Placental leptin gene methylation and macrosomia during normal pregnancy

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Abstract. The present study examined the placental leptin (LEP) DNA methylation and mRNA levels in macrosomic infants from normal pregnancies. In total, 49 neonates with macrosomia, i.e., high birth weights of ≥4,000 g, and 52 neonates with normal birth weights between 2,500 g and 4,000 g were recruited from The Second Affiliated Hospital of Wenzhou Medical University (Wenzhou, Zhejiang) in China. Placental LEP promoter methylation and LEP transcript levels were determined by Sequenom MassARRAY and quantitative PCR, respectively. LEP promoter methylation and mRNA levels were not significantly different between the individuals with macrosomia and the controls. However, stratification revealed that individual CpG dinucleotides were hypermethylated in macrosomia ($P<0.05$) in primiparous females and at 39 weeks of gestation ($P<0.05$). Variations in methylation did not affect placental LEP expression. It was concluded that the methylation of the placental LEP promoter was altered during a specific gestational period in macrosomia following a normal pregnancy and under certain conditions. However, placental LEP expression was not affected.

Introduction

Macrosomia is characterized by a high birth weight of ≥4,000 g (1) and has previously been observed in ~10% of newborns in certain regions of China (2). The incidence of macrosomia is increasing (3). Macrosomia increases the risk of fetal asphyxia, shoulder dystocia, birth trauma and neonatal hypoglycemia (4,5). Furthermore, macrosomia is associated with long-term health problems (6). The developmental origin hypothesis (7) indicates that nutrition and other environmental stimuli affect prenatal and postnatal development, causing permanent changes in the metabolism and increasing susceptibility to chronic diseases. Birth weight is considered to be an indicator of risk for developing future metabolic disorders. Numerous studies have documented associations between birth weight and the increased incidence of metabolic diseases (8-10), including insulin resistance (11), obesity (12) and cancer (13).

The placenta has important functions in controlling fetal growth and development. In particular, the placenta functions as a gatekeeper of nutrient and waste exchange between the mother and the developing fetus, and as a regulator of the intrauterine environment (14). An adverse intrauterine environment may affect fetal birth weight and long-term health outcomes (15). Epigenetic mechanisms regulate gene expression and contribute to adverse intrauterine growth and fetal development. Thus, by investigating epigenetic alterations in the placenta we may gain an improved understanding of the molecular mechanisms behind a number of developmental outcomes (16), including macrosomia, which may be affected by intrauterine conditions.

Leptin (LEP), a 16-kDa protein hormone, was initially identified in adipose tissue and is also known to be expressed in placental and fetal tissues. LEP is considered to be a significant fetal growth factor that maintains energy and metabolic balance during pregnancy (17,18). Studies involving rats and humans have shown that LEP is regulated in part by epigenetic mechanisms, specifically DNA methylation. The CpG islands of the LEP promoter region may be subject to dynamic methylation, which may affect LEP gene expression. The dynamic methylation process may be affected by environmental or endogenous factors. A study by Milagro *et al* reported that a high-fat diet altered the methylation pattern of the LEP promoter in rats, and that the methylation of at least one of the analyzed CpG sites was significant in the regulation of leptin transcription in adipose tissue (19). Melzner *et al* (20) also provided evidence that LEP promoter demethylation induces gene transcription in human adipocytes. Additionally,

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LEP methylation levels in the placenta were associated with maternal glycemia during pregnancy in individuals with gestational impaired glucose tolerance (IGT; 2 h post-oral glucose tolerance test, glycemia of >7.8 mmol/l) (21). However, the placental LEP methylation pattern in macrosomia remains unclear. In the present study, differences between placental LEP promoter methylation in infants with macrosomia and infants with normal birth weights who were born to non-diabetic and/or non-hypertensive mothers were examined. Furthermore, the contribution of placental LEP to macrosomia was investigated.

