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A novel Wiskott-Aldrich syndrome protein (WASP) complex mutation identified in a WAS patient results in an aberrant product at the C-terminus from two transcripts with unusual polyA signals

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Abstract Wiskott-Aldrich syndrome (WAS) is an X-linked recessive disorder characterized by immunodeficiency, thrombocytopenia and eczema. A broad spectrum of mutations in the WASP gene has been identified as causing the disease. In the present paper, we report on a patient affected by WAS with a novel complex mutation, characterized by a small 9 bp deletion followed by an inversion of 151 bp and a gross deletion of 4.3 kb within the Xp11.23 region. The small deletion and the inverted fragment are found in intron 11. The large deletion initiates downstream of exon 11 of the WASP gene, including exon 12, and a genomic region upstream of the promoter of the contiguous SUV39H1 gene. Expression studies of the mRNA of the patient's sample showed the presence of two aberrant transcripts that code for a protein of 519 amino acids. We demonstrate that these two transcripts differ in the 3' UTR region, and result from the use of two alternative polyadenylation signals. The severe phenotype of the patient correlates with the presence of an aberrant protein.

Keywords Wiskott-Aldrich syndrome · WASP · Mutation · Deletion · Inversion

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Introduction

Wiskott-Aldrich syndrome (WAS) is an X-linked recessive immune deficiency disorder (MIM#301000) characterized by thrombocytopenia with small platelets, eczema and recurrent infections. It mostly affects boys, although several cases of female WAS have also been reported (Parolini et al. 1998; Inoue et al. 2002; Lutskiy et al. 2002; Andreu et al. 2003b). The gene involved in the disease is located on the short arm of the X chromosome in region Xp11.22-23 at 5 kb from the SUV39H1 or human homolog of Su(var)3-9, a gene involved in the regulation of higher order chromatin organization (Hagemann et al. 2000). Mutations in the Wiskott-Aldrich syndrome protein (WASP) gene, leading to a loss of function, have been described in patients with WAS and X-linked thrombocytopenia (XLT) (Derry et al. 1994; Zhu et al. 1995; Ochs and Rosen 1999). Gain of function mutations in the same gene have been found to cause X-linked severe congenital neutropenia (Devriendt et al. 2001). The WASP gene has 12 exons and encodes for a cytoplasmatic protein (WASP), predominantly expressed in hematopoietic cells, and it plays a key role in regulating changes in cytoskeletal structure in response to external stimuli and signal transduction processes (Brickell et al. 1998; Snapper and Rosen 1999).

Approximately 300 unique mutations have been reported in the WASP gene, while only five mutational hotspots have been clearly identified (Imai et al. 2004; Jin et al. 2004) (<http://homepage.mac.com/kohsukeimai/wasp/WASPbase.html>). Mutations are found throughout the entire gene, with missense mutations preferentially locating in the amino-terminal part of the protein, and stop and frameshift mutations mainly in the carboxyl-terminal (Schindelhauer et al. 1996; Fillat et al. 2001). Mutations in the WASP gene cause a wide variety of clinical phenotypes, ranging from isolated thrombocytopenia to severe WAS. Phenotype/genotype correla-

tion studies indicate that mutations resulting in the expression of normal-sized mutated protein are associated with a milder phenotype, while mutations leading to a truncated or absent protein correlate with a more severe phenotype (Snapper and Rosen 1999; Jin et al. 2004). However, of all the mutations reported, only approximately one-third involve WASP expression studies at the mRNA or protein level.

In the present study, we describe for the first time a complex WASP mutation that results in the expression of two alternative mRNA products, derived from two different polyadenylation signals. At the genomic level, the mutation involves a small 9 bp deletion followed by a 151 bp inversion and a large deletion of 4.3 Kb, involving intron 11, exon 12 and the 3'UTR of the WASP gene, and the genomic region upstream of the SUV39H1 gene.

