Brief report

# Missense mutations of the *WASP* gene cause intermittent X-linked thrombocytopenia

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**Mutations of the *WASP* gene have been previously shown to be responsible for classical Wiskott-Aldrich syndrome, isolated X-linked thrombocytopenia, and severe, congenital X-linked neutropenia. We report herewith 2 families in which affected males had a history of intermittent thrombocytopenia with consistently reduced platelet volume, in the absence of other major clinical features, and carried missense mutations of the *WASP* gene that allowed substantial protein expression. This observation broadens the spectrum of clinical phenotypes associated with *WASP* gene defects, and it indicates the need for molecular analysis in males with reduced platelet volume, regardless of the platelet number. (Blood. 2002;99: 2268-2269)**



## Introduction

The Wiskott-Aldrich syndrome (WAS, MIM 301 000) is an X-linked disorder characterized by congenital thrombocytopenia with low mean platelet volume (MPV), eczema, increased susceptibility to infections, autoimmune diseases, and malignancies.[1] Cloning of the *WASP* gene, mutated in WAS,[2] has allowed the recognition of attenuated forms of the syndrome, with thrombocytopenia and mild eczema or infections, also referred to as X-linked thrombocytopenia (XLT, MIM 313 900).[3-5] Most patients with XLT have missense mutations within exons 1 and 2, leading to decreased but detectable protein expression,[6,7] whereas a wide spectrum of mutations, most often leading to the absence of protein, have been detected in classical WAS.[7,8]

We have identified 2 families in which affected males have a history of intermittent thrombocytopenia with persistently reduced MPV. Mutation analysis of the *WASP* gene disclosed missense mutations in exon 2 (family A) and exon 11 (family B).

## Study design

### Family A

The index case (patient 1) is a 7-year-old boy in whom petechiae developed at 1 month of age. He had mild and transient antecubital eczema in infancy. The diagnosis of idiopathic thrombocytopenia, based on a platelet count of $38 \times 10^{-9}$/L, was made when he was 2 years of age. Treatment with high-dose intravenous immunoglobulin (Ig), attempted twice, was ineffective. He continued to have intermittent petechiae and occasional epistaxis associated with variability in the platelet count, but he had consistently low MPV. His idiopathic thrombocytopenia was considered chronic.

His 4-year-old brother (patient 2) and a 39-year-old maternal uncle (patient 3) also had histories of intermittent petechiae, without other symptoms. At the time of first evaluation, his younger brother had $130 \times 10^{-9}$/L platelets and an MPV of 5.2 fL. His uncle's most recent platelet count was $64 \times 10^{-9}$/L, with an MPV of 6.4 fL. Searches for antiplatelet antibodies were performed in patients 1 and 2 and were negative. Immunologic evaluation revealed normal serum IgG, IgA, and IgM levels and normal antibody responses to hepatitis B immunization in the 3 affected males. IgE concentrations, performed in patients 1 and 3, were 346 IU/mL and 403 IU/mL, respectively. In vitro lymphocyte proliferation to mitogens was normal in all 3 patients, but it was markedly decreased (4%-15% of normal controls) to anti-CD3 in the brothers and was normal in the uncle.

### Family B

Patient 4, a 7-year-old boy, is the only child of nonconsanguineous parents. At 3 years and 5 months of age, he had petechiae and bruises. Platelet count was $4 \times 10^{-9}$/L. Treatment with intravenous immunoglobulin (800 mg/kg for 1 day) and prednisone (2 mg/kg for 3 weeks) resulted in a transient and moderate increase in platelet numbers ($58 \times 10^{-9}$/L). He has since maintained a low-normal ($106 \times 10^{-9}$/L) to normal ($289 \times 10^{-9}$/L) platelet count and reduced MPV without treatment. Clinical history was unremarkable for eczema and infections. Serum IgG, IgA, and IgM levels were normal.

### Mutation analysis at the *WASP* locus

Genomic DNA was extracted from peripheral blood. Amplification of each of the 12 exons and flanking splice-sites at the *WASP* locus was performed as described.[9] Mutation analysis was accomplished by single-strand conformation polymorphism and direct sequencing using the ABI Prism 310 sequencer (Applied Biosystem, Foster City, CA).

### Analysis of WASP protein expression

The WASP protein was immunoprecipitated from Epstein-Barr virus–transformed lymphoblastoid B-cell lines (LCLs) and from platelets derived from patients and controls, using the 3F3 anti-WASP monoclonal antibody.[10] Briefly, $20 \times 10^6$ LCL cells were lysed in 300 mM NaCl, 50 mM Tris, pH 7.5, 2 mM EDTA, pH 8, 0.5% Triton-X plus protease inhibitors (Buffer A). Platelets were prepared from peripheral blood collected in acid-citrate dextrose, after centrifugation at 500*g* and washing at 700*g* of platelet-rich plasma with one-third (vol/vol) acid-citrate dextrose. Platelets were resuspended in phosphate-buffered saline–.35% bovine serum albumin and were counted; $150 \times 10^6$ platelets from patients

