Butyrylcholinesterase (BCHE) Genotyping for Post-Succinylcholine Apnea in an Australian Population

Tina Yen, Brian N. Nightingale, Jennifer C. Burns, David R. Sullivan, and Peter M. Stewart*

Background: Measurement of plasma butyrylcholinesterase (BChE) activity and inhibitor-based phenotyping are standard methods for identifying patients who experience post-succinylcholine (SC) apnea attributable to inherited variants of the BChE enzyme. Our aim was to develop PCR-based assays for BCHE mutation detection and implement them for routine diagnostic use at a university teaching hospital.

Methods: Between 1999 and 2002, we genotyped 65 patients referred after prolonged post-SC apnea. Five BCHE gene mutations were analyzed. Competitive oligo-priming (COP)-PCR was used for flu-1, flu-2, and K-variant and direct DNA sequencing analysis for dibucaine and sil-1 mutations. Additional DNA sequencing of BCHE coding regions was provided when the five-mutation screen was negative or mutation findings were inconsistent with enzyme activity.

Results: Genotyping identified 52 patients with primary hypocholinesterasemia attributable to BCHE mutations, and in 44 individuals the abnormalities were detected by the five-mutation screen (detection rate, 85%). Additional sequencing studies revealed mutations in eight other patients, including five with novel mutations. The most common genotype abnormality was compound homozygous dibucaine and homozygous K-variant mutations. No simple homozygotes were found. Of the remaining 13 patients, 3 had normal BChE activity and gene, and 10 were diagnosed with hypocholinesterasemia unrelated to BCHE gene abnormalities.

Conclusion: A five-mutation screen for investigation of post-SC apnea identified BCHE gene abnormalities for 80% of a referral population. Six new BCHE mutations were identified by sequencing studies of 16 additional patients.

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Prolonged post-succinylcholine (SC) apnea is an uncommon but serious adverse event that follows administration of SC or mivacurium, with an incidence estimated at 1 per 1800 anaesthetic cases (1). Approximately 65% of cases are attributable to abnormalities of the butyrylcholinesterase enzyme (BChE; EC 3.1.1.8; OMIM 177400) (2, 3), a plasma esterase that hydrolyzes acetylcholine, other choline esters, and choline ester-containing drugs such as SC. Measurement of the biochemical defect for the identification of individuals with inherited abnormalities is routinely performed by enzyme assay and inhibitor-based phenotyping, but diagnosis using these methods is less than ideal. DNA-based approaches can improve diagnostic precision, and a possibility of new drugs on the market possessing the same gene-based determinants of enzyme hydrolysis means that timely implementation of DNA-based methods in routine analysis will be important so that laboratories have a role in the development of diagnostic pharmacogenetics.

Prolonged post-SC apnea occurs with BChE deficiencies of marked severity (impairment >70%), and the majority are attributable to BCHE gene defects (2). Hypocholinesterasemia can occur secondary to pregnancy, liver disease, malnutrition, and chronic debilitating illnesses, but enzyme impairments are usually mild and rarely cause severe deficiency and prolonged apnea (6% of

1 Nonstandard abbreviations: SC, succinylcholine; BChE and BCHE, butyrylcholinesterase gene and enzyme (protein), respectively; nt, nucleotide; COP, competitive oligo-priming; dNTP, deoxynucleotide triphosphate; WT, wild type; MU, mutation; and SUPAMAC, Sydney University/Prince Alfred Macromolecular Analysis Centre.
cases) (2). Hence, the identification of individuals with inherited BChE variants remains within the province of the laboratory because the demonstration of low plasma cholinesterase activity (BChE) and abnormal enzyme phenotype (defined by responses to dibucaine and fluoride inhibitors) quantify the degree of impairment and confirm its hereditary basis.

As early as 1978, the Danish Cholinesterase Research Unit reported that 13% of individuals with abnormal SC responses were unable to be typed by inhibitor-identified alleles (2), an observation reinforced 4 years later by Morgan (4), who suggested that up to 8–10% of patients were potentially misdiagnosed because of difficulties with phenotyping. Phenotype studies are prone to inaccuracy when enzyme amounts are low, but additional problems of variable enzyme response in different substrates and a diagnostic range of 10 phenotypes contribute to difficulties with identification. Inhibitor-based methods are also incapable of differentiating compound heterozygotes (with the exception of heterozygotes with an atypical allele) because inhibitor numbers frequently overlap in the distributions for the wild type and enzyme variants (5).

DNA-based studies have described at least 44 mutations in the BChE gene that cause primary hypocholinesterasemia, with the D70G (Asp70 → Gly), or dibucaine point mutation in exon 2, the most extensively studied. The amino acid change reduces the binding affinity of BChE enzyme for SC and dibucaine, rendering the molecule ineffective in enzyme hydrolysis (6). Three point mutations encode fluoride resistance, flu-1 (T243M; Thr243 → Met), flu-2 (G390V; Gly390 → Val), and the Japanese flu-variant (L330I; Leu330 → Ile) (7, 8), whereas the silent BChE variant may arise from any of 33 known mutations [reviewed by Primo-Parma et al. (9)].

