T.C.
YEDİTEPE UNIVERSITY
INSTITUTE OF HEALTH SCIENCES
DEPARTMENT OF MOLECULAR MEDICINE

DETERMINATION OF RELATIONSHIP OF SOCS3GENE AND MICRO RNA 218 IN CHILDHOOD OBESITY

DOCTORAL THESIS

EMRE MURAT ALTINKILIÇ, MSc Biologist

SUPERVISOR
Prof. Dr. Rukset ATTAR

İSTANBUL-2019

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APPROVAL

This thesis has been deemed by the jury in accordance with the relevant articles of Yeditepe University Graduate Education and Examinations Regulation and has been approved by Administrative Board of Institute with decision dated 22.11.2019 and numbered 2019/18-02

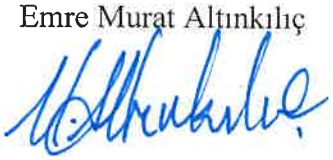
Prof. Dr. Bayram YILMAZ

Director of Institute of Health Sciences

DECLARATION

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which has been accepted for the award of any other degree except where due acknowledgment has been made in the text.

Emre Murat Altınkılıç



DEDICATION

This work is dedicaded to all the obese children around the world.

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LIST OF SYMBOLS AND ABBREVIATIONS

LR: Leptin Resistance

SOCS3: Suppressor of Cytokine Signaling 3

miRNA: Micro RNA

RISC: RNA Induced Silencing Complex

mRNA: Mesenger RNA

UTR: Untranslated Region

SNP: Single Nucleotide Polymorphism

BMI: Body Mass Index

JAK: Janus Kinase

STAT: Signal Transducer and Activator of Transcription

MAPK: Mitogen- Activated Protein Kinase

OD: Optical Density

PCR: Polymerase Chain Reaction

cDNA: Complementary DNA

qPCR: Quantitative PCR

Ct: Cycle Threshold

ΔCt: Ct difference between targeted miRNA and control miRNA

ELISA: Enzyme Linked Immunosorbent Assay

ROC: Receiver Operating Characteristic

ABSTRACT

Altınkılıç, EM. Determination of Relationship of SOCS3 Gene and Micro RNA 218 in Childhood Obesity. Yeditepe University, Institute of Health Science, Department of Molecular Medicine. Doctorate Thesis. İstanbul, 2019.

Obesity is a medical condition which increases the risk of cardiovascular diseases, diabetes, and cancer. It usually results from a combination of genetic, behavioral, metabolic and hormonal factors. Leptin Resistance is one of the important causes of obesity. Some of the mechanisms which underlines its development are mutations in leptin and leptin receptor genes or genetic mutations of proteins in post-receptor pathway and proteins taking part in feedback mechanism on its own synthesis. Also permeability of blood–brain barrier effects leptin function. However its underlying mechanism still needs to be investigated. In this study, we investigated the role of Suppressor of Cytokine Signaling 3 (SOCS3) and circulating miRNAs in leptin resistance in childhood obesity by miRNA target prediction methods. Our preliminary study about miRNA target prediction has shown that mir-218-5p may be involved in the development of leptin resistance by affecting Leptin, Leptin Receptor and SOCS3 genes. Furthermore we investigated serum miR-218-5p expression in two study groups, leptin resistant obese children (Patient Group: n=35) and non-obese children (Control Group: n=30). Our results have shown that serum miR-218-5p expression are significantly increased in leptin resistant obese children in accordance with the serum leptin levels ($p<0.05$). There were no significant difference between obese and non-obese children according to serum SOCS3 levels and SOCS3 genotypes. We state that miR-218-5p can be used as a possible diagnostic parameter for leptin resistance in childhood obesity.

Key Words: Childhood Obesity, Leptin, Leptin Resistance, Serum miRNA, SOCS3.

ABSTRACT (Turkish)

Altınkılıç, EM. Çocuk Obezitesi Olgularında SOCS3 Geni ve Mikro RNA 218'in İlişkisinin Belirlenmesi. Yeditepe Üniversitesi, Sağlık Bilimleri Enstitüsü, Moleküler Tıp Anabilim Dalı. Doktora Tezi. İstanbul, 2019.

Obezite, kardiyovasküler hastalıklar, diabet, yüksek tansiyon ve bazı kanser türleri için görülme riskini arttıran tıbbi bir durumdur. Obezite genellikle genetik, davranışsal, metabolik ve hormonal etmenlerin sonucu olarak şekillenir. Leptin Direnci önemli obezite sebeplerinden biridir. Leptin Direnci gelişiminde rol oynayan faktörler arasında leptin ve leptin reseptörünü kodlayan genlerde ki yada reseptör sonrası yolak proteinlerinde meydana gelen genetik mutasyonlar, leptin sentezi ve sentezin kendini düzenlemesinde görev alan proteinler ve kan-beyin bariyeri geçirgenliği sayılabilir. Bununla beraber altında yatan nedenlerin daha fazla araştırmaya ihtiyacı vardır. Çalışmamızda Sitokin sinyal baskılayıcı 3 (SOCS3) geni ve dolaşımda bulunan miRNA'ların, çocukluk çağı obezitesinde leptin direnci gelişimi üzerindeki rolü miRNA hedef tahmini yöntemleri kullanılarak araştırılmıştır. miRNA hedef tahmini üzerine yaptığımız ön çalışmamız, miR-218-5p'nin Leptin, Leptin Reseptörü ve SOCS3 genleri üzerine etki ederek leptin direnci gelişiminde rol oynayabileceğini göstermiştir. Çalışmamızda leptin direnci görülen obez çocuklar (n=35) ve obez olmayan sağlıklı çocuklar (n=30) olmak üzere iki grup üzerinde, serum miR-218-5p ekspresyonunun leptin direnci gelişimine etkisi incelenmiştir. Elde edilen sonuçlarda miR-218-5p seviyelerinin leptin dirençli obez çocuklarda, sağlıklı obez olmayan çocuklara göre anlamlı şekilde arttığı ve leptin seviyelerinin miR-218-5p ile ilişkili olarak artış gösterdiği ($p<0.05$) görülmüştür. Obez ve obez olmayan çocuklar arasında serum SOCS3 seviyeleri ve SOCS3 genotipleri arasında istatistiksel olarak anlamlı bir farklılık bulunamamıştır. Bulgularımız doğrultusunda, miR-218-5p'nin çocukluk çağı obezitesinde leptin direncinin olası bir tanısal parametresi olarak değerlendirilebileceği görülmüştür.

Anahtar Kelimeler: Çocuk Obezitesi, Leptin, Leptin Direnci, Serum miRNA, SOCS3.

1. INTRODUCTION AND PURPOSE

Leptin is a polypeptide hormone which plays role in regulation of food intake and energy metabolism. Main synthesis and secretion of leptin occurs in adipose tissue and it shows endocrine function (1, 2). Leptin pathway is one of the major pathways which takes part on obesity development. A slight increase in leptin secretion, causes a decrease in body weight by reducing the appatite.

Leptin Resistance (LR) is an endocrine pathology which is seen as elevated serum leptin levels and obesity together. In the case of LR, poor or decreased response causes obesity development and because of obesity development, serum leptin levels increase (3, 4). There is no exact cause of LR. Mouse studies have shown that Suppressor of Cytokine Signaling 3 (SOCS3) protein expression is increased in the hypothalamus, which is the target tissue of leptin (5, 6).

Micro RNAs (miRNA) are approximately 22 nucleotide long single stranded small non-coding RNA molecules which plays role in post-transcriptional gene regulation. miRNAs acts with RNA Induced Silencing Complex (RISC), and binds to the 3' untranslated region (3'UTR) of messenger RNA (mRNA) resulting in translational arrest and/or mRNA degradation (7). Studies on miRNA expression in different tissues have shown that miRNAs also exists in body fluids. Also it is shown that transport of miRNAs in circulation occurs by serum proteins, lipoproteins, exosomes or by free (8).

Besides differing expression profiles of miRNAs by the developing pathologies in different tissues and organs, changes in serum or exosomal miRNA profiles creates the idea of using miRNAs as diagnostic and/or prognostic biomarkers (8, 9, 10). Also single nucleotide polymorphisms (SNP) in 3'UTRs affect miRNA functions and have relation with miRNA dysregulation in different pathologies are known (11).

In this study, we examined serum levels of miRNAs and related SNPs, hypothysing that they may have effect on leptin resistance. Also we investigated the relation between serum leptin, leptin receptor and SOCS3 levels and serum miRNA levels and related SNPs in leptin resistant obese children and non-obese healty controls.

2. GENERAL INFORMATION

2.1. Obesity and Childhood Obesity

2.1.1. Obesity

Obesity is a condition of excessive fat storage in the body which develops due to genetic and environmental factors (12). Besides several pathologic syndromes (Polycystic ovary synd., Bardet-Biedl synd.), the most common cause of obesity is misnutrition (12, 13). Diagnosis of obesity depends on Body Mass Index (BMI) of the patient. Formula for the calculation of BMI is; BMI= weight/height^2, gives a result in kg/m^2 unit. BMI which is between 18.50–24.99 is considered to be normal, BMI between 25.00-29.99 is considered as pre-obese and BMI 30.00 and over is considered as obese.

2.1.2. Childhood Obesity

In addition to obesity in adults, childhood obesity is seen in every 1 in 5 between ages of 9 to 16. Childhood obesity is a condition which effects physical, psychological, social and mental development of children. Also it is a primary risk factor for Type 2 Diabetes and Cardiovascular Diseases (13, 14). Along with all, pathologies like bone and joint diseases, sleep apnea and asthma are seen more frequently in obese children (14, 15). For the diagnosis of Childhood Obesity, BMI percentile is used and children have 5-84.99 percentile are considered as normal, 85-94.99 percentile are considered as overweight and 95 and over percentile are considered as obese (16).

2.2. Structure and Function of Leptin and Its Role in Obesity

2.2.1. Structure and Function of Leptin

Leptin is a polypeptide hormone which is formed by folding of 167 amino acid long polypeptide chain as 4 α-heliks structures. By the cleavage of signal peptide, Leptin passes into the circulation as 146 amino acid lengt, 16Kda weight functioning active form. After its synthesis in white adipose tissue, leptin transports in the circulation to its target tissue hypothalamus. Main function of the Leptin is regulation of food intake and energy metabolism (17).

Exact target of the Leptin is the arcuate nucleus in hypothalamus. Leptin shows its function by binding to Leptin Receptors in arcuate nucleus and activating Janus

Kinase/Signal Transducer and Activator of Transcription (JAK/STAT) and Mitogen-Activated Protein Kinase (MAPK) pathways (Figure 1.). JAK/STAT pathway is a intracellular signalling pathway which shows activity by; activation of JAK by the binding of ligand to its receptor, phosphorylation of receptor by JAK, activation of STAT by phosphorylated receptor and activation of transcription by STAT. As the result of leptin signalling, there is a decrase in food intake, increase in energy expenditure and satiety feeling occurs (18).

