Leptin is a 16 kDa cytokine mainly produced by adipose tissue (1) which contributes to body weight homeostasis by regulating food intake and energy expenditure (2, 3). It has also been implicated in the modulation of many other processes such as reproduction, hematopoiesis, angiogenesis, wound healing, and cell differentiation, and proliferation (4). The human leptin receptor (OBR) belongs to the class I cytokine receptor family, and two main isoforms, resulting from an alternative splicing, have been identified. It is now well established that the long form (OBR-L), mainly expressed in the hypothalamus, is responsible for signal transduction through the activation of Janus-activated kinase 2/signal transducers and activators of transcription 3 and the mitogen-activated protein kinase pathways (5, 6). On the other hand, the short isoform (OBR-S), which is more abundant in peripheral tissues, mainly activates the mitogen-activated protein kinase and seems to be responsible for mitogenic activity (5, 6).

The relationship between leptin and obesity is clearly shown, and it is well established that overweight and obesity are associated with an increased risk of developing cancer such as adenocarcinoma of the esophagus, colon cancer, endometrial cancer, kidney cancer, and breast cancer (7). Several lines of evidence suggest that leptin and its receptor are involved in the development of normal mammary gland and in breast cancer development and progression. Hu et al. (8) reported that genetically obese leptin-deficient Lep<sup>ob</sup>/Lep<sup>ob</sup> and leptin receptor-deficient Lep<sup>ob</sup>/Lep<sup>ob</sup> mice have minimal epithelial development in the mature mammary gland compared with their lean counterparts. Moreover, leptin- or leptin receptor-deficient mouse mammary tumor virus-transforming growth factor-α mice do not develop mammary tumors (9, 10). Finally, we showed that leptin receptors (short and long isoforms) are expressed in normal mammary epithelial cells during pregnancy and lactation (11). In breast cancer cells, leptin (12), and both receptor isoforms (13), are expressed at the mRNA and/or

**Abstract**

**Purpose:** Leptin and obesity are clearly related, and obesity is associated with an increased risk of breast cancer. We therefore measured the expression of leptin and its two main receptor isoforms, OBR-L and OBR-S, in 322 breast cancers. We analyzed their relations with the classical prognostic factors and with survival to establish their links with breast cancer.

**Experimental Design:** The expression of leptin and its receptors was quantified by real-time reverse transcription-PCR, using TaqMan fluorogenic probes and an ABI PRISM 7700 sequence detector system (Applied Biosystems, Courtaboeuf, France). TATA box binding protein was used to normalize expression. The human breast cancer cell, SK-BR-3, expressing the three targets, was chosen as the calibrator sample (i.e., target expression = 1).

**Results:** All the tumors expressed both receptors, and 318 of 322 expressed leptin. These three variables correlated positively with each other and with estradiol and progesterone receptors, whereas they correlated negatively with histoprognostic grading and tumor diameter. OBR-L/ OBR-S expression was inversely correlated with progesterone receptors. Patients with elevated OBR-S expression had longer relapse-free survival (P = 0.008), whereas high OBR-L/OBR-S was associated with a shorter relapse-free survival (P = 0.05). In Cox multivariate analyses, OBR-S maintained its prognostic value (P = 0.02; relative risk, 0.51).

**Conclusions:** This study shows that (a) almost all of the breast cancers coexpress leptin and its two main isoforms of receptors, suggesting that the human epithelial breast cancer cells respond to leptin acting via an autocrine pathway; (b) high expression levels of leptin and leptin receptors are biological markers of a more differentiated phenotype; and that (c) OBR-S is an independent prognostic factor.
protein level. In addition, the proliferation of human breast cancer cells, T47-D, is significantly stimulated by leptin (8, 13). Simultaneously, leptin enhances the phosphorylation of both signal transducers and activators of transcription 3 and extracellular signal-regulated kinase (8) and mitogen-activated protein kinase (13). These observations suggest the autocrine action of leptin in the regulation of breast cancer development and progression.

