petechial lesions, micro thrombocytopenia and a B cell deficiency were suggestive of WAS.

We identified a hemizygous missense mutation in exon 4 of the WASP gene: c. C371A; p.A124E.

HSCT from an HLA-matched sibling donor is planned. After in vitro fertilization and pre implantation HLA-matching, the mother is expecting a baby likely to be a suitable HLA donor for patient 5. This is in accordance with the applicable Turkish Ethics and Law.

PATIENT #6

Patient #6 was clinically diagnosed as having WAS at the age of 6 years. At the age of 6 months, he developed petechiae due to thrombocytopenia. At 16 year of age he experienced Henoch-Schönlein purpura, aortic aneurysmal dilatation, and mediastinal lymphadenopathy. The molecular genetic analysis was not performed, because the patient did not come regular medical control. To date, patient 6 is 20 years old.

RESULTS

CLINICAL AND IMMUNOLOGIC FEATURES

Six patients were included in this study. Age of onset varied from 2 day to 6 years of age, whereas age of diagnosis from 3 months to 11 year of age. Demographic data and clinical characteristics are presented in Table I. The hematological and immunological evaluations were presented in Table II. Genetic features were demonstrated in Table III. The patients presented with petechiae, eczema, bloody diarrhea, and recurrent infection, and the Ochs clinical scores were 5 for patient #1, #3, #4, and #6; and clinical score was 4 for patient #2, clinical score was 3 for patient #5. They were all thrombocytopenic with low MPV, and megakaryocyte number was normal or slightly increased in bone marrow. In 5/6 patients, the serum level of IgE was increased; otherwise in 6/6 patients the serum levels of IgM were decreased. The percentage of CD3+ T cells was decreased or lower limit. The level of CD4+, CD8+, CD19+ and CD16+ CD56+ cells were in normal range or slightly decreased.

Unfortunately, two patients died due to intracranial hemorrhage (patients #3, #4). Patient #1 was referred to another center at the request of the family. Donor selection and preparations for HSCT were in progress for two patients (patients #2, #5).

MUTATION ANALYSIS

Mutations were located in exons 2, 4, 9 and 10 of the *WASP* gene. Two novel mutations were found in the *WAS* gene. Hemizygous deletion mutation in exon 10 is a C to T transition: c.1017delT; p.S339fsX444. Hemizygous mutation in the splice-site of exon 9 is a A to T transversion c.778-2A>T. (Table III) (Fig. 1). Residual expression of WASP was detected in patient #1, #2, #5; WASP was absent in patient #3. Residual expression of a truncated protein was observed in patients #2, and #5.

DISCUSSION

In 1937, Alfred Wiskott (40) identified three brothers with thrombocytopenia, bloody diarrhea, eczema, and recurrent ear infections. The patients died at young age from intestinal bleeding and sepsis; an X-linked nature of WAS syndrome was suspected by Aldrich (3) in 1954. *WASP* mutation can cause different clinical conditions (24). Classic WAS is characterized by micro-thrombocytopenia, eczema and recurrent infections due to cellular and humoral immunodeficiency. Further an increased incidence of autoimmunity and malignancy were noted in patients with XLT/WAS (2, 15). Mutations that result in reduced but not absent, protein expression cause the milder disease X-linked thrombocytopenia (XLT) that is described mainly by thrombocytopenia and occasionally is associated with milder eczema and immunodeficiency (43). A third clinical phenotype X-linked neutropenia (XLN), defined by neutropenia and variable myelodysplasia, has been attributed to activating mutations in the GTPase-binding domain (GBD) of WASP (4, 5, 10).

In the present study, we evaluated the clinical data of 6 children with classical WAS. We detected characteristic clinical findings including in all children, i.e: eczema, bleeding, micro thrombocytopenia and recurrent infections. All patients had an early age of onset except for one patient (patient #2). The first sign in most cases was petechiae. Most patients had recurrent upper respiratory tract infection, otitis media, pneumonia and diarrhea as previously described (17, 42). One patient (patient #3) had CMV interstitial pneumonia, one patient (patient #1) had skin infection due to *Staphylococcus aureus* as well as meningitis and brain abscess caused by *Streptococcus pneumoniae*. Brain abscess had been observed in primary and secondary immunodeficiencies (8, 16, 33 34, 35). Our group recently published 24 patients with brain abscess including the patient #1 (8). In previous studies abdominal and

gluteal abscesses were reported but brain abscess in patients with WAS have not been reported so far (39, 42). We suggested that brain abscesses should be considered as a possible infectious complication in WAS patients. The same patient also experienced herpes *simplex virus type 1 (HSV-1)* skin infections, and herpetic keratitis.

