CASE REPORT
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Two different STAT1 gain-of-function mutations lead to diverse IFN-γ-mediated gene expression

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Signal transducer and activator of transcription 1 (STAT1) regulates multiple biological processes downstream of a variety of cytokine receptors in many cell types. Heterozygous gain-of-function (GOF) mutations in STAT1 have been associated with a diverse phenotype encompassing chronic mucocutaneous candidiasis (CMCC) and declining immunity. There is no clear correlation between STAT1 domain-specific mutations and phenotype, and it remains unclear why GOF mutations in STAT1 result in such a wide spectrum of clinical presentations. To begin exploring this dilemma, we have studied the patterns of gene expression mediated by two different GOF mutations. Analysis of IFN-γ response elements using RNA microarrays in cells transfected with the rare H629Y mutant or the common R274G mutant showed distinct patterns of gene expression. We show here that the impact of GOF mutations in STAT1 is variant-specific. This difference in gene expression may explain the diversity in clinical manifestations experienced by these patients.

INTRODUCTION
Signal transducer and activator of transcription 1 (STAT1) is a transcription factor that controls many biological effects downstream of the type I interferons as well as other cytokine receptors in many cell types.1

Monallelic mutations in STAT1 have been identified in patients with a great diversity of clinical manifestations and immune lesions. Mutations have been predominately associated with chronic mucocutaneous candidiasis (CMCC)2–3, with or without a variety of autoimmune manifestations.4,5 STAT1 mutations have been also associated with a gradual decline in cellular and humoral immunity leading to fatal viral infections.6

Increased STAT1 phosphorylation and DNA binding found in most patients promoted the notion that a gain-of-function (GOF) mechanism underlies all these disorders. Yet, a common mechanism would not appear to explain the diversity of presentations.

We compared here a common mutation in STAT1 with a different mutation identified in a patient that had a novel clinical presentation.

RESULTS AND DISCUSSION
Here we describe a female patient of English descent who is currently 30 years old. She was born preterm at 32 weeks of gestation and suffered necrotising enterocolitis soon after birth. Since the age of 1 year old, she suffered recurrent candida infections, pneumococcal chest infections, complicated by pneumatocele and bronchiectasis that eventually required left lower lobe resection and by 15 years old she was diagnosed with primary ovarian failure.

At 26 years, she had noticed gradual and progressive difficulty walking, right side weakness and spasticity, as well as dysarthria. Extensive neurological investigations, including visualised evoked potentials, brainstem auditory-evoked responses and somatosensory-evoked potentials were normal. Cerebrospinal fluid was negative for infectious agents and no oligoclonal banding was detected. Muscle biopsy showed no inflammation, necrosis or vascular changes. Magnetic resonance imaging of the brain and the spinal cord demonstrated diffuse abnormalities in the periventricular region and the white matter, with extensive wellerian degeneration in the brainstem affecting mainly the pyramidal tract (Fig. 1a, b). While brain aneurisms have been previously reported in association with GOF STAT1 mutations2,4,5,6,7 progressive neurodegenerative manifestations observed in this patient appear unique.

Evaluation of the immune system revealed normal numbers of circulating T, B and natural killer cells. In vitro proliferative responses to mitogens were normal but responses to antigens, including candida, cytomegalovirus (CMV), herpes simplex and herpes zoster were absent (Table 1). Serum immunoglobulin levels were normal but specific antibody responses to tetanus toxoid were non-protective. Analysis of phytohemagglutinin (PHA)-induced cytokine secretion by patient T cells revealed virtually no interleukin (IL)-17 production as compared with control (Fig. 2a, left panel), while interferon-γ (IFN-γ) and IL-2 secretion were markedly increased when compared to control samples (Fig. 2a, middle and right panels).

Suspecting CMCC, we conducted Sanger sequencing for STAT1 and identified a de novo heterozygous c.1885C>T mutation in STAT1, predicting a histidine-to-tyrosine amino-acid change at...
position 629 (H629Y). No other immune-related gene aberration was detected using an immunodeficiency wide panel.

In order to understand why this patient has such a unique clinical pattern we studied gene expression patterns triggered by this mutation and compared it to the common mutant R274G, both designated GOF mutations. This common STAT1 mutation is typically associated with extensive candidiasis of the skin and mucous membranes and autoimmune endocrinopathy, infections and autoimmunity.