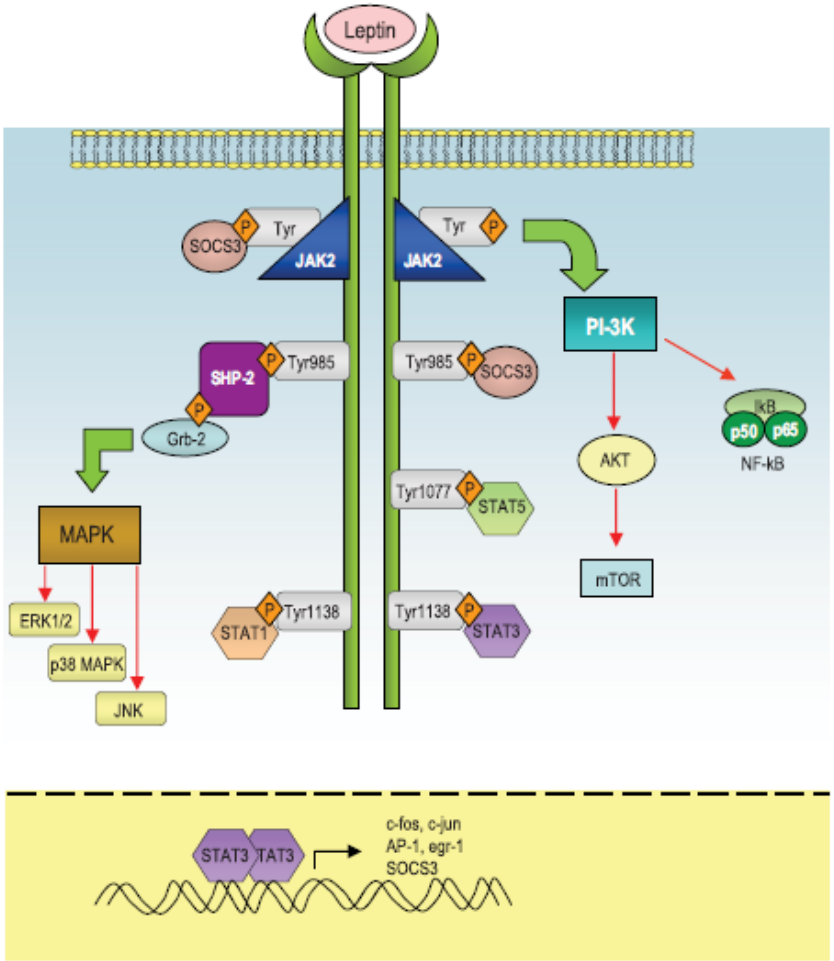


Figure 1. Intracellular Leptin Signalling Pathway (18).

2.2.2. Role of Leptin in Obesity

Leptin has a strong relation with obesity because of its regulatory effects on food intake and energy metabolism. Its first discovery and following animal studies shown that lack of Leptin coding gene (*ob/ob* mutant) results with obesity in mice (19). It is seen that changes in the serum Leptin levels are also have relation with obesity.

Decreased serum Leptin levels and/or reduced biological activity of Leptin are causing obesity, exogenous leptin supplementation like subcutanous injection of Leptin is inducing weight lost and improvement in the obesity releated biological parameters (20, 21). Along with those, elevated serum leptin levels in obese is defined as leptin resistance which is caused by inhibition or reduction of action of biologically active Leptin in receptor or postreceptor pathway level (22).

2.3. Relationship of Leptin Resistance, SOCS3 and miR-218-5p

2.3.1. Leptin Resistance

Without any known exact cause, there are two main hypotheses on development of Leptin Resistance; failure of circulating Leptin molecule to reach the receptor in arcuate nucleus or inhibition of postreceptor pathway (intracellular signal cascade) (23). Experiments with animal models have shown that SOCS3 protein has a important role in the development of Leptin Resistance. It is shown that, SOCS3 expression is increased in hypothalamus of leptin resistant obese mice, furthermore development of Leptin sensitivity and resistance against diet induced obesity in SOCS3 deficient mice (24, 25).

2.3.2. Suppressor of Cytokine Signaling 3 (SOCS3)

SOCS3 functions in the negative feedback mechanism of JAK/STAT pathway and inhibits the signalling cascade. After activation of STAT by the binding of signal molecule to the cell surface receptor, initiates transcription in the cell. With this initiation STAT also induces transcription of SOCS3 and creates negative feedback control on itself (Figure 2.) (26).

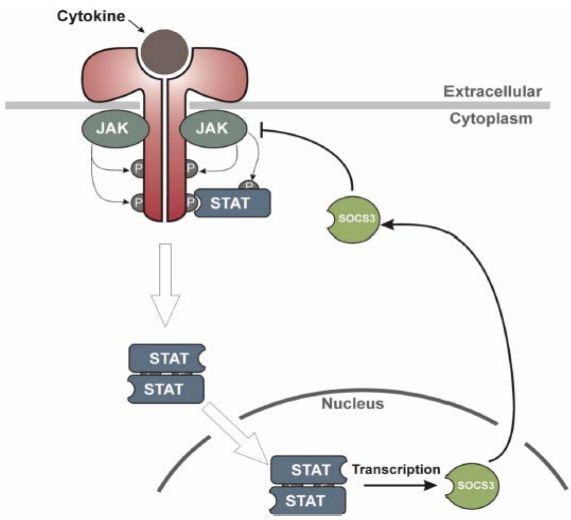


Figure 2. JAK/STAT Pathway and SOCS3 Negative Feedback Mechanism (26).

Inhibition of JAK/STAT pathway by SOCS3 occurs in three steps, first two steps of the inhibition are reversible but the third step is irreversible because of the degradation of receptor protein by SOCS3 induced ubiquitylation (Figure 3.) (26);

- Inactivation of signalling by the binding of SOCS3 to transmembrane receptor protein on phosphate residues,
- Inhibition of activity of JAK by binding of the receptor bound SOCS3 to JAK,
- Induction of ubiquitylation and degradation of JAK and receptor protein by SOCS3 (26).

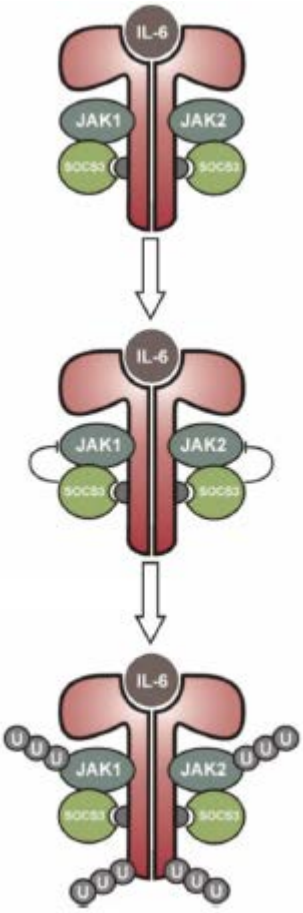


Figure 3. Three Steps Mechanism of Action of SOCS3 (26).

2.3.3. miR-218-5p

Role of miRNAs on different pathologies gives the idea of potential role of miRNAs on Leptin Resistance by effecting SOCS3 and differing expression levels of miRNAs which are targeting Leptin-SOCS3 axis might change the activity of Leptin and Leptin Pathway.

In this study, we investigated the relationship between miRNAs and Leptin, Leptin Receptor and SOCS3. For this purpose, we searched online databases which are scoping miRNAs and their target mRNAs. First, we investigated miRNA sets which are targeting our genes of interest and then tested their reliabilty (existence in serum, relationship with different pathologies and relationship with demographic charactheristics of patients) for this study by literature review and cross-checking on different databases. Results of most cited miRNA target prediction databases (http://www.targetscan.org - http://www.microrna.org/ - http://mirtarbase.mbc.nctu.edu.tw/php/index.php) cross-checked on databases which

are not for target prediction but bringing reliable data about miRNAs (http://www.mirbase.org - http://www.mirdb.org/ - http://mirtarbase.mbc.nctu.edu.tw/php/index.php) and overlapping miRNAs on all databases were included in this study (27).

We searched miRNAs targeting Leptin, Leptin Receptor and SOCS3 mRNAs and categorized and cross-checked with each other to determine the miRNAs which targets all three of the genes synergistycally. As a result of this search, we decided to investigate hsa-miR-218-5p which is a miRNA originating from 5' arm of miR-218 primary transcript. Its mature sequence is 5'-uugugcuugaucuaaccaugu-3' (Figure 4.) (28).

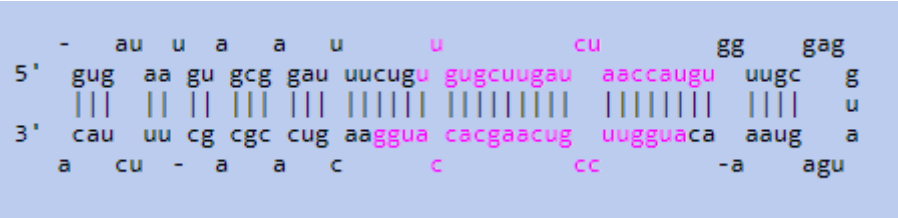


Figure 4. hsa-miR-218-5p primary transcript (28).

Furthermore, 3'UTR sequences of Leptin, Leptin Receptor and SOCS3 genes were examined on genome databases for determining potential binding regions for hsa-miR-218-5p. Also we searched the polymorphisms which may effect binding efficiency of hsa-miR-218-5p. There were no defined polymorphism for leptin and leptin receptor genes. However rs906271000 SNP was determined on the 3'UTR region of SOCS3 gene. rs906271000 is located on the seed sequence of miR-218-5p binding region which may effect the effect of miR-218-5p on SOCS3 (29, 30).

3. MATERIALS AND METHODS

3.1. Description of Patient and Control Groups

All sera and peripheral whole blood samples of patient and control groups were collected at Yeditepe University Hospital Department of Pediatric Endocrinology and inclusion criterias for patient and control groups are;

- Between 2-18 years of age,
- No diagnosed obesity before 2 years of age,
- No diagnosed monogenic obesity in parents,
- Diagnosed obesity for the Patient Group,
- No diagnosed obesity for the Control Group.

65 patients with similar demographic characteristics were included in the study. Ethical Committee Approval was taken from Ethical Committee of Yeditepe University. All the volunteers were physically examined in Yeditepe University Hospital Pediatric Endocrinology Department and divided into Patient and Control Groups after evaluation of their BMI and BMI percentiles.

3.2. Sample Collection

After the physichal examination, 4ml of peripheral blood was collected in EDTA containing tubes for DNA isolation and 5ml of peripheral blood was collected in gel containing tubes for serum seperation for ELISA analysis and miRNA isolation. EDTA containing tubes were stored at +4 ^{0}C until DNA Isolation. Gel containing tubes were let to rest 15 minutes for coagulation by gently rolling around a few times in every 5 minutes. After coagulation period, tubes were centrifuged in 4500rpm for 20 minutes and supernatant aliquoted in eppendorf tubes and stored in -80 ^{0}C'.

3.3. Chemicals, Consumables and Instruments

3.3.1 Chemicals and Consumables

iPrep Purelink gDNA blood kit (Invitrogen, Thermo Fisher Scientific Inc), miRNeasy Serum/Plazma Kit, Qiagen, Trizol (Quiazol, Qiagen), Chloroform (Sigma Aldrich) %100 Ethanol (Sigma Aldrich), cDNA Reverse Transcription Kit (miScript II Kit, Qiagen): microRNA Universal Primer (Qiagen), miScript PreAMP PCR Kit (Qiagen), miScript SYBR Green PCR Kit (Qiagen), microRNA Primer Assay (Qiagen) Qubit 3.0 microRNA Assay Kit (Invitrogen, Thermo Fisher Scientific Inc.) TaqMan

Genotyping Assay (Applied Biosystems, Thermo Fisher Scientific Inc.), TaqMan Genotyping MasterMix (Applied Biosystems, Thermo Fisher Scientific Inc.), EliKine Leptin Elisa Kit (Abbkine Inc.), EliKine Leptin Receptor Elisa Kit (Abbkine Inc.), EliKine SOCS3 Elisa Kit (Abbkine Inc.), 96 well plate (Applied Biosystems, Thermo Fisher Scientific Inc.), 96 well plate optical seal (Applied Biosystems, Thermo Fisher Scientific Inc.) PCR grade water (Invitrogen, Thermo Fisher Scientific Inc).

