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GENETIC AND CLINICAL MARKERS OF LACTASE DEFICIENCY IN ADOLESCENTS OF THE CENTRAL AND SOUTHERN REGIONS OF EASTERN SIBERIA

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Research objective: to establish the genotype frequency of single nucleotide polymorphisms rs4988235 and rs182549 of the MCM6 gene depending on the ethnicity of adolescents in Eastern Siberia (Russians, Khakasses, Tuvans) and to identify the relationship between lactase deficiency (LD) and the clinical characteristics of recurrent abdominal pain (RAP).

Materials and Methods: 449 adolescents aged 11-18 years old were examined at schools in three cities of Siberia (Krasnoyarsk, Abakan, Kyzyl) and in-patient hospital in Krasnoyarsk. Lactase deficiency (LD) was diagnosed by the hydrogen breath test (HBT) after oral lactose load using the Gastrolyzer apparatus (Bedfont, UK). In schoolchildren, genomic DNA was isolated from saliva samples by the sorption method using the DIAtom DNA Prep kits (IsoGen, Russia). In inpatient children, DNA was isolated from whole blood by the sorption method from 0.1 ml of a suspension of leukocytes using the DNA-Sorb-B kit (103-20, AmpliPrime, Russia). Genotyping for the carriage of allelic variants rs4988235 and rs182549 of the MCM6 gene was performed on the basis of TaqMan allelic discrimination technology using real-time polymerase chain reaction (RT-PCR) on a detecting thermal cycler «Rotor-Gene 6000» (Corbett Life Science, Australia).

Results: The CC genotype of the rs4988235 polymorphism of the MCM6 gene occurs almost 5 times more often (93%) with a positive HBT than with a negative HBT (22%), p<0.001. Moreover, carriage of the rs4988235*CC genotype has a high sensitivity for LD diagnostics, i.e. 93 (81-99) %, with a relatively low specificity of 77 (69-85) %, which is likely to be due to the presence of secondary LD. A significantly higher prevalence of CC genotypes of both polymorphisms associated with LD has been observed in Mongoloid adolescents (Khakas - 82% and Tuvans - 91%), compared with Russian adolescents - 49%, p<0.001. There was no relationship between genetic markers of LD and RAP, verified according to the J. Apley and N. Naish criteria.

Conclusion: A high diagnostic significance of the rs4988235*CC genotype for LD diagnostics in Siberian adolescents was established. The CC genotype prevalence of both polymorphisms, associated with LD, in Russian adolescents (49%) does not differ from European data, whereas these genotypes were found in the great majority of Mongoloids examined (82-91%), which can be considered to be "paradoxical", given that the southern regions of Central Siberia are characterized by a historically high level of dairy farming development.

Keywords: lactase deficiency, adolescents, hydrogen breath test (HBT), genetic polymorphisms, recurrent abdominal pain.

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Introduction: Lactase deficiency (LD) is the most common variant of disaccharidase deficiency. LD is realized to be the reduced intestinal lactase activity, i.e. the parietal digestion enzyme that breaks down lactose, composed of glucose and galactose. There are primary (infant), primary constitutional (adult, late), and secondary hypolactasia.

Lactose, the main carbohydrate in

milk, is one of the main energy source during breastfeeding. To implement the energy function, the disaccharide has to be hydrolyzed to glucose and galactose, effected using β-galactosidase (lactase-phlorizin hydrolase, LPH), which is encoded by the lactase (*LCT)* gene localized at chromosome 2q21 [5]. The LPH enzyme is expressed only in the apical region of the villi of the small intestine, with the highest activity appearing to be at an early age. The enzyme activity decreases rapidly in most people in older age, which can cause full dairy intolerance with specific clinical consequences. However, the enzyme activity is preserved for about a third of the human population, i.e., lactase persistence (LP) seems to take place by adult age. There is currently strong evidence for the genetic basis of LP, encoded by at least five single nucleotide polymorphisms in a dominant inheritance pattern. These polymorphisms are localized upstream of the *LCT* gene in the regulatory region that regulates the *LCT* gene expression, i.e. *MCM6* (minichromosome maintenance complex component 6) [7].

The population LP frequency, being the highest in northern European populations, decreases towards the south of Europe and the Middle East, with it being minimal in non-livestock regions of Africa, Asia and the Far North [1]. Currently, population differences in the LP prevalence are considered by most authors to be a consequence of positive natural selection after the domestication of cattle in the Middle East and North Africa 7500-9000 BC. The four most likely hypotheses of the positive selection advantage for individuals with LP-determining mutations in populations with developed dairy farming have recently been provided [3, 8, 9]:

• the ability to take a highly nutritional food product (milk) without restrictions for life, and not only in childhood;

• the possibility of using milk as a liquid energy drink during heat and drought;

• the ability for lactose to enhance calcium absorption, with that being especially important in areas with vitamin D deficiency;

• the ability of milk to positively affect growth and fertility by stimulating insulin-like growth factor-1.