We first examined STAT1 phosphorylation as a determinant of GOF. We found increased STAT1 phosphorylation in H629Y-mutated patient T cells following IFN-α and IL-27 stimulation (Fig. 2b, c). Increased tyrosine phosphorylation was confirmed in STAT1-deficient U3A fibrosarcoma cells transfected with the H629Y mutant (Fig. 2d), comparable to phosphorylation in the R274G-transfected U3A cells, indicating that nuclear transit was not altered.

To further understand the effect of various GOF mutations, RNA microarrays were used to study downstream gene expression. RNA microarray analysis was performed on U3A cells transfected with either WT STAT1, the SH2 H629Y mutant or a ‘typical’ well-studied GOF mutant, R274G. The R274G mutant has been previously shown to increase STAT1 phosphorylation, enhance gamma-activated sequence DNA binding and induce an exaggerated upregulation of several genes in response to IFN-γ. Gene upregulation in this study was defined as a 1.5-fold increase over baseline of gene expression levels. The analysis revealed 24 IFN-γ-upregulated inducible genes in the H629Y mutant as compared with far greater (83 genes) triggered by the R274G mutation, while WT showed an increase of only 22 genes (Fig. 2f and Table 2). Eleven genes including CXCL10, GBP1, GBP2, GBP4, GBP5, TAP1, PARP9, NMI, DTX3L, HLA-E and PSMB9 were commonly increased.

### Table 1. Immune evaluation of the patient

| Parameter                        | Patient | Normal range/ control |
|----------------------------------|---------|-----------------------|
| Lymphocyte immunophenotyping (cells/µL) |         |                       |
| CD3                              | 1903    | 700–2100              |
| CD4                              | 1012    | 300–1400              |
| CD8                              | 784     | 200–900               |
| CD56                             | 348     | 90–600                |
| CD19/20                          | 1012    | 100–500               |
| Responses to mitogens (stimulation index; P/C) |         |                       |
| PHA                              | 358/500 | Stim. index > 300 Patient/control > 50% |
| Responses to antigens (stimulation index; no. responses) |         |                       |
| Candida, CMV, zoster, simplex, tetanus, PPD | All < 20 | Stim. index > 20 Total antigen responses > 2 |
| Immunoglobulins                  |         |                       |
| IgG (g/L)                        | 12.6    | 6.6–15.3              |
| IgA (g/L)                        | 1.1     | 0.54–4.17             |
| IgM (g/L)                        | 0.7     | 0.3–2.3               |
| Specific antibodies              |         |                       |
| Tetanus                          | 0.02    | >0.1                  |
| Measles/mumps/rubella            | +ve/-ve/ +ve/-ve/+ve |
| Isohemagglutinin                 | 1:16    | >1:32                 |

Fig. 1 CNS changes in the patient. Brain magnetic resonance imaging demonstrating white matter periventricular lesions, as shown in the a fluid-attenuated inversion recovery (FLAIR) image, and b T2-weighted image. c Haematoxylin & eosin/luxol fast blue staining of the brain biopsy shows (I) normal architecture with preservation of grey-white differentiation. Immunohistochemical staining with glial fibrillary acidic protein demonstrates white matter and subpial gliosis (II, III). Images were taken at varying magnification with scale bars as shown.
Two different STAT1 gain-of-function mutations lead by WT STAT1 as well as both mutants. WT and R274G shared 11 other genes, which were highly expressed (GBP3, IRF1, SP110, APOL6, AIM2, C1R, HAPLN3, LRRTM2, UBA7, TRIM69 and IRF9). Only 5 IFN-γ-inducible genes (CXCL9, WAR5, SERPING1, UBE2L6 and TRIM21) were commonly upregulated by H629Y and R274G. The H629Y mutant demonstrated uniquely high expression of TLR2, IFI44L, APOL3, TINAGL1, FZD10, OARD1, KLC2 and TRAV8-4, while the R274G mutant induced a rise in an additional 56 genes, which were not increased in WT nor in the H629Y mutant. These results indicated that different GOF mutations can affect IFN-γ-mediated responses by inducing diverse patterns of gene expression, with some shared while others private response genes.
We next investigated whether the magnitude of response of gene amplification was similar among the different mutations for genes, which were increased in both mutants. For these experiments, quantitative PCR was used to compare responses to IFN-γ stimulation. As shown in Fig. 2g, the rise in CXCL10 in the R274G variant was far greater than the increase recorded in the H629Y mutant. Similarly, GBP1 and IRF1 expression was highest in the R274G variant. These results demonstrate that in addition to induction of a unique variant-specific set of genes, GOF variants also differ in the magnitude of the response.

