Alternative Methods to a TaqMan Assay to Detect a Tri-allelic SNP in the *HNFIβ* Gene

JJ Swen, RF Baak-Pablo, H-J Guchelaar and T van der Straaten

ABSTRACT

Background: Several studies report difficulties in genotyping $HNF1\beta$ rs757210 using TaqMan probes. This is possibly due to the tri-allelic nature of this SNP. The aim of the present research was to develop alternative methods for genotyping rs757210.

Material & Methods: Pyrosequencing and High Resolution Melting analysis of small amplicons (HRM) were developed and tested in panels of type 2 diabetes mellitus patients ($n = 258$) and healthy blood donors ($n = 183$). Results were confirmed by Sanger sequencing.

Results: With pyrosequencing, allele frequencies for the A, G and C allele of 0.42, 0.56, 0.02, and 0.37, 0.62, 0.01 were established in the panel of type 2 diabetes mellitus patients and healthy blood donors, respectively. Similar results were found using the more routinely available HRM method. Results for pyrosequencing and HRM were in 99.6% concordance.

Conclusion: Pyrosequencing and HRM can be used to genotype the tri-allelic SNP rs757210 in the $HNF1\beta$ gene and have the advantage over the commercially available TaqMan analysis that they can determine the rare C-allele variant.
INTRODUCTION
Rs757210 is located in the gene that codes for hepatocyte nuclear factor 1β (HNF1β). HNF1β is a transcription factor and alterations in this gene are associated with congenital anomalies of the kidney and urinary tract, as well as an increased risk for developing type 2 diabetes mellitus (T2DM) [1-12]. Rs757210 is an intronic A/G SNP with a reported minor allele frequency of 0.43 (HapMap-CEU, accessed 03-12-2010). Genotyping of rs757210 with commercially available TaqMan probes was found problematic in multiple studies. Stancakova et al. reported genotyping results of 20 risk alleles for T2DM using TaqMan probes in a large cohort of non-diabetic men. HNF1β rs757210 had to be excluded from the analyses because of failure to achieve Hardy-Weinberg equilibrium (HWE) [13]. In an investigation of the influence of T2DM risk alleles on the risk for the development of diabetes after renal transplantation, Ghisdal et al. reported a low call rate for rs757210 compared to a call rate of > 99% for the 10 other genotyped SNPs [14]. Again, rs757210 was the only SNP that deviated from HWE. Holmkvist et al. report that genotyping rs757210 with matrix-assisted laser desorption/ionization-time-of-flight mass spectroscopy (MALDI-TOF) was successful in subjects from the Malmö Preventive Project whereas genotyping rs757210 with a TaqMan assay failed in subjects from the Botnia study. These authors noted that the SNP is tri-allelic what is considered the reason for problematic genotyping [15]. In our own experience, repeated genotyping of rs757210 with a TaqMan assay also failed to meet the pre-defined quality criteria of a call rate ≥ 95% in a recent study on the effect of T2DM associated risk alleles on the response to sulfonylureas in T2DM patients [16].

The finding that rs757210 is tri-allelic has been reported previously [1,15] but has received limited attention in the literature. Furthermore, the NCBI SNP database [http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=757210, accessed 27-06-2011] also does not mention that rs757210 is tri-allelic.

The aim of the present research was to develop alternative methods for genotyping rs757210. First we developed a pyrosequencing method, and since this methodology is not always available, also the feasibility of a high resolution melting method of small amplicons (HRM) to genotype rs757210 was investigated.

RESULTS

Pyrosequencing
Pyrosequencing confirmed rs757210 as being tri-allelic. Using the given nucleotide dispensation order we identified the rare variant C allele, with an allele frequency of 0.02 (95% confidence interval (CI) 0.01-0.03) in a cohort of 258 T2DM patients (Figure 6.1).
Allele frequencies for the A and G allele were 0.42 (95% CI 0.37-0.46) and 0.56 (95% CI 0.52-0.61), respectively. No homozygous carriers of the allele C were found. Results were in Hardy-Weinberg equilibrium (p = 0.30). To ascertain that the pyrosequencing results were valid, genotypes of the 8 patients carrying a copy of the variant C-allele and 13 (5%) other randomly chosen samples were confirmed by conventional Sanger sequencing. Results were in 100% concordance (data not shown). Subsequently, genotype frequencies of 0.37 (95% CI 0.32-0.42), 0.62 (95% CI 0.56-0.67), and 0.01 (0.002-0.03) for the A, G, C allele respectively, were established in a cohort of 183 healthy blood donors. No statistically significant differences were observed between the TDM population and healthy blood donors for any of the alleles (Table 6.1).

