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Short communication

Routine mutation screening of HNF-1α and GCK genes in MODY diagnosis: How effective are the techniques of DHPLC and direct sequencing used in combination?

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Abstract

Aims/hypothesis. Mutations in the hepatocyte nuclear factor (*HNF*)-*1α* and glucokinase (*GCK*) genes are the major causes of monogenic forms of Type II (non-insulin-dependent) diabetes mellitus (Maturity-Onset Diabetes of the Young subtypes, MODY). We evaluated the effectiveness of fluorescent single-strand conformation polymorphism (F-SSCP), denaturing high-performance liquid chromatography (DHPLC) and sequencing based mutation detection in the molecular diagnosis of MODY. Our goal is to identify a rapid, efficient and cost effective mutation detection method for the molecular diagnosis of MODY and other human genetic disorders.
Methods. We evaluated the accuracy of DHPLC in screening for MODY 2 and 3 mutations. In addition, we compared the sensitivity, specificity, cost, handling time and analysis time of fluorescent single-strand conformation polymorphism, denaturing high-performance liquid chromatography and direct sequencing screening methods.
Results. Denaturing high-performance liquid chromatography is a recently developed method for mutation detection. It is cost effective, powerful and reliable and quite suitable for 22 out of the 24 fragments required for MODY 2 and 3 testing. However, exons 1 and 7 of the HNF-1α gene are very polymorphic and so direct sequencing is faster as well as more efficient and reliable.
Conclusion/interpretation. Our results suggest that combining denaturing high-performance liquid chromatography and direct sequencing is a good approach for the routine detection of *HNF-1α* and *GCK* mutations in MODY families. Denaturing high-performance liquid chromatography appears to be a powerful tool in genetic testing and the method could be applied to the molecular diagnosis of other human genetic diseases. [Diabetologia (2001) 44: 775–778]

Keywords Hepatocyte nuclear factor, glucokinase, Maturity-onset diabetes of the young, mutation detection, molecular diagnosis

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Abbreviations: DHPLC, Denaturing high-performance liquid chromatgraphy; F-SSCP, flourescent single-strand conformation polymorphism; HNF, hepatocyte nuclearfactor; GCK, glucokinase.

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Maturity-onset diabetes of the young (MODY) is a monogenic subtype of Type II (non-insulin-dependent) diabetes mellitus characterised by a primary insulin secretory defect and an autosomal-dominant mode of inheritance. Five genes have been identified as causing MODY: *HNF-4a*/MODY1; *glucokinase*/MODY2; *HNF-1α*/MODY3; *IPF1*/MODY4 and *HNF-1β*/MODY5 [1]. In French subjects with MODY the prevalence of MODY 2 and 3 is 63% and 21% [2] while MODY 1, 4 and 5 are rare in French patients. Additional MODY genes that are not known should account for 16% of MODY. In 1997, we established a routine laboratory test for

Table 1. Primer sequences and DHPLC conditions for the molecular screening of the *HNF-1α* and *GCK* genes. Theoretical optimal temperatures as determined by the WaveMaker software and those experimentally chosen for optimal detection are reported. All cycling conditions included an initial denaturation at 96 °C for 12 min and a final extension for 10 min at 72 °C

