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Short communication

A novel mutation in the signal transducer and activator of transcription 3 (STAT3) gene, in hyper-IgE syndrome

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ABSTRACT

The hyper-IgE syndrome (HIES) is a rare primary immunodeficiency characterized by a highly elevated serum IgE, recurrent staphylococcal skin abscesses and cyst-forming pneumonia. Non-immunological abnormalities, including a distinctive facial appearance, hyperextensive joints, scoliosis, fracture following minor trauma, and the retention of primary teeth are also observed in many patients. Recently, it was shown that heterozygous mutations in signal transducer and activator of transcription 3 (STAT3), can cause autosomal-dominant HIES. Here we identify and characterize a novel mutation in the DNA-binding domain of STAT3 in a patient with hyper-IgE syndrome. Sequence analysis revealed a *de novo* heterozygous transition of a G-to-A, causing a substitution of a glycine residue for an aspartic acid in the translated sequence (G342D). The patient has normal levels of STAT3, which is able to translocate to the nucleus upon IL-6 stimulation. However, enzyme-linked DNA–protein interaction analysis showed that the G342D mutation affects the binding ability of STAT3 to target DNA sequences. In addition, as shown by qRT-PCR, the mutation abrogates the STAT3-dependent transcription of the retinoid-related orphan receptor γ t (ROR γ t) gene, an indispensable transcription factor for the commitment of naive CD4⁺ T cells to the Th17 lineage. These data suggest that the novel G342D mutation affects the binding of STAT3 on DNA and the STAT3-dependent expression of ROR γ t mRNA, leading to the HIES phenotype.

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1. Introduction

The hyper-IgE syndrome (HIES; Job syndrome; OMIM 147060) is a rare primary immunodeficiency characterized by recurrent skin and pulmonary abscesses, pneumonia, and markedly increased levels of IgE in serum. In addition to immunologic abnormalities, patients also often exhibit abnormalities in dentition, bones, and connective tissues, suggesting that the hyper-IgE syndrome is a multisystem disorder (Grimbacher et al., 2005). In more detail, manifestations include distinctive facial features, hyperextensibility of the joints, cranial synostosis, scoliosis, abnormal dentition, and pathological fractures (Grimbacher et al., 1999a,b). Central nervous system lesions and coronary artery aneurysms have also been reported as part of the clinical pathology of the syndrome (Ling et al., 2007; Freeman et al., 2007). Most of the cases are sporadic, but both autosomal dominant (AD-HIES) and autosomal recessive (AR-HIES) forms of the syndrome have been described.

Recently, Minegishi et al. (2006) identified a homozygous mutation in the tyrosine kinase-2 gene (*TYK2*) in a patient with a variant form of AR-HIES. However, to date this is the only case of AR-HIES carrying a mutation in *TYK2* that has been described in the literature. In addition, more recent studies have shown dominant-negative mutations in the signal transducer and activator of transcription 3 gene (*STAT3*) as major causes of AD-HIES in both familial and sporadic cases (Minegishi et al., 2007; Holland et al., 2007). STAT3 is a homodimeric transcription factor of the STAT family, with the domain structure shown in Fig. 1D. The first identified mutations associated with HIES were localized in either the DNA-binding domain or the Src homology 2 (SH2) domain. However, newly identified mutations are found to localize in the DNA-binding, Linker, SH2 and trans-activation domains of STAT3. They include single amino acid substitutions, in-frame deletion of more than one amino acid and splicing mutations resulting in exon skipping (Renner et al., 2008; Jiaoa et al., 2008; Kim et al., 2009). In most cases the mutations affect the binding of STAT3 to the DNA or its phosphorylation on tyrosine 705 (Renner et al., 2008; Minegishi et al., 2007). However, from the reported mutations in the literature, few are functionally characterized at the protein level and the exact causative mechanism of the phenotype remains elusive.

