

CASE REPORT

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Prenatal Diagnosis of Chronic Granulomatous Disease in a Male Fetus

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ABSTRACT

Mutations in any of four known NADPH-oxidase components lead to CGD. X-linked CGD (X-CGD) is caused by defects in *CYBB*, the gene that encodes gp91-*phox*. Autosomal recessive (AR) CGD is caused by defects in the genes for p47 *phox*, p22-*phox* or p67-*phox*. The aim of this study was to screen the molecular defect in the fetus of an X-CGD carrier mother and postnatal confirmation of the results.

In a family whose first-born child died from X-CGD, fetal DNA was obtained from an ongoing pregnancy by chorionic villus sampling (CVS). Direct sequencing was used to detect the previously identified *CYBB* gene mutation. The NADPH oxidase activity in the neutrophils from the carrier mother and from the newborn was analyzed by the DHR assay.

Our studies predicted that the fetus in question was not affected by chronic granulomatous disease, which was demonstrated to be correct at birth. For prenatal screening in a pregnant X-CGD carrier, direct sequencing is a good method for detecting the mutation in the fetal DNA. Postnatal confirmation of results with the DHR assay is more practical than mutation screening to show whether the newborn have normal NADPH oxidase activity or does not.

Key words: Chronic granulomatous disease; *CYBB*, X-CGD; DHR assay; NBT; Prenatal diagnosis

INTRODUCTION

Chronic granulomatous disease (CGD) is a rare inherited disorder of the innate immune system. The disease is characterized by a severe susceptibility to bacterial and fungal infections due to the failure of phagocytic leukocytes to produce microbicidal reactive oxygen species (ROS).

The source of these radicals is superoxide (O_2^-), generated by the nicotinamide adenine dinucleotide phosphate, reduced (NADPH) oxidase, a multi-component enzyme specifically expressed in phagocytic leukocytes.¹ Mutations in any of four genes for NADPH-oxidase components will lead to CGD, including defects in *CYBB* (encoding gp91-*phox*) leading to X-CGD and defects in *NCF1* (for p47-*phox*), *CYBA* (for p22-*phox*) or *NCF2* (for p67-*phox*) leading to AR-CGD.¹⁻³

Flow cytometric detection of the conversion of dihydrorhodamine-1,2,3 (DHR) to rhodamine-1,2,3 is a rapid and sensitive assay for the diagnosis of CGD.⁴ Thus,

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neutrophils with an active NADPH oxidase can be readily distinguished by flow cytometry from neutrophils without this activity. Due to lyonization, female carriers of X-CGD generally exhibit two populations of neutrophils, one positive and the other negative for NADPH-oxidase activity.⁴⁻⁶

In the present study, we have used direct sequencing for prenatal diagnosis to screen for the known family mutation in fetal DNA. The DHR assay was used for the functional analysis of the neutrophils of the newborn to exclude CGD and to confirm the molecular results after birth.

CASE REPORT

Family

Mrs. X is 28 years old and the mother of a son with CGD who died at the age of three. The maternal aunt of Mrs. X has two sons with CGD, in whom a c.C736>T mutation was found in *CYBB*. Mrs. X as well as the maternal grandmother, mother and maternal aunt of Mrs. X were found to be carriers of this mutation (Figure 1).^{6,7} At week 10-12 of the second pregnancy of Mrs. X, the fetus was shown by ultrasound (USG) analysis to be male. Transcervical chorionic villus biopsy was performed at week 12 in the second pregnancy of Mrs. X, under ultrasound guidance.

Molecular Analysis

The red chorionic villi were separated from the white villi, and DNA was isolated from both tissues separately. DNA isolated from the blood of Mrs. X, from a healthy, non-related control individual and from a related CGD patient (elder nephew) was also investigated.

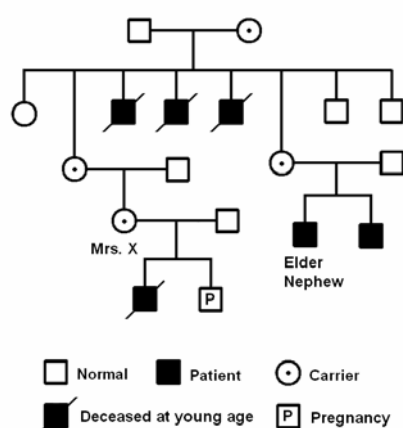


Figure 1. Pedigree of the X-CGD family. Mrs. X and her mother, maternal aunt and maternal grandmother are X-CGD carriers. The maternal aunt of Mrs. X has two sons with CGD.

