Genotyping Standards

Positive genotyping standards for -13907 G/G, -13910 C/C, -13910 T/T, -13913 C/C and -13915 G/G were prepared by GenExpress GmbH (Berlin, Germany) from donors with known genotypes and cloned into a pUC21 vector; plasmid sequences were confirmed by Sanger-sequencing.

Experimental conduction

Real-time PCR amplification with consecutively genotyping by melting curve analysis was performed on a LightCycler 480 Instrument II (Roche Diagnostics, Mannheim, Germany). The PCR-mastermix was prepared with 1 µM of the forward primer (LCTA-S, 5'-TTCAGGAAAAATGTACTTAGACCC-3'), 0.2 µM of the reverse primer (LCTA-A, 5'-AATGCAGGGCTC AAAGAA-3') and 1 µM of the internal fluorescent labelled SimpleProbe oligonucleotide (5'-AGGCCAGGGACTACATTAT CTTATC-block-3'), 3 mM $MgCl_2$ and 1x FastStart DNA Master Hybridization Probes (Roche Diagnostics, Mannheim, Germany). A total reaction volume of 10 µl (8 µl mastermix and 2 µl sample DNA) was heated at 95 °C for 10 min followed by 50 cycles of 5 s at 95 °C, 10 s at 60 °C and 15 s at 72 °C. Melting curve analysis was performed by 30 s at 95 °C and 43 °C for 30 s, followed by a temperature increase of 1.5 °C/s to 75 °C with a continuous acquisition (3 acquisitions per °C) with filters 465-510, and a final cooling step of 30 s at 40 °C.

3.0 RESULTS

First, melting peaks were established for all the genotyping standards and controls (Fig. 1). For these, melting temperatures of 61.3 °C (-13910 T/T, n = 8, SD = 0.3 °C), 55.9 °C (-13910 C/C, n = 8, SD = 0.3 °C), 51.6 °C (-13907 G/G, n = 6, SD = 0.5 °C), 49.9 °C (-13913 C/C, n = 6, SD = 0.6°C) and 52.6 °C (-13915 G/G, n = 6, SD = 0.6 °C), were obtained. Although the three African/Arabian type variants yield distinct melting temperatures, the obtained temperature differences were estimated as too small to use them for a reliable identification of a particular African/Arabian allele in routine analysis of patient samples. However, the appearance of a melting peak in the temperature range of 50 to 52 °C indicated an African/Arabian variant, and the African/Arabian variants as a group can clearly be discriminated from the other variants.

