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The expression of *CCN2, IQSEC, RSPO1, DNAJC15, RIPK2, IL13RA2, IRS1*, and *IRS2* genes in blood of obese boys with insulin resistance

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The development of obesity and its metabolic complications is associated with dysregulation of various intrinsic mechanisms, which control basic metabolic processes via changes in the expression of numerous regulatory genes. We studied the expression of the subset of genes, which responsible for control of cell growth and glucose metabolism, in blood cells of obese boys with normal and impaired insulin sensitivity as well as in normal (control) individuals. It was shown that obesity with normal insulin sensitivity enhances the expression of IRS1, RIPK2, IL13RA2, RSPO1, IQSEC, and CCN2 genes, but decreases the expression level IRS2 and DNAJC15 genes in the blood cells as compared to control group. Insulin resistance in obese boys leads to up-regulation of IRS2, RSPO1, and DNAJC15 gene expressions as well as to down-regulation of IRS1 and RIPK2 genes in the blood cells versus obese patients with normal insulin sensitivity. Results of this study provide evidence that obesity affects the expression of the subset of genes related to cell growth and glucose metabolism in blood cells and that insulin resistance in obesity is associated with changes in the expression level of IRS1, IRS2, RIPK2, RSPO1, and DNAJC15 genes, which contribute to the development of insulin resistance and glucose intolerance and possibly reflect some changes in fat tissue.
Key words: mRNA expression; CCN2; IQSEC; RSPO1; DNAJC15; RIPK2; IL13RA2; IRS1; IRS2; blood; obesity; insulin resistance.

INTRODUCTION

The development of obesity as well as its metabolic complications, the most profound public health problems, is associated with dysregulation of numerous intrinsic mechanisms, which control most key metabolic processes, including cellular growth, glucose and lipid metabolism as well as insulin sensitivity [1–5]. Moreover, obesity as well as metabolic syndrome results from interactions between genes and environmental factors and is associated with changes in gene expressions of regulatory network in various organs and tissues, but preferentially in adipose tissue [5–9]. Adipose tissue growth is in a center of obesity and tightly associated with glucose and lipid metabolism as well as cell proliferation processes and controlled by different interconnected regulatory factors and enzymes [10]. At the same time, the blood reflects numerous changes in different organs and tissues in diseases including obesity [7, 10]. Special interest deserves the key regulatory enzymes and factors, which control glucose and lipid metabolism as well as cell growth [4, 2].

The insulin receptor substrate 1 (IRS1) as well as IRS2 is a cytoplasmic signaling molecule that mediates effects of insulin, insulin-like growth factor 1, and other cytokines by acting as a molecular adaptor between diverse recep-

tor tyrosine kinases and downstream effectors through phosphorylation by the insulin receptor tyrosine kinase upon receptor stimulation as well as other kinases [11, 12]. Moreover, mutations in *IRS1* and *IRS2* genes are associated with type 2 diabetes and susceptibility to insulin resistance because these factors mediate the control of various cellular processes by insulin and dysregulation of these insulin receptor substrates is associated with PDGF-induced cell proliferation [12, 13]. There is data that genetic variation near *IRS1* gene associates with reduced adiposity, decreased IRS1 expression, and an impaired metabolic profile, including an insulin resistance, dyslipidemia, risk of diabetes and decreased adiponectin levels [14]. Furthermore, *IRS1* and *IRS2* genes have both common and different regulatory mechanisms because the double-stranded RNA-dependent protein kinase differentially regulates IRS1 and IRS2 in HepG2 cells [15].

The IL-13RA2 (interleukin-13 receptor-alpha2) is overexpressed in malignant tumors and may induce invasion and metastasis in pancreatic cancer [16, 17]. Receptor-interacting serine/threonine-protein kinase 2 (RIPK2 receptor-interacting protein-like interacting caspase-like apoptosis regulatory protein (CLARP) kinase, is a multifunctional protein, which controls apoptosis [18, 19]. CCN family member 2 (CCN2), also known as CTGF (connective tissue growth factor), is responsible for proliferation, differentiation, and cell adhesion, including mesenchymal stromal cells differentiation into adipocytes [20, 21]. The *IQSEC2* (IQ motif and Sec7 domain 2) gene encodes a guanine nucleotide exchange factor for the ARF family of GTP-binding proteins, which plays multifunctional role including the regulation of cell proliferation. The RSPO1 (R-spondin homolog, Xenopus laevis, regulates WNT signaling and controls beta-cell growth and insulin secretagogue [22, 23]. DNAJC15 (DNAJ (Hsp40) homolog, subfamily C, member 15) functions as co-chaperone of the human TIM23 pre-protein translocase and acts in the import of proteins into human mitochondria as well as promotes c-Jun degradation [24, 25].