**High Resolution Melting**

Figure 6.2 depicts the typical melting curves of the 6 different HNF1β genotypes. Since no patient with the CC genotype was included, the CC melting curve was obtained from genomic plasmid control. HRM results were obtained for all of the 258 TDM patients. Results were in 99.6% concordance with the pyrosequencing results confirming the feasibility of this method.
MATERIALS AND METHODS

DNA samples and isolation

A total of 258 DNA samples was obtained from a panel of T2DM patients. Panel ascertainment and sample collection have been described in detail previously [17]. Briefly, patients that had at least one prescription of the oral antidiabetic drugs tolbutamide, glibenclamide, glimepiride or gliclazide between January 1992 and June 2008, were at least 18 years of age and without insulin use at the time of first sulfonylurea prescription were included. Patients were recruited from four university affiliated primary care centers located in the vicinity of Leiden, The Netherlands. Patients received a written invitation by mail from their general practitioner. All patients provided informed consent and the study was approved by the institutional ethics committee. DNA was extracted from saliva specimens using Oragene kits (Westburg, Veenendaal, The Netherlands). To compare allele frequencies between T2DM patients and healthy volunteers an additional set of 183 anonymized DNA samples was obtained from a panel of residual blood samples obtained from healthy blood donors (Sanquin, Leiden, The Netherlands). DNA was extracted with the Magnapure Compact instrument (Roche, Almere, The Netherlands) according to the manufacturers’ protocol.

Pyrosequencing

First a pyrosequencing method was developed. Oligonucleotides were obtained from Eurogentec (Maastricht, The Netherlands). Primer sequences for polymerase chain reaction (PCR) and pyrosequence reactions are listed in Table 6.2. HotStar PCR mastermix and pyrosequencing reagents were obtained from Qiagen (Hilden, Germany) and PCR reactions were performed on the MyCycler (Biorad, Veenendaal, The Netherlands). The PCR reactions consisted of 10 ng DNA and 5 pmol of each primer in a total volume of 12µl. Cycle conditions were, initial enzyme activation for 15 minutes at 95°C, followed by

Table 6.1 Allele frequencies of HNF1β rs757210 alleles in 258 type 2 diabetes mellitus patients and 183 healthy blood donors determined with pyrosequencing

<table>
<thead>
<tr>
<th>Allele</th>
<th>Type 2 diabetes mellitus patients (n = 258)</th>
<th>Healthy blood donors (n = 183)</th>
</tr>
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<tr>
<td></td>
<td>Mean</td>
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</tr>
<tr>
<td>A</td>
<td>0.42</td>
<td>0.37-0.46</td>
</tr>
<tr>
<td>G</td>
<td>0.56</td>
<td>0.52-0.61</td>
</tr>
<tr>
<td>C</td>
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<td>0.01-0.03</td>
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</tbody>
</table>

95% CI, 95% confidence interval.
Panel A  Mixture of samples from type 2 diabetes mellitus patients and genomic plasmid controls.

Panel B  Typical melting peaks obtained from type 2 diabetes mellitus patients.

Panel C  Typical melting peaks obtained from genomic plasmid controls.

Figure 6.2  Typical melting peaks for the 6 different HNF1β rs757210 genotypes obtained with High Resolution Melting of the small amplicons. Lines represent results for AA (•); GG (•); AG (•); CG (•); AC (•), genotypes respectively, obtained from type 2 diabetes mellitus patients or plasmid controls, and CC (•) genotype obtained from genomic plasmid control.
35 cycles of 95°C-55°C-72°C each for 30 seconds, and a final extension of 10 minutes at 72°C. Samples were genotyped on the Pyrosequencer 96 MA (Biotage, Uppsala, Sweden). The pyrosequence reactions were performed according to the manufacturers’ protocol. Sequence to analyze was C/A/GTGTCCAGGCT and nucleotide dispensation order was TCAGCTGTCA. As a quality control 5% of the samples were genotyped in duplicate and no inconsistencies were observed. To ascertain that pyrosequencing results were valid, genotypes from more than 5% of patients were confirmed with Sanger sequencing, including all patients with the rare third allele.

**High Resolution Melting of small amplicons**

Secondly, the DNA samples from 258 T2DM patients were genotyped based on melting plots obtained by HRM of small amplicons. Results were compared to the results of pyrosequencing. Oligonucleotides used for small amplicon (+ 40 bp) genotyping were chosen adjacent to the SNP. Internal oligonucleotide calibrators were used to improve small amplicon genotyping. Oligonucleotide sequences are listed in Table 6.2. Amplification was performed in a 10 µl reaction volume in a HRM suitable 96-well plate (Thermofast® 96 Skirted, ABgene, Westburg, Leusden, The Netherlands). The reactions included HotStar PCR mastermix (Qiagen, Hilden, Germany), 5 pmol primers, 1x LC-Green® Plus (Bioke, Leiden, The Netherlands), 0.05 mM internal oligonucleotide Calibrator (IDT, Coralville, USA) and 20 ng DNA. Reactions were overlaid with mineral oil (Sigma-Aldrich, Zwijndrecht, The Netherlands). PCR conditions was as follows: 15 minutes at 95°C, followed by 40 cycles of 95°C (10 sec)-55°C (20 sec)-72°C (30 sec) and a final extension of 5 minutes at 72°C followed by 1 minute at 95°C. Finally, the samples were cooled down to room temperature. High resolution melting was performed on the Lightscanner® (HR-96, Idaho Technology, Salt Lake City, USA). Melting was

<table>
<thead>
<tr>
<th>Name</th>
<th>Purpose</th>
<th>Modification</th>
<th>Orientation</th>
<th>Sequence 5’-3’</th>
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<tr>
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<td>reverse</td>
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</tr>
</tbody>
</table>

Table 6.2 Primers for PCR and pyrosequencing of HNF1β rs757210
done from 50°C to 98°C with ramp rate of 0.1°C/sec. Melting curves were analyzed with Lightscanner® Software using Call-IT 2.0. Again, 5% of the samples were genotyped in duplicate and no inconsistencies were observed.

