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Leptin gene $(TTTC)_n$ microsatellite polymorphism in pre-eclampsia and HELLP syndrome

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Abstract

Background: Leptin plays an important role in energy homeostasis. There is polymorphism on the leptin (*LEP*) gene. Our aim was to compare the tetranucleotide repeat $(TTTC)_n$ polymorphism in the 3′-flanking region in the *LEP* gene on DNA samples from patients with pre-eclampsia (PE), hemolysis, elevated liver enzymes, and low platelet (HELLP) syndrome and healthy pregnant controls.
Methods: Blood samples were collected from healthy pregnant women (n=88), patients with PE (n=79) and HELLP (n=77) syndrome. Fluorescent PCR and DNA fragment analysis was performed from the isolated DNA for the detection of (TTTC) repeats. The electrophoretograms were evaluated and patients were assigned to two groups; class I low (<190 bp) or class II high (≥190 bp) PCR fragments.
Results: We observed similar distributions of the class I and class II (TTTC) alleles in the groups studied (class I allele: healthy pregnant 58.5%; severe pre-eclamptic 58.3%; HELLP syndrome 52.6%). We detected a higher frequency of the II/II genotype in HELLP syndrome patients (32.4%) compared to healthy controls (22.7%). However, the difference was not statistically significant.
Conclusions: In an ethnically homogenous population, the *LEP* gene (TTTC) microsatellite polymorphism in the 3′-flanking region does not show a significant difference in the allele and genotype distribution in healthy pregnant, pre-eclamptic and HELLP syndrome patients. Furthermore, we recommend a new classification of the class I and class II alleles based on the distribution of the (TTTC) microsatellites.
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Keywords: hemolysis, elevated liver enzymes, low platelet (HELLP) syndrome; leptin (*LEP*) gene polymorphism; pre-eclampsia; pregnancy.

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Introduction

Leptin (LEP) is a 167-amino acid polypeptide hormone produced in adipose tissue (1, 2). It is secreted into the bloodstream where it circulates bound to other proteins. LEP stimulates or inhibits the production of different neurotransmitters in the brain (3). It is involved in the regulation of energy homeostasis, in the neuroendocrine system and in the reproductive system (4). The expression and secretion of LEP is correlated with body fat content and adipocyte size (5). Elevated LEP concentrations have been found in obese patients (6).

The human *LEP* gene was assigned to 7q31.3 and consists of three exons and two introns ~20 kb in length which encode 3.5 kb of mRNA (1, 7, 8).

LEP plays an important role not only in energy homeostasis, but also in reproduction. It serves as a permissive regulator of reproductive maturity (9–15). LEP also plays a role in the regulation of fetal growth and development (11, 12, 16). An interesting observation is that the placenta and ovaries produce LEP, which then enters the maternal circulation (2, 16). There is further evidence that it stimulates blood vessel formation (1). Also, LEP is reported to enhance vascular endothelial growth factor (VEGF) synthesis in cultured human cytotrophoblast cells (11).

Serum LEP concentrations are higher during pregnancy and peaks in the second trimester (15, 17–19). Shintani et al. (19) identified a highly variable tetranucleotide repeat $(TTTC)_n$ on the LEP 3′-flanking region. Muy-Rivera et al. (8) characterized the polymorphic alleles by size distribution, short repeats (class I allele) and long repeats (class II allele). They classified the alleles into two groups, <160 pb and >160 pb, based on TTTC repeat units. They found correlation between the occurrence of pre-eclampsia (PE) and the presence of the I/II genotype. Contradictions exist among the results of studies on the LEP microsatellite in terms of size and the occurrence of disease. Polymorphic variations of the gene have an effect on obesity, but it is not clear whether it has an impact on the development of cardiovascular disease (20).

PE is a pregnancy-associated syndrome which is a major cause of maternal and perinatal mortality and morbidity. There is a severe form of PE with hemolysis, elevated liver enzymes and low platelets, termed hemolysis, elevated liver enzymes, and low platelet (HELLP) syndrome. Several studies have shown over expression of LEP on microarray experiments in PE (21, 22). We decided to determine the LEP tetranucleotide repeat polymorphism in healthy, pre-eclamptic, and HELLP syndrome patients.

