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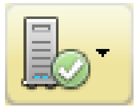

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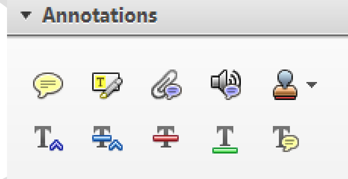

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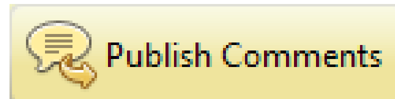

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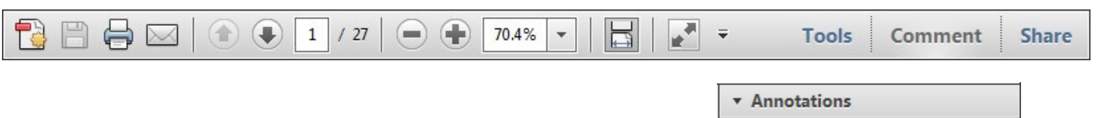

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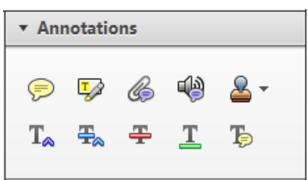

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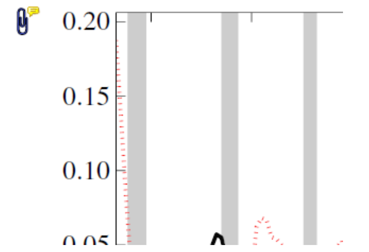

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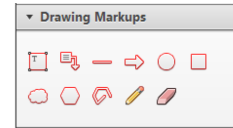

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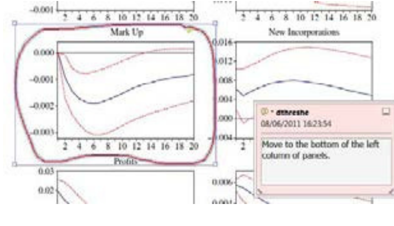

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CASE REPORT

A novel large deletion and single nucleotide insertion in the Wiskott–Aldrich syndrome protein gene

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Abstract

Deletion mutations of *WAS* are relatively rare and the precise localization of large deletions in the genome has rarely been described in previous studies. We report here a 5-month-old boy with a large deletion mutation in *WAS* that completely abolished protein expression. To localize the deletion, a 2816-bp-length sequence that spans between exons 9 and 12 was amplified. PCR amplification of the patient's sample revealed a single band of about 1 kb in contrast to the 2816-bp-amplicon in the control. Genomic DNA sequencing of the patient revealed a 1595-bp-deletion and an adenine insertion (g.5247_6841del1595insA). This large deletion of *WAS* resulted in partial loss of exon 10 and intron 11, and a complete loss of intron 10 and exon 11.

Key words Wiskott–Aldrich syndrome; WAS gene; InDel; gross deletions

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The Wiskott–Aldrich syndrome (WAS; OMIM301000) is a rare, X-linked primary immunodeficiency disorder inherited in an autosomal recessive manner, and it is caused by mutation of the WAS protein gene (*WAS*) resulting in defective expression and function of WASP (1–4). Clinical and hematologic characteristics of WAS include early-onset gastrointestinal bleeding, recurrent infections, eczema, micro-thrombocytopenia, and platelet dysfunction (1, 2, 5). *WAS* has 12 exons and encodes a 502 amino acid hematopoietic cell lineage-specific protein (6–10).

Reports on genotype–phenotype correlation in WAS patients are contradicting which may have been related to the lack of carefully collected clinical data over time. We report here a WAS patient with an InDel mutation and review the previously published literature of gene gross deletions.

