Alanine-scanning mutagenesis of human signal transducer and activator of transcription 1 to estimate loss- or gain-of-function variants

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Background: Germline heterozygous mutations in human signal transducer and activator of transcription 1 *(STAT1)* can cause loss of function (LOF), as in patients with Mendelian susceptibility to mycobacterial diseases, or gain of function (GOF), as in patients with chronic mucocutaneous candidiasis. LOF and GOF mutations are equally rare and can affect the same domains of STAT1, especially the coiled-coil domain (CCD) and DNA-binding domain (DBD). Moreover, 6% of patients with chronic mucocutaneous candidiasis with a GOF *STAT1* mutation have mycobacterial disease, obscuring the functional significance of the identified *STAT1* mutations. Current computational approaches, such as combined annotation-dependent depletion, do not distinguish LOF and GOF variants.

Objective: We estimated variations in the CCD/DBD of STAT1.

Methods: We mutagenized 342 individual wild-type amino acids in the CCD/DBD (45.6% of full-length STAT1) to alanine and tested the mutants for STAT1 transcriptional activity.

Results: Of these 342 mutants, 201 were neutral, 30 were LOF, and 111 were GOF mutations in a luciferase assay. This assay system correctly estimated all previously reported LOF mutations (100%) and slightly fewer GOF mutations (78.1%) in the CCD/DBD of STAT1. We found that GOF alanine mutants occurred at the interface of the antiparallel STAT1 dimer, suggesting that they destabilize this dimer. This assay also precisely predicted the effect of 2 hypomorphic and dominant negative mutations, E157K and G250E, in the CCD of STAT1 that we found in 2 unrelated patients with Mendelian susceptibility to mycobacterial diseases.

Conclusion: The systematic alanine-scanning assay is a useful tool to estimate the GOF or LOF status and the effect of heterozygous missense mutations in *STAT1* identified in patients with severe infectious diseases, including mycobacterial and fungal diseases. (J Allergy Clin Immunol 2016;■■■:■■■-■■■.)

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Signal transducer and activator of transcription (STAT) 1 is a latent cytoplasmic transcription factor belonging to the STAT family. STAT1 is phosphorylated on tyrosine 701 (Y701) by the Janus kinases when a cytokine or growth factor binds to its receptor, allowing STAT1 dimerization. STAT1 can form a homodimer, which is known as IFN-γ activation factor (GAF), after stimulation by IFN-γ, IFN-α/β, or IL-27. GAF translocates to the nucleus and binds to specific DNA sequences known as gamma-activating sequences (GASs) in the promoters of interferon-stimulated genes to induce their transcription.[1] STAT1 also forms a heterotrimer after stimulation by IFN-α/β or IFN-λ, consisting of STAT1, STAT2, and interferon regulatory factor (IRF) 9, which is known as interferon-stimulated gene factor 3 (ISGF3). ISGF3 binds the interferon-stimulated response element, initiating gene transcription.[1] The deactivation of STAT1 is mediated by a nuclear phosphatase thought to be TC45, which dephosphorylates STAT1, allowing its release into the cytoplasm.[2,3] In human subjects STAT1 plays a nonredundant role in IFN-α/β, IFN-γ, IL-27, and IFN-λ signaling.[4]

Inborn errors in human STAT1-based immunity cause 4 types of immune deficiency: (1) autosomal recessive (AR) complete STAT1 deficiency, (2) AR partial STAT1 deficiency, (3) autosomal dominant (AD) STAT1 deficiency, and (4) AD STAT1 gain of activity.[4] Biallelic loss-of-function (LOF) mutations have been identified in patients with complete and partial AR STAT1 deficiency. These patients experience life-threatening viral infections (especially herpes virus infections) because they lack the STAT1-dependent response to IFN-α/β (and perhaps IFN-λ) signaling and experience mycobacterial susceptibility because they lack the response to IFN-γ (and perhaps IL-27).[5] Partial AR STAT1 deficiency is a milder form, and therefore patients with this disorder have mild viral and mycobacterial diseases and impaired but not abolished responses to IFN-α/β, IFN-γ, IL-27, and IFN-λ signaling.[6-8] Heterozygous LOF *STAT1* mutations cause Mendelian susceptibility to mycobacterial diseases (MSMD), which is attributable to the impairment of IFN-γ signaling, and mutations have been identified in the DNA-binding domain (DBD), SH2 domain, and transactivation domain (Fig 1, *A*).[9-14] Heterozygous gain-of-function (GOF) mutations underlie chronic mucocutaneous candidiasis (CMC) and have been most commonly identified in the coiled-coil domain (CCD) and the DBD (Fig 1, *A*).[15-49]

LOF and GOF mutations can affect the same domains in STAT1, obscuring the behavior of a particular mutation based solely on its location. Therefore we systematically investigated the effects of alanine substitutions in the CCD and DBD (CCD/DBD) of STAT1, screening 342 alanine mutants (in a total of 750 residues) with a GAS reporter assay after IFN-γ stimulation. All the LOF MSMD-causing mutations and most (78.1%) GOF CMC-related mutations previously identified in the CCD/DBD were correctly identified in the alanine mutants, suggesting that this technique can be used to establish a reference library of STAT1 variants. Our results clearly demonstrate that the majority of GOF mutations are located at the interface of the antiparallel STAT1 dimer, probably disrupting the dimerization of antiparallel STAT1 structures. We confirmed our results by identifying 2 germline heterozygous hypomorphic and dominant negative mutations in the CCD of STAT1 in patients with MSMD.

Abbreviations used

AD:	Autosomal dominant
AR:	Autosomal recessive
CADD:	Combined annotation-dependent depletion
CMC:	Chronic mucocutaneous candidiasis
DBD:	DNA-binding domain
dbSNP:	Single Nucleotide Polymorphism Database
ExAc:	Exome Aggregation Consortium
GAF:	IFN-γ activation factor
GAS:	Gamma-activating sequence
GOF:	Gain of function
IRF:	Interferon regulatory factor
ISGF3:	Interferon-stimulated gene factor 3
LOF:	Loss of function
MSC:	Mutation significance cutoff
MSMD:	Mendelian susceptibility to mycobacterial diseases
STAT:	Signal transducer and activator of transcription
WT:	Wild-type

METHODS

Functional assay based on systematic alanine-scanning mutagenesis

A vector from which to express HaloTag STAT1 was obtained from the Kazusa cDNA/ORF clone collection (FHC013013). The codons of the vector-encoding residues from L136 to F487 of STAT1, except the 10 alanines (A188, A230, A246, A254, A267, A401, A402, A415, A469, and A479), were individually substituted with GCC, the codon most frequently encoding alanine in human subjects, by using site-directed mutagenesis. The activities of the mutants were measured with a luciferase reporter assay with the pGL4.24 vector (Promega, Madison, Wis) driven by 5 tandem IRF-derived GAS elements (TTCCCCGAA; IRF1 reporter plasmids). Detailed methods of the luciferase reporter assay and the other experiments are available in the Methods section in this article's Online Repository at www.jacionline.org.

Case

Detailed case reports of kindreds A, B, and C (Fig 1) are available in the Methods section in this article's Online Repository. Briefly, 8 patients from 3 unrelated families are included in this study. Six patients received BCG at infancy, and 5 of the 6 had BCGitis. Multifocal osteomyelitis was observed in 5 patients, including 4 with BCGitis. Two patients who did not receive BCG had a history of tuberculosis. One patient who does not have an obvious history of BCGitis had intracranial granuloma with *Mycobacterium avium* complex detectable by means of PCR from brain biopsy specimens. The penetrance of STAT1 mutation was not complete. One family member in kindred A who has no history of BCG vaccination turned out to have STAT1 mutation based on the results of a familial study.

