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Denaturing HPLC Profiling of the *ABCA4* Gene for Reliable Detection of Allelic Variations

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Background: **Mutations in the retina-specific ABC transporter (*ABCA4*) gene have been associated with several forms of macular degenerations. Because the high complexity of the molecular genotype makes scanning of the *ABCA4* gene cumbersome, we describe here the first use of denaturing HPLC (DHPLC) to screen for *ABCA4* mutations.**

Methods: **Temperature conditions were designed for all 50 exons based on effective separation of 83 samples carrying 86 sequence variations and 19 mutagenized controls. For validation, samples from 23 previously characterized Stargardt patients were subjected to DHPLC profiling. Subsequently, samples from a cohort of 30 patients affected by various forms of macular degeneration were subjected to DHPLC scanning under the same conditions.**

Results: **DHPLC profiling not only identified all 132 sequence alterations previously detected by double-gradient denaturing gradient gel electrophoresis but also identified 5 sequence alterations that this approach had missed. Moreover, DHPLC scanning of an additional panel of 30 previously untested patients led to the identification of 26 different mutations and 29 polymorphisms, accounting for 203 sequence variations on 29 of the 30 patients screened. In total, the DHPLC approach allowed us to identify 16 mutations that had never been reported before.**

Conclusions: **These results provide strong support for the use of DHPLC for molecular characterization of the *ABCA4* gene.**

Mutations in the retina-specific ABC transporter (*ABCA4*)[7] gene (MIM 601691; GDB 370748; GenBank accession no. U88667) have been found to be associated with autosomal recessive Stargardt/fundus flavimaculatus disease (STGD; MIM 248200), cone-rod dystrophy, and age-related macular degeneration. Only a restricted number of recurrent mutations have been reported to date, and the majority are rare or private, leading to broad allelic heterogeneity. Because of the high frequency of polymorphisms, which add to the complexity of the molecular genotype, and the relatively large size of the gene, which contains 50 exons, molecular scanning of the *ABCA4* gene is particularly cumbersome. In the past we developed double-gradient denaturing gradient gel electrophoresis (DG-DGGE) conditions to screen the entire coding region of the gene. Although this is one of the most powerful techniques for mutational screening, it is also time-consuming and labor-intensive. The aim of this project was to develop conditions for rapid, automated mutational scanning of the *ABCA4* gene through denaturing HPLC (DHPLC) *(1, 2)*, which is more sensitive and less labor-intensive than the DGGE technique. DHPLC is a well-established mutation detection system with 96–100% sensitivity and specificity in molecular scanning *(3)*. In the present study we established DHPLC analytical conditions to screen for *ABCA4* variants based on theoretical parameters predicted by the dedicated software

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[7] Nonstandard abbreviations: *ABCA4*, retina-specific ABC transporter gene; STGD, Stargardt disease; DG-DGGE, double-gradient denaturing gradient gel electrophoresis; and DHPLC, denaturing HPLC.

and experimental optimization performed against a panel of positive controls, either naturally occurring or artificially created.

Materials and Methods

PATIENTS

Patients were selected from those attending the Department of Ophthalmology and Visual Sciences of the University Hospital San Raffaele, Milan, Italy. This patient sample represents the entire Italian population because it originates from different regions of northern, central, and southern Italy.

A panel of 83 DNA samples carrying 86 known sequence variations previously identified by DG-DGGE or direct sequencing were used to establish experimental DHPLC conditions (see Table 1). For validation of the DHPLC assay, we subjected samples from 23 STGD patients identified through our previous mutational screening *(4)* as carrying 40 different mutations and polymorphisms, for a total of 132 sequence alterations, to DHPLC profiling of the entire coding sequence of the *ABCA4* gene to assess the sensitivity and the efficiency of the DHPLC system in identifying *ABCA4* mutations compared with DG-DGGE.

Subsequently, we subjected samples from a cohort of 30 previously untested patients with various maculopathies, including STGD, fundus flavimaculatus disease, cone-rod dystrophy, and age-related macular degeneration, to DHPLC scanning under the same conditions. After receiving approval from the local ethics committee and informed consent from the patients, we extracted genomic DNA from 10 mL of EDTA-anticoagulated blood by standard phenol–chloroform procedures.

