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ORIGINAL PAPER

Functional annotation of lactase gene and its distal enhancer MCM6 for prediction of metabolically unhealthy obesity

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ABSTRACT

Introduction: Lactose maldigestion associated with single nucleotide variants (SNV) of the genes for lactase (LCT) and its enhancer minichromosome maintenance complex component 6 (MCM6) is one of the key triggers that initiate meta-inflammation in metabolically unhealthy obesity (MUO). The aim is to study the contribution of LCT and MCM6 gene SNV to the development of MUO in children.

Material and methods: 152 obese children aged 6–18 years were genotyped for the LCT/MCM6 genes (RT-PCR, Synevo, Ukraine). The main group (n = 77) according to the IDEFICS 2014 recommendations was represented by children with MUO. The control group (n = 75) consisted of children with metabolically healthy obesity. Whole genome sequencing (NGS, CeGat, Germany) was performed in 27 children of the main and 15 children of the control group. To verify the results, bioinformatics analysis, analysis of nominal data, calculation of Cramer's criterion (V), Pearson's randomness factor (C), and the normalized value of Pearson's coefficient (C') were used.

Results: Among obese children 20 SNV LCT and 11 SNV MCM6 were revealed. Odds ratio (OR) for MUO to detect SNV LCT A/G rs3213891 – 1.75 (95% CI 0.17–18.4); G/A rs3213890 –2.5 (95% CI 0.65–10.06); C/T rs3754689 – 3.4 (95% CI 1–13.6). SNV MCM6 G/A rs1057031 – OR = 2.6 (95% CI 0.65–10). There is a direct correlation between MUO and SNV LCT with genotypes: A/G rs3213891 (V = 0.073; C = 0.072; C' = 0.102); G/A rs3213890 (V = 0.284; C = 0.273; C' = 0.386); C/T rs3754689 (V = 0.278; C = 0.268; C' = 0.379) and SNV MCM6 G/A rs1057031 (V = 0.143; C = 0.142; C' = 0.201), $p < 0.05$.

Conclusions: The greatest contribution to the development of MUO in children out of 20 SNV of the LCT gene identified by us in obesity was found for the three genotypes A/G rs3213891, G/A rs3213890, C/T rs3754689, and SNV MCM6 G/A rs1057031 out of 11 SNV MCM6 diagnosed by us.

KEY WORDS:

children, lactase gene, minichromosome maintenance complex component 6 gene, single nucleotide gene variants, metabolically unhealthy obesity.

INTRODUCTION

Recently, more and more data have been accumulating, indicating that single nucleotide variants (SNV) of genes make an important contribution to phenotypic differences between people, including personal characteristics of the development of compensatory reactions, and also determine the predisposition to the occurrence of a number of chronic diseases [1, 2].

According to the Global Burden of Diseases, Injuries, and Risk Factors Study 2019, children and adolescents in the European Union have seen an increase in eating disorder-related mortality over the past 30 years (32.36% [95% UI 2.25 to 66.96]), and disability associated

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with the increasing incidence of type 2 diabetes mellitus (T2DM) [3–5]. Metabolically unhealthy obesity (MUO), including components such as abdominal obesity, insulin resistance, dyslipidemia, and hypertension, is highly hereditary and is caused by a combination of genetic and environmental factors [6]. One of the key triggers that initiates adipose tissue meta-inflammation in MUO is lactose maldigestion associated with the lactase (LCT) SNV gene [7].

The *LCT* gene is 49.3 kb long and is located on the long (q) arm of chromosome 2 at position 21 (2q21.3); it contains 17 exons and is translated into a 6 kb transcript. The *LCT* gene is transcribed towards the centromere. For efficient transcription of the *LCT* gene, the proximal promoter signal must be complemented by the activity of an enhancer located upstream of the *LCT* gene. It was found that the region located 850 bp upstream of the *LCT* gene has regulatory activity and is a necessary sequence to ensure high expression of the *LCT* gene in differentiated Caco-2 cells [8]. This regulatory region is called cis-regulatory element minichromosome maintenance complex component 6 (MCM6) [9]. The functional role of MCM6 in vertebrates is unknown, but it is associated with the "licensing" of DNA replication during the cell cycle. This association has been confirmed in a study of DNA collected from individuals of Finnish, South Korean, Italian, German, French, White, and African American ancestry [10].