Materials and methods

Case report

The proband is a boy clinically diagnosed as having Wiskott-Aldrich syndrome at 3.5 months of age. On the second day of his life he was already presenting bloody stools. When only 18 days old, he suffered a urinary tract infection and presented anaemia, lymphopenia and thrombocytopenia. At two months of age he developed generalized dermatitis. Occasional bouts of oral candidiasis were observed. His platelet count varied from 17 to 28×10^3 cells/mm³, with a normal mean volume, although when observed under light microscopy, both micro- and macrothrombocytes were identified. Immunological studies revealed a decrease in absolute lymphocyte number (2.200 – 3.270 cells/mm³), with low CD3+ cells (325 – 580 cells/mm³). This CD3 lymphopenia affected both CD4+ and CD8+ cells. However, B and NK cell values were normal. Serum immunoglobulins were also found to be normal. Evaluation of T-cell function indicated a decrease in T-cell proliferation in response to mitogenic stimuli (phytohemagglutinin, concanavalin A, pokeweed mitogen and OKT3 monoclonal antibody anti-CD3). Other types of primary immunodeficiencies, such as adenosine deaminase (ADA) and purine nucleoside phosphorylase (PNP) deficiencies were excluded as the pertinent enzyme activity in erythrocytes was found to be normal, and no changes in the sequences of the common γ chain, the α chain of the IL-7 receptor or Jak3 genes were detected. An allogeneic bone marrow transplant from his HLA-identical sister was performed at the age of ten months. During the post-transplantation period he suffered HV-6 and CMV infections. He is presently 24 months old and has not suffered recurrent infections, but does present psychomotor retardation of unknown etiology. The number and subpopulations of lymphocytes, platelets and neutrophils were found to be normal, and his peripheral blood cells presented 100% chimerism.

Screening for WASP mutation

Informed consent was obtained to perform WASP mutational analysis in blood samples taken from both the patient and his mother. DNA was extracted from peripheral blood leukocytes by standard techniques. The WASP exons were amplified as previously described (Fillat et al. 2001). Exons 1–11 and the flanking splice site of the WASP gene were analyzed by SSCA (Fillat et al. 2001).

Characterization of the breakpoint

To localize the deletion breakpoint, we carried out multiple PCR amplification of nonoverlapping fragments by designing four primer pairs spaced every 1–2 kb along the WASP and SUV39H1 genes. Two primer pairs amplified PCR products from the genomic region between the WASP and the SUV39H1 genes (PCR fragments W and X). The other two primer pairs were used to amplify fragments from the SUV39H1 gene (PCR fragments Y and Z). A complete list of the primers used is shown in Table 1.

To characterize the deletion breakpoint, we used a PCR approach, employing the following primers: E11W-L, which anneals to exon 11 of the WASP gene, and X-R, which anneals to the intergenic region. The PCR product was resolved on 2% agarose gel, purified with the PCR purification kit (Qiagen, Valencia, CA, USA) and DNA-sequenced with BigDye terminator chemistry (Applied Biosystems, Foster City, CA, USA). Mutation was confirmed in two independent polymerase chain reaction (PCR) amplifications.

RT-PCR analysis

An RT-PCR analysis was performed as previously described (Andreu et al. 2003a). Briefly, 1 μ g of total RNA from each sample was reverse-transcribed with a Retroscript RT kit (Ambion, Austin, TX, USA). Five microlitres of cDNA was PCR-amplified with WASP-specific primers (listed in Table 2). We performed different PCR reactions to amplify the WASP cDNA, using the

Table 1 Primers used to characterize the breakpoint

Primer	Sequence
W-F	5'- TAACAACCTCACACACCAGGA-3'
W-R	5'- CCCCACTACCTTTCTCTTTT-3'
X-F	5'- CAAAAATTAGCCAGGTGTG-3'
X-R	5'- AGGCTGAGCAACCATTAAAG-3'
Y-F	5'- TAACGTGATTGGCTAGCAG-3'
Y-R	5'- ATACTTTCCCCCAGAAGAG-3'
Z-F	5'- CCCACAGCATATCACTTAC-3'
Z-R	5'- GTCAAGTCCAGTCAAAACC-3'
E11W-L	5'- ATGCACGTGATGCAGAAGAG-3'