PCR

PCR conditions were optimized to obtain only highly specific amplicons because the presence of additional products could generate artifacts that might be erroneously interpreted as heteroduplexes in the DHPLC elution profile. Primers used for the amplification (without the GC-clamp) were the same as reported previously for DG-DGGE analysis for 36 exons *(4)*. New sets of primers were specifically designed for DHPLC scanning for the remaining exons (listed in Table 1 of the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol50/issue8/). Among these, primers for exons 7, 13, 39, and 40 were chosen to exclude some frequent intronic polymorphisms from the amplicon, and primers for exons 6, 8, 11, and 50 were specifically designed for DHPLC because conditions to analyze these exons could not be developed for DG-DGGE. PCR fragment length varied from 173 to 501 bp. PCR reactions were performed in 50 μL containing 100 ng of DNA, 200 μM each deoxynucleotide triphosphate, 1.5 mM $MgCl_2$, 15 pmol of each primer, and either 1.0 U of AmpliTaq Gold DNA Polymerase together with 1× PCR Buffer II (Applied Biosystems) or OPTIMASE™ Polymerase together with 1× Reaction Buffer (Transgenomic). Standard cycling conditions were as follows: denaturation at 95 °C for 5 min; 35 cycles of 95 °C for 30 s, annealing at the temperature specific for each set of primers for 30 s, and 72 °C for 30 s; and a final elongation step at 72 °C for 5 min. Touchdown protocols *(5)* were used in some cases, as follows: 95 °C for 10 min; 2 cycles of 95 °C for 30 s,

Table 1. Genotypes of previously obtained control samples containing mutated DNA.

Exon	Genotypes[a]	Exon	Genotypes[a]
1b	**M1V (1A>G)** *(11)*	24	3523-28T>C *(12)*
	R18W (52C>T) *(11)*	25	**G1203D (3608G>A)**[b]
3	**250_251insCAAA** *(7)*	27	**R1300X (3898C>T)** *(12)*
	N96K (288C>A)		**R1300Q (3899G>A)** *(11)*
	302 + 26 G>A *(13)*	28	P1380L (4139C>T) *(14)*
4	**P143L (428C>T)** *(10)*		P1401P (4203C>A) *(15)*
5	**R152Q (455G>A)** *(4)*		4253 + 43G>A *(12)*
6	571-1G>T *(4)*	29	4253 + 13G>A *(12)*
	R212H (635G>A) *(16)*		4354-38G>A *(4)*
	C230S (688T>A) *(12)*	30a	4466 + 3G>A *(4)*
	641delG *(9)*	30b	**C1490Y (4469G>A)** *(17)*
10	1240-14C>T *(13)*		**P1512R (4535C>G)** *(4)*
	H423R (1268>G) *(13)*	31	**T1526M (4577C>T)** *(14)*
	1357 + 11delG *(16)*	33/34	**A1598D (4793C>A)** *(4)*
	H423H (1269C>T) *(13)*	35	**4947delC** *(14)*
11	**1387delTT** *(4)*		**5018 + 2T>C** *(7)*
	R500R (1500G>A) *(4)*	39	**H1838Y (5512C>T)** *(14)*
12	**L541P (1622T>C)** *(14)*	40	N1868I (5603A>T) *(13)*
	R572Q (1715G>A) *(17)*		L1894L (5682G>C) *(15)*
13	**Y639X (1917C>G)** *(17)*		**5714 + 5G>A**
	C641S (1922G>C) *(4)*	41	L1938L (5814A>G) *(12)*
14	**R653C (1957C>T)** *(12)*	42	5836-43C>A
	W700X (2099G>A) *(4)*		5836-11G>A *(15)*
	3607 + 49T>C		P1948I (5843C>T) *(15)*
15	**V767D (2300T>A)** *(7)*		P1948P (5844A>G) *(15)*
16	**W821R (2461T>A)** *(14)*		**G1961E (5882G>A)** *(14)*
17	2588-33C>T[b]	43	**L1970F (5908C>T)** *(11)*
	G863A (2588G>C) *(17)*	44	6006-16A>G *(16)*
18	2654-36C>T *(4)*		I2023I (6069C>T) *(14)*
	T897I (2690C>T) *(7)*		**L2027F (6079C>T)** *(14)*
19	R943Q (2828G>A) *(13)*	45	**V2050L (6148G>C)** *(14)*
	Y954D (2860T>G) *(4)*	46	**R2107H (6320G>A)** *(18)*
	N965S (2894A>G) *(14)*		**6386 + 2G>C** *(10)*
20	**G978D (2933G>A)** *(4)*	47	**R2139W (6415C>T)** *(14)*
	L988L (2964C>T) *(4)*		**R2149L (6446G>T)** *(4)*
21	**E1022K (3064G>A)** *(4)*		**C2150Y (6449G>A)** *(19)*
	A1038V (3113C>T) *(14)*	48	**D2177N (6529G>A)** *(17)*
	G1050D (3149G>A) *(4)*		**L2241V (6721C>G)** *(12)*
	3211_3212insGT *(14)*		6729 + 21C>T *(15)*
22	**E1087K (3259G>A)** *(14)*	49	6730-3T>C *(15)*
	R1098C (3292C>T) *(12)*		S2255I (6764G>T) *(13)*
	S1099P (3295T>C) *(4)*		6816 + 28G>C *(4)*
	R1108C (3322C>T) *(14)*		
	R1129L (3386G>T) *(17)*		

