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CASE REPORT

A Novel Mutation of the *CYBB* Gene Resulting in Severe Form of X-Linked Chronic Granulomatous Disease

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SUMMARY We evaluated a boy who had multiple *Salmonella* septicemia, *Aspergillus* pneumonia and brain abscesses. His nitroblue tetrazolium (NBT) test was reportedly abnormal. The dihydrorhodamine (DHR) flow cytometry assay was compatible with typical X-linked chronic granulomatous disease (X-CGD). *CYBB* analysis revealed a novel complex mutation atggacg → ttca in exon 12 (base pairs 1532-1538). As a result, 3 amino acids Tyr 511, Gly 512 and Arg 513 were deleted and replaced by 2 amino acids, Phe and Gln. The DHR and mutation analysis of his mother showed normal DHR pattern and no mutations in exon 12 of *CYBB* gene. In conclusion, any children with multiple *Salmonella* and *Aspergillus* infection should be suspected of CGD. NBT test, DHR assay and gene analysis are helpful tools to confirm the diagnosis even in the case of *de novo* mutation.

Chronic granulomatous disease (CGD, OMIM 306400) is a primary phagocytic disorder involving the reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex which results in defective superoxide formation and intracellular microbe killing.[1] CGD patients present with recurrent life-threatening bacterial and fungal infections, poor wound healing and formation of chronic granulomas.[1] CGD may be inherited in an X-linked (X) or autosomal recessive (AR) manner. X-CGD is far more common, accounting for about 70% of all CGD cases.[2] The X-CGD gene, *CYBB* (GenBank AF469757–AF469769), is localized on chromosome Xp21.1. *CYBB* encodes gp91^{phox}, the β-subunit of cytochrome *b558,* a key transmembrane protein in the phagocyte NADPH oxidase system. This protein and p22^{phox} are the two membrane subunits of flavocytochrome *b558* and are essential components of the phagocyte NADPH oxidase system. AR-CGD is most often associated with a deficiency of p47^{phox} [*neutrophil cytosolic factor-1 (NCF-1)* mutation] or, less frequently, with deficiencies of p67^{phox} (*NCF-2* mutation) or p22^{phox} [α-subunit of cytochrome *b558* (*CYBA*) mutation].[1] The cytosolic proteins p47^{phox} and p67^{phox} are phosphorylated and bind to the cyto-

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chrome upon cellular activation.[1,2]

The diagnosis of X-CGD is made by demonstrating absent or markedly reduced oxidase activity in stimulated neutrophils. In the past, screening for CGD has been done by using the nitroblue tetrazolium (NBT) test. However, it has proved to be subjective, being based on visual inspection of a limited number of cells, and can miss the diagnosis of CGD.[1,3] Flow cytometry with the conversion of dihydrorhodamine (DHR) 123 to rhodamine 123 is a rapid and sensitive assay to diagnose CGD. It can help identify the CGD genotype because in the majority of cases, X-CGD demonstrates virtually no DHR shift, whereas p47^{phox}-AR-CGD shows a modest DHR shift with a broad-based histogram.[3,4] The quantitative methods for evaluating oxidase activity include the measurement of superoxide production by ferricytochrome *c* assay or protein expression characterized by Western blot analysis.[1] These methods are time consuming and in some cases of atypical X-CGD, normal amount of gp91^{phox} protein can be found instead of the complete absence of protein.[4] Therefore, *CYBB* mutation analysis is important to establish the definitive genotype.[1,4]

Over 300 *CYBB* mutations have been registered in an internationally maintained X-CGD database.[5,6] Most mutations are distributed throughout the 13 exons or at exon/intron boundaries, and almost 200 of these mutations are unique.

