Clinical Utility of the ABCR400 Microarray
Basing a Genetic Service on a Commercial Gene Chip
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Objectives: To assess the clinical utility of ABCR400 microarray testing in patients with ABCA4-associated retinopathies and to report on possible issues that could arise should genetic results be delivered without validation.

Methods: One hundred thirty-two probands were genotyped with the microarray. Diagnostic assays were designed to verify all mutations identified in individuals in whom at least 2 causative mutations were found. Mutations were verified in the probands, and wherever possible cosegregation analysis was performed in additional family members.

Results: Eighty-five of the 132 probands (64.4%) genotyped with the microarray had 2 or more disease-associated mutations identified. Verification of the genotyping, however, resulted in only 80 families being able to benefit from genetic result delivery. The remaining families could not receive results owing to the confounding effect of multiple ABCA4 mutations or the incorrect identification of mutations.

Conclusions: The ABCR400 microarray is useful for mutation screening; however, raw data cannot be delivered directly to patients. All mutations should be verified and, whenever possible, investigated in other family members.

Clinical Relevance: Validated ABCR400 results provide an unequivocal molecular diagnosis, allowing family members to be offered diagnostic, predictive, carrier, and prenatal testing. Use of the microarray can inform decision-making and identify candidates for future therapies.


The ABCA4 gene encodes a transmembrane protein that functions in transporting vitamin A derivatives in photoreceptors.1,2 Mutations of this gene are responsible for several autosomal recessive retinal degenerative disorders, including Stargardt disease (STGD), fundus flavimaculatus, retinitis pigmentosa, and cone-rod dystrophy.3 ABCA4 mutations are thought to cause an accumulation of toxic vitamin A derivatives in the retinal pigment epithelium, which subsequently causes the death of photoreceptor cells and vision loss.

Traditional mutation screening methods were laborious due to the size of the ABCA4 gene and the spectrum of sequence variations identified therein. A genotyping microarray (ABCR400) has therefore been designed to test for more than 400 variants in ABCA4.4 Pathogenic mutations and nonpathogenic polymorphisms are included on the ABCR400 microarray based on a “systematic analysis of all published, reported and communicated data.”4 This microarray was more than 98% effective in determining ABCA4 sequence variations and was purported to be applicable for mutation screening (including diagnostic screening) of patients with potential ABCA4-associated retinopathies (AARs).

Many publications have reported the use of the ABCR400 microarray to identify the mutation spectrum and frequency in different populations.5,6 To our knowledge, there have been no reports on the clinical utility of the microarray. Clinical utility has been described as the use of test results to inform clinical decision-making and/or the ability of a test to prevent or alleviate adverse health.7 To develop a diagnostic service in South Africa based on the ABCR400 microarray results, it was necessary to validate the research findings.

METHODS

COHORT SELECTION

Individuals with inherited retinal degenerative disorders and their family members were recruited throughout South Africa to participate in a research program at the Division of Human Genetics, University of Cape Town. Biological material, relevant clinical details, and demographic information from more than 1000 families are currently archived at the Division of Human Genetics, University of Cape Town.
of Human Genetics. Informed consent was obtained according to the tenets of the Declaration of Helsinki (2000). The consent forms and patient information sheets used for recruiting these families were approved by the research ethics committee of the Faculty of Health Sciences, University of Cape Town, in 1999.

One hundred thirty-two probands with a confirmed diagnosis of STGD (n = 124), fundus flavimaculatus (n = 2), or cone-rod dystrophy (n = 6) were genotyped with the ABCR400 microarray (Asper Ophthalmics, Tartu, Estonia).4 These diagnoses were made during ophthalmic examinations by general ophthalmologists.

**MODES OF ANALYSIS**

Following ABCR400 testing, each family was classified as being in 1 of 3 modes of analysis. Those in whom the causative mutations were not fully identified (who thus required further investigation) were classified as being in research mode. Families in whom at least 2 causative mutations were identified were classified as being in potential diagnostic mode. Families in whom mutation cosegregation was proven and/or mutations were verified using a diagnostic assay were classified as being in diagnostic mode.

