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Effect of three common SNPs in 5′-flanking region of *LEP* and *ADIPOQ* genes on their expression in Polish obese children and adolescents

J. Cieslak · M. Bartz · M. Stachowiak · B. Skowronska · K. A. Majewska · J. Harasymczuk · W. Stankiewicz · P. Fichna · M. Switonski

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Abstract Genes encoding adipokines are considered as candidates for human obesity. In this study we analyzed the expression of leptin (*LEP*) and adiponectin (*ADIPOQ*) genes in relation to common 5′-flanking or 5′UTR variants: -2548G>A (*LEP*), 19A>G (*LEP*) and -11377C>G (*ADIPOQ*) in Polish obese children and adolescents. Relative transcription levels in the subcutaneous adipose tissue (real time RT–PCR) and serum protein concentrations (RIA) were measured in 48 obese subjects with known genotypes at three polymorphic sites and in five non-obese controls. None of the studied polymorphisms altered significantly the expression. Significantly elevated relative transcription levels of the *LEP* gene ($P < 0.05$) and serum leptin concentrations ($P < 0.01$) were recorded in obese patients, when compared with the non-obese controls, but such differences were not found for the *ADIPOQ* gene. Interestingly, the leptin to adiponectin protein concentration ratio (L/A) was approximately sevenfold higher in obese children and adolescents when compared with the non-obese controls ($P < 0.001$). Taking into consideration the observed relationship between the genotypes and the gene expression level we suggest that these SNPs are not conclusive markers for predisposition to obesity in Polish children and adolescents. On the other hand, we confirmed that the leptin to adiponectin gene expression ratio (L/A) is an informative index characterizing obesity.

Keywords Obesity · Leptin · Adiponectin · Gene expression · Leptin/adiponectin ratio · SNP

J. Cieslak · M. Bartz · M. Stachowiak · M. Switonski (✉)
Department of Genetics and Animal Breeding,
Poznan University of Life Sciences, Wolynska 33,
60-637 Poznan, Poland
e-mail: switonsk@jay.up.poznan.pl

B. Skowronska · K. A. Majewska · W. Stankiewicz · P. Fichna
Department of Pediatric Diabetes and Obesity,
Poznan University of Medical Sciences, Szpitalna 27/33,
60-572 Poznan, Poland

K. A. Majewska
Department of Pediatric Nursing, Poznan University
of Medical Sciences, Szpitalna 27/33,
60-572 Poznan, Poland

J. Harasymczuk
Department of Pediatric Surgery, Traumatology and Urology,
Poznan University of Medical Sciences, Szpitalna 27/33,
60-572 Poznan, Poland

Introduction

An incidence of obesity varies within a wide range when different countries or ethnic populations are considered. Recent investigations showed that in several countries (e.g. the USA, Australia, Japan and some European countries) the observed epidemic of childhood and adolescence obesity is leveling off, but generally it still remains at a high, unacceptable level [1]. An excessive deposition of adipose tissue, which plays an important endocrine function, is associated with the role of adipokines (e.g. leptin and adiponectin) in the regulation of energy homeostasis [2, 3]. Thus, the search for adipokine gene mutations has become an obvious target in studies on molecular markers predisposing to obesity. Unfortunately, molecular studies revealed that the incidence of causal mutations of adipokine genes is very low. Until now, only several functional nonsynonymous polymorphisms were identified in the leptin gene [4–6]. On the other hand, studies of adipose tissue are also important since the adipocytes are

considered as a promising source of stem cells for potential use in regenerative medicine [7].

An altered endocrine function of the adipose tissue, associated with obesity, can also be caused by an abnormal level of gene expression due to mutations in regulatory sequences of genes encoding adipokines. Analysis of the transcription profile in the fat tissue originating from obese and non-obese subjects revealed wide differences in terms of the number of up- and down-regulated genes (for review see: [8]). There are also reports indicating an association between obesity and some polymorphisms in the 5′-flanking regions of the leptin (*LEP*) and adiponectin (*ADIPOQ*) genes; however, the conclusions are not consistent [9–13]. In our recent studies of obese children and adolescents we detected 9 polymorphic sites in the 5′-flanking region of the *LEP* and 2 in the 5′-flanking region of the *ADIPOQ* genes [14], but the frequency of these variants did not differ in a cohort of obese patients when compared with non-obese ones. The effect of polymorphism in the 5′-flanking regions of genes encoding adiponectin and leptin was reported in several studies. The following polymorphisms were considered: -2548G>A (*LEP*), -11377C>T, -11391G>A (*ADIPOQ*) [12, 15–17]. Also polymorphism in 5′UTR of the *LEP* gene (19A>G) was investigated with regard to the circulating leptin level [18, 19].

