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Two novel mutations identified in the Wiskott-Aldrich syndrome protein gene cause Wiskott-Aldrich syndrome and thrombocytopenia

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Abstract. Wiskott-Aldrich syndrome (WAS) and X-linked thrombocytopenia (XLT) are rare X-linked genetic disorders caused by mutations of the Wiskott-Aldrich syndrome protein (*WASP)* gene. Both disorders are clinically characterized by chronic thrombocytopenia of small platelets. WAS is a more severe form of the disorder and also courses with eczema, and immune dysfunction. In the present study, we investigated two novel mutations of the *WASP* gene in two Spanish families with patients clinically diagnosed as having XLT and WAS, respectively. In one of the families a missense mutation in exon 12 (1488A>G), resulting in the highly conserved glutamic residue changing to glycine at position 485 (D485G), was identified in several members. Notably, a female of this family, with clinical signs of XLT, was determined as the carrier of the mutation and showed a skewed pattern of X-inactivation, preferentially inactivating the X-chromosome carrying the wild-type allele. In the case of the second family, we describe a WAS patient with a single base deletion in exon 2 (266-267delA), resulting in a frameshift (at codon 78) that creates a stop codon at amino acid 127. As a consequence, there was no WASP expression.

Introduction

Wiskott-Aldrich syndrome (WAS) is a rare X-linked genetic disorder (MIM #301000) characterized by the clinical triad: thrombocytopenia with small platelets, eczema, and symptoms of immune dysfunction. Classically the phenotype of affected males is severe, although heterozygous females are usually asymptomatic. However, several cases of WAS carriers with clinical signs have been reported (reviewed in ref. 1).

The *WASP* gene encodes a multidomain protein (WASP) that is expressed predominantly in hematopoietic lineages. The main function of WASP is to couple signals generated at the cell membrane level with the reorganization of the cellular cytoskeleton, ultimately resulting in cell activation and the promotion of cell motility (2).

The gene responsible for WAS is located on the short arm of the X-chromosome in region Xp11.23-p11.22 (3). Approximately 300 unique mutations have been reported in the *WASP* gene, but only five mutational hotspots have been clearly identified (4-6) (http://homepage.mac.com/kohsukeimai/wasp/WASPbase.html). Diagnosed patients show remarkable heterogeneity of clinical symptoms, ranging from isolated thrombocytopenia (X-linked thrombocytopenia, XLT; MIM#313900) to severe disease, including pyogenic and opportunistic infections, autoimmune disease, and malignancy, while microthrombocytopenia was found to be present in all patients (3,7,8). Few *WASP* mutations have been identified to cause any disease other than typical WAS/XLT. Such cases include very mild missense mutations affecting the C-terminus and resulting in an intermittent form of XLT (9), and gain-of-function missense mutations that result in X-linked neutropenia, without signs of WAS/XLT (10,11).

In the present study we described two novel mutations in the *WASP* gene: a missense mutation of D485G on exon 12 (family A) and a deletion on exon 2 (266-267delA, family B) that resulted in XLT and classical WAS, respectively.

Materials and methods

Case report. Family A: Patient I.1. (index case) is a woman originally diagnosed as having immune thrombocytopenic purpura (ITP, MIM#188030). At the time of diagnosis her platelet count was 39,000/mm^3, and following corticosteroid treatment platelet counts reached normal values. She was in good general condition, and no major infections were recorded in her clinical history. Other hematological and immunological

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Abbreviations: WAS, Wiskott-Aldrich syndrome; WASP, Wiskott-Aldrich syndrome protein; XLT, X-linked thrombocytopenia; HUMARA, human androgen receptor

Key words: Wiskott-Aldrich syndrome, X-linked thrombocytopenia, frameshift, missense mutation, X-chromosome inactivation

Table I. Platelet values and immunological characteristics of family A.

