Letter to the Editor

Practical challenges for functional validation of STAT1 gain of function genetic variants

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Abbreviations: CMC: chronic mucocutaneous candidiasis; CVID: common variable immunodeficiency; CXCL-10: C-X-C motif chemokine ligand 10; GOF: gain of function; HC: healthy control; IEI: inborn errors of immunity; IFN-α: interferon-alpha; MFI: mean fluorescence intensity; NGS: next generation genetic sequencing; PBMC: peripheral blood mononuclear cell; PID: primary Immunodeficiency; PMA: phorbol 12-myristate 13-acetate; SD: standard deviation; pSTAT1: phosphorylation of signal transducer and activator of transcription 1; STAT1: signal transducer and activator of transcription 1; VUS: variant of unknown significance.

Wider application of next generation genetic sequencing (NGS) has significantly improved diagnosis for patients with inborn errors of immunity (IEI) and is increasingly a routine part of clinical practice [1, 2]. However, functional validation of genetic variants of unknown significance (VUS) remains a significant challenge for confirming definitive diagnoses, both due to inconsistent access to standardized testing and poor standardization of currently available assays [3]. The challenges are greater for conditions where clinical phenotype is not confirmatory due to heterogeneity or incomplete penetrance. STAT1 gain-of-function (GOF) immunodeficiency typically presents with chronic mucocutaneous candidiasis (CMC), with or without a combination of bacterial, viral or mycobacterial infections, along with other complications including bronchiectasis, autoimmunity and vascular abnormalities [4, 5]. Over 75 different individual genetic mutations have been described to confer gain of function in the STAT1 gene [6] and classically result in increased phosphorylation of STAT1 (pSTAT1) in immune cells, either at baseline or following cytokine stimulation [7]. Therefore, most immunology laboratories that offer validation assays for STAT1 VUS rely on comparing pSTAT1 levels between a potential index case and one or a group of healthy controls. However, levels of pSTAT1 at baseline and upregulation after cytokine stimulation vary considerably in the healthy population and overlap with levels observed in STAT1 GOF patients.

In this study, we performed technically standardized flow cytometry assays using peripheral blood obtained from six patients with five different STAT1 VUS, identified through whole genome or targeted chip panel sequencing for suspected IEI (see Supplementary Table 1 and Methods). We compared these against 4 patients with known pathogenic GOF mutations in STAT1 and 16 healthy controls. A group of six patients with Common Variable Immunodeficiency (CVID) who had no pathogenic mutations identified in currently known IEI genes by NGS and no rare VUS in STAT1 were used as disease controls. Healthy control samples were taken on the same day as patient or disease control samples and assays performed in parallel. STAT1 VUS tested here were rare (allele frequency <10−4 in reference databases) with variable prediction of deleterious impact using in silico prediction tools (Table 1 and Supplementary Fig. 1a and b).

Significant variability was seen in STAT1 levels and in pSTAT1 upregulation in response to interferon-alpha (IFN-α) stimulation for all groups (Supplementary Figs 1c-e and 2a). When considered by group, both STAT1 GOF mutations and STAT1 VUS showed a significant increase in upregulation of pSTAT1 compared with healthy control (Supplementary Fig. 1d), although there was an overlap in the range observed for each group. We then considered each genetic variant individually to determine whether or not VUS could be assigned as GOF. When normalized against their matched healthy control sample, all patients with GOF mutations and 4/6
with VUS demonstrated at least 2-fold higher upregulation in pSTAT1 levels after stimulation (Fig. 1a). Two patients with VUS did not achieve this arbitrary cut off and one of these had a matched healthy control at the upper limit of the healthy control range (Fig. 1b). To reduce the impact of individual healthy control variation, we compared each VUS and GOF mutation against the whole healthy control range and identified that all patients with GOF and 5/6 with VUS upregulated pSTAT1 above a cut off of mean+1SD of the healthy control range (Fig. 1c). Variant location did not appear to impact degree of upregulation (Fig. 1d). Upregulation of total STAT1 was more variable; although all GOF and VUS showed upregulation to at least an arbitrary cut-off of 1.4x healthy control (Fig. 1e), this was considered to be not sufficiently robust to assist diagnosis (Supplementary Fig. 1e). CVID disease controls as a group behaved like healthy controls, with comparable upregulation of pSTAT1 and STAT1 (Fig. 1a, c–e, and Supplementary Fig. 1c–e). To test inclusion controls, with comparable upregulation of pSTAT1 and STAT1 CVID disease controls as a group behaved like healthy control (Fig. 1e), this was considered to be not sufficiently robust to assist diagnosis (Supplementary Fig. 1e). This study highlights the difficulty of definitively assigning GOF status to STAT1 VUS using the single assessment of pSTAT1 and the benefit of including Th17 quantification as an additional parameter. For laboratories performing pSTAT1 assays, highly standardized phosflow protocols and flow cytometer settings together with a local healthy control range are important to reduce variation and improve assessment of index cases. In our hands, pSTAT1 assays only perform reproducibly on fresh blood (taken same day) and older samples are not reliably interpretable. To mitigate against healthy control variation, we suggest that assessment of pSTAT1 is made both against a same-day matched control and the local healthy control range. Future refinement of functional assessment for STAT1 VUS requires development of additional robust assays that can be translated from research to diagnostic laboratories.

Supplementary data

Supplementary data is available at Clinical and Experimental Immunology online.

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**Table 1. Predicted functional impact of STAT1 variants evaluated by in silico analysis and functional assays**

<table>
<thead>
<tr>
<th>Group</th>
<th>SIFT raw (&lt;0.05)</th>
<th>PolyPhen2 Raw</th>
<th>CADD PHRED</th>
<th>Patient/mutation</th>
<th>pSTAT1 Fold change &gt; 2* (normalized for HC)</th>
<th>pSTAT1 &gt; Mean + SD of HC</th>
<th>pSTAT1 &lt; HC of the day</th>
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<td></td>
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<td>No</td>
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<td>No</td>
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<td>No</td>
<td>10/P293S</td>
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</table>

*Normalized for the healthy control of the day; VUS = variant of unknown significance; GOF = gain of function; HC= healthy control; SD = standard deviation.
Consortia

NIHR BioResource – Rare Diseases Consortium:
Thaventhiran, Moira Thomas, Adrian Thrasher (principal investigator), Steve Welch, Lisa Willcocks, Sarita Workman, Austen Worth, Nigel Yeatman, and Patrick Yong.

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Ethics approval and consent to participate

The local ethics committees have approved our study (reference numbers 04/Q0501/119 and 06/Q0508/16) and patients and healthy controls gave written informed consent.

Conflict of interests

S.O.B. has received grant support from CSL Behring and personal fees or travel expenses from Immunodeficiency Canada/IAACI, CSL Behring, Baxalta US Inc. and BioTest. E.C.M. has received honoraria from GlaxoSmithKline, AstraZenica, and Orchard Therapeutics. The rest of the authors declare that they have no conflicts of interest.

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Data availability

The datasets used and analysed in the current study are available from the corresponding author on reasonable request.

Author contributions


References