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Genotyping the Butyrylcholinesterase in Patients with Prolonged Neuromuscular Block after Succinylcholine

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Background: **Succinylcholine remains the standard neuromuscular blocking drug for tracheal intubation in emergency situations. The short duration of action is due to its rapid hydrolytic degradation by butyrylcholinesterase (plasmacholinesterase). Multiple variants of this enzyme are known (A, F, S, H, J, K variants) with different effects on enzyme activity. This study was undertaken to evaluate the use of molecular genetic methods in patients with clinically prolonged neuromuscular block.**

Methods: **Nine patients with a neuromuscular block of 14 min to 5 h were selected. All four exons of the butyrylcholinesterase were amplified by polymerase chain reaction and analyzed by automated sequencing. Molecular genetic results were compared with clinical relaxation time and with biochemical test results (total butyrylcholinesterase activity, dibucaine and fluoride inhibition).**

Results: **Seven of nine patients were mutation carriers. Five of these had more than one mutation. The A and K variants were the most frequent variations. Three of four patients who were homozygous for the A variant were also carriers of the K allele. The authors identified one novel mutation (G1294T) introducing a stop codon at amino acid position 432. The duration of neuromuscular block was substantially different between patients with identical butyrylcholinesterase genotypes.**

Conclusions: **Variations in the genetic sequence of butyrylcholinesterase are frequent in patients with prolonged duration of action of succinylcholine. Direct sequencing of the whole butyrylcholinesterase gene is an appropriate method for genotyping and, accordingly, should be used in future clinical studies with drugs metabolized by this enzyme (*e.g.*, succinylcholine, mivacurium).**

SUCCINYLCHOLINE remains the drug of first choice for facilitating tracheal intubation in emergency patients and in patients with a high risk of gastroesophageal regurgitation.[1–4] The short duration of action of succinylcholine is due to its rapid hydrolization by the enzyme butyrylcholinesterase (BCHE; benzoylcholinesterase, plasmacholinesterase, pseudocholinesterase, or acylcholine acylhydrolase).

A remarkably prolonged duration of action of succinylcholine in some patients led to the discovery of one of the first pharmacogenetic disorders, called *postanesthetic apnea*.[5] Besides the usual (U) variant of BCHE, three qualitative variants with altered hydrolyzing activity were identified: the atypical (A), fluoride-resistant (F), and silent (S) variants. In addition, three quantitative variants (H, J, and K) with decreased enzyme concentration but normal activity have been described (table 1).[6] Biochemical methods use butyrylcholine as an *in vitro* substrate for BCHE, and total BCHE activity as well as enzyme inhibition by dibucaine or fluoride is measured. The A and F variants are characterized by normal total activity *in vitro* but reduced inhibition by dibucaine and fluoride, respectively. Although thousands of patients have been investigated by these biochemical methods and "genotyped" according to BCHE activity and inhibition,[7] genotyping by biochemical methods is known to be inadequate.[8–10]

The identification of a single gene locus encoding for BCHE on chromosome 3q26 allowed molecular genetic techniques to be used for investigations in patients with reduced BCHE activity.[11,12] A variety of mutations responsible for most variants has been published.[6,9] However, only limited data are available describing the relation between clinical data, *i.e.*, duration of neuromuscular block by succinylcholine, biochemical analyses, and molecular genetic investigations.

In the current study, we analyzed the sequence of BCHE gene in patients with prolonged duration of action of succinylcholine. The aim was to investigate the adequacy of patient selection with possible BCHE deficiency on the basis of simple clinical criteria and to compare phenotypic presentation and molecular genetic results in these subjects. We also wanted to determine the feasibility and advantages of full-length sequencing of the BCHE gene over a screening program for known mutations only.

Materials and Methods

Patient Selection

The local ethics committee (ethical commission of Basel, Switzerland) approved the study protocol. Members of the study group selected patients having a prolonged duration of action of succinylcholine during routine anesthetic practice. Neuromuscular function was measured using continuous 1-Hz single-twitch supramaximal stimulation at the ulnar nerve and tactile monitoring of the adductor pollicis longus by the anesthesiologist in charge. The time from injection of succinylcholine until initial twitch response was measured. Duration of more than 10 min was defined as

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Table 1. The Variants of Butyrylcholinesterase

Name	Abbreviation	Mutation	Allele Frequency	Description
Usual	U		0.85	Normal
Atypical	A	A209G	0.018	Reduced activity, dibucaine resistant
Fluoride resistant	F	C728T, G1169T	0.002	Reduced activity, fluoride resistant
Silent	S	Multiple	?	No activity
H	H	G424A	?	Approximately 10% reduced concentration
J	J	A1490T	0.002	Approximately 33% reduced concentration
K	K	G1615A	0.128	Approximately 66% reduced concentration

prolonged. Patients were contacted 24–48 h after administration of succinylcholine and asked for written, informed consent for molecular genetic analyses. In addition, two patients (patient 2 and 3) with a history of prolonged duration of BCHE were referred from other hospitals.

