Leptin is overexpressed in the tumor microenvironment of obese patients with estrogen receptor positive breast cancer

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Abstract. The present study aimed to investigate the potential role of leptin in the progression of breast cancer and the associated cell proliferation signalling pathway(s). A total of 44 female patients diagnosed with breast cancer and 24 healthy donors from Ain Shams University Hospitals (Cairo, Egypt) were enrolled in the present study. The present study assessed leptin expression in breast cancer tissues at the gene and protein level using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and immunohistochemistry. The results demonstrate that the expression of leptin was significantly higher in tissue of breast cancer samples from obese patients than overweight and control samples ($P<0.001$). ELISA results indicated a significant increase ($P<0.001$) of leptin expression in obese patients. To investigate whether there is any difference in leptin expression between the peripheral and tumor microenvironment blood of patients with breast cancer, the concentration of leptin was assessed in plasma from both using ELISA assays. The results demonstrated a statistically significant increase in the level of leptin in plasma samples from the tumor microenvironment of obese patients with estrogen receptor positive (ER^+) breast cancer, compared with peripheral plasma samples. Furthermore, the leptin gene was overexpressed in obese ER^+ breast cancer tissue. RT-qPCR was also performed to assess the expression of genes involved in proliferation pathways including leptin receptor (LEPR), aromatase, mitogen activated protein kinase (MAPK) and signal transducer and activator of transcription-3 (STAT3). A positive association between leptin expression, LEPR, aromatase, MAPK and STAT3 was detected in tissue samples of patients with breast cancer. The current study concluded that leptin may enhance breast cancer progression by inducing the expression of JAK/STAT3, ERK1/2 and estrogen pathways in obese patients breast cancer.

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Abbreviations: LEPR, leptin receptor; BMI, body mass index; CYP19A1, cytochrome P450 family 19 subfamily A member 1; STK11, serine/threonine kinase 11; MAPK, mitogen activated protein kinase; ERK, extracellular signal-related kinases; JAK/STAT3, janus kinase/signal transducer and activator of transcription-3; MMP, matrix metalloproteinase; EGFR, epidermal growth factor receptor; VEGF, vascular endothelial growth factor; LPrA2, leptin receptor antagonist; PR, progesterone receptor; ER, estrogen receptor; HER2, human epidermal growth factor receptor-2; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; IHC, immunohistochemistry; TBST, tris-buffered saline with Tween-20; ASCs, adipose stromal stem cells; obASC, obese adipose stromal stem cells

Key words: obesity, breast cancer, leptin, expression, tumor microenvironment, estrogen receptor

Introduction

Breast cancer leads to 15.4% of cancer-related mortalities among females in developed countries, and is the primary cause of cancer morbidity in poorly developed countries (1). Breast cancer represented 1.7 million cases, 11.9%, of all cancer worldwide in 2015 (1,2), and is the most prevalent cancer among women in Egypt, constituting 32% of female cancer cases leading to death (3).

Obesity is characterized by the accumulation of adipocytes in fat tissues and is considered as a serious health problem due to its association with different disorders including carcinogenesis (4). Obesity degree is measured by body mass index (BMI) and it was estimated that >1.3 billion individuals worldwide are obese according to World Health Organization (WHO) (5,6). Obesity is considered to be a breast cancer risk factor that may rise steadily worldwide, and it is estimated that 21% of all cancer morbidity worldwide is due to obesity (7,8). Among risk factors related to obesity is the accumulation of adipose tissue that secretes adipokines including leptin, resistin, adiponectin and other cytokines (9).

Obese patients with breast cancer are characterized by advanced pathological characteristics including high tumor

grade, advanced tumor stage and lymph node metastasis (10), in addition to cancer recurrence and shorter disease free survival (11). Furthermore, obesity may decrease the efficiency of chemotherapy against breast cancer (12).

Leptin is a polypeptide (16 kDa) product of a gene associated with obesity (13) that mediates its physiological actions through the leptin receptor (LEPR) (14). It is a cytokine hormone that modulates energy balance and weight homeostasis through stimulating the expression level of cytochrome P450 family 19 subfamily A member 1 (CYP19A1), and controlling serine/threonine kinase 11 (STK11) and mitogen activated protein kinase (MAPK) (13,15-17). Furthermore, leptin possesses different biological and physiological functions including immune responses, puberty, lactation, cell proliferation and hematopoiesis (18,19). Leptin and its receptor were previously identified to be associated with aggressive breast tumor proliferation, cell migration and stimulation of angiogenesis and invasion (20). It was demonstrated that leptin is associated with breast cancer development by enhancing the janus kinase/signal transducer and activator of transcription-3 (JAK/STAT3), extracellular signal-related kinases 1 and 2 (ERK1/2) and phoshphoinositide 3-kinase pathways that lead to breast cancer cell proliferation and cell survival *in vitro* studies (21). A number of *in vitro* studies have reported that leptin may stimulate estrogen expression by increasing the expression of the intracellular aromatase enzyme, which has also been implicated in breast cancer development (22,23).