The aim of this study was to compare the transcription level of the *LEP* and *ADIPOQ* genes in the subcutaneous adipose tissue, as well as serum leptin and adiponectin concentrations, in Polish obese children and adolescents with different genotypes at three polymorphic sites in the 5′-flanking or 5′UTR sequences: -2548G>A (rs7799039) (*LEP*), 19A>G (rs2167270) (*LEP*) and -11377C>G (rs266729) (*ADIPOQ*). Moreover, the expression level of both genes as well as the leptin to adiponectin ratio (L/A) were compared in obese patients and non-obese controls.

Materials and methods

Patients

Altogether 53 blood and subcutaneous adipose tissue samples were collected from patients (48 obese children or adolescents and five non-obese children as the control) of the Department of Pediatric Diabetes and Obesity, Poznan University of Medical Sciences (Poznan, Poland) and the study was approved by the local Bio-Ethics Committee at the Poznan University of Medical Sciences. The cohort of obese patients consisted of 23 boys and 25 girls (mean age: 11.9 ± 3.3). The control group consisted of five healthy and non-obese children. After recording of basic parameters (age, gender, body mass and height) their relative BMI (RBMI) was calculated as described before [20]. Mean RBMI for obese patients was 164.2 ± 20.6, while in case of controls was below 110.

Molecular and statistical analyses

Total RNA from the subcutaneous adipose tissue was isolated using an RNasy Lipid Tissue Mini Kit (Qiagen, USA) according to the manufacturer's protocol and 1 μg of RNA was used for reverse transcription (cDNA synthesis). The relative transcription level of the *LEP* and *ADIPOQ* genes was measured (by real-time PCR) in duplicate using a SybrGreen (Roche) detection (LightCycler 2.0, Roche). The relative mRNA abundance was normalized to the *GAPDH* reference gene which reveals high expression stability in human adipose tissue and is commonly used in studies of adipokines genes [21, 22]. For real-time PCR amplification we used the following primer pairs: ADIPOQ-F-ccatctcctc ctcacttcc, ADIPOQ-R-atgaccgggcagagctaata, LEP-F-aaccct gtgcggattcttgtgg, LEP-R-ccggtgactttctgtttggaggag, GAPDH-F- gagccacatcgctcagacac and GAPDH-R-catgtagttgag gtcaatgaa. The primers were designed with the use of Primer3 software (http://frodo.wi.mit.edu/primer3/).

Cycling conditions for all amplified genes were: 95°C for 10 min (preliminary denaturation), 40 cycles of 95°C for 10 s (denaturation), 63°C for 2 s (annealing) and 72°C for 6 s (elongation) each. Serum adiponectin and leptin concentrations in all investigated patients were measured with the application of radioimmunoassay (RIA) using commercial kits (Millipore, USA).

For statistical analysis the R package (version 2.12.0) (http://www.r-project.org/) was applied. Differences in leptin and adiponectin relative mRNA levels, serum proteins concentrations as well as in leptin to adiponectin ratio (L/A) between lean and obese subjects were measured using the Wilcoxon Rank Sum Test. The *LEP* and *ADIPOQ* gene expression between patients carrying different genotypes in the 5′-flanking polymorphic sites was compared in additive, dominant and recessive models using Kruskal–Wallis or Wilcoxon Rank Sum Test. Estimated *P*-values were adjusted for multiple testing using Bonferroni correction.

Results

Analysis of the correlation between *ADIPOQ* and *LEP* genes expression, measured at mRNA and protein concentration level, as well as between their expression and RBMI value did not show highly significant results, putatively due to a relatively small number of investigated patients. The strongest correlation ($r = 0.28$; $P = 0.05$)

was found for relative mRNA (*LEP)* level and serum concentration of leptin. In addition, correlation between adiponectin serum concentration and RBMI was close to significancy ($r = -0.27$; $P = 0.06$).

