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A novel diagnostic test detects a low frequency of the hemicentin Gln5345Arg variant among Northern Irish age related macular degeneration patients

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Purpose: Age related macular degeneration (AMD) is a common cause of severe vision loss. Identification of genes involved in AMD will facilitate early detection and ultimately help to identify pathways for treatment for this disorder. The A16,263G mutation in the HEMICENTIN-1 gene produces a non-conservative substitution of arginine for glutamine at codon 5345 which has been implicated in familial AMD. The aim of this study is to develop a rapid diagnostic assay for the detection of this mutation and to evaluate its frequency in a sample of AMD patients.

Methods: A primer probe set was designed from exon 104 of the HEMICENTIN-1 gene to differentiate between mutant and wild type alleles. A region spanning the mutation was amplified by PCR using a LightCycler (Roche Diagnostic). The mutation was then detected by melt curve analysis of the hybrid formed between the PCR product and a specific fluorescence probe. The frequency of the mutation within the Northern Ireland population was evaluated by assaying 508 affected AMD patients, 25 possibly affected and 163 controls.

Results: This assay clearly discriminates between the A16,263G mutant and wild type HEMICENTIN-1 alleles. The wild type sequence has a single base mismatch with the probe which decreases the stability of the hybrid, resulting in a lower Tm (Tm = 51.27 °C) than that observed for the perfectly matched mutant allele (Tm = 59.9 °C). The mutant allele was detected in only one of the 696 subjects, an affected AMD patient.

Conclusions: We describe a rapid assay for the genotyping of the Gln5345Arg mutation using real-time fluorescence PCR to facilitate rapid processing of samples through combined amplification and detection steps. These characteristics are suitable for a clinical setting where high throughput diagnostic procedures are required. The frequency of this mutation within the Northern Ireland population has been estimated at 0.2%, concurring with previous findings that this mutation is a rare variant associated with AMD. A rapid diagnostic assay will facilitate a reliable and convenient evaluation of the frequency of the Gln5345Arg mutation and its association with AMD within other populations.

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Age related macular degeneration (AMD; OMIM 603075) is a complex, multifactorial disease that leads to bilateral visual impairment. AMD is responsible for half of all cases of blindness in Western populations greater than 65 years of age. AMD is estimated to affect approximately 25-30 million people, with this number expected to treble over the next 25 years [1-4]. A dramatic increase in the size of the aging population makes AMD a significant public health problem and a major focus of research efforts [5]. Apart from studies indicating that nutrient supplementation may be helpful in retarding the progress of dry AMD, no treatment is available for this form of the disorder which accounts for 80% of cases. Although therapy is available for wet AMD, it remains poorly efficacious [4].

AMD is a progressive disease encompassing a broad spectrum of clinical findings that primarily affect the central (macular) region of the retina [6,7]. The disease is characterized clinically by drusen, extracellular deposits of proteins, lipids and cellular debris found between Bruch’s membrane and the retinal pigment epithelium (RPE) and pigmentary abnormalities in the macular region. Small drusen deposits are commonly found in the aging macula but the presence of large drusen is considered to be diagnostic for AMD [8]. Late stage AMD can be subdivided clinically into two distinct forms: (1) dry AMD has been associated with the loss of RPE and photoreceptors resulting in geographic atrophy (GA) and reduced retinal function; (2) wet AMD is an exudative form with formation of choroidal neovascularization (CNV) leading to sub-RPE or subretinal hemorrhage, subretinal fluid, macular edema and eventually fibrotic scarring [9]. It is currently unclear as to whether both forms represent separate clinical disease with distinct genetic etiologies or stages in a single continuum [10].
Determination of the first genetic locus for AMD (ARMD1) on chromosome 1q25-31 was provided by linkage analysis of data from a family with a mainly dry form of the disease that appeared to be inherited in an autosomal dominant manner [11]. Several recent independent genomewide scans have provided evidence for a possible AMD susceptibility locus at 1q [12-17]. A A16,263G change in the HEMICENTIN-1 gene (NM_031935) was reported to be associated with the disease in the family originally found to demonstrate linkage to 1q25-31 [15]. This variation produces a non-conservative substitution of arginine for glutamine at codon 5345 (Gln5345Arg), an amino acid highly conserved at this position in eight other species analysed. This mutation was reported in several additional AMD affected individuals beyond the original family from a total sample size of 1378 individuals, of which 1204 were affected or were related to an affected individual. A subsequent independent investigation failed to detect this mutation in 620 affected AMD patients and 237 unaffected controls [16]. Development of a PCR based assay for detection of this SNP will facilitate a quick estimation of its prevalence among AMD affected populations.

