



Alimentary Tract

Genetic test for lactase non-persistence and hydrogen breath test: Is genotype better than phenotype to diagnose lactose malabsorption?

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Abstract

Background. Adult-type hypolactasia is a widespread condition throughout the world, causing lactose malabsorption. The lactose breath test is a simple tool for diagnosis but the need for prolonged monitoring of hydrogen excretion has led to a genetic test proposal. The aim of this study was to compare the genetic test with the lactose breath test in order to give some insights into the clinical value of genetic testing.

Methods. Thirty-two consecutive functional patients underwent lactose breath test and lactase genetic polymorphism analysis (C/T 13910 and G/A 22018). Intolerance symptoms after lactose load were also monitored.

Results. All patients with positive lactose breath test showed homozygosity for both polymorphisms. Among the nine patients with a negative breath test result, six showed heterozygosity while three showed homozygosity. Intolerance symptoms were present in 16 homozygotic patients but also in one heterozygotic patient. The *k* value for the agreement between the genetic test and the lactose breath test was 0.74.

Conclusion. A positive genetic test for lactase non-persistence indicates whether lactase activity decline may represent a clinical problem for the patient, but does not give information on actual patient symptoms. On the contrary, this information is already available by combining the lactose breath test with intolerance symptom evaluation. Lactose absorption phenotype may be not yet evident until young adult age.

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1. Introduction

Human adult-type hypolactasia is a widespread condition throughout the world due to a genetically determined decline of lactase activity, inherited through an autosomal recessive gene [1]. Its prevalence is highly variable, ranging from 5% in north-west Europe to almost 100% in some Asian populations [2]. On clinical grounds, hypolactasia is responsible for lactose malabsorption, which may cause symptoms such as abdominal pain, bloating, flatulence and diarrhoea, evoked by milk consumption [3] and a consequent exclusion of these products from the diet is often seen, with a high risk for nutritional defects [4].

The monitoring of breath hydrogen excretion after oral lactose load is a simple test for diagnosing lactose mal-

absorption [5]. If symptom onset monitoring is performed, information on lactose intolerance will also be available. Due to the need for prolonged testing, up to 7 h for breath hydrogen excretion [6,7] and even more for symptom onset, genetic testing was recently proposed [8]. A single nucleotide polymorphism, C/T₋₁₃₉₁₀ cosegregates with the persistent/non-persistent trait in Finnish hypolactasia families as all 236 tested hypolactasic family members proved to be homozygotic for C allele [8]. Therefore, the CC genotype indicates lactase non-persistence, the TT genotype indicates lactase persistence and heterozygosity (C/T) is accompanied by an intermediate defect. Several previous papers have studied the correlation between genetic test results and lactase activity detected on intestinal biopsies [9] or lactose malabsorption detected by lactose breath test [10,11]. However, the measurement of lactase activity on intestinal biopsies gave conflicting results [12,13] probably due to the marked effect of small differences in the biopsy site [14]. On the other hand,

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the commonly used diagnostic criterion for H₂ breath test, i.e. an increase of breath H₂ levels ≥ 20 ppm over fasting values, was derived from a study using a 50-g dose of lactose [15], the amount contained in around 1 l of milk. A more recent study [6], evaluating breath H₂ excretion after low and more physiological doses of carbohydrates, showed that this classical criterion had an almost absolute specificity but a sensitivity of less than 60%. On the other hand, both breath H₂ excretion >6 ppm at the 6th hour and the sum of breath H₂ values obtained at the 5th, 6th and 7th hour >15 ppm showed a much better sensitivity without losing specificity both in experimental [6] and clinical conditions [4,7].

The aim of our paper was, therefore, to compare the genetic test with the lactose breath test in order to draw conclusions in regards to correlation of the results and clinical management of the patients.

