Association of leptin receptor gene polymorphisms and meta-inflammation markers with metabolically unhealthy obesity in children

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The aim: to study the contribution of single-nucleotide polymorphisms (SNP) of the leptin receptor (LEPR) gene and meta-inflammation markers to the formation of metabolically unhealthy obesity (MUO) in children.

Materials and methods. A total of 109 obese children aged 6–18 years were examined. Based on the recommendations of the National Heart, Lung, and Blood Institute (NHLBI), 2 observation groups were formed. The main group (n = 56) was represented by patients with MUO. The control group (n = 53) comprised children with metabolically healthy obesity (MHO). Serum levels of interleukin-16 (IL-16) were measured using a chemiluminescent immunosorbent assay (CLIA) method, interleukin-6, leptin, adiponectin – by enzyme-linked immunosorbent assay (ELISA) and the serum level of C-reactive protein were quantified by latex turbidimetric method (Synevo, Ukraine). The method of next-generation sequencing (NGS) (CeXGat, Germany) was used to identify LEPR SNP. Statistical methods were used: analysis of variance, Spearman’s correlation analysis and multiple discriminant analysis.

Results. In obese children aged 6 to 18 years, there was an increase in pro-inflammatory adipokines IL-6 and leptin and a decrease in anti-inflammatory adipocytokine. Statistically significant changes in these indicators were more expressed in the main group: IL-6 – 7.4 ± 0.5 pg/ml (ρ = 0.65; P ≤ 0.001); adiponectin – 3.9 ± 0.8 μg/ml (ρ = -0.27; P = 0.007) among all the children examined, leptin in girls – 47.8 ± 2.4 ng/ml (ρ = 0.65; P ≤ 0.001); adiponectin – 7.7 ± 2.4 μg/ml, leptin in girls – 32.5 ± 4.3 ng/ml, P ≤ 0.05. The most important in the development of MUO were the following SNP of the LEPR gene: rs3790435 (C1230A = 0.939), rs2186248 (C4847T = 0.862), P < 0.05. A strong correlation was found between MUO and serum IL-6 level (ρ = 0.7), LEPR SNP rs3790435 (ρ = 0.7), basal hyperinsulinemia (ρ = 0.72); P ≤ 0.001. The risk of IL-6-dependent meta-inflammation in the presence of SNP rs3790435 of the LEPR gene: OR = 17.11; 95 % CI 2.8–20.4.

Conclusions. Meta-inflammation in MUO is IL-6-dependent. Among the 10 SNPs of the LEPR gene that we identified, SNP rs3790435 of the LEPR gene has a strong association with the formation of MUO. SNP rs2186248 LEPR was described by us for the first time when it was found in 94.1 % of obese children, but it was characterized by the presence of a weak association with MUO.

Key words: leptin receptor gene, polymorphism, interleukin-6, meta-inflammation, obesity, children.

Association of leptin receptor gene polymorphisms with metabolically unhealthy obesity in children

В асоціації поліморфізмів гена лептинового рецептора з метазапаленням у дітей із метаболічно нездоровим ожирінням

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Мета роботи – вивчити внесок однонуклеотидних поліморфізмів гена лептинового рецептора та маркерів метазапалення в формування метаболічно нездорового ожиріння в дітей.

Матеріали та методи. Обстежили 109 дітей віком 6–18 років з ожирінням. Згідно з рекомендаціями National Heart, Lung, and Blood Institute (NHLBI), сформували 2 групи спостереження: основна (n = 56) – пацієнти з метаболічно нездоровим ожирінням; контрольна (n = 53) – діти з метаболічно здоровим ожирінням. Рівень інтерлейкіна-1β (IL-1β) у сироватці вимірювали за допомогою методу хемілюмінесцентного імуноаналізу (CLIA), IL-6, лептин, адипонектин – за допомогою методу геномного секвенування нової генерації, NGS (CeXGat, ФРН). Використовували статистичні методи: аналіз варіацій, кореляційний аналіз Спірмена та множинний дискримінантний аналіз.