Table 2 Primers used for RT-PCR

Primer	Sequence	Position
W1S	5'-GCCTCGCCAGAGAAGACAAG-3'	Exon 1
E6-7W-R	5'-ACTGGAGGGCCTCCTTGGTC-3'	Junction of exon 6 and 7
E4W-L	5'-AAGGAATCAGAGGCAAAGTGG-3'	Exon 4
W10S2	5'-TCCAGCTACTGGACGTTCTG-3'	Exon 10
W10A1	5'-CATGGGTGGCCACCAAGCTC-3'	Exon 10
E11W-R	5'-CTGCAGCGCTGAGCTCTCTG-3'	Exon 11
E11W-L	5'-ATGCACGTGATGCAGAAGAG-3'	Exon 11
W12A	5'-TGGAGGGGGAGCCATGTC-3'	3' UTR

indicated primers. RT-PCR products from exon 1 to 7; exon 4 to 10; exon 10 to 11; exon 10 to 12 and exon 11 to 12 were amplified and run in a 2% agarose gel stained with ethidium bromide. All RT-PCR results were confirmed by two independent RT-PCR amplifications.

3' RACE (rapid amplification of cDNA end)

3' RACE synthesis of first-strand cDNA was performed according to the procedure previously described (Frohman 1990). Briefly, 1 µg of total RNA was mixed with MMLV-Retrotranscriptase (Ambion) and the 5 µM adaptor primer 3' RACE-AS (5'-TAATACGACTCACTATAGGGCGATCC(T)₂₅VN-3'), under the conditions recommended by the manufacturer. One microlitre of the reaction product was amplified in a 25 µl PCR reaction using the PCR enhancer system (Gibco, NY, USA). The primers used to amplify the 3'-end of WASP cDNA were E10W-F2 5' GAGCGC TTTTGGATCAAATC 3' and T7-AS 5' GTAATACG ACTCACTATAGGGC 3'. PCR products were purified by agarose gel electrophoresis and cloned in pGEM-T Easy Vector System I (Promega, Madison, WI, USA). Cloned cDNA fragments were then sequenced with the Big Dye terminator cycle sequencing kit (Applied Biosystems) and an ABIPRISM3100 genetic analyzer (Applied Biosystems).

Western immunoblotting of WASP

Blood peripheral lymphocytes from the patient and a healthy individual were isolated by Ficoll-Hypaque gradient centrifugation. WASP protein analysis was carried out as detailed elsewhere (Martin et al. 2005). Briefly, cells were lysed with 1% NP-40 lysis buffer containing protease inhibitor cocktail (Sigma, St Louis, MO, USA), resolved by SDS-PAGE and electrotransferred to Hybond-P PVDF membranes (Amersham, Piscataway, NJ, USA). Membranes were blocked with 5% non-fat milk and probed for 1 h at RT with 1 µg/ml of the anti-WASP mAb D1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by a similar incubation with HRPO-labeled goat anti-mouse antibody (1:10,000 dilution) (Caltag, Botolph Claydon, UK). The blot was

developed by chemiluminescence (ECL, Amersham) and exposed to autoradiographs (Amersham).

Results and discussion

Using PCR analysis of genomic DNA, we identified a complex mutation in the Xp11.22-23 region of a patient clinically diagnosed with WAS (accession number AM085518). Exon amplification and SSCA analysis of exons 1 to 12 revealed that the patient's sample lacked exon 12, the last exon of the WASP gene. PCR amplification of four nonoverlapping DNA fragments (labeled W, X, Y and Z), spanning the region between the WASP and SUV39H1 genes, showed that fragment W was not present in the patient's sample (Fig. 1a,b). Primer pairs E11W-L and X-R amplified a fragment containing the breakpoint of the large deletion (4,356 bp). The deleted fragment involved 1,347 bp of intron 11, coding exon 12 and 3' UTR of the WASP gene, as well as DNA sequences upstream of the promoter region of the contiguous SUV39H1 gene (Hagemann et al. 2000). Additionally, a small 9 bp deletion and an inversion of 151 bp, with an adenine insertion in nucleotide 148 of the inverted sequence were also identified (Fig. 1c). Family studies in the patient's mother showed that she was not a carrier for the mutation, indicating that this was a de novo mutation (data not shown).

