The Expression of LEP, LEPR, IGF1 and IL10 in Obesity and the Relationship with microRNAs

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Abstract

Obesity is a multifactorial disease, with epigenetic alterations. Have been described modifications in the expression of some microRNAs, and some proteins related to obesity. The objective was to determine and correlate, in obese patients, the gene expression of LEP, LEPR, IGF1, IL10 and of miR-27a, miR-27b, miR-143 and miR-145. RNA was extracted from biopsies of subcutaneous fat, liver and visceral fat of 15 obese subjects submitted to bariatric surgery and of 15 non-obese subjects submitted to cholecystectomy for cDNA synthesis and for RT-PCR. The microRNAs were chosen using the TargetScan software. An increased expression of LEP and IGF1 was detected in the subcutaneous fat of the obese group compared to control, while the expression of IL10 was higher in the control group than in the obese one. MiRNA-27a had a higher expression in the omentum of the obese patients and there was also a correlation in the expression of miRNA-145 and LEPR in the omentum of this group.

Introduction

Obesity is a public health problem that has greatly increased over the last decade. It is accompanied by a series of diseases such as type 2 diabetes mellitus, cardiovascular diseases, and increased visceral fat, among others, which characterize the metabolic syndrome [1–3]. It is also characterized by an increased size and number of adipocytes and is considered to be a chronic inflammatory state since the concentration of many pro inflammatory cytokines increases with increased body fat [4,5]. Adipose tissue is formed by different cell types and has an endocrine function since it secretes substances such as leptin (LEP), insulin-like growth factor 1 (IGF1) and interleukin 10 (IL10) [6–9].

Studies have shown that LEP is a protein hormone mainly synthesized by white adipose tissue which acts through a specific receptor and is directly related to the quantity of adipose tissue [10]. Obese individuals have hyperleptinemia and resistance to leptin, which is caused by changes in leptin receptors [10]. Obese individuals have hyperleptinemia and resistance to lepin receptors (LEPRs) as well as in its isoform or in deficiencies in the blood-brain transport system [11].

The study of the role of IGF1 in cell metabolism has permitted us to associate it with obesity. This is a hormone that plays important roles in the organism, among them the regulation of adipose tissue mass, mediating the proliferation and differentiation of adipocytes during adipogenesis [12]. A study by Grégoire-Nyomba et al. [13] has suggested that both LEP and IGF1 play a role in the regulation of body weight.

Another factor related to obesity is IL10, with a reduction of its concentration being related to the presence of metabolic syndrome [14]. IL10 is an anti-inflammatory cytokine with an important role in the regulation of the immunological system by inactivating pro inflammatory cytokines through the suppression of macrophage function, with a consequent important role in the immunity of the gastrointestinal tract [15].

More recent discoveries have related changes involving some microRNAs to obesity. microRNAs are small non-protein coding RNAs consisting of 18 to 25 nucleotides which regulate gene expression in a negative and post-transcriptional manner [16,17]. They have an important role in the regulation of various biological processes, including adipocyte differentiation, metabolic integration, insulin resistance, and appetite regulation, inhibiting the expression of target genes [18,19].

Tang et al. [20] have suggested that miR-124a is involved in pancreatic cells, acting as a possible regulator of glucose in blood together with miR-375. In addition, it was demonstrated that both miR-143 and miR-145 are involved in cell differentiation [21,22]. The microRNAs miR-143, miR-145, miR-27a and miR-27b were selected for inclusion in this study because, with the use of bioinformatics tool TargetScan (http://www.targetscan.org/), we verified that miR-27a and miR-27b had the genes of LEP, IGF1 and IL10 as targets. We also add the other microRNAs because some other studies showed that miR-143 interferes in glucose metabolism in mice, inducing insulin resistance, and that miR-145 is correlated with human body mass index [23,24].
Study the possible relationship of miR-27a, miR-27b, miR-143 and miR-145 with leptin, leptin receptors, IGF1 and IL10 is important to try to understand the changes that occur in obesity and its physiopathology.

Methods

The study was approved by the Research Ethics Committee of the University Hospital, Faculty of Medicine of Ribeirão Preto, University of São Paulo (HCFMRP/USP) and all patients gave written informed consent to participate (HCRP n° 8052/2010).