The endoplasmic reticulum stress is also recognized as an important determinant of obesity, insulin resistance, and impaired glucose tolerance and contributes to the expression profile of many regulatory genes resulting in peripheral insulin resistance and other obesity complications [6, 10, 26- 28], although detailed molecular mechanisms cannot be ruled out.

It is possible that identification of real mechanisms of metabolic abnormalities in obesity as well as its complications at molecular and cellular levels helps to better understanding why obesity develops and why only a part of the obese individuals develops secondary metabolic disorders. However, a detailed molecular mechanism of the involvement of different genes of regulatory network in the development of obesity and its complications are not clear yet and remains to be determined.

The main goal of this study was to clarify the role of the subset of gene expressions, encoding for important cell growth factors and enzymes, which control glucose and lipid metabolism, in blood cells of obese boys for evaluation of its possible significance to development of obesity and insulin resistance.

METHODS

The 15 boys participate in this study. They were divided into three equal groups (5 subjects in each group): normal individuals as control and patients with obesity and with or without insulin resistance. All participants gave written informed consent and the studies were approved by the local research ethics committees of Institute of Children and Adolescent Health Care of the National Academy of Medical Science of Ukraine.

Clinical characteristics of the study participants are shown in Table 1. The normal (control) participants were individuals with mean age 14 ± 0.7 years and mean body mass index (BMI) 18.7 ± 0.12 kg/m^2. The obese participants with

normal insulin sensitivity as well as the patients with insulin resistance were individuals with mean age (14 ± 0.6 and 14 ± 0.4 years, correspondingly) and mean BMI (31.0 ± 0.40 and 34.2 ± 2.39 kg/m^2, correspondingly).

Thus, BMI, which is a main criteria of obesity, in these last two groups of patients was significantly higher (+66 and +83 %, correspondingly; $P<0.05$ in both cases) as compared to control individuals (Table 1). Moreover, no significant changes were found in insulin resistance index in obese individuals as compared to control group, but in obese patients with impaired insulin sensitivity, versus control boys as well as obese subjects with normal insulin sensitivity, the insulin resistance index is significantly increased (3.7 and 3.2 fold, correspondingly; $P<0.05$ in both cases), but decreased (almost two fold; $P<0.05$) (Table 1). Similar results were observed in the fasting insulin levels: no significant changes in obese individuals and strong increase in obese children with insulin resistance (3.3 fold; $P<0.05$) as compared to control group. Fasting blood glucose levels were similar in all three groups of the study participants, but 2hrs oral glucose tolerant test was increased in obese boys with insulin resistance versus control group (+25 %; $P<0.05$) (Table 1).

RNA isolation. Trisol reagent (Invitrogen, USA) was used for RNA extraction from blood of normal (control) and obese individuals with or without insulin resistance.

Reverse transcription and quantitative real-time polymerase chain reaction analysis. The expression levels of genes related to regulation of cell growth and glucose homeostasis (*CCN2, IQSEC, RSPO1, DNAJC15, RIPK2, IL13RA2, IRS1*, and *IRS2*) were measured in blood cells by real-time quantitative polymerase chain reaction of complementary DNA (cDNA). QuaniTect Reverse Transcription Kit (QIAGEN, Germany) was used for cDNA synthesis. The 7900 HT Fast Real-Time PCR System (Applied Biosystems), Absolute QPCR SYBRGreen Mix (Thermo Scientific, UK) and pair of primers specific for each studied gene (Sigma/Aldrich, USA) were used for quantitative polymerase chain reaction Table 2).

The expression of beta-actin mRNA was used as control of analyzed RNA quantity. The amplified DNA fragments were analyzed on a 2 % agarose gel and that visualized by 5x Sight DNA Stain (EUROMEDEA). An analysis of quantitative PCR was performed using special computer program "Differential expression calculator".

