Comparison of High-Resolution Melting Analysis with Denaturing High-Performance Liquid Chromatography for Mutation Scanning in the *ABCA4* Gene

Jana Aguirre-Lamban,[1,2] *Rosa Riveiro-Alvarez,*[1,2] *Maria Garcia-Hoyos,*[1,2] *Diego Cantalapiedra,*[1,2] *Almudena Avila-Fernandez,*[1,2] *Cristina Villaverde-Montero,*[1,2] *Maria Jose Trujillo-Tiebas,*[1,2] *Carmen Ramos,*[1,2] *and Carmen Ayuso*[1,2]

Purpose. Mutations in the *ABCA4* gene have been associated with autosomal recessive Stargardt disease (STGD), a few cases of autosomal recessive cone-rod dystrophy (arCRD), and autosomal recessive retinitis pigmentosa (arRP). The purpose of this study was to compare high-resolution melting (HRM) analysis with denaturing high-performance liquid chromatography (dHPLC), to evaluate the efficiency of the different screening methodologies.

Methods. Thirty-eight STGD, 15 arCRD, and 5 arRP unrelated Spanish patients who had been analyzed with the ABCR microarray were evaluated. The results were confirmed by direct sequencing. In patients with either no or only one mutant allele, *ABCA4* was further analyzed by HRM and dHPLC. Haplotype analysis was also performed.

Results. In a previous microarray analysis, 37 *ABCA4* variants (37/116; 31.9%) were found. dHPLC and HRM scanning identified 18 different genotypes in 20 samples. Of the samples studied, 19/20 were identified correctly by HRM and 16/20 by dHPLC. One homozygous mutation was not detected by dHPLC; however, the p.Cys2137Tyr homozygote was distinguished from the wild-type by HRM technique. In the same way, one novel change in exon 5 (p.Arg187His) was found only by means of the HRM technique. In addition, dHPLC identified the mutation p.Trp1724Cys in one sample; however, HRM detected the mutation in two samples.

Conclusions. *ABCA4* should be analyzed by an optimal screening technique, to perform further characterization of pathologic alleles. The results seemed to show that HRM had better sensitivity and specificity than did dHPLC, with the advantage that some homozygous sequence alterations were identifiable. (*Invest Ophthalmol Vis Sci.* 2010;51:2615–2619) DOI:10.1167/iovs.09-4518

From the [1]Genetics Department, Fundacion Jimenez Diaz, Madrid, Spain; and [2]Centro de Investigacion Biomedica en Red de Enfermedades Raras (CIBERER), ISCIII (Instituto de Salud Carlos III), Madrid, Spain.

Supported by FIS (Sanitary Research Fund) Grants 06/0027 and 07/90285 and CIBERER (06/07/0036). JA-L was supported by FIS Grant PI06/0027 from the Instituto de Salud Carlos III.

Submitted for publication August 20, 2009; revised October 26, 2009; accepted October 29, 2009.

Disclosure: **J. Aguirre-Lamban**, None; **R. Riveiro-Alvarez**, None; **M. Garcia-Hoyos**, None; **D. Cantalapiedra**, None; **A. Avila-Fernandez**, None; **C. Villaverde-Montero**, None; **M.J. Trujillo-Tiebas**, None; **C. Ramos**, None; **C. Ayuso**, None

Corresponding author: Jana Aguirre-Lamban, Fundacion Jimenez Diaz, Genetics Department, Reyes Catolicos 2, 28.040 Madrid, Spain; jaguirre@fjd.es.

