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Original article

Clinical and molecular characteristics of Thai families with autosomal recessive chronic granulomatous disease

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Background: Autosomal recessive chronic granulomatous disease (AR-CGD) is an inherited defect in neutrophil oxidative burst as a result of mutations in one of the three genes, *NCF1, NCF2*, and *CYBA*, which respectively encode p47-phox, p67-phox and p22-phox subunits of the NADPH oxidase complex.

Objectives: To investigate clinical and molecular characteristics of two unrelated Thai patients with AR-CGD.

Methods: A Thai girl who suffered from pulmonary aspergillosis at the age of two months and another unrelated Thai boy presented with recurrent cutaneous abscesses caused *by Chromobacterium violaceum* since 30 months old, were investigated. The DHR assays revealed abnormalities in both patients but normal results in their mothers, consistent with the diagnosis of AR-CGD. PCR-sequencing of the entire coding regions of *NCF1, NCF2*, and *CYBA* was performed.

Results: A homozygous c.75_76delGT mutation at the beginning of exon 2 of *NCF1* was identified in both individuals. This mutation resulted in a frameshift with premature termination of p47-phox at codon 51 (p.Val25fsX51).

Conclusion: The homozygous GT deletion in NCF1 may be a common mutation in Thai patients with AR-CGD. Unlike all other autosomal recessive disorders, AR-CGD caused by *NCF1* mutations has a unique mutational pattern, in which there is only one mutation responsible for most patients regardless of their ethnic backgrounds.

Keywords: Chronic granulomatous disease, CGD, mutation analysis, *NCF1*.

Chronic granulomatous disease (CGD) is a rare inherited primary immunodeficiency disorder characterized by the inability of phagocytes to kill intracellular organisms via reactive oxygen species due to the defective reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex [1]. Affected individuals usually present in early childhood with recurrent bacterial and fungal infections, typically from catalase-positive bacteria and *Aspergillus spp* [2]. Diagnosis requires demonstration of impaired neutrophil ability to undergo superoxide-generating respiratory burst bursts by using the nitroblue tetrazolium (NBT) test or the flow cytometric dihydrorhodamine-1,2,3 (DHR) assay [3], which can be confirmed by mutation analysis.

The most common form of CGD, accounting for approximately 65% of cases, is caused by a defect in gp91-phox, a membrane-bound NADPH oxidase subunit encoded by the X-linked *CYBB* gene [4]. The rest are inherited in an autosomal recessive manner (AR-CGD) associated with inactivating mutations in one of the three genes, *NCF1 a*t 7q11.23 encoding p47-phox, *CYBA* at 16q24 encoding p22-phox, and *NCF2* at 1q25 encoding p67-phox. This was reported to be responsible for 24%, 6% and 5% of cases, respectively [4-6]. This study aimed to investigate clinical and molecular characteristics of two unrelated Thai families with AR-CGD.

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Materials and methods

Patients

Two Thai families were included in the study. The proband of family 1 was an 18-month-old girl, from non-consanguineous parents, presented with hemoptysis at the age of two months. Physical examination revealed enlarged liver and spleen. Laboratory investigation showed leukocytosis (WBC 30,000 cells/mm^3, neutrophils 56%, lymphocytes 41%). Prothrombin time and partial thromboplastin time were normal and erythrocyte sedimentation rate (ESR) was elevated to 55 mm/hour. Computer tomography (CT) angiography of the chest demonstrated several pulmonary nodular opacities in the right middle lobe and the left upper lobe, with a feeding vessel leading into the right upper lobe consolidation. Galactomannan screening for *Aspergillus infection* was positive. Open lung biopsy was performed and tissue culture confirmed *Aspergillus* fumigatus infection. The proband had a modest DHR shift with a broad-based histogram (patient stimulation index [SI] 31.0, control SI 150.5), while the DHR histogram pattern in the patient's mother was normal (SI 93.9), consistent with autosomal recessive inheritance of CGD (see **Fig. 1A**) [7]. After initial improvement with amphotericin B treatment, she was put on prophylactic therapy with itraconazole and trimethoprim-sulfamethoxazole. However, she later had a relapse of pulmonary aspergillosis and suffered from itraconazole-induced hepatitis, which led to the use of voriconazole as prophylaxis. The patient also had multiple catheter-related infections with *Staphylococcus aureus*, *Serratia marcescens*, and *Proteus mirabilis* during the course of illness.

