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*J Immunol* published online 22 June 2012
http://www.jimmunol.org/content/early/2012/06/22/jimmunol.1200926
Chronic Mucocutaneous Candidiasis Caused by a Gain-of-Function Mutation in the STAT1 DNA-Binding Domain

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Chronic mucocutaneous candidiasis (CMC) is a heterogeneous group of primary immunodeficiency diseases characterized by chronic and recurrent Candida infections of the skin, nails, and oropharynx. Gain-of-function mutations in STAT1 were very recently shown to be responsible for autosomal-dominant or sporadic cases of CMC. The reported mutations have been exclusively localized in the coiled-coil domain, resulting in impaired dephosphorylation of STAT1. However, recent crystallographic analysis and direct mutagenesis experiments indicate that mutations affecting the DNA-binding domain of STAT1 could also lead to persistent phosphorylation of STAT1. To our knowledge, this study shows for the first time that a DNA-binding domain mutation of c.1153C>T in exon 14 (p.T385M) is the genetic cause of sporadic CMC in two unrelated Japanese patients. The underlying mechanisms involve a gain of STAT1 function due to impaired dephosphorylation as observed in the coiled-coil domain mutations.

Materials and Methods

Patients

Patient 1 is a 12-yr-old boy born to nonconsanguineous healthy Japanese parents. He developed severe and recurrent oral thrush since the age of 2 yr and was diagnosed with CMC. He has also had recurrent pneumonia, bronchitis, and otitis media caused by Streptococcus pneumoniae since the age of 3 yr. Chest x-ray and computed tomography scan demonstrated the presence of bronchiectasis at the age of 5 yr. He was noticed to have hypothyroidism with positive anti–thyroid-stimulating hormone receptor Abs, and levothyroxine was initiated at the age of 9 yr.

Patient 2 is a boy born to nonconsanguineous healthy Japanese parents. He had poor body weight gain soon after birth. He was diagnosed with CMC at the age of 6 yr. He also had recurrent bronchitis, pneumonia, and sinusitis caused by S. pneumoniae. He was diagnosed with bronchiectasis at the age of 7 yr. At the age of 13 yr, he developed hemophagocytic lymphohistiocytosis (HLH). He subsequently presented with autoimmune hemolytic anemia with positive direct and indirect Coombs’ tests and thrombocytopenia and was diagnosed as having Evans syndrome. He died suddenly at the age of 14 yr and 5 mo from disseminated intravascular coagulation and pulmonary insufficiency of unknown etiology. These two patients were not related (case reports in preparation).

Patient 3 is a 15-yr-old girl with CMC. Her father had also been diagnosed with CMC and died of cerebral vasculitis (9). She was demonstrated to have the heterozygous R274Q mutation affecting the CC domain of STAT1. Because this mutation was recently reported as a gain-of-function mutation due to impaired dephosphorylation of STAT1 (4), we studied Patient 3 as a control for investigating the mechanisms of the development of CMC in Patients 1 and 2. Informed consent for genetic analysis was obtained from the patients, their family members, and normal controls under a protocol approved by the Institutional Review Board of Hokkaido University Hospital.

Abbreviations used in this article: CC, coiled-coil; CMC, chronic mucocutaneous candidiasis; DBD, DNA-binding domain; HLH, hemophagocytic lymphohistiocytosis; STAT1p, phosphorylated STAT1; Wt, wild-type.

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-generation of EBV-transformed cell lines

EBV-transformed cell lines (EBV-LCLs) were generated by in vitro transformation of human B cells with EBV (strain B95-8), as described elsewhere (10). Based on the results of STAT1 sequence analysis, EBV-LCLs from Patient 1 with T385M and wild-type (Wt) alleles and Patient 3 with R274Q and Wt alleles were designated as T385M/Wt and R274Q/Wt, respectively. Two age-matched control EBV-LCLs, designated Wt-1 and Wt-2, were used as controls. EBV-LCLs from Patient 2 were not obtained.

