Published in final edited form as:

J Clin Immunol. 2017 October ; 37(7): 707–714. doi:10.1007/s10875-017-0431-5.

Detection of Sp110 by Flow Cytometry and Application to Screening Patients for Veno-occlusive Disease with Immunodeficiency

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Electronic supplementary material The online version of this article (doi:10.1007/s10875-017-0431-5) contains supplementary material, which is available to authorized users.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Abstract

Mutations in *Sp110* are the underlying cause of veno-occlusive disease with immunodeficiency (VODI), a combined immunodeficiency that is difficult to treat and often fatal. Because early treatment is critically important for patients with VODI, broadly usable diagnostic tools are needed to detect *Sp110* protein deficiency. Several factors make establishing the diagnosis of VODI challenging: (1) Current screening strategies to identify severe combined immunodeficiency are based on measuring T cell receptor excision circles (TREC). This approach will fail to identify VODI patients because the disease is not associated with severe T cell lymphopenia at birth; (2) the *SP110* gene contains 17 exons, making it a challenge for Sanger sequencing. The recently developed next-generation sequencing (NGS) platforms that can rapidly determine the sequence of all 17 exons are available in only a few laboratories; (3) there is no standard functional assay to test for the effects of novel mutations in *Sp110*; and (4) it has been difficult to use flow cytometry to identify patients who lack *Sp110* because of the low level of *Sp110* protein in peripheral blood lymphocytes. We report here a novel flow cytometric assay that is easily performed in diagnostic laboratories and might thus become a standard assay for the evaluation of patients who may have VODI. In addition, the assay will facilitate investigations directed at understanding the function of *Sp110*.

Keywords

Sp110; Venous-occlusive disease with immunodeficiency; VODI; Flow cytometry; Combined immunodeficiency; Newborn screening; *Pneumocystis*; Primary immunodeficiency

Introduction

Veno-occlusive disease with immunodeficiency (VODI), first described by Mellis and Bale in 1976, is an autosomal recessive severe form of combined primary immunodeficiency (PID) caused by mutations in the gene encoding *Sp110* [1–4]. Patients with VODI often die in the first year of life due to either hepatic failure or fulminant infections [3]. Hematopoietic stem cell transplantation may cure the disease [5], but high transplant-related mortality has been reported, partly because chemotherapy (conditioning) may exacerbate hepatic veno-occlusion.

Because early treatment is critically important for patients with VODI, broadly usable diagnostic tools are needed to detect *Sp110* protein deficiency. Several factors make establishing the diagnosis of VODI challenging: (1) Current screening strategies to identify severe PID are based on measuring T cell receptor excision circles (TREC). This approach will fail to identify VODI which is not associated with severe T cell lymphopenia; (2) although the founder genetic mutation in the Lebanese patient population occurs in exon 5, disease-causing mutations in other exons have been reported [2, 6]. The *SP110* gene contains 17 exons, making it a challenge for Sanger sequencing. The recently developed next-generation sequencing platforms that can rapidly determine the sequence of all 17 exons are available in very few laboratories only; (3) there is no standard functional assay to test the effect of novel *Sp110* mutations; and (4) it has been difficult to use flow cytometry

to identify VODI patients, partly because of the low level of Sp110 protein in peripheral blood lymphocytes.

The ability to quantify the level of SP110 may have applications beyond the detection of patients with VODI. Polymorphisms in *SP110* are associated with increased susceptibility to tuberculosis in humans [7] and increased risk of canine degenerative myelopathy. This latter disease has clinical features that are similar to human amyotrophic lateral sclerosis [8]. Thus, quantifying the level of Sp110 will also assist in exploring the role of Sp110 protein dosage in the pathogenesis of these diseases.

In this study, we have established and validated a novel flow cytometric assay to quantify Sp110 in immune cells.

Methods

Patients

Blood was obtained from patients and controls upon informed consent, according to protocol 04-09-113R, approved by Boston Children's Hospital Institutional Review Board.