Patients

The study was conducted on 15 obese patients with an indication of bariatric surgery according to the criteria of the International Federation for Surgery of Obesity, who were operated upon at HCFMRP/USP [25]. The control group consisted of 15 non-obese individuals submitted to video laparoscopic cholecystectomy at the State Hospital of Ribeirão Preto (HERP). Biopsies of subcutaneous tissue, liver and visceral fat were obtained from all subjects and snap frozen in liquid nitrogen (table 1).

RNA extraction

The RNA extraction was performed using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. All RNA samples were quantified by spectrophotometry (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. For such, we used the following primers: has-miR-143, has-miR-145 has-miR-27a, has-miR-27b (Applied Biosystems). The primers sequences are presented in table 3. The RNU24 and RNU48 genes were used as endogenous controls (housekeeping) for miRNA reactions.

miRNA qPCR analysis

The cDNA was synthesized using 2.5 μg of RNA. The specific looped RT primers for miR-143, miR-145, miR-27a and miR-27b and reagents were included in the High Capacity c-DNA archive Kit (Applied Biosystems), and these components were incubated with 3.8 μU of RNase Inhibitor (Applied Biosystems) for 30 μmin at 16°C, 30 μmin at 42°C, 5 μmin at 85°C, and then held at 4°C. The system TaqMan Assay-on-demand (Applied Biosystems, Foster City, CA, USA) was used to analyze genes: LEPTIN (Assay ID Hs00174877_m1), INTERLEUKIN 10 (Assay ID Hs00174497_m1), LEPTIN RECEPTORS (Assay ID Hs00174497_m1), INTERLEUKIN 10 (Assay ID Hs00174877_m1), and INSULIN-LIKE GROWTH FACTOR 1 (Assay ID Hs00961622_m1). Complementary DNA (cDNA) was synthesized using 2.5 μg of RNA per sample, using a thermociclator to perform DNA denaturation, 30 minutes at 42°C for the strand extension, 5 minutes at 85°C to the enzyme deactivation and then, at 4°C. For the real time PCR, the conditions were pre-heating at 50°C for 2 μmin, denaturation at 95°C for 10 μmin, followed by 40 cycles at 95°C for 15 μs and 60°C for 1 μmin in an ABI Prism 7500 Sequence Detection System using TaqMan Reaction Master Mix (Applied Biosystems), in accordance with the manufacturer’s instructions. For such, we used the following primers: has-miR-143, has-miR-145 has-miR-27a, has-miR-27b (Applied Biosystems). The primers sequences are presented in table 3. The RNU24 and RNU48 genes were used as endogenous controls (housekeeping) for miRNA reactions.

Data were analyzed (for both genes and miRNAs) using the ABI-7500 Sequence Detection System software and the variation of expression among samples was calculated by the 2^ΔΔCt method.

Statistical Analysis

Numerical variables between groups were compared with the Mann-Whitney test. Spearman’s or Pearson’s correlation test were used to verify the correlation between gene expression and microRNAs. Was used GraphPad PRISM software, version 5.0 (GraphPad Software Inc., San Diego, CA, USA) for statistical analysis. Significance was set at p<0.05.

Results

The 15 obese patients had a BMI ≥ 36.8 μg/kg/m² and an abdominal circumference ≥ 119 μcm and the 15 control subjects had BMI ≤ 26.62 μg/kg/m² and an abdominal circumference ≤ 87 μcm. The average expression and p values are shown in Tables 4 and 5, respectively.

