

Research Article

Polymorphism in *LEP* and *LEPR* May Modify Leptin Levels and Represent Risk Factors for Thyroid Cancer

Marjory Alana Marcello,¹ Antonio Ramos Calixto,²
Jacqueline Fatima Martins de Almeida,¹ Mariana Bonjorno Martins,¹
Lucas Leite Cunha,¹ Camila Ayume Amano Cavalari,¹ Elba C. S. Etchebehere,³
Ligia Vera Montalli da Assumpção,⁴ Bruno Geloneze,²
Andre Lopes Carvalho,⁵ and Laura Sterian Ward¹

¹Laboratory of Cancer Molecular Genetics (Gemoca), Faculty of Medical Sciences, University of Campinas (FCM-Unicamp), Rua Tessalia Vieira de Camargo 126, 13083-970 Campinas, SP, Brazil

²Laboratory of Investigation on Metabolism and Diabetes (LIMED), Faculty of Medical Sciences, University of Campinas (FCM-Unicamp), Rua Carlos Chagas 420, 13083-878 Campinas, SP, Brazil

³Service of Nuclear Medicine, University of Campinas, Rua Vital Brasil 251, 13083-888 Campinas, SP, Brazil

⁴Service of Endocrinology, University of Campinas, Rua Vital Brasil 251, 13083-888 Campinas, SP, Brazil

⁵Department of Head and Neck Surgery, Barretos Cancer Hospital, Rua Antenor Duarte Vilela 1331, 14784-400 Barretos, SP, Brazil

Correspondence should be addressed to Marjory Alana Marcello; marjoryam@gmail.com

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Purpose. To understand the role of polymorphisms in the *LEP* (rs7799039 and rs2167270) and *LEPR* (rs1137101 and rs1137100) genes in DTC susceptibility and their effect on leptin levels. **Methods.** We studied 153 patients with DTC and 234 controls through TaqMan SNP Genotyping and ELISA, comparing these data to the clinicopathological data of patients with DTC. **Results.** Patients with AA genotype of rs7799039 had higher levels of serum leptin (9.22 ± 0.98 ng/mL) than those with AG genotype (10.07 ± 0.60 ng/mL; $P = 0.005$). Individuals with AG genotype of rs2167270 also produced higher serum leptin levels (10.05 ± 0.59 ng/mL) than the subjects with GG genotype (9.52 ± 0.79 ng/mL; $P < 0.05$). A multivariate logistic regression adjusted for gender, age, and BMI showed that the AG genotype of rs7799039 was an independent risk for DTC (OR, 11.689; $P = 0.0183$; 95% CI, 1.516–90.119). Similarly, AG and GG genotypes of rs1137101 increased the susceptibility to DTC (OR, 3.747; $P = 0.027$; 95% CI, 1.161–12.092 and OR, 5.437; $P = 0.013$; 95% CI, 1.426–20.729). **Conclusions.** We demonstrated that rs7799039 and rs2167270 polymorphisms modify the serum leptin concentrations in patients with DTC. Furthermore, polymorphisms rs7799039 and rs1137101 increase the risk of DTC development, although they do not correlate with tumor aggressiveness.

1. Introduction

The mechanisms underlying the relationship between cancer and obesity have not been established and may vary according to the primary tumour location [1, 2]. These variations may be attributed to the metabolic and endocrine effects that obesity may exert on the metabolism, hormones, inflammation, and protein production [3], thus leading to the development and progression of cancer.

Our group has previously demonstrated that cytokines produced by adipocytes (adipokines), such as adiponectin, leptin, and resistin, are differentially expressed in malignant and benign thyroid nodules, helping to differentiate not only malignancy but also subtypes of thyroid nodules, which are difficult to identify using PAAF [4]. Of the three adipokines, leptin presents as an interesting diagnostic option for thyroid malignancy; leptin has a 100% accuracy, as well as high sensitivity and specificity. Additionally, the serum leptin

levels differed in follicular lesions, which is currently the primary diagnostic challenge for pathologists, introducing a fascinating clinical perspective to the diagnosis of thyroid malignancy using a simple and robust blood test [4].