Molecular analysis of the *CYBB* gene was performed in the Sanquin Research laboratory in Amsterdam. Exon 7 of the *CYBB* gene of each DNA sample was amplified in two independent PCR reactions, and each PCR product was directly sequenced both in the sense and in the anti-sense direction.⁷⁻⁹

Functional Analysis of Neutrophil

The DHR assay was performed according to a previously described method, with slight modifications.^{6,10,11} For the DHR assay, total white cells were isolated from 100-200 μ l of human peripheral blood by lysis of the erythrocytes in the pellet fraction with a non-fixing lysis solution of 155 mM (isotonic) NH_4Cl , 10 mM NaHCO_3 and 0.1 M EDTA (final concentrations; a 10-times concentrated solution was made and diluted ten times with distilled water) by gently mixing the tube at 37°C for 5 minutes. After a 5-minute spin at 1000 rpm, supernatant was poured out and the cell suspension (leukocytes) were washed with Hank's balanced salt solution (HBSS) without Mg^{2+} , Ca^{2+} and phenol red) and suspended in 400 μ l of HBSS buffer. Then, the leukocytes were loaded with 2 μ l of 29 mM nonfluorescent DHR (Molecular Probes, Eugene, OR, USA) and incubated at 37°C for 5 minutes in the presence of 12.5 I.U./ml catalase (Sigma Chemicals, St. Louis, MO, USA). After DHR loading, the cells were stimulated with 100 pg/ml phorbol myristate-acetate (PMA) and incubated at 37°C for 15 minutes and analyzed by flow cytometry (FACSCalibur, Becton Dickinson, Palo Alto, CA, USA). Stimulation index (SI) was determined as the ratio of the mean fluorescence of the stimulated cells to the mean fluorescence observed in the unstimulated cells. In cases in which two distinct fluorescent populations were observed (i.e., X-linked carriers), the SI for each population was calculated.^{4-6,12}

Cytogenetic Assay

In a cytogenetic assay we found the fetus to be male (positive Y PCR) with a 46,XY karyotype.

DNA Analysis

We analyzed the DNA from a CVS of Mrs. X, mother of a deceased CGD patient with a C736>T mutation in *CYBB*, the X-chromosome-linked gene that encodes the gp91-*phox* component of the leukocyte NADPH oxidase. The DNA samples from the red villi and the white villi were amplified in two independent PCR reactions, and each PCR product was analyzed both

in the sense and in the anti-sense direction. We found that none of these eight assays showed the mutation to be present in the fetal DNA. Thus, we regarded the fetus not to be a CGD patient (Figure 2). As controls, we also analyzed DNA from a healthy control individual (no mutation), from a related X-CGD patient (a nephew of the deceased CGD patient, with the same mutation), and from Mrs. X (heterozygous mutation). A nonsense mutation c.C736>T, which changes the CAA codon for

glutamine-246 into the TAA stop codon was found in exon 7 of the *gp91-phox* gene (*CYBB*) in the carrier mother on one of her two X chromosome alleles and in the related X-CGD patient (Figure 2). The mutation in this carrier mother had been previously detected and reported.^{6,7} The postnatal sequence analyses in the newborn child showed the wild-type sequence to be present in exon 7 of the *gp91-phox* gene (*CYBB*).

