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Relationship Between Infant Non-Infectious Diarrhea and Polymorphism of Lactase Gene c/t13910, Lactase Activity and Intestinal Flora Structure

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YZ, MH and JC conducted the experiments in this study. SW and JX contributed to the design and interpretation of the current study and wrote the article. All authors read, revised, and approved the final manuscript.

Key words
Infant non-infectious diarrhea, Lactose intolerance, 13910 site upstream of lactase gene

ABSTRACT

The objective of this study was to analyze and explore the relationship between lactase activity and intestinal microflora structure in infants under 1-year old with non-infectious diarrhea. the hospital selected 60 infants in the maternal and Child Health Hospital of Haizhu District from August 2019 to September 2020 as the research objects. The infants in the experimental group were diagnosed as lactose intolerant infants, and the control group were normal infants. There were 30 cases in the experimental group and 30 cases in the control group. To understand the relationship between the 13910 gene at the upstream of lactase gene and the activity of lactase and the structure of intestinal flora in infants. there was no difference in lactase gene between the experimental group and the control group in Guangdong population. The number of beneficial bacteria in the intestinal tract of the experimental group was less than that of the control group. If the structure of intestinal flora was corrected and probiotics were increased, the lactase activity could be improved and the symptoms of lactose intolerance could be alleviated. It was concluded that when lactose intolerance occurs in infants, it is necessary to pay attention to the lack of beneficial bacteria in the intestine. Targeted supplementation of probiotics can correct the status of intestinal flora, improve the activity of lactase, and alleviate the symptoms of lactose intolerance.

INTRODUCTION

Infants under 1-year old mainly eat breast milk or dairy products, with lactose as the main component. Lactose is the main energy source of infants and is related to the normal development of the brain (Heyman *et al*., 2006). Lactose is absorbed and utilized in the small intestine after being hydrolyzed by lactase on the mucosal microvilli (Qibtia *et al*., 2022). Lack of lactase makes lactose in breast milk or dairy products unable to be decomposed and absorbed, resulting in increased intestinal osmotic pressure and osmotic diarrhea (Yuan, 2010); after entering the colon, unhydrolyzed lactose is decomposed by intestinal bacteria, producing a large number of short chain fatty acids such as lactic acid and formic acid and hydrogen, causing diarrhea, abdominal distension and abdominal pain, which is clinically called lactose intolerance (Di Costanzo and Canani, 2018). Lactose intolerance is a common cause of chronic diarrhea in infants, which can easily lead to malnutrition, skeletal malformation, anemia, insufficient energy intake, and affect physical and intellectual development (Ana Abad Sinden and Sutphen, 1991). The incidence of lactose intolerance in infants in China is high, about 47-70%. Lactase deficiency is the main cause of lactose intolerance (Yang *et al*., 2000). Some studies found that the lactase gene was located on chromosome 2, and

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the lactase activity was related to the polymorphism of the lactase gene (Kruse *et al*., 1988). In European populations, 89~96% of the t13910 alleles are continuously related to lactase (Itan *et al*., 2009). In the single nucleotide polymorphism (SNP) genotype at 13910 upstream of the lactase gene, CC is continuously negatively related to lactase, while CT and TT are continuously positively related to lactase (Mattar *et al*., 2008). Therefore, in many European countries, the detection of t13910 has become a continuous gene detection method for lactase, but there are differences in human genetics, Therefore, this study focuses on whether there is also a correlation among Guangdong people in China.

