J Clin Immunol (2015) 35:26–31
DOI 10.1007/s10875-014-0117-1

ASTUTE CLINICIAN REPORT

Severe XLP Phenotype Caused by a Novel Intronic Mutation in the *SH2D1A* Gene

B. Tóth · B. Soltész · E. Gyimesi · G. Csorba · Á. Veres · Á. Lányi · G. Kovács · L. Maródi · M. Erdős

Received: 23 September 2014 / Accepted: 27 November 2014 / Published online: 10 December 2014
© Springer Science+Business Media New York 2014

Abstract We describe here a novel c.137+5G > A intronic mutation in the *SH2D1A* gene of the signaling lymphocyte activation molecule (SLAM)-associated protein (SAP) in association with Epstein-Barr virus (EBV)-induced fatal infectious mononucleosis (FIM) in an 8-year-old male patient and his 3-year-old step brother. The mother and the maternal grandmother of the boys are healthy and heterozygous for this sequence variant. Genetic sequencing of blood-cell-derived cDNA in the younger patient revealed a 22 bp deletion in the *SH2D1A* cDNA. Immunoblot and flow cytometry analysis performed in this younger patient showed the lack of SAP protein expression in peripheral blood lymphocytes. These data suggest that the novel c.137+5G > A mutation results in loss of function of SAP protein and leads to typical X-linked lymphoproliferative disease phenotype. We propose that intron 1 and the c.137+5G may be the most frequent intronic hot spot for *SH2D1A* splicing mutation.

Shared last authorship L Maródi and M. Erdős

B. Tóth · B. Soltész · G. Csorba · L. Maródi · M. Erdős (✉)
Department of Infectious Diseases and Pediatric Immunology, Faculty of Medicine, University of Debrecen, Nagyerdei Krt. 98, 4012 Debrecen, Hungary
e-mail: melinda.erdos@yahoo.com

L. Maródi
e-mail: lmarodi@med.unideb.hu

E. Gyimesi
Department of Laboratory Medicine, Faculty of Medicine, University of Debrecen, Debrecen, Hungary

Á. Veres · Á. Lányi
Institute of Immunology, Faculty of Medicine, University of Debrecen, Debrecen, Hungary

G. Kovács
2nd Department of Pediatrics, Semmelweis University, Budapest, Hungary

Keywords XLP · *SH2D1A* · novel intronic mutation

Introduction

X-linked lymphoproliferative disease (XLP) (OMIM #611432) is a recessive primary immunodeficiency disorder (PID) characterized by aberrant immunoregulation [1, 2]. The clinical phenotype of XLP includes Epstein-Barr virus (EBV)-induced fatal infectious mononucleosis (FIM), hypogammaglobulinemia, vasculitis, and lymphoma localized typically to the ileocecal region of the gastrointestinal tract [3–5]. Intriguingly, patients with XLP are not particularly susceptible to infection by viruses other than EBV. Immunological studies of affected individuals have revealed that T lymphocytes and NK cells fail to mediate effective virus-specific cytotoxicity towards EBV-infected B cells resulting in overwhelming lymphoproliferation and macrophage activation [6, 7]. Hypogammaglobulinemia and lymphoma may develop before patients encounter EBV. Transitional $CD24^{+}CD38^{+}$ B cells are present in the blood of patients but these cells fails to develop normally into $CD27^{+}$ memory B cells [8–10]. In the majority of patients, XLP is caused by inactivating mutations in the *SH2D1A* gene encoding the signaling lymphocyte activation molecule (SLAM)-associated protein (SAP) [11, 12]. Mutations in *BIRC4*, the gene encoding X-linked inhibitor of apoptosis protein (XIAP) may also result in XLP [1, 13]. To date, more than 100 different sequence variant in *SH2D1A* including missense, nonsense, frameshift, deletion, insertion, and slice site mutations have been identified in XLP patients [1, 5, 11, 12]. The vast majority of mutations affect exonic sequences; one mutation in intron 3, five mutations in intron 2, and seven mutations in intron 1 have been reported (Fig. 1) [1, 10, 14–21].