Physiological mechanisms may influence the synthesis of leptin and thus lead to variations in the amounts of leptin associated with the fat mass [5]. Notably, several cytokines, such as tumour necrosis factor alpha (TNF- α), increase the expression of leptin through mRNA synthesis [6]. Additionally, recent evidence has indicated that the modulation of leptin's gene expression might be related to the presence of polymorphisms in *LEP* and *LEPR* (leptin receptor) gene [7, 8]. The polymorphisms in these genes have been recently related to an increased susceptibility to several types of cancer, such as prostate, breast, gastric, and lung carcinomas [9–13]. Hence, we hypothesised that polymorphisms in *LEP* and *LEPR* could lead to an increased risk for differentiated thyroid cancer (DTC), thus establishing the link between the observed epidemiological increases in both thyroid cancer and obesity rates.

The objective of the present study was to investigate the polymorphisms in *LEP* and *LEPR* genes in patients with DTC, correlating the obtained data with previously measured leptin serum levels, DTC risk, and clinicopathological features.

2. Subjects and Methods

2.1. Patients. This study was approved by the Research Ethics Committee of the participating centres. All of the participants signed the informed consent forms. We investigated 153 patients (130 females, 23 males, 40.87 ± 13.80 years) diagnosed with DTC and 234 healthy controls (204 females, 30 males, 37.61 ± 13.39 years) with no significant differences in gender, age, and body mass index (BMI). Seventy-four of 153 patients included in this study had been previously described in another report and had serum leptin levels measured [4].

The patients were consecutively referred to the Thyroid Cancer Unit, Division of Endocrinology, Unicamp Teaching Hospital in Campinas, São Paulo, Brazil, and to the Head and Neck Department of the Barretos Cancer Hospital, Barretos, São Paulo, Brazil, between 2009 and 2011, for thyroid nodule diagnostic evaluations. They were submitted to interviews, using a structured in-person questionnaire that allowed the recollection of information, such as demographic, social, economic, and xenobiotic exposures, food intake, and previous disorders. The questionnaire has been used in several publications by our group in recent years [14–17].

To classify BMI, height was measured with the individuals standing barefoot on a stadiometer with a centimetre scale. Weight was measured using a balance with division of 100 g, with all of the individuals wearing light clothes and no shoes. BMI was calculated using the formula $(\text{weight})/(\text{height})^2$. All of the participants were classified using the WHO recommendation for nutritional status as follows: normal weight, $18.5\text{--}24.99 \text{ kg/m}^2$; overweight, $\geq 25\text{--}29.99 \text{ kg/m}^2$; and obese, $\geq 30 \text{ kg/m}^2$ [18]. Underweight patients ($< 18.49 \text{ kg/m}^2$) were excluded from the study because we could not obtain underweight controls.

Anatomopathological data, including nodule size, tumour histological features and image, as well as laboratory data were retrieved from the patients' medical records. All thyroid cancer histological samples were reviewed for diagnostic confirmation by a thyroid pathologist (J. Vassallo). Chronic lymphocytic thyroiditis, investigated in the nonmalignant parenchyma of the contralateral thyroid lobe, was characterised by extensive lymphocytic infiltration with lymphoid follicles, scarring, and follicular regenerative activity in the form of numerous small follicles, frequently lined by Hurthle cells.

Thyroid cancer patients were monitored using periodic total body scans and serum TSH and thyroglobulin (Tg) measurements, according to a standard protocol based on the American Thyroid Association and Latin American Thyroid Association recommendations [19, 20], which included X-ray, ultrasonography, computed tomography scans, and other timely and necessary procedures for detecting distant metastasis for a period of 18–56 months (40.8 ± 16 months). The aggressiveness of the cancer at the time of diagnosis was ascertained using the TNM stage classification system for differentiated thyroid carcinoma [21]. The patients were also classified into low, intermediate, and high risk groups, according to the ATA guidelines [19]. The patients who presented with suspicious or high serum Tg levels ($> 2 \text{ ng/dL}$) underwent intensive imaging procedure. We defined tumours as recurrent and long-distance metastasis according to the above-mentioned parameters. The patients with thyroid cancer were classified as disease-free when they maintained serum Tg levels $< 2 \text{ ng/dL}$ and exhibited no clinical or image suspicion of disease for at least 12 consecutive months after surgery. There were 80 patients (63.5%) who were free of disease and 46 patients (37.5%) with recurrence. Twenty-seven patients could not be classified into any of these two groups and were excluded from any analysis involving outcome. No patient died of the disease during our follow-up.