RESULTS

Functional assay based on systematic alanine-scanning mutagenesis

Alanine-scanning mutagenesis is a widely used technique in the determination of the catalytic or functional role of protein residues. We systematically investigated the effects of alanine substitutions in the CCD/DBD of STAT1 with a GAS reporter assay after IFN-γ stimulation. We generated 176 alanine mutants

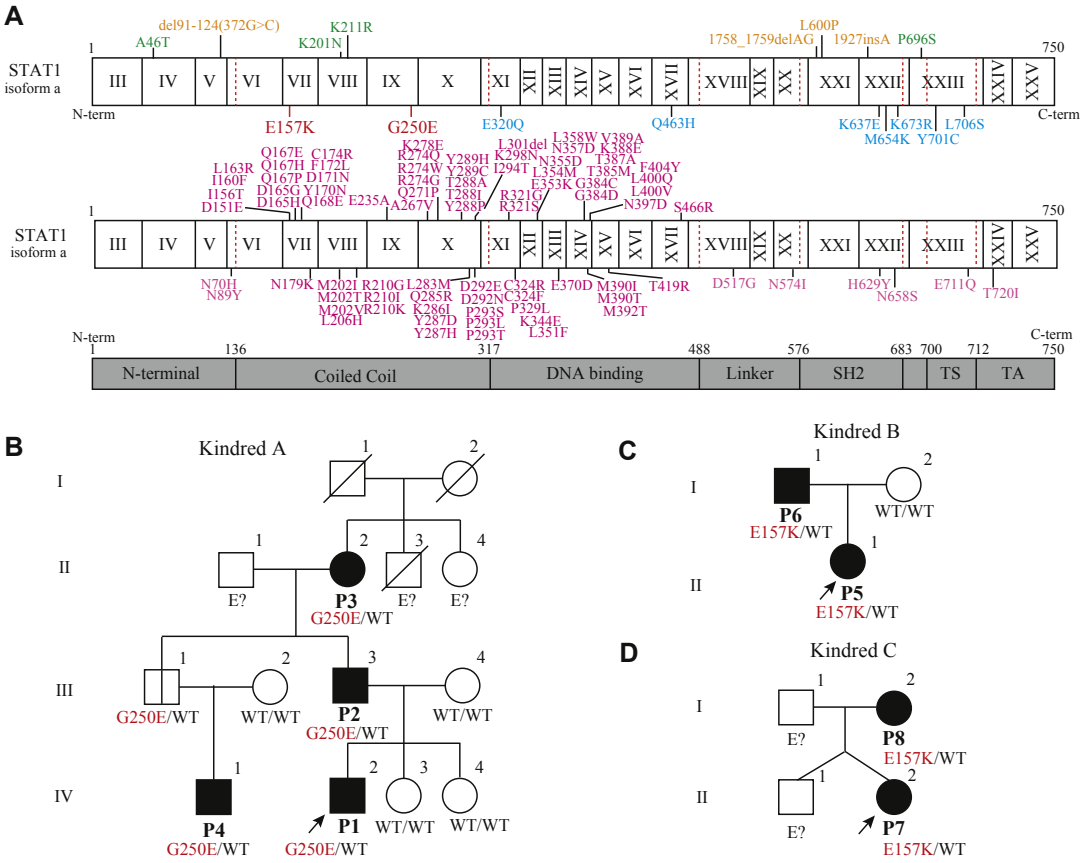

FIG 1. Family trees and known pathogenic STAT1 mutations. **A,** Human STAT1α isoform with its known pathogenic mutations. Mutations identified in patients with an AR form of complete (orange) or partial (green) STAT1 deficiency are shown above. Mutations identified in patients with AD MSMD are shown in red (current study) and blue (previously reported). The reported GOF mutations are shown in magenta below the protein. *TA*, Transcriptional activation domain; *TS*, tail segment. **B-D,** Family trees.

in the CCD and 166 alanine mutants in the DBD. We defined the individual alanine mutants as LOF or GOF if they showed less than 0.3 times or greater than 1.6 times the GAS transcriptional activity of wild-type (WT), respectively (Fig 2). These cutoffs were defined based on the GAS transcriptional activity of known pathogenic mutations. The functional assay based on systematic alanine-scanning mutagenesis (designated the alanine-scanning assay) showed that 34.7% (61/176) of the alanine mutants in the CCD were GOF and 2.2% (4/176) were LOF. In the DBD 30.1% (50/166) of the mutants were GOF and 15.7% (26/166) were LOF. These results are consistent with the clinical observation that most reported STAT1 mutations in the CCD/DBD are GOF. Many CMC-related GOF mutations have been identified in the CCD/DBD of STAT1 in previous studies.[15-44] In contrast, only 2 MSMD-causing LOF STAT1 mutations (E320Q and Q463H) have been identified in the DBD, and no LOF mutation has been reported in the CCD.[10]

The results of the alanine-scanning assay were compared with previously identified GOF and LOF mutations in STAT1 (see Table E1 in this article's Online Repository at www.jacionline.org). The assay allowed us to explain 100% of LOF mutants and 78.1% (86.7% in the CCD and 64.3% in the DBD) of GOF mutants in the CCD/DBD of STAT1. No known LOF mutations were deemed to be GOF mutations. Only 1 GOF mutation was misidentified as an LOF mutation in the alanine-substituted mutants. Fifteen GOF mutations (20.5%) were falsely identified as neutral. We then evaluated STAT1 variants that are occasionally identified in healthy subjects and are listed in the Single Nucleotide Polymorphism Database (dbSNP), 1000 Genomes Project, and/or the Exome Aggregation Consortium (ExAc) database, with the alanine-scanning assay (see Table E2 in this article's Online Repository at www.jacionline.org). This assay estimated 71.1% (27/38), 23.7% (9/38), and 5.4% (2/38) of the variations as neutral, GOF, and LOF mutations, respectively. We next blotted the GOF and LOF alanine mutants in the parallel and antiparallel dimeric STAT1 structures (Fig 3). Intriguingly, the GOF alanine mutations predominantly localized at the interface of the antiparallel dimer (Fig 3, *A*). In contrast, several LOF alanine mutations localized close to the DNA-binding site (Fig 3, *B*). Although the other LOF alanine mutations localized inside the protein, there was no obvious preferential localization.

Mutation significance cutoff effect estimation of *STAT1* mutations and variations in the CCD/DBD

We evaluated the previously identified mutations with mutation significance cutoff (MSC) effect estimation based on their combined annotation-dependent depletion (CADD) scores. The CADD scores of all previously identified GOF and