2. Patients and methods

2.1. Subjects

Thirty-two consecutive patients (25 females, mean age 34 ± 14 , range 17–76) attending our ambulatory clinic due to the presence of functional complaints, such as bloating, flatulence, abdominal pain/discomfort, constipation or diarrhoea, were recruited. In all patients a careful history taking, clinical examination, colonoscopy/barium enema or upper endoscopy where appropriate, routine biochemistry, upper abdominal ultrasonography, fecal occult blood test and microbiological stool tests excluded the presence of organic diseases, which could be responsible for a secondary lactase deficiency. None of the patients had undergone abdominal surgery. None of the enrolled subjects had previously undergone therapeutic courses of antibiotics or drugs interfering with gut motility and sensitivity in the months preceding the study.

The final diagnosis was IBS in 19 patients, functional dyspepsia in 11, and reflux disease in two (non-erosive reflux disease).

All the subjects gave their informed consent to participation in the study. The protocol was approved by the local ethics committee.

2.2. Lactase genetic test (GenoType LCT)

The GenoType LCT (Hain Lifescience GmbH, Nehren, Germany) is a commercial and easy-to-perform assay developed for the combined molecular characterization of positions –13910 and –22018 in the enhancer region of the human lactase (LCT) gene. The test is based on reverse hybridization between amplicons derived from a multiplex PCR and nitrocellulose-bound wild-type and mutated probes for the polymorphisms of interest.

The GenoType LCT line probe assay (Hain Lifescience GmbH, Nehren, Germany) was carried out according to the

manufacturer's instructions with the use of a modified DNA isolation protocol. Briefly 15 μ l of blood sample were pipetted in the middle of the round sample field of a GenoCard (Hain Lifescience GmbH, Nehren, Germany), the GenoCard was dried for 15 min at 37–40 °C in a drying cabinet. After that, a disc of GenoCard was used immediately for amplification by transferring it directly to a prepared PCR reaction tube containing 45 μ l of amplification mix.

Amplification was performed in a thermal cycler (Mastercycler, Eppendorf) according to the following amplification protocol: denaturation at 95 °C for 15 min (*Taq* activation cycle); 10 cycles of denaturation at 95 °C for 30 s and elongation at 58 °C for 120 s; additional 20 cycles of denaturation at 95 °C for 25 s, annealing at 53 °C for 40 s, and elongation at 70 °C for 40 s; and a final extension step at 70 °C for 8 min.

After denaturation, the single-stranded biotin-labelled amplicons are hybridized to membrane-bound probes (four quality control zones and two probes of each polymorphism examined) at 45 °C for 30 min on a thermostatic shaker (Digital Thermostat, 'Arnika'). The strips are washed for 15 min according to the manufacturer's instructions, and hybridization is detected by the addition of a streptavidin-alkaline phosphatase conjugate, followed by an alkaline-phosphatase substrate reaction. The results are available the same day with one-step PCR.

Each strip contains a Conjugate Control zone (CC), a Specificity Control zone (Spec-C) and two Sensitivity Control zone (Sens-13910 and Sens-22018) to document a correct chromogenic reaction, unspecific test conditions and the optimal sensitivity of the reaction for each of the tested gene loci, respectively. The probe –13910T detects a thymine and the probe C-13910 detects a cytosine at position 13910 in the enhancer region of the human LCT gene. The probe –22018A detects an adenine and the probe G-22018 detects a guanine at position 22018 in the enhancer region of the human LCT gene. Strips were interpreted according to the manufacturer's instructions.

2.3. Lactose breath test

In order to avoid prolonged intestinal gas production, due to the presence of non-absorbable or slowly fermentable material in the colonic lumen, the evening before the test day, the subject consumed a meal consisting of only rice, meat and olive oil [16]. This meal was then followed by a 12-h fasting period. Breath testing started between 08.30 a.m. and 09.30 a.m., after thorough mouthwashing with 40 ml of 1% chlorhexidine solution [17]. Smoking [18] and physical exercise [19] were not allowed for 1 h prior to and throughout the test.

Sampling of alveolar air was performed by means of a commercial device (Gasampler Quintron, Milwaukee, WI, USA) that allows the first 500 ml of dead space air to be separated and discarded while the remaining 700 ml of end-alveolar air are collected in a gas-tight bag. Subjects were instructed to avoid deep inspiration and not to hyperventilate.

late before exhalation. A gas chromatograph dedicated to the detection of H₂ and CH₄ in air samples was used for breath samples analysis (Model DP12, Quintron Instrument, Milwaukee, WI, USA). The accuracy of the detector was ± 2 ppm with a linear response range between 2 and 150 ppm of H₂ and between 2 and 50 ppm of CH₄.

