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Clinical heterogeneity of dominant chronic mucocutaneous candidiasis disease: presenting as treatment-resistant candidiasis and chronic lung disease

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ABSTRACT

In gain-of-function *STAT1* mutations, chronic mucocutaneous candidiasis disease (CMCD) represents the phenotypic manifestation of a complex immunodeficiency characterized by clinical and immunological heterogeneity. We aimed to study clinical manifestations, long-term complications, molecular basis, and immune profile of patients with dominant CMCD. We identified nine patients with heterozygous mutations in *STAT1*, including novel amino acid substitutions (L283M, L351F, L400V). High risk of azole-resistance was observed, particularly when intermittent regimens of antifungal treatment or use of suboptimal dosage occurs. We report a case of Cryptococcosis and various bacterial and viral infections. Risk of developing bronchiectasis in early childhood or gradually evolving to chronic lung disease in adolescent or adult ages emerges. Lymphopenia is variable, likely progressing by adulthood. We conclude that continuous antifungal prophylaxis associated to drug monitoring might prevent resistance to treatment; prompt diagnosis and therapy of lung disease might control long-term progression; careful monitoring of lymphopenia-related infections might improve prognosis.

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1. Introduction

In 2011, autosomal dominant gain-of-function (GOF) mutations in signal transducer and activator of transcription 1 (*STAT1*) gene have been identified as a cause of chronic mucocutaneous candidiasis disease (CMCD) [1,2]. To date, more than 100 patients have been reported, with 37 different mutations affecting the coiled–coiled (CCD) or the DNA-binding (DBD) domains of *STAT1*. This transcriptional activator plays a major role in various signaling pathways, particularly of IFNs, IL-27, IL-21, IL-6, and IL-17 immunity [3,4]. *STAT1* loss-of-function (LOF) mutations were already known to account for rare autosomal recessive (AR) immunodeficiencies [5], resulting respectively in fatal mycobacterial and viral diseases (when complete gene deficiency occurs [6]), or milder phenotypes (when deficiency is partial [7]). Autosomal dominant (AD) LOF mutations were identified as a cause of Mendelian susceptibility to mycobacteria infections [8]. GOF-STAT1 mutations were firstly described among patients affected with severe

Abbreviations: GOF, Gain-of-function; STAT1, Signal transducer and activator of transcription 1; CMCD, Chronic mucocutaneous candidiasis disease; CCD, Coiled–coiled domain; DBD, DNA-binding domain; LOF, Loss-of-function; AR, Autosomal recessive; AD, Autosomal dominant; CMC, Chronic mucocutaneous candidiasis; IPEX, Immune dysregulation, polyendocrinopathy, enteropathy, X-linked; WES, Whole exome sequencing; IFN, Interferon; pSTAT1, phosphorylated signal transducer and activator of transcription 1; ISRE(s), Interferon-stimulated response element(s); AIRE, Autoimmune regulator; PBMCs, Peripheral blood mononuclear Cells; EBV, Epstein–Barr virus; GAS, γ-Activated Sequence; MIC, Minimal inhibitory concentration; TSH, Tyrhoid-stimulating hormone; SLE, Systemic lupus erythematosus; TRECS, T-cell receptor excision circles; CT, Computed-tomography; PEP, Positive expiratory pressure; FEV1, Forced expiratory volume in 1 s; FEF 25–75%, Interval 25–75% of the forced vital capacity; G-CSF, Granulocyte-colony stimulating factor; GM-CSF, Granulocyte monocyte-colony stimulating factor; HSCT, Hematopoietic stem cell transplantation; MFI, Mean intensity fluorescence; HD, Healthy donor.

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CMCD of the skin, nails, and mucous membranes, associated to autoimmune diseases; life-threatening conditions such as squamous-cell carcinoma, cerebral and, recently, aortic aneurysms [9] were also rarely reported. Disseminated fungal (coccidioidomycosis, histoplasmosis, mucormycosis) and viral (herpes virus family, a case report of orf virus) infections were also described [10–13]. Mutations of *STAT1* have been identified by next generation sequencing also in patients with IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked)-like features [14], and in patients with severe, fatal phenotypes resembling combined immunodeficiency [10,15]. Any evidence of any genotype–phenotype correlation is emerging, and data about long-term morbidity and prognosis may be controversial. Herein, we report nine patients diagnosed with previously reported and novel GOF-*STAT1* mutations, showing large heterogeneity for age distribution and phenotypes.

2. Material and methods

2.1. Patients

We report nine patients who were diagnosed as affected with CMCD and evaluated for genetic analysis and *in vitro* functional studies from their peripheral blood, according to approved protocols of Spedali Civili in Brescia, Italy. Cases are described in details in [16]. Written informed consent was obtained from all patients or their parents (for minors), and for healthy controls. Medical history and clinical data were retrospectively collected from medical records. In all patients extended immunological assessment was performed. For patients under the care of the Department of Brescia a pulmonary evaluation, including assessment of pulmonary function tests and imaging, was carried out.