Only a few complex mutations have been identified in WAS patients. In a recent study of 227 WAS/XLT families, only 10% of the total mutations studied could be classified as complex, with a broad spectrum of phenotypes (Jin et al. 2004). In the present study the mutation could be associated with a severe form of WAS presenting a clinical score of 4 (Zhu et al. 1995). Progress of the disease could not be studied because the patient underwent bone marrow transplantation at the age of ten months.

To explore the consequences of the mutation effect, expression studies were performed on the patient's RNA samples. RT-PCR analysis with the indicated primers showed that the WASP mRNA contained exons 1 to 11 of the WASP gene (Fig. 2a). In order to identify the complete open reading frame of the aberrant WASP mRNA transcript, we performed 3' RACE experiments.

Interestingly, we detected the presence of two aberrant transcripts of lengths 767 and 557 bp, respectively (Fig. 2b,c). Sequence analysis of the two fragments showed that they were different in the 3' UTR region, but had a putative common coding region. Each transcript used a particular polyadenylation signal, different to that of wild-type WASP. The polyA signal of wild-type WASP, located in exon 12, was absent in the patient's sample as a consequence of the mutation. Alternatively, the patient's mRNA used a polyA signal to generate the longer transcript characterized by the canonical hexanucleotide sequence AAUAAA located downstream of the inverted sequence. Interestingly, the second smaller transcript was produced from the novel polyA signal, generated as a consequence of the break-point sequence and the A insertion, corresponding to hexanucleotide AUUAAA (Fig. 2c,d). In the mammalian system, effective polyadenylation has been found to require two main sequence components: a highly conserved hexanucleotide signal located 10–30 nucleotides 5' from the cleavage site, and a more variable GU element located 20–40 bases 3' from the cleavage site. Several hexanucleotide variants have been identified in the human genome. The two variants identified in the

transcripts of the WAS patient are the two signals most commonly found. In fact, the AAUAAA sequence is considered a canonical signal, and is found to be present in 90% of the mRNA, while the second most frequent variant AUUAAA is found in the remaining 10% (Colgan and Manley 1997; Beaulieu et al. 2000). Moreover, both signals have been reported to possess comparable polyadenylation activity (Zhao et al. 1999).

The two transcripts had the same open reading frame, with a stop codon in intron 11. The aberrant mRNAs encoded for a hypothetical abnormal WASP protein of 519 amino acids. This protein is different to wild-type WASP (502 amino acids) in that it lacks 17 amino acids from exon 12, and includes 34 new amino acids from intron 11. Western blot analysis showed that this protein was expressed and apparently had a similar size to the wild-type protein (Fig. 3). A mutation leading to a protein of 519 amino acids has recently been described in three patients (Imai et al. 2004). However, this mutation was completely different from the one we describe in the present paper, in terms of both the genomic and mRNA changes. Interestingly our results demonstrate that the 519 amino acid protein, derived from two aberrant transcripts, is a stable protein. Nevertheless, the protein

Fig. 1a–c Mutation screening. **a** Schematic representation of WASP and SUV39H1 genes on chromosome Xp11. Structure of the WASP gene (or WASP genomic organization).

Unshaded blocks represent uncoded regions. The 12 exons coding 502 amino acids are represented as *boxes* (and numbered) while intronic sequences are shown as *thin bars*. PCR fragments (W, X, Y and Z) are shown in the scheme. **b** Schematic diagram representing the affected region of the WASP gene. The *arrow* shows the inverted sequence. *Square brackets* represent deleted regions. **c** Wild-type and patient sequences of the affected region. Exon 11 is shown in *bold*. Deleted sequences are between brackets. In *bold* and *italic* type, inverted sequence (151 bp). *Underlined*, the A insertion

