Genotyping of the Lactase-Phlorizin Hydrolase −13910 Polymorphism by LightCycler PCR and Implications for the Diagnosis of Lactose Intolerance, Gerda Bodlaj, Markus Stöcher, Peter Hufnagl, Rainer Hubmann, Georg Biesenbach, Herbert Stelk, and Jörg Berg (1) Institute of Laboratory Medicine and 2 Department of Medicine II, General Hospital Linz, Linz, Austria; 3 Roche Diagnostics, Engelhornsgasse, Vienna, Austria; 4 these authors contributed equally to this work; 5 address correspondence to this author at: Institute of Laboratory Medicine, General Hospital Linz, Krankenhausstrasse 9, A-4020 Linz, Austria; fax 43-732-7806-1815, e-mail joerg.berg@akh.linz.at

Background: Hypolactasia and lactose intolerance are common conditions worldwide. Hypolactasia seems to be strongly correlated with genotype C/C of the genetic variant C→T−13910 upstream of the lactase-phlorizin hydrolase (LPH) gene. We developed a rapid genotyping assay for LPH C→T−13910 and investigated the relationship of positive lactose breath hydrogen test (LBHT) results suggesting lactose intolerance with LPH C→T−13910 genotype.

Methods: Using automated DNA purification on the MagnaPure LC and real-time PCR on the LightCycler, we examined samples from 220 individuals to estimate genotype frequencies; we then determined LPH C→T−13910 genotype in samples from 54 Caucasian patients with a positive LBHT result and symptoms of lactose intolerance.

Results: Genotyping of 220 individuals revealed frequencies of 21.4%, 41.8%, and 36.8% for genotypes C/C, C/T, and T/T. Of the patients with positive LBHT results, only 50% had the C/C genotype suggestive of primary adult hypolactasia in our study population. The other patients had various degrees of secondary hypolactasia or symptoms of lactose intolerance. Patients with C/C genotype had a mean (SD) peak H2 increase in the LBHT [108 (58) ppm] that was significantly higher than in patients with the C/T [65 (54) ppm] and T/T [44 (34) ppm] genotypes.

Conclusions: The new real-time PCR assay provides a rapid, labor-saving means for the genotyping of LPH C→T−13910. Use of the assay may assist in differentiating patients with primary hypolactasia from those with secondary hypolactasia and lactose intolerance, who may need further clinical examinations to diagnose their underlying primary diseases.

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Deficiency of the brush border enzyme lactase-phlorizin hydrolase (LPH), which catalyzes the hydrolysis of lactose to yield glucose and galactose, leads to malabsorption of lactose and to lactose intolerance when unabsorbed lactose reaches the colon and is fermented by bacteria (1, 2). Adult hypolactasia, which reflects declining LPH gene expression (3–5) during maturation, varies in frequency among populations (6).

A single-nucleotide polymorphism (SNP) upstream of the LPH gene at position −13910 (C→T−13910) was reported to perfectly match with phenotypic hypolactasia and lactose malabsorption for the C/C genotype (7). Further studies demonstrated that the C/C genotype matched with low LPH-specific mRNA expression and low lactase activity in intestinal biopsies, suggesting primary adult hypolactasia, whereas the genotypes C/T and T/T matched with high LPH-specific mRNA expression and high lactase activity, strongly suggesting lactase persistence (7–11). A second described SNP, LPH G→A−32018, is thought to be only in linkage disequilibrium with the SNP C→T−13910 (11).

The described SNPs may not necessarily explain all of the genetic variations of LPH gene expression in all ethnic groups (12). In some sub-Saharan African populations, the C/C genotype is frequent and not associated with the epidemiologic data of lactose intolerance (13). In many ethnically diverse populations of Europe and of European descent, however, data on the frequency of the C/C−13910
genotype concur with the prevalence of primary adult hypolactasia (2).

Tests for the diagnosis of hypolactasia consist of biochemical tests such as the lactase breath hydrogen test (LBHT), lactase enzyme activity tests performed with biopsy samples of the small intestine, and most recently, molecular tests to determine the allelic variants of LPH C→T-13910 (14–17).

We developed an automated rapid genotyping assay for LPH C→T-13910 based on real-time PCR on the LightCycler instrument (Roche Applied Science) and used this assay to perform LPH C→T-13910 genotyping on DNA samples from 220 persons and to investigate the relationship between positive LBHT results and genotyping of LPH C→T-13910 in patients with symptoms of lactose intolerance.

DNA was purified from EDTA blood (200 μL) on the MagNa Pure LC instrument (Roche Applied Science) with use of the MagNa Pure LC Total Nucleic Acid Isolation Kit (Roche Applied Science) according to the manufacturer's instructions. DNA was eluted in 50 μL of elution buffer.

