AIMS Medical Science

AIMS Medical Science, 8(4): 301–310.
DOI: 10.3934/medsci.2021025
Received: 09 September 2021
Accepted: 29 October 2021
Published: 05 November 2021

http://www.aimspress.com/journal/medicalScience

Research article

Comparison of Q223R leptin receptor polymorphism to the leptin gene expression in Greek young volunteers

Running title: Leptin polymorphism and leptin gene expression

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Abstract: Objective: The objective of the present study was to identify the leptin gene expression and the leptin receptor polymorphisms in blood samples and to correlate gene expression values with anthropometric characteristics. **Methods**: Blood from 140 Greek young volunteers was subjected to polymerase chain reaction–restricted fragment length polymorphism (PCR–RFLP), for the genomic region of Q223R polymorphism at codon 223 in the leptin receptor gene (*LEPR*) coding region. RNA extraction, cDNA synthesis and Quantitative Real-Time PCR was performed for assessing the expression of the leptin gene (*LEP*). **Results**: Leptin gene was identified in all tested specimens and the gene was expressed in 88.9% of all volunteers with BMI < 25. In addition, it was observed that gene expression is affected by various external factors, such as Body Mass Index (BMI), eating behavior, gender and age. It was also shown that as for the Q223R polymorphism (A to G) allele G occurs with a frequency of 100% in men with BMI > 30 and 75.9% in men and 88.9% in women with BMI 25–30. Volunteers with BMI 25–30 who were homozygous on the G allele were 50% and 77.8% in men and

women respectively. All subjects with a BMI > 30 were homozygous on the G allele at 100%. **Conclusions**: In this small-scale study, results have shown that the leptin gene expression correlates with BMI and that the allele G in Q223R polymorphism is linked to overweight individuals.

Keywords: obesity; leptin gene expression; leptin receptor polymorphisms

1. Introduction

Obesity is a metabolic disorder that results from deregulation between food intake and energy expenditure. Increasing prevalence of obesity worldwide is due to changes in environmental factors, including lower physical activity and genetic factors, which may vary among distinct populations. Leptin is a hormone which is produced predominantly in adipose tissue but has also been found in blood cells, mammary epithelial cells and human saliva and affects the regulation of energy homeostasis, metabolism and neuroendocrine function of the body [1]. About 118 candidate genes are so far associated with obesity [2]. Some of the important candidate genes involved in causing obesity are the genes encoding leptin (LEP), leptin receptor (LEPR), melanocortin 4 receptor (MC4R), adiponectin (ADIPOQ), corticotrophin releasing hormone1 (CRHR1), prohormone convertase1 (PC1), pro-opiomelanocortin (POMC), resistin (RETN) and Tumor necrosis factor (TNFα) [3–7].

Leptin is an important regulator of the mass of adipose tissue and of body weight. Many studies showed that several polymorphisms of the *LEP* and *LEPR* genes are potentially related to the pathophysiology of obesity, metabolic syndrome and diabetes, while in particular, G-2548A in the LEP promoter and Q223R in LEPR variants have been associated with obesity in several populations [8–11]. Even more, the presence of Obesity was correlated to reduced leptin receptor gene expressions in hypothalamus and liver. It was also reported that circulating LEP levels correlate with body adiposity in both adults and children and the high value of LEP levels found in obese individuals are believed to indicate resistance in LEP action [2].

The aim of this study was to investigate if the occurrence of Q223R Leptin Receptor polymorphism is correlated to the expression of leptin gene and the anthropometric characteristics of Greek young volunteers.

2. Materials and methods

2.1. Collection of clinical samples

A total of 140 venous blood samples from 140 Greek young volunteers (82 males and 58 females) with a mean age of 28.2 years old were collected in an EDTA containing vacutainer tube early in the morning. The study period was during 2021. All samples were then transported in sterile tubes and stored at −20 °C immediately after sampling.

2.2. Genomic DNA extraction

DNA was directly extracted from blood specimens using an automatic extractor with the Whole Blood Nucleic Acid Extraction Kit, (ZYBIO Company) following the protocol recommended by the

supplier. The purity and the quantity of extracted DNA was evaluated spectrophotometrically by calculating OD_{260}/OD_{280} (spectrophotometer Epoch, Biotek).

