Impact of molecular diagnosis on treating Mendelian susceptibility to mycobacterial diseases

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Background/Objective: The IL-12–IFN-γ axis is critical for immune defense against mycobacterial infections. Inherited mutations that affect normal activation of this self-amplifying cytokine reaction lead to increased chances of mycobacterial infections, known as Mendelian susceptibility to mycobacterial diseases (MSMD). Delayed diagnosis and difficulty in identifying pathogenic mycobacteria hinder proper treatment of patients, so the aim of this study was to facilitate the diagnosis of mycobacterial infections in MSMD patients using an oligonucleotide array method.

Methods: Peripheral blood mononuclear cells (PBMCs) were isolated from three MSMD patients in the same family. A series of immunologic studies, including testing for cytokine secretion after leukocyte stimulation, cell-surface marker analysis, and cDNA sequencing, were then performed. An oligonucleotide array was used to rapidly identify pathogens.

Results: Cytokine secretion testing showed normal IFN-γ secretion after IL-12 stimulation but low IL-12 secretion after IFN-γ stimulation, which indicates a defect in the IFN-γ receptor or its intracellular signaling. Cell-surface receptor analysis showed IFN-γ receptor 1 overexpression, suggesting an autosomal dominant IFN-γ receptor 1 deficiency. cDNA sequencing identified the IFNGR1 818del4 mutation in three members of the family with known MSMD, and an oligonucleotide array identified Mycobacterium tuberculosis complex and Mycobacterium abscessus as pathogens.

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Introduction

Severe infections caused by low-virulence mycobacteria, including Bacillus Calmette–Guérin (BCG) and environmental non-tuberculous mycobacteria (NTM), often occur in patients with several types of primary immunodeficiency. It has been reported that gene mutations leading to severe combined immunodeficiency (SCID), chronic granulomatous disease (CGD), hyper-IgE syndrome, and a defective interferon-γ (IFN-γ)–IL-12 axis cause increased susceptibility to mycobacteria. Different from people with other primary immunodeficiencies, patients with defects in the IFN-γ–IL-12 axis, termed Mendelian susceptibility to mycobacterial diseases (MSMD), are particularly susceptible to mycobacterial or salmonella infections, while other infections are relatively rare.

Treatment for mycobacterial infections in MSMD patients depends on early identification of the infecting microorganisms and effective antibiotic treatment. However, early diagnosis of the causative microorganisms in these patients can be difficult because isolation of mycobacteria by culture may take up to weeks and is often unsuccessful. A strategy for rapid identification of the infecting microorganisms is therefore crucial for treatment of this group of immunocompromised patients with frequent and severe mycobacterial infections.

Here, we report the molecular characterization of three MSMD patients from the same family. Each of them suffered from different mycobacterial infections. Using the oligonucleotide array method, we identified the infectious mycobacteria in two of the patients and successfully treated them based on the results.

Patients and methods

The proband was a male infant (Patient 1). His older sister (Patient 2) and his mother (Patient 3) were found to have the same disease-causing mutation (Fig. 1A).

Patient 1

A 6-month-old boy was referred to our hospital due to fever and severe cough with profuse sputum for 1 month. Physical examination on admission revealed a febrile and acutely ill-looking infant with tachypnea. Chest auscultation showed bilateral crackles. Palpation revealed hepatosplenomegaly. Generalized erythematous papules were found all over the body surface (Fig. 1B). BCG vaccine was given 5 months before admission shortly after his birth. An unhealed BCG vaccination wound over the left upper arm (Fig. 1C) and left axillary lymphadenopathy were found. A chest X-ray (CXR) examination showed diffuse bilateral pulmonary nodular infiltrates (Fig. 1D). Acid-fast staining of gastric lavage fluid, sputum, and skin biopsy showed the presence of bacilli. Mycobacterial culture was negative after 8 weeks. The absolute numbers and ratios for his lymphocyte subpopulations were within normal limits. Normal respiratory burst was found in his stimulated phagocytic leukocytes. Immunological study and gene sequencing revealed that the patient had a disease-causing mutation on the gene encoding IFN-γ receptor 1. An oligonucleotide array method was used to identify the mycobacteria in a skin biopsy sample. His clinical condition gradually improved after anti-M. tuberculosis complex treatments including isoniazid, rifampin, ciprofloxacin, and amikacin. CXR examination showed clearance of the lung lesions 2 months after the anti-mycobacterial treatment was started (Fig. 1E). The patient is currently on antibiotic and IFN-γ treatment and shows normal growth and development.

