

# Nonsense Mutations of the *CYBB* Gene in Two Thai Families with X-linked Chronic Granulomatous Disease

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**SUMMARY** X-linked chronic granulomatous disease (X-CGD) is an immunodeficiency disorder characterized by defective intracellular killing of microorganisms due to the neutrophils' inability to generate superoxide ions. Although it is always caused by mutations in the *CYBB* gene, clinical and molecular characteristics vary in different ethnic backgrounds. Two unrelated Thai boys presented with severe persistent pulmonary infections at the age of two months. Their abnormal dihydrorhodamine (DHR) flow cytometry assays supported the diagnosis of X-CGD. Mutation analysis was performed by polymerase chain reaction (PCR) amplification and sequencing of the entire coding regions of *CYBB*. Mutations identified were confirmed by restriction enzyme analyses. PCR-sequencing of the entire coding regions of *CYBB* identified nonsense mutations, 271C>T (R91X) in exon 4 and 456T>A (Y152X) in exon 5, in probands of each family. Both of the patients' mothers were found to be carriers. This observation supports that *CYBB* is the gene responsible for X-CGD across different populations and nonsense mutations are associated with severe phenotypes.

Chronic granulomatous disease (CGD, OMIM 306400) is an inherited phagocytic disorder involving the reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex that results in defective superoxide generation and intracellular killing.<sup>1,2</sup> Affected patients usually suffer from severe bacterial and fungal infections leading to granulomatous inflammation with an onset in the early childhood or, less frequently, in adolescence and adulthood.<sup>3</sup> The diagnosis of CGD is made by demonstrating the neutrophils' inability to undergo a respiratory burst to generate superoxide ions. Screening for CGD is done by the nitroblue tetrazolium test or the more sensitive dihydrorhodamine (DHR) flow cytometry 123 assay.<sup>4,5</sup> Seventy percent of patients are X-linked (X-CGD) while the remaining 30% show autosomal recessive inheritance.<sup>6</sup> Most

X-CGD patients demonstrate no activity in the DHR assay. A subset of X-CGD patients have atypical phenotypes with a modest activity in the DHR assay requiring diagnosis confirmation by mutation analysis.<sup>7-9</sup>

The defect in X-CGD is attributable to mutations in the *CYBB* gene (GenBank Accession No. AF469757–AF469769) which encodes cytochrome b-245, beta polypeptide (also known as gp91<sup>phox</sup>), a key transmembrane protein in the phagocyte NADPH oxidase system.<sup>10,11</sup> It is localized on chro-

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mosome Xp21.1, encompasses 30 kb, and contains 13 exons. Over 300 *CYBB* mutations have been identified and registered in an internationally maintained X-CGD database (<http://bioinf.uta.fi/CYBBbase/>). We report the clinical and molecular characterization of two Thai families with X-CGD.

## MATERIALS AND METHODS

### Patients

Two Thai families were included in this study. The proband of family 1 was a 2-month-old boy with severe persistent pneumonia since the age of 1 month. Physical examination revealed hepatomegaly and a huge splenomegaly. Laboratory investigation showed leukocytosis (WBC, 23,070 cells/mm<sup>3</sup>, 62% neutrophils, 22% lymphocytes, hemoglobin 7 g/dl, hematocrit 23.3%, platelets 230,000 cells/mm<sup>3</sup>). Chest radiography revealed two large areas of consolidation at the right upper lobe and the left lower lobe. An abdominal ultrasonogram showed multiple splenic microabscesses. A DHR assay of the proband showed no DHR shift and his mother's showed bimodal distribution supporting a clinical diagnosis of X-CGD in the patient and X-CGD carrier in his mother (Fig. 1). Despite treatment with a broad-spectrum antibiotic, intravenous amphotericin B, the patient still suffered from severe persistent pneumonia that progressed to chronic lung disease. He also had recurrent perianal abscesses, and catheter related infections. His only other sibling an older sister, remains healthy and the other family members are unaffected.

The patient of family 2 was a 2-month-old boy presenting with growth failure, recurrent pneumonia and hepatosplenomegaly at the age of 2 months. Laboratory investigation showed leukocytosis. CT of the chest revealed multiple pulmonary masses of various sizes measuring 0.5-3 cm in diameters. An open lung biopsy showed necrotizing granulomatous formations with evidence of fungal hyphae. A DHR assay of the patient showed no DHR shift and his mother's showed bimodal distribution, supporting a clinical diagnosis of X-CGD in the proband and X-CGD carrier in his mother (Fig. 2). Despite treatment with a broad-spectrum antibiotic, intravenous amphotericin B, and a right upper lung lobectomy, he suffered from recurrent anal abscesses, meningitis, pneumonia and catheter related

infections. This led to his death at the age of 19 months. His older brother who also suffered from recurrent pneumonia and skin abscesses died of *Burkholderia cepacia* septicemia at the age of 18 months.