Materials and methods

Patients

Healthy pregnant controls (n=88), patients with severe PE (n=79) and HELLP syndrome (n=77), at the 1st Department of Obstetrics and Gynecology of Semmelweis University were asked to participate in the study. Table 1 shows the primary demographic parameters of the patients. Control patients were consecutively selected from a group of normotensive, healthy pregnant women who were undergoing routine blood tests, and were excluded if they developed hypertensive disorder. Severe PE was defined as new-onset persistent hypertension ($\geq$160/110 mm Hg) and new-onset proteinuria ($\geq$1000 mg/24 h) following the 20th gestational week, in the absence of urinary tract infection (23).

In HELLP syndrome cases the following criteria were defined: hemolysis, classified by increased lactate dehydrogenase activity (>600 IU/L); elevated activity of liver enzymes including increased aspartate aminotransferase and alanine aminotransferase (>70 IU/L); and thrombocytopenia ($\leq$100 platelets $\times 10^9$/L). The Ethical Committee of the Semmelweis University approved the study. All participants were informed and agreed to their involvement in the study.

DNA isolation

Three mL of peripheral blood was collected from each patient into an ethylenediaminetetraacetic acid (EDTA) tube. Genomic DNA was extracted from 0.2 mL of samples using the High Pure PCR Template Isolation kit (Roche, Mannheim, Germany), according to the manufacturer's instructions (24).

Determination of the $(TTTC)_n$ polymorphism

Fluorescent PCR and DNA fragment analyses was performed using the 5′-6-FAM-AGT TCA AAT AGA GGT CCA AAT CA-3′ forward and 5′-TTC TGA GGT TGT GTC ACT GGC A-3′ reverse primer sequences (8). The PCR mix contained 25 μL of the Qiagen Multiplex PCR mix (Qiagen, Hilden, Germany) and 0.3 μM of primers in a final 50 μL volume. PCR was performed for 32 cycles of 15 s at 95°C, 15 s at 55°C and 20 s at 72°C, with an initial denaturation of 10 min at 95°C and a final extension of 10 min at 72°C. Four μL of the PCR products were added to 19 μL of formamid and 1 μL of GeneScan 500-ROX Size Standard (PE Applied Biosystems, Foster City, CA, USA). The mixture was denatured at 95°C for 3 min and cooled to 4°C for 5 min. Electrophoresis was performed using an ABI 3130 Genetic Analyzer with POP7 gel (PE Applied Biosystems). The results were analyzed with Genescan Analysis software (PE Applied Biosystems) (25).

Statistical analyses of the data

Statistical analyses were performed with the STATISTICA software package (version 8; StatSoft, Inc, Tulsa, OK, USA). The statistical significance of the differences between patient groups was evaluated using the Mann-Whitney non-parametric U-test. A $p<0.05$ was considered to be statistically significant.

Pearson Chi-square (χ^2) test was used for comparing groups of categorical data of allele and genotype frequencies. The observed allele and genotype frequencies of the LEP receptor small tandem repeats (STRs) were tested for Hardy-Weinberg equilibrium.

Results

We established a fluorescent PCR and DNA fragment analysis method for the determination of the *LEP* gene $(TTTC)_n$ microsatellite polymorphism. The capillary electrophoresis system allows the assignment of the fragment size with 1 bp size precision. The alleles were assigned into two groups based on the size distribution of the $(TTTC)_n$ tetranucleotide repeats. Shorter repeats (<190 bp) were classified as class I, and longer repeats as class II alleles ($\geq$190 bp) (19, 26).

Table 2 shows the allele size distribution in the healthy controls, pre-eclamptic and HELLP syndrome patients. We observed a lower frequency of class I alleles in HELLP syndrome patients; 52.6% compared to 58.5% in healthy controls and 58.3% in PE. These differences were not statistically significant.

Table 3 shows the genotype distribution of the *LEP* gene in healthy controls, PE and HELLP syndrome patients. The occurrence of the II/II genotype in the PE (20.3%) and HELLP (32.4%) syndrome patients was compared to healthy controls (22.7%) using statistical analysis. These results did not show a significant difference among the groups studied.

Figure 1 shows the electrophoretogram of a sample with genotype I/II, where the representative short and longer alleles were observed.

Figure 2 shows the allele distribution of the (TTTC) microsatellites. There are two exact groups of alleles in each group of patients.

The Hardy-Weinberg equilibrium was calculated using the Hardy-Weinberg equilibrium calculator (www.changbioscience.com/genetics/hardy.html). The rule was not valid for the patient population studied.

