A new detection method for the K variant of butyrylcholinesterase based on PCR primer introduced restriction analysis (PCR-PIRA)

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Abstract
The K variant of human butyrylcholinesterase is caused by a G/A transition in the butyrylcholinesterase gene, which neither creates nor destroys any restriction site. In an attempt to detect the K variant both simply and rapidly, we developed a two step method of “PCR primer introduced restriction analysis” (PCR-PIRA). The first step was used to introduce a new Fun4HI site into the normal allele for a screening test, while the second step was performed to create a new MaclII site on the variant allele for a specific test. This method thus enabled us to distinguish clearly the K variant from the normal allele, and also showed that the frequency of the K variant allele is 0.164 in the Japanese population.

Materials and methods
Subjects
One hundred and sixteen unrelated Japanese subjects who lived in Beppu City and neighbouring areas were studied. Informed consent was obtained from all participants.

As a positive control, we used DNA from two unrelated persons who had been identified as homozygous or heterozygous for the K variant by DNA sequencing.

Screening test for the K variant
Genomic DNA samples were prepared from peripheral leucocytes. The amplification of genomic DNA (approximately 100 ng) was carried out by PCR in 10 µl total volume which included 10 × PCR buffer (100 mmol/l Tris-HCl, pH 8.8, at 25°C, 500 mmol/l KCl, 15 mmol/l MgCl2, 1% Triton X-100), 25 mmol/l dNTP (mixed dATP, dCTP, dGTP, and dTTP, each 100 mmol/l), 50 pmol/l of each primer, and 1 unit of Tag DNA polymerase (Promega, Madison, WI, USA). Two oligonucleotide primers were synthesized specifically to amplify part of BCHE exon 4 in our laboratory. Screening primer 1 (3′ primer: 5′-CCTGCTTTCCACTCCCATGCTG-3′) binds to the last two bases of codon 539, codon 540–545, and the first two bases of codon 546. It contains a single base mismatch at the fourth position from the 3′ end (fig 1). Screening primer 2 (5′ primer: 5′-CGAAATTA- TTTTCTGATTAAGAAACGATAAA-AATT-3′) binds at 69 to 32 nucleotides upstream from the intron 3/exon 4 junction. The amplification conditions were (30 cycles): denaturation at 94°C for one minute, annealing at 63°C for one minute, with extension at 72°C for one minute. The amplified DNA fragments (106 bp) were digested with 45 units of Fun4HI (New England Biolabs, MA, USA) at 37°C for 12 hours in the buffer supplied by the manufacturer. The digested DNA was separated by electrophoresis on 4.5% NuSieve agarose gel containing ethidium bromide with a DNA molecular weight marker for comparison. This approach thus allowed us to screen for the K variant.

Human butyrylcholinesterase (E.C.3.1.1.8; BChE, serum cholinesterase, pseudocholinesterase) is an enzyme consisting of four identical subunits, and is encoded by a single gene (BCHE) which is composed of four exons. Several genetic variants have been shown to cause prolonged apnoea in patients who were given a standard amount of the muscle relaxant drug succinylcholine. The K variant, named in honour of Werner Kalow, was found to cause a 33% reduction in BChE activity, and it was first recognised using dibucaine inhibition. Although the dibucaine or fluoride numbers have most commonly been used to identify various genetic variants including the K variant, they tend to vary depending on the substrates, temperature, and buffer. Furthermore, the inhibition tests are not able to distinguish the K variant from normal enzymes when the K variant is linked to the atypical variant on one enzyme subunit. Consequently, it is considered important to develop a more specific detection method for the K variant.

Recently, the K variant was found to be caused by a point mutation at nucleotide position (nt) 1615 (GCA/ACA) in exon 4 of BCHE, which might cause the Ala 539 to Thr change. Unfortunately, this mutation neither creates nor destroys any restriction site. Therefore we adopted the method of “PCR primer introduced restriction analysis” (PCR-PIRA) in order to devise a new detection method for the K variant.
SPECIFIC TEST FOR THE K VARIANT
The second PCR was performed on 100 ng genomic DNA samples, which were suspected to have a K variant mutant allele at the first screening test, by using the same amplification conditions except for primers and an annealing temperature of 57°C. The two oligonucleotide primers were newly synthesised. Specific primer 1 (5′ primer: 5′-TTACAGGGATAATTGTAGTGA-3′) binds to the last six nucleotides of the 3′ end of intron 3 and codon 534–538 in exon 4, and introduces a single base substitution at its penultimate position from the 3′ end (fig 2). Specific primer 2 (3′ primer: 5′-GTGTAAAAAAGCTCCTGTATATT-TTGCCCTTGATCTAAG-3′) binds to the non-coding bases (nucleotide 1797 to 1799) in exon 4. The PCR products were digested with 2 units of MaeIII (Boehringer Mannheim Biochemica, Mannheim, Germany) at 65°C for 12 hours in the buffer which was supplied by the manufacturer. The digested fragments were similarly electrophoresed.

