

A review of the LCT gene and research proposal

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BIO352: From Genotype to Phenotype

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Abstract

The declined activity of the lactase-phlorizin hydrolase (LPH) in intestinal cells after weaning results in adult-type hypolactasia, also known as lactose intolerance. The LCT gene provides instructions for making lactase-phlorizin hydrolase (LPH) which digests lactose, a sugar found in milk and other dairy products. A specific DNA sequence within the MCM6 gene called a regulatory element helps control the expression of the LCT gene (MCM6 Gene: MedlinePlus Genetics, n.d.). Since it is unclear how the LCT gene can be dramatically downregulated with age in most individuals, but remain active in some, it is important to investigate the possible causation. In this report, I will introduce past research that investigated LCT and MCM6 genes and epigenetic factors that contributed to lactase persistence and lactase non-persistence among humans and mice.

The Phenotype

Carbohydrates, one of the main nutrients that keep us alive, are found in foods and drinks that we consume and come in a variety of forms depending on the source. Of those, lactose, commonly known as milk sugar, is the main carbohydrate found in dairy products such as milk, cheese, yogurt, and some prepared packaged foods such as bread and pancakes. Lactose is made of two sugar molecules called disaccharides. Since our body uses carbohydrates in the simplest form of monosaccharides for energy, lactose must first be broken down into monosaccharide forms for absorption. When we consume dairy products, lactose is broken down in the intestine by the Beta-galactosidase enzyme called lactase-phlorizin hydrolase (LPH) for digestion (Eating, Diet, & Nutrition for Lactose Intolerance, 2022). Hypolactasia, commonly known as lactose intolerance, occurs when your body can not break down lactose milk sugar due to a lack of LPH enzyme, commonly known as lactase enzyme in your intestine. When undigested lactose passes into your colon, bacteria in your colon break down the lactose and create fluid and gas which results in discomfort such as bloating, abdominal pain, or diarrhea after consuming products containing lactose (Definition & Facts for Lactose Intolerance, 2022). Furthermore, lactose intolerance may also affect your overall health since dairy products are the main sources of calcium and vitamin D which are essential for your bone health (Neville et al., 2019). I am interested in the genes that regulate the level of LPH enzymes because I am lactose intolerant. I hope to gain a better understanding of lactose intolerance by researching the gene that regulates the LPH enzyme.

There are four different forms of lactase enzyme deficiency. Primary lactase enzyme deficiency is the most common type of lactose intolerance across the world. This type of lactase deficiency is due to a genetic mutation that runs in family and develops after weaning around the age of two. During these periods, the body starts to produce less lactase enzyme, which may not be noticeable until reaching adulthood (NHS website, 2021). Secondary lactase deficiency is caused by intestinal disorders like Crohn's disease, ulcerative colitis, or Celiac disease, and radiation therapy used for cancer treatment. These conditions are all known to cause damage to the lining of the intestines, which significantly interferes with lactase enzyme production. This type of lactase deficiency can occur at any age but is often resolved once the underlying cause is treated. Secondary lactase deficiency is the most common cause of lactose intolerance among infants and toddlers in the UK (NHS website, 2021), which can be difficult as they primarily rely on breastmilk and formula for nutrition. Developmental lactase deficiency is seen in babies born prematurely before the 37th week of pregnancy. A baby's small intestine creates cells producing lactase at the end of the third trimester of pregnancy. Therefore, babies born early have underdeveloped small intestines and are not able to produce lactase enzyme, though this generally resolves soon after birth (NHS website, 2021). Lastly, congenital lactase deficiency

(CLD) is a rarer form of the condition that produces very little or no lactase. The baby will develop this symptom if both parents have mutations in the lactase (LCT) gene, therefore impairing the body's ability to make lactase enzymes. In this case, the baby cannot tolerate breastmilk or regular infant formula, as this will cause excessive diarrhea and dehydration. Thus, they must be fed a lactose-free formula (NHS website, 2021).

While most infants can digest lactose, lactose malabsorption tends to develop later in life. According to new research, an astonishing 70% of the global population is intolerant to lactose, however, the estimates vary by ethnicity. African American and Asian ethnicities present a 75% - 95% lactose intolerance rate, while northern Europeans have a lower rate of 18% - 26% lactose intolerance (Neville et al., 2019). Hence, lactose intolerance is most common among East Asians, Native Americans, and Africans who consume fewer dairy products and less common among Europeans who have a long history of consuming a diet rich in milk products. Furthermore, some individuals who frequently consume milk and dairy products may develop lactase persistence which allows them to digest lactose into adulthood successfully (Lactose Intolerance - Symptoms and Causes, 2022).