Recently, Ishikawa et al. (14) reported that leptin and OBR-L protein overexpression correlated with a high risk of tumor recurrence in 76 breast cancers. In keeping with this observation, some authors found that enhanced circulating leptin levels were associated with breast cancer (15, 16). However, Petridou et al. (17) showed that these circulating levels were inversely associated with breast cancer among premenopausal women, whereas other authors reported that circulating leptin was not increased in patients with breast cancer (18–20).

The aim of the present study was to assess the links between mRNA expression of leptin, its receptors (OBR-L and OBR-S), and breast cancer. We therefore analyzed their relations with the classical clinical, pathologic, and biological characteristics as well as with survival in a series of 322 primary breast cancers.

Patients and Methods

Cell lines. The human breast cancer cell line, SK-BR-3 (21), was purchased from the American Type Culture Collection (Manassas, VA). They were cultured in RPMI 1640 supplemented with 10% FCS, 2 mmol/L glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin. The cells were grown at 37°C in a humidified atmosphere of 5% CO2 and collected at subconfluence.

Patients. This study involves 322 breast tumor samples from patients who underwent surgery for locoregional disease in the Centre Oscar Lambret, the Anticancer Center of the North of France, between May 1989 and December 1991 (22, 23). The mean age of the patients was 57.8 years (range, 26-90). There were 151 node-negative and 168 node-positive patients (103 with one to three positive nodes, and 65 with more than three positive nodes). In three cases, the nodal status was unknown. Histopathologic grading (HPG) was determined according to Scarff and Bloom (24). Patients were treated by segmentectomy when tumor was <3 cm wide and by total mastectomy if the tumor was larger or centrally located. Surgery was followed by radiation therapy. Node-positive premenopausal patients, estradiol receptor (ER)–negative and progesterone receptor (PR)–negative postmenopausal patients received adjuvant treatment (six cycles of chemotherapy). The node-positive ER-negative and PR-positive postmenopausal patients received tamoxifen for 2 years. Node-negative patients received no adjuvant treatment (22). The median duration of follow-up for living patients was 77.3 months, there were 80 deaths, and 101 cases of relapse.

ER and PR assay. Both ER and PR were determined by the dextran-coated charcoal method, as described previously (25). Our laboratory is affiliated with the European Organization for Research and Treatment of Cancer Receptor Study Group, which undertook the quality control of the assays (26).

Isolation of total RNA. The tumor samples were frozen in liquid nitrogen and then stored at ~80°C until RNA extraction. The total RNA was isolated (RNasy Mini Kit, Qiagen, Courtaboeuf, France) from 40 mg of each tumor sample. The disruption and the homogenization of the tumor samples was done using a Rotor-Stator Homogenizer (Ribolyzer, Hybaid, Ilkirch, France). The amount of extracted RNA was quantified by measuring the absorbance at 260 nm. The purity of the RNA was checked by the ratio between the absorbance values at 260 and 280 nm and ranged between 1.8 and 2.1, demonstrating the high quality of the RNA. This was confirmed in 52 (16%) randomly selected samples either by electrophoresis on 1.5% agarose gel containing ethidium bromide or using an Agilent 2100 Bioanalyzer.

PCR primers and TaqMan fluorogenic probes. The primers and the TaqMan fluorogenic probes had the following sequences: sense primer 5’-CAGCAAAACCDCTCATAGACAA-3’ (nucleotides 145-166), antisense primer 5’-TAGAAGGGCCAGCGCTGAAA-3’ (nucleotides 378-398), and probe 5’-ACGGACGTGCTCTGTCGAATA-3’ (nucleotides 193-222) for leptin (GenBank accession no. BC060830); sense primer 5’-GATAGCCAGCCAGTTTTCA-3’ (nucleotides 3,283-3,305), antisense primer 5’-ACACACTCTTCTTCTTTAGTGA-3’ (nucleotides 3,443-3,468), and probe 5’-TTTCCCTCCAAATTCCTAAGTCTATC-3’ (nucleotides 3,362-3,390) for OBR-L (GenBank accession no. U43168); sense primer 5’-ATTGTGCAGCTAATTTATTCCCTTCC-3’ (nucleotides 2,533-2,559), antisense primer 5’-CCACCATATGGTAAACTTCAGGAGCTTAC-3’ (nucleotides 2,806-2,834), and probe 5’-GAATGTTCCGAAAACTAAAAGTTT-3’ (nucleotides 2,619-2,644) for OBR-S (GenBank accession no. U50748).