A mutation detection approach can be used for investigation and characterization of BChE variants because the phenotype is defined, the gene is relatively small [73 kb; four exons interrupted by three introns with exon 1 not translated; coding region 1722 nucleotides], and most abnormalities described are point mutations. However, the implementation of DNA-based methods in biochemistry laboratories is slow or, in the transition, is hampered by limited time and resources, lack of technical expertise, and the financial outlay for equipment and technology. Furthermore, paucity of information about the clinical significance of BChE polymorphisms and uncertainty related to the extent of additional DNA investigation when mutations are not detected are ongoing concerns; therefore, the scope of DNA studies would need to be clarified before genotyping can be offered.

Mindful of these issues, we developed a protocol for analysis of five BChE mutations based on competitive oligo-priming (COP)-PCR and DNA sequencing analysis. During 1999–2002, we genotyped 65 individuals referred for investigation of prolonged post-SC apnea. We provide important information on gene defects responsible for inherited hypocholinesterasemia in our referral population and the prevalence of novel mutations in this condition. We also show results from BChE gene sequencing in cases of SC apnea unexplained by the five-mutation screening and suggest alternative causes for their abnormalities.

### Materials and Methods

AmpliTaq DNA polymerase and MgCl₂ were from Applied Biosystems. Ultrapure water was from Fisher Biotec. Dimethyl sulfoxide (100%) was from Ajax Chemicals. The 5× PCR buffer, pH 9.0, was manufactured in-house using Tris-HCl (Sigma) and enzyme grade ammonium sulfate (ICN Biomedicals). Deoxynucleotide triphosphates (dNTPs) were from Promega. Oligonucleotides (Tables 1 and 2) were synthesized and supplied by Genset Pacific Pty. Ltd. (Lismore, Australia).

### Participants

All patients who were referred for investigation after delayed recovery from procedures involving SC or mivacurium or for apnea risk assessment during 1999–2002 were included. Patients with secondary causes of hypo-
The five cop-cpcr instructions. DNA was eluted in 200 reagent set (Qiagen) according to the manufacturer’s
venous blood with use of the QIAamp Blood-DNA mini
Genomic DNA was extracted from EDTA-anticoagulated
dna extraction
dscribed by the manufacturers
lyzer (Roche Diagnostic), according to the method de-
dbutyrylthiocholine as a substrate on a Hitachi 917 ana-
Total plasma cholinesterase (BChE) was measured with
and the rest were Anglo-Celtic Australian.
backgrounds were diverse: two patients were Asian, five
Wales, except for one from South Australia. The ethnic
age range, 4–83 years). Referrals were from New South
ers were removed.
netic testing, and for analysis, all patient-specific identifi-
was included. All participants were living in Australia at
BChE was not a prerequisite for mutation detection.
cholinesterasemia were excluded. Decreased plasma
BChE was not a prerequisite for mutation detection.
When an entire family was genotyped, only the proband
was included. All participants were living in Australia at
the time of testing. Patients consented to diagnostic ge-
for, and analysis, all patient-specific identifi-
ers were removed.
Study participants included 29 males and 36 females
(age range, 4–83 years). Referrals were from New South
Wales, except for one from South Australia. The ethnic
backgrounds were diverse: two patients were Asian, five
were Southern European, one was from the Middle East,
and the rest were Anglo-Celtic Australian.

TOTAL PLASMA BCH E ACTIVITY
Total plasma cholinesterase (BChE) was measured with
butyrylthiocholine as a substrate on a Hitachi 917 an-
lyzer (Roche Diagnostic), according to the method
described by the manufacturers (10). Activity was cal-
culated in kU/L at 37 °C. BChE enzyme activity was stable
in serum and EDTA plasma stored at 4 °C for up to 7 days.
The reference interval for BChE activity in healthy indi-
viduals was 7–16 kU/L, and the interval was rechecked in
2003 using stored samples from healthy individuals. The
mean (SD) was 10.6 (2.0) kU/L.

DNA EXTRACTION
Genomic DNA was extracted from EDTA-anticoagulated
venous blood with use of the QIAamp Blood-DNA mini
reagent set (Qiagen) according to the manufacturer’s
instructions. DNA was eluted in 200 μL of water in the
final step and stored at −20 °C until required.

COP-PCR
The five BCH E mutations analyzed in the mutation screen
were dibucaine (D70G; Asp70→Gly), silent-1 (sil-1;
G117FS; Gly GGT to GGAG), fluoride-1 (flu-1; T243M;
Thr243→Met), fluoride-2 (flu-2; G390V; Gly390→Val),
and K-variant (A539T; Ala539→Thr; Fig. 1), as summarized
by La Du et al. (11). COP-PCR was used for flu-1, flu-2, and
K-variant, and PCR amplification with direct DNA se-
quencing analysis was used for dibucaine and sil-1 mu-
tations (see results of BCH E genotyping).

Competitive oligonucleotide priming of DNA synthe-
thesis has been described previously in detail (12, 13). In
brief, COP-PCR is a method for allele-specific amplifi-
cation in which two oligonucleotide primers of similar
length compete for annealing to DNA. Competitive prim-
ers are usually identical except for the point mutation,
which is positioned at the middle of the primer [hence
designated wild-type- (WT) or mutation (MU)-specific
primers (14)]. When PCR is performed under competitive
conditions, amplification of the better-matched primer is
favored ≈100-fold over amplification of the primer with a
mismatch (13).

