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Molecular diagnosis of Wiskott-Aldrich syndrome in Taiwan

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The spectrum of Wiskott-Aldrich syndrome (WAS) mutation in Han Chinese residing in Taiwan has not been previously reported. We describe a multidisciplinary approach to the molecular diagnosis of WAS which could be applied to clinical diagnosis, carrier prediction, and prenatal diagnosis. A total of 6 male patients, from 6 independent families, were referred for the molecular diagnosis of WAS. The respective methylation status of the 6 X chromosomes in peripheral blood leukocytes from obligatory female carriers was analyzed initially, followed by analysis of the level of expression of WAS protein (WASP) in peripheral leukocytes from patients, using a Western blotting technique. The analysis of the specific WAS gene mutation was done by direct sequencing. Mutations were identified in the WAS gene of all patients. Mutations identified included p.R13X, p.R41X, p.S82P, IVS1-1 G→C, p.L342TFsX493, and a large deletion. Four patients had no WASP expression in peripheral leukocytes obtained before bone marrow transplantation. Several female carriers in the families of the 6 patients with such mutations were confirmed. One prenatal diagnosis was made in a fetus and he did not inherit the mutation. The importance of mutations in the first 2 exons of the WAS gene was demonstrated in this study, which represented 5 of the 6 mutations identified in 6 patients. The use of a multidisciplinary approach including DNA and protein analysis is required for molecular diagnosis and genetic counseling of WAS.

Key words: Chinese, mutation, prenatal diagnosis, Taiwan, Wiskott-Aldrich syndrome, X-inactivation

Wiskott-Aldrich syndrome (WAS) is an X-linked recessive immunodeficiency disease, with an estimated worldwide incidence of 4 per million live male births [1], characteristically associated with eczema, hemorrhagic episodes and recurrent severe infections [2]. This condition typically results from the inability of affected individuals to generate antibody responses to polysaccharide antigens and by the impairment of such patients' T-cell responses to allogenic and selected mitogenic stimuli [3]. Data pertaining to the natural history of WAS has suggested that the disease is uniformly associated with a very high morbidity and early mortality [3]. The only known effective treatment modality for WAS is stem-cell transplantation [4]. An early diagnosis, or even prenatal diagnosis, will typically provide a more favorable outcome for WAS patients.

The gene responsible for WAS, encoding the WAS protein (WASP), is located on chromosome Xp11.22-p11.23, and extends over about 9 kb of genomic DNA and composes 12 exons [5]. Over 100 unique mutations have now been reported for this site [6,7]. Mutations are located throughout the gene, the most common of which involve nucleotide substitutions [7]. The interaction between WASP, the Rho family GTPase Cdc42, and the cytoskeletal-organizing complex Arp2/3 is probably critical to many other functions in signal transductions, which, when disturbed, will translate into measurable defects relating to cell signaling, polarization, motility, and phagocytosis [4, 8]. The potential for a correlation between the levels of WASP and the phenotypes of affected patients has been suggested [2]. In order to elicit the accurate prediction of the condition's clinical course, mutation analysis at the DNA level as well as the investigation of transcription and/or protein levels are required.

Clinical diagnosis of WAS is often difficult because the associated symptoms such as eczema and recurrent infections are not always present at diagnosis and are frequently seen in other medical conditions. Immunological dysfunction in WAS patients also appears to be too variable to be used alone for the purposes of making a definite diagnosis. Direct sequencing of the

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WAS gene aided by mutation-searching techniques including single-strand conformation polymorphism or denaturing high-pressure liquid chromatography are typically effective techniques for the diagnosis of WAS but lack practical applicability. More critically, it is sometimes not possible to confirm the functional relevance of novel mutations, which are common in WAS patients.

This study describes a multidisciplinary approach to the molecular diagnosis of WAS through the analysis of X-inactivation and linkage, the analysis of WASP expression using a Western blot analysis technique, and via direct sequencing of the WAS gene. Three of the 6 mutations identified for Taiwanese WAS-affected families in this study are novel. Only through this multidisciplinary approach were we able to perform an accurate genetic diagnosis in WAS patients, to conduct a precise prediction of the carrier status of patients, and to perform a prenatal diagnosis.

Materials and Methods

Six male patients, from 6 independent families, were referred for the molecular diagnosis of WAS. The ages of the patients ranged from 3 months to 5 years. They were all classified as having severe, classical WAS (as defined by the World Health Organization Committee on Immunodeficiency criteria) [9]. Case 1 received successful related-mismatched bone-marrow transplantation (BMT) from his mother at the age of 2 years, but died 3 months later due to an Epstein-Barr virus-related lymphoproliferative disorder transmitted from his mother. DNA samples were obtained from this patient before the transplantation procedure was conducted. Case 5 also received successful related-matched BMT from his sister at the age of 6 months, and the DNA samples were obtained from his mucosa cells at the age of 3 years. Case 6 received unsuccessful related-mismatched BMT from his father at the age of 3 years, and the DNA samples were obtained from his peripheral blood at the age of 5 years. All studies were conducted after informed consent had been obtained. For the purposes of carrier detection in adult women and for conducting prenatal diagnosis, genetic counseling was performed for participants both prior and subsequent to the tests having been completed.

