GENETICS, PROTEOMICS AND METABOLOMICS

SEQUENCING OF A FRAGMENT OF THE LEPTIN GENE IN ADOLESCENTS WITH DIFFERENT WEIGHT STATUS

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ABSTRACT

Background. *Obesity is a significant social problem among the population of the world. The leptin gene (LEP) is currently considered as a potential candidate gene influencing metabolic disorders associated with predisposition to overweight and obesity. Leptin plays an important role in body weight homeostasis by influencing food intake and energy expenditure and maintaining constant energy stores. A defect in the leptin gene may be one of the causes of obesity and, as a result, of various obesity-associated pathologies.*

The aim of the study. *To search for single-nucleotide polymorphisms (SNP) of the leptin gene in adolescents with different weight status.*

Methods. *The study involved 20 adolescents aged 11–17 years with normal body weight and overweight/obesity. Research methods: assessment of clinical status with anthropometry; Sanger sequencing of the leptin gene fragment localized in the intron of this gene – (5'-AGCCTTGTTTTCATCATCTGGA, 3'-TGGGAGGAATCGCTCTCAGA). We also carried out bioinformatic processing of sequencing results.*

Results. *As a result of the study, the optimal conditions for amplification of the 891 bps leptin gene region were selected for the above mentioned primer pair of the LEP gene (s16_L891, s16_R891). Based on the results of sequencing, 45 single nucleotide substitutions of the LEP gene were identified, including 23 single nucleotide substitutions which were not previously registered in GenBank. In the group of adolescents with overweight and obesity, 14 unregistered single nucleotide substitutions of the LEP gene and 13 registered SNPs were identified in the GenBank database. In the group of adolescents with normal body weight, these SNPs were not found.*

Key words: *leptin gene, sequencing, overweight, obesity, leptin*

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СЕКВЕНИРОВАНИЕ ФРАГМЕНТА ГЕНА ЛЕПТИНА У ПОДРОСТКОВ С РАЗНЫМ СТАТУСОМ ВЕСА

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РЕЗЮМЕ

Ожирение – значимая социальная проблема среди населения всего мира. В настоящее время ген лептина (LEP) рассматривается как потенциальный ген-кандидат, влияющий на метаболические нарушения, ассоциированные с предрасположенностью к избыточной массе тела и ожирению. Лептин играет важную роль в гомеостазе массы тела, влияя на потребление пищи и расход энергии и поддерживая постоянные запасы энергии. Дефект гена лептина может быть одной из причин ожирения и, как следствие, различных патологий, связанных с ожирением.

***Цель исследования**. Поиск однонуклеотидных полиморфизмов (SNP, single-nucleotide polymorphism) гена лептина у подростков с разным статусом веса.*

***Методы.** В исследовании приняли участие 20 подростков 11–17 лет с нормальной массой тела и избыточной массой тела/ожирением. Методы исследования: оценка клинического статуса с антропометрией; секвенирование по методу Сенгера фрагмента гена лептина, локализованного в интроне данного гена – (5'-AGCCTTGTTTTCATCATCTGGA, 3'-TGGGAGGAATCGCTCTCAGA). Также проведена биоинформационная обработка результатов секвенирования.*

***Результаты.** В результате исследования проведён подбор оптимальных условий амплификации участка гена лептина размером 891 п. н. для вышеуказанной пары праймеров гена LEP (s16_L891, s16_R891). По результатам секвенирования идентифицировано 45 однонуклеотидных замен гена LEP, в том числе 23 однонуклеотидные замены ранее не зарегистрированные в GenBank. В группе подростков с избыточной массой тела и ожирением идентифицировано 14 незарегистрированных однонуклеотидных замен гена LEP и 13 зарегистрированных SNP в базе данных GenBank. В группе подростков с нормальной массой тела данные SNP не обнаружены.*

***Ключевые слова:** ген лептина, секвенирование, избыточная масса тела, ожирение, лептин*

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INTRODUCTION

Childhood obesity is a major public health problem. Currently, there is a significant increase in the number of overweight and obese children and adolescents, with a majority of them being obese in adulthood as well [1]. The risk of obesity and its consequences in adulthood is higher if the problem begins in childhood. Obesity is the cause of type 2 diabetes mellitus, non-alcoholic fatty liver disease, cardiovascular and other diseases [2–9].