At the same time, human tolerance to lactose is also closely related to intestinal microecology (Deng *et al*., 2015). Changes in intestinal microecology affect gastrointestinal symptoms of lactose intolerance (Szilagyi, 2015). However, these changes in the nature of primary or secondary colonic flora can lead to the proliferation of some flora, which will produce more gases and short chain fatty acids, and promote the early dissociation of bile acids. The latter can affect the obvious changes in colonic water and electrolyte transport, as well as the motility and sensitivity of the colon. Intestinal flora can also resist the colonization and reproduction of pathogenic microorganisms; Promote the differentiation of intestinal mucosal epithelial cells, stimulate the development of systemic immune system and intestinal immune system, and establish immune tolerance (Khan *et al*., 2021). The structure of intestinal flora in the initial colonization stage of infancy is affected by many factors, such as delivery mode, gestational age, food, feeding mode, antibiotic use, family environment and so on (Fanaro *et al*., 2003). At the age of 1, the structure of intestinal flora begins to approach of adults (Jandhyala *et al*., 2015). Studies have shown that intestinal flora affects the digestion and absorption of lactose, and the changes of intestinal microecology are related to the severity of gastrointestinal symptoms of lactose intolerance (Forsgad, 2019). Compared with normal people, lactose intolerants have different intestinal flora structure, the number of Lactobacillus and Bifidobacterium is reduced, and the composition of intestinal flora changes the pH in the intestine, affecting the activity of intestinal lactase. Therefore, the utilization and decomposition of lactose by intestinal bacteria also affect the metabolism of lactose in the intestine (Firrman *et al*., 2022). In the study, 60 infants under 1-year old were investigated for the single nucleotide polymorphism (SNP) genotype at the 13910 site upstream of the lactase gene, lactase activity and intestinal flora, and the corresponding treatment was given.

MATERIALS AND METHODS

General information

The following two groups of infants have been included in this study.

(i) Lactose intolerant infants: 30 aged 0 ~ 12 months. Patients with typical clinical manifestations of lactose intolerance and positive urine galactose by galactose oxidase method were included. Those who used probiotics and antibiotics 8 weeks before the collection of samples, those with fever, respiratory tract infection and other systemic infections when collecting samples and people with milk protein allergy were excluded from this study.

(ii) Healthy infants: 30 aged 0 ~ 12 months who have regular physical examination in the children's health department, have no diarrhea, have normal stool routine, and have galactose in urine detected by galactose oxidase method were included in this study. Infants with lactose intolerance were excluded.

Experimental method

Urine samples were used for determination of lactose, stool samples were used for determination of sequence of V4-V5 region of 16S rRNA genes of intestinal flora, and venous blood was used for SNP analysis of 13910 sites upstream of lactase gene.

Galactose oxidase method was used for determination of galactose in urine. The subjects drank breast milk or ordinary formula milk according to 10 ml /kg, collected urine for 2 ~ 3 h, and operated according to the instructions. If the color of the sample hole was darker than the standard hole or more consistent, it was judged as lactose intolerance. If the color of the sample hole was lighter than the standard hole or does not develop color, it was lactose intolerance.

For sequence determination of v4-v5 region of 16S rRNA gene of intestinal flora, DNA was extracted from fresh stool samples, PCR amplification of v4-v5 region of 16S rRNA gene of intestinal flora was done. miseq high-throughput sequencing technology was used to obtain sequence information of v4-v5 region of 16S rRNA gene. The sequencing original data was deposited in the NCBI (SRA) database qiime2, uchime and other software were employed to analyze the sequence.

For SNP analysis of 13910 sites upstream of lactase gene, DNA was extracted from 1 ~ 2ml of peripheral venous blood of the subjects and lactase gene was amplified by PCR. PCR products were sequenced, and the nucleotide genotype of 13910 sites upstream of lactase gene was analyzed.

Statistical analyses

SPSS 17.0 statistical software was used to process and compare the data, and X2 test was performed. The difference was statistically significant (P<0.05).

RESULTS

Sequencing results of 13910 loci in the upstream of lactase gene at present, the genotype of 13910 in the upstream of lactase gene in our hospital's control group (normal infants) and experimental group (lactose intolerant infants) is CC, and the frequency of t-13910 allele is 0% (Table I).

Table I. Genotype and allele distribution frequency of 13910 loci in the upstream of lactase gene in normal infants (control) and lactose intolerant infants (experimental).

	Genotype frequency (%)			Allele frequency (%)	
Phenotype	CC	CT	TT	C	T
Control group	30(100)	0(0)	0(0)	30(100)	0(0)
Experimental group	30(100)	0(0)	0(0)	30(100)	0(0)

Comparing the sequencing results of 60 related samples, the single nucleotide polymorphism (SNP) genotype at 13910 sites upstream of the lactase gene is CC type gene in both the experimental group and the control group. No CT or TT type is found, and no other new SNP sites are found temporarily.

Using miseq high-throughput sequencing technology, at least 21054 and at the most 113044 reads of a single sample, with an average of 64363 per sample was recorded. According to the statistics of OTU classification and corresponding species taxonomic pedigrees of each sample, all sequences have 9 phyla, 20 classes, 32 orders, 56 families and 93 genera.