### Mutation analysis

After informed consent was received, peripheral blood (3 ml) was obtained from the patients and their mothers. RNA was extracted from peripheral leucocytes using the QIAamp RNA Blood Mini Kit (Qiagen, Valencia, CA, USA). Reverse transcription was performed using ImProm-IITM reverse transcriptase (Promega, Madison, WI, USA), according to the manufacturer's recommendations. PCR amplification of the *CYBB* complementary DNA (cDNA) exons 1-13 was performed using 1 µl of cDNA, 1x PCR buffer, 1.5 mM MgCl<sub>2</sub>, 0.2 mM dNTP, 0.2 µM primers, and 0.5 U *Taq* DNA polymerase (Fermentas Hannover, MD, USA) in a final volume of 20 µl. The primer sequences were 5'-CCTTCATTGGAAGAAGAAGC-3' and 5'-TCAA-AACCATAGAGCTCCAC-3'. After an initial denaturation step at 94°C for 5 minutes, PCR amplification was conducted for 35 cycles with a denaturation step at 94°C for 45 seconds, annealing at 58°C for 1 minute, extension at 72°C for 2 minutes 30 seconds, and a final elongation step at 72°C for 5 minutes.

Genomic DNA was obtained from whole blood using a standard extraction method. Exons 4 (365 bp) and 5 (318 bp) of the *CYBB* gene were amplified using 1.5 µl of gDNA, 1x PCR buffer, 1.5 mM MgCl<sub>2</sub>, 0.1 mM dNTP, 0.1 µM primers, and 0.4 U *Taq* DNA polymerase in a final volume of 20 µl. The primer sequences for exon 4 were 5'-AGTT-TGCAGGGTGGTTCATGA-3' and 5'-GAAGTGG-CAGAAAGTGTGGT-3' and for exon 5 were 5'-TCATAGAGTCAGAGGCTGTC-3' and 5'-CAAA-GGAGAGGTCTTCACTC-3'. After an initial denaturation step at 94°C for 5 minutes, PCR amplification was conducted for 35 cycles with a denaturation step at 94°C for 30 seconds, annealing at 65°C for 30 seconds, extension at 72°C for 30 seconds, and a final elongation step at 72°C for 5 minutes.

The PCR products were treated with ExoSAP-IT (USP Corporation, Cleveland, OH, USA), according

to the manufacturer’s recommendations, and sent for direct sequencing to Macrogen Inc (Seoul, Korea).

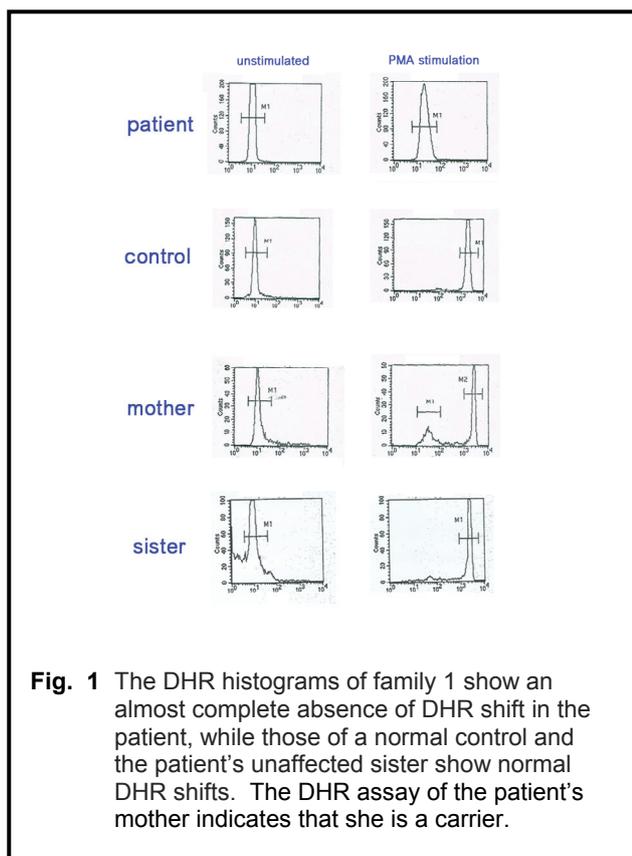
The 271C>T (R96X) on exon 4 and 456 T>A (Y152X) on exon 5 were confirmed by digestion of the PCR products with the restriction enzymes *Bst*BI (New England Biolabs, Beverly, MA) and *Hin*CII (New England Biolabs, Beverly, MA) respectively according to the manufacturer’s recommendations. Digested DNA was analyzed by electrophoresis on 2% agarose and stained with ethidium bromide.

