Journal of
Obesity & Weight Loss Therapy

Wasim and Fakhar, J Obes Weight Loss Ther 2014, 4:4
DOI: 10.4172/2165-7904.1000233

Research Article Open Access

Leptin Gene Mutations in Morbidly Obese and Severely Lean Individuals from Punjab, Pakistan

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Received: August 16, 2014; **Accepted:** October 20, 2014; **Published:** October 25, 2014

Abstract

Obesity has become an epidemic of modern world. Although 6 mutations in the leptin gene have been found to be associated with congenital leptin deficiency, leptin gene polymorphism has not been studied in adult morbidly obese and severely lean individuals. This study involves comparison of obese and lean individuals. In this research leptin gene mutations were studied in both morbidly obese and severely lean adults. Studied individuals belong to Punjab, region of Pakistan. Present study included 25 consenting morbidly obese (BMI>30 kg/m^2) and 25 severely lean (BMI<18 kg/m^2) individuals from Punjab, Pakistan. By using organic method DNA extracted and PCR was done to amplify leptin gene with the help of specific primers. The sequence of leptin gene was analyzed by using SSCP, heteroduplex analysis and automated Sanger sequencing. No genetic mutation was detected in leptin gene in studied morbidly obese and severely lean individuals. The result suggested that in Pakistani population leptin gene may not be a major cause for obesity and leanness. Other genetics as well as environmental factors may be involved.

Keywords: Congenital leptin deficiency; SSCP; Heteroduplex analysis; BMI; Genetics and environmental factors

Introduction

Whether a person is obese or lean is determined by Body Mass Index which is abbreviated as "BMI". If BMI of the individual exceeds from 30 kg/m^2 then person is considered to be obese and lean when less than 18 kg/m^2 (World Health Organization, 2000). Leptin protein codes for a 167 amino acids, finally processed into the 146 amino acids [1]. It is a 16-kDa protein secreted by white adipocytes and dominantly have major role in the body weight regulation [2,3]. Another name of the leptin gene is *Ob* gene. It is located on chromosome no. 7 its cytogenetic location is 7q31.3. In lean adults it circulates in the body range 5-15 ng/mL [4].

Almost 40 chemicals are present for the behavior control in the brain. These chemicals are called neurotransmitters. 12 out of 40 have been found to control eating behavior. Leptin is one of these neurotransmitters. This neurotransmitter signals to the brain mainly in the hypothalamus, when a person stops to eat to maintain his BMI (www.loop.com/%7Ebkrentzman/obesity/genetics.html). Sequence of amino acids of the leptin protein is highly conserved among species so rare chance that mutation will be present in this gene. There is a 67% sequence homology among species [5]. Activity of this gene is started after binding with a receptor known as Leptin Receptor (LEPR). Due to alternative splicing of LEPR, six different isoforms formed. These isoforms are LEPRa, LEPRb, LEPRc, LEPRd, LEPRe and LEPRf. Only the LEPRb is the important and longest isoform that have the capacity of strong signaling so defect in signaling cause severe obesity [6].

Leptin use a pathway to perform functions is known as JAK/STAT pathway. Abbreviation of JAK/STAT is Janus Kinase (JAK) and Signal Transducer and Activator of Transcription (STAT) [7]. Leptin acts via two groups of arcuate neurons which are located in the hypothalamus region of the brain. First group of neurons expresses Agouti-Related Peptide and Neuropeptide Y (NPY) and the 2nd group expresses Pro-Opio-Melano-Cortin (POMC) and Cocaine and Amphetamine-Related Transcript (CART) [8]. Leptin is upregulated by insulin and cortisol and downregulated by catecholamines. In addition to these factors, Tumor Necrosis Factor-α (TNF-α) also serves as paracrine regulator, and increases secretion of leptin [9].

First two mutations have been reported these two mutations were D133G and R105W [10,11]. Rather than these mutations another mutation was also identified in the leptin gene in an Egyptian patient. This Egyptian patient had very high BMI which was 51. Sequencing showed a novel homozygous missense mutation N103K associated with obesity [12]. After that, fourth (L72S) in an Austrian 14 years old female child [13] finally, fifth and sixth mutations were (p.Leu161fsX170 and c.104_106delTCA) found in obese children of Pakistan belonging to Punjab [8]. So it is observed that in Punjab region of Pakistan chances of mutation is high in obese children. This research included 50 adult individuals belonging to Punjab region of Pakistan, 25 morbidly obese and 25 severely lean adult individuals to find out mutations in the leptin gene. Different techniques such as SSCP, heteroduplex analysis and sequencing were used to analyze the leptin gene.