Assessment of patterns of X-inactivation

Patterns of X-inactivation were assessed as either random or non-random via the relative status of methylation of the polymorphic X-linked human androgen receptor genes (HUMARA) as described previously [10]. Genomic DNA was extracted from peripheral blood leukocytes from the test group of patients and their families, including at least 1 obligatory female carrier for each family. The DNA samples were either digested or not by methylation-sensitive endonuclease *Hpa II* first, and then amplified with primers that bracketed a polymorphic CAG repeat sequence located in the second exon of the HUMARA gene. The sizes of the polymerase chain reaction (PCR) products so produced were analyzed by GeneScan using a 377 AutoSequencer (Prokinomi, New York, NY, USA). Non-random inactivation of the X chromosomes was considered present in carriers if 1 of the 2 alleles was preferentially amplified following *Hpa II* digestion of the DNA.

Western blot analysis

Peripheral blood leukocytes from WAS patients and their mothers were sonicated, and the concentrations of soluble protein were determined using a protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). From each sample, 40 μg of protein was loaded together with the appropriate molecular-weight markers onto a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel. After completion of electrophoresis, the protein was transferred to a polyvinylidene fluoride Immobilon-P membrane (Millipore, Bedford, MA, USA). Subsequent to blocking with 5% non-fat milk for 1 hour at room temperature, the membranes were incubated with an anti-WASP antibody (Upstate Biotechnology, New York, NY, USA) according to the manufacturer's instructions. The membrane was then washed and incubated with 1:1000 diluted horseradish peroxidase-conjugated anti-rabbit antibody (Sigma, Poole, UK). The resultant signals were visualized by an enhanced chemiluminescence method (Amersham, Arlington Heights, IL, USA).

Amplification and direct sequencing of the WAS gene

Genomic DNA was prepared from peripheral blood leukocytes by a standard method. Each exon of the WAS gene was amplified as previously described [11], and was then subjected to direct sequencing using a Prism 377 DNA Sequencer (Applied Biosystems, Foster City, CA, USA).

Reverse transcription and polymerase chain reaction

Total cellular RNA was prepared from peripheral blood leukocytes using the TRIzol reagent (Life Technologies,

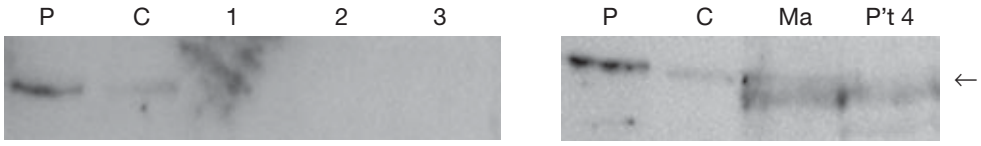

Fig 1. Absent Wiskott-Aldrich syndrome protein (WASP) expression in peripheral leukocytes for WAS patients. Positive control cellular proteins from Jurkat cell lysate (P) and from a normal individual (C) indicate a 65-kD band by Western blot after incubation with anti-WASP antibody, although patients (1-4) reveal no such band. The carrier from patient 4 (Ma) shows a faint band in the normal position (arrow).

Grand Island, NY, USA) according to the manufacturer's instructions. cDNA was synthesized from RNA via reverse transcription reaction by using an AMV reverse transcriptase (Promega, Madison, WI, USA). PCR amplification of *WAS* cDNA exon 1 to exon 5 was performed using primers of 1F (5'-AGACAAGGGCA GAAAGCAC) and 5R (5'-CCCTCCTCTTCTCTCTT CATTG).

Results

Skewed X-inactivation for WAS carriers

Three obligatory females from the first 4 families showed extremely skewed X-inactivation patterns, since 1 of the 2 HUMARA alleles disappeared following *Hpa II* digestion in the assay. We could not assess the X-inactivation patterns for the mother of patient 4 because her HUMARA locus was found to be non-polymorphic. Three additional adult females from the family of patient 1 also exhibited skewed X-inactivation and they were all shown to be carriers when assayed by mutation analysis. One female from the same family showed no evidence of skewed X-inactivation and, as predicted, did not reveal any evidence of WAS mutation. We performed prenatal diagnosis for a subsequent pregnancy of the mother of patient 1. Analysis of a chorionic villi sample at the gestational age of 12 weeks revealed that this male fetus had inherited the normal chromosome from his mother, and therefore was probably not affected by WAS. The fetus was also found to be normal on mutation analysis.