Each COP reaction required a set of three primers (two
competitive primers and one common primer). Duplicate
sets of these primers were synthesized; the first set was
named POS-hex COP-PCR because the MU-specific
primer was labeled with hex fluorophore (6-carboxyfluoro-

<table>
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<th>T° C</th>
<th>Length, bp</th>
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<td>570</td>
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<td>636–618</td>
<td>R: TAC AAT AAC TTC TTC AAC C</td>
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<td>K-EXT</td>
<td>2066–2046</td>
<td>R: CAA TAT TAT CCT TCT GGC ATT</td>
<td>5</td>
<td>47</td>
<td>298</td>
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</tbody>
</table>

a Sequence of human BCHE is GenBank accession no. M16541 J02964. Position +1 is the first nucleotide of exon 1 (TAC, tyrosine, Y).

b Primer annealing site is in intron 1; BC14 is numbered from the intron 1-exon 2 junction (the PCR product from BC14 and BC3 includes the intron-exon junction).

c Primer annealing site is in intron 2 (the PCR product from BC5 and BC13 includes the exon 2-intron 2 junction).

d Primer annealing site is in intron 1; BC14 is numbered from the intron 1-exon 2 junction (the PCR product from BC14 and BC3 includes the intron-exon junction).

e Primer annealing site is in intron 3 (the PCR product of K5 and Kext includes the intron 3-exon 4 junction).

Fig. 1. Schematic diagram of the human BCHE gene.
Arrows show the locations of five BCHE gene mutations; dibucaine (Dib; D70G), Sil-1 (G115FS), Flu-1 (T243M), Flu-2 (G390V), and K-variant (A539T), analyzed by
our protocol. Exons are represented by rectangles, and introns by the interrupted
lines. The hatched portion of an exon denotes the DNA coding region. Exon
length is shown in base pairs below each rectangle.
COP-PCR buffers and reaction mixtures were preoptimized for the detection of the mutation indicated. The flu-1 COP-PCR reaction mixture contained 5× buffer [300 mM Tris-HCl, pH 9.0, 62.5 mM (NH₄)₂SO₄, 2 mM MgCl₂, the four dNTPs (dATP, dCTP, dGTP, and dTTP; 250 μM each), 1 U of AmpliTaq DNA polymerase, and 24 pmol of each primer (Table 1) in a final volume of 20 μL. The flu-2 COP-PCR mixture contained the same 5× buffer, 2 mM MgCl₂, the four deoxyribonucleotide triphosphates, AmpliTaq DNA polymerase, and the flu-2 primers (same amounts as indicated for flu-1). K-variant COP-PCR mixtures contained 5× buffer, 1.5 mM MgCl₂, the four deoxyribonucleotide triphosphates, AmpliTaq DNA polymerase, and K-variant primers (same amounts as for flu-1).

The COP-PCRs for the three mutations were performed simultaneously in a PC-960G gradient thermal cycler (Corbett Research), all with the same cycling conditions. DNA was denatured at 95 °C for 3 min, followed by annealing at 44 °C for 60 s and elongation at 72 °C for 60 s. This was followed by 35 cycles of denaturation (15 s at 95 °C), annealing (30 s at 44 °C), and extension (20 s at 72 °C). The final cycle was followed by 10 min at 72 °C and 1 min at 25 °C. DNA controls with confirmed mutations were verified by DNA sequencing studies.

DNA sequencing of dibucaine and sil-1 loci
An exon 2 region that included the dibucaine (D70G) and sil-1 (G117FS) loci was amplified as a single 570-bp fragment with primers BC14 and BC3 (Table 2). DNA was amplified in 5× buffer [300 mM Tris-HCl, pH 9.0, 62.5 mM (NH₄)₂SO₄, 2 mM MgCl₂, four dNTPs (dATP, dCTP, dGTP, and dTTP; 250 μM each), 1 U of AmpliTaq DNA polymerase, and 8 pmol each of BC14 and BC3 in a final volume of 20 μL. Samples were denatured at 95 °C for 3 min, annealed at 46 °C for 60 s, and elongated at 72 °C for 60 s. This was followed by 35 cycles of denaturation (15 s at 95 °C), annealing (30 s at 46 °C), and extension (20 s at 72 °C). The final cycle was followed by 10 min at 72 °C and 1 min at 25 °C. PCR product was separated by agarose gel electrophoresis to verify the presence of a single 570-bp band, and products were then sent to Sydney University/Prince Alfred Macromolecular Analysis Centre (SUPAMAC) for direct sequencing by the dye dideoxy terminator cycle sequencing system on ABI automated sequencers.

