Chapter 4

Genotyping of the pregnane X receptor A11156C polymorphism with locked nucleic acid containing fluorogenic probes

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Abstract

Background and aim

The discriminative power of oligonucleotide probes to differentiate between two alleles of a biallelic gene locus is an important aspect in polymorphism analysis. This study investigates the enhancement of discriminative power in a fluorescence resonance energy transfer (FRET) assay by incorporation of a locked nucleic acid (LNA) at the polymorphic site applied on genotyping of the pregnane X receptor (PXR) A11156C polymorphism.

Materials and methods

Fifty individuals were genotyped for this PXR A11156C polymorphism. Polymerase chain reaction (PCR) products of the different PXR 11156 allelic variants were sequenced to validate the results of this FRET assay.

Results

It follows that incorporation of the LNA significantly improved the genotyping of the PXR A11156C polymorphism. Especially, heterozygotes could be discriminated clearly by showing two melting peaks, whereas the probes without a LNA were not able to separate melting peaks in the melting curve analysis.

Conclusion

A LNA within a sensor probe may improve the discriminating power of FRET assays significantly.

Introduction

The introduction of molecular technologies that combine the polymerase chain reaction (PCR) with the use of fluorescent hybridisation probes significantly reduced the risk of contamination, since no post PCR steps are involved. Although these techniques have been successfully used for the identification of a huge variety of single nucleotide polymorphisms (SNPs)[1,2], there are target sequences in which the discriminating power of deoxyribonucleic acid (DNA) probes is not sufficient to differentiate between the two alleles of a biallelic gene locus. The nuclear pregnane X receptor (PXR), (also known as SXR, PAR, PRR and NR1I2) is an important component of the body's adaptive defensive mechanism against toxic substances including drugs. Ligand activated PXR is involved in the transcriptional activation of multiple drug resistance gene 1 (MDR1) and cytochrome P450 3A4 (CYP3A4). Both are main regulators in the uptake and transformation of many orally prescribed medicines. Hence, knowledge with regard to known PXR polymorphisms, which influence the expression of MDR1 and/or CYP3A4, is therefore relevant to understand the inter-individual responses towards drugs[3-5]. One of the putative relevant polymorphisms is PXR A11156C because it has been shown that the presence of the 11156C allele is associated with a decreased expression of MDR1 mRNA in intestinal villi cells derived from gut biopsies[4]. In order to determine the genotype of the PXR A11156C polymorphism we developed a real-time PCR fluorescence resonance energy transfer (FRET) assay. Although homozygotes could be discriminated unequivocally, separate melting peaks could not be obtained in putative heterozygote genotypes. To improve the discriminative power of the sensor probe two routes were followed. The first was to shorten the sensor probe and the second route was to modify the sensor probe with a locked nucleic acid (LNA) on the polymorphic position[6-13] . This last mentioned possibility is based on some recently published reports, which documented the high discriminative power of fluorogenic probes containing LNA to differentiate between matched and mismatched duplexes in several techniques, but not yet applied in FRET assays[14-16]. However, FRET assays have become a major technique in the field of polymorphism analysis. It is therefore worthwhile to examine the usefulness of LNA in FRET assays. The present study describes a real-time PCR FRET assay on the LightCycler in which three different sensor probes, of which one contains a LNA, are compared for their ability to differentiate between matched and mismatched duplexes.

Materials and methods

Ethylene diamine tetra-acetic acid (EDTA) whole blood samples of 50 anonymized patients referred for routine haematologic laboratory investigation were collected. The whole blood samples were stored at –20°C until DNA isolation, with a QIAamp blood mini kit (Qiagen, Leusden, the Netherlands) according to the manufacturers'

instructions, was performed. To obtain the different allelic variants of the PXR A11156C polymorphism a real-time PCR FRET assay consisted of a primerset, an anchor probe and the sensor probe Sen [A] is used. The PCR primers were chosen to specifically target the human PXR gene. The primerset and the different fluorogenic probes used were designed and synthesised by TIB MOLBIOL (Berlin, Germany). The primers F22: 5'-CAA TCA gTT AAC ACA CCg gAg-'3 (sense; 81370-391) and R22: 5'-TTT TAT gTT CTT ACg CCg gAg T-'3 (anti-sense; 81766-745) were used to amplify a 397 bp fragment from the human PXR gene which covers the A11156C polymorphism in the 3'-untranslated region (3'-UTR) area (Genbank acces no: AF280107). Detection was carried out using the anchor probe 56Anchor: 5'-TTT Tgg gAA ATg TAg CCC Tgg gT-'3 (sense; 81553-575), which was labelled at the 5'-end with LCRed640 and phosphorylated at the 3'-end to block extension, and combined with one of the three sensor probes shown in Table 4.1.

