Alterations of the X-linked lymphoproliferative disease gene \textit{SH2D1A} in common variable immunodeficiency syndrome

Massimo Morra, Olin Silander, Silvia Calpe, Michelle Choi, Hans Oettgen, Laurie Myers, Amos Etzioni, Rebecca Buckley, and Cox Terhorst

X-linked lymphoproliferative (XLP) disease is a primary immunodeficiency caused by a defect in the \textit{SH2D1A} gene. At least 3 major manifestations characterize its clinical presentation: fatal infectious mononucleosis (FIM), lymphomas, and immunoglobulin deficiencies. Common variable immunodeficiency (CVID) is a syndrome characterized by immunoglobulin deficiency leading to susceptibility to infection. In some patients with CVID, a defective \textit{btk} or \textit{CD40-L} gene has been found, but most often there is no clearly identified etiology. Here, 2 unrelated families in whom male members were affected by CVID were examined for a defect in the \textit{XLP} gene. In one family previously reported in the literature as having progressive immunoglobulin deficiencies, 3 brothers were examined for recurrent respiratory infections, whereas female family members showed only elevated serum immunoglobulin A levels. A grandson of one of the brothers died of a severe \textit{Aspergillus} infection secondary to progressive immunoglobulin deficiency, FIM, aplastic anemia, and B-cell lymphoma. In the second family, 2 brothers had B-lymphocytopenia and immunoglobulin deficiencies. X-linked agammaglobulinemia syndrome was excluded genetically, and they were classified as having CVID. The occurrence of FIM in a male cousin of the brothers led to the \textit{XLP} diagnosis. Because the \textit{SH2D1A} gene was found altered in both families, these findings indicate that \textit{XLP} must be considered when more than one male patient with CVID is encountered in the same family, and \textit{SH2D1A} must be analyzed in all male patients with CVID. Moreover, these data link defects in the \textit{SH2D1A} gene to abnormal B-lymphocyte development and to dysgammaglobulinemia in female members of families with \textit{XLP} disease. (Blood. 2001;98:1321-1325).

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found mutated in some CVID patients, whereas most patients remain without a clearly identified etiologic factor. Here, 10 males of 2 families in whom CVID had been previously diagnosed were found to have alterations in the XLP gene SH2D1A. Our results indicate that mutations in the SH2D1A gene must be studied in all male patients with CVID.

Materials and methods
Detection of mutations in the SH2D1A gene
Genomic polymerase chain reaction. Peripheral blood lymphocytes from families were collected in EDTA-containing test tubes. When lymphoblasts were available, they were grown in RPMI 1640 supplemented with 10% fetal bovine serum under standard culture conditions. DNA was isolated using standard techniques. Coding sequences, 5’ regulatory region (300 nucleotides from the transcription initiation site), and intronic splice-site sequences were amplified by PCR (GeneAmp/XL PCR kit; PerkinElmer, Branchburg, NJ). From each family, at least 2 affected members, 2 carriers, and 2 healthy members were analyzed for mutations in the SH2D1A gene.

PCR was performed in 50 μL with a Genecene PCR System 9700 (PE Applied Biosystems, Foster City, CA), under the following conditions: 94°C for 3 minutes, 94°C for 1 minute, 60°C for 1.5 minutes, 72°C for 1 minute for 35 cycles; 72°C for 10 minutes; 4°C (reo). Primer combinations used were: exon 1 F5’-GTC GCA TTC GGC ATG GCC ACA TAC GAA CAG-3’; exon 1 R5’-GCA GGA GCC CCA GCA GAT AAT GAA ATC CCC AGC-3’; exon 2 F5’-GGA AAG AAC TGT GTT GCA GAT ACA ATG TGG-3’; exon 2 R5’-GGA TAA ACA GCA CTG GGA CCA AAA TTC TC-3’; exon 3 F5’-GCT CCT CTT GCA GGA AAA TTC TTC CAA CC-3’; exon 3 R5’-GCT ACC TCT CAT TTG ACT TGC TGG CAT CTA C-3’; exon 4 F5’-GAG GAC GAC TCA GGC TCA GGC ATC ACA TCA TGC C-3’; exon 4 R5’-ATG TAC AAA AGA GCA GCA TTT CAG CTT TGA C-3’.