Statistical analyses were performed according to Student's *t*-test using OriginPro 7.5 software. All values are expressed as mean ± SEM from five independent experiments; $P < 0.05$ was considered as significant difference.

RESULTS AND DISCUSSION

We studied the expression of *CCN2, IQSEC, RSPO1, DNAJC15, RIPK2, IL13RA2, IRS1*,

Table 1. Characteristics of the study participants.

Variable	Control	Obesity	Obesity + IR
Age at visit (years) (*n*)	14 ± 0.73 (5)	14 ± 0.6 (5)	14 ± 0.38 (5)
Body mass index (BMI) (kg/m^2) (*n*)	18.7 ± 0.12 (5)	31 ± 0.40 * (5)	34.2 ± 2.39 * (5)
Insulin resistance index (HOMA) (*n*)	2.36 ± 0.17 (5)	2.70 ± 0.28 (5)	8.70 ± 1.41*^ (5)
Fasting insulin (μIU/ml) (*n*)	13.0 ± 0.95 (5)	14.1 ± 1.35 (5)	43.4 ± 6.70 *^ (5)
Fasting glucose (mmol/l) (*n*)	4.1 ± 0.22 (5)	4.3 ± 0.14 (5)	4.5 ± 0.08 (5)
2h oral glucose tolerance test (OGTT) glucose (mmol/l) (*n*)	4.28 ± 0.08 (5)	5.08 ± 0.11 (5)	5. 36 ± 0.18 * (5)

Data are means ± SEM; IR – insulin resistance;

*P < 0.05 versus control group; ^ - P < 0.05 versus obese group.

and *IRS2* genes in blood cells of three groups: normal (control) individuals, obese boys with normal insulin sensitivity and obese patients with insulin resistance. As shown in Fig. 1, the expression level of IRS1 gene is increased (+68 %; $P < 0.05$) in blood cells of obese boys with normal insulin sensitivity as compared to the group of control children. At the same time, the expression level of *IRS2* gene is significantly decreased (more than 4fold; $P < 0.05$) in blood cells of this experimental group of obese children (Fig. 1). Moreover, the development of insulin resistance in obese individuals is associated with down-regulation of IRS1 gene expression (-32 %; $P < 0.05$) in blood cells as compared to group of children with obesity and normal insulin sensitivity. We have also shown that the expression level of *IRS2* gene is increased (+29 %; $P < 0.05$) in blood cells of obese children with impaired glucose tolerance versus group of obese boys without insulin resistance (Fig. 1).

This data has clearly demonstrated that obesity leads to significant dysregulation of

Table 2. Characteristics of the primers used for quantitative real-time polymerase chain reaction.

Gene symbol	Gene name	Primer's sequence	Nucleotide numbers in sequence	GenBank accession number
CCN2 (CTGF, IGFBP8)	CCN family member 2 (connective tissue growth factor; insulin-like growth factor-binding protein 8)	F: 5'- actgtcccggagacaatgac R: 5'- tgctcctaaagccacacctt	1198–1217 1527–1508	NM_001901
IQSEC2 (BRAGL1)	IQ motif and Sec7 domain 2 (a Sec7 domain-containing protein BRAG1)	F: 5'- atatggaggctcctgtggtg R: 5'- atatggaggctcctgtggtg	771–790 1012–993	NM_015075
1RS1	insulin receptor substrate 1	F: 5'- agtcccagcaccaacagaac R: 5'- tcatccgaggagatgaaacc	1094–1113 1341–1322	NM_005544
IRS2	insulin receptor substrate 2	F: 5'- acctacgccagcattgactt R: 5'- aacaagggaaagaggcaggt	4469–4488 4725–4706	NM_003749
DNAJC15	DnaJ (Hsp40) homolog, subfamily C, member 15	F: 5'- tgagtaggcgagaagctggt R: 5'- gcatcaatgtttggtggttg	676–695 857–838	NM_013238
RSPO1	R-spondin homolog (Xenopus laevis)	F: 5'- tctgagtgatcgctttggtg R: 5'- atctcagctggggacagaga	416–435 650–631	NM_001038633
RIPK2 (CARDIAK)	receptor-interacting serine/threonine-protein kinase 2 (CARD-containing interleukin-1 beta-converting enzyme-associated kinase)	F: 5'- ttccaattttgggaatttgc R: 5'- atgcgccactttgataaacc	550–569 829–810	NM_003821
IL13RA2 (CT19)	interleukin 13 receptor, alpha 2 (cancer/testis antigen 19)	F: 5'- tcttggaaacctggcatagg R: 5'- tctgatgcctccaaataggg	591–610 742–723	NM_000640
ACTB	beta-actin	F: 5'- ggacttcgagcaagagatgg R: 5'- agcactgtgttggcgtacag	747–766 980–961	NM_001101