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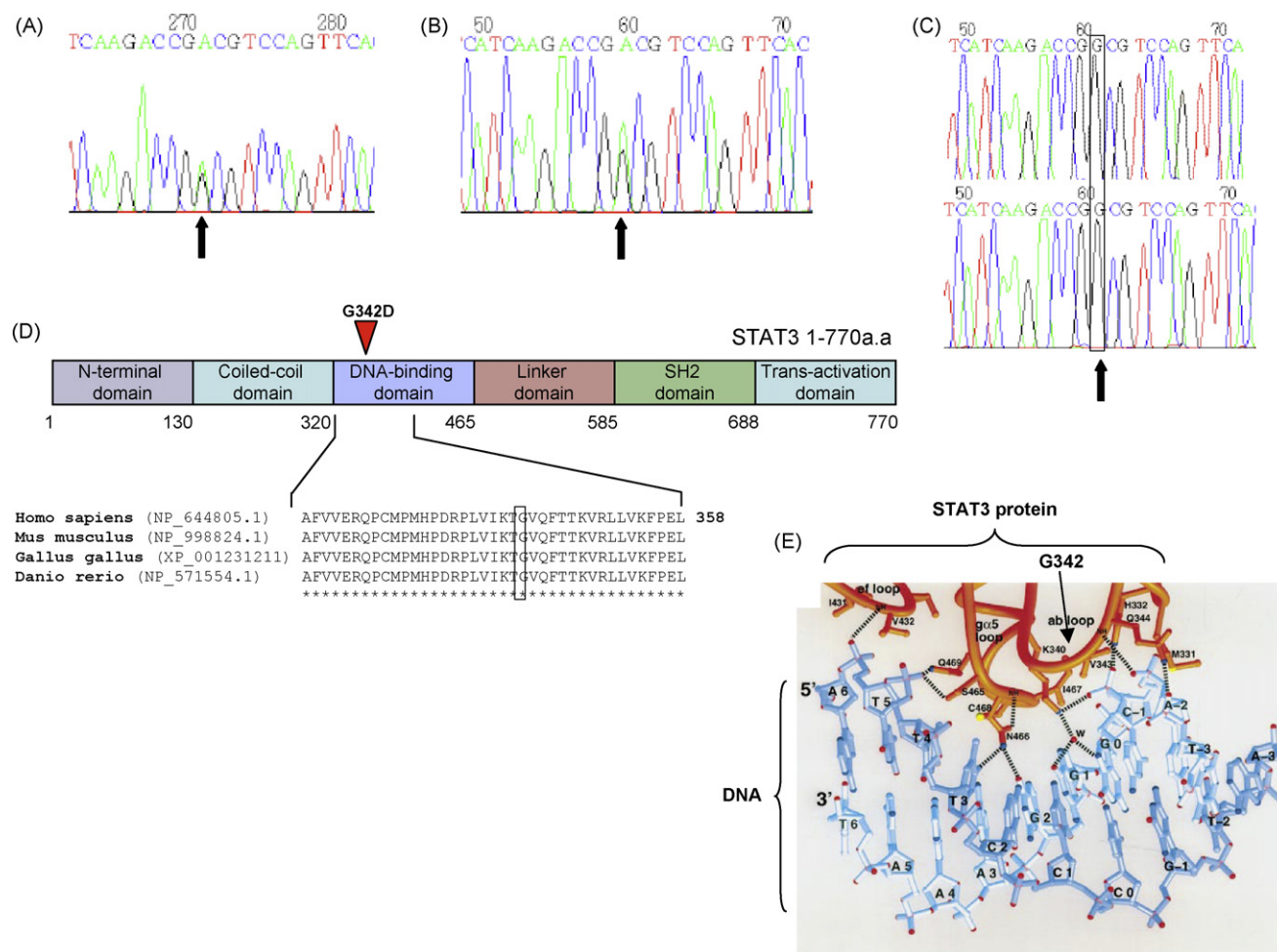


