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CASE REPORT

Molecular characterization of two Malaysian patients with Wiskott-Aldrich syndrome

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Abstract

The Wiskott-Aldrich Syndrome (WAS) is an X-linked immunodeficiency condition characterized by microthrombocytopenia, eczema and recurrent infections. It is caused by mutations in the Wiskott-Aldrich Syndrome protein (*WASP*) gene. We investigated two Malay boys who presented with congenital thrombocytopenia, eczema and recurrent infections. Here we report two cases of *WASP* mutation in Malaysia from two unrelated families. One had a novel missense mutation in exon 1 while the other had a nonsense mutation in exon 2. Both patients succumbed to disease-related complications. A differential diagnosis of WAS should be considered in any male child who present with early onset thrombocytopenia, especially when this is associated with eczema and recurrent infections.

Keywords: Primary immunodeficiency, Wiskott-Aldrich Syndrome, eczema, thrombocytopenia, recurrent infections

INTRODUCTION

Wiskott-Aldrich Syndrome (WAS) is a primary immunodeficiency (PID) characterized by the triad of thrombocytopenia, eczema and immunodeficiency. Patients are also at increased risk for malignancy and autoimmunity. WAS was first described in 1937 by Wiskott as a clinical entity characterized by thrombocytopenia, eczema and bloody diarrhea in male infants.[1] The X-linked inheritance was established by Aldrich in 1954 and subsequently the condition was called Wiskott-Aldrich Syndrome.[1]

WAS is due to a mutation in the Wiskott-Aldrich Syndrome protein (*WASP*) gene. The *WASP* gene was discovered in 1994.[2] It comprises 12 exons that encode 502 amino acids.[3] The gene product, the Wiskott-Aldrich Syndrome protein (WASp) plays an important role in actin cytoskeletal rearrangement.[4] Widely expressed in hematological lineages, defective WASp leads to disturbance in cellular and humoral immunity as well as impaired platelet formation. With greater understanding of the disease, mutations in *WASP* are now known to result in a clinical spectrum which encompasses: (1) Classical WAS with the triad of thrombocytopenia, eczema and recurrent infection, (2) X-Linked Thrombocytopenia (XLT) which is the milder form, and (3) X-Linked Neutropenia (XLN) without features of WAS or XLT.[5] Classical WAS carries the poorest prognosis. While gene therapy provides some promise for the future,[6] the only curative therapy currently is hematopoietic stem cell transplantation.[7,8]

In Asia, various mutations have been described in several countries.[9-14] Here we describe two Malay patients with classical WAS with two different mutations in the *WASP* gene.

MATERIALS AND METHODS

Patients

Patient 1 (P1)

The index case presented with thigh swelling following vaccination at birth. The swelling subsided spontaneously over time. He developed

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eczematous skin eruptions at the age of two weeks. He was admitted for pneumonia at age five months, when thrombocytopenia was noted. That admission was followed by several hospital admissions due to respiratory tract infection. P1 is a child of a non-consanguinous marriage. His mother had an episode of early trimester loss. His family pedigree is shown in Figure 1. He was investigated for WAS when he was 18-months of age.

Patient 2 (P2)
P2 developed bloody stools at the age of two weeks, which resolved after a change to soy-based formula. At the age of three months he was hospitalized with high fever. Thrombocytopenia and an eczematous lesion were noted. Family history revealed two older brothers who died at the age of four years and eleven months due to sepsis. Both of them had eczema and episodes of bloody stool. The mother had two episodes of early trimester fetal loss. The family pedigree is shown in Figure 1. A molecular investigation of WAS was done when the patient was 4-months of age.

Analysis of WASp expression on CD3 cells

Fresh blood was stained with PE (phycoerythrin) conjugated anti-CD3 (Becton Dickinson, San Jose, CA). Cells were then washed, permeabilized and incubated in the presence of FITC (flourescein isothiocyanate) conjugated anti-WASP antibody (sc 2867, Santa Cruz Biotechnology). Multicolour flow cytometry analysis was carried out using a FACSCalibur (Becton Dickinson, San Jose, CA). The expression of WASp was evaluated only in P1 and a healthy adult volunteer as normal control. WASp expression was not performed in P2 due to insufficient blood sample.

RNA and DNA extraction

Total RNA was extracted from blood using the RNeasy Mini Blood Kit (Qiagen, GmbH) as recommended by the manufacturer. First strand cDNA was synthesized with 1ug total RNA using Superscript™ II Rnase H-Reverse Transcriptase (Invitrogen, USA) and random hexamers (Promega, Madison, WI), incubated at 42°C for 1 hour. The QIAamp DNA Blood Mini Kit (Qiagen,GmbH, D-40724, Hilden) was used for DNA extraction, as recommended by the manufacturer.

