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Identification and characterization of a novel splice-site mutation in a patient with Wiskott-Aldrich syndrome

Abstract Wiskott-Aldrich syndrome (WAS) is an X-linked recessive disorder characterized by immunodeficiency, eczema, and thrombocytopenia with small platelets. A wide spectrum of mutations in the Wiskott-Aldrich syndrome protein (*WASP*) gene have been identified as causative of the disease. In the present paper, we report on a family with a boy affected by WAS, with a splice-site mutation caused by a T to G substitution in the $+2$ position of intron 6 (IVS6$+2$T$>$G). Expression studies performed in COS-7 and U-937 cells showed that the mutation affected the normal splicing process. As a consequence, an abnormally long transcript of 38 nucleotides is generated. Such missplicing is probably due to the activation of a cryptic splice donor site located 38 nt downstream of exon 6. The translation of such aberrant mRNA will produce a truncated protein with a premature stop at codon 190. Thus, a novel splice-site mutation is reported in a patient with a mild WAS phenotype.

Keywords Wiskott-Aldrich syndrome · Splice-site mutation · Molecular analysis · Expression studies

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Introduction

Wiskott-Aldrich syndrome (WAS) is an X-linked recessive disorder (MIM #301000) characterized by thrombocytopenia with small platelets, eczema, and recurrent infections. The gene involved in the disease is located on the short arm of X chromosome in region Xp11.23-p11.22, and mutations in the Wiskott-Aldrich syndrome protein gene (*WASP*), leading to 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). More recently, it has been described that gain of function mutations in *WASP* may be responsible for X-linked severe congenital neutropenia (Devriendt et al. 2001). The gene is expressed in hematopoietic lineages and encodes for a cytoplasmic protein (WASP), which has a key role in regulating changes in cytoskeletal structure in response to external stimuli and signal transduction processes (Snapper and Rosen 1999; Badour et al. 2003).

Mutations in *WASP* cause a wide variety of clinical phenotypes, ranging from isolated thrombocytopenia to severe WAS. Mutations were found throughout the entire gene, with a preferential location of missense mutations in the amino-terminal part of the protein and mainly stop and frameshift mutations in the carboxyl-terminal part (Schindelhauer et al. 1996; Fillat et al. 2001). Generally, mutations resulting in the expression of normal-sized or truncated protein are associated with a milder phenotype, while mutations leading to the absence of protein correlate with a more severe phenotype (Ochs and Rosen 1999). However, among the total reported mutations, only in approximately one third of them, expression studies at the mRNA or protein level have been performed.

In the present study, we describe a spanish family with a mutation of intron 6. Molecular studies were performed to identify the effect of the mutation on RNA splicing.

Material and methods

Case report

The patient was clinically diagnosed as having Wiskott-Aldrich syndrome at 6 months of age. From the age of 2 months, he presented thrombocytopenia with small platelets, eosinophilia, mild eczema, and moderate infections. His platelet count was of 41.000/mm^3, with a mean platelet volume of 6.2 fL (normal range 6.5–10.5 fL). Immunological studies revealed a strong lymphopenia with low CD8 lymphocytes (4%). Evaluation of T-cell function indicated a decrease in T-cell proliferation in response to pokeweed mitogen (7,260 cpm). However, the mitogenic response of lymphocytes to concanavalin A, phytohemagglutinin, CD3 antibodies, and tetanus antigen was normal. Serum immunoglobulin evaluation showed a slight decrease in IgM (30 mg/dl), lack of isohemagglutinins, and an increase in IgE (161 UI/ml). The clinical diagnosis was molecularly confirmed with the identification of the mutation reported in the present study. Allogeneic peripheral stem-cell transplantation (PBSCT) from his healthy HLA-compatible brother was performed at the age of 1 year. Clinical and biochemical responses were favorable after transplant. At 2 years of age, he had bronchiolitis, but responded well to treatment with ribavirin. At present, he is 32 months old and remains healthy. Treatment with intravenous immunoglobulins every 3 weeks was established to minimize the risk of infections.

Mutation analysis

DNA was extracted from peripheral blood leukocytes by standard techniques. Mutation analysis of *WASP* was performed by single-strand conformation analysis (SSCA), as previously described (Fillat et al. 2001). Direct DNA sequencing was performed on these samples that revealed a change in SSCA, and mutation was confirmed in two independent polymerase chain reaction (PCR) amplifications. For DNA sequencing, the PCR products were purified with a PCR purification kit (Qiagen) according to the manufacturer's protocol. They were sequenced with an ABI prism dye terminator cycle sequencing ready reaction kit (Perkin Elmer) on an automated ABI 377 DNA sequencer.