2.3. Polymerase chain reaction–restricted fragment length polymorphism (PCR–RFLP) for the genomic region of Q223R polymorphism at codon 223 in LEPR gene coding region

After checking for DNA/RNA purity, PCR was carried out. The chosen primers amplify a size of 440 bp the genomic region of Q223R polymorphism at codon 223 in *LEPR* gene using 0.3 µl of primers forward 5'-ACCC TTT AAG CTG GGT GTC CCAAATGA-3' and reverse 5'-CTA GCAAATA TTTTT GTAA GCAA TT-3'. PCR was performed in 50 µL final volume solution using the Master Mix (Hot Start Promega). The amplification was conducted by a thermal cycler (96 Well thermal cycler applied Biosystems, Singapore), as follows: an Initial denaturation: 95 ℃, 10 min; 40 cycles with the following step-cycle profile: denaturation 95 ℃, 60 s; annealing 53 ℃, 60 s; extension 72 ℃, 60 s; Final extension 72 ℃, 10 min.

PCR products were separated in 2% agarose gel, stained with ethidium bromide (0.5 µg/ml) and documented under UV illumination using MiniBIS Pro device (DNR Bio-Imaging Systems Ltd., Israel).

The 440 bp PCR product was digested with *MspI* (Thermo Scientific) restriction enzyme for 16 h at 65 ℃. Three types of bands were observed—a complete *MspI* cut representing homozygous Q223R G/G, resulting in two fragments of 300 and 140 bp; a partial cut representing heterozygous G/A resulting in three fragments of 440, 300 and 140 bp; and an uncut 440 bp fragment representing homozygous A/A (Figure 1).

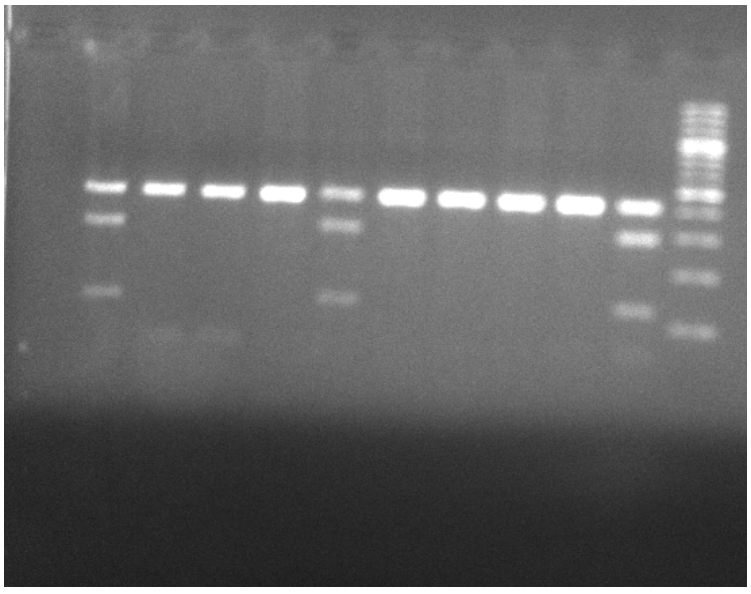

Figure 1. Lanes 2, 6 and 11 the samples had a partial cut with the *MspI* representing heterozygous G/A resulting in three fragments of 440, 300 and 140 bp. Lanes 3–5 and 7–10 had an uncut 440 bp fragment representing homozygous A/A. Lanes 1 negative control and Lane 12 DNA Ladder 100 bp.

2.4. RNA extraction

RNA extraction from blood specimens was performed using the commercial kit Nucleospin®RNA Plus (Macherey-Nagel).

2.5. cDNA

RNA was used for cDNA synthesis following a qRT-PCR (Quantitative Real Time PCR). cDNA was synthesized using Luna Script® RT SuperMix Kit (New England Biolabs). A 5 µL aliquot of purified RNA was added to 4 µL of the Luna Script® RT SuperMix Kit. The reaction was performed in a total volume of 20 µl.

