SPANDIDOS PUBLICATIONS

Leptin and leptin receptor involvement in cancer development: A study on human primary breast carcinoma

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Received September 4, 2007; Accepted October 19, 2007

Abstract. Obesity is associated with an increased risk of breast cancer. Leptin, a hormone synthesised essentially by adipose tissue, may be involved in cancer development. We examined the expression of leptin and leptin receptor (Ob-R) in human primary breast cancer and adjacent non-cancerous tissue. We also analysed their relationships with histological variables such as the oestrogen and progesterone receptors, Ki67 proliferation factor and tumour size. The expressions of leptin and Ob-R were investigated by immunohistochemical staining in 35 primary breast cancers and 17 adjacent non-cancerous tissues. Samples and histological features were obtained from the Anti-Cancer Centre. Expressions of leptin and Ob-R were detected in, respectively, 85 and 75% of the primary breast cancer cases studied. The expression of leptin was significantly correlated with Ob-R detection (p=0.008). In addition, Ob-R expression in primary breast carcinoma was positively correlated with oestrogen receptor expression (p=0.028) and tumour size (p=0.045) but not with Ki67 or progesterone receptor expressions. However, the expression of leptin showed no statistical correlation with these variables. First, the co-expression of leptin and Ob-R in primary breast cancer shows that leptin acts on mammary tumour cells via an autocrine pathway. Second, the co-expression of Ob-R and oestrogen receptors suggests a possible interaction between leptin and oestrogen systems to promote breast carcinogenesis. Finally, the fact that Ob-R expression was positively correlated with tumour size may point to a potential role of leptin as a growth factor and of Ob-R as a new prognostic factor.

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Key words: breast, breast cancer, ductal carcinoma, leptin, leptin receptor, lobular carcinoma, oestrogen receptor, prognostic, tumour size

Introduction

Obesity is a major health problem in developed countries; 280,000 US adults die annually of causes related to obesity (1). This pathology is associated with numerous metabolic disorders (endocrine, cardiovascular and gastrointestinal diseases) and is probably involved in the development of various cancers such as colon, prostate and breast cancers (2). It has been clearly demonstrated that obesity is an important risk factor for breast cancer in postmenopausal women (3,4). In addition, being overweight is associated with increased breast cancer recurrence and mortality (5). One of the mechanisms that might explain the relationship between obesity and hormone-dependent breast cancer development is oestrogen overproduction by adipose tissue derived from elevated androgen aromatisation (6). However, adipose tissue is also able to synthesise and secrete many other compounds, termed adipokines, such as leptin, adiponectin, resistin and interleukins (7).

Leptin, a 16 kDa polypeptidic hormone encoded by the *obese* gene, was first described as a regulator of body weight and energy balance (8). Since its discovery, many other metabolic activities have been demonstrated, leptin interfering with fetal development (9), haematopoiesis (10), reproduction (11) and immunity (12,13). It was initially thought to be synthesised only by adipose tissue (8), but other sources of leptin have been described, including testicles (14), ovaries (15), placenta (16), cartilage and bone cells (17), skeletal muscle (18) and stomach (19). However, adipose tissue remains the main source of leptin in the body. Interestingly, one of factors influencing plasma leptin concentration is adipose tissue mass, circulating leptin levels being positively correlated with body weight and fat mass (20).

Leptin exerts its physiological activity through binding to its receptor, a component part of the class I cytokine receptor family. The leptin receptor (Ob-R) was initially identified in the brain (21), but further work demonstrated its expression in other tissues including immune cells (22), placenta (23), endometrium (24), stomach (25) and lung (26). In addition, Ob-R expression has been detected in pathological tissue, such as acute myeloid leukaemia (27), intracranial (28) and pituitary (29) tumours, hepatocellular carcinoma (30), gastric cancer cells (19) and breast cancer (31,32).

