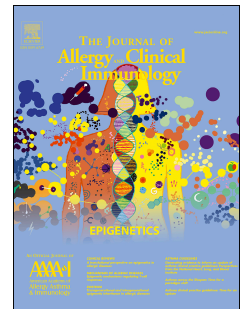


Journal Pre-proof

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Germline gain-of-function mutation of STAT1 rescued by somatic mosaicism in immune dysregulation-polyendocrinopathy-enteropathy-X-linked-like disorder

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Capsule Summary

We report a case of immune dysregulation-polyendocrinopathy-enteropathy-X-linked-like-disorder with a somatic mosaicism which eliminates *de novo* *STAT1* gain-of-function mutation in a proportion of T cells, and speculate that this contributes the autoimmunity in the patient.

Keywords

IPEX-like disorder, *STAT1*, Gain-of-function mutation, Somatic mosaicism

Disclosure of potential conflict of interest: The authors declare that they have no relevant conflicts of interest.

Journal Pre-proof

To the Editor:

Gain-of-function (GoF) mutations of signal transducer and activator of transcription 1 (STAT1) cause broad immune dysfunction ranging from simple immunodeficiency to complex immune disorders.¹ These mutations are frequently enriched in the coiled-coil domain or DNA-binding domain of STAT1 (Fig 1, A).^{2,3} *STAT1* GoF mutations lead to the uncontrolled phosphorylation of STAT1, which disrupts STAT1 dependent clonal expansion and memory formation of CD8⁺ T cells.^{4,5} The most frequent clinical phenotype induced by *STAT1* GoF mutations is simple immunodeficiency, for example chronic mucocutaneous candidiasis (Fig 1, B).⁶ In a very rare circumstance, GoF *STAT1* mutations result in aggressive immune phenotypes, similar to immune dysregulation-polyendocrinopathy-enteropathy-X-linked (IPEX) syndrome caused by *FOXP3* loss-of-function (LoF) mutations. In the IPEX-like disorder (IPEX-LD)¹, severe immunodeficiency and autoimmune phenotypes are combined (Fig 1, B). However, the molecular mechanisms underlying the broad clinical phenotypes of *STAT1* GoF mutations remain elusive (Table E1).

Here we conducted a comprehensive whole genome analyses of an IPEX-LD patient. Medical history and laboratory information were obtained through a medical chart review (Table E2). Over her lifetime, she showed multiple immuno-deficient and autoimmune phenotypes (Fig 1, C).¹ The patient exhibited lower NK cell cytotoxicity and T helper 17 cell count than healthy controls as previously reported (Fig E1; Methods)^{1, E12}. Serum IgE level was within normal range unlike *FOXP3* mutated IPEX syndrome. The patient harbored a *de novo* *STAT1* T385M GoF mutation (chr2:191,851,647 G>A substitution) (Fig 1, D).¹ The mutation was not present in the genomes of either the parents or her sister. The variant allele fraction of the mutation was ~50%, consistent with heterozygous mutations present in all the cells of the proband. The variant was phased to the reference allele of the maternally inherited

heterozygous polymorphism (chr2:191,851,997 T>C substitution rs17817076), suggesting that the mutation was acquired through the maternal germline lineage (Fig 1, D).

To identify additional genomic alterations that conferred the patient's extreme IPEX-LD manifestation, we systematically analyzed her entire genome sequences targeting four variant classes (Table E3-E5). However, given the genomic positions of those alterations, none of (1) germline substitutions/indels and (2) *de novo* mutations seemed to be associated with the disease phenotypes and (3) structural variations (rearrangements and retrotransposon mobilization) were not found. Finally, we explored the impact of (4) somatic mosaicism.

Of the 2,474 somatic mosaicism candidates from whole-genome sequencing (2,043 base substitutions and 431 indels; Table E6), 15 (0.6 %) were located in protein coding regions. The most interesting candidate was a frameshift T insertion in the *STAT1* coding sequence, 11 bp from the *de novo* T385M GoF mutation (chr2:191,851,636 C>CT)(Fig 1, D). Consistent with somatic mosaicism, this variant was not found in other family members and other unmatched normal populations (0 out of 15,708 WGS from gnomAD(v2.1.1) and 0 out of 125,748 whole-exome sequences from ExAC consortium⁷, but was present in 8.1 % of the patient's blood cells (3 supporting reads of the 37 reads from the maternal allele; 95%CI=1.7-23.7%, Poisson Exact Test). Because the T insertion was phased to the T385M mutation (Fig 1, D), the somatic mutation likely abrogates the impact of the *de novo* GoF *STAT1* mutation in the fraction of patient's blood cells.