Materials and Methods

Blood samples were collected from 25 obese and 25 lean adult individuals from different areas of Punjab, Pakistan. Sampling was done by asking different questions about individual's life style, diet, routine work and family background. All the obese adults had the BMI above 30 kg/m^2 and lean adults had the BMI less than 18 kg/m^2 (Figure 1). DNA extraction was done by using organic (PCI) method (Figure 2). Coding regions for exon 2 and 3 of leptin gene

(NG_007450.1) were amplified. The sequences of primers used are given in Table 1.

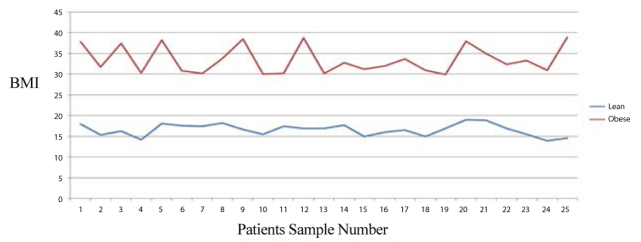

Figure 1: Sampling data of lean and obese individuals.

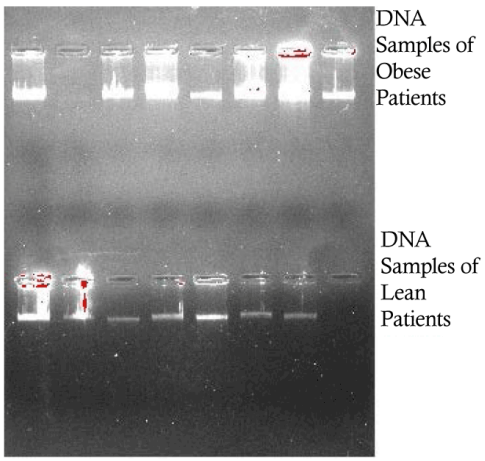

Figure 2: DNA samples of obese and lean patients.

Primer name	Primers	Tm	Product size
Exon 2 (P1)	P1F: CAGTGTGTGGTTCCTTCTGTTT P1R: ACTTTGTCCCCGCATACTCT	59.2°C 58.4°C	224 bp
Exon 3 (P2)	P2F: TGAGCACTTGTTCTCCCTCT P2R: GCAGGAAGAGTGACCTTCAA	57.6°C 58.0°C	417 bp

Table 1: Primer used in study.

Each 25 µl PCR reaction included 50 ng DNA, 1× Taq buffer [75 mM Tris-HCl (pH 8.8 at 25°C), 20 mM $[(NH_4)_2SO_4]$, 2 mM $MgCl_2$, 200 µM of each dNTP, 10 pmoles of each primer and 5U of Taq DNA polymerase (Fermentas BioSciences, USA).

DNA was initially denatured at 95°C for 5 min and then the desired fragments were amplified using 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min followed by a 10 min final extension at 72°C in PCR machine (Bio-Rad Hercules, CA, USA). The process yielded a 224 bp product of exon 2 and a 417 bp product of exon 3. All the PCR products were detected by agarose and analyzed for mutation by SSCP, heteroduplex analysis and Sanger sequencing method.

Results

All the amplified samples (Figures 3 and 4) were screened by Single-Strand Conformation Polymorphism (SSCP) and heteroduplex analysis to ascertain whether any mutation is present in the leptin gene or not. SSCP is a powerful method for identifying sequence variation in amplified DNA. SSCP analysis of DNA has been used for detection of genetic mutations in humans [14].

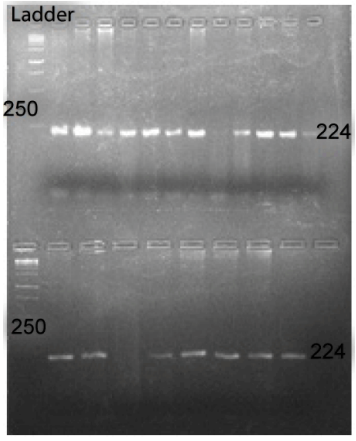

Figure 3: Gel electrophoresis for PCR products (LEP gene exon 2) amplified by P1F/P1R primers. Size of amplified second exon of leptin is 224bp as shown in comparison with 250 bp band in 1 Kb DNA ladder.