We designed primers and probes targeting a region around nucleotide -13910 upstream of the LPH gene (GenBank accession number AY220757; nucleotides 26481–26672) with the LightCycler probe design software 1.0 (Roche; see Fig. 1 in the Data Supplement that accompanies the online version of this Technical Brief at www.clinchem.org/content/vol52/issue1). Real-time PCR, followed by melting curve analysis for genotyping of LPH C→T-13910, was performed on the LightCycler instrument 1.0 (Roche; software version 3.5.3). The master-mix reagent Fast Start DNA Master Hybridization Probes (Roche) was used and supplemented to 4 mmol MgCl₂, 0.2 μM each of the LPH-specific primers (LPH-for, 5'-TGAGTGTAGTTGGACGG-3'; LPH-rev, 5'-CAACCTAAGGAGGAG-AGT-3'), and 0.2 μM each of the LPH-specific fluorescence resonance energy transfer hybridization probes (5'-GCCTCTGCGCTGGCAATACAGATA-fluorescein-3' and 5'-LC-Red705-ATAATGTAGCCCTGGCCT-phosphate-3'). PCR was carried out in 20-μL capillaries (2 μL of sample DNA and 18 μL of master-mix) at 95 °C for 8 min, followed by 40 cycles of 0 s at 95 °C, 10 s at 54 °C, and 15 s at 72 °C. Genotyping was performed by melting curve analysis: 95 °C for 0 s and 40 °C for 30 s, followed by a temperature increase from

| Patient age, years | Sex | Genotype | ΔH₂O ppm | Symptoms at lactose challenge during LBHT | Lactose-free diet | Primary diagnosis | Symptoms at genotyping |
|--------------------|-----|----------|-----------|------------------------------------------|-------------------|------------------|-----------------------|
| 43                 | M   | CT       | 21        | None                                     | Response          | NE*              | No complaints under diet |
| 54                 | M   | CT       | 22        | None                                     | No response       | Pancreas insufficiency | Diarrhea              |
| 74                 | M   | CT       | 22        | None                                     | No response       | None             | None                  |
| 83                 | F   | CT       | 29        | Diarrhea                                  | No response       | Pancreas insufficiency | None                  |
| 27                 | F   | CT       | 30        | None                                     | No response       | Crohn disease      | Diarrhea              |
| 60                 | F   | CT       | 39        | Bloating                                  | No response       | None             | Bloating              |
| 48                 | F   | CT       | 44        | Abdominal pain                            | No response       | Gastric bypass    | Abdominal pain; diarrhea |
| 56                 | F   | CT       | 44        | Abdominal pain; bloating; diarrhea        | No response       | None             | None                  |
| 71                 | M   | CT       | 45        | Abdominal pain; bloating; diarrhea        | Response          | None             | No complaints under diet |
| 71                 | M   | CT       | 47        | None                                     | No response       | Eosinophilic duodenitis | Abdominal pain; diarrhea |
| 26                 | F   | CT       | 62        | Abdominal pain; diarrhea                  | ND                | Celiac sprue      | No complaints with gluten-free diet |
| 82                 | M   | CT       | 66        | None                                     | No response       | None             | Abdominal pain; diarrhea |
| 66                 | F   | CT       | 72        | Abdominal pain; nausea                    | No response       | None             | None                  |
| 81                 | F   | CT       | 133       | Abdominal pain; diarrhea                  | No response       | Abdominal pain; diarrhea | None                  |
| 61                 | F   | CT       | 162       | Abdominal pain; diarrhea                  | No response       | None             | None                  |
| 60                 | M   | CT       | 200       | Abdominal pain; diarrhea                  | No response       | Pancreas insufficiency | Diarrhea              |
| 24                 | F   | TT       | 22        | None                                     | No response       | None             | None                  |
| 40                 | M   | TT       | 22        | None                                     | No response       | Pancreas insufficiency | Diarrhea              |
| 85                 | M   | TT       | 22        | None                                     | No response       | None             | None                  |
| 74                 | F   | TT       | 23        | None                                     | No response       | None             | None                  |
| 65                 | F   | TT       | 28        | None                                     | ND                | Celiac sprue      | No complaints with gluten-free diet |
| 76                 | F   | TT       | 30        | Abdominal pain; bloating; diarrhea        | No response       | Soybean allergy   | Diarrhea              |
| 30                 | F   | TT       | 41        | None                                     | No response       | None             | None                  |
| 18                 | M   | TT       | 43        | Abdominal pain; bloating                  | No response       | None             | Abdominal pain; bloating |
| 83                 | F   | TT       | 49        | None                                     | No response       | Diarrhea         | None                  |
| 81                 | F   | TT       | 64        | Bloating; diarrhea                        | No response       | None             | Bloating; diarrhea    |
| 82                 | F   | TT       | 137       | Bloating; diarrhea                        | No response       | None             | Diarrhea              |

*NE, primary diagnosis not established at time of LBHT; ND, no lactose-free diet.

Table 1. Characteristics of C/T and T/T genotyped patients with positive LBHT and suspected lactose intolerance.
40 °C to 80 °C (0.2 °C/s), with continuous fluorescence recording on channel F3. Each assay run included a reference sample for each of the 3 genotypes and a reagent control containing water instead of sample DNA.

