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ARAŞTIRMA / RESEARCH

Leptin and leptin receptor gene polymorphisms in obese and healthy children

Obez ve sağlıklı çocuklarda leptin ve leptin reseptör gen polimorfizmleri

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Abstract

Purpose: The of this study is to explore the differences between leptin (LEPG2548A), which is considered efficacious in respect of adiposity and leptin receptor gene variants (LEPRQ223R, K109R, K656N). Furthermore, the relationship between these differences and the serum leptin level shall be scrutinized.

Materials and Methods: A total of 300 volunteers (12-17 years of age) joined our study (150controls–150obese). Blood samples obtained from these individuals were used for DNA isolation. An examination was carried out in order to show polymorphisms of the leptin receptor gene increased by RealTime PCR previously. The variations of the leptin gene were ascertained by implementation of restriction fragment length polymorphism method.

Results: Genotype dispersion calculations led to the understanding that the AA-genotype was lower in the K109R polymorphism control group than in the patient group, whereas AG-genotype was higher. The control group of Q223R polymorphism had higher AA-genotype values than the patient group, whereas it showed lower AG-genotype values. Moreover, anthropometric and metabolic results were found to be significantly higher (p in the patient group than in the control group.

Conclusion: The patient group of LEPRQ223R polymorphism showed lower AA-genotype values, a higher AG-genotype dispersion and a higher allele-G value. Therefore, a relationship to adiposity has been assumed.

Keywords: Leptin, leptin receptor, obesity, polymorphism.

Öz

Amaç: Bu çalışmada obezitede etkili olduğu düşünülen leptin (LEP G-2548A) ve leptin reseptör gen (LEPR Q223R, LEPR K109R ve LEPR K656N tek nükleotid polimorfizmleri) varyantlarındaki farklılıkların araştırılması amaçlanmıştır. Ayrıca bu farklılıkların serum leptin düzeyi ile olan ilişkisinin incelenmesi planlanmıştır.

Gereç ve Yöntem: Çalışmamıza toplam 300 (150 obez çocuk, 150 sağlıklı çocuk) gönüllü (12-17 yaş aralığında) katılmıştır. Bu kişilerden alınan kan örneklerinden DNA izolasyonu yapılmıştır. İzole edilen DNA örnekleri RealTime PCR ile çoğaltılmış leptin reseptör geninde polimorfizm bakılmıştır. Leptin genindeki varyasyon ise restriksiyon fragment uzunluk polimorfizmi yöntemiyle saptanmıştır.

Bulgular: Genotip dağılım hesaplamaları sonucunda K109R polimorfizminin kontrol grubunda AA genotipin hasta grubuna göre düşük, AG genotipin ise yüksek görüldüğü belirlenmiştir. Q223R polimorfizminin kontrol grubunda AA genotipin hasta grubuna göre yüksek, AGgenotipin ise düşük olduğu görülmüştür. K656N ve G2548A polimorfizmlerinin hasta ve kontrol gruplarındaki genotip dağılımlarında belirgin bir farklılık bulunamamıştır. Dahası antropometrik ve metabolik sonuçların hasta grubunda kontrol grubuna göre anlamlı derecede yüksekolduğu tespit edilmiştir.

Sonuç: LEPR Q223R polimorfizminin hasta grupta AA genotipinin daha düşük, AG genotipinin daha yüksek dağılımı ve G allelinin daha yüksek oranla görülmüştür. Bu nedenle obeziteyle ilişkili olabileceği düşünülmüştür.

Anahtar kelimeler: Leptin, leptin reseptör, obezite, polimorfizm, real time PCR reaksiyon

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INTRODUCTION

Obesity is a chronic disease commonly seen in developed and developing countries, which can cause various health problems such as high blood pressure, type 2 diabetes and cardiovascular diseases, as a result of an energy intake being higher than the energy consumed [1-3]. It is among the most fatal diseases today[2,4]. It is known that factors such as sex, education, marital status, psychological factors, economic income, smoking, alcohol and drug use, number of births, as well as genetic factors like mutations in many genes or chromosomal regions cause excess weight gain[1,2,5,6].

