Long Polymerase Chain Reaction-Based Fluorescence *in Situ* Hybridization Analysis of Female Carriers of X-Linked Chronic Granulomatous Disease Deletions

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Chronic granulomatous disease (CGD) is a rare inherited disorder in which antimicrobial activity of phagocytes is impaired due to the lack of reactive oxygen species, or oxidative burst, produced by NADPH oxidase. The X-linked form of CGD, representing ~70% of all cases, is caused by mutations in the cytochrome b β subunit (CYBB) gene, which maps to chromosome Xp21.1. CYBB encodes the gp91-phox protein, a necessary component in the NADPH oxidase pathway. A wide variety of mutations have been identified in X-linked CGD patients, all of which lead to deletion of the functional protein and no oxidative burst activity. The mutations vary from single nucleotide substitutions to deletions of the entire gene. In this article, we report a mutation detection method for probands of female relatives at risk for carrier status of large deletions of the CYBB gene. Through fluorescent *in situ* hybridization of metaphase chromosomes, we were able to consistently distinguish carriers from noncarriers using polymerase chain reaction-derived, labeled DNA specific for exons 2 to 13 of the CYBB region at Xp21.1. (J Mol Diagn 2005, 7:183–186)

Chronic granulomatous disease (CGD) is a genetic condition in which phagocytic killing ability is impaired due to the absence of reactive oxidative species generated by NADPH oxidase that is composed of four components. All of these protein components are potent antimicrobial agents essential for the killing of bacteria, fungi, and parasites.¹ Patients with CGD are vulnerable to recurrent, sometimes life-threatening, infections because lymphocytes are capable of phagocytosis, but cannot kill the

infectious agent. The lack of reactive oxygen species, necessary for pathogen eradication, can arise from mutations in the genes encoding for one of the major proteins that comprise the NADPH oxidase pathway.²

Most cases of CGD are diagnosed in pediatric patients before the age of 1 year. The presenting symptom is recurrent infections of the skin and soft tissue, with granuloma development. The most clinically serious sites of infection are the liver, lymph nodes, spleen, and lungs.³ Diagnosis of CGD includes consistent clinical history and testing for impaired respiratory burst capabilities of affected blood cells. Patients are routinely screened by demonstration of the ability of neutrophils and monocytes to produce reactive oxygen species on stimulation. The phagocytic cells are stained with dihydrorhodamine, a dye that becomes fluorescent after contact with H₂O₂ and other intracellular reactive oxygen species.⁴ Additional tests to confirm the diagnosis of CGD include superoxide measurements using reduction of cytochrome c, Western blot, and flow cytometry. Finally, single strand conformation polymorphism and subsequent DNA sequencing can identify mutations in the various genes in the complex.⁵

CGD is inherited as an autosomal recessive or X-linked disorder, with an overall incidence rate of 1 in 200,000 to 1 in 250,000 live births.⁶ All forms of the disease involve mutations in one of the four subunits of NADPH oxidase: p67phox (1q25), p47phox (7q11.23), p22phox (16q24), and gp91phox (Xp21.1).⁷ The X-linked form is more common and occurs in 70% of CGD families.⁸ It is caused by mutations in the CYBB gene that encodes the β subunit of cytochrome b558, also referred to as gp91-phox, an essential component of a functional NADPH oxidase. Cytochrome b558 is a 100- to 135-kd heterodimer composed of an α chain and a β chain, 22 kd and 91 kd, respectively.⁹ Neutrophils of patients with X-linked CGD lack gp91-phox protein product. Female carriers of X-linked CGD lack β cytochrome b558 in approximately half of their neutrophils, but the percentage varies due to nonrandom X-inactivation.¹⁰ Female carriers of the auto-

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somal recessive form of CGD cannot be ascertained through testing of the protein level.^{7,8}

A comprehensive genetic study by Rae and colleagues¹¹ describes 103 specific mutations within 124 kindreds, with no more than seven families possessing the same mutation. Of these mutations, the following frequencies were observed: 11% deletion, 24% frameshift, 23% missense, 23% nonsense, 17% splice-region, and 2% regulatory region mutations in the CYBB gene, which is composed of 13 exons. Currently, more than 327 separate mutations for CGD and 4 polymorphisms in the CYBB gene have been described.^{12,13} Additionally, CGD mutations have been found in exonic regions and exon-intron borders, and also, in intronic regions.¹⁴ The genetic discrimination of X-linked CGD using molecular technology has been inadequate in discerning large deletion mutations. This is a particularly important issue for determining recurrence risks for potential carriers of the disorder.