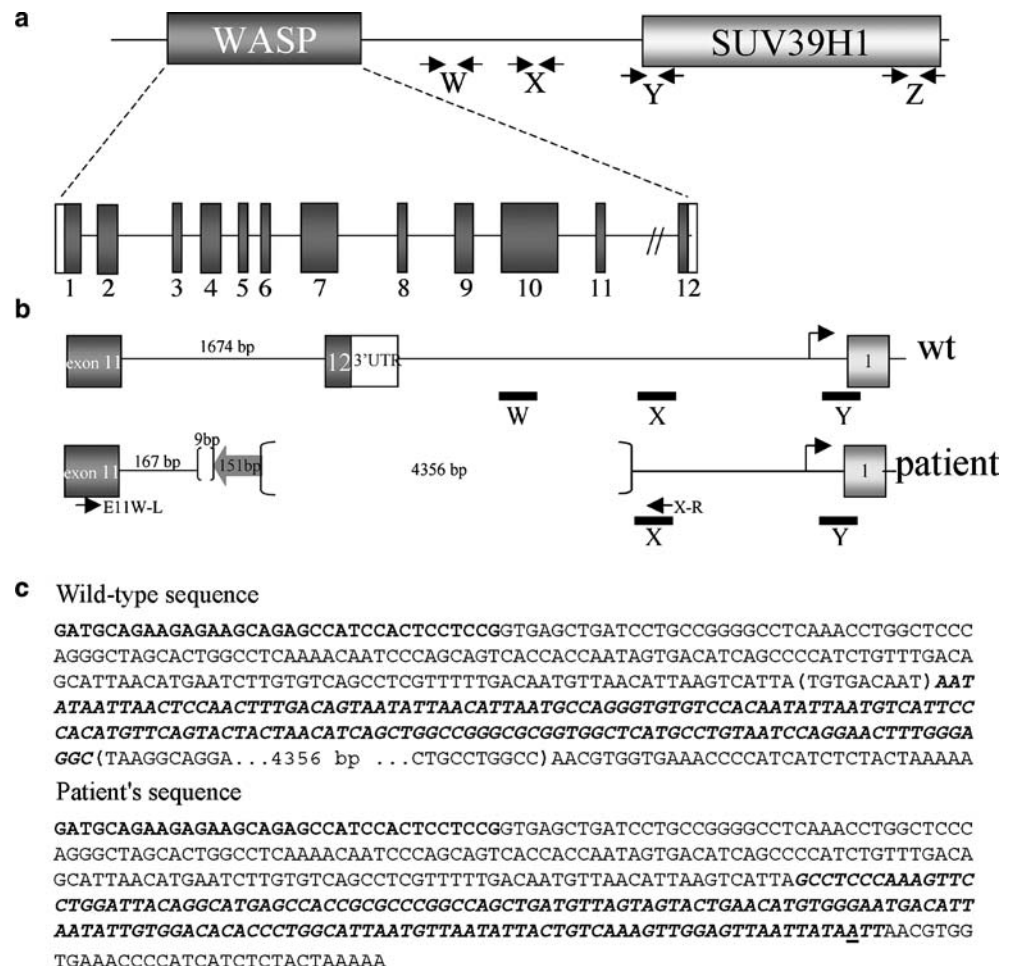
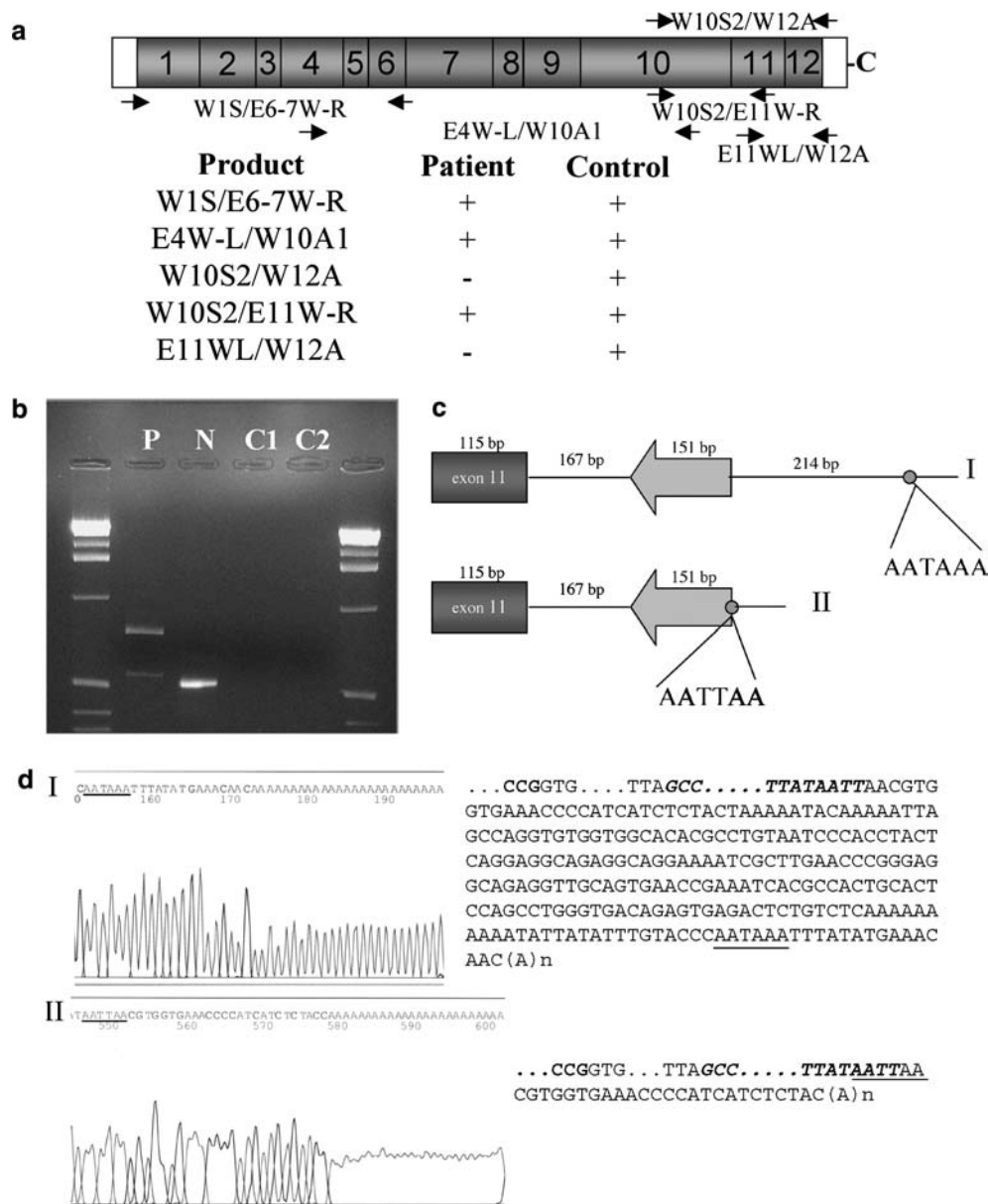