Leptin may induce breast cancer progression through stimulating the adhesion process by enhancing the expression level of E-cadherin in MCF-7 cell lines (24), migration and invasion processes by activating the expression of matrix metalloproteinase 2 and 9 (MMP2 and MMP9) and epidermal growth factor receptor (EGFR) (25). Additionally, leptin may stimulate angiogenesis and cell cycle processes via the activation of vascular endothelial growth factor (VEGF) expression and cyclin D1, respectively (26-28) and inhibiting apoptosis of breast cancer cells (29). It has been indicated that the small peptide leptin receptor antagonist (LPrA2) decreases breast cancer growth in mice (27). The inhibition of leptin signalling provides a target for breast cancer treatment that may be useful in reducing the progression of breast cancer.

Studying the molecular mechanisms of leptin that contribute to breast cancer development may guide the identification of novel therapies to reduce breast cancer progression and/or development. In the present study, leptin expression in patients with breast cancer and the possible proliferation pathway(s) responsible for breast cancer progression were assessed and a significant positive association between leptin expression, LEPR and activation of cell proliferation signalling pathways (aromatase, MAPK and STAT3) in tissue samples of breast cancer patients was observed. Furthermore, the concentration of leptin in plasma of the breast tumor microenvironment and peripheral blood of patients was assessed and the present study demonstrates that the concentration of leptin in plasma from tumor microenvironment blood was significantly higher compared with the leptin in plasma from peripheral blood of obese patients with estrogen receptor positive (ER^+) breast cancer.

Materials and methods

Patient selection. The present study was approved by the Institutional Review Board of the Ain Shams University Hospital Ethics Committee. Each patient signed a consent form prior to participation.

Patients who visited the breast clinic of Ain Shams University Hospital (Cairo, Egypt) and were subjected to medical analysis by clinical examination, mammogram, ultrasound and biopsy were enrolled in the present study.

A total of 44 female patients (age, 34-70 years; weight, 70-120 kg) diagnosed with breast cancer and 24 healthy donors (age, 30-65, weight, 70-100 kg) were enrolled between February 2013 and August 2014. The clinical-pathological characteristics: BMI, menopausal status and tumor invasion were recorded based on pathological reports and medical records. Prognostic factors including tumor grade, tumor size, lymphovascular invasion, progesterone receptor (PR), estrogen receptor (ER), human epidermal growth factor receptor-2 (HER2) and Ki67 were documented by a professional pathologist, to be used as a cell proliferating labelling index.

Subject groups. Patients were divided into groups; group i included obese breast cancer patients (BMI ≥30; n=24), group ii included overweight breast cancer patients (BMI between 25 and 30; n=20) and group iii was control group of healthy donors (n=24). These groups were subdivided according to menopausal status into the following sub groups: Postmenopausal obese patients (group iA; n=18), premenopausal obese patients (group iB; n=6), postmenopausal overweight patients (group iiA; n=11) and premenopausal overweight patients (group iiB; n=9). The control group was subdivided into the following subgroups; (group iiiA; n=6), premenopausal obese controls (group iiiB; n=6), postmenopausal overweight controls (group iiiC; n=6) and premenopausal overweight controls (group iiiD; n=6). Furthermore, patients were subdivided according to estrogen receptor into subgroups; obese patients positive for estrogen receptor (group iC; n=20), obese patients negative for estrogen receptor (group iD; n=4), overweight patients positive for estrogen receptor (group iiC; n=12) and overweight patients negative for estrogen receptor (group iiD; n=8). Tissue samples were collected from conservative breast surgery or modified radical mastectomy and divided into 2 halves; one fixed for 24 h at room temp in 10% neutral buffered formalin for immunohistochemistry and second snap frozen in liquid nitrogen for molecular studies.

Plasma sample preparation. A total of 10 ml plasma was isolated from peripheral blood and blood collected from tumor microenvironment prior to and during surgical operation for each patient in EDTA tubes as previously described (30). In patients with breast cancer, venous withdrawal from the breast may include cells of immunological importance, including tumor cells and other biological factors obtained from the tumor microenvironment. Therefore, biological characteristics of breast tumor microenvironment may be defined by collecting axillary tributaries during modified radical mastectomy prior to dilution in circulation (30). A further 10 ml peripheral blood was withdrawn from the antecubital vein from healthy

Table I. Primer sequences of target genes for reverse transcription-quantitative polymerase chain reaction.

