Featured Article

Enhanced Expression of Leptin and Leptin Receptor (OB-R) in Human Breast Cancer

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ABSTRACT

Purpose: **To evaluate leptin and leptin receptor (OB-R) expression in human breast cancer and determine whether it could be effective for the prevention and treatment of breast cancer.**

Experimental Design: **Immunohistochemical staining using specific antibodies was used to evaluate the protein expression of leptin and OB-R in 76 invasive ductal carcinomas and 32 samples of corresponding normal mammary gland, and the relationship between the expression of OB-R and leptin and clinicopathological features was analyzed.**

Results: **Normal mammary epithelial cells did not express a significant level of Ob-R, whereas carcinoma cells showed positive staining for OB-R in 63 (83%) cases. Both normal epithelial cells and carcinoma cells expressed a significant level of leptin. However, overexpression of leptin, as determined by staining intensity, was observed in 70 cancers (92%) but in no normal epithelium. The expression of OB-R showed a significant correlation with the level of leptin expression. Interestingly, distant metastasis was detected in 21 (34%) of 61 OB-R-positive tumors with leptin overexpression, but in none of the 15 tumors that lacked OB-R expression or leptin overexpression ($P < 0.05$). Consequently, patients with the former tumors showed significantly lower survival than those with the latter.**

Conclusions: **Leptin may have a promoting effect on the carcinogenesis and metastasis of breast cancer, possibly in an autocrine manner. Functional inhibition of leptin may be effective for the prevention and treatment of breast cancer.**

INTRODUCTION

The adipocyte-derived cytokine, leptin, is thought to play a key role in the control of satiety, energy expenditure, food intake, and various reproductive processes (1–3). Leptin has 167 amino acids with a molecular mass of 16 kDa and is produced mainly by adipocytes (4). The plasma leptin level is representative of body fat mass (5–8) and increases in a logarithmic fashion with an increase in body mass in mice (9). Additionally, leptin has been shown to control metabolism by affecting the metabolic, neuroendocrine, reproductive, and hematopoietic systems (10). Although initially thought to be exclusively expressed in and secreted by adipocytes, leptin has been identified in additional tissues, such as placenta (11), gastric (12) and colonic mucosa (13), as well as mammary epithelial cells (14).

Leptin exerts its physiological action through the leptin receptor (OB-R), a member of the cytokine family of receptors. OB-R was initially identified in the brain, which explained the negative feedback mechanism of controlling food intake and body weight (15). Further studies, however, have demonstrated that OB-R is also expressed in many other tissues, including brain, placenta, pancreas, adrenal gland, hematopoietic cells, liver, lung, and heart (11, 13, 16–22). OB-R has also been identified in malignant cells of diverse origins, including lung and gastric carcinomas and leukemic cells (22–24). In addition, leptin can regulate the proliferation and invasiveness of colonic and renal epithelial cells (13, 25), and the expression of leptin in pituitary adenomas showed a positive correlation with the invasiveness of tumors (26). However, the expression of OB-R in various cancers has not been fully investigated, and the precise role of leptin in the development and promotion of cancer remains unknown.

Recently, increased body weight has been shown to be associated with increased death rates for cancers at multiple specific sites (27). Previous studies have consistently shown a positive association between adiposity and increased risk of cancer of the endometrium, kidney, colon, and gallbladder in women, and breast cancer in postmenopausal women (28–34). These results encouraged us to evaluate the expression of leptin and its receptor in breast cancers because leptin plays a major role in the regulation of weight and adiposity. In this study, we characterized the expression pattern of leptin and OB-R in breast cancer specimens by immunohistochemical study, and delineated the possible role of this cytokine in the tumorigenesis and spread of breast cancer.