Malabsorption of lactose can be diagnosed by the hydrogen breath test which measures hydrogen and methane excretion in the breath after ingesting a specific dose of lactose. A lactose intolerant patient will have an increased amount of hydrogen gas in their breath after ingesting lactose. This is due to undigested lactose traveling down to the large intestine where it is fermented by bacteria that produce hydrogen. On the other hand, the lactose intolerance test requires patients to drink lactose solution, and their blood samples are taken to measure the amount of blood glucose their blood contains. In this case, patients with lactose intolerance will not experience a rise in blood glucose levels since they are not able to break down lactose into glucose. Clinical testing and a history of symptoms such as bloating, abdominal cramps, and diarrhea after ingesting lactose products are used to diagnose lactose intolerance (NHS website, 2021).

Currently, there is no cure for lactose intolerance; however, most people are able to manage their symptoms by cutting down or avoiding food and drinks containing lactose and replacing them with lactose-free alternatives (NHS website, 2021). Over-the-counter medicine, such as Lactaid, contains lactase enzyme derived from *Aspergillus oryzae* which can aid in the digestion of lactose. When taken together with dairy products, Lactaid will break down the lactose into glucose and galactose for digestion and patients will not experience side effects from ingesting dairy products (Dajani, 2004).

The activities of LPH enzymes are maximal at birth when we heavily rely on breast milk for survival. However, the majority world's population experiences a decline in the production of LPH enzymes from the toddler years to young adulthood (Olds, 2003). Why do most individuals become lactase non-persistence (LNP) and others maintain lactase persistence (LP) throughout their life? What affects the amount of LPH enzymes produced in the small intestine? Two main genes control the production of LPH enzymes: lactase gene (LCT) and minichromosome maintenance complex component 6 (MCM6) gene, however, much of the mechanism remains incompletely understood (OMIM).

The Molecular Function of the Gene Product(s) and a Mouse Model

Lactose is a disaccharide that is made of two sugar molecules of glucose and galactose held together by glycosidic linkage. LPH enzymes, which lie in the brush border membrane of the small intestine, hydrolyze lactose to produce D-glucose and D-galactose and are actively absorbed to be exported to the liver for metabolism (Kuchay, 2020). The initial polypeptide that forms from this gene is called preprolactase (prepro-LPH) which is made of a long chain of 1,927 amino acids. Prepro-LPH has five domains: a 19-amino-acid cleaved signal sequence, a large prosequence domain that is not present in mature lactase, the mature lactase segment, a membrane-spanning hydrophobic anchor, and a short hydrophilic carboxyl terminus (Mantei et al., 1988). The synthesis of the LPH enzymes begins when the signal sequence from the prepro-LPH is cleaved in the endoplasmic reticulum and results in prolactase (pro-LPH). Pro-LPH is then transported to the Golgi apparatus and glycosylated and proteolytically converted into

its mature form of LPH enzymes. However, only 60% of the initial pre-pro-LPH was expressed as LPH enzymes in the brush border membrane (Mantei et al., 1988). The end product, LPH enzyme, is a homotetramer protein complex made of four identical subunits, each with 1023 amino acid residues for a total of 4092 amino acid residues. Its enzyme is oriented with the N-terminus outside the cell and the C-terminus in the cytosol of the intestinal epithelial cell of the brush border membrane (Naim et al., 1987).

The LCT gene, which encodes for LPH enzymes, has both lactase activity and phlorizin hydrolase activity. It is found in a single genetic locus on 2q21.3 and expressed exclusively by small intestine enterocytes (Mantei et al., 1988). A study from Van Wering et al. in 2002 revealed that proteins such as GATA5 (611496) and HNF1- α (TCF1; 142410) play a critical function in lineage differentiation and embryonic development and were required for synergistic activation of the human LCT promoter. Within their study, complete loss of transcriptional activity resulted in the deletion of the HNF1- α activation domain or interruption of the HNF1-binding sites in the LCT promoter. Whereas deletion of the GATA5 activation domains or interruption of the GATA-binding site was reduced, the transcriptional activity remained (Wering, 2002).