Healthy individuals included in the control group presented neither history of malignancy in the family nor familial history of thyroid diseases. These individuals were blood donors of the Hematology and Hemotherapy Center, located in the University of Campinas.

2.2. Serum Analysis. The 74 individuals investigated for serum leptin levels have undergone similar blood collection procedures, based on the previously described method [22–24]; that is, 4 mL of whole blood was collected in red plastic tubes that had no anticoagulant factors and contained a gel with intermediate density between blood cells and serum. The serum samples were then separated by centrifugation at 3000 rpm for 10 min and stored at -20°C , until analysis could be performed. We used commercial ELISA assays to quantify the circulating leptin levels (R&D Systems, Minneapolis, MN, USA).

2.3. Genotype Analysis. Approximately 4 mL of peripheral blood was collected into EDTA (ethylenediaminetetraacetic acid) coated tubes. Genomic DNA was extracted from leucocytes separated from whole blood, using a standard

TABLE 1: Comparisons of different histopathological types according to their leptin serum expression.

Analysed groups	Leptin	P
Malignant versus benign	9.89 ± 0.63 versus 1.92 ± 0.69	<0.001
CPTC versus goiter	9.69 ± 0.89 versus 1.88 ± 0.73	<0.001
CPTC versus FA	9.69 ± 0.89 versus 2.11 ± 0.47	<0.01
CPTC versus FC	9.69 ± 0.89 versus 9.49 ± 0.50	N.S.
FVPTC versus CPTC	10.02 ± 0.57 versus 9.69 ± 0.89	N.S.
FVPTC versus goiter	10.02 ± 0.57 versus 1.88 ± 0.73	<0.001
FVPTC versus FA	10.02 ± 0.57 versus 2.11 ± 0.47	<0.001
FVPTC versus FC	10.02 ± 0.57 versus 9.49 ± 0.50	N.S.
FC versus FA	9.49 ± 0.50 versus 2.11 ± 0.47	<0.001
FC versus goiter	9.49 ± 0.50 versus 1.88 ± 0.73	<0.01
FA versus goiter	2.11 ± 0.47 versus 1.88 ± 0.73	N.S.

CPTC: classic type PTC; FVPTC: follicular variant PTC; FC: follicular carcinoma; AF: follicular adenoma; N.S.: not statistically significant.

protocol, which includes leucocyte lysis, SDS treatment, phenol-chloroform extraction, and ethanol precipitation.

The DNA samples were genotyped for *LEP* (rs7799039 and rs2167270) and *LEPR* (rs1137101 and rs1137100) genes using TaqMan SNP genotyping assays (C_1328079_10, C_15966471_20, C_8722581_10, and C_518168_20, resp.) (Applied Biosystems, CA, USA) in the 7500 *Real Time* PCR System (Applied Biosystems, CA, USA). The reactions were carried out using a 25 μ L total volume containing 20 ng of sample DNA, 12.5 μ L of TaqMan universal PCR Master Mix, 0.625 μ L of TaqMan assay, and 8.875 μ L of milli-Q water. The reactions were then analysed using the allelic discrimination endpoint analysis of the sequence detection software package, *Sequence Detection Software* (SDS) Version 1.3 (Applied Biosystems). These SNPs were selected because previous studies showed that they were associated with other cancers, obesity-related diseases, and leptin expression [9, 25–30].

