olive oil was submitted. Direct methanolic water extracts without any further treatment or extracts submitted to an acidic treatment yielded similar amounts of tyrosol present in olive oil: 216 μg (in 50 mL). From this value, urinary recoveries of tyrosol were unrealistic because they were 131.5–327.8% of the dose administered. When the tyrosol content was estimated after NaOH digestion and sonication of olive oil, the total tyrosol content was 1650 μg (in 50 mL). From this value, recoveries of tyrosol in the 24-h urine were 17–43%, with a mean recovery of 24.7% ± 8.5% (mean ± SD). These values are likely to be a better estimate of the real bioavailability of this compound. The differences in recoveries between the extraction procedures probably reflect differences in the extent to which HCl and NaOH hydrolyze esterified forms of tyrosol present in olive oil (15).

The results from the present study clearly show for the first time that urinary tyrosol concentrations are responsive to dietary intake of virgin olive oil. The method developed for urinary tyrosol determination has the sensitivity and accuracy to detect tyrosol in amounts typically present in the urine of nonsupplemented individuals. These results also underline the relevance of adequate estimation of administered phenolic doses in studies linking them to biological effects. In a recent report, recoveries of ~20% in urine from tyrosol-enriched olive oil were described (18). These recoveries were obtained after urine samples were submitted to enzymatic hydrolysis by β-glucuronidase. Taking into account the similar recoveries of tyrosol when different hydrolytic procedures (enzymatic vs acidic) were used, it is very likely that tyrosol is excreted in the urine mainly as the glucurononoconjugate. From a theoretical perspective, studies after the administration of pure phenolic compounds or enriched foods have some interest. However, studies in which phenolic ingestion is closer to typical dietary patterns may be more appropriate for estimating bioavailability. Slight details in our study design may have some relevance in experimental results. As an example, subjects who ingested olive oil with bread had a tyrosol recovery (20% ± 3%; n = 6) lower than that observed in those who directly drank the olive oil (37.5% ± 5.5%; n = 2).

The present work is the first description of the bioavailability in humans of a phenolic compound present in olive oil administered in its natural form. We found that tyrosol was absorbed from virgin olive oil administered and that one part of the dose was excreted over the next 24 h. This study also shows the feasibility of controlled bioavailability studies of phenolic compounds from dietary sources in humans.

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References

Novel Mutation and Polymorphisms of the HMBS Gene Detected by Denaturing HPLC, Ching-Wan Lam, Priscilla Miu-Kuen Poon, Sai-Fan Tong, Anthony Wing-Ing Lo, Chi-Kong Lai, Kin-Lam Choi, Sau-Cheung Tiu, Yan-Wo Chan, and Chi-Chung Shek (1 Department of Chemical Pathology, The Chinese University of Hong Kong, Prince of Wales Hospital, Hong Kong, China; 2 Department of Pathology, Princess Margaret Hospital, Hong Kong, China; Departments of 3 Medicine and 4 Pathology, Queen Elizabeth Hospital, Hong Kong, China; * author for correspondence: fax 852-2636-5090, e-mail chingwanlam@cuhk.edu.hk)

Acute intermittent porphyria (AIP) is an autosomal dominant, inborn error of the metabolism of heme biosynthesis caused by partial deficiency of hydroxymethylbilane synthase (HMBS). This enzyme catalyzes the condensa-
tion of four molecules of porphobilinogen to a tetrapyrrole, hydroxymethylbilane. AIP is characterized by acute attacks of a neurological disorder manifesting as abdominal pain, hypertension, tachycardia, peripheral neuropathy, and mental dysfunction. Biochemical diagnosis of AIP relies on increased urinary porphobilinogen and normal fecal porphyrin excretion (1). Identification of presymptomatic AIP carriers in families with affected individuals is of clinical importance because avoidance of precipitating agents (e.g., drugs and alcohol) can prevent the occurrence of the first porphyric attack, which may be life-threatening. However, there is a significant overlap between the enzyme activities of healthy individuals and patients with AIP (2). In addition, there is a variant of AIP, in which the red blood cell (RBC) HMBS activity is normal (3). DNA-based diagnosis of presymptomatic AIP has been more reliable than diagnosis by RBC HMBS activity, and, thus, direct detection of mutations in presymptomatic AIP is now the definitive approach.

The human HMBS gene spans ~10 kb of DNA on chromosome 11q24.1-24.2 and contains 15 exons (4). Extensive allelic heterogeneity has been demonstrated in this gene, and 159 mutations have been identified in the HMBS gene (5). Most of these mutations are found only in individual families, except those found in Dutch (R116W) (6) and Swedish (W198X) (7) AIP families. Although DNA sequencing can identify all the mutations, this approach is both labor-intensive and time-consuming (8). Several methods that accelerate mutational screening before sequencing have been developed, such as single-strand conformation polymorphism analysis (9), heteroduplex gel analysis (10), and denaturing gradient gel electrophoresis (11, 12). Recently, denaturing HPLC (DHPLC) appears to be more sensitive than other methods in mutation detection, as exemplified in the detection of TSC1 (13), TSC2 (14), BRCA1 (15, 16), BRCA2 (15), CFTR (13), EXT1 (17), EXT2 (17), and HPRT (18). Here, we describe the use of DHPLC for mutation scanning in a family with AIP.