To take into account the variation in RNA quantity and quality, the expression of leptin and OBR was normalized to the endogenous TATA box binding protein (TBP) gene (27). In contrast with the widely used glyceraldehyde-3-phosphate dehydrogenase gene (28), the use of TBP as a control RNA was relevant in these studies investigating prognosis because we observed that its expression was not associated with tumor aggressiveness (data not shown). The sequences of the primers and probe used for TBP were as follows: sense primer 5’-CAGCAAC-CACCGCAGCTGAT-3’, antisense primer 5’-TTTCTGTGCTGAGCTGTT-3’, and probe 5’-TGCTGCAAAGGCTGTAAG-3’ (GenBank accession no. X54993).

The size of the PCR products was 254, 186, 302, and 89 bp for leptin, OBR-L, OBR-S, and TBP, respectively. In order to confirm the total gene specificity of the sequences chosen for the primers and probes, we did BLASTn searches against the nonredundant set of GenBank, European Molecular Biology Laboratory, and DNA Data Bank of Japan sequences.

Reverse transcription-PCR conditions. Reverse transcription and PCR were done in a one-step method on 50 ng of total RNA using a 7700 ABI PRISM sequence detector system (Applied Biosystems, Courtaboeuf, France). For leptin and OBR-L, the reaction mixture (20 μl final volume) contained 2.7× Master mix (LC RNA Master hybridization probe, Roche Diagnostics, Meylan, France) including TaqMan fluorogenic probes, deoxyxynucleotide triphosphate (3.25 mmol/L of Mn(OAc)2), 8 units of RNase inhibitor, 200 nmol/L of primers, and 200 nmol/L of probe (Proligo, Saint Quentin Fallavier, France). Reverse transcription was done at 61°C for 20 minutes. After incubation for 30 seconds at 95°C, PCR was done for 15 seconds at 95°C, and 1 minute at 60°C for 50 cycles.

For OBR-S, the reaction mixture (20 μl final volume) contained 5× Master mix (LC FastStart DNA Master hybridization probe, Roche Diagnostics) including FastStart Taq DNA polymerase, deoxyxynucleotide triphosphate, and MgCl2, 8 units of RNAse inhibitor, 5 units of MuLV reverse transcriptase (Applied Biosystems), 400 nmol/L of primers, and 200 nmol/L of probe (Proligo). Reverse transcription was done at 42°C for 30 minutes. The activation of the FastStart Taq DNA polymerase (10 minutes at 95°C) was followed by PCR (15 seconds at 95°C and 1 minute at 60°C for 50 cycles).

For TBP, the reaction mixture (50 μL final volume) contained 2× Master mix (TaqMan Universal Master Mix No AmpErase UNG; Applied Biosystems) including Taq Gold DNA polymerase, deoxyxynucleotide triphosphate and MgCl2, 20 units of RNAse inhibitor, 12.5 units of MuLV reverse transcriptase (Applied Biosystems), 200 nmol/L of primers and probe (Proligo). Reverse transcription was done at 48°C for 30 minutes. The activation of the Taq Gold DNA polymerase for 10 minutes at 95°C was followed by PCR (15 seconds at 95°C and 90 seconds at 60°C for 40 cycles). A nontemplate control was included in each experiment. The nontemplate controls and the samples were assayed in duplicate.

