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Evidence of an Autocrine Role for Leptin and Leptin Receptor in Human Breast Cancer

UMAR WAZIR[1,2], WAEL AL SARAKBI[1], WEN G. JIANG[3] and KEFAH MOKBEL[1,2]

[1]*The London Breast Institute, Princess Grace Hospital, London, U.K.;*
[2]*Department of Breast Surgery, St. George's Hospital and Medical School, University of London, London, U.K.;*
[3]*Metastasis & Angiogenesis Research Group, University Department of Surgery, Cardiff University School of Medicine, Cardiff University, Heath Park, Cardiff, Wales, U.K.*

Abstract. *We studied potential associations between the expression of leptin, leptin receptor (LEPR) and clinicopathological parameters of breast cancer, and the correlation of leptin with human telomerase reverse transcriptase (hTERT) and cyclooxygenase 2 (COX2) Patients and Methods: A total of 153 specimens were studied. Transcript levels were determined using quantitative polymerase chain reaction (qPCR), and were correlated with clinicopathological data collected for over 10 years. Additionally, leptin and LEPR expression was studied in several breast cell lines. Results: Both leptin and LEPR mRNA expression were higher in malignant samples (p=0.0011 and 0.0014 respectively). Both were also expressed in MDA436 and MCF7 cell lines. Leptin showed a significant correlation with LEPR (r=0.504, p=0.0000000222). Leptin did not correlate significantly with clinical stage, tumour grade, or with the expression of hTERT or COX2. Conclusions: This study demonstrates that leptin and LEPR are more readily expressed in cancerous tissues suggesting a possible autocrine role in mammary carcinogenesis, which may be independent of hTERT and COX2 genes.*

Leptin is a protein that plays a key role in regulating energy intake and energy expenditure, including appetite and metabolism. There is recent evidence that leptin stimulates proliferation in MCF-7 breast cancer cells. Both cyclooxygenase 2 (COX2) and human telomerase reverse transcriptase (hTERT) have been proposed as mediators of the action of leptin.

Correspondence to: Kefah Mokbel, The London Breast Institute, Princess Grace Hospital, London, U.K. E-mail: kefahmokbel@hotmail.com

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Our objective was to determine, using quantitative PCR, whether the mRNA expression levels of leptin and leptin receptor (*LEPR*, alias OB-R) were consistent with a tumour-proliferative function and whether these levels were significantly associated with clinical outcome in breast cancer. The potential correlations with *hTERT* and *COX2* gene expression were also investigated.

In addition, we studied leptin and LEPR expression in two breast cancer cell lines (MCF7 and MDA436).

Patients and Methods

Samples. Tissue samples were collected after informed consent with ethical approval as per contemporaneous institutional guidelines. Immediately after surgical excision, a tumour sample was obtained from the tumour area, while in some cases another was taken from associated non-cancerous tissue (ANCT) within 2 cm of the tumour, without affecting the assessment of tumour margins. Breast cancer tissues (n=120) and normal background tissues (n=33) were collected and stored at −80˚C, in liquid nitrogen, until the commencement of this study.

All the patients were treated according to local guidelines, following discussions in multidisciplinary meetings. Patients undergoing breast-conserving surgery also underwent radiotherapy. Hormone-sensitive cases were given tamoxifen. Hormone-insensitive cases, high-grade cancer, and node-positive cases were treated with adjuvant therapy. Clinicopathological data (Table I) were collected from the patient charts, and were collated in an encrypted database.

RNA extraction kits and reverse transcription kits were obtained from AbGene Ltd. (Surrey, UK). PCR primers were designed using Beacon Designer (Palo Alto, CA, USA) and synthesized by Invitrogen Ltd. (Paisley, UK). Custom-made hot-start Master Mix for quantitative PCR was from AbGene Ltd.