**DIAGNOSTIC ASSAY DEVELOPMENT**

Diagnostic assays were designed (Table) to verify the 11 most common mutations (occurring in ≥3 probands each) and rarer mutations. Polymerase chain reaction amplification was performed using standard conditions. Probands were tested using restriction enzyme digests to verify the presence of each mutation. If no restriction site was altered, direct sequencing was used. When possible, cosegregation analysis was performed on samples from family members (including the proband sample, amplified in a separate experiment to the initial verification). Cosegregation studies were performed using restriction enzyme digests, denaturing high-performance liquid chromatography analysis (WAVE Nucleic Acid Fragment Analysis System; Transgenomic Inc, Omaha, Nebraska), allele-specific polymerase chain reaction, or direct sequencing (ABI Prism 3100 automated sequencer; Applied Biosystems, Foster City, California).

Of the 132 probands genotyped with the ABCR400 microarray, 30 were identified as having a single heterozygous mutation and Exon

<table>
<thead>
<tr>
<th>Mutation and Exon</th>
<th>Primer 5’-3’</th>
<th>PCR Condition</th>
<th>Diagnostic Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1490Y; exon 30</td>
<td>Forward: 5’GCAGCACTTTTGAGGCTG 3’; Reverse: 5’TCCCTCTGTGAGGACGAG 3’</td>
<td>25 Cycles at 60°C</td>
<td>Verification and cosegregation studies: Rsal digest</td>
</tr>
<tr>
<td>R602W; exon 13</td>
<td>Forward: 5’AGCTACAAAACGCCCTACG 3’; Reverse: 5’CTATTGACTGATCATGAGG 3’</td>
<td>25 Cycles at 60°C</td>
<td>Verification and cosegregation studies: Mapl digest</td>
</tr>
<tr>
<td>L2027F; exon 44</td>
<td>Forward: 5’GAAGCTTTGTCGACCTGACG 3’; Reverse: 5’TGCACCTCTACTGAAACAGG 3’</td>
<td>28 Cycles at 60°C</td>
<td>Verification and cosegregation studies: FruxHl digest</td>
</tr>
<tr>
<td>V256V; exon 6</td>
<td>Forward: 5’GGTGCCTTCTCACCACAG 3’; Reverse: 5’AGGATACACCTGCAATTGG 3’</td>
<td>30 Cycles at 55°C</td>
<td>Verification: direct sequencing using forward primer</td>
</tr>
<tr>
<td>IVS38-10T&gt;C; exon 39</td>
<td>Forward: 5’GCCCAACCCCGGCTGAAAG 3’; Reverse: 5’TCCACGCTTTGGACACGG 3’</td>
<td>30 Cycles at 55°C</td>
<td>Cosegregation: dhPLC analysis</td>
</tr>
<tr>
<td>G863A; exon 17</td>
<td>Forward: 5’CTGGGTAAAGGAAGATAGG 3’; Reverse: 5’ACACCGTTTTACATAGAGGGG 3’; G863A-RevC: 5’TATTTGGAAGGGTCTCATTGACAG 3’; G863A-RevS: 5’GGCTGCTGGGTAGTAAAGGCTGAGTGTCATGACAG 3’</td>
<td>28 Cycles at 60°C</td>
<td>Verification: direct sequencing using reverse primer</td>
</tr>
<tr>
<td>R152X and R152Q; exon 5</td>
<td>Forward: 5’GACCATTTCTCCTCCACG 3’; Reverse: 5’AGGCTGTTGGTATTCTCCCTCACTGATACCTGATAACTCCCTTATTGCAG 3’</td>
<td>28 Cycles at 60°C</td>
<td>Cosegregation studies (R152Q): direct sequencing using reverse primer</td>
</tr>
<tr>
<td>P1380L; exon 28</td>
<td>Forward: 5’CCACAGCAGGGGTGGTATG 3’; Reverse: 5’CCCACACCCCCACAGGAGG 3’</td>
<td>28 Cycles at 55°C</td>
<td>Cosegregation studies (R152Q): direct sequencing using reverse primer</td>
</tr>
<tr>
<td>N965S; exon 19</td>
<td>Forward: 5’TGCGGCTAGTAAATTGACG 3’; Reverse: 5’TGGGAAAAAGGAGTAAAGCAGGCGG 3’</td>
<td>28 Cycles at 55°C</td>
<td>Cosegregation studies (R152Q): direct sequencing using reverse primer</td>
</tr>
<tr>
<td>G1961E; exon 42</td>
<td>Forward: 5’GTCAGAATCTGACTCCCGG 3’; Reverse: 5’GGGAGAAGAGGCAAGGACG 3’</td>
<td>28 Cycles at 60°C</td>
<td>Various</td>
</tr>
<tr>
<td>Rare mutations</td>
<td>Previously published primers, except exon 14 forward: 5’CCCTGCCTCTCTCTCTCCCATC 3’; exon 14 reverse: 5’TCTTGAGGTGTCCTCCACGG 3’; exon 24 forward: 5’ATGGTTGCTCTGCAACTCTGGAGA 3’; exon 24 reverse: 5’GTCTACCAACAGGAAAGCAG 3’</td>
<td>Various</td>
<td>Verification and cosegregation analysis: direct sequencing using primer farthest from mutation</td>
</tr>
</tbody>
</table>