Variable	Subject no.				Normal range
	I.1.	II.1.	II.2.	II.3.	
Platelets[a]	39,000-313,000	115,000-152,000	187,000-192,000	211,000-250,000	140,000-400,000
MPV (fl)	5.5-7.3	5.7-6.1	6.1-7.2	7.0-8.2	7.5-11.0
Serum immunoglobulins (mg/dl)					
IgG	1090	882	699	796	723-1685
IgA	337	296	421	475	69-382
IgM	165	105	85	192	40-230
IgE	1290	60	<2	6	3-160

[a]Number of platelets/mm^3.

parameters of subject I.1., as well as of other family members, are shown in Table I. The possibility of XLT was eventually considered. Her sons (II.1 and II.2) had X-linked thrombocytopenia with small platelets, whereas her daughter (II.3) had normal platelet values.

Family B: The propositus is a boy, clinically diagnosed with Wiskott-Aldrich syndrome. From the age of 4 months he presented thrombocytopenia with small platelets, eosinophilia and eczema. His platelet count was 38,000/mm^3, with a mean platelet volume of 6.8 fl (normal range 7.5-11 fl). His clinical history was unremarkable in terms of infection. Immunological studies indicated that lymphocyte counts and serum immunoglobulins were normal. The clinical diagnosis was molecularly confirmed with the identification of the mutation reported in the present study. An allogeneic peripheral stem cell transplant (PBSCT), from an unrelated HLA-compatible donor, was performed at the age of 16 months. The number of subpopulations of hematopoietic cells present was found to be normal, and his peripheral blood cells presented complete chimerism. Following the transplant he suffered an Epstein-Barr viral infection and responded well to ganciclovir and intravenous immunoglobulin. At present, he remains healthy. Treatment with intravenous immunoglobulin was established to minimize the risk of infection.

Screening for WASP mutation. Genomic DNA was obtained from peripheral blood leukocytes by the salting out method (12). The 12 exons and the flanking splice site of the *WASP* gene were amplified and analysed by single-strand conformation analysis (SSCA) (13). Direct DNA sequencing was performed on the samples that revealed a change in SSCA. For DNA sequencing, the PCR products were purified and sequenced with the BigDye terminator cycle sequencing kit (Applied Biosystems) on an ABI PRISM 3100 genetic analyser (Applied Biosystems). The mutations detected were confirmed by sequencing in the opposite direction and using two independent polymerase chain reaction (PCR) amplifications.

HUMARA assay. A semi-quantitative analysis of X-chromosome inactivation was performed by the Human Androgen Receptor (*HUMARA*) assay, as previously reported (1).

Computational methods. ClustalW (http://www.ebi.ac.uk/clustalw) was used to align the WASP protein sequences of four mammalian species represented in the GenBank and of *Xenopus laevis* and *Danio rerio*.

Results

In family A, sequencing analysis revealed a single base substitution of A for G at nucleotide 1488 in exon 12 of the *WASP* gene (1488A>G). This missense mutation changes codon 485 from GAC to GGC; the amino acid glutamic changes to glycine at position 485 of the WASP protein (D485G). The mutation was detected in woman I.1. (Fig. 1), her sons (II.1. and II.2.) and daughter (II.3.), indicating that both women were carriers of the mutation. No other changes to the *WASP* gene were recorded.

The amino acid change D485G occurred within the Verprolin Central Acidic (VCA) domain of the *WASP* gene, at a highly conserved residue of the WASP gene, as identified by the multiple sequence alignment of the VCA domains of WASPs of different species (Fig. 1B). The data suggest that such a modification was very likely to have produced protein changes which in turn would have led to the development of the disease. Moreover, no such nucleotide change was detected in over 100 normal X-chromosomes studied, thus eliminating the possibility that the change could have been a polymorphism.

Notably, woman I.1. had clinical signs of thrombocytopenia, with a reduced platelet volume. Since changes in the pattern of X-chromosome inactivation have already been reported to cause the disease in carriers of X-linked diseases, we decided to study the X-chromosome inactivation patterns of the family (Fig. 1C). The pattern was found to be different for each female family member. We observed a random pattern of inactivation in girl II.3. (57% of cells had a *WASP* mutated active allele), whereas woman I.1. had a skewed pattern of X-inactivation, preferentially inactivating the X-chromosome carrying the wild-type allele (262 bp) (85% of