Fig. 1. Sequence chromatograms of *STAT3*, depicting the presence of the heterozygous mutation (c.1025G>A) (black arrow) in patient's cDNA (A), and genomic DNA (B), respectively. (C) Chromatograms of the *STAT3* sequence of the patient's father (upper panel) and mother (lower panel), showing the wild-type alleles (boxed). (D) Modular structure of *STAT3* showing the affected residue G342, in the DNA-binding domain (red arrowhead). Alignment of the *STAT3* protein sequence among various species (CLUSTALW) showing the G342 residue perfectly conserved (black box). The numbers indicate amino acid positions based on NP_644805.1. (E) Ribbon diagram of the interactions of one monomer of *STAT3* with DNA. The arrow depicts the approximate position of G342 in the *ab* loop and its close proximity to the major groove of the DNA (reprinted by permission from Macmillan Publishers Ltd.: Becker et al., 1998). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

The immunodeficiency phenotype produced from *STAT3* mutations in patients with HIES, is attributed to the differentiation impairment of T helper cells (Milner et al., 2008; Ma et al., 2008). The commitment of naive CD4⁺ T cells to the Th17 lineage requires exposure to TGF- β and either IL-6 (Mangan et al., 2006; Veldhoen et al., 2006) or IL-21 (Nurieva et al., 2007). This step is a *STAT3*-dependent procedure and a functional *STAT3* protein is required to induce the expression of retinoid-related orphan receptor γ t (*ROR* γ t), the critical transcription factor necessary for the development of Th17 cells (Ivanov et al., 2006; Harris et al., 2007; Milner et al., 2008).

However, other features of the HIES phenotype, such as hyper-extensibility of the joints, cranial synostosis, scoliosis, abnormal dentition and others, cannot be explained by the impairment of Th17 cell differentiation. It seems that *STAT3* mutations can affect either the DNA-binding efficiency or the phosphorylation status of the protein or in a more general way they affect the functionality of the protein as a transcription factor, resulting in the abrogation of *ROR* γ t expression and other important *STAT3* target genes (Minegishi et al., 2009).

Here we identify and characterize a novel mutation in the DNA-binding domain of *STAT3* gene in a patient with HIES.

2. Materials and methods

2.1. Patient

A 12-year-old Greek girl was admitted because of fever, and cough for a week. Her parents were not consanguineous. She had no remarkable perinatal problems and had been vaccinated as scheduled. She had a history of atopic dermatitis since 6 months of age. Past medical history also revealed recurrent infections, including multiple episodes of skin abscesses and pneumonia. She had a history of two hospitalizations for pneumonia with cyst formation in the right lung and numerous visits at the clinic for the treatment of skin abscesses caused by *Staphylococcus aureus*. Family history revealed no members with relevant allergic or immunologic diseases.

On physical examination, her height was 118 cm (under the 5th percentile), and her weight was 25.8 kg (under the 5th percentile). She had coarse facial features with facial asymmetry, and prominent forehead. She was also diagnosed with scoliosis with maximal curvature of 14°.

Complete blood cell counts demonstrated a high total eosinophil count (max 4000 cells/ μ l). The serum IgE level was increased at

Table 1

National Institutes of Health score for the patient.

Clinical findings	Point										
	0	1	2	3	4	5	6	7	8	9	10
Highest serum IgE level (IU/ml)											x
Skin abscesses									x		
Pneumonia (episodes over lifetime)					x						
Parenchymal lung anomalies							x				
Retained primary teeth									x		
Scoliosis, maximum curvature			x								
Fractures with minor trauma	x										
Highest eosinophil count (cells/ μ l)							x				
Characteristic face						x					
Midline anomaly						x					
Newborn rash	x										
Eczema (worst stage)			x								
Upper respiratory tract infections/year		x									
Candidiasis		x									
Other serious infections	x										
Fatal infections	x										
Hyperextensibility					x						
Lymphoma	x										
Increased nasal width		x									
High palate			x								
Young age correction	x										

74,000 IU/ml. All other laboratory data examined, such as serum immunoglobulin level, the oxidative burst of granulocytes and complement components, were normal with the exception of slightly increased IgD titer (300 mg/l). Lymphocyte subset analyses revealed normal levels of both CD4⁺ and CD8⁺ T cells (883 and 850 cells/ μ l, respectively). According to the HIES scoring system (Grimbacher et al., 1999b), the patient gained 65 points ("affected") (Table 1).