Patient characteristics including mutations, clinical complications, and treatment are summarized in Table 1. Patient-derived peripheral blood mononuclear cells (PBMC) were obtained after informed consent following approval by the local institutional review board. PBMC-derived T cell blasts were expanded in 96-well plates (Axonlab) with initial stimulation using PHA (5 µg/ml, Sigma Aldrich) and human IL-2 (200 units/ml, Novartis). Fresh IL-2 was added to the culture every 5–7 days.

Western Blot

The preparation of adenovirus vectors encoding Sp110 and Sp140 has been previously described [9]. Recombinant adenovirus encoding green fluorescent protein (GFP) was purchased from Vector Biolabs (Malvern, PA). Rabbit anti-Sp110 antiserum was obtained from Proteintech (Rosemont, IL), and rabbit antiserum directed against Sp140 has been previously described [10]. Extracts were prepared from adenovirus-infected HEp-2 cells by boiling the cells in 1× Laemmli sample buffer. Proteins were fractionated by electrophoresis using 8% Bis-Tris polyacrylamide gels (NuPage, Invitrogen, Grand Island, NY) and transferred to polyvinylidene fluoride membranes. The membranes were incubated in blocking solution (Odyssey Blocking Buffer (TBS), LI-COR, Lincoln, NE) for 1 h at room temperature, followed by rabbit antibodies directed against Sp110 or Sp140 in blocking solution. After washing, the membranes were incubated with IRDye 800CW donkey anti-rabbit IgG (LICOR, Lincoln, NE), and images were captured using LICOR Odyssey detection system. Wild-type Jurkat T cells and Sp110-deficient Jurkat cells (Sp110 Jurkat) were tested for Sp110 expression by western blot using a mouse monoclonal IgG2 anti-Sp110 (clone: JR-16, Santa Cruz).

Immunofluorescence

For immunofluorescent staining, HEp-2 cells were grown and infected in eight-well Lab-Tek II chamber slides (Nunc Inc., Naperville, IL), fixed in 2% paraformaldehyde in PBS at room

temperature for 10 min, and permeabilized by treatment with ice cold, 100% methanol for 7 min. Cells then were incubated with rabbit anti-Sp110 (Proteintech) or anti-Sp140 antiserum each diluted 1:200 in PBS. To permit detection of PML-Sp100 nuclear bodies, previously described human serum containing anti-Sp100 antibodies, but lacking anti-Sp140 and anti-Sp110 antibodies [9], was co-incubated with rabbit antisera. Unbound antibodies were removed by three successive washes with PBS. Bound primary antibodies were detected with Alexa Fluor 488-conjugated donkey anti-rabbit IgG and Alexa Fluor 610-conjugated donkey anti-human IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) [9].

Flow Cytometry

PBMCs were fixed with Fix/Perm Buffer (BD Biosciences) for 20 min at room temperature and permeabilized with Perm III Buffer (BD Biosciences, San Jose, CA, USA) for 30 min at 4 °C. Primary, polyclonal anti-Sp110 antibody (Proteintech Group Inc., Rosemont, IL, USA) was added at a dilution of 1:100 in FACS Buffer (PBS, 2% FCS) for 45 min at room temperature. Secondary Alexa647-conjugated polyclonal goat anti-rabbit antibody (Cat #: 111-606-047, Jackson ImmunoResearch) was added subsequently after dilution (1:1000) in FACS Buffer for 30 min at RT. Rabbit IgG was used as a nonspecific staining control (clone: DA1E, Cell Signaling). To analyze Sp110 expression specifically in T cells, Alexa488-conjugated anti-human CD3 (clone: UCHT1, Biolegend) was added. SP110 fluorescence was measured with an Accuri 2-laser-cytometer (BD Biosciences, San Jose, CA, USA), and data were analyzed with FlowJo software (FlowJo LLC, Ashland, OR, USA). In an alternative protocol, primary and secondary antibodies were diluted in Perm Buffer (BD Biosciences) to stain cells that were fixed with Fix/Perm Buffer (BD Biosciences).

siRNA Knock down

For transient knockdown of *SP110*, 2×10^6 Jurkat cells/condition were transfected with 500 nM SP110-specific small interfering RNA (siRNA) (Qiagen) or nonspecific control siRNA using an AMAXA Nucleofection Kit V (Lonza Group Ltd., Basel, Switzerland) and default program X-001. Cells were incubated in 2 ml fresh, prewarmed RPMI medium for 24 h and subsequently analyzed for Sp110 expression as described previously.