2.4. Statistical Analysis. The statistical analysis was conducted using the SAS statistical software (Statistical Analysis System, version 8.1, 1999–2000, Cary, NC, USA). Associations were assessed using 2×2 or $2 \times n$ contingency table analyses, and χ^2 or Fisher's exact test was used to examine homogeneity between cases and controls. Kruskal-Wallis and Mann-Whitney tests were used to compare the mean ages of patients and controls and to compare the mean serum leptin expression among genotypes. The odds ratio (OR) and 95% CI provided the measurements of association strength. The variables that were significantly associated with DTC using univariate analysis were analysed using a multiple logistic regression model for evaluating the effect of all genotypes and clinical risk factors. To analyse the primary factors related to the disease-free interval, Cox regression analysis was performed. All of the tests were conducted at the $P < 0.05$ significance level.

3. Results

The majority (82%) of the patients were females, aged 14 years to 76 years (41.02 ± 15.54 years) and 87% had an anticipated

diagnosis of papillary thyroid carcinoma (PTC). Multifocality was observed in 55% of the patients and 59% presented invasion of the capsule. Stage I (40%) and stage II (35%) were more frequent, although at the reference hospitals involved in this study there were a large proportion of stage III (10%) and stage IV (15%) cases. In fact, 37 patients presented metastasis at the time of diagnosis. Moreover, concurrent thyroiditis was diagnosed in 48% of the patients. The majority (63.5%) of the patients evolved free of disease after a 9-year follow-up period (101.5 ± 57.6 months). However, 46 patients (36.5%) presented recurrence or metastasis during the follow-up.

3.1. Serum Analysis. As we have previously described, leptin presented outstanding accuracy in the diagnosis of thyroid nodule malignancy [4]. All of the parameters considered for a diagnostic test were noticeable. For a cut-off point of 7.24 ng/mL, the serum levels of leptin differentiated all of the malignant lesions from the benign lesions, with sensitivity, specificity, and positive predictive value and negative predictive value of 100% ($P = 1 \times 10^{-6}$). Additionally, leptin serum levels differentiated the follicular variant of papillary thyroid cancer (FVPTC) from the follicular adenomas (FA) ($P < 0.001$) and from goiters ($P < 0.001$). They also distinguished FA from the follicular carcinomas (FTCs) ($P < 0.001$) and from the classic PTC (CPTC) ($P < 0.001$) [4]. These results are shown in Table 1. Although leptin was differentially expressed among thyroid lesions, it did not correlate with any clinicopathological feature (Table 2).

3.2. Genotyping Analysis. A multivariate logistic regression adjusted for gender, age, smoking, and BMI showed that the AG genotype of *LEP* rs7799039 was an independent risk for DTC (OR, 11.689; $P = 0.0183$; 95% CI, 1.516–90.119). Similarly, AG and GG genotypes of *LEPR* rs1137101 represented independent risks for DTC (OR, 3.747; $P = 0.027$; 95% CI, 1.161 to 12.092, and OR, 5.437; $P = 0.013$; 95% CI, 1.426 to 20.729, resp.).

The AA genotype of *LEPR* rs1137100 was more frequent among patients with FTC (93.8%) than among patients with PTC (60.2%; chi-square = 8.219, $P = 0.016$). There was no association between the genotypes and the presence of

TABLE 2: Leptin serum concentrations and LEP and LEPR SNPs genotypes compared to clinicopathological features.

