

# Two novel mutations of Wiskott–Aldrich syndrome: the molecular prediction of interaction between the mutated WASP L101P with WASP-interacting protein by molecular modeling

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## Abstract

Wiskott–Aldrich syndrome (WAS) is an X-linked disorder characterized by eczema, thrombocytopenia and increased susceptibility of infections, with mutations of the *WAS* gene being responsible for WAS and X-linked thrombocytopenia. Herein, two novel mutations of WAS at T336C on exon 3, and at 1326–1329, a G deletion on exon 10, resulting in L101P missense mutation and frameshift mutation 444 stop, respectively, are reported. The affected patients with either mutation showed severe suppression of WAS protein (WASP) levels, T cell proliferation, and CFSE-labeled T cells division. Because WASP L101 have not shown direct nuclear Overhauser effect (NOE) contact with the WASP-interacting protein (WIP) in NMR spectroscopy, molecular modeling was performed to evaluate the molecular effect of WASP P101 to WIP peptide. It is presumed that P101 induced a conformational change in the Q99 residue of WASP and made the side chain of Q99 move away from the WIP peptide, resulting in disruption of the hydrogen bond between Q99 WASP and Y475 WIP. A possible model for the molecular pathogenesis of WAS has been proposed by analyzing the interactions of WASP and WIP using a molecular modeling study.

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**Keywords:** Wiskott–Aldrich syndrome (WAS); WAS protein (WASP); WASP-interacting protein (WIP); Molecular modeling

## 1. Introduction

Wiskott–Aldrich syndrome (WAS) is an X-linked disorder characterized by eczema, thrombocytopenia and immunodeficiency [1,2]. Classical WAS is often associated with the absence of WAS protein (WASP) caused by mutations in the *WAS* gene [3], which consists of 12 exons located on the X-chromosome at Xp11.22–p11.23 [4]. WASP is a signal

transduction protein that is expressed in hematopoietic lineage cells, plays a central role in promoting actin polymerization [5] and has a possible role in motility [6], phagocytosis [7] and activation of T [8] and B cells [9].

*WAS* gene encodes a 502-amino-acid protein which consists of a WASP homology 1 (WH1) domain, basic region (BR), GTPase-binding domain (GBD), polyproline (PP) domain, verprolin-cofilin homology (V/C, or WH2) domain, and a carboxy-terminal acidic (A) domain [1,2]. WASP can bind to a variety of proteins [1,2]; the WH1 domain at the N-terminal region of WASP interacts with the WASP-interacting protein (WIP) [10,11], which is important for NF-AT/AP-1-mediated gene transcription by cooperating with Vav [12]; for actin-tail formation [11], BR and GBD interact with GTP-bound Cdc42,

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phosphatidylinositol-4,5-bisphosphate, the PP domain with several signaling and adaptor molecules, such as Nck, Grb2, Fyn, CrkL, Bruton's tyrosine kinase (Btk) and proline-serine-threonine phosphatase-interacting protein (PSTPIP). The V/C and A domains of WASP also recruit actin-related protein (Arp) 2/3 complex for actin polymerization [1,13].

Recently, NMR spectroscopy and computer modeling studies to WASP WH1 and N-WASP EVH1, which are highly homologous WASP WH1 domains, have been able to predict the structure of WASP WH1 and its molecular interaction with WIP peptide [14,15]. The pathogenesis of WAS could be understood in detail by analyzing the missense mutation events of WASP WH1 through the structural basis of WASP WH1 to WIP.

In the present study, two novel mutations of *WAS* gene, a missense mutation of L101P WASP on exon 3 (family A; patient 1 and 2) and a deletion frameshift on exon 10 (family B; patient 3), both of which caused defects in CFSE-stained T cell division, are reported. We here analyzed the molecular interaction of the mutated P101 WASP with WIP peptide using molecular modeling because L101 WASP was not the direct binding site to WIP in the NMR spectroscopy [14] and the WASP expression was only decreased but not absent. P101 WASP was found to make the side chain of Q99 WASP move away from the WIP peptide and disrupt the hydrogen bond between Q99 of WASP and Y475 of WIP.