Sp110 Over-expression

A plasmid encoding Sp110 and a P2A-linked GFP (Addgene, Cambridge, MA, USA) was used to reconstitute the protein in SP110-deficient primary T cells from a VODI patient or in Sp110 Jurkat T cells. AMAXA Nucleofection Kit V (Lonza Group Ltd., Basel, Switzerland) and default program X-001 were used to transfect DNA into primary cells (5×10^6 cells) or Sp110 Jurkat T cells (2×10^6 cells). In some experiments, Sp110 Jurkat T cells were transfected with a plasmid encoding mutant Sp110 (p.Ile27Leu) [4]. Twenty-four hours after transfection, cells were flow-sorted for GFP-positive and GFP-negative cells and analyzed for Sp110 expression by flow cytometry (described above) or real-time PCR.

Production of a Sp110-Knockout Jurkat T Cell Clone (Sp110 Jurkat)

For deleting Sp110 in Jurkat T cells, 2×10^6 cells/condition were transfected with an individually designed CRISPR/Cas9 plasmid (Santa Cruz Biotechnology Inc., Dallas, TX,

USA), encoding for a specific guide RNA, the Cas9 nuclease, and a P2A-linked GFP. After 24 h, cells were sorted based on GFP fluorescence and subsequently seeded into 96-well plates via the single cell sort function of an Aria 5-laser sorting cytometer (BD Biosciences, San Jose, CA, USA). Cells were then expanded by incubation in tissue culture medium for 2–3 weeks. Messenger RNA was isolated from individual clones and incubated with reverse transcriptase and oligo(dT) primers to prepare complementary DNA (cDNA). *Sp110* cDNA of individual clones was then amplified by PCR and sequenced by Sanger sequencing. Sequencing was used to identify a Jurkat T cell clone that contained a frameshift insertion within *Sp110*, and these cells were tested by flow cytometry for expression of Sp110 and were subsequently used for Sp110 reconstitution experiments.

Results

A commercially available rabbit polyclonal anti-Sp110 antibody (see “Methods”) was evaluated for its ability to detect Sp110. To confirm that the polyclonal antibody indeed reacts with Sp110, we used an adenovirus vector (Ad.Sp110) to express Sp110 in HEp-2 cells, which normally contain very low levels of this protein (Fig. 1a). Using immunoblot assays, the antibody reacted with Sp110 contained in a lysate prepared from Ad.Sp110-infected—but not from control—HEp-2 cells (Fig. 1a). To show that the anti-Sp110 antibody did not cross-react with Sp140, a protein with regions of homology with Sp110, HEp-2 cells were infected with Ad.Sp140 as a control. Sp140 was detected by immunoblot using rabbit anti-Sp140 antiserum, but not the rabbit anti-Sp110 antiserum (Fig. 1a). When tested by indirect immunofluorescence, Sp110 was detected in a finely speckled nuclear pattern in HEp-2 cells infected with Ad.Sp110, but not in control HEp-2 cells (Fig. 1b). When co-expressed with Sp140, Sp110 was detected in nuclear bodies (Fig. 1b), as previously reported. In cells infected with Ad.Sp140 alone, the anti-Sp110 antibody did not stain nuclear bodies (Fig. 1b).

To determine whether the rabbit anti-Sp110 antiserum could detect Sp110 in lymphocytes by flow cytometry, the antiserum was used to stain T cell blasts from (i) a healthy individual, (ii) two VODI patients, and (iii) the parent of a VODI patient carrying a heterozygous *SP110* mutation (patient characteristics, Table 1). The mean Sp110 fluorescence intensity (MFI) of T cell blasts from the two VODI patients was lower than that of T cell blasts from the healthy control (Fig. 1c). The intermediate Sp110 MFI from T cell blasts derived from the parent was in keeping with the heterozygous carrier state (Fig. 1c and Supplementary Fig. 5). There was no difference in CD3 expression in all T cell blasts tested (Fig. 1c). A nonspecific rabbit IgG antibody showed similar fluorescence in all samples tested (Fig. 1c). To confirm that the rabbit anti-Sp110 antiserum could detect the presence of varying amounts of Sp110 by flow cytometry, we next decreased Sp110 expression in Jurkat T cells using siRNA-mediated knockdown. Twenty-four hours after treatment with Sp110-specific siRNA, Sp110 messenger RNA (mRNA) expression was reduced to 50% of normal (Supplemental Fig. 1). This led to a decrease in the Sp110 flow cytometric signal, compared to control Jurkat T cells treated with nonspecific siRNA (Fig. 1d). The level of CD3 expression was unaffected by Sp110 siRNA knockdown (data not shown). These results demonstrate that flow cytometry can be used to measure the level of Sp110 in lymphocytes. Of note, flow cytometry was sufficiently sensitive to detect a decrease in Sp110 protein