3.3.2. Instruments

iPrep Pure Link Nucleic Acid Isolation Instrument (Invitrogen, Thermo Fisher Scientific Inc), ABI 7500 Fast Real Time Real Time PCR Instrument (Applied Biosystems, Thermo Fisher Scientific Inc.), T100 Thermalcycler (BioRad) Qubit 3.0 Flourometer (Applied Biosystems, Thermo Fisher Scientific Inc.), Plate Centrifuge (Hettich), Centrifuge (Centrifuge 22R, Beckman Coulter), Microplate Reader (WHYM201, Poweam Medical Co.), Microplate Autowasher (WHYM200, Poweam Medical Co.), NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific Inc), +4 ^{0}C Fridge (Haier), −20 ^{0}C Deepfreeze (Haier), −80 ^{0}C Ultra Deepfreeze (Haier) Micropipette Set (Denville Scientific), Ultra Pure Distilled Water Instrument (Purelab option Q, Elga).

3.4. Nucleic Acid Isolation

3.4.1. DNA Isolation from Peripheral Blood and Purity and Concentration Measurements

DNA isolation from peripheral blood were performed with iPrep Pure Link (Invitrogen) Nucleic Isolation Instrument. 350µl peripheral whole blood sample and iPrep Pure Link gDNA Blood Kit (Invitrogen) cartridge were used for each volunteer. This instrument is using Dynabead (Invitrogen) technology for nucleic acid isolation. During isolation procedure all pippetting processes were performed by the instrument, blood cells were lysed, nuclear matter in the solution seperated from proteins, free nucleic acids bound to magnetic beads which can change their surface polarity by the enviromental pH changes and seperated from cellular impurities by a magnet respectively. Isolated DNA samples were eluted in 100µl as final volume.

Sample purity and concentration measurements were performed by spectrophotometrically by NanoDrop 2000 (Thermo Fisher Scientific Inc.) instrument.

For the Polymerase Chain Reaction (PCR) samples in the range of OD260/ OD280= 1.7-1.9 purity and 50-150 ng/µl concentrations were used.

3.4.2. miRNA Isolation from Sera

Samples stored in –80^{0}C were thawed on ice and then heated up to 37^{0}C. For the isolation 200µl of serum semple used for each volunteer, which is the maximum volume recommended by the manifacturer. Before the isolation procedure, essential solutions of the isolation kit were prepared. Through the directions of the manifacturer, 30ml and 22ml 100% ethanol added in RWT and RPE buffers respectively and mixed by gently inverting several times.

For each volunteer 200µl serum sample added into a clean eppendorf tube and mixed with 1000µl Qiazol Lysis Solution by pippetting and the mixture let rest for 5 minutes in room temperature. After that, 3,5µl Spike in Control (Ce_miR-39_1) and 200µl added into each mixture, mixed by vortexing and let rest for 3 minutes in room temperature. After the rest, all samples centrifuged +4^{0}C and 12.000g for 15 minutes. After centrifugation lysate fragmented into 3 phases, upper aquous phase (supernatant, apprx. 600µl) were taken into a clean eppendorf tube, 1.5 times of (apprx. 900µl) 100% ethanol added and mixed by pipetting several times. For the capturing of miRNAs in the mixture, silica-membrane based spin columns were used. Columns were placed into 2ml waste tubes, 700µl (which is the maximal column volume) of ethanol-supernatant mixture added in columns and centrifuged at 8000g, room temperature for 15 seconds. Liquid collected in waste tubes were discarded and this step repeated for total of the ethanol-supernatant mixture with maximum 700µl portions. For the purification of miRNAs captured in silica-membrane, spin columns were washed in three steps. For the first washing step 700µl RWT buffer added in spin column and centrifuged at 8000g in room temperature for 15 seconds and liquid collected in waste tubes were discarded, second washing done by 500µl RPE buffer and spin column centrifuged at 8000g in room temperature for 15 seconds and liquid collected in waste tubes were discarded, third washing done by 500µl 80% Ethanol and spin column centrifuged at 8000g in room temperature for 15 seconds and liquid collected in waste tubes were discarded. For to prevent a possible impurity, spin columns placed in new waste tubes and centrifuged with their lids open at 14000g in room temperature for 5 minutes to dry the silica membranes and move away the ethanol. After drying silica membranes, spin

columns were placed in sample collection tubes, 14µl RNAse free water pipetted on the silica membrane which is at the center of the spin column and centrifuged at 14000g in room temperature for 1 minute. After centrifugation approximately 12µl of purified miRNA collected in collection tubes.

3.4.3. Purity Measurement and Determination of miRNA Concentration of Isolated miRNA Samples

Sample purity measurement were performed by spectrophotometrically by NanoDrop 2000 (Thermo Fisher Scientific Inc.) instrument and OD260/ OD280: 2.0 accepted as pure RNA.

Concentration of miRNA samples were measured with Qubit 3.0 microRNA Assay Kit and Qubit 3.0 instrument (Invitrogen, Thermo Fisher Scientific Inc.). For the concentration measurement, Qubit microRNA Reagent diluted in Qubit microRNA Buffer with 1:200 ratio. For each sample 198µl mixture and 2µl sample mixed in 0.5ml optical grade PCR tubes, incubated in dark in room temperature for 2 minutes and read with the instrument. Min., Max. and Mean values of isolated miRNA concentrations are given below (Table 3.4.3.-1);

Table 3.4.3.-1. Min., Max. and Mean values of Isolated miRNA concentrations.

Min.	Mean	Max.
0,31 ng/µl	1,06 ng/µl	2,86 ng/µl

3.5. cDNA Synthesis

Isolated miRNA samples were converted to DNA molecules which is much more stable and safe to store than RNA molecule. This convertion were performed with conventional PCR method with an enzyme called Reverse Transcriptase (miScript II Reverse Transcription Kit, Qiagen) which is synthesizing DNA by using RNA as a guide. For the synthesis of Complementary DNA (cDNA) molecules 10 ng of miRNA in total volume were used. PCR mixture and protocol are given below (Table 3.5.2.-1, Table 3.5.2.-2)

Table 3.5.2.-1. Reverse Transcription PCR Mixture.

Component	Volume
5x miScript Hiflex Buffer	4 µl
10x miScript Nucleic mix	2 µl
miScript Reverse Transcriptase Mix	2 µl
RNase Free Water	1 µl
Sample cDNA	10 µl
Total Volume: 20 µl	

Table 3.5.2.-2. Reverse Transcription PCR Protocol.

Temperature	Time
37 °C	60 min.
95 °C	5 min.
95 °C	∞

3.6. Determination of Serum miR-218-5p Gene Expression Levels

Serum miRNA levels measured with Quantitative Real Time PCR (qPCR) method. In this method, SYBR Green flourescent dye (which emits green light when bound to double stranded DNA) is added in PCR mixture and during the PCR reaction flourescent emissions are measured to determinate reaction characteristics. In every cycle of the protocol flourescent emissions are measured and Cycle Tresholds (Ct) for each reaction well is calculated by the software of the instrument (Figure 5.). Ct values are dependent on sample amounts in the reaction well and can not give exact copy numbers but, comparison with a “House Keeping” gene gives relative expression change.

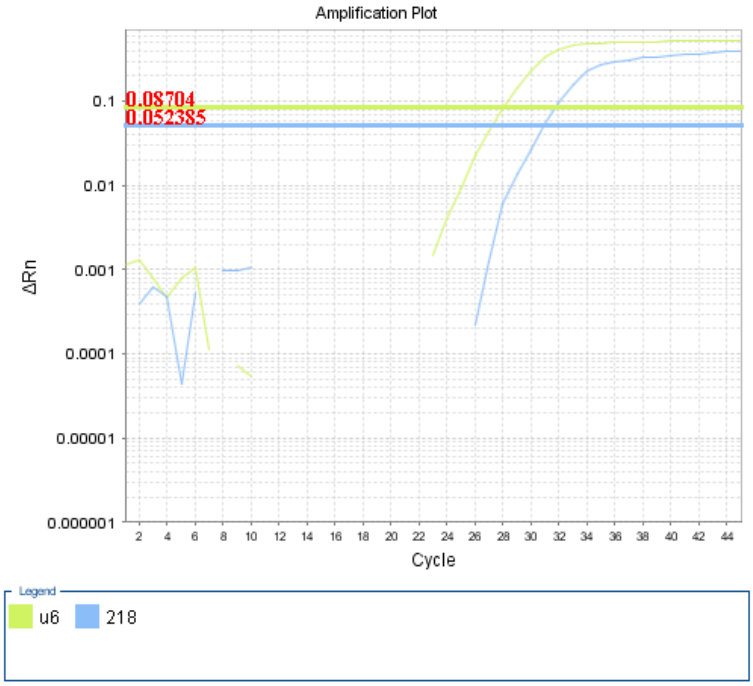


Figure 5. Amplification Plots and Cycle Tresholds of Targeted miRNAs.

Because of low yields of miRNA isolation, preamplification were performed for each cDNA sample. For the preamplification reaction miScript PreAMP PCR Kit (Qiagen) were used. Before preamplification reaction, cDNA samples were 5 fold diluted according to the manifacturers recommendations. For the preamplification reaction, PCR mixture and protocol are given below (Table 3.6.-1, Table 3.6.-2);

Table 3.6.-1. Preamplification PCR Mixture.

Component	Volume
5X miScript PreAMP Buffer	5 µl
HotStartTaq DNA Polymerase	2 µl
miScript Preamp Primer Mix	5 µl
RNase- free water	7 µl
miScript Preamp Universal Primer	1 µl
Diluted template cDNA	5 µl
Total Volume 25 µl	

Table 3.6.-2. Preamplification PCR Protocol.

Step	Temperature	Time	
Enzyme Activation	95 ^{0}C	15 min.	
Denaturation	94 ^{0}C	30 sec.	12 Cycles
Annealing / Extention	60 ^{0}C	3 min.	

After preamplification reaction, samples were diluted 20 fold according to the manifacturers recommendations.

For the measurement of miR-218-5p expression levels, Real Time PCR mixture and protocol are given below (Table 3.6.-3, Table 3.6.-4);

Table 3.6.-3. Real Time Quantitative PCR Mixture.

Component	Volume
SYBR Green PCR Mix	12.5 µl
miScript Universal Primer	2.5 µl
miScript Primer Assay	2.5 µl
PCR Grade Water	5 µl
Sample cDNA	2.5 µl
Total Volume: 25 µl	

Table 3.6.-4. Real Time Quantitative PCR Protocol.

Step	Temperature	Time	
Enzyme Activation	95^{o}C	15 min.	
Denaturation	94^{o}C	15 sec.	40 Cycles
Annealing	55^{o}C	30 sec.	
Elongation	70^{o}C	30 sec.	

After PCR reactions, targeted miRNA and house keeping Ct differences (ΔCt) were calculated to see the relative expression changes between samples.

3.7. Determination of SOCS3 3'-UTR Genetic Variants

SOCS3 gene 3’-UTR variants were determined with Real Time PCR method by using TaqMan Genotyping Assay, TaqMan Genotyping MasterMix (Thermo Fisher Scientific Inc.) and ABI 7500 Fast Real-Time PCR (Applied Biosystems). In this method, there are sequence spesific flourescent hybridization probes are used additional to conventional PCR methods. During PCR reaction, flourescent emissions of hybridization probes are measured and depending on the different dyes bound on the probes, sample genotypes determined by the software of the instrument (Figure 6., Figure 7.). For the undetermined samples, genotypes determined by examining amplification plots manually.

