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ASSOCIATION OF LEPTIN GENETIC POLYMORPHISM -2548 G/A WITH GESTATIONAL DIABETES MELLITUS

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ABSTRACT: ***The aim of this study was to investigate possible associations of -2548 G/A polymorphism in leptin gene promoter and pregnancy-associated diseases with abnormal fetal growth such as preeclampsia and gestational diabetes. The study was also focused on whether it is rather maternal or fetal variants that determines the pathological growth status. Peripheral or cord blood samples obtained from 49 preeclamptic women and their 39 newborns, 53 healthy controls and their 53 healthy newborns and 48 patients with gestational diabetes mellitus were evaluated for leptin gene (LEP) locus -2548 genotypes. The significantly higher risk for gestational diabetes mellitus was observed in the presence of an allele (AA and AG genotypes) against carriers of GG genotype (OR=2.84, 95%CI 1.14-7.07, p=0.02). There is a significant risk of diabetes mellitus associated to A allele (OR=1.79, 95%CI 1.02-3.14, p=0.03). Furthermore, evaluations of preeclamptic patients´ data revealed a significant association of genotype distribution and delivery and spontaneous abortion rate, where the GG carriers performed the highest pregnancy rate while the AG carriers performed the lowest spontaneous abortion rate. Our results support the hypothesis for -2548 G/A leptin gene polymorphism involvement in ethiopathogenesis of pregnancy-associated diseases with abnormal fetal growth, especially gestational diabetes mellitus.***

KEY WORDS: Gestational Diabetes Mellitus, Leptin, Polymorphism, and Preeclampsia

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INTRODUCTION

Leptin, a small peptide produced by adipocytes, is implicated in a great number of endocrine regulations, including obesity, satiety regulation and fertility. This 167 amino acid protein transcribed from the *ob* gene, was originally cloned in the mouse during research directed at identifying the molecular defect in an obesity-prone strain, the *ob/ob* mouse (Zhang Y et al, 1994). Human leptin gene is located to 7q31 and contains three exons. At first, leptin was considered to be a signaling molecule limiting food intake and increasing energy expenditure (Zhang et al, 1994). This was essentially supported by the fact that rodents with genetic (Campfield et al, 1995; Halaas et al, 1995; Pelleymounter et al, 1995; Stephens et al, 1995; Weigle et al, 1995) or diet induced (Campfield et al, 1995) obesity that were injected with leptin had manifested with decrease in bodyweight and improvement of metabolic parameters. Furthermore, hypothalamus has been identified as the most probable critical target for the satiety effect of leptin that can be transported through the blood/brain barrier via a saturable transport system (Baumann et al, 1996; Golden et al, 1997).

Recently, human placentas have been identified as a major source of leptin and the existence of placenta specific upstream enhancer indicates that placenta leptin may be regulated differently to that of adipose origin (Green et al, 1995; Bi et al, 1997; Masuzaki et al, 1997). Furthermore, the placenta leptin localization suggests it could be released into both maternal and fetal blood. The localization of the leptin receptor on the maternal side of the placenta supports the hypothesis that placenta leptin may have an autocrine role on the placenta itself as well as an endocrine role in the mother (Lea et al, 2000). The recent reports suggest that leptin may exert physiological effects on the placenta and conception, including fetal and placenta angiogenesis, fetal growth and development, embryonic hematopoiesis (Holness et al, 1999; Henson and Castracane, 2000).

Taking into account that hyperinsulinemia and hypoxia induce partially overlapping pathophysiological disturbances during pregnancy and both of them are known to induce leptin secretion, we may ask what elements of the leptin promoter are responsible for these effects. Recently, evidence was provided that insulin