	Forward primer	Reverse primer	Size (bp)	% B	Theoretical temperatures (°C)	DHPLC temperatures (°C)
HNF-1α promoter 1	agccagcactgttcttgg	agggacagggagctatg	376	64	63 and 64	64 and 65
HNF-1α promoter 2	tcccatcgcaggccatagctc	ccgtctgcagctggctcagtt	364	65	61 and 65	63 and 64
HNF-1α exon 1	ggcaggcaaacgcaacccacg	gaaggggggctcgttaggagc	483	67	65 and 66	64 and 66
HNF-1α exon 2	catgcacagtccccaccctca	cttccagcccccacctatgag	389	62	64	64 and 65
HNF-1α exon 3	tgagagtggccagtaccc	caaaccagcactgtttcc	263	63	63	62 and 63
HNF-1α exon 4	cagaaccctccccttcatgcc	ggtgactgctgtcaatgggac	397	62	63 and 66	66 and 67
HNF-1α exon 5	ggcagacaggcagatggccta	gcctccctagggactgctcca	346	63	60 and 64	62 and 64
HNF-1α exon 6	tggagcagtccctagggaggc	gttgccccatgagcctcccac	322	65	65 and 66	63 and 64
HNF-1α exon 7	ggtcttgggcaggggtgggat	ctgcaatgcctgccaggc**acc**	347	62	65	63 and 65
HNF-1α exon 8	gaggcctgggactagggctgt	ctctgtcacaggccgagggag	229	61	63 and 66	65 and 66
HNF-1α exon 9	cctgtgacagagcccctcacc	cggacagcaacagaaggggtg	287	62	65 and 66	65 and 66
HNF-1α exon 10	gtacccctagggacaggcagg	acccccaagcaggcagtaca	251	62	64	64 and 65
GCK promoter	atggggatggaggctctttg	tgtggggcttagtgtccttc	324	63	61 and 63	62 and 63
GCK exon1a	tccacttcagaagcctactg	tcagattctgaggctcaaac	195	58	61 and 64	63 and 64
GCK exon 1b	ggggcagagtatttgagcag	tgcccccagccttagttttg	346	64	63 and 64	63 and 64
GCK exon 1c	c tccacatctacctctccag	aggggctgaggagaggaaca	196	58	63	62 and 63
GCK exon 2	atggcgtgtgggggagat	tcgggctggctgtgagtc	385	64	64 and 65	63 and 64
GCK exon 3	taatatccggctcagtcacc	ctgagatcctgcatgccttg	295	62	64	64 and 65
GCK exon 4	gtgtccctgaggaatagctt	tacatttgaaggcagagttc	292	62	64	63 and 64
GCK exon 5	tccagatatgttagcagcca	ggagaaaggcaggcagtg	209	61	63 and 67	62 and 63
GCK exon 6	ccagcactgcagcttctgtg	gagcctcggcagtctggaag	176	59	61 and 64	61 and 63
GCK exon 7	agtgcagctctcgctg**acag**	catctgccgctgcaccaga **g**	288	65	65	64 and 65
GCK exon 8	gccctccctcgtgcctgctg	tcgccctgagaccaagtctg	279	62	61 and 64	65 and 66
GCK exon 9	actgtcggagcgacactcag	cttggagcttgggaaccgca	367	67	65 and 66	65 and 67
GCK exon 10	gtcgactgcgtgcagggcgc	tgtggcatcctccctgcgct	261	63	65 and 66	65 and 66

% B, the percentage of buffer B in the elution buffer; bp, base pairs

MODY screening. Mutation detection in the *HNF-1α* and *GCK* genes was performed by fluorescent single-strand conformation polymorphism (F-SSCP) [3] and later by direct sequencing [4]. Although the sensitivity of F-SSCP was high, it did not offer a 100% detection rate and the false positive rate (5%) was not negligible. New developments in chemistry (i.e. energy transfer dichloro-rhodamine) allowed the dilution of reagents reducing the cost of sequencing. Although sequencing is accurate for the screening of mutations, it remains expensive and time consuming.

A new mutation detection technique was developed: denaturing high-performance liquid chromatography (DHPLC) [5] discriminating homoduplices and heteroduplices in PCR products by ion pair reverse-phase chromatography under partially denaturing conditions. Its sensitivity is high (96–100%) compared with denaturing gradient gel electrophoresis, SSCP and gel electrophoresis-based heteroduplex analysis [6,7]. These reports prompted us to evaluate a DHPLC screening protocol of the *HNF-1α* and *GCK* genes. In the context of a routine laboratory we compared fluorescent SSCP, direct sequencing and DHPLC for MODY2 and 3 diagnosis.

Subjects and methods

DNA extraction was performed from EDTA whole-blood samples using the Puregene kit (Gentra, Minneapolis, Minn., USA).

DHPLC protocol. The primers and DHPLC conditions are described in Table 1. Polymerase chain reactions were performed using AmpliTaq Gold and a Gene-Amp PCR system 9700 thermocycler (Perkin Elmer, Foster city, Calif., USA). The PCR products were denatured at 95 °C for 5 min and allowed to cool to 65 °C. The DHPLC was performed on a Wave nucleic acid fragment analysis system and DNASep column (Transgenomic, SanJose, Calif., USA). Buffers A and B contained respectively 0.1 mol/l triethylammonium acetate (TEAA) and 0.1 mol/l TEAA, 25% acetonitrile. Elutions were performed with a mixture of buffers A an B. Initial proportions of buffers and temperature were determined for each PCR fragment (Table 1). Elution was at a flow rate of 0.9 ml/min with a buffer B gradient increase of 2% per min for a 4 min period.

The time needed for the DHPLC analysis of one sample was 5 min. Homoduplices and heteroduplices were detected by 260 nm absorbance. The sample chromatogram was compared with a reference pattern. If additional or abnormal peak shapes were detected, direct sequencing identified the nucleotide variation.