MATERIALS AND METHODS

Patients and Materials. Seventy-six cases of invasive ductal carcinoma, which was surgically resected in the Department of Surgery, The University of Tokyo, from 1992 to 1999, were included in this study. In all cases, ipsilateral axillary lymph node dissection was performed. In all cases, serial-step sections 3-mm wide were cut, fixed in 10% formalin solution, then embedded in paraffin. All of the resected primary tumors and regional lymph nodes were stained with H&E and histologically examined according to the International Union Against Cancer Tumor-Node-Metastasis classification (35). Several discrete histological parameters, including lymphatic invasion, venous invasion, and lymph node metastasis, were additionally

Received 12/16/03; revised 3/23/04; accepted 3/25/04.
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examined. The outcome of these patients was followed up for 3–10 years (mean 5.2 years).

Immunohistochemical Study of OB-R and Leptin. The expression of OB-R and leptin was investigated by immunohistochemical staining using affinity-purified goat polyclonal antibodies against OB-R (M-18; Santa Cruz Biotechnology, Santa Cruz, CA; Ref. 13 and 22) and rabbit polyclonal antibodies against leptin (A20; Santa Cruz Biotechnology; Ref. 13 and 22), respectively. Sections (3-μm thick) were deparaffinized in xylene, hydrated through a graded series of ethanol, and heated in a microwave oven for two 7-min cycles (500 W). After rinsing in PBS, endogenous peroxidase activity was inhibited by incubation with 0.3% hydrogen peroxide in 100% methanol for 30 min. After three washes in PBS, nonspecific reaction was blocked by incubation with PBS containing 5% skimmed milk for 30 min at room temperature, and then the sections were incubated with normal rabbit or goat serum for 30 min. The sections were incubated overnight at 4°C in humid chambers with the primary antibody to leptin at 1:70 dilution or to OB-R at 1:100. After three washes in PBS, the sections were incubated with biotinylated rabbit antigoat or rabbit immunoglobulin for 30 min. After washing again with PBS, the slides were treated with peroxidase-conjugated streptavidin for 30 min, and developed by immersion in 0.01% H_2O_2 and 0.05% diaminobenzidine tetrahydrochloride for 3 min, followed by light counterstaining with Mayer's hematoxylin. Assessment of immunoreactivity was performed by two evaluators without knowledge of the background features. In cases with leptin staining, the samples were subdivided into two groups according to their immunoreactivity. When both investigators agreed that the staining intensity of normal epithelial or carcinoma cells was apparently less than that of adipocytes, those tumors were categorized as having low expression, and when cells were stained at a similar level or more strongly than adipocytes, those tumors were categorized as having high expression.

Statistical Analysis. All statistical calculations were carried out using StatView-J 5.0 statistical software (SAS Institute). The relationship between the expression of OB-R and leptin and clinicopathological features was examined by Student's *t* test, Kruskal-Wallis test, and Spearman rank correlation. Differences with a P value < 0.05 were considered to be statistically significant.

RESULTS

Immunohistochemical Detection of OB-R and Leptin. The expression of leptin and OB-R was evaluated in 76 invasive ductal carcinomas and 32 samples of adjacent normal mammary gland and is summarized in Table 1. Leptin was positively stained in all of the carcinoma cells and normal mammary epithelial cells (Fig. 1, *A-C*). In these samples, the cytoplasm of epithelial and carcinoma cells was homogeneously stained, and heterogeneity was rarely observed. However, the expression level showed a significant difference between normal and carcinoma cells. In all of the 32 cases, the staining level of leptin was markedly weaker in normal epithelial cells than in adipocytes in adjacent adipose tissue of the same sample (Fig. 1*A*). However, in 70 (92%) cases of breast cancer, most of the carcinoma cells were stained as strongly as adipocytes (Fig. 1*C*). In the other six cases, the staining intensity of carcinoma cells was similar to that of normal epithelium and markedly less than that of adipocytes (Fig. 1*B*). These staining intensities were classified as strong and weak expression of leptin, respectively. From these results, it is presumed that leptin production is enhanced in the majority of breast cancers.