Patient 2

A 19-month-old girl who had suffered from recurrent fever for 3 weeks was referred to our hospital 9 years ago. A gallium inflammation scan and magnetic resonance imaging (MRI) revealed osteomyelitis over L1–2 and L5 (Fig. 1F). After empiric antibiotic treatment for 8 weeks, intermittent fever persisted. Follow-up laboratory data and MRI revealed no improvement in her osteomyelitis. Acid-fast staining of a bone biopsy showed the presence of bacilli, so antituberculosis drug therapy was initiated with isoniazid, rifampin, pyrazinamide, and ethambutol. Mycobacterial culture revealed Mycobacterium avium complex 12 weeks later. We then changed the anti-mycobacterial drugs to clarithromycin and rifampin. The patient’s clinical condition gradually improved after the above treatment. cDNA sequencing revealed the same disease-causing mutation (Fig. 1A) as in Patient 1. She is currently on prophylactic antibiotic treatment with azithromycin. Genetic counseling on the autosomal dominant (AD) inheritance was provided for the family.

Patient 3

A 35-year-old woman had an enlarging wound (size on examination, 8 × 8 cm²) on her right knee for 8 years (Fig. 1G). She had been given many different treatments, including empiric antibiotics, wound debridement, hyperbaric oxygen therapy, and skin grafting, before she presented at our hospital. Wound biopsies revealed granulomatous inflammation. However, acid-fast staining and mycobacterial culture produced negative results.
repeatedly. After *Mycobacterium abscessus* was identified by an oligonucleotide array, she was treated with azithromycin and ciprofloxacin, along with IFN-γ therapy. cDNA sequencing revealed the same disease-causing mutation as in Patients 1 and 2. The wound condition gradually improved, with apparent granulation tissue formation on the central area and scar formation on the margin 2 months after initiation of the treatment (Fig. 1H).

**Figure 1.** Clinical characteristics of patients with MSMD. (A) Pedigree of this family. (B) Multiple erythematous papules on the left leg of Patient 1. (C) An unhealed BCG vaccination wound on the left arm of Patient 1. (D) Diffuse bilateral pulmonary nodular infiltrates in the CXR of Patient 1. (E) Improvement in CXR findings after 2 months of anti-BCG treatment of Patient 1. (F) Multiple vertebral osteomyelitis over L1–2 and L5 (shown by arrows) in MRI results for Patient 2. (G) A large and difficult-to-heal wound on the right knee of Patient 3. (H) The wound improved after 2 months of antibiotic treatment against *Mycobacterial abscessus*.

**Stimulated cytokine production by peripheral blood mononuclear cells**

Peripheral blood mononuclear cells (PBMCs) from healthy control individuals and patients were obtained by gradient centrifugation of peripheral blood on Ficoll-paque (GE Healthcare, Piscataway, NJ, USA). Separated PBMCs were prepared at $1 \times 10^6$/mL in RPMI1640 with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA). The cells were
then stimulated with phytohemagglutinin (PHA, 1%; Sigma, St. Louis, MO, USA) or lipopolysaccharide (LPS, 200 ng/mL; Sigma) with or without IL-12 (1 ng/mL; ProSpec-Tany TechnoGene, Rehovot, Israel) or IFN-γ (1000 U/mL; ProSpec-Tany TechnoGene). Supernatants were collected after 48 hours of incubation at 37°C and stored at –20°C before cytokine detection.

**Anti-cytokine autoantibody detection**

Plasma samples from healthy control individuals and patients were diluted 50 and 5000 times and incubated 1:1 with fixed concentrations of recombinant IL-12 and IFN-γ at 37°C for 1 hour. IL-12 and IFN-γ levels were detected using a Bio-plex cytokine assay (Bio-Rad, Hercules, CA, USA) system and a Luminex 200 analyzer (Luminex, Austin, TX, USA).

**Cytokine determination**

Bio-plex cytokine assays were used to determine cytokine levels in supernatants from PBMC stimulation or anti-cytokine autoantibody detection experiments. IL-12, IFN-γ, TNF-α, IL-1β and IL-6 were detected. In brief, supernatants were incubated with beads coupled to anti-cytokine capture antibodies. After 30 minutes of incubation and a washing procedure, the beads were incubated with detection antibodies for another 30 minutes. Finally, the beads were labeled with streptavidin-PE and then analyzed on a Luminex 200 analyzer.

**Cell-surface IFNGR1 (CD119) expression**

Detection of IFNGR1 by flow cytometry on fresh blood cells was performed using a carboxyfluorescein (CFS)-conjugated mouse anti-CD119 monoclonal antibody (R&D, Minneapolis, MN, USA). Whole blood samples were treated with BD Lysing Solution (BD Biosciences, San Diego, CA, USA) and then incubated with 10 μL of antibody at 4°C for 30 minutes. After incubation, unreacted antibody was removed by washing the cells twice with ice-cold phosphate buffer solution (PBS). As a control, cells in a separate tube were treated with non-binding fluorescein-labeled mouse IgG1 antibody.