We have identified a previously unknown c.137+5G > A intronic sequence variant in a male patient who died of FIM

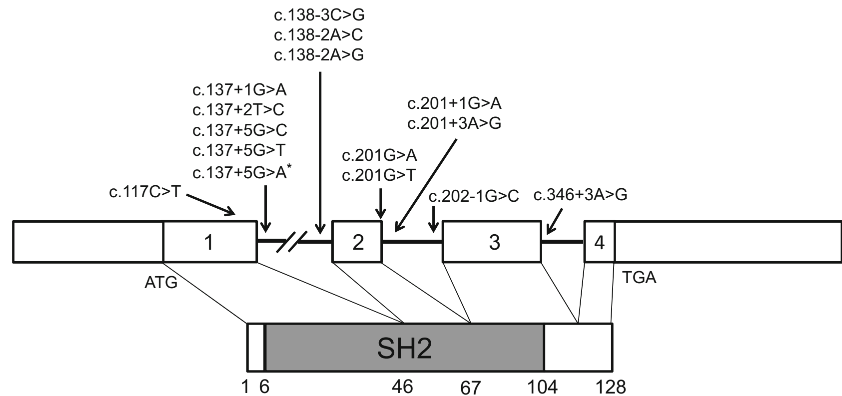

Fig. 1 Fifteen splicing mutations in the *SH2D1A* gene which have been reported before and the one published in this paper (*). Exons are indicated with arabic numbers in squares

(Patient 1). This mutation carried by his mother and maternal grandmother was also detected in his step brother (Patient 2). We have performed detailed genetic and molecular analyses of the novel mutation in Patient 2. We report here that the c.137+5G > A intronic single nucleotide change resulted in a 22 bp deletion in the *SH2D1A* cDNA sequence. We propose that intron 1 and the c.137+5G position may be the most frequent intronic hot spot for *SH2D1A* splicing mutation.

Materials and Methods

Patients

All studies reported here were approved by the Regional Ethics Committee of the University of Debrecen. Informed consent was obtained from the mother of the patients.

Patient P1 was born in 2000 to healthy Hungarian parents. He received routine immunization with no complications. He developed non-itching eczematous rush in early childhood over the wrists, elbows, and ankles which persisted for years and were treated with local agents. At age 6 he developed pain and limitation of movement in several joints including the knees, uncles, and wrists, as well as the proximal interphalangeal, distal interphalangeal and metacarpophalangeal joints. Despite the lack of swelling and warmth he was put on metothrexate and methylprednisolone after evaluation by a rheumatologist. Side effects developed soon and methylprednisolone treatment was stopped 2 months and metothrexate after 6 months of therapy. Joint pain and weakness recurred and histopathological examination of a soft tissue sample including skin, fascia and muscle tissues taken from the right thigh was performed which revealed vasculitis. At age 8 he presented with exudative tonsillopharyngitis and prolonged, high fever and was treated with amoxicillin, amoxicillin-clavulanic acid, and cefzil with no clinical improvement. Chest and sinus maxillaris X-ray showed no signs of sino-pulmonary infection. After 2 weeks of recalcitrant fever, liver enzymes started to elevate (GLT, 137 U/L; GOT, 101 U/L) and he developed thrombocytopenia (platelet number, 30 G/L), neutropenia (granulocyte number, 0.29 G/L) and anemia (hemoglobin, 97.2 g/L). His general condition and cardiorespiratory status rapidly deteriorated, he developed multiple organ failure and pancytopenia and bone marrow smears showed hemophagocytosis suggesting FIM. EBV DNA copy number in the plasma and the cerebrospinal fluid were 97,320/ml and 5830/ml, respectively; EBV gDNA in peripheral blood mononuclear cells (PBMCs) and bone marrow cells were 16.1 μg/g and 8.0 μg/g, respectively. He died 3 weeks after the onset of febrile disease. Sequence analysis of the *SH2D1A* gene revealed hemizygous c.137+5G > A intronic mutation for which the mother and maternal grandmother were both heterozygous.

Patient 2 was born in 2011 from uneventful pregnancy and the second marriage of the mother. He received immunization with no complication over the first 3 years of life and has had no remarkable clinical infection or other disease manifestation. Genetic analysis revealed that he was also hemizygous for the c.137+5G > A intronic mutation. Thus, we performed detailed molecular analysis to define if this intronic sequence variant might be pathological. This patient underwent successful hematopoietic stem cell transplantation by using matched-unrelated donor cells.