Quality Control of the column. An important parameter for a successful mutation detection was the quality control of the DNASep column (Transgenomic, SanJose, Calif., USA). Quality control was periodically performed with a standard mutation from a 209 bp fragment (at the DYS271 locus) from Transgenomic. A 1:1 mixture of the wild type and mutant

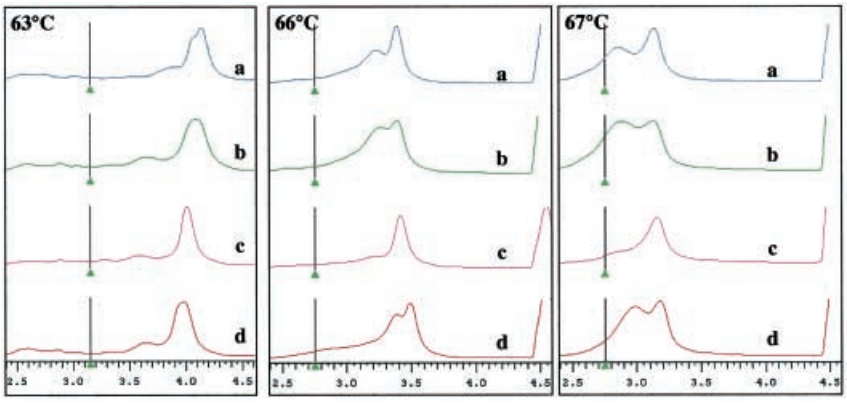

Fig. 1. Resolution of genetic variant detection in exon 4 of *HNF1 a* according the Wave Maker predicted temperatures (63 and 66 °C) and the experimentally chosen temperature (67 °C). P291fsinsC heterozygote mutation (a), P291fsinsC heterozygote mutation and G288G homozygote polymorphism (b), wild type (c), G288G homozygote polymorphism (d)

PCR fragments was injected weekly into the DHPLC system. Thus discrimination of heteroduplices and homoduplices was a criteria of reliability for the column. In our experiment, 8000 injections were processed without a loss of efficiency.

Mutation detection by direct sequencing. The protocol was performed using a 3700 DNA sequencer (Applied Biosytems, Foster City, Calif., USA) as previously described [4].

Results

To evaluate the accuracy of DHPLC, we tested 43 known genetic variants, previously identified by F-SSCP and direct sequencing, in the *HNF-1α* and *GCK* genes [2, 3, 8, 9]. DHPLC optimal temperatures (T), critical for suitable detection, were defined with the Wave Maker software. A PCR product from the sample was codenatured with a wild type product and loaded on DHPLC. Primer sequences and DHPLC conditions are reported in Table 1. All mutations were detected at the temperatures predicted by the WaveMaker software. However, the detection of these mutations was evaluated to be in the range of T-2 °C to T + 2 °C (i.e. for exon 4 of the *HNF1α* gene the range was 61 °C to 68 °C). Thus we selected one or two temperatures presenting the best discrimination between homoduplices and heteroduplices (Fig. 1). The results confirmed the high sensitivity of the DHPLC previously described [6, 7]. For PCR fragments with one frequent polymorphism (*GCK* promoter G > A –187, intron 9 T > C + 8 or *HNF1 a* intron 1 G > A –42, intron 2 C > T –23, G288G, T515T, intron 9 T > C –24), a homozygote and a heterozygote PCR product for the polymorphism were loaded as internal controls, thus polymorphisms could be detected on the basis of their characteristic DHPLC profiles even if the retention time was slightly different from one experiment to another (maximal variation of the retention time of ± 0.1 mins). However, in a second step the sample on its own (without wild type product) was analysed by DHPLC to discriminate between heterozygosity and homozygosity at the relevant polymorphism. The PCR products for exons 1 and 7 of the *HNF-1α* gene contain respectively 2 and 3 frequent polymorphisms. Exon 1 of *HNF1α* contains 2 frequent polymorphisms in codon 17: CTC-CTG (C 0.43/G 0.57) codon 27; ATC-CTC (A 0.65/C 0.35). Exon 7 of *HNF1α* contains 3 frequent polymorphisms in codon 459: CTG-TTG (C 0.72/ T 0.28) codon 487 (A 0.47/ G 0.53) and a: A-G n + 7 (A 0.51/G 0.49). From our data of direct sequencing of more than 200 patients, only 26 % were wild type for polymorphisms of exon 1 and 15 % for polymorphisms in exon 7. The various complex haplotypes produced many different profiles. Although DHPLC was able to discriminate the patterns [7], analysis of these complex DHPLC patterns was time consuming and misinterpretation cannot be excluded. Thus for exons 1 and 7 of the *HNF1α* gene, molecular screening by direct sequencing appeared faster and more efficient. As included in the optimisation process, the 43 reference polymorphisms were not screened blind. However 25 probands were screened by DHPLC and direct sequencing. Both methods identified 100 % of the polymorphisms. Applying the optimised DHPLC method to the screening of *GCK* and *HNF1α* genes in 150 probands suspected to have MODY led to the detection of 11 more genetic variants than the previous 43 tested: C > T nt + 16 intron 2, IVS4 –1 G > A, C > A nt + 26 intron 4, Y215Y, E256 K, A > T nt + 22 intron 7 in the GCK gene, C > T nt-302 promoter, D135fsdelA, C > T nt-25 intron 5, S535R, A586Y in the HNF1α gene. IVS4 –1 G > A (*GCK*), S535R and A586Y. Some of these *HNF1α* variants have not yet been reported in the literature and their association with MODY diabetes is still under investigation.