The sparse curve is completed by the qiime2 diversity alpha rarefaction plug-in, which randomly samples the counting sub of each sample to the specified sampling depth. The algorithm resamples the counts in each sample without replacement, so that each sample in the result table has the total count of the minimum sample size. The sampling depth is selected to include all samples in the diversity analysis. Samples with a total count less than this value were deleted from the diversity analysis. Choosing the best sampling depth is always difficult, because too low a value will lead to the wrong interpretation of the lack of some low abundance or rare species in the flora. Therefore, each sample retains more sequences, while excluding a small number of samples to complete the retention of all or as many metadata groups as possible. The plug-in calculates all specified alpha diversity indicators between step 1 and maximum depth. At each sampling depth step, a sparse table is generated, and the diversity index of all samples in the table is calculated. If the line in the figure looks flat (i.e. close to zero slope) at a certain sampling depth along the X axis, it indicates that collecting other sequences beyond this sampling depth is unlikely to lead to the observation of other functions (Fig. 1).

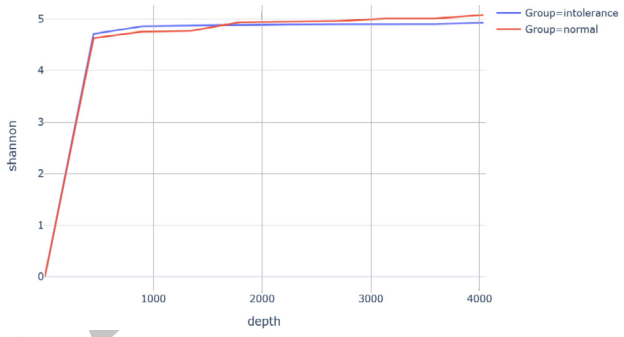

Fig. 1. Sparse curve.

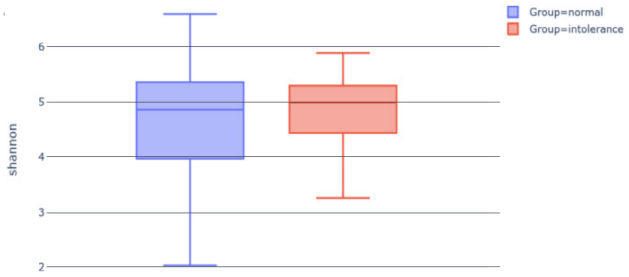

Fig. 2. Alpha diversity differences between groups.

Using the provided sample grouping information or metadata, use the qiime2 diversity Alpha group significance plug-in to test the association between metadata categories and alpha diversity. All previously calculated alpha diversity indicators are included in this correlation test. Figure 2 shows an example of the results of the correlation test. This set of significance tests allows researchers to visualize which metadata categories are most strongly related to differences in microbial community richness and evenness. In addition, Kruskal Wallis statistical test was conducted to show whether the identified group differences were statistically significant.

The analysis method based on linear discriminant analysis effect quantity (lefse) is a tool for screening important features among samples. In addition to population comparison, lefse also allows researchers

to identify features with rich differences, which are also consistent with biologically meaningful categories (subgroups or subclasses). The genus level feature table was used in lefse analysis, and the features with LDA effect greater than 2 were extracted. The classification hierarchy chart can be found in the lefse directory (Fig. 3). Comparing the intestinal flora levels of infants in lactose intolerance (test) and normal (control) groups, the proportion of beneficial flora of Clostridium is more than that of lactose intolerance infants, and the difference is statistically significant ($P<0.05$).

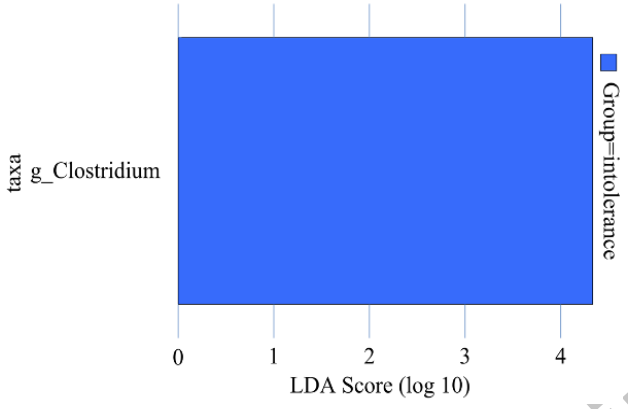

Fig. 3. The comparison results between various groups.