**Gene sequencing**

Polymerase chain reaction (PCR) of the IFNGR1 cDNA coding region was performed and the PCR products were directly sequenced by the Center for Genomic Medicine of National Cheng Kung University.

**DNA extraction**

DNA was extracted from tissue biopsies obtained from the patients. Six paraffin sections were dissolved in 1 mL of xylene and centrifuged at 12,000 rpm for 10 minutes. The pellet was washed with 1 mL of absolute alcohol and then centrifuged at the same speed for 10 minutes. DNAs in the resulting precipitate and in the tissue biopsy were extracted with a Qiagen DNeasy Blood and Tissue kit (Hilden, Germany) according to the manufacturer’s instructions.

**Identification of mycobacteria by oligonucleotide array**

A membrane array (Blue Point, Sancordon Co., Taichung, Taiwan) was used to identify mycobacteria in the extracted DNA according to the manufacturer’s instructions. The array was capable of identifying Mycobacterium tuberculosis complex and 21 NTM species. The procedures of array hybridization consisted of PCR amplification of a region of the rRNA operon, hybridization of the digoxigenin-labeled amplicon to probes on the array, and then reaction with enzyme-conjugated anti-digoxigenin antibodies and enzyme substrate. The hybridized spot (0.4 mm in diameter) was read by the naked eye. An isolate was identified to the species level with the positive control probe (mycobacteria-specific) and species-specific probe (or probes) being hybridized at the same time.

**Results**

**PBMCs from our patients secreted low levels of IL-12 and TNF-α after IFN-γ stimulation**

We investigated IL12- or IFN-γ-induced cytokine secretion by activated PBMCs from our patients. IL-12 stimulation induced equivalent IFN-γ secretion by PHA-activated cells from our patients and healthy control individuals (Fig. 2A). However, IFN-γ stimulation did not effectively induce IL-12 or TNF-α secretion in LPS-treated PBMCs from the patients, while cells from healthy control individuals produced higher amounts of IL-12 or TNF-α (Fig. 2B and C). By contrast, IL-6 secretion after LPS and IFN-γ stimulation was equivalent in cells from the patients and the control individuals (Fig. 2D). These findings indicate that IFN-γ-induced PBMCs activation was defective in our patients.

It has been reported that an auto-antibody against IFN-γ is a cause of susceptibility to NTM infection,7–10 so we tested serum for auto-antibodies against IL-12 (Fig. 2E) and IFN-γ (Fig. 2F). We diluted serum samples 100 or 10,000 times with PBS and measured their activity for neutralization of IFN-γ or IL-12 recombinant proteins. Serum from a patient with strong neutralizing activity against IFN-γ, as previously reported,11 was used as a positive control (Fig. 2E and F). We did not find significant neutralizing activity in serum samples from healthy control individuals and our patients. These findings indicate that the susceptibility to NTM infection observed in our patients is probably due to an intrinsic defect in IFN-γ-induced leukocyte activation.

**Cell-surface receptor expression and molecular diagnosis revealed that the patients have an AD IFNGR1 mutation**

We analyzed IFNGR1 (CD119) expression on monocytes from our patients by flow cytometry. CD119 levels were higher in
patients than in healthy control individuals (Fig. 3A). This finding is consistent with previous reports of patients with AD IFNGR1 deficiency. We then sequenced the complete cDNA of the IFNGR1 gene and found a deletion of four nucleotides in exon 6, designated 818del4, which leads to a truncated IFNGR1 peptide (Fig. 3B).

Oligonucleotide array for *Mycobacterium* species identification

In the two recently infected patients, we used an oligonucleotide array method to detect and identify mycobacteria in their tissues. *M. tuberculosis* complex was identified in skin biopsy tissue (paraffin sections) from Patient 1. *M. abscessus* was identified in wound biopsy tissue from patient 3 (Fig. 4).

**Discussion**

Our three patients with MSMD were infected by different atypical mycobacteria due to an inherited defect in response to IFN-γ. Their different clinical courses highlighted the difficulty in clinical decision-making in patients with primary immunodeficiencies. Even when a genetic
diagnosis of MSMD is made, timely microorganism identification, which is essential for effective infection control, can be a difficult and time-consuming process. In this family with an AD IFNGR1 mutation, the present study underscores use of the oligonucleotide array method to determine the pathogenic microorganism.