Table 4.1 Melting curve analysis obtained by genotyping fifty individuals with three different sensor probes.

Name[a]	Sequence sensor probe[b]	$Tm_{[A]}$[c] (°C)	$Tm_{[C]}$[d] (°C)	$\Delta Tm_{[A]/[C]}$[e] (°C)
Sen [A]	Agg CAT TCC ACA CCT A**A**g AAC TA-F	62.4 ± 0.18	59.3	Nd
Sen [A]s	CAT TCC ACA CCT A**A**g AAC TA-F	57.2 ± 0.08	53.1	Nd
Sen [A]sL	CAT TCC ACA CCT A**A**g AAC TA-F	58.7 ± 0.08	51.9	7.5 ± 0.10

[a] "s" is the short variant of the sensor probe; "L" short variant with a locked nucleic acid (LNA) on the polymorphic site; [b] Probe position in human PXR sequence AF280107 is sense between 81529-551 bp; the polymorphic nucleotide is underlined and expressed in bold; F is the position of the labelled fluorescein; [c] The mean melting temperature (Tm) ± standard deviation (SD) of 33 homozygote PXR 11156 [A]/[A]; [d] The melting temperature (Tm) of a homozygote PXR 11156 [C]/[C]; [e] The mean melting temperature difference (ΔTm) ± SD of 16 samples with the PXR 11156 [A]/[C] genotype; Nd is not discriminable.

PCR and melting curve analysis were performed on the LightCycler (Roche Diagnostics, Almere, the Netherlands). The PCR mixture contained 3.0 mM $MgCl_2$, 2.0 μl LC Faststart DNA Master Hybridization Probes Mix (Roche Diagnostics GmbH, Mannheim, Germany), 0.50 μM of each primer, 0.20 μM of the anchor probe and 0.20 μM of the sensor probe. After adding 2.0 μl containing 50-100 ng genomic DNA to the PCR mixture the total volume was 20 μl. The PCR protocol included the following steps: a) denaturation for ten minutes at 95°C b) 45 cycles at 95°C for ten seconds; 55°C for ten seconds and 72°C for 16 seconds. After amplification was completed, a melting curve was recorded by cooling to 45°C at a ramp rate of 20°C/second, holding at 40°C for one minute and then heating slowly to 70°C at 0.2°C/second. The derivative melting curves are read in channel 2 of the LightCycler for the detection of the PXR fragment. The PCR products were sequenced according to a direct sequence procedure performed with the capillairy sequencer ABI 3100® (Applied Biosystems, Fostercity, USA) using the Bridge version 1.1 sequence kit.

Results and discussion

Fifty individuals were genotyped for the PXR A11156C polymorphism by a real-time PCR FRET assay using the sensor probes illustrated in Table 4.1. Since sensor probe Sen [A] is complementary to the PXR 11156 [A] allele, melting of the PCR product homozygous for this 11156 [A] allele produced a melting peak at 62.4 ± 0.18°C, whereas for a PCR product homozygous for the PXR 11156 [C] allele the melting peak was obtained at 59.3°C. In accordance with the melting temperatures obtained, 33 samples were genotyped as PXR 11156 [A]/[A] and one sample was genotyped as PXR 11156 [C]/[C]. A heterozygous sample contains both types of targets and should therefore generate both peaks. However, the real-time PCR FRET assay using the sensor probe Sen [A] produced for 16 samples one unspecific melting peak around 60.0 ± 0.66°C (see Figure 4.1A), which in our hands under no conditions could be resolved into two separated peaks. Most likely, these samples are heterozygous for the PXR A11156C polymorphism. Hence, the allele frequency for the 11156 [A] polymorphism is 84 % and for the 11156 [C] 16% which is in line with the other study[4]. The PCR products of three different PXR 11156 allelic variants were sequenced to confirm the results obtained by the real-time PCR FRET assays. The results provided by the sequence analysis matched exactly with the genotypes as classified by the melting peaks. The discriminating power of SNP detection by FRET probes is based on the difference in melting behaviour of mismatches *versus* perfect matches to the target probe. The greater the difference between the melting temperature (Tm) of the matched *versus* mismatched duplexes (ΔTm), the better is the discriminating potential of the sensor probes in the FRET assay. This ΔTm is dependent upon the length of the sensor probe, the type of mismatch and the neighbouring nucleotides. The longer the sensor probe, the smaller the effect of a single-base mismatch on overall duplex stability, hence the ΔTm between match and mismatch duplexes decreases. Therefore to improve the melting curve analysis the sensor probe was shortened by three nucleotides which resulted in Sen [A]s. The Tm of both homozygotes was lowered as to be expected. The difference between the Tm of both homozygotes was 4.1, which is 1°C more than obtained with Sen [A] (Table 4.1). However the melting curve analysis of heterozygotes did not yield two separate melting peaks, this is illustrated in Figure 4.1B. The differential stability of a match *versus* mismatch duplex is the main limitation of the use of FRET probes for detection of SNPs. Fluorogenic probes containing LNAs have shown strong affinity for their complementary targets. The main structural feature of these probes is the presence of an additional methylene bridge linking the 2'-hydroxyl group of a RNA monomer to the 4'-carbon of the ribose ring. The presence of this bridge "locks" the sugar ring in one fixed conformation (3'-endo), which is the conformation observed for the ribose ring in RNA or DNA hybrids. Based on NMR studies of LNA/DNA duplexes, it has been concluded that the main factor contributing to the extra-ordinary high stability of LNA containing duplexes is a local change of the phosphate backbone geometry that favours a much higher degree of base stacking[6-13]. Thus, the

