Molecular Testing for Hereditary Retinal Disease as Part of Clinical Care

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Objective: To describe clinical molecular testing for hereditary retinal degenerations, highlighting results, interpretation, and patient education.

Methods: Mutation analysis of 8 retinal genes was performed by dideoxy sequencing. Pretest and posttest genetic counseling was offered to patients. The laboratory report listed results and provided individualized interpretation.

Results: A total of 350 tests were performed. The molecular basis of disease was determined in 133 of 266 diagnostic tests; the disease-causing mutations were not identified in the remaining 133 diagnostic tests. Predictive and carrier tests were requested for 9 and 75 nonsymptomatic patients with known familial mutations, respectively.

Conclusions: Molecular testing can confirm a clinical diagnosis, identify carrier status, and confirm or rule out the presence of a familial mutation in nonsymptomatic at-risk relatives. Because causative mutations cannot be identified in all patients with retinal diseases, it is essential that patients are counseled before testing regarding the benefits and limitations of this emerging diagnostic tool.

Clinical Relevance: The molecular definition of the genetic basis of disease provides a unique adjunct to the clinical care of patients with hereditary retinal degenerations.

RETINAL DYSTROPHIES ARE A phenotypically and genotypically heterogeneous group of diseases that are inherited in autosomal dominant, autosomal recessive, X-linked, mitochondrial, and complex modes. The clinical signs and symptoms of retinal diseases cover a broad continuum within and between specific disease entities. The complex and overlapping nature of these phenotypes can prove challenging to the clinician in making the diagnosis. Molecular confirmation of the diagnosis can afford the ophthalmologist the opportunity to make an unequivocal diagnosis, verify etiology, provide prognosis, and calculate the recurrence risk. Once the familial mutation is identified, a molecular diagnosis can rule in or rule out the presence of a causative mutation for the patient and other at-risk family members.

Clinical molecular testing is available to aid in the evaluation of more than 950 genetic conditions, including ocular diseases, syndromes whose phenotypes include ocular findings, and others with no known ocular involvement. Molecular genetic tests can be requested for diagnostic, predictive, carrier, prenatal, and preimplantation testing. Diagnostic testing is used to confirm the molecular basis of disease in patients exhibiting signs of disease. Predictive testing can be provided to nonsymptomatic individuals whose family history puts them at risk of developing the disease. Carrier testing can identify males and females who have 1 mutation for a disease inherited in an autosomal recessive mode and identify females who have 1 mutation for a disease inherited in an X-linked recessive mode. At the University of Michigan W. K. Kellogg Eye Center's Ophthalmic Molecular Diagnostic Laboratory, we have offered Clinical Laboratory Improvement Amendment (CLIA)--approved molecular diagnostic testing for the past 5 years. In this report, we describe our initial experience regarding the utility, scope, and limitations of this testing and the importance of patient education and counseling.
Blood samples were submitted with a genetic test request form that included patient name, date of birth, sex, ethnic background, test(s) needed, indication for referral, family history depicted in pedigree format, and the DNA testing consent form signed by the patient or the parent or guardian of a pediatric patient. Referrals for molecular testing were received from clinicians within and outside the institution. Most referrals were received from ophthalmologists and genetic counselors in several parts of the United States; a few requests for testing were received from outside the country. The laboratory director (R.A.) and genetic counselor (K.D., K.B., or B.M.Y.) were available to provide pretest consultation regarding the testing protocol and the likelihood that the diagnostic test will confirm the molecular basis for the retinal disease in question. The laboratory clinical hereditary retinal specialist (J.R.H. or P.A.S.) was available to review ocular clinical records in cases in which the clinician had questions about which test to order. On-site pretest and posttest genetic counseling was available to patients and their family members.