CASE REPORT

A 15 month-old Thai boy presented with past medical history of pneumonia and lymphadenitis at 1 month of age, prolonged fever and hepatosplenomegaly at 4 months of age and 4 episodes of *Salmonella* septicemia at 7, 8, 9, and 13 months of age. The patient was the only child of the family. There was no family history of recurrent infections, hepatosplenomegaly, death in the young, immunodeficiency, or consanguinity. Physical examination revealed a sick boy with weight and height in the 3rd and 10th percentile, respectively. He had fever (38.3˚C), hepatomegaly (span 12 cm), splenomegaly (span 7 cm), and 3 perianal fistulas. Laboratory investigation revealed complete blood count: hemoglobin 4.8 gm/dl, hematocrit 14%, WBC 13,500 cells/mm^3 (neutrophils, 73%; lymphocytes, 24%; monocytes, 3%), platelets 100,000 cells/mm^3, liver function tests: total bilirubin 2 mg/dl, direct bilirubin 0.8 mg/dl, alkaline phosphatase 453 U/l, AST 171 U/l, ALT 87 U/l, GGT 221 U/l, albumin 2.4 gm/dl, and globulin 6.3 gm/dl. Electrolytes, blood sugar, renal function and urine analysis were within normal limits. An anti-HIV antibody was not detectable. TORCHS titers were negative. Blood culture was positive for *Salmonella* group D. Pus culture from perianal fistulas was positive for *E. coli* and *K. pneumoniae.* Computerized tomography (CT) of abdomen showed hepatosplenomegaly without space occupying lesion, granuloma or lymphadenopathy. Immunoglobulin levels, numbers of T, B and NK cells as well as complement levels were normal. NBT test was abnormal. DHR assay was performed according to the previously described methods.[7] The DHR histogram of the patient and his mother's granulocytes were shown in Fig. 1. The histogram and stimulation index (SI) of the patient's granulocytes (Fig. 1a) demonstrated a typical X-CGD pattern. However, the DHR of his mother's granulocytes (Fig. 1b) demonstrated a normal histogram, as were the normal subject's granulocytes (Fig. 1c). These results suggest that the mother is not a carrier and the disease is a *de novo* mutation within the patient.

To confirm this hypothesis, the genomic DNA sequence of the patient and his mother were examined for mutation of the *CYBB* gene by the previously described method.[4] The 13 *CYBB* exons and exon/intron boundaries were sequenced. Exon 1 analysis included the promoter/enhancer region up to nucleotide position -181 relative to the start of *CYBB* exon 1 as described in GenBank AF469757. To detect potential PCR artifacts, the mutation was confirmed by sequencing a second PCR product amplified from DNA from the original subject and his mother; no discrepancies were encountered. Sequence analysis of our patient (*CYBB*base accession number A0540) revealed, in exon 12, a novel complex mutation atggacg → ttca (base pairs 1532-1538). When translated, 3 amino acids (aa) Tyr 511, Gly 512 and Arg 513 were deleted and replaced by 2 aa, Phe and Gln, as demonstrated in Fig. 2. Furthermore, the mutation was a *de novo* mutation since sequence analysis of genomic DNA of mother showed normal *CYBB* gene in both alleles (Fig. 2). The DNA of the mother, father and patient was sent for pater-

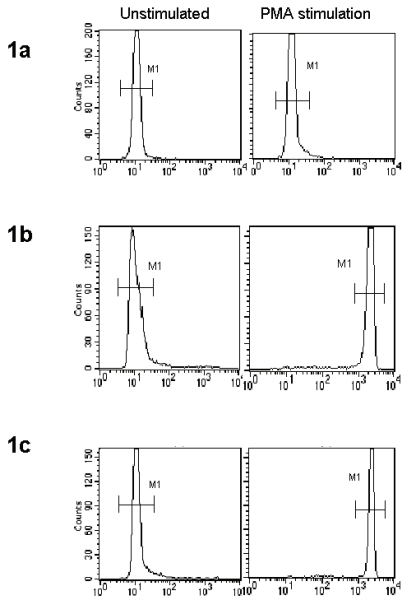

Fig. 1 Histograms of the dihydrorhodamine (DHR) assay of granulocytes from patient, his mother and a normal subject. DHR assay of the patient's granulocytes (1a) revealed almost absence of fluorescence upon granulocyte stimulation. The stimulation index (SI) was 1.14 which was compatible with X-CGD. The DHR assay of granulocytes from the patient's mother (1b) and a normal subject (1c) showed normal histogram with the SI of 193.08 and 202.54, respectively.

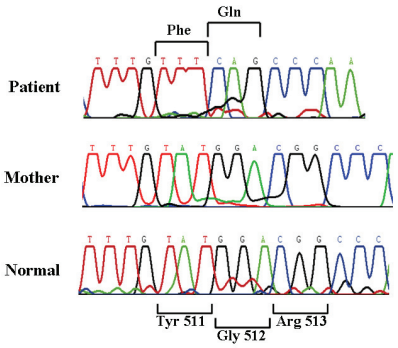

Fig. 2 Analysis of *CYBB* exon 12 of the patient, his mother and a normal subject. Chromatogram of *CYBB* exon 12 of the patient showed a novel complex mutation atggacg → ttca in exon 12 (base pairs 1532-1538). As a result, 3 amino acids Tyr 511, Gly 512 and Arg 513 were deleted and replaced by 2 amino acids, Phe and Gln.

nity test at the Department of Forensic Medicine, Siriraj Hospital. The result of PCR-based short tandem repeat (STR) systems indicated that the patient is a son of his parents.