Tissue processing, RNA extraction and cDNA synthesis. Approximately 10 mg of cancerous tissue were homogenised. A larger amount of ANCT (20-50 mg) was used as its high fat content made it difficult to obtain sufficient RNA for analysis. The concentration of RNA was determined using a UV spectrophotometer (Wolf Laboratories, York, UK) to ensure adequate amounts of RNA for analysis. Reverse transcription was carried out using a reverse

Table I. *Clinical data showing the number of patients in each category.*

Parameter	Category	Number
Node status	Node-ositive	65
	Node-negative	55
Tumour grade	1	23
	2	41
	3	56
Tumour type	Ductal	88
	Lobular	14
	Medullary	2
	Tubular	2
	Mucinous	4
	Other	4
TNM staging	1	69
	2	40
	3	7
	4	4
Clinical outcome	Disease-free	81
	With local recurrence	5
	Alive with metastasis	7
	Died of breast cancer	20

transcription kit (AbGene Ltd) with an anchored oligo (dT) primer using 1 mg of total RNA in a 96-well plate to produce cDNA. The quality of cDNA was verified using β-actin primers (5'-ATGATATCGCCGCGCTCGTC-3' and 5'-CGCTCGGTGAGGATCTTCA-3').

Cell line studies. RNA was similarly extracted from subconfluent cultures of breast cancer lines (MCF7 and MDA436, supplied by the European Collection of Animal Cell Cultures, Salisbury, UK). The concentration of RNA was determined using a UV spectrophotometer (Wolf Laboratories) to ensure adequate amounts of RNA for analysis. cDNA was synthesised using reverse transcription, which was carried out using a reverse transcription kit (AbGene Ltd).

Quantitative analysis. Transcripts of cDNA library were determined using real-time quantitative PCR based on Amplifluor technology. The PCR primers were designed using Beacon Designer software (Premier Biosoft International Ltd., Pal Alto, CA, USA), but an additional sequence, known as the Z sequence (5'-ACTGAACCTGACCGTACA-3'), which is complementary to the universal Z probe (Intergen Inc., Oxford, UK) was added to the primer. The primers used are detailed in Table II.

The reaction was carried out under the following conditions: 94˚C for 12 min and 50 cycles of 94˚C for 15 s, 55˚C for 40 s, and 72˚C for 20 s. The levels of each transcript were generated from a standard plasmid which contained the specific DNA sequence that was simultaneously amplified within the samples.

With every run of the PCR, a negative and positive control was employed, using a known cDNA sequence (1).

Statistical analysis. Transcript levels were normalised using the expression of cytokeratin 19 (CK19). Analysis of the data was performed using the Minitab 12 statistical software package (Minitab Ltd., Coventry, UK) using a custom-written macro (Stat06e.mtb). Medians were compared using the Mann-Whitney *U*-test, while means were compared using the two-sample *t*-test. The transcript levels within the breast cancer specimens were compared to those of the ANCT and correlated with clinicopathological data collected over a 10-year follow-up period.

p-Values less than 0.05 were considered significant, whereas *p*-values between 0.05 and 0.10 were considered marginally significant.

Associations between the expressions of different molecules assessed using Spearman's correlation.

For purposes of the Kaplan–Meier survival analysis, the samples were divided arbitrarily into high and low transcription groups, with the value for the moderate prognostic group as defined by Nottingham Prognostic Index (NPI) serving as the dividing line. Survival analyses were performed using PSAW18 (SPSS Inc., Chicago, IL, USA).

Results

The levels of *leptin* and *LEPR* mRNA were significantly higher in malignant tissue samples (p=0.0011 and 0.0014, respectively). This difference remained statistically significant when comparing levels between normal samples and all malignant subgroups according to their NPI, grade, stage, local recurrence, and distant metastasis in both oestrogen receptor (ER)-positive and- negative samples. It was also present across all histological types for both leptin and its receptor.

There was no significant change in mRNA transcription levels correlating with tumour grade, stage, disease-free survival, or development of locoregional recurrence.