and hypoxia act as agonists on the human leptin transcription but on two different regulatory elements. It has been published that hypoxia induces leptin transcription by a hypoxia-inducible-factor-1 (HIF-1) dependent mechanism (Meissner et al, 2003) by identifying at least one hypoxia-responsive element, located -120 bp to -116 bp in the leptin promoter being involved in this HIF-1-mediated effect on the transcriptional regulation. Moreover, it was reported (Grosfeld et al, 2001) that the human leptin promoter carries a potential insulin response element, located in the region from -720 bp to -150 bp. Therefore, it could be suggested that placenta leptin synthesis can be stimulated by the combination of local (e. g., hypoxia) and generalized factors (e. g., hyperinsulinism). This is in agreement with recent finding that leptin gene expression and production are markedly elevated in placenta of diabetic women treated with insulin. The previous findings provide strong evidence that leptin production can be regulated in uteri and emphasize the role of placenta leptin in human pregnancy (Lepercq et al, 1998). In keeping with these findings, disturbances in leptin plasma levels occur not only in diabetic pregnancies with tendency to fetal macrosomia, but also in pregnancies representing the opposite trend – intrauterine growth retardation (IUGR, Jaquet et al, 1998).

As previously described, DNA polymorphisms in leptin gene (LEP) are linked to extreme obesity (Jaquet et al, 1998). Unlike the other polymorphic sites, the G-2548A polymorphism in the 5′ region of the LEP gene was reported not only to be associated with overweight (Clement et al, 1996; Mammes et al, 2000) but also to have a strong influence on leptin gene expression and adipose tissue secretion (Hoffstedt et al, 2002). Thus, we suppose it might also influence leptin levels during pregnancy, especially when taking into account that the polymorphic site is located approximately 1800 bp from the insulin response element within the leptin promoter.

From the personal history of patients whose fetuses suffer from IUGR we knot that some women are prone to have IUGR pregnancy while in others pregnancies with IUGR can alternate with normal birth weight pregnancies. As IUGR can in these cases be considered a maladaptive maternal-fetal genotype, attention was focused on investigating possible genetic background of intrauterine growth restriction in preeclampsia not only on mothers but also on the newborns from these pregnancies.

Based on these observations and the fact the G-2548A polymorphism has proved to influence leptin gene expression, possible associations between the -2548 G/A leptin gene polymorphism variants and pregnancy associated diseases implicating abnormal leptin status such as preeclampsia and gestational diabetes mellitus were set out to be identified.

MATERIALS AND METHODS

Subjects

Forty-nine women (group A; median age 29, age range 19-46 years) with preeclampsia, thirty-nine newborns of these preeclamptic women (group B, median of birth weight 1950 g, birth weight range 700-2300 g, 23 of these newborns were diagnosed intrauterine growth restriction (IUGR)), fifty-three healthy women with non-complicated single pregnancy without positive history of pregnancy or delivery complications and without serious internal disease (group C; median age 28, age range 21-45 years), fifty-three newborns of these healthy women (group D, median of birth weight 3650g, birth weight range 2350-4600 g) and forty-eight pregnant women with gestational diabetes mellitus (group E; median age 30, age range 24-39 years) were enrolled in study. To ensure homogeneity of the genetic background, the healthy controls, originating from a regional Czech population, were enrolled by random selection. Women with polycystic ovary syndrome, hirsutism or menstrual cycle disturbances and women previously treated for infertility were excluded from both the study and control groups.

In nine cases of children coming from preeclamptic pregnancies we did not succeed in obtaining the cord blood sample. All individuals in the study were Caucasians; the pregnant women were followed-up and their children delivered at Gynecology and Obstetrics Clinic, University Hospital Brno, Czech Republic.

All individuals in the study had given informed consent prior to their inclusion in the study expressing their agreement to the fact their blood samples and blood samples of their children would be included in the study. The study was approved by the Committee for Ethics of Medical Experiments on Human Subjects, Faculty of Medicine, Masaryk University Brno.

Glucose and diagnostic criteria for GDM

A standard OGTT protocol was used. After a 12-h overnight fasting, venous plasma samples were collected fasting, 1-h and 2-h post-oral 75-g glucose. The diagnosis of GDM was based on the criteria of the World Health Organization (plasma glucose thresholds mmol/l: 0h, 7.0; 2h, 7.8). This OGTT was performed routinely between 24 and 28 weeks gestation, but occasionally performed at other stages of gestations if clinically warranted. Women with type 1 or type 2 diabetes diagnosed before the pregnancy were excluded from the study.