The proband of family 2 was a four-year-old boy, born to non-consanguineous parents, presenting with recurrent cutaneous abscesses and generalized lymphadenopathy since two and a half years of age. Laboratory studies showed mild leukocytosis (WBC 16,950 cells/mm^3, neutrophils 61%, lymphocytes 31%) and increased ESR of 48. Incision and drainage were performed and *Chromobacterium violaceum* was the only organism identified from pus cultures. The hemoculture grew no organism. Abdominal ultrasonography did not show any evidence of intraabdominal collection or abscess. The DHR histograms of the patient and his mother showed similar patterns to those of family 1 (patient SI 43.7, control SI 157.7, mother SI 132.0; see **Fig. 1B**). The patient was successfully treated with trimethoprim-sulfamethoxazole, and subsequently started on antibiotic prophylaxis using the same drug.

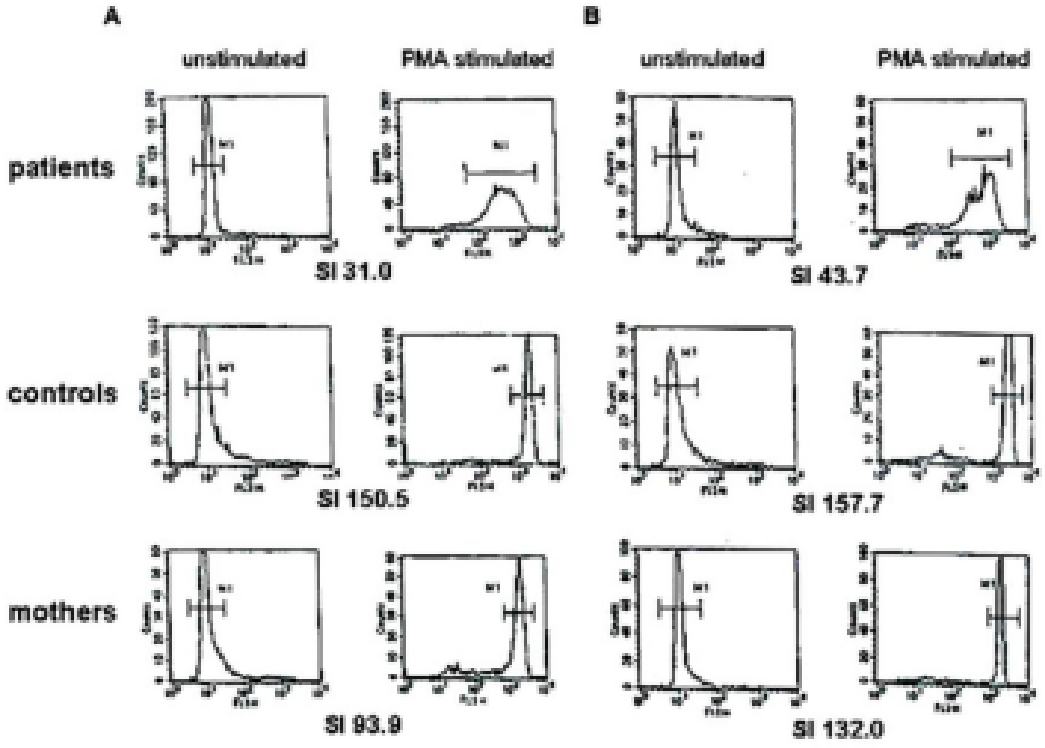

Fig. 1 DHR histograms of both families with AR-CGD. **A:** family 1. **B:** family 2. The patients demonstrated a modest DHR shift with a broad-based histogram after PMA stimulation (upper panel), while both patients' mothers showed a normal pattern (lower panel) similar to that of the control (middle panel**). SI**: stimulation index, **PMA:** phorbol 12-myristate-13-acetate.

Mutation analysis

Blood samples were collected from both CGD patients and parents of family 1 after informed consent was obtained. Samples from parents of proband 2 were unavailable. RNA was isolated from peripheral blood leukocytes using QIAamp RNA Blood Mini Kit (Qiagen, Valencia, USA). Reverse transcription was performed using ImProm-II ™ reverse transcriptase (Promega, Madison, USA), according to the manufacturer's recommendations. Polymerase chain reaction (PCR) amplifications of *NCF1* exons 1-11, *NCF2* exons 1-15, and *CYBA* exons 1-6, were performed using 1 μL of cDNA, 1X PCR buffer, 1.9 mM $MgCl_2$ (except for 2.3 mM $MgCl_2$ for *CYBA*), 0.2 mM dNTP, 5% DMSO, 0.15 μM of each primer (except for 0.1 μM of primers for *NCF1*), and 0.5 U *Taq* DNA polymerase (Fermentas Hannover, USA) in a final volume of 20 μL. Genomic DNA (gDNA) was extracted from whole blood using a standard extraction method. Exon 2 (81 bp) of the *NCF1* gene was PCR-amplified using 3 μL of gDNA, 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.2 μM of each primer, and 0.5 U *Taq* DNA polymerase in a final volume of 20 μL.