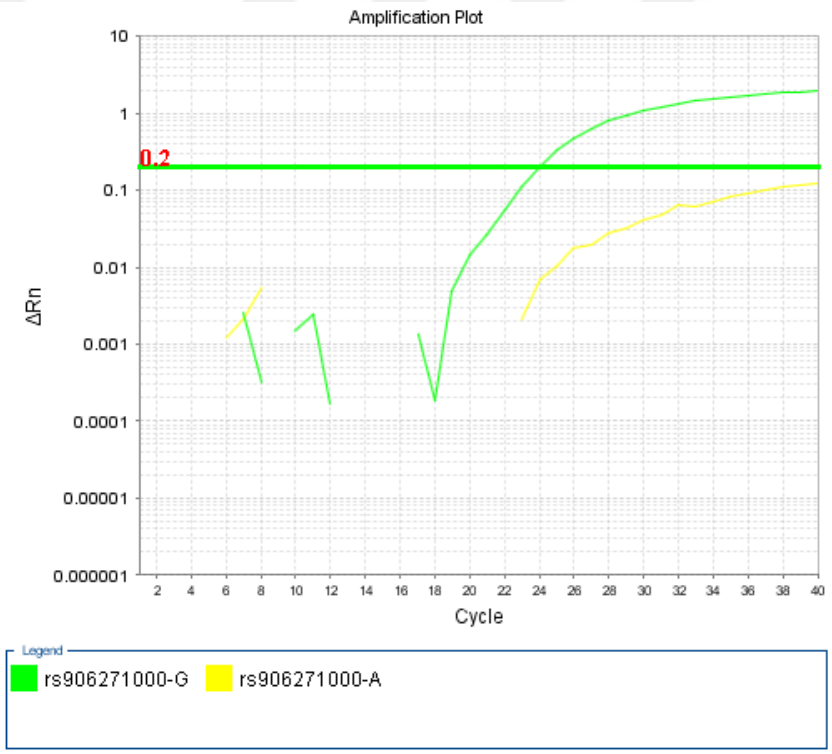


Figure 6. Emission Plot of Hybridization Probes.

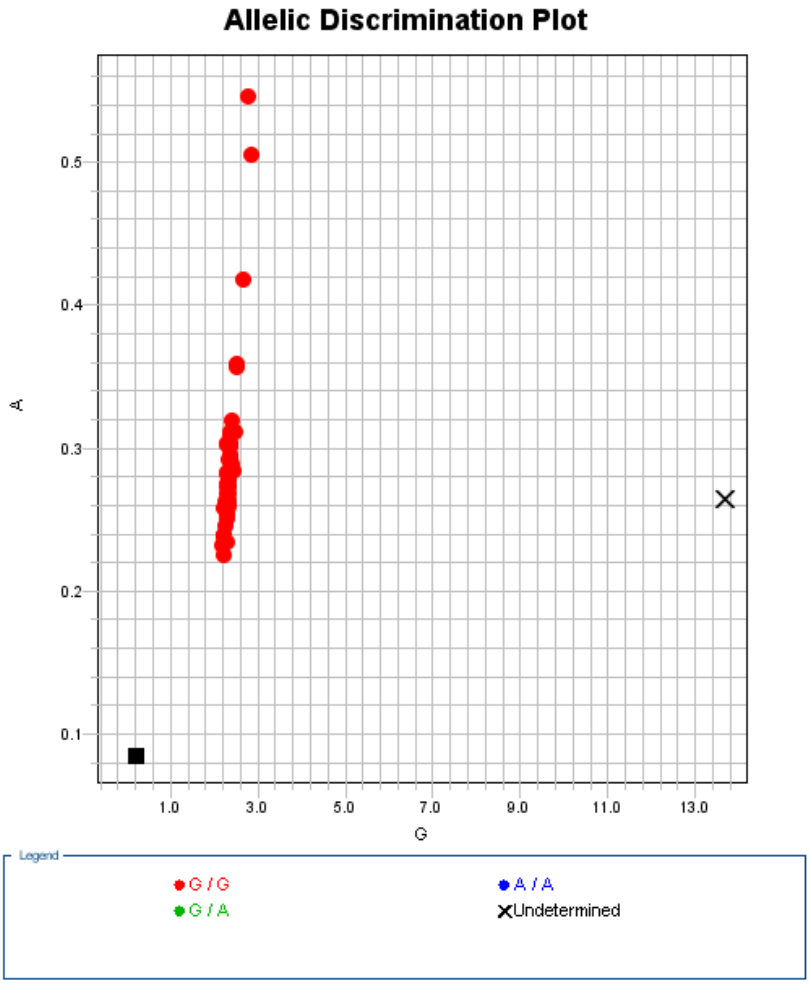


Figure 7. Allelic Discrimination Plot.

Real Time PCR mixture and protocol for genotyping PCR are given below (Table 3.7.-1, Table 3.7.-2);

Table 3.7.-1. Real Time Genotyping PCR Mixture.

Component	Volume
TaqMan Genotyping MasterMix	10 µl
TaqMan Genotyping Assay	0.5 µl
PCR Grade Water	8.5 µl
Sample DNA	1 µl
Total Volume: 20 µl	

Table 3.7.-2. Real Time Genotyping PCR Protocol.

Step	Temperature	Time	
Enzyme Activation	95°C	10 min.	
Denaturation	92 °C	15 sec.	40 Cycles
Elongation	60°C	1 min.	

3.8. Measurement of Serum Leptin, Leptin Receptor and SOCS3 Levels

Serum Leptin, Leptin Receptor and SOCS3 levels were measured with Enzyme Linked Immunosorbent Assay (ELISA) method by using EliKine (Abbkine Inc.) ELISA kits. All three kits are depending Sandwich ELISA principle which is working with two antibodies (one for stablising the molecule on bottom of the well and one for enzymatic marking) binding from different epitopes on the target molecule. Reaction wellplates are precoated with stabilisation antibodies (spesific for target molecule) and after stabilisation marking antibodies are applied on target molecules in reaction wells. All EliKine (Abbkine Inc.) ELISA kits are highly standardised and for the measurements of Leptin, Leptin Receptor and SOCS3 levels single protocol have used as the manifacturer recommended.

Serum samples which are stored in -80^{0}C were thawed in room tepmerature and ELISA kits were heated up to room temperature, stock solutions (Wash Buffer, Sample Diluent, Assay Buffer, Standart Solutions, Leptin Antibody and Streptavidin-HRP Conjugate) diluted and serum samples diluted ½ with Sample Diluent as 50µl Serum 50µl Sample Diluent with final volume of 100µl before procedure.

100µl of samples and standarts added in wells which are precoated with and antibody for our target molecule, sealed with an adesive film and incubated in room temperature for 2 hours on microplate shaker in medium speed for the immobilisation of our target molecule. After incubation, liquid in wells aspirated and all wells washed with 250µl of wash buffer twice, after second wash wash buffer aspirated and microplate tapped gently on absorbend paper for avoiding any wash buffer remains. After washing, 100µl of Leptin Antibody added and wells sealed with an adesive film incubated in room temperature for 1 hour on microplate shaker in medium speed. After incubation, liquid in wells aspirated and all wells washed with 250µl of wash buffer twice, after second wash wash buffer aspirated and microplate tapped gently on absorbend paper for avoiding any wash buffer remains. After washing, 100µl of HRP

Substrate solution added in wells, sealed with an adesive film, wrapped with tin foil to protect from light and incubated in room temperature for 15 minutes on microplate shaker in medium speed. After incubation, 50µl Stop solution added in wells, gently shaked with hand to mix the solution and read in microplate reader at 450nm wavelenght.

Concentration calculations have done by standard curve method depending on absorbances of the standards.

3.9. Statistical Analyses

Statistical analyses of the study were performed with SPSS 25.0 (IBM) software by using Fisher's Exact Test, Student's T-Test, Pearson Correlation, Logistic Regression Analysis and MedCalc (BVBA) Software by using ROC Analysis. It was considered significant when *p* value was below 0.05.

4. RESULTS

In this study, there were two groups of volunteers (Patient, n=35 – Control, n=30) with similar demographic characteristics. All demographic data were collected at Yeditepe University Hospital Department of Pediatric Endocrinology during physical examination.

4.1. Demographic Characteristics of Patient and Control Groups

Demographic characteristics of the patient and control groups are given in Table 4.1.-1.

Table 4.1.-1. Demographic Characteristics of the Patient and Control Groups.

	Patient (n=35)	Control (n=30)	*p* -Value	Odds Ratio	%95 CI
Age, x̄± SD (Years)	10,5409 ±2,30654	10,3493±3,04938	0,782	1.028	0.850-1.243
Sex Male/Female	17 (48.6 %) / 18 (51.4%)	14 (46.7 %) / 16 (53.3%)	0,878	1.079	0.406-2.866
BMI, x̄± SD (kg/m²)	25,3871±3,47301	17,6744±2,94957	**0,000***	2.319	1.450-3.708
BMI Percentile, x̄± SD	98,3315±1,30644	47,6708±30,88618	**0,000***	1.816	1.057-3.119

n= Number of Samples, x̄± SD : Mean Value ± Standard Deviation, The Values Marked with * and bold are showing statistically significance, Student's t-test have used for to show the difference of Age, BMI and BMI Percentile parameters and Chi-Square test have used for to show the difference of Gender parameter between two groups, Odds Ratios were calculated with Logistic Regression Analysis.

Between patient and control groups, there were no statistically significant difference in age and gender parameters, however BMI and BMI percentiles were highly significant (Table 4.1.-1.), which shows that demographic characteristics of both groups were appropriate for the study.

4.2. SOCS3 3'-UTR Genotypes of Patient and Control Groups

Genotypes according the SOCS3 Gene rs906271000 polymorphism are given in Table 4.2.-1.

Table 4.2.-1. Genotypes and Allelic Discriminations of Patient and Control Groups.

Genotype	Patient (n=35)	Control (n=30)	*p* -Value
GG	35 (100 %)	30 (100 %)	*p*>0.05
GA	0 (0 %)	0 (0 %)	*p*>0.05
AA	0 (0 %)	0 (0 %)	*p*>0.05
Allele			
G	70 (100 %)	60 (100 %)	*p*>0.05
A	0 (0 %)	0 (0 %)	*p*>0.05

n= Number of Samples, Chi-Square test have used for to show the difference of Genotype and Allele distributions between two groups.

rs906271000 polymorphism is a G>A transition, as in case G allele is wildtype and A allele is mutant. Our results have shown that all the volunteers in patient and control groups have GG genotype. There were no mutant allele (A) seen in both study groups. Thus there were no statistically significant difference between patient and control groups according to SOCS3 Gene rs906271000 polymorphism .

4.3. Serum Leptin, Leptin Receptor and SOCS3 Levels of Patient and Control Groups

Serum leptin, leptin receptor and SOCS3 levels are given in Table 4.3.-1.. There was no statistically significant difference between patient and control groups according to serum leptin receptor and SOCS3 levels, but leptin levels were significantly higher in patient group compared to control. These results showed that our patient group had leptin resistance.

Table 4.3.-1. Serum Levels of Leptin, Leptin Receptor and SOCS3

	Patient (n=35)	Control (n=30)	*p* -Value	Odds Ratio	%95 CI
Leptin, x̄± SD (ng/ml)	44,5042 ± 26,8873	11,1745 ± 16,0820	**0,000***	1.080	1.038-1.124
Leptin Receptor, x̄± SD (µg/L)	34,8033 ± 38,3641	27,7809 ± 25,9974	0,399	1.007	0.990-1.024
SOCS3, x̄± SD (pg/ml)	268,4463 ± 163,2283	226,2149 ± 45,7568	0,175	1.008	0.996-1.019

n= Number of Samples, x̄± SD : Mean Value ± Standard Deviation, The Values Marked with * and bold are showing statistically significance, Student's t-test have used for to show the difference of serum levels of Leptin, Leptin Receptor and SOCS3 between two groups, Odds Ratios were calculated with Logistic Regression Analysis.

Furthermore, there was a slight increase in leptin receptor and SOCS3 levels in patient group compared to control.

We also analysed the relation between demographic characteristics and the serum levels of leptin, leptin receptor and SOCS3 in patient and control groups have shown that ;

- In patient group, BMI and BMI percentiles were positively correlated with serum leptin levels (Table 4.3.-2.) (Figure 8., Figure 9.),
- In control group, BMI was positively correlated with serum leptin levels (Table 4.3.-2.).

as expected.