The minichromosome maintenance complex component 6 (MCM6) gene is another gene that is known to affect the expression of LPH enzymes. The MCM6 gene is known as a regulatory element and cis-acting (non-coding) enhancer for the LCT gene which is located 14kb upstream of the LCT gene (OMIM). Research from Enattah et al. in 2002 revealed that the sequence analyses of the coding and promoter region of the LCT gene had no DNA variations correlating with lactase non-persistence. Instead, the study suggested that a cis-acting element contributes to the lactase non-persistence phenotype. Furthermore, their study of the linkage disequilibrium (LD) and haplotype analysis in humans resulted in the identification of the genetic variants C/T -13910 and G/A -22018 upstream of the human LCT gene. Moreover, those variants were associated with hereditary lactase persistence/non-persistence among Finnish families. The variant-13910bp was located 5' to the LCT gene within intron 13 of the adjacent MCM6 gene and had a single nucleotide polymorphism of C to T. The authors concluded that homozygosity for the C variant correlates with the lactase non-persistence phenotype and the T allele for the lactase persistence phenotype.

In a similar study conducted by Olds and Sibley in 2003, the genetic human variants C/T -13910 or G/A -22018 were cloned upstream of the 3.0 kb rat lactase gene promoter in a luciferase reporter construct. Then, the human intestinal Caco-2 cells, known to have similar properties to enterocytes found in the brush border membrane in the small intestine, were transfected with the lactase variant promoter-reporter constructs and assayed for promoter activity. The C -13910 genetic variant, associated with lactase non-persistence resulted in a 2.2-fold increase in lactase promoter activity, and the T -13910 variant, associated with lactase persistence, resulted in an even greater 2.8-fold increase. For the second variant, G/A -22018, the G-22018 variant resulted in a minimal 1.2-to-1.4-fold induction of promoter activity and the A-22018 variant resulted in an 0.8-fold reduction in promoter activity. Hence, the authors concluded that an approximately 200-kb region surrounding the C/T (-13910) polymorphism in MCM6 intron 13 (601806.0001), the region known for lactase persistence/non-persistence variant, functioned in vitro as a cis element capable of enhancing differential transcriptional activation of the lactase promoter. The G/A -22018 variant region had minimal enhancement of lactase promoter activity.

Epigenetic mechanisms, such as DNA methylation, modification of histone, and small RNAs, could contribute to the transcriptional variation among the same cell type since such modifications are important in regulating gene transcription (MedlinePlus Genetics, n.d.). A study conducted by Oh et al., in 2017 analyzed epigenetic profiles involved with the in vivo transcriptional gradient of the mouse LCT gene which is expressed in enterocytes along the proximal to distal axis of the small intestine. First, they examined whether a phenotype-related environmental change such as lactose exposure could modify transcription and induce epigenetic changes in enterocytes in the small intestine. The mice were either fed lactose-containing milk (LAC+) or lactose-free milk (lac-) for 60 days. The researchers learned that the mice that were fed lactose-containing milk (LAC+) increased LCT mRNA levels in the enterocytes of the distal intestinal segment

by 1.3- to 2-fold compared to mice in the lactose-free group. From this, they reasoned that the environment modifies the LCT transcriptional gradient and induces epigenetic alterations. Additionally, to investigate whether transcriptional gradients could be established from age-associated epigenetic changes, they compared the LCT mRNA levels in enterocytes across the small intestine of infant mice (P6) and adult mice (P60). The LCT mRNA levels were consistent across the intestinal segment in infant mice and were 5-15-fold higher than in adult mice. Therefore, they concluded that the difference in the LCT gene transcription in the small intestine increase with age, and over time LCT gene becomes mainly suppressed in enterocytes of the proximal segments of the small intestine of the adult mice.