Genomic DNA from the Raji human cell line (American Type Culture Collection, Manassas, VA) was used as a positive control, and distilled water was used as a negative control. PCR products were visualized on a 1% agarose gel and subjected to direct sequencing procedure or to subcloning followed by sequencing (samples from female donors).

For direct nucleotide sequencing, PCR products were purified using Microcon-PCR centrifugal filters (Amicon-Millipore, Danvers, MA) and subcloning followed by sequencing (samples from female donors).

Reverse transcription–PCR. Total RNA was isolated from peripheral blood lymphocytes of patients, carriers, and healthy persons by TRIzol Reagent (BRL, Gaithersburg, MA). One microgram total RNA was reverse-transcribed using a one-step reverse transcription (RT)-PCR system (Access RT-PCR kit; Invitrogen). The primer combinations used were: F5’-GCC TGG CTG CAG TAG CAG CGG CAT CCC TC-3’ and R5’-ATG TAC AAA AGT CCA TTT CAG CTT TGA C-3’. Annealing temperature for both primer pairs was 60°C.

Measurement of serum immunoglobulin levels
Immunoglobulin isotypes G, A, and M were determined using standard laboratory procedures. Part of the data reported in Table 1 were obtained from Buckley and Sidbury. Measured values were considered normal, above average, or below average relative to the standards of the laboratory in which measurements were taken. Patient studies were conducted in accordance with the Helsinki protocol.

Results
Clinical and immunologic presentation of the patients
Family 1. Patient C.L. (Figure 1A) was born in 1996. After the first 6 months of life, the patient was examined for recurrent infections of the upper and lower respiratory tracts (bronchitis, pneumonia, and otitis media) and of the gastrointestinal tract. Determination of serum immunoglobulin levels indicated only elevated IgA at 6 months and slightly low IgM at 14 months (Table 1). Because of the family history of immunodeficiency, he did not receive live vaccines. At the age of 17 months, serum concentrations of IgG and IgA declined, and he had almost no detectable antibody titer against tetanus and diphtheria toxoids despite repeated immunizations. B- and T-cell numbers were normal. Lymphocyte proliferation tests at the age of 18 months indicated normal responses to phytohemagglutinin and concanavalin A, with a low response to pokeweed mitogen. At the age of 19 months, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, and γ-glutamyltransferase liver enzyme levels were elevated. Abdominal ultrasonography results were normal, and findings were negative for hepatitis A, B, and C, cytomegalovirus, and human immunodeficiency virus. Two months later, the patient was admitted to the hospital because of fever, pneumonia, a diffuse morbilliform rash, and an enlarged liver. Absolute neutropenia and thrombocytopenia then developed, and the patient was found to be EBV positive by PCR testing of his blood and cerebrospinal fluid. He was treated with acyclovir, granulocyte transfusions, and intravenous immunoglobulin (IVIG), but he died 5 weeks after admission because of an overwhelming Aspergillus infection.

Table 1. Immunoglobulin levels in members of family 1 and family 2A

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age (y)</th>
<th>IgG (mg/dL)</th>
<th>IgA (mg/dL)</th>
<th>IgM (mg/dL)</th>
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<td>06/12</td>
<td>391</td>
<td>74†</td>
<td>75</td>
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<td></td>
<td>12/12</td>
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<td>35</td>
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<tr>
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<td>37</td>
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<td>31</td>
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<td>17/12</td>
<td>248</td>
<td>11</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td>18/12</td>
<td>264</td>
<td>10</td>
<td>97</td>
</tr>
<tr>
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<td>66/12</td>
<td>760</td>
<td>690</td>
<td>114†</td>
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<tr>
<td></td>
<td>86/12</td>
<td>410</td>
<td>67</td>
<td>44</td>
</tr>
<tr>
<td></td>
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<td>10</td>
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<td>423</td>
<td>253</td>
<td>59</td>
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<td>290</td>
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<td>18</td>
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<td></td>
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<td>223</td>
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<td>33†</td>
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<td>1274</td>
<td>30†</td>
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<td>1000</td>
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</tr>
<tr>
<td></td>
<td>43</td>
<td>900</td>
<td>1000</td>
<td>39</td>
</tr>
<tr>
<td>C.A.* (father)</td>
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<td>450</td>
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<td>64</td>
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<td>A.C.</td>
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<td>120</td>
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<td>A.B.</td>
<td>010/12</td>
<td>90</td>
<td>0</td>
<td>4</td>
</tr>
</tbody>
</table>

† indicates value above normal for age; ††, value below normal for age. For normal ranges, see “Materials and methods.”