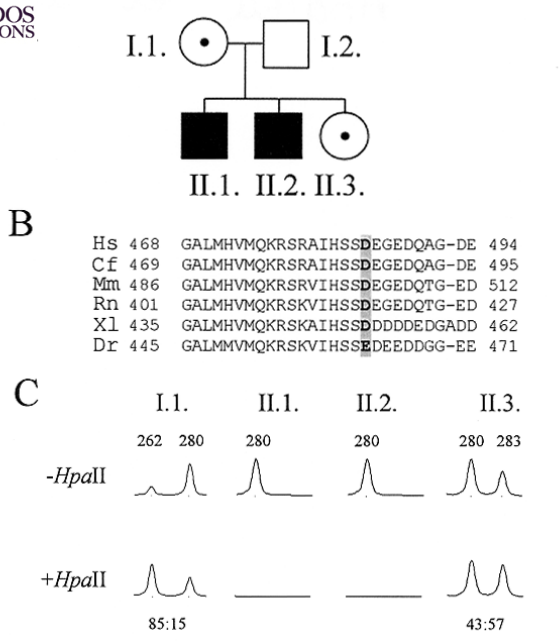

Figure 1. Family A: Pedigree and molecular analysis of the *WASP* gene. (A) Pedigree of family A. Filled squares indicate affected boys (II.1. and II.2.). Circles with a dot represent female carriers (I.1. and II.3.). Open square indicates non-affected male (I.2). (B) Alignment of amino acid sequences for genes orthologous to *WASP*: Hs, *Homo sapiens*; Cf, *Canis familiaris*; Mm, *Mus musculus*; Rt, *Rattus norvegicus*; Xl, *Xenopus laevis*; and Dr, *Danio rerio*. ClustalW alignment of residues 468-494 of human WASP with dog, mouse, rat, zebra fish (*Danio rerio*) and *Xenopus* orthologs. D485 is highlighted in grey. (C) X-chromosome inactivation study. DNA samples extracted from whole blood cells were directly amplified by PCR with specific primers that flank the *HUMARA* gene (labelled as -*Hpa*II), or digested with methylation-sensitive enzyme *Hpa*II, followed by PCR amplification of the *HUMARA* gene (labelled + *Hpa*II). The alleles amplified after *Hpa*II digestion correspond to the inactive X-chromosome. The samples were analysed with GeneScan software. Boys (II.1 and II.2) amplified only one allele in non-digested conditions (280 bp), corresponding to the active chromosome which carries the *WASP* mutation. All female members were heterozygous at the *HUMARA* gene, with a total of three bands detected (262-, 280- and 283-bp alleles).

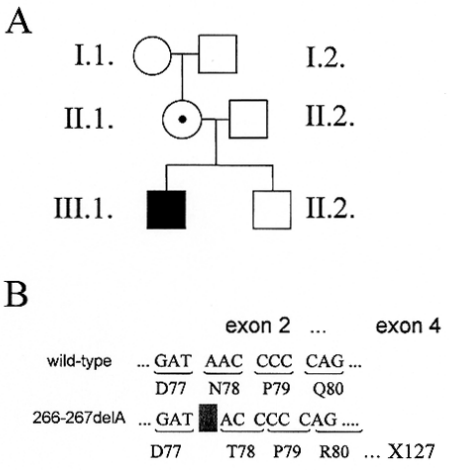

Figure 2. Family B: Pedigree and molecular analysis of the *WASP* gene. (A) Pedigree of family B. The filled square indicates the WAS patient (III.1.). The circle with a dot represents the asymptomatic female carrier (II.1.). The circle represents the healthy female (I.1.). Open squares indicate the non-affected males (I.2, II.2 and III.2). (B) Explanation of the frameshift mutation.

cells). As expected, in the case of the WAS boys (II.1. and II.2.), the X-chromosome that carried the mutation (280 bp) was active. Abnormalities in the X-inactivation process have been associated with a point mutation in the minimal promoter of *XIST* (14). We performed screening for cytosine-to-guanine mutation in the *XIST* minimal promoter for the female members of the family, but no mutation was detected (data not shown).