Biochemical Investigations

Determination of total BCHE activity, dibucaine inhibition, and fluoride inhibition was performed in routine laboratories of the institutions referring the patients. Because there were three laboratories with different ranges of normal values, BCHE activity is indicated as percentages of the reference value.

Molecular Genetic Investigations

Blood samples were collected in 7.5-ml tubes containing potassium-EDTA. The blood samples were stored at −80°C until DNA isolation. Genomic DNA was isolated from whole blood samples using a QIAamp DNA blood mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. DNA was eluted in 100 μl Tris-buffer and stored at −20°C until required. DNA concentration was calculated by spectrophotometry.

The noncoding exon 1 and the other three exons covering the coding sequence of the mature protein of BCHE were amplified by polymerase chain reaction (PCR) using a set of six primer pairs as indicated in table 2. Because of its size, exon 2 was covered by three primer pairs. PCR was performed in a 25-μl reaction volume with approximately 100 ng genomic DNA, 0.4 μM of each primer, and 2.5× Hot Start Master Mix (Eppendorf AG, Hamburg, Germany). The cycling program was started with a denaturation step of 5 min at 95°C, subjected to 40 cycles of 30 s at 94°C, 45 s at annealing primer-specific temperature (table 2), and 1 min at 72°C, followed by a final extension of 5 min at 72°C. The PCR products were purified with Qiaquick PCR purification kit (Qiagen GmbH) and eluted in 50 μl EB buffer. Purified PCR product was screened by direct sequencing using an ABI PRISM 3730 sequencer (Applied Biosystems, Foster City, CA) with a Big Dye Terminator Sequencing kit (Applied Biosystems). The reaction mixture contained 4 μl purified PCR product, 2 pmol primer, and 2 μl sequencing reagent mix. The reactions were subjected to 25 cycles of 30 s at 94°C, 30 s at 50°C, and 4 min at 60°C. PCR primers were used as sequencing primers. Both strands were analyzed and aligned together with the wild type sequence (Genbank NT_005612) using the Staden sequence analysis package available through the World Wide Web.# If a novel mutation was identified, the PCR reaction and the sequencing were repeated to confirm the sequence variation.

Results

Patient Characteristics

Clinical data of the nine patients included in the study are summarized in table 3. None of the patients received drugs known to interact with BCHE activity. Because inhalation anesthetics interact with neuromuscular transmission, their use was limited to a maximum of 1 minimal alveolar concentration.

Laboratory Results

Clinical data, results of biochemical tests, and results of genetic analysis are summarized in table 4. All but one patient (patient 7) revealed pathologic values in the biochemical tests. In patient 5, biochemical analyses

Table 2. PCR Primer Sequences and Conditions for Amplification of All Four BCHE Exons

Pair	Primer	Size, bp	Annealing, °C	Sequence 5′-3′
1	F1	261	53	AACAGATTTCAAGTTGCTGCTG
	R1			TCATCCCACAGAATGAGCTTT
2	F2-1	450	56	TCTTTTGCTCTGCATGCTTATTG
	R2-1			CTTTCAACCCGAGCCAGAAA
3	F2-2	623	60	TGGATTCCAGCACCTAAACC
	R2-2			TATGTCTGGCATGTCAGTGAGA
4	F2-3	614	62	AACTTTGGTCCGACCGTGGAT
	R2-3			AAAACGGATCAAACCAAGCCAG
5	F3	407	62	AGCCCAGGTTCACATACGTT
	R3			CACCGTGCCTTGGAGAGTAT
6	F4	750	60	AAAATGGCTTTTGTATTCGAAATTA
	R4			GTGGCTGAGCCTCTCATTTT

The labeling of the primers indicates the direction (forward/reverse) and the exon amplified. Because of its size, exon 2 is covered by three primer pairs.

BCHE = butyrylcholinesterase; bp = base pair; PCR = polymerase chain reaction.

Staden sequence analysis package. Available at: http://www.mrc-lmb.cam.ac.uk/pubseq/. Accessed January 19, 2005.