*Data reprinted with permission from Pediatric Research.

24 Measured values were considered normal, above average, or below average relative to the standards of the laboratory in which measurements were taken. Patient studies were conducted in accordance with the Helsinki protocol. Arbitrary initials were given to protect the patients’ identities.
Buckley and Sidbury, affected by a variety of large B-cell lymphoma involving peripancreatic lymph nodes only liver congestion, cholestasis, and peritoneal serous effusion; and thymopoiesis; acute and organizing splenic infarcts; centrilobular LMP and EBNA-2); severe thymic atrophy with no evidence of and moderate number of T cells) positive for the EBV antigens of thoracic and abdominal lymph nodes (necrosis with rare B cells bronchopneumonia; hypocellular bone marrow; lymphodepletion intestines, pericardium, and diaphragm); acute and organizing pergillosis (involving lungs, kidney, esophagus, large and small secondary to aplastic anemia. Autopsy showed disseminated aspergillosis (involving lungs, kidney, esophagus, large and small intestines, pericardium, and diaphragm); acute and organizing bronchopneumonia; hypocellular bone marrow; lymphodepletion of thoracic and abdominal lymph nodes (necrosis with rare B cells and moderate number of T cells) positive for the EBV antigens LMP and EBNA-2); severe thymic atrophy with no evidence of thymopoiesis; acute and organizing splenic infarcts; centrilobular liver congestion, cholestasis, and peritoneal serous effusion; and large B-cell lymphoma involving peripancreatic lymph nodes only (LMP and EBNA-2 positive).

C.L.’s family is a well-studied sibship, previously reported by Buckley and Sidbury (Figure 1A), affected by a variety of progressive immunoglobulin abnormalities in male and female members. C.L.’s grandfather (C.G.), together with his 2 brothers C.E. and C.F., were first seen at Duke University Medical Center in 1963 because all 3 were affected with frequent respiratory infections that were particularly severe in C.E. C.G. had only a late onset of mild infections. When he was first seen at the age of 6.5 years, C.G.’s findings were reported to be normal, but by the time he was 8.5 years of age, splenomegaly and a low lymphocyte count were noted. Immunologic studies over a period of 4 years showed a progressive deficiency of all 3 immunoglobulin isotypes (Table 1). He had a normal number of B cells initially, but this declined with progressive deficiency of all 3 immunoglobulin isotypes (Table 1). Nevertheless at age 10 months, pneumonia and hypogammaglobulinemia developed (Table 1). IVIG treatment was begun, and, like his brother, he is now doing well. A mutation in btk was ruled out (courtesy of Dr M. E. Conley), and, thus, CVID was diagnosed.

In 1999, their cousin B.C. (age 2 years) was admitted to the hospital with clinical signs and symptoms compatible with FIM. He had marked hepatosplenomegaly and rapid deterioration of liver function. Anti–viral caspid antigen (VCA) IgM was positive, and liver biopsy showed typical features of FIM. He was treated with high-dose methylprednisolone and VP-16, but, unfortunately, he died before bone marrow transplantation could be performed. Family history (Figure 1B, 2-B) revealed that 2 other brothers died of FIM at approximately the same age range. In one of them, EBV was detected by biopsy of the liver. His 2 sisters were healthy.

Analysis of the SH2D1A gene in the 2 families

Family 1. Clinical and autopsy findings of C.L. led us to consider the possibility that he and his ancestors could have had XLP. Genomic DNA of C.G. and C.L. was extracted from B lymphocytes immortalized with EBV virus. DNA sequencing results demonstrated marked polyclonal IgA hyperglobulinemia, selective unresponsiveness to blood group B substance injections, and poor responses to immunization with diphtheria and polio vaccines. Two maternal aunts (C.C. and C.D.) also had IgA hyperglobulinemia and low isohemagglutinin titers. The father of the 3 boys (C.A.) was healthy and had normal levels of serum immunoglobulins (Table 1). There was no history of conditions similar to those of the boys on either side of the family.