There was also no statistically significant difference in the levels of expression of both leptin and its receptor when comparing ER-positive to- negative cases (p=0.74 and 0.27 respectively).

Leptin levels were significantly positively correlated with LEPR (r=0.504, p=<0.0001). We observed no significant correlations between leptin and hTERT or COX2 expression. Both leptin and LEPR were expressed in MCF7 and MDA436, representing both ER-negative and ER-positive forms of breast neoplasia (Table IV).

Discussion

The effects of lesions at certain nuclei of the hypothalamus on weight and satiety have been observed since the 1940s (2). The existence of a weight-regulating factor was inferred from experiments by Hervey, in which he connected the circulatory systems of a healthy mouse, and a mouse with a surgically-damaged hypothalamus. The previously healthy mouse reduced its food intake, whilst its counterpart became obese (3).

The satiety factor, termed leptin, was isolated in 1995, along with LEPR was initially isolated in the satiety centre of the hypothalamus (4). Subsequently, at least five isoforms

Table II. *Primers used in real-time quantitative polymerase chain reaction analysis.*

Gene	Sequence
*LEPTIN*F1	ATGACACCAAAACCCTCAT
*LEPTINZ*R1	ACTGAACCTGACCGTACAAACCGGTGACTTTCTGTTT
*LEPR*F1	TGGTGAAATAAAATGGCTTAG
*LEPRZ*R1	ACTGAACCTGACCGTACATTGGGTAAAGACTGAACTGG
Beta-actin Forward	ATGATATCGCCGCGCTCGTC
Beta-actin Reverse	CGCTCGGTGAGGATCTTCA

Table III. *Mean leptin and leptin receptor (LEPR) mRNA expression levels (copy number) in a cohort of 120 breast cancer patients showing the comparison between breast cancer tissue and associated non-cancerous tissue.*

	Mean (BCT vs. ANCT)	Median (BCT vs. ANCT)	95% CI	*p*-Value
Leptin	979 *vs.* 969	0. 1 *vs.* 63.4	2.1 to 82.6	0.0011
LEPR	280124 *vs.* 28891	1 *vs.* 87	–4 to 152	0.0014

BCT: Breast cancer tissue, ANCT: associated non-cancerous tissue, CI: confidence interval.

of LEPR were identified (5), which have been found to be expressed in various tissues, including the reproductive tract (6), breast tissue (7), liver (8), and the alimentary canal (9).

Leptin is an adipocytokine encoded by the OB (*LEP*) gene on chromosome 7 (10). It is largely secreted by adipose tissue, and its level in serum has been found to correlate closely with the proportion of body fat (11). In addition to control of nutritional state and satiety, it has also been found to play a role in cell proliferation, inflammation, bone development, angiogenesis, breast development and reproduction (12).

Obesity has been associated increased risk of cancer of the liver, cervix, uterus, colon, gall bladder, bladder, kidney and breast, as well as lymphoma (13, 14) . Several pathways activated in obesity have been implicated in the pathogenesis of various neoplasias, including breast cancer. These pathways include the insulin–leptin–adiponectin axis, transforming growth factor and the epidermal growth factor receptor (15, 16). Increased serum leptin levels have been found to be significantly associated with an increased risk of breast cancer. The association of circulating leptin levels with breast cancer risk has been found to persist after adjustments for age and obesity (17, 18). Leptin and LEPR are expressed in normal and cancerous breast tissue. Evidence with regards to correlation of LEPR with clinicopathological parameters has been contradictory. Kim *et al*. found no correlation between prognosis and LEPR expression (19), whilst Xia *et al*. did find a find correlation between LEPR and leptin expression and clinicopathological features (20). Miyoshi *et al*. found an association between intra-tumoral LEPR expression and worsening prognosis in breast cancer in a subset of patients with high serum or high tissue leptin expression (21). Some authors have associated specific polymorphisms of LEPR gene and leptin with breast cancer risk in certain populations (22, 23). Such polymorphisms may account for the findings of Artac *et al*., who demonstrated the waist-height ratio to be a prognostic marker in metastatic breast cancer, if associated with serum leptin levels above 19,400 pg/ml (24).