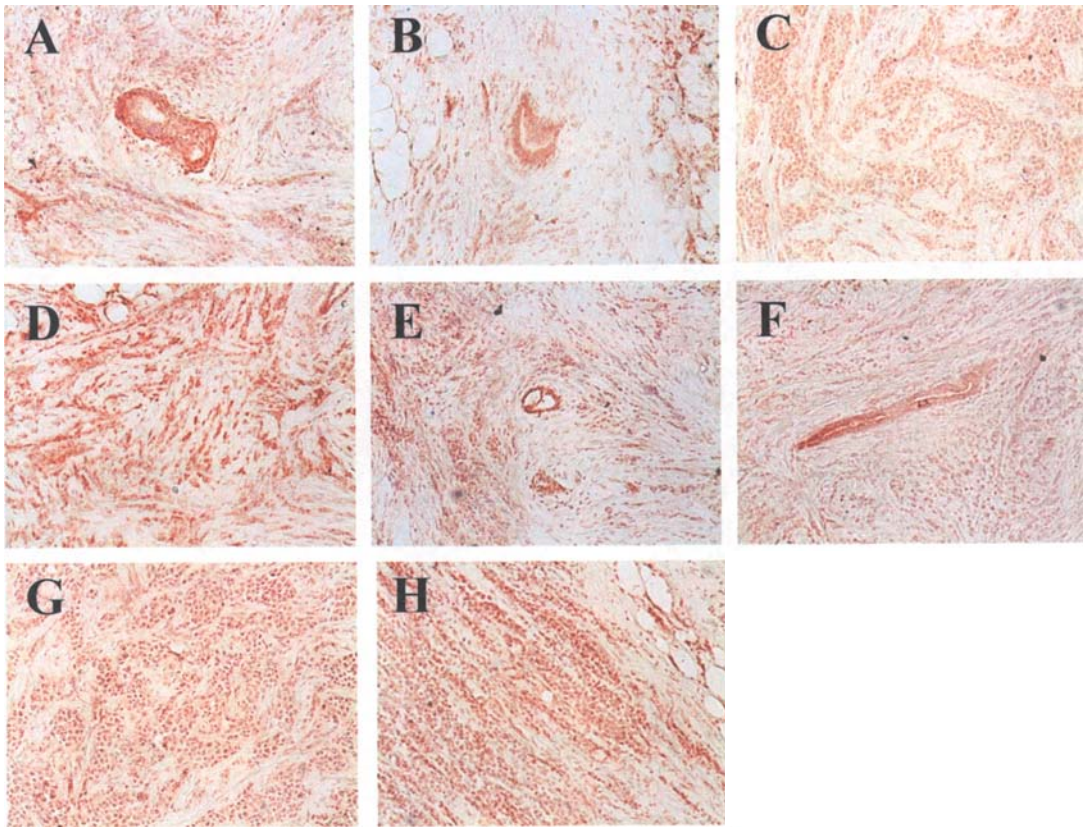

Figure 1. Immunohistochemical detection of leptin receptor (A-D) and leptin (E-H). In these images, leptin receptor detection is observed in normal tissue adjacent to invasive breast ductal carcinoma (A) or invasive lobular carcinoma (B), in invasive breast ductal cancer cells (C) and in invasive breast lobular cancer cells (D). Leptin immunostaining is seen in normal tissue adjacent to invasive breast ductal carcinoma (E) or invasive lobular carcinoma (F), in invasive breast ductal cancer cells (G) and in invasive breast lobular cancer cells (H). Magnification, x80.

Through binding to Ob-R, leptin may influence cancer development. Several authors have found evidence that leptin *in vitro* promotes angiogenic processes (33), suppresses apoptosis (34) and stimulates proliferation of malignant cell lines of various origins including prostate (35), oesophagus (36) and stomach (37).