Table 3. Patients' Clinical Data

Patient No.	Sex	Age, yr	Surgery	Succinylcholine, mg/kg	Relaxation, min	Additional Information
1	M	22	Bleeding after tonsillectomy	1.2	15	
2	M	77	Knee surgery	1.2	120	Surgery was 25 yr previously
3	F	21	Cesarean delivery	1.5	300	
4	F	7	Maxillary fracture	1.5	150	
5	M	27	Shoulder surgery	1.2	120	
6	M	73	Peripheral vascular surgery	1.1	45	Revision 4 hours after first surgical intervention with a blood loss of 2,000 ml
7	M	82	Femoral fracture	1.1	14	Prostate cancer
8	M	73	Thoracic surgery	0.8	60	Pleural mesothelioma
9	M	23	Pelvic fracture, appendicitis	1.0	17, 12	Two anesthesias within 1 week

were not performed. Molecular genetic investigations revealed mutations of the BCHE gene in seven of the nine patients. Five patients had more than one mutation in their BCHE genes. All carriers of the atypical (A) variant were homozygous; three of these also carried the mutation responsible for the K variant. In addition, we discovered a new mutation at position 1294, where a guanine is replaced by a thymidine. This base exchange at the end of exon 2 leads to the stop codon TAA at amino acid position 432, resulting in a shorter and probably inactive protein. The duration of relaxation was found to differ in patients carrying the same genotype.

Discussion

Molecular genetic analyses have shown biochemical methods to be imprecise in determining BCHE genotypes.[7–10] La Du[8] found 20–25% of all persons diagnosed as homozygous for the A variant (genotype AA) by biochemical methods to be compound heterozygous for the A and S variants (genotype AS) when investigated by molecular genetic methods. In addition, biochemical testing cannot distinguish between primary and secondary BCHE deficiency, the latter caused by drugs, hepatic disease, pregnancy, and carcinomas. We selected patients with possible BCHE deficiency based on simple clinical criteria. Seven of nine patients with an interval of more than 10 min from succinylcholine injection until initial twitch response carried at least one mutation in the BCHE gene. There were remarkable differences in the duration of neuromuscular block after succinylcholine administration in patients with identical BCHE genotypes. Our results show that full-length sequencing of the BCHE gene is feasible, and we found a novel mutation at nucleotide position 1294. Shortcomings in this investigation include the small sample size and that the lack of a control group did not allow for calculation of allele frequencies or phenotypic characterization of certain mutations. In addition, neuromuscular monitoring was only based on tactile train-of-four. Although objective measurements using acceleromyometry are desirable to establish recovery from nondepolarizing neuromuscular blocking drugs, tactile train-of-four is still a valuable method for intraoperative monitoring of neuromuscular function.[13]

The K variant had a very high prevalence (table 1). Bartels *et al.*[14] demonstrated linkage disequilibrium between the K and A variants; the K variant was present in 17 out of 19 genes bearing the A variant. This was also reflected in our patients, because the A and K variants

Table 4. Patient Characteristics and Laboratory Results in Patients with Prolonged Duration of Action of Succinylcholine

Patient No.	Relaxation, min	Total BCHE Activity, %	Dibucaine Inhibition, %	Fluoride Inhibition, %	A Variant	K Variant	T305AG	Other Mutations
1	15	64	66	36	−/−	−/−	+/−	C551T
2	120	9	0	0	−/−	−/−	+/−	G1294T
3	300	22	20	26	+/+	+/+	−/−	
4	150	94	21	ND	+/+	+/+	−/−	
5	120	ND	ND	ND	+/+	+/−	−/−	
6	45	67	63	35.4	−/−	−/−	−/−	
7	14	100	73	43	−/−	−/−	−/−	
8	60	55	22	ND	+/+	−/−	−/−	
9	17, 12	76	71	40	−/−	+/−	−/−	

Total butyrylcholinesterase (BCHE) activity is indicated as percentages of lower normal limit. Presence (+) or absence (−) of the A, K, or S (T305AG) allele are indicated. Patient 9 had two anesthesias within 1 week; therefore, two durations are indicated.

ND = not done.

