Rapid and safe determination of human apolipoprotein E genotypes by miniaturised SDS-PAGE in non-insulin dependent diabetes mellitus

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Abstract

Aims—To present a non-isotopic procedure for the analysis of the different apolipoprotein E genotypes in normal subjects and patients with non-insulin dependent diabetes mellitus.

Methods—Apolipoprotein E genotypes were detected following polymerase chain reaction and miniaturised sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (PhastSystem Pharmacia).

Results—The time taken from extraction of DNA from 6 µl whole blood to the final result was eight hours. The allele frequencies in patients with diabetes mellitus in our series were similar to those found in the control subjects.

Conclusions—SDS-PAGE is rapid, reliable and safe with few drawbacks, can be used as a routine procedure and may easily replace apolipoprotein E phenotyping.

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Of the different constituents of lipoprotein, apolipoprotein E is essential for the catabolism of lipoproteins by receptor mediated endocytosis. Apolipoprotein E is a structural component of several classes of plasma lipoproteins such as chylomicrons and their remnants, very low density lipoprotein (VLDL) and its intermediate density lipoprotein (IDL) remnants, and a high density lipoprotein (HDL) subpopulation. Apolipoprotein E is also involved in the conversion of VLDL to low density lipoprotein (LDL) by lipoprotein lipase and is a ligand for two types of high affinity receptors on most cell plasma membranes: the apolipoprotein E receptor and the LDL (B/E) receptors.^{1,2} Thus, apolipoprotein E mediates hepatic elimination of potentially atherogenic remnant particles generated by the lipolysis of triglycerides rich in lipoproteins (chylomicrons, VLDL). Apart from its role in lipoprotein metabolism, apolipoprotein E is synthesised in most cells and organs. Apolipoprotein E synthesis is increased following injury and is implicated in immunoregulation, repair and modulation of cell growth.³⁻⁶ A recent paper even suggested a functional pathogenic role for a particular apolipoprotein E isoform in Alzheimer's disease.⁵

The human apolipoprotein E gene spans 3.7 kilobases including four exons and three introns and is located on chromosome 19.⁷⁻⁹ Structural variants of apolipoprotein E were first identified using isoelectric focusing (IEF). Now, either cloned DNA fragments, acting as probes, or synthesised oligonucleotides can be used to detect DNA mutations and/or polymorphisms which are associated, genetically linked or causally related to lipoprotein disorders. Three co-dominant alleles ε2, ε3 and ε4 encode three common major isoforms of apolipoprotein E: from acidic to basic, E2, E3 and E4, respectively. Rare variants have been identified using IEF and sequencing. These three alleles determine six different apolipoprotein Es: three homozygous phenotypes E2/E2, E3/E3, E4/E4 and three heterozygous phenotypes: E2/E3, E2/E4, E3/E4, all relatively common in the population. These variations result from Arg-Cys interchanges at residues 112 and 158 of apolipoprotein E and are caused by a single nucleotide alteration at two loci in the fourth exon of the apolipoprotein E gene. These allelic variations affect receptor binding and influence total, LDL cholesterol and triglyceride concentrations.

Apolipoprotein E3 is the ancestral protein and the most common of these isoforms. It has a Cys residue at position 112 and an Arg residue at position 158 in the receptor binding region of apolipoprotein E. The mutant E2 isoform (112 Cys, 158 Cys) binds with reduced affinity to cellular receptors.¹ It is associated with decreased cholesterol concentrations and increased triglyceride concentrations. E2/E2 phenotypes are detected in more than 90% of patients with type III hyperlipoproteinaemia.¹⁰ The E4 isoform (112 Arg, 158 Arg) is associated with increased concentrations of total and LDL cholesterol, and decreased triglyceride concentrations. Thus, because of the high apolipoprotein E polymorphism in the general population and its relation with a potentially atherogenic lipid profile, there is growing interest in developing a method for the rapid analysis of apolipoprotein E polymorphism.

Given the higher risk of cardiovascular disease in patients with non-insulin dependent diabetes mellitus (NIDDM), which is partially related to their lipid disorders, the forms of apolipoprotein E occurring frequently in this population are of particular interest. In previous studies, Snowden *et al*¹¹ and Wenham *et al*¹²

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Table 1 Electrophoresis protocol

Step 1	Pre-run	250V	10 mA	3 W	1 Vh
Step 2	Sample application	250V	10 mA	3 W	1 Vh
Step 3	Run	250V	10 mA	3 W	98 Vh

emphasised discrepancies between the results of genotyping and phenotyping of apolipoprotein E in normal control and diabetic populations (13% to 17%). IEF can lead to the erroneous assignment of an apolipoprotein E phenotype even after pretreatment with neuraminidase. Thus, genotyping by DNA analysis is the preferred method for determining apolipoprotein E status.

