Whole Wiskott-Aldrich syndrome protein gene deletion identified by high throughput sequencing

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Abstract. Wiskott-Aldrich syndrome (WAS) is a rare X-linked recessive immunodeficiency disorder, characterized by thrombocytopenia, small platelets, eczema and recurrent infections associated with increased risk of autoimmunity and malignancy disorders. Mutations in the WAS protein (WASP) gene are responsible for WAS. To date, WASP mutations, including missense/nonsense, splicing, small deletions, small insertions, gross deletions, and gross insertions have been identified in patients with WAS. In addition, WASP-interacting proteins are suspected in patients with clinical features of WAS, in whom the WASP gene sequence and mRNA levels are normal. The present study aimed to investigate the application of next generation sequencing in definitive diagnosis and clinical therapy for WAS. A 5 month-old child with WAS who displayed symptoms of thrombocytopenia was examined. Whole exome sequence analysis of genomic DNA showed that the coverage and depth of WASP were extremely low. Quantitative polymerase chain reaction indicated total WASP gene deletion in the proband. In conclusion, high throughput sequencing is useful for the verification of WAS on the genetic profile, and has implications for family planning guidance and establishment of clinical programs.

Introduction

Wiskott-Aldrich syndrome (WAS) is a rare X-linked recessive primary immunodeficiency disease recognized by symptoms including eczema, thrombocytopenia, immune deficiency and bloody diarrhea (1). It is also known as eczema-thrombocytopenia-immunodeficiency syndrome, in line with Aldrich's original description in 1954 (2). The molecular defects of WAS were discovered in 1994 by Derry et al (3), who isolated the pathogenic gene by positional cloning. The gene that encodes the WAS protein (WASP) is located in the short arm of X chromosome (XP11.22-11.23) and is ~9 kb, containing 12 exons and encoding 502 amino acids.

Epidemiological studies have demonstrated that the incidence of neonatal WAS in the developed countries is 1/1,000,000-10/1,000,000 (4), and WAS is caused by a mutation in the WASP gene. WASP is a hematopoietic system-specific intracellular signal transduction molecule, which is proline rich, expressed only in hematopoietic cell lines. It is well documented that WASP gene mutations affect the expression of WASP, thereby resulting in disorders in the response of non-red blood cells to external stimuli, which in turn leads to issues in signal transduction and cytoskeleton dysfunction. This further influences the number, size and aggregation of platelets, hence leading to defects in lymphocytes migration, signal transduction and immunological synapse formation (5).

WASP has been revealed to be critical for cell-signaling (6), actin polymerization (7), synapse formation (8), cell/cell interaction (9), chemotaxis (10), and Treg function (11). As a result, clinical manifestations of patients with alterations of the WASP gene show a great heterogeneity. Categorized by the mutations in the WASP gene, WAS can be divided into typical classic WAS, X-linked thrombocytopenia (XLT) and X-linked neutropenia (XLN). Classic WAS is characterized by triad of thrombocytopenia/micro-platelets, recurrent infections, and eczema. The milder XLT variant behaves predominantly as thrombocytopenia, sometimes occurring intermittently. Patients with congenital neutropenia but without the clinical findings characteristic of WAS or XLT are classified as XLN (12).

Different mutations of WASP could lead to the heterogeneity of WAS in patients. In addition to WASP, WASP interacting proteins have been demonstrated to be involved in the onset of WAS (13-15). Disruption of the binding of WASP to its interacting proteins could also lead to patients with clinical features of WAS, in whom the WASP gene sequence and mRNA levels are normal (16-18). The binding of WASP with its interacting proteins have been confirmed by crystallography (19-21).

It is not difficult to diagnose WAS according to the clinical features; however, as the clinical manifestations of patients demonstrate a great heterogeneity, definite diagnosis...
is required in many cases. Previously, Sanger sequencing of the WASP gene has been applied for the confirmation of WAS diagnosis (22,23), as WASP gene mutations account for the majority of WAS cases. However, as WAS may be caused by mutations of other WASP interacting proteins, other genes may be involved; therefore, performing only WASP gene detection may lead to misdiagnosis. Therefore, it is necessary to identify multiple genes simultaneously. In the present study, next generation sequencing was performed on a WAS patient so as to give the definite conclusion and provide evidence for therapy based on the genetic profile.