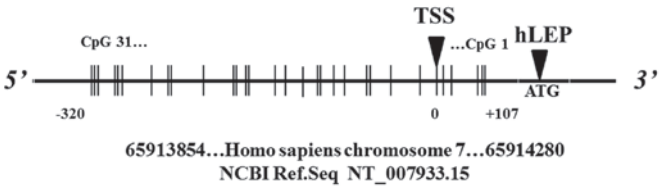

Figure 1. Schematic of the 3' LEP promoter region that was investigated, which spans between -320 and +107 with respect to the TSS. The translation initiation site is also indicated. Black vertical lines represent the 31 CpGs that were identified. The region of human chromosome 7 to which this part of the LEP promoter localizes has been reported and is annotated in the NCBI GenBank database. LEP, leptin; TSS, transcription start site.

Materials and methods

Study population. The subjects were recruited between April 2011 and March 2012 at The Second Affiliated Hospital of Wenzhou Medical University in Wenzhou (Zhejiang), China. The Wenzhou Medical University Ethics Committee approved the study. Informed written consent was obtained from each subject, i.e., the mother. Samples were collected from females between the ages of 18 and 42 years old whose infants were full-term (≥37 weeks), viable without known genetic disorders and from normal pregnancies. Normal pregnancies were defined by a lack of hypertension, hepatitis, heart disease, psychological disorders, gestational diabetes and IGT. Newborns were immediately weighed following delivery. Infants with birth weights ≥4,000 g were considered macrosomic infants. An infant with a normal birth weight was randomly selected as a control within three days of the birth of the macrosomic infant. In total, 101 infants, including 49 macrosomic babies and 52 control newborns, were selected.

Placental sampling. Placental biopsies from 101 deliveries were obtained within 15 minutes of the delivery from mothers who were considered to be full-term. A chorionic villous biopsy (~1 g) was excised, obtained from the maternal side of the placenta 2 cm from the umbilical cord insertion site. Biopsies that were free of maternal decidua were washed and rinsed in sterile phosphate-buffered saline. Biopsies were cut into small sections, suspended at a ratio of 5:1 in RNAlater solution (Ambion, Austin, TX, USA), incubated at 4˚C overnight and stored at -80˚C until nucleic acid extraction was performed.

DNA methylation measurements. Genomic DNA was extracted from placental tissues with the Cell and Tissue DNA kit (BioTek, Beijing, China), according to the manufacturer's instructions. DNA quality was based on purity and concentration, which were determined by measuring the absorbance at 260 and 280 nm. Genomic DNA (200 ng) from each sample was treated with bisulfite using the EZ 96-DNA methylation kit (Zymo Research, Orange, CA, USA), according to the standard overnight bisulfite treatment instructions. A total of 99 DNA samples (excluding two degraded DNA samples) were treated with bisulfite on two 96-well plates. DNA samples from macrosomic and normal-weight newborns were equally distributed on the plates. Samples from male and female infants were also equally distributed on the two plates. The targeted region of the LEP (gene ID, 3952) promoter includes several CpG sites of which the methylation status affects transcription (22) and associates with glucose levels in females with IGT (21). The methylation level was determined with the gold standard Sequenom MassARRAY platform (CapitalBio, Beijing, China). This system combines matrix-assisted laser desorption/ionization time-of-flight mass spectrometry with RNA base-specific cleavage. A detectable pattern was analyzed to determine the methylation status. PCR primers were designed with Methprimer (http://epidesigner.com) and the following primers were used based on the reverse complementary strand of LEP (forward, 5'-ATTTAGAGTTGTGTGGGGTTTTGT-3'; reverse, 5'-CACCTTCCCAAAAAACTAATCCTTA-3') to amplify base pairs 65913854-65914280 of the Homo sapiens chromosome 7 genomic contig, GRCh37.p2 reference primary assembly (NCBI reference sequence, NT_007933.15). A total of 31 CpG sites, which were divided into 16 CpG units, were examined in the LEP promoter, with the exception of the 15th and 18th CpG sites (Fig. 1), which did not exhibit signals. The spectra methylation ratios were generated with Epityper software version 1.0 (Sequenom, San Diego, CA, USA).