In the same way, leptin enhances proliferation of different types of breast lines such as MCF-7, T-47D and ZR-75-1 carcinoma cells (38-40). Exploring tumour biopsies, we previously reported that leptin was expressed by benign breast cells and different grades of mammary ductal lesions (41). We have also reported that leptin is expressed in normal epithelial tissue in the vicinity of breast cancer cells but not in breast epithelial cells in healthy women (41). In addition, leptin expression by peritumoural breast adipose tissue increased in patients with breast cancer (42).

To elucidate the relationship between leptin and breast cancer development, we investigated the pro-carcinogenic potential of leptin by exploring variations in both leptin and Ob-R expressions in different grades of ductal and lobular breast lesions by immunohistochemistry. We also analysed the relationships between leptin and its receptor and clinical breast cancer features.

Materials and methods

All chemicals were purchased from Sigma (Saint-Quentin-Fallavier, France) except for anti-leptin and anti-Ob-R antibodies (R&D, Abingdon, UK), the avidin/biotin blocking kit, the Vectastain ABC kit, the diaminobenzidine (DAB) substrate and the Vectamount mounting medium (Vector Laboratories, Abcys, Paris, France).

Patients. In total, 35 women aged 30-80 years, not treated by radiotherapy or chemotherapy, were surgically resected in the Department of Surgery, Centre Jean-Perrin, France. Primary breast cancer tissue samples were immediately frozen, cut into 3 μm wide sections, deposited on glass microscope slides and maintained at -20˚C until immunohistochemical analysis. This experiment was approved by the local ethics committee.

Tissue classification. The diagnosis was made on alcohol-formalin-acetic acid fixed paraffin-embedded tissue sections after haematoxylin-eosin-saffron staining. Routinely, the expression of oestrogen receptors (ER), progesterone receptors (PR) and Ki67 were evaluated and scored as previously described (43). Tissues were classified according to histological subtypes as malignant lesions corresponding to ductal *in situ* carcinoma (n=8), lobular *in situ* carcinoma (n=2), invasive ductal carcinoma of different grades [grade 1 (n=4), 2 (n=16) or 3 (n=8)] and invasive lobular carcinoma (n=7). Invasive tumours were evaluated according to the SBR grade classification modified by Elston and Ellis (44).

Immunohistochemistry. The expressions of leptin and Ob-R were investigated by immunohistochemical staining using

Leptin and leptin receptor expressions in normal tissue adjacent to primary ductal and lobular breast cancer.

	Normal tissue adjacent to		
	Primary breast cancer	Ductal breast cancer	Lobular breast cancer
Breast cancer cases			
n	17	12	5
Leptin receptor			
n[a]	10	6	4
(%)	60	50	80
Leptin			
n[b]	13	9	4
(%)	75	75	80

n, total number of cases studied; n[a], number of cases expressing leptin receptor; n[b], number of cases expressing leptin; (%), percentage of cases expressing leptin receptor or leptin.

affinity-purified goat polyclonal biotinylated antibodies against leptin and Ob-R.

Sections were thawed for 1 h at room temperature before fixing with acetone for 10 min. Non-specific binding sites were blocked using the avidin/biotin kit for 30 min. Slides were then incubated overnight at 4˚C in a humid chamber with the anti-leptin and anti-Ob-R biotinylated antibodies (1 μg/ml). Endogenous peroxidase activity was inhibited with 0.3% hydrogen peroxide for 5 min. Visualisation was carried out using a Vectastain ABC peroxidase-conjugated streptavidine kit for 30 min. The sections were then treated with DAB substrate for 10 min to give staining. Finally, slides were contrasted using haematoxylin, dehydrated and mounted using the Vectastain mounting medium. For each assay, control samples without the anti-leptin or anti-Ob-R antibody or without the peroxidase revelation kit were used to establish the specificity of the immunohistochemical analysis.

Microscopic examination. Assessment of immunostaining was performed by a pathologist blinded to the clinical data. The expression of leptin and Ob-R in cancerous and normal adjacent tissues was classified as negative (<5% labelled cells) or positive (≥5% labelled cells). The total percentage of cases with leptin or Ob-R expression was determined.