ADDITIONAL SEQUENCING OF BCHE GENE CODING REGIONS
For patient samples in which the five-mutation screen indicated no mutation or the mutations found were inconsistent with BCHE activity, additional BCHE coding region analysis was performed (exons 2–4; Fig. 1). Because the BC14/BC3 region was sequenced for the five-mutation screen, only five additional PCR reactions were required: flu-1com/flu-2com, BC11/BC12, BC5/BC13, BC8/BC9, and K5/Kext (Table 2). For simplicity, the five amplification reactions were performed with the same buffer, MgCl₂, dNTPs, and AmpliTaq as for BC14/BC3. Only the primers and annealing temperatures were different (Table 2). PCR products were sequenced by SUPAMAC, and the electropherogram results were compared with the BCHE exon sequence on the Human Genome Mutation Database (accession no. M16541; J02964; human butyrylcholinesterase mRNA complete coding sequence). All DNA abnormalities were compared with the list of known BCHE mutations. Novel mutations were confirmed by sense and antisense sequencing, with clinical follow-up in family members.

RESULTS
DESIGN OF COP-PCR FOR BCHE MUTATION ANALYSIS
COP-PCR is a method with high specificity for detection of single base-pair changes because annealing between MU-specific and WT-specific primers under competitive conditions overwhelmingly favors amplification of the closest matched primer. The original method used different fluorophore labels for identification of primers (Fig. 2). We chose a single fluorophore and used hex-labeled primer in competition with unlabeled primer (Fig. 3). This modification produced discordant migration of amplimers on the nondenaturing polyacrylamide gel. PCR products with a hex attachment had reduced mobility, and the product appeared 2- to 5-bp larger than its equivalent nonlabeled counterpart. Fluorophore dye attachment is known to affect the electrophoretic migration of DNA fragments (16), but the effect is usually imperceptible with standard laser detectors. In our system, the differences were visible because of the higher resolution of the Corbett analyzer system (~1-bp), and we were able
to use fragment size to deduce the identity of the annealed primer and, hence, the mutation locus.

Because both WT- and MU-specific primers could be tagged with fluorophore, the COP-PCRs were analyzed in duplicate. The two PCRs were arbitrarily called POS-hex and NEG-hex (POS denoting the PCR with the MU-specific primer tagged, and NEG indicating fluorophore on the WT primer; Fig. 3).

Interpretation of the genotype was based on the number and sizes of bands on the gel (shown schematically in Fig. 4A). WT/unaffected DNA samples amplified in POS-hex reactions yielded a single PCR product at the predicted molecular weight. In NEG-hex reactions, the amplimers carried the hex label; therefore, the fragment was 2 bp larger. Thus, after gel separation, the appearance of a lower band in POS-hex and an upper band in NEG-hex samples indicated a WT/unaffected genotype (Fig. 4B). Likewise, patients who were homozygous/affected yielded PCR products with the reversed pattern (one upper band in POS-hex, one lower band in NEG-hex). Heterozygous-affected DNA characteristically revealed two PCR products in approximately equal amounts in POS-hex and NEG-hex lanes because both the MU-specific and WT alleles were present.

**Fig. 2.** COP-PCR for detection of single nucleotide mutations. (A), template DNA is mixed with two oligonucleotide primers that differ by a single DNA base. Under competitive conditions, the correctly matched primer anneals preferentially to the template DNA over the mismatched primer. Primers are labeled with different fluorophores (flu-1 and flu-2). (B), the common primer and the successful COP primer are incorporated in the PCR product. The correctly matched primer is identified by its fluorophore label. Identification of the incorporated COP primer reveals the identity of the template sequence.

**Fig. 3.** Schematic representation of COP-PCR procedure for mutation detection. Patient DNA is amplified in two separate PCRs that contain the primers shown. For simplicity, the primers are designated MU-POS and MU-NEG (MU-NEG is represented as the WT primer). Amplified products are separated by polyacrylamide gel electrophoresis and stained with ethidium bromide. Insert shows the relative positions of hex and unlabeled PCR products. Note that hex-labeled amplimers appear to have larger molecular masses as a result of fluorescent tag-altered mobility.

**INTERPRETATION OF flu-1, flu-2, AND K-VARIANT MUTATIONS BY COP-PCR**

Interpretation of genotype at the flu-1 and flu-2 loci by COP-PCR was straightforward, and from our patient population, four heterozygous/affected patients were identified. In contrast, K-variant was difficult to optimize for COP-PCR under the conditions established for flu-1 and flu-2 detection. K-variant PCR products from POS-hex amplifications adhered to the predicted pattern and specified the correct genotype, as verified by DNA sequencing (not shown), but amplification products from NEG-hex reactions migrated abnormally to the higher molecular weight position irrespective of the patient’s genotype (Fig. 4B).

These anomalies are usually the result of interference, or a partial mismatch from suboptimal cycling conditions, and although they can be eliminated by adjustment of PCR conditions, the cycling conditions in our assay were defined in order to coordinate amplifications at flu-1 and flu-2. Adjustment of pH and Mg\(^{2+}\) concentrations did not improve the specificity (not shown), and because other strategies substantially increased the number of steps and
turnaround time of COP-PCR, the interpretation of K-variant was based on POS-hex reaction products alone (NEG-hex reaction products were not used). Results are shown here for completeness.