Methods

Isolation of Genomic DNA (gDNA) and RNA EDTA blood from patients, family members, and controls was obtained and gDNA was isolated by using Gen ELUTE Blood Genomic DNA mini kit (Sigma-Aldrich Ltd., Germany). Total RNA was extracted from peripheral blood leukocytes using Trizol reagent (Invitrogen Life Technology, Carlsbad, Calif.), and RNA was extracted following the manufacturer's instructions. The RNA was treated with RNase-free DNase (Thermo Scientific, Rockford, IL, USA).

Preparation of cDNA The RNA was reverse-transcribed with Superscript III first-strand synthesis supermix (Invitrogen). The prepared cDNA was used for the subsequent polymerase

chain rection. Primers were designed to amplify exons 1–4 of *SH2D1A*. The RT-PCR products were separated on 1 % agarose gels, and directly sequenced.

Targeted Gene Sequencing Primer sequences used to amplify exons 1–4 and the flanking intron regions of *SH2D1A* are available on request. Mutational analysis was performed by using the BigDye Terminator Cycle sequencing kit, and an ABI 3130 analyzer (Applied Biosystems, Foster City, CA, USA) [22]. The DNA mutation numbering was based on the cDNA sequence (Ensembl: ENST00000371139) and the first nucleotide is A in the translation initiation codon.

Real-time RT-PCR The RNA samples were reverse transcribed (RT) with the Superscript III first-strand synthesis supermix (Invitrogen) using random hexamer primers (Applied Biosystems, Darmstadt, Germany). Quantitative real-time PCR was performed with TaqMan assay (Hs00158978_m1: Applied Biosystems). All PCR reactions were performed in triplicates and specificity of PCR reaction was controlled by template and no RT controls. The comparative Ct method was used to quantify transcripts and to normalize for GAPDH.

Analysis of SAP Expression by Western Blotting (WB) PBMCs were isolated from anti-coagulated peripheral blood samples by Ficoll gradient centrifugation (Ficoll-Paque PLUS, GE Healthcare, Little Chalfont, UK). PBMCs were seeded on 6-well plates in 5 ml complete RPMI medium in 10 % FBS with Penicillin, 10,000 IU /mL, and Streptomycin,10,000 μg/mL (PAA Laboratories GmbH, Pasching, Austria) at 1×10^6 density and incubated in the presence or absence of 2000 ng/ml phytohaemagglutinin (Life Technologies, Carlsbad, CA, USA) for 72 h. Cells were subsequently lysed in 200 μl 2X Laemli buffer (62.5 mM Tris–HCl, pH 6.8, 25 % glycerol, 2 % SDS, 0.01 % Bromophenol Blue). Cell lysates were boiled for 5 min and clarified by centrifugation (16,000 g, 10 min at room temperature) and aliquots corresponding to 5×10^5 cells were run on 15 % SDS/PAGE gels followed by standard WB. The presence of SAP was detected by a SAP-specific affinity purified rabbit serum. The affinity purified rabbit anti-SAP antibody specific to C-terminal 25 amino acids of SAP has been described [11]. After addition of horseradish peroxidase-conjugated donkey anti-rabbit Ig antibody (GE Healthcare, Little Chalfont, United Kingdom, 1:5000 dilution), signals were visualized by enhanced chemiluminescence (ECL, Super Signal West Pico (Thermo scientific, Rockford, IL, USA). Expression of actin was detected in parallel in each sample by an actin-specific rabbit polyclonal antibody (Sigma St. Louis, MO, USA, 1: 2000 dilution), as a loading control [23].

Detection of Intracellular SAP Protein by Flow Cytometry One hundred microliters of whole blood was incubated with peridinin chlorophyll protein-conjugated mouse anti-CD3 (Becton Dickinson, San Jose, CA, USA), phycoerythrin-conjugated mouse anti-CD8 (Sigma Aldrich) and allophycocyanin-conjugated mouse anti-CD56 (Becton Dickinson) for the surface staining for 20 min at room temperature. The samples were fixed with Fix & Perm "A" (Invitrogen, Camarillo, CA, USA) for 20 min at room temperature in dark place [24]. After the washing step Fix & Perm "B" (Invitrogen) was added to the samples for permeabilization and the cells were incubated with purified rat immunoglobulin G1 (IgG1) isotypic control (Beckton

