

ORIGINAL PAPER

Methylation status of leptin receptor gene promoter in obese children

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ABSTRACT

Obesity in children is a growing problem in many countries around the world. Molecular processes have been identified that may lead to epigenetic patterns contributing to obesity, such as DNA methylation in promoters of specific genes. The leptin receptor gene (*LEPR*) has been associated with obesity, and the aim of our study was to evaluate the methylation status of the *LEPR* promoter in the white blood cells (WBC) of 50 children with obesity. Leptin receptor gene promoter methylation was analysed using Combined Bisulfite Restriction Analysis (COBRA). In all patients, the *LEPR* promoter was found to be unmethylated. The results may indicate that methylation of CpG islands in the *LEPR* promoter is not associated with obesity in children.

KEY WORDS:

obesity, children, methylation.

INTRODUCTION

The problem of childhood overweight and obesity has become one of the main challenges for public health in many countries around the world. According to a recent World Health Organization (WHO) report, nearly 41 million children under the age of five years were overweight or obese. If this trend continues, the number of overweight or obese infants and young children globally could rise to 70 million by 2025 [1]. Obesity can affect every system in the body and can lead to many serious and potentially life-threatening conditions including cardiovascular disease, diabetes, and musculoskeletal disorders [2]. Therefore, it is important to elucidate the mechanisms of obesity that would help define novel targets for preventative interventions.

Among the causes of obesity in children and adolescents, genetic, hormonal, and environmental factors have been implicated. There is strong evidence for the involvement of genetic and environmental epigenetic factors in the development of obesity in children [3]. The genetic determinants of obesity have been of interest to researchers for many years. In 1994, the first direct evidence of a genetic role in the formation of obesity was reported in studies of the mouse obesity gene (*ob*) and its product protein leptin [4]. The obesity gene locus was found to be located in humans on the q31.3 section of chromosome 7 [5, 6]. Subsequent studies have identified the leptin receptor as belonging to the family of class I cytokine receptors, and its gene has been cloned [6–8]. Detection of the *ob* gene has demonstrated that adipose tissue performs important regulatory functions

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in the body because the leptin concentration is directly proportional to the body fat mass. Leptin is a multidirectional protein with neurohormonal, metabolic, and immunomodulatory functions [9–12]. Leptin activity at the cellular level is associated with the presence of membrane receptors (Ob-R, also known as *LEPR*) that are widespread in targeted organs (brain tissue, heart, lungs, kidneys, liver, pancreas, intestines, placenta, gonads, spleen, and thymus). The membrane receptor is a glycoprotein similar in structure to the class I cytokine receptors. The gene coding for the leptin receptor is located on chromosome 1p32 and is responsible for the synthesis of at least five isoforms: long (long form-OB-Rb) – which can transmit an intracellular signal, three short (OB-Ra, OB-Rc, OB-Rd), and one isoform of a soluble receptor (OB-Re). Within the OB-Rb isoform, present in the extracellular domain, are binding sites for leptin, and in the transcellular domain, sites of interaction for Janus kinase (JAK) and signal proteins STAT (signal transducers and activators of transcription) [10, 13–17]. The Ob-Re short isoform is a form of the receptor lacking endothelial and intracellular subunits. According to some researchers, it is a secretory isoform deprived of the ability to transduce signal and thus not playing a role in the biological function of leptin and only acting as a transporter (as leptin binding protein) of this hormone in the blood serum, while promoting its biostability. Currently, only long isoforms are considered as functional receptors of leptin, and in particular, the Ob-Rb isoform represented in the hypothalamus, which can participate in the transmission of the cellular signal where the anorectic action of leptin is expressed the most [18]. Leptin has, therefore, a weight-controlling effect, which is conditioned by both the normal structure of the hormone itself and the functionality of its receptor.

