January 2009 *Volume 3 Issue 1*

Trade Science Inc.

BioTechnology

An Indian Journal

FULL PAPER

BTAIJ, 3(1), 2009 [04-08]

Leptin and leptin receptor gene polymorphisms in polycystic ovary syndrome

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Received: 12th August, 2008 ; Accepted: 17th August, 2008

ABSTRACT

Polycystic ovary syndrome (PCOS) is a heterogeneous disorder affecting female infertility originally described as early as 1935. Genetic studies have identified a link between PCOS and disordered insulin metabolism, and indicate that the syndrome may be the presentation of a complex genetic trait disorder. Leptin is also important in regulating the onset of puberty. Extremely thin women often stop ovulating and abnormally thin adolescent women enter puberty later than their heavier counter parts, indicating that fat tissue may produce a signal that regulates reproduction, this factor may be leptin. Treatment of mice with leptin accelerates the maturation of the female reproductive tract and leads to an earlier onset of the estrous cycle and reproductive capacity. Serum leptin concentration in women with PCOS has been reported to be higher than or similar to those in weight-matched controls. Mutations of the LEP gene lead to obesity in ob/ob mice. In the present study, LEP (-2548) G/A and LEPR Q223R genotypes in a series of PCOS cases and normal controls were analyzed. The prevalence of the alleles was different in the 2 groups, control and PCOS women. For the LEP (-2548) G/A, the overall distribution of the 3 genotypes in the PCOS patients (GG 19%; GA 56%; AA 25%) was comparable with the distributions found in related subjects of normal control (GG 28%; GA 50%; AA 22%). Exonic polymorphisms in the LEPR gene, namely Q223R polymorphism on the leptin concentration variable in a selected population of PCOS patients and normal control were studied. These polymorphisms cause a change in charge (Glutamine[Q] to Arginine[R]) at codon 223. For the Q223R polymorphism, the overall distribution of the 3 genotypes in the PCOS patients (QQ 45%; QR 41%; RR 14%) were compared with the distribution found in related subjects of normal control (QQ 40%; QR 57 %; RR 3%). Findings suggest that, in this sample, regulatory sequence primarily predisposing to early onset obesity was not in close linkage equilibrium with these polymorphisms.

KEYWORDS

PCOS;
Leptin.

INTRODUCTION

Polycystic ovary syndrome (PCOS) is the disorder in which numerous benign cysts form on the ovaries under a thick, white covering. It is most common in women under thirty years of age and many ovarian cysts

disappear without treatment. Manifestation of PCOS ranges from infertility as a result of chronic anovulation and menstrual irregularities such as amenorrhea or oligomenorrhea, obesity in combination with a preponderance of fat localized in the upper body region and several dermatological features such as seborrhea, alopecia, acne or hirsutism as a result of incessant hyperandrogenism[1].

Women with PCOS seldom ovulate because follicle growth arrests all the small antral stage, and large pre-ovulatory follicles rarely develop. PCOS is frequently associated with obesity and insulin resistance[2], symptoms reminiscent of those observed in leptin deficient ob/ob mice.

Leptin is a 16 KDa polypeptide hormone produced predominantly by white adipose tissue[3] that plays an important role in body weight homeostasis, through effects on food intake and energy expenditure[4]. In addition to the regulation of body weight, leptin also influences hematopoiesis, reproduction, angiogenesis and immune processes[5]. Leptin may be an important signal indicating the adequacy of nutritional status for reproductive function[6]. Genetic variation at the leptin and receptor gene locus may play an important role in the pathophysiology of human obesity, a leptin resistant state. Single nucleotide mutations of the leptin gene (LEP) (resulting in a truncated protein)[3] or the leptin receptor (LEPR) gene (resulting in a premature termination of the intracellular domain)[7] are responsible for the morbid obesity seen in mouse models of obesity (ob/ob and db/db mice, respectively) as well as few rare cases of obesity in humans. Although human obesity is generally not thought to be monogenic disorder, leptin levels increases with increasing amounts of fat mass[8], suggesting that obesity is a leptin resistant state in humans. Therefore, it has been previously suggested that genetic variation at the leptin receptor locus and/or post receptor defects may play a significant role in the pathophysiology of human obesity. A deletion of the guanine nucleotide in codon 133 has been described[9] which caused leptin deficiency and severe obesity in two children of the same consanguineous pedigree.

MATERIALS AND METHODS

Collection of blood samples

The study population consisted of 88 women aged between 24-34 years (mean ± SD: 28.92 ± 4.03), with PCOS, who have never given birth up to the time of the present investigation of undesirable infertility. All the examined blood samples were collected at the beginning of the infertility therapy when PCOS women were not taking any medications.

Sixty normal ovulatory women, aged between 24-34 years (mean ± SD: 25.12 ± 2.95) were selected as normal subjects. The normal women included in this study were selected on the basis of not having hirsutism or signs of androgenization and all had normal ovulatory menstrual cycles.