## 2. Patients, materials and methods

### 2.1. Patients

#### 2.1.1. Family A

Patient 1 (the index case), a 29-year-old man, had chronic idiopathic thrombocytopenia diagnosed at 7 years of age (Fig. 1A). He had a splenectomy for the treatment of idiopathic thrombocytopenic purpura (ITP), and was admitted due to pneumonia, meningoencephalitis and bacterial meningitis. *Streptococcus pneumoniae* type 6A was identified in a blood culture and *Acinetobacter baumannii* and vancomycin-resistant enterococci were identified in his sputum. His platelet count and MPV were  $30 \times 10^9/l$  and 8.5 fL, respectively. Platelet aggregation tests showed depressed responses to ADP, collagen, epinephrine and ristocetin. No secondary response wave was observed, revealing a pattern of storage pool deficiency. The immune cells were 41% of CD4+ T cells, 23.6% of CD8+ T cells, and 11.2% of B cells. The serum IgG, IgA and IgM levels were normal. His clinical score [16] was 4 and he died of intracerebral hemorrhage during the treatment. His brother, who suffered with ITP, died at an early age due to suspicious infections. His nephew (patient 2), a 4-year-old boy, was admitted due to recurrent intussusception. The platelet count was  $30 \times 10^9/l$  and the clinical score was 3. The nephew's brother was also diagnosed with ITP and died at 8 years of age.

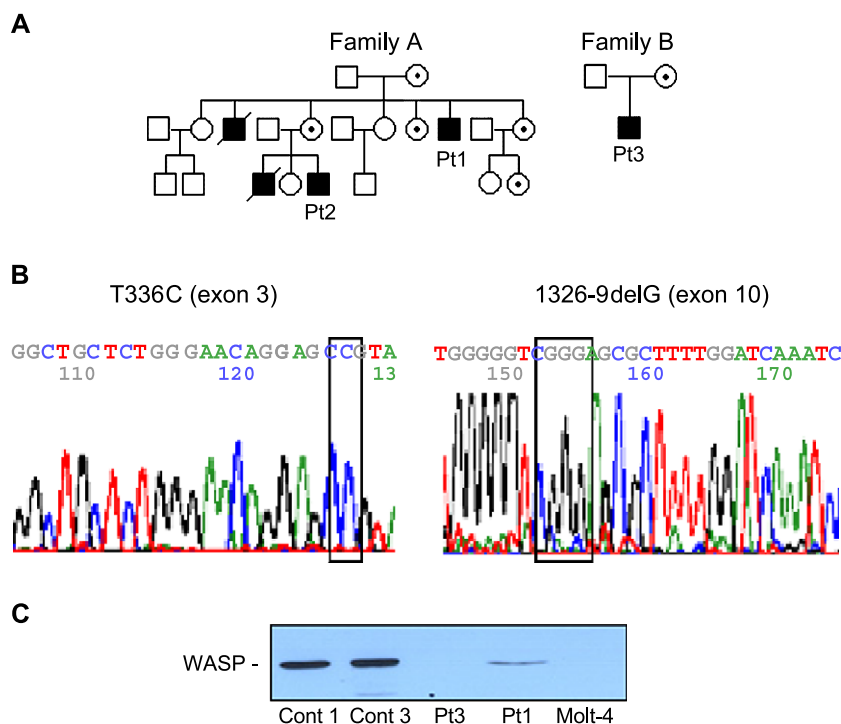


Fig. 1. Analysis of WASP expression. Pedigrees of families A and B with *WAS* patients (A) and their genomic DNA analyses for the WASP mutations of patients 1 and 2 (B left) and of patient 3 (B right) are shown. Each brother of patients 1 and 2 died at an early age. (C) The Western blot analysis of WASP. PBMCs were derived from patients (Pt) 1 and 3, normal controls (Cont) 1 and 3. Molt-4, a T cell line which expressed barely or not detectable WASP [36], was used as control cells. (For color see online version).

### 2.1.2. Family B

Patient 3 was an 18-month-old boy of non-consanguineous parents. He had eczema, intermittent petechiae with bruising and a clinical score of 4. The platelet count and MPV were  $14.7 \times 10^9/l$  and 7.7 fL of the low-normal limit, respectively. The serum IgG, IgA and IgM levels were normal, but the IgE level was highly elevated to 2310 IU/ml (normal range; <17 IU/ml). He had a congenital cytomegalovirus (CMV) infection confirmed by positive anti-CMV IgM in the serum and CMV DNA PCR of urine samples, but no chorioretinitis or hearing loss was observed. The immune cells were 11.7% of CD4+ T cells, 28.5% of CD8+ T cells, 3.3% of NK cells, and 53.8% of B cells. HIV RNA was not detected in the serum sample by PCR. His bone marrow finding of myeloid to erythroid cells was increased, with fully matured myeloid cells, abundant number of megakaryocytes and marked lymphocytosis.

