

## Brief Report

## X-LINKED WISKOTT-ALDRICH SYNDROME IN A GIRL

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**T**HE Wiskott-Aldrich syndrome is a life-threatening X-linked recessive disorder. Affected males present with recurrent infections, eczema, and thrombocytopenia with small platelets. The immune defect involves both humoral and cellular immunity and increases in severity with age.<sup>1</sup>

The gene involved in this disease, located on the short arm of the X chromosome in the region Xp11.22-23, was recently cloned and named the Wiskott-Aldrich syndrome protein (*WASP*) gene.<sup>2,3</sup> Different mutations or deletions within the *WASP* gene have been described in patients with the Wiskott-Aldrich syndrome and X-linked thrombocytopenia.<sup>4</sup> The gene is expressed in early progenitor cells as well as in differentiated cells of various hematopoietic lineages.<sup>5,6</sup> Evidence has been presented that *WASP* might be involved in cytoskeleton organization and signal transduction.<sup>7</sup>

Female carriers of the disorder have no clinical signs of the gene defect because of the preferential selection of the normal, nonmutated X chromosome in their hematopoietic cells.<sup>8-10</sup> In contrast, in skin fibroblasts and cells from the buccal mucosa of female carriers there is random inactivation of the X chromosome.<sup>8,10</sup> There have been few reports of sporadic cases of females with a clinical disorder similar to Wiskott-Aldrich syndrome.<sup>11-13</sup> When evaluated, the pattern of X-chromosome inactivation was found to be random, and the existence of an autosomal disorder that is clinically similar to classic Wiskott-Aldrich syndrome was postulated.

We describe an eight-year-old girl with typical features of Wiskott-Aldrich syndrome. Molecular anal-

ysis revealed a spontaneous mutation in exon 4 of the *WASP* gene on the paternally derived X chromosome, associated with a nonrandom pattern of inactivation of the maternally derived X chromosome. These findings show how X-linked recessive diseases may occur in females.

## CASE REPORT

In 1989 a two-month-old girl was admitted to the hospital because of stomatitis and thrombocytopenia-related petechiae. A bone marrow aspirate revealed no signs of a malignant disorder, and since all other blood values were within the normal range, a diagnosis of idiopathic immune thrombocytopenia was proposed. The patient subsequently had repeated episodes of mild bleeding that were associated with platelet counts of 5000 to 20,000 per cubic millimeter and clinical signs of immunodeficiency, manifested by repeated ear infections and several episodes of pneumonia. A varicellavirus infection had an unusually severe course, with concurrent viral keratitis and skin lesions resulting in persistent scars. The patient had eczema, which resolved over time. All blood measurements were normal with the exception of persistently low platelet counts and platelet volume (Table 1).

Serum immunoglobulin measurements at eight years of age revealed elevated IgA levels and low IgM levels (Table 1). Specific antibodies to tetanus toxoid, Epstein-Barr virus nuclear antigen, and rubella virus were detected in the serum, but no antibodies against measles or mumps were found despite previous vaccination. Specific antibodies to *Haemophilus influenzae* type b were detected, although levels were in the low-normal range. The karyotypes of the patient and her mother were normal. Review of the family history revealed neither bleeding disorders nor immunodeficiency. The triad of thrombocytopenia with small platelets, eczema in early life, and subsequent clinical signs of immunodeficiency led to a tentative diagnosis of the Wiskott-Aldrich syndrome, and detailed molecular and immunologic diagnostic procedures were performed.

## METHODS

Lymphocyte phenotyping was performed by standard direct immunofluorescent techniques and evaluation with a FACStar cell sorter (Becton Dickinson, San Jose, Calif.).

Analysis of X-chromosome inactivation was performed as previously described.<sup>14</sup> Briefly, DNA was extracted from peripheral-blood cells and cells from buccal swabs with a QIAamp tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. An aliquot of DNA was digested with the methylation-sensitive enzyme *HpaII* (New England Biolabs, Schwalbach, Germany) and amplified by the polymerase chain reaction (PCR) at the human androgen receptor (*HUMARA*) gene locus with specific primers as described previously.<sup>14</sup> The PCR products were subjected to electrophoresis on 3 percent agarose gel and stained with ethidium bromide.

For the synthesis of complementary DNA (cDNA), total cellular RNA was isolated from peripheral-blood cells with the RNeasy Total RNA kit (Qiagen), according to the manufacturer's directions. Approximately 1  $\mu$ g of total RNA was used for the synthesis of cDNA (SuperScript II reverse transcriptase kit, GIBCO-BRL, Gaithersburg, Md.). The primers and PCR conditions used for the amplification of cDNA have been described previously.<sup>6</sup> Each of the 12 exons of the *WASP* gene with flanking splice sites was amplified by PCR as previously described.<sup>15</sup> The PCR products were purified and sequenced with the ABI PRISM dye terminator cycle-sequencing kit (Perkin-Elmer Cetus, Norwalk, Conn.) and analyzed with a DNA sequencer reader (model 373A, Applied Biosystems, Foster City, Calif.).