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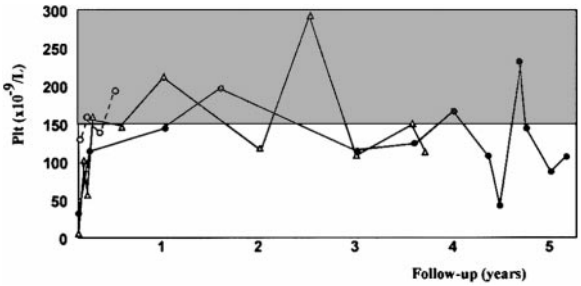


**Figure 1. Profile of platelet count in males with intermittent thrombocytopenia and *WASP* gene mutations.** Profile of platelet count in patient 1 (closed circle), patient 2 (open circle), and patient 4 (open triangle) since they were first seen at the Department of Pediatrics, University of Brescia. The shaded area represents the normal range for platelet count. Only recent data were available for patient 3 (not shown), who had thrombocytopenia and a small mean platelet volume.

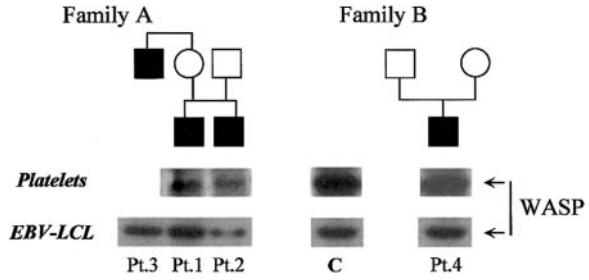


**Figure 2. Analysis of WASP protein expression in 4 males with intermittent X-linked thrombocytopenia.** (top) Pedigrees of the 2 families with intermittent X-linked thrombocytopenia. (bottom) Western-blot analysis of WASP protein expression in platelets and in Epstein-Barr virus–transformed lymphoblastoid B cell lines (LCL) derived from the patients and from a normal control (C).

and controls were lysed with Buffer A. For LCL and for platelets, lysates were normalized for protein amount using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein were incubated with protein G–Sepharose coupled with 3F3 monoclonal antibody and were run on 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis at 80 V for 16 hours. The gel was transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA) and was blotted with rabbit polyclonal antibody against WASP (H250-SC8353; Santa Cruz Biotechnology, Santa Cruz, CA) using a secondary anti-rabbit IgG POD (Hoffmann-LaRoche, Basel, Switzerland), and then it was revealed with enhanced chemiluminescence (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom).

## Results and discussion

As depicted in Figure 1, patients 1, 2, and 4 showed variability of platelet numbers, ranging from very low to normal, whereas the MPV was consistently reduced, regardless of the platelet count (ranges, 5-6.3 fL for patient 1; 5.2-5.4 fL for patient 2; 4.7-6.4 fL for patient 4; normal range, 7-10 fL). Only limited information is available for patient 3, whose most recent platelet counts were low (37-64 $\times$ $10^{-9}$/L) and who had a reduced MPV (6.2-6.4 fL). Clinically, the platelet counts correlated with the appearance of petechiae. No major episodes of bleeding were recorded, and no transfusions were required. Because of the history and the reduced platelet count and volume, mutation analysis at the *WASP* locus was performed.

In family A, a C207G nucleotide substitution in exon 2 was identified in all 3 affected males, resulting in a Pro58Arg amino acid change. Heterozygosity for this mutation was detected in the mother of the 2 boys. In family B, a T1476A point mutation in exon 11 was detected in the affected boy, resulting in an Ile481Asn amino acid substitution. The mother was found to be a carrier for this mutation. It is unlikely that these genetic abnormalities are polymorphisms because the nucleotide changes were not detected in more than 300 normal X chromosomes.

Mutations of the *WASP* gene have been previously shown to be responsible for 3 different clinical phenotypes: classical WAS, XLT, and X-linked congenital neutropenia.[1,6,11] The spectrum of phenotypes associated with abnormalities of the *WASP* gene reflects heterogeneity of the mutations. In particular, XLT usually entails missense mutations in exons 1 and 2 and decreased amounts of mutated protein, whereas classical WAS is associated with a variety of genetic defects that usually result in the absence or truncation of WASP.[6,8] Our 2 families had missense mutations involving exon 2 (C207G) and 11 (T1476A), respectively. As shown in Figure 2, these mutations allow substantial protein expression. Reduced amounts of WASP protein were detected in LCL and platelets from patient 2 and in platelets from patient 1, whereas normal amounts were detected in patient 4 (for LCL and platelets) and in patient 3 (for LCL).

The 2 families with intermittent thrombocytopenia had in common consistently small platelet size, minimal if any bleeding, and, in most members, no eczema or increased susceptibility to infections. Two of the 3 affected males from family A had low proliferative responses to anti-CD3 in vitro and moderately elevated serum IgE levels. These immunologic abnormalities are typical of WAS/XLT.[12,13]

The intermittent thrombocytopenia reported herewith represents the mildest consequence of *WASP* mutations. Because none of the affected males had serious problems, no long-term treatment was indicated. In view of our findings, males with persistently low MPV must be considered for mutation analysis at the *WASP* locus, regardless of the platelet count.

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