The individual of this study and her family were referred for counselling and treatment to our Department of Pediatrics. The protocol has been approved by the Institutional Ethical Board, and written informed consent was obtained from the parents of the patient.

2.2. Peripheral blood mononuclear cell (PBMC) and primary skin fibroblast isolation, cell cultures and cytokine cell stimulation

Cells from whole blood were isolated through ficoll gradient centrifugation and kept in RPMI1640, with 10% (v/v) fetal bovine serum (FBS, Invitrogen) and 1% (v/v) penicillin/streptomycin (P/S, Sigma). After 16 h, cells were treated with 1 μ g/ml phytohemagglutinin (PHA, Sigma–Aldrich) or 10 ng/ml phorbol 12-myristate 13-acetate (PMA) and 1 mg/ml ionomycin (Sigma–Aldrich) for 2, 4 or 8 h or left untreated.

Primary skin fibroblasts were isolated from skin biopsies and grown in Dulbecco's modified Eagle's medium (Invitrogen) with 10% (v/v) fetal bovine serum (Invitrogen) and 1% (v/v) P/S (Sigma–Aldrich) and used for experimental procedures between passages 7 and 13.

For skin fibroblasts, stimulation with human recombinant interleukin 6 (IL006, Chemicon) was performed at 100 ng/ml in Dulbecco's modified Eagle's medium (Invitrogen) with 10% (v/v) FBS (Invitrogen) and 1% (v/v) P/S (Sigma–Aldrich), before cells were processed for immunofluorescence or for the enzyme-linked DNA–protein interaction assay, as described below.

2.3. DNA, RNA isolation and cDNA synthesis

From our patient, her parents and nine control subjects DNA was extracted from blood samples and skin fibroblast cultures with the Flexi gene kit (Qiagen) according to the manufacturer's instructions. RNA was extracted from our patient and three age-gender

matched controls from PBMCs and skin fibroblasts by using the RNeasy Kit (Qiagen) according to the manufacturer's instructions. We carried out cDNA synthesis using the Superscript II cDNA synthesis kit (Invitrogen) from 500 ng RNA.

2.4. Direct sequence analysis and RT-PCR

PCR was performed on cDNA and genomic DNA from PBMCs and skin fibroblasts, using LA Taq (Takara) and specific primers for the whole coding region of *STAT3*, *STAT4* and *TYK2* genes. Also gene-specific primers were used for exon 10 of *STAT3* on genomic DNA. Sequencing was done with a 3730xl DNA analyzer (Macrogen).

ROR γ t expression was analysed by qRT-PCR using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) and gene-specific primers. *GAPDH* was used as the reference gene. Relative expression level of the gene of interest was calculated with the $2^{-\Delta\Delta Ct}$ method, with expression level in unstimulated healthy cells assigned an arbitrary value of 1. Three healthy donors were used as controls and all the samples (controls and patient) were analysed in triplicate with the Rotor-Gene 3000 (Corbett Research).

2.5. Western blotting

Primary skin fibroblasts from patient and controls were harvested and cell lysates were prepared, after lysing cell pellets directly in SDS-PAGE loading buffer and boiling. Western blot analysis was performed using specific polyclonal rabbit antibodies against STAT3 (sc-482, Santa Cruz Biotechnology) and phospho-STAT3-Tyr (sc-7993, Santa Cruz Biotechnology), using anti-tubulin (Sigma–Aldrich) as control. Antibodies were used at the following dilutions for Western blotting: anti-STAT3 1:600, polyclonal anti-phospho-STAT3 1:300, anti-tubulin 1:10,000.