To understand the quantitative impact and the cell type distribution of the secondary *STAT1* mutation, we confirmed the burden of mutations using ultra-high depth of amplicon sequencing (>1,000,000-fold) from different cell types including buccal swabs and specific blood cells. The mutation was present at variable fractions in different cell types, i.e. buccal epithelial cells (~0%, 217/645,926), CD8⁺ (15.8%, 93,048/589,788, 95%CI=15.7-15.9%),

CD4⁺ (7.0%, 47935/682,072, 95%CI=6.96-7.09%), CD19⁺ (2.5%, 17,535/692,530, 95%CI=2.49-2.57%) and CD14⁺ (0.6%, 5,348/874,933, 95%CI=0.59-0.62%) cells (Fig 1, E). We further looked for other somatic mutations in CD8⁺ cells using 70-fold WGS, but no remarkable mutations other than the *STAT1* T insertion were identified.

Our findings suggest that at least two different sets of blood cells were present in the IPEX-LD patient: (1) single T385M *STAT1*-mutant cells and (2) double mutant (T385M and T insertion) *STAT1*-mutant blood cells (Fig 1, D and Fig 2). In theory, the single-mutant cells will be non-functional due to the impaired STAT1 signaling, leading to immunodeficiency. In contrast, the double-mutant T cells with restored STAT1 signaling could be more reactive to antigens than single-mutant cells because the T385M GoF *STAT1* mutation is not expressed. Because normal STAT1 signaling confer cytotoxic activity, we believe that some, if not all, of the double *STAT1*-mutant CD8⁺ T cells may contribute to the multiple extreme autoimmune phenotypes manifested in the IPEX-LD patient.

Experimental studies suggest that the double-mutant cells are functionally distinct from the single-mutant cells. U3A cells with frameshift T insertion in combination with *STAT1* T385M GoF mutation exhibited a full inactivation of *STAT1* T385M mutant alleles in response to interferon- γ (IFN- γ) (Fig 1, F). In addition, Jurkat T cells transfected with *STAT1* harboring both the T385M and the T insertion showed the higher cellular proliferation rate after IFN- γ stimulation than cells transfected with only the T385M mutation ($P=0.0066$), which is close to the rate observed in the wild type *STAT1* cells (Fig 1, G).

The somatic mutation was not identified in the cells of endodermal origin (buccal cells), but was present in mesodermal blood cells. This suggests that the somatic mosaicism likely emerged after the formation of the three germ layers in embryogenesis but earlier than the cell fate determination of pluripotent hematopoietic stem cells (Fig 2). The reason

underlying the higher frequency of double-mutant clones in CD8⁺ T cells is unclear. In theory, it can be caused by (1) more frequent fate determination into CD8⁺ T cells for double *STAT1*-mutant hematopoietic stem cells, (2) moderate clonal expansion of double *STAT1*-mutant CD8⁺ T cells by restoration of *STAT1*-dependent inhibition of CD8⁺ T cell proliferation in periphery, or both.⁸ Of note, we found a few TCR clonotypes of minor contributions (<3%) from bulk RNA-seq of peripheral blood mononuclear cells (Table E7) although their *STAT1* genotypes are unclear.

The incidence and functional importance of early somatic mosaicisms is emerging.⁹ Given the mutation rate in early embryogenesis (~3 base substitutions per cell per cell doubling), a 16-cell embryo will collectively acquire ~90 somatic base substitutions, which will be present in most cell types of the individual. In addition, much later mutations can also affect a substantial proportion of specific cell types if they were enriched in founder cells of a specific cell type. However, due to technical limitations, studies targeting somatic mosaicisms are lacking. Our study demonstrates that comprehensive whole genome analyses are necessary to understand the frequency and the functional roles of somatic mosaicisms in the phenotypic diversity of rare human diseases.

We thank to professor Sei Won Yang, Choong-Ho Shin, and Young Ah Lee of Seoul National University Children's Hospital for the care of this patient for a long time. Most of all, we give special thanks to the dedicated efforts of patient and her family who have made this study possible. We thank Ji Yong Choi for her technical assistance.

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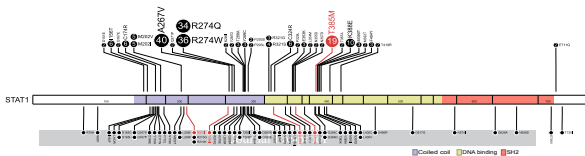
Figure Legend

Figure 1. *STAT1* mosaicism observed from an IPEX-LD patient. (A) Known *STAT1* GoF mutations. Number of cases reported in the previous literatures are represented in the circle. Loci associated with IPEX-LD are colored in red. (B) Spectrum of clinical manifestations of *STAT1* GoF mutation. (C) Medical history of the IPEX-LD patient. CMV, cytomegalovirus; DKA, diabetic ketoacidosis; DM, diabetes mellitus; NTM, non-tuberculous mycobacterium. (D) Pedigree and mutational status of *STAT1* of the patient. Maternal alleles are colored in red. (E) Fraction of double mutant cells upon specific immune cell types. (F) IFN- γ stimulated *STAT1* proteins in U3A cells transfected with wild type or different *STAT1* mutations. DM, T385M and T insertion double mutant. (G) Proliferation of Jurkat T cell lines transfected with wild type or different *STAT1* mutations. * $p < 0.05$; ** $p < 0.005$.