Abbreviations: dhPLC, denaturing high-performance liquid chromatography; PCR, polymerase chain reaction.
gous mutation (22.7%) and 17 were found to have no mutations (12.9%). These individuals were classified as remaining in research mode. The remaining 85 probands had 2 or more mutations (64.4%) and were classified as being in potential diagnostic mode.

SIMPLEX CASES

No additional family members were available for 43 of 85 probands in potential diagnostic mode, and cosegregation analysis could not be performed. Of these 43 probands, 39 had 2 mutations verified, 3 had 3 mutations verified, and 1 (RPS1111.1) had a homozygous mutation disproved.

Case RPS1111.1, a Black African Xhosa-speaking individual with STGD, was found not to carry the homozygous c.2565G>A (W855X) mutation that was detected using the chip. Sequencing of exon 16 instead revealed a homozygous 8–base pair deletion spanning codon 855. The presence of this deletion was confirmed by non-denaturing high-performance liquid chromatography analysis (results not shown). The 2565delG-TACCTTG variation has not been reported previously, to our knowledge, and is predicted to cause a stop codon at position 855 (Figure 1). Because this is a novel variation, in a population not previously shown to exhibit a founder effect for ABCA4 mutations, the homozygosity observed in this individual could be due to uniparental isodisomy or a gross deletion spanning the ABCA4 gene. This individual exhibited no heterozygosity at any locus tested by the microarray. Blood from additional family members has been requested and additional research, such as cosegregation analysis and the screening of ethnically matched controls for this variation, is required to accurately define whether this deletion is a pathogenic mutation. Forty-two families (each represented by a single individual) could thus benefit from delivery of these results via a diagnostic molecular service and be reclassified as being in diagnostic mode.

FAMILY INVESTIGATIONS

Family members were available for 42 of 85 probands in potential diagnostic mode, and cosegregation analysis was performed in addition to mutation verification. In the pedigrees shown, all recruited individuals from whom biological material was available were tested, and genotypes are listed below their symbols. For 30 of 42 probands, compound heterozygosity of 2 mutations or homozygosity of a single mutation was confirmed to be causative of disease within the family (Figure 2). Two families had 3 mutations identified in the probands, and cosegregation analysis indicated which mutations were transmitted on a single parental allele (Figure 3).
For 5 families, it was determined that a third (unknown) mutation was present in addition to the 2 mutations initially identified in the proband. The presence of a third mutation did not confound results that could be delivered to some members of each family. However, a second affected individual requires ABCR400 genotyping to fully characterize the molecular basis of disease in the entire family (Figure 4).

One proband was confirmed to have inherited the heterozygous R2107H mutation from her father. This individual did not, however, carry the heterozygous 4537delC (Q1513FSX12) mutation reported. Upon sequencing of the proband, it was determined that an unknown mutation was present, which could be delivered to some members of each family. However, a second affected individual requires ABCR400 genotyping to fully characterize the molecular basis of disease in the entire family (Figure 4).

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The presence of multiple mutations did not confound diagnostic service delivery when cosegregation analysis determined which 2 mutations were on a single allele (Figure 3) or when individual results could be given to several family members regardless of the third familial mutation being uncharacterized (Figure 4). In addition, the founder chromosomes underlying AARs in South Africa occasionally allowed the identification of both causative mutations in an individual other than the proband. When performing cosegregation analysis, all available members of a family were tested for all mutations identified in the proband. Occasionally, affected family members in different generations were found to have the same mutation complement of the common mutations (Figure 2).

