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A heterozygous dominant-negative mutation in the coiled-coil domain of STAT1 is the cause of autosomal-dominant Mendelian susceptibility to mycobacterial diseases

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ABSTRACT

Heterozygous dominant-negative mutations of *STAT1* are responsible for autosomal-dominant Mendelian susceptibility to mycobacterial diseases (AD-MSMD). So far, only 7 mutations have been previously described and are localized to 3 domains: the DNA-binding domain, the SH_2 domain, and the tail segment. In this study, we demonstrated the first coiled-coil domain (CCD) mutation of c.749G>C, p.G250A (G250A) in *STAT1* as a genetic cause of AD-MSMD in a patient with mycobacterial multiple osteomyelitis. This *de novo* heterozygous mutation was shown to have a dominant-negative effect on the gamma-activated sequence (GAS) transcriptional activity following IFN-γ stimulation, which could be attributable to the abolished phosphorylation of STAT1 from the wild-type (WT) allele. The three-dimensional structure of STAT1 revealed the G250 residue was located distant from a cluster of residues affected by gain-of-function mutations responsible for chronic mucocutaneous candidiasis.

1. Introduction

Mendelian susceptibility to mycobacterial diseases (MSMD) is one of the primary immunodeficiency diseases characterized by susceptibility to weakly virulent mycobacteria such as Bacillus-Calmette-Guerin (BCG) or non-tuberculosis mycobacteria and unsusceptibility to other microbial infections [1]. So far, mutations in 9 genes responsible for MSMD have been reported, which are related with IL12-IFN-γ circuit: *STAT1*, *IFNGR1*, *IFNGR2*, *IKBKG*, *IL12RB1*, *IL12B*, *CYBB*, *IRF8*, and *ISG15* [2–14].

STAT1 is both a signal transducer and a transcriptional activator regulating specific gene expression. In response to IFN-γ, STAT1 is phosphorylated by Janus kinase 1 and 2, and is then translocated into the nucleus after forming homodimers. STAT1 homodimers bind the gamma-activated sequence (GAS) to induce expression of their targeted genes. In contrast, IFN-α/β stimulation induces phosphorylation of STAT1 and STAT2. Phosphorylated STAT1 and STAT2 form heterotrimers with IRF9, migrate into the nucleus to bind IFN-stimulated response element (ISRE), and induce specific gene expression. IFN-γ-mediated responses are crucial for protection from mycobacterium or other intracellular microorganisms, whereas IFN-α/β signals are crucial for protection from most viruses.

Heterozygous dominant-negative mutations of *STAT1* are responsible for autosomal-dominant MSMD (AD-MSMD). These mutants were demonstrated to have dominant-negative effects on the GAS transcriptional activity following IFN-γ stimulation, but not on the ISRE transcriptional activity following IFN-α stimulation. Previous reports have demonstrated 7 *STAT1* mutations responsible for AD-MSMD: 2 in the DNA-binding domain (DBD), 3 in the SH2 domain, and 2 in the tail segment [7,11–14].

In this study, we demonstrated the first coiled-coil domain (CCD) mutation of c.749G>C, p.G250A (G250A) in *STAT1* as a genetic cause of AD-MSMD in a patient with mycobacterial multiple osteomyelitis. This heterozygous *de novo* mutation was associated with a dominant-negative effect on the GAS transcriptional activity following IFN-γ stimulation, which could be attributable to abolished phosphorylation of STAT1 from the WT allele.

Abbreviations: AD-MSMD, autosomal-dominant Mendelian susceptibility to mycobacterial diseases; GAS, gamma-activated sequence; ISRE, IFN-stimulated response element; WT, wild-type; CCD, coiled-coil domain; DBD, DNA-binding domain; PBMC, peripheral blood mononuclear cells; CMCD, chronic mucocutaneous candidiasis; qRT-PCR, quantitative RT-PCR.

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2. Materials and methods

2.1. Patient

Patient is a 3-y-old girl born to nonconsanguineous healthy Japanese parents. She received BCG vaccination at the age of 4 months and presented with left axillary lymphadenitis and skin nodules 3 months later. Although isoniazid therapy for 6 months led to resolution of the lesions, she developed submaxillary swelling at the age of 2 years and 5 months. On admission, she was afebrile but her laboratory data demonstrated elevated WBC of 18,860/μl with 47% neutrophils, 42% lymphocytes with normal subsets, and CRP of 8.08 mg/dl. The findings of magnetic resonance imaging (MRI) suggested multifocal osteomyelitis in the left clavicle, 8th rib, mandible bone, and cranial bones, and granulomatous lesions in liver, spleen, and subcutaneous tissues. Specimens from the mandible bone and skin nodules were positive for acid-fast stain, and *Mycobacterium bovis* BCG was identified by PCR of the strain grown in culture. Treatment with isoniazid and rifampicin for 3 months improved the MRI findings of most of the lesions except the mandible bone, which is also resolving during the course of the treatment. The patient is still under the same treatment regimen for 2 years, and she will be reevaluated at the end of the regimen. She has not suffered from other bacterial, viral, or fungal infections.

2.2. DNA and total RNA isolation, PCR and RT-PCR, TOPO-TA cloning, and sequence analysis

These procedures were performed following the methods described elsewhere [15].

2.3. Generation of EBV-transformed cell lines

EBV-transformed cell lines (EBV-LCLs) were generated by *in vitro* transformation of human B cells with EBV (Stain B95-8) as previously described [15]. EBV-LCLs from patient with a heterozygous G250A mutation was designated as G250A/WT. Two EBV-LCLs from healthy controls were designated as WT1 and WT2.