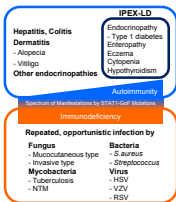
Figure 2. Mutational history of *STAT1* mutations in the lifetime of the IPEX-LD patient.

Figure 1

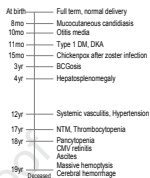
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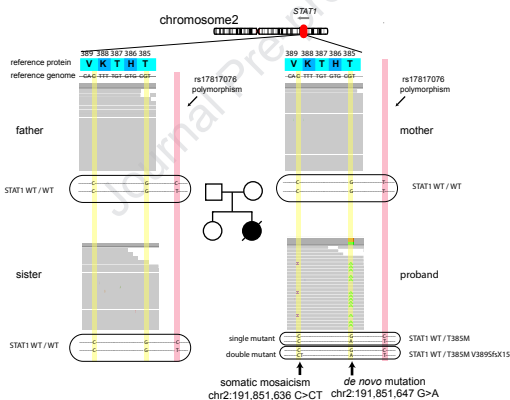
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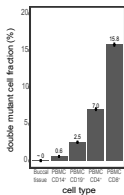
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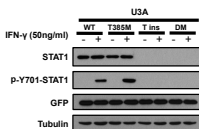
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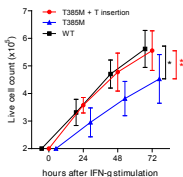
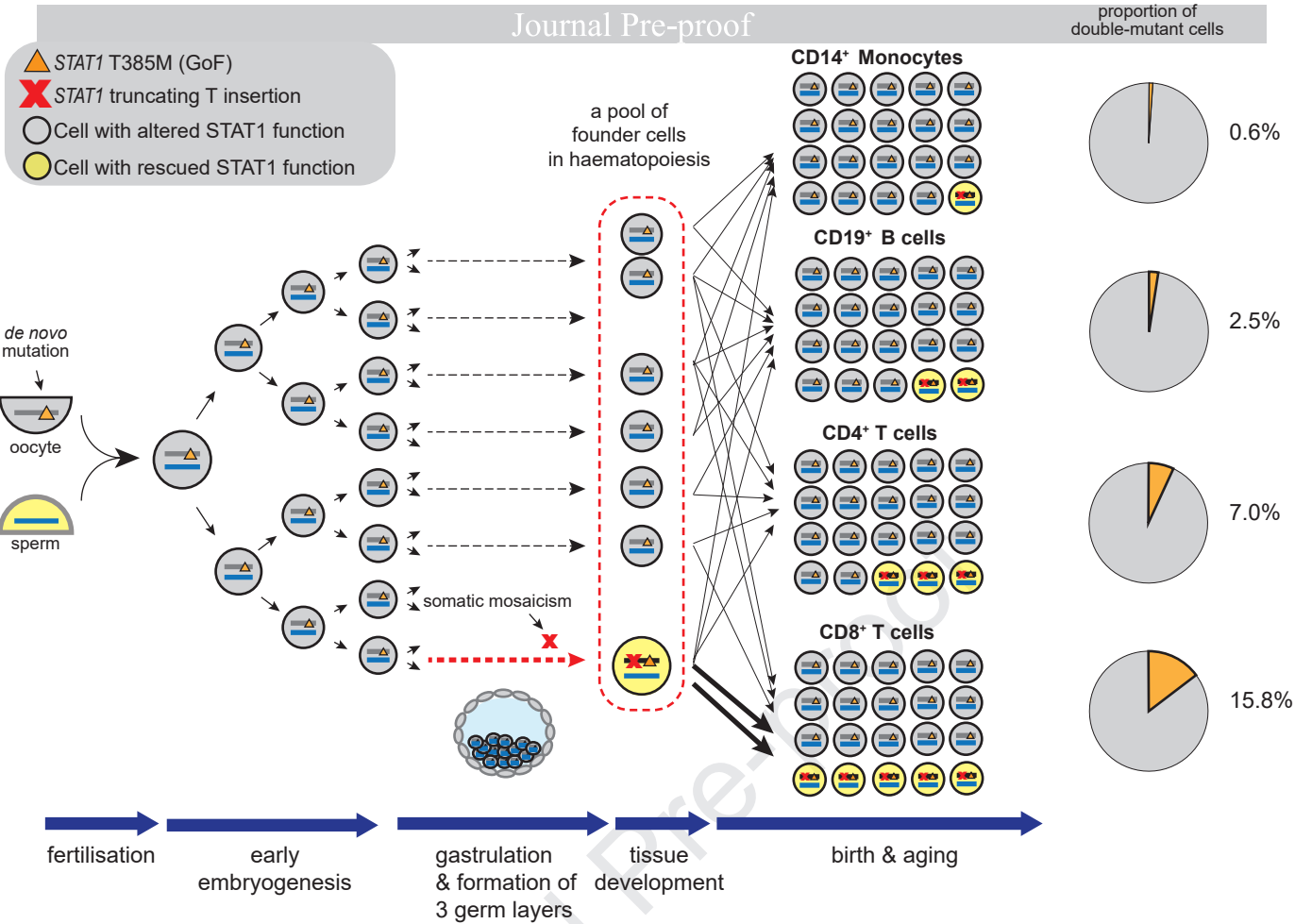