The presence of multiple mutations confounded diagnostic service delivery in 4 cases. In 3 families, the presence of a third (unknown) mutation resulted in only the proband being fully characterized, with all other affected individuals in the family unable to receive results. For example, a homozygous mutation was identified in the proband of a consanguineous family. However, 2 affected siblings showed heterozygosity for this mutation (Figure 6). A second affected individual from these families should therefore be tested with the microarray to identify the unknown mutation. In the fourth family, the mutations could have been misinterpreted had cosegregation analysis not been performed. Analysis revealed that the father carries both mutations and has no retinal phenotype, indicating that both mutations were transmitted on a single parental allele (Figure 7). Due to microarray genotyping of 132 affected probands from families with STGD, fundus flavimaculatus, or cone-rod dystrophy resulted in the identification of 85 families in potential diagnostic mode. Verification of the ABCR400 results in our laboratory, however, resulted in only 80 families actually being reclassified to diagnostic mode. The 2 main confounding issues in the translation of results were (1) multiple mutations leading to the potential misinterpretation of alleles or missed alleles, and (2) incorrect identification of mutations.
to the recessive nature of AARs, mutations on both alleles must be identified. Because both identified mutations in RPS593 occur on 1 allele, the mutation on the second allele is unknown. No other mutation was identified by the array, indicating that novel and full-length gene screening is required for its characterization. Owing to the difficulties in interpreting these multiple mutations, these 4 families remain in research mode.

The second main confounding issue was the incorrect identification of 2 mutations. The 4535insC mutation was incorrectly identified as 4537delC, despite the insertion causing C/A heterozygosity at position 4538 instead of 4537 (Figure 5). If the microarray probes position 4537 and the sequence containing the insertion displays a C/C genotype at position 4537, no mutation should
have been detected. Nevertheless, the correct mutation in this individual could be identified during the verification process. The novel 2563delGTACCTTG variation was also incorrectly identified by the ABCR400 microarray as the W855X mutation (Figure 1). The presence of this deletion causes the sequence at position 855 to be that expected from the W855X mutation (TGG→TGA). In this case, the pathogenicity of the variant identified during verification is uncertain and the family remains in research mode.

The ABCR400 microarray is a useful tool in the first step of a translational research program, ie, mutation screening. It allowed the rapid, cost-effective identification of causative mutations in more than 60% of our cohort. Less than half of the original cohort thus remains, requiring full-length gene screening to identify the causative novel mutations of ABCA4. It must be stressed, however, that raw data cannot be delivered directly to patients without verification and familial investigation. Because more than half of the families with results are represented by a single affected individual, cosegregation analysis could not be performed and mutations were simply verified. As a result, when 3 mutations were verified, it was impossible to determine which 2 shared a single allele; when 2 mutations were verified, it was impossible to confirm that they were located on separate alleles. In result-delivery counseling sessions, the limitations of the tests were discussed and probands were given the option of recruiting their parents or additional family members for testing to confirm their own results. In the future, the need to test family members (preferably parents) should be explained up front to the patient, rather than at the result-delivery stage. The researcher should consider issues such as the incorrect interpretation and incorrect identification of mutations, and every caution should be taken to ensure accuracy and completeness of results when the ultimate goal is translation and service delivery.

In terms of evaluating clinical utility, validated ABCR400 results can be delivered to individuals with important outcomes. Families can be molecularly diagnosed as having an AAR, rather than being given the clinical diagnosis of STGD, fundus flavimaculatus, retinitis pigmentosa, or cone-rod dystrophy. Overlapping phenotypes prove challenging to diagnosticians, and the progression of disease results in updated or altered diagnoses, however, the molecular diagnosis is unequivocal. Identification of the exact molecular defect in patients with AARs means that diagnostic, predictive, carrier, prenatal, and preimplantation testing can be offered. Spouses can be offered carrier testing using the chip and (accompanied by careful genetic counseling) make informed reproductive decisions. An accurate molecular diagnosis may be a requirement for future gene-based therapies. In addition, patient candidates are identified for early intervention. Our patients with AARs are advised to wear sunglasses and avoid vitamin A supplementation, despite some of them having been prescribed vitamin A by their ophthalmologists or general practitioners. Future intervention may include treatment with drugs designed to reduce the levels of toxic vitamin A derivatives that cause photoreceptor death.

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REFERENCES