We have developed a fluorescence *in situ* hybridization (FISH) test using a long polymerase chain reaction (PCR) strategy to fashion a probe from the CYBB gene exons for hybridization to metaphase chromosomes. Individuals with large deletions can be identified as carriers using this methodology. A control probe for the chromosome X centromere is included in the dual color probe cocktail, assisting in easy visualization of deletions of the targeted gene regions.

FISH is a well-established technique for the resolution of chromosomal microdeletion syndromes such as Velocardio facial syndrome, Williams syndrome, and Prader-Willi syndrome¹⁵ and has been shown to be applicable for the identification of carriers of the X-linked disorders, Duchenne and Becker muscular dystrophy.¹⁶ X-linked carrier status of a large DNA deletion can be most easily resolved with FISH because both X chromosomes can be visualized within the same cells, a qualitative measure of the mutation. The alternative approach is densitometric analysis of X-linked carrier DNA, a quantitative method requiring gene dosage assessment, which is more subjective.

Materials and Methods

Probe Development

The CYBB gene probes were amplified from human genomic DNA in three separate segments, each encompassing ~10 kb of the gene, using the Expand Long Template PCR System 3 (Roche Molecular Biochemicals, Indianapolis, IN). The IMT Bioinformatics website is www.uta.fi/imt/bioinfo/CYBBbase/ to determine exon sequences. Genomic DNA was isolated from whole blood stored in ethylenediamine tetraacetic acid using the Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN). The three PCR reactions were based on exons 2 to 4, 4 to 8, and 8 to 13. Amplification from exons 2 to 4 was accomplished using a forward primer starting at the eighth bp of exon 2 (cDNA 2F) and a reverse primer starting at the 36th bp of exon 4 (cDNA 4R). Exons 4 to 8

were amplified using a forward primer starting at the third bp of exon 4 (cDNA 4F) and a reverse primer starting at the 79th bp of exon 8 (cDNA 8R), and exons 8 to 13 were amplified using a forward primer starting at the 22nd bp of exon 8 (cDNA 8F3) and a reverse primer starting at the 87th bp of exon 13 (cDNA 13R). The following method was used for the amplification of exons 2 to 4 and 4 to 8: an initial denaturation at 95°C for 3 minutes followed by 10 cycles of 95°C for 15 seconds, 60°C for 30 seconds, and 68°C for 8 minutes, and 20 cycles at 95°C for 15 seconds, 60°C for 30 seconds, and 68°C for 8 minutes with cycle elongation of 20 seconds per cycle. A final extension was performed for 7 minutes at 68°C. Exons 8 to 13 were amplified using the following method: an initial denaturation at 95°C for 3 minutes followed by 10 cycles of 95°C for 15 seconds, 62°C for 30 seconds, and 68°C for 8 minutes, and 20 cycles at 95°C for 15 seconds, 62°C for 30 seconds, and 68°C for 8 minutes with cycle elongation of 20 seconds per cycle. A final extension was performed for 7 minutes at 68°C. PCR-amplified fragments were purified using a QIAquick PCR purification kit (Qiagen, Valencia, CA). Primer sequences were: cDNA 2F, GGCTGGGGTTGAACGTC; cDNA 4R, CCTGTC-CAGTTGTCTTCGAAC; cDNA 4F, CTGCTCAACAA-GAGTTCGAAG; cDNA 8R, CCTCTGTTGAGATCGCCA; cDNA 8F3, GTGAGAGGTTGGTGCGGT; and cDNA 13R, GGGCCAGACTCAGAGTTGG.

Probe Labeling

DNA prepared by PCR from exons 2 to 13 of the CYBB region DNAs were directly labeled with Spectrum Orange (Vysis, Downer's Grove, IL) by nick translation and mixed with COT-1. Individual exon probes were hybridized separately and mixed to make a composite probe including all segments of CYBB gene. In addition, a Spectrum Green-labeled X-centromeric probe was used as an X chromosome control (Vysis).

