Original Article

Role of Leptin (rs7799039) and Leptin Receptor (rs1137101) Gene Polymorphisms in the Development of Uterine Leiomyoma

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Kuwait Medical Journal 2017; 49 (2): 129 - 134

ABSTRACT

Objective: To evaluate whether there is an association between leptin (rs7799039) and leptin receptor (rs1137101) gene polymorphisms and risk of Uterine Leiomyoma (ULM) development
Design: Controlled prospective study
Setting: Department of Obstetrics and Gynecology and Medical Biology School of Medicine, Mugla Sitki Kocman University, Mugla, Turkey
Subjects: This cross-sectional, clinical study included 103 perimenopausal patients who had ULM and 82 age-matched healthy perimenopausal controls. Serum estradiol (E29, Follicule Stimulating Hormone FSH) and hemoglobin levels were measured.
Intervention: Leptin and leptin receptor gene polymorphisms were determined by using polymerase chain reaction-based restriction fragment length polymorphism (PCR-RFLP) methods.
Main outcome measures : Genotype of leptin gene
Results: Median FSH level was significantly higher in the control group (62.13 Vs 32.46; $p < 0.001$), whereas Median E2 level was higher in the ULM group (114.94 Vs. 29.08; $p<0.0019$). According to genotype and alleles analyzing, GA genotype of leptin rs7799039 polymorphism ($p = 0.004$, OR = 0.380, 95% CI = 0.173 - 0.837), AA genotype of leptin receptor rs1137101 polymorphism ($p = 0.020$, OR = 0.326 CI = 0.144 - 0.736) and A Allele of leptin receptor rs1137101 polymorphism ($p = 0.003$, OR = 0.537, 95% CI = 0.346-0.803) presented protective effects for ULM development. In correlation analysis, AA genotype of leptin gene polymorphism was found to be significantly related with increased E2 levels in ULM patients ($p = 0.032$) but not in control group.
Conclusion: Leptin and leptin receptor gene polymorphisms together with increased estrogen levels might affect susceptibility to ULM development.

KEY WORDS: hysterectomy, multiple myomas, perimenopausal patients, polymerase chain reaction, single nucleotide polymorphism

INTRODUCTION

Uterine Leiomyoma (ULM) is a common non-malignant tumor in the female genital system[1]. ULMs are the most common benign neoplasm of the reproductive organs in women of reproductive age. During its growth, a myoma compresses the surrounding structures (the myometrium and connective tissue), causing the progressive formation of a sort of pseudocapsule, rich in collagen fibers, neurofibers and blood vessels. These tumors occur in up to 30% of premenopausal women, an incidence that is arguably higher than any other type of gynecological neoplasm[2]. Most common symptoms are pelvic pain, infertility, menorrhagia and recurrent pregnancy loss. ULMs, which are mostly asymptomatic, can grow from a few millimeters to 30 cm and are the most frequent cause of hysterectomy[3]. Despite their high prevalence, little is known about the pathogenesis of these tumors. Genetic factors can play a significant role in ULM development. The growth of multiple myomas in the same uterus implies that heritage plays an important role in myoma development[4].

Leptin is a 16-kD protein encoded by the *ob gene* (7q32.1) in adipose tissue. Leptin is involved in the etiology of obesity, angiogenesis, and carcinogenesis

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and is the cornerstone of the female reproductive system regulation[5-6]. In humans, leptin synthesis is increased by estrogen and inhibited by testosterone, which is mediated by leptin receptors in the brain and peripheral tissue[5]. Leptin receptor is a single-transmembrane-domain receptor member of the cytokine receptor family encoded by 1p31.3. Leptin receptor mRNA was demonstrated in the granulosa cells, preovulatory follicular cumulus cells in oocytes and in human placental trophoblastic cells[6-8]. It was suggested that by acting through those female reproductive system autocrine-paracrine mechanisms, leptin may be involved in the development of ULM[8-14].

The aim of our study was to determine whether there are relationships between leptin (rs7799039), and leptin receptor (rs1137101) gene polymorphisms and the risk of ULM development.