Patients and methods

Patient data. A 5-month old male pediatric patient with thrombocytopenia for 3 months and rash for 2 months was admitted to Hunan Provincial People's Hospital (Changsha, China). His mother was a 25-year old gravida 1 para 1 healthy woman without familial diseases. This child was identified to have thrombocytopenia in a local hospital 35 days after birth, when he was hospitalized due to a lung infection. Gamma globulin protein shock treatment was ineffective and his platelet levels maintained at (30-36)×10⁹/L. When he was 2 months old, repeated eczematous rashes appeared. His parents confirmed the family history. The present study was approved by the ethics committee of Hunan Provincial People's Hospital, and informed consents were obtained from the parents of the participant and healthy controls.

Investigation of WASP-interacting proteins. To investigate potential proteins that may interact with WASP, the online server STRING (www.string-db.org/) was applied. This server provides a database of known and predicted protein-protein interactions (24). The network analysis was performed strictly according to the user documentation. The search was initiated using the Single Protein by Name/Identifier selection of the website.

Next-generation sequencing (NGS). For specimen preparation, genomic DNA from 2 ml peripheral blood was extracted from the trio of the proband following the instruction of BloodGen Midi kit (CWBIO, Beijing, China) and the DNA was sheared by sonication. The sheared genomic DNA was then hybridized to NimbleGen 2.0 probe sequence capture array (Roche Diagnostics, Basel, Switzerland). The captured DNA was firstly applied for exonic DNA enrichment (Roche Diagnostics) and the libraries were then tested for enrichment by quantitative polymerase chain reaction (qPCR) using the Agilent Bioanalyzer 2100 (Agilent Technologies, Inc., Santa Clara, USA). The samples were thereby sequenced on an Illumina Hiseq2500 system (Illumina, Inc., San Diego, CA, USA).

Raw image files were processed by the BclToFastq (Illumina, Inc.) for base calling and raw data generating. The low-quality variations were filtered out using quality score ≥20 (Q20). The sequencing reads were aligned to the NCBI human reference genome (hg19) using the Burrows-Wheeler Alignment tool (25). The Genome Analysis Toolkit (26) was used to analyzed single nucleotide polymorphism and insertion-deletion of the sequences. The genes that encoded WASP interacting proteins predicted by STRING were analyzed in detail.

PCR amplification. PCR amplification was performed using whole genome DNA of the proband and a 25-year-old female healthy control. Whole genome DNA was extracted from peripheral blood using DNAzol™ BD Reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Specific primers covering most exons of the WASP gene were designed using Primer premier 5.0 software (Premier Biosoft International, Palo Alto, CA, USA). DNA polymerase was purchased from Takara Bio, Inc. (Otsu, Japan). The primer sequences and relative product lengths are presented in Table I. The PCR conditions were as follows: Initial denaturation at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, elongation at 72°C for 30 sec, and extension at 72°C for 5 min. PCR products were applied to 1% agarose gel electrophoresis and analyzed by image acquisition and analysis system (Tanon Science and Technology Co., Ltd., Shanghai, China).

qPCR amplification. qPCR amplification was performed using whole genome DNA extracted from peripheral blood of the mother and a 25-year-old female healthy control using DNAzol™ BD Reagent (Thermo Fisher Scientific, Inc.). Specific fluorescence quantitative primers were designed for the N terminal, middle and C terminal of the WASP gene with Primer premier 5.0 software. The primer sequences and relative product lengths are presented in Table II. The primers were synthetized by Sangon Biotech Co., Ltd. (Shanghai, China). The PCR conditions were as follows: Initial denaturation at 95°C for 1 min, followed by 40 cycles of denaturation at 95°C for 15 sec, annealing at 60°C for 40 sec and elongation at 68°C for 30 sec. For each sample, two parallel reactions were performed and tyrosine-protein kinase ABL1 (ABL1) served as a reference gene. Homogenization of the PCR products were firstly performed using the output of ABL. Amplification efficiency was calculated according to the comparative Cq method (27) by drawing the standard curve of each pair of primers. The copy number ratio of each truncated gene was calculated by comparing with that of the control.