Recent years have brought the discovery of epigenetic mechanisms that play a key role in adapting the phenotype under the influence of environmental factors without changing the genetic code. The term epigenetics describes processes that alter gene expression without modifying the primary DNA sequence [19–21]. These changes include DNA methylation, histone modifications (i.e. acetylation, phosphorylation, ubiquitination) and synthesis of non-coding microRNAs (miRNAs). The most common epigenetic change is DNA methylation, which involves the transfer of a methyl group ($-CH_3$) from the donor – most commonly S-adenosyl methionine (SAM) to the carbon atom at the 5-position of the cytosine pyrimidine ring, in a reaction catalysed by methyltransferase DNA [24]. S-adenosyl methionine arises through the direct activation of methionine supplied with food or as a result of homocysteine transformations involving folates. This process takes place with the participation of DNA methyltransferase: DNMT1, DNMT3A, and DNMT3B. Methyltransferases are re-

sponsible for *de novo* methylation and methylation associated with the transmission of epigenetic information to daughter cells arising after mitotic division [23]. In mammals, methylation of cytosine occurs almost entirely within the dinucleotide CpG (cytosine-phosphate-guanine). This cytosine methylation is a frequent method of epigenetic communication used by cells to “turn on and off” genes. Methylation changes the ability to transcribe genes without modifying the basic DNA sequence. Abnormal DNA methylation relies on hypermethylation or hypomethylation of the CpG sequence. Hypermethylation leads to the repression of transcription, while hypomethylation induces the transcription activation in silent genes [22]. Methylation is a process dependent on co-factors and donors of methyl groups.

Leptin plays an important role in body-weight regulation, and this mechanism is due to both the normal structure of the hormone and the function of its receptor. Abnormalities in leptin production and leptin receptor insensitivity often lead to overweight and obesity [24]. The purpose of this study was to determine whether *LEPR* methylation at the promoter region is associated with the occurrence of obesity in children.

MATERIAL AND METHODS

Fifty obese and overweight children hospitalised in the Department of Paediatric Endocrinology were enrolled in the study. In all children, the secondary cause of obesity was excluded. All children were weighed and measured in their underwear three times, and the mean of these measurements was calculated. The measurements were carried out with the use of a RADWAG WPT 60/150 device (RADWAG, Radom, Poland). Body mass index (BMI) was calculated, and children were classified as obese based on age and gender BMI percentiles according to the Polish OLAF study [25]. Overweight was defined as BMI > 85 pc and obesity > 95 pc. Data on overweight and obesity in parents was also collected.

The study group included 26 girls (25 with obesity, one overweight) and 24 boys (16 with obesity, eight overweight) between 4 and 16 years of age (SD 7.67 ± 4.50). The differences in boys' and girls' group sizes were statistically not significant ($p < 0.05$). Also, differences between the ages of both groups were statistically not significant ($p < 0.05$). The average BMI of children participating in the study was 28.7 kg/m² (SD 29.1 ± 9.21).

Some of children in this study were also diagnosed with hypercholesterolaemia (six boys and seven girls) and hypertriglyceridaemia (two boys and two girls). Two children, on the basis of oral glucose tolerance test (OGTT), were diagnosed with impaired glucose tolerance. Insulin resistance was defined in the HOMA-IR (value > 2.67 in boys and > 2.22 in girls before puberty, and at puberty > 5.22 in boys and > 3.82 in girls). Insulin resistance was

TABLE 1. Primer design for PCR-based methylation analysis

Gene name	Primer sequences	Product size (bp)
<i>LEPR</i>	Forward primer: GTTATGTGAAGGTAGATAGG Reverse primer: CAACCCAATACCCTAAACCC	330

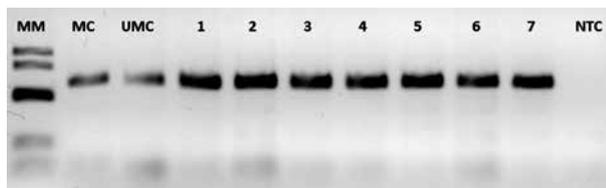


FIGURE 1. Polymerase chain reaction (PCR)-amplification of *LEPR*. Amplicons were analysed by 2% agarose gel electrophoresis. MM: DNA molecular mass marker (500, 400, 250, 100, 50 bp); MC: methylated control DNA; UMC: unmethylated control DNA; 1–7 samples from obese children; NTC: negative template control. PCR product 330 pz