Body weight regulation is a complex process of neurohumoral regulation involving various neurotransmitters, including leptin. Leptin is a polypeptide hormone secreted by adipose tissue and provides regulation of energy, neuroendocrine and metabolic processes in the body. Leptin has an impact on the processes of energy intake and consumption, fat and carbohydrate metabolism, and the production of hypothalamic and pituitary hormones [10].

The leptin gene (*LEP*) is located on chromosome 7 and consists of three exons separated by two introns [11, 12]. According to the National Center for Biotechnology Information (NCBI), 4,239 single nucleotide substitutions have been described for this gene [13]. Today, 2 polymorphisms (*rs2167270, rs7799039*) are of the greatest interest, the contribution of which to the formation of metabolic disorders in obese patients has been proven [14].

The search for other, including new, polymorphisms of the leptin gene, their contribution to the formation of metabolic disorders in patients of different races, populations, and different age groups is still relevant.

THE AIM OF THE STUDY

To search for single-nucleotide polymorphisms (SNP) of the leptin gene in adolescents with different weight status.

METHODS

The study included 20 adolescents aged 11 to 17 years (14.8 ± 0.45 years), including 14 girls and 6 boys, with different weight status: 12 overweight and obese adolescents (standard deviation score (SDS) of body mass index (BMI) > 1), 8 adolescents with normal body weight (SDS BMI < 1). All teenagers are representatives of European ethnicity (at the example of Russians). The characteristics of the groups are presented in Table 1.

Thus, the groups are comparable in gender and age. The mean values of anthropometric indices are statistically significantly different between groups with different weight status – normal and overweight.

In working with adolescents, we observed the ethical principles required by the World Medical Association Declaration of Helsinki (World Medical Association Declaration of Helsinki, 1964, as revised in 2013; amended at the 64th General Assembly of the World Medical Association, Brazil) and paragraph 5 of Article 24, "Rights of Minors" Fundamentals of Legislation of the Russian Federation about the Protection of Citizens' Health of 22.07.1993 No. 5487-1 (as amended on 20.12.1999). All participants have informed parental consent (if the age of the subject is less than 14 years old) or informed consent of the surveyed sample (if the age of the subject is 14 years or older).

At study inclusion, adolescents had their linear height and body mass (BM) measured, then body mass index (BMI; kg/m^2) was calculated. The trial subjects were weighed with an accuracy of 0.1 kg in standard light clothing and without shoes on a platform hand scale. Height was measured with a stationary height meter with an accuracy of 0.1 cm. BMI was calculated as a person's weight in kg divided by his height in m^2. The height and weight parameters of adolescents were evaluated using WHO reference values using the AnthroPlus calculator [15]. Standard deviation values from population mean values were determined for BMI – SDS was calculated using the Auxology 1.0 b17 computer software application (Pfizer, USA). BW was con-

TABLE 1

CHARACTERISTICS OF STUDIED GROUPS OF ADOLESCENTS

Indicators	Control group (n = 8), Me [Q_1; Q_3]	Overweight and obese group (n = 12), Me [Q_1; Q_3]	p
Age (years)	16.5 [14.75; 17]	14.5 [13.5; 16]	0.97
Height (cm)	167.2 [161.825; 183.1]	158.5 [155.25; 163.125]	0.031*
Weight (kg)	63 [57.95; 66.25]	74.8 [72; 82.975]	0.001*
BMI (kg/m^2)	20.4 [19.625; 21.575]	30.05 [27.80; 33.15]	0.000016*
SDS BMI	0.04 [–0.49; 0.9225]	2.845 [2.335; 3.065]	0.000016*

Note. * – the noted criteria are statistically significant at the level of $p < 0.05$, Mann – Whitney U test was used in the comparison.

sidered excessive at a BMI in the range of 25.0–29.9 kg/m^2, obesity – at a BMI = 30 kg/m^2 [16].

Blood samples were collected into 5 ml vacuum tubes containing K_3-ethylenediaminetetetraacetic acid (K_3EDTA) in order to analyse DNA. DNA isolation was carried out by the sorbent method using the DNA sorb-B kit (Federal Budget Institution of Science Central Research Institute of Epidemiology of Rospotrebnadzor, Russia).

The primers were selected using the Primer-BLAST software [17] (Fig. 1). Oligonucleotides were synthesized in Eurogen CJSC.

One of the primer pairs was matched to an 891 bps fragment of the leptin gene localised in the intronic part of this gene:

1) s16_L891 – 5'-AGCCTTGTTTTCATCATCTGGA (absolute number of nucleotides – 22, molecular weight 6670 (Mw)g/mol);

2) s16_R891 – 3'-TGGGAGGAATCGCTCTCAGA 9 (absolute number of nucleotides – 20, molecular weight 6163 (Mw)g/mol).