Fig. 2a–d Expression of the WASP gene. **a** Diagram of WASP mRNA and a table summarizing RT-PCR results. **b** 3' RACE analysis showing the alternative transcripts. *P* Patient sample, *N* normal individual, *C1* RT – from patient sample, *C2* RT – from sample of normal individual. **c** Scheme illustrating the two transcripts with the alternative polyA signals. **d** Chromatograms and sequences from transcript I and II. The hexanucleotide motifs of the polyA signals are underlined



may be nonfunctional, due to the important domains affected. It is known that the C-terminal region of WASP, coded by exon 11 and 12, contains a verprolin central acidic (VCA) domain that interacts with

monomeric actin and the nucleator actin complex Arp2/3 (Orange et al. 2004). The mutation in the WAS patient affecting the VCA domain may result in the impairment of such interactions, strongly affecting actin polymerization and leading to alterations in the biological processes in which WASP is involved. As a consequence, the reported mutation would appear to correlate with the severe phenotype observed in the WAS patient.

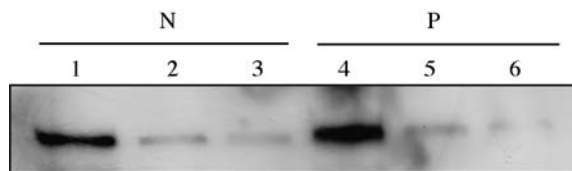


Fig. 3 Anti-WASP Western blot analysis from peripheral blood mononuclear cells. *N* normal individual, *P* patient. Lysates from 10^6 cells were loaded onto lanes 1 and 4, and serial dilutions were loaded onto lanes 2, 5 and 3, 6

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