In addition, monogenic disorders such as leptin deficiency, leptin receptor mutations, proopiomelanocortin (POMC) deficiency and melanocortin4 receptor mutations, and some important genetic hormonal disorders are known to cause monogenic obesity[4,7].

Leptin is a (adipo) cytokine produced by adipose tissue that produces a satiety signal when it binds to its receptor in the hypothalamus[8]. This peptide, expressed by white adipose tissue, has a molecular weight of 16 kD, a length of 167 amino acids amd is found in many different tissues such as placenta, skeletal muscles, stomach, lymph tissue, milk and pituitary gland[9-11]. The leptin gene is encoded in the ob/ob gene, which is located in the long arm of chromosome 7 (7q31.3), consisting of 3 exons and 2 introns[10-12].

The main role of leptin is by binding to the long – form receptor (LEPR-B) in the brain to activate the JAK/STAT pathway and thereby regulate energy homeostasis[9,13,14]. Leptin is known to have important roles not only in nutrition and energy balance but also in many systems such as reproduction, cardiovascular system, neuroendocrine, T lymphocyte systems and immune functions, glucose, lipid and bone metabolism[15,16].

There are six isoforms of the leptin receptor encoded by the LEPR gene, which consists of 18 exons and 17 introns, localized on chromosome 1p31 in humans[17-19]. These isoforms are different in regard of length, location and function.

Body resistance to leptin occurs in the absence of leptin or due to functional disorders of its receptors; it prevents leptin from performing its important functions in the body and causes many problems, especially obesity[16,20].

Deficiency is compensated by leptin replacement therapy, amylin-leptin combination or metreleptin[21].

This study aims to explore the differences between leptin, which is considered efficacious in respect of adiposity and leptin receptor gene variants.

To the best of our knowledge; this is the first large-sized study conducted in Turkish population in terms of sample size and number of polymorphisms examined. Therefore, it was decided to investigate leptin and leptin receptor gene variants, which are thought to be closely related to obesity and overweight, and were examined previously in other countries but have not been studied in a large sample of the Turkish population. In addition, when the results of the study conducted were examined, some publications associated these polymorphisms with obesity and overweight, while others concluded that they had no effect[22]. It was aimed to eliminate the deficiency in this matter and to clarify the uncertainty.

MATERIALS AND METHODS

Sample

The patients who were eligible for the study were first invited by the responsible and assisting doctors to participate in the study, and the patients and their parents who asked to participate in the study were asked to sign the informed consent form and the Ethical Committee of the Dokuz Eylul University has approved the study (Protocol Number: 3894-GOA, Approval Number: 2018/08-26).

In March 2018, a study was conducted with a total of 300 volunteers, as 150 of which consists the control group and the 150, the obese In the study, 350 cases were screened, but 50 subjects were excluded from the study because of their chronic diseases, histories of drug use (steroid, antipsychotic, etc.) and endocrine pathologies that are detected. LEP G2548A and LEPR Q223R, K656N, K109R polymorphisms were studied in all individuals included in the study shown in Table 1.

Table 1. Featuresof the aample

Group	Obese Group	Control Group
Female	75	75
Male	75	75
Total	150 (average age 14.3)	150 (average age 14.8)
Serum Leptin Concentration	74	78

The patient group consisted of exogenous obesity cases with; body mass index (BMI) percentile - according to 2000 CDC (Centers for Disease Control and Prevention)- data>95p, without endocrine (hypothyroidism, Cushing's syndrome, etc.) and non-endocrine diseases (hypothalamic dysfunction, drug use, syndromic diseases). The control group consisted of healthy volunteers who were admitted to the general pediatric clinic for any reason, for routine healthy child examination, similar to the patient group in age and gender, and without chronic diseases or obesity (BMI 3-85 percentile).