### 2.2. Mutation analysis at the WAS gene

Genomic DNA was extracted from peripheral blood and the 12 exons, containing flanking splice sites at the *WAS* locus, were amplified as described [17]. DNA sequencing was performed in both directions using an ABI Prism 310 sequencer (Applied Biosystem, Foster City, CA, USA).

### 2.3. Analysis of WASP expression

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-hypaque gradients. The cells were lysed using 1% Nonidet P-40 buffer, containing a protease inhibitor cocktail (Sigma), and the concentrations of cytoplasmic protein measured by the Bradford assay (Biorad). After normalization of the concentrations of cytoplasmic protein, the protein lysates underwent 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and were transferred to a nitrocellulose membrane. Western blot analysis was performed using the monoclonal anti-WASP to amino acids 1–250 of WASP of human origin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) with HRP-labeled rabbit anti-mouse Ig used as a secondary antibody. The signal was revealed with enhanced chemiluminescence (Pierce, Rockford, IL, USA).

### 2.4. Proliferation assay

PBMCs, in 10% FBS-RPMI1640 (Gibco), containing 2 mM L-glutamine, 100  $\mu$ g/ml streptomycin and 100 U/ml penicillin, were plated at  $1 \times 10^5$  cells/well in 96-well U-bottom plates. Phytohemagglutinin (PHA) was added to the wells at the concentrations of 1 or 10  $\mu$ g/ml and incubated for 72 h. The cells were labeled with [ $^3$ H]-thymidine for 18 h. Thymidine incorporation was measured in triplicates by liquid scintillation counting, with the mean values used for the analysis.

### 2.5. Analysis of T cell division

The PBMCs were labeled with 5  $\mu$ M CFSE dye (Molecular Probes, Eugene, OR, USA) for 10 min at 37 °C [18], washed three times with 0.1% BSA-PBS and cultured with PHA at 1  $\mu$ g/ml for 90 h. The cells were washed and incubated with PE-conjugated anti-CD3 (BD Biosciences, San Jose, CA, USA) at 4 °C for 30 min. The cells were incubated with propidium iodide to exclude dead cells, and then analyzed by flow cytometry (Beckman Coulter, Miami, FL, USA).

### 2.6. Molecular modeling

Primary sequences of human WASP and WIP were retrieved from Swiss-Prot [19]. In order to search the template for homology modeling, PSI-BLAST was carried out with the WASP and WIP sequences against the Protein Data Bank (PDB, <http://www.rcsb.org/pdb>) [20]. The homology modeling was performed using the InsightII2000

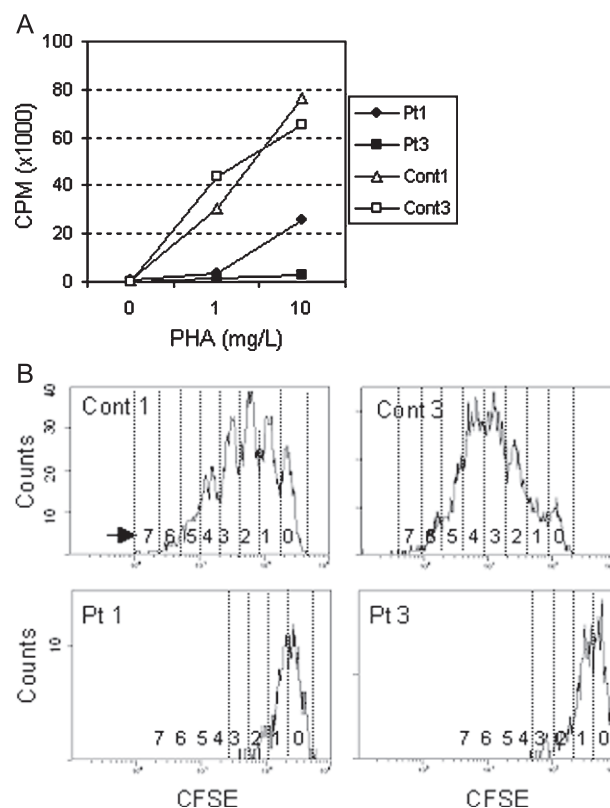


Fig. 2. T cell proliferation. (A) PBMCs ( $1 \times 10^5$  cells/well) were incubated with PHA at concentrations of 1 or 10  $\mu$ g/ml for 72 h, and the cells labeled with [ $^3$ H]-thymidine for a further 18 h. The thymidine incorporation was measured and the mean cpm values used for the analysis. The black and white circles and squares represent the patients and age-matched normal controls, respectively. (B) In vitro tracking of live CD3+ T cells. CFSE-labeled PBMCs (Pts 1 and 3, and Cont 1 and 3) were cultured with 1  $\mu$ g/ml PHA for 90 h, the cells harvested and then stained with PE-conjugated anti-CD3. The cells were stained with propidium iodide and the live CD3+ T cells analyzed by flow cytometry. The arrow indicates the number of cell divisions.