In family B, sequence analysis of genomic DNA from the affected boy (III.1.) revealed the presence of one base pair deletion, 266-267delA, on exon 2 (Fig. 2). This change resulted in a frameshift (at codon 78) and created a stop codon at amino acid 127 (exon 4). No other changes were detected in the *WASP* gene of this patient. The same nucleotide change was detected in his mother (II.1.) in heterozygosis, indicating that she was a carrier of the mutation. In contrast, his grandmother (I.1.) was not a carrier (Fig. 2), indicating that the mutation originated *de novo* in the mother (II.1). We also analysed the expression of the *WASP* gene in RNA samples from the affected boy (III.1.), using RT-PCR, and no abnormal products were detected (data not shown). However, Western blot analysis showed no expression of the *WASP* gene, thus suggesting that the truncated protein was unstable (data not shown).

Discussion

The majority of mutations identified in the *WASP* gene pertain to the group of missense mutations, and mostly localise in the four first exons. The second most common type of *WASP* mutations are splice site mutations and occur predominantly in the downstream part (introns 6-11) of the *WASP* gene. Along the whole gene, but with reduced frequency, deletions, insertions, nonsense and complex mutations are also to be found (4-6).

Interestingly, for the first time, in the present paper we describe a family with a missense mutation in exon 12 of the *WASP* gene, that has been associated with XLT. Until now only 6 different mutations have been reported on exon 12 (Table II): two missense mutations in the last codon of the WASP protein (X503R and X503S) (5,15), two deletions (1519delT and 1543delAGTG) (16,17), a splice defect (4) and a complex mutation (18). All these mutations resulted in aberrant WASP protein or an absence of WASP expression, and have been associated with a severe WAS phenotype. The reduced number of mutations identified in the exon could be partially accounted for by the fact that nucleotide changes resulting in amino acid substitutions in these region might coincide with a very mild phenotype, thus escaping a diagnose of WAS or XLT. In fact it is well documented that X-linked thrombocytopenia (XLT) is sometimes misdiagnosed as chronic idiopathic thrombocytopenic purpura (ITP) (19,20).

The molecular basis of the described mutation is unknown. Interestingly, in the WASP protein, preceding amino acid 485, there are two serine residues, S483 and S484. Their phosphorylation has been reported to be required for efficient *in vitro* actin polymerization by WASP (21). One could speculate that the nucleotide substitution (D485G) may introduce a conformational change that affects serine phosphorylation resulting in a less functional protein.

Table II. Summary of published *WASP* mutations found in exons 2 and 12.

Exon	Mutation	Effect	Type	Reference	Protein	Protein[d]
2[a]	168C>T	T45M	Missense	(26)	N.D.	R or -
2	171T>C	L46P	Missense	(27)	N.D.	
2	174C>A	A47D	Missense	(7)	N.D.	R
2	177C>T	T48I	Missense	(28)	N.D.	R
2	185G>T	V51F	Missense	(6)	-	
2	190G>C	Q52H	Missense	(29)	N.D.	
2	201C>T	A56V	Missense	(30)	N.D.	R
2	201C>G	A56V	Missense	(31)	N.D.	
2	206C>G	P58A	Missense	(5)	R	R[e]
2	207C>T	P58L	Missense	(26)	N.D.	
2	207C>G	P58R	Missense	(9)	+	+
2	224T>C	W64R	Missense	(32)	N.D.	
2	242G>T	G70W	Missense	(29)	N.D.	
2	251T>C	C73R	Missense	(33)	-	
2	252G>A	C73Y	Missense	(34)	R	R
2	255T>C	F74S	Missense	(5)	N.D.	
2	257G>A	V75M	Missense	(16)	N.D.	R or +
2	261A>C	K76T	Missense	(5)	N.D.	
2	263G>C	D77H	Missense	(5)	R	
2	264A>G	D77G	Missense	(35)	R	R
2	278T>C	S82P	Missense	(16)	N.D.	R
2	279C>T	S82F	Missense	(36)	N.D.	
2	282A>G	Y83C	Missense	(33)	+	
2	284T>C	F84L	Missense	(37)	-	-[f]
2	288T>C	I85T	Missense	(38)	N.D.	
2[a]	290C>A	R86S	Missense	(39)	N.D.	N.D.
2[a]	290C>G	R86G	Missense	(5)	R	
2[a]	290C>T	R86C	Missense	(16)	N.D.	R or -
2[a]	291G>A	R86H	Missense	(3)	N.D.	R or -
2[a]	291G>T	R86L	Missense	(28)	N.D.	-
2[a]	291G>C	R86P	Missense	(27)	N.D.	
2	300G>A	G89D	Missense	(37)	+	
2	196C>A	Y54X	Nonsense	(40)	N.D.	-[g]
2	272C>T	Q80X	Nonsense	(16)	N.D.	
2	283C>A	Y83X	Nonsense	(41)	-	
2	298C>A	Y88X	Nonsense	(42)	-	
2	186-196del	51fs/58X	Deletion	(5)	-	
2	201-213del	56fs/71X	Deletion	(5)	N.D.	
2	206-210delC	59fs/75X	Deletion	(43)	N.D.	N.D.
2	211delT	59fs/75X	Deletion	(3)	N.D.	-
2	211delT	59fs/75X	Complex	(5)	N.D.	
2	211 T>C	P59P	(2 Mutations)			
2	237-246del	68fs/72X	Deletion	(5)	-	
2	248delG	72fs/75X	Deletion	(36)	N.D.	
2	260-261delA	76fs/126X	Deletion	(4)	-	
2	268delC	77fs/127X	Deletion	(16)	N.D.	
2	279-280delC	82fs/126X	Deletion	(5)	N.D.	
2	283delCTTCA	84fs/119X	Deletion	(4)	-	
2	218-219insG	62fs/78X	Insertion	(44)	N.D.	
12[b]	1507T>A	X503R, 581 aa protein	Missense	(15)	-	
12	1519delT	Fs/extend polyA	Deletion	(17)	-	
12	1542G>C	X503S	Missense	(5)	-	
12	1543delAGTG	Extend polyA	Deletion	(16)	N.D.	
12	No mutation in coding region	Normal and abnormal cDNA	Ins intron 11 (118 bp)	(4)	+	
12[c]	Complex mutation	519 aa protein	Inversion/deletion	(18)	+	