DIRECT DNA SEQUENCING FOR DIBUCAIN AND S1-1 MUTATION ANALYSIS

The dibucaine and sil-1 mutations were analyzed by DNA sequencing because their relative proximity enabled them to be coamplified in a single 570-bp product. Interpretation of DNA sequencing results was straightforward. At first, the dibucaine and sil-1 mutations were also analyzed by COP-PCR (with DNA sequencing confirmation of affected individuals), but it became more expeditious for us to proceed directly to sequencing analysis, given the high frequency of dibucaine abnormalities in our population. Interestingly, the sequencing results revealed additional mutations in three patients (N106FS, N96Y, and F28I; described in the “New Mutations” section).

BCHE GENOTYPES IDENTIFIED FROM A PATIENT POPULATION

Sixty-five patients who presented to our service between 1999 and 2002 were genotyped, and 52 were diagnosed with primary hypocholinesterasemia attributable to BCHE mutations (80%; Table 3, with details available in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol49/issue8/). For 44 patients, the five-mutation screen provided complete diagnosis (85% diagnostic capability for this test in our population). BCHE coding region sequencing studies identified additional defects in eight others. Primary hypocholinesterasemia was excluded in 13 patients after the assays failed to identify pathogenic defects in the BCHE gene.

In the 52 patients with hereditary hypocholinesterasemia, the most common DNA abnormalities were dibucaine and K-variant mutations: 47 patients carried the dibucaine mutation, whereas 48 were identified with the K-variant (allele frequencies, 0.72 and 0.74 respectively; Tables 3 and 4). Because of known differences in atypical allele frequencies in difference races [reviewed in Ref. (17)], patient ethnicity was recorded. Because the numbers were low (Table 5), ethnicity had no significant impact on the prevalence of the mutations identified. The allele frequency of the dibucaine mutation in Australia was consistent with observations from studies in Caucasian populations.

GENOTYPES IN PRIMARY HYPOCHOLINESTERASEMIA

Primary hypocholinesterasemia-causing BCHE deficiency has autosomal recessive inheritance, and all 52 patients diagnosed had compound gene defects (i.e., compound heterozygotes and compound homozygotes). No homozygotes with fluoride or silent mutations were found, and none of the patients had an isolated homozygous dibucaine defect. Co-inheritance of dibucaine and K-variant occurred in 88% of patients with primary hypocholinesterasemia.

The most common genotype responsible for inherited hypocholinesterasemia was compound homozygous dibucaine/homozygous K-variant (AAKK; 44%). The other three dibucaine/K-variant combinations were next in

<table>
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<th>Diagnosis</th>
<th>n</th>
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<td>Primary hypocholinesterasemia</td>
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<tr>
<td>AAKK</td>
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<td>Total</td>
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</table>

* A denotes the dibucaine mutation; K denotes the K-variant mutation.
frequency: compound heterozygous dibucaine/heterozygous K-variant (AK; 13%), compound homozygous dibucaine/heterozygous K-variant (AAK; 7.7%), and compound heterozygous dibucaine/homozygous K-variant (AKK; 7.7%; Table 5 with details in the online Data Supplement). In total, 73% of patients with primary hypocholinesterasemia had abnormalities at the dibucaine and K-variant loci only. The remaining 27% of patients had various genotypes, such as dibucaine and K-variant plus a third mutation, unusual BCHE mutations, or rare combinations where dibucaine and K-variant linkage was absent. Five patients had sil-1, flu-1, or flu-2 mutations, collectively representing 10% of all cases (allele frequencies, 0.01, 0.02, and 0.02, respectively; Table 4).

For the diagnosis of primary hypocholinesterasemia, a correlation between gene defect and the magnitude of enzyme impairment was required. Fifty of 52 patients diagnosed had genotypic abnormalities that supported the degree of BChE enzyme impairment discovered. Two patients had BChE activities below the mean and SD of activities calculated from the other patients in their genotype group. Patients 57 and 65 had compound heterozygous dibucaine/heterozygous K-variant and compound heterozygous dibucaine/homozygous K-variant abnormalities (see the online Data Supplement at http://www.clinchem.org/content/vol49/issue8/), which are genotypes consistent with primary hypocholinesterasemia. However, the severity of enzyme deficiency appeared atypical for the genotype in both cases (4.8 and 1.7 kU/L, respectively; Table 5). Furthermore, no other BCHE gene abnormalities were found on sequencing analysis to account for the discrepancy.

RESULTS FROM BCHE SEQUENCING, INCLUDING NEW MUTATIONS

Gene sequencing of BCHE coding regions (exons 2–4) revealed additional BCHE mutations in 8 patients, increasing the total number with primary hypocholinesterasemia to 52 (see Table 3 and the detailed results in the online Data Supplement). Two mutations are well described; Ile6FS del-A (also known as sil-2) is a frameshift mutation that creates a new stop codon that prematurely truncates polypeptide synthesis [described in Ref. (9)]. The G115D mutation (Gly 115 Asp; also named sil-7) encodes a BChE variant with marked instability (18). Our sequencing analysis also uncovered six new BCHE mutations: N106FS, R424X, E460K, F28I, N96Y, and R386C (Fig. 5; patients are also listed in the online Data Supplement).