2.2. Molecular genetic analysis

Whole exome sequencing (WES) was performed at Children's Mercy Hospital and Clinics, Kansas City, Centre for Pediatric Genomic Medicine (Professor Kingsmore Stephen F) for P1. *STAT1* mutation was subsequently confirmed by Sanger sequencing in both P1 and his mother P2. In patients P3–P9 Sanger sequencing was used to analyze *STAT1*. DNA was isolated from whole blood using QIAamp DNA Blood Mini Kit (Qiagen). *STAT1* gene was amplified by PCR and products were sequenced using BigDye Terminator Kit (Applied Biosystems). Sequences were analyzed with 310 Genetic Analyzer (Applied Biosystems). Sequence variants were identified relative to a reference sequence, GenBank accession no. ENST00000361099 for the *STAT1* cDNA, in which the c.1 position corresponds to the A of the ATG translation initiation codon. Mutations are designated as recommended by den Dunnen and Antonarakis [17].

2.3. Analysis of phosphorylated STAT1 (pSTAT1) by flow cytometry

Peripheral blood was left unstimulated and stimulated with IFNγ (1000 U/ml), or IFNα (40,000 U/ml), for 30 min. Cells were lysed, permeabilized, and stained, as indicated by the manufacturer (BD Phosflow). Specific phycoerythrin-labeled antibody for phosphorylated STAT1 (pSTAT1) (pY701; BD Biosciences) was used. pSTAT1 was evaluated in both lymphocyte and monocyte gates. Cells were acquired using FACSCalibur (BD Bioscence) and analyzed by FlowJo version 7.5 Software (TreeStar).

2.4. Cytoplasmic and nuclear extracts preparation, Western blot and EMSA

After stimulation with IFNγ or IFNβ for 30 min, cells were lysed in cold buffer (150 mM NaCl, 50 mM Tris–HCl pH 7.4, 1% NP40, and protease inhibitors -Roche-containing 0.2 μg of aprotinin, leupeptin and 1 mmol/L of sodium orthovanadate) on ice for 15 min. For Western blot analysis, cytoplasmic extracts were resolved on 8% polyacrylamide and subjected to immunoblots by standard procedures. Nitrocellulose membranes were first blocked for 1 h at room temperature in TBST containing 5% BSA, and then incubated overnight at 4 °C with specific primary Abs (pSTAT1, STAT1) in the same buffer. Antibodies against phospho-tyrosine STAT1 were purchased from Cell Signaling Technologies (Denver, MA, USA). Antibodies against total STAT1 (sc-346) were obtained from Santa Cruz, and antibodies against β-actin were obtained from Sigma-Aldrich. Detection was carried out using HRP-conjugated anti-mouse or anti-rabbit IgG (Amersham Biosciences), and revealed using ECL system (Amersham Biosciences). For EMSA, after stimulation with IFNγ and/or IFNβ for 30 min, EBV transformed B cells (5×10^6/condition) were diluted in ice-cold PBS and centrifuged twice at 300 X g for 5 min at 4 °C. Nuclear extracts were prepared using a modification of the method of Dignam *et al.* [18]. Transcription factor-binding analyses were performed as described previously [18]. Nuclear extracts were incubated in binding buffer in the presence of the labeled oligonucleotide STAT-binding probe from the GRR element located within the promoter of the FcγRI/CD64 gene (5-CTTTTCTGGGAAATACATCTCAAATCCTTGAA ACATGCT-3) or from the interferon-stimulated response element (ISRE) (5-GATCGGGAAAGGGAAACCGAAACTGAA-3).

2.5. Mutagenesis assay

eGFP STAT1 WT vector (Addgene) was used to obtain mutated STAT1 form carrying L351F and L400V variants. Mutations were generated by site direct mutagenesis QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies).

2.6. Luciferase reporter assay

U3C cells were seminated into 96-well plates (1×10^4/well) and transfected with 100 ng/well reporter plasmids and plasmids carrying alleles of STAT1 (L351F and L400V) or a mock vector with Arrest-in (Thermo Scientific). After 6 h, cells were transferred into medium containing 10% FBS and cultured for 24h. Cells were stimulated with IFNγ at different concentration (10, 100, 1000 UI/ml), and IL-27 (100 ng/ml) for 16h. Luciferase production was assesed with Dual-Glo luciferase assay system (Promega) and normalized with respect to Renilla luciferase activity. Data are expressed as fold inductions with respect to unstimulated cells.

2.7. Statistical analysis

Statistical significance of *in vitro* immunological studies was analyzed by nonparametric two-side Mann–Whitney U-test with 95% confidence bounds. For all analyses $p < 0.05$ was considered statistically significant. Statistical analyses were performed using GraphPad Prism Version 5.0 (GraphPad Software, San Diego, CA).

3. Results

3.1. Genetic findings

We identified a novel familial *STAT1* mutation (L283M) in P1 (by WES) and in his mother P2. Other two previously unreported sporadic mutations were detected respectively in P3 and P9 (L351F), and in P6 (L400V). P3 had been previously analyzed for *AIRE* (autoimmune regulator) gene, showing a heterozygous variation 769C > T (R257X) he inherited from his healthy mother. Other mutations were: T385M in P4 and P5 [19], A267V in P7 [2], and T387A in P8 (Pignata personal communication; Higgins *et al.* [20]). All are heterozygous missense mutations (Table 1). Novel *STAT1* mutations affect both CCD and DBD domains.

3.2. Novel mutations result in increasing STAT1 phosphorylation

We investigated IFNα- and IFNγ-induced pSTAT1 by cytometry from peripheral blood mononuclear cells (PBMCs) of our patients compared to healthy controls (Fig. 1 in [16]). Overall, we observed

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