Relative quantification of leptin and OBR expression. The relative quantification of target gene expression was done using the comparative
cycle threshold (CT) method (29), where the CT variable is defined as the cycle number at which the fluorescent signal generated by cleavage of the dual labeled probe is first detectable. This method is based on the use of a calibrator sample (i.e., 1× sample) which permits the quantification in the unknown samples. The human breast cancer cell line, SK-BR-3, which expresses the three target genes, was chosen as the calibrator sample (i.e., target expression = 1).

The relative target expression was given by the formula: $2^{-\Delta\Delta CT}$, where $\Delta CT = CT$ patient sample $- CT$ calibrator sample; with $\Delta CT = CT$ TBP $- CT$ TBP.

For the $\Delta\Delta CT$ calculation to be valid, the PCR efficiencies of target genes and TBP must be approximately equal. To check that leptin, OBR-L, and OBR-S had the same PCR efficiency than TBP, we looked at how $\Delta CT$ varied with template amount (i.e., the absolute value of the slope of log input amount versus $\Delta CT$ should be <0.1). The slopes passed this test ($-0.016$ for leptin, 0.09 for OBR-L, and 0.023 for OBR-C).

### Statistical analyses

All the statistical analyses were done using the SPSS software (version 11.5). Relationships between qualitative variables were determined using the χ² test (with Yates’ correction when necessary). Correlations between variables were assessed according to the Spearman nonparametric test. Nonparametric Mann-Whitney and Kruskal-Wallis tests were used to compare the expression of leptin, OBR-L, and OBR-S in subgroups of patients and tumors. Overall survival and relapse-free survival (RFS) were studied by Kaplan-Meier method analysis. Comparison between curves was carried out by the log rank test. The proportional hazard regression method of Cox (30) was used to assess the prognostic significance of the variables taken in association.

### Results

#### Expression of leptin, OBR-L, and OBR-S in human breast cancer

All the tumors tested expressed OBR-L and OBR-S, and leptin was detectable in 318 of 322 cases. The distribution of the expression of these variables in the tumors was not normal (Gaussian) but those of leptin was log-normal. The median expression of these variables in the tumors was not normal either (Table 1). A positive correlation was also observed between leptin, OBR-L, and ER and between OBR-S and PR. In contrast, OBR-L and OBR-S correlated negatively with HPG, whereas OBR-L and leptin correlated negatively with tumor diameter. Interestingly, when looking at the OBR-L/OBR-S ratio, an inverse correlation was found with PR.

When looking at the median expression levels according to the classical prognostic factors (Table 2), we observed that the expression of leptin was higher in postmenopausal than in premenopausal patients ($P < 0.001$), in lobular than in other tumor types ($P = 0.016$), and in smaller tumors ($P = 0.039$). With regard to OBR-L, its median expression value was significantly lower in tumors with the highest HPG ($P = 0.023$). As observed for OBR-L, the median expression of OBR-S was lower in high HPG tumors ($P = 0.001$), and it was higher in lobular carcinomas than in other histologic types ($P < 0.001$), as observed for leptin.

#### Prognosis studies

**Relapse-free survival.** Three cutoff points (i.e., median value, lower, and upper quartiles) were tested for their ability to distinguish between two populations of tumors with different prognoses. Regardless of the threshold tested, neither leptin nor OBR-L were prognostic factors. In contrast, OBR-S had a prognostic value. The best OBR-S cutoff for prognosis was 4.45, corresponding to the upper quartile. Patients with elevated OBR-S expression had longer RFS (Fig. 2). Interestingly, the OBR-L/OBR-S ratio was associated with a shorter RFS (Fig. 2). The best OBR-L/OBR-S threshold was 2.69, corresponding to the median value. The other prognostic factors for RFS were node involvement and tumor diameter (Table 3). In multivariate analyses, OBR-S maintained its prognostic value aside from node involvement and tumor diameter (Table 3).