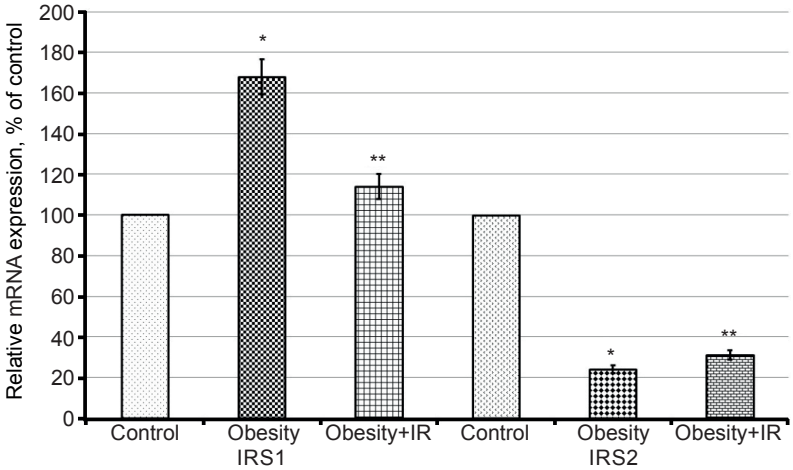

Fig. 1. Relative expression level of insulin receptor substrate 1 (IRS1) and IRS2 mRNA in blood cells of normal boys (Control) and obese individuals with normal insulin sensitivity (Obesity) and obese patients with insulin resistance (Obesity + IR). The values of IRS1 and IRS2 mRNA expression were normalized to the beta-actin mRNA and are expressed as mean ± SEM and represented as a percent of control (100 %); $n = 5$; * - $P < 0.05$ vs group of control individuals; **$P < 0.05$ vs group of obese boys with normal insulin sensitivity

IRS1 and *IRS2* gene expressions in blood cells, being more robust for *IRS2* gene and that this dysregulation of insulin receptor substrate genes is contributed to the development of insulin resistance and enhanced cell proliferation. Moreover, we have shown that development of impaired insulin sensitivity in obese individuals is associated with down-regulation of both *IRS1* and *IRS2* genes and that obesity without insulin resistance and obesity with impaired insulin sensitivity introduces diverse changes in *IRS1* and *IRS2* gene expressions. This data correlates to results of Yang et al. [15] that IRS1 and IRS2 are differentially regulated by the double-stranded RNA-dependent protein kinase in HepG2 cells and other authors [12, 13] that both of these genes may participate in the development of obesity and insulin resistance [30, 31].

Investigation of the expression level of receptor-interacting serine/threonine-protein kinase 2 gene in blood cells of obese boys with normal insulin sensitivity shown significant up-regulation of this gene expression (+69 %; $P < 0.05$) as compared to the group of control children (Fig. 2). At the same time, the expression level of *RIPK2* gene is slightly decreased in blood cells of obese children, which have insulin resistance, versus group of obese boys with normal insulin sensitivity. As shown in Fig. 2, the expression level of other gene encoded interleukin 13 receptor-alpha 2 is also strongly increased in blood cells of obese children (+82 %; $P < 0.05$), but development of insulin resistance does not change significantly the level of this gene expression.

It is possible that overexpression of *IL13RA2* and *RIPK2* genes in obesity reflects its contribution to development of obesity as regulatory factors controlling cellular growth, apoptosis, invasion and metastasis, because interleukin-13 receptor-alpha2 is overexpressed in malignant tumors [16, 17]. Moreover, receptor-interacting serine/threonine-protein kinase 2 as multifunctional protein controls both apoptosis and cytokine responses [18, 19].