Результати. У дітей віком 6–18 років з ожирінням визначили підвищення прозапальних адипокінів IL-6 і лептину, а також зниження противапального адипонектину. Статистично значущі зміни цих показників зафіксували основній групі: IL-6 – 7,4 ± 0,5 пг/мл (ρ = 0,65; P ≤ 0,001); адипонектин – 3,9 ± 0,8 μг/мл (ρ = -0,27; P = 0,007) у всіх обстежених; лептин у дітей – 47,8 ± 4,4 нг/мл (ρ = -0,28; P = 0,003). Результати пацієнтів контрольної групи: IL-6 – 4,3 ± 0,3 пг/мл, адипонектин – 7,7 ± 2,4 μг/мл, лептин у дітей – 32,5 ± 4,3 нг/мл, P ≤ 0,05. Найбільш значимі зміни у розвитку метаболічно нездорового ожиріння мають такі однонуклеотидні поліморфізми гена лептинового рецептора: rs3790435 (C1230A = 0,939), rs2186248 (C4847T = 0,862), P < 0,05. Виявили сильну кореляційну зв’язок між метаболічно нездоровим ожирінням і рівнем IL-6 (ρ = 0,7), однонуклеотидним поліморфізмом rs3790435 гена лептинового рецептора (ρ = 0,7), базальною гіперглікемією (ρ = 0,72), P ≤ 0,001. Ризик формування IL-6-залежного метазапалення за наявності однонуклеотидного поліморфізму rs3790435 гена лептинового рецептора – ВШ = 17,11; 95 % ДИ 2,8–20,4.
Obesity in children, adolescents, and subsequently in adults is one of the most serious public health problems in the 21st century. The spread of obesity is pandemic. The number of children and adolescents (5–19 years) living with obesity, predicted by the early 2025, will be 206 million [1]. Molecular genetic features of obesity-induced meta-inflammation are of particular practical interest in the context of an obesity pandemic in the human population, due to the fact that it is the obesity phenotype that determines the degree of cardiovascular risk [2]. The phenotype of metabolically healthy obesity (MHO) is defined by The American Academy of Pediatrics [3] as a condition in which, despite the excess of physiological body weight over the 95th percentile, there are no risk factors such as insulin resistance, dyslipidemia and arterial hypertension, in contrast with the phenotype of metabolically unhealthy obesity (MUO) [4–6].

The development of meta-inflammation in MUO is characterized by a change in the spectrum of products synthesized by adipocytes due to excessive fat accumulation as a result of an increase in the synthesis of pro-inflammatory cytokines and a decrease in the level of adiponectin secretion. The previously presented data on the difference in the pathophysiological processes of the meta-inflammation formation in MHO and MUO require further study on the role of candidate genes responsible for the formation of insulin resistance [7,8]. One of the main genomic representatives involved in the regulation of energy consumption is the leptin receptor (LEPR) gene. It has been proven that SNPs of the LEPR gene are found in 21.9 % of patients with various obesity phenotypes and lead to a decrease in the activity of the anorexigenic cascade and deregulation of the LEPR axis, increased food intake and fat deposition [9,10].

Analysis of the scientometric PubMed database showed that the most studied association between obesity and type 2 diabetes are the following SNPs of the LEPR gene: rs3790435 [11] and missense mutations rs1137100 [12,13], rs1137101 [14,15], rs1805094 [16], rs8179183 [17], which are located in gene regions encoding functionally significant domains of the LEPR protein.

Genetic studies have demonstrated a high degree of association of non-synonymous polymorphisms of the LEPR gene with the development of monogenic obesity [18]. However, data on the role of intron mutations in the formation of excess fat mass are presented in isolated works and only in some variants [19,20]. In addition, present, there are no studies demonstrating the relationship between the LEPR SNP and the development of meta-inflammation in various obesity phenotypes.
Using new generation of the complete genome sequencing method opens the potential for studying the role of SNP variants of the LEPR gene and demonstrates the different functional significance of these conformations in the central regulation of energy balance in polygenic obesity [21]. Isolation of the key pro-inflammatory factors associated with obesity will allow the use of drug-induced silencing in personalized prevention and treatment of metabolic disorders that pose a threat to the health and life of patients.