Gene	Direction	Sequence
HPRT	Forward	5'-CTCCTCCTGAGCAGTCAGC-3'
	Reverse	5'-GTCATAACCTGGTTCATCATCACT-3'
Leptin	Forward	5'-AAAGATAGGGCCAAAGCCAC-3'
	Reverse	5'-GTAGGAATCGCAGCGCC-3'
Leptin receptor	Forward	5'-CCCAATGTAACAAAACCACACA-3'
	Reverse	5'-CTTATGCTGGGATGTGCCTT-3'
Aromatase	Forward	5'-TCTCGATTCGGCAGCAAACT-3'
	Reverse	5'-TGACCATACGAACAAGGCCG-3'
MAPK	Forward	5'-GGGGCTGATTTTCTTGATAGC-3'
	Reverse	5'-ACCAACCTCTCGTACATCGG-3'
STAT-3	Forward	5'-CTGCTCCAGGTACCGTGTGT-3'
	Reverse	5'-CCTCTGCCGGAGAAACAG-3'

HPRT, hypoxanthine-guanine phosphoribosyltransferase; MAPK, mitogen activated protein kinase; STAT-3 signal transducer and activator of transcription-3.

volunteers in anticoagulant tubes as a control (30). Blood was then centrifuged at 2,000 x g for 10 min at room temperature for plasma preparation. Plasma was aliquoted and stored at -80˚C until use.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RNA was extracted from 29 tissue samples from patients with breast cancer and 8 normal tissues using the GeneJET RNA Purification kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocol. A total of 1 μg total RNA was converted into cDNA using a Revert aid cDNA synthesis kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. PCR was performed using the Maxima SYBR-Green Master Mix kit (Thermo Fisher Scientific, Inc.) to amplify leptin, LEPR, aromatase, MAPK and STAT3 genes using hypoxanthine-guanine phosphoribosyltransferase (HPRT) as a housekeeping control gene. Primers used for qPCR were commercially synthesized from Macrogen, Inc. (Seoul, Korea) and are listed in Table I. qPCR was performed in applied Biosystems Step One Plus (Thermo Fisher Scientific, Inc.) and reactions were performed in duplicate. Each sample was initially denatured at 95˚C for 5 min, then subjected to 40 cycles of the following: Denaturation at 95˚C for 50 sec, and annealing and extension at 60˚C for 1 min. Each sample was exposed to a final extension at 72˚C for 10 min and finally held at 4˚C followed by amplification and melting curves. Following qPCR, Cq values were measured, ΔΔCq and fold expression were calculated to quantify the results (31).

Immunohistochemistry (IHC) for leptin. The expression of leptin in breast tissue was evaluated in 23 female patients with breast cancer from the obese (n=13) and overweight (n=10) groups and compared with obese and overweight control samples (n=6) from healthy donors.

The paraffin embedded blocks were sliced using a microtome into 4 μm-thick tissue sections. Tissue sections were initially stained with hematoxylin and eosin, mounted using positive charged slides and air-dried overnight. Following de-waxing (by immersing in xylene for 5 min) and hydration (by embedding slides in graded concentrations of alcohol; 100, 95, 80 and 50%; (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), positive slides were incubated in citrate buffer (pH=6; 2.1 g citric acid dissolved in 1 l distilled water) in a water bath for 1 h at 99˚C. Slides were subsequently kept at room temperature and dipped with two changes of Tris-buffered saline with Tween-20 (TBST; 0.05 mol/l tris-HCl, pH 7.6, 0.15 mol/l NaCl and 0.05% Tween-20) for 5 min of washing. The slides were blocked using 3% hydrogen peroxide for 10 min (Dual Endogenous Enzyme block, K4065; Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) and were washed with TBST. Slides were then incubated at room temperature overnight with rabbit polyclonal primary antibody against leptin (ab3583; 1:50; Abcam, Cambridge, UK). The slides were rinsed in TBS two times for 5 min and incubated with 100 μl horseradish peroxidase-labelled polymer rabbit (catalogue number not supplied; EnVision+ Dual link system-HRP DAB+; 1:50; Dako; Agilent Technologies, Inc.) for 45 min at room temperature and in TBST for 5 min. Diaminobenzidine with substrate/chromogen was put on the slides and incubated at room temperature for 5-10 min, depending on the appearance of a brown color, then slides were washed in distilled water. Mayer's hematoxylin was added to the slides for counterstaining. The slides were washed in tap water, following dehydration and clearing steps, and were covered using DPX mounting media (Thermo Fisher Scientific, Inc.).

An immunohistochemical score of 0 was considered negative, + represented faint staining, ++ represented moderate staining and +++ was considered to be strong staining. Leptin status was assessed as positive and negative for patients. The staining was described as negative if no cancer cells were stained and positive if cancer cells were stained and subsequently examined using a light microscope (Optika S.r.l, Ponteranica, Italy) (<37 or 10 or >10%).