Table 4.3.-2. Correlation of Serum Leptin Levels Between BMI and BMI Percentiles in Patient and Control Groups

Correlation	Patient (n=35)		Control (n=30)	
	r^2	*p* value	r^2	*p* value
Leptin-BMI	0,555	***p*=0,004***	0,558	***p*=0,004***
Leptin-BMI Percentile	0,448	***p*=0,045***	0,370	*p*=0,069

n= Number of Samples, The Values Marked with * and bold are showing statistically significance, Pearson Correlation Analysis have used for to show correlation between serum levels of Leptin, BMI and BMI Percentile.

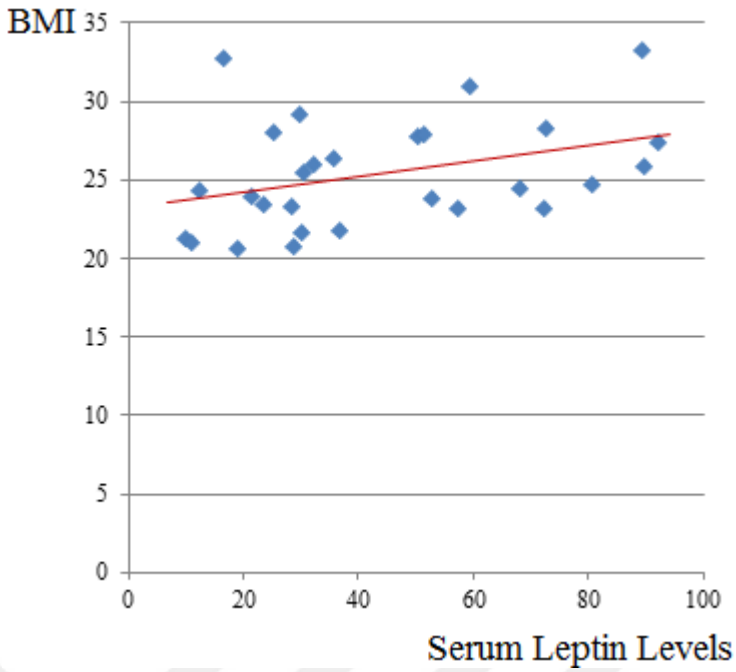


Figure 8. The Distribution of BMI and Serum Leptin Levels in Patient Group

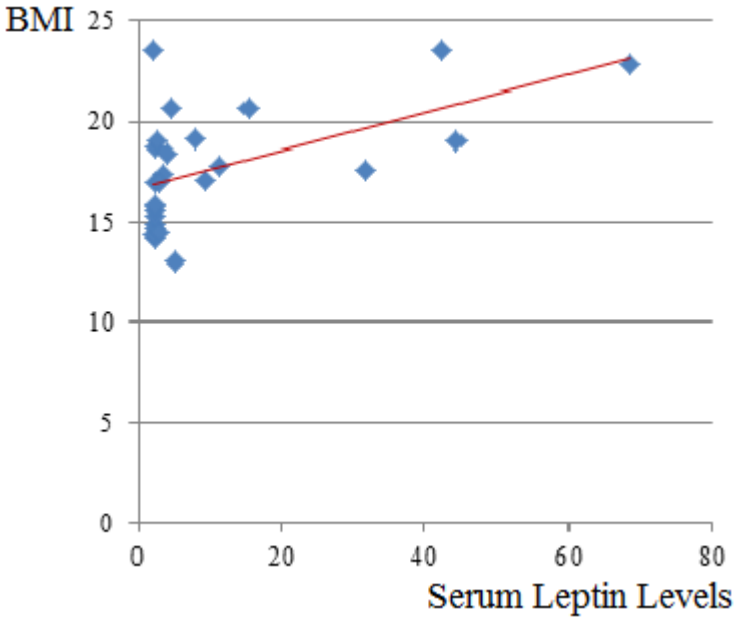


Figure 9. The Distribution of BMI and Serum Leptin Levels in Control Group

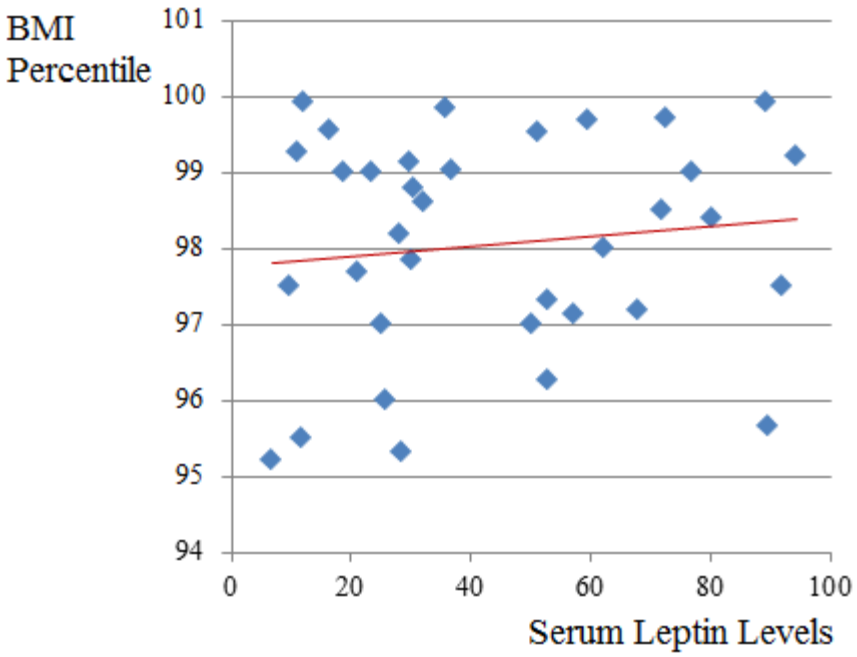


Figure 10. The Distribution of BMI Percentiles and Serum Leptin Levels in Patient Group

Furthermore, there were significant correlation between serum SOCS3 levels and BMI percentiles (p=0,008) and serum leptin receptor Levels (p=0,001) in patient group but not in control group.

4.4. Expression Levels of Serum miR-218-5p in Patient and Control Groups

Serum miR-218-5p Levels of patient and control groups are given in Table 4.4.-1..

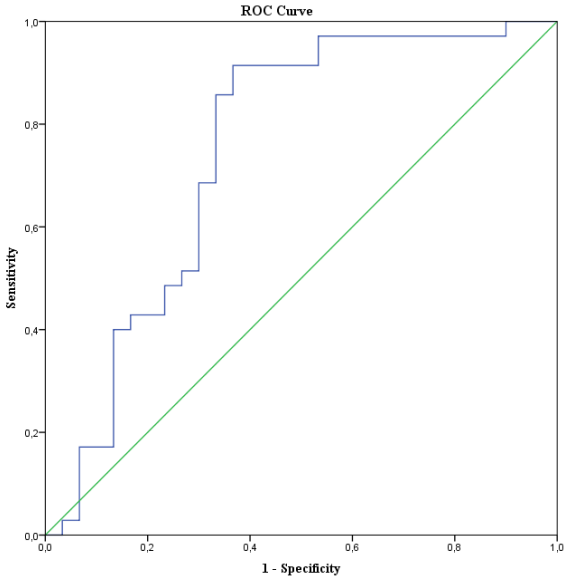
Table 4.4.-1. miR-218-5p ΔCt Values of Patient and Control Groups

	Patient (n=35)	**Control (n=30)**	***p* -Value**
miR-218-5p, x̄± SD (ΔCt Values)	0,01854 ± 1,2754	-1,5336 ± 2,0255	**0,000***

n= Number of Samples, x̄± SD : Mean Value ± Standard Deviation, The Values Marked with * and bold are showing statistically significance, Student's t-test have used for to show the difference of serum levels of miR-218-5p between two groups.

There was a statistically significant diffence between patient and control groups in serum miR-218-5p expression levels. It was significantly increased in patient group (Table 4.4.-1.). As a result of our linear logistic regression analysis, it was seen that increased miR-218-5p levels were 1.756 times more likely to obesity in children (Odds Ratio: 1.756, 95% CI= 1.236- 2.496; p=0.002).

For Receiver Operating Characteristic (ROC) Analysis was performed to evaluate the reliability as a biomarker and/or diagnostic parameter. According to the results of ROC Analysis, miR-218-5p has a reliable value as a biomarker and/or diagnostic parameter (*p*= 0.001, Area Under Curve= 0.748, %95CI=0.124-0.381)



Area Under Curve= 0.748, %95CI=0.124-0.381, *p*= 0.001.

Figure 11. miR-218-5p ROC Curve

Results of the statistical analysis according to relationship of the serum miR-218-5p expression levels and demographic characteristics between patient and control groups are given in Table 4.4.-2..

Table 4.4.-2. Correlation of the miR-218-5p Expression Levels and Demographic Characteristics Between Patient and Control Groups

Correlating Parameter	**miR-218-5p Expression Levels in Patient Group (n=35)**		**miR-218-5p Expression Levels in Control Group (n=30)**	
	r^2	***p* value**	**r^2**	***p* value**
Age	0.176	p=0.335	-0.046	p=0.811
BMI	0.219	p=0.262	0.245	p=0.237
BMI Percentile	0.063	p=0.756	0.305	p=0.138
Correlating Parameter	**miR-218-5p Expression Levels in Allover Study Group (n=65)**			
	r^2		***p* value**	
Age	0.044		p=0.736	
BMI	0.458		**p=0.001***	
BMI Percentile	0.487		**p=0.000***	

n= Number of Samples, The Values Marked with * and bold are showing statistically significance, Pearson Correlation Analysis have used for to show correlation between serum levels of miR-218-5p and Demographic Characteristics.

There was no correlation between miR-218-5p expression levels and demographic characteristics in patient and control groups (p>0.05). When we focus on correlation between miR-218-5p levels and demographic characteristics of whole study group, there was a strong correlation between miR-218-5p levels and BMI (p=0.001) (Figure 12.) and BMI percentile (p=0.000) (Figure 13.)

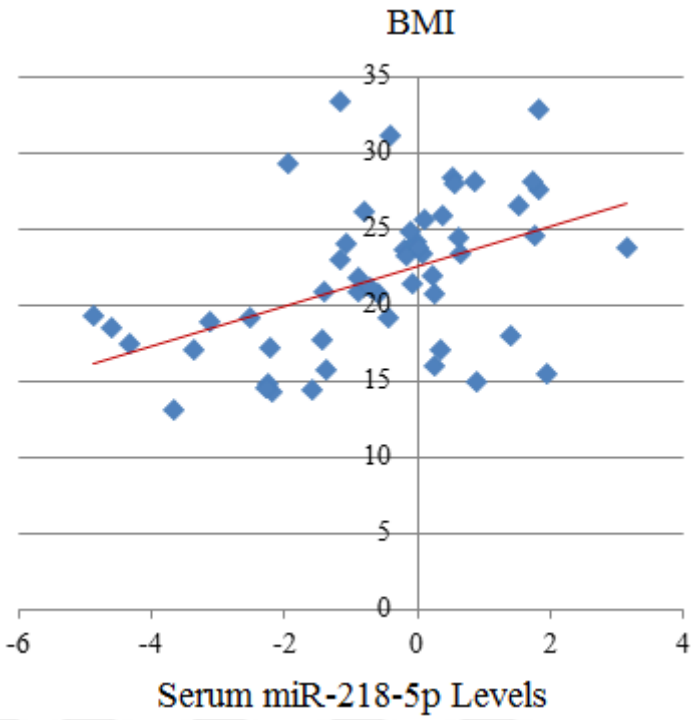


Figure 12. The Distribution of BMI and Serum miR-218-5p Levels in Entire Study Population.