**Aim**

To study the contribution of SNP of the LEPR gene and meta-inflammation markers to the formation of MUO in children.

**Material and methods**

All the procedures performed in studies involving human participants were in accordance with the ethical standards of the Institutional and/or National Research Committee and with the 2000 Helsinki declaration (52nd WMA General Assembly, Edinburgh, Scotland) and its later amendments or comparable ethical standards. The submissions were reviewed by the Ethics Committee of Dnipro State Medical University (meeting minutes No. 7 of December 11, 2019 and meeting minutes No. 5 of September 3, 2020).

An informed consent was obtained from each individual participant enrolled in the study.

At the Children’s Endocrinology Department of the Communal Non-profit Enterprise “Dnipro City Clinical Hospital No. 9” of the Dnipro City Council, 109 children aged 6–18 years (mean age 12.24 ± 0.14) with a diagnosis of obesity were examined. To verify the diagnosis, the classification of obesity recommended in clinical practice was used: Order of the Ministry of Health of Ukraine No. 254 of 27.04.2006 “Protocol for the provision of medical care to obese children”.

Inclusion criteria: body mass index (BMI) exceeding the 95th percentile or 2 SD [22].

Exclusion criteria: monogenic and secondary forms of obesity; hereditary syndromes accompanied by obesity; diseases, the treatment of which requires the use of medications that affect the metabolism of carbohydrates and lipids; pregnancy.

The main group (n = 56) included patients with the MUO phenotype, the control group (n = 53) comprised children with the MHO phenotype. The recommendations of the NHLBI expert group (USA) were used as the criteria for MUO in children and adolescents [4].

For inclusion in the main study group, the presence of abdominal obesity and two of the presented criteria were taken into account: 1) fasting glycemia ≥5.6 mmol/L [23]; 2) high-density lipoprotein (HDL) ≤1.03 mmol/L; 3) triacylglyceride (TAG) ≥1.7 mmol/L; 4) systolic blood pressure (SBP) above the 90th percentile for a given age, sex and height [6]. The abdominal type of obesity was determined according to the consensus of the International Diabetes Federation (IDF), based on the waist circumference exceeding the 90th percentile for children aged 6–15 years or more than 94 cm for boys aged 16–18 years and more than 80 cm for girls aged 16–18 years [24–26].

To study the contribution of SNP variants of the LEPR gene to the formation of MUO, a molecular genetic examination was conducted using new generation of the complete genome sequencing (NGS) method according to the American College of Medical Genetics and Genomics (ACMG) recommendations [29]. We examined 35 patients (18 children from the main group and 17 children from the control group) aged 6–18 years with obesity via venous blood sampling in a certified CeXGat Laboratory (Tubingen, Germany) using the Illumina CSPro® Certified service provider platform. The mean age of patients in the study groups was 12.06 ± 1.25 years. The proportion of boys in the main group was 61.1 ± 5.5 % (35/56), while in the control group was 53.5 ± 6.1 % (29/54). The mean age of patients in the study groups was 12.06 ± 1.25 years. The proportion of boys in the main group was 61.1 ± 5.5 % (35/56), while in the control group was 53.5 ± 6.1 % (29/54). 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Bioinformatic analysis – demultiplexing of the sequencing reads was performed with Illumina bcl2fastq (version 2.20). Adapters were trimmed with Skewer, version 0.2.2 [30]. 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 [31]. ABRA, version 2.18 [32] was used for local restructurin of readings in target regions to improve more accurate detection of indels in the genome during mutagenesis. Proprietary readout tools, alignment with more than one locus with the same alignment score, were used; duplicate reads were discarded.

Variant calling: additional proprietary software was used to detect variants of polymorphisms, including variants with low frequencies (Observed frequency of the alternative allele in the range, OFA up to 2 % of sequenced readings). The mutation variants were annotated based on various publicly available databases (Ensembl v100, RefSeq Curated (20200723), CCDS r22, dbsNP154, GnomAD 2.1 (exonic) and 3.0 (genomic), Gencode 34). All synonymous SNP types were excluded from the study.