DNA was isolated from blood samples using standard protocols. For diagnostic tests of the ABCA4 gene (the adenosine triphosphate–binding cassette, subfamily A, member 4 gene), preliminary analysis for known mutations and polymorphisms was performed by an outside laboratory using the ABCA4 chip, as described previously. Mutations identified in this manner were confirmed by sequencing relevant exons in the laboratory according to our clinical protocol following CLIA guidelines. If fewer than 2 causative mutations were identified using the ABCA4 chip, and for analysis of all other genes, sequencing was performed by amplification of all exons and at least 20 base pairs of flanking intronic sequence using primers described previously. In cases of predictive or carrier testing in which the familial mutation was known, only the exon in question was analyzed. Amplicons were sequenced in both directions using polymerase chain reaction primers and a cycle-sequencing reaction, and were separated using a genetic analyzer (ABI Prism 3100; Applied Biosystems, Foster City, Calif), as described earlier. Current technology can detect 1 or a few nucleotide substitutions, minor deletions, and minor insertions in the coding region. Large deletions, large insertions, and mutations in noncoding regions are types of mutations that cannot be detected by current methods and, thus, would not be identified. To determine if a newly identified sequence change was pathogenic, DNA, when available, from the patient’s parents or affected blood relative(s) was screened for the change in question. Sequence changes, novel and previously published, were compared with DNA analysis of at least 100 chromosomes from control subjects of similar ethnicity (also A.J.K. and R.A., unpublished data, 2006).

A written report provided test results and interpretation. The laboratory methods used, published detection rate when available, and references. Sequence variations were classified in the report as previously reported disease-causing mutations (causative mutations), previously reported polymorphisms (neutral changes), novel sequence changes believed to be disease causing (potentially pathogenic), novel sequence changes believed to be noncausative variations (potentially neutral), or changes of unknown significance. Novel changes were classified as potentially pathogenic or potentially neutral polymorphic changes based on the expected effect of the change on the amino acid sequence and/or gene structure.

We describe the results of our initial 350 molecular tests. Table 1 lists the 8 genes tested with their correspond-

Table 1. Retinal Diseases Associated With the 8 Genes Tested

<table>
<thead>
<tr>
<th>Gene*</th>
<th>No. of Exons</th>
<th>Retinal Disease</th>
<th>Mode of Inheritance</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCA4</td>
<td>50</td>
<td>Stargardt macular degeneration</td>
<td>AR</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fundus flavimaculatus</td>
<td>AR</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cone-rod dystrophy</td>
<td>AR</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Retinitis pigmentosa</td>
<td>AR</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Late-onset retinal degeneration with early-onset anterior zonules</td>
<td>AD</td>
</tr>
<tr>
<td>C10orf5</td>
<td>3</td>
<td>Malattia leventinese</td>
<td>AD</td>
</tr>
<tr>
<td>CTRP5</td>
<td>12</td>
<td>Doyne honeycomb dystrophy</td>
<td>AD</td>
</tr>
<tr>
<td>EFEMP1</td>
<td>6</td>
<td>Dominant Stargardtlike macular degeneration</td>
<td>AD</td>
</tr>
<tr>
<td>ELOVL4</td>
<td>5</td>
<td>Pattern dystrophy</td>
<td>AD</td>
</tr>
<tr>
<td>RDS</td>
<td>11</td>
<td>Butterfly macular dystrophy</td>
<td>AD</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adult-onset foveomacular dystrophy</td>
<td>AD</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bull’s-eye maculopathy</td>
<td>AD</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adult-onset Best disease</td>
<td>AD</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Late-onset dominant macular degeneration</td>
<td>AD</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Additional RDS phenotypes</td>
<td>AR or AD</td>
</tr>
<tr>
<td>TIMP3</td>
<td>5</td>
<td>Sorsby fundus dystrophy</td>
<td>AD</td>
</tr>
<tr>
<td>VMD2</td>
<td>11</td>
<td>Best macular degeneration, childhood onset</td>
<td>AD</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adult-onset vitelliform macular degeneration</td>
<td>AD</td>
</tr>
<tr>
<td>RS1</td>
<td>6</td>
<td>X-linked retinoschisis</td>
<td>X linked</td>
</tr>
</tbody>
</table>

Abbreviations: AD, autosomal dominant; AR, autosomal recessive.
*The ABCA4 gene is the adenosine triphosphate–binding cassette, subfamily A, member 4 gene; C10orf5/CTR5, C10 tumor necrosis factor–related protein-5 gene; EFEMP1, epidermal growth factor–containing fibulilnine extracellular matrix protein 1 gene; ELOVL4, elongation of very long chain fatty acid 4 gene; RDS, peripherin gene; TIMP3, tissue inhibitor of metalloproteinase 3 gene; VMD2, viteliform macular dystrophy 2 gene; and RS1, retinoschisin gene.