[a] Bold indicates disease-causing mutations. References for previously published genotypes are given.

[b] Our unpublished results.

59 °C for 30 s, and 72 °C for 30 s; 2 cycles of 95 °C for 30 s, 57 °C for 30 s, and 72 °C for 30 s; 2 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s; 35 cycles of 95 °C for 30 s, 53 °C for 30 s, and 72 °C for 30 s; and a final step at 72 °C for 5 min (Table 2 in the online Data Supplement). Before DHPLC analysis, heteroduplexes were formed by denaturing the PCR product at 95 °C for 5 min and cooling it to 56 °C over 1 h.

DHPLC ANALYSIS

DHPLC sample screening *(3)* was performed on a WAVE™ DNA Fragment Analysis System (Transgenomic). The PCR amplicons were loaded (5 μL) on a C_{18} reversed-phase column based on nonporous poly(styrene/divinyl-benzene) particles (DnaSep™ column; Transgenomic). Hetero- and homodimer analysis was carried out with an acetonitrile gradient formed by mixing buffers A and B (WAVE Optimized™; Transgenomic). The optimized program gradients used to elute the different amplicons were those calculated by the WAVE MAKER program (Ver. 4.1; Transgenomic). The flow rate was 0.9 mL/min, and DNA was detected at 260 nm. A chromatographic run lasted 8.0 min, including the steps for washing and equilibrating the column. For each DNA region, DHPLC conditions were established by a titration analysis 1–3 °C above and below the mean melting temperature predicted by software simulation.

Because DHPLC does not usually differentiate the wild-type from the homozygous mutant sample, all unknown samples were analyzed both singularly and mixed in a 1:1 proportion with another unknown sample at the end of each PCR session and before heteroduplex formation. Mixing of samples with each other instead of with a wild-type sample was feasible because of the scarcity of homozygous mutated STGD patients [<3% in our population *(4)*], which makes the probability of mixing two samples homozygous for the same mutation low enough to be negligible. This strategy greatly shortens screening time while still allowing accurate investigation.

MUTAGENESIS

Because of the lack of naturally occurring mutations in exons 1a, 2, 7, 8, 9, 26, 37, 38, and 50, we generated mutagenized amplicons *(6)* for each of these DNA regions by introducing a conservative transversion (C↔ G or A↔ T) in the portion of the fragment displaying the higher melting profile. Mutagenized control samples were also designed for the high-melting domains of exons 1b, 3, 5, 13, 24, 25, 32, 33, 34, 45, and 49, where available variants were located within the lower melting domains. Mutagenesis was achieved by dividing each amplicon into two fragments. The 5′ fragment was then amplified with the original forward primer and a mutagenizing reverse primer introducing a conservative transversion. In parallel, the 3′ fragment was amplified with the original reverse primer and a mutagenized forward primer introducing the same nucleotide variation as above (see Table 3 in the online Data Supplement). This led to the production of two partially overlapping fragments, which were each gel-eluted in a final volume of 50–100 μL of distilled water to eliminate nonincorporated primers. We mixed 2 μL of each eluted solution together; each mixture was then elongated for 15 cycles in the presence of the PCR reaction mixture containing all reagents but primers. A 1- to 2-μL aliquot of the resulting full-length centrally mutagenized fragment was further PCR amplified for 20–30 cycles by addition of the complete PCR mixture, including the two original wild-type forward and reverse primers. Direct sequencing confirmed that the desired nucleotide change was introduced into the mutagenized control. After DHPLC analysis at 50 °C under nondenaturing conditions, all mutagenized and wild-type fragments were mixed in a 1:1 proportion based on the measurements of the areas corresponding to each elution peak to generate a perfectly matched artificial heterozygous control sample.