A comparison of relative transcription levels in subcutaneous fat depots as well as the analysis of LEP and ADIPOQ protein serum concentrations revealed the significantly elevated expression of the first in the group of obese children and adolescents. Such differences were not observed for adiponectin mRNA and protein. However, we found that the leptin to adiponectin serum concentration ratio (L/A) was significantly (approximately sevenfold, $P = 0.000311$) higher in the group of obese patients. This ratio was also higher (approximately 20-fold, $P = 0.0216$) when the relative mRNA level was concerned (Table 1).

For all the 48 patients genotypes at five polymorphic sites (three in *LEP* and two in *ADIPOQ*) in the 5′-flanking regions of both genes were established in our earlier study [14]. The comparison of gene expression, including all the three genotypes (two alternative homozygotes and a heterozygote) was possible only for the three most common SNPs, i.e. -2548G>A, 19A>G (*LEP)* and -11377C>G (*ADIPOQ).* No significant differences were found in any of the studied polymorphisms between the genotypes (Table 2). The lowest relative mRNA level, as well as serum leptin concentration, were observed for the GG (19A>G) and AA (-2548G>A) genotypes. On the other hand, the 5′-flanking polymorphism (-11377C>G) in the *ADIPOQ* gene showed the lowest expression (mRNA and serum protein) for the GG genotype.

Discussion

The expression of adipokine genes in obese subjects is usually altered—elevated in case of leptin and decreased for adiponectin [23, 24]. Our observations concerning leptin gene expression in obese and non-obese patients confirmed this relationship. In case of adiponectin the observed differences were not significant, may be due to a small control group of non-obese children ($n = 5$) included in our study. However, it should be pointed out that some discrepancies between adiponectin mRNA and protein levels were recently described also in the subcutaneous adipose tissue collected from obese Tunisians [25]. The authors observed significantly decreased serum adiponectin level in overweight and obese subjects whereas at transcription level no significant differences were noticed.

An abnormal gene expression level can be caused by polymorphisms in 5′-flanking sequences, thus adipokine gene polymorphisms may alter their expression and thus are considered as potential candidates contributing to obesity predisposition. The studied polymorphisms, due to their occurrence within or in a close vicinity to binding sites for transcription factors, may play a functional role. Earlier studies showed that haplotype including polymorphic site -11377C>G and two other SNPs in the *ADIPOQ* gene promoter can interfere with NKXH, CART and zinc–finger binding sites and thus has a potential impact on serum adiponectin concentration [26]. The -2548G>A polymorphism in the leptin gene is not localized within the consensus site for any known transcription factor, but it was suggested that this SNP may affect the function of a

Table 1 A comparison of leptin and adiponectin gene expression between obese subjects and the control group of non-obese children

Measurement	Obese children and adolescents ($n = 48$) Median (1st; 3rd quantile)	Control group ($n = 5$) Median (1st; 3rd quantile)	P value[a]
Relative mRNA leptin level (adipose tissue)	0.037 (0.025; 0,079)	0.006 (0.001; 0.006)	**0.0296**[b]
Serum leptin concentration (ng/ml)	21.850 (16.875; 32.025)	3.580 (3.470; 4.510)	**0.000276**
Relative mRNA adiponectin level (adipose tissue)	0.034 (0.025; 0.055)	0.031 (0.019; 0.075)	0.9152
Serum adiponectin concentration (μg/ml)	4.590 (3.205; 8.650)	6.110 (4.640; 7.710)	0.5842
Relative mRNA leptin to adiponectin level ratio (L/A)	1.280 (0.717; 1.925)	0.061 (0.060; 0.327)	**0.0216**[b]
Serum leptin to adiponectin concentration ratio (L/A)	4.288 (2.508; 8.714)	0.623 (0.568; 0.973)	**0.000311**

[a] Based on Wilcoxon Rank Sum Test, significant differences are shown in bold

[b] Non-significant after Bonferroni correction for multiple testing (adjusted P value = 0.0083)

Table 2 Expression of the leptin and adiponectin genes in obese subjects, in relation to genotypes at three polymorphic sites