Patient 35. Patient 35 had two novel mutations. N106FS-insA was a single A-nucleotide insertion in exon 2 that changed the codon for Asn106 to Lys, and shifted the reading frame +1 (Fig. 5A), creating a new stop codon 23 amino acids downstream at position 129. Truncation was upstream to the active center Ser198, therefore, catalytic activity was absent, and this variant reached only 22.5% of the length of the mature protein. The A insertion occurred

### Table 4. BCHE allele frequencies. a

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<tr>
<td>sil-1</td>
<td>0.010</td>
<td>0.008</td>
</tr>
<tr>
<td>sil-2</td>
<td>0.010</td>
<td>0.008</td>
</tr>
<tr>
<td>sil-7</td>
<td>0.029</td>
<td>0.023</td>
</tr>
<tr>
<td>flu-1</td>
<td>0.019</td>
<td>0.015</td>
</tr>
<tr>
<td>flu-2</td>
<td>0.019</td>
<td>0.015</td>
</tr>
</tbody>
</table>

a BCHE mutation allele frequency was determined by counting the abnormal alleles in the population: primary hypocholinesterasemia, n = 52; total patient population, n = 65.

### Table 5. BCHE genotypes: Group characteristics.

<table>
<thead>
<tr>
<th>Genotype a</th>
<th>AAKK</th>
<th>AK</th>
<th>AAK</th>
<th>AKK</th>
<th>Three mutations b</th>
<th>New mutations</th>
<th>sil/flu c</th>
<th>Not primary d</th>
</tr>
</thead>
<tbody>
<tr>
<td>BChE activity, kU/L</td>
<td>3.32</td>
<td>8.13</td>
<td>3.1</td>
<td>6.25</td>
<td>2.1</td>
<td>5</td>
<td>3</td>
<td>12</td>
</tr>
<tr>
<td>Mean</td>
<td>1.8–5.3</td>
<td>6.7–9.4</td>
<td>2.9–3.6</td>
<td>5.4–7.3</td>
<td>1.3–3.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>0.89</td>
<td>1.29</td>
<td>0.34</td>
<td>0.82</td>
<td>0.75</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>Total patients, n</td>
<td>23</td>
<td>7</td>
<td>4</td>
<td>4</td>
<td>6</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>Males, n</td>
<td>13</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>4</td>
<td>3</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Females, n</td>
<td>10</td>
<td>5</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>Race/Ethnicity e</td>
<td>e2, me1</td>
<td>e1</td>
<td>e2, a1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


b Three mutations of BCHE gene identified.

c Genotyped with sil-1, flu-1, or flu-2 mutations.

d Individuals with primary hypocholinesterasemia were excluded.

e BCHE activity reference interval, 7–16 kU/L.

f Race/ethnicity (race, number of individuals): e, European; me, Middle Eastern; a, Asian.
within a train of A repeats, sites known to be predisposed to frameshifts. The truncated product of N106FS-insA was the same length as the sil-1 mutation polypeptide product (G117FS; Gly -GGT to -GGAG), except for 11 additional mistranslated amino acids in the N106FS polypeptide (N106FS was 11 codons upstream of G117FS).

The second novel mutation (R424X) was a C-to-T substitution that changed Arg424 to a stop codon (Fig. 5B). Although the product was truncated close to the COOH terminus (80% of full length), enzyme function was unlikely because deletion constructs have shown a 525-amino acid polypeptide is the minimum length for BChE compatible with function (19).

Pedigree studies on the patient’s two daughters indicated both are carriers of the N106FS-insA but not R424X (Fig. 6A). The daughters had mild hypocholinesterasemia, suggesting that the muta-
tions were interallelic, with a nonfunctional protein encoded by R424X.

Patient 56. Patient 56 had a novel mutation (E460K) that was a G-to-A substitution that changed Glu460 to a Lys (Fig. 5C). She also carried the sil-7 mutation (Fig. 6B), which accounted for her severe hypocholinesterasemia.

Patient 39. Patient 39 had a novel mutation (F28I), which was a T-to-A substitution close to the NH2 terminus that converted Phe28 to Ile (Fig. 5D). This proband was heterozygous for dibucaine and K-variant, hence, compound heterozygous for three mutations (Fig. 6C). The severity of BChE deficiency indicated that F28I encoded a pathogenic variant because family studies revealed that F28I was separate from dibucaine and K-variant.

Patient 51. Patient 51 had a new mutation (N96Y), which was an A-to-T substitution that changed Asn96 to Tyr (Fig. 5E).

Patient 8. Patient 8 had a heterozygous R386C mutation, which was a C-to-T substitution that changed Arg386 to Cys (Fig. 5F). Family studies have yet to be performed.

PRIMARY HYPOCHOLINESTERASEMIA EXCLUDED
From 65 patients genotyped, 13 had primary hypocholinesterasemia excluded. Three had normal BChE activities with a wild-type \( BCHE \) genotype (patient details are available in the online Data Supplement at http://www.clinchem.org/content/vol49/issue8/). Three patients had hypocholinesterasemia with \( BCHE \) gene defects: two K-variant homozygotes (patients 6 and 21), and one...
dibucaine heterozygote (patient 4; shown in the Data Supplement). Their enzyme activities were low compared with the reduction of 30% usually observed for K-variant homozygotes and the 17–20% expected for dibucaine heterozygotes (20); however, neither genotype causes primary hypocholinesterasemia. Additional sequencing studies failed to demonstrate other abnormalities; therefore, secondary deficiencies were suggested. The last seven patients had hypocholinesterasemia with no corroborating BCHE mutations, despite sequencing of BCHE coding regions (patient details are available in the online Data Supplement).