Patients with primary immunodeficiency are prone to develop autoimmune diseases and cancer (29, 30, 32, 35). Previous studies show that up to 40% of patients with WAS develop autoimmune disease and malignant tumors. Lymphoma is the most common malignancy in WAS and its incidence is correlation with age (37). In WAS, autoimmune hemolytic anemia (14%), vasculitis (13%), kidney diseases (12%), transient arthritis (11%), chronic arthritis (10%), HSP (5%) are the most common encountered autoimmune conditions. In addition, inflammatory bowel disease, autoimmune neutropenia, uveitis, recurrent angioedema, cerebral vasculitis, dermatomyositis, myositis, autoimmune hepatitis, pyoderma gangrenosum, erythema nodosum, and vasculitis have been described (11, 14, 20, 22, 37). In our study two patients showed autoimmune disorders (leukocytoclastic vasculitis, Henoch-Schönlein purpura, auto-immune hemolytic anemia). Immunological correlates were a positive direct Coombs test, and autoimmune antibody positivity (ANA, ANCA) (%33) (Table I). In addition, aortic dilatation and aneurysma are detected in patient #1 and patient #6 (14). These are possible sequelae of vasculitis of the vasa vasorum of the aorta and vasculitis of the aorta itself.

Clinical severity score for WAS as developed by Ochs (25) ranges from 1-5. A score from 1-3 corresponds to XLT or patients with mild WAS. A score of 4-5 corresponds to severe WAS. A score of 5 is associated with severe disease (platelet refractoriness (see below), and/or autoimmunity and/or inflammation and/or malignancy) (2, 26). The scores of the presented patients varied from 3 to 5 (Table I). The clinical severity of patients' condition with WAS is correlating to the Ochs score in our cohort; and patients with higher Ochs score (patient #3, #4) experienced severe bleeding episodes and died early (2, 12, 25). In the Ochs score system, the degree and persistence of thrombocytopenia was only used to grade severity of the disease and associated with the mildest form of the disease, intermittent X-linked thrombocytopenia (score 1) (26). However, persistent and severe thrombocytopenia is associated with a significant risk of hemorrhage. In particular, in a large, multi-institutional retrospective study of patients with WAS, life-threatening bleeding had been recorded in nearly one third of the patients (37). Therefore, Mahlaoui *et al.* (19) have decided to modify the clinical severity scoring system by attributing a score of 5 also to

patients with severe refractory thrombocytopenia, defined as platelet count persistently $\leq 10 \times 10^9$ cells/L. Notarangelo *et al.* (23) recently underlined the importance of thrombocytopenia in patients with WAS. We detected platelet refractoriness in one of our patient; and vincristine, immunoglobulin, and methylprednisolone were given to the patient #4 as reported by Young and Angiolillo (41). Unfortunately two brothers (patients 3, #4) died due to refractory thrombocytopenia in their early life. Patient 3 had a hemizygous mutation in Exon 9 affecting the splice site (c.778-2A>T). Splice site mutations are found predominantly in exons 6-11. With exceptions, correlation between WAS genotype-phenotype leads to the general finding, that patients with absent WASP have a more severe clinical features than patients with residual WASP expression (18). The occurrence of refractory thrombocytopenia leading to fatal intracranial hemorrhage in 2 brothers suggests a similar genetical, environmental or treatment-associated effect. However, a WAS genotype-phenotype correlation with respect to refractory thrombocytopenia can only be hypothesized, because of the limited numbers of the patients. Jin Y *et al.* (13) identified of 141 unique mutations, 71 not previously reported, from 227 WAS/XLT families with a total of 262 affected members. In this study the most common mutations were missense mutations (n=80 families) followed by splice site mutations (n=44), deletions (n=40), and nonsense mutations (n=35). Insertions and complex mutations made up less than 13% of the WAS mutations. Most patients with WAS showed deletions or insertions affecting exon 10. We identified in our patient two missense mutations (affecting exon 2 and exon 4), one deletion mutation (affecting exon 10), one splice-site mutation (affecting exon 9) (Table III). As previously reported a majority of WAS patients have moderately decreased serum IgM levels, increased serum levels of IgA and IgE, and normal levels of serum IgG (43). In our study, 5/6 patients had increased levels of IgA and IgE; while IgG levels remained normal or elevated and IgM levels were reduced in 5/6 patients. The percentage of CD3+ T cells was decreased or in the lower limit. The level of CD4+, CD8+, CD19+ and CD16+ CD56+ cells were in normal range in the majority of the patients.

Traditional and supportive treatments include use of prophylactic antibiotics and antiviral agent such as trimethoprim sulfamethoxazole and aciclovir respectively, and use of platelet transfusions to treat major bleeding episodes. Intravenous immunoglobulin (IVIG) therapy is indicated in the presence of significant antibody deficiency. Immunosuppressive treatment may be required if autoimmune manifestations are present (18, 27). In our study two patient (patient #1 and patient #3)

were treated with immunosuppressive agents including cyclosporine A and vincristin for vasculitis and refractory thrombocytopenia respectively. Patient #3 and patient #4 died because of intra-cranial hemorrhage, which did not respond to the platelet suspension and other immunosuppressive agents including IVIG, methylprednisolone, and vincristin. The only curative therapy for WAS is hematopoietic stem cell transplantation although gene therapy for WAS is under study (7, 21, 28).

In conclusion, we report two novel mutations in patients with WAS. Clinicians managing patients with ITP must carefully evaluate differential diagnosis including XLT/WAS. To date refractory thrombocytopenia remains a challenge in patients with WAS.

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