Gene expression

As shown in table 4, the gene expression of LEP in subcutaneous fat was significantly increased in the obese group compared to control (p = 0.00, Mann-Whitney test), whereas no difference was

Table 2. Values of Ct B-ACTIN.

|                         | Subcutaneous Fat | Visceral Fat | Liver |
|-------------------------|------------------|--------------|-------|
| Obese                   | 27.54            | 28.54        | 29.47 |
| Control                 | 31.58            | 26.86        | 28.67 |

Table 3. Primer sequences used for the microRNA’s study.

| miRNA | Primer sequences       |
|-------|------------------------|
| miR-27a | UUCACAGUGCCUAAGUCCGC  |
| miR-27b | UUCACAGUGCCUAAGUCCGC  |
| miR-143 | UGAGUAUGACGACUGUAGGC  |
| miR-145 | GUCAGUUUCUCAGGAUCCCC  |

Table 1. Characteristics of obese and control patients.

|                         | OBESE | CONTROL |
|-------------------------|-------|---------|
| Age (years)             | 42.6  | 39.86   |
| Waist circumference (cm)| 131.4 | 78.13   |
| BMI (kg/m²)             | 47.46 | 24.04   |
| Men                     | 2     | 3       |
| Women                   | 13    | 12      |

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observed between groups in the expression of this gene in the liver and the omentum (p = 0.43; p = 0.21, respectively, Mann-Whitney test) (table 4). The gene expression of LEPR in subcutaneous fat, liver and visceral fat did not differ significantly between groups in the tissues tested (p = 0.10; p = 0.12; p = 0.55, respectively, Mann-Whitney test) (table 5).

The gene expression of IGF1 was higher in the subcutaneous fat of the obese group compared to control (p = 0.04, Mann-Whitney test), and in the visceral fat of the control group compared to the obese group (p = 0.00, Mann-Whitney test). No significant difference between groups was observed in the liver (p = 0.26, Mann-Whitney test) (table 5).

The gene expression of IL10 in subcutaneous fat did not differ between groups (p = 0.56, Mann-Whitney test). Similarly, its expression did not differ between groups in the liver and visceral fat (p = 0.18; p = 0.90, Mann-Whitney test) (table 5).

microRNA expression
The expression of miR-27a was higher in the visceral fat of the obese group compared to control (p = 0.01, Mann-Whitney test) but did not differ between groups in subcutaneous fat (p = 0.33, Mann-Whitney test) or in the liver (p = 0.47, Mann-Whitney test) (table 5).

The expression of miR-27b in subcutaneous fat, liver and visceral fat did not differ between the obese and control groups (p = 0.43, p = 0.40, p = 0.27, respectively, Mann-Whitney test) (table 5).

The expression of miR-143 in subcutaneous fat, liver and visceral fat did not differ between the obese and control groups (p = 0.19, p = 0.56, p = 0.94, respectively, Mann-Whitney test) (table 5).

Similarly, the expression of miR-145 in subcutaneous fat, liver and visceral fat did not differ between the obese and control groups (p = 0.10, p = 0.74, p = 0.98, respectively, Mann-Whitney test) (table 5).

Correlation
Was only observed a negative correlation between miR-145 and LEPR gene expression in the visceral fat of obese patients (r = -0.5780, and p = 0.0304) (figure 1). The p values for the correlations of the microRNAs with the genes, in the studied tissues, are showed on Tables 6, 7 and 8.

| Table 5. P values of the comparison of gene expression in the studied tissues between obese patients and the control group. |
|-----------------|-----------------|-----------------|
|                 | Subcutaneous Fat | Visceral Fat     | Liver            |
| LEP             | <0.01           | 0.21            | 0.43            |
| LEPR            | 0.18            | 0.53            | 0.12            |
| IGF1            | 0.04            | <0.01           | 0.26            |
| IL10            | 0.56            | 0.90            | 0.18            |
| miR-27a         | 0.33            | <0.01           | 0.47            |
| miR-27b         | 0.43            | 0.27            | 0.40            |
| miR-143         | 0.19            | 0.84            | 0.56            |
| miR-145         | 0.10            | 0.98            | 0.74            |

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Discussion
Obesity is a growing worldwide public health problem accompanied by health complications such as the development
of type 2 diabetes mellitus, cardiovascular problems and sleep apnea, among others. Taken together, these factors characterize the metabolic syndrome [28]. The syndrome also involves genetic, hormonal, inflammatory, environmental and behavioral dysfunctions [28]. In order to contribute to the elucidation of the molecular pathways involved in the pathogenesis of obesity, we used real time PCR to quantify the expression of the genes LEP, LEPR, IGF1 and IL10 and of the microRNAs: miR-27a, miR-27b, miR-143 and miR-145 in the abdominal subcutaneous fat, the liver and omentum of individuals with morbid obesity of the central type compared to non-obese individuals.