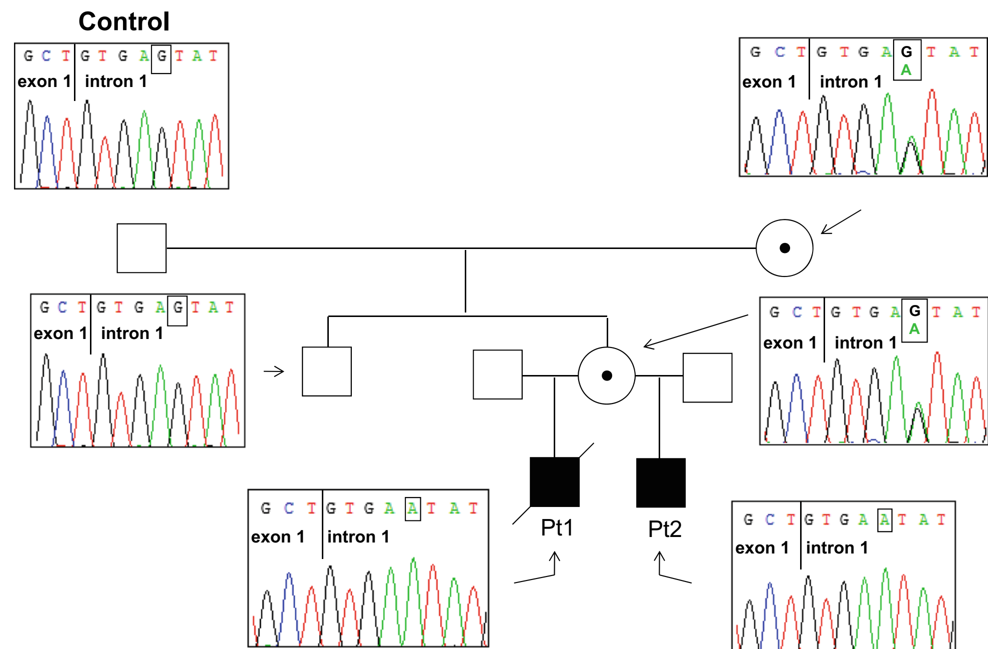

Fig. 2 The identification of the mutation c.137+5G > A mutation in intron 1 of *SH2D1A*. Direct sequencing of exon 1 and part of intron 1 demonstrated hemizygous (Patients; dark squares) and heterozygous (mother and grandmother) single base substitution of G > A at position c.137+5. Pt, Patient

Dickinson) or rat anti-SH2D1A unconjugated monoclonal antibody (clone: KST-3) for 20 min at room temperature. After washing, fluorescein isothiocyanate-conjugated goat anti-rat IgG1 antibody diluted in Fix & Perm "B" was added (Becton Dickinson) for 20 min at room temperature. After washing, the cells were resuspended in 1 % paraformaldehyde for the analysis by FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) [18].

Results and Discussion

Analysis of SH2D1A gDNA and cDNA by Using PCR Amplicons

Sequencing of the SH2D1A gene ofthe whole blood gDNA revealed a novel mutation in intron 1 (c.137+5G > A) in both patients and heterozygosity for this sequence variant in the mother and maternal grandmother (Fig. 2). The father of Patient 1 was wild-type. The father and grandfather of Patient 2 were not available for genetic testing. Complete cDNA amplification from blood leukocytes of Patient 2 demonstrated a smaller band (352 bp) compared with that of the mother and healthy control (374 bp) (Fig. 3a). Sequencing of the cDNA demonstrated a 22 bp deletion (116_137del22bp) in exon 1 of Patient 2 (Fig. 3b). This deletion could not be detected in cDNA isolated from a normal control and from the mother (Fig. 3c and d). The c.137+5G > A nucleotide change may result in altered donor splice site, deletion of cDNA, change of cDNA sequence, and as a consequence a truncated SAP protein. Therefore, the amino acid sequence of the protein between positions 39 and 72 couldalso be altered. Real-time-PCR analysis showed a complete lack of SH2D1A cDNA expression in Patient 2 and decreased expression in the mother (Fig. 4).