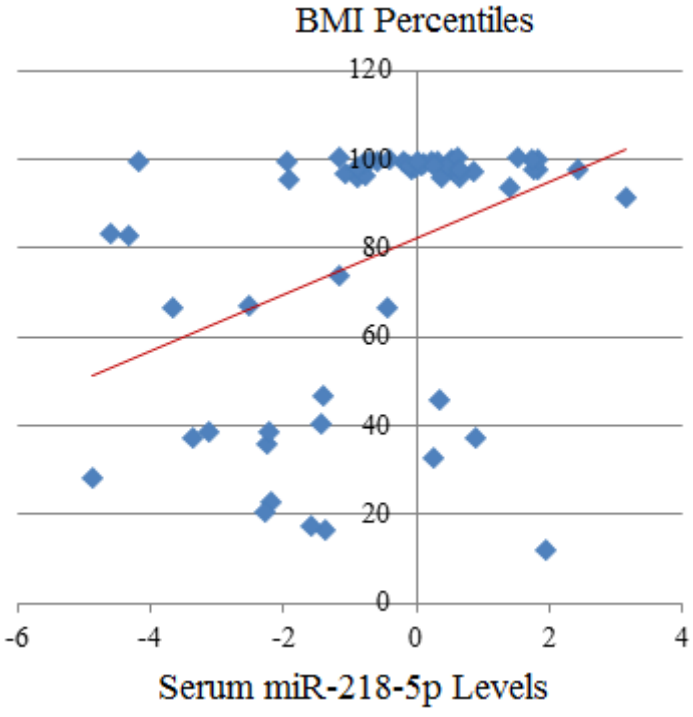


Figure 13.The Distribution of BMI Percentiles and Serum miR-218-5p Levels in the Entire Study Population.

4.5. Determination of Relationship among Serum miR-218-5p Levels, SOCS3 3'-UTR Genotypes and Serum Leptin, Leptin Receptor and SOCS3 Levels and Their Comparison Between Patient and Control Groups

Results of the statistical analysis on the relationship between serum miR-218-5p levels and serum levels of Leptin, Leptin Receptor and SOCS3 are given in Table 4.5.-1..

Table 4.5.-1. Correlation Between Serum miR-218-5p Levels and Serum Levels of Leptin, Leptin Receptor and SOCS3

Correlating Parameter	**miR-218-5p Expression Levels in Patient Group (n=35)**		**miR-218-5p Expression Levels in Control Group (n=30)**	
	r^2	***p* value**	**r^2**	***p* value**
Leptin	0.008	*p*=0.962	0.309	*p*=0.097
Leptin Receptor	-0.029	*p*=0.871	-0.372	***p*=0.043***
SOCS3	-0.048	*p*=0.785	0.68	*p*=0.068
Correlating Parameter	**miR-218-5p Expression Levels in Allover Study Group (n=65)**			
	r^2		***p* value**	
Leptin	0.348		***p*=0.004***	
Leptin Receptor	-0.114		*p*=0.368	
SOCS3	-0.013		*p*=0.919	

n= Number of Samples, The Values Marked with * and bold are showing statistically significance, Pearson Correlation Analysis have used for to show correlation between serum expression levels of miR-218-5p and serum levels of Leptin, Leptin Receptor and SOCS3.

There was no correlation between miR-218-5p levels and serum levels of leptin, leptin receptor and SOCS3 in Patient Group. However there was a correlation between miR-218-5p and leptin receptor levels in the control group. When we examine the correlation between miR-218-5p expression levels and demographic characteristics of whole study group, there was a strong correlation between miR-218-5p expression levels and serum leptin levels (*p*=0.004) (Figure 14.).

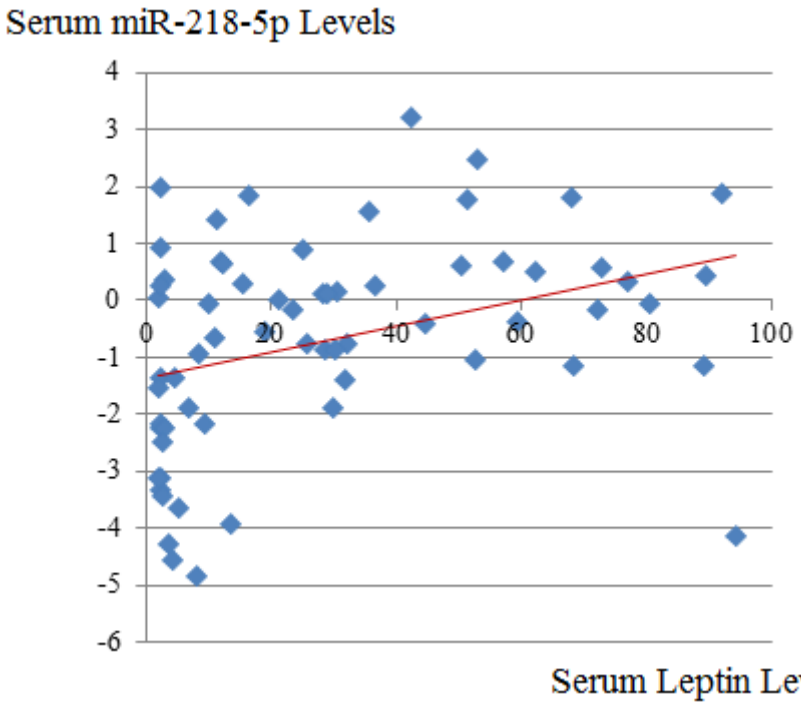


Figure 14. The Distribution of Serum Levels of Leptin and miR-218-5p Expression in Entire Study Population.

We couldnt find any relation between miR-218-5p expression, serum levels of leptin, leptin receptor, SOCS3 and SOCS3 Gene rs906271000 polymorphism as there was no mutant genotype/allele have seen in Study Population.

5. DISCUSSION AND CONCLUSION

Obesity is a metabolic pathology which adversely effects physical, mental and social development of children during developmental ages. There are several causes of childhoold obesity such as genetic, socio-cultural and/or obesity development depending on another pathology. Among them leptin resistance is one of the major causes of the obesity.

There are many causes of leptin resistance. The main cause of leptin resistance known in humans is mutations (monogenic obesity) in specific genes in leptin pathway such as Leptin (Biologically Inactive Leptin), Leptin Receptor which causes early-onset obesity (<2 years old), familial obesity, and eating disorders (31). However these mutations are rare. Beyond spesific mutations, underlying mechanism of leptin resistance is still unknown. In this study we investigated idiopathic type of leptin resistance. For this purpose, children who developed obesity after 2 years of age and had no family history selected for the study.

In the published literature according to miRNA effects on leptin in obesity, there are several publications in adults which show that different miRNA levels in adipose tissue are in correlation with obesity parameters, especially serum Leptin levels (32, 33, 34). In addition, Heneghan et al. have shown that, besides the adipose tissue, serum miRNA levels also differ between obese and non-obese subjects (35). In their study, Heneghan et al. have compared miRNA levels in omental and sub cutaneous fat tissue with blood samples. All three results were not only compatible with each other but also were in correlation with obesity parameters (35). Although data on leptin resistance is missing in their study, their results about circulating miRNA effects on tissues were similar to our results.

Development of leptin resistance in severe obesity is more familiar than the development of obesity due to leptin resistance (36). To distinguish these conditions, we focused on childhood obesity. In the childhood obesity, serum leptin levels and obesity parameters are seem far from obesity-induced leptin resistance.

According to the literature in the scope of childhood obesity and leptin resistance, it is shown that serum leptin levels of obese children are significantly higher than non-obese children (37, 38). Hassink et al. showed that serum leptin levels are

positively correlating with BMI in obese children (37). In our study, serum Leptin levels were in positive correlation with both BMI (p=0.004) and BMI Percentiles (p=0.004) in Patient Group. Moreover, there was a positive correlation of serum Leptin levels and BMI in Control Group (p=0.045), which is compatible with the published literature (37, 38).

There are number of publications (39, 40, 41, 42) in the scope of miRNA effect on childhood obesity. In the study of Prats-Puig et al. 15 circulating miRNAs (miR-486-5p, miR-486-3p, miR-221, miR-28-3p, miR-142-3p, miR-130b, miR-423-5p, miR-532-5p, miR-125b, miR-140-5p, miR-16-1, miR-328, miR-222, miR-363, miR-122) differed between Patient and Control Groups which correlates with obesity parameters (39). In our study, we have shown that serum miR-218-5p levels were significantly increased in Patient Group in correlation with obesity parameters (p=0.000). In the same study of Prats-Puig et al., they specified that decrease in serum levels of miR-221 and miR-28-3p may be involved in hypothalamic resistance of circulating satiety factors (39). However, the data about serum leptin levels and leptin resistance is lacking. We did an online search about the possible targets of miR-221 and miR-28-3p. We have seen that miR-221 also targets SOCS3 which was supporting our theory. Furthermore, we investigated possible targets of circulating miRNAs which are in correlation with obesity parameters, miR-486-3p and miR-130b are also targeting SOCS3.

There are several publications which examined the role of miRNAs in cardiovascular risk, lipid metabolism, and potential risk of future adult diabetes in childhood obesity (40, 41, 42). We wanted to compare our results with their results, but the limitation was patient histories and demographic characteristics (according to leptin resistance) were not compatible with our study.

Yang et al. showed that serum miR-218-5p levels decreased in hepatocellular carcinoma patiens, which may imply miR-218-5p serum levels can differ under pathological conditions (43). Li et al. also showed that miR-218-5p had an effect on SOCS3 and decrease in miR-218-5p expression resulted in increased expression levels of SOCS3 in tissue level (44). In our study we have found that serum miR-218-5p expression levels are significantly increased leptin resistant obese children and may have effect on SOCS3. So far, these resulst are compatible with literature (43,44).

In our study**,** we hypothesised miR-218-5p which is targeting Leptin, Leptin Receptor and SOCS3 may be involved in Leptin Resistance in childhood obesity and the results are confirming this hypothesis. 35 obese and 30 non-obese controls were included in the study. Our findings were;

- miR-218-5p expression are significantly increased in the circulation of patients,
- increased miR218-5p expression is strongly correlating with Leptin levels in entire study group (n=65) –no correlation have seen in seperated groups more likely to caused by small sample sizes,
- there were no significant difference between patient and control groups according to serum leptin receptor levels –according to the published data, leptin receptor has a restrictive effect on leptin action (45), thus our results are showing that leptin resistance seen in patient group are independent from circulating leptin receptor,
- furthermore, there were no correlation between miR-218-5p expression and serum leptin receptor levels neither in entire study group, nor in patient group but within control group which shows possible protective effect of miR-218-5p against leptin resistance in healty subjects by circulating leptin receptor,
- serum SOCS3 leves have investigated as a factor to increase reliability of miR-218-5p as a diagnostic parameter but there were no significant results have found –there are significant correlationn between serum SOCS3 levels and BMI percentiles and serum leptin receptor levels in patient group, but these results more likely to coincidental and independent from serum miR-218-5p levels, thus there were no publication according to the relationship of serum and tissue levels of SOCS3 and transport of SOCS3 through blood brain barrier,

these five major findings have shown that miR-218-5p have a role on leptin levels and likely involving to leptin resistance.

In the light of this knowledge, we state that the hypothesis about possible effect of serum miR-218-5p expression on leptin, leptin receptor and SOCS3 sustains its reliability.

In conclusion, we believe that miR-218-5p can be evaluated as a potential therapeutic target when we consider involvement of circulating miRNAs to metabolism

regulation, synergistic function of miRNAs and potential role of SOCS3 as an inducer in leptin resistance. Also it can be a diagnostic parameter. However, one has to keep on mind that, target tissue of the Leptin is arcuate nucleus in hypothalamus which means that serum miR-218-5p and SOCS3 levels investigated in this study may be a trace of hypothalamic regulation of leptin signalling. Elevated serum expression levels of miR-218-5p may not be showing direct effect of miR-218-5p on SOCS3 but may be the results of an indirect effect.