**Discussion**

Characterization of inherited BChE variants by inhibitor-based phenotyping is a well-described technique capable of identifying atypical, fluoride, and silent alleles. Difficulties arise with detection of compound heterozygotes or patients who possess rare or new mutations. This is because phenotyping lacks specificity (as shown when different genotypes encode the same inhibitor phenotype, e.g., AA and AS) and has relative insensitivity (especially for mutations that do not affect the regions recognized by common inhibitors). A DNA-based approach to diagnosis may be of value, but to date BCHE genotyping has been reported after analysis of one or a few mutations. Furthermore, it is unknown whether the expense and effort of genotyping yields results that are significantly more informative than phenotyping on a population scale or as clinically important as they are purported to be in family studies after gene sequencing is used to identify mutations. Our approach to BCHE genotyping considered issues of time- and cost-effectiveness, with a method for identification of common abnormalities and optional supplementary analysis by DNA sequencing when the five-mutation screen was inconclusive. We characterized the BCHE gene abnormalities in 65 patients from our referral population and report the prevalences of mutations and genotypes identified, with the clinical and laboratory implications of the distributions found.

The importance of the dibucaine mutation in patients with primary hypocholinesterasemia was undisputable. All of the homozygous patients and 19 of 24 compound heterozygotes possessed dibucaine abnormalities, indicating a frequency of 90% in patients with inherited hypocholinesterasemia. However, none of the patients had simple homozygous genotypes. Patients were either compound dibucaine homozygotes or compound heterozygotes, with both groups in approximately equal numbers (Table 6). This is the first report of the compound genotype-prevalence in this condition.

The mutation relationship responsible for most compound genotypes was coexpression of dibucaine and K-variant (88% of patients). Compound homozygous dibucaine/homozygous K-variant was the most common genotype, accounting for 44% of inherited hypocholinesterasemia. A significant role for K-variant was not surprising given the allele frequency of 13–22% in the general population (homozygote frequency, 1 in 65) (21). The K-variant, G-to-A base substitution that changes Ala539 to Thr, manifests as a 30% reduction in circulating BChE in K-variant homozygotes (21). In the presence of the dibucaine mutation, which impairs the affinity of BChE for SC, a K-variant mutation contributes significant additional enzyme abnormality (21). The mutations are linked, as shown previously by Bartels et al. (21), with the K-variant identified in 89% of individuals with the dibucaine allele. The prevalences of K-variant and dibucaine were remarkably similar in our population (allele frequencies, 0.72 and 0.74 for dibucaine and K-variant, respectively), and co-association was slightly more frequent (K-variant present in 96% of patients with the dibucaine mutation).

Although linkage is well described, the number of dibucaine and K-variant mutations did not always correlate in the genotypes of our patients. Identical numbers of dibucaine and K-variant alleles occurred in 30 of 52 patients, indicating that 22 patients exhibited a discordant number of dibucaine or K-variant mutations or that one of the mutations was identified in combination with other mutations. Because it appeared that the K-variant dosage was not universally predicted by the number of dibucaine mutations, we determined the contribution of K-variant to BChE activity in patients coexpressing both mutations. BChE activities in dibucaine homozygotes were unaffected by K-variant hetero- or homozygosity (Table 5), but K-variant homozygosity and heterozygosity altered the activity of BChE in dibucaine heterozygotes (6.25 vs 8.13 kU/L, respectively), which is similar in magnitude to the

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**Table 6. BCHE mutation frequencies.**

<table>
<thead>
<tr>
<th>Group/category</th>
<th>Primary hypocholinesterasemia</th>
<th>Patient population</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>Homozygous dibucaine</td>
<td>28</td>
<td>54</td>
</tr>
<tr>
<td>Heterozygous dibucaine</td>
<td>19</td>
<td>36</td>
</tr>
<tr>
<td>WT/unaffected dibucaine</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>Mutations</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dibucaine</td>
<td>47</td>
<td>90</td>
</tr>
<tr>
<td>K-variant</td>
<td>48</td>
<td>92</td>
</tr>
<tr>
<td>sil-1</td>
<td>1</td>
<td>1.9</td>
</tr>
<tr>
<td>sil-2</td>
<td>1</td>
<td>1.9</td>
</tr>
<tr>
<td>sil-7</td>
<td>3</td>
<td>5.8</td>
</tr>
<tr>
<td>flu-1</td>
<td>2</td>
<td>3.8</td>
</tr>
<tr>
<td>flu-2</td>
<td>2</td>
<td>3.8</td>
</tr>
<tr>
<td>Genotype profile</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dibucaine and K-variant</td>
<td>46</td>
<td>88</td>
</tr>
<tr>
<td>Dibucaine or K-variant</td>
<td>49</td>
<td>94</td>
</tr>
<tr>
<td>Additional mutations</td>
<td>8</td>
<td>15</td>
</tr>
<tr>
<td>BCHE with three mutations</td>
<td>6</td>
<td>12</td>
</tr>
<tr>
<td>BCHE novel mutation</td>
<td>5</td>
<td>10</td>
</tr>
</tbody>
</table>

*Frequencies of BCHE mutations and mutation categories are shown for patients according to population. Primary hypocholinesterasemia, n = 52 patients; total patient population, n = 65.*
activities reported by Bartels et al. (21). Hence, the K-variant locus was important for genotyping because the prevalence of the K-variant was high, its allele dosage was unpredictable, and the magnitude of its effect on enzyme phenotype was sufficiently large to alter the interpretation.