The leptin (LEP) hormone is mainly synthesized in white adipose tissue and is related to the quantity of body fat. It acts on the control of food intake through specific receptors, LEPR located in the hypothalamus and in peripheral organs such as the liver. This hormone acts on the central nervous system, promoting reduced food intake and energy expenditure [29]. In a study on the blood of obese and lean individuals, Pardina et al. [30] observed higher LEP levels in morbidly obese individuals compared to lean individuals. The authors also reported that LEP levels were reduced one year after bariatric surgery, becoming identical to those of the control group. Esteghamati et al. [31] also observed that obese individuals with metabolic syndrome have higher leptin levels, suggesting that hyperleptinemia is associated with this syndrome.

In the present study, as expected, we observed a higher LEP expression in the subcutaneous fat of the obese group compared to control, whereas no difference was observed in the liver or omentum. The profiles of gene expression of subcutaneous abdominal fat and of the omentum are different, including the expression of some proteins, LEP among them [32]. Studies conducted on samples of abdominal subcutaneous fat and omentum have shown that the gene expression of LEP is higher in the abdominal subcutaneous fat, a result observed both in men and in women and in obese and normal weight individuals [33–37]. Since we did not detect a significant difference in LEP expression in the omentum of the obese group compared to control, we may assume that this was due to the fact that the highest production of LEP occurs in subcutaneous. In addition, Carmina et al. [38] observed that LEP expression decreases with increasing BMI both in the omentum and in the visceral subcutaneous fat. We may also suggest that another reason for the lack of a difference between groups in the expression of this gene in the omentum is that obese individuals have a lower mRNA production for this protein in the omentum and that this production is further reduced with increasing BMI.

Elevated circulating leptin concentration has been associated with obese patients with insulin resistance compared to normal weight individuals. In contrast, the circulating levels of leptin receptors are lower in obese individuals than in normal weight individuals. Also, the proportion of free circulating levels of LEP and LEPR is significantly higher in obese than in non-obese individuals [39]. Séron et al. [40] showed that expression of mRNA (long isoform) was elevated in subcutaneous fat of lean women and diminishes around 70% in the obese group. In visceral fat of both groups, the expression of this receptor is low. In the present study we did not detect a significant difference in the gene expression of LEPR in the obese group compared to control in any of the tissues analyzed. A possible explanation for this observation is the probably small number of individuals studied.

Obesity is also known to be associated with abnormal IGF1 secretion. A 1997 study by Nam et al. [41] detected no significant difference in total IGF1 between lean and obese individuals, but showed a higher free IGF1 concentration in the obese group. Gomez et al. [42] observed a lower IGF1 concentration in obese individuals, as also reported later by Pardina et al. [30]. The present results showed that IGF1 is more expressed in the subcutaneous fat of obese subjects compared to control, whereas its expression is higher in the omentum of the control group. In the liver there was no difference in expression compared to control patients.

Another change detected in obesity is a chronic low grade inflammation, a state characterized by increased inflammatory markers. Literature data have demonstrated that circulating IL10 levels are higher in obese women than in lean women and are lower in women presenting any component of the metabolic syndrome [14]. In another study, Bassols et al. [43] observed higher serum IL10 concentrations in obese individuals compared to controls. Changes in circulating IL10 concentration have been demonstrated in other studies in which polymorphisms of the promoter region of its gene reduce this concentration [44]. It has also been reported that rats with LEP and IL10 deficiency have a lower body weight gain than rats having only the absence of LEP [45].