All the subjects underwent a preliminary evaluation of intestinal gas production capacity and oro-cecal transit time by H₂ breath test after the ingestion of 400 ml of an iso-osmotic solution containing 20 g of lactulose, a non-absorbable disaccharide, which is fermented by colonic flora. Breath samples were taken at fasting and every 10 min for a 4-h period. Subjects were considered low H₂ excretors if no breath sample contained an H₂ concentration exceeding 20 ppm [20] and the test was considered a false negative if an increase of breath H₂ excretion greater than 20 ppm over the baseline was not evident [21]. None of the patients fulfilled these criteria.

Breath methane (CH₄) excretion, the most important metabolic pathway of H₂ consumption [22], was also measured and the prevalence of CH₄-producers, i.e. subjects with

mean breath CH₄ excretion higher than 5 ppm, was evaluated. Only one subject proved to be a methane producer.

After at least a 3-day period, the H₂/CH₄ breath test after lactose ingestion was performed. The test solution consisted of 400 ml of semi-skimmed milk, containing 20 g of lactose. Breath samples were taken at fasting and every 30 min for a 7-h period. The test was considered as indicative of the presence of lactose malabsorption when the sum of breath H₂ values obtained at the 5th, 6th and 7th hour was higher than 15 ppm [6,7].

All the subjects were asked to record the occurrence of intolerance symptoms experienced during a 24-h period from the beginning of the test. Bloating, abdominal pain or cramps, constipation or diarrhoea and flatulence were ranked as follows: 0 = absence of the symptoms, 1 = trivial symptoms, 2 = mild symptoms, 3 = moderate symptoms, 4 = strong symptoms, 5 = severe symptoms [23]. The individual scores were then added together and a mean of the individual values was given. The individual maximum score was 5. However, for the purpose of the present study, only the presence or absence of symptoms was considered, as we were only inter-

Table 1

Lactase gene polymorphism, lactose malabsorption and lactose intolerance in 32 functional patients.

Pts	Age	C-13910	G-22018	H ₂ -breath test	Symptoms
1	19	Homozygosis (CC)	Homozygosis (GG)	Pos	Yes
2	20	Homozygosis (CC)	Homozygosis (GG)	Pos	Yes
3	20	Homozygosis (CC)	Homozygosis (GG)	Pos	Yes
4	21	Homozygosis (CC)	Homozygosis (GG)	Pos	Yes
5	22	Homozygosis (CC)	Homozygosis (GG)	Pos	Yes
6	23	Homozygosis (CC)	Homozygosis (GG)	Pos	Yes
7	24	Homozygosis (CC)	Homozygosis (GG)	Pos	Yes
8	25	Homozygosis (CC)	Homozygosis (GG)	Pos	Yes
9	26	Homozygosis (CC)	Homozygosis (GG)	Pos	Yes
10	28	Homozygosis (CC)	Homozygosis (GG)	Pos	Yes
11	37	Homozygosis (CC)	Homozygosis (GG)	Pos	Yes
12	41	Homozygosis (CC)	Homozygosis (GG)	Pos	Yes
13	47	Homozygosis (CC)	Homozygosis (GG)	Pos	Yes
14	50	Homozygosis (CC)	Homozygosis (GG)	Pos	Yes
15	51	Homozygosis (CC)	Homozygosis (GG)	Pos	Yes
16	66	Homozygosis (CC)	Homozygosis (GG)	Pos	Yes
17	25	Homozygosis (CC)	Homozygosis (GG)	Pos	No
18	32	Homozygosis (CC)	Homozygosis (GG)	Pos	No
19	36	Homozygosis (CC)	Homozygosis (GG)	Pos	No
20	36	Homozygosis (CC)	Homozygosis (GG)	Pos	No
21	46	Homozygosis (CC)	Homozygosis (GG)	Pos	No
22	50	Homozygosis (CC)	Homozygosis (GG)	Pos	No
23	76	Homozygosis (CC)	Homozygosis (GG)	Pos	No
24	17	Homozygosis (CC)	Homozygosis (GG)	Neg	No
25	22	Homozygosis (CC)	Homozygosis (GG)	Neg	No
26	23	Homozygosis (CC)	Homozygosis (GG)	Neg	No
27	18	Heterozygosis (CT)	Heterozygosis (GA)	Neg	No
28	28	Heterozygosis (CT)	Heterozygosis (GA)	Neg	No
29	30	Heterozygosis (CT)	Heterozygosis (GA)	Neg	No
30	40	Heterozygosis (CT)	Heterozygosis (GA)	Neg	No
31	45	Heterozygosis (CT)	Heterozygosis (GA)	Neg	No
32	50	Heterozygosis (CT)	Heterozygosis (GA)	Neg	Yes