Stimulation reagents

For stimulation, 1:1000 diluted recombinant human IFN-γ (Shionogi, Osaka, Japan; 1000 IU/mL, 200 ng/mL), 1500 U/mL recombinant human IFN-α (Biosource International, Camarillo, CA), 20 ng/mL IL-27 (R&D Systems, Minneapolis, MN), and 100 µg/mL Curdlan (Wako, Osaka, Japan) were used.

DNA isolation, PCR, and sequence analysis of PCR products and TOPO-TA clones

These procedures were performed following the methods described elsewhere (10).

Measurement of CXCL10 (IP-10) concentration in supernatant of monocyte-derived macrophages and EBV-LCLs using Cytometric Bead Array

To accurately evaluate STAT1 function by studying supernatant IP-10 production from macrophages, monocytes were first purified from PBMCs with CD14 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) to avoid contamination of other cells. A total of 5 × 10⁶ cells/ml monocytes was then differentiated into macrophages by culturing for 7 d in RPMI 1640 containing 10% FBS in the presence of 5 ng/mL M-CSF (R&D Systems). To determine the effect of IFN-γ, differentiated macrophages in triplicate were not stimulated, were stimulated with 1 µg/mL LPS (Sigma, St Louis, MO), or were prestimulated with 1000 U/mL (200 ng/mL) IFN-γ (Shionogi) for 2 h and then stimulated with 1 µg/mL LPS for 24 h (IFN-γ–LPS stimulation), and supernatant was harvested for IP-10 measurement. For studying IP-10 production from EBV-LCLs, 1 × 10⁶ cells/ml EBV-LCLs were cultured in the presence of 1000 U/mL IFN-γ for 6 h. The concentration of IP-10 in the supernatant was measured with Cytometric Bead Array (BD, San Diego, CA), following the manufacturer’s instructions. Data from triplicate independent experiments are reported as the mean ± SD.

Preparation of nuclear extract

Nuclear extract was prepared essentially as described previously (11). Briefly, harvested cells were washed with Ca²⁺ and Mg²⁺-free PBS and pelleted by centrifugation at 1500 × g for 5 min at 4°C. The resulting cell pellets were resuspended in cytoplasmic extract buffer (10 mM HEPES [pH 7.9], 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM Na₃VO₄, 1 mM NaF [pH 8]) with the addition of the recommended volume of dissolved protease inhibitor mixture tablets (Roche). After incubation on ice for 15 min, a 1:16 volume of 10% Nonidet P-40 was added. The suspension was vortexed and then centrifuged at 1500 × g for 5 min at 4°C. The pellets were washed again with cytoplasmic extract buffer without Nonidet P-40, resuspended with nuclear extract buffer (20 mM HEPES [pH 7.9], 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM Na₃VO₄, 1 mM NaF [pH 8]) with the addition of protease inhibitor, and incubated at 4°C for 30 min. After centrifugation at maximum speed for 5 min at 4°C, the supernatant was saved as nuclear extract. Protein concentration was measured by Protein Assay (Bio-Rad, Hercules, CA).

Western blot analysis

After addition of SDS sample buffer, 10 µg nuclear extract was separated by 7.5% polyacrylamide gels and transferred to Immobilon-P Transfer Membranes (Millipore, Billerica, MA). Anti-lamin A Ab (BioLegend, San Diego, CA) was used as a loading control for nuclear extract. All of the primary Abs were used at the final concentration of 1 µg/mL. HRP-conjugated anti-mouse IgG secondary Abs (GE Healthcare, Buckinghamshire, U.K.) were used at 1:2000 dilution. The blots were then visualized by Pierce Western blotting Substrate (Thermo, Rockford, IL).