Statistical analysis. The statistical analysis was performed using Stat View statistical software (SAS Institute). The association between leptin and Ob-R expressions was determined using the χ^2 test (with Yates' correction). Relationships between leptin or Ob-R expressions and histological features were analysed using Spearman rank correlation. In this study, because the number of cases was limited, we combined ductal and lobular carcinomas for Spearman rank correlation. Differences with $p<0.05$ were considered to be statistically significant.

Results

Ob-R and leptin expressions in normal tissue adjacent to breast cancer. The expression of Ob-R was examined using a polyclonal antibody recognising the extracellular domain of Ob-R, similar for all Ob-R isoforms.

In normal primary breast cancer tissue, positive Ob-R and leptin immunostaining was observed in respectively 60 and 75% of cases studied (Fig. 1, Table I). More specifically, in invasive ductal carcinomas, normal adjacent tissue expressed Ob-R in 6 out of 12 (50%) cases studied and leptin in 9 out of 12 (75%). Ob-R and leptin were also detected in normal tissue in the vicinity of breast lobular invasive carcinomas in 4 out of 5 (80%) cases studied.

Ob-R expression in breast cancer tissue. Positive Ob-R immunostaining was noted in 75% of primary breast cancer cases (Fig. 1, Table II). In ductal carcinomas, Ob-R was expressed in 6 out of 8 (75%) *in situ* cases studied. In invasive tissue, Ob-R expression was detected in respectively 75 and 80% of grade I and II cases and was found in only 50% of grade III cases. In lobular carcinomas, the percentage of Ob-R expression was 100% in *in situ* tissue and 85% in invasive tissue. In both ductal and lobular breast cancer, Ob-R expression was essentially nuclear (60% of cancer cases studied) but was also cytoplasmic (40% of cancer cases studied). The expression of Ob-R was not detected in breast tissue when immunostaining was carried out without biotinylated antibody.

Leptin expression in breast cancer tissue. In ductal and lobular breast carcinomas, positive immunoreactivity for leptin was detected in 85% of cases studied (Fig. 1, Table II). The expression of leptin was higher in ductal invasive carcinomas (90% of cases studied) than Ob-R expression (70% of cases studied), but was similar in *in situ* ductal carcinomas (75% of cases studied). In addition, leptin was strongly expressed in all three grades of ductal cancers (90% of cases studied). In lobular carcinomas, the percentage of leptin expression was 100% in *in situ* tissue and 70% in invasive tissue. In contrast to Ob-R, leptin expression was cytoplasmic and nuclear (in 60% of cases studied). Leptin immunoreactivity was not detected in control samples when the biotinylated antibody was omitted.

Co-expression of Ob-R and leptin in breast cancer. The expression of leptin in breast cancer showed a significant correlation with the expression of Ob-R (p=0.008) (Table III). Thus out of 34 carcinomas with positive Ob-R expression, 32 also expressed leptin (95%). By contrast, out of 38 carcinomas with positive leptin immunostaining, 32 expressed Ob-R (85%).

Relationships between expressions of leptin or Ob-R and histological variables. Ob-R was positively correlated with ER (p=0.028, r=0.264) and tumour diameter (p=0.045, r=0.269)

Table II. Leptin and leptin receptor expressions in primary ductal and lobular breast cancer.

Tissue type	Total no. of cases studied n	Leptin receptor expression		Leptin expression	
		n^a	(%)	n^b	(%)
Primary breast cancer	35	26	75	30	85
Ductal *in situ* carcinoma	8	6	75	6	75
Invasive ductal carcinoma	28	20	70	25	90
Grade I	4	3	75	4	100
Grade II	16	13	80	14	90
Grade III	8	4	50	7	90
Lobular *in situ* carcinoma	2	2	100	2	100
Invasive lobular carcinoma	7	6	85	5	70

n, total number of cases studied; n^a, number of cases expressing leptin receptor; n^b, number of cases expressing leptin; (%), percentage of cases expressing leptin receptor or leptin.