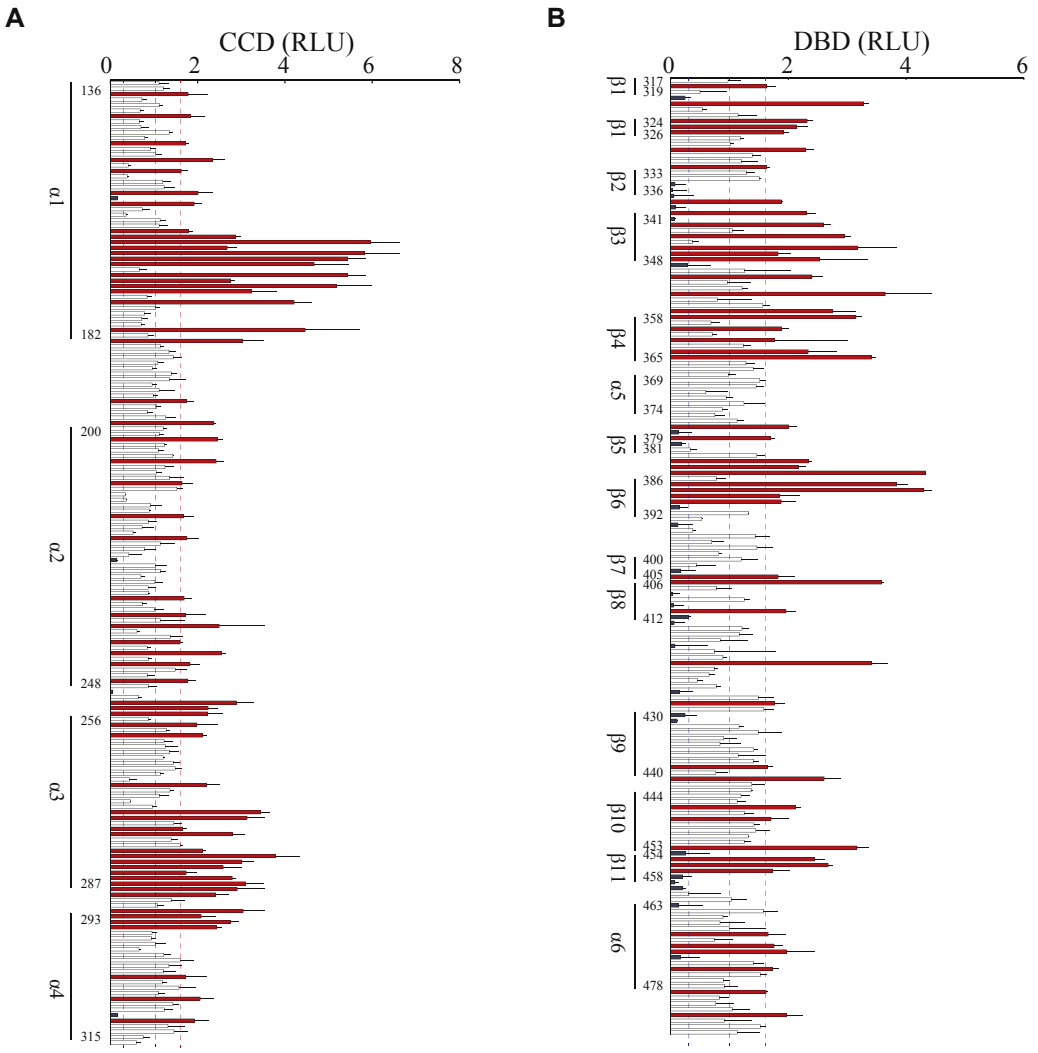

FIG 2. GAS transcription assay based on systematic alanine-scanning mutagenesis. GAS transcriptional activity in response to IFN-γ was measured in 176 STAT1 proteins mutated in the CCD **(A)** and 166 STAT1 proteins mutated in the DBD **(B)**. Of alanine mutants, 34.7% (61/176) and 30.1% (50/166) were deemed to be GOF mutations, whereas 2.2% (4/176) and 15.7% (26/166) were deemed to be LOF mutations in the CCD and DBD, respectively. Experiments were performed in triplicate, and data are expressed in relative luciferase units *(RLU)*.

LOF mutations, except E235A and R321G, in the CCD/DBD of STAT1 were greater than 7.736 (the benign/damaging cutoff specific for STAT1) and were therefore estimated to have a high effect (see Table E1). However, 89.7% (35/39) of natural variations in the CCD/DBD of STAT1 listed in the dbSNP, 1000 Genomes Project, and ExAc databases were also estimated to have a high effect. We also detected no significant differences in the CADD scores of the LOF and GOF mutations in *STAT1*.

Identification of *STAT1* mutations

The *STAT1* mutation in the proband (P1) was identified with the candidate gene approach for MSMD. We identified a novel heterozygous mutation, 746G>A (G250E), in *STAT1* with Sanger sequencing (see Fig E1 in this article's Online Repository at www.jacionline.org). No mutation was identified in *IFNGR1*, *IFNGR2*, *IKBKG*, *CYBB*, *IRF8*, or *ISG15* in P1. Another novel heterozygous mutation in *STAT1*, 469G>A (E157K), was identified in unrelated patients (P5 and P7) by using whole-exome sequencing and confirmed with Sanger sequencing. Neither the E157K nor G250E mutation was found in the National Center for Biotechnology Information, Ensembl, dbSNPs, or ExAc databases or in our own in-house database of 3000 exomes. Nor were they detected in the 1052 control samples from 52 ethnic groups in the Centre d'Etude du Polymorphisme Humain and Human Genome Diversity panels.[50,51] Therefore they are rare MSMD-causing variants rather than irrelevant polymorphisms. The G250E mutation was identified in P1, P2, P3, and P4, which is consistent with AD inheritance. The same mutation was also identified in an asymptomatic uncle (A.III.1), who had not been vaccinated with BCG. The E157K mutation was identified in P5 and P6 in kindred B and in P7 and P8 in kindred C. Thus we identified 2 novel heterozygous mutations, E157K and G250E, in the CCD of STAT1 in 7 patients from 3 families (Fig 1, *B* and *D*).

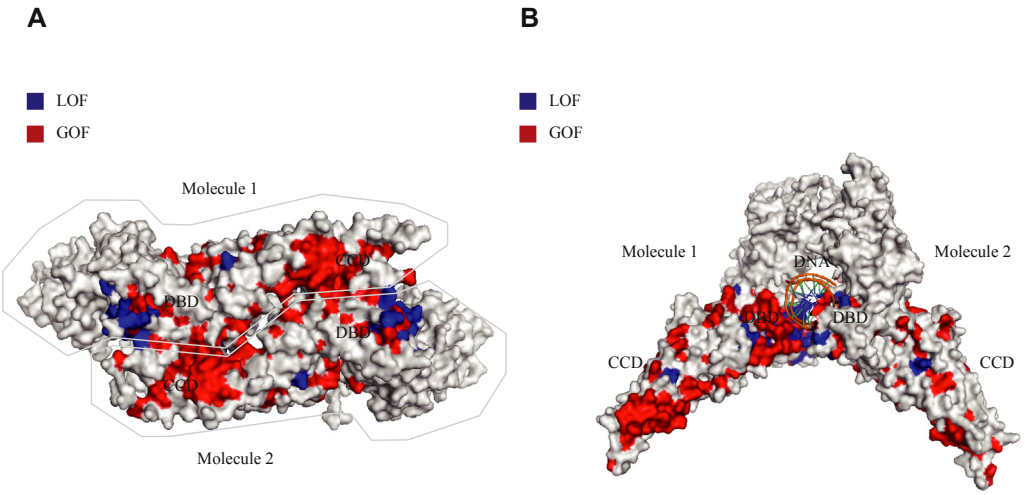

FIG 3. Distributions of LOF and GOF mutations in antiparallel and parallel STAT1 dimers. GOF (red) and LOF (blue) alanine substituents were mapped to the antiparallel **(A)** and parallel **(B)** dimeric structures of STAT1. Fig 3, *A*, GOF alanine mutants predominantly localized at the interface of the antiparallel dimer, whereas LOF alanine mutants localized inside the protein. Fig 3, *B*, Many LOF alanine mutants localized close to the DNA-binding site.

E157K and G250E in STAT1 partially impair its phosphorylation, GAS binding, and GAS transactivation in response to IFN-γ

We transiently introduced WT and/or mutant *STAT1*-containing plasmids into U3C cells, a STAT1-null fibrosarcoma cell line, with lipofection and analyzed the expression and Y701 phosphorylation of STAT1 after IFN-γ stimulation, with immunoblotting (Fig 4, *A*, and see Fig E2, *A*, in this article's Online Repository at www.jacionline.org). Both E157K and G250E STAT1 and the K673R dominant negative hypomorphic mutant that causes AD STAT1 deficiency were expressed normally but showed partially impaired phosphorylation after IFN-γ stimulation.[12] In contrast, the R274Q mutation, a common GOF mutation in patients with CMC, showed increased phosphorylation in response to IFN-γ.[15] Consistent with previous reports, Q463H STAT1 was normally phosphorylated, and Y701C abolished STAT1 phosphorylation after IFN-γ stimulation.[10,11]

We then analyzed the intrinsic DNA-binding ability of the STAT1 proteins with an electrophoretic mobility shift assay. U3C cells expressing WT or mutant STAT1 were stimulated with IFN-γ and subjected to electrophoretic mobility shift assay with a GAS probe. E157K and G250E, as well as K673R, STAT1 displayed markedly lower levels of GAF binding to GAS (Fig 4, *B*, and see Fig E2, *B*). Y701C STAT1 had no DNA-binding ability, whereas R274Q STAT1 showed increased DNA-binding ability.