Studies of STAT1 phosphorylation state and staurosporine and pervanadate treatment of cells

We assessed dephosphorylation with the tyrosine kinase inhibitor staurosporine in EBV-LCLs. A total of 1 × 10⁶ cells/ml EBV-LCLs was stimulated with IFN-γ for 30 min and then incubated with 1 µM staurosporine (Alomone Labs, Jerusalem, Israel) for 15, 30, or 60 min. The phosphorylase inhibitor pervanadate was prepared by mixing 200 mM sodium orthovanadate (Wako, Osaka, Japan) and 100 mM H₂O₂ at a 2:1 ratio for 15 min at 22°C. EBV-LCLs were treated with pervanadate (0.8 mM orthovanadate and 0.2 mM H₂O₂) for 5 min and then stimulated with IFN-γ for 30 min. The nuclear extract from each condition was subjected to SDS-PAGE. The phosphorylation state of STAT1 was evaluated with anti-human STAT1 (pY701) Ab purchased from BD. The membrane was then stripped and reprobed with anti-human STAT1 (BD) and anti-lamin A (BioLegend) Abs.
Flow cytometric analysis of intracellular IL-17A expression in CD4+ cells

PBMCs at a density of $1 \times 10^6$ cells/ml were stimulated with 20 ng/ml PMA plus 500 ng/ml ionomycin for 6 h in the presence of GolgiStop (BD). Harvested PBMCs were washed and stained with PECy5-conjugated anti-human CD4 Ab (BioLegend) for 20 min at 4˚C. Cells were washed three times and fixed and permeabilized with Cytofix/Cytoperm solution (BD) for 20 min at 4˚C. Cells were then washed, incubated for 30 min with PE-conjugated anti-human IL-17A (BioLegend) or FITC-conjugated anti-human IFN-γ Abs (BioLegend), washed, and analyzed with a FACSCalibur (BD).

Results

A possible DBD mutation in STAT1

We first performed direct sequence analysis of the genes responsible for CMC in our patients: AIRE, CLEC7A, CARD9, IL17RA, IL17F, IL2Rα, and STAT1 (4–6, 12–17). This study demonstrated that Patient 1 and Patient 2 have the same heterozygous base change in STAT1 (c.1154C>T, p.T385M) (Fig. 1A), which was confirmed by the sequence analysis of TOPO-TA clones (Fig. 1B, data not shown). This base change has not been reported either as a mutation or as a single nucleotide polymorphism in the National Center for Biotechnology Information database, Ensembl database, or the Single Nucleotide Polymorphism Database, and it was not present in the family members of Patient 1 (Fig. 1C) or in 108 normal healthy controls (data not shown). Furthermore, the affected residue was evolutionarily conserved, as shown in Fig. 1D. The polymorphism phenotype-2 (PolyPhen-2) algorithm (http://genetics.bwh.harvard.edu/pph2/index.shtml), a structure sequence-based amino acid substitution-prediction method, predicted p.T385M as probably damaging, with a score of 1.000 (sensitivity: 0.00; specificity: 1.00). The sort intolerant from tolerant algorithm (http://sift.jcvi.org/) also predicted this amino acid substitution as deleterious. These results strongly indicate that c.1153C>T (p.T385M) is a de novo disease-causing mutation. Patient 1 was also shown to have an unreported heterozygous base change in CARD9 (c.661G>A, p.K221E). However, this base change was also detected in his healthy father (data not shown). Additionally, PBMCs from Patient 1 showed normal IL-6 production in response to IFN-γ.
FIGURE 4. T385M was associated with hyperphosphorylation of STAT1 due to impaired dephosphorylation. (A) STAT1p expression in EBV-LCLs stimulated with IFN-γ for 30 min and then incubated with 1 μM staurosporine for 15, 30, or 60 min. (B) STAT1p expression in EBV-LCLs treated with pervanadate for 5 min and then stimulated with IFN-γ for 30 min.

To β-1,3-glucan stimulation with Curdlan (data not shown), indicating that the base change of c.661G>A, p.K221E in CARD9 is not a disease-causing mutation but a single nucleotide polymorphism. The rest of the genes studied were demonstrated to be normal in both patients.