Gene mutations leading to MSMD have been found in genes encoding IFNGR1, IFNGR2, IL-12B, IL-12RB1, NF-kB essential modulator (NEMO), and signal transducer and activator of transcription-1 (STAT1), which are all crucial for the IL-12–IFN-γ axis.3 We showed that the gene mutation leading MSMD in our patients is an AD IFNGR1 deficiency caused by a small deletion of four nucleotides in a mutation hotspot.13 This gene mutation leads to a premature stop codon in the proximal intracellular domain and produces a truncated receptor peptide lacking the domains for signal transduction and receptor recycling. Consequently, leukocytes from these patients showed a three- to fivefold increase in IFNGR1 (CD119) expression in flow cytometry analysis. The more abundant mutant IFNGR1 pairs with itself other or wild-type IFNGR1 and inhibits the signals triggered by IFN-γ in a dominant-negative manner.13,14

Two key points are crucial for successful treatment of MSMD. First, the gene mutations responsible for the defective immunity against mycobacterial infection should be identified through effective differential diagnoses and laboratory examinations. A newly characterized disease entity with anti-cytokine autoantibodies that lead to susceptibility to NTM infections has been reported in several groups, including our own.7,10,11 An anti-IFN-γ autoantibody inhibits the IFN-γ-mediated immune response against mycobacteria and leads to severe NTM infections in patients, especially those of Southeast Asian origin. The present study started by excluding the possibility of the presence of anti-cytokine autoantibody in our patients by performing cytokine neutralization assays (Fig. 2E and F) before pinpointing the defect in IFNGR1.

Since IFN-γ is critical for macrophage activation, the failure of infected macrophages to kill intracellular bacteria in patients with MSMD may play an important role in the particular susceptibility to mycobacteria. However, IFNGR is also expressed on lymphocytes and other immune cells. The production of other cytokines and small-molecule mediators, including reactive oxygen species (ROS) and reactive nitrogen species (RNS), which are sensitive to IFN-γ stimulation and crucial for defense against mycobacteria,15,16 may also be responsible for the immune defects. In other MSMD gene mutations such as IL-12p40 deficiency and STAT1 deficiency, the involvement of different leukocyte populations and cytokines, including IL-17, may complicate the immunodeficiency in these patients and change the spectrum of infections.17,18 Molecular diagnosis of the immunodeficiency should therefore be performed as one of the top priorities in patients with suspected MSMD.19

Figure 3. The defective response to IFN-γ in leukocytes from the patients is due to a mutation in IFN-γ receptor 1. (A) Peripheral blood mononuclear cells were isolated from normal control individuals and the patients. Surface expression of CD119 was analyzed by CD119-CFS cell surface staining and flow cytometry. A histogram profile for cells from Patient 1 is shown. Similar results were found for cells from Patients 2 and 3. (B) Sequencing of cDNA revealed a small deletion (818del4) in one allele of IFNGR1 exon 6, which leads to dual signals after nucleotide 818. Similar results were found for samples from Patients 2 and 3.

Figure 4. Mycobacteria in tissue samples were identified with an oligonucleotide array test. DNAs extracted from tissue samples from the patients were tested using a mycobacterial oligonucleotide array. (A) Negative control. (B) Patient 1. (C) Patient 3. The arrows indicate positive hybridization signals on the arrays. Dots in the first row and column are position markers. Spots corresponding to Mycobacterium spp. are indicated.
The other key point for treating infections in MSMD patients is to identify the microorganism responsible for the infection as early as possible. Because immunodeficient patients tend to have faster infection progression, the pathogen must be identified rapidly so that appropriate antibiotics can be given. In two of our three patients, the mycobacterial culture produced negative results, which might be attributable to previous antibiotic treatment. We hence used an oligonucleotide array to identify the mycobacteria and guide the use of antibiotics.

Traditionally, microscopic examination after acid-fast staining and culture using specific media are the cornerstones of diagnosis for mycobacterial infections. However, direct microscopy has lower sensitivity and cannot identify mycobacterial species. Mycobacterial culture may take up to weeks because of the slow growth rates of many non-tuberculosis microorganisms.20 Because timely detection and identification of mycobacterial species and other slow-growing microorganisms are important for clinical treatment, DNA-based molecular methods, including the oligonucleotide array technique, have been developed for their sensitivity and specificity.21–23 For our oligonucleotide array test, the entire testing procedure, including DNA extraction, PCR, DNA hybridization, and signal detection, can be finished within 7 hours. The oligonucleotide array should be an important tool for diagnosing mycobacterial infections in MSMD patients in the future.

In conclusion, invasive mycobacterial infections are common in patients with MSMD. The molecular diagnosis of primary immunodeficiency should be pursued diligently and oligonucleotide arrays may be a useful and rapid method for identifying the pathogens in these patients.

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References