2.6. Immunofluorescence

For immunofluorescence, patient and control skin fibroblasts grown on coverslips, were fixed in 4% paraformaldehyde for 10 min, washed twice with phosphate-buffered saline (PBS), and permeabilized with 0.3% Triton X-100 in PBS. Non-specific binding was blocked by incubating cells in blocking buffer with 3% (w/v) BSA and 10% (v/v) FBS in PBS. Fixed cells were incubated with primary anti-STAT3 (1:200, sc-482, Santa Cruz Biotechnology), diluted

in blocking buffer. Cells were washed with PBS containing 0.1% Tween 20 three times and incubated with secondary antibody, Alexa 488-conjugated goat anti-rabbit (Molecular Probes, Invitrogen). After washing, cells were mounted in mounting medium (Vectashield, Vector Laboratories) containing 4,6-diamidino-2-phenylindole (DAPI).

2.7. Enzyme-linked DNA–protein interaction assay

Binding of STAT3 to the target DNA was measured using the TransAM STAT3 assay kit (Active Motif Carlsbad, CA) according to the manufacturer's instructions and Milner et al. (2008). Briefly, 5–15 µg of nuclear lysate from both stimulated (100 ng/ml IL-6) and unstimulated cells were assayed in the presence or absence of a competitor oligonucleotide or a mutated oligonucleotide. Histograms represent the absorbance at 450 nm after subtraction of the background seen when competing with soluble wild-type oligonucleotide. The binding of STAT3 was assayed at the optimized temperature of 37 °C.

3. Results

3.1. A novel STAT3 mutation in the binding domain of STAT3

Direct sequencing analysis of the coding region of the STAT3 gene was performed on cDNA synthesized from RNA isolated from PBMCs and skin fibroblasts, of our patient. As a result, we identified a heterozygous mutation in the STAT3 DNA-binding domain; it was a G-to-A transition at the nucleotide position 1025 (c.1025G>A, based on NM.139276.2) leading to substitution of a glycine residue at the amino acid position 342 (based on NP.644805.1) to aspartic acid (p.G342D) (Fig. 1A). G342D was not present in the single nucleotide polymorphism database (dbSNP; www.ncbi.nlm.nih.gov/projects/SNP/) nor has it been identified previously as a mutation in patients with HIES. The heterozygous mutation (G342D) was also confirmed through direct sequencing analysis on genomic DNA (exon 10) extracted from PBMCs and

skin fibroblasts of our patient (Fig. 1B). No other sequence alterations were detected in any other parts of STAT3. We also sequenced directly TYK2 and STAT4 genes for sequence changes in the coding region, but no alterations were detected.

Family study, on genomic DNA from PBMCs, revealed that none of the patient's parents had the mutation (Fig. 1C), and her four siblings were also homozygous for the wild-type allele of the STAT3 gene.

Functional prediction of the variation with the PolyPhen (Polymorphism Phenotyping) program (<http://genetics.bwh.harvard.edu/pph/>) and the Sorting Intolerant From Tolerant program (<http://sift.jcvi.org/>) predicted “possibly damaging” (PSIC score difference, 1.857) and “affect protein function” (SIFT score 0.05) values, respectively.

3.2. Functional analysis of the STAT3 (G342D) mutated protein

The G342D mutation of the DNA-binding domain did neither affect the quantity of the protein nor the extent of its phosphorylation, as judged from western blot analysis of cell extracts from IL-6 treated and untreated fibroblasts from patient and controls (Fig. 2A). In addition, nuclear translocation of the mutated STAT3 protein upon IL-6 stimulation was comparable to that of control cells (Fig. 2B and C).

3.3. Decreased DNA-binding of STAT3 mutant

Comparative analyses performed by aligning STAT3 amino acid sequences from different species showed that the G342 residue in the DNA-binding domain is perfectly conserved (Fig. 1D). In addition, the reported mutation introduces a negatively charged residue (aspartic acid, D) substituting a non-polar as G342, in the tip of the loop *ab* of the DNA-binding domain of STAT3 (Fig. 1E), as seen from the structural model reported by Becker et al. (1998).