We describe the results of our initial 350 molecular tests. Table 1 lists the 8 genes tested with their correspond-

Table 2 lists the 266 diagnostic, 9 predictive, and 75 carrier tests performed. The molecular basis of disease was confirmed in 133 of the 266 diagnostic tests. Of the 9 predictive tests performed, 4 determined that the nonsymptomatic patients did share the genotype of an affected relative; the remaining 5 did not inherit the familial mutation. Of the 75 females who underwent XLRS1 gene (the gene responsible for X-linked juvenile retinoschisis) carrier testing, 47 were carriers. These data represent test results of patients referred for genetic testing without restriction based on phenotypic inclusion and exclusion criteria; in addition, members of the same family referred for diagnostic testing were included in these results. Therefore, these figures do not reflect the true detection rates.

We identified 44 novel sequence changes (Table 3). Of these changes, 30 were potentially pathogenic and 14 were classified as potentially neutral polymorphisms or changes of unknown significance.

**ABCA4 GENE**

The ABCA4 gene is composed of 50 exons, and more than 320 pathogenic sequence changes have been reported. Diagnostic tests were ordered for 152 patients whose differential diagnosis included Stargardt macu-
lar degeneration, fundus flavimaculatus, and/or cone-rod dystrophy. Because these are all autosomal recessive dis-
eases, the identification of 2 causative mutations is neces-
sary to confirm the molecular basis of disease. We identi-
fied 2 or more causative mutations or potentially pathogenic changes in 73 of the 152 samples submitted for diagnostic
testing, thus confirming the molecular basis for disease in 48% of those tested. While we found exactly 2 causative mutations in 60 of these 73 samples, we identified 3 caus-
active mutations in 10 samples and 4 causative mutations in 3 samples. Among all the changes detected, we identi-
fied 24 novel potentially pathogenic changes and 12 novel changes that were potentially neutral changes or changes of unknown significance (Table 3). Of the remaining 79 samples in which the molecular basis of disease was not defined, a single previously reported or potentially patho-
genic change was identified in 34 samples, and none in 45 samples. Of these 79 patients, 8 were subsequently re-
ferred for diagnostic testing. A missense mutation in
EFEMP1 gene (the epidermal growth
factor–containing fibulinlike extracellular matrix protein 1 gene) has been implicated in the dominant dis-
ease Doyne honeycomb dystrophy or malattia leventi-
nese. Of the 3 samples submitted for diagnostic testing, a causative mutation was identified in 1, thus confirm-
ing the clinical diagnosis. In addition, we detected 1 novel potentially neutral polymorphic change in another pa-
tient (Table 3).

**EFEMP1 GENE**

A missense mutation in EFEMP1 (the epidermal growth factor–containing fibulinlike extracellular matrix protein 1 gene) has been implicated in the dominant disease Doyne honeycomb dystrophy or malattia leventin-
nese. Of the 3 samples submitted for diagnostic testing, a causative mutation was identified in 1, thus confirming the clinical diagnosis.

**RDS GENE**

Mutations in the RDS gene (the peripherin gene) have been reported to be associated with a broad range of au-
tosomal dominant retinal dystrophies listed in Table 1. Diagnostic RDS testing was requested for 13 patients. In 3 of the 13 patients, the molecular basis of disease was identified by detecting a change that was previously re-
ported to be a causative mutation, and in a fourth pa-
tient, a novel potentially pathogenic change was found.