**Overall survival.** Regardless of the threshold tested, neither leptin nor both receptor isoforms had a prognostic value. In Cox univariate analyses, PR ($P = 0.009$; relative risk (RR), 0.548], tumor size ($P = 0.006$; RR, 1.796), and HPG ($P = 0.021$; RR, 1.56) were prognostic factors. In contrast, OBR-S had a prognostic value. The best OBR-S cutoff for prognosis was 4.45, corresponding to the upper quartile. Patients with elevated OBR-S expression had longer RFS (Fig. 2). Interestingly, the OBR-L/OBR-S ratio was associated with a shorter RFS (Fig. 2). The best OBR-L/OBR-S threshold was 2.69, corresponding to the median value. The other prognostic factors for RFS were node involvement and tumor diameter (Table 3). In multivariate analyses, OBR-S maintained its prognostic value aside from node involvement and tumor diameter (Table 3).

In the population studied, 72.2% of the samples were ER-positive and 71.5% were PR-positive. The classical correlations (Spearman test) observed in breast cancer between ER and PR ($P < 0.001, r = 0.60$), ER and age ($P < 0.001, r = 0.30$), and PR and age ($P = 0.029, r = 0.123$) were found. Leptin, OBR-L, and OBR-S were all strongly positively correlated with each other (Table 1). A positive correlation was also observed between leptin, OBR-L, and ER and between OBR-S and PR. In contrast, OBR-L and OBR-S correlated negatively with HPG, whereas OBR-L and leptin correlated negatively with tumor diameter. Interestingly, when looking at the OBR-L/OBR-S ratio, an inverse correlation was found with PR.

#### Relationships with the pathologic, clinical, and biological variables

In the population studied, 72.2% of the samples were ER-positive and 71.5% were PR-positive. The classical correlations (Spearman test) observed in breast cancer between ER and PR ($P < 0.001, r = 0.60$), ER and age ($P < 0.001, r = 0.30$), and PR and age ($P = 0.029, r = 0.123$) were found. Leptin, OBR-L, and OBR-S were all strongly positively correlated with each other (Table 1). A positive correlation was also observed between leptin, OBR-L, and ER and between OBR-S and PR. In contrast, OBR-L and OBR-S correlated negatively with HPG, whereas OBR-L and leptin correlated negatively with tumor diameter. Interestingly, when looking at the OBR-L/OBR-S ratio, an inverse correlation was found with PR.

### Fig. 1. Distribution of 322 primary breast cancer samples as a function of the expression of leptin, OBR-L, and OBR-S. The expression of the SK-BR-3 cell line is used as the calibrator.
In the present article, we show that almost all (98.7%) of the breast cancer samples studied coexpressed leptin and its two main isoforms of receptors, OBR-L and OBR-S. In most breast cancers, the mammary epithelial tumor cells are the major tissue component, the other cell types being stromal cells and endothelial cells. This observation supports the hypothesis that the transcripts of leptin and leptin receptors quantified by real-time reverse transcription-PCR are actually produced by the tumor cells. This is in agreement with previous studies reporting that leptin and both receptor isoforms are expressed at the mRNA and/or protein level in human breast cancer cell lines and breast malignant tumors. We already showed, by in situ hybridization studies, that both the long and short forms of leptin receptor mRNA were localized in proliferating epithelial cells from human breast cancer tissue (13). Similarly, using a polyclonal antibody against OBR-L, Ishikawa et al. (14) observed that this receptor isoform was expressed in most of the epithelial carcinoma cells in 63 of 76 breast cancers, whereas none of the 32 samples of adjacent normal mammary glands showed immunoreactivity for OBR-L. In the same study, leptin was expressed in all of the breast cancer cells and normal mammary epithelial cells. However, overexpression of leptin, as determined by staining intensity, was observed in 92% of the carcinomas, but in none of the normal epithelium. Moreover, leptin protein has been identified by indirect immunofluorescent staining in the T47D cell line (12). All these observations show that the human epithelial breast cancer cells could respond to leptin acting via an autocrine pathway.