Functional annotation of the *LCT* gene and its distal enhancer *MCM6* opens up promising opportunities for predicting the effects of SNV in these regions in determining dietary and metabolic phenotypes: arterial hypertension (24–37%), dyslipidemia (58–66%), type 2 diabetes mellitus (26–69%) and obesity (40–70%) [11]. The results of the MiBioGen study, which involved 18,340 people from 11 countries, demonstrated that it is the 2q21.3 locus, which includes the *LCT* gene and 12 other genes associated with the synthesis of the LCT enzyme, that also determines the composition of the intestinal microbiome, thus, probably indirectly affecting human metabolism [12]. In this connection, the aim of our work was to study the contribution of SNV of the *LCT* gene and its distal enhancer *MCM6* in the development of MUO in children.

MATERIALS AND METHODS

Ethical approval. Participants provided written informed consent, and research protocols and procedures were approved according to the ethical standards of the Helsinki Declaration 2013 and by the Human Research Ethics Committee of Dnipro State Medical University (meeting minutes No. 7 of December 11, 2019). Time of data collection: January 2020 – July 2022.

Study design: observational, analytical, longitudinal, cohort study [13]. Inclusion criteria: children with polygenic obesity (body mass index – BMI, ≥ 97th percentile) 6–18 years old. Exclusion criteria: children with monogenic and/or syndromic obesity, pregnancy, overweight children (BMI = 85–96th percentiles).

To test the hypothesis about the association of the studied SNV with obesity phenotypes, an analysis of the frequency of *LCT/MCM6* genetic variants, along with measurements of anthropometric and biochemical parameters, according to the recommendations of IDEFICS 2014, was carried out in a cohort of 152 obese children aged 6–18 years in the children's endocrinology department of CNE Dnipro Clinical Hospital No. 9 of the Dnipro City Council (children from an urban obesity clinic) [13, 14]. For the examination of children, the consent of their parents was obtained. The main group (n = 77) was represented by children with MUO. The control group (n = 75) consisted of children with metabolically healthy obesity (MHO). Each participant was identified by a code used in the database.

For inclusion in the main observation group, the presence of abdominal obesity and two of the following criteria were taken into account:

- fasting glycaemia ≥ 5.6 mmol/l [15],
- high-density lipoprotein cholesterol (HDL-C) ≤ 1.03 mmol/l or less than 10th percentile of the age norm [16],
- triglycerides (TG) ≥ 1.7 mmol/l or more than the 90th percentile of the age norm,
- systolic blood pressure (SBP) above the 90th percentile for a given age, gender and height [17].

The abdominal type of obesity was determined according to the consensus of the International Diabetes Federation, based on the excess of the waist circumference over the 90th percentile for children 6–15 years old or more than 94 cm for boys aged 16–18 years and more than 80 cm for girls 16–18 years old [18–20].

Anthropometric measurements were made in a child in underwear and without shoes. Height (cm) was measured using a Heightronic Digital Stadiometer to the nearest 0.1 cm. Weight (kg) was measured using a Tefal Bodysignal body composition analyzer (France). Waist circumference and hip circumference were measured using a standardized anthropometric tape, measuring the circumference at the midpoint between the top of the iliac crest and the lower part of the lateral rib cage to the nearest 0.1 cm. Body mass index was converted to SDS by means of the current World Health Organization growth references [21].

Systolic and diastolic blood pressure were measured using a digital oscillometric device, Dinamap ProCare (GE Healthcare).