We next investigated the effect of the E157K and G250E mutations on the transcriptional activity of GAS in assays with GAS reporter plasmids. Like K673R STAT1, both E157K and G250E STAT1 severely impaired STAT1-mediated signaling but did not abolish it, as Q463H and Y701C STAT1 did, disrupting GAS transcriptional activity after IFN-γ stimulation (Fig 4, *C*). Both E157K and G250E STAT1, as well as the other previously reported dominant negative STAT1 mutations, had dose-dependent negative effects in cotransfection experiments, suggesting that both mutants exert a dominant negative effect on IFN-γ–induced WT STAT1–mediated GAS activation (Fig 4, *D*, and see Fig E3 in this article's Online Repository at www.jacionline.org).

E157K and G250E in STAT1 partially impair nuclear localization

We analyzed the nuclear translocation of STAT1 in U2OS cells stably expressing V5-tagged WT, E157K, G250E, Q463H, K673R, Y701C, or R274Q STAT1 proteins.[10-12,15,16] STAT1 was mainly observed in the cytoplasm of unstimulated cells (Fig 5, *A*, and see Fig E2, *C*). After IFN-γ stimulation, STAT1 was mainly observed in the nuclei of cells expressing WT, Q463H, or R274Q STAT1, whereas Y701C STAT1 remained in the cytoplasm (Fig 5, *B*, and see Fig E2, *D*). E157K, G250E, and K673R STAT1 displayed partially impaired nuclear translocation.

E157K STAT1 forms a hydrogen bond with E394 in its antiparallel partner

STAT1 forms both parallel and antiparallel dimers.[52,53] Phosphorylated STAT1 forms parallel dimers (active form), whereas unphosphorylated STAT1 preferentially forms antiparallel dimers (inactive form). When STAT1 forms antiparallel dimers, the CCD and DBD associate reciprocally.[2] We mapped the positions of E157K and G250E, together with 6 other well-characterized mutations (GOF: D165G and R274Q in the CCD and N355D and K388E in DBD; LOF: E320Q and Q463H in the DBD) in both the parallel (see Fig E4, *A*, in this article's Online Repository at www.jacionline.org) and antiparallel STAT1 dimers (see Fig E4, *B*). In the parallel structure G250E was located close to the linker domain, which plays an important role in DNA binding.[54] This mutation also localized close to E320Q, which impairs STAT1 phosphorylation and the binding of GAF to GAS.[10] The Q463H mutation, which specifically impairs DNA binding, was located very close to the DNA-binding site. In contrast, the E157K mutation localized far from the DNA-binding site and close to the GOF mutations D165G and R274Q in the CCD. When we examined these mutations in the antiparallel structure, all 4 GOF mutations were located at the dimerization interface. The G250E, E320Q, and Q463H mutations were located inside the protein, relatively

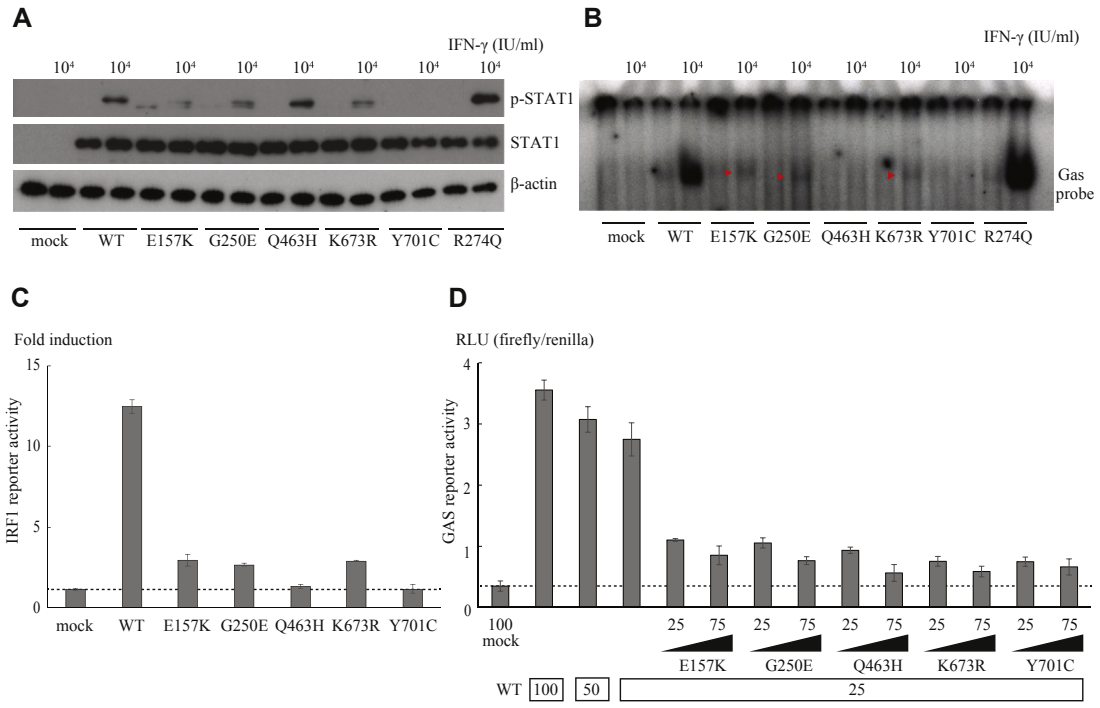

FIG 4. Functional assays of E157K and G250E STAT1 identified in patients with MSMD. **A,** Levels of pSTAT1 were reduced in the E157K and G250E mutants in response to IFN-γ to levels similar to those in the K673R mutant. **B** and **C,** E157K and G250E STAT1, as well as the K673R hypomorphic mutant, severely impaired the ability of GAF to bind GAS (Fig 4, *B*) and induce IRF1 reporter activity after IFN-γ stimulation (Fig 4, *C*). In contrast, Q463H and Y701C STAT1 completely abolished DNA binding to GAS (Fig 4, *B*) and IRF1 activation (Fig 4, *C*). **D,** All the mutants exerted a dose-dependent negative effect on WT STAT1–mediated GAS activation after IFN-γ stimulation. The amounts of plasmids used are shown below the *x-axis*.

far from the dimerization interface, in the antiparallel dimer. However, the E157K LOF mutation and other GOF mutations were located at the dimeric interface in the antiparallel structure.

We next mapped known pathogenic mutations (35 GOF and 4 LOF mutations) in the CCD/DBD in the parallel (see Fig E5, *A*, in this article's Online Repository at www.jacionline.org) and antiparallel (see Fig E5, *B*) dimers. As in the alanine-scanning assay, the GOF mutations were preferentially observed at the molecular interface in the antiparallel dimer. These observations suggest that some specific mechanism underlies the LOF of E157K STAT1. Therefore we analyzed E157K STAT1 *in silico* by using the protein modeling software MOE (Fig 6, *A* and *B*, and see Fig E6 in this article's Online Repository at www.jacionline.org). The mutant K157 residue was predicted to form a hydrogen bond with E394 of its antiparallel partner, stabilizing the antiparallel STAT1 dimer.

Strength of interaction between residues E157 and E394 affects GAS transcriptional activity

We then investigated the functional significance of mutation E157K by focusing on the interaction between residues E157 and E394. An MOE analysis predicted that the mutant K394 residue forms a hydrogen bond with E157 (see Fig E6). Therefore we generated 2 STAT1 mutants, E394K and E157K+E394K (double mutant), and investigated their functions (Fig 6, *C* and *D*). As expected, E394K STAT1 showed LOF in a GAS reporter assay, like the E157K mutant (Fig 6, *E*). However, surprisingly, the E157K+E394K double mutant showed GOF, with markedly increased GAS transcriptional activity (Fig 6, *E*). Glutamic acid is a negatively charged amino acid, whereas lysine is positively charged. Therefore we inferred that the electrostatic repulsion between residues K157 and K394 underlies the GOF of the E157K+E394K double mutant (Fig 6, *D*). To confirm this inference, we substituted residues E157 and E394 with arginine, another positively charged amino acid (see Fig E7 in this article's Online Repository at www.jacionline.org), and tested the functional significance of these changes in a GAS reporter assay (Fig 6, *E*). Like the lysine-substituted mutants, both E157R and E394R STAT1 displayed LOF, whereas the E157R+E394R double mutant displayed GOF.