Base on all investigations, he was diagnosed as CGD with *Salmonella* septicemia and perianal fistulas. He was treated with ciprofloxacin and metronidazole and fistulectomy. He was put on co-trimoxazole (trimetroprim, TMP 10 mg /kg/day) and itraconazole (6.5 mg/kg/day) for long-term prophylaxis. The patient was not given IFN-γ because it was not available in Thailand.

At 17 months of age, he was hospitalized for *Aspergillus* pneumonia and pleural effusion. Bronchoscopy revealed granuloma at the right main bronchus. He was treated with cefotaxime for 2 weeks, ciprofloxacin for 3 months, increased dose of itraconazole (13 mg/kg/day) and the same dose of co-trimoxazole prophylaxis. At 2 years of age, he was hospitalized for *Burkholderia cepacia* septicemia. He was successfully treated with ceftazidime for 3 weeks. From 2 to 4 years of age, he had occasional staphylococcal lymphadenitis and perianal abscess that responded well to cloxacillin. At 4 years of age, he was hospitalized for right hemiparesis and focal seizure of the right arm and leg. Neurological examination showed hypertonia, hyper-reflexia and decreased motor power of the right arm and leg. The CT brain showed ill-defined hypodensity area at the left fronto-parietal area with minimal gyral enhancement and midline shift to the right. The brain biopsy showed fungal hyphae compatible with *Aspergillus*. He was treated with vericonazole and depakine. He is currently on the 5th month of vericonazole, anticonvulsant and co-trimoxazole prophylaxis. His neurological function has gradually improved.

DISCUSSION

The clinical spectrum of CGD is heterogeneous, ranging from a mild disorder to a very severe form. We demonstrated a case of severe CGD who had multiple *Salmonella* septicemia, *Aspergillus* pneumonia and brain abscesses. The frequent infections with intracellular organisms and granuloma formation warranted investigation for a phagocytic defect such as CGD. Although the abnormal NBT

test was suggestive for CGD, it was possibly a false positive result. Therefore, we performed DHR flow cytometry assay which has proved to be a rapid and sensitive screening test for CGD.[7] This assay can differentiate between X-CGD and p47^{phox}-AR-CGD in the majority of patients, based on distinctive DHR histogram SI and pattern.[3,4,7] Vowells, *et al*.[3] demonstrated that the geometric mean SI of granulocytes from normal subjects was 127.9 (range, 85.2 to 264.6), whereas the SIs from patients with CGD with defective gp91^{phox} (X-CGD) and p47^{phox} (AR-CGD) were 1.3 (range, 0.9 to 2.2) and 13.2 (range, 3.5 to 52.1), respectively.

The *CYBB* gene lies on the short arm of the X chromosome at Xp21.1, encompasses 30 kb, and contains 13 exons. *CYBB* encodes 570 aa which consists of four domains (N-terminal domain from aa 1–277, FAD-binding domain from aa 278–397, NADPH-binding domain from aa 398–483 and 504–570, as well as a loop over the NADPH-binding domain from aa 484–503).[6] It has been shown that X-CGD may result from mutations in any of these gp91^{phox} domains. The frequency of mutations is proportional to the length of the domains (N-terminal domain, 61%; FAD-binding domain, 20%; NADPH-binding domain, 17%; and the loop over the NADPH-binding domain, 2%).[6] Most common mutations found in *CYBB* gene is single-nucleotide substitution, followed by deletion, insertion, and combinations of small deletions and insertions.[6]

It should be noted that the coding sequence numbering of the mutated CYBB cDNA sequence reported in this paper was the systematic name position, based on recommendations by Human Genome Variation Society (HGVS) for systematic names (http://www.hgvs.org/mutnomen/). According to the recommendations, the coding sequence (A of ATG) of NM_000397 would start at position 15 in the systematic name position.

The complex mutation in this patient resulted in deletion of 3 aa Tyr 511, Gly 512 and Arg 513 which was replaced by 2 aa, Phe and Gln. This mutation occurred in the NADPH-binding domain of gp91^{phox}. It was possible that the mutations increased gp91^{phox} mRNA turn over, decreased protein half-life (decreased stability) or altered protein function presumably related to the NADPH binding domain.

In conclusion, we reported a novel mutation within exon 12 of *CYBB* gene which was on the NADPH-binding domain in a CGD patient. Functional defect was demonstrated by almost absence of fluorescence upon stimulation of granulocytes on DHR histogram. This defect leads to a severe form of X-CGD. Identification of the molecular defect is helpful for genetic counseling to the patient and his family. The nucleotide sequence data used in this report have been submitted to *CYBB*base with the accession number A0540.

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