Table 1 Expression of Ob-R[a] and leptin in breast carcinoma and normal mammary gland

	Ob-R expression		Leptin expression	
	Positive	Negative	Strong	Weak
Normal mammary gland	0	32	0	32
Breast carcinoma	63	13	70	6

[a] Ob-R, obesity receptor.

In contrast to leptin, none of the normal mammary glands showed significant immunoreactivity for OB-R (Fig. 1*D*), whereas OB-R was expressed in most of the carcinoma cells in 63 (83%) carcinomas (Fig. 1*F*). In these carcinoma cells, OB-R could be detected in the cytoplasm as well as the cell membrane but not in the nucleus. Some interstitial cells were positively stained, but the signals were not as strong as those of carcinoma cells. The staining pattern was similar to that in the gastric or colonic epithelium in previous reports (13, 23). However, in the other 13 cases, most of the carcinoma cells were negative for OB-R, with only a few tumor cells showing faint staining, and thus these were distinguished as OB-R-negative tumors (Fig. 1*E*).

Relation between Expression of Leptin or OB-R and Clinicopathological Features. The relation between leptin/OB-R expression and clinicopathological data are shown in Table 2. The expression levels of both leptin and OB-R tended to increase as tumor size or TMN stage increased, although the relation was not significant. Interestingly, in the 63 OB-R-positive tumors, hematogenous metastasis was detected preoperatively in 4 (6.3%) patients, and 17 (27.0%) patients developed recurrence in distant organs during the follow-up period of 1–8 years. On the contrary, none of the 13 patients with OB-R-negative tumors was associated with distant metastasis, and this difference was statistically significant ($P < 0.05$). Similarly, distant metastasis was detected in 30% (21 of 70) of tumors with strong leptin expression, but in none of the six tumors with weak leptin expression. However, the expression of leptin or OB-R showed no significant correlation with lymphatic invasion, lymph node metastasis, or levels of the following tumor markers, estrogen receptor and progesterone receptor expression, tumor size, and pathological type.

The relationship between the expression patterns of OB-R and leptin is presented in Table 3. Of 63 carcinomas with positive OB-R expression, 61 also expressed leptin strongly, whereas only 2 carcinomas (3.2%) expressed leptin weakly. Of the 13 carcinomas that lacked OB-R expression, 4 (31%) expressed leptin weakly. Hence, the expression of leptin and OB-R in breast cancer was significantly correlated ($P < 0.01$).