**TIMP3 GENE**

TIMP3 gene (tissue inhibitor of metalloproteinase 3 gene) testing was ordered for 11 patients, all of whom had a differential diagnosis that included Sorsby fundus dys-
trophy. Sorsby fundus dystrophy is inherited in an au-
tosomal dominant mode and is characterized by late-
onset retinal degeneration and choroidal neovascular mem-
brane. It is often misdiagnosed as age-related macu-
lar degeneration, particularly if there is no report of family history consistent with dominant inheritance.\textsuperscript{12,24} Causative mutations were identified in 3 of the 11 patients. One polymorphism was identified in a fourth sample. Five patients underwent predictive testing, and familial mutations were identified in 2 patients. A patient with a family history of late-onset macular dystrophy resembling Sorsby fundus dystrophy showed no TIMP3 mutation. Subsequent to the diagnostic testing, this patient and family members participated in genetic research, and a pathogenic mutation in the \textit{RDS} gene was identified.\textsuperscript{17}

\textbf{VMD2 GENE}

Mutations in the \textit{VMD2} gene (the vitelliform macular dystrophy 2 gene), which encodes the bestrophin protein, have been associated with childhood-onset Best macular degeneration and adult-onset vitelliform macular degeneration.\textsuperscript{25} Because these conditions are inherited in an autosomal dominant mode, detection of a single causative mutation can confirm the diagnosis. Molecular diagnosis was requested for 12 patients with diagnoses of autosomal dominant childhood-onset Best macular degeneration (Table 2). The molecular basis for disease was identified in 10 of the 12 patients. A single mutation was identified in 8 of these 10 patients. Two mutations were detected in the ninth and tenth patients; in one case, both mutations were previously reported causative mutations, and in the other case, both were novel. Among the changes observed in the \textit{VMD2} gene, 6 were previously reported causative mutations and 5 were potentially pathogenic novel changes (Table 3). In addition, 6 previously described polymorphisms were detected.

\textbf{RS1 GENE}

Of the genes included in our testing for retinal degenerations, \textit{RS1} (the retinoschisin gene) is unique in being the only one responsible for an X-linked disease (ie, X-linked juvenile retinoschisis [\textit{XLR5}]).\textsuperscript{15} We have provided diagnostic testing for 50 males, carrier testing for 75 females, and 1 predictive test for an at-risk male. Of the 50 diagnostic tests, 39 were positive for a causative mutation. The 1 predictive test ruled out the familial mutation. The 75 females tested, 47 proved to be carriers, while the remaining 28 did not carry the familial mutation.

\textbf{CASE REPORT 1: PRETEST EDUCATION AND GENETIC COUNSELING}

The index case was a 10-year-old Caucasian boy whose differential diagnosis included pattern dystrophy, cone dystrophy, cone-rod dystrophy, rod-cone dystrophy, and Stargardt macular degeneration. His ophthalmologist made a referral for \textit{ABCA4} diagnostic testing and genetic counseling. The 3-generation family history elicited during the pretest genetic counseling session was negative for ocular disease; there was no known consanguinity. The younger son had been seen by another ophthalmologist; his phenotype differed from his brother’s phenotype. The parents hoped for a specific diagnosis and cause of their older son’s eye disease and for a determination of whether their younger son was affected with the same disease or a mild unrelated vision problem. The counseling provided the parents the opportunity to discuss the implications of recessive inheritance, develop realistic expectations for the information the test could provide, and prepare for all possible test results. The \textit{ABCA4} test result identified 2 previously described mutations in the heterozygous state in both samples; thus, the results were consistent with

\begin{table}
\caption{Novel Sequence Changes}
\begin{tabular}{|c|c|c|}
\hline
Gene* & Potentially Pathogenic Changes & Amino Acid Change \\
\hline
\textit{ABCA4} & 164A → C & H55P \\
 & 611G → A & A204T \\
 & 868C → T & R290W \\
 & 1699G → A & V676M \\
 & 1726G → C & D576H \\
 & 1817G → A & G606A \\
 & 1964T → G & L725I \\
 & 2173C → A & F655C \\
 & 2297G → A & G766D \\
 & 2385C → G & S795R \\
 & 2401G → A & A801T \\
 & 3076T → C & F1026L \\
 & 3137T → G & L1046W \\
 & 3414T → C & L1138P \\
 & 4256T → C & M1419T \\
 & 4535C → A & P1512H \\
 & 4849G → A & V1617M \\
 & 4870T → G & W1624G \\
 & 5026A → C & T1676P \\
 & IVS36 – 3G → C & Splice site change \\
 & 5701G → A & F1900L \\
 & 5885T → A & V1962D \\
 & 6718A → G & T2240A \\
 & IVS42 + 1G → A & Intronic change \\
 & RDS & 667G → C & C222S \\
 & 174A → G & Y29C \\
 & 660T → C & L191P \\
 & 738G → T & L217F \\
 & 948G → A & W287X \\
 & 974A → G & N296D \\
 & \textit{VMD2} & 320C → A & R107R \\
 & 1692A → G & P562P \\
 & 2283T → C & C641T \\
 & 4869C → G & G1623G \\
 & 5318C → T & A1773V \\
 & 5390T → C & C1797C \\
 & IVS15 – 13T → C & Intronic change \\
 & IVS24 + 46A → T & Intronic change \\
 & IVS35 + 8G → A & Intronic change \\
 & IVS40 – 35A → C & Intronic change \\
 & IVS47 + 29T → C & Intronic change \\
 & IVS50 – 131ins/del & Intronic change \\
 & 399C → A & G133G \\
 & IVS3 + 13C → T & Intronic change \\
\hline
\end{tabular}
\end{table}