Stargardt disease (STGD, MIM [Mendelian Inheritance in Man; National Institutes of Health, Bethesda, MD] 248200;) is the most common hereditary macular dystrophy, with an estimated prevalence of 1:10,000.[1] It is characterized by onset in the juvenile to young adult years, decreased central vision, progressive bilateral atrophy of the retinal pigment epithelium (RPE), and the appearance of orange-yellow flecks around the macula and/or midperiphery of the retina.[2]

The locus for recessive STGD was mapped to the short arm of chromosome 1 (1p21-p13).[3] Mutations in *ABCA4* have been described in several inherited retinal disorders, including autosomal recessive STGD (arSTGD),[4] autosomal recessive retinitis pigmentosa (arRP),[5] autosomal recessive cone–rod dystrophy (arCRD),[6] and age-related macular degeneration (AMD).[7]

Up to now, ~500 disease-causing mutations have been identified in *ABCA4*. The mutation spectrum ranges from single-base substitutions to deletions of several exons, although most of the reported changes are missense mutations.

Despite all the efforts of many research teams, there is a variable and often unsatisfactory mutation detection rate, and many mutations remain undiscovered. Therefore, new mutation detection methods or the combination of technical approaches, together with the knowledge of the genetic background of a given population, are important for the determination of novel mutations and for the genetic characterization of these patients.

A subset of Spanish patients previously tested for variants on the ABCR genotyping microarray described by Jaakson et al.[8] and updated over the years, in whom none or only one allele could be identified, were selected for further analysis. In a previously study, dHPLC was used, and 12 novel mutations were identified. Moreover, the mutation detection was increased in 21%.[9]

In this study, we compared the denaturing high-pressure liquid chromatography (dHPLC) and high-resolution melting analysis (HRM) techniques, to evaluate the specificity, sensitivity, and efficiency of the two methodologies and to determinate the technique that would allow us to further analyze the *ABCA4* gene in cases of STGD, arCRD, and arRP.

Methods

Recruitment of Subjects

STGD, arCRD, and arRP patient groups were prestudied. This molecular study was reviewed and approved by the ethics committee of the hospital (Fundacion Jimenez Diaz) and adhered to the tenets of the Declaration of Helsinki. Informed consent was obtained from all patients after the nature of the procedures to be performed was explained fully.

Clinical Evaluation

Ophthalmic and electrophysiological examinations were performed according to preexisting protocols. Examinations included the history

of the patient and his or her family, visual acuity testing with the best correction, central and peripheral visual field testing, fluorescein angiography, electro-oculography, and electroretinography, incorporating the protocols recommended for vision testing by the International Society for Clinical Electrophysiology of Vision and Color.[10,11] Diagnosis of STGD, arCRD, and arRP were based on the following criteria: (1) Diagnosis of STGD was determined according to a history of a recessive mode of inheritance; bilateral central vision loss with a beaten-bronze appearance and/or the presence of orange-yellow flecks in the retina from the posterior pole to the mid periphery; a typical dark choroid observed by fluorescein angiography; and normal-to-subnormal electroretinograms (ERGs). (2) Diagnosis of CRD was based on initial reports of blurred central vision without a history of night blindness, poor visual acuity, impairment of color vision, funduscopic evidence of atrophic macular degeneration, peripheral disturbances including pigment clumping and/or pigment epithelial thinning, and greater or earlier loss in cone than in rod ERG amplitude. (3) RP was diagnosed in patients who developed night blindness early in life, peripheral vision loss, pigmentary retinal degeneration and markedly reduced scotopic ERGs.

Molecular Methods

DNA Extraction. Peripheral blood samples were obtained, and genomic DNA was extracted with an automated DNA extractor (BioRobot EZ1; Qiagen, Hilden, Germany).

Genotyping Microarray. DNA samples from the selected 58 patients had been screened for variants on the ABCR microarray (Asper Biotech, Tartu, Estonia) over the past 7 years.[12] The 50 exons of the *ABCA4* gene, including the intron–exon junctions, were amplified by using previously described PCR primers.[13]

Direct Sequencing. The sequencing reaction was performed with dye-termination DNA sequencing (Big-dye Terminator Kit; Applied Biosystems, Inc. [ABI], Foster City, CA) with sequence product resolution (Prism 3130; ABI).