SEQUENCING

Direct sequencing was performed in both directions with use of the DYEnamic ET Terminator Cycle Sequencing reagent set (Amersham Pharmacia Biotech) and a ABI Prism 3100 Genetic Analyzer (PE Biosystems).

Results

The temperature for successful heteroduplex separation was selected starting from the mean melting temperature predicted by software simulation (Wavemaker). For each DNA fragment, under the optimum combination of denaturing temperature and gradient of elution buffers, all mutated or mutagenized control samples displayed altered elution profiles attributable to the presence of heteroduplex species with respect to the wild-type control. For 19 of 50 exons, the final temperatures chosen for analysis based on efficient heteroduplex separation in mutated or mutagenized control samples varied by 1–3 °C from the range predicted by the software. The availability of a mutagenized control sample located in a region with a higher melting temperature has also been useful for selecting the appropriate denaturing conditions for exons for which a natural mutated control sample located in a region with a lower melting temperature was available (Fig. 1A). This was particularly crucial in those cases in which the optimized denaturing temperature differed from that predicted by the software (Fig. 1B). Because of the presence of two melting domains, amplicons encompassing exons 1b and 33 + 34 required a double analysis under two different temperatures. The optimized elution profiles and melting temperatures of the entire coding sequence and flanking regions of the *ABCA4* gene are presented in Table 2 of the online Data Supplement.

Retrospective DHPLC analysis of samples from 23 patients identified all 21 mutations and 19 heterozygous polymorphisms (23 of which were also present in mutated control samples), for a total of 132 samples carrying