We also studied the expression of *CCN2* gene encoded connective tissue growth factor as well as *IQSEC2* gene encodes a guanine nucleotide exchange factor for the ARF family of GTP-binding proteins in blood cells of obese children with and without insulin resistance. As shown in Fig. 3, the expression of *CCN2* and *IQSEC2* genes is increased in obesity, being more robust for *IQSEC2* gene (+19% for *CCN2* gene

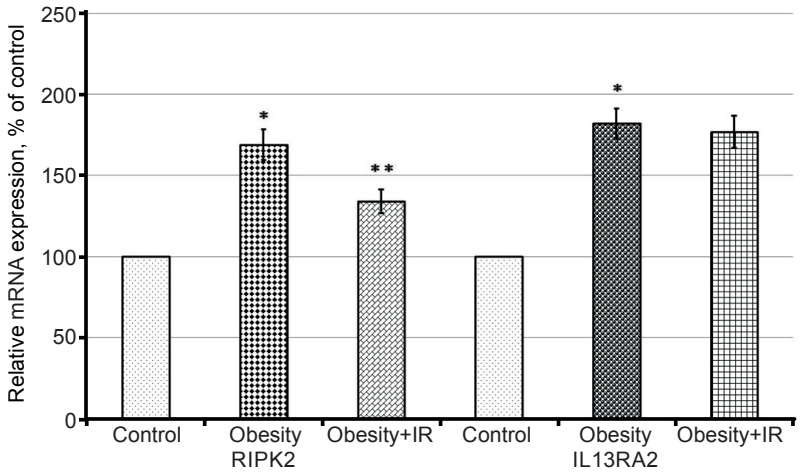

Fig. 2. Relative expression level of receptor-interacting serine/threonine-protein kinase 2 (RIPK2) and interleukin 13 receptor-alpha 2 (IL13RA2) mRNA in blood cells of normal boys (Control) and obese individuals with normal insulin sensitivity (Obesity) and obese patients with insulin resistance (Obesity + IR). The values of RIPK2 and IL13RA2 mRNA expression were normalized to the beta-actin mRNA and are expressed as mean ± SEM and represented as a percent of control (100 %); $n = 5$; *P < 0.05 vs group of control individuals; **P < 0.05 vs group of obese boys with normal insulin sensitivity

and +57 % for *IQSEC2* gene; both P < 0.05), though development of insulin resistance does not change significantly the expression level of both these genes. Enhanced expression of both *CCN2* and *IQSEC2* genes possibly contributes to obesity, because there is data that CCN2 gene is responsible for proliferation, differentiation, and cell adhesion, including mesenchymal stromal cells differentiation into adipocytes [20, 21].

We next tested whether obesity also affects the expression of *RSPO1* and *DNAJC15* genes. As shown in Fig. 4, the expression level of *RSPO1* gene is up-regulated in blood cells of obese boys with normal insulin sensitivity (+35 %; P < 0.05). Furthermore, the development of insulin resistance in obese individuals leads to additional increase of this gene expression (+12 %; P < 0.05). At the same time, *DNAJC15* gene has

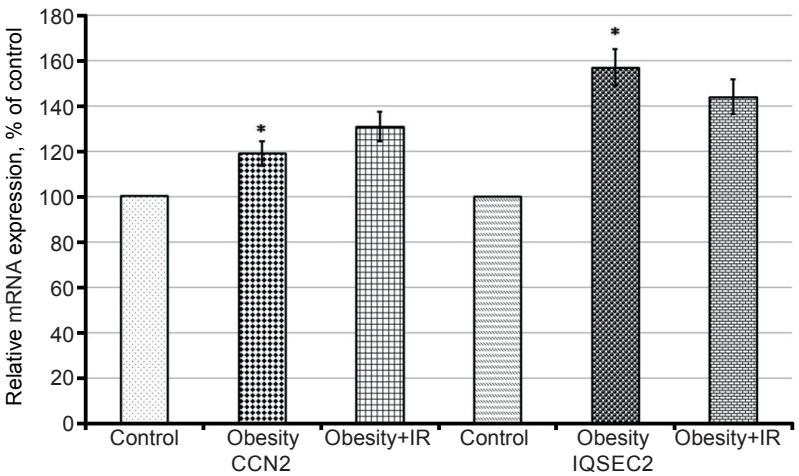

Fig. 3. Relative expression level of CCN family member 2 (CCN2), also known as connective tissue growth factor (CTGF), and IQ motif and Sec7 domain 2 (IQSEC2) mRNA in blood cells of normal boys (Control) and obese individuals with normal insulin sensitivity (Obesity) and obese patients with insulin resistance (Obesity + IR). The values of CCN2 and IQSEC2 mRNA expression were normalized to the beta-actin mRNA and are expressed as mean ± SEM and represented as a percent of control (100 %); $n = 5$; *P < 0.05 vs group of control individuals