Diagnostic criteria for preeclampsia

Preeclampsia was defined as the development of hypertension and new-onset proteinuria (>300 mg of urinary protein in 24 h) in women with no proteinuria at baseline. Hypertension was defined according to current guidelines that accept 140 and/or 90mmHg of systolic and diastolic pressure, respectively, or higher, as hypertension, when measured on two consecutive occasions at least 24 h apart. Women with chronic hypertension were excluded from the study.

Intrauterine growth restriction diagnostic criteria

IUGR was defined as infants whose birth weight is below the 10th percentile of birth weight adjusted for sex and

gestational age; children chromosomal abnormalities, fetal infections and multigestations were excluded from the study.

Genotyping of LEP -2548 G/A by PCR-Restriction Fragment Length Polymorphism (RFLP)

After venous blood sample (5-10 ml) or umbilical cord blood sample (1-5 ml) collection from each subject, white cell fraction was used to extract DNA according to standard procedure using proteinase K. The -2548 G/A polymorphism of the LEP gene was analyzed by RFLP as previously described (Mammes et al, 2000). The method was performed by PCR amplification using following primers: forward 5′-TTTCCTGTAATTTTCCCGTGAG and reverse 5′-AAAAGCAAAGACAGGCATAAA.

The PCR was followed by incubation of the resulting 242 bp at 37°C/overnight with *Hin6I* restriction enzyme, an isosquizomer of *Cfo I*. The restricted fragments were separated by electrophoresis on 2% agarose gels with ethidium bromide staining. The polymorphism was defined by presence (G allele) or absence (A allele) of a restriction site. To assess genotyping reliability double sampling in more than 20% of the samples was performed and found no differences. A quality control and negative controls were always used to identify possible false positive.

Statistical Analysis

The differences in genotype and allelic distributions as well as consistency of genotype distribution with Hardy-Weinberg equilibrium were tested using the $\div^2$ test. Statistical differences between mean values of groups were evaluated using the unpaired ANOVA test (Kruskal-Wallis). The observed number of each genotype was compared with that expected for a population in Hardy-Weinberg equilibrium using $\div^2$ test. The cases were analyzed according to age, weight, BMI, parity, delivery rate and spontaneous abortion history. The data analysis was performed using Statistica v. 6.0 (Statsoft Inc., Tulsa, USA) program package. The expected genotype distributions for LEP -2548 G/A under Hardy-Weinberg equilibrium were calculated for both patients and controls and they were compared with the observed distributions. This strategy confirmed whether the observed distributions were consistent with the Haedy-Weinberg equillibrium or not.

RESULTS

Borderline significant differences in genotype distributions between patients with preeclampsia and healthy controls (p=0.06) and patients with gestational diabetes mellitus and healthy controls (p=0.07) were found. This is presented in Table 1 as well as the genotype distributions and allelic frequencies in the two groups of children. The significantly higher risk for gestational diabetes mellitus was observed in presence of A allele (AA and AG genotypes) against carriers of GG genotype (OR=2.84, 95%CI 1.14-7.07, p=0.02).

The main preeclamptic patient characteristics according to LEP -2548 G/A genotypes are shown in Table 2. A statistically significant association between number of pregnancies and genotype distributions was found (p=0.04).

A statistically significant association between the number of spontaneous abortions in anamnesis of the patient and genotype distribution of examined polymorphism (p=0.03) was observed. In Table 3, we present the main personal characteristics of healthy women according to LEP -2548 G/A genotypes. No statistically significant differences were found between LEP -2548 G/A genotypes and age, weight or BMI. No statistically significant association of personal characteristic with a genotype of LEP -2548 G/A polymorphism in the group of gestational diabetes mothers was observed (Table 4).

