examined for the presence of macroprolactin by precipitation with PEG.

It is interesting to note that the PRL assay on the Roche Enzymun system reacts less strongly with macroprolactin (7) but uses the same pair of antibodies as the Elecsys assay. However, the antibodies are coupled to different solid phases and use different signal-generation systems in the two assays.

We thank Roche Diagnostics (Lewes, UK) for providing the reagents for this work.

References

Rapid Detection of the C3435T Polymorphism of Multidrug Resistance Gene 1 Using Fluorogenic Hybridization Probes, Markus Nauck,* Ulrike Stein, Sabine von Karger, Winfried März, and Heinrich Wieland (Department of Clinical Chemistry, University Hospital, Hugstetter Strasse 55, 79106 Freiburg i. Br., Germany; * author for correspondence: fax 49-761-270-3444; e-mail msnauck@med1.ukl.uni-freiburg.de)

Genetic polymorphisms in drug-metabolizing enzymes, transporters, receptors, and other drug targets have been linked to interindividual differences in the efficacy and toxicity of many medications (1, 2). Knowledge of an individual’s drug metabolism characteristics can help in avoiding adverse reactions or therapeutic failure and thus enhance therapeutic efficiency. One gene of particular interest in pharmacogenetics is the human multidrug resistance-1 (MDR1) gene. The MDR1 gene encodes an integral membrane protein, P-glycoprotein (PGP), whose function is the energy-dependent export of substances from the inside of cells to the outside (3). Many drugs are substrates of PGP. Therefore, expression and functionality of the MDR1 gene product can directly affect the therapeutic effectiveness of such compounds. This is of specific importance in cancer therapy, where high expression of MDR1 makes cancer cells refractory to treatment with many agents that are PGP substrates (4).

MDR1 is also expressed on nonmalignant cells in various organs, e.g., the intestine and at the blood-brain...
barrier. Modulation of MDRI expression in these tissues can influence the activity and bioavailability of drugs (5). Recently, a polymorphism of the MDRI gene has been shown to be correlated with intestinal PGP expression and activity in vivo (6). This polymorphism consists of a C-to-T exchange at cDNA position 3435 located in exon 26 of the MDRI gene. Although this base exchange does not affect the amino acid sequence of PGP, the T allele appears to be associated with markedly lower MDRI expression compared with the C allele. Hoffmeyer et al. (6) were able to consistently show that plasma concentrations of digoxin, which reflect PGP activity in vivo, were significantly higher in individuals harboring the T allele compared with carriers of the C/C genotype.

Because of its silence on the protein level and its location in a nonregulatory region of the MDRI gene, it is conceivable that this particular polymorphism is not causative for differences in PGP expression. It is rather likely that this polymorphism is linked to other, as yet unidentified, changes in regions of the MDRI gene that control expression, e.g., in the promoter/enhancer region or in regions that are relevant for mRNA processing. Nevertheless, the C3435T polymorphism appears to allow the differentiation of alleles with distinct MDR-1 expression and activity. Therefore, genotyping of the C3435T polymorphism may provide a basis for treating patients more effectively with agents that are substrates of the MDR-1 gene product, e.g., anticancer drugs such as vincristine and doxorubicin or HIV protease inhibitors such as ritonavir and saquinavir (3).

We present here a single-step method for homogeneous genotyping of the C3435T polymorphism of the MDRI gene that combines rapid-cycle PCR and fluorescent probe melting point analysis on the LightCycler™ (Roche Molecular Biochemicals) (7). Fluorescent melting point analysis is a technique that detects mutations by differences in the melting temperature ($T_m$) of fluorescent oligonucleotides hybridized to different alleles (8–11).

For the present study, genomic DNA from 126 individuals was isolated from whole blood using the High-Pure PCR Template Preparation method from Roche Molecular Biochemicals. The DNA was resuspended at a concentration of 40 mg/L in 10 mmol/L Tris, pH 7.4, containing 0.1 mmol/L EDTA. The primers 5'-TGTITTCAGCTGCTT-GATGG-3' (sense, intron 25/exon 26) and 5'-AAGGCAT-GATGTGGCCCTC-3' (antisense, exon 26) were used to amplify a 197-bp fragment of the human MDRI gene, which harbors the polymorphic site at nucleotide (nt) position 3435 of the cDNA (GenBank Accession No. M29445) (12).

The detection probe was a 20mer oligonucleotide, labeled at the 5' end with LC-Red 640 and phosphorylated at the 3' end to block extension. The sequence 5'-GGAAGATGCTGTAGGGCCAG-3' (nt 3426–3445) is complementary to the antisense strand of the wild-type allele, with the polymorphic nucleotide (underlined) 10 bases at the 3' end. The 5'-fluorescein-labeled anchor probe was a 21mer that binds with a distance of two bases 5' to the detection probe (5'-GACAACAGGCAGGTTGTT-GTCA-3'; nt 3403–3423). The unlabeled primers for amplification and the hybridization probes were synthesized by TIB MOLBIOL.