In this study we present a sensitive, easy, rapid, non-isotopic procedure for the analysis of the different apolipoprotein E genotypes which can be used in routine practice.

Methods

The study population comprised 104 unrelated French patients with NIDDM (58 men and 46 women) aged between 31 and 79 years with a mean (SD) age of 57 (11) years, all attending the Reims Diabetes Centre.

The control group comprised 85 non-diabetic subjects (50 men and 35 women with a mean (SD) age of 55.4 (9.3) years. The study was approved by the local ethics committee.

Statistical analysis of genotype distributions was performed by testing the difference in allele frequencies between the NIDDM and control populations. We could not use the χ^2 test because of the small size of our study population and therefore applied Fisher's exact test. Significance was set at the 5% level.

DNA was extracted from 189 peripheral blood samples using the InstaGene DNA purification matrix (732-6030; BIO-RAD, Ivry-sur-Seine, France). The InstaGene matrix permits fast and easy preparation of polymerase chain reaction (PCR) amplifiable DNA by eliminating the labour intensive phenol/chloroform extraction steps. A simple cell lysis step achieved by boiling in the presence of matrix is sufficient. This is possible because the matrix efficiently absorbs cell lysis products that interfere with the PCR amplification process. Moreover, the InstaGene bottle is cheap (£40 for 100 extractions from 6 µl whole blood). Extraction takes one hour.

Briefly, 3 to 6 µl whole blood were mixed with 1 ml water, incubated for 20 minutes at room temperature and centrifuged at 10 000 rpm for three minutes. The InstaGene matrix (200 µl) was added to the pellet and incubated for 20 minutes at 56°C. The sample was then vortexed, incubated for eight minutes at 100°C,

and spun for two minutes; 20 µl of the resulting supernatant fluid were used per 50 µl PCR reaction. The remainder was stored at -20°C.

Apolipoprotein E PCR products 244 base-pairs long, containing amino acid positions 112 to 158, were generated. Non-labelled primers F4 (5'-ACAGAATTCGCCCCGGCCTGGTA C A C-3'), and F6 (5'-TAAGCTTGGCACGGCTGTCCAAGGA-3'), proposed by Emi *et al.*¹ in standard conditions in a final volume of 30 µl were used to amplify the DNA. The PCR was performed on approximately 0.2 µg genomic DNA from peripheral blood samples.

The PCR mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin (w/v), 200 µM each of the four deoxynucleotides, and 10 pM each of the non-labelled primers. The DNA samples were first denatured at 95°C for six minutes and then 1 unit Taq DNA polymerase (BioTaq, Bioprobe Systems, Montreuil-sous-bois, France) was added. Then these samples were denatured again at 95°C for one minute, annealed with PCR primers at 60°C for one minute, extended at 70°C for two minutes in a thermocycler (Trioblock, Eurogentec, Seraing, Belgium) for 30 cycles, with a final extension of seven minutes.

PCR products were digested with 5 units of *HhaI* (Eurogentec) for three hours or overnight at 37°C. Electrophoresis was carried out using PhastSystem PAGE, on preformed polyacrylamide gels (43 × 50 × 0.45 mm). These gels have a 13 mm stacking zone, at a concentration of 7.5% polyacrylamide and cross-linking of 3%, and a 32 mm homogenous zone of 20% with 2% cross-linking (Pharmacia 17-0624-01). Moreover, we chose a discontinuous buffer system (Native at the anode and SDS buffer at the cathode) rather than a standard continuous buffer (Native buffer at both the anode and cathode). Like the buffer system in the gels, we used 0.112 M acetate (leading ion) (Pharmacia 17-0517-01) and 0.112 M Tris (pH 6.4) (anode) as the Native buffer and SDS-PAGE buffer (0.20 M tricine (Pharmacia 17-0516-01), 0.20 M Tris and 0.55% SDS, pH 7.5) (cathode). The discontinuous buffer system gave the same results as the continuous buffer system, but was faster. Three microlitres of restriction enzyme digestion products were mixed with 0.05% Bromophenol Blue and 0.05% Xylene Cyanol. This mixture was loaded on to the 20% gel at the cathode. Environmental conditions (table 1), with a constant gel temperature during electrophoresis (30 minutes), are critical and were optimal at 15°C for a total of 98 volts/hours (AVh) (maximal total of 100 AVh). PCR products were detected using the silver staining method, with a slightly modified Development Technique (file no. 210 in the PhastSystem) (table 2).

