

# X-LINKED DELETION ANALYSIS IN CHRONIC GRANULOMATOUS DISEASE

Chronic granulomatous disease (CGD) is a rare genetic disorder which can involve deletion mutations in the cytochrome b beta subunit (CYBB) gene on chromosome Xp21.1. Most patients present at 1 year of age with recurrent life threatening infections in the skin and soft tissues, such as the spleen, lung, and lymph nodes. Female carriers of the disorder are difficult to ascertain as biochemical methods used for making a diagnosis in affected patients are not sensitive enough for carrier detection. The Lombardi Cancer Center lab has developed a fluorescence *in situ* hybridization (FISH) test for determining carrier status that can be used once the index case mutation is determined to be a deletion. We are currently performing FISH on metaphase chromosomes from 2 separate families and deletions by a second will also be detected by a second method, quantitative polymerase chain reaction (QPCR), or real-time PCR performed by our collaborators. Two familial cases collected will be tested by both methods and the results will be compared and analyzed. This is an important step in carrier-based testing in x-linked CGD cases. Once a familial mutation has been identified, other at risk females can be offered genetic counseling and testing.

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## INTRODUCTION

Of the different types of chronic granulomatous disease (CGD), autosomal recessive and x-linked, x-linked is more common, and also, more serious.<sup>1</sup> In the x-linked form of CGD, there can be deletion mutations in the cytochrome b beta subunit (CYBB) gene on chromosome Xp21.1.<sup>2,3</sup> Patients with CGD present with nearly chronic infections in the skin, spleen, lung, lymph nodes and other soft tissue organs. Patients with CGD must undergo constant treatments and often experience pain. A problem faced by many in CGD families is knowing whether they are carriers of the x-linked recessive trait. It is difficult to correctly identify the large deletion in female carriers of the disorder using biochemical methods normally used to diagnose affected patients.<sup>2,3</sup> Using fluorescence *in situ* hybridization (FISH) testing, carrier status of females can be determined.<sup>4</sup> This can only be done, however, once the index case in the family has been confirmed as a deletion of the CYBB gene. Collaborators in Italy at the Clinica Pediatrica in Universita Degli Studi di Roma have identified the deletions using quantitative polymerase chain reaction (QPCR), or real-time PCR, which has also been effective in identification of female carriers of Duchenne and Becker muscular dystrophies.<sup>5,6</sup> As a method to confirm the deletions in the CYBB gene, we have performed FISH on metaphase chromosomes from two families and are comparing the results with those of our collaborators.

## METHODS

Three slides of lymphoblastoid cells from the patients in Italy were sent to

Lombardi Cancer Center for FISH testing. The FISH was done in accordance with Pinkel et al.<sup>7</sup> The slides were pretreated by placing them in 2 × SSC, pH 7.0 at 37°C for 30 minutes in a coplin jar. After the 30 minutes, the slides were dehydrated for two minutes each in a coplin jar with 70% ethanol, then 80% ethanol, and then 90% ethanol. The slides were then air dried and slide denaturation began immediately. In a microfuge tube, 70 uL of form amide and 30 uL of 2 × SSC were mixed well on a vortex and 100 uL of the solution as well as a coverslip were placed on each slide, which were then placed in a Hybrite at 80°C for two minutes. The coverslips were then removed and the dehydration series was repeated with each slide.

Probe development preceded the entire FISH process. It is a lengthy process in which CYBB gene probes were amplified from human genomic DNA in three separate segments. As described in Simon et al, the genomic DNA was isolated using the Puregene DNA isolation kit (Gentra Systems, Minneapolis, Minn). The PCR reactions were based on exons 2 to 4, 4 to 8, and 8 to 13 of the CYBB gene. All the PCR-amplified fragments were purified by using a QIAquick PCR purification kit (Qiagen, Valencia, CA). The probes were then labeled with Spectrum Orange (Vysis, Downer's Grove, Ill) using nick translation and mixed with COT-1. Also, a Spectrum Green-labeled x-centromeric probe was used as the x-chromosome control.

The probes and a buffer were warmed in a 37°C degree water bath for 5 minutes; then centrifuged and added to the slides to be hybridized. To each slide, 2 uL of probe was added as well as 2 uL dh20 and 7 uL of Vysis

buffer, which had been centrifuged and denatured in a 72°C water bath.

After adding the 10  $\mu$ L of probe to each slide and placing coverslips on each, the slides were then sealed with rubber cement and placed in a 37°C humidified incubator overnight. After the hybridization process was complete, the stringency wash began. The next day, the slides were carefully stripped of rubber cement and the coverslips were gently removed. The slides were then inserted into a coplin jar with 4  $\times$  SSC and Tween at 72°C for two minutes. The slides were then placed in a coplin jar with 2  $\times$  SSC for 45 seconds at room temperature. They were then placed in a dark drawer to dry for 15 minutes.

Once the slides were dry, the counterstain was added. One drop of DAPI counterstain (Vector Shield) was added to the middle of each slide using the eye dropper that comes with the counterstain. Coverslips were then added, and the slides were left in a drawer to dry for at least 10 minutes before they were viewed on an Olympus fluorescence microscope equipped with an Applied Imaging System, Cytovision.

## RESULTS

After viewing the slides with the fluorescence microscope, it was determined that one of the slides, which had only one green signal and no red signal on that chromosome, was a male patient suffering from CGD. Because the green probe is specific for the X-centromere, it was apparent that the patient is a male

because males only have one X-chromosome, and therefore, there was one green signal. Since males only have one X-chromosome, they are the sex affected with X-linked CGD. The next slide, the mother of that patient, had two green signals and only one of those chromosomes had the red signal, indicating only one CYBB gene present. The last slide, from a case in a different family, had both green signals, as she was female, and both CYBB genes lit up in spectrum orange, indicating that she is normal with respect to the region tested.

## DISCUSSION

After comparing the results of the FISH analysis to the ones attained by QPCR, the FISH was proven to support the results of the QPCR. The first case, the Italian boy labeled CL, proved to be a sufferer from CGD, which helped us see that the FISH analysis and QPCR were successful in their tests. The mother (CM), earlier identified as a carrier of the deletion causing CGD, was also found to be a carrier by the FISH analysis. The last slide, labeled RA, was tested for carrier status of CGD. This assay will be able to accurately confirm carriers with deletions in exons 2 to 13. Because FISH analysis is established as an effective way of carrier-based testing, it can help to validate the QPCR method of carrier-based testing. The reports of the FISH tests have been sent to Italy and the clinic that can perform QPCR will be able to confirm its testing on CGD cases. In the future, the clinic in Italy may establish its method as an effective

way to test for CGD carrier status and genetic counseling will be able to take place and help unsure families who visit the Clinica Pediatrica in Italy. Once a familial mutation has been identified, other at risk females can be offered genetic counseling and testing.

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