The primer sequences and PCR conditions to amplify *NCF1*, *NCF2*, *CYBA* cDNAs, and exon 2 of *NCF1* are shown in **Table 1**. All PCR products were treated with ExoSAP-IT (USP Corporation, Cleaveland, OH), according to the manufacturer's instructions, and sent for direct sequencing at Macrogen Inc (Seoul, Korea). The sequence was analyzed using Sequencher (version 4.2, Gene Codes Corporation, Ann Arbor, USA).

Results

Sequence analysis of the cDNA derived from both probands' samples showed homozygosity for a GT deletion at nucleotide position 75/76 (c.75_76delGT) of the coding sequence of *NCF1* (data not shown). The mutation was confirmed by the presence of a homozygous GT deletion at the start of exon 2 of *NCF1* in gDNA of both patients (**Fig. 2A, B**), predicting a frameshift and premature termination at codon 51 (p.Val25fsX51). The gDNA sequence of an unaffected control (**Fig. 2C**) revealed double sequence pattern as a result of co-amplification of the GTGT-containing *NCF1* functional gene and its pseudogenes, which lack GT at the beginning of exon 2. The superimposed sequence was similarly detected in gDNA of both parents of proband 1, although the ratio of the mutant (pseudogene) sequence to the wild type (functional) sequence was higher than that of the unaffected control (data not shown). No other mutations were identified in cDNAs of *NCF1*, *NCF2*, and *CYBA* of both probands.

Table 1. Oligonucleotides and PCR Conditions for *NCF1*, *NCF2*, and *CYBA* mutation analysis.

NCF1 cDNA	F	CGACTTCCTCTTTCCAGTGC	55
	R	GACGCCAGGCTCTATAGAAC	
NCF2 cDNA	F	GAAGTTATCTTAAGGGAGG	53
	R	GTACAGTATACAGCAGAAGG	
CYBA cDNA	F	TCCCAGCCGGGTTCGTGTCG	54
	R	GGCTTCGCTGCATTTATTGC	
Exon 2 of *NCF1*	F	ACTTGGGTCCACGTTTGTGC	52
	R	CCCCTTTAACTGTGGTCTGG	

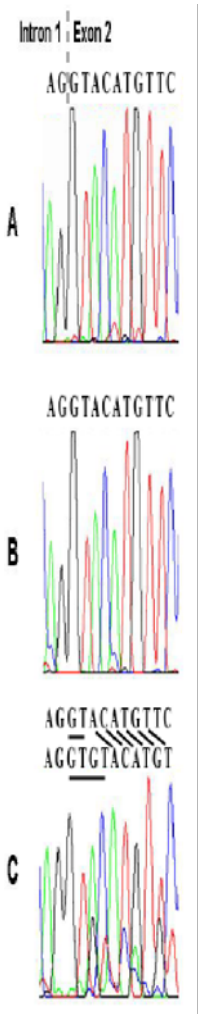

Fig. 2 *NCF1* mutation analysis. Genomic DNA sequencing of (**A**) proband 1, and (**B**) proband 2, revealing homozygosity for a dinucleotide GT deletion at the start of exon 2; (**C**) an unaffected control revealing a superimposed (pseudogene) sequence on the wild-type sequence (double sequence pattern) due to co-amplification of the *NCF1* functional gene and its pseudogenes.

Discussion

Our study described the clinical and genetic features of two unrelated Thai patients affected with CGD who manifested with different degrees of clinical severity at variable age of onset. The first subject exhibited an earlier onset of serious pulmonary infection with *Aspergillus fumigatus*, the major microorganism involved in CGD, during her first few months of life. The other patient presented in his third year with relatively milder symptoms of recurrent cutaneous abscesses caused by Chromobacterium violaceum. Both patients were found to have a homozygous GT deletion (ΔGT) at the start of exon 2 of the NCF1 gene (c.75_76delGT), resulting in a frameshift with premature stop codon at amino acid position 51 (p.Val25fsX51).