Table 1 Clinical characteristic of the study patients.

	Controls n=88	Pre-eclampsia n=79	HELLP n=77	Level of significance (p)
Average age, years	28.1	29.0	28.5	NS
Primipara	42 (47.7%)	38 (48.1%)	41 (53.2%)	NS
Systolic blood pressure	110 (105–120)	170 (140–220)	160 (130–220)	<0.01
Diastolic blood pressure	70 (60–80)	104 (100–115)	102 (90–140)	<0.01
Gestational age at delivery, weeks	40 (39–40)	32 (25–39)	32 (23–39)	<0.05

Mann-Whitney U-test was performed. HELLP, hemolysis, elevated liver enzymes, and low platelet.

Table 2 Leptin $(TTTC)_n$ tetranucleotid allele distribution in healthy pregnant, severe pre-eclamptic and HELLP syndrome patients.

Subjects	$(TTTC)_n$ allele		Level of significance (p)
	Class I	Class II	
Healthy pregnant (n=88)	103 (58.5%)	73 (41.5%)	0.9564[a]
Severe pre-eclamptic (n=79)	92 (58.3%)	66 (41.7%)	0.3174[b]
HELLP syndrome (n=77)	81 (52.6%)	73 (47.4%)	0.2796[a]

Pearson χ^2-test was performed. [a]Healthy pregnant controls compared to severe pre-eclamptic or HELLP syndrome patients. [b]Severe pre-eclamptic compared to HELLP. HELLP, hemolysis, elevated liver enzymes, and low platelet.

Table 3 Leptin $(TTTC)_n$ tetranucleotid genotype distribution in healthy pregnant, severe pre-eclamptic and HELLP syndrome patients.

Subjects	Genotype I/I	Genotype I/II	Genotype II/II	Level of significance (p)
Healthy controls (n=88)	35 (39.8%)	33 (37.5%)	20 (22.7%)	0.7639[a]
Severe pre-eclamptic (n=79)	29 (36.7%)	34 (43.0%)	16 (20.3%)	0.227[b]
HELLP syndrome (n=77)	28 (36.4%)	25 (31.2%)	24 (32.4%)	0.468[a]

Pearson χ^2-test was performed. [a]Healthy pregnant compared to severe pre-eclamptic or HELLP syndrome patients. [b]Severe pre-eclamptic compared to HELLP. HELLP, hemolysis, elevated liver enzymes, and low platelet.

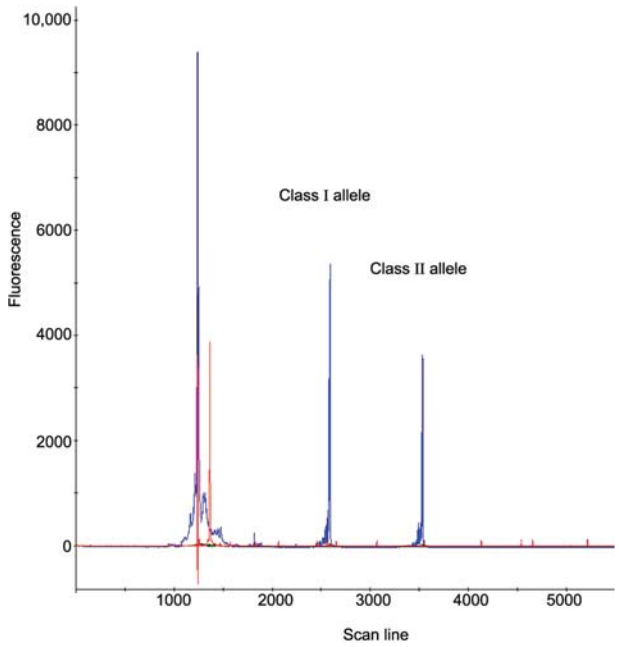

Figure 1 Electrophoretogram showing the detection of leptin $(TTTC)_n$ polymorphism.
PCR fragments with low molecular weight (class I) and high molecular weight (class II) alleles are shown on the Figure, representing a I//II genotype sample.