Laboratory examination for the formation of observation groups for obesity phenotypes included general clinical methods. Blood samples were obtained after an overnight fast by venipuncture in vacutainer gel tubes, and serum was separated from cells by centrifugation in a certified laboratory ("Synevo", Ukraine) using an analyzer and a Cobas 6000 test system; Roche Diagnostics (Swit-

zerland). The analysis of serum glucose was carried out by the hexokinase method; the determination of TG and HDL-C of blood plasma was carried out by the enzymatic-colorimetric method. The determination of the level of basal insulin was carried out by electrochemiluminescent immunoassay. The level of basal insulin in the venous blood was considered normal: 2.6–24.9 μU/ml.

Genotyping *LCT/MSM6* – 13910 (PCR-RT, Synevo, Ukraine) was performed in all examined children. DNA from peripheral blood mononuclear cells was isolated using the DNeasy Blood and Tissue Kit (Qiagen). TaqMan oligonucleotide primers and probes for LCT/MCM6 – 13910 genotypes were developed and synthesized by Applied Biosystems, USA (ID: C_15769614_10). Fluorescence data were analyzed with 7500 Allele Recognition Software, v.2.0.2. (Applied Biosystems, USA).

The sample population examined by whole genome sequencing (NGS, Illumina CSPro, CeGat, Germany) consisted of 27 children of the main and 15 children of the control group and was qualitatively homogeneous in relation to the general population. The average amount of DNA in samples was 0.875 μg. Library Preparation: Quantity used 50 ng. Library Preparation Kit: Twist Human Core Exome plus Kit (Twist Bioscience). Sequencing parameters: NovaSeq 6000; 2 × 100 bp.

Bioinformatic analysis – demultiplexing of the sequencing reads was performed with Illumina bcl2fastq (version 2.20). Adapters were trimmed with Skewer, version 0.2.2 [22]. DNA-Seq: Trimmed raw reads were aligned to the human reference genome (hg19-cegat) using the Burrows-Wheeler Aligner, BWA – mem version 0.7.17-cegat [23]. ABRA, version 2.18 and Genotype Harmonizer v.1.4.20 were used for local restructuring of readings in target regions to achieve more accurate detection of indels in the genome during mutagenesis [24, 25].

To evaluate the functional effects of SNV *LCT/MCM6* in the development of MUO, nominal data analysis was performed using odds ratio (OR), 95% confidence interval (CI), Pearson correlation coefficients (C), normalized Pearson coefficient (C'), Cramer's test (V), and Spearman's criterion (r), where *p*-values less than 0.05 were considered statistically significant. Statistical processing of the results was performed using Microsoft Excel (Office Home Business 2KB4Y-6H9DB-BM47K-749PV-PG3KT) and STATISTICA 6.1 software (StatSoft Inc, no. AGAR909E415822FA).

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RESULTS

The average age of children in the main group was 12.09 ±0.59 years, in the control group 12.27 ±0.79 years. By gender, boys predominated in clinical groups among children with MUO, and their relative number exceeded that among children with MHO (72.7% vs. 61.5%), while girls predominated among MHO children, the percentage of which was 58.33% versus 41.66% boys, but these differences was not statistically significant ($p > 0.05$).

The mean BMI (in percentiles) in the main group was 99.54 ±0.31, while in the control group it was 98.74 ±0.39 ($p = 0.12$) and it was not statistically significant.

As shown by the results of the nominal analysis, the development of MUO is due to the influence of a number of factors (Table 1).

According to the data obtained, the factors contributing to the formation of MUO are: a high level of basal insulinemia (18.36 μU/ml and above); hereditary burden for metabolic syndrome; daily consumption of red meat, sausages, potatoes, rice, margarine, sugary carbonated drinks; "wild" genotype *LCT/MCM6*-13910.

Factors that reduce the risk of developing MUO can be considered: prolonged mealtime (20 minutes or more) and daily consumption of up to 2–3 servings of fresh vegetables and fruits, which is consistent with other literature data [26].

According to the data of *LCT/MCM6* gene genotyping by PCR, the frequency of MUO ($r = 0.22$; $p = 0.02$) and extreme obesity ($r = 0.22$; $p = 0.022$) was higher in children with the "wild" genotype *LCT/MCM6*-13910 and was respectively OR = 80% (95% CI 66.96–88.76) and OR = 54% (95% CI 40.4–67.03), compared with carriers of mutant genotypes ($r = –0.37$; $p < 0.001$) (Table 2).