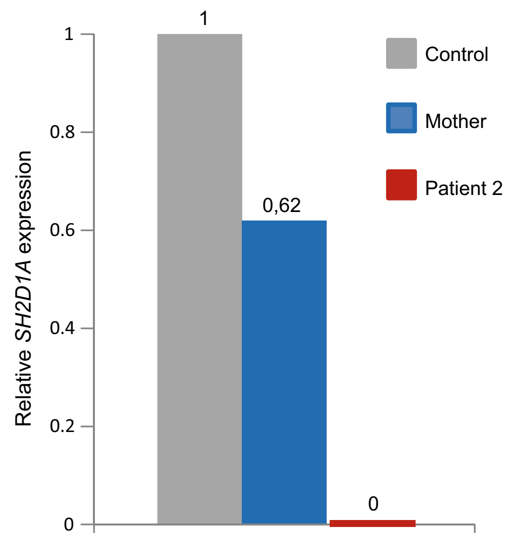

Fig. 4 Real-time PCR analysis showed a complete lack of *SH2D1A* cDNA expression in white blood cells from Patient 2 and intermediate expression in cells from the mother

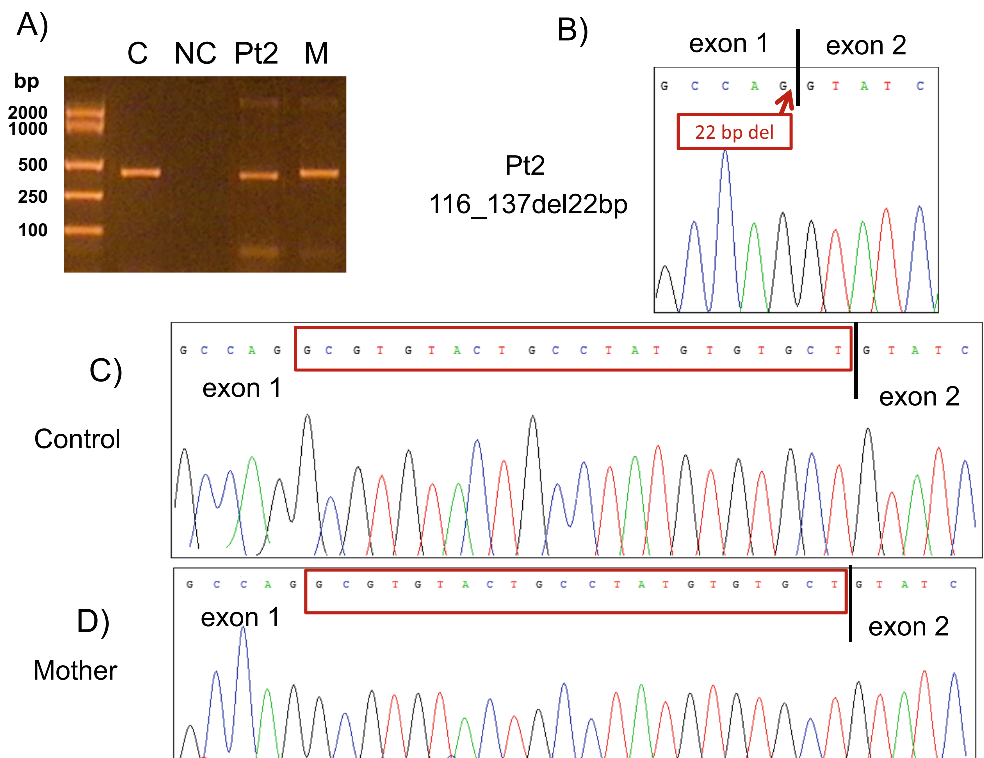

Fig. 3 Identification of a 22 bp deletion in exon 1 of cDNA from Patient 2. **a**) cDNA amplicons of Patient 2 and his mother. **b–d**) Indication of the 22 bp deletion in Patient 2 in exon 1. *C* healthy control, *NC* negative control

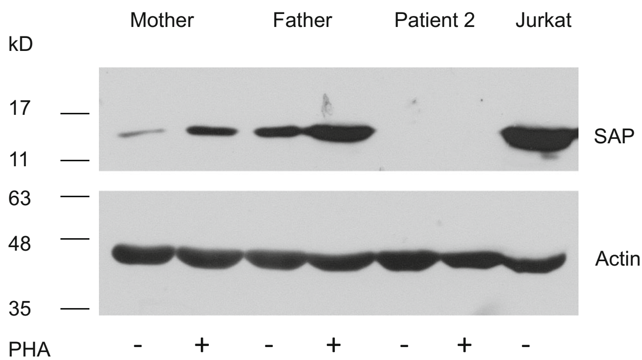

Fig. 5 The c.137+5G > A intronic mutation abrogates expression of SAP in phytohemagglutinin (*PHA*)-induced blasts from Patient 2. Peripheral blood mononuclear cells isolated from Patient 2, his mother and father were activated with 2000 ng/mL PHA. Expression of SAP was tested by Western blot analysis of whole cell lysates corresponding to 5×10^5 cells. Actin expression was detected as an internal loading control. Jurkat cells were used as positive controls