To the best of our knowledge, this is the first study which investigates the relation between leptin resistance and serum miRNA expression levels and miR-218-5p, leptin, leptin receptor and SOCS3. In our future studies, we are aiming to investigate the mechanism of action of miR-218-5p across tissues in leptin resistance.

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ETHICAL APPROVAL

T.C. YEDİTEPE ÜNİVERSİTESİ

Sayı : 37068608-6100-15-1421 **18/01/2018**
Konu: Klinik Araştırmalar
Etik kurul Başvurusu hk.

İlgili Makama (Emre Murat Altınkılıç)

Yeditepe Üniversitesi Biyokimya, Tıbbi Biyoloji ve Moleküler Tıp Anabilim Dalı Prof. Dr. Turgay İsbir ve Yeditepe Üniversitesi Hastanesi Çocuk Endokrinolojisi Anabilim Dalı Uzm. Dr. Belma Haliloğlu'nun sorumlu olduğu **"Çocuk Obezitesi Olgularında SOCS3 Geni ve MikroRNA 218'in İlişkisinin Belirlenmesi"** isimli araştırma projesine ait Klinik Araştırmalar Etik Kurulu (KAEK) Başvuru Dosyası (**1393** kayıt Numaralı KAEK Başvuru Dosyası), Yeditepe Üniversitesi Klinik Araştırmalar Etik Kurulu tarafından **17.01.2018** tarihli toplantıda incelenmiştir.

Kurul tarafından yapılan inceleme sonucu, yukarıdaki isimi belirtilen çalışmanın yapılmasının etik ve bilimsel açıdan uygun olduğuna karar verilmiştir (**KAEK Karar No: 785**).

Prof. Dr. Turgay ÇELİK
Yeditepe Üniversitesi
Klinik Araştırmalar Etik Kurulu Başkanı

Yeditepe Üniversitesi 26 Ağustos Yerleşimi, İnönü Mahallesi Kayışdağı Caddesi 34755 Ataşehir / İstanbul
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APPENDICES

Appendix 1. Informed Consent Form

Asgari Bilgilendirilmiş Gönüllü Olur Formu

Sayın Hastamız,

- Bu belge bilgilendirilme ve aydınlatılmış onam haklarınızdan yararlanabilmenizi amaçlamaktadır.
- Size gerçekleştirilebilecek klinik araştırmalar amaçlı girişimler konusunda, tüm seçenekler ile bu girişimlerin yarar ve muhtemel zararları konusunda anlayabileceğiniz şekilde **bilgi alma hakkınız ve bir kopyasını isteme hakkınız** vardır.
- Yasal ve tıbbi zorunluluk taşıyan durumlar dışında **bilgilendirmeyi reddedebilirsiniz**. Yazılı bildirmek koşulu ile bilgi almama veya yerinize güvendiğiniz bir kimsenin bilgilendirilmesini talep etme hakkına sahipsiniz.
- klinik araştırmalara katılım konusunda bilgilendirildikten sonra bunu kabul edebilirsiniz. Ya da **karar verebilmek için uygun zaman talep edebilirsiniz.**
- Hayatınız veya hayati organlarınız tehlikede olmadığı sürece onamınızı (yazılı talep etme koşulu ile) **dilediğiniz zaman geri alabilir** ya da önceden kabul etmediğiniz herhangi bir tanı/tedavi amaçlı girişimi **tekrar talep edebilirsiniz**.
- Hastanemizde verilen hizmetleri **Hastane Tanıtım Broşüründen** edinebilirsiniz. Ayrıca Hastane personeli hakkında http://www.veditepehastanesi.com.tr/ web sayfalarından daha detaylı bilgilere ulaşabilirsiniz.
- Burada belirtilenlerden başka sorularınız varsa bunları yanıtlamak görevimizdir.

TANIMLAMA

Araştırmanın Adı : Çocuk Obezitesi Olgularında SOCS3 geni ve MikroRNA 218'in İlişkisinin Belirlenmesi

Araştırmaya Katılımcı Sayısı: 50 Hasta / 50 Kontrol, Toplam 100 Gönüllü

Bu araştırmanın Amacı: Çocuk Obezitesi Olgularında SOCS3 geni ve MikroRNA 218'in İlişkisinin Belirlenmesi

Süresi: 2 Yıl

İzlenecek Yöntem/Yöntemler:

miRNA İzolasyonu: Gönüllülerden düz kan tüplerine alınan kan örnekleri 4500 rpm de 15 dakika santrifüj edilecek ve serum ayrılacak, ayrılan serum örnekleri izolasyon -80C° buzdolabında muhafaza edilecektir. Serum miRNA izolasyonu miReasy Serum/Plasma kit (Qiagen) Kullanılarak yapılacaktır.
DNA İzolasyonu: Gönüllülerden EDTA'lı tüplere alınan kan örnekleri +4Co buz dolabında muhafaza edilecek ve DNA izolasyonu iPrep Purelink DNA izolasyon robotu (Invitrogen) ile yapılacaktır.
miRNA ekspresyon seviyelerinin belirlenmesi: İzole edilen miRNA'lar dan ters transkriptaz reaksiyonu ile cDNA sentezi ve Gerçek Zamanlı PZR reaksiyonu ile miRNA ekspresyon seviyeleri belirlenecek ve ekspresyon farklı $2^{(\Delta\Delta Ct)}$ yöntemi ile belirlenecektir.
Genotip Analizi: miRNA'lar ile lişkili SNP'ler için genotip analizi Gerçek Zamanlı PZR reaksiyonu ile gerçekleştirilecektir.

Serum Leptin ve Leptin Reseptörü Seviyelerinin Belirlenmesi: Serum Leptin ve Leptin Reseptörü Seviyeleri ELISA yöntemi ile belirlenecektir.
Deney sonuçlarının istatistik analizi: IBM SPSS Statistics programıyla yapılacaktır.

Araştırma Sonunda Beklenen Fayda: Çocuk Obezitesi Olgularında SOCS3 geni ve MikroRNA 218'in İlişkisinin Belirlenmesi

Bu Çalışmada Herhangi Bir Alternatif Tedavi yada Girişimde Bulunulmayacaktır.

Bu Araştırma Gönüllüler İçin Hiçbir Risk Teşkil Etmemekte ve Hiçbir Rahatsızlığa Sebep olmamaktadır.

ONAM (RIZA)

Bilgilendirilmiş Gönüllü Olur Formundaki tüm açıklamaları okudum. Bana, yukarıda konusu ve amacı belirtilen araştırma ile ilgili yazılı ve sözlü açıklama aşağıda adı belirtilen hekim tarafından yapıldı. Araştırmaya gönüllü olarak katıldığımı, istediğim zaman gerekçeli veya gerekçesiz olarak araştırmadan ayrılabileceğimi ve kendi isteğime bakılmaksızın araştırmacı tarafından araştırma dışı bırakılabileceğimi biliyorum. Bu durumda hastanenin çalışma düzeni ve hastalara verilen bakımda aksaklık olmayacağı konusunda bilgilendirildim. Bu araştırmaya katılırken zorlama, maddi çıkar ve ast üst ilişkisine dayalı herhangi bir baskı olmaksızın bu çalışmaya katıldığımı beyan ederim. Bu bilimsel çalışmanın devamı esnasındaki süreçle ilgili olarak ayrıca eklenen çalışma protokolü ile bilgilendirildim. Tarafımdan alınan kan ve doku örneklerinin daha sonra başka araştırma çalışmalarında kullanılmasında herhangi bir sakınca yoktur.

Söz konusu araştırmaya, hiçbir baskı ve zorlama olmaksızın kendi rızamla katılmayı kabul ediyorum.

Gönüllünün:

Adı Soyadı:

İmzası:

Tarih:

Açıklamaları Yapan Kişinin:

Adı Soyadı:

İmzası:

Tarih:

Gerekiyorsa Olur İşlemine Tanık Olan Kişinin:

Adı Soyadı:

İmzası:

Tarih:

Gerekiyorsa Yasal Temsilcinin :

Adı Soyadı:

İmzası:

Tarih:

Klinik Araştırma Sorumlu Araştırmacı İletişim Bilgileri:

Adı Soyadı: Belma Haliloğlu

Uzmanlık alanı: Çocuk Endokrinolojisi

Kurumu: Yeditepe Üniversitesi Hastanesi

E-posta adresi: belma.haliloglu@yeditepe.edu.tr

Telefon numarası: 0216 578 0000

Appendix 2. Case Report Form

Ad :

Soyad :

Yaş :

Doğum Tarihi :

Boy :

Kilo :

Bel Çevresi :

Obezite Görüldüğü Yaş:

2 Yaş Öncesi Obezite : ☐ Var ☐ Yok

Kan Değerleri;

Total Kol.	LDL	HDL	VLDL	Trigliserit	Glukoz	İnsulin	ALT	AST

Bilinen Bir Hastalığı : ☐ Var ☐ Yok

Bilinen Bir Hastalığı var ise;

Sürekli Kullandığı Bir İlaç: ☐ Var ☐ Yok

Sürekli Kullandığı Bir İlaç var ise;

Puberte Evresi;

☐ Preadolesan ☐ Erken Adolesan ☐ Orta Adolesan ☐ Geç Adolesan ☐ Erişkin

Anne ve Baba'nın;

Anne;	Baba;
Yaş :	Yaş :
Boy :	Boy :
Kilo :	Kilo :
Bel Çevresi :	Bel Çevresi :

Anne ve Baba da;

Akrabalık : ☐ Var ☐ Yok

Akrabalık Derecesi:

Appendix 3. Raw Data

Group Statistics

	Grup	N	Mean	Std. Deviation	Std. Error Mean
Yaş	Kontrol	29	10,3493	3,04938	,56626
	Hasta	32	10,5409	2,30654	,40774
Leptin	Kontrol	30	11,1745837	16,08207918	2,93617251
	Hasta	35	44,5042114	26,88735713	4,54479286
LepR	Kontrol	30	27,7809618	25,99744963	4,74646320
	Hasta	35	34,8033483	38,36418963	6,48473162
SOCS3	Kontrol	30	226,2149868	45,75680097	8,35401068
	Hasta	35	268,4463371	163,2283571	27,59062810
miR_218_DeltaCT	Kontrol	30	-1,53364667	2,025578488	,369818343
	Hasta	35	,01854857	1,275460412	,215592159
BMI	Kontrol	25	17,6744	2,94957	,58991
	Hasta	28	25,3871	3,47301	,65634
Percentile	Kontrol	25	47,6708	30,88618	6,17724
	Hasta	27	98,3315	1,30644	,25143

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Yaş	Equal variances assumed	2,827	,098	-,278	59	,782	-,19163	,68835	-1,56901	1,18575
	Equal variances not assumed			-,275	51,949	,785	-,19163	,69778	-1,59186	1,20861
Leptin	Equal variances assumed	16,938	,000	-5,936	63	,000	-33,32962776	5,61447077	-44,54925233	-22,11000320
	Equal variances not assumed			-6,160	56,720	,000	-33,32962776	5,41075329	-44,16563227	-22,49362325
LepR	Equal variances assumed	2,461	,122	-,849	63	,399	-7,02238645	8,27230482	-23,55326855	9,50849565
	Equal variances not assumed			-,874	59,999	,386	-7,02238645	8,03620912	-23,09720300	9,05243010
SOCS3	Equal variances assumed	1,822	,182	-1,370	63	,175	-42,23135032	30,81869133	-103,817591	19,35489004
	Equal variances not assumed			-1,465	40,125	,151	-42,23135032	28,82763003	-100,488533	16,02583211
miR_218_DeltaCT	Equal variances assumed	7,997	,006	-3,751	63	,000	-1,552195238	,413844345	-2,379197171	-,725193305
	Equal variances not assumed			-3,626	47,392	,001	-1,552195238	,428071940	-2,413176876	-,691213600
BMI	Equal variances assumed	,828	,367	-8,659	51	,000	-7,71274	,89077	-9,50103	-5,92445
	Equal variances not assumed			-8,740	50,885	,000	-7,71274	,88248	-9,48450	-5,94099
Percentile	Equal variances assumed	65,569	,000	-8,521	50	,000	-50,66068	5,94504	-62,60166	-38,71971
	Equal variances not assumed			-8,194	24,080	,000	-50,66068	6,18235	-63,41820	-37,90317