Genotype	Relative mRNA level in subcutaneous adipose tissue median (1st; 3rd quantile)	Serum concentration median (1st; 3rd quantile)
LEP 19A>G		ng/ml
AA	0.044	29.650
($n = 4$)	(0.035; 0.085)	(24.600; 33.175)
AG	0.052	28.850
($n = 22$)	(0.031; 0.077)	(19.250; 33.850)
GG	0.030	18.650
($n = 22$)	(0.016; 0.077)	(16.025; 25.325)
P value[a]	0.162	0.189
***LEP* -2548G>A**		ng/ml
AA	0.035	19.000
($n = 11$)	(0.027; 0.061)	(16.450; 25.150)
AG	0.033	20.800
($n = 23$)	(0.024; 0.081)	(14.100; 32.050)
GG	0.045	29.050
($n = 14$)	($\pm$0.034; 0.077)	(20.775; 33.650)
P value[a]	0.739	0.395
***ADIPOQ* -11377C>G**		μg/ml
CC	0.040	6.180
($n = 29$)	(0.027; 0.047)	(3.100; 8.680)
CG	0.033	3.920
($n = 15$)	(0.023; 0.058)	(3.615; 6.300)
GG	0.018	3.430
($n = 4$)	(0.012; 0.035)	(2.688; 5.045)
P value[a]	0.297	0.452

[a] Estimated differences were non-significant also if dominant or recessive models were applied

binding site for the Sp1 transcription factor which is localized in the neighborhood [27]. Also several studies of 19A>G polymorphism, localized in 5'UTR of the *LEP* gene, indicated its association with serum leptin concentration [18, 19]. Interestingly in our study both SNPs in the leptin gene were in a strong linkage disequilibrium ($D' = 0.91$). In obese patients four haplotypes were observed with the following frequencies: -2548A/19G (0.455), -2548G/19A (0.299), -2548G/19G (0.233) and -2548A/19A (0.014) but analyses of their putative association with leptin gene expression, at mRNA and protein levels, did not show any significant results ($P > 0.05$).

Our recent study showed that the above mentioned SNPs are also distributed in Polish children and adolescents [14]. Thus, we decided to analyze the relationship between the observed genotypes and gene expression level, but we did not find any significant association. However, there is a report showing reduced level of circulating leptin in extremely obese (BMI $\geq$ 40) French patients with the GG genotype at 19A>G site [18]. Such a relationship was not confirmed in comparative studies of African-American and Caucasian cohorts [19].

The effect of the -2548G>A polymorphism in the *LEP* gene on its expression also remains unclear. In a study of Brazilian women a positive relationship between the G allele and plasma leptin concentration was reported [11]. A similar tendency was observed in obese Tunisians [16]. On the other hand, there are reports indicating that allele A is related with the elevated leptin expression. Such a relationship was also found in Polish obese adults [28] and healthy Greek women [15]. In our studies the highest expression was observed in GG patients, but the differences were not significant.

In case of the *ADIPOQ* gene polymorphism (-11377C>G) there are several reports indicating a decreased expression of the adiponectin gene in GG subjects [17, 18, 29]. Also in our studies the lowest expression level in GG patients was observed, but the differences were again not significant.

An interesting observation concerns the ratio of leptin to adiponectin serum concentrations (L/A). This ratio was significantly elevated in obese patients than in non-obese subjects. It was already reported that the leptin to adiponectin ratio is an informative marker of atherosclerosis in obese patients with type 2 diabetes [30]. Later it was suggested that the L/A ratio may also be considered as a useful biomarker of insulin resistance [31] and a parameter characterizing the hormonal profile of obese children and adolescents, as well as a predisposition to obesity and the metabolic syndrome in normal-weight patients [32]. Our observations also showed that the L/A ratio for serum concentration is significantly elevated in obese children and adolescents. A similar tendency was observed with regard to the relative mRNA level, but the ratio was only two-fold higher in obese subjects.

Concluding, our study showed that the studied SNPs are not conclusive markers for predisposition to obesity in Polish children and adolescents. On the other hand, we confirmed that the L/A expression ratio in serum (protein) and subcutaneous adipose tissue (mRNA) is an informative index characterizing obesity.

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