Optimal amplification conditions were selected for this pair.

Stock solutions with a concentration of 100 µmol/ml were prepared from lyophilised oligonucleotide samples by dilution with deionised water and further diluted to a final concentration of 20 µmol/ml.

Two approaches were used to select the annealing temperature of the primers. The first one is calculated using the formula: T = 2(AT) + 4(GC) (instructions for the ScreenMix reagent), where T is the annealing temperature of the primers; AT is the number of nucleotides adenine and thymine; GC is the number of nucleotides guanine and cytosine included in the primer. The second – by using the online calculator Thermo Fisher (Thermo Fisher Scientific, USA) [19].

Experimental polymerase chain reaction (PCR) assays were performed in a volume of 10 µl of reaction mixture. The PCR components were mixed in the sequence and volumes shown in Table 2, according to the protocol for the ScreenMix reagent (5X ScreenMix, Eurogen CJSC, Russia).

TABLE 2

SEQUENCE AND VOLUMES OF PCR MIX COMPONENTS APPLIED FOR 1 DNA SAMPLE AND TOTAL PCR MIX VOLUME OF 10 ML

Components	Quantity (µL)
Sterile water	5.2
Screen Mix	2.0
PCR primer 1 (L – left)	0.4
PCR primer 2 (R – right)	0.4
DNA matrix	2.0
Total PCR mixture volume	10.0

PCR was performed using the amplifier DT prime (NPO DNA-Technology LLC, Russia) according to the ScreenMix manufacturer's software program presented in Table 3.

The amplicon fragment sizes for each sample were estimated by electrophoresis in a 1.5 % agarose gel in 1xTAE buffer admixed with ethidium bromide, at an electrical voltage of U = 146 V, over a period of 2 hours.

BigDye™ Terminator v. 3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, USA) reagents were used for sequencing according to the manufacturer's instructions. The reaction products were purified by precipitation with 75 % isopropyl alcohol. The nucleotide sequences were determined using the NANOPHOR 05 automatic sequencer (Syntol LLC, Russia). This study has been performed using the equipment of the Center for the Development of progressive personalized Health Technologies of the Scientific Centre for Family Health and Human Reproduction Problems (Irkutsk).

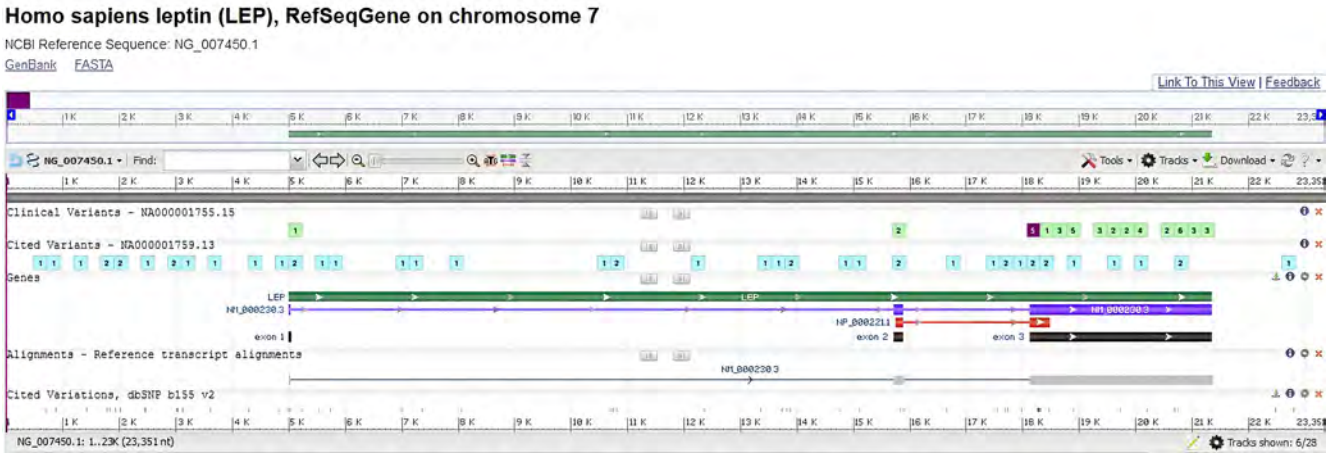

FIG. 1.