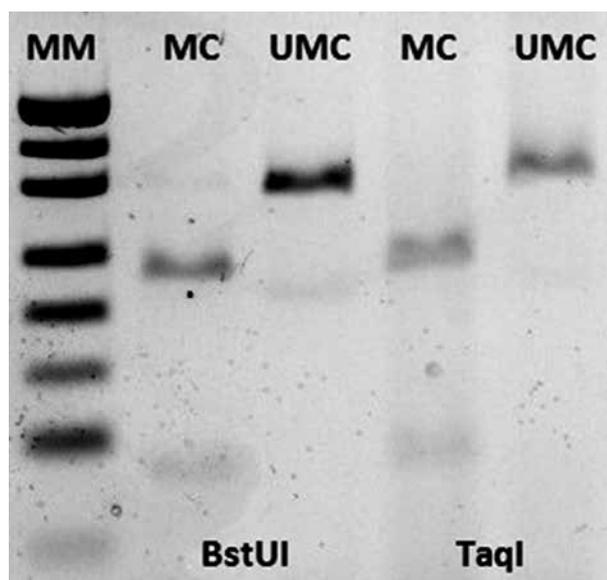


FIGURE 2. Combined Bisulfite Restriction Analysis (COBRA) of *LEPR*. PCR amplicons were subjected to restriction analysis with BstUI resulting in 232 and 98 bp fragments (lane 2, 3), TaqI resulting in 234 and 96 bp fragments (lane 4, 5). MM: DNA molecular mass marker (501, 404, 331, 242, 190, 147, 110, 67 bp); MC: methylated control DNA; UMC: unmethylated control DNA

found in four boys and three girls. In 28 children, obesity also affected their parents; nine of 28 children had both an obese mother and father.

METHYLATION ANALYSIS

Peripheral blood was collected from 50 patients. The leptin receptor promoter methylation (*LEPR*) was analy-

sed using the Combined Bisulphite Restriction Analysis (COBRA) method. The method was designed using the MethPrimer software 2.0, which allows designing primers for bisulphite-conversion-based PCR and predicting CpG islands in DNA sequences [26]. Our results were checked using the BiSearch Primer Design and Search Tool to avoid non-specific amplification products [27]. For the analysis, the QIAamp DNA Mini Kit (Qiagen) was used to isolate and purify DNA from whole blood. Measurement of the concentration and quality of the isolated DNA was performed by a spectrophotometric method. Bisulphite conversion was performed using the EZ DNA Methylation-Gold kit (Zymo Research). Converted DNA (50 ng) was stored at -20°C and amplified using TaKaRa EpiTaq[™] HS (for bisulphite-treated DNA) (Clontech) as follows: $1 \times$ buffer, 0.2 mM dNTPs, 1.75 mM MgCl_2 , 0.5 U polymerase, and 0.5 μM designed primers (Table 1) in a total volume of 20 μl . The optimal conditions were determined to be: initial denaturation at 94°C for 15 min followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 57°C for 45 s, extension at 72°C for 45 s, with a final extension at 72°C for 7 min. PCR amplifications were performed in a (T100[™] Thermal Cycler) thermal cycler (Bio-Rad). The amplification products were separated by electrophoresis through 2% agarose gel (PB Genoplast Biochemicals) and stained with Midori Green Stain (Nippon Genetics). For the promoter of the receptor leptin, a single band of 330 bp was observed (Fig. 1). Next, 5 and 7 μl of PCR product was used to perform a restriction digest of the amplified sequence. EpiTect Control DNAs, completely methylated, and completely unmethylated bisulphite converted DNAs were used for DNA methylation analysis. For the restriction analysis, two restriction enzymes were used: BstUI (ThermoFisher) and TaqI (ThermoFisher). Restriction digestions were performed using 10 U of BstUI and 10 U of TaqI in a total volume of 16 μl at 65°C for 18 h. After this, the reaction was stopped by incubation at 80°C for 20 min. The digestion reaction products (15 μl) were electrophoresed in a 3% high-resolution agarose gel (Blirt DNA) and visualised by Midori Green staining. For the methylated regions, two bands were observed: 98 bp and 232 bp for BstUI or 96 bp and 234 bp for TaqI (Fig. 2).