Analysis of SAP Expression by Western Blotting and Flow Cytometry

Figure 5 shows the immunoblots of SAP expression in PHA-induced blasts of Patient 2 and the heterozygous mother. In concert with the clinical, immunological and genetic data, SAP protein could not be detected with Western blot in the patient's cells in contrast to that seen in the father. Protein expression in the blast cells from the mother was decreased as indicated by the thickness of the bands (Fig. 5). Flow cytometry analysis of CD3+CD8+CD56- (CD8), CD3-CD56+ (NK) and CD3+CD56+ (CD56+ T) cells revealed that SAP expression in Patient 2 was virtually abrogated compared to the expression of this protein in these cells from healthy control (Fig. 6).

The novel intronic mutation we presented here is predicted to result in splicing defect leading to reduction of SAP level in T cells. Based on molecular analysis performed in this study we suggest that the c. 137+5G > A mutation is disease-causing and may result in severe XLP phenotype. Data presented in this report further support the concept the deep intronic mutation in the *SH2D1A* gene may lead to XLP. In a recent report an intronic c. 137+5G > C mutation was found to associate with normal length and sequence of mRNA and low level of SAP protein expression detected by Western blot [10]. In contrast, we present here a 22 bp deletion in the *SH2D1A* mRNA, and a complete lack of SAP protein expression.

Intronic mutations are mostly localized in consensus donor or acceptor splicing sites. easily detected by the analysis of exon-intron boundaries of genomic DNA, however the mutation deeper in the introns may not be considered pathologic unless cDNA and protein analysis is applied. The identification of c. 137+5G replacements may reflect the existence of