Denaturing High-Performance Liquid Chromatography. dHPLC sample screening was performed on a fragment-analysis system (WAVE; Transgenomic Inc., San Jose, CA). The PCR amplicons were loaded (5 μL) on a C_{18} reserved-phase column based on nonporous poly (styrene/divinyl-benzene) particles (DNA Sep column; Transgenomic, Inc.). Hetero- and homodimer analyses were performed with an acetonitrile gradient formed by mixing buffers A and B (Wave Optimized; Transgenomic, Inc.). The optimized program gradients used to elute the different amplicons were those calculated by the system program (Wave Maker, ver. 4.1; Transgenomic, Inc.). The flow rate was 0.9 mL/min, and DNA was detected at 260 nm. A chromatographic run lasted 8 minutes, including the steps for washing and equilibrating the column.

Because dHPLC does not usually differentiate the wild-type from the homozygous mutant samples, all unknown samples were analyzed both singularly and mixed in a 1:1 proportion with a wild-type sample at the end of each PCR session and before heteroduplex formation.

High-Resolution Melting Analysis. Melting curve analysis with the high-resolution melting instrument (LightCycler480; Roche Applied Science, Mannheim, Germany) was a sensitive and specific tool for the detection of variations in DNA.

PCR amplification mixture included 2.0 mM $MgCl_2$, 2× 10 μL master mix, 5.4 μL H_2O (from the Reaction Mix Kit; Roche Applied Science), 0.2 mM primers. We added 25 ng DNA template. PCR amplification began with the first denaturation step at 95°C for 10 minutes, followed by 30 cycles of denaturation at 95°C for 20 seconds, annealing at temperature corresponding to each exon for 20 seconds, and extension at 72°C for 40 seconds. After amplification, a program for melting curve generation was performed at 95°C for 1 minute, 40°C for 1 minute, and 65°C for 1 second followed by a cooling program at 40°C for 30 seconds.

Haplotype Analysis. Haplotypes were constructed by using three microsatellite markers flanking the *ABCA4* gene (TEL-*D1S435-D1S2804-ABCA4-D1S236*-CEN). After amplification by PCR, fluorescence-labeled products were mixed and electrophoresed (Prism 3130; ABI). For haplotype reconstruction, an informatic program was used (Cyrillic ver. 2.1; Cyrillic Software, Wallingford, UK).

Results

Further Analysis of Partially Characterized Families with dHPLC and HRM

A total of 58 Spanish patients, who had been tested for mutations with the ABCR microarray as a first approach, were further analyzed with two different techniques: dHPLC and HRM. In 37 of 58 patients, one disease-associated allele was detected by the genotyping microarray and in the remaining cases, none variant was detected by the chip (21/58 patients).

The melting curves and dHPLC traces of the amplified samples were compared with wild-type samples, to assess the sensitivity and specificity of each method.

In 20 patients (19 STGD and 1 CRD), 18 different genotypes were identified (Table 1). Except p.Arg187His and p.Tyr954Ser variants, the rest of changes were previously reported as disease-associated allele.[14] The p.Arg187His and p.Tyr954Ser variants were not found in 100 ethnically matched control chromosomes. All the mutations identified in the 20 patients except one were detected by HRM for sensitivity of 95%; however, only 16 variants were identified by dHPLC for sensitivity of 80%.

Homozygous sequence alteration (p.Cys2137Tyr) could be identified from wild-type by HRM analyses (Fig. 1). In contrast, dHPLC did not distinguish any homozygous mutation, except when we mixed it, in a 1:1 proportion, with a previously sequenced wild-type sample at the end of each PCR session and before heteroduplex formation.

The exon 5 included one novel heterozygous single-nucleotide substitution (p.Arg187His) identified in two STGD patients. The amplicon melted in two domains: one between 75°C and 80°C and another between 83°C and 88°C. The melting curves of both samples in both domains were highly reproducible (data not shown). The normalized and shifted melting curve of the novel mutation was different from the wild-type control sample (Fig. 2A).

The dHPLC traces for exon 5 were best resolved at 55.8°C and 57.5°C after empiric optimization (Table 2). The p.Arg187His trace was similar to the control sample, resulting in a false-negative result for dHPLC (Fig. 2B).