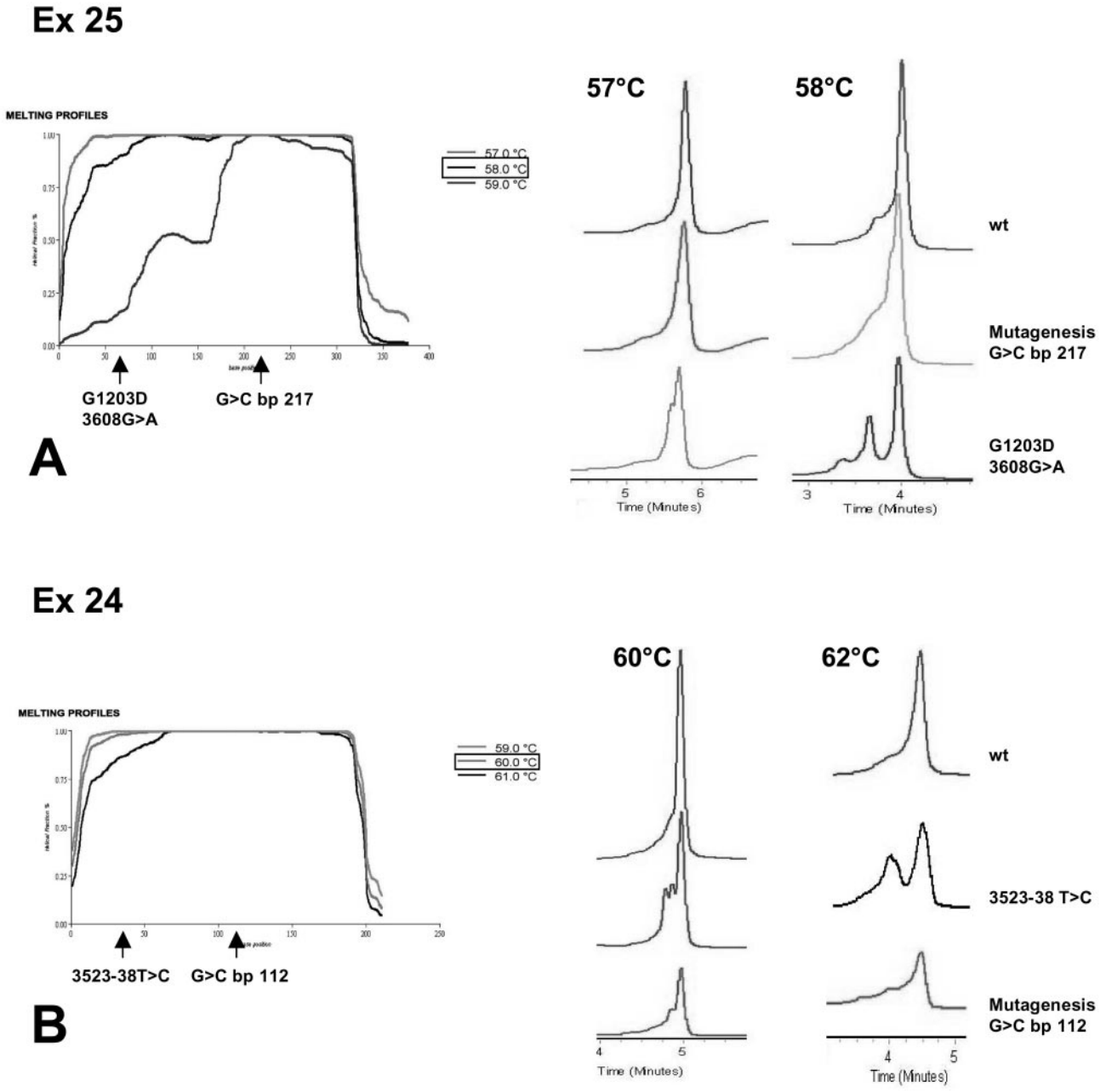

Fig. 1. Development of the optimized DHPLC conditions based on natural and mutagenized control samples.

(*A*), *left*, melting profile of exon 25 at a temperature range predicted by the software, displaying the locations of natural (*G1203D*) and mutagenized (*G>C bp 217*) control samples within the amplicon. The corresponding chromatograms (*right*) show that 58 °C is the optimum temperature to differentiate between the wild-type and both mutated profiles, whereas at 57 °C the mutagenized profile may be erroneously interpreted as wild type. (*B*), *left*, melting profile of exon 24 at a temperature range different from that predicted by the software (62–64 °C), displaying the location of natural (*3523-38 T>C*) and mutagenized (*G>C bp 112*) control samples within the amplicon. The profiles obtained at 60 °C (*center*) allow differentiation between the wild-type and both mutated samples, whereas at a higher temperature (62 °C; *right*) resolution of the mutagenized sample is worse.

sequence variations previously detected by DG-DGGE mutational scanning, which indicated complete concordance between results obtained by the two methods (Table 2). Among these mutations, three had not been reported previously: N96K (288C>A) in exon 3, T2253A (6757A>C) in exon 49, and a 9-bp deletion at position 6730-10 in intron 48. Additionally, DHPLC profiling singled out the presence of the mutations D108V (323A>T) in exon 4, V767D (2300T>A) in exon 15 *(7)*, R2030Q (6089G>A) in exon 44 *(8)*, and 6817-1G>A and a 3329-25G>A polymorphism in five STGD patients that DG-DGGE had failed to detect. Among these, the D108V mutation in exon 4 and the mutation 6817-1G>A in intron 49 had not been described previously.

Under the same experimental conditions, DHPLC identified 26 different mutations and 29 polymorphisms

Table 2. Genotypes and phenotypes of patients screened retrospectively for DHPLC validation, previously analyzed by DG-DGGE.[a]

Sample	Genotype[b]	Exon
A	No mutation found	
B	R2030Q (6089G>A)[c]	44
C	G1050D (3149G>A)	21
	6817–1G>A[c]	50
D	G978D (2933G>A)	20
	T1526M (4577C>T)	31
E	T1526M (4577C>T)	31
	A1598D (4793C>A)	34
F	G1961E (5882G>A)	42
G	P1512R (4535C>G)	30b
H	W821R (2461T>A)	16
	V767D (2300T>A)[c]	15
I	W821R (2461T>A)	16
J1[d]	Y1652X (4956T>G)	35
J2[d]	R2149T (6446G>T)	47
K1[d]	1358-8delTT	11
K2[d]	G1961E (5882G>A)	42
L	250_251insCAAA	3
	D108V (323A>T)[c]	4
M	No mutation found	
N	Y954D (2860T>G)	19
	G1050D (3149G>A)	21
O	R212H (635G>A)	6
	T230S (688T>A)	6
	E1022K (3064G>A)	21
P	5018 + 2T>C	35
Q	Y954D (2860T>G)	19
R	**N96K (288C>A)**	3
	A1598D (4793C>A)	34
S	No mutation found	
T	**T2253A (6757A>C)**	49
	6730-10del9	49
U	Q876X (2626C>T)	17
V	No mutation found	
Z	5714 + 5G>A	40
	R1108C (3322C>T)	22

[a] The 50 *ABCA4* exons were analyzed in a total of 23 DNA samples.
[b] Bold indicates mutations identified in this study.
[c] Mutation that DG-DGGE analysis failed to detect.
[d] Because it was not possible to amplify samples J1 and K1 for the whole *ABCA4* gene because of the long period of time since they had been collected, they were replaced by samples J2 and K2, which were analyzed for the remaining exons.