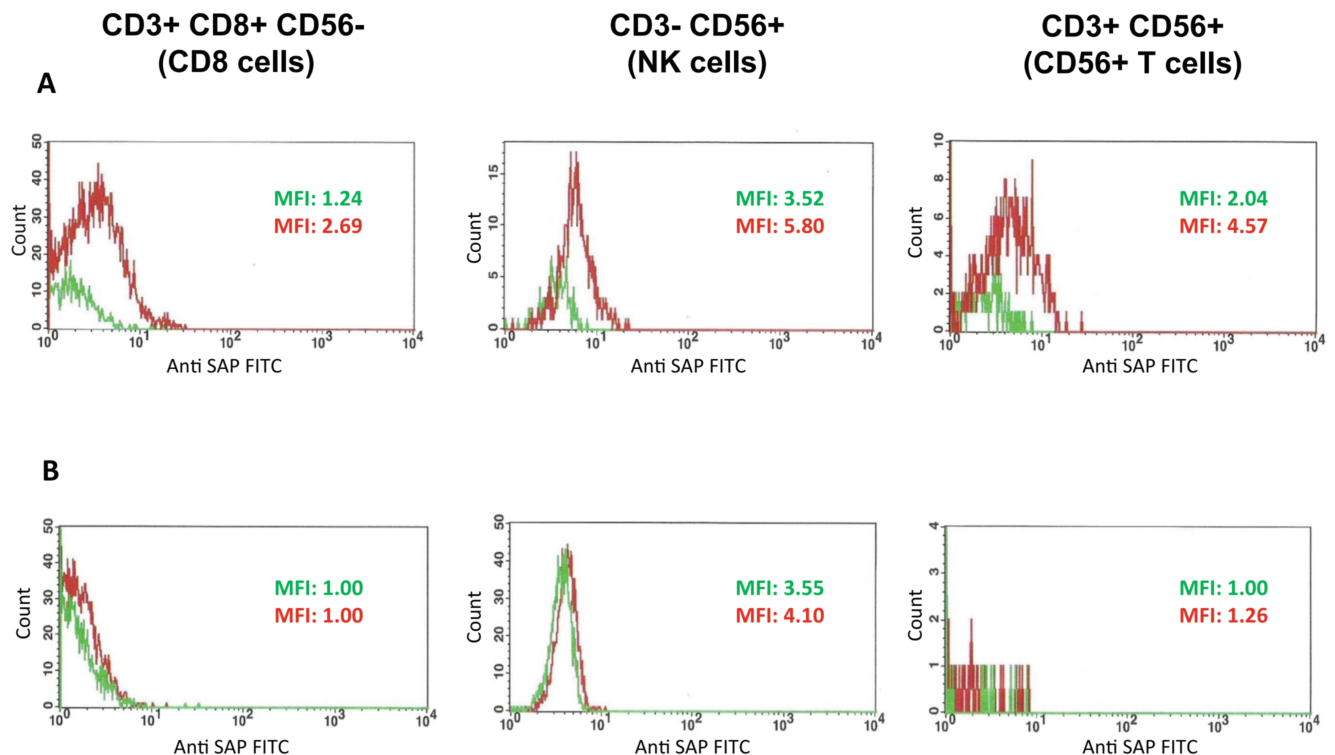

Fig. 6 Intracellular SAP expression in Patient 2 with XLP. Analysis of intracellular SAP expression by flow cytometry in cells from healthy controls (**a**) and Patient 2 (**b**). Lymphocytes were gated with anti-SAP antibody (*red*) or IgG1 isotype control (*green*) and with FITC-conjugated anti-rat IgG1 antibody. *MFI* median fluorescence intensity

mutational hot spot in *SH2D1A*. In the *SH2D1A* gene, 15 splicing mutation were known, including three exonic and 12 intronic sequence variant have been reported including the one published here. Eight out of the 12 intronic mutations affect intron 1 of *SH2D1A*. Based on published data it is possible that this intronic mutation may be a recurrent hot spot in different ethnics groups. Thus screening for cDNA 137+5G mutation may provide early molecular diagnosis of XLP.

In conclusion, our results indicate that analysis of cDNA isolated from blood leukocytes or blast cells to detect intronic mutation must be considered for XLP patients who have undetectable *SH2D1A* mutation in exonic sequences.

Acknowledgments We thank J. Sümegi, R. Marsch and G. Kriván for help. This work was supported by the TÁMOP 4.2.2.A-11/1/KONV-2012-0023 “VÉD-ELEM” project grant to LM.

Conflicts of interest The authors have no competing financial interests to declare.