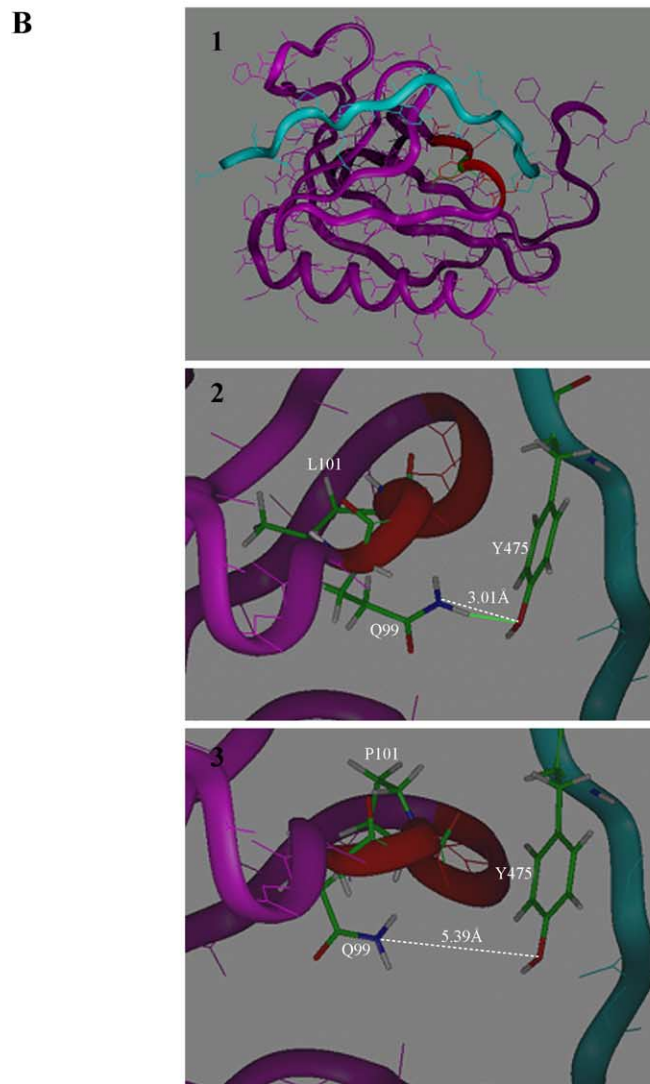
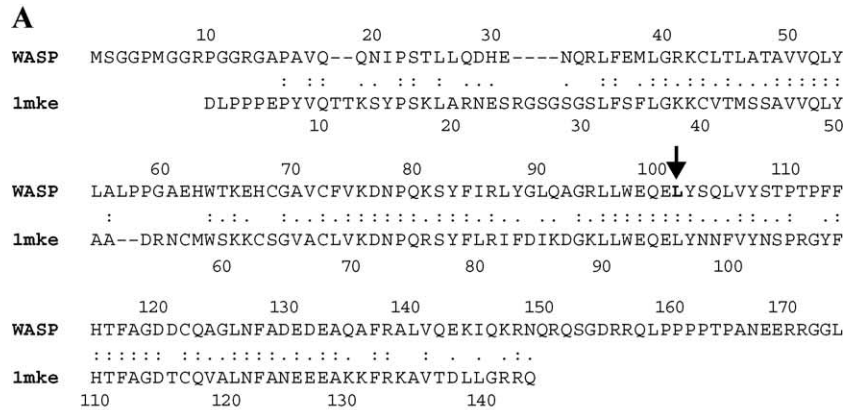


Fig. 3. Molecular modeling of WASP L101P with WIP peptide. (A) Sequence alignment of human WASP with the PDB code of 1 mke. Identities are shown with the (:) symbol, and similarities (or conservative replacements) with the (.) symbol. The arrow is the mutation site of WASP L101. (B-1) The model structure of the human WASP–WIP peptide complex. Purple, human WASP (residues R34–R148); cyan, WIP peptide (residues D461–P476); green, residue L101; red, residues Q99, E100, Y102 and S103. Molecular modeling of L101 wild (B-2) and P101 mutant (B-3) WASP with WIP peptide. The hydrogen bond between Q99 in wild WASP and Y475 in WIP is shown by the green line (B-2) and the distances (white dotted line) between the side chain nitrogen of Q99 and the side chain oxygen of Y475 were 3.01 and 5.39 Å in wild WASP and L101P mutant, respectively. Blue, nitrogen; red, oxygen; green, carbon.