Table 2. Comparison of fluorescent SSCP, direct sequencing and DHPLC for the routine mutation screening of the HNF-1α and GCK genes for the diagnosis of MODY diabetes

	Fluorescent SSCP	Direct sequencing	DHPLC
Label	Yes	Yes	No
Cost* 1 exon and 1 sample	2 Euros	4 Euros	1.2 Euros
Sensitivity	95 %	100 %	95–100 %
False positives rate	5–6 %	0 %	< 1 %
Handling time 96 samples	150 min	165 min	90 min
Time to look at and score 96 samples	120 min	240 min	60 min

* Cost includes consumables (reagents for PCR, mutation detection . . .) and maintenance of automatic DNA sequencer or DHPLC devices

Discussion

It is noteworthy that, in contrast to F-SSCP, the DHPLC analysis did not require either label or internal fluorescent size standard. The time required for the three methods was 24 hours from 96 genomic DNA samples to the completion of computer data files suitable for analysis. However, DHPLC required less handling time (90 mins) compared with F-SSCP (150 mins) and direct sequencing (165 mins) (Table 2). Moreover the time to look at and score 96 DHPLC patterns (60 mins) was shorter compared with 96 F-SSCP profiles (120 mins) or 96 sequencing files (240 mins). We compared the cost of the methods, including consumables and maintenance of automatic DNA sequencer or DHPLC. The cost of DHPLC (1.2 Euros per fragment analysed) is less than F-SSCP (2 Euros) and direct sequencing (4 Euros). As in our F-SSCP screening protocol [3], when a suspect DHPLC pattern was detected, direct sequencing was used to ascertain the genetic variation. Because DHPLC produced fewer false positives (< 1 %) than F-SSCP (5 %), fewer false variants had to be sequenced. Our experience in molecular screening of MODY genes by SSCP and DHPLC is in agreement with the previously reported sensitivities of these methods : 85–95 % for SSCP and 96–100 % for DHPLC [6, 7, 10]. The most sensitive remains sequencing-based detection. If false positives are rare with automatic software analysis of the sequencing data (< 1 false positive /100 nucleotides) [4], a precise inspection of forward and reverse profiles allows up to a 100 % sensitivity excluding false positives. Although new sequencing chemistries offer a more cost effective detection of mutation, sequencing remains expensive and the precise analysis of the data is time consuming. Thus the method did not appear satisfactory for a quick and accurate routine genetic testing of large samples.

In conclusion, DHPLC is an efficient tool for the routine diagnosis of human genetic diseases, especially for the detection of heterozygous mutations. The method is cost effective and requires less handling and interpretation time than SSCP or direct sequencing. However, in our study, dye-terminator cycle sequencing appeared more suitable for PCR products containing at least 2 or 3 frequent polymorphisms. A total of 15 patients could be screened per week for *HNF1α* and *GCK* genes, with one DHPLC device including PCR, two temperatures DHPLC runs, sequencing of variants and interpretation. An experienced technician requires 10–12 hours/week to perform this screening and interprete the results. The cost of the routine diagnosis of both MODY 2 and MODY 3 genes is 34.4 Euros per sample. Combining DHPLC and direct sequencing screening provides a sensitive, accurate, rapid and lower cost tool for the molecular diagnosis of human genetic disorders.

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