Table 3. Phenotypes and disease-causing mutations identified in patients not previously screened who were screened only by DHPLC.

Sample	Phenotype	Genotype[a]	Exon
1	AMD[b]		
2	STGD	**G690V (2069G>T)**	14
		5714 + 5G>A	40
3	STGD	R653C (1957C>T)	14
4	STGD		
5	FFM	Y1652X (4956T>G)	35
6	STGD	5714 + 5G>A	40
		6748delA	49
7	AMD		
8	STGD		
9	FFM	250_251insCAAA	3
10	STGD	P1380L (4139C>T)	28
11	STGD		
12	CD		
13	STGD	5714 + 5G>A	40
14	STGD	R18W (52C>T)	1b
		4466 + 3G>A	30a
		C1490Y (4469C>A)	30b
15	AMD		
16	STGD	**D498E (1494C>A)**	11
		3970delG	27
		5714 + 5G>A	40
17	STGD	**Q2187P (6560A>G)**	48
		W700X (2099G>A)	14
18	STGD		
19	AMD	D1204N (3610G>A)	25
20	STGD	R1108H (3323G>A)	22
		A1762D (5285C>A)	37
21	AMD		
22	STGD		
23	STGD	**4667 + 1G>A**	32
		H1838N (5512C>A)	39
24	STGD	R653C (1957C>T)	14
25	STGD	**K223Q (667A>C)**	6
		S1071L (3212C>T)	22
		G1203R (3607G>A)	24
26	STGD	G550R (1648G>A)	12
27	STGD	**A246T (736G>A)**	6
		G1961E (5882G>A)	42
28	STGD		
29	STGD		
30	STGD	**Y245X (735T>G)**	6

[a] Bold indicates mutations identified in this study.
[b] AMD, age-related macular degeneration; FFM, fundus flavimaculatus disease.

(37 of which were present on both mutated control samples and retrospectively analyzed patients), for a total of 203 samples carrying sequence variations that occurred in 29 of 30 patients not previously screened for different maculopathies. Patient genotypes and phenotypes are displayed in Table 3.

Eleven of these mutations—K223Q (667A>C), A246T (736G>A), and Y245X (735T>G) in exon 6; D498E (1494C>A) in exon 11; G690V (2069G>T) in exon 14; G1203R (3607G>A) in exon 24; 3970delG in exon 27; 4667 + 1G>A in exon 32; A1762D (5283C>A) in exon 37; H1838N (5512C>A) in exon 39; and Q2187P (6360A>G) in exon 48—had not been reported previously. DHPLC elution profiles and sequence analysis for all 16 novel mutations identified in this study are shown in Fig. 1 of the online Data Supplement. Because of the high frequency of polymorphisms in the *ABCA4* gene, some regions displayed altered chromatographic patterns. DH-

PLC analysis of exons 10, 40, 42, 45, 46, and 49 showed complex elution profiles that were generated by two or three polymorphisms occurring in almost all fragments. For these exons, the amplicon could not be placed differently on the gene to reduce the complexity and thus enhance the ease of interpretation. Some polymorphisms were located within the coding region (exons 10, 40, 45, and 46). Concerning the amplicon encompassing exons 42 and 49, exclusion of the farther intronic polymorphism would not have substantially reduced the complex profile of these regions because they contain additional exonic variations (see Table 4 in the online Data Supplement). Some examples of the complex elution pattern in amplicons featuring two or more single-nucleotide polymorphisms are shown in Fig. 2.

Discussion

The perspectives for molecular diagnostics of macular degeneration based on the identification of mutations in *ABCA4* are complicated by the large size of the gene and the marked allelic heterogeneity. In addition, all PCR-based methods are hampered by high melting profiles of most exons, probably attributable to the high GC content. Previously we had developed a DG-DGGE assay, an improved version of conventional DGGE, that provided sharper heteroduplex resolution and better identification of conservative transversions *(4)*. In the present study, in an attempt to progress to an automated, high-throughput strategy, we established DHPLC conditions for all 50 exons of the *ABCA4* gene and compared these assays with DG-DGGE to assess their effectiveness as screening methods for gene variants.