TABLE 1. The frequency distribution of the genotypes of LEP -2548 polymorphism among mothers and children

MOTHERS	GG	GA	AA	PG	G	A	PA
Preeclampsia	12/24.5%	24/49.0%	13/26.5%		0.490	0.510	
HCMs	21/39.6%	24/45.3%	8/15.1%	0.06*	0.623	0.377	0.02**
GDs	9/18.8%	28/58.3%	11/22.9%		0.479	0.521	
HCMs	21/39.6%	24/45.3%	8/15.1%	0.07*	0.623	0.377	0.04**
GDs	9/18.8%	28/58.3%	11/22.9%		0.479	0.521	
Preeclampsia	12/24.5%	2449.0%	13/26.5%	0.775*	0.490	0.510	0.882**

CHILDREN	GG	GA	AA	PG	G	A	PA
CPG	12/30.8%	20/51.3%	7/17.9%		0.564	0.436	
HCC	15/28.3%	25/47.2%	13/24.5%	0,771*	0.519	0.481	0.543**

HCMs – healthy controls mother, GDs – gestational diabetes mother, CPG – newborn from preeclamptic pregnancy, HCC – healthy control child

** - Analysis for linear trend according to the presence of null, one or two A-alleles*

*** - Chi-square test*

P_g – probability of difference in genotype distributions between preeclampsia and HCMs, GDs and HCMs and GDs and preeclampsia

P_a - probability of difference in allelic frequencies between preeclampsia and HCMs, GDs and HCMs and GDs and preeclampsia

TABLE 2. Preeclamptic women Characteristics and LEP -2548 G/A Genotypes

	AA	AG	GG	P-VALUE
Age /means	29.40±4.24	30.08±4.44	33.80±6.01	0.136
Weight /means	71.00±19.19	73.56±19.18	67.00±11.38	0.579
BMI /means	25.78±6.84	25.80±5.86	24.23±3.88	0.783
Spontaneous abortion rate /median, (min+max)	1.0 (0.0-2.0)	0.0 (0.0-1.0)	0.0 (0.0-1.0)	0.03
Number of pregnancies/median, (min+max)	1.5 (1.0-5.0)	1.0 (0.0-4.0)	2.5 (1.0-6.0)	0.04

LEP – leptin, BMI – body mass index, Mean ± SEM, P- probability of difference in genotype distributions

TABLE 3. Healthy women Characteristics and LEP -2548 G/A Genotypes

	AA	AG	GG	P-VALUE
Age	30.25±7.28	30.04±4.48	26.67±4.09	0.070
Weight	65.75±14.35	70.43±11.19	71.76±11.47	0.581
BMI	23.18±3.75	25.14±3.88	25.66±3.43	0.321

LEP – leptin, BMI – body mass index, Mean ± SEM

TABLE 4. Diabetic women Characteristics and LEP -2548 G/A Genotypes

	AA/MEANS	AG/MEANS	GG/MEANS	P-VALUE
Age	31.63±4.80	30.50±3.98	29.88±1.96	0.686
Weight	77.18±27.54	71.00±17.32	73.77±20.53	0.976
BMI	27.40±9.43	25.38±5.65	26.67±6.66	0.974

LEP – leptin, BMI – body mass index, Mean ± SEM

TABLE 5. Comparative study of Genotype Frequencies in Normal Controls from Different Ethnic Populations

		GENOTYPES				
STUDY GROUP REF.	NO.	AA	AG	GG		P-VALUE
Caucasian-European France	314	63 (20.1)	165 (52.5)	86 (27.4)	0.185*/0.635**	Mammes et al, 2000
Japanese	237	144 (60.8)	86 (36.3)	7 (3.0)	<0.001/<0.001	Hamajima et al, 2002
Caucasian-European Germany	152	24 (15.8)	84 (55.3)	44 (28.9)	0.338*/0.341**	Nieters et al, 2002
Caucasian-European Czech - Mothers	53	8 (15.1)	24 (45.3)	21(39.6)	0.331***	
Caucasian-European Czech - Children	53	13 (24.5)	25 (47.2)	15 (28.3)	referent	

Chi-square analysis comparing reported genotype frequencies with the present study

** - genotype frequencies of mothers*

*** - genotype frequencies of children*

**** - genotype frequencies of mothers against children*

DISCUSSION

GMD represents a heterogeneous disorder with both genetic and environmental component, as women with GMD tend to have more often family history of diabetes and as GMD is more frequent in some ethnic groups, independent of BMI (Dornhorst et al, 1992).