The LightCycler system enables the detection of such single nucleotide polymorphisms and is widely used in clinical testing as it combines PCR amplification and detection into a single step. The platform enables the real-time detection of a specific PCR product followed by melting curve analysis of hybridization probes. The technology is based on the detection of two adjacent oligonucleotide probes, whose fluorescent labels communicate through fluorescence resonance energy transfer (FRET). The molecular concept of single nucleotide polymorphism (SNP) detection is simple: one of the probes serves as a tightly bound anchor probe and the adjacent sensor probe spans the region of sequence variation. During the melting of the final PCR product, the sequence alteration is detected as a change in the melting temperature of the sensor probe. For a typical homozygous wild type sample, a single melting peak is observed; for mixed alleles, two peaks are observed; and for a homozygous mutated sample, a single peak at a temperature different from the wild type allele is observed. The temperature shift induced by one mismatched base is usually between 5 and 9 °C and easily observable. The aim of this study was to develop a rapid and high throughput detection method for the determination of the Gln5345Arg mutation. We have developed a real-time PCR protocol using hybridization probes for detecting polymorphisms between mutant and wild type alleles by simultaneously identifying their characteristic melting behavior with the use of sequence specific fluorescent labeled oligonucleotide hybridization probes.

**METHODS**

**Study cohort:** Patients with AMD were primarily ascertained and recruited following attendance and examination at The Department of Ophthalmology, Royal Victoria Hospital, Belfast, the tertiary referral center for retinal conditions in Northern Ireland. Ethical approval for the study was granted (QUB 147/01). For the purpose of this study AMD was defined as stage 3 or greater according to the Rotterdam study classification [18]. Stage 3 is defined as soft indistinct drusen (>125 µm) or reticular drusen with pigmentary abnormalities. The predominant type of AMD however was Stage 4 wet AMD which was present in 90% of the affected patients. All patients who were diagnosed as having AMD and consented to take part in the study had color fundus photography and fluorescein angiography as part of their routine clinical management. Patients were included in the AMD affected group if they had stage 3 or beyond on the Rotterdam scale and their media was clear enough to obtain gradable fundus photographs. Patients without AMD were therefore not included within the affected group. Control patients were identified from lists from general care practitioners. These included 41 patients over 85 years of age who were examined and shown to be free of AMD. The remainder was representative of the normal population and although these control patients were not examined, they were selected as having no past history of known visual problems. It is therefore unlikely that controls had end stage AMD but is likely that they would have the same rate of early AMD changes as that expected within the general population.

A 10 ml blood sample was collected from 696 patient samples comprising 508 with AMD, 25 possibly affected sib-pairs awaiting ophthalmic examination, 41 AMD free normal samples (of age 85 and above) and 122 members of the general population. DNA was extracted using a Wizard® Genomic DNA Extraction Kit (Promega, Southampton, UK) and tested for the presence or absence of the Gln5345Arg SNP.

