Cholinesterase variants: rapid characterisation by PCR/SSCP and evidence for molecular homogeneity

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Abstract

We have applied the technique of PCR-SSCP (polymerase chain reaction – single stranded conformation polymorphism) to characterise the molecular basis of cholinesterase deficiency and variants in a Jordanian family. PCR-SSCP proved to be a quick and sensitive method of screening cholinesterase variants in a clinical setting. An AG insertion at position 351 was found to cause a silent allele, for which the parents were heterozygous and three children homozygous. In addition, the father and two sons were heterozygous for an A to G transition at position 209, known to cause the dibucaine resistant variant. No linkage to the K variant was found, which has been reported previously in white populations. These findings suggest considerable homogeneity in the molecular basis of CHE variants between different ethnic groups.

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Serum cholinesterase (CHE, butyrylcholinesterase) is a cholinester hydrolysing enzyme that is synthesised in the liver. Its activity is measured in routine laboratory tests to assess liver function and monitor intoxication with organophosphates. The physiological function of the enzyme is unknown, but patients with acholinesterasaemia or certain cholinesterase variants are known to have prolonged duration of inhibition of neuromuscular transmission induced by succinylcholine.¹

The molecular bases of some structural variants of the enzyme have been identified.^{2–4} The atypical or dibucaine resistant variant is caused by a point mutation at nucleotide 209 (A to G, allele frequency 0.983/0.017).³ This variant appears to be closely linked to the K variant defined by a polymorphism at nucleotide position 1615 (G to A, allele frequency 0.875/0.125).⁴ The less common allele codes for the K variant which is characterised by a reduction of 30% in enzyme activity. In patients with very low or absent cholinesterase activity a frameshift mutation at position 351 (GGT→GGAG) has been identified as the basis of one type of silent mutation.³ In another family inactivation of the cholinesterase gene was caused by an Alu insertion in exon 2.²

Studies of CHE gene alterations are a valuable model for other gene mutations in man.

The entire coding region of the gene can be amplified with only a few PCR primers using genomic DNA. The consequences of mutations are easily assessed by measuring the CHE activity with routine laboratory tests.

To characterise the CHE variant of a patient with acholinesterasaemia and her family, we developed a strategy based on PCR amplification and genomic DNA sequence analysis. We selected four pairs of primers from the published CHE genomic sequence⁵ covering the three exons that code for the mature protein. The amplified DNA was subjected to single stranded conformation polymorphism (SSCP) analysis to detect possible mutations. Finally, direct PCR sequencing of the PCR product exhibiting a variation in the SSCP pattern was used to define the type of mutation.

Methods

PATIENTS

A 20 year old woman from Jordan was admitted to our hospital because of acute hepatitis B. A very low CHE activity was found in her serum. After informed consent, blood was also taken from her parents, her sister, and her three brothers. DNA was isolated according to standard methods. Serum cholinesterase phenotypes were determined from the rate of benzoylcholine hydrolysis, the dibucaine inhibition number,¹ and the fluoride inhibition number.⁶ Inhibition numbers could not be determined in one brother, one sister, and the patient herself because of the very low CHE activity.

PCR AMPLIFICATION

Four pairs of oligonucleotide primers were chosen according to the published CHE genomic sequence from flanking intron sequences⁵ as shown in the table. The downstream primer of each primer pair was biotinylated. With these primers all three exons coding for the mature CHE protein can be amplified. PCR was performed in a thermocycler (9600, Perkin Elmer Corp, Norwalk, CT) with 100 ng of genomic DNA, 25 pmol of each primer, 200 µmol total dNTP, and 2 U *Taq* polymerase (Boehringer, Mannheim) using a “touchdown” protocol.⁷ The first six cycles were carried out at decreasing annealing temperatures in 1°C steps for one cycle each from 65° to 60°C, followed by 25 cycles using the following conditions: 30 seconds 94°C; one minute 59°C; one minute 72°C. For primers 45/43 and 55/53 a different

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Primers used in the study

Primer	Sequence (5'-3')	Position	Size (bp)
15	TGGTTTCITTTGCTCTGCAT	-42--23	465
13	TACAATAACTCTTTCAACCCG	423-403	
25	CATGTTTATGATGGCAAG	376-393	1088
33	CAGAGAACAATGACAAAA	Intron 2*	
45	CAATTTTACTATAATGTCTC	Intron 2*	237
43	TCTATTAAATAACCAACAC	Intron 3	
55	TGATTAATACAACTTATTCC	Intron 3*	189
53	GAAATATGTTCTATAAAGGG	3' untranslated region	
55/5	TTTCAGTTAATGAAACAGAT	Intron 3*	
seq5	GCCACAAAATATGCAAATCTT	172-192	

* Intron primers are all located approximately 30 bp within the intron.

programme without touchdown was carried out, which used an annealing temperature of 50°C for all 30 cycles.