\*The genes are described in the second footnote to Table 1.
\ †This change was considered a potentially neutral polymorphism after screening controls of similar ethnicity and determining that this change appeared in more than 17% of those screened.
the diagnosis of Stargardt macular degeneration in both children.

CASE REPORT 2: INCONCLUSIVE TEST RESULT

A 38-year-old white man was seen with a chief complaint of progressively worsening vision. Clinical examination and electroretinographic results were consistent with a diagnosis of Stargardt macular degeneration. His ophthalmologist ordered ABCA4 testing. No causative mutations were detected in this individual; however, 4 previously reported polymorphic changes were observed in the heterozygous state (Table 4). Although the molecular basis of disease was not identified, this is not a negative test result (ie, the clinical diagnosis can neither be confirmed nor ruled out based on these results). The test result was inconclusive.

<table>
<thead>
<tr>
<th>Nucleotide Change</th>
<th>Effect on Amino Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVS10 + 5delG</td>
<td>This deletion is in a noncoding region.</td>
</tr>
<tr>
<td>5603A → T</td>
<td>The sequence change alters the amino acid at position 1868, from asparagine to isoleucine.</td>
</tr>
<tr>
<td>5682G → C</td>
<td>The sequence change does not alter the amino acid, leucine.</td>
</tr>
<tr>
<td>5843C → T</td>
<td>This sequence change alters the amino acid at position 1948, from proline to leucine.</td>
</tr>
</tbody>
</table>

*This table is based on data found in articles by Stone,18 Maugeri et al,26 and Rivera et al.21

The most common use of molecular diagnostic information of patients with retinal disease is to confirm a clinical diagnosis. The clinical presentation often was not straightforward, and the differential diagnosis could include several diseases. Molecular diagnostics does not replace the necessary expertise of the ophthalmologist; rather, it adds a new tool to the ophthalmologist’s diagnostic arsenal.

Molecular testing offers unique advantages and novel challenges. One advantage is that it is available to patients at remote sites by the simple submission of a blood sample and appropriate clinical referral. In addition, once the familial mutation is identified, DNA from blood relatives may be tested for the specific mutation without sequencing the entire gene. Translating knowledge derived from the genetic research literature to the clinical paradigm necessitates considering the applicability to the individual patient. In cases in which the familial mutations are known, results are typically either positive or negative (ie, they confirm or rule out the presence of the known mutations). In contrast, when the familial mutation is not known, the likelihood of detecting the pathogenic mutation can only be estimated. Caution must be exercised in extracting research detection rates for use for the individual patient. In the clinical setting, patients may not match research subjects for a variety of characteristics, including phenotype, family history, and racial and ethnic background.28 Detection rates in the genetic research literature may be reported as the percentage of subjects in whom any mutation was found. However, in cases of recessive diseases, the report of a single mutated allele does not confirm the diagnosis; thus, the detection rate reported in the research literature may be higher than the rate of confirmation of disease.