Exon 36 included the p.Trp1724Cys mutation detected in two STGD patients. The HRM technique was able to identify the variant in both patients, but the dHPLC technique detected the mutation in only one patient. The exon 36 PCR products resolved well when the melting points (T_ms) predicted by the commercial software were used (Table 2).

Exon 8 was amplified as a longer PCR amplicon (400 bp) and included one frame-shift variant caused by insertion of one nucleotide (c.1029_1030insT). In this case, the melting curve of the patient sample was similar to that of the control sample (Fig. 3A); however, the dHPLC trace for exon 8 showed multiple peaks, whereas the wild-type control sample had a single peak (Fig. 3B). Therefore, we obtained a false-negative result for HRM.

Additional previously described mutations, which have not yet been included in the gene chip (Asper Biotech), were detected easily by HRM and dHPLC scanning without false-negatives or -positives. The p.Tyr954Ser variant had not been reported and was not found in 100 ethnically matched control chromosomes. Segregation analyses were performed in all families in which samples from additional family members were available.

TABLE 1. Mutations Analyzed

Family	Exon	Genotype		Mutation Detected by dHPLC	Mutation Detected by HRM
		Nucleotide Change	Amino Acid Change		
ARDM-167	**5**	**c.560G>A**	**p.Arg187His**	No	Yes
ARDM-257	**5**	**c.560G>A**	**p.Arg187His**	No	Yes
ARDM-164	6	c.700C>T	p.Gln234X	Yes	Yes
ARDM-135	8	c.1029_1030insT	p.Asn344fsX	Yes	No
ARDM-240	15	c.2285C>A	p.Ala762Glu	Yes	Yes
ARDM-248	**19**	**c.2861A>C**	**p.Tyr954Ser**	Yes	Yes
ARDM-90	—	IVS21-2A>T	—	Yes	Yes
ARDM-40	27	c.3943C>T	p.Gln1315X	Yes	Yes
ARDM-158	30	c.4537delC	p.Gln1513fsX1525	Yes	Yes
ARDM-38	33	c.4739delT	p.Leu1580fs	Yes	Yes
ARDM-163	36	c.5172G>T	p.Trp1724Cys	Not	Yes
ARDM-197	36	c.5172G>T	p.Trp1724Cys	Yes	Yes
ARDM-181	—	IVS38+5G>A	—	Yes	Yes
ARDM-125	40	—	p.KNLFA1876dup	Yes	Yes
ARDM-183	43	c.5929G>A(False−)	p.Gly1977Ser(False−)	Yes	Yes
ARDM-146	44	c.6140T>A	p.Ile2047Asn	Yes	Yes
ARDM-174	—	IVS44+2T>A	—	Yes	Yes
ARDM-247	47	c.6410G>A	p.Cys2137Tyr*	Yes	Yes
ARDM-84	47	c.6410G>A	p.Cys2137Tyr†	No	Yes
ARDM-225	48	c.6559C>T	p.Gln2187X	Yes	Yes

Previously unreported mutations are shown in bold.
* Mutation in heterozygous.
† Mutation in homozygous.

Evaluation of the Efficiency of Different Methodologies

In this study, the sensitivity of HRM was 95%, and so this tool detected all the mutations except one in the 20 patients. However, the sensitivity of dHPLC was 80%, and it identified only 16 variants. The specificity was 100% for both methodologies because false-positives were not present in our study.