SUBJECTS AND METHODS

Study Design

This cross-sectional, clinical study was carried out in the Obstetrics and Gynaecology Department of Mugla Sıtkı Kocman University School of Medicine between January 2012 and November 2014. Ethical approval for the study was taken from the ethics committee of Mugla Sıtkı Kocman University Health Sciences (2012-99) and each patient signed a written informed consent. This study had been carried out in accordance with the principles of the Helsinki Declaration of 1975, revisions made in 2000 were taken into consideration during the study.

The present study included 103 female perimenopausal (40 - 51 years old) patients who had ULM, which was verified by abdominal or transvaginal ultrasonography, and 82 age matched healthy perimenopausal (40 - 51 years old) controls (without ULM) from the same geographic area. Descriptive parameters such as age, and serum hemoglobin levels were noted for each patient. Patients with diabetes mellitus, thyroid disorders, oral contraceptive use and history of myomectomy and obesity were excluded from the study.

Determination of hormonal levels

Follicle stimulating hormone (FSH) and estradiol (E2) levels were obtained in the follicular phase of menstrual cycle and analyzed using the electrochemiluminescence immunometric assay (ECLIA) method. ECLIA method was evaluated and compared to a previous semiquantitative immunoassay. The ECLIA test was performed using a Cobas E601 analyzer (Roche Diagnostics).

Genotyping

2 - 3 cc venous blood samples were collected into vacutainer plastic tubes containing sodium/potassium EDTA. DNA was extracted with a Genejet Genomic DNA purification kit (Thermo Scientific K0772) with spin colon method. For all genotyping, PCR was performed in a 25 μl volume with 100 ng DNA, 100 μm dNTPs, 20 pmol of each primer, 1.5 mM $MgCl_2$, 1× PCR buffer with $(NH_4)_2SO_4$ and 2 U Taq DNA polymerase (Thermo Scientific EP0401). Amplification was performed on an automated thermal cycler (Techne Flexigene, Cambridge, UK) (Table 1). Polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) conditions of polymorphisms of leptin (rs7799039) and leptin receptor (rs1137101) genes were determined by fragment separation at 120 V for 40 – 50 min on 3.5% agarose gel containing 0.5 mg/ml ethidium bromide (Table 1). A 100-bp DNA ladder (Thermo SM0241) was used as a size standard for each gel lane. The gel was visualized under UV light using a gel electrophoresis visualizing system (Vilber Lourmat E-BOX VX5).

Statistical analysis

The Hardy-Weinberg equilibrium was verified using the chi-square test and by estimating the expected genotypic frequencies on the basis of the development of the square of the binomial for these polymorphisms. Allelic and genotypic distributions among the different groups were compared using the likelihood-ratio chi-square test or Fisher's exact test. Categorical variables were compared using Pearson's Chi square test. Continuous variables were compared

Table 1: PCR–RFLP conditions and RFLP enzymes of polymorphisms leptin, and leptin receptor genes

Gene	Polymorphism	Primers	Temperature of annealing	Restriction endonuclease	PCR products	Reference
Leptin	rs7799039	P1 P2 P3	50 °C	CfoI	G Allele: 181 bp, 61 bp A Allele: 242 bp A allele: 421 bp	Matsuoka *et al* (1997)
Leptin receptor	rs1137101	P4	55 °C	MspI	G allele: 294 bp, 127 bp	Mammès *et al* (2000)

P1: 5′-TTCCTGTAATTTTCCCGTGAG-3; P2: 5′-AAAAGCAAAGACAGGCATAAA-3′
P3:5′CCCTTTAAGCTGGGTGTCCCAAATAG-3′; P4: 5′-AGCTAGCAAATATTTTTGTAAGCAATT-3′

by an independent sample t test or the Mann–Whitney U test for two groups. The Bonferroni-adjusted Mann–Whitney U test was used as a post hoc test after the Kruskal-Wallis test. P-values less than 0.05 were considered statistically significant for all tests. Haplotype analysis was used to evaluate the effect of the genes.