PCR reaction and sequence analysis

WASP cDNA was amplified in three overlapping fragments. Primers selection is adopted from Zhu *et al* with modification.[15] The primers used for each fragment are as follows :
Fragment 1 (exon 1- 7) : Forward sequence 5'-GCCTCGCCAGAGAAGACAAG-3'
& Reverse sequence 5'-AGCCCCAGGGAGAGCGGACC-3'
Fragment 2 (exon 5-10) : Forward sequence 5'-GGCAAAGTGGAGACAGACGC-3'
& Reverse sequence 5'-GCAATCCCCAAAGGTACAGG-3'
Fragment 3 (exon 9-12) : Forward sequence 5'-ACGACTTCATTGAGGACCAG-3'
& Reverse sequence 5'-TGAGTGTGAGGACCAGGCAG-3'

For all the fragments, samples from patients, mothers, and unrelated, healthy adult volunteers as normal controls, were incubated at 94°C for 3 minutes followed by 35 cycles at 94 °C for 1 minute, 64 °C for 1 minute and 72 °C for 1.5 minutes with a final elongation step of 72 °C for 10 minutes. For fragment 1 and 2, the Amplitaq® DNA Polymerase with Gene Amp (Applied Biosystem, Life Technologies, USA) was used. As for fragment 3, the Expand Long Template PCR system (Roche, Germany) was used.

Any mutations at the cDNA were confirmed by direct sequencing of gDNA at the site affected exons. Primers for exon 1 are 5'-GGTCTAAGCAGTCAAGTGG-3' and 5'-GGAAGGGTGGATTATGACG-3'. Primers for exon 2 are 5'-CGTCATAATCCACCCTTCC-3' and 5'-CTTGAAGCTATGGACACATATG-3'. For exon 1 and 2, gDNA samples from patients,

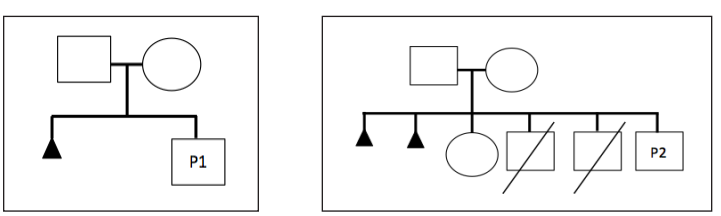

FIG. 1: Family pedigree for P1 and P2

mothers, and unrelated healthy adult volunteers as normal controls were incubated at 94°C for 3 minutes followed by 35 cycles at 94 °C for 30 seconds, 59 °C for 30 seconds and 72 °C for 1 minute with a final elongation step of 72 °C for 10 minutes. Primers and PCR condition for gDNA were adapted from Jones *et al*.[16]

The nucleotide position is in accordance with the *WASP* mRNA (Genbank Accession No. NM_000377).

RESULTS

Clinical characteristics and immunological features

Both patients had clinical features consistent with classic WAS, including thrombocytopenia, eczema and recurrent infections. The initial clinical presentations in both cases were a bleeding tendency. Thrombocytopenia and low mean platelet volume was detected in both cases. P1 had a platelet count of 22,000/uL and MPV of 6.1 fL. P2 had a platelet count of 28,000/uL and MPV 5.9fL.

Table 1 summarizes the immunological parameters of both patients. P1 had low CD19, CD4 and CD8 counts while P2 had a slightly low CD4 count. Assessment of humoral immunity revealed that both patients had markedly elevated levels of IgE. As for other immunoglobulin classes, P1 had low IgM while P2 had low IgG.

WASp expression

Only P1 was subjected to flow cytometric analysis of WASp expression. P1 had reduced WASp expression on T lymphocytes, compared to a healthy normal control. As shown in Figure 2, the normal control showed 94% expression of WASp on T lymphocytes as compared to 31% expression in P1.

Mutation analysis

PCR-sequencing showed two different WAS-causing mutations in our two cases. P1 showed a novel mutation of c.28C>T in exon 1 (Figure 3a). P2 had a nonsense mutation of c.264C>A in exon 2 (Figure 3b). In both cases, the mothers expressed both normal and mutant alleles, indicating that they were carriers.

DISCUSSION

WAS is estimated to affect between 1 to 10 per million male newborns.[17] This number is likely to be an underestimation as facilities for definitive diagnosis of WAS are not widely available, especially in developing nations.