The physiological role of leptin in GMD pregnancy has yet to be entirely clarified. Leptin is known to be produced in large amounts by the placenta, which may explain why some researches described correlation between leptin levels and preeclampsia. However, in most of the studies, the results were not adjusted for maternal age or BMI. Previously, there were several conflicting reports on leptin levels in GMD pregnancy, with authors reporting similar (Simmons and Brier, 2002), reduced (Festa et al, 1999) or elevated (Hoffman et al, 1998) leptin levels in GMD women compared to healthy pregnancy controls.

The -2548 G/A polymorphism was first described as a sequence variant in the 5′ flanking region of the leptin gene (Li et al, 1999) and was previously associated to obesity. In 2002, it was reported that this polymorphism increase gene expression and adipose secretion of leptin (Hoffstedt, 2002).

As large for gestational age (LGA) infants coming mainly from diabetic pregnancies as well as those with IUGR associated rather with preeclampsia were reported to have different leptin expression profiles (Meissner et al, 2003; Jaquet et al, 1998; Hoffman et al, 1998), the possible associations of promoter polymorphism LEP gene -2548 G/A variants and maternal states with abnormal fetal growth such as preeclampsia and gestational diabetes mellitus were investigated.

Based on our data, it can be suggested that the AA and AG genotype carriers (that are supposed to have higher transcriptional activity of the LEP gene) have a significantly higher risk for gestational diabetes mellitus against those carrying the GG genotype, which supports the hypothesis for leptin involvement in ethiopathogenesis of GMD. Furthermore, evaluation of preeclamptic patients′ anamnestic data revealed a statistically significant association of genotype distribution and delivery and spontaneous abortion rate, where the GG carriers had the highest pregnancy rate while the AG carriers had the lowest spontaneous abortion rate. This suggests the leptin could also be involved in fertility regulation.

As it is known that women with a history of GMD have a high risk of progression to type 2 diabetes mellitus, it would be interesting to know whether A or G allele could be protective or risky in respect to type 2 diabetes risk. However, it definitely requires further investigation and patients′ follow-up to estimate whether the GG carriers are in higher risk of in the elderly or not, even if it is known that hyperleptinaemia in type 2 diabetes often goes hand in hand with insulin resistance, which supports the hypothesis leptin is involved in GMD as well as type 2 diabetes ethiopathogenesis. Still, we have to take into account that GMD as well as type 2 diabetes definitely are complex multifactorial disorders with both genetic and environmental component. A number of epidemiological studies examined serum leptin concentration in diabetic and non-diabetic subject. For example, among US Pima Indians, subjects suffering from type 2 diabetes had lower leptin concentrations than non-diabetic subjects, independently of percentage body fat (Fox et al, 1999). Even if it has been discussed previously that insulin resistance associated with GMD results in increased leptin secretion (Malmstrom et al, 1996, Utriainen et al, 1996), the prognosis of GMD patients in respect to type 2 diabetes still remains unclear.

The allelic distribution of -2548 G/A polymorphism of leptin gene promoter varies essentially among the European and Japanese population (Table 5). These findings proved to be consistent with allele frequencies reported recently for the German and French population (Mammes et al, 2000, Nieters et al, 2002). The A allele in the Japanese population showed essentially higher frequency (60.8%, Hamajima et al, 2002) than in the Czech Republic, which confirms previous findings.

A larger clinical study should be undertaken with a larger population sample to investigate the real meaning of correlations between leptin polymorphisms and preeclampsia, IUGR and gestational diabetes, supporting evidence for leptin gene polymorphism as a genetic factor on gestational diabetes risk. This is believed to be the first study focused on association between -2548 G/A leptin gene polymorphism and risk for gestational diabetes or preeclampsia.

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