The frequency of compound heterozygotes in primary hypocholinesterasemia was surprisingly high (representing almost one-half of all patients), and compound heterozygous dibucaine/heterozygous K-variant was the second most frequent genotype identified. However, compound heterozygosity was not limited to dibucaine/K-variant combinations. Other BCHE mutations contributed to the compound genotype in 27% of cases, including rare mutations (sil-1, flu-1, and flu-2), a multiplicity of mutations, and novel mutations. The diversity and complexity of these mutation combinations clearly explained the inability of inhibitor-based phenotypic criteria to define some variants. It also indicated that DNA-sequencing instrumentation or access to a commercial referral service is required to achieve the diagnostic success shown here. In fact, analysis of a limited number of mutations could be ill advised because our findings suggested up to 27% of genotype results would be equivocal in the absence of DNA sequencing studies.

BCHE genotyping was useful for confirming the absence of BCHE gene defects in a small number of patients. Patients with the wild-type BCHE and normal BChE activity were usually individuals referred for risk assessment because of a known family history of SC sensitivity. Occasionally patients are genotyped on the strength of clinical suspicion, despite a BChE activity within reference values, but to date no BCHE mutations have been identified in our patients with BChE activity within our reference interval. Explanations for prolonged apnea with normal BChE activity are SC overdose, poor intubation technique, electrolyte instability, and respiratory insufficiency from other medical causes.

We identified patients with hypocholinesterasemia and nonpathogenic BCHE mutations, where the diagnostic problem was enzyme impairment disproportionate to the nonpathogenic genotype. Inconsistencies or small exaggerations in BChE activity measurements may indicate the presence of secondary factors or prompt a review if measurements were supplied from a different laboratory.

The final group of patients had unexplained hypocholinesterasemia; we found no BCHE defects in these patients, even after exhaustive DNA sequencing. Few explanations exist, apart from failure to exclude secondary hypocholinesterasemia or sample collection too soon after exposure to SC. Abnormalities in noncoding regions of the gene is one possibility, particularly because our sequencing studies were limited to the gene coding regions. To date, however, only 1 of 44 BCHE mutations discovered is in a noncoding region (intron-2/exon-3 boundary, −8nt G) (9). Two other abnormalities have been described, an exon 1 defect (nt −116) and an exon 4 untranslated region mutation (nt −1914), both in linkage disequilibrium with the K-variant; however, their pathologic significance has yet to be shown (21). Some authors have attributed hypocholinesterasemia to involvement of another, unknown protein or factor that modifies the expression of BChE enzyme activity. This was suggested by Dey et al. (22) when no mutation explanation could be found for 2 of 10 individuals with severe hypocholinesterasemia, in spite of complete BCHE gene sequencing. The seven patients with unexplained hypocholinesterasemia in our study represented 11–15% of the patient population and resembled the 20% undiagnosed by Dey et al. (22); therefore, a study of other proteins affecting BChE function may be worthy of future investigation.

The prevalences of mutations identified in our study compared favorably with those described with use of biochemical inhibitors, even with slight differences in patient composition. In 1978, Viby-Mogensen and Hanel (2) typed 225 Danish patients with silent phenotypes and determined that the phenotype composition was 47% homozygous atypical allele, 13% heterozygous atypical allele, 9% heterozygous silent allele, 3% heterozygous fluoride allele, 6% rare genotypes, and 13% who could not be classified diagnostically by biochemical criteria. Our referral population was composed of 43% dibucaine homozygotes, 31% dibucaine heterozygotes, 8% silent mutations, 6% fluoride mutations, 12% rare mutations, and 11% with genotypes that could not be resolved. The relative agreement between populations was remarkable and suggested that the majority of patients would not have their phenotype classification altered by genotyping and could benefit from the increased diagnostic precision of mutation detection strategies.

In conclusion, our work genotyping patients with prolonged post-SC apnea revealed high prevalences of dibucaine and K-variant defects in primary hypocholinesterasemia; therefore, a mutation screening approach was suitable for diagnosis in these individuals. However, we also showed that BCHE mutation heterogeneity complicated the genetic diagnosis for 27% of patients, particularly because six novel mutations were identified during this study. We used a five-mutations screening approach, which was cost-effective, produced results in 14–21 days, and was suitable for a hospital diagnostic laboratory. We also showed that with additional DNA sequencing, a full diagnostic service can be provided and that a combination of approaches was the most efficient use of DNA detection strategies currently available.

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References