Homology module (Accelrys). The WASP L101P mutant was constructed by replacement of the corresponding residue. To refine the model structures, each structure was energy-minimized using the steepest descents for 500 steps and conjugate gradients for 5000 steps with a distance-dependent dielectric constant ( $4^*r$ ). All molecular mechanics calculations were carried out using the Discover2.98 program (Accelrys) with AMBER force field using a nonbonded cutoff of 12 Å running on a Silicon Graphics O2 workstation.

### 3. Results

Patients 1 and 2 showed T336C nucleotide substitution on exon 3 of the *WAS* gene, resulting in L101P missense mutations (Fig. 1A and B). Heterozygosity for this mutation was detected in three sisters, the mother and one niece of patient 1. Patient 3 showed a G deletion on 1326–1329 of exon 10, resulting in a frameshift mutation that eventually stop at amino acid 444 (Fig. 1A and B). Both mutations were novel findings, with all three patients in this study having classical WAS phenotypes. The expression of WASP in unstimulated PBMCs was severely suppressed to 42% of control 1 in patient 1 and undetected in patient 3 (Fig. 1C).

The *in vitro* lymphocyte proliferation to 1 µg/ml PHA was markedly decreased to 5–15% (patient 1) and 2–10% (patient 3) of those of the age-matched normal controls, and low proliferative responses, even at high concentration of 10 µg/ml PHA (Fig. 2A). To investigate the cell division processing of T cells, PBMCs labeled with CFSE dye were cultured with 1 µg/ml of PHA for 90 h and then the cells stained with anti-CD3 Ab and propidium iodide. The numbers of live CD3+ T cell division generations were severely decreased two to three times in patients 1 and 3 with both mutations, compared to the six to seven times of the age-matched normal controls (Fig. 2B). The numbers of mitotic events of the live T cells, from a total of 20,000 CD3+ T cells gated, were 944 (patient 1), 627 (patient 3), 6061 (control 1) and 12,437 (control 3), and the percentages for patients 1 and 3 were 15.6% and 5.0% of the normal controls, respectively (data not shown). The percentages of live CD3+ T cells among the total CD3+ T cells after polyclonal activation were only 11.6% and 8.1% (Pt 1 and 3, respectively), compared to the 44.4% and 52.5% (Cont 1 and 3, respectively) (data not shown), indicating susceptibility of T cell deaths after T cell activation.

The L101 of WASP was identified as a highly conserved hydrophobic amino acid in multiple sequence alignment of the WH1 domains of the WASPs from several different species [15]. However, it did not show direct NOE contact with the WIP peptide, although the neighboring amino acids of Q99, E100, Y102 and S103 showed interactions with the WIP peptide on NMR spectroscopy [14]. How the novel L101P WASP missense mutation influences the interaction with the WIP peptide was analyzed using molecular mod-

eling, based on the NMR structure of the N-WASP EVH1 (pdb code of 1 mke)–WIP complex [14]. The sequence alignment of human WASP with 1 mke (sequence identity of 47.5%) was obtained from a PSI-BLAST search (Fig. 3A). This alignment suggested that 1 mke could be a good template for homology modeling [21,22]. The human WASP–WIP peptide complex structure was built by homology modeling using 1 mke as a template (Fig. 3A). WASP WH1 from R34 to R148 and WIP peptide from D461 to P476 were used for the modeling. It seems that the L101P WASP mutation has no effect on the WASP–WIP interactions, as it does not contact with the WIP [14]. According to our data, the model structure of the human WASP–WIP complex showed that the amino acid residues (Q99, E100, Y102, and S103) around L101 have interactions with WIP, but not L101 (Fig. 3B-1). In order to investigate the effect of the L101P mutation, molecular modeling studies were carried out on wild and L101P mutant WASP. The models achieved from the molecular modeling suggest that the L101P mutation makes the side chain of Q99 move away from the WIP peptide and disrupts the hydrogen bond between Q99 of WASP and Y475 of WIP (Fig. 3B-3), although the wild WASP maintained the hydrogen bond (Fig. 3B-2). The distances between the side chain nitrogen of Q99 of WASP and the side chain oxygen of Y475 of WIP peptide are 3.01 and 5.39 Å in wild WASP and L101P mutant, respectively (Fig. 3B-2 and B-3).