Patients and methods

The male patient studied in detail in this work was born from the second, uneventful pregnancy of his mother. The parents are clinically healthy. The mother's first pregnancy ended in spontaneous abortion at 5 wk of gestation. From 3 wk of age, he presented with bloody diarrhea, and erythematous maculopapular rash appearing on his face. By the age of 5 wk, the rash spread to the cervical region and the superior part of his trunk, and he continued to have recurrent bloody stools. At 6 wk of age, physical examination revealed pallor, eczema, mucosal petechiae, and oral candidiasis. Laboratory examination showed anemia (hemoglobin, 6.5 g/dL), thrombocytopenia (platelets, $35 \times 10^3/\mu L$), and elevated C-reactive protein level (260 mg/L). At the age of 7 wk, he was still thrombocytopenic (platelets, $21–85 \times 10^3/\mu L$), and the mean platelet volume (MPV) was between 7.5 and 9.9 fL. Serum immunoglobulin levels were: IgA, 0.6 g/L; IgG, 3.95 g/L; IgM, 0.34 g/L. Bone marrow examination showed abundant thrombocytopoiesis and thrombocytic anisocytosis. At the age of 3½ months, the MPV decreased to 6.5–6.9 FL, and the patient continued to have recurrent bloody diarrhea. Despite several platelet transfusions, he presented with persistent micro-thrombocytopenia (platelets, $12 \times 10^3/\mu L$; MPV<7 FL) at age

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1 of 5 month. The serum immunoglobulin G level gradually
2 decreased and the patient was treated with 400 mg/kg
3 IVIG on two subsequent days. Blood lymphocyte subset
4 analysis revealed decreased CD8+ T cell count (0.07–
5 0.12 × 10³/μL), B cell count (0.11 × 10³/μL), and NK
6 cell number (0.17 × 10³/μL). The number of CD19+ lym-
7 phocytes decreased from 1.16 × 10³/μL to 0.56 × 10³/μL
8 at 11 months of age; CD4+ T cell count decreased from
9 1.34 × 10³/μL to 0.69 × 10³/μL, CD8 + T cell number
10 lowered from 0.12 × 10³/μL to 0.05 × 10³/μL; CD4/CD8
11 ratio was constantly elevated; the NK number was vari-
12 able, normal, or low. After informed consent was obtained
13 from the parents, blood samples were collected from the
14 patient, the mother, and healthy controls. All the studies
15 described here were approved by the Regional Ethics
16 Committee of the University of Debrecen and by the insti-
17 tutional review boards of the centers at which the patients
18 were managed.
19
20
21 **Genomic DNA analysis**
22 Genomic DNA (gDNA) was extracted from blood leuko-
23 cytes and immortalized B cells according to standard pro-
24 tocols. gDNA sequences were analyzed by amplifying
25 exons 1–12 and the flanking intronic regions of *WAS* by
26 PCR. Sequence variations were described with respect to
27 a reference sequence, NCBI NG_007877.1 for *WAS*. To
28 identify the location of deletion, a 2816-bp-length
29 sequence was amplified that included exons 9 and 12
30 using AccuTaq LA DNA Polymerase (Sigma-Aldrich Ltd.,
31 5 Germany) with the primers 9–12 F (CGACGCCGAGA
32 CCTCTAAACTTATC) and 9–12 R (CAGCAAGTAACTC
33 AGCCACTCAGTC).
34
35 **Flow cytometry**
36
37 Whole blood cells were blocked with Human IgG Blocking
38 solution (Sigma-Aldrich, St. Louis, MO, USA). The cells
39 were stained with phycoerythrin (PE)-conjugated CD3 anti-
40 body (BD, San Jose, CA, USA) and fixed with Fix Medium
41 A (Invitrogen Corp., Carlsbad, CA, USA), washed with
42 phosphate buffered saline containing 0.5% bovine serum
43 albumin and 2% fetal bovine serum (PBS/BSA/FBS). Cells
44 were permeabilized with Perm Medium B (Invitrogen) and
45 were or were not (negative control) stained with anti-human-
46 WASP rabbit monoclonal antibody (Abcam, Cambridge,
47 MA, USA) and isotype-matched control rabbit IgG mAb
48 (Sigma-Aldrich). After 30 min incubation, cells were washed
49 with PBS/BSA/FBS and labeled by Alexa Fluor 488 conju-
50 gated anti-rabbit-IgG (Cell Signaling, Eugene, OR, USA) as
51 secondary antibody for 30 min in Perm Medium B. Samples
52 were washed twice with PBS/BSA/FBS, fixed in 1% para-
53 formaldehyde containing PBS and immediately analyzed
54 with an AccuriC6 cytometer.