Statistical analysis. Statistical analysis was performed using SPSS software version 17.0 (SPSS, Inc., Chicago, IL, USA). The rations of the truncated WASP genes of the mother were calculated to that of the control and then expressed as mean ± standard deviation. The value was then compared with the estimated value of 0.5 using single-sample Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Clinical features of the patient. Following hospitalization, routine examinations of the blood, urine and stools were performed. Physical examination revealed scattered needlepoint to small grain-sized papules mixed with a few pinpoint-sized bleeding points across the whole body. Parts of the eruptions were accompanied by a little secretion, with peri-auricular as the most serious. Purulent secretion was identified in the left ear. The liver was enlarged 6 cm below the right costal margin and the spleen could be palpated 2 cm under the left costal margin. Blood routine examination revealed that the...
The white blood cell count was 14.28x10^9/l, the neutrophil count was 7.42x10^9/l, hemoglobin was 116 g/l, the platelet count was 26x10^9/l, the thrombocytocrit was 0.01%, and the platelet mean volume was 5.8 fl. Stool and urine routine tests indicated that liver function, renal function, myocardial enzyme, blood glucose, electrolytes, coagulation and C-reactive protein all were roughly normal. The erythrocyte sedimentation rate was 37 mm/h. The fecal occult blood test was negative. Examination of immunoglobulin revealed that immunoglobulin (Ig)A was 0.76 g/l, IgG was 9.73 g/l [after intravenous gammaglobulin (IVIG)] and IgM was 5.88 g/l; all were higher than healthy limits. In addition, total IgE was within the normal range (11.36 IU/ml). Lymphocyte subsets detection demonstrated that cluster of differentiation (CD)3+ T cells accounted for 38.4% of lymph, CD3+CD4 helper/inducer T cells were 32.9% of lymph, and the percentage of CD3+CD8+ suppressor/cytotoxic T cells was 1.3%, which were a little lower than that of normal values. The percentage of CD3-CD56+CD16+ natural killer cells and CD3-CD19+ B cells were 16.2 and 35.9% respectively, which were a little higher compared with normal limits.

Respiratory virus antigen examination (influenza A, influenza B, respiratory tract virus, adenovirus, parainfluenza virus 1, 2 and 3) and Torch analysis were negative. Cytomegalovirus-DNA was <1.00E+03 copies and blood culture was negative. A chest X-ray revealed scattered patchy shadow in both lungs with a fuzzy edge, whereas no obvious abnormalities in cardiophrenic angle were identified. A cranial plain X-ray was normal and an abdominal ultrasound revealed hepatosplenomegaly. A bone marrow examination revealed that proliferation of bone marrow was active. A large number of megakaryocytes were observed, and there was megakaryocyte mature hindrance in cell classification.

Acute feverish and shortness of breath occurred 3 days after the child was admitted to hospital. He was transferred to the intensive care unit for treatment as he was diagnosed severe pneumonia, respiratory failure and sepsis. In line with the above symptoms, the child was clinically diagnosed as typical WAS. As his condition aggregated, his parents abandoned treatment and the child was followed up. WASP-interacting proteins identification results. In order to gain insight into the proteins that interact with WASP, the online server STRING was used. The predictive result is represented in Fig. 1. Together, there were 10 proteins that
were considered to be associated with WASP directly or indirectly. The results indicated that >one proteins are enrolled in the interaction of WASP; NGS may reveal novel insights into the genetic profile of WAS.