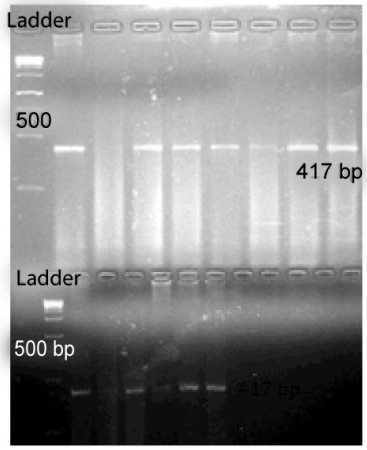

Figure 4: Gel electrophoresis for PCR products (LEP gene exon 3) amplified by P2F/P2R primers. Size of amplified second exon of leptin is 417bp as shown in comparison with 500 bp band in 1 Kb DNA ladder.

SSCP analysis

For SSCP analysis, 5 µl (amplified product) were added to 15 µl of stop solution (95% formamide, 10 mM NaOH and 0.05% bromophenol blue). The samples were heat-denatured at 95°C for 5 min and chilled on ice immediately for 5 min, and loaded onto a 10% PAGE for exon 2 (6-7 hours) and 12% for exon 3 (10-12 hours). SSCP analysis showed no mutation in exon 2 and 3 of the leptin gene (Figures 5 and 6).

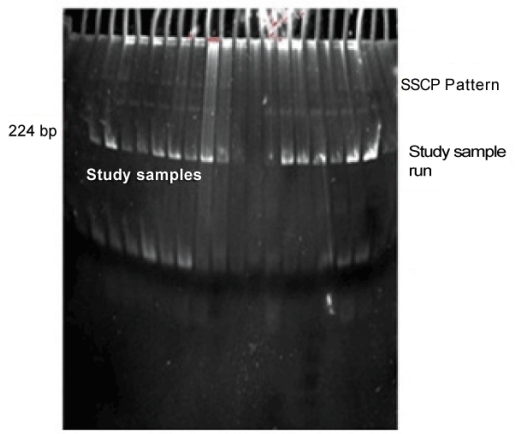

Figure 5: Polyacrylamide gel electrophoresis for SSCP of PCR product (LEP gene exon 2). Samples with standard normal samples have been compared showing no difference in banding patterns.

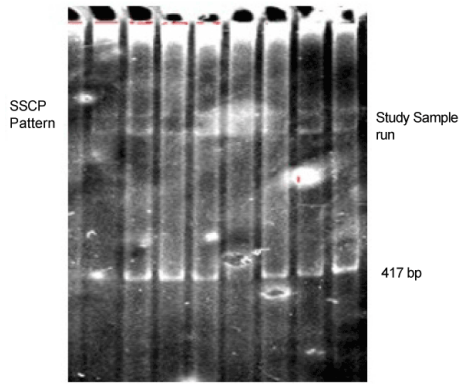

Figure 6: Polyacrylamide gel electrophoresis for SSCP of PCR products (LEP gene exon 3). Samples with standard normal samples have been compared, showing no difference in banding patterns.

Heteroduplex analysis

5 µl standard and 5 µl studied samples were mixed and denatured at 95°C for 5 min in Thermo-cycler by following the step of 30 min at 37°C and then added 3 ul bromophenol dye. At the end, products were loaded onto a 10% PAGE. All the studied samples showed no pattern in the heteroduplex analysis which means no mutations in the studied samples. For confirmation of the technique used standard samples for the analysis as shown in Figure 7.

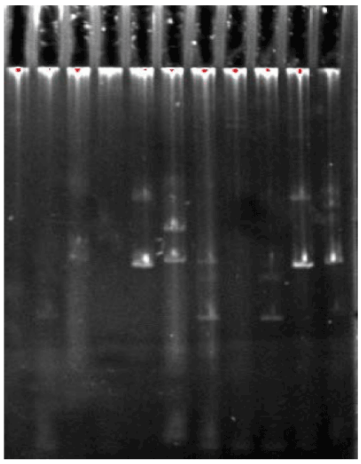

Figure 7: Polyacrylamide gel electrophoresis for heteroduplex analysis of PCR products (LEP gene). Same exon was amplified with two types of primers. Different combinations were made of these amplified products and run on the gel. Results showed that there is a pattern developed for hetero-duplex eg., slower moving DNA is hetero and faster moving DNA is homo duplex.

Sequencing

These PCR products were sequenced using ABI Prism 3100 genetic analyzer (Applied Biosystems, Inc., Foster City, CA, USA). Sequences were first aligned using the 2-sequence nucleotide blast facility provided online by NCBI and then every nucleotide in these sequences was visually inspected in Chromas 2.4 to differentiate between the controversial labeling and true mutations (Figures 8 and 9). After sequencing analysis result showed that there was no mutation present in the exon 2 and 3 of the leptin gene.