DNA regions containing high-temperature melting domains are particularly difficult to analyze with the DGGE and DG-DGGE systems; several high-temperature denaturing urea-formamide gradients need to be tested to identify the appropriate conditions for identification of sequence variations. One can hypothesize that the cooperative effect of an increased GC content may favor a rapid transition from fully or predominantly double-stranded molecules to highly denatured single-stranded species, which may occur rapidly within a narrow increase of denaturant. We had failed to develop DG-DGGE conditions for the analysis of four exons of the *ABCA4* gene, which had to be sequenced directly without a previous prescreening method. Conversely, we were able to successfully develop DHPLC conditions for the analysis of these exons, which suggests that the DHPLC approach has a higher resolving power than DGGE. This may be ascribed to more sophisticated (or refined) temperature control in the DHPLC system, which allows selection of more effective conditions by inspection in a few minutes of a wide panel of temperatures varying by increments. as small as 0.5–1 °C.

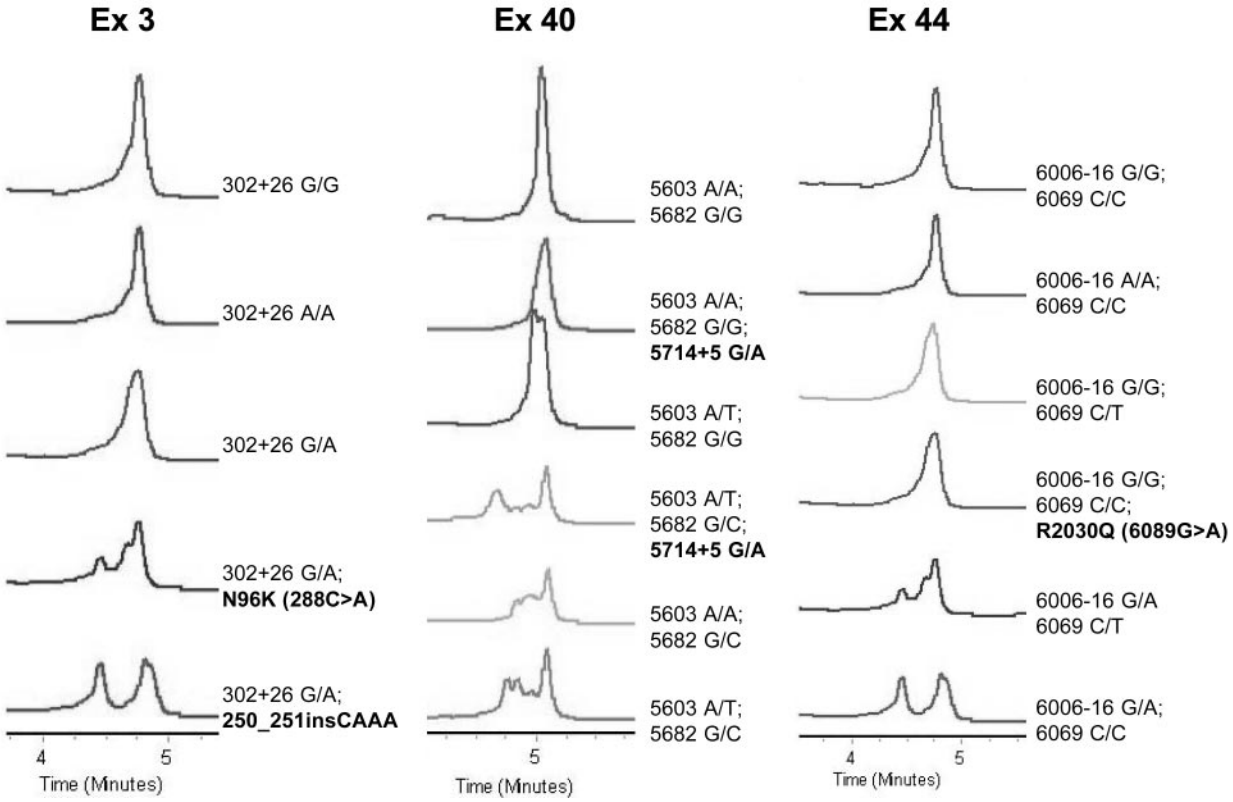

Fig. 2. Examples of complex elution profiles.

For each exon (*Ex*), different combinations of polymorphisms and mutations (in *bold*) are shown.