mRNA expression measurements. Total RNA was extracted from the placental tissue using TRIzol reagent (cat. no. 15596-026; Invitrogen, Carlsbad, CA, USA). RNA quality was assessed by agarose gel electrophoresis and by measuring the absorbance at 260 and 280 nm. Total RNA (1 μg) was reverse-transcribed with the ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan), following the manufacturer's instructions. LEP mRNA levels were quantified with THUNDERBIRD SYBR qPCR mix (Toyobo) and an Applied Biosystems Step One Plus Real-Time PCR system (Applied Biosystems, Foster, CA, USA). The following PCR primer sequences were synthesized by Sangon (Shanghai, China). LEP (NM_000230.2) forward, 5'-ATTTCACACACGCAGTCAGTCT-3' and reverse, 5'-TCT TGGATAAGGTCAGGATGG-3'. LEP expression levels were normalized to glyceraldehyde-3-phoshate dehydrogenase expression as an internal control. Expression levels were calculated for macrosomic and control babies with the mean ± standard deviation (SD) $2^{-\Delta Ct}$ method (23).

Statistical analysis. The quantitative data are expressed as the mean ± SD. Anthropometric and pregnancy characteristics and DNA methylation levels demonstrated normal distributions and were analyzed by one-way analysis of variance and unpaired t-tests. LEP mRNA expression ($2^{-\Delta Ct}$) did not exhibit normal distribution. Thus, differences in LEP expression between macrosomic and control groups were assessed

Table I. Characteristics at birth and during pregnancy.

Characteristic	Macrosomia (n=49)	Control (n=52)	P-value
Maternal age in years, mean ± SD	28.9±4.2	29.4±4.1	0.556
Gestational age in weeks, mean ± SD	39.2±1.3	39.0±1.1	0.352
Maternal pre-pregnancy weight in kg, mean ± SD	55.8±6.8	53.0±6.8	0.049
Height in meters, mean ± SD	1.60±0.05	1.59±0.04	0.433
Body mass index in kg/m^2, mean ± SD	21.8±2.3	20.9±2.5	0.075
Education status in years, mean (%)			0.593
<6	12 (24.5)	16 (30.8)	
6-12	8 (16.3)	7 (13.5)	
>12	25 (51.0)	29 (55.7)	
Missing	4 (8.2)	0 (0.0)	
Parity, n (%)			0.432
Primiparity	34 (69.4)	31 (59.6)	
Multiparity	15 (30.6)	21 (40.4)	
Birth weight in g, mean ± SD	4307.9±206.6	3526.6±345.8	<0.001
Infant gender, n (%)			0.305
Male	34 (69.4)	30 (57.7)	
Female	15 (30.6)	22 (42.3)	
Alcohol during pregnancy, n (%)			0.593
No	48 (98.0)	50 (96.2)	
Yes	1 (2.0)	2 (3.8)	
Tobacco during pregnancy, n (%)			<0.001
No	49 (100.0)	52 (100.0)	
Yes	0 (0.0)	0 (0.0)	
Weight gain during pregnancy in kg, mean ± SD			
Total	19.5±4.5	17.7±5.6	0.093
1-3 months	3.8±4.0	2.4±2.6	0.052
3-6 months	9.1±8.6	9.3±10.3	0.913
6-9 months	9.3±10.0	9.0±8.9	0.868
Delivery method, n (%)			0.001
C-section	44 (89.8)	31 (59.6)	
Vaginal	5 (10.2)	20 (40.4)	

Tests for differences in specific clinical or demographic factors between the two groups (macrosomia and control). Significant differences in continuous variables were examined by unpaired t-tests and an analysis of variance. Categorical variables were examined by χ^2 test.

with the Mann-Whitney rank sum test, in which the results were presented as the median and interquartile ranges. Similar results were obtained with unpaired t-tests following log transformation. Significant differences in categorical variables were examined by chi-squared test. A statistically significant difference was indicated by P<0.05, and all the P-values reported were two-tailed. Statistical analyses were performed with SPSS version 14.0 software (SPSS, Inc., Chicago, IL, USA).