Western blotting

Peripheral blood mononuclear cells (PBMCs; 3×10^6) were treated with lysis buffer and protease inhibitor cocktail (Sigma-Aldrich). Samples were mixed with equal volumes of Laemmli loading buffer (Sigma-Aldrich) and incubated at 100°C for 10 min. Total protein (10 μg) was electrophoresed on 10% sodium dodecilsulfate-polyacrylamide gel and electro blotted into a polyvinylidene fluoride membrane. Next, anti-human-WASP rabbit monoclonal antibody (Abcam, Cambridge, MA, USA), and for actin detection anti-Actin rabbit monoclonal antibody (Sigma-Aldrich) were added and incubated overnight at 4°C. Next day, membranes were incubated with Horseradish peroxidase (HRP)-labeled affinity-purified goat anti-rabbit IgG (Sigma-Aldrich) as secondary antibody. WASP and actin bands were visualized by incubation with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, Rockford, IL, USA).

Results

Genetic findings

PCR amplification of the 12 exons of *WAS* was performed by using gDNA. Agarose gel electrophoresis of exons 1–9 and 12 revealed appropriate length bands and normal sequences of nucleotides was found by sequencing (Fig. 1). 6 In case of exon 10 and 11, the amplicons were undetectable. To determine the boundaries of the *WAS* deletion gDNA between exons 9 and 12 was amplified. In a normal control, we detected a 2816-bp-length amplicon whereas the patient

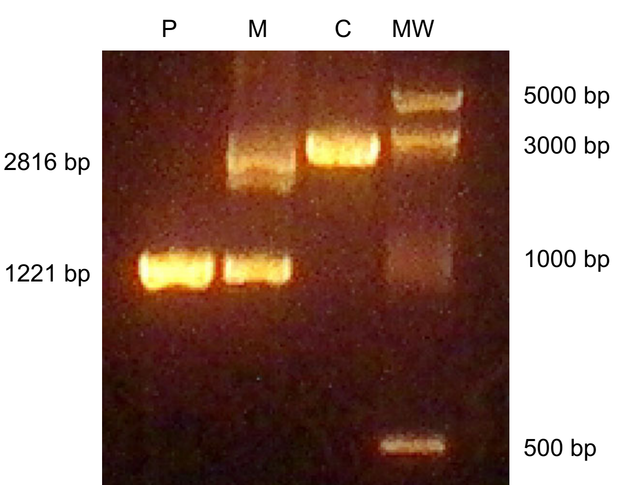

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Figure 1 Agarose gel electrophoresis showing deletion of WAS in the patient (P) and his mother (M). Agarose gel electrophoresis of a 2816-bp-sequence of *WAS* from exon 9 (forward primer) to exon 12 (reverse primer) was performed in a healthy control individual (C). In the patient's sample, a single band of about 1 kb was detected (P). In the mother's sample, agarose gel electrophoresis showed two amplicons indicating that she was carrier for the deletion mutation (M).

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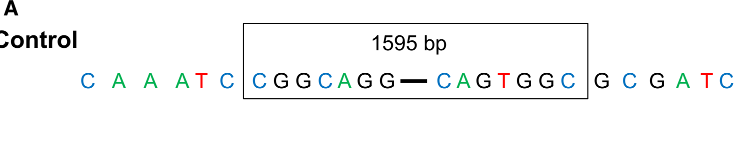