Table III. Relationship between leptin and leptin receptor expressions in *in situ* and invasive ductal and lobular breast cancer (χ^2 test, n=45).

	Leptin expression		p-value
	Positive	Negative	
Leptin receptor expression			
Positive	32	2	
Negative	6	5	0.008

Table IV. Correlations between leptin or leptin receptor expressions and histological variables in primary ductal and lobular breast cancer (Spearman test, n=35).

	p-value	r
Ob-R, ER	0.028	0.264
Ob-R, PR	0.293	0.028
Ob-R, Ki67	0.305	-0.326
Ob-R, tumour diameter	0.045	0.269
Leptin, ER	0.154	0.106
Leptin, PR	0.656	0.015
Leptin, Ki67	0.194	-0.391
Leptin, tumour diameter	0.309	0.059

(Table IV). However, Ob-R expression showed no significant correlation with Ki67 or PR expressions. The expression of leptin showed no statistically significant correlation with ER, PR or Ki67 expressions, or tumour diameter.

Discussion

Several lines of evidence have suggested that leptin, a hormone synthesised essentially by adipose tissue, may be involved in cancer development and progression. Numerous studies have demonstrated *in vitro* that leptin stimulates cancer cell proliferation (35-37), inhibits apoptosis (34) and induces angiogenesis (33). Similar results have been described in breast cancer where leptin stimulates the proliferation of breast cancer cell lines (38-40). In the same way, recent studies have demonstrated that high serum leptin levels are associated with an increased risk of breast cancer (45), and that high expression of Ob-Rl and Ob-Rs mRNA in breast cancer tissue predicts poor prognosis (46). However, the role of leptin in breast cancer development is not fully understood.

In human breast cancer tissue, the implication of leptin in mammary carcinogenesis using an immunohistochemical approach has been explored in only a few studies. Initially, O'Brien *et al* (47) showed leptin expression in the T47D breast cancer cell line. Some authors reported that leptin and Ob-R were overexpressed in primary breast cancers, but their conclusions on the relationships between leptin or Ob-R and clinicopathological data were conflicting (31,32).

Thus our objective was first to determine whether leptin and Ob-R were expressed in primary breast cancer and normal tissue adjacent to breast lesions using frozen tissue sections for immunohistochemical analysis. We chose this procedure because it was as specific as the paraffin-embedded tissue method but more sensitive (48,49). Second, we investigated the relationship between leptin/Ob-R and histological variables, such as oestrogen and progesterone receptors, Ki67 proliferation factor and tumour size.

We have shown that primary ductal and lobular breast cancer strongly express leptin and Ob-R. These data were consistent with the results of Ishikawa *et al* (31), whereas another study demonstrated that Ob-R was poorly expressed in breast cancer cases (41.2%) (32). A recent report revealed that

l Ob-R mRNA expressions were detected in 98.7% % of mammary tumours, respectively (50). In addition, leptin and Ob-R mRNA have been detected in numerous breast cancer cell lines, such as MCF-7 and T47D (47,51). All these observations confirm that leptin can act not only by endocrine and (or) paracrine action on mammary tumour cells, but also via an autocrine pathway. Additionally, we observed that leptin and Ob-R expressions were significantly correlated in breast cancer tissue, in agreement with similar earlier observations (31,32).

A recent hypothesis was that leptin and Ob-R might be overexpressed in breast cancer cells, contrary to benign tissue or normal mammary gland adjacent to breast lesions (31,32). In the present study, we observed no such effect, leptin and Ob-R expression levels being comparable in breast cancer and in normal adjacent tissue. Likewise, we recently showed that leptin expression in mammary tumour was similar in benign and normal adjacent tissue (41). However, we originally explored normal breast tissue in healthy women and showed that leptin was not expressed by normal epithelial cells (41). Consequently, we first hypothesised that leptin expression was enhanced in breast cancer compared with normal tissue in healthy women. Second, we suggested that leptin expression in normal tissue adjacent to breast cancer was induced during mammary carcinogenesis. The hypothesis that cancerous tissue may interfere with adjacent tissue was approached by Tessitore *et al* (42), who showed that leptin mRNA expression was significantly enhanced in adipose tissue adjacent to breast cancer.