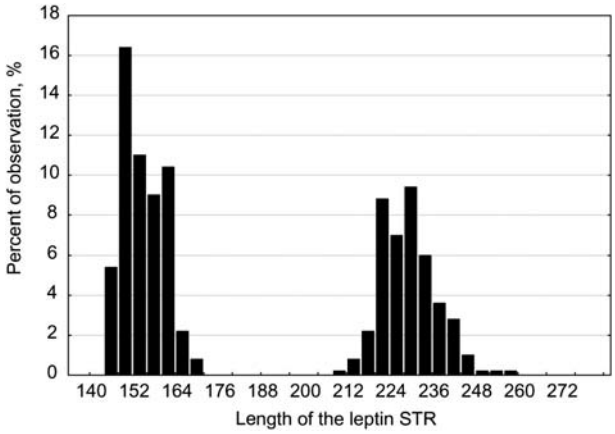

Figure 2 Leptin $(TTTC)_n$ allele distribution in the samples studied.
Figure showing the leptin $(TTTC)_n$ allele distribution in the samples studied. The cut-off at 190 bp is clearly visible.

Discussion

We determined LEP $(TTTC)_n$ polymorphism in healthy pregnant controls, pre-eclamptic and HELLP syndrome patients. We did not find any differences in the distribution of the (TTTC) microsatellite repeats among the different study groups. However, the frequency of the allele class II and the genotype II/II were higher in HELLP syndrome patients.

We classified the alleles based on their molecular weight. Class I had the PCR fragment size <190, while class II had ≥ 190 bp. The fluorescent PCR and DNA fragment analysis makes determination of the LEP $(TTTC)_n$ polymorphism fast and reliable.

Muy-Rivera et al. (8) studied 40 pre-eclamptic and 39 controls and detected higher frequencies of genotype I/II in PE. They did not find any II/II genotype in their DNA samples from pre-eclamptic patients. There are several differences between our study and the above-mentioned American study. One of the differences was that our study involved an ethnically homogeneous population, Caucasian Hungarians, and we had a higher number of cases with PE. Based on a search of the literature, our study is the first performed on the $(TTTC)_n$ tetranucleotide polymorphism in patients with HELLP syndrome. Muy-Rivera et al. (8) discussed discrepancies and inconsistent result in previous publications (19, 27). Porreca et al. (20) studied the relationship between the LEP microsatellite polymorphism and the risk of cardiovascular disease, but they did not find any correlation. They concluded

that LEP microsatellite polymorphism does not appear to have an effect on plasma LEP concentrations, body mass index (BMI), or hypertension. We assume ethnic differences in the frequency of the *LEP* gene (TTTC) microsatellite length should be evident, and it could cause the discrepancies observed in the different groups.

We suggest reclassification of the allele classes used by Muy-Rivera et al. (8). The distribution of the microsatellites shows two peaks. There is a loop from 180 bp to 210 bp. Thus, the two groups are separated better using the new classification with the limit at 190 bp. There should be a consensus for the new classification of I and class II alleles.

Masuzaki et al. did not find any correlation between plasma LEP concentrations and BMI in pregnant women (2). On the contrary, Considine et al. found significant correlation between normal-weight and obese individuals (28). Our department serves as a regional center for high-risk pregnancies. Since our patients receive infusion therapy and other treatments upon arrival to our department, determination of LEP concentrations is not reliable. We are not able to compare our genotypes with the LEP concentration measurements in the population studied.

Some authors have hypothesized and suggested that hyperleptinemia results from insulin resistance (29), tissue hypoxemia (30) or chronic systemic inflammation (31). Numerous authors found higher concentration of LEP in pre-eclamptic pregnancies and a linear relationship between the severity of PE and LEP concentrations (32). It is suspected that this increase may be caused by hypoxia. Lu et al. showed increased serum LEP concentrations and slight decreases in LEP receptor concentrations (33). They interpreted this as due to greater bioavailability of LEP in PE. Muy-Riviera et al. stated that regardless of the mechanisms of the function of LEP, it is a potential marker for risk of PE (8).

Irrespective of the contradictions in the effect of LEP $(TTTC)_n$ polymorphism on different diseases, it remains an exciting target for research. We did not find a difference in the allele and genotype distribution in PE and HELLP syndrome compared to healthy pregnant individuals. It seems to not play a role in the development of this disease. We recommend using a new classification of the two allele subgroups based on their distribution. This may help solve the discrepancies seen in previous studies.

Conflict of interest statement

None of the author has any financial or other conflict of interests. None has been or is presently employed or supported by any company.

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