Consequently, we tested the ability of the patient's STAT3 to bind specific oligonucleotides that comprise target sequences for STAT3, through an enzyme-linked DNA–protein interaction assay. Nuclear

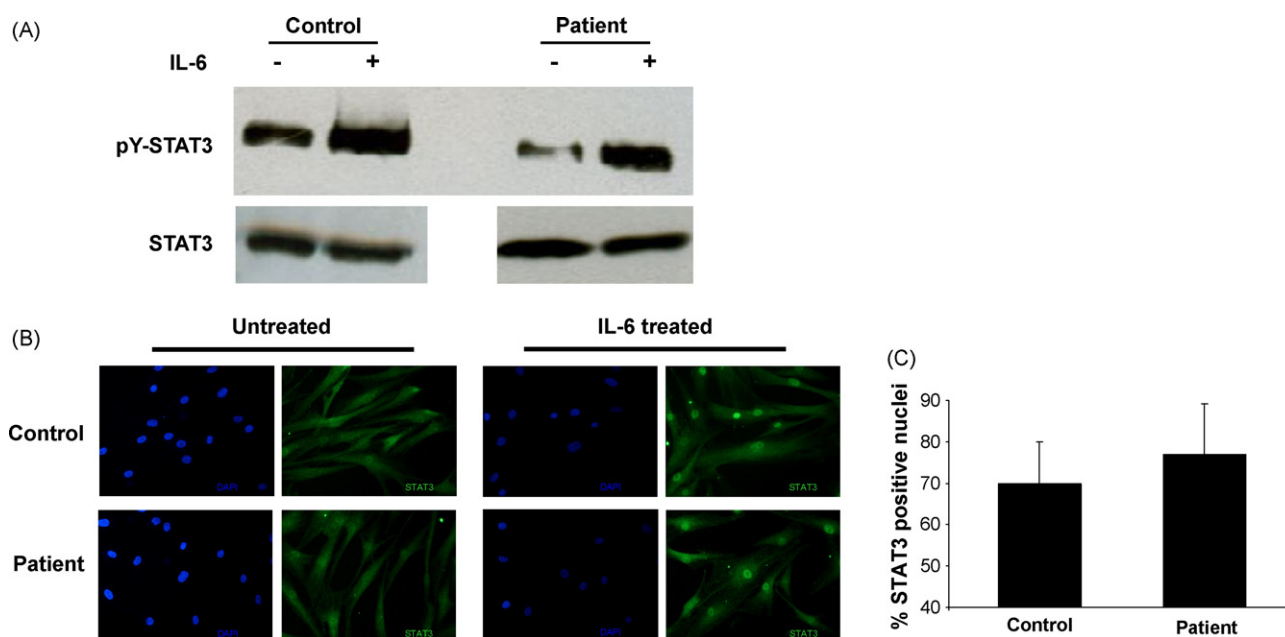


Fig. 2. (A) Total and tyrosine-phosphorylated (pY-) STAT3 proteins detected by Western blot analysis in fibroblast extracts of a control subject and the HIES patient before and after IL-6 stimulation. (B) Immunofluorescently stained fibroblasts for total STAT3, from a control subject and the HIES patient before and after IL-6 stimulation (30 min). (C) Percentage of STAT3-positive (green) nuclei in control and patient fibroblasts after IL-6 stimulation for 30 min. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