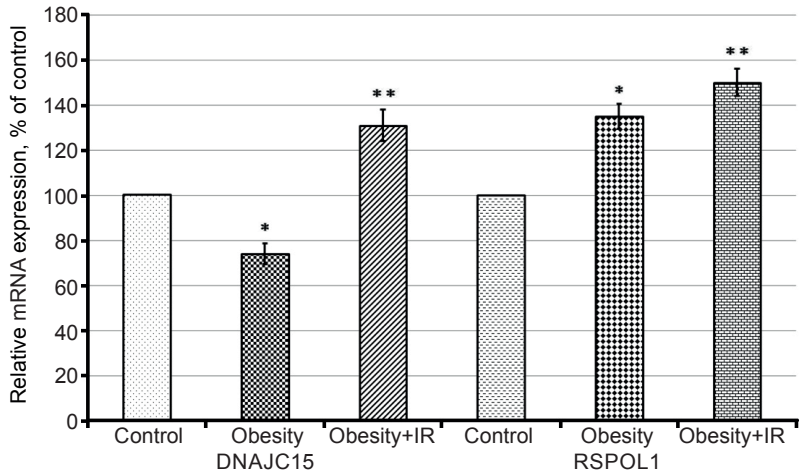

Fig. 4. Relative expression level of DNAJC15 (DnaJ (Hsp40) homolog, subfamily C, member 15) and RSPO1 (R-spondin homolog, Xenopus laevis) mRNA in blood cells of normal boys (Control) and obese individuals with normal insulin sensitivity (Obesity) and obese patients with insulin resistance (Obesity + IR). The values of DNAJC15 and RSPO1 mRNA expression were normalized to the beta-actin mRNA and are expressed as mean ± SEM and represented as a percent of control (100 %); $n = 5$; *P < 0.05 vs group of control individuals; **P < 0.05 vs group of obese boys with normal insulin sensitivity

been shown to exhibit distinct pattern of expression in blood cells in obesity with and without insulin resistance (Fig. 4). Thus, the expression level of *DNAJC15* gene is decreased in obese children with normal insulin sensitivity (-26 %; P < 0.05) and strongly increased in boys with obesity and insulin resistance (+77 %; P < 0.05).

There is data that obesity is associated with insulin resistance and hyperinsulinemia [29] and increased expression of RSPO1 is agreed with this data, because this protein regulates WNT signaling and controls beta-cell growth as well as insulin secretagogue [22, 23].

Thus, results of this study provide evidence that obesity affects the expression of the subset of genes related to cell growth, apoptosis, and glucose metabolism in blood cells and that impaired insulin sensitivity in obesity is associated with changes in the expression level of *IRS1, IRS2, RIPK2, RSPO1,* and *DNAJC15* genes, which possibly contribute to the development of insulin resistance and glucose intolerance as well as reflect some changes in other tissues, including fat tissue.

CONCLUSIONS

1. Obesity (with normal insulin sensitivity) enhances the expression of *IRS1, RIPK2, IL13RA2, RSPO1, IQSEC,* and *CCN2* genes, which control glucose metabolism and cell growth, in the blood cells versus control.

2. The expression of *IRS2, RSPO1,* and *DNAJC15* genes is up-regulated in blood cells of obese boys with insulin resistance versus obese patients with normal insulin sensitivity; however, the expression of *IRS1* and *RIPK2* genes is down-regulated.

3. This study has demonstrated that obesity affects the expression of the subset of genes related to the control of glucose metabolism and cellular growth and that insulin resistance in obesity is associated with changes in the expression level of *IRS1, IRS2, RIPK2, RSPO1,* and *DNAJC15* genes in blood cells contributing to the development of the obesity and its complications.