The quality of FASTQ files was analyzed using FastQC, version 0.11.5-cegat [33]. The plots were created with ggplot2 [34] in R version 3.6.1 [35]. When interpreting the data of bioinformatic analysis, the combined annotated – dependent depletion (CADD) was calculated for each identified SNP of the LEPR gene [36], and the software products FILTUS [37], SeqVISTA [38], Mutation assessor [39] were used.

Statistical processing of the results using parametric and nonparametric methods included: analysis of variance with the calculation of the Student’s test (t); Spearman correlation analysis with calculation of Spearman’s rank correlation coefficient (ρ), multiple discriminant analysis with the calculation of the coefficients of the standardized canonical discriminant function (C_i).

The critical value of the statistical significance level (P) for all types of analysis was taken at the level of P < 0.05 (5 %). Statistical processing of the results was performed using Microsoft Excel (Office Home Business 2KB4Y-6H9DB-BM47K-749PV-PG3KT) and Statistica 6.1 software (StatSoftInc, no. AGAR909E415822FA).

Results

The mean age of patients in the main and control groups was 12.14 ± 0.08 and 12.34 ± 0.76, respectively. The proportion of boys in the main group was 57.14 ± 6.61 %, while in the control group, the proportion of boys in the study population was 47.17 ± 6.86 %, P ≥ 0.05.

As a result of molecular immunological studies, the serum levels of pro-inflammatory and anti-inflammatory adipokines and cytokine IL-6 was detected in obesity (Table 1).

In obese children, regardless of the sex and obesity phenotype, there was a significant increase in serum leptin levels (P < 0.05). At the same time, changes in the leptin concentration moved towards being sex-dependent with the development of metabolic disorders. In males of both study groups, the level of leptin did not differ from each other, and in females with MUO, the concentration of leptin was 1.5 times higher than that in the representatives with MHO and amounted to 47.8 ± 4.4 ng/ml.

Table 1. Mean concentration (M ± m) and median (Me)* of serum inflammatory markers values in children with different obesity phenotypes

| Indicator, units | Reference values | Patients with MUO (n = 56) | Patients with MHO (n = 53) | P |
|------------------|------------------|-----------------------------|-----------------------------|----|
|                  | M ± m            | Me                          | M ± m                      | Me |
| IL-1β, pg/ml     | 0–5              | 2.5 ± 0.3                   | 1.9                        | 1.8 ± 0.7    | 1.7            | >0.05         |
| IL-6, pg/ml      | 1.5–7.0          | 7.4 ± 0.5                   | 6.8                        | 4.3 ± 0.3    | 3.4            | ≤0.05         |
| C-reactive protein, mg/L | 0–5              | 2.6 ± 0.7                   | 2.3                        | 2.1 ± 0.4    | 2              | >0.05         |
| Leptin, ng/ml    | Boys             | 2.0–5.6                     | 29.3 ± 8.9                 | 26.0 ± 6.4   | 24.4           | >0.05         |
|                  | Girls            | 3.7–11.1                    | 47.8 ± 4.4                 | 45.2 ± 3.2   | 32.5 ± 4.3    | <0.05         |
| Adiponectin, µg/ml | ≥10              | 3.9 ± 0.8                   | 3.1                        | 7.7 ± 2.4    | 6.5            | <0.05         |

* Me with 95 % CI median

Children with the MUO phenotype differed from MHO by a significantly lower serum level of adiponectin, which was 3.9 ± 0.8 µg/ml versus 7.7 ± 2.4 µg/ml (P < 0.05).

One of the most important differences in the cytokine status of obesity phenotypes was the level of IL-6. In the MUO phenotype, a significantly higher serum concentration of IL-6 was observed reaching 7.4 ± 0.5 pg/ml, than in the MHO phenotype (4.3 ± 0.3 pg/ml, P < 0.05). At the same time, the serum levels of IL-1β and CRP in the main and control groups corresponded to the reference values and did not differ statistically from each other.