The alanine-scanning assay showed that E157A STAT1 is an LOF mutant. Therefore we investigated the effect of A157 with MOE. Although LOF E157A STAT1 formed no hydrogen bond with E394, a conformational change was predicted that allowed amino acid H158 to form a hydrogen bond with E394 (see Fig E8 in this article's Online Repository at www.jacionline.org).

DISCUSSION

We systematically evaluated the effect of amino acid substitutions (342 alanine mutants) in the CCD/DBD of STAT1 using a GAS reporter assay. The GAS reporter assay is an accurate and practical method with which to assess LOF and GOF STAT1 mutations.[11,12,15,19-21] This assay allowed us to explain 100% of known LOF mutations and 78.1% (86.7% in the CCD and 64.3% in the DBD) of known GOF mutations in the CCD/DBD of STAT1 by using alanine substituents of the same residues. It

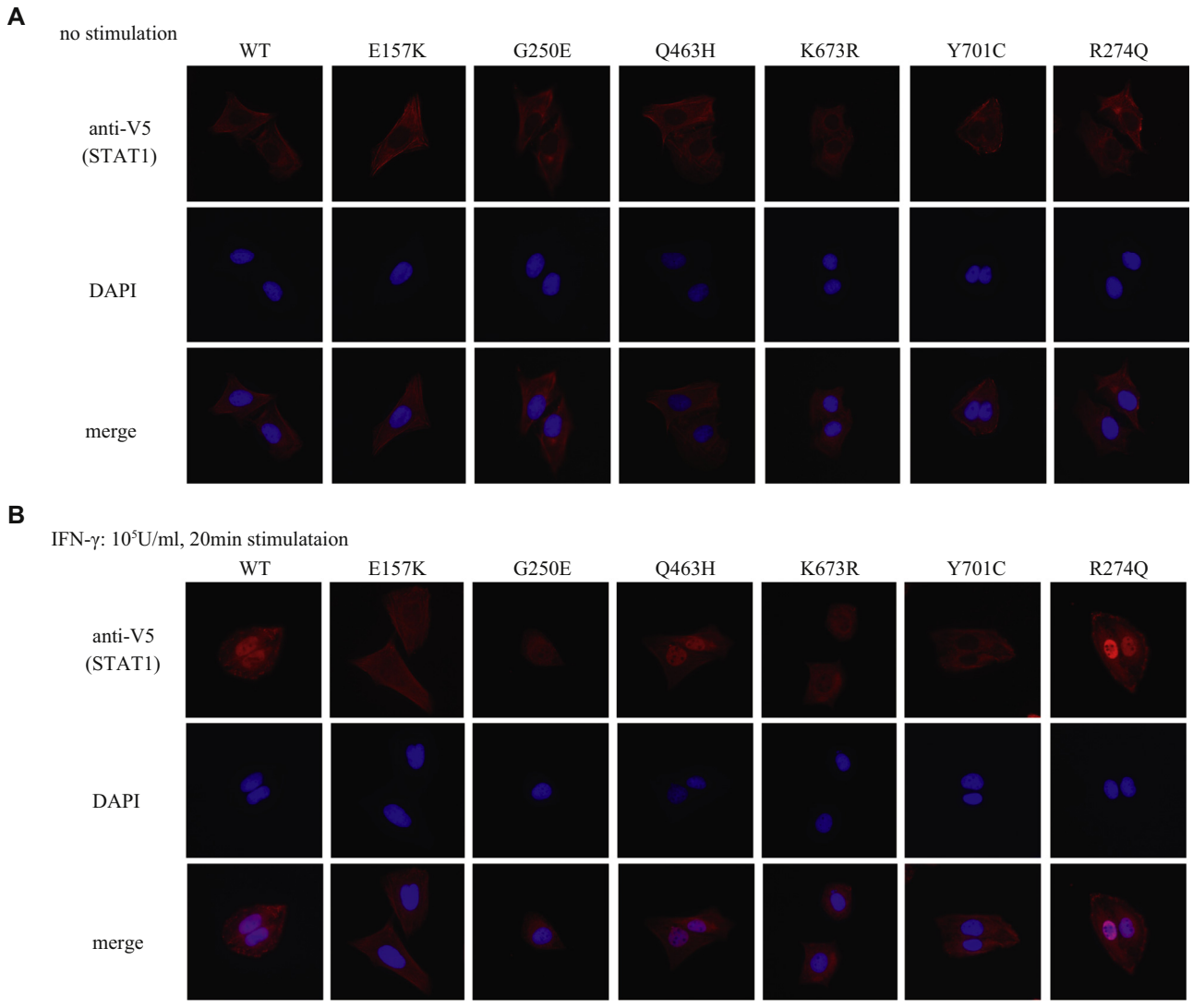

FIG 5. Subcellular localization of WT and mutant STAT1. U2OS cells stably expressing V5-tagged WT or mutant STAT1 were stimulated with IFN-γ for 20 minutes and subjected to immunostaining. **A,** Before IFN-γ stimulation, the WT and all mutant STAT1 proteins were localized to the cytoplasm. **B,** Like the K673R mutant, the nuclear translocation of E157K and G250E STAT1 was partially impaired in cells expressing either protein. In contrast, Y701C completely abolished the nuclear translocation of STAT1 after IFN-γ stimulation.

showed that 34.7% (61/176) and 30.1% (50/166) of alanine mutants were GOF, whereas 2.2% (4/176) and 15.7% (26/166) were LOF in the CCD and DBD, respectively. This is consistent with previous clinical observations that most reported disease-causing mutations in the CCD/DBD are GOF and are associated with CMC.[4,15,16,22,36] In contrast, only 2 AD MSMD-related LOF mutations, E320Q and Q463H, both in the DBD, have been reported in those domains.[10] The alanine-scanning assay revealed that 4 of 176 alanine substituents (E157A, T224A, G250A, and Q311A) in the CCD were LOF mutations. This result was confirmed by the identification of 3 familial cases of a patient with AD MSMD carrying LOF mutations in the CCD. The functional significance of the E157K and G250E mutations, which were newly identified in those patients, were correctly predicted with the alanine-scanning assay, confirming the efficiency and reliability of this assay system.

The alanine-scanning assay also showed that the GOF alanine mutants predominantly localized to the molecular interface of the antiparallel dimer. This assay has contributed to the evaluation of protein-protein interfaces by disturbing the roles of the side-chain functional groups at specific positions and the energetic contributions of individual side chains to protein binding.[55] Therefore the distribution of the GOF alanine mutants suggests that the disruption of the antiparallel dimer is a key molecular mechanism underlying the GOF STAT1 mutations. *In vitro* and *in silico* analyses of 2 STAT1 residues, E157 and E394, strongly supported this hypothesis. Both E157K and E394K STAT1 were evaluated as LOF mutations by using the GAS reporter assay and were predicted to stabilize the antiparallel dimerization of STAT1 by forming an additional hydrogen bond with MOE. In contrast, the E157K+E394K double mutant was identified as a GOF mutant with the GAS reporter assay. These findings were confirmed with arginine substitutions at residues E157 and