Sen [A]s was modified with a LNA on the polymorphic site, resulting in Sen [A]sL. The melting curve analysis showed an increment in the temperature of the melting peaks of homozygotes 11156 [A]/[A]. This could be attributed to the improved binding of the LNA probe. The melting peak for the homozygote 11156 [C]/[C] seemed to be somewhat lowered. Figure 4.1C shows these melting curves. The heterozygotes now yielded two separated peaks for with a ΔTm of 7.5°C (Table 4.1). From the present study it is obvious that by introducing a LNA on the polymorphic location in a sensor probe the discriminative power of a real-time PCR FRET assay may increase enormously. Therefore, we conclude that the incorporation of a LNA in the sensor probe of a FRET assay is an additional tool to improve further the analysis of SNPs.

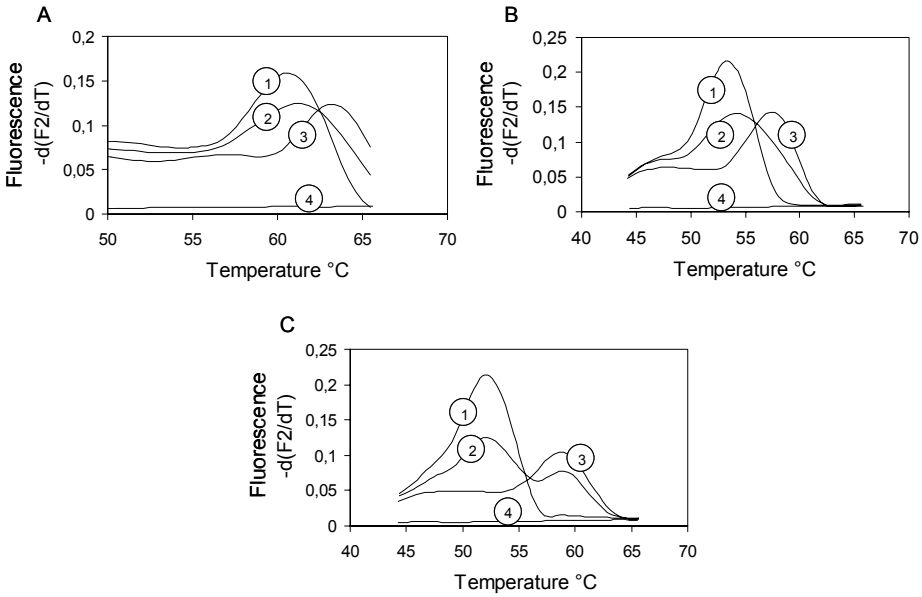

Figure 4.1 Genotyping of the A11156C polymorphism of the PXR gene with allele specific fluorescent probes using (A) Sen [A], (B) Sen [A]s and (C) Sen [A]sL by derivative melting curve plots. Representative derivative melting curves are plotted for the sample homozygous for the (1) 11156 [C] allele; (2) a heterozygous sample; (3) a homozygous sample for the 11156 [A] allele; (4) a no template control.

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