Such individuals under 12 and over 17 years old, with a history of any kinds of cardiovascular, gastrointestinal, respiratory and oncological chronic diseases, a history of drug use, endocrine pathology (Cushing's syndrome, hypothyroidism, pseudohypoparathyroism etc.), diagnosed with syndromic obesity (Prader Willi, Alström Cases with Laurence - Moon Biedl syndrome), with an active focus of infection and who did not sign the informed consent form were not included in the study.

Genotyping of LEP and LEPR variants

A total of 6 ml of peripheral blood (2 ml (for DNA isolation) and 4 ml (serum)) was collected from the volunteers at Dokuz Eylul University Faculty of Medicine, Pediatric Endocrinology policlinic. DNA was isolated from the blood samples collected in the clinic according to the procedure with commercial isolation kits (NucleoSpin Blood DNA Extraction Kit - Macherey Nagel).

Table 2. Primers and probes sequences of the LEPR and LEP gene polymorphism

LEPR* K109R	
Forward	5'AGTGGTACTCACTTTTCTAACTTATC 3'
Reverse	5'GAATTAAAAAACATTGTTCAATACA 3'
Sensor	5'AACATTGAAGGAA(A/G)GACATTTGTT-Fluorescence
Anchor	LC640-CAACAGTAAATTCTTTAGTTTTTCAACAAATAGG-Phosphate
Tm	WT 65 °C, variant 55 °C
Annealing Temp.	58 °C
LEPR* Q223R	
Forward	5'CAGCCAAACTCAACGACACT 3'
Reverse	5'CCACTCTTAATACCCCCAGTACTA 3'
Sensor	5'CATTAGAGGTGAC(T/C)GGAAAATTAC-Fluorescence
Anchor	LC640-CCACCAGATGTGATTTTCAAACACATAAGG-Phosphate
Tm	WT 63 °C, variant 56 °C
Annealing Temp	62 °C
LEPR* K656N	
Forward	5'CAACTTGTCATTTTGCAGTTCCTA 3'
Reverse	5'GCTTTCCGAAGATTAATAACAGGAT 3'
Sensor	5'TGACATTTTTCTC(C/G)TTTTTCATAGTATC-Fluorescence
Anchor	LC640-CCATTAATTATTCTCCAAAATTCAGGTCCT-Phosphate
Tm	WT 63 °C, variant 58 °C
Annealing Temp	60 °C
LEP** G2548A	
Forward	5'TTTCCTGTAATTTTCCCGTGAG 3'
Reverse	5'AAAGCAAAGACAGGCATAAAAA 3'
Annealing Temp	55 °C

* LEPR – Leptin receptor; **LEP – Leptin

The LEPR Q223R, K109R, K656N polymorphisms were analyzed under usage of the Real Time PCR method (Roche LightCycler 2.0) by melt curve analysis. The primers and probes indicated in Table 2 were synthesized by Eurofins. The PCR program and the melting curve analysis parameters were optimized.

LEP G2548A polymorphism was examined by RFLP PCR. After PCR with appropriate primer sequences (Eurofins, Table 2); enzyme digestion was performed with the restriction enzyme HhaI (ThermoScientific). The results were obtained by displaying the band sizes by gel electrophoresis method. The A allele was expected to form a band of 242 bp and the G allele of 181 + 61 bp.

Anthropometric parameters and biochemical measurements

Serum leptin concentrations were measured by an Enzyme Immunoassay kit based on the principle of standard sandwich enzyme immunoassay (ELISA Kit, Boster Biological Tech). All anthropometric and biochemical measurements were performed as indicated in the reference research article[23].

Statistical analysis

SPSS 25.0 (IBM, Armonk, NY) program was used for statistical analysis. Results were evaluated at 95% confidence interval. Differences between the control and obese groups were determined by the Chi-square test and the allelic distribution using the Fischer's Exact test. metabolic and T test was used in the analysis of anthropometric measurements, and Mann – Whitney U test was used in cases that did not comply with the normal distribution.