Results

The F4/F6 primers gave amplified DNA fragments 244 base pairs long with six possible *HhaI* restriction sites⁷ and two polymorphic sites (fig 1). Figures 2A and 2B illustrate the char-

Table 2 Silver staining protocol

1	in 1 out 0	t: five minutes	T: 50°C	Trichloroacetic acid 20%
2	in 2 out 0	t: five minutes	T: 50°C	Glutaraldehyde 5%
3	in 3 out 0	t: two minutes	T: 50°C	H ₂ O
4	in 3 out 0	t: two minutes	T: 50°C	H ₂ O
5	in 4 out 0	t: eight minutes	T: 40°C	AgNO ₃ (0.5%)
6	in 3 out 0	t: 30 seconds	T: 30°C	H ₂ O
7	in 3 out 0	t: 30 seconds	T: 30°C	H ₂ O
8	in 5 out 0	t: 30 seconds	T: 30°C	Developer
9	in 5 out 0	t: five minutes	T: 30°C	Developer
10	in 6 out 0	t: two minutes	T: 50°C	Reductor
11	in 7 out 0	t: three minutes	T: 50°C	Glycerol (20%)

Figure 1 Partial restriction map of the polymorphic region of the apolipoprotein E (apo E) gene and locations of constant HhaI cleavage sites (small arrows) and polymorphic sites (large arrows). The distances between polymorphic HhaI site are in base pairs.

Figure 2 A: Diagrammatic representation of the major banding patterns of six apolipoprotein E genotypes obtained on 20% polyacrylamide Phast-Gel; B: electrophoretic pattern of the five NIDDM apolipoprotein genotypes. A, ϵ_2/ϵ_2 ; B, ϵ_2/ϵ_3 ; C, ϵ_3/ϵ_4 ; D, ϵ_4/ϵ_4 ; E, ϵ_3/ϵ_3 ; F, pBR 322 ladder digested by Hae III.

Table 3 Genotype counts and frequencies in French patients with NIDDM

Types	ϵ_2/ϵ_2	ϵ_2/ϵ_3	ϵ_2/ϵ_4	ϵ_3/ϵ_3	ϵ_3/ϵ_4	ϵ_4/ϵ_4	Total
NIDDM (n)	1	13	0	69	19	2	104
NIDDM (%)	1	12.5	0	66.3	18.2	2	100
Controls (%)	1.2	11.8	1.2	67.0	17.6	1.2	100

acteristic and reliable patterns of each genotype from 91 base pairs to the smallest fragments (16, 18 and 19 base pairs) on the silver stained polyacrylamide gels.

The French apolipoprotein E genotype frequencies for NIDDM are given in table 3 and the allele frequencies for the patients with NIDDM and controls are presented in table 4.

Table 4 Allele frequencies in the two French groups

Alleles	ϵ_2	ϵ_3	ϵ_4
French patients with NIDDM (n=208)	7.2	81.7	11.1
French control population (n=170)	7.6	81.8	10.6
	NS	NS	NS

As expected, the ϵ_3/ϵ_3 genotype was the most common and >81% of our samples had at least one ϵ_3 allele. The next most common allele was ϵ_4 . Heterozygotes for ϵ_2/ϵ_4 were not detected in our series. The allele frequencies in patients with NIDDM were similar to those in the control subjects.

Discussion

Apolipoprotein E single gene locus polymorphisms appear to have an important effect on lipid profile. Data on the status of apolipoprotein E in several populations are estimated differently. Most authors have analysed the frequencies of apolipoprotein E phenotypes in patients with NIDDM.¹⁴⁻¹⁷ Snowden *et al*¹¹ and Shriver *et al*¹³ compared the genotypes of patients with NIDDM with the phenotypes of normal control subjects. These various studies did not reveal any differences in the isoform frequencies in patients with NIDDM compared with a non-diabetic population, with the exception of the study by Snowden *et al*.¹¹ However, when the genotypes of patients with NIDDM were compared with the phenotypes of the control population, the apolipoprotein E status was similar in the two populations.^{11,13}

Nevertheless, the discrepancies observed between genotypes and phenotypes in normal or diabetic subjects^{11,12} may lead to the erroneous estimation of the apolipoprotein E status. Moreover, phenotypic analysis may lack discriminative power and perhaps reliability.¹⁸ IEF cannot distinguish rare variants of apolipoprotein E that have the same net charge as the three common isoforms. Electrophoretically silent structural variations may also escape detection.⁷ Minor glycosylated isoforms complicate the interpretation of phenotypes on IEF gels, especially with the superimposition of glycosylated and non-glycosylated forms.^{1,19} All of these drawbacks make phenotyping cumbersome, difficult to interpret and time-consuming.