### Table 1. Correlations between the mRNA expression of leptin, leptin receptors and the pathologic, clinical, and biological variables (Spearman test, n = 322)

<table>
<thead>
<tr>
<th></th>
<th>Leptin</th>
<th>OBR-L</th>
<th>OBR-S</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pr</td>
<td>r</td>
<td>Pr</td>
</tr>
<tr>
<td>Leptin, OBR-L</td>
<td>&lt;0.001</td>
<td>0.387</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Leptin, OBR-S</td>
<td>&lt;0.001</td>
<td>0.387</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>OBR-L, OBR-S</td>
<td>&lt;0.001</td>
<td>0.387</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Leptin, ER</td>
<td>0.026</td>
<td>0.125</td>
<td>0.026</td>
</tr>
<tr>
<td>Leptin, tumor diameter</td>
<td>0.026</td>
<td>0.125</td>
<td>0.026</td>
</tr>
<tr>
<td>OBR-L, ER</td>
<td>0.022</td>
<td>0.128</td>
<td>0.022</td>
</tr>
<tr>
<td>OBR-L, HPG</td>
<td>0.007</td>
<td>0.159</td>
<td>0.007</td>
</tr>
<tr>
<td>OBR-L, tumor diameter</td>
<td>0.05</td>
<td>0.11</td>
<td>0.05</td>
</tr>
<tr>
<td>OBR-S, PR</td>
<td>0.024</td>
<td>0.126</td>
<td>0.024</td>
</tr>
<tr>
<td>OBR-S, HPG</td>
<td>&lt;0.001</td>
<td>0.219</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>OBR-L/OBR-S, PR</td>
<td>0.033</td>
<td>0.119</td>
<td>0.033</td>
</tr>
</tbody>
</table>

### Table 2. Expression of leptin, OBR-L and OBR-S according to the clinical, histologic, or biological variables

<table>
<thead>
<tr>
<th></th>
<th>Leptin (arbitrary units)</th>
<th>OBR-L (arbitrary units)</th>
<th>OBR-S (arbitrary units)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median (10-90%) P</td>
<td>Median (10-90%) P</td>
<td>Median (10-90%) P</td>
</tr>
<tr>
<td>Menopausal status*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Premenopausal</td>
<td>13.1 (1.28-306.7) &lt;0.001</td>
<td>6.19 (2.22-27.47)</td>
<td>2.24 (0.72-6.81)</td>
</tr>
<tr>
<td>Postmenopausal</td>
<td>45.1 (1.80-636.5) &lt;0.001</td>
<td>6.59 (2.78-18.56) 0.932</td>
<td>2.60 (0.89-7.45) 0.776</td>
</tr>
<tr>
<td>Positive lymph nodes*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>29.5 (2.62-462.1) &lt;0.001</td>
<td>6.21 (2.52-19.9)</td>
<td>2.27 (0.76-5.99)</td>
</tr>
<tr>
<td>1-3</td>
<td>41.9 (1.27-753.1) &lt;0.001</td>
<td>6.88 (2.97-20.47) 0.932</td>
<td>2.59 (0.97-8.27)</td>
</tr>
<tr>
<td>&gt;3</td>
<td>26.5 (1.21-631.2) 0.510</td>
<td>7.14 (2.88-23.48) 0.538</td>
<td>2.66 (1.07-7.94) 0.388</td>
</tr>
<tr>
<td>HPG*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>47.9 (2.25-294.2) &lt;0.001</td>
<td>7.98 (2.63-45.07) 0.538</td>
<td>3.85 (1.09-10.77)</td>
</tr>
<tr>
<td>2</td>
<td>35.1 (1.55-543.7) &lt;0.001</td>
<td>7.39 (3.14-20.02)</td>
<td>2.90 (0.82-8.46)</td>
</tr>
<tr>
<td>3</td>
<td>22.2 (1.58-549.5) 0.476</td>
<td>5.64 (2.51-14.34) 0.023</td>
<td>1.97 (0.87-4.52) 0.001</td>
</tr>
<tr>
<td>Tumor type*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ductal</td>
<td>28.2 (1.65-438.8) &lt;0.001</td>
<td>6.43 (2.85-17.46)</td>
<td>2.06 (0.85-5.57)</td>
</tr>
<tr>
<td>Lobular</td>
<td>105.2 (5.20-1196.7)</td>
<td>9.3 (2.44-26.6) 4.868</td>
<td>115.9-9.7</td>
</tr>
<tr>
<td>Others</td>
<td>36.1 (0.84-638.4) 0.016</td>
<td>6.57 (2.54-37.47) 0.263</td>
<td>3.19 (1.07-10.38) &lt;0.001</td>
</tr>
<tr>
<td>Tumor diameter (cm)*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;2</td>
<td>72.7 (1.90-835.4) &lt;0.001</td>
<td>9.71 (3.02-32.7) 2.23</td>
<td>0.83-3.83) &lt;0.001</td>
</tr>
<tr>
<td>2-5</td>
<td>38.18 (1.89-558.9) &lt;0.001</td>
<td>6.32 (2.65-21.89)</td>
<td>2.61 (0.86-7.52)</td>
</tr>
<tr>
<td>&gt;5</td>
<td>19.16 (1.39-496.4) &lt;0.001</td>
<td>6.28 (2.63-15.79) 0.097</td>
<td>2.31 (0.88-6.96) 0.799</td>
</tr>
<tr>
<td>ER (fmol/mg protein)*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;10</td>
<td>22.16 (1.89-520.9)</td>
<td>5.66 (2.84-17.33)</td>
<td>2.03 (0.80-5.96)</td>
</tr>
<tr>
<td>≥10</td>
<td>38.32 (1.70-575.2)</td>
<td>7.24 (2.69-21.11) 0.074</td>
<td>2.67 (0.87-7.58) 0.154</td>
</tr>
<tr>
<td>PR (fmol/mg protein)*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;10</td>
<td>23.18 (1.42-658.1)</td>
<td>5.84 (2.49-23.11)</td>
<td>2.00 (0.83-5.67)</td>
</tr>
<tr>
<td>≥10</td>
<td>35.94 (1.86-548.6) 0.365</td>
<td>6.90 (2.83-20.17) 0.344</td>
<td>2.68 (0.87-7.65) 0.076</td>
</tr>
</tbody>
</table>

