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Four Common Mutations of the Cystathionine β-Synthase Gene Detected by Multiplex PCR and Matrix-assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry

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Background: **A deficiency of cystathionine β-synthase (CBS) is the most frequent cause of homocystinuria. The effect of therapy is related to the underlying *CBS* genotype, which makes early diagnosis of this genetic defect important. Our aim was to develop a fast and reliable method based on matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry for the determination of common mutations of the *CBS* gene.**
Methods: **We used MALDI-TOF mass spectrometry to detect four common *CBS* mutations (G307S, T272M, I278T, and V320A). The method is based on multiplex PCR of exons 7, 8, and 9, followed by single nucleotide extension in the presence of dideoxy NTPs of four primers targeted at the separate mutation sites. The extension products, as well as the 3-hydroxypicolinic acid matrix, were incubated with cation-exchange beads to remove disturbing salt contaminants.**
Results: **The above-mentioned mutations were determined in samples from 12 homocystinuria patients. The MALDI-TOF spectra allowed unambiguous discrimination between primers and extension products (>9 Da) in the mass range between 4500 and 7500 Da. No labeled primers or ddNTPs were required. The genotyping was verified by reference technique.**
Conclusion: **Our results demonstrate fast, simple, and unambiguous multiplex genotyping of four common *CBS* mutations by MALDI-TOF mass spectrometry.**

A deficiency of cystathionine β-synthase (CBS) is the most frequent cause of homocystinuria, an autosomal recessive disease that leads to highly increased concentrations of plasma total homocysteine *(1)*. These patients suffer from premature occlusive vascular disease, dislocation of the lens, and neurological defects *(2)*. The plasma total homocysteine concentrations can be lowered and the clinical symptoms prevented by treatment with pyridoxine alone or pyridoxine combined with betaine and folic acid *(1, 3, 4)*. The effectiveness of the therapy is related to the underlying *CBS* genotype *(5)*, which makes early diagnosis of this genetic defect important.

The *CBS* gene has been mapped to chromosome 21q22.3 *(6)*. It was cloned in 1993 *(7)*, and to date, >60 mutations have been reported *(8)*. The prevalence of the various mutations differs markedly between countries, and screening for homocystinuria should be optimized accordingly *(5, 9)*. In a recent study, we found that the most common mutations in Norway are located on exons 7, 8, and 9 *(9)*.

Conventional methods for mutation detection are based on gel or capillary electrophoresis. These methods are time-consuming and provide no direct molecular information. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry *(10, 11)* for the analysis of DNA *(12, 13)* has become an attractive alternative because of advances in both sample preparation and instrument performance. Reduction of cation adduction of oligonucleotides *(14–16)*; minimization of DNA degradation, including depurination *(17–19)*; and selection of proper matrices *(20–24)* are important to obtain high sensitivity, and the delayed extraction technology *(25)* has reduced ion fragmentation and increased spectral resolution. Thus, MALDI-TOF mass spectrometry represents a fast method (seconds to minutes) for the analysis of oligonucleotides up to ~2 kbp *(26)*. The spectral data provide information about base sequence *(27, 28)*, and resolution is now at the base level when DNA fragments of several kDa are analyzed. Several methods for mutation detection with MALDI-TOF have

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been described recently *(29–34)*, but only a few involve multiplex genotyping *(35, 36)*.

We present here a simple and reliable method based on MALDI-TOF mass spectrometry for the determination of common mutations of the *CBS* gene. The technique involves a primer extension reaction, as originally described by Haff and Smirnov *(35)*, and simultaneously detects four point mutations on three different exons: exon 7, T262M (C785T); exon 8, G307S (G919A) and I278T (T833C); exon 9, V320A (T959C).

Materials and Methods

REAGENTS

The QIAAmp Blood Kit was obtained from Qiagen. The Taq polymerase (DyNAzyme™) and reaction buffer for PCR were from Finnzymes; the dNTPs and ddNTPs were from Pharmacia Biotech. The primers were designed with the software Oligo 4.0 (National Biosciences) and synthesized by Eurogentec. PCR products were purified with the QIAquick PCR Purification Kit (cat. no. 28106) from Qiagen. The enzyme and the reaction buffer (ThermoSequenase™) for the primer extension reaction were obtained from Amersham Life Sciences.

