Rapid genotyping of the human renin (REN) gene by the LightCycler® instrument: Identification of unexpected nucleotide substitutions within the selected hybridization probe area

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Abstract. Preeclampsia is a serious disorder affecting nearly 3% of all pregnancies in the Western world. It is associated with hypertension and proteinuria, and several lines of evidence suggest that the renin-angiotensin system (RAS) may be involved in the development of hypertension at different stages of a preeclamptic pregnancy. In this study, we developed rapid genotyping assays on the LightCycler® instrument to allow the detection of genetic variants in the renin gene (REN) that may predispose to preeclampsia. The method is based on real-time PCR and allele-specific hybridization probes, followed by fluorescent melting curve analysis to expose a change in melting temperature (Tm). Ninety-two mother-father-child triads (n = 276) from preeclamptic pregnancies were genotyped for three haplotype-tagging single nucleotide polymorphisms (htSNPs) in REN. All three htSNPs (rs5705, rs1464816 and rs3795575) were successfully genotyped. Furthermore, two unexpected nucleotide substitutions (rs11571084 and rs61757041) were identified within the selected hybridization probe area of rs1464816 and rs3795575 due to aberrant melting peaks. In conclusion, genotyping on the LightCycler® instrument proved to be rapid and highly reproducible. The ability to uncover additional nucleotide substitutions is particularly important in that it allows the identification of potentially etiological variants that might otherwise be overlooked by other genotyping methods.

Keywords: Preeclampsia, renin, LightCycler, real-time PCR

1. Introduction

Preeclampsia is a heritable complication affecting nearly 3% of all pregnancies in Western populations [1]. Although the etiology remains to be elucidated, reduced placental perfusion, oxidative stress, activation of thrombosis and the renin-angiotensin system (RAS) are probably involved at different stages of a preeclamptic pregnancy [2–5]. Family studies have shown that genetic factors contribute to the development of preeclampsia, but the exact mechanism is still unknown despite extensive studies [6–8]. The presence of renin (REN) in the placenta indicates a local synthesis and suggests that REN may be involved in the regulation of maternal blood pressure [9,10]. Therefore,
2. Materials and methods

2.1. Biological samples

Ninety-two women who delivered at the Stavanger University Hospital in Norway in the period January 1993 to December 1995 and who fulfilled the diagnostic criteria for preeclampsia were included in the study [11, 15]. Umbilical cord blood was collected from delivered neonates and peripheral blood was drawn from mothers and fathers post partum [11]. The study was approved by the Regional Committee for Ethics in Medical Research and the Norwegian Data Inspectorate. Written informed consent was obtained from the parents. The present work was undertaken as a part of a larger study examining genetic risk factors for preeclampsia [16].

2.2. SNP selection and DNA extraction

A 40 kb-long region containing REN on chromosome 1q32 (NT 004487.18) was downloaded from HapMap (http://www.hapmap.org) and imported into Haploview v.3.0 [17] for the selection of htSNPs and the evaluation of marker metrics. Three htSNPs on chromosome 1 (rs5705, rs1464816, and rs3795575) were chosen using the “pairwise tagging” algorithm. All nucleotide positions are according to the National Center for Biotechnology Information (NCBI) assembly 34 of the human genome (Table 1). To capture the genetic variation encompassing REN, we used a minor allele frequency (MAF) cut-off of at least 10% and an r^2 greater than 0.8 for linkage disequilibrium within haplotype blocks. Genomic DNA from 276 samples was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany).

2.3. PCR primers and hybridization probes

PCR primers and hybridization probes were designed and synthesized by TIB MOLBIOL (Berlin, Germany). Details are provided in Table 1.

2.4. Genotyping

To genotype rs5705, rs1464816 and rs3795575, three separate real-time PCR reactions were performed followed by fluorescent melting curve analyses on the LightCycler® 2.0 instrument (Roche Diagnostics, Mannheim, Germany). The reaction mixtures contained 2.5 µL of DNA (approximately 10–50 ng), 4 µL of LightCycler FastStart DNA MasterPLUS HybProbe (Roche Diagnostics), PCR primers, hybridization probes and sterile water to a final volume of 10 µL. Asymmetric PCR conditions were used. PCR primers and hybridization probes were added at the following concentrations: for rs5705, 0.2 µM RENe2S, 0.4 µM RENe2A, 0.2 µM sensor C, and 0.4 µM Anchor REN; for rs1464816, 0.2 µM RENi4S, 0.4 µM RENi4A, 0.2 µM Sensor wt and 0.4 µM of Anchor REN; and, finally, for rs3795575, 0.2 µM RENi8A, 0.5 µM RENi8S, 0.2 µM REN mut and 0.4 µM of REN LC.