* Mann-Whitney test.

† Kruskal-Wallis test.
Using the Spearman test, we showed that leptin expression was positively correlated with those of its receptors, as already reported by Ishikawa et al. (14) for leptin and OBR-L. Considering the links with the classical clinicopathologic and biological features, we observed that leptin, OBR-L, and OBR-S were positively correlated with ER or PR. This result is corroborated by our observation that tumors from postmenopausal patients, which are known to be rich in steroid hormone receptors (31), express higher leptin levels than tumors from premenopausal patients. A similar relationship between circulating leptin levels and steroid receptor levels has already been reported in patients with gynecologic and breast cancers (32), however, recently, high plasma levels have been reported to be associated with ER or PR negativity (33). On the other hand, we found negative correlations between leptin, its receptors, and tumor diameter or HPG. This result is not unexpected because it is well established that large tumors and high-grade undifferentiated tumors are more likely ER- and PR-negative. As OBR-L is known to be mainly expressed in the hypothalamus whereas OBR-S is more abundant in peripheral tissues, we looked at the transcriptional ratio between both types of receptor isoforms in breast cancer. Interestingly, OBR-L/OBR-S was found to be positively correlated with the lack of PR, suggesting that an increase in OBR-L and/or a decrease in OBR-S could be a marker of tumor aggressiveness. All these associations between leptin, its receptor isoforms, and steroid hormone receptors suggest a crosstalk between these two pathways. Indeed, in MCF-7 cells, leptin enhances the expression of aromatase, resulting in an increase in estrogen production (34, 35). Moreover leptin induces the functional activation of estrogen receptors in MCF-7 cells via the ERK1/ERK2 signal (36). Additionally, estrogens seem to be able to activate leptin promoter in MCF-7 cells, this activation being dependent on coactivators present in leptin-producing cells (37). Finally, all the effects of the antiestrogen ICI 182,780 on MCF-7 cells, such as inhibition of cell proliferation, rapid ERα degradation, inhibition of nuclear ERα expression, and reduced ERα-dependent transcription from ERE-containing promoters, are significantly attenuated by simultaneous treatment with leptin (38).