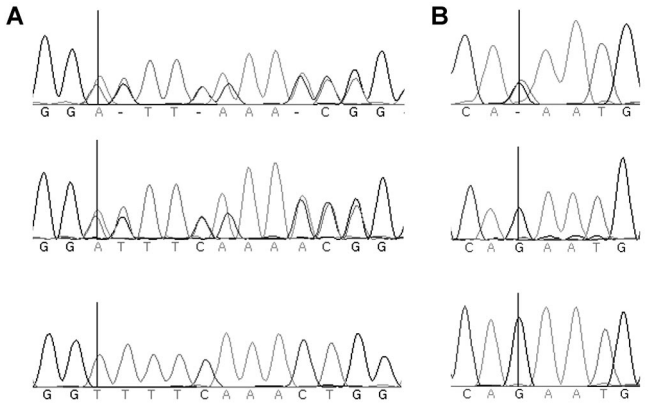

Fig. 1. Representation of DNA sequencing electropherograms. Each peak represents a DNA nucleotide. Double peaks show heterozygous alleles. (*A*) Sequence region of a silent variant (mutation T305AG), which leads to a shift of the reading frame. (*B*) Sequence region of mutation G1294T. A *vertical bar* indicates the position of the mutation. The *top row* represents sequences of patient number 2, the *middle row* represents those of his sister, and the *bottom row* represents sequences of a control (wild-type) person.

were the most frequent variations and three of four patients who were homozygous for the A variant also carried the K allele. Patients who are homozygous for a BCHE mutation have less enzyme activity than those who are heterozygous. In addition, patients carrying compound mutations (*i.e.*, carriers of different mutations in the homozygous or heterozygous state) have lower enzyme activity than carriers of single mutations.[14]

In patients 1 and 2, a frame shift mutation at position 117 (T305AG) was identified. This mutation leads to a premature stop codon at amino acid position 129, known to be responsible for the S variant of BCHE. In both patients, a second mutation was present: mutation C551T in patient 1 and mutation G1294T in patient 2. Mutation C551T was described earlier and characterized as undetectable by conventional biochemical methods.[15] Because relatives were unavailable for genotyping, we could not determine whether the mutations T305AG and C551T are on the same or different alleles. Patient 2, who carried the S variant T305AG, was compound heterozygous for mutation G1294T. This mutation leads to a premature stop codon (TAA) at amino acid position 432. The resulting protein is shorter than the minimal length required for a functional BCHE of 525 amino acids.[16] The sister of this patient also carried the S variant, whereas mutation G1294T was not identified (fig. 1). Therefore, mutation G1294T and the S variant probably reside on different alleles in patient 2. Both BCHE alleles in this patient would encode for inactive proteins, a finding that correlates with the eightfold longer clinical relaxation time compared with patient 1.

Secondary deficiency of BCHE activity, such as pregnancy, chronic illness, and acute major blood loss, might have contributed to differences in the duration of neuromuscular relaxation in patients with the same genotype, such as patients 3 and 4, as well as in patients 6 and 7, who had normal molecular genetic investigations.

Today, molecular genetic methods are the only appropriate techniques for genotyping. The choice of a molecular genetic method must be efficient and cost effective. Screening for only one mutation[17] or sequencing the whole gene only if known BCHE mutations are absent[18] might be cost efficient, but these methods are not complete. With such an approach, we would have certainly missed the mutations C551T and G1294T in the patients carrying the S variant T305AG in our study.

The rapid recovery of neuromuscular function after succinylcholine administration is commonly thought to prevent severe hypoxia in the case of impossible ventilation. This margin of safety was challenged by a calculation of Benumof *et al.*,[19] who predicted significant hemoglobin desaturation before functional recovery. Heier *et al.*[20] confirmed hemoglobin desaturation below 80% in 4 of 12 healthy volunteers after administration of 1 mg/kg succinylcholine. As a consequence, several authors investigated the influence of dose reduction on the duration of action of succinylcholine. Kopman *et al.*[21] measured onset time and recovery after succinylcholine doses of 0.4, 0.6, and 1 mg/kg in 45 intubated patients. The times to 90% twitch recovery were 6.6, 7.6, and 9.3 min, but maximal recovery times were 10, 10.5, and 11 min, respectively. El-Orbany *et al.*[22] investigated the influence of dose reduction on intubation conditions and the time until spontaneous ventilation in 115 patients. In all of these investigations, BCHE genotypes of the patients or probands were unknown. The high prevalence of BCHE sequence variations, especially the allele frequency of 0.128 of the K variant,[14] might contribute to the variability of measured parameters. This variability makes the choice of an optimal individual dose of succinylcholine very difficult.[23]

Our data support the selection of patients to be included in molecular genetic studies of BCHE on the basis of simple clinical parameters. Full-length sequencing of the BCHE gene in these patients has the potential to reveal novel mutations. Future clinical investigations on drugs metabolized by BCHE (*e.g.*, succinylcholine, mivacurium) in healthy probands should include molecular genetic analyses to cover at least the most frequent variations in this gene.

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