Novel Complex ABCA4 Alleles in Brazilian Patients With Stargardt Disease: Genotype–Phenotype Correlation

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PURPOSE. To analyze the presence of complex alleles of the ABCA4 gene in Brazilian patients with Stargardt disease and to assess the correlation with clinical features.

METHODS. This was an observational cross-sectional study. Patients with a diagnosis of Stargardt disease who presented three pathogenic variants of the ABCA4 gene or who had variants previously described as complex alleles were included. The relatives of these probands were evaluated in the segregation analysis. The patients were evaluated based on age at symptom onset and visual acuity, and the clinical characteristics were classified according to the findings observed on autofluorescence examination.

RESULTS. Among the 47 families analyzed, approximately 30% (14/47) presented complex alleles. The segregation analysis in 14 families with cases of Stargardt disease identified three novel complex alleles and one previously described complex allele. The known complex allele p.[Leu541Pro; Ala1038Val] was identified in two families. The novel complex alleles identified were p.[Leu541Pro; Arg1443His] in five families, p.[Ser1642Arg; Val1682_Val1686-del] in seven families, and p.[Pro1761Arg; Arg2106Cys] in one family. Furthermore, four new variants (p.Lys22Asn, p.Asp915Asn, p.Glu1447Val, and p.Pro1761Arg) were identified in the second allele of the ABCA4 gene.

CONCLUSIONS. Segregation analysis is important in order to confirm the molecular diagnosis of patients with Stargardt disease, given the frequency of complex alleles in the ABCA4 gene. The various pathogenic variation combinations observed in this study were associated with different phenotypes.

Keywords: Stargardt disease, macular degeneration/genetics, retinal dystrophy, eye diseases, hereditary, ABCA4 protein, human, complex allele
RESULTS

Fourteen families suspected of having complex alleles were identified in a cohort of 47 families who previously underwent sequencing of the ABCA4 gene (approximately 30%). The probands from these 14 families presented three genetic variants, including pathogenic or unknown variants, or presented variants previously reported as segregating in cis. A total of 55 individuals from these 14 families were included in the segregation analysis.

From the initial group of 47 families, 5 families were identified as carrying the complex allele p.[Leu541Pro; Arg1443His] (5/47, 10.6%). The known complex allele p.[Leu541Pro; Ala1038Val] was identified in two families (2/47, 4.2%). The complex allele p.[Ser1642Arg; Val1682 Val1686del] was found in seven families (7/47, 14.9%), and p.[Pro1761Arg; Arg2106Cys]:: was identified in one family (1/47, 2.1%). The molecular analysis results and the clinical characteristics of the Stargardt patients are presented in Table 1 and Figure 1.

Segregation Analysis

The segregation analysis results are shown in the pedigrees in Figure 2. Individuals BII2, DII1, DII2, and JII1 were described by the probands as having Stargardt disease but were not clinically evaluated.

Family D had a history of consanguinity. The matriarch (DII1) and the brother (DII2), who were also reported to have Stargardt disease, were tested only for segregation. For this reason, the pathogenic variant in the second allele was not found.

Individual JII1 was tested only for segregation of the variants found in proband JII2 in this family; therefore, a second variant was not found. The complex allele p.[Ser1642- Arg; Val1682_val1686del] was also found in these individuals (JII1 and JII2) as well as in JI3.

Individuals KII and NI2, who have Stargardt disease, underwent only segregation analysis, and the molecular diagnosis concluded that they carry the same pathogenic variants as their siblings.

Pathogenicity of Novel Variants

Four novel variants were identified in this study (p.Lys22Asn, p.Asp915Asn, p.Glu1447Val, and p.Pro1761Arg), and their effects were previously unknown (Table 2). Only the p.Asp915Asn variant had been previously described as a rare variant in the ExAC database. None of the other three novel variants were found in the population databases used as references.

The p.Lys22Asn variant (family L) was classified as deleterious by three predictive software programs: CADD (score 24), MutationTaster (score 1.000), and PolyPhen-2 (score 0.701). In contrast, it was classified as tolerated by SIFT (score 0.124).