Cycling conditions were 95 °C for 10 min, followed by 45 cycles of 95 °C for 5 s, 52 °C, 56 °C and 58 °C for 10 s for rs5705, rs1464816 and rs3795575 respectively, then 72 °C for 15 s, with a ramp rate of 20 °C/s. Melting curve analysis was performed as follows: 95 °C for 10 s, cooling to 55 °C and 45 °C for 30 s each, 40 °C for 2 min, then heating to 75 °C (0.2 °C/s). Thirty pa-
PCR primers and hybridization probes used for genotyping REN

<table>
<thead>
<tr>
<th>SNP</th>
<th>Nucleotide position*</th>
<th>Alleles</th>
<th>PCR primers (5'-3')</th>
<th>Hybridization probes (5'-3')*</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs5705</td>
<td>202397809</td>
<td>A/C</td>
<td>RENe2S: CAAGAGAATGCCCTCAATCC</td>
<td>Sensor C: ATGAAGAGGCTGACCCFL-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>RENe2A: CAAGACCTCAAGTCCCATAG</td>
<td>Anchor REN: 640-ACACCACTCCTCGTGTGACCC-p</td>
</tr>
<tr>
<td>rs1464816</td>
<td>202395477</td>
<td>G/T</td>
<td>RENi4S: GCTTTTTTGGTCTGTGG</td>
<td>Sensor wt: TGAGGCTGACCCFL-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>RENi4A: TCTCTGTGCTTCATATGCTC</td>
<td>Anchor REN: 640-CTTACCCGATTTCTGTGACCC-p</td>
</tr>
<tr>
<td>rs3795575</td>
<td>202391712</td>
<td>C/T</td>
<td>RENi8S: AAGAAGCCAAAAGGGAAAG</td>
<td>REN mut: GCCTTCTTGATGGAAAGAACATTCAGCFL-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>RENi8A: GAAAGAGATGGAAGCGTGATG</td>
<td>REN LC: 640-GACAAAGAGGTCTTGCGCTGCTGGC-p</td>
</tr>
</tbody>
</table>

*Nucleotide positions according to the National Center for Biotechnology Information (NCBI) human assembly 34.

**The position of the polymorphic site is underlined.

Table 2

Genotype distribution of rs5705, rs1464816 and rs3795575 in the human renin gene (REN) among 92 preeclampsia mother-father-child triads

<table>
<thead>
<tr>
<th>SNP</th>
<th>Genotype</th>
<th>Count (%)</th>
<th>$T_m$ °C (± SD)*</th>
<th>$\Delta T_m$ °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs5705</td>
<td>A/A</td>
<td>217 (78.6)</td>
<td>58.4 (0.24)</td>
<td>8.2</td>
</tr>
<tr>
<td></td>
<td>A/C</td>
<td>59 (21.4)</td>
<td>58.4 (0.24) and 66.4 (0.23)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C/C</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs1464816</td>
<td>G/G</td>
<td>112 (40.6)</td>
<td>63.0 (0.40)</td>
<td>10.1</td>
</tr>
<tr>
<td></td>
<td>G/T</td>
<td>139 (50.4)</td>
<td>63.0 (0.40) and 53.1 (0.44)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T/T</td>
<td>25 (9.0)</td>
<td>53.1 (0.44)</td>
<td></td>
</tr>
<tr>
<td>rs3795575</td>
<td>C/C</td>
<td>212 (76.8)</td>
<td>65.0 (0.38)</td>
<td>5.1</td>
</tr>
<tr>
<td></td>
<td>C/T</td>
<td>64 (23.2)</td>
<td>65.0 (0.38) and 70.0 (0.27)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T/T</td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$T_m$: melting temperature; $\Delta T_m$: difference in $T_m$ between the wild-type and mutant genotype.

*Mean $T_m$ ± standard deviation.

2.5. Sequencing

To assess genotyping consistency, DNA from 30 randomly selected individuals was sequenced for each htSNP (PCR primers are provided in Table 1). Amplification products were electrophoresed on 2% agarose gel and visualized by ethidium bromide staining. PCR fragments were purified using QIAquick® PCR Purification Kit (Qiagen) followed by cycle sequencing using ABI PRISM® BigDye® Terminator v1.1 Cycle Sequencing Kit (AB Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s recommendations. Finally, the sequencing reactions were cleaned using Centri-Sep spin columns (Princeton Separations, Inc, Adelphia, NJ) according to the manufacturer’s instructions and analyzed on an ABI PRISM® 310 Genetic Analyzer instrument (AB Applied Biosystems). Resulting sequences were aligned in BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for confirmation.

3. Results

DNA samples from preeclamptic women, their partners and children (276 subjects in total) were genotyped for three htSNPs in REN. Each of the htSNPs showed a clear difference in $T_m$ between the wild-type and mutant genotype (between 5.1 °C and 10.1 °C). All three genotyping assays were performed in duplicate and no inconsistencies between the genotypes were observed. The results are summarized in Table 2. When genotypes from the LightCycler® analysis were compared with the results of DNA sequencing in a random set of 30 patient samples, identical genotypes were obtained for each htSNP.