Table IV. *Mean leptin and leptin receptor (LEPR) mRNA expression levels (copy number) in breast cancer cell lines.*

Breast cancer cell line	Leptin	Leptin receptor
MCF7	0.064	20833.3
MDA436	326.9	0.184

Whilst correlations of breast cancer with circulating leptin are well-known, the role of leptin expressed within the tissue is relatively obscure. Here we demonstrated an increased expression of both LEPR and leptin in tumour tissue, as compared to normal tissue, with a strong correlation between the two. This was also demonstrated in a previous smaller study by Karaduman et al, who also failed to find an association between leptin and LEPR expression and clinicopathological data (25). This is highly suggestive of an autocrine role of leptin in breast cancer pathogenesis, which may account for the previously-cited association of LEPR with breast cancer risk independently of obesity (26).

LEPR has been characterized as a member of the cytokine class I family of receptors, exerting its effects mainly *via* Janus kinase, and the signal transducers and activators of transcription (STATs). Additionally, it has also been found to impact on extracellular signal-regulated kinase (ERK), and protein kinase B (PKB, also known as AKT) (27). One of the known effectors of this pathway is telomerase, which is believed to be a factor in cell immortality in cancer. Its catalytic component, which is a reverse transcriptase (hTERT), is significantly associated with clinicopathological parameters in breast cancer (28). Studies in MCF-7 breast cancer cells have suggested that leptin may up-regulate hTERT expression and activity in a dose-dependent fashion through a pathway involving phosphorylation of STAT3 (29). Rahmati-Yamchi *et al*. found an association between serum leptin and tissue hTERT expression, as well as between body mass index and hTERT expression (30). In our study, no significant association was found between tissue leptin and LEPR expression and hTERT. This may suggest that the endocrine and autocrine effects of leptin in breast cancer may be distinct, and may involve different mechanisms of actions meriting closer examination (26).

Increased COX2 activity and expression have long been implicated in colonic and breast cancer. COX2 is up-regulated in response to a diverse range of stimuli, including interleukin 6 (IL-6), tumour necrosis factor, the rat sarcoma (RAS) – ERK and RAS–mitogen-activated protein kinase kinase kinase 1, E3 ubiquitin protein ligase (MAP3K1) – Jun N-terminal Kinase (JNK) signal transduction pathways, obesity and dietary factors. Products of its activity include superoxides and mutagens known for their oncogenic effects (31-33). Recent studies in breast cancer cell lines have also demonstrated that increased COX2 activity could lead to genomic instability (34). COX2 expression has been found to be of prognostic significance in both early- and late-breast cancer (35, 36). It may have role in bone metastasis in breast cancer, possibly by stimulating the production of IL-11 (37, 38). Additionally, COX2 has been implicated in the development of resistance to Herceptin and other anticancer treatments (39). Non-steroidal anti-inflammatory drugs have been demonstrated to reduce the risk of breast cancer recurrence (40). Celecoxib, a selective inhibitor of COX2, has shown promise in breast cancer. However, its utility has been reduced by its cardiovascular side-effects (41-43).

COX2 is evidently one of the many down-stream mediators of obesity-related carcinogenesis, for which circulating, endocrine leptin could be taken as a surrogate. The lack of association with tissue-expressed leptin emphasises the distinction between the proposed endocrine and autocrine roles of leptin in breast cancer.

In conclusion, in view of the literature and our results, there is compelling evidence for leptin acting as a local mediator in the pathogenesis of breast cancer, distinct from its well-attested role as an endocrine mediator of obesity-related cancer risk. This merits further investigation in order to gain a better understanding of the role of dietary and metabolic factors in breast cancer.

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