A total of 300 subjects (150 obese, 150 control) were planned to be included in the study according to the power analysis result (80% power, 0.05 significance coefficient, and the Leptin gene TT allele frequency difference, which was found to be significant in obese and control subjects, was calculated as 10.0%). It was planned to study serum leptin levels in 100 patients from each group (power analysis (80%), alpha error: 0.05, calculated by taking the mean difference of leptin levels as 2.22 ng/L). However, in our study, it was decided

RESULTS

LEPR gene polymorphisms were determined by melting curve analysis and LEP gene polymorphism was determined by the RFLP PCR method in a total of 300 volunteers. However, serum leptin analyzes could be analyzed in 74 obese and 78 healthy persons due to data deficiencies (Table 1).

Anthropometric (BMI SDS, height SDS, weight SDS, fat mass, percent of fat) and metabolic results (serum leptin, fasting insulin, HDL-C, triglyceride, systolic blood pressure SDS and diastolic blood pressure SDS) were significantly higher in the patient group compared to the control group ($p \leq 0.001$) (Table 3)

Table 3. The anthropometric and metabolic measurement results of patient and control groups

	Obese Group (n=150)	Control Group (n=150)	P Value
BMI-SDS**	2.71 (2.01 to 4.34)	-0.22 (-1.83 to 1.19)	< 0.001[1]
Height-SDS	0.50 (-2.28 to 3.36)	-0.26 (-2.58 to 2,84)	< 0.001[1]
Weight-SDS*	3.11 (1.19 to 30.6)	-0.45 (-0.52 to 1.85)	< 0.001[2]
Fatt mass (kg)	37.5 (17.5 to 66)	10.2 (1.4 to 43)	<0.001[1]
Percent of Fat (%)	39.8 (21.9 to 63.1)	18.3(3 to 34.4)	<0.001
Leptin* (pg/mL)	9.2 (5.5–14.1)	4.3 (3.1–5.8)	<0.001[2]
Glucose (mg/dL)	87.7 ± 7.5	87.5 ± 9.1	0.897[1]
Fasting insülin* (uIU/mL)	23.8 (4.1 to 116)	7 (2.81 to 80)	<0.001[2]
Total Cholesterol* (mg/dl)	136.7 (12to 254)	109.5 (78 to 205)	0.003[2]
HDL-C (mg/dL)**	44.6 (29 to 70)	50.0 (25 to 78)	<0.001[1]
LDL-C* (mg/dL)**	135.4 (41 to 400)	111.3 (37 to 139)	0.009[2]
Triglyceride* (mg/dL)	145.4 (34 to 534)	96.1 (37 to 286)	<0.001[2]
Systolic Blood Pressure-SDS	1,48 (-2.33 to +2.33)	0.36 (-2.33 to +2.33)	< 0.001[1]
Diastolic Blood Pressure-SDS	1,18 (-0.81 to +2.33)	0.77 (-1.28 to 2.33)	< 0.001[1]

[1]Independent Samples t test, [2]Mann–Whitney U test.
**SDS - standardised; BMI-SDS – standardised Body Mass Index; HDL-C - high density lipoprotein cholesterol; LDL-C - low density lipoprotein cholesterol

Table 4. Genotype and allele distribution of polymorphisms

Polimorphisms		Obese Group	Control Group	P Value
K109R	AA	72 (%47.7)	41 (%26.8)	0.001
	AG	74 (%49.7)	103 (%69.1)	0.001
	GG	4 (%2.7)	6 (%4.0)	0.750
	A	218 (%72.5)	184 (%61.1)	0.348
	G	82 (%27.5)	116 (%38.9)	<0.001
Q223R	AA	99 (%66.4)	122 (%81.9)	0.003
	AG	49 (%32.2)	25 (%16.1)	0.002
	GG	2 (%1.3)	3 (%2.0)	1.000
	A	247 (%82.3)	270 (%89.9)	1.000
	G	53 (%17.7)	30 (%10.1)	0.004
K656N	GG	27 (%18.1)	15 (%10.1)	0.066
	GC	86 (%57.7)	97 (%65.1)	0.234
	CC	37 (%24.2)	38 (%24.8)	1.000
	G	141 (%47.0)	129 (%42.6)	0.692
	C	159 (%53.0)	171 (%57.4)	0.051
G2548A	AA	30 (%20.1)	36 (%24.2)	0.486
	AG	71 (%47.0)	70 (%46.3)	1.000
	GG	49 (%32.9)	44 (%29.5)	0.617
	A	131 (%43.6)	141 (%47.3)	0.473
	G	169 (%56.4)	159 (%52.7)	0.349