Results

Sample characteristics. Variations in the methylation levels of the LEP promoter region in 99 placental samples and in the LEP mRNA expression in 101 placental samples obtained from full-term infants were examined. The demographics data of the total study population (n=101) are listed in Table I. Infants were grouped according to birth weights as normal-weight newborns (n=52) and macrosomic infants (n=49). The distributions of maternal age, gestational age, infant gender and alcohol and smoking status during pregnancy were not significantly different between the groups. As expected, females with higher body weights prior to pregnancy tended to give birth to macrosomic babies by cesarean section. The amount of weight gained during pregnancy did not differ between the groups.

LEP DNA methylation. DNA methylation analyses focused on a 426-bp human LEP promoter region, which included 31 cytosine CpG dinucleotides (Fig. 1). Differences in the

Table II. Mean level of methylation of four CpG dinucleotides (only statistically significant data are shown) in the LEP promoter region in macrosomic (n=34) and control (n=31) groups within the primiparity group.

CpG dinucleotides	Macrosomia, % (mean ± SD)	Control, % (mean ± SD)	P-value
CpG1.2.3	65.0±8.0	61.3±7.0	0.045
CpG9.10	21.5±19.0	13.3±9.0	0.034
CpG26.27.28	65.0±8.0	61.3±7.0	0.045
CpG29.30.31	63.6±14.0	55.4±11.0	0.014

P-values obtained by unpaired t-tests. LEP, leptin.

Table III. Mean levels of methylation of seven CpG dinucleotides (only statistically significant data are shown) in the LEP promoter region in macrosomic (n=14) and control (n=17) groups at the gestational age of 39 weeks.

CpG dinucleotides	Macrosomia, % (mean ± SD)	Control, % (mean ± SD)	P-value
CpG4.5.6	60.9±10.0	51.6±6.0	0.045
CpG7	14.0±5.0	8.8±6.0	0.019
CpG8	38.2±8.0	32.1±7.0	0.035
CpG9.10	22.6±15.0	11.8±6.0	0.008
CpG16.17	38.2±8.0	32.1±7.0	0.035
CpG22	18.2±8.0	12.3±6.0	0.025
CpG23.24.25	41.8±11.0	32.9±8.0	0.015
Mean	38.0±7.0	32.0±6.0	0.016

P-value obtained by unpaired t-tests. LEP, leptin.

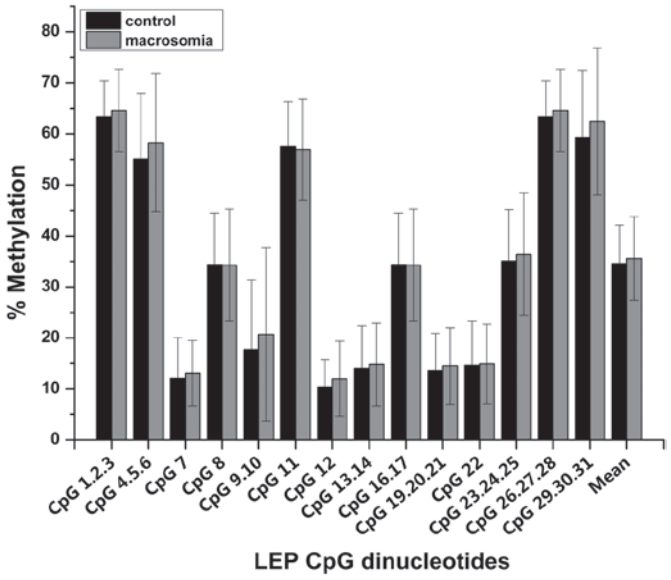

Figure 2. Comparison of the mean levels of methylation in 14 CpG dinucleotides in the LEP promoter regions in macrosomic (n=48) and control (n=51) placentas, as determined by the Sequenom MassARRAY. LEP, leptin.