We obtained 3 DNA reference samples, genotyped for the LPH C→T_{13910} SNP by PCR followed by restriction fragment length polymorphism analysis, from the Clinical Institute of Medical and Laboratory Analysis, Medical University Graz, Austria (15). LPH C→T_{13910} Genotyping of the reference DNA samples and all additional DNA samples led to distinct melting temperatures at [mean (SD)] 64.5 (0.3) °C (n = 20) and 56.0 (0.2) °C (n = 20) for the C and T alleles, respectively (see Fig. 2 in the online Data Supplement).

We tested the robustness of the assay in the routine clinical laboratory by assaying samples from 220 persons in a screening setup. All samples returned an unequivocal genotyping result with genotypes C/C, C/T, and T/T in 47, 92, and 81 samples, representing frequencies of 21.4%, 41.8%, and 36.8%, respectively. These frequencies are similar to those found in comparable European populations (15, 16). In addition, our measured C/C genotype frequency matched the frequency in persons with phenotypic lactose malabsorption identified by use of the LBHT in an earlier study of apparently healthy adult Austrians (18).

In a retrospective study, we randomly selected 54 European Caucasian outpatients (18 men and 36 women; General Hospital Linz, Austria) with a mean (SD) age of 55.8 (20.5) years (range 18–87 years) for LPH C→T_{13910} genotyping based on a positive LBHT result obtained after the appearance of abdominal symptoms suggesting lactose intolerance, such as diarrhea (19). Routine LBHT was performed with 50 g of lactose, and breath hydrogen (H2) excretion was recorded on the EC60 Gastrolyzer 2 (Bedfont Scientific Ltd) (20). We began recording within the first hour after the participants ingested lactose, then recorded every 15 min, and thereafter every 30 min, for 4 h. The mean H2 concentration of 2 breath samples measured before lactose ingestion was used as the baseline value, and an increase in excess of 20 parts per million (ppm) was considered indicative of LBHT positivity (20).

Of the 54 patients with a positive LBHT result, 27 (50%) were found to have genotype C/C, 16 (29.6%) genotype C/T, and 11 (20.4%) genotype T/T. Patients with the C/C genotype had a mean (SD) H2 peak increase in LBHT of 108 (58) ppm, which was significantly higher than that in patients with the C/T [65 (54) ppm] and T/T [44 (34) ppm] genotypes when compared by the independent Student t-test (significance at P <0.05). The difference in the mean peak H2 increases between patients with genotypes C/T and T/T was not significant. Our results contrast with those of 2 recent reports showing a 97% and a 91.4% correlation of the C/C genotype with positive LBHT results in patients with suspected lactose malabsorption (21, 22). In those 2 studies, patients were selected for LBHT and genotyping on the basis of history and clinical symptoms, whereas we selected our patients exclusively on the basis of their positive LBHT results.

Thus, patients with secondary hypolactasia or with other causes for symptoms of lactose intolerance leading to a positive LBHT result were more likely to be present in our study population. In fact, previous studies have reported that up to 57% of individuals who experience symptoms of lactose intolerance do not exhibit hypolactasia (23).

Of our 27 patients with either the C/T or T/T genotype, 10 patients had a diagnosis concomitant with secondary hypolactasia (Table 1). Five other patients exhibited a small increase, just above the threshold, of H2 in LBHT and presented with no complaints at the time of genotyping. We considered those 5 patients and 3 other patients who no longer had complaints at the time of genotyping to have had false-positive results or suffered lactose intolerance symptoms for causes other than hypolactasia. The remaining 9 patients still complained of chronic diarrhea and other abdominal symptoms at the time of genotyping, and we advised their referring physicians to perform further diagnostic testing. Thus, the genotyping of LPH C→T_{13910} in our study population, identified those patients who needed further follow-up because the diagnosis of lactose intolerance appeared to be not sufficiently substantiated.

Lactose intolerance usually leads to avoidance of milk and dairy products, often leading to lower calcium intake. Reduced calcium intake has correlated with reduced bone mineral density and increased risk for bone fractures (24). In postmenopausal women, the C/C genotype was correlated with higher bone fracture incidence and lower bone mineral density than in women with C/T and T/T genotypes (18). In addition, the C/C genotype has been correlated with an increased frequency of bone fractures in older individuals in European populations (18, 25). Because milk and dairy product restrictions can probably be revoked in patients with secondary hypolactasia and other causes of lactose intolerance symptoms after successful diagnosis and therapy of their primary diseases, it is important to distinguish these patients from those with primary adult hypolactasia.

Larger studies are warranted to further clarify the diagnostic value of LPH C→T_{13910} genotyping in lactose intolerance in patients of diverse ethnic populations, particularly those of non-European descent. Our almost fully automated assay may assist in those studies because it is rapid, robust, suitable for the screening of large numbers of samples, and considerably less labor-intensive than previously reported assays based on PCR and restriction fragment length polymorphism analysis. Costs for consumables and reagents per sample are higher to some extent but are balanced by less labor time; hands-on time in our assay was only 1 h for the analysis of 32 samples, including DNA purification.

In conclusion, this real-time PCR assay provides a rapid, labor-saving means for the genotyping of LPH C→T_{13910}. The genotyping LPH C→T_{13910} may assist in differentiating patients with primary hypolactasia from those with secondary hypolactasia and lactose intolerance.
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