*According to genotype and allele distributions, AA genotype of LEPR K109R polymorphism was significantly higher and AG genotype was lower in obese group compared to control group. When LEPR Q223R polymorphism was examined, the obese group showed 66.4% AA, 32.2% AG and 1.3% GG genotypes whereas the control group showed 81.9% AA, 2% GG and 16.1% AG genotypes. This polymorphism's AA genotype was higher in the control group compared to the obese group and the AG genotype was lower. No significant differences were found in the genotype distributions of the other polymorphisms examined in the obese and control groups.

Table 5. Comprasion of LEPR K109R, Q223R, K656N and LEP G2548A Polymorphisms with Serum Leptin Concentrations

LEPR* K109R			
	AA	AG+GG	P Value
Obese	10.2	9.0	0.353
Control	5.6	4.7	0.100
LEPR* Q223R			
	AA	AG+GG	P Value
Obese	9.2	10.1	0.545
Control	4.9	5.8	0.164
LEPR* K656N			
	GG	GC+CC	P Value
Obese	9.5	9.5	0.995
Control	3.9	5.1	0.213
LEP** G2548A			
	GG	AG+AA	P Value
Obese	10.0	9.2	0.581
Control	5.6	4.8	0.221

* LEPR – Leptin receptor;**LEP – Leptin

According to genotype and allele distributions, AA genotype of LEPR K109R polymorphism was significantly higher and AG genotype was lower in obese group compared to the control group. When LEPR Q223R polymorphism was examined, the obese group showed 66.4% AA, 32.2% AG and 1.3% GG genotypes whereas the control group showed 81.9% AA, 2% GG and 16.1% AG genotypes. This polymorphism's AA genotype was higher in the control group compared to the obese group and the

AG genotype was lower. No significant differences were found in the genotype distributions of the other polymorphisms examined in the obese and control groups (Table 4).

The relationship of polymorphisms examined in our study with serum leptin concentrations was obtained by comparing the genotype groups' patient and control groups by anthropometric and metabolic measurements. Serum leptin concentrations of 74 obese and 78 healthy individuals were examined. Accordingly, the measurements in the patient group were found to be higher than in the control group, but their relationship with polymorphisms was not detected (Table 5).

DISCUSSION

In our study, polymorphisms in LEP and LEPR genes which are thought to cause monogenic obesity were investigated. The aim of this study was to investigate the effects of LEP G2548A, LEPR Q223R, K656N and K109R polymorphisms on childhood obesity in children and adolescents in Turkish population. LEPR Q223R polymorphism is thought to be associated with obesity due to lower AA genotype and higher AG genotype distribution in the obese group.

The sample of our study consists of equal numbers of male and female cases and reflects the Turkish population, but when determining the individuals, attention was not paid to them being born in the same region. In a study conducted by Huvenne and colleagues in France in 2015, the relationship between LEPR gene polymorphisms and obesity was examined. Seven new mutations were discovered in twelve patients. The occurrence of one of these mutations in six obese individuals living in the same region revealed the idea that the mutation may be site-specific[24]. As in this study, the polymorphisms examined may be more common in some regions or may only be seen in that region. We plan to examine this in our future studies.