**Design of primers and fluorogenic probes:** Primer and probe sets were designed, synthesized and purified by reversed phase high performance liquid chromatography by Tib MolBiol (Berlin, Germany). Sequences for the primers and probes used in this study are listed in Table 1. Two stem-loops were identified within the region of the mutation that might negatively influence the amplification and detection of the mutant allele (Figure 1). To improve probe binding within the assay, a primer was designed to include a mutation, reducing the effects of the 62 °C stem-loop. The sensor probe was designed to span the mutation and to be separated from the anchor probe by one nucleotide. The melting temperature (Tm) of the sensor probe was designed to be approximately 5 °C lower than that of the anchor probe. The 5’-end of the sensor probe placed

<table>
<thead>
<tr>
<th>Identifier</th>
<th>Sequence</th>
<th>Reference ID</th>
<th>Tm</th>
</tr>
</thead>
<tbody>
<tr>
<td>FIBL-6 F mut</td>
<td>CAGCTTCAAGTGTTATCTACCC</td>
<td>463599</td>
<td>51.7 °C</td>
</tr>
<tr>
<td>FIBL-6 A</td>
<td>ATGCTGTTGAGGTTGATAGTT</td>
<td>463600</td>
<td>50.7 °C</td>
</tr>
<tr>
<td>FIBL-6 FL</td>
<td>CAATCCAGGCGAGATTCCCC-FL</td>
<td>463602</td>
<td>61.6 °C</td>
</tr>
<tr>
<td>Sensor C</td>
<td>LC-TCGCTGAAATACGCTGCGGCTGCGG</td>
<td>463603</td>
<td>56.2 °C</td>
</tr>
</tbody>
</table>

The nucleotide sequences and melting temperatures of the primers (FIBL-6 F mut and FIBL-6 A) and sensor (Sensor C) and anchor (FIBL-6 FL) probes employed (see Figure 1) are shown. The reference ID numbers may be used to identify specific probes for resynthesis by the manufacturer (Tib MolBiol; Berlin, Germany). The positions of fluorescein (FL) and LC Red 640 (LC) labels and the phosphorylated 3’-end (p) of the sensor probe are indicated.
downstream from the anchor probe was labeled with LC Red 640 and the 3'-end phosphorylated. The 3'-end of the anchor probe was labeled with fluorescein. Only when both probes are hybridized to their adjacent target sequences can fluorescence resonance energy transfer (FRET) occur between their fluorophores.

**Rapid cycle PCR and melting curve analysis:** PCR and melting curve analysis were performed in a 32 sample rapid fluorescent thermal cycler with three color fluorescence monitoring capability (LightCycler). A 136 bp fragment of exon 104 from the HEMICENTIN-1 gene was amplified from 10 ng of genomic DNA in a 10 µl reaction volume with 0.25 µmol/l each primer, 0.1 µmol/l anchor and sensor probe, and 5 µl of 2X Platinum® Quantitative PCR Supermix-UDG, containing Platinum® Taq DNA polymerase, Taq PCR buffer, a dNTP mixture, and MgCl₂, at a concentration of 3 mM. Samples were loaded into composite plastic/glass disposable capillaries, capped, briefly centrifuged and placed in the LightCycler sample carousel.

**Table 2. LightCycler experimental conditions**

<table>
<thead>
<tr>
<th>Program</th>
<th>Segment 1</th>
<th>Segment 2</th>
<th>Segment 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50 °C for 120 s</td>
<td>95 °C for 120 s</td>
<td>50 °C for 120 s</td>
</tr>
<tr>
<td>UDG Denaturation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amplification 40</td>
<td>95 °C for 5 s</td>
<td>50 °C for 5 s</td>
<td>72 °C for 5 s</td>
</tr>
<tr>
<td>Melting 1</td>
<td>95 °C for 30 s</td>
<td>40 °C for 120 s</td>
<td>95 °C for 0 s</td>
</tr>
<tr>
<td>Cooling 1</td>
<td>40 °C for 30 s</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The LightCycler protocol consists of four programs, each involving one or more incubation period or segment. In the first UDG Denaturation program, incubation at 50 °C for 120 s enables uracil-DNA glycosylase (UDG) to destroy any contaminating dU containing PCR product from previous reactions. Following denaturation at 95 °C, forty cycles of amplification are performed. The temperature transition rate between segments is 20 °C/s throughout, except between segments 2 and 3 of the melting program when the temperature is slowly raised at a rate of 0.1 °C/s to enable accurate discrimination between mutant and wild type alleles.