In the present study we did not detect a difference in IL10 expression between groups. Since the obese group studied here had central obesity, which is considered to be a component of metabolic syndrome, we expected to find a lower expression in these individuals compared to control, as observed in the study by

| Table 6. P values of the correlation between micro RNAs and genes in the subcutaneous fat of the groups. |
|---------------------------------|---------|---------|---------|---------|
| miR-27a | miR-27b | miR-143 | miR-145 |
| LEP     | 0.72    | 0.24    | 0.26    | 0.35    |
| LEPR    | 0.27    | 0.33    | 0.48    | 0.61    |
| IGF1    | 0.73    | 0.07    | 0.09    | 0.06    |
| IL10    | 0.36    | 0.43    | 0.83    | 0.83    |

| Table 7. P values of the correlation between micro RNAs and genes in the liver of the groups. |
|---------------------------------|---------|---------|---------|---------|
| miR-27a | miR-27b | miR-143 | miR-145 |
| LEP     | 0.60    | 0.24    | 0.70    | 0.53    |
| LEPR    | 0.12    | 0.33    | 0.10    | 0.31    |
| IGF1    | 0.11    | 0.07    | 0.10    | 0.14    |
| IL10    | 0.25    | 0.43    | 0.59    | 0.42    |

| Table 8. P values of the correlation between micro RNAs and genes in the visceral fat of the groups. |
|---------------------------------|---------|---------|---------|---------|
| miR-27a | miR-27b | miR-143 | miR-145 |
| LEP     | 0.77    | 0.55    | 0.47    | 0.84    |
| LEPR    | 0.52    | 0.09    | 0.97    | 0.03    |
| IGF1    | 0.68    | 0.25    | 0.16    | 0.38    |
| IL10    | 0.40    | 0.21    | 0.15    | 0.22    |

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11. Romero CEM, Zanesco A (2006) The role of leptin and ghrelin on the genesis of diabetes mellitus, suggesting that this microRNA is related to adipose tissue dysfunction. Kim et al. (2007) studied this same microRNA in rats and observed that its expression is increased in white adipose tissue of subcutaneous fat but is reduced during adipocyte differentiation. Studies have demonstrated that miR-27a is less expressed in the subcutaneous fat of individuals with type 2 diabetes mellitus [48]. In the present study we observed a higher expression of miR-27a in the omentum of the obese group compared to control. Although there was a lower expression of miR-27a in the omentum of the obese group and no differences of the expression of LEP between groups, the correlation test didn’t show any relationship between this microRNA and LEP, when there was expected a negative correlation, mainly because LEP is one of the targets of miR-27a. Like miR-27a, miR-27b also participates in metabolism, Ji et al. (2010) observed in a culture of rat hepatic stellate cells that these two microRNAs regulate in a negative manner the metabolism of fat and the proliferation of this cell type. In our study the expression of miR-27b did not differ between the group of obese individuals (subcutaneous fat, liver and omentum) and non-obese (control) individuals. Still regarding microRNAs in obesity, increased miR-143 expression is known to be associated with increased body weight and with increased mesenteric fat [50]. A study carried out in order to determine the expression of this miR by microarray in samples of human subcutaneous adipose tissue demonstrated that its expression is increased in differentiating fat cells [46]. Another microRNA related to cell differentiation and proliferation is miR-145. La Rocca et al. (2011) demonstrated that miR-145 expression is increased during cell differentiation. A 2009 study conducted on cell cultures of pre-adipocytes and differentiating adipocytes of obese individuals demonstrated that the expression of miR-145 was greatly reduced during cell differentiation [23]. In the present study, the expression of miR-143 and miR-145 did not differ between obese individuals (subcutaneous fat, liver and omentum) and non-obese controls. We expected to detect an increased expression of miR-143 and miR-145 in all tissues of the obese group compared to control since these microRNAs are related to increased cell differentiation and proliferation, but this was not the case. On the other hand, there was a negative correlation between the expression of miR-145 and LEP in the omentum of obese patients. In a critical evaluation of this article, the limited number of patients included in each group, mainly because of the limited grant dedicated to it, must have interfered in the statistical analysis. New study with more patients is now being planned and also with the possibility to include other microRNAs. On the basis of the present data, we may conclude that there was a negative correlation between the expression of miR-145 and LEP in the omentum of obese patients. This allows us to affirm that miR-145 may play a role in the regulation of obesity. New studies, with larger casuistic, are needed to confirm the real involvement of the microRNAs with obesity.

Author Contributions

Performed the experiments: RVAC WSJ DPCT JSS. Analyzed the data: RVAC WSJ DPCT. Contributed reagents/materials/analysis tools: RVAC WSJ DPCT JSS. Wrote the paper: RVAC WSJ.
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