Flow Cytometry is a Reliable Tool in the Diagnosis of STK4 Deficiency

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ABSTRACT

Objective: STK4 (serine-threonine protein kinase 4) deficiency is categorized under combined immunodeficiencies, which are a subgroup of primary immunodeficiencies that profoundly affect T cells. Autosomal recessive STK4 deficiency is characterized by recurrent bacterial and viral infections, mucocutaneous candidiasis, and CD4+ T cell lymphopenia. Autoimmune comorbidities including hemolytic anemia and idiopathic thrombocytopenic purpura, and cardiac problems have been reported in STK4-deficient individuals. Current diagnostic tools for STK4 deficiency include next-generation sequencing (NGS) and Sanger sequencing that detect DNA sequence variations, and western blotting, which evaluates altered protein expression. However, all of these assays are time-consuming; and in particular, NGS is a costly clinical use tool for a diagnostic laboratory. In comparison, flow cytometry is a rapid and reliable system commonly employed in the diagnosis of a wide range of primary immunodeficiency disorders.

Materials and Methods: This study aimed to evaluate STK4 protein expression by flow cytometry among four patients with genetically confirmed STK4 gene mutations and seven healthy individuals. We calculated ΔMFI/cell values to investigate differences in protein expression among the subjects.

Results: STK4 protein expression was reduced in the peripheral blood mononuclear cells obtained from all STK4-deficient patients compared to those from the healthy controls. Flow cytometry data was validated by western blotting.

Conclusion: Flow cytometry is a rapid and reliable method to detect STK4 protein expression, qualified as a diagnostic tool to study STK4 deficiency.

Keywords: STK4, flow cytometry

INTRODUCTION

STK4 (serine-threonine protein kinase 4), also known as MST1 (Macrophage Stimulating 1), was first found in Drosophila as a member of the Hippo pathway, which regulates proliferation and cell survival (1). In murine organisms, STK4 also regulates cell death, lymphocyte egress from the thymus, and cell survival and proliferation. Human STK4 is principally discovered as a constitutively expressed kinase, structurally homologous to the Drosophila Hippo, and plays roles in vital biologic processes such as morphogenesis, proliferation, apoptosis, and stress response (1). Although STK4 has both proapoptotic and antiapoptotic functions in the immune system, it is mainly associated with apoptotic machinery due to its relationship with the members of the extrinsic pathway of apoptosis such as caspases (2,3).

Primary immunodeficiencies (PIDs) are heterogeneous genetic diseases of the immune system (4). Combined immunodeficiencies (CIDs) are classified in PIDs and mostly characterized by impairment of two main cellular components of the adaptive immune system, T and B lymphocytes. With the improvements in the field of gene sequencing, researchers have had access to the description of more than 430 genes related to PIDs (5,6,7). STK4
Deficiency was first defined in 2012 by 3 separate groups as causing a novel autosomal recessive CID, which is characterized by a profoundly decreased level of CD4+ T cells with the concomitant tendency to recurrent viral and bacterial infections and mucocutaneous candidiasis (8-10).

Diagnosis of STK4 deficiency currently depends on gene sequencing via NGS (Next-generation sequencing) panel, Whole-exome sequencing (WES) or Sanger sequencing, and rarely western blotting (8-15). Early diagnosis of the diseases allows for better prognosis and improved quality of life due to exact treatment options. Therefore, the requirement of a rapid, accurate, sensitive and cost-effective assay for diagnosis is uncontroversial. This study aimed to develop a flow-cytometry-based reliable method to detect STK4 deficiency based on intracellular STK4 expression in the peripheral blood mononuclear cells (PBMCs).

**MATERIAL and METHODS**

**Patients**

We studied four patients with homozygous STK4 gene mutations who were clinically agreeable with IUIS criteria (Table I) (7). P1 and P2 were newly diagnosed patients. P3 and P4 were siblings, and their clinical/laboratory findings were reported in Clinical Immunology in 2015 (11). Four female and three male healthy individuals’ PBMCs were used as control, and the median age was 24 years. We also studied the father of P1 and the mother of P2 to examine how heterozygous parents differ from the controls concerning STK4 protein expression. In addition, we extracted protein from the cells obtained from the father of P2 for the evaluation of STK4 protein expression utilizing western blotting.

**Flow cytometry**

For flow cytometry studies, blood samples were collected from patients and the controls after receiving a signed informed consent. This study was approved by the Ethics Committee Review Board of Hacettepe University (No: TSA-2016-9087). Peripheral blood mononuclear cells were separated by density gradient centrifugation over Biocoll solution following centrifugation at 2000 rpm for 20 minutes (Biochrom, USA).

Following the buffy coat’s collection to a fresh tube, cells were washed once in a solution of PBS containing 5% FBS (wash buffer; WB), which was then centrifuged. The cell pellet was resolved in 1 ml WB, and 100 ul of cell suspension was transferred to a flow cytometry tube. CD45 leukocyte marker antibody was added and incubated at room temperature for 20 min (BD, USA). Cells were washed in WB and centrifuged. After fixation with 100 ul fixation buffer for 15 min, cells were permeabilized with permeabilization buffer for 15 min and washed (BD, USA). Anti-human STK4 antibody (Atlas Antibodies, USA) was used as the primary antibody at dilution 1:200, and anti-rabbit FITC conjugated secondary antibody (Thermofisher Scientific, USA) was applied at a dilution of 1:500 to the cells for 30 min at room temperatures, respectively. Then,

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<th>Table I. Clinical and laboratory findings in the patients.</th>
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<td><strong>Age (year)</strong></td>
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the cells were washed and analyzed using the FACSDiva program in a FACS CANTO II flow cytometer (BD, USA). The tube containing only secondary antibody was used as an isotype control. Healthy donors were used as healthy controls for the study.