Reference Sequence LEP screenshot [18]

TABLE 3
AMPLIFICATION PROGRAM

	Stage	Temperature, °C	Incubation time	Number of cycles
1	Pre-denaturation:	95°	5 min	1
	Denaturation	95°	30 s	
2	Annealing of primers	52–70°	30 s	40
	Elongation	72°	30 s	
3	Storage	4°	–	–

The sequencing results have been bioinformatically processed. The quality of chromatograms was assessed using the UGENE software (Unipro, Russia). The chromatograms were processed using the R v. 4.2.3 software. The obtained nucleotide sequences were aligned to the reference sequence of the *LEP* gene – NG_007450.1 RefSeqGene – and compared with each other in the MEGA 11 software (Pennsylvania State University, USA).

Ethical review

The conduct of this study was approved by the Ethics Committee of the Scientific Centre for Family Health and Human Reproduction Problems (Protocol No. 9 dated October 8, 2014).

RESULTS

At the first stage, the selection of PCR temperature conditions for the analyzed pair of primers (oligonucleotides s16_L891, s16_R891) was carried out. According to the above formula for selecting the annealing temperature of primers, the temperature for both left (s16_L891) and right (s16_R891) primers was 62 °C. A similar calculation using the online calculator Thermo Fisher Scientific (USA) showed an annealing temperature of 63.2 °C. It is known that the annealing temperature gradient should start at a temperature 6–10 °C below the calculated annealing temperature; therefore, three variants of primer annealing temperature have been determined at the first stage of the experiment: 56 °C, 58 °C, and 60 °C.

PCR was performed with 5 DNA samples. The reagents were introduced in the sequence and volumes shown in Table 2. The PCR was performed using the software program shown in Table 3. An electrophoregram with the results of the experiment is shown in Figure 2.

Amplification was satisfactory in all three experimental PCR assays. PCR products with a size of 891 bps are observed in the gel in tracks with DNA amplicons. The annealing of primers at a temperature of 60 °C was the most optimal, because in this mode of amplification, the least amount of non-specific amplification products is observed in the gel.

As a result, the next step in selecting the amplification conditions for the primer pair under study was an experiment in which the number of primers was reduced by 10 % of that indicated in Table 2, the primer annealing temperature was gradiently increased by 2 °C (62 °C annealing) and 4 °C (64 °C annealing) (Fig. 3).

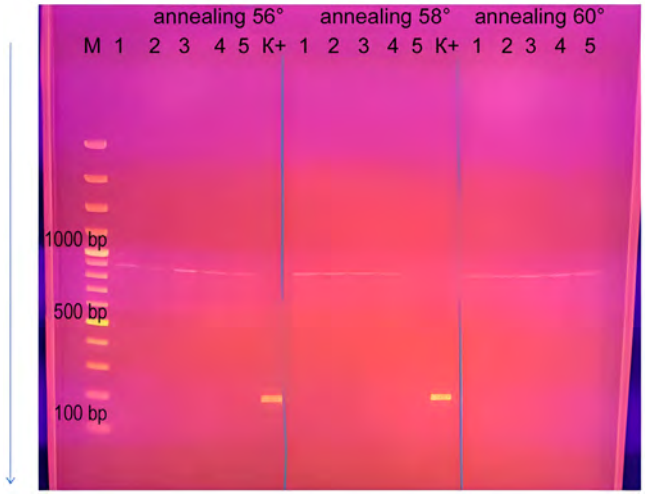

FIG. 2.
Electrophogram: the LEP gene primers – s16_L891, s16_R891; three variants of the temperature shelf for the annealing stage – 56 °C, 58 °C and 60 °C

Analysis of the separation of PCR products shown in Figure 3 showed that a 10 % decrease in the number of primers with primer annealing at all temperature conditions led to the expected result – specific PCR products with a size of 891 bps are present in the in tracks with DNA

amplicons and there are no products of non–specific amplification.