LEP G2548A polymorphism was not found to be associated with obesity and overweight in the Turkish population, according to the results of our study. The relationship of LEP G2548A and LEPR Q223R polymorphism with obesity was investigated in a study conducted by Şahin et al. in a less comprehensive sample of the Turkish population (127 patients, 105 control group). The LEP G2548A polymorphism was not correlated similar to our results[25]. Also In Poland, a comprehensive study was conducted with 128 individuals who were over a hundred years of age and 414 control group members in which the association of some polymorphisms in the leptin and leptin receptor genes with long life, type 2 diabetes and myocardial infarction were investigated. Accordingly, the GG genotype of LEP G2548A polymorphism was highly distributed only in the group of individuals with over one hundred years of age, who weren't diagnosed with type 2 diabetes and myocardial infarction. Thus, the researchers reached the conclusion that this polymorphism is not related to obesity. When the results of our study were examined, the distribution of this genotype in the obese group was higher, but there was no statistically significant relationship with obesity[26]. According to Khosropour et al., the LEP-G2548A polymorphism on the leptin gene promoter region is thought to be associated with obesity because of its association with leptin production and secretion. However, contrary to this view, according to the results of our study, no significant difference was found between obese and control groups in terms of LEPG2548A polymorphism[22].

According to our findings, the AA genotype of the LEPR K109R polymorphism shows a high distribution in the patient group and the GG genotype in the control group. In the study conducted by Angel-Chavez et al., LEPR K109R, Q223R and K656N polymorphisms were investigated in a total of 128 Mexican children between the ages of 6-17 years. This study is in line with our results; when genotype distribution was analyzed, AA genotype of LEPR K109R polymorphism showed high distribution in patient group and GG genotype in control group[27]. On the contrary, in another comprehensive study conducted in Japan, the relationship between leptin and leptin receptor gene polymorphisms and fondness for sweet things and obesity was investigated. In this study, it was concluded that the GG genotype of LEPR K109R polymorphism caused obesity by causing fondness for sweets[28]. As a result it is thought that the genotype and allele distributions of this polymorphism, which pose a risk for obesity and overweight, differ according to the population and sample size.

In the same study, the GG genotype of LEPR Q223R polymorphism was also found to be associated with obesity. This genotype was also found to be highly distributed in individuals with fondness for sweets[28]. LEPR Q223R polymorphism is the only

polymorphism in which we reached results directly related to obesity and overweight in the sample we examined genetically. In a study conducted in a Brazilian population in 2013, it was aimed to investigate the relationship between obesity and metabolic parameters such as leptin, glucose, lipid and LEP and LEPR polymorphisms. According to this, LEPR Q223R polymorphism showed increased serum leptin and serum lipid values in patients carrying GG genotype it was observed that the risk of obesity decreased in patients carrying GG genotype of LEPR K109R polymorphism. Contrary to this result, no correlation was found between serum leptin concentration and any of the polymorphisms in our study. However, in our study, it was concluded that the risk of obesity was reduced in patients with G allele[29]. According to Maðrginean et al, In the Romanian population AG + GG genotype distribution of LEPR223 gene polymorphism was found to be significantly more common in obese children, similar to the results of Q223R polymorphism[30].

Since obesity is a multifactorial disease, it is difficult to determine the effect of heredity on the disease and this limits our study.

LEPR K109R, K656N and LEP G2548A polymorphisms were not shown to be associated with obesity. However, in the obese group, LEPR Q223R polymorphism was found to have lower rate of AA genotype, higher rate of genotype and higher rate of G allele, suggesting that this polymorphism could be considered susceptible to obesity. In addition, leptin levels in obese and control groups were not significantly different according to polymorphism genotype distribution. More comprehensive and meaningful results will be obtained with studies to be conducted in a wider sample range.

Yazar Katkıları: Çalışma konsepti/Tasarımı: AKY, SK; Veri toplama: AKY, KR, TK; Veri analizi ve yorumlama: AKY, KR, TK; Yazı taslağı: AKY, SK; İçeriğin eleştirel incelenmesi: AKY, SK, AA; Son onay ve sorumluluk: AKY, KR, GÇ, TK, AA, SK; Teknik ve malzeme desteği: GÇ, AA; Süpervizyon: SK, GÇ, AA; Fon sağlama (mevcut ise): yok.
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