methylation levels of these 31 CpG sites are shown for macrosomic and control placentas (Fig. 2). The average DNA methylation levels were 35.6% and 34.6% for the macrosomic and control placentas, respectively, which was not significantly different (P=0.538). Similarly, the two groups were not significantly different in the extent of methylation of individual CpG dinucleotides.

A stratified analysis was performed to reduce the effect of heterogeneity. First, the analysis was restricted in parity. Significant differences were identified in the methylation of certain CpG dinucleotides in the LEP promoter of macrosomic and control groups of primiparous females. A higher methylation level was identified in the macrosomic group of primiparous females (Table II). Second, weekly stratification of the gestational age from 37 to 41 weeks demonstrated that no significant differences were identified between the two groups at any gestational age, with the exception of 39 weeks. The mean and individual CpG dinucleotide methylation levels were higher in macrosomic infants than those in the control group at the gestational age of 39 weeks (P<0.05; Table III).

mRNA expression. Quantitative PCR was performed to understand the contribution of placental LEP to infant birth weight. No significant differences in LEP mRNA expression levels were identified between the macrosomic and control groups (Fig. 3). The stratified analysis did not indicate significant differences, although the methylation status was significantly different.

Discussion

LEP promotes the proliferation and invasiveness of trophoblast cells and also affects local angiogenesis. LEP may have an

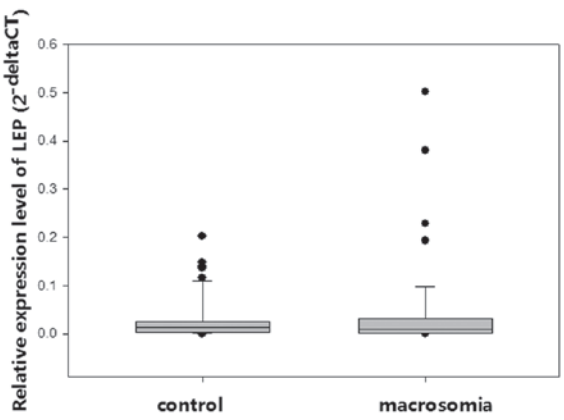

Figure 3. Relative LEP mRNA expression levels ($2^{-\Delta Ct}$) between macrosomic and control groups. Data were normalized to glyceraldehyde-3-phosphate dehydrogenase mRNA levels by the $2^{-\Delta Ct}$ method. The statistical significance of the difference between the two groups was determined by the Mann-Whitney rank sum test. The black lines represent the median values. The lower edges represent the 25th and 75th percentiles and the upper edges represent the 5th and 95th percentiles. The bars and dots inside or outside of the boxes represent the data below the 5th percentile and above the 95th percentile, respectively. LEP, leptin.

affect on the outcome of pregnancy and fetal growth by interfering with placental development (24). In the present study, significant differences were identified in the DNA methylation of individual CpG dinucleotides in the placental LEP promoter at the gestational age of 39 weeks for macrosomic infants who were born to primiparous females. These results indicate that the methylation status of LEP in macrosomia may be altered within a specific gestational period or during certain maternal conditions.