Expression constructs

Two expression vectors containing either a fragment of the wild-type *WASP* (pLN) or a fragment with the mutated allele, IVS6 + 2T > G (pLIVS6mut), were generated. A genomic fragment of *WASP,* spanning exons 4–7, was PCR amplified from the total genomic DNA from the patient sample (carrying the mutation), or from his father who had no *WASP* mutations. Primers W4S (5'-GTGTGGAGAGGAGATGGGAA-3') and W7EA (5'-AG-GAATCTGTGGGTCCACTG-3') were used and the 1322-bp PCR product was cloned in pGEM-T vector (Promega). Confirmation of the correct sequence was performed by direct sequencing of the recombinant plasmid with universal primers T7 and SP6. A *Not* I fragment, containing either the wild type or the mutated fragment, was cloned to a *NotI* I site at the pFLAG-CMV-2 expression vector (Sigma).

Cell lines and transfection

COS-7 cells, maintained in DMEM medium (Gibco-BRL, Invitrogen) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 mg/ml), and glutamine (2 mM) (Gibco-BRL, Invitrogen), were transfected with pLN or pLIVS6mut vectors using Superfect transfection reagent (Qiagen) according to the manufacturer's protocol. Forty-eight hours post-transfection, the cells were collected and total RNA isolated.

The human promonocytic U-937 cells were cultured in RPMI medium (Gibco-BRL, Invitrogen) supplemented with 10% heat-inactivated FBS, penicillin (100 U/ml) and streptomycin (100 mg/ml) (Gibco-BRL, Invitrogen). U-937 cells (2×10^7) were transiently transfected with pLN or pLIVS6mut vectors by electroporation, at 300 V, 960 μF using BioRad electroporator. Twenty-four hours post-transfection, the cells were collected and total RNA isolated.

RT/PCR analysis

Total cellular RNA was prepared, either from peripheral blood or cultured cells, with Tripure reagent (Roche) according to the manufacturer's protocol. One microgram of total RNA from each sample was reverse transcribed with Retroscript RT kit (Ambion). Five microliter of cDNA was PCR amplified with *WASP*-specific primers E4W-L (5'-AAGGAATCAGAGGCAAAGTGG-3') and E7W-R (5'-TCTTCCCTGAGCGTTTCTTA-3'). Analysis of RT-PCR products was performed in a 6% 19:1 acrylamide/bisacrylamide gel followed by silver staining. Bands were extracted from the gel and sequenced with the above-mentioned primers.

Results

Sequence analysis of genomic DNA from the affected boy revealed the presence of a IVS6 + 2T > G mutation in *WASP*, affecting the splice site. The same nucleotide change was detected in his mother, indicating that she was an asymptomatic carrier of the mutation (Fig. 1). This particular change was not found to be present in 100 randomly screened alleles from the general population, thus proving the mutation to be causative of the disease.

To analyze the impact of the mutation on RNA splicing, genomic DNA fragments spanning exons 4–7 from control and patient samples were cloned into eukaryotic expression vector pFLAG-CMV-2. The generated vectors pLN and pLIVS6mut were transiently transfected into COS-7 and U-937 cells, and the total RNA from the transfected cells was used as a template for RT-PCR analysis with WASP-specific primers. RT-PCR analysis was also performed on RNA isolated from mononuclear cells of the patient after transplant, the patient's mother, and a healthy, unrelated individual. A 251-bp band was detected in all control samples. However, a higher molecular weight band of 289 bp was identified in the mutated PCR product (Fig. 2A). Sequence analysis of the different PCR fragments indicated that products from the control transfected cells and control mononuclear cells spliced RNA normally. However, sequence analysis of the RT-PCR product containing the IVS6 + 2T > G mutation showed the presence of the first 38 nucleotides from intron 6 spliced onto exon 7 (Fig. 2B).