The pathogenicity of variant p.Asp915Asn (family A) was rated as tolerated/benign by SIFT (score 0.273) and PolyPhen-2 (score 0.001). However, it was classified as probably deleteri-
### Table 1. Genotyping of ABCA4 Gene and Clinical Characteristics of Patients With Stargardt Disease

<table>
<thead>
<tr>
<th>Family</th>
<th>Patient</th>
<th>Sex</th>
<th>Allele 1</th>
<th>Allele 2</th>
<th>Age at Onset, Years</th>
<th>Age at Examination, Years</th>
<th>Duration of Disease, Years</th>
<th>RE Visual Acuity</th>
<th>LE Visual Acuity</th>
<th>Classification in Autofluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fam A</td>
<td>II 1</td>
<td>M</td>
<td>p.[Leu541Pro; Arg1443His]</td>
<td>p.Asp915Asn</td>
<td>8</td>
<td>13</td>
<td>5</td>
<td>20/200</td>
<td>20/200</td>
<td>II</td>
</tr>
<tr>
<td>Fam B</td>
<td>II 1</td>
<td>F</td>
<td>p.[Leu541Pro; Arg1443His]</td>
<td>p.Arg602Trp</td>
<td>7</td>
<td>31</td>
<td>24</td>
<td>CF 1.5m</td>
<td>CF 1.5m</td>
<td>III</td>
</tr>
<tr>
<td>Fam C</td>
<td>III 1</td>
<td>M</td>
<td>p.[Leu541Pro; Arg1443His]</td>
<td>p.Arg1129Leu</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>I</td>
</tr>
<tr>
<td>Fam D</td>
<td>II 1</td>
<td>M</td>
<td>p.[Leu541Pro; Arg1443His]</td>
<td>p.[Leu541Pro; Arg1443His]</td>
<td>N/A</td>
<td>35</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Fam E</td>
<td>II 1</td>
<td>F</td>
<td>p.[Leu541Pro; Ala1038Val]</td>
<td>c.3329-2A&gt;T</td>
<td>7</td>
<td>59</td>
<td>52</td>
<td>HM</td>
<td>CF 10cm</td>
<td>III</td>
</tr>
<tr>
<td>Fam F</td>
<td>II 1</td>
<td>M</td>
<td>p.[Leu541Pro; Ala1038Val]</td>
<td>c.3329-2A&gt;T</td>
<td>8</td>
<td>65</td>
<td>57</td>
<td>HM</td>
<td>HM</td>
<td>III</td>
</tr>
<tr>
<td>Fam A</td>
<td>II 1</td>
<td>F</td>
<td>p.[Ser1642Arg; Val1682_Val1686del]</td>
<td>p.Glu1447Val</td>
<td>14</td>
<td>37</td>
<td>25</td>
<td>20/150</td>
<td>20/150</td>
<td>II</td>
</tr>
<tr>
<td>Fam I</td>
<td>II 1</td>
<td>M</td>
<td>p.[Ser1642Arg; Val1682_Val1686del]</td>
<td>p.Arg602Trp</td>
<td>8</td>
<td>26</td>
<td>18</td>
<td>CF 50cm</td>
<td>CF 2m</td>
<td>III</td>
</tr>
<tr>
<td>Fam J</td>
<td>II 2</td>
<td>M</td>
<td>p.[Ser1642Arg; Val1682_Val1686del]</td>
<td>p.Thr1019Met</td>
<td>5</td>
<td>26</td>
<td>21</td>
<td>CF 2m</td>
<td>CF 1m</td>
<td>III</td>
</tr>
<tr>
<td>Fam K</td>
<td>II 2</td>
<td>M</td>
<td>p.[Ser1642Arg; Val1682_Val1686del]</td>
<td>p.Gly1961Glu</td>
<td>32</td>
<td>32</td>
<td>0</td>
<td>20/80</td>
<td>20/80</td>
<td>I</td>
</tr>
<tr>
<td>Fam L</td>
<td>I 1</td>
<td>F</td>
<td>p.[Ser1642Arg; Val1682_Val1686del]</td>
<td>p.Lys22Asn</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>III</td>
</tr>
<tr>
<td>Fam M</td>
<td>II 1</td>
<td>M</td>
<td>p.[Ser1642Arg; Val1682_Val1686del]</td>
<td>p.[Leu541Pro; Arg1443His]</td>
<td>N/A</td>
<td>33</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Fam N</td>
<td>II 1</td>
<td>M</td>
<td>p.[Pro1761Arg; Arg2106Cys]</td>
<td>p.Trp782</td>
<td>15</td>
<td>25</td>
<td>10</td>
<td>20/400</td>
<td>20/400</td>
<td>I</td>
</tr>
<tr>
<td>Fam N</td>
<td>II 2</td>
<td>F</td>
<td>p.[Pro1761Arg; Arg2106Cys]</td>
<td>p.Trp782</td>
<td>N/A</td>
<td>26</td>
<td>N/A</td>
<td>20/200</td>
<td>20/200</td>
<td>N/A</td>
</tr>
</tbody>
</table>