Whole genome sequencing using the NGS method allowed us to identify 20 SNV of the *LCT* gene among obese children: rs3816088, rs748841, rs6719488, rs3213890, rs2236783, rs2278544, rs375845174, rs147652514, rs3739022, rs2322659, rs3213891, rs116951780, rs140994860, rs17699796, rs35093754, rs2304371, rs148298513, rs3754689, rs4954449, rs147290601 (Table 2) *и* 11 SNV *MCM6:* rs61752701, rs141448886, rs201537325, rs2289049, rs3087353, rs1057031, rs143348934, rs3087348, rs4988270, rs2070068, rs141917101 (Table 3).

According to our data, the most significant functional effect on the development of MUO is caused by three SNV genotypes of the *LCT* gene: A/G rs3213891, Chr. 2: 136552371 (GRCh37); G/A rs3213890, Chr. 2:136552188 (GRCh37); C/T rs3754689, Chr. 2:136590746 (GRCh37) and one SNV genotype of the *MCM6* gene – G/A rs105703,Chr. 2:136633962 (GRCh37). The risk of developing MUO among obese children in the presence of such SNV genotypes of the *LCT* gene as A/G rs3213891 increased by 1.75 times (95% CI 0.17–18.4); G/A rs3213890, 2.5 times (95% CI 0.65–10.06); C/T rs3754689 – 3.4 times (95% CI 1–13.6) and in

TABLE 1. Odds ratio of the influence of the studied risk factors on the development of metabolically unhealthy obesity

Studied factor	OR	95% CI	*p*-value
The age of the child at the time of examination is 12–17 years	6.00	2.51–14.37	**< 0.001**
Pathological course of pregnancy in the mother	3.23	1.35–7.68	**0.008**
Presence of excess weight at an early age	2.58	1.08–6.19	**0.034**
Time of introduction of supplementary food 0–4 months	16.21	0.9–291.34	**0.059**
History of pneumonia	6.67	1.81–24.50	**0.004**
Transferred chickenpox in the anamnesis	3.79	1.71–8.39	**0.001**
Hereditary burden of metabolic syndrome	11.61	3.67–36.70	**< 0.001**
Average duration of eating 20 minutes or longer	0.11	0.04–0.29	**< 0.001**
Serving volume 1–2 palms	0.45	0.08–2.59	**0.374**
Prevalence of fast food	2.36	1.09–5.08	**0.029**
Daily consumption of up to 2–3 servings of fresh fruits and vegetables	0.20	0.07–0.55	**0.002**
Daily consumption of red meat, sausages, potatoes, rice, margarine, sweet drinks	10.95	4.31–27.86	**< 0.001**
Multiplicity of physical activity – only in physical education classes	1.96	0.83–4.65	**0.127**
Non-academic computer/TV time is more than 3 hours	6.87	2.91–16.24	**< 0.001**
Presence of clinical symptoms of hypolactasia	3.24	1.48–7.10	**0.003**
The level of physical development of the child is more than 67.34 percentiles	3.12	1.39–7.00	**0.006**
The presence of acne vulgaris	5.34	1.66–17.16	**0.005**
Initiation of puberty outside age norms	5.88	2.16–15.99	**0.001**
Genotype of the LCT/MCM6 C/C – 13910 gene	10.75	4.37–26.44	**< 0.001**
The level of basal insulinemia from 18.36 μU/ml	93.85	5.51–1598.54	**0.002**

the presence of the SNV genotype of the *MCM6* gene G/A rs105703 – 2.6 times (95% CI 0.65v10).

We have established a direct correlation between SNV *LCT* and the risk of MUO in the A/G rs3213891 genotype (V = 0.073; C = 0.072; C' = 0.102) of weak strength and in the G/A rs3213890 genotypes (V = 0.284; C = 0.273; C' = 0.386), C/T rs3754689 (V = 0.278; C = 0.268; C' = 0.379) of medium strength. There was also a direct correlation between SNV *MCM6* G/A rs1057031 (V = 0.143; C = 0.142; C' = 0.201) and the risk of moderate-strength MUO ($p < 0.05$).