Discussion

Mutations leading to missplicing and exon skipping have been proposed to account for 15–20% of mutations that lead to human disease, with splice-donor sites more
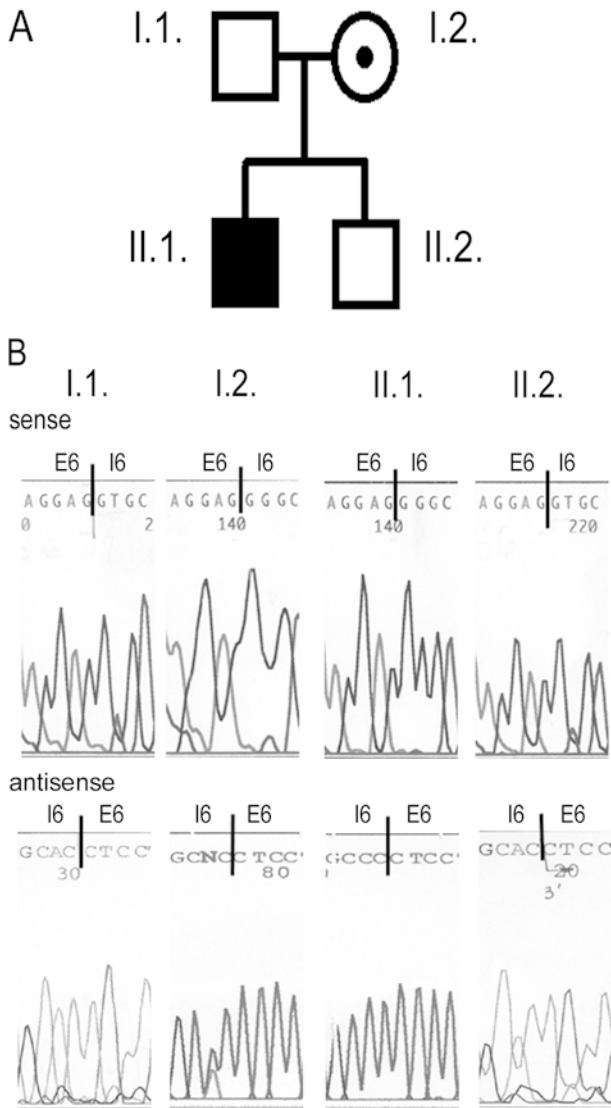
Fig. 1A, B Molecular analysis of the Wiskott-Aldrich syndrome protein (*WASP*) gene. **A** Pedigree of the family. *Open squares* denote healthy males (I.1, II.2). The *filled square* indicates the WAS patient (II.1) The *circle with a dot* represents the asymptomatic female carrier (I.2). **B** WASP IVS6 + 2 T > G splicing mutation. Sequence analysis of exon/intron 6 borders of the WASP gene from the four family members. The sense sequences, T to G transition at position 2 of intron 6, and the antisense sequences A to C transition, are shown in the chromatograms of the boy affected (II.1). The carrier is heterozygous (T/G); (A/C) at this position (I.2)

Fig. 2A, B Effects of IVS6 + 2 T > G mutation on RNA splicing. **A** Electrophoresis of the RT-PCR products of RNA from: *lane 1*, COS-7 cells transfected with pLN; *lane 2*, COS-7 cells transfected with pLIVS6mut; *lane 3*, U937 cells transfected with pLN; *lane 4*, U937 cells transfected with pLIVS6mut; *lane 5*, U937 cells; *lane 6*, mononuclear cells from transplanted patient; *lane 7*, mononuclear cells from asymptomatic carrier; *lane 8*. mononuclear cells from a healthy unrelated individual. The molecular-weight marker is indicated to the *left* of the gel photograph. **B** Diagram showing RNA processing. *1* normal splicing, *2* abnormal transcript. Chromatograms obtained from normally processed RNA–pLN and mononuclear cells from the transplanted patient (*MN*) – and from the abnormal transcript with the 38 bp insertion (pLIVS6mut)