The patient was a 40-year-old woman presenting with frequent acute episodes of abdominal pain and convulsions. In one episode of acute attack, urine porphobilinogen was 117 μmol/mmol of creatinine (reference limit <10 μmol/mmol of creatinine). Reversed-phase high-performance thin-layer chromatography showed a normal pattern of fecal porphyrin excretion (19, 20). The patient was diagnosed to have AIP, but the RBC HMBS activity, measured at a time when she was anemic, was within the reference interval (35 nmol/mL of RBC per hour; reference interval, 28–66 nmol/mL of RBC per hour). The RBC HMBS activity of the mother was low (27.5 nmol/mL of RBC per hour), whereas the father had normal activity (52.4 nmol/mL of RBC per hour). On the basis of these data, the mother was an asymptomatic carrier and the patient was a manifesting individual of AIP.

Genomic DNA was extracted from whole-blood samples of the patient and the family members by a commercially available method (QIAamp; Qiagen). The study was performed in accordance with the principles of the Declaration of Helsinki. Informed consent was obtained from all subjects. All of the exons and the flanking introns of the HMBS gene were amplified using previously described primers (21). Exon 15 of the HMBS gene was amplified into two fragments [i.e., exon 15A (nucleotide positions 9051–9469) and exon 15B (nucleotide positions 9373–9700)]. Heteroduplex analysis was performed on a WAVETM DHPLC instrument (Transgenomic Inc.). Analysis on the system was performed at a temperature sufficient to partially denature (melt) the DNA heteroduplexes. The partially denatured heteroduplexes were resolved from the corresponding homoduplexes by ion-pair reversed-phase HPLC (22). The stationary phase consisted of 2-μm nonporous alkylated poly(styrene-divinyl-
benzene) particles packed into a 50 × 4.6 mm (i.d.) column (DNASep column; Transgenomic Inc.). Crude PCR product (10 μL) was loaded on the column and eluted from the column by an acetonitrile gradient in 0.1 mol/L triethylammonium acetate (TEAA; pH 7.0) buffer at a constant flow rate of 0.9 mL/min. The buffers were prepared from TEAA buffer concentrate to give the following: buffer A, 0.1 mol/L TEAA; buffer B, 0.1 mol/L TEAA containing 250 mL/L acetonitrile. The gradient was created by mixing buffers A and B. The recommended gradient for mutation detection was a slope of 2% increase in buffer B per minute. Eluted DNA fragments were detected with ultraviolet absorbance at a wavelength of 260 nm. The WAVE utility software helped determine the correct temperature for mutation scanning based on the sequence of the wild-type DNA. This approach was modified during the analysis of DNA from individuals carrying two identical mutant alleles (homozygous mutation). The PCR product spanning the homozgyous mutation was mixed with equal amounts of wild-type amplified DNA and hybridized. After this treatment, a sample contained a mixture of hetero- and homoduplexes.

PCR products were purified by Microspin S300 columns (Amersham Pharmacia), and both strands were sequenced using the amplification primers as sequencing primers and BigDyeDeoxy™ terminator cycle sequencing reagents according to the manufacturer’s instructions (PE Biosystems). Products of sequencing reactions were purified by Centri-Sep™ spin columns (Princeton Separations). Purified sequencing fragments were separated by capillary electrophoresis and detected via laser-induced fluorescence on an ABI PRISM 310 Genetic Analyzer (PE Biosystems). Sequencing results were compared with the established human HMBS sequence (GenBank Accession No. M95623).

For the proband (II2), the DHPLC chromatograms of exons 11 (Fig. 1A), 14, 15A, and 15B (data not shown) showed the presence of heteroduplex peaks. Direct DNA sequencing of exon 11 showed a double heterozygous pattern (data not shown). The mutation is a one-nucleotide deletion, 8193delC. This mutation alters the reading frame of the encoded protein such that a stop codon, TGA, is generated at codon 254 (i.e., L254X). The predicted mutant HMBS is a truncated protein of 253 amino acids, compared with 361 residues for the wild-type protein. Screening of the family members by DHPLC and subsequent sequencing confirmed that only the mother (I2) had a 8193delC mutation. This 8193delC mutation was not detected in 50 apparently healthy Chinese individuals. This is the first disease-causing mutation identified in the HMBS gene in Chinese individuals. Direct sequencing of both exon 14 and exon 15A and the flanking introns of the proband showed a novel transition in intron 14, at position 9103, changing the base from guanine to adenine, i.e., 9103G→A (Fig. 1B). The size of intron 14 was only 88 bp, and the two PCR products both contained the nucleotide at 9103. When we analyzed 50 apparently healthy Chinese individuals, we found that 49 were 9103G/G and 1 was 9103G/A. The allele frequency of 9103G thus was 0.99, and that of 9103A was 0.01. Direct sequencing of exon 15B and the flanking introns showed our previously described single nucleotide polymorphism (SNP), 9678G→A (23).