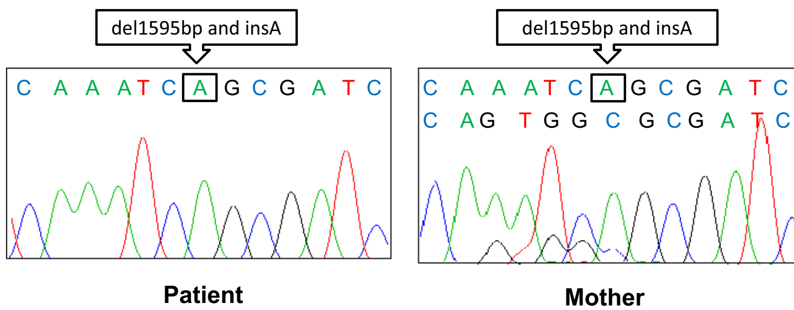

Figure 2 (A) Mutation analysis of the InDel mutation of *WAS*. Electropherogram of genomic DNA sequencing of the patient shows a 1595-bp-deletion and a single adenine insertion (shown in brackets) at the site of the deletion. Electropherogram of the mother shows heterozygosity for the InDel mutation. (B) Schematic representation of InDel mutation on the *WAS* gene and protein in the patient. The 1595-bp-deletion located in exon, intron 10 and exon, intron 11. The mutation caused a frame shift, and the order of amino acid was damaged from the position 439. PH, pleckstrin homology; WH, WAS homology; GBD, GTP-ase binding; PR, proline rich; VD, verprolin homology; and CD, cofilin homology domain.

a smaller, about 1000-bp-length amplicon was found. gDNA sequencing revealed a 1595-bp-deletion of *WAS* and a single adenine insertion in the patient (g.5247-6841del1595insA; Fig. 2A). In the mother, two bands were identified, one representing the normal and one representing the mutant alleles (Fig. 2A). The InDel mutation affected exons 10–11 and caused a frame shift (R439 fs) (Fig. 2B).

WASP expression in white blood cells

WASP expression in blood cells of the patient, his mother, and healthy controls was studied by flow cytometry after intracellular staining. WASP expression (red line) was compared to that of isotype control (blue line). Cells isolated from the mother and healthy controls expressed WASP, whereas negligible amount of WASP was detected in PBMC of the patient (Fig. 3A). Western blot analysis showed that PBMC derived from the mother contained full length WASP (66 kDa) similarly to that of travel and healthy control cells (Fig. 3B). In contrast, WASP was not detectable in PBMC of the patient.

Discussion

The Wiskott–Aldrich syndrome may result from missense and nonsense mutations, small or large deletions, insertions, and splice anomalies of the *WAS* gene. In some of the cases described before, the mutation leads to complete loss of the WASP, while in others a truncated or mutated protein is expressed (WASP positive patients). Deletions in *WAS* were in most cases identified as short (1–12 bp) whereas the occurrence of large deletions have been rare (11–22) (Fig. 4). These genetic aberrations can span from a single exon to larger regions involving two or more exons with the encompassed introns or, in certain cases, the entire gene can be deleted (15). Here, we review the literature describing large deletions together with their genomic and clinical features and extend the list with a newly identified deletion mutation.

We report here the identification of a mutation affecting WAS in a Romanian patient based on laboratory and clinical findings. His mutation proved to be a novel InDel localized