The 3-hydroxypicolinic acid, ammonium acetate, and ammonium citrate were purchased from Sigma Aldrich. The cation-exchange beads (AG50W-X8 resin) were from Bio-Rad. The beads were activated by incubation in a 1 mol/L ammonium acetate solution and then washed 5 times with deionized water.

ASSAY DESIGN

The initial step involved multiplex PCR amplification of exons 7 (T262M), 8 (G307S, I278T), and 9 (V320A) containing the mutation sites *(9)*. Using the amplified DNA as template, the four mutation sites were targeted by site-specific primers (Table 1), which were extended by one matching ddTNP. The four extension primers were designed so that the primers and their extension products spanned a mass range between 4500 and 7500 Da. They were sized to avoid mass interference between primers, extension products, and depurination products.

DNA SAMPLES AND PCR

Blood samples were obtained from 12 patients with known mutations in the *CBS* gene. DNA was isolated with the QIAAmp Blood Kit. Amplification of exons 7, 8, and 9 of the *CBS* gene was accomplished by multiplex PCR, using the primers 5′-CCAGGCAGGGACCCAAGAAT-3′ and 5′-CCACTCCGCACTGTCCCTCT-3′ for exon 7, 5′-TTTGGCCGGGCTCTGGACTC-3′ and 5′-TTTCTGGCCTTGAGCCCTGAA-3′ for exon 8, and 5′-CTGACGGGCTGTGGTGGGGTCC-3′ and 5′-CGCACAGCAGCCCCTCTTGCGC-3′ for exon 9. The reaction was performed in a total volume of 100 μL containing 100 ng of template DNA, 1.5 μL of Taq DNA polymerase (1 U/μL), 1.5 mmol/L $MgCl_2$, 125 μmol/L dNTPs, and 20 pmol of each primer. The cycling conditions were 95 °C for 2 min, followed by 36 cycles at 94 °C for 30 s, 64 °C for 40 s, and a final extension at 72 °C for 6 min.

To remove excess primers and dNTPs, the amplification product was purified with the QIAquick PCR Purification Kit according to the instructions given by the manufacturer. The product was eluted in 45 μL of Tris-EDTA buffer (10 mmol/L Tris, 1 mmol/L EDTA, pH 8.0).

PRIMER EXTENSION REACTION

The multiplex extension reaction was performed with a combination of 10 pmol of each of four specific extension primers (Table 1). The total reaction volume of 60 μL contained 10 μmol/L of each ddNTP, 8 U of ThermoSequenase DNA polymerase, 2 μL of ThermoSequenase reaction buffer, and the total multiplex PCR reaction product concentrated down to a volume of 45 μL. The cycling conditions were 40 cycles at 94 °C for 30 s, 64 °C for 30 s, and 72 °C for 1 min. The PCR and extension reactions were performed on a Perkin-Elmer Thermal Cycler 480.

SAMPLE PREPARATION

The extension reaction products were lyophilized, dissolved in 10 μL of distilled water, and then incubated for at least 3 h with cation-exchange beads. 3-Hydroxypicolinic acid (0.5 mol/L in water), used as matrix, was incubated separately with beads. To avoid formation of and interference from adducts, the ammonium acetate-treated beads were washed extensively with deionized water before use. The steel surface of the MALDI probe was exposed to 50 mmol/L ammonium citrate for 10 min to avoid sodium contamination of the target. The probe was not exposed to beads. Matrix solution (1 μL) was then applied to the probe and allowed to dry before deposition of 1 μL of primer extension sample. This sequential procedure led to higher ion yields than those observed when a mixture of matrix and sample was deposited on

Table 1. Sequences and sizes of the DNA oligonucleotides used as extension probes.