**Western Blot**

PBMCs were collected and washed in ice-cold 1X PBS buffer. Cell lysis buffer was added and incubated on ice for 15 minutes, and then centrifuged at 15000 rpm for 7 min at 4 °C. Supernatants were collected and heat-inactivated at 95 °C for 5 min after adding Laemml buffer. Protein samples were run at 60 V for 1 hour and transferred to the PVDF membrane at 70 V using the wet transfer method. The anti-human STK4 antibody was added at 1: 1000 concentration and incubated overnight at 4 °C. The anti-rabbit secondary antibody was added at 1:2000 concentration for 1 hour at room temperature. Membranes were visualized using the SuperSignal West-Femto Substrate under a CCD camera (Syngene, India).

**Statistics**

For statistical evaluation, the Mann-Whitney U test was performed using Graphpad-Prism 8.2.1. p<0.05 was accepted as statistically significant.

**RESULTS**

**Flow cytometric analysis of STK4 protein expression and data analysis**

Following the gating of PBMCs using forward scatter – side scatter plots, the CD45 + cells were gated. CD45, also known as protein tyrosine phosphatase, receptor type C, is expressed on leukocytes. Then, STK4 expression was analyzed in CD45+ gated cells (16, 17). ΔMFI/cell values for the STK4 stain were calculated separately in four patients, seven healthy controls, and two parents as follows:

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\text{ΔMFI/cell: MFI of STK4 expression in positive control} - \text{MFI of STK4 expression in negative control}
\]

ΔMFI/cell values per cell of STK4 expression in the patients were 0.46–1.64. In healthy control samples, ΔMFI/cell values for STK4 were 2.14–8.97. There was a significant decrease in ΔMFI/cell values in patients compared to healthy donors (p<0.05) (Figure 1B). The ΔMFI/cell value was diminished in the heterozygous father to 1.45 and the control sample had a value of 2.54 ΔMFI/cell. The ΔMFI/
cell value was 1.09 in the relevant patient (P1). ΔMFI/cell was 1.65 in P2, 1.92 in her mother, and 8.97 in the healthy control. P3 and P4 had a ΔMFI/cell value of 0.46 and 0.5, respectively, and the relevant healthy control had a ΔMFI/cell value of 2.13.

The reduced STK4 protein expression in P1, P3, and P4 was statistically significant compared to the healthy controls (Figures 1A, B).

Evaluation of STK4 protein expression by using western blot

We compared the STK4 protein expression levels obtained from flow cytometry and western blot. In P2, STK4 protein expression was lower than in the healthy control sample and the father in the western blot experiment (Figure 2A). The heterozygous father had a half decrease of STK4 protein expression compared to the healthy control sample. Like Western blot experiments, flow cytometric analysis of the STK4 protein of the same patient showed the lowest protein expression. The patients' mother had a half decrease of STK4 protein expression compared to the healthy control (Figure 2B).

DISCUSSION

STK4 deficiency is a rare, heterogeneous autosomal recessive disease classified under the CIDs (8-10). A wide range of the clinical spectrum is observed in STK4 deficient patients ranging from recurrent infections and autoimmune manifestations to a very rare form of malignancies such as primary cardiac lymphoma as well as EBV-associated lymphoma. Until now, 18 patients were diagnosed with STK4 deficiency with nine different novel mutations, and they were diagnosed at least after 6 years old even with early onset of their diseases and clinical severity (8-15). Methods performed in the diagnosis of STK4 deficiency such as NGS (panels, WES, WGS) need confirmation by Sanger sequencing, western blotting, and some functional assays that require extra time for the final decision (8-15). Sanger sequencing confirmation and western blotting have been the only methods used in the diagnosis of STK4 deficiency since its statement (11-15). For instance, we evaluated STK4 mRNA and protein levels by using real-time PCR and western blot, respectively, in the first study describing STK4-deficient CID published by our group after identification of STK4 deficiency by 3 separate groups in 2012 (11). Schipp C et al. recently published a study related to EBV-negative lymphoma and autoimmune lymphoproliferative syndrome caused by STK4 deficiency. They also performed western blot to show STK4 protein expressions (14). Western blot is an old, reliable, but exhausting method to use in clinical diagnosis and takes at least two days. In contrast, flow cytometry is a rapid, reliable, and easy method to detect intracellular STK4 protein expression in cells. STK4 protein is expressed in all healthy tissues as well as PBMCs and its expression is abrogated due to pathogenic mutations (8-11,18,19). Therefore, flow cytometric analysis could be applied in patients' PBMC samples without stimulation of a specific pathway. In the clinic, possible CID patients could be easily checked for suspicious STK4 deficiency following physical examination and evaluation of the laboratory findings.

Figure 2. A) Representative western blot image of STK4 protein expression in healthy control, father, and P2. B) Representative flow cytometry image of P2, mother, and healthy control. IC: Isotype control, HC: Healthy control.
However, flow cytometry shows STK4 protein expression levels only quantitatively and it cannot give an idea about truncated proteins. Besides, in some cases, STK4 protein expression can be detected at normal levels depending on the type of mutation, e.g., a truncated nonfunctional form of the protein due to the premature stop codon mutation. In that case, depending on the antibody binding site, protein expression can be detected independently from the technique used in the laboratory. Despite these challenges, flow cytometry should be the first-step analysis in suspected patients for screening STK4 protein deficiency.

In conclusion, flow cytometry is a quantitative and rapid method to detect STK4 deficiency.

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