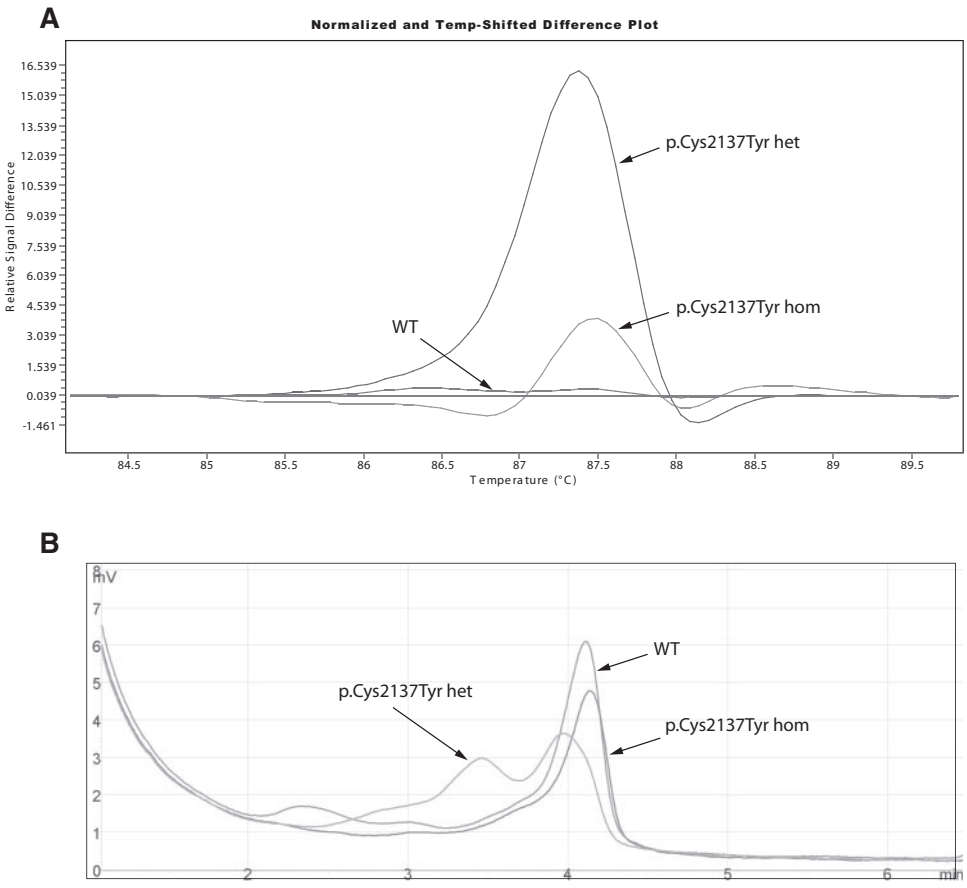

FIGURE 1. HRM and denaturing dHPLC analysis of exon 47 of the *ABCA4* gene. (**A**) Melting curves for two wild-type (WT), one p.Cys2137Tyr heterozygous (het), and one p.Cys2137Tyr homozygous (hom) sample. (**B**) dHPLC profile of the heterozygous (het) p.Cys2137Tyr, the homozygous (hom) p.Cys2137Tyr, and the WT control sample.