Family 2. A 2.5-year-old boy (A.C.) (Figure 1B, 2-A) was brought to the Rambam Medical Center (Haifa, Israel) in 1988 after several episodes of pneumonia and Escherichia coli sepsis starting when he was 1 year old. His B-lymphocyte count was very low (1%-2%), as were his serum IgG and IgM levels, and serum IgA was undetectable (Table 1). A presumptive diagnosis of XLA was made, and he was started on IVIG therapy. B cell levels rose to 7% to 8% over years. He had no major medical problems until the present; he is now 12 years of age. In 1993, his brother (A.B.) (Figure 1B, 2-A) was born and was found to have a normal number of B cells. Nevertheless at age 10 months, pneumonia and hypogammaglobulinemia developed (Table 1). IVIG treatment was begun, and, like his brother, he is now doing well. A mutation in btk was ruled out (courtesy of Dr M. E. Conley), and, thus, CVID was diagnosed.
alterations in the \textit{SH2D1A} gene. DNA sequencing (Figure 2B) indicated that the male family members B.B., B.C., A.B., and A.C. had an 8-base pair (bp) deletion located in the third exon (nucleotides 548 to 555). This alteration in the \textit{SH2D1A} gene was previously unreported. Curiously, the sequence deleted in these patients (GCATTTCAG) is repeated twice in the third exon, and this deletion is situated adjacent to an internal splice acceptor site located in the third exon. This low-frequency splicing acceptor site generates a physiologically shorter form of the \textit{SH2D1A} protein, named SAPA55, which is found in all healthy persons. Because of a shift in the reading frame, this deletion leads to a premature stop codon (at a position corresponding to residue 100). This premature stop codon results in a short, altered \textit{SH2D1A} protein of 99 amino acids (Y100X) (Figure 2C). The same \textit{SH2D1A} gene microdeletion was also found in the 2 mothers (B.A. and A.A.), identifying them as genetic carriers.

Another brother, an asymptomatic 11-month-old (B.D.) (Figure 1B, 2-B) tested positive for the same \textit{SH2D1A} gene deletion. In December 1999 the patient underwent bone marrow transplantation (BMT) from a completely matched donor (a 9-year-old sister). No complications occurred during or after BMT.

\textbf{Discussion}

CVID is a heterogeneous syndrome both clinically and immunologically.\textsuperscript{19,20} A precise clinical and laboratory definition of the disease has been difficult because of the heterogeneity in phenotypes. In a large study of 248 patients with CVID,\textsuperscript{20} 40% had impaired T-cell proliferation to mitogens. Based on B-lymphocyte responses to plate-bound \alpha-IgM, patients with CVID were divided into 4 subgroups.\textsuperscript{19} Numerous studies have attempted to establish diagnostic criteria for the disease and to determine molecular etiologies. Recently, guidelines for the evaluation of CVID have been published.\textsuperscript{26} A much stricter definition of the disease must now include the genetic exclusion of mutations in \textit{btk}, \textit{CD40-L}, \textit{AID}, and \textit{SH2D1A} genes.\textsuperscript{19,21,27}

Cellular immunologic alterations in patients with XLP are not well understood. T and B lymphocytes undergo sustained proliferation in XLP. Extensive tissue infiltration and multi-organ failure are the primary causes of death in these patients.\textsuperscript{6} The failure to eliminate EBV-transformed B cells in XLP does not seem to be caused by a defect in the B cell.\textsuperscript{28} \textit{SH2D1A} expression in B lymphocytes is probably limited only to certain subpopulations.\textsuperscript{12} Moreover, no major B-lymphocyte defects have been found in \textit{SH2D1A} null mice (C. Gullo, C. Terhorst, personal communication). On the contrary, variable defects in T cells and natural killer cells of patients with XLP have been reported. \textit{SH2D1A}-deficient natural killer cells are unable to lyse appropriate target cells.\textsuperscript{29-33} B-lymphocyte developmental abnormalities were detected in one member of the 2 families. Such a defect in B cells has been described in the past.\textsuperscript{34} Whether these B-lymphocyte abnormalities and abnormal immunoglobulin levels result from a \textit{SH2D1A} deficiency in B cells or from abnormal T–B lymphocyte interactions among SLAM-family members is unknown at this time. The \textit{SH2D1A}-interacting molecules SLAM and CD84 and the 2B4-ligand CD48 are highly expressed in B cells,\textsuperscript{35} and their expression increases after cell activation or EBV infection. In particular, SLAM has been demonstrated to play a role in B-lymphocyte proliferation and immunoglobulin synthesis after ligation by its soluble form (sSLAM).\textsuperscript{36} The complex network of interactions among \textit{SH2D1A}, \textit{EAT-2}, and their ligands SLAM, 2B4, CD84, and Ly-9 may account for the clinical variability of manifestations in XLP. Recent data (M.M. et al, manuscript submitted, 2001) indicate that EAT-2 is probably the \textit{SH2D1A}-like molecule functional in B lymphocytes. One could predict that mutations of EAT-2 might give rise to CVID.