The CGD FISH probe was first tested to assure robust signal production and positive Xp hybridization signal on five separate normal control female and five normal male specimens. Blood samples were then obtained from four separate CGD families, each with a male index case and at least two female family members participating in the probe analysis study. All families had suspected CYBB deletions based on dihydrorhodamine analysis and lack of PCR amplification with CYBB-specific primers (spanning the coding region) in the probands. Samples were grown in RPMI for 72 hours and harvested for metaphase accumulation using standard protocols. FISH hybridization of control and composite test probes was conducted as in Stratakis and colleagues.¹⁷ Ten metaphase spreads were scored for the presence of the X chromosome control and the test signal for each patient. Male proband metaphase FISH analysis was also conducted using the same experimental conditions as the female relatives. Images were analyzed with a Zeiss Axioskop (New York, NY) microscope equipped with a Cytovision imaging system (Applied Imaging, Pittsburgh, PA). The imaging system allows for separate exposures of the fluorochromes

(A)

(B)

(C)

Figure 1. FISH using the CGD CYBB gene probe composed of exons 2 to 13 (red) and centromere control probe (green) on three separate study samples. **A:** Female relative of a CGD patient:noncarrier. **B:** Carrier of CYBB deletion showing no red signal on one X chromosome. **C:** CGD-affected male with CYBB deletion showing green control, but no red signal on X chromosome.

which assists in visualization of smaller red signal relative to the larger control green fluorochrome.

Results

All female relatives with CYBB deletions showed deletion of one X chromosome for the CYBB exons 2 to 13 at Xp21.1 for all 10 metaphases (Figure 1), whereas non-carriers showed a signal on both X chromosomes for all 10 metaphases counted. Males affected with the disorder with deletion in exons 2 to 13 of the CYBB gene showed one X chromosome marker and no signal for the CYBB exons. We were also able to discern images from FISH tests using smaller 2- to 5-exon individual probes; however all patients tested thus far have demonstrated larger deletions discerned with the composite probe. Biochemical assays were unable to distinguish carriers from non-carriers with whole gene deletions of CYBB. The results of gene sequencing studies and FISH studies performed on the at risk family members were as follows: six female carriers demonstrated normal CYBB sequencing results, but FISH deletions; three female relatives had normal sequencing and normal FISH results; two affected males had deletions on both sequencing and FISH tests.

Discussion

X-linked CGD is a rare disease attributable to many different types of mutations, including up to 12% caused by deletions in the CYBB gene. The majority of all mutations seen in the X-linked form of CGD in the CYBB gene, whether point mutations, frameshift, or deletion all lead to the same phenotype: classic severe CGD. The exceptions are rare and have involved missense mutations or regulatory region mutations that allow some normal protein to be synthesized and some oxidative function to remain intact. These rare variant X-linked CGD cases have a milder phenotype, but they are the exception. All of the other mutations lead to the same result: no protein and no function. Another noteworthy category of mutations is contiguous gene deletion syndromes of the p arm of the X chromosome, in which additional genes are deleted resulting in corresponding health issues. These additional genes may be dystrophin (Duchenne muscular dystrophy), Kell antigen (McLeod phenotype), retinitis pigmentosa, and others.

X-linked CGD usually presents at an early age in affected males, whereas carrier detection does not occur until a symptomatic proband is identified. Confirmation of the likelihood of a female relative of an X-linked CGD patient being a carrier, accurate testing procedures are essential to individuals who can potentially pass on this mutation to offspring. This ability is essential for detecting carriers with whole gene deletions because current DNA sequencing methods are not sufficient. We have developed an assay for accurately confirming carriers with deletions in exons 2 to 13 of the CYBB gene.

We performed CGD FISH on proband chromosomes after sequencing results were obtained for the CYBB

gene. Once the deletion mutation was established by FISH in the index case, we analyzed metaphase chromosomes of sisters and mothers to determine whether they possessed one or two gene copies.

Most recently, we have come to depend on the DNA sequencing for diagnosis and carrier status. Unfortunately, DNA sequencing of X-linked carriers of some genetic diseases has a blind spot. A carrier of a large deletion will always show normal sequence of the CYBB gene because of amplification of the normal copy of the gene in the homologous X chromosome. The proposed analytic approach for mutation studies of X-linked CGD families is DNA sequencing of male probands, followed by FISH of the proband using the largest composite probe, followed by smaller exon region probes. If the FISH studies demonstrate deletions, potential carrier females can also be tested by the FISH analysis using the same probe as the family index case. Detection of deletions smaller than the cases in our study is also possible because the smaller probes for exons 2 to 4, 4 to 8, and 8 to 13, were visualized, reliably. This test could also be used for confirmation of CGD diagnosis on interphase cells from fetal autopsy material in which cells are not successfully cultured.

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