T385M is associated with gain of STAT1 function

Gain-of-function mutations in STAT1 were very recently shown to be the genetic cause of autosomal-dominant or sporadic CMC (4–6). The reported mutations have been exclusively localized in the CC domain, leading to gain of STAT1 function due to impaired STAT1 dephosphorylation (4). To study whether the base change of c.1153C>T, p.T385M affecting the DBD of STAT1 also leads to gain of STAT1 function, the production of the downstream target of STAT1, IP-10, was studied following IFN-γ stimulation.

IP-10 production was significantly higher in monocyte-derived macrophages from Patient 1 (T385M/Wt) and Patient 3 (R274Q/Wt) than in the matched control macrophages after IFN-γ–LPS stimulation (Fig. 2A). IP-10 production was also significantly higher in EBV-LCLs from Patient 1 (T385M/Wt) and Patient 3 (R274Q/Wt) after IFN-γ stimulation (Fig. 2B). These results indicated that T385M is a mutation leading to gain of STAT1 function.

STAT1 T385M leads to STAT1 hyperphosphorylation in response to IFN-γ, IFN-α, and IL-27 stimulation, which is due to impaired dephosphorylation

We then studied the STAT1 phosphorylation state in EBV-LCLs to determine the mechanisms of the gain of STAT1 function. Expression of phosphorylated STAT1 (STAT1p) protein following IFN-γ stimulation was higher in T385M/Wt and R274Q/Wt EBV-LCLs than in Wt EBV-LCLs (Fig. 3A). The hyperphosphorylated state of STAT1 was also observed following stimulation with IFN-α and IL-27 (Fig. 3B, 3C). Additionally, expression of total STAT1 in nuclear extract tends to be higher in T385M/Wt and R274Q/Wt EBV-LCLs than in Wt EBV-LCLs, especially without stimulation (Fig. 3). The mechanisms underlying STAT1 hyperphosphorylation in T385M/Wt EBV-LCLs were further explored with the tyrosine kinase inhibitor staurosporine and the phosphatase inhibitor pervanadate. The dephosphorylation of IFN-γ-ac-
tivated T385M/Wt EBV-LCLs was impaired in the presence of staurosporine, as observed in R274Q/Wt EBV-LCLs (Fig. 4A). In contrast, with pervanadate treatment, the phosphorylation of STAT1 in T385M/Wt EBV-LCLs was similar to that seen in Wt EBV-LCLs (Fig. 4B). Therefore, the mechanisms underlying STAT1 hyperphosphorylation in T385M/Wt EBV-LCLs involve impaired dephosphorylation of STAT1, as observed in R274Q/Wt EBV-LCLs.

**Patient 1 with the heterozygous T385M mutation in STAT1 had deficient Th17 cells**

Deficient development of Th17 cells was documented to be associated with the development of CMC. CMC patients with gain-of-function mutations of STAT1 affecting the CC domain have shown this defect (4). Therefore, we studied the proportion of CD4+IL-17A+ cells among CD4+ cells in our patients after PMA plus ionomycin stimulation for 6 h. We also studied the population of CD4+IFN-γ+ cells to evaluate Th1 development. Patient 1 with the heterozygous T385M/Wt mutation of STAT1 was reproducibly demonstrated to have dramatically reduced CD4+IL-17A+ cells (0.2% of CD4+ cells), and Patient 3 with the heterozygous R274Q mutation had significantly reduced, but a little higher, CD4+IL-17A+ cells (0.7% of CD4+ cells) (Fig. 5). The p value estimated using the Mann–Whitney U test was 0.039 between controls and the two patients. In contrast, both patients and controls had comparable percentages of CD4+IFN-γ+ cells (Fig. 5).