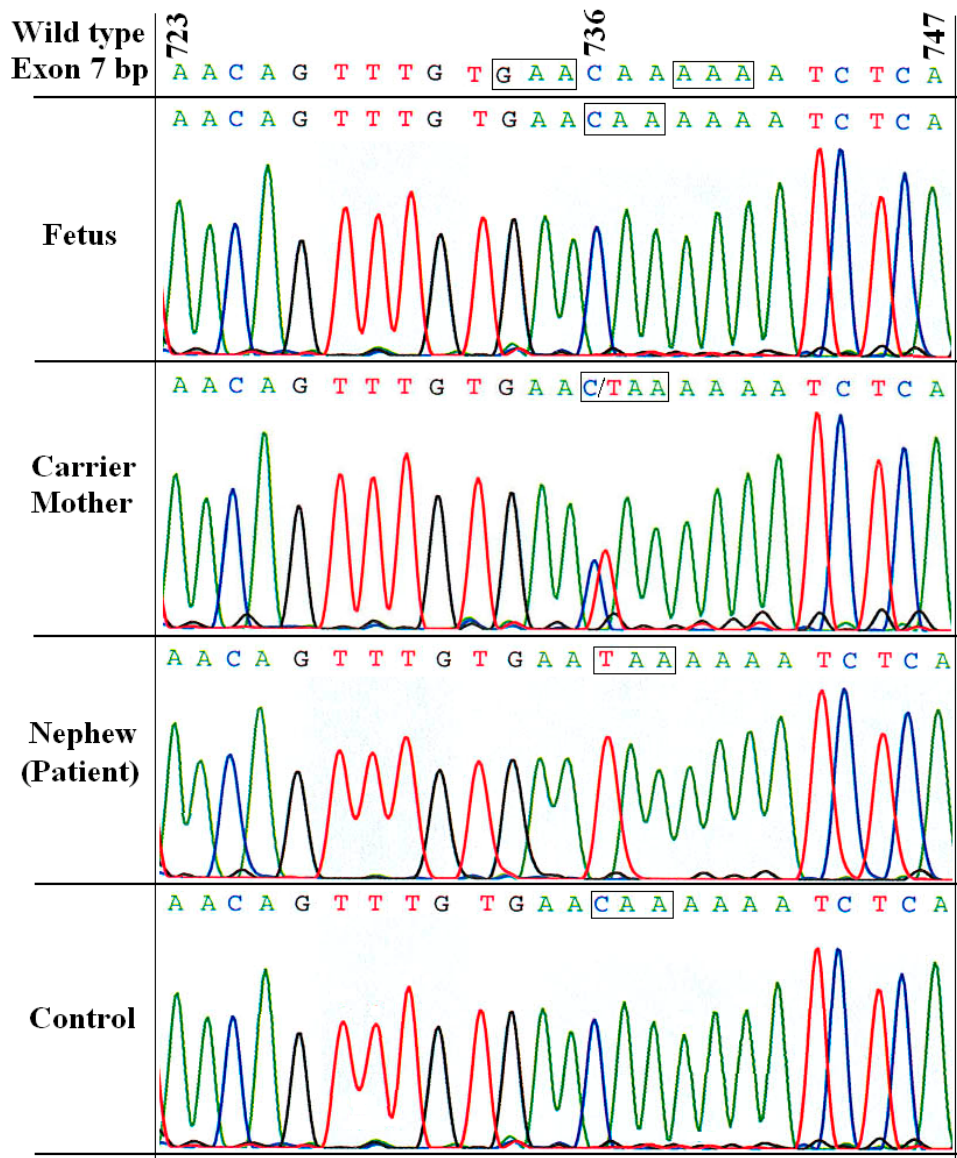


Figure 2 Nucleotide sequence of *CYBB* from A723 to A747 in the fetus, the carrier mother and a nephew of the deceased CGD patient, with the same mutation, as well as in a control individual. Fetus and control show only wild-type c.C736 sequence, carrier mother shows both c.C736 and c.T736 sequences, and the nephew shows only c.C736>T transition.