Single-strand conformation polymorphism (SSCP) analysis was performed with the Phast electrophoresis system (Pharmacia, Uppsala, Sweden). After PCR amplification the DNA samples

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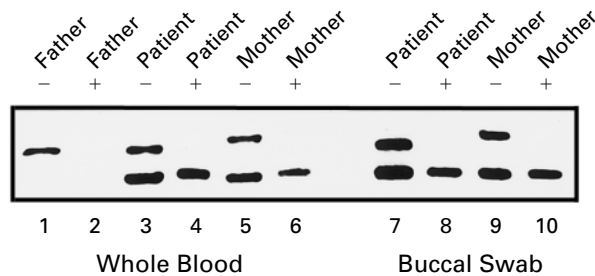
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**TABLE 1.** IMMUNOLOGIC AND HEMATOLOGIC CHARACTERISTICS OF THE EIGHT-YEAR-OLD PATIENT.

VARIABLE	PATIENT	NORMAL RANGE
Serum immunoglobins (mg/dl)		
IgA	554	65–240
IgG	965	730–1410
IgM	38	68–175
Platelets*		
Count ( $\times 10^{-3}/\text{mm}^3$ )	14.6 $\pm$ 2	130–400
Volume (fl)	4 $\pm$ 0.3	7.2–11
Lymphocyte phenotype (%) <sup>†</sup>		
CD3	62	49–75
CD4	23	14–50
CD8	29	9–38
CD19	9	2–23
CD56	9	2–11
$\alpha/\beta$ T-cell receptor	42	37–69
$\gamma/\delta$ T-cell receptor	21	0–13

\*Plus-minus values are the means  $\pm$ SD of the three determinations.

<sup>†</sup>The values are the percentages of mononuclear cells positive for these markers.



**Figure 1.** Analysis of the Pattern of X-Chromosome Inactivation in the Patient and Her Parents.

DNA was extracted from whole blood or oral mucosal cells from the patient and her parents and amplified by PCR with specific primers that flank the *HUMARA* locus (lanes 1, 3, 5, 7, and 9; labeled with a minus sign). In addition, the DNA was digested with the methylation-sensitive enzyme *HpaII* before PCR amplification (lanes 2, 4, 6, 8, and 10; labeled with a plus sign). The samples were analyzed on 3 percent agarose gel and stained with ethidium bromide.

were prepared as described previously<sup>15</sup> and subjected to electrophoresis on 12.5 percent homogeneous Phast gel (Pharmacia) at 15°C for 150 volt-hours. The gels were stained with silver as described previously.<sup>15</sup>

## RESULTS

### Immunologic and Hematologic Characteristics

Table 1 shows selected immunologic and hematologic characteristics of our patient. Serum IgA levels were consistently elevated above normal ranges, whereas serum IgM levels were lower than normal.

Platelet counts and volumes were consistently low. The number of T cells positive for  $\gamma/\delta$  T-cell receptors was increased on repeated tests over a two-year period. Quantitative analyses of other subpopulations of peripheral-blood lymphocytes were normal (Table 1). The response of T cells and cytokines to various mitogenic stimuli varied but was at some times normal (data not shown).

### Analysis of X-Chromosome Inactivation in Cells from Peripheral Blood and Buccal Mucosa

We analyzed the pattern of X-chromosome inactivation in the patient by evaluating the pattern of methylation of the *HUMARA* gene, as previously described.<sup>14</sup> The first exon of this gene contains a highly polymorphic trinucleotide repeat and two *HpaII* sites that are methylated on the inactive X chromosome but not on the active X chromosome.<sup>16</sup>

Both the patient and her mother appeared to be heterozygous at the *HUMARA* locus, as shown by the presence of two bands of different sizes after PCR amplification (lanes 3 and 5, respectively, in Fig. 1). In the patient, the upper DNA band represents the paternally derived allele, whereas the lower one is inherited from her mother. In the case of random inactivation of the X chromosome, as occurs in normal females, a portion of both the maternally and paternally derived X chromosomes will not be digested and both alleles will be amplified. Alternatively, if there is nonrandom inactivation of the X chromosome, the allele on the active X chromosome will be completely digested and only the other allele will be amplified.