As a result of NGS, 10 types of SNP in the LEPR gene were identified among 35 obese children: rs3790435, rs1137100, rs2186248, rs70940803, rs79639154, rs1359482195, rs1137101, rs1805094, rs13306520, rs13306522. Multiple discriminant analysis with the C_i calculation showed the greatest contribution to the development of MUO of the following two SNPs: rs3790435 (C_i = 0.939), rs2186248 (C_i = 0.862), P < 0.05. In obese children, the incidence of SNP rs3790435 was 71.4 %, SNP rs2186248 – 91.4 %.

The study on the role of the LEPR gene SNP in various phenotypes of obesity formation demonstrated the following results. The presence of the “wild” CC genotype rs3790435 was more common in children with MHO (52.9 %), and less often – in children with MUO (16.7 %), P < 0.05. Meanwhile, the “wild” CC genotype rs2186248 was significantly more often identified in the group of children with MUO (100 %), and less often – in the group of children with MHO (88.2 %), P < 0.05.

23 factors of the clinical, immunological and genetic parameters analyzed were found to be significantly associated with MUO (P < 0.05). Among them, according to the strength of relationship with the risk of MUO, 3 groups were distinguished.

1) Highly significant factors (0.7 ≤ |ρ| < 1): the level of basal insulinemia (ρ = 0.72; P < 0.001; serum IL-6 level (ρ = 0.7; P < 0.001) and the presence of the LEPR gene SNP rs3790435 (ρ = 0.7; P < 0.001).

2) Moderately significant factors (0.3 ≤ |ρ| < 0.7): daily consumption of red meat, sausages, potatoes, rice, margarine, sugary drinks (ρ = 0.52; P < 0.001); duration of non-academic time spent at the computer/TV (ρ = 0.5; P < 0.001); fast food intake (ρ = 0.47; P < 0.001); family history of metabolic syndrome (ρ = 0.45; P < 0.001); early introduction of complementary foods (ρ = 0.38; P < 0.001); serving size (in the child’s palms) (ρ = 0.37; P < 0.001); age norms for puberty timing violation
(\(p = 0.35; P = 0.001\)); child’s age at the time of examination (\(p = 0.33; P = 0.001\)); daily consumption of up to 2–3 servings of fresh vegetables and fruits (\(p = -0.32; P = 0.001\)); pneumonia in a past medical history in puberty (\(p = 0.3; P = 0.001\)).

3) The least significant factors (0 < \(|p| < 0.3\)): the presence of pyoderma (\(p = 0.29; P = 0.003\)); acanthosis nigricans (\(p = 0.29; P = 0.003\)); hyperleptinemia (\(p = 0.28; P = 0.003\)); serum adiponectin levels (\(p = -0.27; P = 0.007\)); frequency of physical activity (\(p = -0.26; P = 0.006\)); level of physical development of a child in percentiles (\(p = 0.26; P = 0.006\)); pathological antenatal period (\(p = 0.26; P = 0.006\)); LEPR gene SNP rs2186248 (\(p = 0.25; P = 0.006\)); being overweight at an early age (\(p = 0.21; P = 0.031\)); prevalence of fast food in diets (\(p = 0.21; P = 0.028\)).

The patients with the LEPR gene SNP rs3790435 was found to have a 17 times higher chance of developing meta-inflammation associated with increased IL-6 serum levels than those without this SNP of the LEPR gene (OR = 17.11; 95 % CI 2.8–20.4), \(P \leq 0.05\).

Discussion

The studied factors relating to the lifestyle (the level of physical development of a child in percentiles, the frequency of physical activity, the duration of non-academic time spent at the computer/TV and inappropriate nutrition (duration of food intake, period of introducing complementary foods, lack of daily consumption of up to 2–3 serving of fresh vegetables and fruits, serving size and daily consumption of red meat, sausages, potatoes, rice, margarine, sugary drinks, fast food) demonstrate a moderate association with MUO and confirm the data of the previous studies on the importance of physical activity and nutritional intervention in the prevention of metabolic complications [4,40]. In contrast to the previous works, the gradient presentation of the results of rank correlation analysis in our study contains data on the diagnostic significance of not only modifiable environmental factors, but also immunological, molecular genetic markers associated with the LEPR gene SNPs and causing the formation of meta-inflammation in MUO.

