The Value of MLPA in Waardenburg Syndrome

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ABSTRACT

Waardenburg syndrome (WS) is an autosomal-dominant neurocristopathy characterized by sensorineural hearing loss, pigmentary abnormalities of the iris, hair, and skin, and is responsible for about 3% of congenital hearing loss. Point mutations in PAX3 have been identified in more than 90% of affected individuals with WS Type 1/WS Type 3. MITF point mutations have been identified in 10–15% of individuals affected with WS Type 2 (lacking dystopia canthorum). Multiplex ligation-dependent probe amplification (MLPA) is now a standard technology in the molecular genetics laboratory to detect copy number changes in targeted genes. We employed MLPA for PAX3 and MITF in a cohort of patients submitted with a diagnosis of WS1, 2 or 3 who were sequence negative for PAX3 and/or MITF. All coding exons of PAX3 and exons 1, 2, 3, and 10 of MITF were included in the MLPA assay. MLPA on 48 patients with WS 1 or 3 revealed 3 PAX3 whole gene deletions (2 WS1; 1 WS3), 2 PAX3 partial gene deletions [WS1, exon 1 and promoter (1st report); WS1, exons 5–9], and 1 partial MITF deletion (“WS1”, exons 3–10) (6/48 ≈ 12.5%). MLPA on 41 patients with WS2 and 20 patients submitted with a diagnosis of either WS1 or WS2 revealed no copy number changes. The detection of both partial and whole gene deletions of PAX3/MITF in this clinical cohort increases the mutation detection yield by at least 6% and supports integrating MLPA into clinical molecular testing primarily for patients with WS1 and 3.

INTRODUCTION

Waardenburg syndrome (WS) is an autosomal-dominant neurocristopathy characterized by sensorineural hearing loss, pigmentary abnormalities of the iris, hair, and skin, and is responsible for about 3% of congenital hearing loss (Milunsky, 2006). WS can be divided into four types on the basis of clinical diagnostic criteria. WS1 and 2 can be differentiated by the absence of dystopia canthorum in WS2. WS3 is characterized by features seen in WS1 in addition to upper limb anomalies, including hypoplasia or contractures of the limb muscles or joints, carpal bone fusion, or syndactyly. WS4 is characterized by features of WS1 in addition to Hirschsprung disease.

Point mutations in PAX3 have been identified in more than 90% of affected individuals with WS1 or WS3 who meet stringent clinical diagnostic criteria (Hoth et al., 1993; DeStefano et al., 1998; Milunsky, 2006). MITF point mutations have been identified in 10–15% of individuals affected with WS2 (Liu et al., 1995; Nobukuni et al., 1996). Mutations in the endothelin-B receptor gene (EDNRB), endothelin-3 (EDN3), and the SOX10 gene have been found in patients with WS4 (Edery et al., 1996).

Clinical testing is available for patients with a clinical diagnosis of WS1 and WS3, and WS2 by sequencing the PAX3 and MITF genes, respectively.

Multiplex ligation-dependent probe amplification (MLPA) is now a standard technology in the molecular genetics laboratory to detect copy number changes in targeted genes (Schouten et al., 2002). In an attempt to elucidate the clinical utility of MLPA in Waardenburg syndrome molecular analysis, we performed a retrospective study utilizing MLPA in a cohort of patients with a clinical diagnosis of WS1, 2, and 3 who were sequence negative for PAX3 and/or MITF.

MATERIALS AND METHODS

We performed a retrospective analysis of those patient DNA samples submitted to our laboratory for WS testing. Chart review revealed 109 patients with a clinical diagnosis of WS1, 2,
or 3 who were sequence negative for PAX3 and/or MITF. The patient samples that did not have information providing a clinical diagnosis of WS1, 2, or 3, were not included in this study. Of the 109 patients in our cohort, 48 patients were clinically diagnosed with WS1 or 3, 41 patients were diagnosed with WS2, and 20 patients were submitted with a diagnosis of either WS1 or 2.