RE, right eye; LE, left eye; CF, count fingers; HM, hand movement; N/A, not available. Bold indicates novel variants.
ous by CADD (score 19.92) and as a disease-causing variant by MutationTaster (score 0.914063).

Likewise, because the pathogenicity of the p.Glu1447Val variant (family H) was previously unknown, this variant was evaluated in silico using the same four software tools. It was identified as benign by PolyPhen-2 (score 0.021), SIFT (score 0.062), and MutationTaster (score 0.949215) but as a deleterious variant by CADD (score 23.1).

The p.Pro1761Arg variant (family N) was consistently identified as a disease-causing variant, with scores of 27 in CADD (deleterious), 1.000 in MutationTaster (disease-causing), 0.001 in SIFT (deleterious), and 0.926 in Polyphen-2 (probably damaging).

**DISCUSSION**

Since the identification of the ABCA4 gene, several variants (both pathogenic and benign) have been described and correlated with Stargardt disease. Because this gene is polymorphic and not highly conserved, categorizing new ABCA4 variants as pathogenic is likely to be important for molecular diagnosis in some patients. Currently, the identification of complex alleles in ABCA4 gene and their correlation with specific populations have enabled changes in the interpretation of molecular analyses of this gene. In this study of Brazilian patients with Stargardt disease, four pairs of pathogenic variants were observed to cosegregate in the ABCA4 gene.

**p.Leu541Pro Associated With p.Arg1443His**

The pathogenic variants p.Leu541Pro and p.Arg1443His were found in families A, B, C, D, and M and were inherited as complex alleles. Variant p.Arg1443His was described as pathogenic by Rivera et al. In the same study, Rivera et al. described variant p.Arg1443Val in a patient who also presented the pathogenic variants p.Leu541Pro and p.Ala1038Val but who did not undergo segregation analysis.

This case shows the importance of segregation in the presence of three pathogenic variants in the same gene. Cosegregation of the pathogenic variants p.Leu541Pro and p.Arg1443His was identified for the first time in the present study and was present in five families.

**p.Leu541Pro Associated With p.Ala1038Val**

The complex allele p.[Leu541Pro; Ala1038Val] was previously identified in several families and had a founder effect associated with German ancestry. Sciezynska et al. also found a high frequency of this complex allele in the Polish population. This combination was found in families E and F in this study.

The paternal grandmother in family E was of Polish origin. The segregation analysis of this family suggested a paternal origin for this set of pathogenic variants (Fig. 2). The segregation analysis of family F identified complex allele p.[Leu541Pro; Ala1038Val] in the maternal lineage of Italian descent.

These variants previously described as disease-causing may present separately. Variant p.Ala1038Val is frequent among patients with Stargardt disease and may be the only pathogenic variant in one allele.