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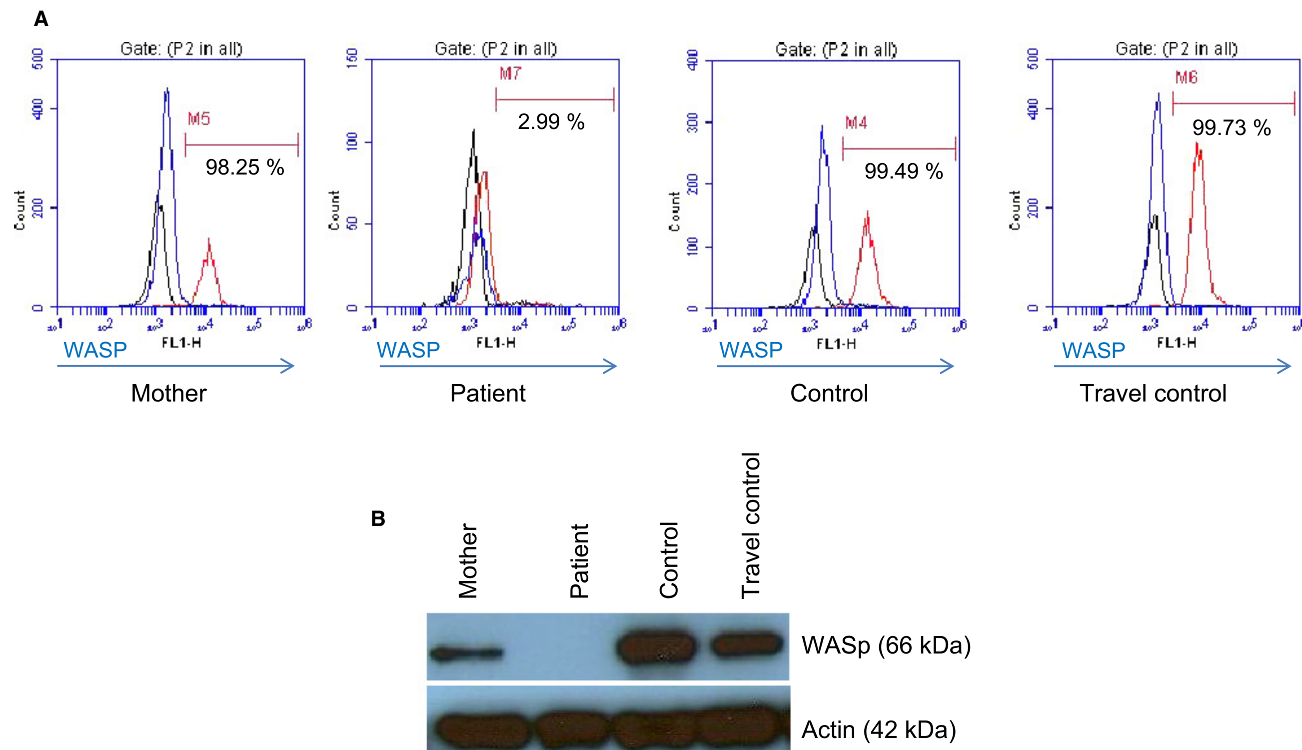

Figure 3 Detection of WASP expression in patient, his mother, and healthy controls. (A) Analysis of intracellular Wiskott–Aldrich syndrome protein (WASP) expression by flow cytometry. WASP expression (red line), isotype control (blue line), and negative control (black line) data are shown. Expression of WASP was in cells from the mother and healthy controls was comparable, and negligible WASP expression was detected in the patient's sample. (B) Analysis of WASP expression by Western blot. Normal WASP expression was identified from cell lysates in the patient's mother and healthy controls in contrast that no protein expression in patient's sample.

to the 3′ end of the *WAS* gene. Genetic analysis revealed that a breakpoint occured in exon 10 at nucleotide position 5246 of *WAS* and resulted in a 1595-bp-deletion with a single adenine insertion at the site of the deletion. The aberration causes the loss of part of exon 10, lack of the entire intron 10 and exon 11 and partial loss of intron 11. Flow cytometry and Western blot analysis showed no WASP expression in peripheral lymphocytes of the patient. The antibody we used recognizes the C-terminus of the human WAS protein (exon 12), and the identified mutation is located upstream from the epitope. Therefore, our data suggest that due to the mutation, the normal size and functional protein was not presented in the patient's cells.

In a Taiwanese patient, the regulatory region of *WAS* gene was also affected, the proximal promoter and the first two exons of the gene were found to be deleted (11, 12). As a result of this unique mutation, WASP was not detectable in the patient's lymphocytes and caused impaired mitogen antigen induced cell proliferation and NK cell activity. Low level of CD4+ memory cells and defected lymphocyte proliferation increased the susceptibility of the patient to infections and led to the development of a severe WAS phenotype (11). In another Taiwanese patient, the first 4 exons could not be amplified from genomic DNA derived from peripheral lymphocytes and WASP expression was undetectable. Lack of WASP expression also resulted in a severe form of classical WAS (13). In a Caucasian patient, a 15 800-bp-long *Alu*-mediated deletion was described, comprising exons 1–6, a part of intron 6 and 13 kb of the upstream region, including the proximal and distal *WAS* gene promoters. WAS protein in PBMCs of the patient could not be detected and a severe phenotype of WAS was observed (14).