This study was conducted according to the Declaration of Helsinki on “Ethical Principles for Medical research in Humans” and has the approval of the Ethics Committee of the University of Rzeszow, Poland.

RESULTS

Samples were analysed from 50 children with obesity. Restriction analysis using BstUI and TaqI enzymes did not bind the cutting sites in any sample (Figs. 3 and 4), nor were there differences in the methylation of the *LEPR* promoter region for two sites CpG.

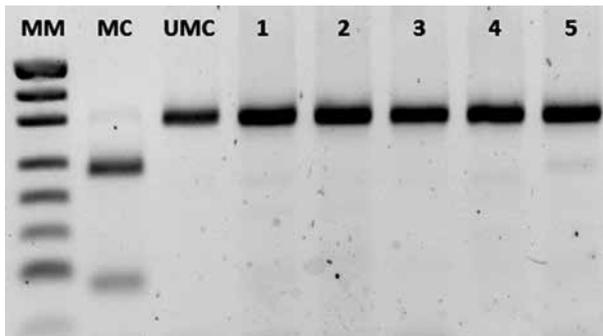


FIGURE 3. COBRA assay using BstUI enzyme to detect methylation of CpG site in the leptin receptor promoter region. The sample 1–5 (lane 4–8), lane 1: MM – DNA molecular mass marker (501, 404, 331, 242, 190, 147, 110, 67 bp); lane 2: MC – methylated control DNA; lane 3: UMC – unmethylated control DNA. No restriction enzyme cutting site, electrophoresis in 3% gel HR agarose

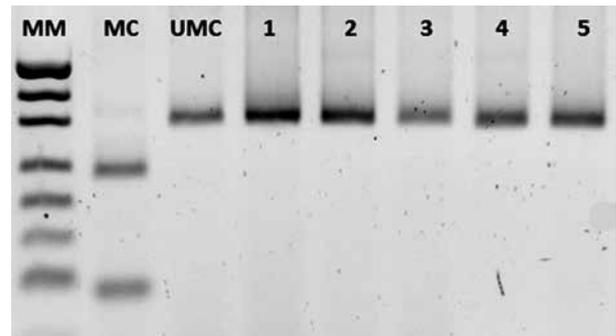


FIGURE 4. COBRA assay using TaqI enzyme to detect methylation of CpG site in the leptin receptor promoter region. The sample 1–5 (lane 4–8), lane 1: MM – DNA molecular mass marker (501, 404, 331, 242, 190, 147, 110, 67 bp); lane 2: MC – methylated control DNA; lane 3: UMC – unmethylated control DNA. No restriction enzyme cutting site, electrophoresis in 3% gel HR agarose

DISCUSSION

Epigenetic regulation of the leptin signalling circuit could be a potential mechanism of leptin function disturbance.

The aim of this research project was to assess the incidence of epigenetic changes in the methylation of the *LEPR* gene in obese children. So far, this problem has rarely been addressed in the literature; most studies have focused on the epigenetic influences in the leptin gene alone.