DNA extraction

Blood samples were received and processed at the Center for Human Genetics, Boston University School of Medicine. Genomic DNA was extracted using the AUTOPURE, automated DNA extractor (Gentra Systems, Minneapolis, MN) according to manufacturer’s instructions.

MLPA analysis

A gene-specific MLPA kit #P186 (MRC, Holland) was used according to the instructions provided. All coding exons of PAX3 and exons 1, 2, 3, and 10 of MITF were included in the MLPA assay. Briefly, a multiplex of probes (a combination of gene-specific and outside control regions) were hybridized overnight to template DNA. The next day, the probes, which contain a common sequence tail, were ligated, and subsequently a PCR reaction was performed to amplify the hybridized probes. This tail sequence is to equalize the amplification efficiency of the probes. The products are of different lengths, with up to 40 products present in the amplification reaction. This reaction was then run on an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA) to separate the fragments. The results were analyzed using GENEMARKER (SoftGenetics, State College, PA).

Extra-long PCR amplification and analysis of genomic DNA

One of our deletion cases required further analysis with Extra Long (XL) PCR and sequencing to determine more precisely the extent of the deletion observed. In this case, increasingly longer regions upstream of exon 1 of PAX3 were PCR amplified to detect an altered sized fragment. Multiple-size ampli-

FIG. 1. A: Typical pattern observed for a PAX3 whole-gene deletion. Visually, the patient peaks for PAX3 are \(~1/2\) the height of the control peaks. B: The height ratio versus size grid shows normal copy number between 0.75 and 1.3, whereas a deletion is \(~0.5\). C. This box shows the locus, fragment size, and ratio of sample peak area to control peak area.
cons ranging from 1 kb to 10 kb in size were generated. GeneAmp XL PCR kit (Applied Biosystems, Foster City, CA) was used. PCR reactions of 100 μl were performed according to manufacturer’s instructions. Cycling conditions were: 94°C for 5 min for one cycle, 30 cycles (94°C for 30 sec, 63°C for 30 sec, 68°C for 12 min), and one cycle of 68°C for 5 min. Amplifications were performed in Tetrad-2 thermocycler (MJ Research, Watertown, MA). PCR amplicons were electrophoresed on a 1% low-melt agarose gel. The gel was stained with ethidium bromide and photographed. Altered-size amplicons were excised from the gel and sequenced. The method used to sequence utilizes M13 sequence-tailed primers in the original PCR reaction and then takes advantage of the tailed primers to sequence the amplicons. Primer sequences for generating amplicons are derived from the NCBI PAX3 reference sequence.

Sequence analysis

The PCR reactions were cleaned up using the ExoSAP-IT (USB Corp. Cleveland, OH) method. Sequence reactions were performed in a 10-μl reaction volume using 2 μl of 3–10 ng of ExoSAP-IT PCR amplicon, 3 μl of sterile deionized water, 1 μl of either M13 forward or reverse primer (3.2 pmol/μl), 4 μl of BigDye Terminator Ready Reaction Mix v3.1 (Applied Biosystems, Foster City, CA) under the following conditions: 96°C for 1 min for one cycle, 25 cycles (96°C for 10 sec, 50°C for 5 sec, 60°C for 4 min). The sequencing reactions were then purified using an EDTA/ETOH method and resuspended in 10 μl of Hi-Di Formamide. The reactions were then electrophoresed on an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA). Sequence analysis was compared to the National Center for Biotechnology Information (NCBI) reference.

RESULTS

MLPA on 48 patients with WS1 or 3 revealed 3 PAX3 whole-gene deletions (2 WS1; 1 WS3) (Fig. 1), 2 PAX3 partial gene deletions (WS1, exon 1; WS1, exons 5–9), and 1 partial MITF deletion (“WS1”, exons 3–10) (6/48 ~ 12.5%). MLPA on 41 patients with WS2 and 20 patients submitted with a diagnosis of either WS1 or WS2 revealed no copy number changes.