Absent WASP expression in peripheral leukocytes of WAS patients

WASP was visible for normal control samples as a clearly defined band at 65 kDa. However, analyses of the 4 affected boys all disclosed no WASP expression on Western blot analysis of leukocytes (Fig. 1).

Analysis of WAS gene in patients, at-risk family members, and fetuses

The clinical presentation and WAS mutations identified in the 6 patients are summarized in Table 1. The

Table 1. Summary of clinical and laboratory data in 6 patients with Wiskott-Aldrich syndrome

Patient no.	Age at onset	Platelet count (/μL)	IgG (mg/dL)	IgM (mg/dL)	IgA (mg/dL)	Mitogen response	Family history	Age at diagnosis	WASP expression	Mutation	Clinical presentation
1	10 days	5000	820	<20	123	Impaired	Yes	1.5 years	Negative	IVS1-1G→C	Bloody stool
2	7 days	5000	787	31.4	97	Impaired	No	2 years	Negative	c.245C→T (p.S82P)	Bloody stool, eczema, repeated infection
3	1 month	14,000	504	178	105	Not performed	Yes	9 months	Negative	c.1021insC (p.L342TfsX 493)	Bloody stool, repeated infection
4	18 days	5200	1540	146	103	Not performed	No	3 months	Negative	Large deletion	Repeated infection
5	3 days	25,000	1058	80	43	Not impaired	No	7 months	ND	c.121C→T (p.R41X)	Gum bleeding, bloody stool, repeated infection
6	1 days	28,000	ND	ND	ND	Not impaired	No	3 years	ND	c.37C→T (p.R13X)	Subgaleal hematoma, repeated infection

Abbreviations: IgG = immunoglobulin G; IgM = immunoglobulin M; IgA = immunoglobulin A; WASP = Wiskott-Aldrich syndrome protein; ND = not detected

mutations identified in case 2, case 5 and case 6 were previously reported as disease-causing mutations [7].

Through analyses of the WAS gene exons, case 1 was found to have an IVS 1-1 G→C in the intron 1 acceptor site, and there appeared to be no detectable evidence of WAS mRNA expression in the patient's leukocytes (Fig. 2). By analyzing this mutation, we further confirmed 4 female carriers in 5 female members of this patient's family. The patient's mother also had another 2-base insertion at the site of IVS2+12 CC, which proved to be a polymorphism because the mother still had normal mRNA expression (Fig. 2). The mother complained about having developed petechiae when she was pregnant, at which time her platelet count was found to be 90,000/μL. Although the other carriers all had normal mRNA in apparently normal amounts, they had all developed eczema and decreased platelet counts at some time in their lives. In the prenatal diagnosis of the next pregnancy of this patient's mother, analysis of the WAS gene in cells from chorionic villi revealed a normal sequence at the supposed mutation site. This result was compatible with that from linkage analysis, thus confirming that the fetus was not affected.

Case 3 exhibited a one-base insertion in exon 10 (c.1021insC→ p.L342TfsX493), which resulted in a frame-shift change after Leu342, and would thus create a premature termination at a location 153 amino acids subsequent to the site of the insertion. To the best of our knowledge, this mutation has not been described previously.

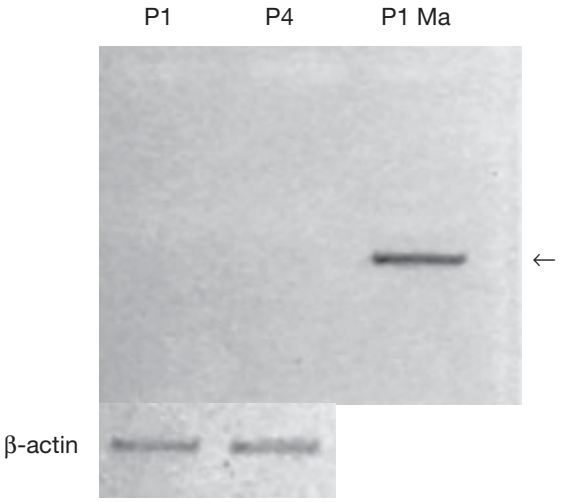

Fig 2. mRNA expression for patient 1. Although cell lysates from both patient 1 (P1) and patient 4 (P4) show normal β-actin mRNA expression, neither of these patients show normal Wiskott-Aldrich syndrome mRNA expression as was the situation for the mother of patient 1 (P1 Ma) [arrow].