2.6. qRT-PCR

5 µL cDNA was added to 20 µL of reaction mixture containing 500 nM of each primer. The primer sequences used were forward 5'-CCA TCC AAA AAG TCC AAG ATG ACA CCA AAC C-3' and REV 5' GGT ATC TCC AGG ATT GAA GAG CAT TGC ATG G-3'. The chosen primers amplify a size of 300 bp the genomic region of leptin gene. 25 µL reaction was setup, containing 5 µL of cDNA, and 12.5 µL Melt Doctor master mix, which includes HRM dye (Melt Doctor Applied Biosystem).

Amplification was conducted by a thermal cycler (96 Well thermal cycler applied Biosystems, Singapore), as follows: an Initial denaturation: 95 ℃, 10 min; 40 cycles with the following step-cycle profile: denaturation 94 ℃ 15 s; annealing 54 ℃ 35 s; extension 72 ℃ 15 s; Final extension 72 ℃, 10 min.

2.7. PCR test for cDNA quality

As an optional step, a cDNA quality test was performed after cDNA synthesis to verify the appropriate synthesis of the cDNA from each sample. *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) gene of *Homo sapiens* with primer set FORW 5' CAA-TGA-CCC-CTT-CAT-TGA-CC. 3' and REV 5' TTG-ATT-TTG-GAG-GGA-TCT-CG was used for the human IPC. In this PCR protocol, 5 µL of cDNA was used with the following PCR cycling conditions: 94 ℃ for 3 min, 35 cycles of 94 ℃ for 30 s, 60 ℃ for 40 s, and 72 ℃ for 1 min, with the final elongation step at 72 ℃ for 5 min. Each primer was used at a concentration of 500 nM in 2× PCR premix reagent (Promega Hot Start Green Master Mix). The amplicons were subjected to electrophoresis in a 2% agarose gel at 130 V for 20 min and visualized under UV light giving an amplicon with 159 bp.

Some samples were chosen randomly to amplify them with a Real Time PCR protocol using 12.5 µL Melt Doctor master mix, which includes HRM dye (Melt Doctor Applied Biosystem), 5 µL cDNA and the same concentration of primers in a total volume of 25 µL. The cycling conditions were the same with the conventional PCR (Figure 2).