Decreases in serum immunoglobulin levels with time in patients C.L., C.E., C.G., and C.F. (family 1) suggest that a cumulative effect of sequential environmental factors must play a strong role in determining the expression of the \textit{SH2D1A} mutations. Because SLAM has been recently identified as another receptor for the measles virus,\textsuperscript{37} a role for measles virus as a potential precipitant of disease expression in \textit{SH2D1A}-deficient patients can be presumed. Dysgammaglobulinemia complicated by disseminated measles has been described in the past.\textsuperscript{38,39}

Of particular interest is the fact that female members of family 1 had abnormal immunoglobulin levels. Female carriers of XLP have been reported to have abnormal antibody responses to EBV.\textsuperscript{40} In male patients with XLP, IgG and IgM serum levels are often low with elevated IgA and IgM classes.\textsuperscript{41} Therefore, in females with 1 of 2 altered \textit{SH2D1A} alleles, a modest reduction in \textit{SH2D1A} protein levels could result in mild laboratory alterations, such as the hyper-IgA reported in family 1. Decreased cellular levels of the \textit{SH2D1A} protein could lead to immunoglobulin dysregulation through alterations in the T–B lymphocyte network. Patients with XLP who have reduced \textit{SH2D1A} protein levels have been described. One patient had a critically reduced \textit{SH2D1A} wild-type
protein level because of a regulatory mutation in the 5′ splicing acceptor site of the second exon. Family 1 is of further interest because all 3 major phenotypes developed in C.L. in only few months, and he died before the age of 2 years. The other affected male relatives had hypogammaglobulinemia or hypogammaglobulinemia and malignant lymphoma and lived until 15 years and 38 years. This clinical variation in patients with the same SH2D1A mutation indicates that other host or environmental factors are important in determining disease expression. Environmental factors are not limited to EBV infection because XLP phenotypes may develop in its absence. Age may be a critical factor in determining disease severity—hypogammaglobulinemia developed in C.L. at 17 months of age, and he succumbed of FIM and aplastic anemia at 20 months of age.

In conclusion, the work reported here indicates the presence of SH2D1A mutations in patients diagnosed with CVID. Therefore, together with [btk], CD40-L, and AID genes, we suggest that SH2D1A must be included in the molecular diagnosis of CVID. Because of the high rate of new mutations occurring in other human X-linked immunodeficiencies, such as XLA, the SH2D1A gene should be studied in all male patients with CVID. Clinically polarized XLP presentations must be considered when patients with CVID are encountered. A similar conclusion could be drawn from data published elsewhere. This is particularly true when more than one male member of a family is affected. Besides allowing genetic counseling, a correct diagnosis of XLP will allow for the selection of more aggressive therapy (such as BMT) because the prognosis for XLP is much worse than for CVID syndrome in general.

References

21. Farrington M, Grosmaire L-S, Nonoyama S, et al. T cells with the same SH2D1A mutation indicates that other host or environmental factors are important in determining disease expression. Environmental factors are not limited to EBV infection because XLP phenotypes may develop in its absence. Age may be a critical factor in determining disease severity—hypogammaglobulinemia developed in C.L. at 17 months of age, and he succumbed of FIM and aplastic anemia at 20 months of age. In conclusion, the work reported here indicates the presence of SH2D1A mutations in patients diagnosed with CVID. Therefore, together with [btk], CD40-L, and AID genes, we suggest that SH2D1A must be included in the molecular diagnosis of CVID. Because of the high rate of new mutations occurring in other human X-linked immunodeficiencies, such as XLA, the SH2D1A gene should be studied in all male patients with CVID. Clinically polarized XLP presentations must be considered when patients with CVID are encountered. A similar conclusion could be drawn from data published elsewhere. This is particularly true when more than one male member of a family is affected. Besides allowing genetic counseling, a correct diagnosis of XLP will allow for the selection of more aggressive therapy (such as BMT) because the prognosis for XLP is much worse than for CVID syndrome in general.
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