Amino acid substitution	Exon	Sequence	Mass, Da		
			Unextended	Wild type, extended	Mutation type, extended
T262M	7	5′-CGGGCGGCACCATCA-3′	4563	4836 (+ddC)	4851 (+ddT)
G307S	8	5′-TACGAGGTGGAAGGGATC-3′	5629	5942 (+ddG)	5926 (+ddA)
I278T	8	5′-ACCCTTCGGGATCCACCCCA-3′	5983	6280 (+ddA)	6296 (+ddG)
V320A	9	5′-TTTTTGCTGGACAGGACGGTGG-3′	6837	7135 (+ddA)	7151 (+ddG)

the probe. Because of the inhomogeneous crystallization of the 3-hydroxypicolinic acid matrix, each sample was analyzed at five different positions.

MALDI-TOF mass spectrometry

The MALDI-TOF instrument was a Bruker Reflex III (Bruker-Franzen Analytik) with a two-stage gridless reflector. The spectrometer was equipped with a nitrogen laser (Laser Science) and a pulsed-ion extraction source. All analyses were run in the reflector mode with negatively charged ions of 20 kV energy. To avoid detector saturation from matrix ions, masses of <2000 Da were suppressed by an ion deflector. The typical number of shots was 50–100. A synthetic oligonucleotide (31-mer; Eurogentec) was used as external standard.

Results

Detection of four *CBS* mutations

Identification of the various alleles was based on the differences in mass shift caused by extension of the primer with two different ddNTPs. The masses of the four ddNTPs were 297.21 (ddA), 273.19 (ddC), 313.21 (ddG), and 288.20 (ddT) Da. Fig. 1 shows the results of three patient samples harboring all eight alleles of the four mutation sites. Fig. 1A shows the mass spectrum of a sample heterozygous for the I278T mutation (extended by ddA and ddG), and wild type for T262M, G307S, and V320A. Fig. 1B shows a sample heterozygous for both G307S and V320A, extended by the pairs ddG/ddA and ddA/ddG, respectively. Fig. 1C shows the extension by both ddC and ddT of a sample heterozygous for the T262M mutation. In all traces in Fig. 1, the extension products were resolved from the primers. With this system, we genotyped blood from 12 homocystinuria patients, and the results (data not shown) were identical to those obtained with reference techniques *(9)*.

Each blood sample was PCR amplified and processed independently three times, and each preparation deposited on the MALDI probe was analyzed at five different positions. Essentially the same results were obtained.

Additional weak signals corresponded to mass peaks of ammonium adducts. There were essentially no sodium adducts. Peaks without labels were minor and could be assigned to depurination (−132 Da) or the presence of an unknown species of 60 Da.

Resolution and peak pattern

The peak doublet shown in the inset of Fig. 1C shows the resolution of the MALDI-TOF system in the mass range of 4800–4900 Da. The mass difference between T and C is 15 Da, and the peaks were baseline resolved. The smallest possible mass difference between two bases is 9 Da, which is the difference between T and A. This situation does not exist for the *CBS* mutation investigated here, but the baseline resolution at a 15-Da difference suggests that an A-T difference of 9 Da would be clearly distinguished.

Although the peak intensities of all extension primers and products varied depending on sample and analysis position, all spectra showed roughly the same peak pattern as shown in Fig. 1. After 40 thermocycles, three of the extension primers showed only weak mass signals, whereas the V320A primer gave a signal equaling that of the extension products.

Discussion

We have developed a MALDI-TOF mass spectrometry procedure for the simultaneous detection of four common mutations in the *CBS* gene. All four mutations were