To the best of our knowledge, the earliest description of WAS in Malaysian patients was in 1979 of two male Malaysian Chinese patients.[18] In 2004, three more cases of Malaysians with WAS were reported, involving mutations in exon 2, 4 and 10.[19] The ethnic background of these

TABLE 1: Immunological findings of two Malay patients with classic WAS (numbers in bracket refers to normal range).

Number of lymphocytes					
Patient	CD3 ($x10^6$/L)	CD19 ($x10^6$/L)	CD4 ($x10^6$/L)	CD8 ($x10^6$/L)	NK ($x10^6$/L)
P1	2368 (1800-3000)	63 (700-1300)	322 (1000-1800)	269 (800-1500)	283 (200-600)
P2	5523 (1700-3600)	1091 (500-1500)	1466 (1700-2800)	3953 (800-1200)	2924 (300-700)

Immunoglobulin levels				
Patient	IgG (mg/dL)	IgA (mg/dL)	IgM (mg/dL)	IgE (kU/L)
P1	2390 (550-1000)	110 (35-75)	32 (40-80)	>5000 (3.2 ± 13)
P2	281 (300-600)	90 (10-50)	225 (30-60)	>5000 (1.0 ± 4.1)

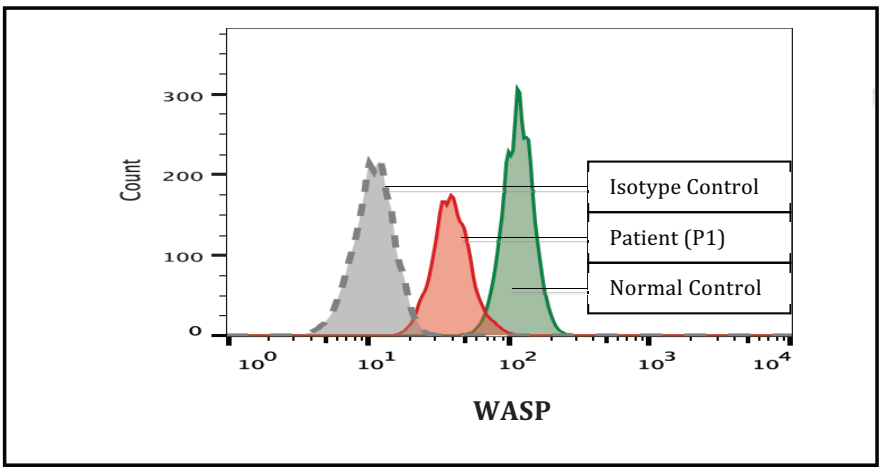

FIG. 2: WASp expression by flow cytometry in P1 showing reduced expression of WASp in P1 (red histogram) as compared to normal healthy control (green histogram) and isotype control is represented by grey histogram.