Together, these findings indicate that STAT1 GOF mutants manifest in distinctively different patterns of gene expression with possible implications to the clinical phenotype. However, the relevance of this variable response to clinical manifestations cannot be deduced from this limited study of two mutations. A far larger study, including multiple different mutations as well as more extensive analysis of patients’ genome will likely be needed to address this important question.

METHODS

Patient and blood samples

Data were compiled prospectively and retrospectively from medical records and were entered into the Canadian Centre for Primary Immunodeficiency Registry and Tissue Bank, which has been approved by the SickKids Research Ethics Board (protocol no. 1000005598). All patients provided written informed consent.

Serum concentration of immunoglobulins and specific antibodies

Nephelometry was used to determine serum concentrations of immunoglobulins (IgG, IgA and IgM), and radioimmunoassay utilised to measure IgE levels (IgE PRIST kit, Pharmacia Diagnostics, Dovral, Canada). Serum antibody levels to tetanus toxoid and pneumococcus (pneumococcal capsular polysaccharide) were quantified by enzyme-linked immunosorbent assay (ELISA), as per the manufacturer’s instructions (Binding Site, Birmingham, UK). Serum antibodies to measles, mumps and rubella were also measured by ELISA (Euroimmune kits, Gross-Groenau, Germany). Serum isohemagglutinin titres are reported as the dilution at which macroscopic agglutination is observed (antiglobulin phase), and were determined by twofold serial dilution with erythrocytes.

T-cell and B-cell proliferative responses

Lymphocyte proliferative responses to mitogens, including PHA, anti-CD3 and anti-CD28 antibodies, as well as a panel of recall antigens (candida, tetanus toxoid, herpes zoster, and CMV) were determined by thymidine incorporation, as reported previously. All assays were performed in triplicate and were compared with random normal controls.

Sequencing analysis

Patient genomic DNA was amplified by PCR and sequenced for the STAT1 gene. Primers were designed to amplify fragments up to 60 bp upstream and downstream of each transcript exon of the STAT1 gene. PCR conditions consisted of 94 °C for 30 s, 55 °C for 30 s and 68 °C for 2 min, for a total of 35 cycles using Elongase (Invitrogen). PCR products were electrophoresed on 0.8% agarose gels. Each PCR fragment was subsequently cut and puriﬁed and fragments were sequenced by DTCs Quick Kit on an automated sequencer (Beckman-Coulter CEQ 8000).

This mutation was conﬁrmed in clinical lab setting whereby a primary immunodeﬁciency panel of 274 genes were analysed using next-generation sequencing methodology.

Cytokine determinations

Ficoll-separated peripheral blood mononuclear cells from patients or controls were stimulated with PHA for 48 h or as indicated, and culture supernatants collected for analysis by ELISA. IL-2, IFN-γ and IL-17 cytokine analysis kits were obtained from R&D Systems (MN). Experiments were performed in triplicates.

Cell lines and transfection

U3A cells (American Type Culture Collection) were grown at 37 °C in a humidified 5% CO2 atmosphere in complete growth media. STAT1-deﬁcient U3A cells were cultured in Dulbecco’s modiﬁed Eagle’s medium (PAA Laboratories) supplemented with 10% foetal calf serum (Gibco).
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AUTHOR CONTRIBUTIONS
C.M.R. conceived the study. A.O., N.S. and C.M.R. designed the study. A.O. and N.S. performed the experiments. A.O. and C.M.R. wrote the manuscript. A.O. and C.M.R. recruited the patients. C.H., S.L. and C.M.R. clinically evaluated the patients. A.O. and C.M.R. interpreted the data. All authors critically revised and approved the final version of the manuscript.

ADDITIONAL INFORMATION
Supplementary information accompanies the paper on the npj Genomic Medicine website (https://doi.org/10.1038/s41525-018-0063-6).

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