Interestingly, DHPLC chromatograms of exon 11 of the other family members (II1, II2, and II3) also showed the presence of heteroduplex peaks. However, the elution profiles were different from that of 8193delC (Fig. 1A). Direct sequencing of the PCR products showed a transversion in intron 10, at position 8164, changing the base from cytosine to adenine, i.e., 8164C→A (data not shown). The 8164C→A transversion is not present in the patient or her mother, thus confirming that 8164C→A is a neutral polymorphism. When we analyzed 50 apparently healthy Chinese individuals, we found that 11 were 8164A/A, 28 were 8164A/C, and 11 were 8164C/C. The allele frequency of 8164A thus is 0.50, and that of 8164C is 0.50. Using the three genomic SNPs, we constructed complete haplotypes for all family members. The haplotypes for the chromosome carrying the disease-causing mutation are 8164C, 9103G, and 9678G. Thus, only the mother and the proband are carriers, and the other family members do not have the mutant alleles (Fig. 1B). The results of haplotype analysis are consistent with the results of mutation detection.

DNA-based diagnosis of AIP was particularly useful in the proband as RBC HMBS activity is uninterpretable in the presence of hemolysis. In this study, we used DHPLC to identify a novel HMBS mutation that causes AIP, and together with the three SNPs, we have determined the haplotypes for the mutation. The haplotypes are informative for this family. We can use either direct mutation detection or linkage analysis to determine whether other family members carry the AIP chromosome.

DHPLC has several advantages over other mutation detection methods. DHPLC is fully automated, eliminating the time and labor in gel preparation, loading, analyzing, and photographic documentation. The PCR products are loaded directly from a 96-well plate without any prior purification. Heteroduplex DNA is resolved from homoduplex DNA in a matter of minutes. The technology detects single-base changes as efficiently as short deletions and insertions. Heteroduplex peaks elute earlier than homoduplexes and can be observed as separate peaks or as shoulders on the leading edge of homoduplex peaks (22). Elution characteristics of the heteroduplex peaks are influenced by the specific base mismatch present and the melting characteristics of the surrounding bases. As demonstrated in this study, even mutations and SNPs located in the same PCR fragment can be distinguished by the characteristic elution profiles.

Although single-base mutations have been detected in 1.5-kb fragments by DHPLC (24), PCR product <100 bp containing a mutant allele with the C→G transversion cannot be resolved from the wild-type allele (25). To achieve the highest accuracy, it is better to amplify large exons into 150- to 450-bp fragments. In addition, cases of possible homozygous mutations require premixing of
References


It is well recognized that circulating prolactin may exist in several forms, including little (monomeric), big, and big, big (macroprolactin) prolactin with molecular masses of 23, 50, and 150–170 kDa, respectively (1).

We report the case of a 30-year-old woman who initially attended her primary care physician because of the onset of painful irregular periods. Her cycle usually was regular, but she had had an 8-week interval of amenorrhea, followed by a particularly painful bleed for which she sought medical advice. Before this, and subsequently, her menstruation had been completely regular with a 28-day cycle. She had no other problems.

The patient’s initial serum prolactin was recorded as 15 800 mIU/L (−530 µg/L) in a Bayer Immuno 1™ assay (Bayer Corporation). Other investigations at the time were entirely normal.

When the subject was monitored 2 months later, she was symptomless and menstruating regularly; her serum prolactin, using the same assay as before, was 8440 mIU/L (−270 µg/L). Pituitary imaging by magnetic resonance was normal. She had, of choice, never been pregnant.

Because of the patient’s lack of symptoms, normal pituitary imaging, and regular cycles, further analytical investigations were carried out on a sample of her serum. After polyethylene glycol (PEG) precipitation, the recovery of prolactin was low, indicating the presence of macroprolactin (2), and 15% of her total prolactin was estimated to be monomeric prolactin.

The patient’s serum prolactin concentration was remeasured using a Wallac Delfia™ assay (EG & G Wallac) and

subject DNA with wild-type DNA. This is, however, less of a problem in autosomal dominant diseases. Although an initial capital investment in the HPLC instrument is required, the combination of low running costs and the tremendous reduction in the effort of sequencing make the DHPLC technique a suitable method for mutation detection (26, 27).