SSCP ANALYSIS

The PCR product from the amplification of primers 25/33 was purified by adsorbition to glass milk (Geneclean; BIO 101, La Jolla, CA) and digested with *HphI* to obtain fragments smaller than 400 bp. The digested fragments, as well as the other PCR products, were denatured with formamide by heating at 80°C for three minutes and kept on ice until loading. The mixture was separated in a 0.75 mm thick MDE gel in 0.6 × TBE buffer (AT Biochem, Malvern, PA), polymerised on Gel-Bond PAG film

(Pharmacia LKB, Freiburg) at room temperature. The gel was electrophoresed at 400 V for 10 to 18 hours.^{8,9} The DNA was visualised by silver staining, as previously described with minor modifications.¹⁰

GENOMIC DNA SEQUENCING

For direct sequencing of PCR amplified products, single stranded DNA was obtained using streptavidin conjugated magnetic beads (Dyna, Norway). The sequencing reaction was carried out on single stranded DNA using the T7 Sequencing Kit (Pharmacia LKB, Freiburg) and α-[³⁵S]dATP (Du Pont NEN, Dreiech).

Results

Results of CHE standard activity assay and inhibition tests are shown in fig 1 indicating the codominant inheritance of the CHE alleles. The very low CHE activity in our patient (arrow), one brother, and one sister is characteristic of persons who are homozygous for silent alleles. The very low dibucaine inhibition number and the reduced CHE activity of the father suggest that he is either homozygous for the dibucaine resistant variant or heterozygous for this variant and a silent allele. Consequently the two brothers with normal CHE activity have to be heterozygous for the dibucaine resistant variant as suggested by the dibucaine inhibition number.

SSCP analysis of PCR fragments 55-53, 45-43, and of the *HphI* digested fragments 25-33 (data not shown) did not show any differences between the DNA of the control subject and the family members. Characteristic SSC polymorphisms were obtained using primers 15 and 13, which amplify the 5' end of the protein coding region from nucleotide -42 to 423 (fig 1). SSCP patterns reflect exactly the inheritance of the CHE alleles. All four bands in the three children with very low CHE activity differ from the control, suggesting that both their CHE alleles are different from the wild type. The band pattern of the mother appears to be a combination of the control and the deficient children presumably caused by heterozygosity for the wild type and a mutated allele. A similarly composed pattern can be observed for the two brothers with normal CHE activity. The patient and the control samples show four distinct bands instead of the expected

Figure 1 Family pedigree illustrating inheritance of CHE variants. The patient is indicated by an arrow. Serum cholinesterase activities (normal 3.0-7.5 kU/l) as well as dibucaine numbers¹ (normal 80-88, atypical heterozygous 60-68, atypical homozygous 12-25) and fluoride numbers¹¹ (normal 40-60, atypical heterozygous 50-66, atypical homozygous 74-84) are shown. Inhibition numbers could not be determined in one brother, one sister, and the patient herself because of the very low CHE activity. Inhibition numbers were not determined (nd) in the person on the bottom left side of the pedigree. Silent alleles are represented by solid symbols, open symbols denote normal alleles, and hatched symbols indicate the dibucaine resistant allele. The photo shows the SSCP analysis of fragment 15-13. Four characteristic patterns can be distinguished (C= control).

Figure 2 DNA sequence of CHE exon 2 (father, coding strand) obtained by PCR amplification with primers 15/13 and sequencing with primer 15. The A to G mutation causes dibucaine resistance.

two for homozygotes. This phenomenon is occasionally observed in SSCP. It is probably explained by the fact that strands with the same sequence can have different stable conformations in non-denaturing polyacrylamide electrophoresis.⁸

Using primers 15/13 and restriction enzyme digestion with *Sau3A*, the father and the two sons with normal CHE activity showed an additional band at 274 bp (data not shown) on a polyacrylamide gel. Therefore this fragment was selected for detailed DNA sequence analysis.

Genomic DNA sequence analysis of this fragment using PCR primer 15 as sequencing primer showed an adenine to guanine mutation a nucleotide 209, causing a change of aspartate (GAT) to glycine (GGT) at position 70. The father and the two sons with the normal CHE were found to be heterozygous for this mutation (fig 2). This mutation is known to cause the dibucaine resistant variant⁸ and the loss of a *Sau3A* restriction site (GATC→GGTC).

Figure 3 Direct sequencing (coding strand) with primer seq5 of DNA amplified with primers seq5/13. (A) Mother heterozygous for the GA insertion at position 351. (B) Patient (arrowed in fig 1), homozygous for the insertion.