One drop of blood was collected in a sterile filter paper disc (Whatman filter paper) and kept in sterile eppendorfs

Isolation of DNA from one drop of blood (Split second™ DNA preparation method

Patient's finger was wiped with 70 % ethanol and pricked with a sterile lancet. The blood was blotted with a Whatmann filter paper disc and placed in a sterile eppendorf.200µl of Sol A was added to the eppendorf. Boiled this for 10 minutes in a hot water bath. Later, cooled this in freezer for 5 minutes. To this 20µl of Sol B was added. Centrifuged at 10,000 rpm for 3 minutes. The supernatant with template DNA was taken into a fresh sterile eppendorf.1: 5 dilution template DNA was prepared by taking 20µl of the template DNA and 80µl of sterile Millipore water in a sterile eppendorf. 3 µl of diluted template DNA was used for PCR amplification

Polymerase chain reaction

Specific pairs of oligonucleotide Primers were selected from the literature[10]. Primer for Leptin (LEP) (242bp) is F-5'TTT CTG TAA TTT TCC CGT GAG 3′ and R-5′AAA GCA AAG ACA GGC ATA AAAA 3′. Primer for Leptin receptor (LEPR) (80 bp) is F-5′AAA CTC AAC GAC ACT CTC CT T3' and R-5'TGA ACT GAC ATT AGA GGT GAC 3′. Primers (LEP F and LEPR), (LEPR F and LEPR R) each 5µM concentration was mixed and diluted with TE Buffer. Multiplex PCR master mix, template DNA and primer mix were thawed to room temperature. The solutions were mixed completely before use. The PCR reaction mix was prepared with 8µl Multiplex PCR master mix, 5 µl Multiplex primer mix, 3µl Template DNA and the final volume is 16µl. The PCR reaction mix was thor-

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oughly mixed by a short spin in a microfuge. The thermal cycler was programmed according to the reaction conditions, initial activation for 5 min at 94^{0}C, Denaturation for 30 sec at 94^{0}C, Annealing for 30 sec at 55^{0}C, Extension for 4 min at 65^{0}C for 35 cycles. There is a final extension for 4 min at 65^{0}C and cooling for 10 min at 4^{0}C. The PCR tubes were placed in the thermal cycler and cycling program was started.

Restriction digestion of PCR Products (PCR–RFLP)

PCR amplified DNA was taken in eppendorfs. The restriction enzymes along with the buffer and Millipore water from the deep freezer was removed and kept in an icebox.16µl of Millipore water was added to all the eppendorfs having the DNA. 2µl of enzyme buffer was added to all the eppendorfs. 2µl of the Cfo I enzyme was added to the 242bp amplified LEP PCR product and Msp I enzyme to the 80bp amplified LEPR PCR product. All the tubes were kept for a short spin at 10,000 rpm for a min. Incubated all the tubes on a floater inside water bath maintained at 37^{0}C for two hours. The tubes were taken out; 3µl of DNA gel loading dye was added to all tubes and mixed well. Loaded the 80bp restricted fragments in 4% agarose gel and 242bp restricted fragments in 2 % agarose gel. Turned on the power supply and was run at 100 volts. Terminated the run when the tracking dye was about to leave the gel.Transferred the gel on an Uvidoc viewed the gel under ultraviolet light (300nm). Photographed as soon as the gel has been checked for the presence of bands.

Polymorphism in LEP and LEPR genes

The polymorphism at position-2548 of LEP gene was defined by the presence (G) or absence (A) of the Cfo I restriction site. Digestion of the 242 bp PCR product with Cfo I produced fragments of the following size: 181 and 61 bp in homozygote (G/G); 242,181 and 61bp in heterozygote (G/A) and 242bp in homozygote (A/A). The polymorphism at position 223 of LEPR gene was defined by the presence (Q) or absence (R) of the Msp I restriction site. Digestion of the 80 bp PCR products with MspI restricted fragments are of the following size: 57 and 23 bp in homozygote (Q/Q); 80, 57 and 23 bp in heterozygote (Q/R) and 80bp in homozygote (R/R).

RESULTS

In the present study, LEP (-2548) G/A and LEPR Q223R genotypes in a series of PCOS cases and normal controls were analyzed. The prevalence of the alleles was different in the 2 groups, control and PCOS women. Overall, the distribution of the 3 genotypes in the PCOS patients (GG 19%; GA 56%; AA 25%) were comparable with the distributions found in related subjects of normal control (GG 28%; GA 50%; AA 22%) (TABLE 1).