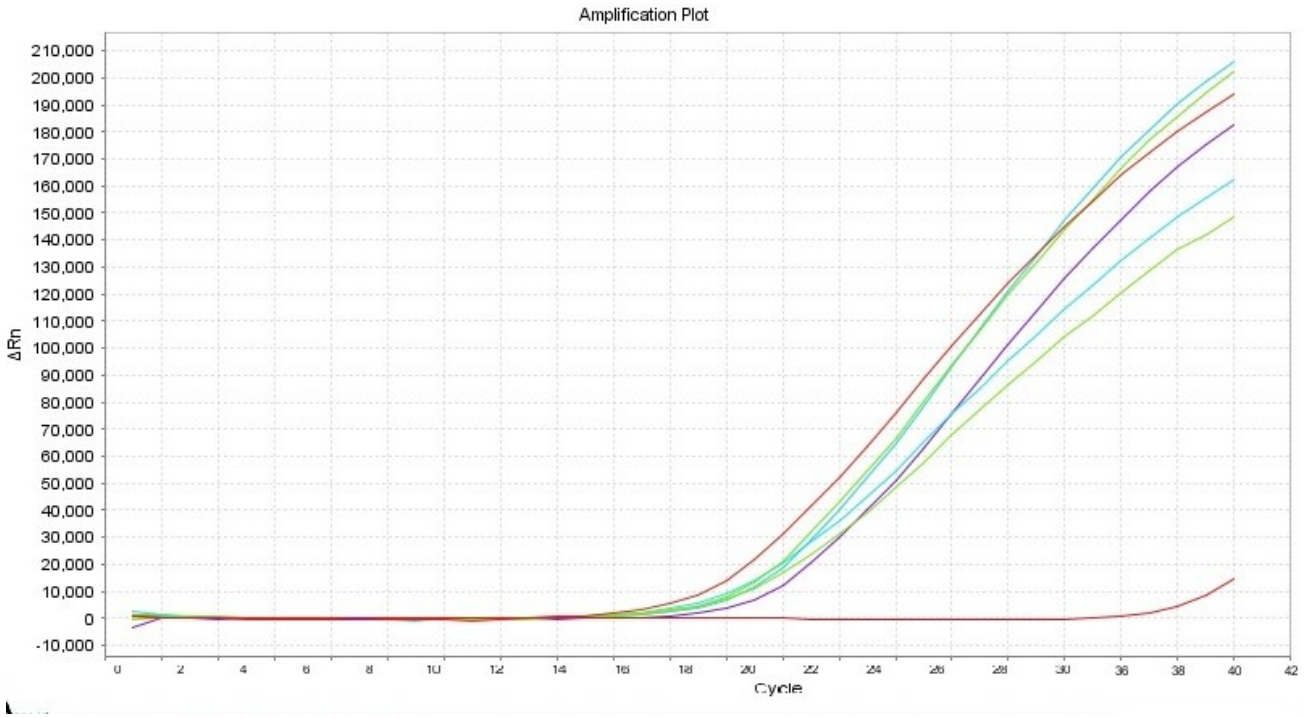

Figure 2. Real Time PCR of some samples for the GADBH gene (Internal Control).

2.8. Questionnaire

The study evaluated demographics (age, BMI), nutritional habits (healthy life style, exercise) and compared them to expression of leptin gene and the polymorphism of the receptor of leptin.

2.9. Biomedical ethics issues

Institutional ethical approval was received prior to the study and the collection of all epidemiological data, were conducted in such a way as to fully guarantee and preserve anonymity and with confidentiality. All the questionnaires were collected with the consent of the volunteers.

2.10. Statistical analysis

The null hypothesis is accepted, since $p > 0.05$. The p value can be calculated in Excel (Microsoft, Redmond, Wash) with the formula 1 – CHISQ.DIST(X; df; T), where X is the value of the χ^2-test, df is degrees of freedom, and $T(RUE)$ corresponds to the cumulative χ^2 distribution. To find the correct value at the χ^2 distribution table, we need the degrees of freedom, which are calculated by the following formula: df = (r−1) × (c−1), where c is the number of columns, and r is the number of rows in our 2 × 2 table. The df = (2−1) × (2−1) = 1.

The allele frequency among all participants is in accordance to the Hardy-Weinberg equilibrium.

3. Results

The demographic characteristics of all individuals investigated are shown in Table 1. The mean age of participants was 28.2 years old. Most of the individuals in our sample (n = 108, 77.1%) had a normal BMI < 25 kg/m^2, while 26 (18.6%) could be classified as overweight (BMI = 25–30) and 6 (4.3%) were obese (BMI > 30 kg/m^2). The BMI was equally distributed in both genders. All volunteers with normal weight claimed a healthy nutrition and everyday life style. The 32 volunteers with higher BMI (>25) were referred without a healthy life style and lack of exercise. As shown in Table 1 there were significant differences ($p < 0.05$) between the genders only in volunteers with BMI > 25.

All RNA samples tested, were positive for the human gene (*GADPH*) which was included as internal control to evaluate the quality of clinical specimens and nucleic acid extraction. The positive mRNA (detectable levels RNA) of the leptin gene was observed in most of the volunteers with BMI < 25 (n = 96, 88.9%). However, fourteen out of 18 overweight females (77.8%) had negative mRNA (nondetectable levels RNA) of the leptin gene, whereas the remaining 4 revealed a lower expression with positive mRNA (Tables 1 and 2). The 14 female participants with negative mRNA of the leptin gene had a mean BMI of 28.3 ± 0.8. All 14 males with BMI > 25 had negative mRNA of the leptin gene as well. As shown in Table 2 there were significant differences ($p < 0.05$) within BMI groups.