**p.Ser1642Arg Associated With p.Val1682_Val1686del**

The probands of families G, H, I, J, K, and M and case I1 of family L were heterozygous for the pathogenic variant p.Ser1642Arg and the p.Val1682_Val1686del (c.5044_5058del15) deletion. The repetition of these two variants in these patients, who were not related, raised the possibility of their cotransmission. This suspicion was confirmed by the results of the segregation analysis. Bertelsen et al. found the pathogenic variants p.Ser1624Arg and p.Val1682_Val1686del in the same patient, but they did not describe the segregation pattern. Riveiro-Alvarez et al. described cases in which the p.Val1682_-Val1686del deletion was associated with other genetic variants.
The novel variant p.Pro1761Arg, which was predicted to cause disease by CADD, SIFT, Polyphen-2, and MutationTaster, was found in family N. However, its frequency in the population has not been described. This variant was found to cosegregate with p.Arg2106Cys in family N. The combination of the cosegregating variants p.Pro1761Arg and p.Arg2106Cys was confirmed in the segregation analysis for this family. Variant p.Arg2106Cys is known to be pathogenic and is described in the reference databases.

Family N was the only family carrying the complex allele p.[Pro1761Arg; Arg2106Cys] in this study. Nevertheless, the identification of this variant may encourage investigators to search for this potential combination in other families.

Challenges in the Interpretation of Molecular Diagnosis

The identification of the complex allele p.[Arg1443His; Leu541Pro] in family A required a determination of pathogenicity in the other allele to make conclusions about the molecular diagnosis. The novel variant p.Asp915Asn was identified in the other allele in this family. As this is a novel variant with a frequency that has not been described in the population and is considered pathogenic by the CADD and MutationTaster prediction software programs, its presence may be related to the presentation of Stargardt disease in this case.

Similarly, in family H, the presence of the complex allele p.[Ser1642Arg; Val1682_Val1686del] does not allow for a conclusive molecular diagnosis. The novel variant p.Glu1447Val was found in the second allele. However, this variant was predicted to be deleterious only by CADD software. Thus, to infer the molecular diagnosis of this family, it is important to either confirm the pathogenicity of variant p.Glu1447Val or identify another pathogenic variant in this allele.

In family L, the novel variant p.Lys22Asn was predicted to be pathogenic by both CADD and MutationTaster, and its frequency in the population is unknown. This variant may be related to the presentation of Stargardt disease in two individual cases. In individual I1, this variant was associated with the complex allele p.[Ser1642Arg; Val1682_Val1686del], whereas in individual II2, this variant was associated with the p.Gly1901Glu variant. This repetition may support the hypothesis that the variant is indeed pathogenic.

Genotype–Phenotype Correlation

Cases in which the onset of Stargardt disease occurs during the first decade of life tend to present with a more rapid loss of central vision and more extensive retinal pigment epithelium atrophy. The pathogenicity of the variant in terms of protein function and/or structure may be directly related to the early onset of disease symptoms. Wiszniewski et al. suggested that pathogenic variants with more deleterious effects on protein function resulted in earlier symptoms of Stargardt disease. Westeneng-van Haaf et al. also suggested that more deleterious pathogenic variants caused more severe phenotypes. In this study, six cases with symptom onset before 10 years of age were classified as type III by autofluorescence. These individuals currently have had the disease for 18 to 57 years. In contrast, in six patients in whom symptoms of decreased visual acuity presented after the age of 10, the autofluorescence classification was type I.

The observed phenotypic classification in this study was the same among siblings, indicating the same pathogenic effects for each variant. This concordance of autofluorescence results among siblings was also described by Fujinami et al.
However, in a study evaluating the intrafamilial characteristics of the phenotypic manifestations of Stargardt disease, Lois et al. described different ages of symptom onset, different visual acuity, and different autofluorescence findings among siblings. No concordance of phenotypic manifestations was observed for the four complex alleles described herein. This may be due to different combinations of pathogenic variants that can alter the phenotype of Stargardt patients. Additional
studies of ABCA4 protein activity may provide insights into the phenotypic characteristics of each case. 5,6

Segregation analysis is important in order to confirm the molecular diagnosis of patients with Stargardt disease, given the high frequency of complex alleles of the ABCA4 gene. The identification of four novel variants and three novel complex alleles in the Brazilian population draws attention to the importance of analyzing molecular diagnoses in different populations. A better understanding of complex alleles may change approaches to sequence analysis and therefore affect genetic counselling practices.

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