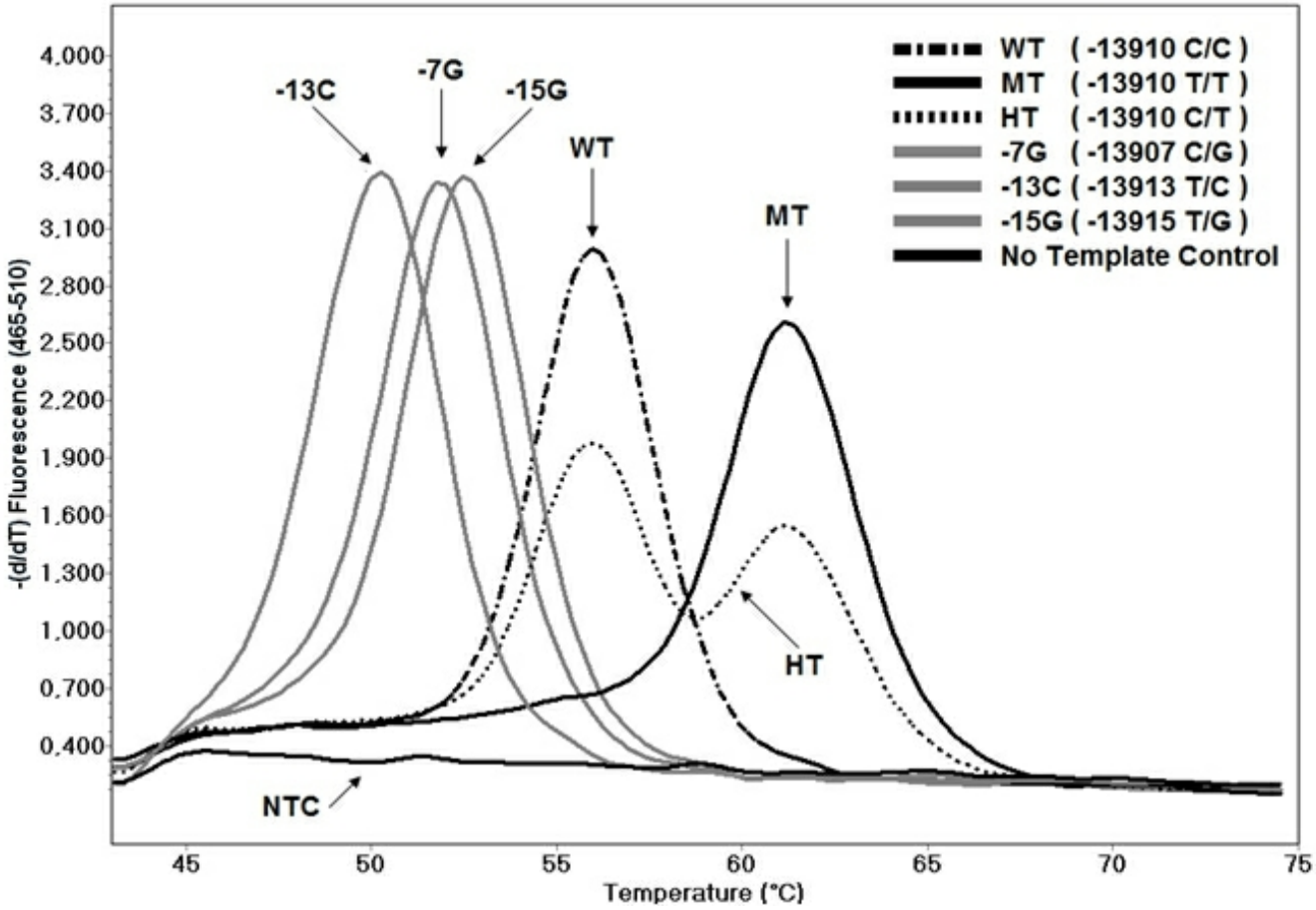

Fig. 1. Melting curve analysis of Polymerase Chain Reaction (PCR) amplicons covering the enhancer region located in intron 9 of the MCM6 gene that includes the Single Nucleotide Polymorphisms (SNPs) controlling adult lactose tolerance.

4.0 CLINICAL VALIDATION

Subsequently, the real-time PCR assay was validated on a set of 100 patients and the results were verified by sequencing. Two hundred microliter of EDTA blood samples from 100 donors were collected by TIB Molbiol S.r.I (Genova, Italy) and purified on a Roche MagNAPure instrument using the MagNAPure LC Total Nucleic Acid Isolation Kit according to the manufacturer's instructions. The purified DNA was eluted in 100 µl elution buffer and added undiluted into the real-time PCR assay. Genotyping standards -13910 C/C (wild-type), -13910 T/T (mutant) and an equimolar mixture of -13910 C/C and -13910 T/T imitating the heterozygous genotype were used as reference samples.

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5.0 DISCUSSIONS

Out of the 100 tested patients, 74 were found carrying the genotype -13910 C/C (wild-type), 23 -13910 C/T (heterozygous), two -13910 T/T (mutant) and one patient, which was classified as 'Unknown' by the LightCycler 480 analysis software. According to the melting temperature of 52.0 °C this sample (S3874) was classified as -13915 G/G. Sequencing was performed by GenExpress on an ABI Prism 310 instrument (Applied Biosystems, Weiterstadt, Germany). 100 percent concordance was obtained with the outcome of the real-time PCR assay. For sample S3874 the assumed genotype -13915 G/G could be confirmed.

To test the robustness of the assay another panel of 20 patient samples of genomic DNA was tested at Copenhagen University Hospital (Roskilde), Denmark, using similar assay conditions, but including a -13915G genotyping standard. The panel, consisting of genomic DNA purified from EDTA blood using the Qiasymphony DNA Midi Kit was kindly donated from Skejby University Hospital, Denmark. According to the routine inhouse-validated TaqMan SNP genotyping assay (Applied Biosystems) the panel represented six of each of the Caucasian MCM6 genotypes -13910 C/C, -13910 C/T and -13910 T/T as well as two African/Arabian type variants, presumed to represent MCM6 -13915G.

For the 18 Caucasian genotypes 100 % concordance was obtained. Also, both of the two African variants showed the expected melting peak corresponding to the African/Arabian -13915G genotyping standard.

6.0 CONCLUSIONS

In conclusion, the new SimpleProbe LightCycler assay provides a reliable method to diagnose hereditary lactose intolerance.

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REFERENCES

1. Ingram CJ, Mulcare CA, Itan Y, Thomas MG, Swallow DM. Lactose digestion and the evolutionary genetics of lactase persistence. Hum Genet 2009;124:579-91
2. Tishkoff SA, Reed FA, Ranciaro A, Voight BF, Babbitt CC, Silverman JS et al. Convergent adaptation of human lactase persistence in Africa and Europe. Nat Genet 2007;39:31-40
3. Itan Y, Powell A, Beaumont MA, Burger J, Thomas MG. Tanaka, Mark M. The origins of lactase persistence in Europe. PLoS Comput. Biol 2009;5:e1000491
4. Olds LC, Ahn JK, Sibley E. 13915*G DNA polymorphism associated with lactase persistence in Africa interacts with Oct-1. Hum Genet. 2011;129(1):111-3
5. Bodlaj G, Stöcher M, Hufnagl P, Hubmann R, Biesenbach G, Stekel H, Berg J. Genotyping of the lactase-phlorizin hydrolase C13910 polymorphism by LightCycler PCR and implications for the diagnosis of lactose intolerance. Clin Chem 2006;52:148-51
6. Nilsson TK, Olsson LA.: Simultaneous genotyping of the three lactose tolerance-linked polymorphisms LCT -13907C>G, LCT -13910C>T and LCT -13915T>G with Pyrosequencing technology. Clin Chem Lab Med 2008;46:80-4
7. Tag CG, Oberkanins C, Kriegshäuser G, Ingram CJ, Swallow DM, Gressner AM et al. Evaluation of a novel reverse-hybridization StripAssay for typing DNA variants useful in diagnosis of adult-type hypolactasia. Clin Chim Acta. 2008;392:58-62

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