Figure 2



METHODS

Study design, setting, and participants

This study was approved by the Institutional Review Board of Seoul National University Hospital (Reference #1606-116-771) and written informed consents were obtained from the patient and her apparently healthy family members for publication of clinical and genetic data.

Genome analyses

Genomic DNA specimens for whole-genome sequencing (WGS) were extracted from whole blood of the patient (proband), elder sister and both parents. WGS data were obtained from the proband patient (overall 130-fold genome-wide coverage; 60-fold coverage from whole-blood and 70-fold coverage from sorted CD8⁺ T cells) and her family members (30-fold coverage each from whole-blood) with an Illumina HiSeq 4000 system. Short-reads were aligned to human reference genome (GRCh37) using BWA-MEM software.^{E1} Germline substitutions and indels were initially called by Varscan2 and their Mendelian inconsistencies were explored and then corrected by in-house scripts.^{E1} *De novo* mutations and somatic mosaicism were detected using in-house scripts modified from our previous report.^{E1} Copy number variation and genomic rearrangements were analysed using Sequenza and Delly softwares as previously described.^{E2, E3} Somatic L1 mobilisation was explored using MELT2 software.^{E4} All the functional mutation candidates were visually inspected using IGV.^{E5} The identified *STAT1* mosaicism (T insertion) was validated using ultra-high depth amplicon sequencing (>104-fold) by Illumina MiSeq system (Primer sequence: GGAAACTGATGTCCCTACCAG (forward), AGGGTGTACTGGTGCTGGTG(reverse)). To determine the distribution of mutant cells in various cell types, we determined variant

allele fraction (VAF) of the mutation from ultra-high depth amplicon sequencing in buccal epithelial cells as well as specific immune cells (CD4⁺, CD8⁺, CD14⁺, and CD19⁺ peripheral blood mononuclear cells) sorted by magnetic activated cell sorting technology (Miltenyi Biotec,).

RNA sequencing

An mRNA sequencing library was constructed according to the TruSeq protocol (Illumina, San Diego, CA) with 1 µg of mRNA materials, and then sequenced on a HiSeq 2500 machine with a target throughput of 10 Gb. We conducted sequencing of PBMC mRNAs of the patient using methods previously published.^{E6} We have analyzed the frequency of TCR repertoires using a combination of MixCR and in-house analyses.^{E7} Briefly, the read-depth of each assembled CDR3 sequences were compared with the read-depth of total TCR region (constant regions). Expression levels of RORγt were compared between our patient and healthy donors using previously published data.^{E8-E10}

Cellular experiments

STAT1 single mutant (T385M GoF mutation), double mutant (T insertion with T385M GoF substitution), or wild-type constructs were transfected in bi-allelic *STAT1*-null U3A cell line. Transfectants were subsequently examined for STAT1 phosphorylation (using Western blot) following 30 minutes incubation with 50ng/ml of IFN-γ.^{E11} Those 3 types of *STAT1* mutants were also transfected in Jurkat T cell line. To evaluate the impact of those *STAT1* mutants on the T cell line, the live cell counts were enumerated by automated fluorescent viability cell counter (Cellometer Auto 2000, Nexcelom Bioscience) 0, 24, 48, and 72 hours after *in vitro* incubation with 50ng/ml of IFN-γ. Each group included 6 independent experiments and the difference among the 3 mutant groups were compared by two-way ANOVA method.

NK cytotoxicity and Th17 frequency

Previous study using our patient's PBMC had shown that she had decreased NK cytotoxicity (less than 5% killing in K562 lysis assay) when compared to healthy control (more than 20% in K562 lysis assay) (patient 14 of reference E12, right panel of FIG 2B)^{E12}.

Another previous using our patient's PBMC had shown that she had lower frequency of Th17 cells (0.3% among CD4⁺ T cells) than healthy control (1-3% among CD4⁺ T cells)) (patient 3 of reference 1, right middle panel of FIG 3C)¹.

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