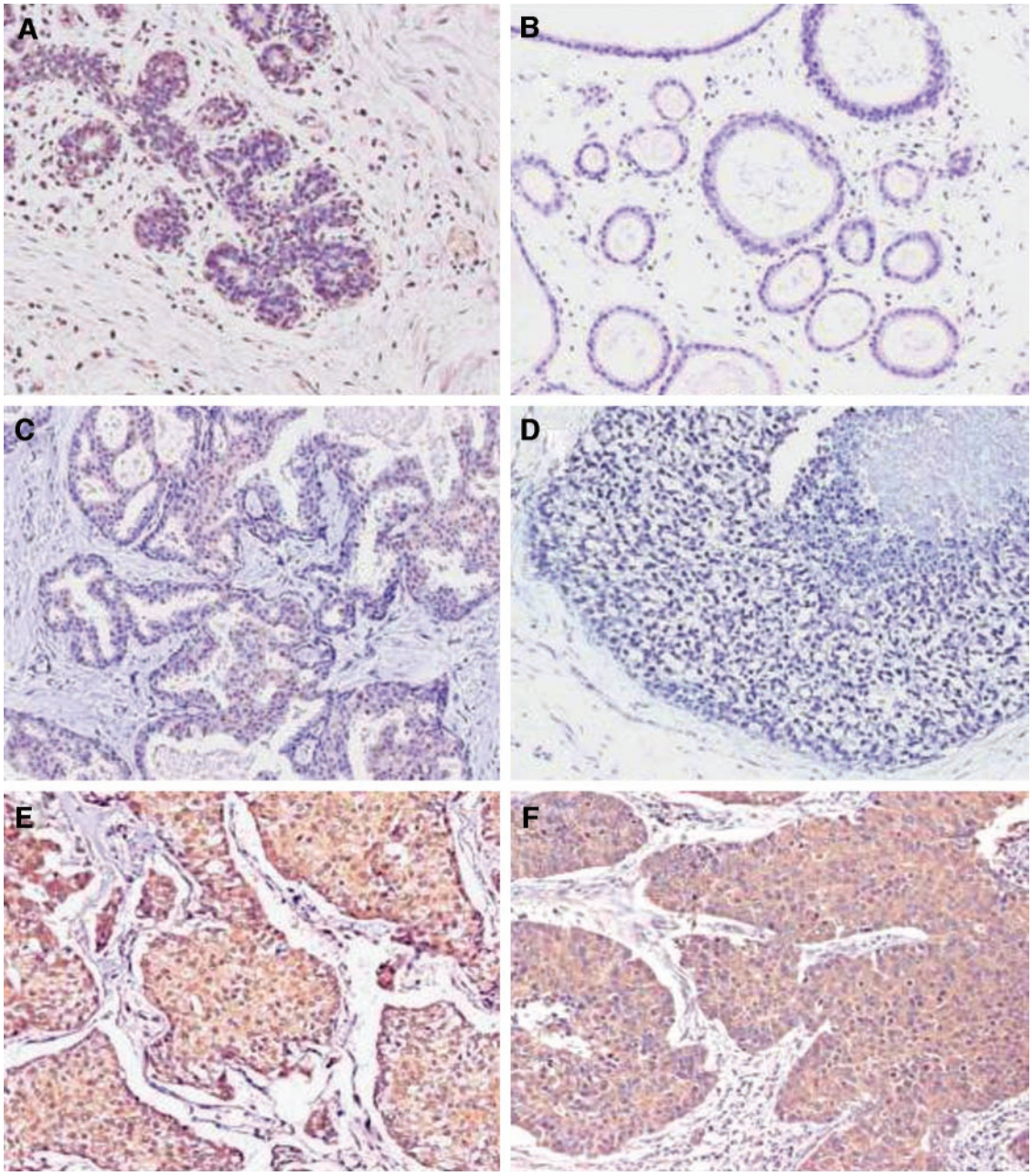

Fig. 1 Immunohistochemical staining of leptin (*A-C*) and Ob-R (*D-F*). Staining of leptin was very weak in normal epithelial cells (*A*). Most of the carcinoma cells were stained as strongly as adipocytes (*C*). In some cases, the staining intensity of carcinoma cells was weak (*B*). Normal mammary glands showed no significant immunoreactivity for OB-R (*D*). OB-R was expressed in most of the carcinoma cells (*F*), but was not expressed in some cases (*E*).

The survival rates of these patients are presented in Fig. 2. All of the 13 patients with OB-R-negative tumors were alive without recurrence of breast cancer at the end of the observation period. Similarly, all 6 patients with low expression of leptin were alive and free from tumor recurrence. In contrast, 10 of 61 patients who had tumors with positive OB-R and enhanced leptin expression died of breast cancer within 7 years. According to this result, breast cancers with and without overexpression of leptin and OB-R were categorized as high- and low-risk groups for tumor recurrence, respectively.

DISCUSSION

Epidemiological studies have shown that obesity is a risk factor for breast cancer in postmenopausal women (32–34). High levels of serum insulin and estrogen, derived from in-

Table 2 The expression of leptin and Ob-R and the clinicopathological characteristics of patients