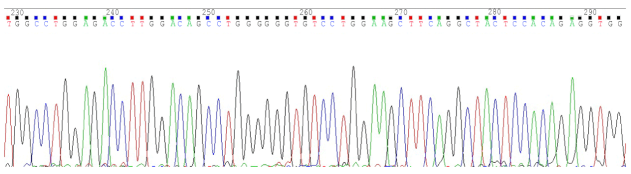

Figure 8: Sequencing results showing normal leptin gene sequence.

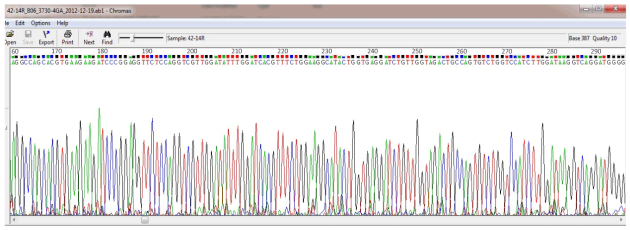

Figure 9: Sequencing results showing normal leptin gene sequence.

Discussion

In this study, leptin gene is employed for its mutational analysis. The samples were taken from the obese and lean adults. The objective of this study was to genetically characterize the leptin gene to identify the SNPs as future biomarkers for the early diagnosis of obesity and leanness. In most of the cases of congenital obesity from Pakistan, a mutation was found to be present in 6 G stretch of exon 3 of leptin gene; this (GGGGGG) stretch is very conserved region so chances of mutation being present in this region is very low.

In a study, mRNA of 68 obese and 38 lean subjects from the adipose tissue was examined to find out mutations in the *Ob* gene. Mutations were not found in human *Ob* gene in 105 subjects. But only in one individual G to A base change was identified. Due to this mutation amino acid changes from valine to methionine at the position 94 of coding region. Result suggested that mutations in the *leptin* gene were not the primary cause of human obesity [15]. In this study, all the samples were screened by using SSCP, heteroduplex and sequencing; after analysis no mutation in all the samples of both second and third exon of leptin gene was found. Third exon of the leptin gene is considered as hotspot for point mutations which cause obesity. In this exon, 6 G stretch region is present as already mentioned it is much conserved region of leptin gene (Figures 8 and 9). Mutation in the 6 G stretch has been observed in Pakistani obese children [8]. Until now only 6 pathogenic leptin mutations have been reported. All the 6 mutations are given in Table 2. Leptin sequence comparison of different species showed that gene is conserved so there is rare chance to find a mutation in the leptin gene (Figure 10).

Sr. No.	Mutations	Individuals	References
1	p.Gly133fsX145	Two Pakistani cousins	Montague et al. [10]
2	R105W	4 members from Turkish family	Strobel et al. [11]
3	N103K	2 Egyptian children	Mazen et al. [12]
4	L72S	14 years old female child	Fischer-Posovszky et al. [13]
5	p.Leu161fsX170	1 obese child from Pakistan	Fatima et al. [8]
6	c.104_106delTCA	1 obese child from Pakistan	Fatima et al. [8]

Table 2: Six pathogenic mutations in chronological order.

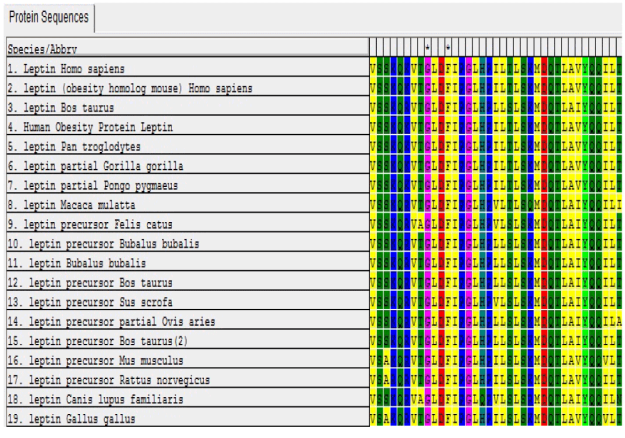

Figure 10: Leptin sequence comparison of different species.

Conclusively, mutation is not present in the leptin gene of studied Pakistani population. Obesity and leanness may mainly be caused by the other genetics and environmental factors. Leptin gene is much conserved so mutation rate is very low as compared to other genes.

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