In univariate and multivariate Cox analyses, our results reveal that OBR-S mRNA expression has a favorable prognostic significance on RFS, with a median duration of follow-up of 77.3 months. In contrast with our results, it has recently been reported that both intratumoral long and short leptin receptor mRNA isoforms were significantly associated with poor prognosis, for a subset of breast cancer patients with high serum leptin or high intratumoral leptin mRNA levels (39). Indeed, it might seem paradoxical that a tumor containing high levels of receptor for leptin, which stimulates breast cancer cell growth (8, 13), has a better prognosis than a tumor without. However, such a relationship is well known for ER and PR (40). Similarly, we have also previously reported the same observation for several growth factor receptors including insulin-like growth factor I receptors (41), fibroblast growth factor II receptors (42), type I growth factor receptors (22), and nerve growth factor receptors (23). An explanation could be that...
tumors with OBR-S have retained some physiologic growth control, which could explain the better prognosis. This hypothesis is sustained by the positive links observed between OBR-S, steroid hormone receptors, and low HPG, which are variables associated with a better prognosis. In contrast, in univariate Cox analyses, patients with an elevated OBR-L/OBR-S ratio in their tumor have a worse prognosis of DFS than patients with a low ratio. Although both isoforms are associated with a more differentiated phenotype, it is noteworthy that an increase in the expression of the isoform known to be mainly expressed in the hypothalamus, OBR-L, or a decrease in the isoform known to be more abundant in peripheral tissues, OBR-S, is related with tumor aggressiveness. These results are in agreement with both the positive correlation observed between the OBR-L/OBR-S ratio and the lack of PR, and with the observation that the mitogenic activity of OBR-L is higher than those of OBR-S.

It is considered that breast tumor cells still retained some features of fully differentiated mammary epithelial cells as well as physiologic growth control at the beginning of the disease and progressively exhibit an undifferentiated phenotype associated with growth autonomy, resulting from the accumulation of genetic alterations (43). However, recent data suggests that despite increasing genetic alterations, the “portrait” of breast tumors remains stable during progression towards the metastatic state (44). The expression of leptin has been reported (12, 14, 45) in human normal epithelial mammary cells, and several lines of evidence suggest that tumorigenesis is associated with an increase in leptin expression by epithelial mammary cells. O’Brien et al. (12) reported that leptin mRNA expression was higher in MCF-7, MDA-MB-231, and T47-D human breast cancer cells than in adipose tissue. In keeping with this result, Ishikawa et al. (14) showed that the expression of leptin, studied by immunohistochemistry, was significantly stronger in epithelial carcinoma cells than in normal epithelium, as judged by its staining intensity. In the present study, we show that a higher expression of leptin and leptin receptors is associated with variables linked to a weak tumor aggressiveness and a more differentiated phenotype, such as high levels of steroid receptors, small tumor diameter, and low HPG. It cannot be excluded that this higher expression of leptin and OBR in well-differentiated, less aggressive, ER- and/or PR-positive tumors could be maintained during the progression of breast cancer. Furthermore, our data shows that an increase in the OBR-L/OBR-S ratio is associated with a more undifferentiated phenotype, suggesting that an imbalance between both receptor isoforms promotes breast cancer aggressiveness.

In conclusion, the coexpression of leptin, OBR-L, and OBR-S in almost all breast cancers shows that the human epithelial breast cancer cells respond to leptin acting via an autocrine pathway. High expression levels of leptin and leptin receptors are biological markers of a more differentiated phenotype, OBR-S being an independent prognostic factor. In contrast, an elevated OBR-L/OBR-S ratio is a marker of tumor aggressiveness.

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Messenger RNA Expression of Leptin and Leptin Receptors and their Prognostic Value in 322 Human Primary Breast Cancers

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