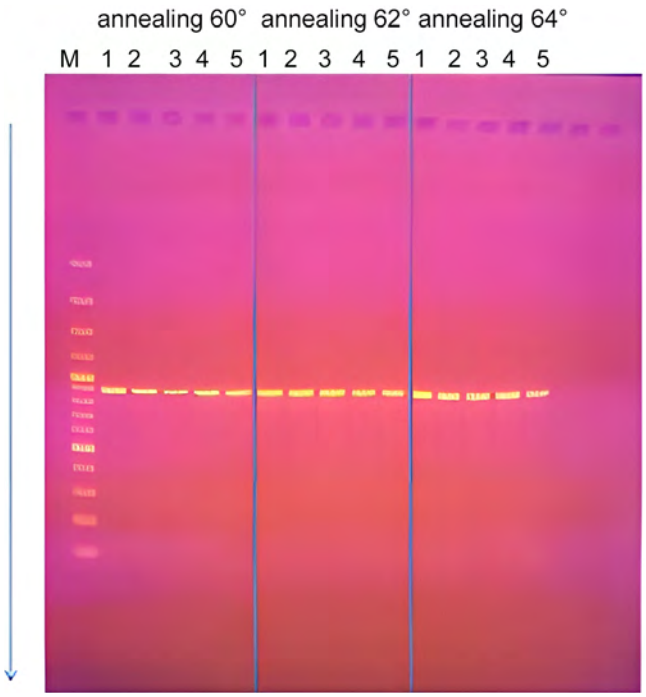

FIG. 3.

Electrophogram: LEP gene primers – s16_L891, s16_R891; the number of primers reduced by 10 %; three variants of the temperature shelf for the annealing stage – 60 °C, 62 °C and 64 °C

Thus, optimal conditions for PCR have been selected for the analyzed fragment of the leptin gene.

The next step of work with the analysed primer pair is amplification of 20 DNA samples using the selected conditions in the volume of reaction mixture equal to 50 µl. Reagents for PCR mixtures were introduced in sequence and volumes, according to Table 4. DNA samples were added at the rate of 10 µl each. The result of PCR is shown in Figure 4.

TABLE 4

SEQUENCE AND VOLUMES OF PCR MIXTURE COMPONENTS APPLIED FOR 20 DNA SAMPLES AND A TOTAL PCR MIXTURE VOLUME EQUAL TO 50 ML

Component	Quantity (µL)
Sterile water	528
Screen Mix	200
PCR primer 1 (L – left)	36
PCR primer 2 (R – right)	36

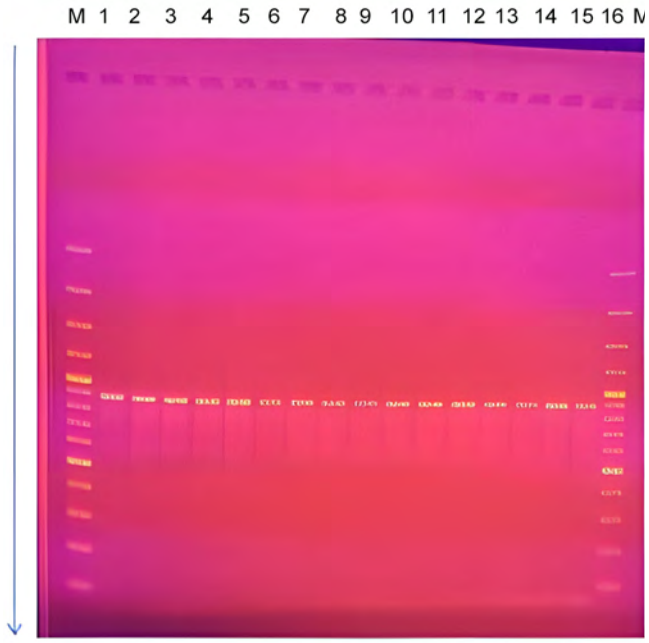

FIG. 4.

Fragment of the electrophogram: LEP gene primers – s16_L891, s16_R891; number of primers reduced by 10 %; primer annealing at 60 °C; DNA samples from 1 to 16

The obtained amplicons were used for sequencing the *LEP* gene using the Sanger method, according to the protocol of the reagent kit manufacturer.

At the next stage, chromatograms were analyzed to search for the SNP leptin *LEP* gene in Caucasian adolescents with different weight status.

We identified 45 SNPs, including 22 SNPs previously reported and 23 SNPs not previously reported in GenBank (01.02.2022) (Table 5), based on the results of aligning the obtained data to the reference sequence of the leptin gene from the NCBI database (NG_007450.1).

Therefore, 14 SNPs of the leptin gene not registered in the GenBank database were found in the group of overweight and obese adolescents.

Moreover, according to the results of sequencing primers s16_L891 and s16_R891 of the *LEP* gene, we identified 12 new nucleotide substitutions in 22 previously registered SNPs (Table 6).