It has already been reported that the sensitivity and specificity of DHPLC may be suboptimal in high-melting domains flanked by low-melting sequences *(3)*. Our experience in establishing DHPLC assays also revealed that in some cases the optimum oven temperature suggested by the software allowed correct differentiation of sequence variations located in low-melting regions but failed to detect sequence alterations located in high-melting domains. Thus, to develop the most efficient protocols, we designed mutagenized positive controls for all exons for which no natural mutation located in a higher, more stable melting domain was available, including those for which one or more natural mutations located in lower melting domains were present. Mutagenesis was specifically designed to introduce a conservative transversion. These substitutions are notoriously the most difficult sequence variations to visualize because they do not change the overall nucleotide composition and have little effect on the melting temperature of the fragment.

In a substantial number of exons (19 of 50; 38%), the availability of mutated or mutagenized controls was crucial to assess the efficacy of the system and to establish the final temperatures for optimum analysis, which varied by 1–3 °C from the range predicted by the software. It is interesting to note that the availability of three different mutated controls carrying four mutations spread throughout exon 6 (see Table 1) allowed us to ascertain effective temperature conditions differing 2 °C from the range predicted by the software. This led to the identification of three new mutations in this exon, of which one, K223Q, is located in the high-melting domain and the others, A246T and Y245X, are located in the low-melting domain.

Our study showed that DHPLC is a more effective screening tool for *ABCA4* mutation detection than is DG-DGGE. In fact, retrospective DHPLC analysis of samples from 23 STGD patients not only detected all sequence variations previously identified by DG-DGGE but also revealed five additional changes that DG-DGGE had missed. Among these, two variants, D108V and 6817-1G>A, had not been described previously. The D108V mutation involves a conservative A>T transversion at position 323 of exon 4. The 6817-1G>A mutation in intron 49 is the first mutation reported to date in the region amplified by primers for exon 50, which includes only six nucleotides and had not been analyzed by DG-DGGE. Among the previously reported mutations that DG-DGGE failed to identify, V767D is a T>A change in exon 15 and R2030Q is a G>A substitution in exon 44. It is interesting to note that both V767D and D108V are conservative A>T transversions, which are notoriously the most difficult variations to detect by DG-DGGE. Under the same DG-DGGE conditions, two additional mutations in exon 4, 323_324insT, involving a T insertion at the same nucleotide position as D108V, and P143L, involving a C>T substitution at position 428, and three additional sequence variations in exon 44 (6006-16A>G, I2023I, and L2027F) were readily detected. In particular, L2027F is a C>T substitution at position 6079, located 10 bp from the variation involved in the R2030Q mutation. This demonstrates that both the porosity of the denaturing gradients and the migration time had been correctly established for these exons and, therefore, can explain the lower sensitivity of DG-DGGE compared with DHPLC.

In total, DHPLC identified 121 different sequence variants, including those detected during the development of assay conditions, during retrospective study and during molecular scanning of new patients. These include 16 A↔T or G↔C conservative transversions (13%), 12 deletions (10%), and 16 new mutations. The latter include 2 mutations detected during the validation study that had been missed by DG-DGGE and 12 identified in a cohort of patients evaluated only by DHPLC.

Our results further establish the DHPLC approach as more effective than other conventional methods; the wider range of experimental conditions possible for DHPLC permit reliable scanning of any genomic sequence *(3, 9)*. After PCR amplification, complete DHPLC screening of the *ABCA4* gene in 96 samples (in a 96-well plate format) can be performed in less than 1 month (10 analysis/h). Additional upgrades, such as faster software programs, can further reduce this time *(3)*.

The lack of a rapid, automated scanning methodology for the detection of unknown sequence alterations has meant that general molecular testing for STGD has previously been performed by only a very few, specialized centers. Recently, a reliable and comprehensive mutation detection tool, the *ABCA4* gene chip (ABCR400), was developed, which contains all known genetic variations in the *ABCA4* gene *(10)*. However, because of the high frequency of rare mutations reported in this gene, which may also be specific to some geographic areas, mutational scanning should be carried out by DHPLC, whereas genotyping is best done on the array. DHPLC is especially applicable for those STGD patients for whom the chip found one or no mutations. Moreover, the two techniques are complementary with each other because any new sequence variation identified by the DHPLC method could be easily included on the *ABCA4* gene chip.

Studies on the prevalence of *ABCA4* mutations in patients with age-related macular degeneration have been limited their analysis to a subset of mutations that are prevalent in certain ethnic groups. The availability of a robust DHPLC protocol coupled with chip genotyping could greatly improve molecular characterization of the *ABCA4* gene, leading to high-throughput testing of large patient cohorts and providing more accurate estimates of the prevalence of *ABCA4*-associated diseases in the general population.

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