PCR was performed in a reaction volume of 10 µL with 0.3 µmol/L each primer, 0.2 µmol/L anchor and detection probes, and 40 ng of genomic DNA. As a reaction buffer, the LightCycler DNA Master Hybridization Probes buffer (Roche Molecular Biochemicals) was used. The final Mg²⁺ concentration in the reaction mixture was adjusted to 2.5 mmol/L. The samples were loaded into composite plastic/glass capillaries (Roche Molecular Biochemicals), centrifuged, and placed in the LightCycler sample carousel. After an initial denaturation step at 94 °C for 30 s, amplification was performed using 45 cycles of denaturation (94 °C for 0 s), annealing (55 °C for 30 s), and extension (72 °C for 4 s), and the temperature transition rates were programmed at 20 °C/s for all three temperatures. Fluorescence was measured at the end of the annealing period of each cycle to monitor amplification with fluorescence gains for F1 = 1, F2 = 10, and F3 = 1. After amplification was complete, a final melting curve was recorded by cooling to 45 °C at a ramp rate of 10 °C/s, holding at 45 °C for 2 min, and then heating slowly to 75 °C at 0.1 °C/s. Fluorescence was measured continuously during the slow temperature ramp to monitor the dissociation of the fluorophore-labeled detection probe.

The fluorescence signals (F) were then converted to melting peaks by plotting the negative derivative of the fluorescence with respect to temperature against temperature (−dF/dT vs $T$).

For confirmation of genotypes, allele-specific restriction enzyme site analysis (ASRA) was performed using the same amplification primers as for the fluorescence protocol. Aliquots (10 µL) of the PCR mixture were digested for 4 h at 37 °C with 5 U of DpnII and the restriction buffer recommended by the manufacturer (New England Biolabs).

The C3435T substitution destroys an restriction enzyme cleavage site for DpnII. Accordingly, after digestion with DpnII, the fragments of the 3435C allele measured 158 and 39 bp, whereas the amplicon of the 3435T allele remained uncut (197 bp). The digested products were electrophoresed on 3.0% agarose (FMC Bioproducts), visualized with 0.5 mg/L ethidium bromide, and examined under ultraviolet illumination.

During amplification, when fluorescence was measured at the end of each annealing phase, the fluorescence signal increased above background after 30 cycles as product accumulated. Even 2 ng of genomic DNA yielded a sufficient fluorescence signal for mutation detection.

The process of hybridization and melting of the detection probe to the target was monitored by melting curve analysis. Melting of the sample homozygous for the 3435C allele produced a melting peak at 64.5 °C, whereas in the sample homozygous for the 3435T allele, a melting peak was obtained at 56 °C. The heterozygous sample contained both types of targets and thus generated both peaks (Fig. 1). With different samples showing different
amplification efficiencies, the derivative melting curves were highly reproducible. The CV for the melting point of the 3435C allele, based on the genotyping of 117 alleles that were analyzed in four different analytical runs on 4 different days, was 0.55%. Likewise, the CV for melting point analysis of the 3435T allele (n = 135) was 0.43%. In addition, for heterozygous samples (n = 59), the difference between the two melting temperatures (mean = 8.43 °C) had a CV of 1.1%. These data document the robustness and accuracy of the assay and provided the basis for the easy and unambiguous assignment of genotypes to the respective melting curves for all samples.

To evaluate the reliability of the fluorescence genotyping, 126 human DNA samples were genotyped for the C3435T polymorphism by both ASRA using DprII and the fluorescence method. The fragments obtained by ASRA were of the expected sizes, and the genotypes determined with both methods were in 100% concordance. Twenty-nine samples were homozygous for the 3435C allele, 59 were heterozygous, and 38 were homozygous for the 3435T allele. This genotype distribution is consistent with the previously published results obtained in a Caucasian population (6).

Routine determination of disease-causing mutations requires accurate, rapid, reliable, and low-cost methods. The potential benefits of homogeneous detection systems for the identification of mutations have long been recognized, and recently, several fluorescence-based methods for typing bi-allelic systems have been described that facilitate sample processing (9, 10, 13, 14). The protocol presented here allows fluorescence genotyping in 32 samples in less than 40 min without any need for enzyme digestion or electrophoresis. Because this method is performed in a closed system with no postamplification processing, potential problems with sample tracking errors and end-product contamination are eliminated. When we compared the costs for reagents and disposable material required for both methods, we found that at a cost of $4 US per typing reaction, the fluorogenic method costs ~50% more than the ASRA. However, because the "hands-on" time for setting up the homogeneous assay is short and manual intervention is required after the LightCycler is loaded, in our opinion, the higher costs for reagents are more than compensated and the homogeneous method still permits genotyping in an economic manner. In addition, results may be provided with short turnaround time, allowing for rapid intervention.

In summary, because of its robustness, speed, and accuracy, this assay is suitable to determine the MDR1 C3435T polymorphism in both small and large numbers of samples.

References