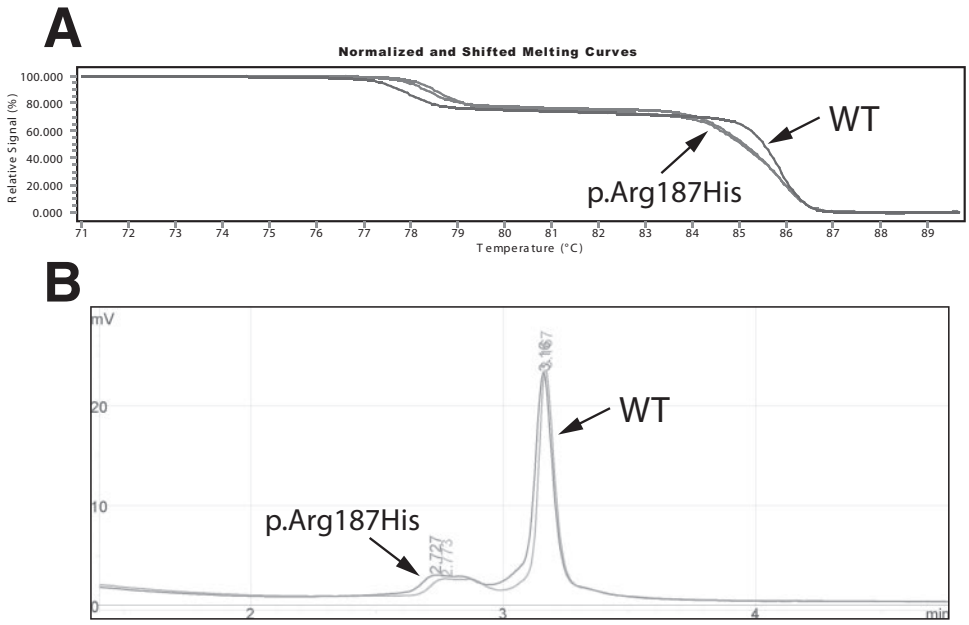

FIGURE 2. HRM and dHPLC analysis of exon 5 of the *ABCA4* gene. (**A**) Melting data normalized and temperature shifted shown 2 p.Arg187His heterozygote (het), and 1 wild-type (WT) sample. (**B**) The dHPLC profile of the heterozygous (het) p.Arg187His and the WT control sample.

DISCUSSION

Fifty-eight Spanish families were analyzed for mutations in *ABCA4*. This gene encodes the ABCA4 protein, a member of the ATP-binding cassette (ABC) transport superfamily. It is involved in the transport of vitamin A derivates across the membrane of the outer segment discs of photoreceptors.[14,15]

In this study, we compared two screening methodologies: dHPLC and HRM analysis, to determinate which would allow us to further analyze the *ABCA4* gene in STGD, arCRD, and arRP cases with one disease-associated allele or no variant, previously analyzed with the ABCR microarray, a reliable tool for detecting reported mutations.

Recently, HRM analysis with saturating DNA dyes has been proposed as a scanning tool to detect variants in the DNA sequence. It is considered a new tool for genotyping. We provide the first comparison of HRM analysis with dHPLC, one of the most used platforms for mutation scanning, and it has been used to scan the *ABCA4* gene.[9,16]

In this work, all sequence alterations were detected by HRM analysis except c.1029_1030insT (exon 8), and so the sensitivity of this tool was 95% for our data set. The false-negative detected was in exon 8, a PCR product of 400 bp. In a previous study, all the errors were made with PCR products 400 bp or larger, suggesting a dependence on product length.[17]

In exon 5, HRM analysis detected a previously unreported mutation (p.Arg187His) in two STGD patients. In these cases, the PCR product was 220 bp in length, and the melting curve showed two melting domains. When fragments contain more melting domains, chances increase that not all variants will be detected.[18] In this case, a difference was observed between the control sample and mutated samples in both domains.

Previous analysis by dHPLC of the exon 5 did not allow the detection of the p.Arg187His mutation. Therefore, multiple column temperatures may be needed to detect new heterozygous sequence alterations.

The p.Trp1724Cys mutation was identified in two STGD patients. HRM analyses detected the variant in both patients; however, dHPLC scanning identified the variant only in one patient. High-resolution instruments were necessary because, in this case, the resolution of the dHPLC technique is limited. The p.Tyr954Ser variant was not found in 100 ethnically matched control chromosomes, suggesting the presence of a disease-associated allele.

Although HRM analysis is similar to dHPLC, in that both methods identify heteroduplexes, melting analysis scans through a range of temperatures. In contrast, dHPLC depends on a specific temperature that requires optimization.[19]

It generally is accepted that routine dHPLC scanning does not detect homozygous sequence alterations, but the situation for melting analyses is less clear.[20] In this study, a homozygous mutation (p.Cys2137Tyr) was detected by HRM.