2.4. Cytometric Bead Array analysis of supernatant CXCL10/IP-10 (IP-10) and/or IL12p70 concentration in EBV-LCLs and monocyte-derived macrophages

To evaluate STAT1 function, we studied IP-10 production from EBV-LCLs and monocyte-derived macrophages. These procedures were described elsewhere [16]. Briefly, 1×10^6 cells/ml of EBV-LCLs from patient and controls (WT1 and WT2) in RPMI 1640 with 10% FBS with Penicillin-Streptomycin (RPMI 10% FBS) were left unstimulated or stimulated with 1000 U/ml (200 ng/ml) of IFN-γ (Shionogi, Osaka, Japan) for 6 h at 37 °C in a CO_2 incubator and the supernatant was stored for measuring IP-10 concentration. IP-10 concentration was studied by Cytometric Bead Array (BD, San Diego, CA) following manufacturer's instruction. For studying IP-10 and IL12p70 production from monocyte-derived macrophages, monocytes were first purified from peripheral blood mononuclear cells (PBMC) with CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). To obtain macrophages, 5×10^5 cells/ml monocytes were incubated for 7 d in RPMI 10% FBS containing 5 ng/ml M-CSF (R&D Systems, Minneapolis, MN). To see the effect of IFN-γ, differentiated macrophages in triplicate were unstimulated, stimulated with 1 μg/ml of LPS (Sigma, St Louis, MO), or first prestimulated with 1000 U/ml of IFN-γ for 2 h and then stimulated with 1 μg/ml of LPS for 24 h (described as "IFN-γ-LPS" stimulation). Supernatant from each sample was stored for studying IP-10 and IL12p70 concentration. Data from triplicated independent experiments were shown as the mean ± SD.

2.5. Quantitative RT-PCR (qRT-PCR) of CXCL9 and CXCL10

These procedures were performed following the methods described elsewhere [17]. Briefly, first strand cDNA was generated from total RNA isolated from EBV-LCLs after IFN-γ stimulation or without stimulation, and was then analyzed with ABI PRISM® 7000 (Applied Biosystems, Waltham, MA) using the SYBR Premix EX Taq™ II (Takara Bio, Kusatsu, Japan). Fluorescence threshold value (Ct value) was calculated using ABI PRISM® 7000 analysis software. Relative expression was calculated by the comparative ΔΔCt method. The sequence of primers was as follows; *CXCL9*, forward, 5′-TTTTCCTCTTGGGCATCATC-3′; reverse, 5′-TTGGGGCAAATTGTTTAAGG-3′; *CXCL10*, forward, 5′-CCACGTGTTGAGATCATTGC-3′; reverse, 5′-ATTTTGCTCCCCTCTGGTTT-3′; *GAPDH*, forward, 5′-AATGACCCCTTCATTGACCTC-3′; reverse, 5′-ATGGGATTTCCATTGATGACA-3′. Data from triplicated independent experiments were shown as the mean ± SD.

2.6. Luciferase assay

These procedures were performed following the methods previously reported with some modification [18]. To assess the dominant-negative effects of the G250A mutant on the transcriptional activity of STAT1 targets, we transiently cotransfected with luciferase reporter plasmids (Cignal GAS and ISRE Reporter Assay kit; SA Biosciences, Hilden, Germany) and plasmids expressing EGFP-STAT1 WT or the G250A mutant into HeLa cells using the TransIT-LT1 reagent (Mirus, Madison, WI). We also transfected with the EGFP-STAT1 L706S mutant which disrupts phosphorylation of the mutant itself [7]. After 24 h incubation, cells were left unstimulated or stimulated with 1000 U/ml IFN-γ or 1500 U/ml IFN-α (Biosource International, Camarillo, CA) for 16 h. Cells were lysed and analyzed by Dual-Luciferase Reporter Assay System (Promega, Madison, WI) following manufacturer's instructions. Data from triplicated independent experiments were shown as the mean ± SD.

2.7. Preparation of nuclear extract and Western blot analysis for the study of STAT1 phosphorylation state

These procedures were performed following the methods previously reported [16,19]. We used anti-human STAT1 and STAT1 (pY701) antibodies from BD, and an anti-Lamin A antibody from Cell Signaling Technology, Danvers, MA.

2.8. Transient transfection or cotransfection of plasmids expressing STAT1-fusion protein into HeLa cells for evaluation of phosphorylation state of STAT1 WT and each mutant itself

Firstly, to evaluate the phosphorylation state of STAT1 G250A mutant itself after IFN-γ stimulation, we transiently transfected HeLa cells with 2.5 μg of pEGFP-C2 construct expressing STAT1 WT, G250A, or L706S mutants fused with EGFP (EGFP-STAT1) using TransIT-HeLa monster kit (Mirus). After incubation for 24 h at 37 degrees C in a CO_2 incubator, phosphorylated STAT1 was studied by Western blot following 1000 U/ml of IFN-γ stimulation for 30 min. To study whether the STAT1 G250A mutant has a dominant-negative effect on the phosphorylation of STAT1 from the WT allele, we then cotransfected HeLa cells with 0.8 μg of pEGFP-C2-STAT1WT and 4 μg of pcDNA3-Histidine-construct expressing STAT1 (His-STAT1) WT or either G250A or L706S mutant at a weight ratio of 1:5, so that the HeLa cells expressing EGFP-STAT1 WT could have more chance to express cotransfected His-STAT1 WT or a mutant. We harvested them after IFN-γ stimulation for 30 min, and analyzed STAT1 phosphorylation.