Clinical pathological features	Leptin serum concentration (ng/mL)	P	LEP rs7799039 (N)		P	LEP rs2167270 (N)		P	LEPR rs137101 (N)		P	LEPR rs137100 (N)		P	
			AA	AG		GG	AA		AG	GG		AA	AG		GG
Age at time of diagnosis															
≤45	9.81 ± 0.58	N.S.	11	39	40	8	48	39	24	47	24	65	20	3	N.S.
>45	10.07 ± 0.80		10	33	20	11	32	24	19	30	18	33	25	6	
Gender															
Male	9.88 ± 0.60	N.S.	17	57	44	11	63	46	29	56	35	69	37	6	N.S.
Female	9.90 ± 0.80		4	8	10	2	7	13	7	10	5	18	3	1	
Smoking habit															
Smokers	9.98 ± 0.76	N.S.	7	20	12	2	18	20	12	17	11	26	10	2	N.S.
Never smoked	9.82 ± 0.58		13	45	40	11	50	38	23	48	28	59	30	4	
Tumour size															
<2 cm	9.99 ± 0.58	N.S.	14	31	29	9	34	31	21	35	18	44	21	5	N.S.
2-4 cm	9.73 ± 0.77		8	33	25	6	38	23	13	38	16	42	16	2	
>4 cm	9.95 ± 0.38		1	15	14	3	17	11	9	13	9	16	9	0	
Extrathyroidal invasion															
Yes	9.94 ± 0.70	N.S.	8	30	15	5	31	19	16	22	17	32	17	1	N.S.
No	9.87 ± 0.62		16	38	17	46	20	3	8	29	34	7	34	30	
Capsule															
Yes	9.80 ± 0.76	N.S.	8	17	14	5	17	17	11	17	11	24	12	1	N.S.
No	9.96 ± 0.57		1	17	10	1	20	7	5	17	6	19	6	1	
Multifocality															
Yes	9.82 ± 0.67	N.S.	15	27	29	9	34	29	19	37	16	44	19	4	N.S.
No	9.88 ± 0.65		7	34	22	5	31	28	16	27	21	42	18	1	
Metastasis at time of diagnosis															
Present	9.86 ± 0.83	N.S.	3	16	17	2	23	13	7	18	13	24	9	3	N.S.
Absent	9.89 ± 0.58		16	48	32	11	45	40	30	45	21	60	31	1	
Stage															
I and II	9.60 ± 0.74	N.S.	6	32	29	3	34	32	19	29	21	42	22	2	N.S.
III and IV	10.17 ± 0.75		2	16	5	4	14	5	6	13	4	13	9	0	
Thyroiditis															
Present	9.96 ± 0.64	N.S.	9	23	12	4	21	20	9	25	11	28	11	2	N.S.
Absent	9.84 ± 0.39		5	24	24	7	25	22	20	21	13	34	19	0	
Outcome															
Disease-free	9.88 ± 0.61	N.S.	14	43	28	8	40	37	27	40	18	49	30	2	N.S.
Recurrence	9.89 ± 0.82		4	21	21	5	28	15	9	23	16	35	9	2	

N.S.: not statistically significant.

TABLE 3: Mean and median leptin serum expressions according to the genotypes of the studied SNPs.

Gene-rs (N)	Mean expression (ng/mL)	Standard deviation	Median (ng/mL)	P
<i>LEP</i> rs7799039 (78)				0.012*
AA (12)	9.22	0.98	9.37	<0.05 [#]
AG (36)	10.07	0.60	10.07	>0.05 [§]
GG (30)	9.75	0.56	9.84	>0.05 [§]
<i>LEP</i> rs2167270 (78)				0.018*
AA (8)	9.89	0.49	9.80	>0.05 [#]
AG (38)	10.05	0.59	10.00	<0.05 ^{&}
GG (32)	9.52	0.79	9.58	>0.05 [§]
<i>LEPR</i> rs1137101 (78)				N.S.
AA (20)	9.75	0.59	9.83	
AG (40)	9.89	0.76	9.85	
GG (18)	9.74	0.74	9.96	
<i>LEPR</i> rs1137100 (73)				N.S.
AA (48)	9.88	0.76	9.96	
AG (20)	9.77	0.72	9.84	
GG (5)	9.76	0.42	9.86	

* AA × AG × GG; [#] AA × AG; [&] AG × GG; [§] AA × GG; N.S.: nonsignificant.

features that could be related to aggressiveness, such as multifocality, invasion of the capsule, and concomitant thyroiditis. However, the GG genotype of rs2167270 of *LEP* gene was more frequent among the patients diagnosed with the less advanced stage (I and II, 46.2%) than among the patients diagnosed with the more advanced stage (III and IV, 22.7%, chi-square = 6.311; $P = 0.043$), as shown in Table 2.