References

1. Filipovich AH, Zhang K, Snow AL, Marsh RA. X-linked lymphoproliferative syndromes: brothers or distant cousins? Blood. 2010;116:3398–408.
2. Dupre L, Andolfi G, Tangye SG, Clementi R, Locatelli F, Arico M, et al. SAP controls the cytolytic activity of CD8 T cells against EBV-infected cells. Blood. 2005;105:4383–9.
3. Purtilo DT, Cassel CK, Yang JP, Harper R. X-linked recessive progressive combined variable immunodeficiency (Duncan’s disease). Lancet. 1975;7913:935–40.
4. Seemayer TA, Gross TG, Egeler RM, Pirruccello SJ, Davis JR, Kelly CM, et al. X-linked lymphoproliferative disease: twenty-five years after the discovery. Pediatr Res. 1995;38:471–8.
5. Sümegi J, Seemayer TA, Huang D, Davis JR, Morra M, Gross TG, et al. A spectrum of mutations in SH2D1A that causes X-linked lymphoproliferative disease and other Epstein-Barr virus-associated illnesses. Leuk Lymphoma. 2002;43:1189–201.
6. Ma CS, Hare NJ, Nichols KE, Dupré L, Andolfi G, Roncarolo MG, et al. Impaired humoral immunity in X-linked lymphoproliferative disease is associated with defective IL-10 production by CD4+ T cells. J Clin Invest. 2005;115:1049–59.
7. Parolini S, Bottino C, Falco M, Augugliaro R, Giliani S, Franceschini R, et al. X-linked lymphoproliferative disease. 2B4 molecules displaying inhibitory rather than activating function are responsible for the inability of natural killer cells to kill Epstein-Barr virus-infected cells. J Exp Med. 2000;192:337–46.
8. Cuss AK, Avery DT, Cannons JL, Yu LJ, Nichols KE, Shaw PJ, et al. Expansion of functionally immature transitional B cells is associated with human-immunodeficient states characterized by impaired humoral immunity. J Immunol. 2006;176:1506–16.
9. Chaganti S, Ma CS, Bell AI, Croom-Carter D, Hislop AD, Tangye SG, et al. Epstein-Barr virus persistence in the absence of conventional memory B cells: IgM+IgD+CD27+ B cells harbor the virus in X-linked lymphoproliferative disease patients. Blood. 2008;112:672–9.
10. Recher M, Fried AJ, Massaad MJ, Kim HY, Rizzini M, Frugoni F, et al. Intronic SH2D1A mutation with impaired SAP expression and agammaglobulinemia. Clin Immunol. 2012;146:84–9.
11. Latour S, Veillette A. Molecular and immunological basis of X-linked lymphoproliferative disease. Immunol Rev. 2003;192:212–24.
12. Nichols KE, Harkin DP, Levitz S, Krainer M, Kolquist KA, Genovese C, et al. Inactivating mutations in an SH2 domain-encoding gene in X-linked lymphoproliferative syndrome. Proc Natl Acad Sci U S A. 1998;95:13765–70.
13. Pachlopnik Schmid J, Canioni D, Moshous D, Touzot F, Mahlaoui N, Hauck F, et al. Clinical similarities and differences of patients with X-linked lymphoproliferative syndrome type 1 (XLP-1/SAP deficiency) versus type 2 (XLP-2/XIAP deficiency). Blood. 2011;117:1522–9.
14. Sumazaki R, Kanegane H, Osaki M, Fukushima T, Tsuchida M, Matsukura H, et al. SH2D1A mutations in Japanese males with severe Epstein-Barr virus–associated illnesses. Blood. 2001;98:1268–70.
15. Brandau O, Schuster V, Weiss M, Hellebrand H, Fink FM, Kreczy A, et al. Epstein-Barr virus-negative boys with non-Hodgkin lymphoma are mutated in the SH2D1A gene, as are patients with X-linked lymphoproliferative disease (XLP). Hum Mol Genet. 1999;8:2407–13.
16. Sümegi J, Huang D, Lányi A, Davis JD, Seemayer TA, Maeda A, et al. Correlation of mutations of the SH2D1A gene and epstein-barr virus infection with clinical phenotype and outcome in X-linked lymphoproliferative disease. Blood. 2000;96:3118–25.
17. Sayos J, Wu C, Morra M, Wang N, Zhang X, Allen D, et al. The X-linked lymphoproliferative-disease gene product SAP regulates signals induced through the co-receptor SLAM. Nat. 1998;395:462–9.
18. Tabata Y, Villanueva J, Lee SM, Zhang K, Kanegane H, Miyawaki T, et al. Rapid detection of intracellular SH2D1A protein in cytotoxic lymphocytes from patients with X-linked lymphoproliferative disease and their family members. Blood. 2005;105:3066–71.
19. Debeljak M, Podkrajsek KT, Aplenc R, Jazbec J. X-linked lymphoproliferative disease with a novel SH2D1A gene mutation. Pediatr Blood Cancer. 2008;50:187.
20. Coffey AJ, Brooksbank RA, Brandau O, Oohashi T, Howell GR, Bye JM, et al. Host response to EBV infection in X-linked lymphoproliferative disease results from mutations in an SH2-domain encoding gene. Nat Genet. 1998;20:129–35.
21. Yin L, Ferrand V, Lavoué MF, Hayoz D, Philippe N, Souillet G, et al. SH2D1A mutation analysis for diagnosis of XLP in typical and atypical patients. Hum Genet. 1999;105:501–5.
22. Erdős M, Uzvölgyi E, Nemes Z, Török O, Rákóczi E, Went-Sümegi N, et al. Characterization of a new disease-causing mutation of SH2D1A in a family with X-linked lymphoproliferative disease. Hum Mutat. 2005;25:506.
23. Nagy N, Cerboni C, Mattsson K, Maeda A, Gogolak P, Sümegi J, et al. SH2D1A and SLAM protein expression in human lymphocytes and derived cell lines. Int J Cancer. 2000;88:439–47.
24. Soltész B, Tóth B, Shabashova N, Bondarenko A, Okada S, Cypowyj S, et al. New and recurrent gain-of-function STAT1 mutations in patients with chronic mucocutaneous candidiasis from Eastern and Central Europe. J Med Genet. 2013;50:567–78.