**Discussion**

To our knowledge, this study shows for the first time that the de novo heterozygous mutation of c.1153C>T in exon 14 (p.T385M), affecting the DBD of STAT1, is the genetic cause of sporadic CMC in two unrelated Japanese patients. The underlying mechanisms involve gain of STAT1 function due to impaired STAT1 dephosphorylation, as observed in the CC domain mutations (4).

Recent extensive studies of the STAT1 molecule reveal the association between the mutations affecting DBD and gain of STAT1 function. Based on crystallographic analysis, Darnell’s group (7, 8) proposed a model of reorientation of phosphorylated “parallel” STAT1 dimers to an “antiparallel” form after leaving the DNA, which allows for reciprocal association of the CC domain and a pocket residue of the DBD for dephosphorylation. They further demonstrated in direct mutagenesis experiments that mutations of the pocket residues of the DBD, Q340A or Q340W, G384A or G384W, and Q408A or Q408W, resulted in impaired dephosphorylation of STAT1 (8). The fact that T385, the amino acid altered in two of our patients, is evolutionarily conserved and is positioned next to the pocket residue G384 may indicate that it is also critical in the reciprocal association with the CC domain for stabilizing the antiparallel structure and for dephosphorylation. It is also possible that this mutation of the DBD leads to impaired dissociation from the DNA, which may also cause a resistance to dephosphorylation of the STAT1 molecule. Higher expression of STAT1 in nuclear extracts from T385M/Wt and R274Q/Wt EBV-LCLs than from Wt EBV-LCLs may reflect impaired nuclear export due to a resistance to dephosphorylation of the mutant STAT1 molecule (18), although the precise mechanisms were not determined in this study.

There may be more patients with CMC who carry gain-of-function mutations affecting the DBD of STAT1, given that significant numbers of patients with STAT1 mutations are reported from all over the world (4–6). Additionally, crystallographic analysis and mutagenesis studies showed that mutations in the N-terminal domain (aa 1–130) also resulted in persistent phosphorylation (7, 8). This suggests that mutations affecting the N-terminal domain may also be a genetic cause of CMC.

We demonstrated deficient Th17 cells (0.2% of CD4+ cells) in Patient 1 with the heterozygous T385M mutation, which was similar to or more severe than the defect observed in Patient 3 with the heterozygous R274Q mutation (0.7% of CD4+ cells). Deficient development of Th17 cells may explain the increased susceptibility to *Candida* infection. IFN-γ, IFN-α, and IL-27 are potent inhibitors of Th17 cell development via STAT1 in mice and/or humans (19–21). Therefore, gain of STAT1 function in response to IFN-γ, IFN-α, or IL-27, which was observed in our patients, could be associated with deficient Th17 cell development. However, it remains to be determined precisely how gain of STAT1 function leads to deficient Th17 cells.

It is unclear whether there are differences in the clinical spectrum or severity of the disease between patients with the DBD mutations and the CC domain mutations. It might be worth noting that the two patients with the DBD mutation of T385M developed bronchiectasis in their early childhood, and one of them eventually developed HLH; these have not been described in patients with CC domain mutations.

With regard to HLH, administration of an anti–IFN-γ Ab was recently shown to have a therapeutic effect in two murine models of human hereditary HLH: perforin-deficient and Rab27a-deficient mice (22). Careful evaluation of the results indicates that T385M could be associated with higher expression of STAT1p in response to various stimulations (Fig. 3). Therefore, CMC patients with the DBD mutation of T385M may be more susceptible to the conditions presumably associated with enhanced IFN-γ–STAT1 signals, such as HLH. Detailed investigations of the clinical spectrum of these two populations should be conducted.

**Acknowledgments**

We thank Dr. D.M. Stewart (Metabolism Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD) for reviewing the manuscript, the patients and their families for participation in this study, and Dr. H. Kanegane (Department of Pediatrics, Graduate School of Medicine, University of Toyama, Toyama, Japan) for coordinating patient recruitment.

**Disclosures**

The authors have no financial conflicts of interest.

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