As illustrated in case 2, when the familial mutation is not known, lack of identification of pathogenic mutations does not rule out the diagnosis. The explanation for why mutations in the gene tested may not be detected could be because of limitations in our knowledge and available technology. Although it is commonly accepted that a single polymorphism does not cause abnormal gene functioning, it is not yet known what effect multiple polymorphisms in the same or different alleles may have on the functioning of the gene. Another consideration is the possibility that gene-gene interaction, whereby a change in another gene in combination with 1 or more of these polymorphisms, would result in disease.29,30

Typically, sequence alterations that do not change the amino acid are considered to be neutral polymorphic changes; however, there have been rare cases in which a nucleotide substitution that does not change the amino acid has been known to be disease causing.31 Current methods could fail to identify large deletions, large insertions, or mutations in noncoding regions. Alternatively, the disease may be caused by mutations in another gene or may not have a genetic cause.

The concept of an inconclusive result is often counterintuitive to the patient and is an important component of pretest education and counseling.32 Identifying and addressing the individual patient’s expectations before testing can alleviate potential pitfalls when reporting results to the patient.33 Before testing, providing written consent for DNA testing in conjunction with a face-to-face discussion of concerns and questions and proactively addressing unique concepts in the interpretation of molecular genetic test results promotes patient satisfaction and alleviation of misunderstanding and distress. This necessitates allocation of time and expertise to explain in lay language the scope and limitations of the test, assess clinical relevance and patient expectations, convey results, and answer questions regarding interpretation.34-42

With more than 100 retinal genes already cloned and the number increasing, one of the hurdles in providing efficient affordable molecular testing is the existing technical limitations. The development of technology that can provide high-throughput mutation detection will afford a significant breakthrough in promoting the practical utility of molecular testing. Microarray chips with known mutations have been used successfully to identify known mutations in patients seen with Stargardt macular degeneration, cone-rod dystrophy, autosomal recessive retinitis pigmentosa, and Leber congenital amaurosis.9,43,44 A promising development is a sequencing microarray chip developed by Mandal et al45 that screens 11 genes associated with early-onset retinal degeneration diseases. These sequencing ar-
rays allow for simultaneous genotyping of any or all of the 11 genes at one time, and can identify novel mutations and those that have been previously described. This technology also affords the opportunity of investigating the interaction of multiple sequence variations and mutations that may occur in more than one gene, thus providing valuable tools in analyzing complex modes of inheritance while increasing productivity and lowering cost.

Future molecular testing for ophthalmic diseases may include prenatal applications, molecular-based clinical trials, and genotype-specific treatment options. Indeed, advances in the molecular diagnosis of retinoblastoma have allowed for at least 1 clinical case of preimplantation genetic diagnosis.46-53 There are animal models illustrating success in genotype-based treatment of retinal degeneration.47-53 As future therapies are designed to treat specific genetic ocular diseases, knowledge of the individual patient’s genotype will be essential in prescribing the appropriate treatment.

Molecular testing will likely become a standard of practice for the ophthalmologist. Because ocular molecular testing is still in its infancy, the ophthalmologist may encounter direct patient requests for testing and will need to determine when to order tests. Clinical molecular diagnostically oriented laboratory personnel, including the laboratory director, the genetic counselor, and the clinical ophthalmic genetic specialist, can be of great value to the ophthalmologist by providing consultation before and after molecular genetic testing.

Submitted for Publication: June 30, 2006; final revision received August 30, 2006; accepted August 31, 2006.

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Author Contributions: Dr Ayyagari had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Financial Disclosure: None reported.

Funding/Sponsor: This study was supported by grants EY11671 (Ms Downs and Dr Richards) and EY13198 (Dr Ayyagari) and core grants EY07003 and EY07060 (Department of Ophthalmology and Visual Sciences, University of Michigan) from the National Institutes of Health; by the Foundation Fighting Blindness (Drs Weleber, Heckenlively, and Ayyagari); and by Research to Prevent Blindness (Dr Ayyagari).

Additional Information: Patient fees varied according to the gene tested and whether the familial mutation was previously confirmed. These fees covered a portion of the costs of testing and provision of genetic counseling.

Acknowledgment: We thank the physicians and genetic counselors who contacted us to determine if genetic molecular testing was appropriate for their patients and who submitted samples for clinical testing.

REFERENCES

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