Table 1. Anthropometric characteristics of 41 (58.6%) female and 29 (41.4%) male volunteers.

	Female	Male	p
Mean	82 (58.6%)	58 (41.4%)	
Age (years)	27.1 ± 0.6	29.3 ± 0.5	>0.05
BMI < 25	64 (78%)	44 (75.9%)	>0.05
BMI > 25	18 (22%)	14 (24.1%)	>0.05

Note: BMI: Body mass index.

Table 2. Percentage of volunteers with positive mRNA (detectable levels of RNA) of the leptin gene.

	BMI < 25	BMI > 25	p
Male	38 (86.4%)	0 (0%)	<0.05
Female	58 (90.6%)	4 (22.2%)	<0.05
Total	96	4	<0.05

Note: BMI: Body mass index.

3.1. Genotyping of the leptin receptor Q223R

The analysis of Q223R polymorphism detects an A to G single nucleotide polymorphism of the leptin receptor gene at position 668 (*LEPR* 668A>G) which is associated with a glutamine (Q) change to arginine (R) at codon 223 of the gene. Individuals can be either A/A (homozygous with a Q/Q

phenotype), or A/G (heterozygous with a Q/R phenotype), or G/G (homozygous with a R/R phenotype). It was shown (Table 3) that allele G occurs with a frequency of 75.0% and 100% in males with BMI 25–30 and >30 respectively. Allele G occurs with a frequency of 88.9% in women with BMI 25–30. Participants with BMI < 25 who were homozygous on the G allele were the 22.7% of males and the 12.5% of females. Volunteers with BMI 25–30 who were homozygous on the G allele were 50% and 77.8%, in males and females respectively. The group with BMI > 30 were homozygous on the G allele at a level of 100%. There were statistically significant differences between the different BMI groups ($p < 0.05$). The allele A occurs in the population with a BMI < 25 at a frequency of 68.2 and 78.1% in males and females respectively. In the BMI group of 25–30 the allele A occurs with a frequency of 25% and 11.1% in male and female volunteers respectively. Those with a BMI < 25 who were homozygous on the A allele were 59.1% (males) and 68.8% (females). Volunteers with BMI 25–30 who were homozygous on the A allele were 0%. Statistically significant differences were also detected between the different BMI groups ($p < 0.05$). Volunteers with BMI < 25 were heterozygous in 18.2% of males and 18.7% of females and with BMI 25–30 in 50% of males and 22.2% of females. As shown in Table 4 the young volunteers with BMI < 25 that had positive mRNA of the leptin gene were the participants that demonstrated higher frequency of A allele ($p < 0.05$). Instead, the young volunteers that have negative mRNA of the leptin gene were the participants that demonstrated higher frequency of G allele ($p < 0.05$) (Table 5).

Table 3. Frequencies of alleles of the leptin receptor Q223R polymorphism.

BMI	GG	GA	AA	A	G
Male (BMI < 25)	10 (22.7%)	8 (18.2%)	26 (59.1%)	68.2%	31.8%
Male (BMI > 25)	10 (71.4%)	4 (28.6%)	0 (0%)	14.3%	85.7%
Female (BMI < 25)	8 (12.5%)	12 (18.7%)	44 (68.8%)	78.1%	21.9%
Female (BMI > 25)	14 (77.8%)	4 (22.2%)	0 (0%)	11.1%	88.9%

Note: BMI: Body mass index.

Table 4. Frequencies of alleles of the leptin receptor Q223R polymorphism comparison with positive mRNA (detectable levels of RNA).

BMI	Positive mRNA	G	A	*p*
Male (BMI < 25)	38	21.1%	78.9%	<0.05
Female (BMI < 25)	58	13.8%	86.2%	<0.05
Female (BMI > 25)	4	50%	50%	>0.05 (small size of samples)

Note: BMI: Body mass index.

Table 5. Frequencies of alleles of the leptin receptor Q223R polymorphism-comparison with negative mRNA (nondetectable levels of Mrna).