A large deletion encompassing exons 1–7 was identified which led to lack of WASP expression in mononuclear cells or EBV-transformed B cell lines derived from a patient and resulted in severe WAS phenotype (15). In another patient, an approximately 2-kb-long deletion was described, involving exons 3–7 and seemed to have been created by the fusion of introns 2 and 7. WASP could not be detected in cell extracts from this patient, and he had a classical severe WAS phenotype with autoimmunity (16, 17). A 322-bp-long deletion was detected in a Russian patient who spanned introns 3–4 and exons 4–5 (18). A single exon deletion (exon 7) was reported in a Japanese patient who caused lack of WASP expression and severe phenotype accompanied by malignancy (16). In one Argentinian patient, deletion started also from exon 7 and was found to extend to intron 11 (19),

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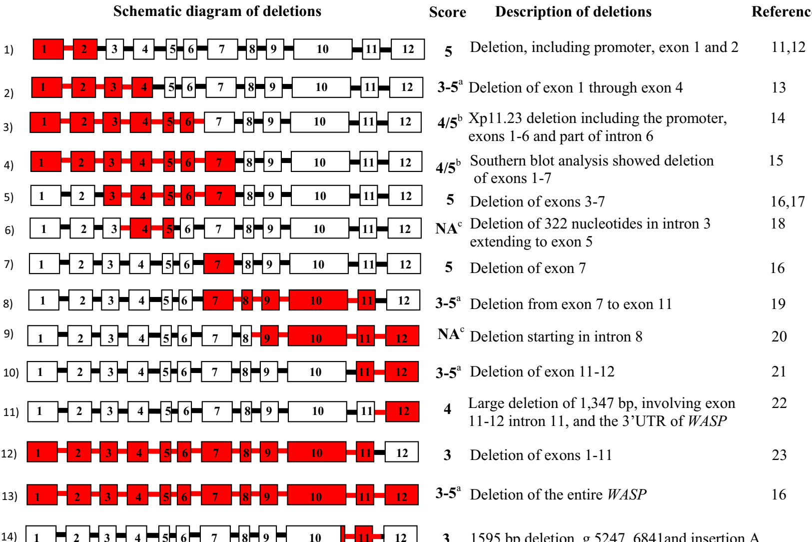

Figure 4 Schematic representation and short description of deletion mutations published before and described in this report. NA, none data. [a]Patient had classical form of WAS, score was not available. [b]Patient had severe form of WAS, score was not available. [c]Data about symptoms were not available.

while in another patient the deletion covered the 3′ terminal region of *WAS* gene from intron 8 to 3′ UTR (20).

There have been two different large deletions reported affecting the 3′ end of the *WAS* gene. In the first case deletion spans, the last two exons of the *WAS* gene with the proximal breakpoint located within intron 10. This caused the development of classical WAS phenotype (21). The second happened to be a complex mutation as the deleted 4.3 kb fragment covered intron 11, coding exon 12 and 3′ UTR of the *WAS* gene, as well as DNA sequences upstream of the promoter region of the subsequent *SUV39H1* gene. Additionally, upstream from the large deletion, a small 9-bp-deletion and the inversion of a 151-bp-long region, involving an adenine insertion at position 148 of the inverted sequence, were also identified. The polyA signal of wild type *WAS*, located in exon 12, was absent in the patient's sample as a consequence of the mutation. Stable truncated but nonfunctional protein expression was also detected inducing classical WAS phenotype (22). In another two intriguing cases, the deletions affected large part of the *WAS* gene. In the first patient, only exon 12 could be amplified from genomic DNA, while in the second patient the entire *WAS* gene was documented to be missing (16, 23). Consequently, WASP expression could not be detected in either patient.

Among all the mutations that have been identified in *WAS*, large deletions constitute an extremely small fraction. Based on this limited number of known cases, no preferentially affected gene regions can be identified. All of the large deletions led to either the total loss of WASP or to the expression of a nonfunctional truncated variant. These were manifested without exception in severe classical WAS phenotype. Therefore, early detection of large deletion mutation is crucial to initiate therapies including stem cell transplantation or gene therapy.

Acknowledgements

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Conflict of interest

We disclose no conflict of interest.

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