An application has been submitted for registration in GenBank of 23 leptin gene polymorphisms newly diagnosed in the world.

SUMMARY

Sequencing of the *LEP* gene fragment for a pair of primers (s16_L891, s16_R891) revealed both new and previously described single nucleotide substitutions. A total of 45 single nucleotide substitutions were identified in the study groups. SNPs previously registered in GenBank

TABLE 5

LIST OF SNPS OF THE LEPTIN GENE NEWLY DIAGNOSED (NOT REGISTERED IN GENBANK) IN ADOLESCENTS WITH DIFFERENT WEIGHT STATUSES

Seq No.	Position in the gene	Identified nucleotide substitution	The number of adolescents with SDS BMI < 1 (abs.)	Number of adolescents with SDS BMI > 1 (abs.)
1	Ch 7:128254003	G>C,T	2	1
2	Ch 7:128254031	G>T	3	0
3	Ch 7:128254086	A>T,G	3	0
4	Ch 7:128254131	A>T	0	3
5	Ch 7:128254135	G>T	1	3
6	Ch 7:128254139	C>T	0	3
7	Ch 7:128254145	A>T	0	2
8	Ch 7:128254146	G>T,C	2	2
9	Ch 7:128253548	C>T	0	1
10	Ch 7:128253560	A>T	0	1
11	Ch 7:128253561	A>T	0	1
12	Ch 7:128253565	T>C	0	1
13	Ch 7:128253877	G>A	0	1
14	Ch 7:128254051	G>A	1	0
15	Ch 7:128254064	G>C	1	0
16	Ch 7:128254070	C>T	0	1
17	Ch 7:128254083	G>C	1	0
18	Ch 7:128254084	G>A	0	1
19	Ch 7:128254089	G>T	0	1
20	Ch 7:128254094	C>G	1	0
21	Ch 7:128254121	C>T	0	1
22	Ch 7:128254133	C>T	0	1
23	Ch 7:128254138	G>T	0	1

TABLE 6

NUCLEOTIDE SUBSTITUTIONS IN IDENTIFIED LEP GENE POLYMORPHISMS IN ADOLESCENTS WITH DIFFERENT WEIGHT STATUS

Seq No.	Single nucleotide polymorphism	GenBank-registered nucleotide substitution	Identified nucleotide substitution
1	rs1795301406	G>A	G>C
2	rs1795303962	G>A	G>T
3	rs1795304377	A>C	A>T
4	rs1795306221	G>A	G> T
5	rs1795306653	G>T	G> T
6	rs1033530971	C>T	C>A
7	rs1795307023	A>G	A>C
8	rs1010492815	A>G	A>T
9	rs188857788	C>A	C>G
10	rs1795307445	G>A	G>C,T
11	rs1795307524	A>G	A>T
12	rs1430150874	G>A	G>T

were identified in obese adolescents: rs917105894 G>T, rs1795301406 G>A, rs1795303962 G>A, rs1795304377 A>C, rs1795304438 G>T, rs1318987243 G>T, rs3793162 G>A,C,T, rs1795306653 G>T, rs1795307023 A>G, rs1010492815 A>G, rs1795307445 G>A, rs1795307524 A>G, rs1430150874 G>A. SNPs not previously registered with GenBank were identified: Ch 7:128254131, Ch 7:128254139, Ch 7:128254145, Ch 7:128253548, Ch 7:128253560, Ch 7:128253561, Ch 7:128253565, Ch 7:128253877, Ch 7:128254070, Ch 7:128254084, Ch 7:128254089, Ch 7:128254121, Ch 7:128254133, Ch 7:128254138. This study should be continued to evaluate the contribution of these SNPs to metabolic disorders in overweight and obese adolescents by expanding the study cohorts of adolescents and further analysing clinical and metabolic parameters in these study groups.

CONCLUSIONS

Optimal conditions for PCR for a pair of primers (5-AGCCTTGTTCATCATCTGGA, 3-TGGGAGGAATCGCTCTCAGA) of the leptin gene: primer concentration – 0.18 μM; primer annealing at a temperature of 60 °C. Based on the results of *LEP* gene sequencing, 12 new nucleotide substitutions were identified in the SNPs previously registered in GenBank. In the analysed intron fragment of the leptin gene (5-AGCCTTGTTCATCATCATCTGGA, 3-TGGGAGGAATCGCTCTCTCTCAGA), 23 SNPs were identified for the first time.

Conflict of interest

The authors declare no conflict of interest.

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