Prenatal Diagnosis of X-CGD

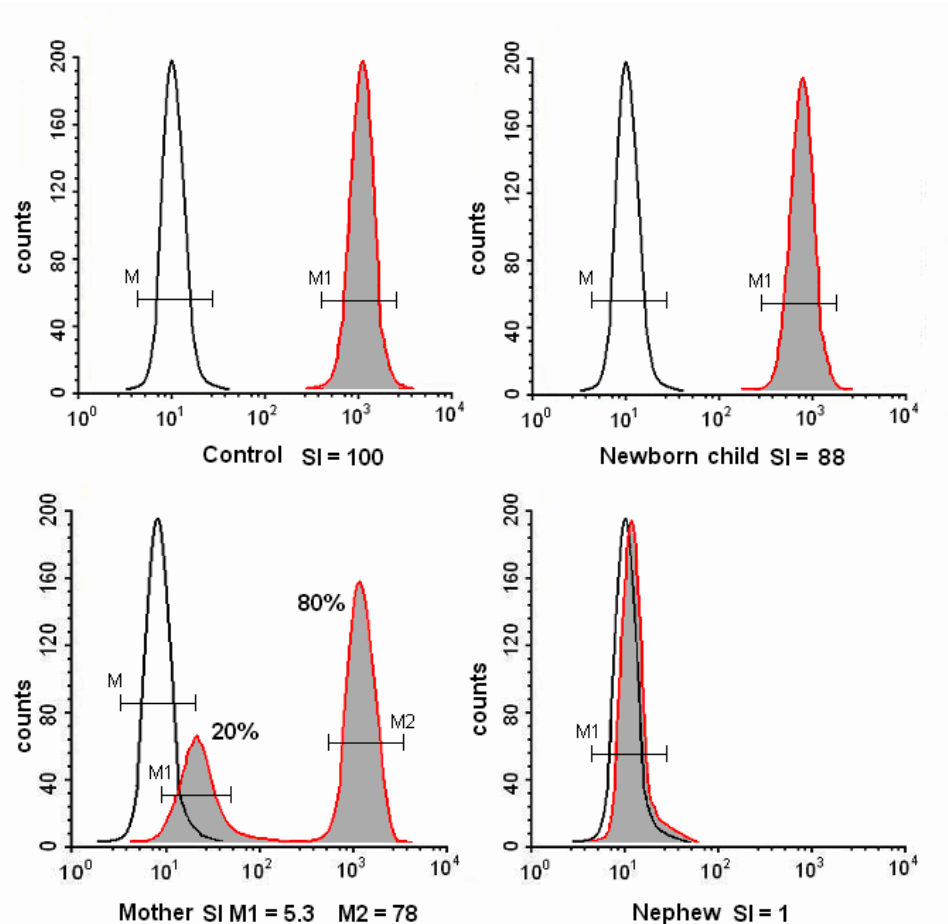


Figure 3. The histograms of DHR assay. The newborn child and the control individual show normal NADPH-oxidase activity, mother shows a mixture of cells with normal and defective oxidase activity, and the nephew (patient) shows neutrophils without NADPH-oxidase activity.

Postnatal Functional Analysis of Neutrophils with DHR Assay

We screened the neutrophils of the newborn for CGD with the DHR assay in the first week after birth. The results showed that the newborn child and the control individual had neutrophils with a normal NADPH-oxidase activity. The mother had a bimodal histogram pattern, showing her neutrophils to be a mixture of cells with normal NADPH-oxidase activity (80% of the neutrophils) and cells without this activity (20% of the neutrophils) (Figure 3). SI of left and right peaks were 5.3 and 78, respectively. The low oxidase activity in the left peak (neutrophils with defective oxidase activity) is probably due to some “carry-over” of hydrogen peroxide from the cells with normal oxidase activity to the cells without oxidase activity. The nephew (patient) had a

histogram pattern showing his neutrophils to be without NADPH-oxidase activity (Figure 3).

DISCUSSION

Mutation analysis with direct sequencing is a good method for detecting a mutation in fetal DNA when CGD is suspected. We used direct sequencing because we knew the mutation in this family and because it is more reliable than haplotype screening. Our results showed the wild-type sequence in a healthy control individual and in the fetus, and a nonsense mutation c.C736>T in exon 7 of the gp91-*phox* gene (*CYBB*) in a related X-CGD patient (a nephew of the deceased CGD patient, with the same mutation) and in the carrier mother (heterozygous mutation) (Figure 2).^{6,7}

Richardson *et al.*¹² predict that the DHR assay can be used for antenatal diagnosis. But that needs fetal blood sampling with cordocentesis when the umbilical cord is sufficiently developed, after 18 weeks of gestation.¹³ In prenatal diagnosis with CVS, mutation analysis can be performed already after 12 weeks of gestation.

We used the DHR assay after birth to confirm that the newborn child had normal NADPH-oxidase activity in his neutrophils (Figure 3). This assay can be performed with a very small volume of peripheral blood (100 µl) compared with mutation analysis, for which 1-2 ml is needed. Moreover, the DHR assay is more sensitive than the nitroblue tetrazolium (NBT) test, because it can give the result of a few thousands of neutrophils and the neutrophils can be gated to compare the NADPH-oxidase activity of stimulated and unstimulated cells.^{12,14} The NBT test may yield false normal results in cases of p47^{phox}-deficient CGD and variant X-CGD, in which low levels of reactive oxidants are produced.^{5,15} Thus, in circumstances of doubt, a more sensitive method like the DHR assay should be preferred for evaluating oxidase activity.

The DHR assay can be completed in less than 1 hour and it is low in costs. It is also easy to perform in many centers that do not possess the qualification for sequence analysis. So, the DHR assay is a practical method, sensitive and faster than NBT, to confirm that a newborn child has normal neutrophils or not. It makes the process of postnatal screening easy to manage in CGD families.

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