associated with a 50% decrease in mRNA which mimics the condition in individuals who carry a heterozygous mutation in this gene. To further confirm the specificity of the Sp110 antiserum, we generated a Jurkat T cell clone that contained targeted loss-of-function mutations in Sp110 using the CRISPR/Cas9 system [11] (Sp110-Jurkat; the altered *SP110* sequence of the targeted Jurkat clone is depicted in Supplemental Fig. 2). The Sp110-Jurkat T cell clone lacked Sp110 protein as assessed by immunoblot (Supplemental Fig. 3). When compared to control Jurkat T cells, the Sp110-Jurkat T cell clone had lower flow cytometric signal, confirming the ability of the polyclonal anti-Sp110 antibody to specifically differentiate presence vs. absence of Sp110 by flow cytometry (Fig. 1e). This was independent of the method used to permeabilize cells (Supplemental Fig. 4).

We used an eukaryotic expression plasmid to reconstitute Sp110 in Sp110-Jurkat T cell clones (Fig. 2a), as well as in Sp110-deficient T cell blasts derived from a VODI patient (Fig. 2b). Sp110 reconstitution was associated with a specific increase in the Sp110 flow cytometric signal (Fig. 2a, b). To confirm that Sp110 antiserum and flow cytometric analysis of peripheral blood-derived immune cells can be used to identify patients with VODI, we obtained blood samples from three healthy controls, a Sp110-deficient VODI patient, and a heterozygous carrier (patient characteristics, Table 1). The samples were shipped to the laboratory at ambient temperature in a process that required 48 h. Of the five samples, CD3⁺ T cells from the VODI patient had the lowest Sp110 signal. There was no difference in mean fluorescence between the five samples when the cells were stained with a control, nonspecific rabbit IgG (Fig. 2c). The heterozygous carrier had a Sp110 mean fluorescence intensity signal that was between the values produced by the VODI patient and the three healthy controls (Supplementary Fig. 5). The absence of a Sp110-specific flow cytometric signal was confirmed in blood-derived CD3⁺ T cells from two additional VODI patients carrying the homozygous 642delC mutation (Fig. 2d, e, patient characteristics, Table 1) as well as in PBMCs from a VODI patient carrying a different homozygous Sp110 frameshift mutation (373delA) [5].

Flow cytometry was able to identify four VODI patients (patients 1–4, Table 1) who had single nucleotide deletions in *SP110* leading to a frameshift and absence of Sp110 protein. A unique VODI patient with a two nucleotide insertion/deletion Sp110 missense variant (c. 78_79delinsAT, encoding a Sp110 protein with a single amino acid change at position 27 (Sp110^{Ile27Leu})) has been described [6]. Sp110^{Ile27Leu} was previously reported to be unstable [4]. To consider the possibility that cells carrying this Sp110 missense mutation might be detected by flow cytometry, we transfected Sp110-Jurkats with plasmids encoding wild-type Sp110, Sp110^{Ile27Leu}, or vector alone. Each of these plasmids contained DNA encoding noncolinear GFP. Twenty-four hours after transfection, GFP-positive cells were flow-sorted and analyzed either for Sp110 mRNA expression by real-time PCR or for Sp110 protein by flow cytometry (Fig. 3). Compared to cells transfected with wild-type Sp110, cells transfected with Sp110^{Ile27Leu} produced very low levels of Sp110 protein as assessed by flow cytometry. In contrast, the level of Sp110 mRNA was similar in cells transfected with wild-type Sp110 compared to Sp110^{Ile27Leu} (Fig. 3). This does not formally prove the utility of the here described Sp110-specific flow cytometry assay to detect this unique Sp110 missense mutation in a clinical sample. However, the results confirm that Sp110^{Ile27Leu} is an unstable protein [6] and that flow cytometry is capable of detecting the

decreased Sp110 level associated with this particular Sp110 missense mutation relative to wild-type Sp110 expression levels.