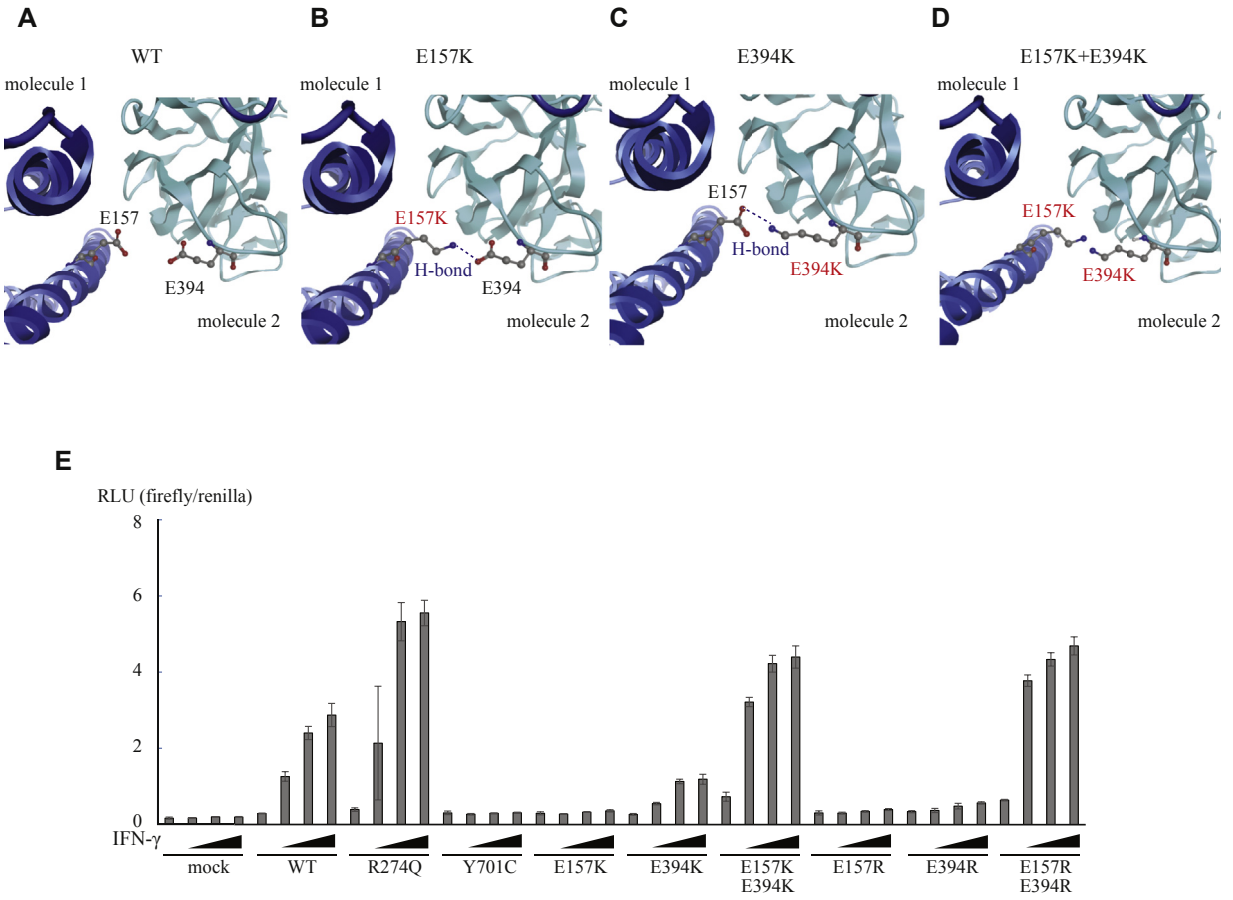

FIG 6. Interaction between E157 and E394 residues affects GAS transcriptional activity. **A-C,** E157K STAT1 was analyzed *in silico* with MOE. Unlike WT STAT1 (Fig 6, *A*), the E157K (Fig 6, *B*) and E394K (Fig 6, *C*) mutants were predicted to form hydrogen bonds with E394 and E157 in their dimeric partners, respectively. **D,** The E157K+E394K double mutant was predicted to allow access to their side chains, leading to electrostatic repulsion between the two. **E,** Both E157K and E394K were LOF mutations, whereas the E157K+E394K double mutant was predicted to be a GOF mutant by using the reporter assay. Similar results were observed with arginine substitutions.

E394. Because both lysine and arginine are positively charged, the disruption of the antiparallel dimer by the electrostatic repulsion between residues 157 and 394 of the double mutants was suspected to be the molecular basis of GOF. These observations are nicely explained by previous studies in which the antiparallel form of STAT1 was shown to facilitate the access of a phosphatase by presenting pY701 at both ends of the antiparallel dimer for ready dephosphorylation.[2,53]

Our results, together with those of previous studies, clearly support the hypothesis that the disturbance of the antiparallel STAT1 dimer affects its activity by altering its phosphorylation at Y701. The mutations that disrupt the antiparallel STAT1 dimer are GOF mutations, whereas the mutations that stabilize the antiparallel dimer are LOF mutations.

Although the alanine-scanning assay can be considered a good system for estimating the functional significance of STAT1 mutations, it has some limitations. First, this system cannot evaluate STAT1 mutations involving WT alanine residues, such as A267V, a known GOF mutation.

Second, the alanine-scanning assay does not always estimate the effect of STAT1 mutations appropriately. Indeed, this assay system misidentified 21.9% of GOF mutations and 28.9% of natural variants. This might have occurred because the alanine mutants cannot mimic the size effects or the hydrophilic or hydrophobic effects exerted by other substituted amino acids. Consequently, the establishment of a reference database of STAT1 variants, which measures the activity of all possible STAT1 substitutions (totaling 14,231 STAT1 mutants), might be a future goal to achieve an accurate evaluation of the functional significance of all STAT1 variants.

Next, to evaluate the applicability of the alanine-scanning assay, we compared the results of this assay system with the computational impact estimates of the MSC method, in which a CADD benign/damaging cutoff specific for STAT1 was used.[56-59] MSC showed a high true-positive estimation rate, with 97.3% (71/73) of known pathogenic GOF and LOF mutations in the CCD/DBD identified as deleterious. However, MSC misestimated 89.7% of the natural variants as damaging. The high rate of false-positive estimates, which was recently demonstrated empirically,[60] is a common problem of current computational effect estimates. Although MSC permits a low false-positive rate,[57] our results show that its false-positive rate was still 89.7% in evaluating the STAT1 variants. The allele frequencies of the STAT1 variants analyzed in the present study are quite

low in the general population (see Table E2), which could explain the high false-positive rate because the allele frequency correlates strongly with the effect estimate.[57,59,61] Our results stress the difficulty in the computational estimation of the mutations and variations in *STAT1* and support the utility of the alanine-scanning assay in their estimation.

The alanine-scanning assay, together with the identification of 3 familial cases of AD MSMD, have shown that LOF and GOF mutations coexist in both the CCD and the DBD of *STAT1*. AD partial STAT1 deficiency and AD STAT1 gain of activity are distinct primary immune deficiencies based on different molecular mechanisms. However, there is considerable overlap in their clinical manifestations. Although CMC is a prominent clinical manifestation in patients with GOF mutations, nearly 6% of these patients are also susceptible to mycobacterial infection.[45] Additionally, many patients with GOF mutations present with broad manifestations associated with bacterial and viral infections.[45] Furthermore, some patients with a GOF mutation have been reported to have combined immunodeficiency disease and immune dysregulation, polyendocrinopathy, enteropathy, X-linked–like syndrome, with severe and/or atypical clinical manifestations.[29,32]

Considering the clinical overlap and phenotypic diversity of their symptoms, we must carefully evaluate any unknown variations in the CCD/DBD of STAT1. The current advances in deep sequencing and genotyping methods have furthered the identification of the genetic cause of many diseases. Simultaneously, the evaluation of unknown variants has become increasingly important in clarifying disease-causing mutations. The current study is the first to establish a reference database for estimating the pathogenesis of naturally occurring genetic variations in a central disease-causing gene based on a systematic alanine-scanning assay. This study also demonstrated that the establishment of a reference database based on the alanine-scanning assay would be a useful tool for evaluating unknown STAT1 variations found in genetic study of patients with primary immune deficiency.