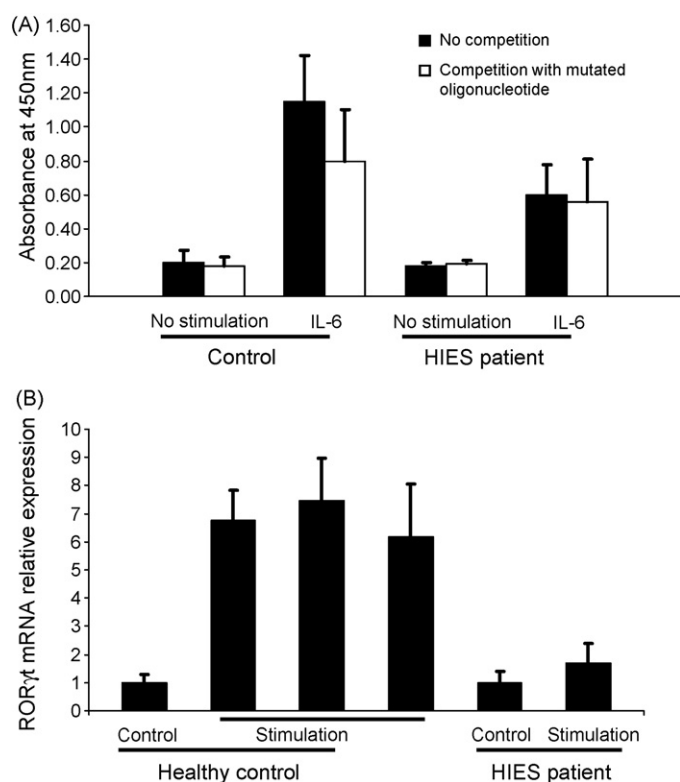


Fig. 3. (A) Ability of STAT3 within nuclear cell lysate to bind to a target oligonucleotide analyzed by an enzyme-linked DNA–protein interaction assay. Both IL-6 stimulated and unstimulated cells were assayed in the absence (white bars) or presence (black bars, demonstrating specificity of binding) of a soluble mutated competitor oligonucleotide. (B) Determination of *ROR γt* mRNA expression by quantitative RT-PCR in three healthy control subjects and the HIES patient. Values shown are mean relative expression levels of triplicate samples. Error bars are standard deviations.

extracts from the patient's fibroblasts stimulated with IL-6 contained lower amounts of active STAT3 able to bind the target DNA compared with nuclear extracts from the control cells. However, the DNA-binding activity of STAT3 in the IL-6 stimulated patient cells was not totally abrogated, but it was approximately half of that of control cells (Fig. 3A). Thus, the genetic mutation (G342D) identified in the HIES patient seems to result in a partial impairment of the DNA-binding activity of STAT3.

3.4. Patient's peripheral blood leukocytes fail to up-regulate *ROR γt* mRNA

The immunological abnormalities that characterize the hyper-IgE syndrome are mainly attributed to the absence of IL-17 producing T helper (Th17) cells. Differentiation of Th17 cells is determined by the function of *ROR γt* and induction of this key transcription factor is regulated by STAT3 protein (Milner et al., 2008; Ma et al., 2008). Therefore, we tested for a possible defect in the expression of *ROR γt* mRNA in our patient's PBMCs. After 8 h stimulation with either PHA or PMA/ionomycin, the patient's cells failed to up-regulate the expression of *ROR γt* mRNA, in comparison with control cells (Fig. 3B).

4. Discussion

In this study, we report the identification and characterization of a novel mutation in the DNA-binding domain of STAT3 in a patient with HIES and a high NIH clinical score (65 points), expanding the spectrum of heterozygous STAT3 mutations observed in AD-HIES.

This novel mutation is located in the DNA-binding domain at position 342 of the amino acid sequence, substituting a glycine residue (G) to an aspartic acid (D). Models based on the crystal structure of STAT3b (Becker et al., 1998) revealed that one of the DNA strands is bound by residues almost exclusively from loop *ab*. More specifically, the STAT3 residues between K340 and Q344 comprise the main part of the loop, entering the major groove of the DNA, forming hydrophobic interactions with backbone sugars. Residue 342 is positioned in the tip of the loop *ab*, in close contact with the major groove of the DNA. Its substitution by a negatively charged residue (G342D) may destabilize the interaction of the loop *ab* with the DNA and as a result the binding of STAT3 on the DNA.

Most STAT3 mutations identified to date are missense mutations (Renner et al., 2008; Jiaoa et al., 2008; Kim et al., 2009). The novel missense mutation reported here results in an amino acid change that most likely impairs function. This is because the affected residue is highly conserved among various species, is not reported as a single nucleotide polymorphism, and also it was not identified in nine normal controls. In addition, using the PolyPhen algorithm and the Sorting Intolerant From Tolerant program, the identified mutation was characterized as “possibly damaging” and “affect protein function”, respectively.