The LightCycler protocol for sequence specific detection and analysis of DNA with hybridization probes contains four component programs: initial denaturation of template DNA, amplification of target DNA, melting curve analysis (for identification of mutated sequences) and cooling of the instrument. The protocol used in this diagnostic assay is shown in Table 2.

**RESULTS**

One hybridization probe, labeled with fluorescent dye, hybridizes to a target sequence adjacent to the mutation site and functions as an anchor probe. The other sensor probe labeled with another fluorescent dye spans the mutation site. In the case of the wild type allele the mismatch of the sensor probe with the target, destabilizes the hybrid. If the mutation is present no mismatches will occur, and the hybrid has a higher Tₘ. This assay is designed to detect the A16,263G mutation, therefore a wild type DNA template will result in a mismatch giving a lower Tₘ than that observed for the mutant allele. The first negative derivative of the fluorescence (-dF/dT) against temperature graph shows peaks with different Tₘ’s allowing easy differentiation of the two alleles (Figure 2). The single base mismatch in the wild type allele decreases the stability of the probe-allele structure, resulting in a lower Tₘ (Tₘ=51.27 °C) than that of the mutant allele (Tₘ=59.9 °C) allowing easy differentiation of the two alleles.

The Gln5345Arg mutation was not detected among any of the 41 AMD free normal samples, 122 other normal samples or 25 possibly affected sib-pairs. It was detected in 1 affected AMD patient and this was confirmed through conventional PCR and DNA sequence analysis. The affected patient was an 83-year-old male smoker with bilateral wet AMD and a history of cataract and disciform scarring in the right eye (Figure 3). No definitive family history of AMD was evident but his mother had “gone blind” in later life. He subsequently presented with a subfoveal choroidal neovascular membrane in the left eye.

![Figure 1. Primer and probe design. The region of the HEMICENTIN-1 gene containing the A16,263G variant was amplified with the primers FIBL-6 F mut and FIBL-6 A (blue arrows). The position of an A in the FIBL-6 F mut that was introduced to disrupt a predicted stem-loop structure (shaded boxes) is indicated with a solid triangle. The 3' fluorescein labeled anchor probe (FIBL-6 FL) is indicated by an orange arrow and the 5' LC Red 640 labeled sensor probe (Sensor C) by a green arrow. The sensor probe spans the site of the A/G variation (indicated by a solid triangle). Pink arrows indicate a predicted stem-loop.](http://www.molvis.org/molvis/v10/a82/)
In this paper, we describe a rapid single-step method for the genotyping of the HEMICENTIN-1 Gln5345Arg mutation using real-time fluorescence PCR. The advantage of this method is that it is rapid, simple and very specific. These characteristics may be particularly suitable in the clinical setting where high throughput diagnostic procedures are required. The PCR amplification and allele detection (for up to 32 samples) are combined into a single program which is completed within 45 min. Furthermore, the assay does not require post-PCR electrophoresis and is specific because it is unaffected by the formation of spurious PCR amplification products. In contrast to previous assays to determine the presence or absence of this mutation, which have been time consuming and labor intensive, the amplified products are identified from the melting peak, allowing more reliable distinction between genotypes. In this study we used a Lightcycler (Roche) quantitative PCR machine, which has been available for many years, with the cost continually decreasing and is now widely accessible. The assay could also be easily adapted to other widely available quantitative PCR platforms. Now that the design fee has been covered the reagent costs for this assay per sample are much reduced. Previous studies used denaturing HPLC [11] and Allele specific oligo hybridization (ASO) to detect the mutation. dHPLC requires specialized apparatus (Transgenomics) and the ASO method involves laborious labelling and hybridization procedures. Direct sequencing [16] has the advantage of providing more data but takes longer and is more expensive.