Considering the link with histological parameters, we show that Ob-R was positively correlated with ER. This finding is consistent with the results of Révillion *et al* (50), who noted a positive relationship between Ob-R mRNA and ER expression. This was expected since several authors have reported evidence of leptin and oestrogen interactions. Leptin exhibited oestrogen-producing activity by enhancing aromatase mRNA expression, aromatase protein content and its enzymatic activity in MCF-7 breast cancer cells (52). Similar results were obtained in breast human adipose stromal cells where leptin stimulated aromatase activity (53). Also, leptin induced a direct functional activation of ER in MCF-7 cells (54). Reciprocally, the oestrogens were able to modulate the leptin system. In this way, it was demonstrated that 17ß-estradiol *in vitro* increased leptin release and leptin mRNA expression in human adipose tissue (55,56). Also, leptin expression in adipose tissue and serum leptin levels were decreased in ovariectomised rats, and estradiol supplement reversed these effects (57). All these findings suggest that both signalling systems may act via a loop pathway and may contribute to breast carcinogenesis in post-menopausal women or in obesity pathology. Finally, it is interesting to note the interaction between leptin and anti-oestrogen breast cancer treatment. Thus in MCF7 breast cancer cells, leptin interfered with the effects of anti-oestrogen ICI 182,780 by increasing nuclear ER α expression and cell proliferation, decreasing ER α degradation and inducing ER α-dependent transcription from oestrogen response element-containing promoters (58). In the same way, recent epidemiological studies reported increased serum leptin levels in women receiving tamoxifen, an anti-oestrogen, as treatment for breast cancer (59,60).

The relationship between leptin and tumour size is still unclear. Using the Spearman test, we showed that Ob-R expression was positively correlated with tumour size. In contrast, Révillion *et al* (50) found Ob-R mRNA expression to be negatively correlated with tumour size, and other studies revealed no relationship between these variables (31,32). However, leptin, acting via the leptin receptor, was able to stimulate the proliferation of breast cancer cell lines such as MCF-7, T47D and ZR-75-1 (38-40). Using genetically obese leptin receptor-deficient $Lepr^{db}Lepr^{db}$ mice, Cleary *et al* (61) demonstrated that these animals did not develop oncogene-induced mammary tumours, whereas homozygous $Lepr^{+}Lepr^{+}$ and heterozygous $Lepr^{+}Lepr^{db}$ lean mice had incidence rates of 69 and 82% respectively. Similar results had previously been obtained from genetically obese leptin-deficient $Lep^{ob}Lep^{ob}$ mice (62). Another recent study in mice demonstrated that inhibition of leptin signalling using leptin peptide receptor antagonist dramatically reduced the growth of implanted mammary tumours (63). Together, these results point to the potential function of leptin, acting via Ob-R, as a growth factor for breast cancer, and the possible role of Ob-R as a new prognostic factor.

In conclusion, we have shown that leptin and Ob-R are strongly co-expressed in both primary breast ductal and lobular cancer, suggesting that leptin acts on breast cancer cells via an autocrine pathway. Also, the co-expression of Ob-R and oestrogen receptors in breast cancer may enhance the interaction between leptin and oestrogen systems, contributing to breast cancer development. Finally, high expression levels of Ob-R are associated with high tumour size, suggesting that leptin may be a growth factor for breast cancer and that Ob-R may be a future prognostic factor. Acting on leptin signalling may thus offer a new approach to breast cancer prevention and treatment.

Acknowledgements

This study received financial support from the 'Ligue Contre le Cancer du Puy-de-Dôme', France. Thierry Jardé is supported by fellowship from the Ministry of Research and Technology.

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