ested in the presence of lactose intolerance and not in the severity of intolerance symptoms.

2.4. Data analysis

The agreement between hydrogen breath test and lactase genotyping was assessed by *k* statistics. A *k* value between 0.4 and 0.6 indicates a moderate agreement and a value between 0.6 and 0.8 indicates good agreement. Age was expressed as mean $\pm$ S.D.

3. Results

In Table 1, the results are shown. In all the patients, a complete concordance between the two polymorphisms was evident. In 26 out of 32 subjects, homozygosis for the lactase non-persistence gene was present: 23 of them showed a positive lactose breath test but three proved to be negative. Among the 23 patients with positive lactose breath test, as expected, a subgroup of these patients experienced symptoms with lactose administration and a subgroup proved to be tolerant. The three patients with homozygosis for the non-persistence gene of lactase and negative lactose breath test were tolerant to oral lactose. Six patients showed heterozygosis for lactase non-persistence polymorphism and all of them proved to be negative for the lactose breath test. However, one of them showed intolerance symptoms after oral lactose.

Considering the whole group of patients, the *k* value for the agreement between genetic test and lactose breath test was 0.74 ($p < 0.00001$), suggesting a good agreement. However, the *k* value for the subgroup of patients under 30 years old was 0.56, suggesting a moderate agreement ($p = 0.0049$), but 1.00 for the subgroup over 30 years old ($p = 0.0001$), as all the subjects were positive to the lactose breath test and showed homozygosis for the non-persistence genotype.

4. Discussion

Our data show that the correlation between the genetic test and the lactose breath test is good, but not perfect. This discrepancy is expected as the two tests simply do not give information on the same parameter. In fact, while the lactose breath test indicates the actual presence of lactose malabsorption, the genetic test gives information on the intestinal lactase expression genotype. Even if the number of patients studied is low, very important suggestions emerge from our results and before a widespread application of the genetic test for lactose malabsorption some considerations are needed. Our aim was not the evaluation of diagnostic accuracy on a large scale basis, but only to show how to interpret the test results. In fact, a recent paper [11] on a group of selected 30 patients was not conclusive about the clinical value of the genetic test. The results were compared with the lactose tolerance

test, based on blood glucose level modifications after oral lactose administration, an old test, characterized by a very low accuracy already shown in 1975 [24], and with the lactose breath test performed according to an old protocol with a 50 g dose of orally administered lactose also characterized by a very low accuracy [6]. No discussion on the possible role for genetic test in clinical practice was present in this paper.

Among patients with homozygosis for the non-persistence genotype, three out of 26 showed a negative lactose breath test, indicating the actual absence of lactose malabsorption, but a future onset of lactase activity expression decline.

Lactose digestion capacity was not impaired among patients with heterozygosis for the lactase non-persistence gene, as all of them showed a negative lactose breath test: a lactase activity expression level lower than patients with homozygosis for persistence phenotype is thus not actually a clinical problem for any of them. However, we cannot express any opinion on the future of these patients as we do not know how “partial” their defect is.

As expected, intolerance symptoms were present in a subgroup of lactose malabsorbers with homozygosis for the lactase non-persistence gene. Moreover, a patient with heterozygosis for lactase non-persistence and a negative lactose breath test showed intolerance symptoms, probably due to dyspeptic syndrome, unrelated to lactose digestion.