Currently, there have been known five single nucleotide polymorphisms associated with LP, evidenced by transfection and clinical studies, i.e. −13910:C>T (rs4988235), −13907:C>G (rs41525747), −13915:T>G (rs41380347), −14009:T>G (rs869051967), −14010:G>C (rs145946881). The most ancient and studied polymorphism is rs4988235, which almost completely determines LP in European populations. Other polymorphisms are likely to determine LP in populations of the Middle East and Africa [1]. In recent years, 18 more rare polymorphisms of the MCM6 gene, associated with LP in various relatively small populations have been described [1]. Among them is the mutation −22.018:G>A (rs182549) described, showing full linkage with the rs4988235 polymorphic region.

In the given study, we aimed to establish the genotype frequencies of single nucleotide polymorphisms rs4988235 and rs182549 of the MCM6 gene depending on the ethnicity of adolescents in Eastern Siberia (Russians, Khakasses, Tuvans) and to identify the relationship between LD and the clinical RAP characteristics.

Materials and methods: Lactase deficiency was diagnosed by determining the concentration of hydrogen in the exhaled air after an oral lactose load using the clinically approved apparatus the Gastrolyzer (Bedfont, UK). The basal hydrogen concentration in the exhaled air was measured, then a child drank a specially prepared lactose solution, followed by measurement series of hydrogen in the exhaled air carried out every 30 min. within 2 hours. Gastrointestinal symp-

Table 1

Clinical characteristics and genotype distribution of single nucleotide polymorphisms rs4988235 and rs182549 of the MCM6 gene depending on the results of the hydrogen breath test (HBT) with a lactose load

Clinical characteristics	negative HBT (n=115)	positive HBT (n=43)	p
Age (years)	12.6±0.29	12.7±0.43	0.956
Boys/girls	50/65	25/18	0.110
Weight (kg)	50.8±2.8	49.8±2.5	0.599
Height (cm)	156.1±1.9	159±2.6	0.304
Dyspeptic symptoms after a lactose load	29 (25)	26 (61)	<0.001
MCM6 (rs4988235) genotypes			
TT	18 (16)	2 (5)	0.067
CT	70 (61)	1 (2)	<0.001
TT+CT	88 (77)	3 (7)	<0.001
CC	27 (23)	40 (93)	<0.001
MCM6 (rs182549) genotypes			
TT	18 (16)	2 (5)	0.067
CT	71 (62)	1 (2)	<0.001
TT+CT	89 (78)	3 (7)	<0.001
CC	26 (22)	40 (93)	<0.001

Table 2

Indicators of diagnostic significance for the carriage of the rs4988235*CC genotype for diagnosing lactase deficiency compared with the hydrogen breath test ("gold standard") in adolescents of Eastern Siberia

Indicator	Value	95% confidence interval
Sensitivity	93	81-99
Specificity	77	69-85
Positive Likelihood Ratio	4.11	2.91-5.83
Negative Likelihood Ratio	0.09	0.03-0.27
Positive Predictive Value	61	52-69
Negative Predictive Value	97	91-99
Accuracy	82	74-87

toms were assessed during and after the test. The test was considered to be positive when the concentration of hydrogen in the exhaled air was more than 10 ppm from the basal one.

Genomic DNA was isolated from saliva samples collected using special containers “Saliva DNA Collection and Preservation Devices” (Norgen Biotek Corp., Thorold, ON, Canada) by the sorption method using the Diatom DNA Prep kits (IsoGene, Russia) according to the manufacturer’s instructions.