**Genotype controls**

HRM requires the use of well characterised controls. Therefore plasmid controls with a 639 bp insert of the HNF1β gene, with the three variants of the SNP at position 240 were created. Preparation has been described in detail previously [18]. In short, plasmid controls were made by ligation of HNF1β PCR product into pGEM-T easy vector (Promega, Leiden, The Netherlands) and plasmid DNA was isolated by standard procedures. Genotypes of the plasmid controls were determined by conventional sequencing on the ABI 3130 analyser using BigDye® Terminator v3.1 Cycle Sequencing Kit From Applied Biosystems (Applied Biosystem, Nieuwerkerk aan de IJssel, The Netherlands).

**Statistical analysis**

The data were analyzed using the SPSS statistical package (version 17.0, SPSS, Chicago, IL, USA). Possible deviation from Hardy–Weinberg equilibrium was tested by the χ² test. A p-value < 0.05 was considered a deviation. Difference in allele frequencies between patients with T2DM and healthy blood donors were tested with an unpaired Student’s t-test. A p-value < 0.05 was regarded as statistically significant.

**DISCUSSION**

In this study, we showed the feasibility and accuracy of pyrosequencing and HRM to genotype the tri-allelic rs757210 in the HNF1β gene. With the pyrosequencing method the presence of the rare variant C allele was confirmed and the allele frequencies were established in T2DM patients and healthy blood donors. To the best of our knowledge, this is the first study to report pyrosequencing and HRM as useful alternatives methods to TaqMan to genotype rs757210 with the latter method having the disadvantage to fail to determine the rare C-allele variant.

The presence of the tri-allelic nature of rs757210 was noted by Winckler et al. recently [1]. Holmkvist et al. established genotype frequencies in a Swedish population of 2293 healthy individuals of whom 132 developed type 2 diabetes [15]. Reported allele frequencies were 0.36, 0.61, and 0.03 for the A, G, and C allele respectively. These results are comparable to the pyrosequencing results reported in this article. However, genotyping by Holmkvist et al. was performed by MALDI-TOF. These techniques are not standard for most laboratories. For this reason we extended our investigations to
test the feasibility of HRM of small amplicons to detect these variants. HRM of small amplicons is an attractive genotyping method because it is fast, relatively cheap (no labelled primers are required), no sample processing is required after amplification and most new generation real-time machines, present in many laboratories performing genotyping studies, are equipped with this possibility making it more accessible for other investigators [19,20].

The problems of genotyping rs757210 with the commercially available TaqMan assays probably originate from the fact that the assay was designed without knowledge of the tri-allelic nature of the SNP. Both pyrosequencing and HRM are more flexible concerning probe design and therefore better suited to genotype this SNP.

A possible limitation of our study is that no homozygous carriers of the C allele were included. Therefore we were unable to test the suitability of the pyrosequencing and HRM method to genotype subjects with this genotype. Based on the frequency of the C allele, 0.04% of the population is expected to be homozygous carrier of the C allele and as a result very large cohorts are required to include them. Also, both methods were perfectly capable of identifying heterozygous carriers.

Carriers of the A allele of rs757210 have an increased risk to develop T2DM (OR 1.12 p = 5*10^{-6}) [1,13]. Models that use HNF1β genotype and other genetic markers can explain approximately 5-10% of T2DM heritability [21]. In our cohort no statistically significant difference in allele frequencies was observed between patients with T2DM and healthy blood donors. This is most likely due to the limited sample size of our study. Post-hoc power analysis revealed that the power to detect a statistically significant difference of 0.05 in allele frequency with an α of 0.05, was 48.8%.

HNF1β is an important gene. Besides its contribution to T2DM risk prediction it might also provide opportunities for new therapeutic interventions and management i.e. pharmacogenetics. Currently there are insufficient data to support the latter, but the knowledge of the pharmacogenetics of oral anti-diabetic drugs has increased significantly in recent years [22-24]. Combined with the ever increasing incidence of T2DM worldwide, these developments might anticipate a greater clinical role for genotyping HNF1β and other genetic variants, and endorse the relevance of easily available genotyping methods such as our pyrosequencing or HRM method.

**Conclusion**

In conclusion, we have shown that pyrosequencing and HRM can both be successfully applied to genotype the tri-allelic SNP rs757210 in the HNF1β gene.
REFERENCES