The CADD indicators calculated by us for SNV *LCT/MCM6* were characterized as follows: G/A rs3213890-0.216 (mutation in the intron region of the *LCT* gene); A/G rs3213891-2.787 (mutation in the intron region of the *LCT* gene); G/A rs1057031-9.898 (mutation in the 5'-untranslated region with a basic change in the 13th intervening sequence (IVS13 c.-13910) of the *MCM6* gene, regarded as a variant of uncertain significance, according to the GnomAD browser); C/T rs3754689-10.09 (missense mutation in the *LCT* gene).

DISCUSSION

Metabolic disorders associated with MUO have a significant impact on the health of the younger generation [19, 27]. The lack of generally accepted criteria for the verification of the obesity phenotype required the search for new markers for identifying disorders of various metabolic pathways that would allow one to reliably distinguish between MHO and MUO.

We found a high correlation between an increased level of basal insulinemia and the development of MUO, which is caused by hyperlipidemia and cytokine adiposopathy and leads to the development of insulin resistance. Prolonged insulin resistance is accompanied by depletion of the possibilities of insulin secretion by β-cells, the secretion level of which becomes insufficient to maintain the physiological level of glucose in the blood, which leads to the development of stable hyperglycemia and increases the risk of developing T2DM, as shown earlier, by 5–20 times, compared with individuals with physiological body weight [28].

Along with other authors, we detected a high risk of MUO among children who regularly violate dietary recommendations and consume high-calorie foods from the "red zone" of the food traffic light system, as well as sugary carbonated drinks, in their daily diet [29, 30].

Despite the significant contribution of exofactors, a key role in the development of MUO is played by a genetic predisposition to the metabolic syndrome associated with adult-type hypolactasia due to the presence of mutations in the *LCT* gene or LCT non-persistence associated with a mutation in the *MCM6* gene [31].

An excess of lactose in the modern human diet can initiate the development of meta-inflammation and insu-

TABLE 2. Characterization of the lactase single nucleotide variants in obesity phenotypes in children

№	Position	*n*	GnomAD_maxPOP	SNV	Ref/Alt	Zygosity	Consequence	CADD	RawScore
1	136587238	2	AFR	rs3816088	G/C	HET	Synonymous	0.009	–0.799136
2	136558157	29	NFE	rs748841	C/T	HET	Intronic	0.027	–0.659869
3	136575199	29	NFE	rs6719488	G/T	HET	Synonymous	0.098	–0.485584
4	136552188	17	OTH	rs3213890*	G/A	HET	Intronic	0.216	–0.375943
5	136594158	29	NFE	rs2236783	G/A	HOM	Synonymous	0.365	–0.300101
6	136546110	30	NFE	rs2278544	A/G	HET	Synonymous	0.389	–0.291058
7	136566101	1	NFE	rs375845174	G/A	HET	Synonymous	0.469	–0.263561
8	136581454	1	AMR	rs147652514	A/T	HET	Intronic	0.49	–0.257192
9	136562472	14	SAS	rs3739022	A/G	HET	Synonymous	0.521	–0.248015
10	136555659	29	NFE	rs2322659	T/C	HET	Missense	2.615	–0.000555
11	136552371	4	EAS	rs3213891*	A/G	HET	Intronic	2.787	0.010692
12	136567154	1	SAS	rs116951780	C/T	HET	Synonymous	3.834	0.074448
13	136567034	1	AMR	rs140994860	G/A	HET	Synonymous	6.35	0.235382
14	136562454	4	NFE	rs17699796	A/G	HET	Synonymous	7.702	0.338832
15	136574968	2	AFR	rs35093754	G/C	HET	Synonymous	8.756	0.433993
16	136561557	37	NFE	rs2304371	G/A	HET	Synonymous	9.667	0.528501
17	136564984	1	AMR	rs148298513	G/A	HET	Intronic	9.746	0.537108
18	136590746	19	AFR	rs3754689*	C/T	HOM/HET	Missense	10.09	0.574319
19	136575534	42	EAS	rs4954449	T/C	HOM	Missense	13.82	1.058787
20	136567206	1	EAS	rs147290601	C/T	HET	Missense	23.8	3.093064