We have, first of all, to consider that lactose intolerance is a condition necessarily linked to lactose intake and, therefore, the first step in selecting patients for diagnostic tests (whatever they are) is a detailed case history evaluation aimed at recognizing consumption of milk and dairy products. Even if taken for granted, this pivotal step is often not considered, and in our experience several patients who do not usually consume lactose due to an unacceptable taste undergo the lactose breath test at the suggestion of both general practitioners and gastroenterologists.

When dairy products are part of the patient's diet, the amount of lactose ingested is a further matter of debate. In fact, even if an increase of the prevalence of sugar malabsorption was demonstrated by increasing the sugar load [25], colonic adaptation may have a role in preventing symptom onset [26]. Other known variables are the ingestion of solid foods together with lactose and the lipid content of lactose-containing foods, as they are both able to slow gastric emptying time [27]. All these points may interfere with symptom onset and they may therefore reduce the correlation between the genetic test result and clinical aspects.

But what mainly makes the genetic test unreliable is undoubtedly a time factor: lactase activity decline is a process that takes place progressively over time. In most mammals, the specific activity of lactase-phlorizin hydrolase is high at birth and during nursing and then declines at weaning to low adult levels [28–30], showing a further decline in old age [31–33]. In humans, in a cohort of 80 healthy Caucasian

women aged 40–79, the prevalence of lactose malabsorption was significantly higher in subjects aged 60–79 (50%) than in subjects aged 40–59 (15%) [34], and we recently showed that in a cohort of healthy, well-nourished subjects aged 23–94 years, the prevalence of lactose malabsorption increases significantly after the age of 74 years [35]. In patients with functional complaints such as diarrhoea, bloating and flatulence, the genetic test is therefore a photograph at a time point at which the rate of decline has not necessarily already reached the threshold for symptom induction after oral lactose load. Accordingly, our three patients with a CC genotype, but a negative breath test were young patients, aged 17, 22 and 25. Therefore, CC or CT genotypes may suggest, in that individual patient, that functional symptoms could be related to a state of lactase non-persistence, but they do not give us the certainty that any alteration is actually present, as in all our CC patients with a positive breath test.

In our cohort, genetic test concordance with breath test results was perfect in the group over 30 years and worst in subjects younger than 30 years. More in general, it can be suggested that the concordance should be low in adolescents and young adults, to improve in adult age but it can also be suggested that it declines again in advanced age due to a lower expression at brush border level caused by enterocyte immaturity: in fact, it was previously shown that in the elderly, a normal architecture of small bowel villi is possible at the expense of a higher enterocyte turnover which causes the presence of less mature enterocytes at the tip of villi [36].

It was suggested, in Finnish [9] and Sardinian [37] populations that the decline of lactase activity and the onset of adult-type hypolactasia is evident from the age of 8–12 years. Our data disagree with these two studies, suggesting that in unselected Italian cohort genetic test may give different results than hydrogen breath test also in older patients.

It is therefore, possible that a CC or CT result of the genetic test could lead the physician to a wrong diagnosis of lactose malabsorption, considered to be present before the activity decline could have reached the thresholds of lactase activity for symptom onset. So patient symptoms could be attributed to a not yet developed lactose malabsorption, thus inducing a diagnostic delay of the condition really causing the symptoms. On the contrary, in the final analysis, only a TT genotype ensures a clinically promptly usable result since this result makes it possible to exclude the role of lactase deficiency in the pathophysiology of patient symptoms. It is already possible to have this information through the low cost, simple lactose breath test.

In conclusion, the genetic test for diagnosis of lactose malabsorption is in good agreement with the lactose breath test, in particular in young adults, but it does not seem to be the new gold standard for lactose malabsorption and it is not yet possible to hypothesize a widespread clinical application.

Practice points

- The agreement between the genetic test and the lactose breath test is good but not perfect.
- The genetic test doesn't represent the new gold standard for lactose malabsorption.
- The genetic test is characterized by a high negative predictive value, like lactose breath test.

Research agenda

- Further studies are needed to verify the agreement between the two tests in different age groups.

Competing interest

All authors declare no competing interest.

Conflict of interest statement

None declared.

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