**NGS results.** To confirm the diagnosis of WAS and unravel the underlying genetic mutations in WASP and other genes, genomic DNA from the proband and his parents underwent NGS. A total of 8.7 G clean data was obtained from the three samples. The average of GC content was 42.92% and the Q20 was >95%. Quality control files demonstrated that the data was reliable and adequate for further analysis. Coverage and mean depth for the child, his mother and his father were 0.24/0.6, 1/32.9 and 1/48.4, respectively. No mutation that could support the determination of other disease besides WAS was identified. The results indicated that the WASP gene was deviant or even totally lost in the proband, and confirmed the diagnosis of WAS.

**PCR results indicate the whole WASP gene is lost in the proband.** To assess the deletion information in the WASP gene in the proband, PCR was performed. As presented in Fig. 2, no bands were observed in all the lanes (1, 3, 5, 7, 9 and 11) of PCR products from the patient, whereas relative bands were observed in all lanes (2, 4, 6, 8, 10 and 12) from that of the healthy control. The results indicated that the WASP gene was deviant or even totally lost in the proband, and confirmed the diagnosis of WAS.

**qPCR results of the mother of the proband.** As the father was healthy, the lost mutation could only be inherited from his mother or caused de novo. To reveal the etiology so as to guide reproduction to this family, the whole genomic DNA of the mother underwent qPCR analysis. As presented in Fig. 3, the ratios of the target truncated WASP genes to that of the control all were ~0.5, with a mean of 0.58±0.10. There were significant differences in the mean ratio value for the mother (t=1.453, P=0.284). The results demonstrated that the WASP gene of the mother was heterozygous, and the mutation of the proband was inherited from his mother.

**Discussion**

At present, >300 kinds of WASP gene mutations have been reported, which are distributed in the whole WAS gene, and are more concentrated in exon 1-4s, 7 and 10 (28). Six sites are regarded as mutational hotspots, accounting for ~25% of all mutational sites, including three splice site mutations and three point mutations in the coding region (29). The mutation type of the WAS gene determines the expression of WASP, which is closely associated with the clinical phenotype of WAS. A missense mutation in exons 1-3 usually leads to the XLT phenotype, loss of or truncated WASP expression of the 10th exon could induce typical WAS, and missense mutations in GTPase binding domain (L270P, S272P and 1294T) could lead to XLN (30). Peng et al (31) observed that from January 1991 to October 2013, there were 12 articles focusing on gene diagnosis of WAS including 54 case studies. Mutations consisting of
In a retrospective study containing 50 cases from 40 pedigrees by Catucci et al. (32), missense and fusion mutations manifested as WASP positive expression, whereas nonsense mutations, deletion mutations and insertional mutations led to WASP-negative expression. Among the 10 cases of deletion mutations, 3 cases were large fragment deletions, and 1 case was whole exon deletion. In the present study, the patient had whole exon 1-12 deletion, which is rare. To the best of our knowledge, this is the first case of whole exon deletion reported in China, and the second case in the world.

WAS is a combined immunodeficiency disease, with different degrees of humoral and cellular immune deficiency. As the abnormal immune function of WAS is gradual, the immune deficiency symptoms increase with age. Therefore, immunological examination results vary markedly. In most cases, it is reported that serum IgM decreases, IgG only slightly decreases or remains normal, IgA and IgE elevate, and T and B cell proliferation is reduced in 50% patients in vitro (4). In the present study, T cell counts reduced whereas B lymphocyte numbers increased, which is consistent with other studies (4,33). However, there was difference in the humoral immunity result. Serum IgE was normal and IgA and IgG levels were elevated, which may be associated with intravenous Ig therapy. IgM levels increased significantly, which may be associated with repeated intrauterine and postnatal infection by combing with liver and spleen enlargement. This indicates that the immune level of the patient may be associated with age, disease course, infection and other factors that affect disease progression. Therefore, WAS diagnosis should not only be based on the outcome of immunodetection.