### 4. Discussion

Herein, two novel mutations of L101P WASP missense and frameshift mutation of WASP 444 stop, respectively, are reported. The WASP 444 stop, by a G deletion in 1326–1329, which deleted a partial region of the V/C and the whole A domain of WASP, is predicted to interfere the Arp2/3-mediated actin polymerization [1,13]. This effect is similar to that seen with 1030–1035 del G, 1109–1113 del C or 1301–1305 del G, each of which has a stop codon at the 444th residue [23,24].

In the case of the L101P missense mutation of WASP, how the P101 WASP influences the interaction was predicted with WIP at the molecular level, although the L101 WASP is found not to be the site of the NOE with the WIP peptide in the NMR spectroscopy study [14]. WASP expressions in PBMCs is correlated with that of T cells [25], and it is necessary to analyze the interaction of WASP and WIP, specifically, how residual L101P WASP in T cells disrupts the interaction with WIP. And the observation of T cell proliferation defect and impaired IL-2 production by anti-CD3 stimulation in WIP-deficient mice suggested that WIP is important for T cell activation [26]. Considering that human WIP residues 461–485 wrap around the N-WASP EVH1 domain amino acids 26–147, a highly homologous protein to the human WASP WH1 domain [14], the L101P missense mutation induces WAS by weakening the interactions be-

tween WASP and WIP, even if residue L101 (or P101) has no direct interaction with WIP. Therefore, it is suggested that the hydrogen bond between Q99 WASP and Y475 WIP is one of important interactions, and its breakage by the mutation at Q99, or the neighboring amino acid which influences its side chain, can disrupt the WASP–WIP interaction. We observed that the Q99R WASP mutation [27] weakened the WASP–WIP interactions in our modeling study (data not shown). In the study of the WASP–WIP interaction using the yeast two-hybrid system and *in vitro* WASP–WIP binding assay [28,29], WASP missense mutants such as R86H, Y107C, A134T, and R138P impaired the interaction with WIP in quantitative level, suggesting that the configuration is necessary for the interaction. For the future work in our study, the functional studies such as co-transfection or co-immunoprecipitation in cells will be helpful to show the consequences of L101P mutation with WIP.

The genotype–phenotype correlation in WAS/XLT was reported independently by several investigators, and is still controversial. Greer et al. [27] and Schindelbauer et al. [17] suggested no strict correlations. In contrast, Zhu et al. [23] showed that missense mutations in exons 1–3 caused mild disease permitting WASP expression. Recently, Imai et al. [30] analyzed the clinical phenotype and course of 50 WAS/XLT patients longitudinally. They demonstrated that WASP-positive WAS patients had better hematological and immunological findings, such as bleeding tendency, episodes of infection and severity of eczema, and also had a longer survival time than WASP-negative patients. In L101P missense mutation, detectable WASP expression was shown but the patient showed a clinically WAS phenotype. It is presumed that the low level of WASP expression and the weakening of WASP–WIP interaction by the disruption of hydrogen bond were involved in the disease progression. And there is a possibility of destruction of the binding between WASP WH1 and unknown ligand to be considered.

T cells of the affected patients with either mutation had dysfunctions of the continuous cell divisions, and T cell viabilities were severely decreased after PHA stimulation. In the study of the unselected 94 WAS patients, 66% of WAS patients were reported to have normal PHA response although 34% of the patients had decreased response [31], indicating the heterogeneity of the PHA response. The several reasons for defective T cell division and T cell viability were probably due to the decreased IL-2 production [32,33] or the increased apoptosis by Fas up-regulation [34]. In the regulation of T cell activation, NF-AT/AP-1 gene transcription is important in mediating normal signals from the T cell receptor, and Vav is a potent regulator of the IL-2 promoter by regulating the activity of the NF-AT/AP-1 transcriptional factor complex [35]. Savoy et al. [12] demonstrated that a functional WASP–WIP complex is required to enhance Vav-mediated activation of NF-AT/AP-1 gene transcription. So it would be helpful to observe the NF-AT/AP-1 activity that is triggered upon WIP interaction with WH1 domain of WASP.

In conclusion, a possible model for the molecular pathogenesis of WAS was proposed first on the aspect of the configuration change of the tertiary structure, as far as we know, by analyzing the interactions of WASP and WIP using a molecular modeling study.

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