Discussion

Our results demonstrate that Sp110 expression in human T lymphocytes can be assessed by flow cytometry and that this test can be used to screen patients with suspected VODI. The assay was able to identify patients carrying different frame-shift *SP110* mutations, and the assay could also detect the so far only described missense mutation Sp110^{Ile27Leu}. The latter was possible because this particular missense mutation appears to produce an unstable Sp110 protein [6]. Analysis of Sp110 protein expression by flow cytometry may be helpful also in other conditions. Sp110 is an interferon-inducible gene and is part of the signature for autoimmune diseases such as SLE and Sjögren's syndrome [12]. The ability to quantify Sp110 in peripheral blood leukocytes may serve as a marker for disease activity in these patients. Sp110 has also been shown to be expressed in activated B cell lymphomas, but not germinal center B cell lymphomas. The ability to assess expression of Sp110 in peripheral blood lymphocytes may therefore assist in the diagnosis and characterization of malignant lymphoproliferative diseases [13, 14]. We anticipate that a low level of Sp110 expression in lymphocytes might correlate with susceptibility to opportunistic infections, including Pneumocystis, either in the presence or absence of co-existing HIV infection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

MR was supported by the Swiss National Science Foundation (PP00P3_144863). MR and CH were supported by the Gebert Rüf Foundation (GRS 058/14). FW was supported by the Deutsche Forschungsgemeinschaft (DFG Wu 841/1-1). CTB was supported by the Swiss National Science Foundation (P3_148000). This work was partially supported by the Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does the mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

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Fig. 1.

a Rabbit anti-Sp110 antiserum reacts with Sp110, but not with Sp140, in immunoblot assays. An adenovirus encoding Sp110 was used to express the protein in HEp-2 cells. Sp110 was detected in cells expressing either Sp110 alone or Sp110 together with Sp140. A very low level of Sp110 (or a cross-reacting, unrelated co-migrating protein) was detected in HEp-2 cells that were infected with a control adenovirus (lane labeled GFP). Rabbit anti-Sp110 antiserum did not cross-react with Sp140. Rabbit anti-Sp110 and anti-Sp140 antisera detected larger isoforms of the corresponding proteins, suggesting the presence of posttranslational modifications of both proteins. *Dark arrows* indicate the presence of the major isoform of both proteins, with *smaller arrows* indicating slower migrating, presumably posttranslationally modified, Sp110 and Sp140. **b** Rabbit anti-Sp110 reacts with Sp110, but not with Sp140, as detected by indirect immunofluorescence. In HEp-2 cells infected with an adenovirus encoding Sp140, the protein was detected in nuclear bodies (*green, a*) and co-localized in nuclear bodies with Sp100 (*red, b*). Ad.Sp110 infection of HEp-2 cells in the absence of Sp140, and subsequent staining with rabbit anti-Sp110 antibody, revealed the presence of Sp110 in fine speckles throughout the nucleus (*green, d*). Sp110 did not co-localize with Sp100 (*red, e*). Co-expression of Sp140 and Sp110 in HEp-2 cells resulted in localization of Sp110 to nuclear bodies (*green, g*) and co-localization with Sp100 (*red, h*).