Previous studies have indicated that placental LEP methylation may be altered by certain maternal conditions during pregnancy and lactation, including prenatal famine exposure (25), gestational IGT (21), a low-protein diet (26) or diet-induced obesity (19). Moreover, Hogg *et al* (27) demonstrated that placental LEP is hypomethylated in early-onset pre-eclampsia. However, no significant differences were identified in the mean methylation level of the LEP promoter between macrosomic and normal-weight babies of primiparous mothers. Potential differences in the methylation of individual CpG dinucleotides may have been diluted by the overall number of CpG sites, as methylation changes may not occur at all CpG sites within a promoter. Hogg *et al* (27) conjectured that CpG sites proximal to SP1 or C/EBP binding motifs are more likely to be methylated. In the present study, the methylation variations identified occurred proximal to these motifs, supporting this hypothesis.

In the present study, placental LEP mRNA expression was not significantly different between the groups. These results were consistent with previous findings from dually perfused human placentas (28,29), which indicated that maternal levels of circulating LEP during pregnancy are determined by placental LEP production, but do not correlate with fetal weight. Rather, the fetus was proposed to regulate its own energy metabolism and appetite (30,31). The present results also indicated that placental LEP did not directly contribute to fetal growth. In addition, the results that were obtained in females who had normal pregnancies differed from findings showing that placental LEP expression is upregulated during pregnancy-related pathological conditions, including gestational diabetes (with or without fetal overgrowth) and pre-eclampsia (with or without fetal growth restriction) (32,33). These differences may be explained by the increased demands that are placed on the placenta to deliver nutrients during pathological conditions.

The present results revealed a lack of correspondence between LEP promoter methylation and LEP expression. These data indicated that methylation was not a main factor contributing to placental LEP expression during normal pregnancies. However, LEP expression may be regulated by promoter methylation during other conditions. A previous study identified various regulatory elements within the LEP promoter, e.g., cAMP and glucocorticoid response elements and CCATT/enhancer and SP1 binding sites (34). Methylation at CpG sites proximal to or within these regulatory elements affected LEP expression in adipocytes (20,35) and during pregnancy-related pathological conditions, including IGT (21), early-onset pre-eclampsia (27) and prenatal famine exposure (25). Furthermore, Hogg *et al* (27) observed monoallelic expression of placental LEP during normal pregnancies and a trend towards biallelic LEP expression during early-onset pre-eclampsia. This study also indicated that the loss of normal monoallelic LEP expression was associated with hypomethylation, which increased the overall level of LEP expression. We hypothesize that placental methylation is one of the mechanisms regulating LEP expression. Various degrees of promoter hypomethylation alter the proportion of cells with biallelic expression, causing variations in the overall levels of LEP expression. These variations may exist to adapt to various physiological and pathological conditions.

Epidemiological evidence shows that multiparity is a risk factor for macrosomia, but that primiparity is associated with an increased risk of low birth weight (36). The present study identified hypermethylation of individual CpG dinucleotides in the placental LEP promoter for macrosomic infants of primiparous females. These data may provide an epigenetic basis for the aforementioned epidemiological data discussed. Additionally, a previous anatomical study demonstrated that the proportion of non-muscular tissues increased with parity, such that the first pregnancy caused permanent anatomical changes in the spiral arteries. These changes may modify vascular remodeling during subsequent pregnancies (37). Further research is required to investigate the correlation between variations in LEP promoter methylation and anatomical changes.

In the present study, the mean methylation level and methylation of several CpG dinucleotides were significantly increased in the macrosomia group at the gestational age of 39 weeks. Increased methylation was observed at the SPI and TATA box, which are significant for LEP transcription (35). However, this difference did not appear at other gestational ages. Moreover, LEP mRNA levels were not significantly different between groups according to stratification by gestational age. A similar result was observed in the study by Hogg *et al* (27), in which gestational age was considered a confounding factor for the direct comparison of LEP DNA methylation between controls and early-onset pre-eclampsia

cases. The present results indicated that gestational age was not a predictor of LEP methylation and vice versa.

In conclusion, macrosomia is a multifactorial condition. The present study indicated that placental LEP methylation in macrosomic infants may be affected by maternal conditions or by a specific gestational period. This data provides valuable information with regard to the contribution of placental LEP to macrosomia.

Acknowledgements

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