	Leptin expression			Ob-R expression		
	Strong (70)	Weak (6)	*P* value	Positive (63)	Negative (13)	*P* value
Age (year)	56 ± 12.5	47 ± 9.7	0.07	54 ± 12.6	40 ± 9.4	0.11
Sex						
Male	1	0		0	1	
Female	69	6	0.77	63	12	0.03
Tumor marker						
CEA	6.7 ± 9.3	83.9 ± 177.2	0.33	14.2 ± 45.9	3.3 ± 2.5	0.066
CA15-3	22.1 ± 16.2	38.6 ± 45.7	0.41	21.6 ± 21.2	23.9 ± 17.7	0.99
Estrogen receptor						
Positive	26	2		23	5	
Negative	19	1	0.76	19	1	0.18
Progesterone receptor						
Positive	28	2		25	5	
Negative	15	1	0.96	15	1	0.3
Tumor size						
T_1	28	4		26	6	
T_2	34	2		29	7	
T_3	8	0	0.28	8	0	0.46
Pathological type						
Papillotubular	23	1		21	3	
Solid tubular	9	1		8	2	
Scirrhous	35	4		32	7	
Other	3	0	0.57	2	1	0.46
TNM stage						
1	18	3		17	4	
2A	20	0		17	3	
2B	19	2		15	6	
3A	10	1		11	0	
4	3	0	0.6	3	0	0.46
Lymphatic invasion						
Positive	35	3		30	8	
Negative	35	3	1	33	5	0.36
Venous invasion						
Positive	22	0		19	3	
Negative	48	6	0.103	44	10	0.61
Lymph node metastasis						
Positive	36	3		31	8	
Negative	34	3	0.95	32	5	0.42
Distant metastasis						
Positive	21	0		21	0	
Negative	49	6	0.11	42	13	0.014

creased adipose tissue, are considered to contribute to the pathogenesis of breast cancer in those obese patients. However, because circulating leptin is an essential factor regulating fat metabolism, it can be hypothesized that leptin itself might be involved in the development of breast cancer. In fact, serum leptin levels have been shown to be significantly elevated in breast cancer patients compared with controls (36, 37), although not in premenopausal patients (38).

Therefore, we first examined leptin receptor expression in breast cancer tissues. Thus far, six isoforms derived from OB-R transcription have been identified, and a long isoform, OB-Rb, is reported to be responsible for signal transduction (39). In this study, we used a polyclonal antibody that specifically reacts with OB-Rb (13, 23) and performed immunostaining of human breast cancers. Our experiments clearly recognized cytoplasmic as well as membranous expression of functional leptin receptor (OB-R) in most of the breast carcinoma cells but not in normal epithelium. Recent studies have shown positive expression of OB-R in human normal or malignant breast epithelial cell lines and that leptin can stimulate the proliferation of both normal and malignant breast epithelial cells (40, 41). This is inconsistent with our finding of negative expression of OB-R in normal epithelium. In the study by Hu *et al.* (41), normal gland-derived HBL100 cells also expressed OB-R and responded to leptin. Although this cell line was established from maternal milk, it is uncertain whether normal mammary epithelial cells express a significant level of OB-R, because long-term culture might alter the protein expression pattern. However the growth-stimulating

Table 3 Relationship between the expression of Ob-R[a] and leptin

	Ob-R expression		
	Positive (63)	Negative (13)	*P* value
Leptin expression			
Strong (70)	61	9	
Weak (6)	2	4	<0.01

[a] Ob-R, obese receptor.