Recent studies have demonstrated that leptin promoter methylation plays an important role in leptin expression. *In vivo* studies in humans and mice have indicated that leptin promoter methylation is normally imposed during postzygotic development and that this epigenetic mark may function in modulating the leptin expression [29]. Melzner *et al.* reported that CpGs in the proximal leptin promoter was highly methylated in pre-adipocytes. During maturation toward terminally differentiated adipocytes, this promoter region was found to be highly demethylated [28]. This suggests that the methylation of CpGs inhibits leptin expression, whereas demethylation activates leptin expression. Therefore, it was interesting to assess the methylation status of the leptin receptor gene in obese children. Plagemann *et al.* and Krechowec *et al.* suggest that prenatal nutrition can shape future susceptibility to obesity through alterations in leptin sensitivity and changes in energy metabolism during adult life. Humans lacking leptin or functional leptin receptors develop severe obesity and hyperphagia [30, 31]. Moreover, the manipulation of the postnatal diet has been shown to limit the adverse outcomes of foetal programming, as a postnatal diet enriched with n-3 fatty acids prevented pre-programmed hyperleptinaemia and hypertension [32]. Khalyfa *et al.* showed in a mouse model that staying on a high-fat diet in late pregnancy leads to an increased risk of insulin resistance and hyperlipidaemia resulting

from epigenetic changes, i.e. an increase in the methylation of adiponectin and leptin receptors, and a decrease in leptin gene methylation in offspring [34].

In the recent study, Glendining *et al.*, in an animal model, found that diet-induced maternal obesity upregulates *LEPR* expression in the female offspring hippocampus and can alter histone binding at the *LEPR* promoter [33]. In this study *in vitro* IL-6 reproduced epigenetic deregulation of *LEPR* in hippocampal neurons from females and not males.

In our study, we examined the methylation status in the promoter of the leptin receptor. We tested *LEPR* promoter in peripheral blood white cells in obese school children and found no evidence of methylation. In our study, methylation of the *LEPR* promoter region in two CpG sites was not observed. The results obtained by us showed no correlation between the level of methylation of the leptin receptor gene and BMI.

In an analysis of the results of our research, we offer several explanations that might account for the lack of detection of methylated DNA in our study. Perhaps the methylation of other CpG islands in the leptin receptor gene promoter regulates its expression, leading to the exclusion of this gene and, consequently, leptin dysfunction in energy regulation leading to overweight and obesity. It is possible that changes could not be identified because the tested material collected from children was blood and not fat tissue. The most desirable tissue for methylation status studies would be adipose tissue, and more precisely adipocytes. However, the process of obtaining these cells for research remains difficult because the accessibility of tissues such as fat from biopsies is difficult and painful. Dunstan *et al.* measured methylation status in saliva. The results suggest that saliva may be a useful tissue for large-scale studies of epigenetics concerning obesity and the *LEPR* gene. This study showed an association between DNA methylation in each CpG site of the *LEPR* gene with

obesity-related measures in children aged 10–15 years. In addition, parental BMI was associated with both leptin methylation and obesity [36]. High-throughput identification of methylation patterns in various tissues would make it possible to establish genome-wide epigenetic profiles and to correlate them with the vulnerability of an individual to diseases and obesity [35].

LIMITATIONS

The lack of DNA methylation may be due to the selection of a method in which only two CpG sites in the leptin receptor gene promoter were tested. It can be assumed that epigenetic changes may occur in other CpG islands. Another limitation may also be the fact that the material for testing was blood (the DNA was isolated from white blood cells – WBC) rather than adipose tissue. The studies were carried out in a population of children, and perhaps exposure to an unhealthy high-fat diet for a longer time is necessary for the methylation process. It would be warranted to assess the methylation status of the CpG islands in the same patients in several years if they, unfortunately, do not curb their eating habits and are still obese. We also think that the use of a complementary research method such as the microarray method would allow for further verification of our results.

CONCLUSIONS

Our results indicate that the *LEPR* promoter in the CpG islands isolated from WBC is not methylated in obese children. To gain insight into the link between methylation status in the promoter region of the leptin receptor gene in obese children, more detailed studies of the epigenetic changes associated with leptin resistance in obesity are warranted.

DISCLOSURE

The authors declare no conflict of interest.

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