More recently, studies using CRISPR-CAS9 genome editing techniques have been utilized to research the relations of LCT gene expression. In a study conducted by Labrie et al. in 2016, they performed a comprehensive epigenetic study of the human and mouse intestine and used CRISPR-CAS9 genome editing technology to determine whether the differentially modified DNA regions indeed contribute to LCT regulation. First, researchers deleted the intronic region in both LCT and MCM6 genes and measured the effect on LCT mRNA. In particular, they deleted the LCT intron 2 region, which had evidence of cross-species lactase gene regulation, and deleted another location in MCM6 intron 13, which played an important role in human LCT regulation. In both cases, the C57BL/6N mice, a common inbred strain of laboratory mice, was used. The genetic deletion in the MCM6 intron13, LCT intron 2, and LCT intron 1 was generated by using the CRISPR-Cas9 genome editing technique. For a positive control, they deleted the LCT intron 1 region where it overlapped with the LCT promoter element. For a negative control, they measured MCM6 mRNA levels in mice carrying MCM6 intron 13 deletion. The study revealed that deletion in LCT intron 1 and intron 2 both resulted in 3 to 8-fold downregulation of LCT mRNA throughout the duodenum and jejunum in the small intestine of the mice. On the other hand, the deletion of MCM6 intron13 resulted in less than 2-fold segment-specific LCT mRNA reduction and had no effect on the MCM6 mRNA level which ensured that CRISPR-Cas9 deletion only affected the LCT gene. Based on the comparison from the result of the intron deletions, the researcher concluded that the mutation in LCT intron 2 produced a significantly greater reduction of 2-fold reduction in LCT mRNA in adults than in infant mice. This suggested that LCT intron 2 had a greater impact and control of LCT mRNA in adulthood. Next, the researcher examined on what extent the epigenetically regulated regions impacted human LCT expression using Caco-2 cell which was derived from cultured human intestinal epithelial cells. The LCT gene was assayed in Caco-2 cells carrying a CRISPR-CAS9n-induced deletion in the regulatory elements in MCM6 intron 13 or LCT intron 2. For a negative control, they deleted a region not overlapping a regulatory element in LCT intron1. The cells were then evaluated on day 6 before confluence (undifferentiated) and day 15 at post-confluence (differentiated to intestine epithelial state). The researcher found that deletion had no effect on LCT mRNA expression in the undifferentiated cells on day 6. However, at day 15, when the cells became an epithelial-like state, the deletion in MCM6 intron 13 or LCT intron 2 resulted in a great reduction in LCT mRNA levels. This confirmed the importance of the regulatory region for LCT expression in humans. Thus, epigenetic factors are causing the changes in the regulation of the human and mouse LCT gene that contribute to lactase non-persistence/persistence which can lead to lactose intolerance.

Experiment for the future

Epigenetic factors such as lactose exposure and aging have been known to modify the transcription level of the LCT gene expression (Oh et al., 2017). However, the research on the regulation of the LCT gene, which codes for the LPH enzymes, is focused on the age between weaning to young adulthood. How does the rate of LCT gene expression level change from epigenetic factors such as aging and diet? Does the rate of the LCT gene transcription in the small intestine differ between weaning to young adult, and from young adult to older adult? Can adult mice maintain LCT gene expression when they age if they were fed a diet consisting of lactose? To answer these questions, I propose mouse model research comparing the LCT mRNA expression level in the enterocytes of the small intestine of the mice from the different age groups with lactose in their diet..

This experiment aims to analyze the relationship between the epigenetic factors of aging and a phenotype-related environmental change, specifically a lactose-containing diet. Epigenetic factors such as aging and diet are known to change the expression level of the LCT gene which produces the LPH enzyme responsible for digesting milk products (Oh et al., 2017). The result from the previous study by Oh et al in 2017 concluded that mice that were fed lactose increased LCT mRNA levels in the enterocytes in the small intestine compared to mice without lactose in their diet. Moreover, their study discovered that LCT gene transcription in the small intestine can be suppressed by epigenetic factors such as aging. Therefore, this experiment will further explore how the LCT mRNA expression level changes with age and whether post-adult mice that are fed a lactose-containing diet can maintain their LCT gene expression when they age. In addition, this experiment can also help determine which epigenetic factors (aging vs diet) are more likely to induce the change in LCT gene expression among mice. The result of the proposed experiment may support one of the hypotheses listed below.

H1: The LCT mRNA expression level decrease with age. Age is a stronger epigenetic factor than a diet.

H2: The LCT mRNA expression level increased with age. Diet is a stronger epigenetic factor than age.

H3: The LCT mRNA expression level maintained constant with age.

H0: No difference in the level of the LCT mRNA expression level between adults and senior mice.