Table 2: The demographic and clinical characteristics of patients with uterine myoma and controls

Variables	Groups		p-Value
	Control (n = 82)	Myoma (n = 103)	
Age (years)	45 (40 - 51)	44 (41 - 52)	0.674
Hemoglobin (g/dl)	13.17 (5.8 - 16.1)	12.53 (11.1 - 14.1)	0.133
Follicle stimulating hormone (mIU/ml)	62 (5.00 - 64.2)	32.46 (0.53 - 81.2)	< 0.001
Estradiol (pg/mL)	29.08 (2.11 - 134.7)	114.94 (7.03 - 563)	< 0.001
BMI	27.72 (19.3 - 43.8)	27.83 (19.1 - 42.3)	> 0.05
Parity	2.0 (0 - 4)	2.0 (0 - 4)	> 0.05
Gravidity	2.0 (0 - 7)	2.0 (0 - 7)	> 0.05

BMI = body mass index

RESULTS

A total of 103 patients with ULM (median age 45; 40 - 51 years) and 82 age matched controls (median age 44; 41 - 52 years) were compared. Age, serum hemoglobin levels, FSH, E_2 levels, BMI status, gravidity and parity numbers are given in Table 2. There were no remarkable differences between control and ULM groups in terms of age (p = 0.674) and serum hemoglobin levels (p = 0.133). FSH and E2 levels were significantly different between two groups (p < 0.001). FSH levels were significantly higher in the control group while E_2 levels were significantly higher in the ULM group.

Genotype distributions of leptin rs7799039 and leptin receptor rs1137101 polymorphisms in control and ULM groups were consistent with the Hardy-Weinberg equilibrium. Genotypes of these distributions are shown in Fig 1 and Table 3. It was determined that GA genotype of leptin rs7799039 polymorphism (p = 0.004, OR = 0.380, 95% CI = 0.173 - 0.837) and AA genotype of leptin receptor rs1137101 polymorphism (p = 0.020, OR = 0.326 CI = 0.144 - 0.736) presented protective effects for ULM development (Table 3). In other words, regarding the GA genotype as reference, GG genotype of leptin rs7799039 polymorphism increased the risk of ULM development by 2,631 times. Furthermore, GG genotype of leptin receptor rs1137101 polymorphism also increased the risk of ULM development by 3.67 times more than AA

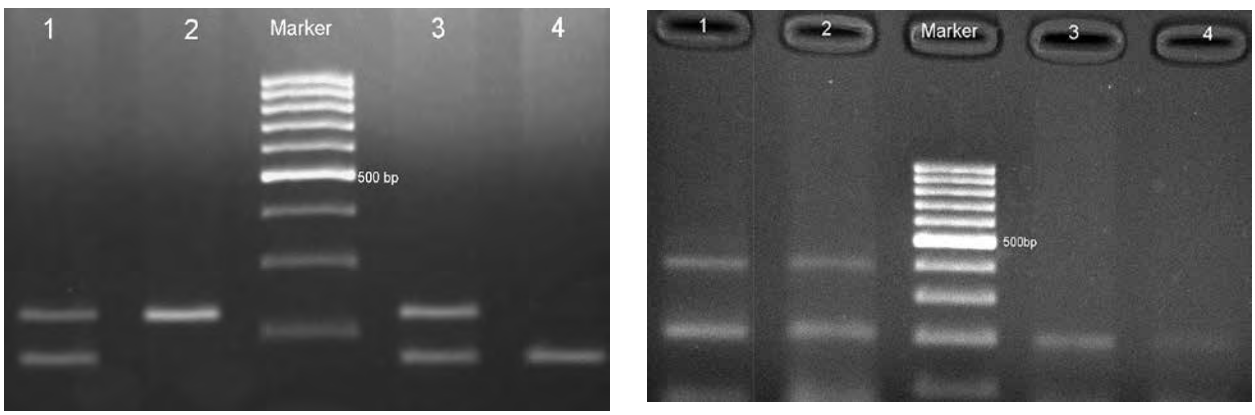

Fig 1. A) shows that Leptin gene rs7799039 polymorphism. 1 and 3 is GA genotypes, 2 AA genotype and 4 GG genotype. **B)** shows Leptin receptor gene rs1137101 polymorphism. 1 and 2 GA genotypes, 3 and 4 GG genotypes

Table 3: Leptin (rs7799039) and leptin receptor (rs1137101) genes genotype frequencies in patients with uterine leiomyoma and controls

Genotype		Groups		X^2 p-value	Odds ratio (95% CI)
		Control (n = 82) n (%)	Myoma (n = 103) n (%)		
Leptin rs7799039	GG	19 (23.2)	30 (29.1)	0.004*	Reference
	GA	35 (42.7)	21 (20.4)		0.380 (0.173 - 0.837)
	AA	28 (34.1)	52 (50.5)		1.176 (0.564 - 2.455)
Leptin receptor rs1137101	GG	24 (29.3)	48 (46.6)	0.020*	Reference
	GA	35 (42.7)	40 (38.8)		0.571 (0.293 - 1.114)
	AA	23 (28.0)	15 (14.6)		0.326 (0.144 - 0.736)