The survival analysis showed no association between the disease-free interval and gender, ethnicity, smoking, multifocality, invasion of capsule, and thyroiditis. None of the genotypes investigated was associated with the disease-free period.

3.3. Leptin Concentrations versus Genotypic Profile. *LEPR* polymorphisms did not correlate with the serum concentrations of leptin as shown in Table 3. In contrast, we observed a good correlation between *LEP* gene genotype and serum leptin levels. The patients who presented the AA genotype of rs7799039 in *LEP* gene had lower serum levels of leptin (9.22 ± 0.98 ng/mL) than those with the AG genotype (10.07 ± 0.60 ng/mL; $P = 0.005$). The individuals with the AG genotype of SNP rs2167270 also produced higher serum leptin levels (10.05 ± 0.59 ng/mL) than the subjects with the GG genotype (9.52 ± 0.79 ng/mL; $P < 0.05$).

4. Discussion

It is recognised that leptin is a component of a series of important thyroid cell processes, including the regulation of TSH expression [31] and the development of autoimmunity [32, 33], and that serum leptin levels may be related to the presence of insulin resistance in hypothyroidism [34] and to other diseases that may be associated with thyroid disorders,

such as obesity and insulin resistance. However, the present study is the first to investigate the occurrence of genetic changes in *LEP* and *LEPR* genes in DTC, which might be a likely link between obesity and thyroid cancer.

To the best of our knowledge, we demonstrate for the first time that polymorphisms in *LEP* (rs7799039) and *LEPR* (rs1137101) may increase the risk of DTC development.

The SNP rs7799039 in *LEP* gene has been associated with an increase in BMI, overweight, and even with variations in serum leptin concentration [27, 35, 36]. A meta-analysis that included 5 articles related to this SNP and colorectal carcinoma risk and 3 studies related to prostate cancer susceptibility concluded that this alteration in *LEP* may increase the risk for prostate cancer but not for colorectal carcinoma [9]. Additionally, several authors have attempted unsuccessfully to establish a relationship between this polymorphism and breast cancer [37].

We demonstrated that serum leptin concentration varies according to specific inherited genotypes. Hoffsted et al. [38] showed that individuals with the AA genotype of rs7799039 had higher serum leptin concentrations than the AG or GG genotypes carriers. Our data confirmed significant differences of this cytokine depending on the genotype, although with more discrete variations. Because this SNP is located in the promoter region, this polymorphism may affect gene expression at a transcriptional level, leading to more or less leptin production. These findings may also explain the fact that there was no correlation between serum leptin and BMI in our population, allowing us to hypothesise that changes in this gene may be even more important than the effects of obesity in the secretion of this hormone.

Regarding SNP rs1137101, which we demonstrated to be related to an increased risk for DTC, reports in the

literature have shown that variants of *LEPR* are related to the following: increased BMI, insulin resistance, and correlation with HOMA-IR (an index used for the assessment of insulin resistance), metabolic syndrome, increased fat mass and adipocytes, increased waist circumference, obesity, development of type 2 diabetes mellitus (T2DM), and serum concentrations of circulating leptin [39–52]. This polymorphism has been associated with breast, prostate, and lung cancers and polycystic ovary syndrome [53–59].

We described the relationship of rs2167270 of *LEP* with different serum levels of leptin. Friedlander et al. [60] correlated *LEP* rs2167270 genotypes with increased body mass and waist circumference in women, and Jiang et al. [27] reported that this polymorphism is associated with increased BMI. The AA genotype of rs2167270 was associated with a lower risk of colorectal cancer [25]. Although the variations in *LEP* may alter the amount of leptin produced, no relationship was found between rs2167270 and increased risk for DTC.

In conclusion, we demonstrated that rs7799039 and rs2167270 polymorphisms of *LEP* modified the serum concentrations of leptin in patients with DTC. Furthermore, the polymorphisms rs7799039 in *LEP* and rs1137101 in *LEPR* increased the risk for developing DTC, although they did not appear to correlate with tumour aggressiveness.

Conflict of Interests

The authors declare that they have no competing interests to disclose.

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