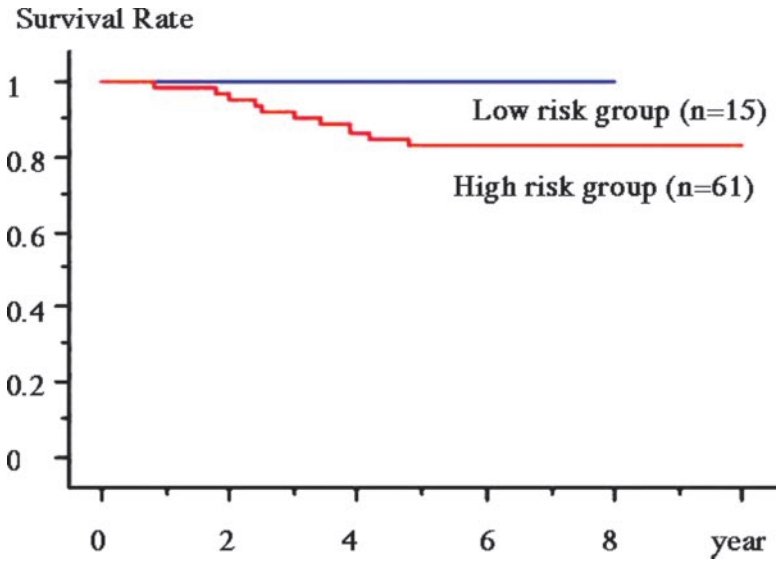

Fig. 2 Survival rate of patients with breast carcinoma in high risk group and low risk group.

effect of leptin on HBL100 was much less than that on a breast cancer cell line, T-47D. This result, together with our data, suggests that the expression of OB-R is induced during the tumorigenesis of breast cancer.

In contrast to OB-R, leptin was positive in both normal epithelial and carcinoma cells. However, the expression of leptin was significantly stronger in carcinoma than in normal epithelium as judged by its staining intensity. The expression of leptin in normal and malignant mammary glands has been reported at the mRNA level (14, 42). O'Brien *et al.* (42) reported that leptin mRNA expression was found to be higher in three different breast cancer cell lines (MCF-7, T470, and MDAMB 231) than in adipose tissue. This finding agrees with our result and suggests enhanced leptin production by the epithelium of mammary glands during tumorigenesis. The relationship between obesity and breast cancer susceptibility has been long verified in mouse experiments (43, 44). Recently, Clear *et al.* (45) have shown a direct contribution of leptin to the development of mammary tumors in a transforming growth factor-α-transgenic and leptin-deficient mouse model. Our data are consistent with these *in vivo* results as well as previous epidemiological studies and strongly support the positive contribution of leptin to the development of breast cancer.

Another important finding in this study is that the expression levels of leptin and OB-R were correlated with distant metastasis. A significant percentage of OB-R-positive tumors showed hematogenous metastasis or recurrence in distant organs within 5 years, and all of the tumors were categorized as having high leptin expression, whereas no tumor that lacked OB-R expression and up-regulation of leptin expression was associated with distant metastasis. Consistently, patients with OB-R-negative and low leptin-expressing tumors showed an extremely good outcome, and the survival rate tended to be lower for patients with OB-R-positive or high leptin-expressing tumors. Although our data on a small sample size are preliminary in nature, this finding strongly suggests that the leptin signal may be involved in the metastatic process as well as carcinogenesis of breast cancer.

Thus far, there is no definite proof on the role of leptin in cancer metastasis. However, leptin has been reported to stimulate proliferation, an essential element for tumor metastasis, in many cancer cells including breast cancer (41, 46–49). Furthermore, leptin has been shown to promote invasiveness of renal and colonic epithelial cells via phosphatidylinositol 3′-kinase-, rho-dependent, and rac-dependent cascades (13, 25). Others have also reported that leptin can augment matrix metalloproteinase production in trophoblasts and endothelial cells (50–52), although this has not been reported in cancer cells. In addition, an epidemiological study has also indicated that breast cancer in obese women is more aggressive, with a poor prognosis (53). These facts, together with our results, support the possibility that the leptin-OB-R signal can play positive roles in breast cancer metastasis. A recent study by Coskun *et al.* (54), however, has shown that serum leptin levels are similar in patients with metastatic and non-metastatic breast cancer. This may rather suggest that leptin functions in an autocrine manner at the tumor site to support the development of metastasis of breast cancer.

In addition, the circulating leptin level is observed to increase during pregnancy in humans and mice (55–57). Pregnancy-associated breast cancer tends to be found at an advanced stage having a poor prognosis, although the tumors often lack estrogen receptors (58, 59). These facts suggest the possibility that leptin may have an important role especially in the pathophysiology of pregnancy-associated breast cancer. Functional inhibition of the leptin signal may be a hopeful strategy for the prevention and treatment of breast cancer.

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