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Key messages

- **A systematic alanine-scanning assay correctly estimated known LOF mutations (100%) and GOF mutations (78.1%) in the CCD/DBD of STAT1.**
- **A systematic alanine-scanning assay correctly estimated 71.1% of nonpathogenic natural variants.**
- **An alanine scanning assay provided better estimation of STAT1 variants compared with computational estimation.**

REFERENCES

1. Stark GR, Kerr IM, Williams BR, Silverman RH, Schreiber RD. How cells respond to interferons. Annu Rev Biochem 1998;67:227-64.
2. Mertens C, Zhong M, Krishnaraj R, Zou W, Chen X, Darnell JE Jr. Dephosphorylation of phosphotyrosine on STAT1 dimers requires extensive spatial reorientation of the monomers facilitated by the N-terminal domain. Genes Dev 2006;20:3372-81.
3. Meyer T, Hendry L, Begitt A, John S, Vinkemeier U. A single residue modulates tyrosine dephosphorylation, oligomerization, and nuclear accumulation of stat transcription factors. J Biol Chem 2004;279:18998-9007.
4. Boisson-Dupuis S, Kong XF, Okada S, Cypowyj S, Puel A, Abel L, et al. Inborn errors of human STAT1: allelic heterogeneity governs the diversity of immunological and infectious phenotypes. Curr Opin Immunol 2012;24:364-78.
5. Dupuis S, Jouanguy E, Al-Hajjar S, Fieschi C, Al-Mohsen IZ, Al-Jumaah S, et al. Impaired response to interferon-alpha/beta and lethal viral disease in human STAT1 deficiency. Nat Genet 2003;33:388-91.
6. Chapgier A, Kong XF, Boisson-Dupuis S, Jouanguy E, Averbuch D, Feinberg J, et al. A partial form of recessive STAT1 deficiency in humans. J Clin Invest 2009; 119:1502-14.
7. Kong XF, Ciancanelli M, Al-Hajjar S, Alsina L, Zumwalt T, Bustamante J, et al. A novel form of human STAT1 deficiency impairing early but not late responses to interferons. Blood 2010;116:5895-906.
8. Kristensen IA, Veirum JE, Moller BK, Christiansen M. Novel STAT1 alleles in a patient with impaired resistance to mycobacteria. J Clin Immunol 2011;31: 265-71.
9. Dupuis S, Dargemont C, Fieschi C, Thomassin N, Rosenzweig S, Harris J, et al. Impairment of mycobacterial but not viral immunity by a germline human STAT1 mutation. Science 2001;293:300-3.
10. Chapgier A, Boisson-Dupuis S, Jouanguy E, Vogt G, Feinberg J, Prochnicka-Chalufour A, et al. Novel STAT1 alleles in otherwise healthy patients with mycobacterial disease. PLoS Genet 2006;2:e131.
11. Hirata O, Okada S, Tsumura M, Kagawa R, Miki M, Kawaguchi H, et al. Heterozygosity for the Y701C STAT1 mutation in a multiplex kindred with multifocal osteomyelitis. Haematologica 2013;98:1641-9.
12. Tsumura M, Okada S, Sakai H, Yasunaga S, Ohtsubo M, Murata T, et al. Dominant-negative STAT1 SH2 domain mutations in unrelated patients with Mendelian susceptibility to mycobacterial disease. Hum Mutat 2012;33:1377-87.
13. Sampaio EP, Bax HI, Hsu AP, Kristosturyan E, Pechacek J, Chandrasekaran P, et al. A novel STAT1 mutation associated with disseminated mycobacterial disease. J Clin Immunol 2012;32:681-9.
14. Bustamante J, Boisson-Dupuis S, Abel L, Casanova JL. Mendelian susceptibility to mycobacterial disease: genetic, immunological, and clinical features of inborn errors of IFN-gamma immunity. Semin Immunol 2014;26:454-70.
15. Liu L, Okada S, Kong XF, Kreins AY, Cypowyj S, Abhyankar A, et al. Gain-of-function human STAT1 mutations impair IL-17 immunity and underlie chronic mucocutaneous candidiasis. J Exp Med 2011;208:1635-48.
16. van de Veerdonk FL, Plantinga TS, Hoischen A, Smeekens SP, Joosten LA, Gilissen C, et al. STAT1 mutations in autosomal dominant chronic mucocutaneous candidiasis. N Engl J Med 2011;365:54-61.
17. Toth B, Mehes L, Tasko S, Szalai Z, Tulassay Z, Cypowyj S, et al. Herpes in STAT1 gain-of-function mutation [corrected]. Lancet 2012;379:2500.
18. Aldave JC, Cachay E, Nunez L, Chunga A, Murillo S, Cypowyj S, et al. A 1-year-old girl with a gain-of-function STAT1 mutation treated with hematopoietic stem cell transplantation. J Clin Immunol 2013;33:1273-5.
19. Soltesz B, Toth B, Shabashova N, Bondarenko A, Okada S, Cypowyj S, et al. New and recurrent gain-of-function STAT1 mutations in patients with chronic mucocutaneous candidiasis from Eastern and Central Europe. J Med Genet 2013;50:567-78.
20. Mekki N, Ben-Mustapha I, Liu L, Boussofara L, Okada S, Cypowyj S, et al. IL-17 T cells' defective differentiation in vitro despite normal range ex vivo in chronic mucocutaneous candidiasis due to STAT1 mutation. J Invest Dermatol 2014;134: 1155-7.
21. Mizoguchi Y, Tsumura M, Okada S, Hirata O, Minegishi S, Imai K, et al. Simple diagnosis of STAT1 gain-of-function alleles in patients with chronic mucocutaneous candidiasis. J Leukoc Biol 2014;95:667-76.
22. Takezaki S, Yamada M, Kato M, Park MJ, Maruyama K, Yamazaki Y, et al. Chronic mucocutaneous candidiasis caused by a gain-of-function mutation in the STAT1 DNA-binding domain. J Immunol 2012;189:1521-6.
23. Yamazaki Y, Yamada M, Kawai T, Morio T, Onodera M, Ueki M, et al. Two novel gain-of-function mutations of STAT1 responsible for chronic mucocutaneous candidiasis disease: impaired production of IL-17A and IL-22, and the presence of anti-IL-17F autoantibody. J Immunol 2014;193:4880-7.
24. Shamriz O, Engelhard D, Rajs AP, Kaidar-Shwartz H, Casanova JL, Averbuch D. Mycobacterium szulgai chronic multifocal osteomyelitis in an adolescent with inherited STAT1 deficiency. Pediatr Infect Dis J 2013;32:1345-7.
25. Frans G, Moens L, Schaballie H, Van Eyck L, Borgers H, Wuyts M, et al. Gain-of-function mutations in signal transducer and activator of transcription 1 (STAT1): chronic mucocutaneous candidiasis accompanied by enamel defects and delayed dental shedding. J Allergy Clin Immunol 2014;134:1209-13.e6.
26. Kilic SS, Puel A, Casanova JL. Orf Infection in a Patient with Stat1 Gain-of-Function. J Clin Immunol 2014;35:80-3.