The strategy of this two step method is illustrated in fig 3.

Results
The first PCR product of 106 bp derived from the normal sequence was completely digested into fragments of 85 and 21 bp by Fnu4HI (fig 4, lane 2). The PCR product from a person who had previously been identified as being homozygous for the K variant by sequencing was found to be resistant to Fnu4HI digestion (fig 4, lane 4). A heterozygote of the K variant showed the two predicted restriction fragments of 106 and 85 bp (fig 4, lane 3).

The second PCR generated the predicted product of 203 bp. The product from a homozygote for the K variant was completely digested into fragments of 186 and 17 bp by MaeIII (fig 4, lane 8). A heterozygote for the K variant yielded two bands of 203 and 186 bp (fig 4, lane 7), and a normal subject was found to be resistant to digestion (fig 4, lane 6). In this study, 35 samples which had been screened as resistant to Fnu4HI digestion all had the PCR-PIRA with MaeIII digestion performed on them, and the predicted products were obtained without any difficulty. The results clearly showed that all alleles which had shown resistance to Fnu4HI digestion contained a G/A transition at the first position of BCHE codon 539. This was then later confirmed by direct sequencing of that DNA region.
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PCR of genomic DNA with specific primer 1

**Screening test**

Yes  No

Normal

PCR of genomic DNA with specific primer 1

**Specific test**

**Maell digestion**

Yes  No

K variant  Other mutation

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**Figure 3** The flow chart of a two step PCR-PIRA method for the K variant.

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We found 32 (27.6%) of the 116 Japanese subjects to be heterozygous for the K variant, with three subjects (2.6%) homozygous. The allele frequency was determined to be 0.164 for this variant.

**Discussion**

Recently, a molecular method for detecting the K variant by using allele specific biotinylated oligonucleotide probes was reported by La Du et al. This method had remarkable advantages, but it still appears to be quite difficult to obtain such optimal experimentation conditions.

PCR-PIRA allows the detection of a mutation which neither creates nor destroys a restriction site. We therefore applied this strategy to the development of a new method for detecting the K variant of BCHE. Although modified primers either contain a single base mismatch at the fourth position or at the penultimate position from the 3' end, each PCR at a selective annealing temperature can produce the predicted products which are then subjected to restriction analysis. In our case, the first PCR was achieved at a higher annealing temperature than is normally used.

In the presence of a G/A transition at the first position of BCHE codon 539, the first PCR-PIRA prevents the formation of the Fnu4HI restriction site. As a means of screening for the K variant, this test depends on the assumption that all samples which are resistant...
to Fun4HI digestion contain the G/A transition. However, this test is not specific for the K variant since the introduction of the Fun4HI restriction site would also be destroyed by other possible mutations at nt 1615. It is certain that a mutation at nt 1616 would prevent amplification from occurring and an A to C mutation at nt 1619 could not be distinguished from a normal allele. Any mutation at the annealing site with the primer in the original DNA template would also prevent any amplification and if the first PCR product is made, then only a mutation at nt 1615 would prevent the formation of a Fun4HI site. On the other hand, the second PCR-PIRA with MaeIII digestion is specific for the K variant. However, the second PCR-PIRA prevents the formation of the MaeIII restriction site in the presence of a different mutation at nt 1615 or nt 1616, while a lack of amplification might indicate a mutation at the annealing site. The Fun4HI(−)/MaeIII(+) PCR-PIRA genotype can only arise from the K variant. Thus, this two step PCR-PIRA procedure clearly enables us to distinguish the K variant from the normal sequence at nt 1615.

The allelic frequency of the K variant has genotypically been determined to be 0.1125[12] and 0.128[8] in the US population, and phenotypically to be 0.115[13] and 0.133[7] in the British population. Our estimation of the allelic frequency of the K variant in the Japanese population was therefore similar to that of whites.

It is unknown why the K variant should cause a reduction of BChE activity. Although it has been reported that an atypical variant or polymorphisms, such as those at nt-116 or 1914, are present on the same K variant allele, these are not always related to the K variant phenotype.[8] Therefore, it is important to examine whether the K variant might also exist in the gene and thus be linked to other mutations responsible for the reduction of activity. Furthermore, the RFLP analysis based on PCR-PIRA would simultaneously permit the rapid and simple analysis of many samples and have wide applicability in detecting many other mutations which neither create nor destroy any restriction site.

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