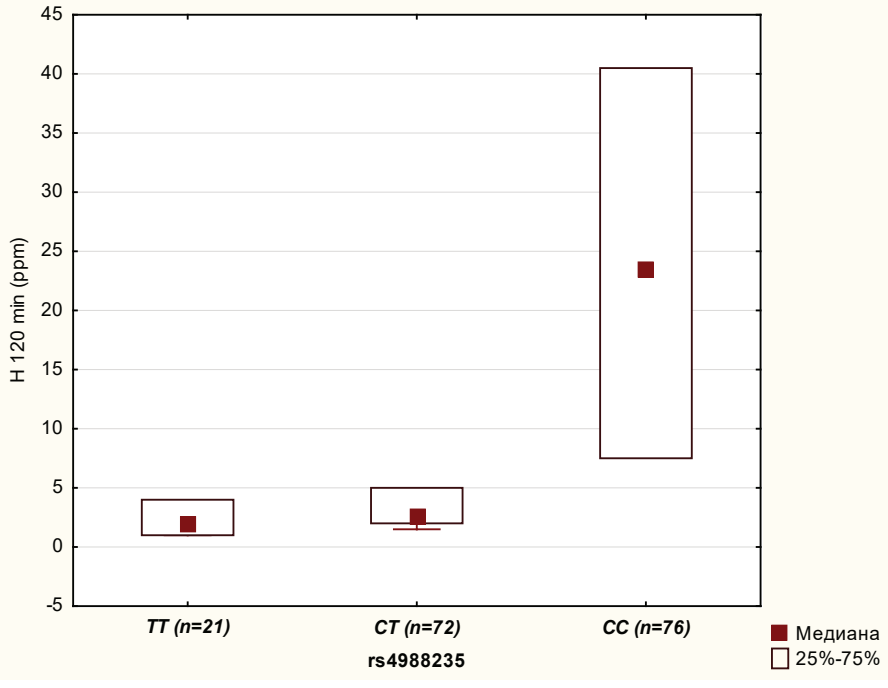
Whole blood was used as a source of genomic DNA in a separate group of experimental subjects (n = 170). Blood sampling in a volume of 3-5 ml was taken aseptically in vacuum tubes "IMPROVACUTER" (Guangzhou Improve Medical Instruments Co., Ltd, Китай) containing 0.5 M EDTA solution. Isolation of genomic DNA was carried out by the sorption method from 0.1 ml of a suspension of leukocytes using the DNA-Sorb-B kit (103-20, AmpliPrime, Russia) according to the manufacturer's instructions.

Genotyping for the carriage of allelic variants rs4988235 and rs182549 of the MCM6 gene was carried out on the basis of TaqMan allelic discrimination technology using real-time polymerase chain reaction (RT-PCR) on a detecting thermal cycler «Rotor-Gene 6000» (Corbett Life Science, Australia). The master mix included a 2.5-fold reaction mixture containing 2.5-fold buffer B (KCl, TrisHCl (pH8.8), 6.25 mM MgCl2), SynTaq DNA polymerase, deoxy-Nucleoside triphosphates, glycerol, Tween 20 (M-428, Synthol, Russia), ddH_2O (M-428, Synthol, Russia), primers and fluorescent probes (DNA-Synthesis, Russia). Amplification was performed in 25 µl of reaction mixtures containing approximately 30 ng of DNA, according to the following protocol: 95°C - 3 min; 95°C - 15 s, 55°C - 30 s, 72°C - 30 s (50 cycles). Each experiment included a negative control, where the DNA template was replaced with distilled water.

Results and discussion: To identify the relationship between genetic markers of LD and the HBT results, 158 adolescents were tested. The results are presented in Table 1.

As follows from the data presented in Table 2, with a positive HBT result, the presence of the CC genotype of the rs4988235 polymorphism of the MCM6 gene occurs almost 5 times more often than with a negative HBT, i.e. in 93% and 22%, respectively (p<0.001).

Additionally, indicators of diagnostic significance for the carriage of the rs4988235*CC genotype for diagnosing lactase deficiency compared with the hydrogen breath test (“gold standard”) in adolescents of Eastern Siberia were calculated. The results are presented in Table 2.



Hydrogen concentration in exhaled air 120 - minutes after experiment started, depending on MCM6 (rs4988235) genotype (K-W test, p<0.001).

The data given in Table 2 have shown the high diagnostic significance for the carriage of the rs4988235*CC genotype for diagnosing lactase deficiency in adolescents of Eastern Siberia.

However, a relatively low carrier specificity of the rs4988235*CC genotype for LD to be diagnosed may be due to the presence of secondary LD caused, for example, by intestinal infections or celiac disease, as well as other genetic loci and/or epigenetic mechanisms of primary LD.

Additional evidence of diagnostic significance may be the estimation of hydrogen concentration in the exhaled air 120 - minutes after experiment started, depending on the MCM6 (rs4988235) genotype. As illustrated in Figure 1, the rs4988235*CC genotype is associated with significantly higher exhaled hydrogen concentrations after lactose load when compared to TT and CT genotypes.

To assess the population frequencies of the genetic marker distribution of LD, there were tested 449 adolescents aged 12-18 years of three nationalities - Russians, Khakasses and Tuvans, living in the central (Krasnoyarsk) and southern (Abakan and Kyzyl) regions of Eastern Siberia.