BMI	Negative mRNA	G	A	*p*
Male (BMI < 25)	6	100%	0%	<0.05
Male (BMI > 25)	14	85.7%	14.3%	<0.05
Female (BMI < 25)	6	100%	0%	<0.05
Female (BMI > 25)	14	100%	0%	<0.05

Note: BMI: Body mass index.

4. Discussions and conclusions

In our study, we reported the association between the leptin receptor gene Q223R polymorphism and the mRNA of the leptin gene in a sample of Greek young volunteers. The leptin receptor is a single transmembrane protein belonging to the superfamily of cytokine receptors and has several alternatively spliced isoforms (one long isoform and several short isoforms) that are distributed in many tissues. The biologically active long isoform is abundantly expressed in the hypothalamus, where it activates the Janus kinase-signal transducer and the activation of transcription (STAT) system to alter the expression of hypothalamic neuropeptides. Single nucleotide mutations (SNP's) of the leptin receptor (LEPR) gene (resulting in a premature termination of the intracellular domain) and is responsible for the obesity. Although human obesity is generally not thought to be a monogenic disorder, leptin levels increase with increasing amounts of fat mass suggesting that obesity is a leptin-resistant state in humans. The Q223R polymorphisms are associated with amino acid substitutions in the extracellular region of the LEPR and have potential functional consequences. One polymorphism causes a conservative change [glutamine to arginine at codon 223] [10,12,13]. In this study frequency of the homozygous allele G form of Q223R polymorphism was found to be higher in volunteers with BMI > 25. The genotypic distribution of the Q223R polymorphism alone, showed significant difference between normal weight, overweight, and obese volunteers. In all subject that had negative mRNA expression had a higher frequency of G allele. Our results are in accordance with previous studies where the QR and RR phenotypes of Q223R polymorphism were highly associated with obesity and the presence of the variant "G allele" of the LEPR Q223R polymorphism is associated with greater BMI in different ethnic groups [12,13]. Similarly, in a study with Greek students, in Q223R polymorphism, there was a higher prevalence of the R223 allele in the homozygous form among overweight-obese subjects *versus* normal weight subjects [14]. In addition, following an evaluation of the effect of 11 SNPs detected to be associated with high childhood BMI on the annual rate in weight and height gain, the presence of the *LEPR* Q223R SNP was identified as a weight growth predictor during childhood and the GG homozygous was shown with the highest percentage of obesity and overweight status [15]. Moreover, in Q223R polymorphism the subjects with the R allele were at risk of the metabolic syndrome [11,16]. Thus, it can be speculated that the polymorphism Q223R may act as a marker in the local population of Greece as well. On the other hand, other studies have failed to demonstrate an association of the Q223R polymorphism to the increased BMI values and obesity [17–19]. Also, conflicting results have been shown by earlier studies in which subjects carrying the A allele had significantly higher BMI [20,21]. In addition, in Mexican adolescents 223Q carriers were found

with increased insulin and leptin levels, as well as augmented body fat percentages [22]. The diverse results may arise from interactions of Q223R polymorphism with other polymorphisms in leptin and/or leptin receptor genes, the sample size of the studied populations, or due to the model used for statistical analysis. This discrepancy implies the possibility that the Q223R appear in relation with other genetic or environmental factors. Finally, limitations of the study include the small sample size and the lack of exploratory investigation of the effects of diet and exercise training on the self-reported information of the volunteers. Future larger studies should be considered necessary, investigating different age groups as well. Therefore, while the primary objective of our study was to investigate certain polymorphisms, we did not focus on the quantification of the express of mRNA in several tissues where the expression is greater. The positive findings regarding the leptin gene expression detecting in blood samples provide a motivation for further investigation of the dominant polymorphisms.

Authors contributions

Study concept and design: PH, AS, PK, ER, MP and DH; acquisition of data: DV, ER, MT, and MP; analysis and interpretation of data: PH, AS, PK, and DH; drafting of the manuscript: PH, AS, PK, AB and DH; critical revision of the manuscript: PH, AS, PK, AB and DH; statistical analysis: DH; and study supervision: DH.

Conflict of interest

All the authors declare that there are not biomedical financial interests or potential conflicts of interest in writing this manuscript.

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