Exonic polymorphisms in the LEPR gene, namely Q223R polymorphism on the leptin concentration variable in a selected population of PCOS patients and normal control were studied. These polymorphisms cause a change in charge (Glutamine[Q] to Arginine[R]) at codon 223. For the Q223R polymorphism, the overall distribution of the 3 genotypes in the PCOS patients (QQ 45%; QR 41%; RR 14%) were compared with the distribution found in related subjects of normal control (QQ 40%; QR 57 %; RR 3%) (TABLE 2).

DISCUSSION

In human, several polymorphisms have been identified in the LEP and LEPR genes; a G to A substitution at-2548 upstream of the ATC start site[11] in the LEP gene 5' promoter region, and A, G substitution at 668 from the start codon 223 in exon 6 (Q223R) of the LEPR gene coding for the extracellular region common to all isoforms of LEPR[12]. The G to A substitution at -

TABLE 1 : χ^2 test result for genotypic and allelic variations at exon polymorphisms in the LEP gene between normal controls and PCOS subjects

G -2548 A Polymorphism					
Normal control (n=60)			PCOS subjects (n=88)		
Genotypes Co dominant model	No. of cases	%	No. of cases	%	χ^2 / P
GG	17	28	17	19	
GA	30	50	49	56	
AA	13	22	22	25	2.03 (0.36)
Dominant model					
GG	17	28	17	19	1.16 (0.28)
GA + AA	43	72	71	81	
Recessive model					
GG + GA	47	78	66	75	
AA	03	22	22	25	0.16 (0.69)

G-Wild type alleles; A-Variant alleles, P-One tailed probability of chi-squared distribution, P- Values are not significant

TABLE 2: $\chi 2$ test result for genotypic and allelic variations at exon polymorphisms in the LEPR gene between normal controls and PCOS subjects

Q223R Polymorphism					
Normal control (n=60)			PCOS subjects (n=88)		
Genotypes	No. of cases	%	No. of cases	%	χ^2 / P
Co dominant model					
QQ	24	40	40	45	
QR	34	57	36	41	4.1 (0.13)
RR	02	03	12	14	
Dominant model					
QQ	24	40	40	45	
QR+ RR	36	60	48	56	0.42 (0.52)
Recessive model					
QQ + QR	58	97	76	86	
RR	02	03	12	14	1.51 (0.22)

Q- Wild type alleles,R-Variant alleles, P-One tailed probability of chi-squared distribution. P-Values are not significant

2548 of the LEP gene was associated with leptin production[13]. The Glutamine to Arginine substitution occurs in the first of the two putative leptin binding regions and may be associated with impaired signaling capacity of the leptin receptor[14]. Enhanced gene expression and increased circulating leptin levels have been reported in subjects carrying the LEPR 223R or LEP (-2548) A alleles[13-15]. The LEP (-2548) A allele has also been associated with a two fold increase of leptin secretion adipocytes when compared to secretions by adipocytes bearing only the LEP (-2548) G alleles[13].

The difference noted in the present investigation was not statistically significant. These findings suggest that, in this sample, regulatory sequence primarily predisposing to early onset obesity was not in close linkage equilibrium with these polymorphisms. These results do not support the hypothesis that the LEP-2548 genotype is associated with changes in leptin production. The frequency distribution of the A and G alleles were not significantly different between normal controls and obese patients with PCOS. Rarely studies had been performed in the 5' non-coding region[11], hence decision was made to screen for allelic variations, which may be responsible for variation in leptin levels in humans and associated with some phenotypes of obesity. Our results were conflicting with the literature[15] that in both cohorts leptin levels were approximately 20-25 % lower in the - / - LEP -2548 obese girls.

The present data has shown that LEPR -2548 A/A and G/A genotype are not significantly associated with high leptin in PCOS. This is contradictory with the report[11], that the G-2548A variant in the promoter of LEP indicates a differential response to a low calorie diet. Certain genotype-specific effects must exist between circulating leptin and body fatness[15] A functional study also influenced leptin mRNA expression, possibly at the transcriptional level[13].

R223 allele was found more in PCOS subjects. But this variation was not significantly different from that of normal controls. Similarly negative results were reported for Q223R polymorphism in different Caucasian populations, including American[16] British[12] and Danish[17] groups. Negative results have also been reported for other racial populations including Japanese, blacks and Pima Indians. This may be due to differing statistical models or analysis, the fact that allelic frequencies vary significantly among different races[18-20], and/or the possibility that the physiological effect of the Q223R polymorphism has a significant racial component[14]. Also in population of white Australian women, the LEP and LEPR Gln 223Arg polymorphisms were not associated with longitudinal changes in body weight, fat mass, percentage body fat or BMI[21]. These results are not surprising, considering the polygenic nature of most human obesity, according to which each individual gene is expected to contribute in a minor way to the phenotypic variation, and combinations of several genes are likely to contribute or predispose to obesity[22]. Evidence of a significant effect of the Q223R polymorphism on human body composition has been reported in two other studies[18].

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