For our patient with the G342D mutation, both parents and siblings were available for analyses, and all had only wild-type STAT3, identifying this as a *de novo* STAT3 mutation. Furthermore, patient's STAT3 protein showed diminished binding to DNA and was unable to induce the mRNA expression of the retinoid-related orphan receptor *γt* (*ROR γt*), the critical transcription factor necessary for the development of Th17 cells (Ivanov et al., 2006; Harris et al., 2007; Milner et al., 2008). Finally, as there were no other mutations in either STAT3, or in *TYK2* and *STAT4* we conclude that the novel STAT3 mutation in the index case is pathogenic.

The exact molecular mechanism by which heterozygous mutations in the STAT3 gene cause HIES remains unclear. Data from complete *Stat3* knockout or *Stat3*^{+/-} heterozygote mice suggest that haploinsufficiency is not sufficient to cause the symptoms of HIES, whereas complete insufficiency is lethal. According to the above, the autosomal-dominant inheritance predicted for HIES, suggests a dominant-negative mechanism by which mutant STAT3 fully or partially abrogates the function of wild-type STAT3 expressed by the normal allele. In addition, none of the mutations identified up to date are nonsense or frameshift alterations, further supporting the notion that AD-HIES is the result of a dominant-negative effect as suggested by Minegishi et al. (2007).

The discovery of STAT3 mutations has improved our understanding of the aetiology of HIES and the central role of STAT3 in numerous signalling pathways in different tissues. However, there are reports in the literature of HIES not linked to STAT3 mutations (Minegishi et al., 2007; Renner et al., 2008). This underlies the complex nature of hyper-IgE syndrome and the need for further studies at the genomic but also at the protein level in order to fully elucidate the mechanisms causing this multisystem disorder.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molimm.2010.01.010.

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Primers used for PCR and quantitative RT-PCR.

ST3GMF1	AGCCCATCTTCTCTTTCTC	Primers for genomic DNA, surrounding exon10 of STAT3
ST3GMR1	ATTCAACCCCGCAACAGTG	
ST3CDF1	TGACTGGAAGAGGCGGCAAC	Primers for the whole STAT3 cDNA.
ST3CDR1	TGGTGGTGGAGGAGAACTGC	
ST3CDF2	TAACATTCTGGGCACAAACAC	
ST3CDR2	TTTGGCTGTGTGAGGGGTGG	
ST3CDF3	TCGGCCTCTGCCGGAGAAAC	
TYK2F1	AGCTGCAGGTGGGGGAGGGGG	Primers for the whole TYK2 cDNA
TYK2F2	ATTGGGTCTGGGGGTCTTTGG	
TYK2R1	TTAGCACAGAGTCAGACCAGC	
TYK2CDF1	TTGCTTGAGTTGACACAGGG	
TYK2CDR1	AACCCTCCTCCTTGTTCCACC	
TYK2CDF2	TGACAGGCACTGGTGGCATCC	
TYK2CDR2	AGGCTGGCTGTCTCGTAGAAG	
TYK2CDF3	GCAAGATGGATGACGAGGAC	
TYK2CDR3	GGCACGTACTIONCATGACCAG	
TYK2CDF4	GCTGGAAGCAGGAGATTGAC	
TYK2CDR4	ATCCCCCTCTTGGTTCATC	
ST4CDF1	AGGGACTGTGAGGGGCGCTTC	Primers for the whole STAT4 cDNA
ST4CDR1	GCTGTCGCTCAACCACAAATG	
ST4CDF2	TGATCCCATTCCAATGCAAAG	
ST4CDR2	TTCCTCCGAGATGGCTTTCAC	
ST4CDF3	AGGAACGGCTGTTGCTAAAGG	
STCDR3	TCCTTTCTTGGTGCGTCAGAG	
PRORGTF	CAGCGCTCCAACATCTTCT	Primers for qRT-PCR, for the detection of ROR γ t mRNA.
PRORGTR	CCACATCTCCCACATGGACT	
rtGAPDHF	CTCTGCTCCTCCTGTTTCGAC	Primers for qRT-PCR, for the detection of the reference gene GAPDH.
rtGAPDHR	ACGACCAAATCCGTTGACTC	