In our patient, there was skewed inactivation of the maternally derived X chromosome in both peripheral-blood cells and buccal mucosal cells (lanes 4 and 8, respectively, in Fig. 1). Her healthy mother also had complete nonrandom inactivation of X chromosomes in both cell populations (lanes 6 and 10, respectively, in Fig. 1). Peripheral-blood cells from the patient's maternal grandmother also showed nonrandom inactivation (data not shown). This pattern of skewed X-chromosome inactivation does not appear to be the result of cytogenetic abnormalities because both the patient and her mother had a normal karyotype (data not shown).

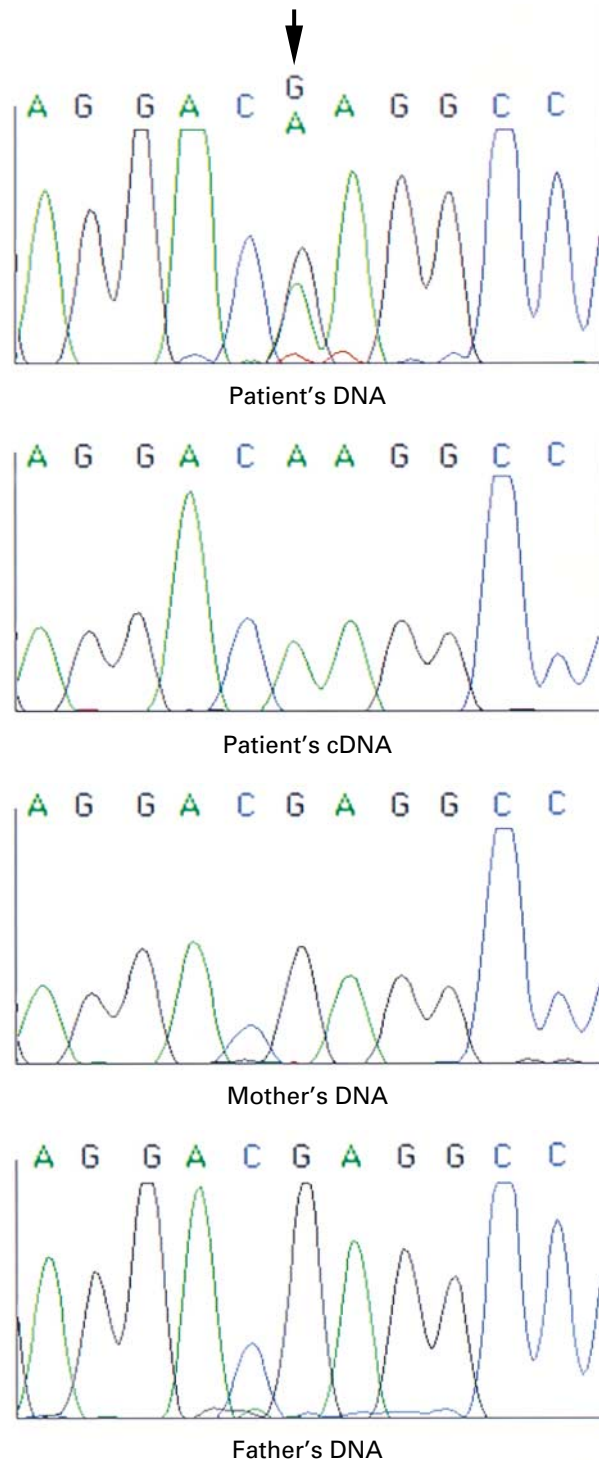
### Identification of a Missense Mutation in the *WASP* Gene

Direct sequencing of the amplified products of the 12 exons of the *WASP* gene and flanking splice sites revealed a normal sequence throughout the patient's gene except at position 431 in exon 4, at which signals for both guanine (G) and adenine (A) were obtained. This result indicates the presence of two alleles differing at this nucleotide (Fig. 2). When the PCR product of exon 4 was cloned and 14 clones were sequenced, 6 had the wild-type G and 8 had the mutant A.

Direct sequence analysis of *WASP* cDNA in our

**Figure 2.** Sequence Analysis of Exon 4 of the *WASP* Gene in the Patient and Her Parents.

Sequence analysis revealed a missense mutation in the patient's DNA (arrow) and cDNA at position 431 of the *WASP* gene, but not in DNA of her mother or father. The PCR-amplified products of exon 4 were sequenced directly, without subcloning, with the same primers used for PCR amplification. Automated fluorescence-based sequencing was performed, and the sequences are shown, with the corresponding DNA base indicated above. At the site of the mutation the sequence of genomic DNA of the patient reveals two signals — one for the wild-type guanine (G) and one for the mutant adenine (A) — indicating heterozygosity. In contrast, only the signal for the mutated adenine is detected in the patient's cDNA.



patient revealed only the abnormal allele (Fig. 2). This finding together with the observed pattern of skewed X-chromosome inactivation indicates that the mutation resides on the active X chromosome. Since DNA analysis of both of her parents revealed a normal sequence in exon 4 (Fig. 2), the mutation found in the patient must have occurred spontaneously.