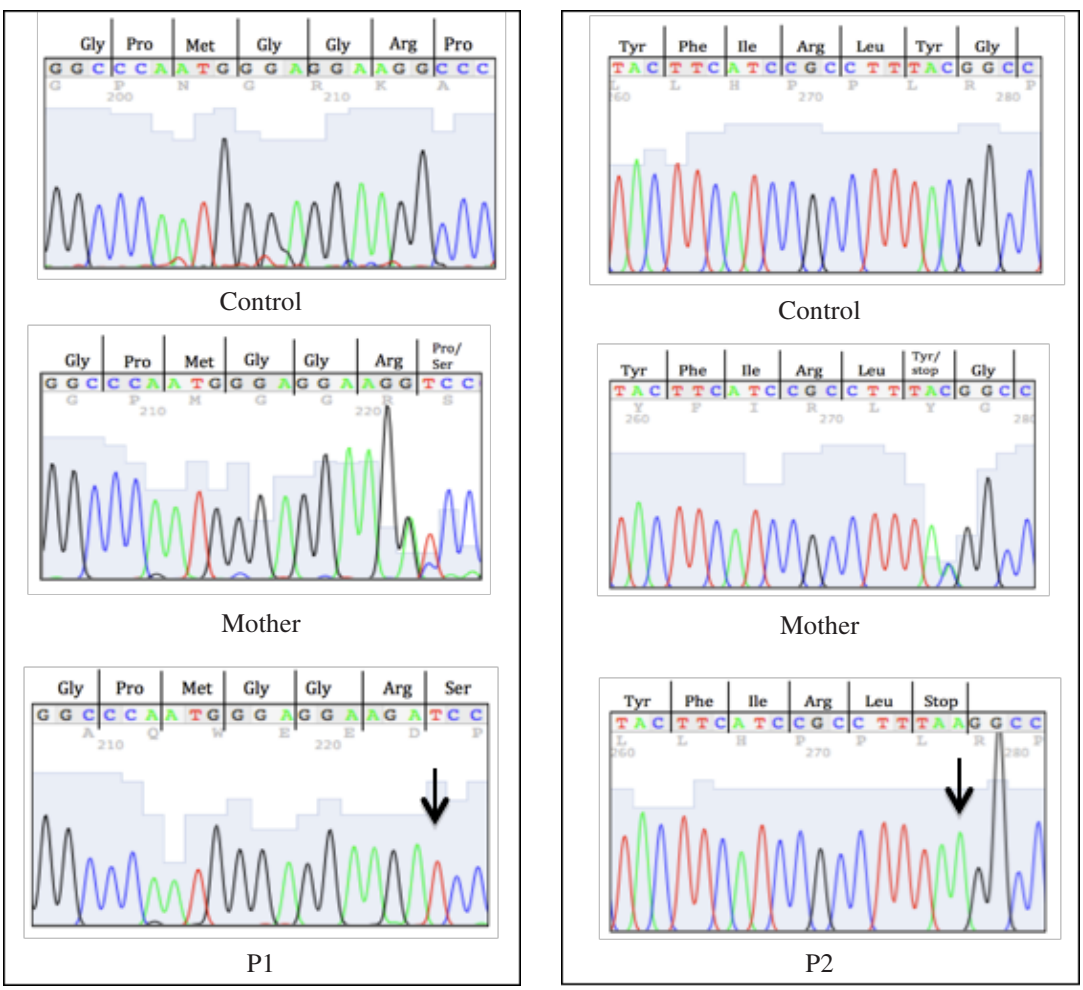

FIG. 3: (a) Mutational analysis for P1 showing a C>T mutation and biallelic condition in his mother, that is C/T. (b) Mutational analysis for P2 showing a C>A mutation and carrier state in mother, that is C/A(* indicate biallelic status , arrow down indicate nucleotide changes)

three cases is not known. Here we report two more cases of WAS in Malay patients, involving mutations in exon 1 and 2.

According to guidelines agreed by PAGID (PanAmerican Group of Immunodeficiency) and ESID (European Society for Immunodeficiency), congenital thrombocytopenia is regarded as the key criteria in diagnosis of WAS.[20] Both our cases may have early onset thrombocytopenia. P1 developed a haematoma following neonatal vaccination while P2 developed spontaneous bloody diarrhea. Unfortunately, the platelet count was not documented during these episodes, and records were only available when the patients were five and four months of age respectively.

Patients with WAS have evidence of both cellular and humoral immune dysfunction.[3] Like most patients with WAS, both of our cases had very high levels of IgE (>5000 kU/L). The immunological feature is more severely presented in P1. P1 had reduced levels of CD19, CD4, CD8 and IgM. In contrast, there was no cellular deficiency in P2, although the level of IgG is low. Nevertheless, the immunoglobulin measurement in P2 was performed at the age of 4 months, and the IgG may be of maternal origin.

More than 300 mutations of the *WASP* have been reported, mainly of missense/nonsense mutations, among various ethnic groups (Human Gene Mutation Database, accessed January 2015). These mutations have been reported in all 12 exons with the majority involving the first three exons. In this case report, our results revealed a novel mutation of c.28C>T in P1. This mutation resulted in a change of the 10th amino acid from proline to serine. The nonsense mutation of c.264C>A in P2 however, had been reported before in the year 1997.[21] The previously reported case manifested with severe clinical phenotype which was also seen in P2. Often missense mutations of the first three exons in *WASP* gene are associated with milder clinical manifestation, but this is not the case in P2. In fact, he succumbed to disease related complication before the age of three years. As for P2, he died before the age of one year.

In view of the different clinical spectrum resulting from mutations of the *WASP* gene, several investigators have examined the correlation between phenotype and genotype. It has been found that clinical phenotype is strongly influenced by the effect of mutation on WASp expression.[19,22,23] In our patients, reduced WASp expression was seen in P1. Unfortunately we were unable to study the phenotype-genotype correlation in P2.

Primary immunodeficiency (PID) is an emerging concern among clinicians in Malaysia. According to a report by Noh *et al*, between 1987 and 2006, a total of 52 PID cases were diagnosed.[24] Antibody related deficiencies were found to be the most common form of PID in Malaysia. Many PID cases such as WAS that take longer to diagnose are missed from this statistics.

In summary we report the clinical manifestations and molecular characteristics of two unrelated Malay children with Wiskott-Aldrich Syndrome. One had a novel c.28C>T mutation in exon 1 and the other had a c.264C>A in exon 2, which has been reported in previous study.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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