Data on genotype distribution of single nucleotide polymorphisms rs4988235 and rs182549 of the MCM6 gene, depending on the ethnicity of adolescents, are presented in Table 3. The results obtained have shown a significantly higher prevalence of CC genotypes for both polymorphisms associated with LD in Mongoloid adolescents, i.e. both Khakasses and Tuvans, compared with Russian ones (p<0.001). Therefore, the

Table 3

Genotype distribution of single nucleotide polymorphisms rs4988235 and rs182549 of the MCM6 gene depending on the ethnicity of adolescents (Russians, Khakasses, Tuvans)

Genotypes	Russians (n=231)	Khakasses (n=66)	Tuvans (n=152)
MCM6 (rs4988235) genotypes			
TT	17 (7)	2 (3)	0 (0)
CT	101 (44)	10 (15)*	13 (9)*
TT+CT	118 (51)	12 (18)*	13 (9)*
CC	113 (49)	54 (82)*	132 (91)*
MCM6 (rs182549) genotypes			
TT	17 (7)	2 (3)	0 (0)
CT	105 (46)	11 (17)*	13 (9)*
TT+CT	122 (53)	13 (20)*	13 (9)*
CC	109 (47)	53 (80)*	132 (91)*

Note: * - statistical significance of differences when compared with the ethnic group “Russians” < 0.001.

rs4988235*CC genotype was found in about half (49%) of Russian adolescents, whereas this genotype appeared to take place in the great majority of Khakasses and Tuvans examined (82% and 91%, respectively). Thus, genetic markers for lactase persistence (LP) seems to take place by adult age only in 20% of Khakass adolescents and 9% of Tuvans, with that being 53% in Russian adolescents of Eastern Siberia (determined by the frequencies for rs4988235 TT + CT, Table 1), corresponds to frequencies found in Caucasoids of Central Europe [1]. These data can be considered to be "paradoxical", taking into account that the southern regions of Eastern Siberia (Khakassia and Tyva) are characterized by a historically high level of dairy farming development and high consumption of dairy products.

Extremely low prevalence rates of LP genetic markers have previously been given for other populations, including those in regions with historically high consumption of dairy products [1]. It is not surprising that the extremely low LP prevalence among the Nenets from the Russian Far North (10%), who did not eat milk until the beginning of the 20th century [7]. The same low LP prevalence (14%) was also recorded, e.g., in northern Yakuts, characterized by low dairy farming [9]. However, similar low LP prevalence was also registered in the southern regions with a high consumption of dairy products, namely, among Kazakhs (21%), Kyrgyzes (12%), Buryats (18%), Mongols (13%) [5].

The low LP prevalence we have found for the Khakassia and Tyva populations, as well as for the other populations of the South Asian regions mentioned above, can be represented to be a combination of several cultural, nutritional, and environmental factors. Features of keeping animals (farming or predominant grazing in meadows, seasonal factors), food traditions in some populations leading to the need for fermentation of dairy products (for example, as cheese, koumiss) or mixing milk with other products (for example, drinking milk with tea and salt, characteristic of Tuvans), reducing the influence of evolution factors on lactase-producing objects [8]. There may also be a modifying effect of gut microbiota, characteristic of certain populations, on the intra-intestinal fermentation of lactose. [2, 4, 6].

Finally, migration processes could result in populations mixing and positive selection leveling of lactase-producers [1]. We have not found a relationship between genetic markers of LD and recurrent abdominal pain (RAP), verified according to the criteria of J.Apley and N.Naish (three or more episodes of abdominal pain in the last three months, disrupting the child's daily activities). Therefore, in Russian adolescents, the frequency of carriage of the CC variant of polymorphism (rs4988235) in children without RAP was 51%, and in children with RAP, that was 43% (p=0.515); in Khakass adolescents that was 78% and 93%, respectively (p= 0.446); among Tuvan adolescents - 93% and 85%, respectively (p=0.221). Thus, the etiopathogenetic relationship between LD and RAP in the total unbiased sample of adolescents seems to be unlikely.

Conclusion: The high diagnostic significance of the rs4988235*CC genotype for LD diagnostics in Siberian adolescents was established. The CC genotype prevalence of both polymorphisms genotypes associated with LD in Russian adolescents (49%) does not differ from European data, whereas these genotypes are found in the great majority of Mongoloids examined (82-91%), which can be considered to be "paradoxical", given that the southern regions of Central Siberia are characterized by a historically high level of dairy farming development.

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