* Statistically significant results; CI = confidence Interval

Table 4: Allele frequencies of leptin and leptin receptor gene polymorphisms in patients with uterine myoma and controls

Allele		Groups		χ^2 p-value	Odds ratio (95% CI)
		Control (n = 82) n (%)	Myoma (n = 103) n (%)		
Leptin rs7799039	Allele G	73 (44.5)	81 (39.3)	0.340	1.238
	Allele A	91 (55.5)	125 (60.7)		(0.817 - 1.876)
Leptin receptor rs1137101	Allele G	83 (50.6)	136 (66)	0.003*	0.527
	Allele A	81 (49.4)	70 (34)		(0.346 - 0.803)

*Statistically significant result; CI = confidence interval

Table 5: Haplotype analysis of leptin-leptin receptors gene polymorphisms in patients with uterine leiomyoma and controls

Haplotype		Groups		Odds ratio 95% CI (Reference)
		Control (n = 82) n (%)	Myoma (n = 103) n (%)	
Leptin / Leptin receptor	AA	51 (31.1)	45 (21.8)	
	GG	43 (26.2)	56 (27.2)	1.476 (0.839 - 2.596)
	GA	30 (18.3)	25 (12.1)	0.944 (0.486 - 1.837)
	AG	40 (24.4)	80 (38.8)	2.267 (1.305 - 3.937)

CI = confidence interval

genotype. Allele frequencies of leptin and leptin receptor polymorphisms in patients with ULM and controls are shown in Table 4. It was found that A Allele of leptin receptor rs1137101 polymorphism showed a protective effect (p = 0.003, OR = 0.537, 95% CI = 0.346 - 0.803). Leptin and leptin receptors gene polymorphisms (rs7799039, rs1137101) were combined for haplotype analysis (Table 5). According to this analysis, AG haplotype increased the risk of ULM development by 2.26 times (OR = 2.267, 95% CI = 1.305 - 3.937).

We investigated the correlation between leptin and leptin receptor genes polymorphisms (rs7799039, rs1137101) and E2 levels in ULM and control group patients. In correlation analysis, leptin and leptin receptor genes polymorphisms (rs7799039, rs1137101) were not found to be related with E2 levels in control group patients (p > 0.005) (not shown in table). However, in ULM group patients, AA genotype of rs7799039 leptin gene polymorphism was found to be significantly related with increased E2 levels (p = 0.032).

DISCUSSION

Many factors accounted for the etiology of ULM but the definite cause and exact pathogenesis are still unknown. The genetic factors which are thought to be involved in the development of ULM are being investigated. Chan *et al*[11] investigated the serum leptin levels in ULM patients and revealed that serum leptin levels were independent of body mass index and were significantly lower in women with ULM than normal women. After that study in 2007, Dingiloglu *et al*[12] examined the influence of leptin in women with ULM and could not find any significant difference in serum leptin levels between ULM patients and controls, but proposed that leptin might have an indirect effect on ULM pathogenesis since many factors might affect serum leptin levels such as body mass index, menstrual phase, dietary fat intake and exercise habits. In the present study, we moved this debate into genetical basis and revealed that leptin and leptin receptor gene polymorphisms might be associated with the risk of ULM development.

Markowska *et al*[8-10] studied the relation between leptin and ULM in three studies. In the first study, they showed the expression of leptin by PCR and Western blotting methods and reported that leptin was expressed in ULM but not in the adjacent normal myometrium, whereas leptin receptors were expressed in tissues of both ULM and normal myometrium. These results were the first statement for the role of leptin in the development of ULM through paracrine or autocrine mechanisms. Right after that study, Markowska *et al*[9] tested the expression of the leptin gene and leptin receptor gene in the myometrium of healthy women, and in myomas and the surrounding myometrium of women with benign tumors. They stated that absence of leptin genes and leptin proteins in the myometrium of healthy women suggests the involvement of leptin in the development of ULMs. Finally, Markowska *et al*[9] aimed to test if treatment with GnRH analogue, which leads to a significant reduction in myoma

volume, changes expression of leptin genes and gene coding leptin receptor isoforms in uterine myomas and in the surrounding unaltered myometrium. GnRH analogue administration to patients with ULM showed that there was no relationship between leptin gene expression in ULM and the levels of estrogen, progesterone and leptin in blood.