All the results were confirmed by direct sequencing, which is the only method confirmed as the gold standard for clinical sample testing. False positives were not detected in our data set, and so the specificity was 100% in both methods. However, the sensitivity of dHPLC was 80%, and the sensitivity of HRM was higher (95%). Therefore, the HRM analysis had a

TABLE 2. Parameters Used in dHPLC

Exon	Amplicon (bp)	Volume (μL)	Predicted Melting Temperature (°C)			Optimized Melting Temperature (°C)		Flow Rate (mL/min)	Buffer B (%)	Time Shift
5	220	5	55.8	56.8	57.8	55.8	57.5	0.90	51.7	0.0
8	400	5	55.8	56.8	57.8	57.0	57.7	0.90	58.6	0.0
36	303	5	60.7	61.7	62.7	61.0	61.7	0.90	54.8	0.0
47	219	5	61.4	62.4	63.4	60.4	62.4	0.90	51.8	0.0

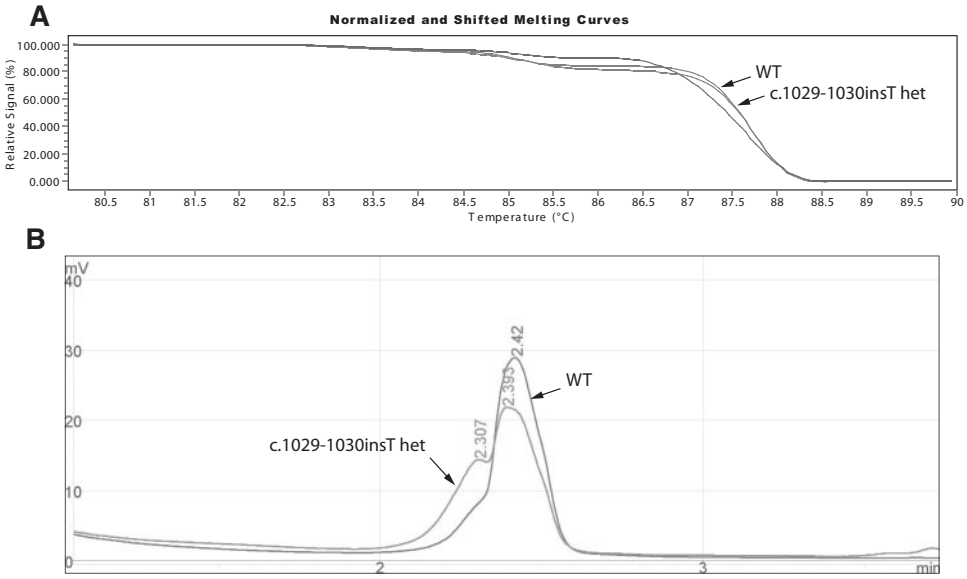

FIGURE 3. HRM and dHPLC analysis of exon 8 of the *ABCA4* gene. (**A**) Melting data normalized and temperature shifted shown 1 c.1029-1030insT heterozygous (het) and 1 wild-type (WT) sample. (**B**) the dHPLC profile of the heterozygous (het) c.1029-1030insT and the WT control sample.

higher resolving power than dHPLC, perhaps ascribable to more refined temperature control in the HRM system.

Our results further establish the HRM approach as more effective than the dHPLC technique. Moreover, the HRM analysis is a rapid (1–2 minutes) closed-tube method, requires no processing or automation, and can examine 96 samples (in a 96-well plate format) 1.5 hours. However, 8.0 min/sample is necessary for dHPLC.

Although, both screening techniques had false-negatives among the samples tested, melting analysis provides a convenient way to detect new mutations in a large gene, such as *ABCA4*, without performing full gene sequencing. However, more studies are needed to define the limits of HRM for mutation screening.

We propose that *ABCA4* should be analyzed by optimal combination of high-throughput screening techniques: microarray first, HRM second, and finally, if none or one mutation is identified by the first two screenings, direct sequencing.

Acknowledgments

The authors thank all patients and control subjects for participating in the study.