Alt – alternative allele, AFR, AMR, EAS, FIN, NFE, SAS and OTH – represent African, American, East Asian, Finnish, Non-Finnish European, South Asian and other populations, CADD – Combined Annotation Dependent Depletion, Consequence – functional consequence of the variation in relation to the transcript, GnomAD_maxPOP – frequency distribution of LCT, MCM6 mutations, Ref – reference allele, SNV – single nucleotide variants

** SNV MCM6 associated with metabolically unhealthy obesity*

The nucleotide change and position relative to the coding sequence of the affected transcript in HGVS nomenclature: c. CDS Position Reference Base > Alternative Base. Example: c.223A > T. This column is empty if the variant is intergenic.

TABLE 3. Characterization of the minichromosome maintenance complex component 6 single nucleotide variants in obesity phenotypes in children

№	Position	*n*	GnomAD_maxPOP	dbSNP (SNV)	Ref/Alt	Zygosity	Consequence	CADD	RawScore
1	136598550	1	SAS	rs141917101	T/C	HET	Intronic	5.239	0.160458
2	136624155	1	AFR	rs2070068	G/A	HET	Intronic	7.639	0.333665
3	136602196	1	NFE	rs4988270	G/A	HET	Synonymous	8.453	0.405136
4	136624314	1	EAS	rs3087348	A/C	HET	Intronic	8.066	0.370008
5	136605608	1	AMR	rs143348934	G/A	HET	Intronic	9.362	0.495651
6	136633962	18	SAS	rs1057031*	G/A	HET/HOM	5_prime_UTR	9.898	0.554078
7	136623717	1	EAS	rs3087353	C/T	HET	Synonymous	11.55	0.749133
8	136624123	1	AFR	rs2289049	G/A	HET	Intronic	13.66	1.034057
9	136633927	1	AMR	rs201537325	G/A	HET	Synonymous	14.01	1.090330
10	136620315	2	NFE	rs141448886	T/C	HET	Missense	23.3	2.866344
11	136627912	1	OTH	rs61752701	G/A	HET	Missense	32	4.570014

Alt – alternative allele, AFR, AMR, EAS, FIN, NFE, SAS and OTH – represent African, American, East Asian, Finnish, Non-Finnish European, South Asian and other populations, CADD – Combined Annotation Dependent Depletion, Consequence – functional consequence of the variation in relation to the transcript, GnomAD_maxPOP – frequency distribution of LCT, MCM6 mutations, Ref – reference allele
** SNV MCM6 associated with metabolically unhealthy obesity*

lin resistance. Violation of lactose degradation (MedGen UID: 75659), due to a deficiency in LCT activity, leads to an increase in the level of LCT in the blood serum [32]. So, lactose, by binding to galectin 9 (Gal-9), prevents activation of the Tim-3 receptor, which has an inhibitory effect on Th1 and Th17 cells [33, 34].

Unlike previous investigators, we have identified functional effects of previously undescribed SNV *LCT* (rs3213890, rs3213891, rs3754689) and *MCM6* (rs1057031) in MUO formation [35–37].

CONCLUSIONS

The formation of MUO is caused by the following negative factors: the level of basal insulinemia is more than 18.36 μU/ml; hereditary burden for metabolic syndrome; daily consumption of red meat, sausages, potatoes, rice, margarine, sugary drinks; "wild" genotype *LCT/MCM6*-13910.

Among genetic biomarkers, three of the following genotypes provide a significant contribution to the development of MUO in children out of 20 SNV *LCT* that we identified in obesity: A/G rs3213891, G/A rs3213890, C/T rs3754689. Among the 11 SNV *MCM6* diagnosed by us, G/A rs1057031 makes the greatest contribution to the development of MUO in children.

DISCLOSURE

The authors declare no conflict of interest.

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