27. Kumar N, Hanks ME, Chandrasekaran P, Davis BC, Hsu AP, Van Wagoner NJ, et al. Gain-of-function signal transducer and activator of transcription 1 (STAT1) mutation-related primary immunodeficiency is associated with disseminated mucormycosis. J Allergy Clin Immunol 2014;134:236-9.
28. Sampaio EP, Hsu AP, Pechacek J, Bax HI, Dias DL, Paulson ML, et al. Signal transducer and activator of transcription 1 (STAT1) gain-of-function mutations and disseminated coccidioidomycosis and histoplasmosis. J Allergy Clin Immunol 2013;131:1624-34.
29. Sharfe N, Nahum A, Newell A, Dadi H, Ngan B, Pereira SL, et al. Fatal combined immunodeficiency associated with heterozygous mutation in STAT1. J Allergy Clin Immunol 2014;133:807-17.
30. Higgins E, Al Shehri T, McAleer MA, Conlon N, Feighery C, Lilic D, et al. Use of ruxolitinib to successfully treat chronic mucocutaneous candidiasis caused by gain-of-function signal transducer and activator of transcription 1 (STAT1) mutation. J Allergy Clin Immunol 2015;135:551-3.
31. Romberg N, Morbach H, Lawrence MG, Kim S, Kang I, Holland SM, et al. Gain-of-function STAT1 mutations are associated with PD-L1 overexpression and a defect in B-cell survival. J Allergy Clin Immunol 2013;131:1691-3.
32. Uzel G, Sampaio EP, Lawrence MG, Hsu AP, Hackett M, Dorsey MJ, et al. Dominant gain-of-function STAT1 mutations in FOXP3 wild-type immune dysregulation-polyendocrinopathy-enteropathy-X-linked-like syndrome. J Allergy Clin Immunol 2013;131:1611-23.
33. Hori T, Ohnishi H, Teramoto T, Tsubouchi K, Naiki T, Hirose Y, et al. Autosomal-dominant chronic mucocutaneous candidiasis with STAT1-mutation can be complicated with chronic active hepatitis and hypothyroidism. J Clin Immunol 2012;32:1213-20.
34. Wang X, Lin Z, Gao L, Wang A, Wan Z, Chen W, et al. Exome sequencing reveals a signal transducer and activator of transcription 1 (STAT1) mutation in a child with recalcitrant cutaneous fusariosis. J Allergy Clin Immunol 2013; 131:1242-3.
35. Lee PP, Mao H, Yang W, Chan KW, Ho MH, Lee TL, et al. Penicillium marneffei infection and impaired IFN-gamma immunity in humans with autosomal-dominant gain-of-phosphorylation STAT1 mutations. J Allergy Clin Immunol 2014;133:894-6.e5.
36. Depner M, Fuchs S, Raabe J, Frede N, Glocker C, Doffinger R, et al. The Extended clinical phenotype of 26 patients with chronic mucocutaneous candidiasis due to gain-of-function mutations in STAT1. J Clin Immunol 2016; 36:73-84.
37. Dhalla F, Fox H, Davenport EE, Sadler R, Anzilotti C, van Schouwenburg PA, et al. Chronic mucocutaneous candidiasis: characterization of a family with STAT-1 gain-of-function and development of an ex-vivo assay for Th17 deficiency of diagnostic utility. Clin Exp Immunol 2016;184:216-27.
38. Giardino G, Somma D, Cirillo E, Ruggiero G, Terrazzano G, Rubino V, et al. Novel STAT1 gain-of-function mutation and suppurative infections. Pediatr Allergy Immunol 2016;27:220-3.
39. Kataoka S, Muramatsu H, Okuno Y, Hayashi Y, Mizoguchi Y, Tsumura M, et al. Extrapulmonary tuberculosis mimicking Mendelian susceptibility to mycobacterial disease in a patient with signal transducer and activator of transcription 1 (STAT1) gain-of-function mutation. J Allergy Clin Immunol 2016;137:619-22.e1.
40. Martinez-Martinez L, Martinez-Saavedra MT, Fuentes-Prior P, Barnadas M, Rubiales MV, Noda J, et al. A novel gain-of-function STAT1 mutation resulting in basal phosphorylation of STAT1 and increased distal IFN-gamma-mediated responses in chronic mucocutaneous candidiasis. Mol Immunol 2015;68:597-605.
41. Nielsen J, Kofod-Olsen E, Spaun E, Larsen CS, Christiansen M, Mogensen TH. A STAT1-gain-of-function mutation causing Th17 deficiency with chronic mucocutaneous candidiasis, psoriasiform hyperkeratosis and dermatophytosis. BMJ Case Rep 2015;2015.
42. Tanimura M, Dohi K, Hirayama M, Sato Y, Sugiura E, Nakajima H, et al. Recurrent inflammatory aortic aneurysms in chronic mucocutaneous candidiasis with a gain-of-function STAT1 mutation. Int J Cardiol 2015;196:88-90.
43. Zheng J, van de Veerdonk FL, Crossland KL, Smeekens SP, Chan CM, Al Shehri T, et al. Gain-of-function STAT1 mutations impair STAT3 activity in patients with chronic mucocutaneous candidiasis (CMC). Eur J Immunol 2015;45: 2834-46.
44. Zerbe CS, Marciano BE, Katial RK, Santos CB, Adamo N, Hsu AP, et al. Progressive multifocal leukoencephalopathy in primary immune deficiencies: stat1 gain of function and review of the literature. Clin Infect Dis 2016;62:986-94.
45. Toubiana J, Okada S, Hiller J, Oleastro M, Lagos Gomez M, Aldave Becerra JC, et al. Heterozygous STAT1 gain-of-function mutations underlie an unexpectedly broad clinical phenotype. Blood 2016;127:3154-64.
46. Baris S, Alroqi F, Kiykim A, Karakoc-Aydiner E, Ogulur I, Ozen A, et al. Severe early-onset combined immunodeficiency due to heterozygous gain-of-function mutations in STAT1. J Clin Immunol 2016;36:641-8.
47. Dotta L, Scomodon O, Padoan R, Timpano S, Plebani A, Soresina A, et al. Clinical heterogeneity of dominant chronic mucocutaneous candidiasis disease: presenting as treatment-resistant candidiasis and chronic lung disease. Clin Immunol 2016;164:1-9.
48. Smeekens SP, Plantinga TS, van de Veerdonk FL, Heinhuis B, Hoischen A, Joosten LA, et al. STAT1 hyperphosphorylation and defective IL12R/IL23R signaling underlie defective immunity in autosomal dominant chronic mucocutaneous candidiasis. PLoS One 2011;6:e29248.
49. Al Rushood M, McCusker C, Mazer B, Alizadehfar R, Grimbacher B, Depner M, et al. Autosomal dominant cases of chronic mucocutaneous candidiasis segregates with mutations of signal transducer and activator of transcription 1, but not of Toll-like receptor 3. J Pediatr 2013;163:277-9.
50. Cann HM, de Toma C, Cazes L, Legrand MF, Morel V, Piouffre L, et al. A human genome diversity cell line panel. Science 2002;296:261-2.
51. Jakobsson M, Scholz SW, Scheet P, Gibbs JR, VanLiere JM, Fung HC, et al. Genotype, haplotype and copy-number variation in worldwide human populations. Nature 2008;451:998-1003.
52. Mao X, Ren Z, Parker GN, Sondermann H, Pastorello MA, Wang W, et al. Structural bases of unphosphorylated STAT1 association and receptor binding. Mol Cell 2005;17:761-71.
53. Zhong M, Henriksen MA, Takeuchi K, Schaefer O, Liu B, ten Hoeve J, et al. Implications of an antiparallel dimeric structure of nonphosphorylated STAT1 for the activation-inactivation cycle. Proc Natl Acad Sci U S A 2005;102:3966-71.
54. Lim CP, Cao X. Structure, function, and regulation of STAT proteins. Mol Biosyst 2006;2:536-50.
55. Moreira IS, Fernandes PA, Ramos MJ. Hot spots—a review of the protein-protein interface determinant amino-acid residues. Proteins 2007;68:803-12.
56. Kircher M, Witten DM, Jain P, O'Roak BJ, Cooper GM, Shendure J. A general framework for estimating the relative pathogenicity of human genetic variants. Nat Genet 2014;46:310-5.
57. Itan Y, Shang L, Boisson B, Ciancanelli MJ, Markle JG, Martinez-Barricarte R, et al. The mutation significance cutoff: gene-level thresholds for variant predictions. Nat Methods 2016;13:109-10.
58. Itan Y, Casanova JL. Can the impact of human genetic variations be predicted? Proc Natl Acad Sci U S A 2015;112:11426-7.
59. Itan Y, Shang L, Boisson B, Patin E, Bolze A, Moncada-Velez M, et al. The human gene damage index as a gene-level approach to prioritizing exome variants. Proc Natl Acad Sci U S A 2015;112:13615-20.
60. Miosge LA, Field MA, Sontani Y, Cho V, Johnson S, Palkova A, et al. Comparison of predicted and actual consequences of missense mutations. Proc Natl Acad Sci U S A 2015;112:E5189-98.
61. Gussow AB, Petrovski S, Wang Q, Allen AS, Goldstein DB. The intolerance to functional genetic variation of protein domains predicts the localization of pathogenic mutations within genes. Genome Biol 2016;17:9.