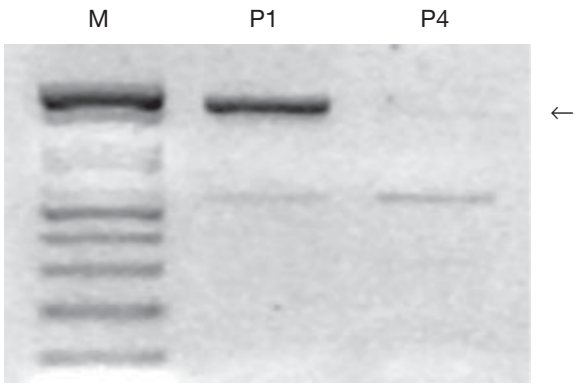

Fig. 3. A large fragment deletion for patient 4. Using the same amplification conditions, a band around 2-kb in length was amplified from genomic DNA of patient 1, but no such band could be amplified for patient 4 (arrow).

For case 4, a large deletion or rearrangement of the WAS gene was found. The deletion may have encompassed exon 1 through exon 4 since amplifications of these exons yielded no DNA product (Fig. 3). This is also a novel mutation.

Discussion

We studied 6 Taiwanese WAS-affected families and found 3 novel mutations responsible for the disease. Five of the 6 mutations were located in exon 1 to exon 2. Our data are similar to those of previous reports and support the importance of the N-terminal of WASP in its function due to the clustering of WAS mutations within the initial 4 exons of the gene [3], a region encoding the WASP homology 1 (WH1) domain [12]. This distinguished motif has been implicated in the coupling of cell stimulation to nuclear response and also to cytoskeletal change [12] through binding to actin monomers in addition to the ARP2/3 complex to catalyze branching of filamentous actin (F-actin) [13]. Since both T-cell activation as well as natural killer cell activation require actin polymerization [14], mutations of the WAS gene result in a reduction in actin polymerization and filopodia formation, thus impairing phagocytosis and chemotaxis [8], although the detailed mechanism of WASP production remains unclear.

Carrier status detection for the female relatives of WAS patients in this study was performed successfully by way of genetic marker linkage revealing a resultant X-chromosomal inactivation pattern coupled with mutation analysis. This method is quite simple, sensitive and effective. WAS carriers present non-random inactivation of the X chromosome at the hematopoietic

stem-cell stage because WASP is necessary for survival in all hematopoietic cells [15]. One limitation in studying for X-inactivation is that the polymorphic markers we used for this purpose are occasionally non-informative, as was evidenced by case 4. A skewing of the X-chromosomal activation pattern is also not exceptional in normal female study subjects, and it would appear that the incidence of an excessive degree of such skewing increases with age [16]. A potential risk exists when applying this method as the only tool for prenatal diagnosis because of the substantial genetic distance and therefore likelihood of recombination in the fetus between the androgen receptor allele and the WAS gene. Therefore, a more direct method for diagnosis should always be coupled with X-inactivation assay, such as an additional technique including detecting WASP expression or conducting direct mutation analysis.

Previous reports have suggested a correlation between the level of WASP expression and the variation in expressed phenotypes of clinically affected patients [2]. In this study, all patients were classified as having either severe or classical WAS, and none revealed WASP expression. Mutations which alter splice sites, as was the situation for patient 1, can lead to the synthesis of alternatively spliced transcripts, and the corresponding truncation of the affected protein. However, in-frame or even normal transcripts may still occur in such patients and therefore occasionally are associated with a milder phenotype. This further augments the discordance between mutations and phenotypes.

Rapid protein-based diagnostic protocols for the investigation of patients with suspected WAS have been reported [17,18]. Most patients do not have any abnormal expression of WASP, and the level of protein expression is related to the specific clinical phenotypes present [6]. An alternative strategy for the rapid determination of WASP both for patients and carriers is flow cytometric analysis using a WASP antibody [17,19]. However, flow cytometry lacks the ability to distinguish normal from truncated WASP, the latter potentially exhibiting an altered mobility on immunoblot assay.

In summary, the data presented here provide further information about the spectrum of WAS mutations responsible for classical WAS. A novel WAS mutation with new functional relevance was identified in this series and the results of this study emphasize the importance of the first 2 exons of the WAS gene to the WASP function. Our findings demonstrate that WASP detection using immunoblot analysis is a simple and effective diagnostic technique, especially for patients with no positive family history of WAS. The observation of skewed X-inactivation provides further support for the diagnosis. X-inactivation assessment is also a rapid and simple technique for the determination of WAS carrier status. These 2 tests provide a good indication of the presence of WAS, and allow mutation analysis and subsequent appropriate medical treatment to be planned. The ever-increasing quantity of reported data pertaining to WAS gene mutations in patients with a variety of clinical phenotypes will likely shed further light upon the role of WASP.

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