Further clinical information on those cases determined to have deletions was obtained. All of the PAX3 whole or partial gene deletion samples submitted for PAX3 sequencing were from individuals who fulfill the cardinal diagnostic criteria for WS1. The WS3 patient met the cardinal diagnostic criteria for WS3. The patient with a MITF partial deletion had deafness, hypopigmentation, and bright blue irides, and had a mother with deafness. The sample was submitted for PAX3 analysis only; however, no W index was calculated. The WS1 sample with only exon 1 deleted was further characterized to determine the extent of the deletion. A series of long PCR fragments of increasing size were generated ranging from 1 to 10 kb in size. The 5-kb fragment showed both the normal 5-kb band and a shorter 1-kb band, indicating that the deletion was contained in this amplicon (Fig. 2). A series of normal controls with no hear-
ing loss or diagnosis of WS were tested (3 shown in Fig. 2) and revealed only the normal 5-kb band. Sequence analysis of the 1-kb band revealed that the upstream promoter elements such as the CAAT and TATA boxes along with exon 1 were deleted. The deletion was determined to span 4.07 kb.

**DISCUSSION**

Since PAX3 was cloned (Baldwin et al., 1992), no other gene has been discovered as an etiology for WS 1 or 3. Point mutations in PAX3 have been identified in more than 90% of affected individuals with WS 1 or 3. In contrast, WS2 is genetically heterogeneous, with only 10–15% of affected individuals having a point mutation in MITF. Although several other genetic loci have been postulated, no additional genes have been found that cause the majority of the WS2 phenotype.

Whole-gene sequencing enables discovery of point mutations and small alterations in the gene, but cannot reliably detect whole-exon or whole-gene copy number changes. Exon and/or whole-gene copy number changes have been reported for many genes resulting in a specific genetic disorder. Although there are several technologies available to detect copy number changes, MLPA is now a standard technology in the molecular genetics laboratory to detect efficiently and accurately copy number changes in targeted genes. As the percentage of partial and whole-gene copy number changes was not known for WS, we performed a retrospective analysis using MLPA for PAX3 and MITF in a cohort of patients with a clinical diagnosis of WS1, 2, or 3, who were previously sequence negative for PAX3 and/or MITF. We found three PAX3 whole-gene deletions and two PAX3 partial gene deletions in 47 patients with a clinical diagnosis of WS 1 or 3 (5/47, ~10%). One partial MITF deletion was found in a patient thought to have WS1. There was no discernable difference in severity between whole-gene or partial-gene deletions and the clinical spectrum reported for PAX3 or MITF mutations (Milunsky, 2006). No copy number changes were found in 41 patients with WS2 and 20 patients submitted with a diagnosis of either WS1 or 2. The 1 “WS1” patient with a partial MITF deletion highlights the fact that it is sometimes difficult to distinguish WS1 from WS2, even when using the W index. The MITF MLPA may be underestimating the frequency of copy number changes, as only four of the ten exons are included in the assay.

Recently, we were recontacted by the family shown in Fig. 2 whose original DNA we had collected more than 20 years ago for WS1 gene mapping and eventual PAX3 sequencing. The MLPA analysis on a new sample from the hearing proband revealed a deletion of only exon 1 of PAX3. We performed XL PCR and sequencing of the junction fragment from the proband’s DNA and demonstrated for the first time in WS that the entire PAX3 promoter region in addition to exon 1 was deleted. This 1-kb junction fragment was also found in the proband’s affected father (hearing) and affected brother (deaf). This further demonstrates the variable expression of WS1, even within families with the same mutation.

From this retrospective study, it can now be estimated that whole gene or partial gene deletions of PAX3 may account for approximately 10% of affected individuals with WS1 or WS3 that do not have mutations identified by sequencing. It does not appear that partial-gene or whole-gene MITF copy number changes contribute significantly to the etiology of WS2, but further study is necessary. Adding the remaining MITF exons to the MLPA kit may increase the number of copy changes identified. In clinical molecular practice it is important to have the combined PAX3/MITF MLPA kit due to the potential diagnostic difficulty separating WS1 from WS2. The detection of both partial-gene and whole-gene deletions of PAX3/MITF in this clinical cohort increases the mutation detection yield by at least 6% and supports integrating MLPA into clinical molecular testing especially for patients with WS 1 or 3.

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**REFERENCES**


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