To our knowledge, no work has been carried out on the comparison between genotypes of apolipoprotein E in patients with NIDDM and those of non-diabetic control subjects. In the present study, we chose this homogeneous approach, but did not find any significant difference in the distribution of apolipoprotein E polymorphisms between these two populations.

PCR procedures are largely applied for genotyping. PCR methods amplify the sequences containing ϵ_2 and ϵ_4 allelic substitution sites at positions 112 and 158.¹²⁰ The close proximity of the variable DNA sites of the apolipoprotein E gene permits their co-amplification within a single DNA fragment. Some authors have extended their PCR re-

Table 5 Comparison of the different methodologies used for genotyping. We have assumed that the same protocols for DNA extraction and PCR have been performed for the three methodologies

Methodology	PCR and dot blot with radiolabelled ASO	PCR and restriction isotyping. Analysis on classic PAGE	PCR and restriction isotyping. Analysis on the PhastSystem
DNA Sensitivity	0.1–1 pg	1–5 ng (ethidium bromide)	75 pg (AgNO ₃)
Duration from blood sampling to the final result	48 hours	36 hours	eight hours
Effective handling	two to three hours	two hours (two gels)	one hour (two gels)
Cost of reactants (excluding the PCR set-up) for one assay	£16	£1	£3
Toxicity	Use of radiolabelled probes Use of formamide in the hybridisation mixtures	Use of ethidium bromide Handling of acrylamide powder	None

action by using other primers which cover other domains (receptor binding domain)¹, and others amplify with biotin labelled primers.³ PCR amplified DNA products can be subjected to dot blot analysis with allele specific oligonucleotide (ASO) probes.¹ Most frequently, separate hybridisations are necessary with each of the four radiolabelled oligonucleotides to distinguish 112 Cys, 112 Arg, 158 Cys, and 158 Arg codons. A nested primer approach to PCR is also possible.^{3, 21} ASO probes and dot blots are accurate, but expensive, methods, are time-consuming and involve several steps. They are also particularly sensitive to non-specific binding and background radioactivity, so that a particular sample often requires multiple testing to obtain an unambiguous hybridisation pattern.

Two more rapid analyses of known mutations can be performed by direct electrophoresis of PCR mixtures: the ARMS (amplified refractory mutation system) method, which requires some non-labelled oligonucleotides with mismatches close to the 3' end.²² Hybridisation with labelled ASO probes is not necessary and the analysis is carried out by agarose gel electrophoresis. The second technique incorporates restriction isotyping. Accurate genotyping is achieved by examination of the *HhaI* or the *CfoI* restriction products generated from PCR amplified DNA using classic PAGE.^{3, 7, 20}

We improved the SDS-PAGE technique by using the PhastSystem (Pharmacia). A micro-processor automatically regulates all of the parameters during the separation and development runs. This system provides preformed polyacrylamide gels with their appropriate buffer strips. Gels are processed with an optimised silver staining method. The method is safe and designed to give reproducible, highly sensitive staining without background. Therefore, each genotype can be directly visualised by silver staining of a unique and reliable combination of restriction fragments for homozygotes and heterozygotes. The silver staining technique is 10 times more sensitive than traditional electrophoresis with ethidium bromide. This system enables detection of quantities as small as 75 pg of DNA. Moreover, even the smallest DNA fragments (16, 18 and 19 base pairs) can be visualised on the preformed gels. This system avoids the use of toxic ethidium bromide and acrylamide powder. Moreover, analysis of the single stranded conformation polymorphisms of the PCR products can be performed using the PhastSystem.²³

Miniaturised SDS-PAGE, including sep-

aration and silver staining, can be completed within 60 minutes. The time taken from extraction of DNA from 6 µl whole blood to the final result is eight hours. SDS-PAGE incorporating the PhastSystem is more expensive than classic PAGE, but is less time-consuming and therefore is more profitable (table 5).

There is a growing interest in apolipoprotein E genotyping for routine purposes in many fields of pathology. This technique using the PhastSystem may easily replace apolipoprotein E phenotyping. In the future, sequencing of the apolipoprotein E gene may permit the analysis of new variants with linkage to polymorphic restriction sites. Our methodology could be easily used to detect other nucleotide substitutions.³

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