often affected than splice-acceptor sites (Krawczak et al. 1992; Nakai and Sakamoto 1994). However, this is likely to have been underestimated, given that the analysis was limited to mutations in the classical splice-site sequences, and it is presently known that aberrant splicing is also caused by mutations that disrupt exonic splicing elements (Faustino and Cooper 2003). This may also apply to Wiskott-Aldrich syndrome, in which splice-site mutations have been reported to account for 13% of total mutations (http://uwcmml1s.uwcm.ac.uk/uwcm/mg/search/120736.html).
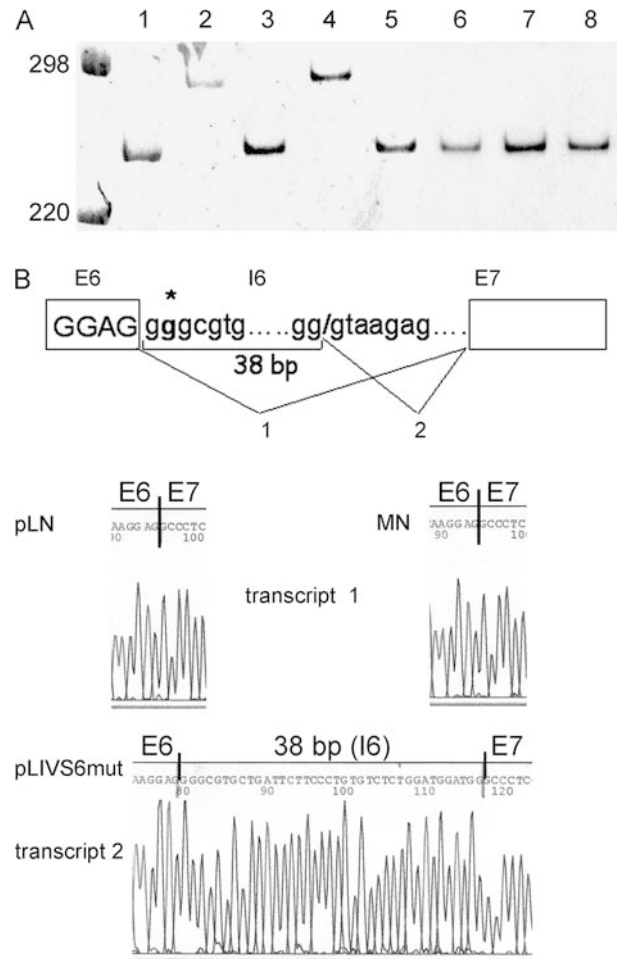
In the present paper, we report a novel splice-site mutation affecting the donor site, which results from a T to G nucleotide substitution in the +2 position of intron 6 (IVS6 + 2T > G). Said nucleotide change may alter the normal splicing process, which appears to be determined by the invariant GT and AG dinucleotides present at the 5' and 3' exon/intron junctions respectively, together with extensive consensus sequences spanning 5' and 3' splice sites (Padgett et al. 1986). Based on this, Shapiro and Senapathy (1987) introduced the consensus value (CV) that reflects the similarity of any splice site to the consensus sequences drawn up through a comprehensive survey of primate genes. At any given site, the CV may

vary between 0 and 100; the most commonly used splice sites have a CV of >70. The CV calculated for the normal eight nucleotide (−2 to +6) of the donor splice recognition sequence of the exon 6/intron 6 of *WASP* for control individuals was 85.2, whereas the CV corresponding to the mutated sequence was 66.9. According to this prediction, an alteration of the normal mRNA splicing process of the mutated sequence was expected.

Four types of abnormal splicing events may result from splice-site mutations: exon skipping, activation of a cryptic splice site, creation of a novel splice site, or intron retention (Maquat 1996). Transfection experiments on the COS-7 and U-937 of plasmids containing either the mutated or control sequence revealed that the mutation resulted in the activation of a cryptic splice site (CV of 86,5), with the consequent incorporation of the first 38 nucleotides from intron 6 spliced onto exon 7. The translation of such aberrant mRNA will produce a truncated protein with a premature stop at codon 190. Activation of this cryptic splice site in *WASP* has already been reported (Lemahieu et al. 1999). Specifically, mutation IVS6+5G>A has been shown to alter normal RNA splicing, with the generation of three different transcripts: a major transcript (70%), containing the 38 nucleotides from intron 6; a minor transcript (2%), including the 38 nt insertion and skipping of exon 7; and a normal transcript (28 %), that leads to very reduced levels of normal-sized protein.

No generation of normally spliced mRNA was detected in COS-7 cells transfected with the pLIVS6mut carrying the IVS6+2T>G mutation. In U937 cells transfected with the pLIVS6mut, only a small amount of normal transcript was determined when RT-PCR experiments were performed using a primer corresponding to the junction site of exon 6–7 (data not shown). This normal spliced product was most probably the result of the processing of the endogenous *WASP* gene present in this hematological cell line. However, we cannot ignore the possibility that some normal transcript could have been present in the patient's RNA. In fact, from the clinical point of view, the patient could be classified as a case of mild classical WAS, with a score of 3 on the system proposed by Zhu et al. (1995). This would be in agreement with the presence of a protein that may maintain some of its functional activities, or the presence of an amount of normal-sized protein. Unfortunately, because the proband underwent PBSCT very early, and only DNA was extracted from the pretransplant sample, we were unable to study RNA processing. Nevertheless, expression studies in cell lines have been extensively used as a strategy to validate mutation consequences. Thus, in the present study, we report the identification of a novel WAS splice-site mutation that leads to the abnormal mRNA processing.

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