During fluorescent melting curve analysis, unusual melting peaks around rs1464816 (Fig. 1) and rs3795575 (Fig. 2) were observed. For rs1464816, seven of the patient samples revealed atypical melting peaks, one positive (heterozygous target) and one negative (no-template) control (n = 32) were analyzed simultaneously. All samples (276 in total) were analyzed in duplicate. The wild-type and mutant genotypes were differentiated by their characteristic melting peaks, which were generated by plotting the negative derivative of the fluorescence signal with temperature versus temperature (T) \([-dF/dT]\) versus T].
peaks, with a $T_m$ of 61.1°C, 1.9°C ± 0.12 (mean ± SD) lower than the $T_m$ of the wild-type genotype G/G (Fig. 1). For rs3795575, 16 samples revealed atypical melting peaks, with a $T_m$ of 59.8°C, 5.2°C ± 0.28 lower than the $T_m$ of the wild-type genotype C/C (Fig. 2). To confirm that the atypical melting peaks were not due to error, we reanalyzed DNA from patients exhibiting atypical melting peaks. Identical results were observed. When we sequenced these samples, additional nucleotide substitutions were discovered in the hybridization probe areas; namely, rs11571084 [A/G] (Chr1: 202395455), 22 nucleotides upstream of rs1464816, and rs61757041 [A/G] (Chr1: 202391716), 4 nucleotides downstream of rs3795575, respectively.

4. Discussion

Detection of etiologically relevant SNPs is a powerful diagnostic test for inherited disorders. In this study, we developed genotyping assays for $REN$ by allele-specific hybridization probes followed by fluorescent melting curve analysis on the LightCycler® instrument. The technique proved to be rapid and reliable. Thirty patient samples, together with positive and negative controls, were genotyped in less than an hour, and the results were highly reproducible when cross-checked against direct sequencing.

Genotyping on the LightCycler® instrument is particularly advantageous compared with other traditional genotyping techniques [11,12] in that it does not require any post-PCR sample processing, no hazardous reagents are involved, and the entire reaction takes place in a close-tube system, which minimizes the risk of contaminations. All these factors are important to consider in a moderate-sized lab-facility. Furthermore, the unanticipated detection of rs11571084 and rs61757041 within the hybridization probes areas designed for rs1464816 and rs3795575 demonstrates the ability of the technique to distinguish patient samples with additional nucleotide substitutions. Samples from patients carrying these unexpected nucleotide substitu-
Fig. 2. Typical melting peaks obtained after genotyping rs3795575 by the LightCycler® instrument. Patients with the wild-type C/C genotype revealed a single melting peak with a T$_m$ at 65.0°C (red) and patients with the heterozygous C/T genotype revealed two melting peaks, one at 65.0°C and the other at 70.0°C (blue). An atypical melting peak with T$_m$ at 61.1°C is shown in green, and the no-template control is shown in black. The DNA sequence (forward direction) is from a patient who is homozygous wild-type C/C for rs3795575 (blue arrow) and simultaneously heterozygous A/G for rs61757041 (highlighted by the black arrow).

**Meltiing Peaks**

- Wild-type genotype (C/C)
- Atypical melting peak
- Heterozygous genotype (C/T)

The impact of these two nucleotide substitutions on the development of preeclampsia is unknown and future studies are warranted to determine the frequency and association of these variants with disease development and progression. The sample size clearly needs to be increased in order to gauge the impact of these additional nucleotide substitutions on the risk of preeclampsia. According to the dbSNP database (www.ncbi.nlm.nih.gov/sites/entrez), two other SNPs (rs11571116 and rs61757042) have recently been reported in the vicinity of rs1464816 and rs3795575. Rs11571116 has not been typed in the CEPH sample of Northern-European origin, while data for rs61757042 have not yet been released in all the HapMap test populations (www.hapmap.org).

Several diagnostic assays have been developed on the LightCycler® instrument, enabling the identification of genotypes by characteristic melting peaks and T$_m$ shifts [20–22]. Some of these well-established diagnostic assays have reported atypical melting peaks due to the presence of new nucleotide substitutions in the hybridization probe area beside those the assays were originally designed for. These additional nucleotide substitutions have shown varying clinical effects [23–26]. Atypical melting peaks are usually detected by T$_m$ shifts of 5–6°C or higher and may therefore be easily overlooked if the T$_m$ shifts are less than 1°C, or if there is an overlap in T$_m$ [27,28]. However, if the hybridization probe area is particularly ambiguous or has not been reported in the public databases, validation with an alternative method and/or direct sequencing of patient samples in diverse populations is warranted.

In conclusion, genotyping using the LightCycler® instrument allows highly specific detection of SNPs in
the targeted DNA sequence. In particular, the discovery of new nucleotide substitutions within the hybridization probe area represents a significant advance over other conventional genotyping platforms. This, coupled with relatively low cost and minimum hands-on time, makes the LightCycler® technique particularly attractive for cost-effective and rapid genotyping in smaller lab-facilities.

Acknowledgements

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Conflict of interest

There are no conflicts of interest.

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