WASP expression in the described patient: positive (+), negative (-), reduced (R), or not determined (N.D.). [a]WASP hotspot mutations. [b]WASP mutation described in the original article as 1507T>A, however the T nucleotide in codon 503 corresponds to position 1541. [c]Complex mutation: 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 contiguous *SUV39H1* gene. [d]WASP protein expression in new patients not included in the first report. The data was obtained from http://homepage.mac.com/kohsukeimai/wasp/WASPbase.html or from references [e](6), [f](45), and [g](46).

e carriers of X-linked disorders are, in most cases, ıatic. This is explained by a skewed X-inactivation pattern with preferential selection of the normal, non-mutated X-chromosome as the active allele in the target cells. This could result from somatic selection after X-inactivation, leading to the survival of the lineages bearing the wild-type allele. In WAS carriers a defect in the migration of WASP-deficient hematopoietic stem cells has been proposed as the cause of such skewed X-inactivation (22). However, several reports of females with clinical signs of WAS have been described many of whom were associated with alterations in the X-inactivation pattern (reviewed in ref. 1).

In family A we described a new female case of XLT with clinical signs. She presented a skewed pattern of the X-chromosome inactivation, but with preferential activation of the mutated allele. Her daughter, completely free of XLT symptoms, was also heterozygous for the *WASP* mutation, but presented a random pattern of X-chromosome inactivation. Females with clinical signs of WAS and a random pattern of X-inactivation have also been described, although in all cases the causative mutation presented a strong WAS phenotype in the male offspring of the family, thus suggesting a major impact of the mutations in WASP protein alteration (23-25). The data appear to indicate that in XLT mutations an aberrant strong skewed X-inactivation pattern is required to develop clinical symptoms in female carriers.

In family B, a novel mutation 266-267delA in the *WASP* gene was identified and associated with the absence of WASP protein (data not shown), presumably because of the resulting frameshift and the formation of a truncated protein. Exon 2 is one of the exons in which more mutations, ~50, have been identified (Table II). Moreover, two of the five hotspots described so far are located in exon 2. The most common mutations in the region are missense followed by small deletions. As with the case described in this study, these deletions have been associated with a severe phenotype and, in patients whose protein expression has been studied, a lack of protein was also reported.

To summarise, we described two novel mutations in the *WASP* gene. A missense mutation in exon 12 associated with XLT, and a frameshift mutation in exon 2 associated with classical WAS. Importantly, a new case of a female with clinical signs of WAS were described in association with XLT.

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