Materials and Methods

C57BL/6, a common inbred strain of laboratory mice that typically lives up to 26–30 months, will be housed under different conditions. To investigate how diet is associated with epigenetic changes in the LCT gene, two conditions will be used. Under condition 1 (LAC++), mice will be supplied with ad libitum sterile food and 2% lactose-containing milk 3 times a day for 60 days. Condition 2 (LAC--) will be used as a negative control and the mice will be supplied with ad libitum sterile food and water 3 times a day for 60 days. To investigate how age is associated with epigenetic changes in the LCT gene, the mice under each condition will be separated into three age groups: Young Adult (3 months), Middle Age (10 months), and Old Age (18 months). Each group will consist of 12 mice, and 12 additional mice (6 days postnatal) will be used to obtain a baseline for LCT mRNA transcription level. Under each group and condition, an equal number of male and female mice will be used for comparison of the gene of interest expression. The outline of the experimental conditions is written below.

Group 1: YM (LAC++) (N=12; 6f, 6m)

MA (LAC++) (N=12; 6f, 6m)

OA (LAC ++) (N=12; 6f, 6m)

Group 2: YM (LAC--) (N=12; 6f, 6m)

MA (LAC--) (N=12; 6f, 6m)

OA (LAC --) (N=12; 6f, 6m)

Baseline: PN (N=12, 6f, 6m)

After 60 days, mice will be weighed and the weight difference between Group 1 (LAC++) and Group 2 (LAC--) will be used to confirm the LCT gene expression in mice's intestinal epithelial cells located in the brush border membrane of the small intestine. An increase in weight is expected in Group 1 since those mice received additional nutrients from lactose in their diet compared to the non-lactose diet mice in Group 2. Mice will then be euthanized under anesthetic via cardiac perfusion. Next, enterocytes

from three intestinal segments (duodenal, jejunal, and ileal segments) from the mice will be collected and homogenized. The total RNA will be extracted from the samples using the Qiagen RNeasy Mini Kit with Quagen Rnase-free DNase I. Purified RNA will then be converted to cDNA using the High Capacity RNA-to cDNA Kit. Lastly, the LCT mRNA levels will be quantified and recorded. The data of the mice's LCT mRNA expression levels will be compared among mice under different dietary conditions (LAC++, LAC-), different age groups (Young Adults, Middle Age, Old Adults), and sex.

For this experiment, I predict that if aging is a stronger epigenetic factor influencing the level of LCT mRNA expression, a diet rich in lactose (milk products) may not increase LCT expression. Therefore, the level of LCT mRNA expression will decrease as the age of the mice increases. If the diet is a stronger epigenetic factor influencing the level of LCT mRNA expression, aging will not affect the level of LCT mRNA expression as long as the mice are continuously fed a diet with lactose (milk). An increased level of LCT mRNA with age may indicate that the body is accommodating long-term lactose exposure for absorption. If there are no differences in the level of the LCT mRNA expression level among the age group, it may indicate that the LCT mRNA expression level will remain constant regardless of aging and diet (milk).

Experimental Proposal

Adult-type lactose intolerance is more common among East Asians, Native Americans, and Africans but much lower occurrence among European due to the historical difference in diet. To examine how and to what extent diet affects lactase persistence and non-persistence in humans, further in-depth analysis of LCT gene expression including age, sex, and lactose exposure level among different races is needed. This can be done by comparing the level of human LCT expression using Caco-2 cells derived from cultured human intestinal epithelial cells from different races and obtaining a dietary history from the participants. In addition, some study has shown that individuals that frequently consume milk and dairy products can build up lactose tolerance (Lactose Intolerance - Symptoms and Causes, 2022). Therefore, a further study investigating how much lactose exposure is needed for lactose intolerant individuals to build up lactose tolerance. To do this, mice will be exposed to different amounts of lactose in their diet and the level of LCT gene expressed in their enterocytes of the small intestine will be quantified. The data will be helpful to determine the effectiveness of building lactose tolerance through lactose exposure over time.

Conclusion

LCT and MCM6 genes play a crucial role in the regulation of LPH enzyme produced in the small intestine required for the digestion of lactose-containing dairy products. Thus, mutations in LCT and MCM6 genes and epigenetic factors such as age and diet can contribute to lactase persistence (LP) and non-persistence (LNP) among humans. Further research is needed to understand how genetic and environmental factors affect the production of the LPH enzyme responsible for absorbing dairy products in our diet.

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