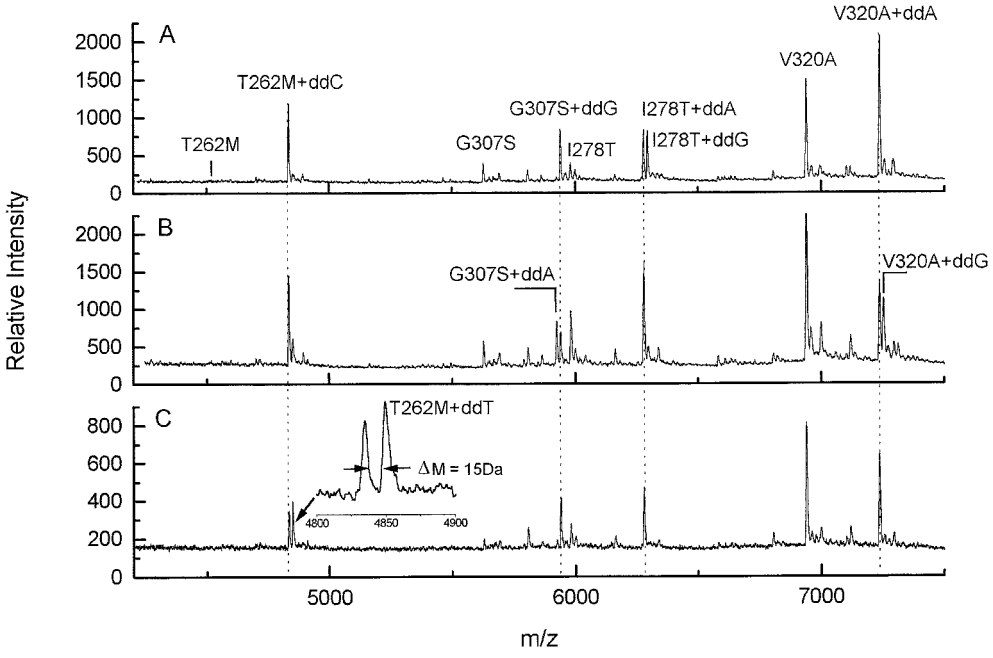

Fig. 1. MALDI-TOF spectra of four *CBS* mutations.

Spectra of samples from three patients harboring all four mutations are shown. The extension products covered a mass range of 4500–7500 Da. Primers and extended primers are assigned by peak labels. The *inset* in *panel C* shows the peak resolution corresponding to 15 Da. All spectra were acquired in the negative ion mode.

unambiguously determined for the 12 homocystinuria patients.

To date, the purification of samples to allow successful detection of oligonucleotides has required tedious and lengthy procedures. These approaches involve ethanol precipitation *(37)* and various solid-phase methods combined indirectly *(14, 15)* or directly *(16, 38, 39)* with MALDI-MS. In 1992, Nordhoff et al. *(14)* described a procedure for removing alkali cations from nucleic acid samples based on a treatment of the matrix and the deposited analyte/matrix droplet by cation-exchange beads. We have used cation-exchange beads for separate desalting of the matrix and analytes. Incubation times of 3 h gave high quality and reproducible mass spectra. If salt adducts were detected, incubation overnight was sufficient. No beads were required on the sample stage. Additional procedures to improve stability *(17, 18)* or to optimize the charge state of the DNA *(19)* were not necessary.

The performance of our system in terms of resolution equals that reported recently for a delayed extraction MALDI-TOF system used for single-nucleotide polymorphism identification *(36)*. A clear separation of oligonucleotides with only an A-T (9 Da) difference in mass should be possible within the mass range investigated (4500–7500 Da). No mass-tagged ddNTPs *(34)* or primers *(35)* were required to carry out multiplex detection of the four *CBS* mutations.

Signal interference usually did not obscure interpretation of spectra. However, peak doublets ddA/ddG and ddC/ddT caused by heterozygous mutations should be evaluated to avoid misinterpretation of ammonium adducts (with a mass increase of 17 Da). In our experience, the doublets related to sample heterozygosity have a balanced height, whereas ammonium adducts usually cause smaller peaks.

The mass spectra usually showed good reproducibility, allowing unequivocal interpretation of data, but small variations between sample preparations or different analyses of the same sample preparation were occasionally observed. This may be the result of unbalanced PCR, variation in primer extension efficiency, inhomogeneous spatial distribution of oligonucleotides and matrix at the MALDI probe, variability in ionization/desorption, and detection probability of the oligonucleotides.

In conclusion, we used MALDI-TOF mass spectrometry with pulsed ion extraction and in the reflector mode for multiplex genotyping of four common mutations in the *CBS* gene. The technique is based on primer extension without DNA strand separation or labeling of primers or ddNTPs. The sample purification step is simple and relatively inexpensive and involves treatment of the extension products and the matrix with cation-exchange beads. This procedure ensures sensitive oligonucleotide analysis of high quality without mass interference or sodium adduct formation. Our results demonstrate unambiguous multiplex genotyping by MALDI-TOF mass spectrometry.

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