Sequencing of fragment seq5/13, which covers 252 bp at the 3' end of fragment 15/13, with sequencing primer seq5 showed a frameshift mutation (GGT→GGAG) at position 351, resulting in the appearance of a stop codon at nucleotide 384. Both parents were found to be heterozygous for this mutation. Two sisters and one brother with very low CHE activity were homozygous for the insertion (fig 3). This mutation is known to cause a silent variant.³

A guanine to adenine polymorphism at nucleotide position 1615 has been found to cause the K variant, which is characterised by a reduction of about one third in cholinesterase activity.^{4,11,12} This polymorphism appears to be in close linkage to the mutation at position 209, which causes the dibucaine resistant variant. SSCP analysis of fragment 55/53, which covers the K variant coding region, did not show any difference between family members and the control. We therefore decided to sequence this fragment with primers 55/5 and 53 using primer 55/5 as sequencing primer. Sequencing results showed guanine at position 1615 in all family members (data not shown).

Discussion

We have applied PCR-SSCP analysis to characterise the molecular defect in a family with acholinesterasaemia. This technique proved to be a rapid and powerful approach to screen the CHE gene for mutations. The mutations detected, the G to A transition at nucleotide 209 as well as the AG insertion at nucleotide 351, are within fragment 15-13. They both caused characteristic SSCP resulting in four different band patterns for the entire family (fig 1). The SSCP technique is most sensitive when examining DNA fragments of 100 to 300 bp, showing mobility shifts of at least one strand in 70 to 99% of mutations.⁸ The sensitivity of the method decreases with the size of the PCR product and is less than 50% when the fragment is >400 bp. To overcome this size limitation we digested fragment 25-33 (1088 bp) with *HphI* before SSCP analysis.

A number of CHE variants have been described and their molecular bases have been characterised.²⁻⁴ Our interest in this family arose from two observations. Cholinesterase activities in the parents did not seem to fit well with current knowledge of CHE variants. In addition, family members were Jordanian Arabs and little is known about the molecular basis for a silent allele as three children were expected to be homozygous for a silent allele (fig 1). Despite the suspected heterozygosity the mother had a normal CHE activity. The dibucaine inhibition number of the father suggested that he was either homozygous for the dibucaine resistant variant or heterozygous for dibucaine resistance and a silent variant. In either case two of the sons had to be heterozygous for the dibucaine resistant variant, despite their high CHE activity, because they were not carriers of a silent allele.

The mother and the father were found to be heterozygous and three children homozygous for a GGT to GGAG frameshift mutation. This mutation leads to a stop codon at nucleotide

384, generating a truncated protein that lacks the active catalytic site.³ No data are available on the frequency of the silent allele, but it is probably very rare.² Its presence in the mother and the father in this family is most likely because they are cousins.

Furthermore the father was heterozygous for a point mutation at nucleotide 209, known to cause the dibucaine resistant variant.³ The two sons with normal CHE activity were also heterozygous for this mutation. The dibucaine resistant variant shows a reduced affinity for binding cholinester substrates such as succinylcholine and a reduced degree of inhibition by dibucaine.¹³ This variant is clinically important because the muscle relaxant succinylcholine is frequently used in anaesthesia. In whites the atypical allele has a gene frequency of 0.017¹⁴ and about 3 in 10 000 are homozygous for the atypical variant.

In a North American population the dibucaine resistant variant has been found to be closely linked to a polymorphism at position 1615 resulting from a G to A transition (GCA→ACA).⁴ The frequencies of the polymorphic alleles are 0.875 (GCA) and 0.125 (ACA). ACA codes for the K variant, a quantitative variant with an activity reduced by 33%.^{4,12} It is believed that the linkage to the K variant accounts for the fact that atypical serum contains one third less esterase protein than is present in usual serum.^{4,11} SSCP analysis of fragment 55–53, which contains this polymorphism, did not show any differences between the control and family members. We therefore proceeded to sequence this fragment with primer 55/5. We found GCA coding for alanine in all four alleles of the parents. This finding probably explains the normal CHE activity in the two sons despite their being heterozygous for the dibucaine resistant variant. In addition, it supports the hypothesis of Bartels *et al*⁴ that the K variant is responsible for the observed reduction in CHE activity in atypical serum.

As CHE variants and deficiencies have no influence on health apart from prolonged

muscle relaxation after succinylcholine they presumably could evolve without selection pressure to eliminate certain CHE variants from the population. Most of the work on the molecular basis of CHE variants has been done in North American patients.^{3,4} Unfortunately, the authors do not state the ethnic origin of these patients. We could identify the same mutations in this family of Jordanian Arabs, which leads us to suppose that there is considerable molecular homogeneity between ethnic groups. With regard to the dibucaine resistant variant our family did not show the linkage to the K variant that has been reported from North America.⁴

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