To the Editor:

We have read the comments by Goulielmos, *et al*¹ regarding the structural significance of the signal transducer and activator of transcription 1 (*STAT1*) gain-of-function (GOF) c.970T>C (p.C324R) mutation, in the DNA binding domain (DBD) of *STAT1* protein. Based on the results of an *in silico* analysis, Goulielmos, *et al* have proposed that the replacement of this position by a positively charged arginine residue triggers a heavily modified interaction of *STAT1* with the DNA residues through the change of loop 325–332 structure. First, we are very grateful for the comments by Goulielmos, *et al* because their structural data¹ strengthen our understanding of the pathogenesis of several clinical phenotypes of our case.

To date, 12 patients carrying the C324R mutation in *STAT1* have been identified^{2,3,4,5}. However, this mutation has not been characterized in detail in previous studies. Thus, we investigated the functional significance of C324R mutation by interferon- γ (IFN- γ)-activated sites (GAS) reporter assay as previously described⁶. The wild-type or mutant *STAT1*-containing plasmids, with GAS reporter plasmids (Cignal GAS Reporter Assay Kit; SABiosciences), were introduced with Lipofectamine LTX Reagent (Thermo Fisher Scientific) into U3C *STAT1* null fibrosarcoma cell lines. Subsequently, the cells were stimulated with IFN- γ for 16 h and subjected to luciferase assay. Upon IFN- γ stimulation, the C324R mutation and a R274Q-known GOF-*STAT1* mutation showed enhanced GAS transcriptional activity (Figure 1). In contrast, IFN- γ -induced GAS activation was completely abolished by Y701C mutation, which is a known *STAT1* loss-of-function mutation identified in patients with mendelian susceptibility to mycobacterial disease. These results confirmed that C324R mutation is a GOF mutation that leads to enhanced GAS activation upon IFN- γ stimulation.

GOF mutation was originally discovered in a coiled coil domain (CCD) of *STAT1* in patients with chronic mucocutaneous candidiasis disease^{7,8}. A subsequent large cohort study showed that the patients with GOF-*STAT1* mutation present broad clinical manifestations with varying severity, and identified GOF mutations are located not only in the CCD (62%) but also in the DBD (35%), transactivation (1%), N-terminal (1%), and SH2 domains

(1%)². In contrast, two-thirds of patients with severe clinical manifestations, who were treated with hematopoietic stem cell transplantation, have GOF mutations in DBD⁹. These clinical observations suggest that GOF mutations in DBD may be related to severe clinical manifestations. In addition, some GOF mutations are known to be related to severe clinical symptoms. For example, T385M mutations can cause combined immune deficiency^{3,10} and severe autoimmunity that is sometimes described as an immune dysregulation-polyendocrinopathy-enteropathy-X-linked-like syndrome^{9,11}. Regarding C324R mutation, one patient with this mutation was reported as having fatal combined immune deficiency³, and another patient presented multiplex autoimmune manifestations by developing type 1 diabetes, Sjögren syndrome, and hypothyroidism⁴. In addition, severe early-onset combined immunodeficiency associated with C324F, which is a different amino acid substitution at C324, has been previously reported¹⁰. Together with these previous observations, fatal autoimmune manifestations reported in our case⁵ suggest that C324R, which is now a functionally proven GOF mutation in DBD of *STAT1*, can occasionally cause severe clinical manifestations.

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Figure 1. Luciferase reporter assay evaluating transcriptional activation by C324R mutation *STAT1*. The *STAT1* mutant leads to enhanced luciferase GAS-induced activity. IFN- γ (white bar, nonstimulated; black bar, 1000 U/ml) were added to U3C cells transfected with the mutant or wild-type (WT) *STAT1* and cultured for 16 h before GAS-induced activity was measured. Known GOF *STAT1* mutation (R274Q) and loss-of-function *STAT1* mutation (Y701C) were also used as controls. IFN- γ : interferon- γ ; GAS: IFN- γ activated sites; GOF: gain-of-function; RLU: relative light units.

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