DISCUSSION

According to the research data, lactose intolerance in infants and young children is mainly divided into two types: primary and secondary lactose intolerance. Primary lactose intolerance infants are mostly infants within 6 months, mainly due to the congenital lack or insufficient activity of lactase in their intestines, but with the growth of age, there is a gradual improvement; Secondary lactose intolerance is caused by infectious factors or other factors, such as damage or decreased activity of lactase in the intestine, which leads to secondary lactose intolerance, which is mostly short-term. In recent years, there have been many studies on infantile diarrhea (Hu *et al*., 2016; Parashar *et al*., 2013; Imtiaz *et al*., 2007; Ingram *et al*., 2007). In addition to acute gastrointestinal diseases, lactose intolerance is also one of the more common reasons, which is likely to lead to malnutrition and growth disorders in infants for a long time (Sunita and Neha, 2011). Because the lack of lactase varies greatly among different ethnic groups, there is a good correlation between the 13910 locus upstream of the lactase gene and the persistence of lactase in Europe (Enattah *et al*., 2007). The persistence of lactase can be diagnosed by detecting its gene locus. However, various studies have shown that different races and genetic backgrounds may have different genetic polymorphisms that determine the persistence of lactase. We studied the c/t-13910 gene locus and found that they were all CC type, and the frequency of t-13910 allele was 0%. No SNP locus related to lactase was found temporarily. Relevant references show that for adults in the north, the frequency of c/t-13910 gene is 3.75% (Sun *et al*., 2007), because the gene has a certain stability, we can infer that there may also be a small number of infants with c/t-13910 genotype in the north, but in the literature, the samples in the north are from Mongolian, Kazak, Manchu and other minorities, while the population in the south, especially in Guangdong, is mainly Han. Therefore, it may lead to completely different genotyping results in the north and South populations. According to the current research, c/t-13910 gene may have no correlation with lactase deficiency in Guangdong population.

Although our study has not found SNPs related to the persistence of lactase activity near 13910 gene locus, there may be regulation of other gene loci, which still need to be further studied. In the case of the same lactase genotype, our study found that the intestinal flora of lactose intolerant children is different from that of normal infants. The proportion of beneficial flora such as Clostridium in normal infants is higher than that of lactose intolerant infants. If the children with lactose intolerance can recover the intestinal flora, the dominant bacteria in the children's intestines can be dominated by beneficial bacteria, the reconstruction speed of the body's immune barrier can be accelerated, the invasion of pathogenic bacteria and the killing of pathogenic microorganisms can be resisted, the digestive and absorption functions of the intestines can be restored, and the symptoms of dyspepsia and nutritional status of children can be alleviated. At the same time, because some intestinal probiotics contain lactase, such as Bifidobacterium, Lactobacillus, etc., the main components of *Clostridium butyricum* live powder are *Clostridium butyricum* and *Bifidobacterium infantum*. Almost all bifidobacteria contain lactase that can decompose lactose into glucose and galactose, and the activity of bifidobacteria is significantly higher than that of other intestinal bacteria. Therefore, when infants lack or are insufficient in lactase, supplementing an appropriate amount of Bifidobacterium can effectively avoid the occurrence of lactose intolerance.

CONCLUSION

The c/t-13910 locus of lactase gene may not be related to the lack of lactase in Guangdong population, and the detection of t-13910 locus cannot predict the persistence of lactase in Guangdong population. In addition to adding

lactase, the microbial health of intestinal flora is also crucial after lactose intolerance in children. By adjusting intestinal flora and adding beneficial bacteria such as Clostridium, diarrhea, vomiting and other gastrointestinal symptoms in children can be reduced, which is worthy of clinical promotion.

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IRB approval

This study has been approved by the Haizhu District Maternal Association of Guangzhou City, Guangzhou Haizhu District Maternal and Child Health Hospital, Guangzhou Health Hospital.

Ethical approval

The study was carried out in compliance with guidelines issued by ethical review board committee of Guangzhou Haizhu District Maternal and Child Health Hospital. The official letter would be available on fair request to corresponding author.

Statement of conflict of interest

The authors have declared no conflict of interest.

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