**RESULTS**

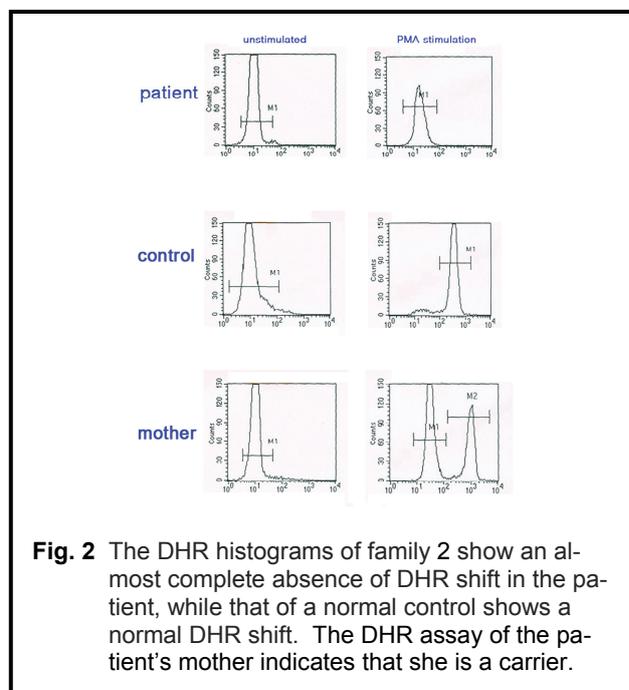
Sequencing analysis of the PCR products of cDNA from the patient of family 1 revealed that the boy was hemizygous for the C>T mutation at the nucleotide position 271 [271C>T] of *CYBB* (data not shown). The mutation was confirmed by the presence of 271C>T in *CYBB* exon 4 of the patient’s genomic DNA (Fig. 3A, left panel) and by digestion of the PCR products with the restriction enzyme *Bst*BI, in which its recognition site is removed by the muta-

tion (Fig. 3C, left panel). The DNA change was expected to result in a conversion of an arginine (CGA) to a stop codon (TGA) [R91X]. This mutation was also detected in the genomic DNA of his mother by digesting the PCR products with restriction enzyme *Bst*BI (Fig. 3C, left panel). No other sequence variants for the remainder of the *CYBB* gene were found in the patient’s cDNA.

Sequencing analysis of the PCR products of the cDNA from the patient of family 2 revealed that the boy was hemizygous for the T>A mutation at the nucleotide position 456 [456 T>A] of *CYBB* (data not shown). The mutation was confirmed by the presence of 456 T>A in *CYBB* exon 5 of the patient’s genomic DNA (Fig. 3A, right panel) and by digestion of the PCR products with the restriction enzyme *Hin*CII, in which its recognition site is added by the mutation (Fig.3C, right panel). The DNA change was expected to result in a conversion of a tryptophan (TAT) to a stop codon (TAA) [Y152X]. This mutation was also detected in the genomic DNAs of his older brother and mother by digesting the PCR products with restriction enzyme *Hin*CII, confirming that the older brother was also affected with X-CGD and the mother was a carrier (Fig. 3C, right panel). No other sequence variants for the remainder of the *CYBB* gene were found in the proband’s cDNA.



**Fig. 1** The DHR histograms of family 1 show an almost complete absence of DHR shift in the patient, while those of a normal control and the patient’s unaffected sister show normal DHR shifts. The DHR assay of the patient’s mother indicates that she is a carrier.



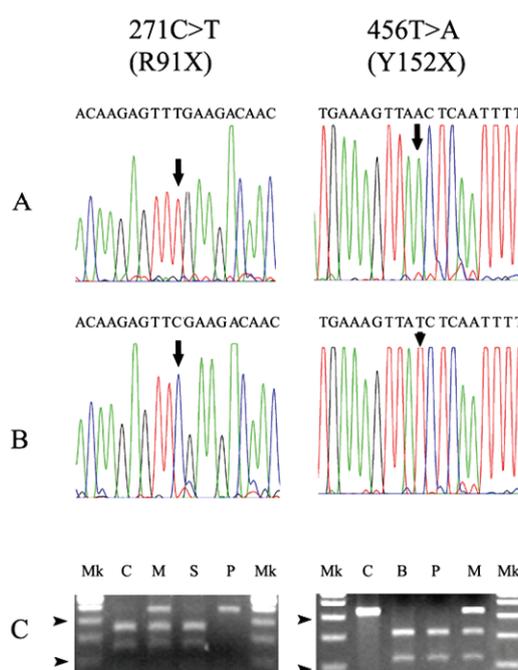
**Fig. 2** The DHR histograms of family 2 show an almost complete absence of DHR shift in the patient, while that of a normal control shows a normal DHR shift. The DHR assay of the patient’s mother indicates that she is a carrier.