Exclusive segregation of Gln5345Arg with the disease haplotype in the family studied by Schultz et al. [15], amino acid conservation of glutamine at that position among mammals, the non-conservative nature of the substitution and similarities to EFEMP1 provide convincing evidence that HEMICENTIN-1 is the ARMD1 gene in the family studied. Previous reports have detected Gln5345Arg at a low frequency (0.4%) [15] or not at all [16,17] among AMD affecteds. Among the 696 subjects tested we detected Gln5345Arg in one of 508 AMD affects and in none of the 41 AMD free normal samples, 122 other normal samples or 25 possibly affected sib-pairs. The frequency of the Gln5345Arg mutation among Northern Irish AMD patients (0.2%) therefore concurs with previous findings in other populations. Of the ten affected individuals examined by Schultz et al. [15], all had large drusen and six had advanced AMD. Although five of the six individuals with advanced AMD had advanced dry AMD (geographic atrophy),

![Figure 2. Differentiation of mutant and wild type alleles following PCR and melting curve analysis. The peaks indicate the temperatures at which rapid decreases in fluorescence occur. These represent the melting temperatures of the sensor probes for the wild type (51 °C) and mutant alleles (60 °C). As the probes melt off their target they are no longer adjacent to the anchor probe, Fluorescence Resonance Energy Transfer (FRET) can therefore no longer take place and the fluorescence emitted drops. The red line is a homozygous wild type sample with a large peak at the melting temperature of the wild type probe. The blue line is a heterozygote, with a decreased wild type peak and an additional peak indicating presence of the mutant allele.](image)

![Figure 3. Clinical phenotype of AMD patient with hemicentin Gln5345Arg mutation. Red free image of right eye with a disciform scar and significant lens opacities (A) and left eye with a subfoveal choroidal neovascular membrane (B). Fluorescein angiogram of left eye at 25 s with early filling of a subfoveal choroidal neovascular membrane (C), 57 s (D), and 3 min (E). Late stage fluorescein of the right eye with a disciform scar (F).](image)
one had wet AMD (pigment epithelial detachments), consistent with the phenotype of the AMD patient with the Gln5345Arg mutation discovered in this study. The 122 normal samples and the 25 possibly affected sib-pairs were not examined and were assumed to have the same rate of early AMD changes as that expected within the general population. The 41 AMD free normal samples (of age 85 and above) were investigated ophthalmologically and showed no AMD. They therefore represent a valuable control population and sequence variations among these samples are unlikely to be involved in causing AMD. Although the number is too small to draw conclusions from the lack of Gln5345Arg in this group, its presence would have questioned its role in AMD.

Initial estimates of the ARMD1 locus suggested that it accounts for between 7-15% [13] and 41% [12] of all AMD cases. The discrepancy between the observed frequency of the Gln5345Arg mutation and the estimates of the number of families linked to this region suggests that other variants which are more commonly associated with AMD may exist in HEMICENTIN-1 or another gene located nearby on 1q31. This possibility is supported by SNP analysis of the HEMICENTIN-1 gene [17].

The highly conserved glutamine at codon position 5345 suggests that it may have an important functional role. This glutamine to arginine variation changes both the size and charge of the amino acid side chain within a very highly conserved calcium-binding EGF-like (cbEGF) domain, likely to lead to a reduction or disruption of the domain function. Similar mutations within cbEGF-like domains have been previously reported in association with other retinal degenerative disorders. These include EFEMP1 (Arg345Trp) which causes Malattia Leventinese and Doyne honeycomb retinal dystrophy [19], two diseases phenotypically similar to AMD and CRB1 (Ala161Val) causing retinitis pigmentosa and (Val1162Met) associated with pigmented paravenous chorioretinal atrophy [20].

In conclusion, the LightCycler is a good system for analyzing SNP variation in a reliable and fast way. Sample handling time and the possibility of carry-over contamination is minimized, an important feature in high throughput routine testing. Its main advantages are: the short time necessary for the analysis, the robustness and accuracy of the method and especially the sensitivity, which allows the detection of extremely low concentrations of DNA. The rapid diagnostic assay described in this paper has enabled us to screen for Gln5345Arg in 696 samples and its detection in an AMD affected patient is consistent with this variant having a role in AMD, although this possibility remains uncertain. This assay will help to resolve this question by providing the scientific community with a reliable and convenient method to evaluate the frequency of Gln5345Arg within other AMD and unaffected populations and therefore establish its association with AMD.

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