Our data indicate a strong association between MUO and the level of IL-6 and a weak association between MUO and the level of leptinemia in girls (no significant changes in boys) and the serum adiponectin concentration in both sexes. This serum content of pro-inflammatory cytokines indicates that meta-inflammation in obesity is IL-6-dependent. This is probably due to the fact that about 35 % of the circulating IL-6 pool is produced in adipose tissue and is responsible for its macrophage infiltration [41–43]. Corina Piercean et al. [44] also found an increase in the secreted IL-6 and leptin in saliva during obesity in children, but unlike our work, despite such an advantage as minimally invasive selection of research material, they did not take into account gender division into subgroups in the study of the level of leptinemia, as well as data on the presence or absence of inflammatory processes in the oral cavity in the examined children. The increased concentration of IL-6 in children with MUO, in our opinion, not only reflects the activity of meta-inflammation, but also carries the risk of developing insulin resistance, since IL-6, in addition to its pro-inflammatory effect, has an inhibitory effect on insulin-associated signaling pathways, in particular, in hepatocytes [45]. In addition, IL-6 in combination with TGF-β ensures the differentiation of naive CD4+ T-cells into Th17-cells, and inhibits TGF-β-stimulated differentiation of Treg-cells [46]. Namely, IL-6-mediated imbalance in Th1/Treg-cells contributes to the development of autoimmune and chronic inflammatory diseases [47].

We present data on the relationship between two SNPs (rs3790435, rs2186248) of the LEPR gene, which were characterized by a high frequency of occurrence in the cohort of obese patients (71.4 % and 91.4 % of individuals, respectively) and the risk of MUO formation. One of them, the LEPR gene SNP rs3790435, was previously described by Juan Li et al. [48] being associated with the development of some features of the obesity course. As a study result, Juan Li et al. found that the presence of the CC genotype rs3790435 in obese individuals reduced the risk of obstructive sleep apnea syndrome compared to people with the TT/CT genotype. At the same time, our results confirm the data of Juan Li et al. about the higher frequency of the CC genotype rs3790435 of the LEPR gene in the cohort of children with the MHO phenotype. In addition, we have demonstrated for the first time a strong relationship between the LEPR gene SNP rs3790435 and the risk of MUO formation, and IL-6-dependent meta-inflammation. An important result of our work is the discovery of the previously undescribed LEPR gene SNP rs2186248, which was found in 91.4 % of the examined obese children, being characterized by the weak correlation with the formation of MUO.

Conclusions

1. The triggering factors for the risk of MUO in association with a genetic predisposition require initial modification in children through a reduction in the duration of non-academic time spent at a computer/TV and daily consumption of red meat, sausages, potatoes, rice, margarine, and sugary drinks.

2. Meta-inflammation in MUO is IL-6-dependent. Pro-inflammatory IL-6 is one of the potential mediators linking obesity-induced meta-inflammation with insulin and leptin resistance.

3. Among the 10 SNPs of the LEPR gene that we identified, two of them are associated with the MUO phenotype development. SNP rs3790435 of the LEPR gene, identified in 71.4 % of obese children examined, is characterized by the strong association with the MUO phenotype. The CT genotype rs3790435 is the risk factor for the development of MUO. Children with the CT SNP rs3790435 genotype of the LEPR gene have the 17 times higher chance of developing IL-6-dependent meta-inflammation than children with the CC genotype. The newly identified SNP rs2186248 of the LEPR gene is weakly associated with the MUO phenotype. However, its TT genotype is significantly more common in children with the MUO phenotype.

Prospects for further research. Our results require further study and confirmation in broader patient populations, which will give us a better and more complete understanding of the relationship between the LEPR gene polymorphism and the MUO risk.
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