References

1. Blacharski PA. Fundus flavimaculatus. Newsome DA, eds. *Retinal Dystrophies and Degenerations*. New York: Raven Press; 1988; 135–159.
2. Stargardt K. Uber familiäre, progressive degeneration in der makulagegend des auges. *Graefes Arch Ophthalmol.* 1909;71:534–550.
3. Kaplan J, Gerber S, Larget-Piet D, et al. A gene for Stargardt's disease (fundus flavimaculatus) maps to the short arm of chromosome 1. *Nat Genet.* 1994;6:214.
4. Allikmets R, Singh N, Sun H, et al. A photoreceptor cell-specific ATP-binding transporter gene (ABCR) is mutated in recessive Stargardt macular dystrophy. *Nat Genet.* 1997;15:236–246.
5. Martínez-Mir A, Paloma E, Allikmets R, et al. Retinitis pigmentosa caused by a homozygous mutation in the Stargardt disease gene ABCR. *Nat Genet.* 1998;18:11–12.
6. Cremers FP, van de Pol DJ, van Driel M, et al. Autosomal recessive retinitis pigmentosa and cone-rod dystrophy caused by splice site mutations in the Stargardt's disease gene ABCR. *Hum Mol Genet.* 1998;7:355–362.
7. Allikmets R, Shroyer NF, Singh N, et al. Mutation of the Stargardt disease gene (ABCR) in age-related macular degeneration. *Science.* 1997;277:1805–1807.
8. Jaakson K, Zernant J, Külm M, et al. Genotyping microarray (gene chip) for the ABCR (ABCA4) gene. *Hum Mutat.* 2003;22: 395–403.
9. Aguirre-Lamban J, Riveiro-Alvarez R, Maia-Lopes S, et al. Molecular analysis of the ABCA4 gene for reliable detection of allelic variations in Spanish patients: identification of 21 novel variants. *Br J Ophthalmol.* 2009;93(5):614–621.
10. Marmor MF, Zrenner E. Standard for clinical electro-oculography. International Society for Clinical Electrophysiology of Vision. *Arch Ophthalmol.* 1993;111(5):601–604.
11. Marmor MF, Zrenner E. Standard for clinical electroretinography. International Society for Clinical Electrophysiology of Vision. *Doc Ophthalmol.* 1998–1999;97(2):143–156.
12. Valverde D, Riveiro-Alvarez R, Bernal S, et al. Microarray-based mutation analysis of the ABCA4 gene in Spanish patients with Stargardt disease: evidence of a prevalent mutated allele. *Mol Vis.* 2006;12:902–928.
13. Rivera A, White K, Stöhr H, et al. A comprehensive survey of sequence variation in the ABCA4 (ABCR) gene in Stargardt disease and age-related macular degeneration. *Am J Hum Genet*. 2000;67: 800–813.
14. Sun H, Molday RS, Nathans J. Retinal stimulates ATP hydrolysis by purified and reconstituted ABCR, the photoreceptor-specific ATP-binding cassette transporter responsible for Stargardt disease. *J Biol Chem.* 1999;274:8269–8281.
15. Weng J, Mata NL, Azarian SM, Tzekov RT, Birch DG, Travis GH. Insights into the function of Rim protein in photoreceptors and etiology of Stargardt's disease from the phenotype in abcr knockout mice. *Cell.* 1999;98:13–23.
16. Maia-Lopes S, Aguirre-Lamban J, Castelo-Branco M, et al. ABCA4 mutations in Portuguese Stargardt patients: identification of new mutations and their phenotypic analysis. *Mol Vis.* 2009;15:584–591.
17. Gudrun HR, Wittwer CT. Sensitivity and specificity of single-nucleotide polymorphism scanning by high-resolution melting analysis. *Clin Chem.* 2004;50:1748–1754.
18. Vossen Rolf HAM, Aten E, Roos A, Dunnen JT. High-resolution melting analysis (HRMA): more than just sequence variant screening. *Hum Mutat.* 2009;30:1–7.
19. Chou LS, Lyon E, Wittwer CT. A comparison of high-resolution melting analysis with denaturing high-performance liquid chromatography for mutation scanning: cystic fibrosis transmembrane conductance regulator gene as a model. *Am J Clin Pathol.* 2005; 124:330–338.
20. Liew M, Pryor R, Palais R, et al. Genotyping of single-nucleotide polymorphism by high-resolution melting of small amplicons. *Clin Chem.* 2004;50:1156–1164.