In the present study, the genotypes of 103 patients with ULM and 82 healthy controls were examined. We found that the distribution of genotypes of leptin rs7799039 was significantly different with a decreased proportion of GA carriers, and the distribution of genotypes of leptin receptor rs1137101 was significantly different with an increased proportion of GG carriers in the ULM group. Allele A frequency of rs1137101 polymorphism in control group is significantly greater than ULM group. As a result of that, allele A might be a protective factor for ULM. Furthermore, when haplotype of rs7799039/ rs1137101 polymorphisms was analyzed, AG haplotype was found to be significantly increased in patients with ULM. According to our results, leptin and leptin receptor genes polymorphisms [rs7799039, rs1137101] might be associated with the risk of ULM development.

The functional role of leptin in uterus and placenta is still not understood precisely. Ramos *et al*[13] indicated that embryo implantation was disconcerted by the prevention of leptin receptors signaling in mouse endometrium. This finding gave the researchers an impression of an autocrine feature of leptin in reproduction and placentation. It has been later reported that leptin stimulates these procedures by modifying integrin, leukemia inhibitory factor, interleukin 1, vascular endothelial growth factor and metalloproteinases expression[13-15]. Leptin have been found in the human and murine uterus[16,17]. Moreover, expression of leptin gene was detected in the pig uterus[18,19]. Genes and the proteins of the leptin and leptin receptors were found in human and rodent placenta[19,20]. Furthermore, leptin and its receptor were localized in the placental tissues obtained from animals in pregnancy[20]. All these findings confirmed the hypothesis that leptin and leptin receptors in placenta might regulate the functions of this organ.

Eren *et al* reported that A allele of Leptin receptor gene rs1137101 polymorphism showed protective effects against gynecomastia[21]. The common factors in etiopathogenesis of both ULM and gynecomastia, like E_2, might be accused of this concurrent finding. In accordance with this hypothesis, median E_2 levels in gynecomastia patients[21] and in ULM patients in our study were significantly higher than control patients. However, we could not find any significant relationship between A allele of Leptin receptor gene rs1137101 polymorphism and E_2 levels in ULM and control group patients.

Estrogen has an important role in etiopathogenesis of ULM[22] and has been shown to increase the production of leptin in women and also in animal studies[23]. Dingiloglu *et al* reported significantly increased E2 levels together with increased, but not statistically significant, levels of leptin in ULM patients[12]. In our study, E2 levels in ULM group was statistically higher than control group and also in correlation analysis, AA genotype of rs7799039 leptin gene polymorphism was found to be significantly related with increased E2 levels in ULM group patients. In a recent study, it was identified that leptin increased lumbar and renal sympathetic nerve activity only in female rats with high levels of estrogen and had no effect in female rats with low levels of estrogen[24]. This new finding is consistent with our results in which rs7799039 leptin gene polymorphism increases the susceptibility to ULM in patients with increased E2 levels. The results of the present study have yielded that the genotype of leptin [rs7799039] gene polymorphisms with high levels of estrogen and leptin receptor [rs1137101] gene polymorphisms may be linked with ULM pathogenesis.

Recently, many studies examined the associations of Leptin receptor gene polymorphisms with morbid obesity and type II diabetes with obesity. According to those studies, leptin receptor gene polymorphisms were found to be associated with morbid obesity and type II diabetes[25-26]. But according to our knowledge, this is the first study examining the association between leptin and leptin receptor gene polymorphisms and risk of ULM development.

CONCLUSION

The results of the present study have yielded that the genotype of leptin [rs7799039] and leptin receptor [rs1137101] gene polymorphisms might be associated with the risk of ULM development. Utility of this polymorphic variant as a diagnostic or prognostic marker in ULM and determination of the exact mechanisms of estrogen and leptin gene polymorphisms on susceptibility for ULM warrants further randomized, prospective, controlled trials on larger series.

ACKNOWLEDGMENT

Disclosure: The authors declare no competing interest.

Funding: No financial support was received for this study.

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