Autoimmune diseases often occur in children with WAS, and in the United States and the European population the incidence is as high as 40-72% (32). Autoimmune hemolytic anemia, vasculitis, arthritis and kidney disease are the most common symptoms. In a retrospective study, Imai et al (33) demonstrated that there is no statistically significant difference between WASP negative and positive groups in autoimmune disease incidence (22 vs. 26%, P>0.05) (33). Five cases were with IgA nephropathy and the age of onset was rather late, from 10 to 20 years old. In addition, 5 out of the 50 reviewed patients were complicated by tumors, and they all were WASP negative. In the present study, neither autoimmune or tumor diseases were identified; this may be associated with the young age of the patient and the short duration of the disease. Zhao et al (34) suggested that the clinical phenotype of WAS evaluation should follow the dynamic principle. In line with their opinion, whether this patient's disease may be associated autoimmune diseases and tumors requires further follow-up observation.

Therapeutic methods of WAS should be planned according to clinical severity, duration, mutations of the WASP gene and WASP expression. Therefore, it is necessary to identify the etiology on genetic profile. Routinely, Sanger sequencing is performed to detect mutational situation in the WASP gene. However, as WAS may be caused by WASP-interacting proteins, it is essential to identify the mutations in these proteins simultaneously so as give systemic identification. In view of this, the present study performed whole exome sequencing for this patient.

Before sequencing, the STRING online database was used to identify WASP-interacting proteins, and the result demonstrated that 10 proteins are included. Among the 10 proteins, actin-related protein 2/3 complex subunit (Arp2/3) is reported to interact with WASP, Cortactin, and cytoplasmic protein NCK1 (13,14). NCK1 is linked to glucose tolerance and insulin signaling within certain tissues, and serves important roles in insulin signaling and c-Jun N-terminal kinase signaling pathway (39). Cdc42 is involved in diverse cellular functions, including cell morphology, migration, endocytosis and cell cycle progression (40). The functions of these proteins are associated with that of WASP, indicating that it is necessary for detecting the status of these genes. The sequencing results indicated that there were no abnormal WASP-interacting protein coding genes, but only whole loss of the WASP gene. No novel mutations in WASP-interacting protein coding genes were identified, indicating that NGS was indispensable for WAS diagnosis.

Supportive treatment and antibiotic prophylaxis are required for typical WAS; however, it is necessary to perform IVIG and platelet transfusion and splenectomy. As for typical WAS, hematopoietic stem cell transplantation is the most effective method for early stage of onset. Without hematopoietic stem cell transplantation, WAS patients will eventually die because of infections, bleeding, malignant tumors and other complications (41). As the patient in the present study has severe WAS, hematopoietic stem cell transplantation is the first choice for the treatment; however, his parents terminated treatment because of economic reasons. To date, this patient is followed up. As the genetic profile had been perfectly revealed, the result serve a significant role for family planning guidance for the parents.

In conclusion, this proband has a typical clinical phenotype of WAS; however, at Sanger sequencing was insufficient to determine the pathogenesis, therefore NGS was performed. NGS indicated whole exon deletion of the WASP gene, and the result was further confirmed by Sanger sequencing. Although once reported, whole exon deletion of this gene is very rare. The one reported case was from Japan and this case is in China, which may indicate that whole exon deletion is prone to occur in Asians. WAS is a rare immunodeficiency disease; due to the diversity of WASP gene mutations, the clinical manifestations are very different, and therapeutic methods of WAS should be planned according to clinical severity. The traditional diagnostic approach for WAS is Sanger sequencing, which is flawed as WASP mutation types are varied and other genes in addition to WASP could also induce WAS. Therefore, it is necessary to perform NGS for pathogenic identification and subsequent family planning guidance. The present study aimed to identify mutations in other genes beside the WASP gene to demonstrate the powerful functionality of NGS both for genotyping and for treatment scheme planning; however, this study identified only whole exon deletion. However, in line with the gene deletion type, it is presumed that the prognosis will be very poor, thereby hematopoietic stem cells transplantation is the first
choice for treatment, which highlights the importance of NGS as a diagnostic tool.

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