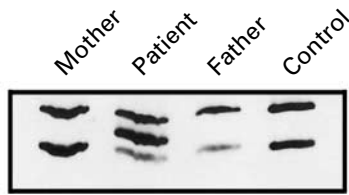
To obtain further independent confirmation of these results, we performed SSCP analysis. When exon 4 of the *WASP* gene was amplified from DNA of a normal subject, the patient, and her parents, an altered migration pattern, indicating the presence of a mutation, was observed only in the patient's DNA (Fig. 3).

Interestingly, although previous mutations identified in patients with the Wiskott–Aldrich syndrome are very heterogeneous and are present throughout the entire gene, the same change from G to A, causing the substitution of a lysine for the conserved wild-type glutamic acid at codon 133, has previously been described in three unrelated males with typical Wiskott–Aldrich syndrome.<sup>3,17</sup> This is probably due to the fact that the mutation occurs at a cytosine–guanine (CG) dinucleotide known to be a hot spot for new mutations.

### DISCUSSION

We describe a child with classic Wiskott–Aldrich syndrome, who had thrombocytopenia with small platelets, eczema in early life, clinical immunodeficiency, and a missense mutation in the *WASP* gene. This case is unique because the patient is a female, and this X-linked recessive disease normally affects males.

Early in embryogenesis, one of the two X chromosomes in all somatic cells of females is inactivated.<sup>18</sup> Although the X chromosome inactivated is picked at random, the same X chromosome is subsequently inactivated in all progeny of that cell. As a result, the normal woman is a mosaic: in some of her cells the paternally derived X chromosome is active, and in others the maternally derived X chromosome is active. If one of the two X chromosomes carries a defect



**Figure 3.** SSCP Analysis of Exon 4 of the *WASP* Gene in the Patient, Her Parents, and a Control Subject.

Genomic DNA was amplified, and after denaturation the PCR products of exon 4 of the *WASP* gene were separated on a non-denaturing polyacrylamide gel. Under these conditions, single strands of DNA migrate according to their conformation, which is dependent on the DNA sequence. The presence of an extra band in the sample from the patient indicates that the genomic DNA has both the normal allele and a mutated one with a different pattern of migration.

that impairs cell proliferation or survival, then by default, all the cells of that lineage will be derived from precursors with the nonmutant X chromosome as the active one. In carriers of the Wiskott–Aldrich syndrome, analysis of X-chromosome inactivation in hematopoietic cells shows that only the X chromosome that does not carry the defect is active.<sup>10</sup> Therefore, a female who is heterozygous for a mutation in the *WASP* gene can become symptomatic only if an additional event prevents the normal *WASP* allele from being active, as occurred in our patient.

Thus, this case appears to be the unfortunate result of two rare, independent genetic events. Clinical manifestations of other X-linked disorders, such as Hunter's syndrome,<sup>19</sup> hemophilia,<sup>20</sup> Duchenne's muscular dystrophy,<sup>21</sup> and the Lesch–Nyhan syndrome,<sup>22</sup> have been described in heterozygous female carriers with normal karyotypes and with a skewed pattern of X-chromosome inactivation. Together, these reports suggest that a skewed pattern of X-chromosome inactivation increases the risk of X-linked diseases in females.

The reason for the unbalanced pattern of X-chromosome inactivation in our patient is unclear. Recent evidence suggests that the inactivation process itself is genetically determined<sup>23,24</sup> and that it can be inherited as a mendelian trait.<sup>25</sup> In our patient, the possibility of inherited unbalanced inactivation is supported by the demonstration of a skewed pattern of X-chromosome inactivation in her mother as well as in her mother's mother. A leading candidate gene with respect to control of the process of X-chromosome inactivation is the X-inactivation-specific transcript (*XIST*) gene, which is expressed exclusively by the inactive X chromosome.<sup>23,24</sup> In mice, the inactivation process is also modified by the X-controlling element (*Xce*) locus.<sup>23,24</sup> Therefore, it is tempting to speculate that an alteration within one of these controlling elements may result in a skewed pattern of

inactivation. A mutation in the promoter region of the *XIST* gene causing nonrandom X-chromosome inactivation in a family has recently been described.<sup>26</sup>

The present report should alert clinicians to the possibility of recessive X-linked diseases in females. The pattern of X-chromosome inactivation can be identified by relatively simple and rapid techniques and may add valuable information in cases in which a female patient is suspected of having an X-linked recessive disorder.

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