## DISCUSSION

Our two patients were boys presenting with severe persistent pulmonary infections and hepatosplenomegaly at the age of two months, consistent with a severe form of X-CGD. DHR assays of the patients showed no DHR shift and their mothers' showed bimodal patterns demonstrating one peak similar to their sons' DHR results and the other peak similar to results seen with unaffected control cells. This makes the possibility of autosomal recessive (AR) CGD unlikely as it usually demonstrates a modest DHR shift with a broad-based histogram. These results rather support a diagnosis of X-CGD in the patients and of X-CGD carriers in their mothers.

Even though the majority (83%) of patients with X-CGD show a unique DHR assay pattern, some X-CGD patients (17%) may have a DHR assay histogram that overlaps with that of p47<sup>phox</sup>-AR-CGD; they require additional testing to identify the genotype.<sup>7</sup>

Both patients were hemizygous for nonsense mutations, R91X in exon 4 and Y152X in exon 5. This is consistent with a previous observation that the most common mutations in this gene leading to X-CGD are nonsense mutations, followed by missense mutations, frameshift deletions, intron mutations, frameshift insertions, and inframe deletions.<sup>12</sup> Both mutations were previously described<sup>10,13</sup> and



**Fig. 3** Mutation analysis. The left and right panels relate to patients 1 and 2, respectively. *A*, Electropherograms of the patients, showing 271C>T and 456T>A (arrows) in patients 1 and 2, respectively. *B*, Electropherograms of female controls showing normal genotypes at codons 271 as CC and 456 as TT (arrows). *C*, Restriction enzyme digestion analysis. B: brother, C: control, M: mother, Mk: 100-bp marker, P: patient, and S: sister. The arrow heads indicate the 100 and 300-bp bands. In the left panel, *BstBI* digested the 365-bp product of the control and the patient's sister into 131 and 234-bp products. The 271C>T mutation in patient 1 eliminates the *BstBI* site, leaving the undigested 365-bp product, indicating that the patient is hemizygous for the mutation. In the mother, the 365-bp undigested product was present with the digested 131 and 234-bp products of the normal allele, indicating that she is a heterozygous carrier. In the right panel, *HinCII* does not have a restriction site in the normal alleles of the control, leaving the 318-bp PCR product intact. The 456T>A mutation creates a *HinCII* restriction site. Therefore, the PCR products of the mutant alleles of the patient and his brother are digested into 114-bp and 204-bp products, indicating that both patient 2 and his brother are hemizygous for the mutation. In the mother, the digested 114- and 204-bp products are present along with the undigested 318-bp product of the normal allele, indicating that she is heterozygous for the 456T>A mutation.

localized in the N-terminal domain of gp91<sup>phox</sup>, a region that probably serves multiple purposes including the binding of gp91<sup>phox</sup> to the membrane, an interaction with p22<sup>phox</sup>, and binding with the heme groups.<sup>10</sup>

Mutations in the *CYBB* usually lead to a lack of gp91<sup>phox</sup> protein expression due to an instability of the mRNA and/or instability of the protein.<sup>10</sup> This results in a total absence of NADPH oxidase activity in activated neutrophils which is generally associated with a severe clinical phenotype.<sup>10</sup> This phenotype of X-CGD is called X91<sup>0</sup>. Nonetheless, almost 10% of X-CGD cases have mutations that allow some expression of the gp91<sup>phox</sup> protein ranging from 1 to 25% (X91<sup>-</sup>).<sup>14</sup> In some X-CGD cases, normal amounts of non- or partially functional gp91<sup>phox</sup> protein are present (X91<sup>+</sup>). Both X91<sup>-</sup> and X91<sup>+</sup> forms have a more variable clinical phenotype.<sup>15-18</sup> The R91X found in patient 1 was previously shown to result in an absence of gp91<sup>phox</sup> protein expression (X91<sup>0</sup>).<sup>10</sup> Our data support a previous observation that nonsense mutations always induce the X91<sup>0</sup> phenotype of X-CGD.<sup>10</sup>

In conclusion, we identified nonsense mutations of the *CYBB* gene that are responsible for X-CGD in 2 unrelated Thai families. This will be a basis for further molecular studies of X-CGD to enhance diagnosis confirmation, carrier identification, genetic counseling and prenatal or preimplantation diagnosis in Thailand. In addition, because CGD is a candidate for gene therapy, the precise molecular identification will gain importance in the future.

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