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ЕКСПРЕСІЯ ГЕНІВ *CCN2, IQSEC, RSPO1, DNAJC15, RIPK2, IL13RA2, IRS1* **ТА** *IRS2* **У КРОВІ ДІТЕЙ ЧОЛОВІЧОЇ СТАТІ ЗА УМОВ ОЖИРІННЯ ТА РЕЗИСТЕНТНОСТІ ДО ІНСУЛІНУ**

Розвиток ожиріння та його метаболічних ускладнень зумовлений дисрегуляцією ключових механізмів, що контро-

люють основні метаболічні процеси за допомогою змін в експресії численних регуляторних генів. Ми дослідили експресію групи генів, які відповідальні за контроль росту клітин та метаболізму глюкози, у дітей чоловічої статі з ожирінням, що мали нормальну або порушену чутливість до інсуліну, а також у нормальних (контроль) індивідуумів. Показано, що ожиріння без порушення чутливості до інсуліну призводить до посилення експресії генів *IRS1, RIPK2, IL13RA2, RSPO1, IQSEC та CCN2,* але знижує рівень експресії генів *IRS2* та *DNAJC15* у клітинах крові при порівнянні з контрольною групою дітей. Порушення чутливості до інсуліну у дітей з ожирінням призводить до посилення експресії генів *IRS2, RSPO1 і DNAJC15* та до зниження експресії генів *IRS1* і *RIPK2* у клітинах крові у порівнянні з пацієнтами, що мали ожиріння і нормальну чутливість до інсуліну. Результати цієї роботи свідчать про те, що ожиріння змінює у клітинах крові експресію групи генів, які контролюють ріст клітин та метаболізм глюкози, і що резистентність до інсуліну за умов ожиріння асоціюється зі змінами в рівні експресії генів *IRS1, IRS2, RIPK2, RSPO1* та *DNAJC15*, які роблять певний внесок у розвиток цієї резистентності та порушення толерантності до глюкози і можливо віддзеркалюють певні зміни в інших тканинах.

Ключові слова: експресія мРНК; *CCN2; IQSEC; RSPO1; DNAJC15; RIPK2; IL13RA2; IRS1; IRS2*; кров; ожиріння; резистентність до інсуліну.

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ЭКСПРЕССИЯ ГЕНОВ *CCN2, IQSEC, RSPO1, DNAJC15, RIPK2, IL13RA2, IRS1* И *IRS2* В КРОВИ ДЕТЕЙ МУЖСКОЙ СТАТИ ПРИ ОЖИРЕНИИ И РЕЗИСТЕНТНОСТИ К ИНСУЛИНУ

Развитие ожирения и его метаболических осложнений обусловлено дисрегуляцией ключевых механизмов, что контролируют основные метаболические процессы путем изменений в экспрессии многочисленных регуляторных генов. Ми изучили экспрессию группы генов, которые ответственны за контроль роста клеток и метаболизм глюкозы, у детей мужского пола с ожирением, что имели нормальную или нарушенную чувствительность к инсулину, а также у нормальных (контроль) индивидуумов. Показано, что ожирение без нарушения чувствительности к инсулину приводит к усилению экспрессию генов *IRS1, RIPK2, IL13RA2, RSPO1, IQSEC и CCN2*, но снижает уровень экспрессии генов *IRS2* и *DNAJC15* в клетках крови при сравнении с контрольной группой детей. Резистентность к инсулину у детей с ожирением приводит к усилению экспрессии генов *IRS2, RSPO1* и *DNAJC15* и к снижению экспрессии генов *IRS1* и *RIPK2* в клетках крови в сравнении с пациентами, что имели ожирение и нормальную чувствительность к инсулину. Результаты этой работы свидетельствуют о том, что ожирение изменяет в клетках крови экспрессию группы генов, контролирующих рост клеток и метаболизм глюкозы. Более того, резистентность к инсулину при ожирении ассоциируется с изменениями в уровне экспрессии генов *IRS1, IRS2, RIPK2, RSPO1* и *DNAJC15*, которые вносят определенный вклад в развитие этой резистентности и нарушение толерантности к глюкозе и возможно отражают определенные изменения в других тканях.

Ключевые слова: экспрессия мРНК; *CCN2; IQSEC; RSPO1; DNAJC15; RIPK2; IL13RA2; IRS1*; *IRS2*; кровь; ожирение; резистентность к инсулину.

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