Coefficient Correlations[a]

Model			Percentile	LepR	SOCS3	Leptin	BMI
1	Correlations	Percentile	1,000	,239	-,316	-,038	-,692
		LepR	,239	1,000	-,514	,010	-,213
		SOCS3	-,316	-,514	1,000	-,057	,254
		Leptin	-,038	,010	-,057	1,000	-,462
		BMI	-,692	-,213	,254	-,462	1,000
	Covariances	Percentile	,000	1,872E-5	-6,748E-6	-4,637E-6	-,001
		LepR	1,872E-5	4,863E-5	-6,829E-6	7,878E-7	,000
		SOCS3	-6,748E-6	-6,829E-6	3,636E-6	-1,180E-6	3,982E-5
		Leptin	-4,637E-6	7,878E-7	-1,180E-6	,000	,000
		BMI	-,001	,000	3,982E-5	,000	,007

a. Dependent Variable: miR_218_DeltaCT

Correlations

		Sıra	Yaş	Leptin	LepR	SOCS3	miR_218_DeltaCT	BMI	Percentile
Sıra	Pearson Correlation	1	,013	-,516**	-,399**	-,222	-,311*	-,614**	-,479**
	Sig. (2-tailed)		,924	,000	,001	,075	,012	,000	,000
	N	65	61	65	65	65	65	53	52
Yaş	Pearson Correlation	,013	1	,322*	-,084	-,084	,044	,431**	,073
	Sig. (2-tailed)	,924		,012	,518	,518	,736	,001	,605
	N	61	61	61	61	61	61	53	52
Leptin	Pearson Correlation	-,516**	,322*	1	,046	,059	,348**	,682**	,556**
	Sig. (2-tailed)	,000	,012		,714	,641	,004	,000	,000
	N	65	61	65	65	65	65	53	52
LepR	Pearson Correlation	-,399**	-,084	,046	1	,482**	-,114	,071	,005
	Sig. (2-tailed)	,001	,518	,714		,000	,368	,612	,974
	N	65	61	65	65	65	65	53	52
SOCS3	Pearson Correlation	-,222	-,084	,059	,482**	1	-,013	,024	,174
	Sig. (2-tailed)	,075	,518	,641	,000		,919	,864	,218
	N	65	61	65	65	65	65	53	52
miR_218_DeltaCT	Pearson Correlation	-,311*	,044	,348**	-,114	-,013	1	,458**	,487**
	Sig. (2-tailed)	,012	,736	,004	,368	,919		,001	,000
	N	65	61	65	65	65	65	53	52
BMI	Pearson Correlation	-,614**	,431**	,682**	,071	,024	,458**	1	,783**
	Sig. (2-tailed)	,000	,001	,000	,612	,864	,001		,000
	N	53	53	53	53	53	53	53	52
Percentile	Pearson Correlation	-,479**	,073	,556**	,005	,174	,487**	,783**	1
	Sig. (2-tailed)	,000	,605	,000	,974	,218	,000	,000	
	N	52	52	52	52	52	52	52	52

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Correlations

		Sıra	Yaş	Leptin	LepR	SOCS3	miR_218_DeltaCT	BMI	Percentile
Sıra	Pearson Correlation	1	,008	-,085	-,626**	-,177	-,084	-,177	,171
	Sig. (2-tailed)		,969	,656	,000	,350	,658	,398	,414
	N	30	29	30	30	30	30	25	25
Yaş	Pearson Correlation	,008	1	,336	,049	,027	-,046	,529**	,004
	Sig. (2-tailed)	,969		,075	,801	,888	,811	,007	,983
	N	29	29	29	29	29	29	25	25
Leptin	Pearson Correlation	-,085	,336	1	-,029	,136	,309	,555**	,370
	Sig. (2-tailed)	,656	,075		,878	,473	,097	,004	,069
	N	30	29	30	30	30	30	25	25
LepR	Pearson Correlation	-,626**	,049	-,029	1	,259	-,372*	,099	-,285
	Sig. (2-tailed)	,000	,801	,878		,166	,043	,637	,167
	N	30	29	30	30	30	30	25	25
SOCS3	Pearson Correlation	-,177	,027	,136	,259	1	-,338	,070	,263
	Sig. (2-tailed)	,350	,888	,473	,166		,068	,739	,203
	N	30	29	30	30	30	30	25	25
miR_218_DeltaCT	Pearson Correlation	-,084	-,046	,309	-,372*	-,338	1	,245	,305
	Sig. (2-tailed)	,658	,811	,097	,043	,068		,237	,138
	N	30	29	30	30	30	30	25	25
BMI	Pearson Correlation	-,177	,529**	,555**	,099	,070	,245	1	,725**
	Sig. (2-tailed)	,398	,007	,004	,637	,739	,237		,000
	N	25	25	25	25	25	25	25	25
Percentile	Pearson Correlation	,171	,004	,370	-,285	,263	,305	,725**	1
	Sig. (2-tailed)	,414	,983	,069	,167	,203	,138	,000	
	N	25	25	25	25	25	25	25	25

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Correlations

		Sıra	Yaş	Leptin	LepR	SOCS3	miR_218_DeltaCT	BMI	Percentile
Sıra	Pearson Correlation	1	,117	-,174	-,405*	-,167	,141	-,141	,204
	Sig. (2-tailed)		,522	,318	,016	,338	,419	,473	,308
	N	35	32	35	35	35	35	28	27
Yaş	Pearson Correlation	,117	1	,457**	-,208	-,162	,176	,609**	-,159
	Sig. (2-tailed)	,522		,009	,253	,376	,335	,001	,428
	N	32	32	32	32	32	32	28	27
Leptin	Pearson Correlation	-,174	,457**	1	-,019	-,084	,008	,371	-,153
	Sig. (2-tailed)	,318	,009		,913	,633	,962	,052	,445
	N	35	32	35	35	35	35	28	27
LepR	Pearson Correlation	-,405*	-,208	-,019	1	,535**	-,029	-,113	-,080
	Sig. (2-tailed)	,016	,253	,913		,001	,871	,567	,693
	N	35	32	35	35	35	35	28	27
SOCS3	Pearson Correlation	-,167	-,162	-,084	,535**	1	-,048	-,258	-,499**
	Sig. (2-tailed)	,338	,376	,633	,001		,785	,184	,008
	N	35	32	35	35	35	35	28	27
miR_218_DeltaCT	Pearson Correlation	,141	,176	,008	-,029	-,048	1	,219	,063
	Sig. (2-tailed)	,419	,335	,962	,871	,785		,262	,756
	N	35	32	35	35	35	35	28	27
BMI	Pearson Correlation	-,141	,609**	,371	-,113	-,258	,219	1	,409*
	Sig. (2-tailed)	,473	,001	,052	,567	,184	,262		,034
	N	28	28	28	28	28	28	28	27
Percentile	Pearson Correlation	,204	-,159	-,153	-,080	-,499**	,063	,409*	1
	Sig. (2-tailed)	,308	,428	,445	,693	,008	,756	,034	
	N	27	27	27	27	27	27	27	27

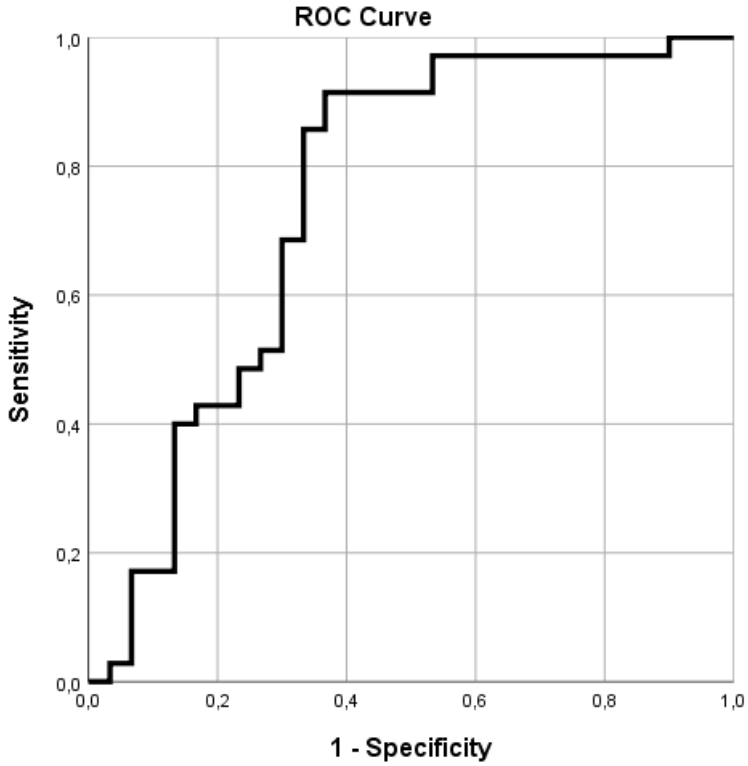
*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

Variables in the Equation

		B	S.E.	Wald	df	Sig.	Exp(B)	95% C.I.for EXP(B) Lower	Upper
Step 1[a]	miR_218_DeltaCT	,563	,179	9,857	1	,002	1,756	1,236	2,496
	Constant	,552	,300	3,379	1	,066	1,737		

a. Variable(s) entered on step 1: miR_218_DeltaCT.



Area Under the Curve

Test Result Variable(s): miR_218_DeltaCT

Area
,748

CURRICULUM VITAE

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Doctorate	Yeditepe University, Molecular Medicine Doctorate Program	2019
Masters in Science	Yeditepe University, Molecular Medicine Masters Program	2015
Bachelor in Science	İstanbul University Faculty of Science Department of Biology	2011
High school	Maltepe Anatolian High school	2006

Foreign Languages	**YDS**	**Other**
English	78,75	YökDil: 88,75

Yayınları/Tebligleri Sertifikaları/Ödülleri

Articles

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5. Aydemir D,Yalbir E, Gul T, Altinkilic EM, Yilmaz SG, İsbir S, Kunt AT, Findik O, İsbir T. Analysis of GCK-MODY (MODY2) Gene Polymorphism on Chr.744149424 in Coronary Artery Disease Patients. VI. International Congress of Molecular Medicine 22-25 of May 2017, İstanbul, Turkey
6. Yilmaz SG, Gormus U, Yildiz BP, Altinkilic EM, Dalan AB, İsbir T. Next Generation Sequencing and Detection of Common Mutations in Lung and Colon Cancers. VI. International Congress of Molecular Medicine 22-25 of May 2017, İstanbul, Turkey
7. Gazi Yıldırım, Rukset Attar, Emre Murat Altınkılıç, Hande Atasoy, Özlem Timirci Kahraman, Seda Güleç Yılmaz, Özge Kızılkale Yıldırım, Altay Burak Dalan, Elif Kök, Ceren Öztunç, Turgay İsbir. Endometrium kanseri ile PON1-55 Polimorfizmi ilişkisinin Türk popülasyonunda araştırılması. 40. ULUSAL HEMATOLOJİ KONGRESİ, EKİM 2014
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1. Gormus U, Yilmaz SG, Altinkilic EM and Isbir T. The Relationship of Embryogenesis, Carcinogenesis and Angiogenesis. Tumor Angiogenesis, Avid Science, 2016

Hobbies