Chromobacterium violaceum is a gram-negative saprophyte, which is generally considered to be of low virulence and rarely causes diseases in immunocompetent hosts. Individuals with chromobacterial infections should therefore undergo an immunologic workup to identify the underlying defects in host defenses. The organism can produce catalase [8], a key enzyme responsible for the protection of intraphagosomal bacteria against toxic oxygen radicals generated by the host's phagocytes. This could explain the increased susceptibility to chromobacterial infections in patients with CGD, in which reactive oxygen species production is impaired. In a review by Sirinavin et al. [9], about one-third (9/25) of children with invasive *C. violaceum* infection, were diagnosed with CGD. Skin was found to be the

most important portal of entry for this saprophyte, which is commonly found in soil and water. Cellulitis, abscesses of skin and visceral organs were the major clinical manifestations, and could lead to bacteremia and sepsis if the treatment was delayed [9]. Fortunately, our patient exhibited only cutaneous involvement without evidence of dissemination. Although patients with CGD are considered to be at risk for chromobacterial infection, the prevalence is rare. According to the report from the national registry of CGD in the USA [10], between November 1993 and September 1997, only 1 of 368 CGD patients was found to be infected with *C. violaceum*. We hereby reported a case of molecularly proven AR-CGD with *C. violaceum* infection.

In family 1, the affected child was female, which favors the autosomal recessive mode of transmission. This was supported by the impaired phagocyte oxidative burst activity in her DHR result, while it was normal in the mother's [7]. In boys with CGD, like our proband 2, one might expect a more common X-linked mode of inheritance, which was previously reported in Thai patients [11]. However, most cases of X-linked CGD tend to have more severe clinical symptoms early in life. The fact that he had a milder severity and later age of onset led us to hypothesize that he might have the AR form. This was supported by his DHR result which showed modest activity (SI 43.7) with a broad-based histogram [7]. However, it is noted that, 17% of male patients with X-linked CGD have modest DHR shift overlapping with the AR-CGD pattern [7]. Thus, additional studies to help establish the genotype are warranted. In our male patient, the definite diagnosis of AR-CGD was later confirmed by the normal DHR histogram pattern in the mother and also supported by mutation analysis.

Sequence analysis of our patients revealed defect in NCF1, the most frequently mutated gene in the AR form, leading to p47-phox subunit deficiency. In agreement with previous reports in racially diverse populations [12-14], both probands were found to have a homozygous GT deletion (ΔGT) at the start of exon 2 of the *NCF1* gene (c.75_76delGT), resulting in a frameshift with premature stop codon at amino acid position 51 (p.Val25fsX51) [15]. This mutational hot-spot accounts for the great majority (>97%) of p47-phox-deficient patients [4, 15, 16], unlike the other AR or X-linked forms of the disease, in which there is substantial heterogeneity among mutations [4]. The predominance of this single defect is most likely explained by recombination events between the wide-type *NCF1* gene and at least one of its pseudogenes located near the functional gene at chromosome 7q11.23 [16, 17]. The pseudogenes show a high degree of sequence identity (approximately 99%) to the functional gene, but contain the GT sequence instead of GTGT at the beginning of exon 2 [18]. Apparently, the pseudogenes are highly expressed at the mRNA level [16-18], predicted to produce a truncated nonfunctional protein of 50 amino acids. Consistent with previous studies, our findings supported the presence of these pseudogenes in unaffected individuals. The pseudogenes are co-amplified and co-sequenced with the functional gene, appearing as a superimposed sequence. This thus complicates the diagnosis of carrier status when using standard PCR sequencing methods. For accurate carrier detection, other techniques including the gene-scan method could be used [19].

Besides p47-phox deficiency, several other autosomal recessive disorders such as Gaucher disease [20], 21-hydroxylase deficiency [21], and von Willebrand disease [22], have gene-pseudogene recombination as a source of pathogenic mutations. These other recessive disorders have diverse mutations and the most common mutations among different populations were dissimilar. Conversely, p47-phox deficiency has a distinctive feature of overrepresentation of a single defect. This could be partly explained by the findings of the GT deletion as the only deleterious mutation identified in the pseudogenes of *NCF1* in contrast to other recessive disorders, where several deleterious mutations are present within their pseudogenes. Considering the distribution of mutations across different populations in genes responsible for most of the autosomal recessive disorders, such as, the cystic fibrosis transmembrane conductance regulator gene associated with cystic fibrosis [23], and the β globin gene associated with β thalassemia [24], there is great mutational heterogeneity. Some mutations can reach higher frequencies in some populations as a result of founder effect or genetic drift. To our knowledge, AR-CGD caused by *NCF1* mutations would be a disorder with a unique mutational pattern, in which there is only one mutation responsible for most patients regardless of their ethnic backgrounds and the existence of pseudogenes.

In conclusion, the homozygous GT deletion in *NCF1*, which has been commonly reported

irrespective of ethnic origins, was also found in Thai patients with AR-CGD. Our study emphasized the unique mutational pattern of *NCF1*, which is unlike all other autosomal recessive diseases regardless of the existence of pseudogenes.

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The authors have no conflict of interest to declare.

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