To further test whether rabbit anti-Sp110 antiserum was specific for Sp110 and did not cross-react with Sp140, HEp-2 cells were infected with Ad.Sp140 at a multiplicity of infection sufficient to infect more than 75% of the cells. Rabbit anti-Sp140 antiserum detected Sp140 in nuclear bodies (*green, j*) and Sp140 co-localized with Sp100 (*red, k*). Rabbit anti-Sp110 antiserum did not stain the nuclei of Ad.Sp140-infected HEp-2 cells (*green, m*). Merge of panels *a* and *b*, *d* and *e*, *g* and *h*, *j* and *k*, and *m* and *n* is shown, together with DAPI staining to indicate the location of nuclei (*blue*), in panels *c*, *f*, *i*, *l*, and *o*, respectively. White arrows in *g–i* indicate representative nuclear bodies that contain both Sp110 and Sp100. *White bar* indicates 10 μ m. **c** T cell blasts from two patients with VODI, from a healthy control, and from the mother of a VODI patient were stained with rabbit anti-Sp110 antibody and analyzed for Sp110 expression by flow cytometry. CD3 was stained in parallel as indicated. Individual histograms are shown. **d** Sp110 was stained by flow cytometry in Jurkat T cells following siRNA knockdown of Sp110 (*blue*) or treatment with nonspecific siRNA (*red*) as a control for 24 h. CD3 was stained in parallel as indicated. **e** Sp110 was stained by flow cytometry in Jurkat T cells that lack Sp110 expression due to CRISPR/Cas9-mediated knockout (*blue*) or nonmodified control Jurkat T cells (*red*)

Fig. 2.

a, b Sp110-Jurkat T cells (**a**) or Sp110-deficient T cell blasts from a patient with VODI (**b**) were transfected with an expression plasmid encoding Sp110 (and co-expressing green fluorescent protein, GFP, as a marker of successful transfection) for 24 h. GFP-positive (or GFP-negative, nontransfected cells used as a control) were flow-sorted and assessed for Sp110 expression by flow cytometry. Sp110-competent (wild-type) Jurkat T cells or Sp110-competent T cell blasts from healthy controls are shown as a control. **c–f** EDTA-treated peripheral blood (**c–e**) or frozen PBMCs (**f**) from VODI patients (patients 1–4, see Table 1 for patient characteristics) and controls were shipped by courier in a process that required 48 h. Following Ficoll-based isolation of PBMCs (**c–e**) or resting of thawed PBMCs in medium overnight (**f**), PBMCs were stained for Sp110 or nonspecific rabbit IgG as a control and analyzed by flow cytometry after gating on CD3⁺ T cells. Disease control refers to a patient with recurrent infections but normal SP110 gene sequence

Fig. 3.

Sp110-Jurkat T cells were transfected with an empty plasmid or Sp110 expression plasmids expressing either wild-type *SP110* or a missense-mutated *SP110* (c.78_79delinsAT, Sp110^{Ile27Leu}). Each of the plasmids co-expressed green fluorescent protein, *GFP*, which was used as a marker of successful transfection. GFP-positive cells within the “live” cell gate based on forward/sideways scatter characteristics were flow-sorted and assessed for Sp110 mRNA by real-time PCR and for Sp110 protein by flow cytometry. The Sp110^{Ile27Leu} mutation was associated with very low Sp110 protein expression as assessed by flow cytometry. SP110 mRNA expression was similar to that produced by wild-type Sp110

Table 1

Patient characteristics

Patient	SP110 mutation	Gender	Age at diagnosis	Current age	Treatment	Clinical complications
1	642delC/642delC	Female	3 months	8 years	Sulfamethoxazole/trimethoprim, intravenous immunoglobulins	Elevated liver values, no veno-occlusion, <i>Pneumocystis</i> pneumonia Hepatosplenomegaly, failure to thrive, currently stable
2	642delC/642delC	Female	4 months	4.7 years	Intravenous immunoglobulins, sulfamethoxazole/trimethoprim, low-dose heparin, low lipid diet	Recurrent pulmonary and intestinal infections. Subacute pancreatitis
3	642delC/642delC	Male	28 months	Died	Bone marrow transplantation	Liver fibrosis, elevated portal pressure, tracheal stenosis, bronchiectasis, multiple lung infections (CMV, <i>Pseudomonas</i>)
4	373delA/373delA	Female	3 months	Died	Bone marrow transplantation	Veno-occlusive disease of the liver
5	78_79delinsAT/78_79delinsAT	Female	3 years	11 years	Sulfamethoxazole/trimethoprim, intravenous immunoglobulins	Fever, veno-occlusive disease of the liver, pancytopenia, <i>Pneumocystis</i> pneumonia, currently stable