Table II. Patient and tumor characteristics.

Characteristics	Data
Age (years)	
Mean ± standard deviation	51.66±1.506
Range	34-70
Menopausal state, n (%)	
Premenopausal	15 (34.1)
Postmenopausal	29 (65.9)
BMI, kg/m^2 (%)	
≥30	24 (54.54)
<30	20 (45.46)
Tumor size (cm)	
Mean	28.6
Range	0.17-110
Tumor grade, patient no. (%)	
Grade II	43 (97.7)
Grade III	1 (2.3)
Metastatic lymph nodes, n (%)	
≤4	35 (79.55)
>4	9 (20.45)
Lymph vascular invasion, n (%)	
Positive	8 (18.2)
Negative	36 (81.8)
Estrogen receptor, n (%)	
Positive	32 (72.73)
Negative	12 (27.27)
Progesterone receptor, n (%)	
Positive	32 (72.73)
Negative	12 (27.27)
HER-2, n (%)	
Positive	10 (22.7)
Negative	34 (77.3)

BMI, body mass index; HER-2, human epidermal growth factor receptor-2.

ELISA assay. Concentration of leptin in plasma from peripheral blood and blood collected from the tumor microenvironment were determined using the Leptin (Sandwich) ELISA kit (EIA 2395; Qiagen AB, Sollentuna, Sweden) following the manufacturer's protocol.

Statistical analysis. The data were analysed using SPSS software version 18.0 (SPSS, Inc., Chicago, IL, USA). Data were expressed as mean ± standard deviation and correlations between categorical variables were assessed using Spearman correlations test and Student's *t*-test. P≤0.01 represents statistically significant differences.

Results

Clinical and pathological characteristics of patients. Clinical and pathological characteristics are presented in Table II and include age, BMI, menopausal status, tumor grade, tumor size, lymph node metastasis, lymph vascular invasion and expressions of ER, PR and HER2 as explained below.

The present study was applied in patients with median age 51.66±1.506 years (range, 34-70). Among 44 female patients, 29 (65.9%) were postmenopausal and 15 (34.1%) were premenopausal. BMI between 18.5 and <25 is considered to be normal, between 25 to <30 as overweight and ≥30 as obese according to the WHO (5). A total of 24 female patients (54.54%) were obese and 20 patients (45.46%) were overweight. The mean tumor sizes ranged from 0.17-110 cm (mean size 28.6 cm). Among patients, 88.6% were negative for lymph vascular invasion and 11.4% were positive for lymph vascular invasion. Tumor grade staging was as follows: 97.7% of patients were classified as grade 2, while 2.3% were classified as grade 3.

Expression of leptin in obese breast cancer patient tissues. The mRNA expression level of leptin in the tissue of patients with breast cancer was assessed. Leptin was significantly overexpressed in obese patients compared with overweight patients and healthy donors by 3.1-fold and 8.3-fold, respectively (P<0.001; Fig. 1A and B). The expression of leptin was higher in postmenopausal and premenopausal obese patients than postmenopausal and premenopausal overweight patients by 3.28-fold and 2.8-fold, respectively (Fig. 1C).

The same findings were obtained when the protein expression of leptin in tissue was assessed by immunohistochemistry (Table III and Fig. 2). The association between leptin expression and clinical data of patients was assessed and indicated that there is a positive correlation between expression of leptin in breast cancer patient tissues and BMI using Student *t*-test (r=0.916), whereas no significant association was identified between leptin expression and menopausal status (r=0.373; Table III). A positive association was identified between the expression of leptin and ER expression in obese patients (Table III). Conversely, a negative correlation was detected between the expression of leptin and ER in overweight patients (r=0.9 and r=-0.346 respectively; Table III). No significant correlation was identified between the expression level of leptin and PR or HER2 (r=0.182 and r=0.171 respectively; Table III). Ki67 is a cell proliferating label index that serves an important role in cell proliferation. The correlation between the expression level of leptin and expression of Ki67 in tissues from patients with breast cancer was assessed. There was no significant correlation between the expression level of leptin and the expression of Ki67 in tissues from patients with breast cancer (r=0.283; Table III).

Assessment of leptin protein expression in plasma of the tumor microenvironment and peripheral plasma. The results of the present study indicate that there was a non-significant increase in the concentration of leptin in plasma from the tumor microenvironment blood in obese patients (Fig. 3A). Furthermore, a significant difference was observed between the concentration of leptin in peripheral plasma of obese and overweight patients with breast cancer compared with that of obese and overweight volunteers, respectively (both P<0.001; Fig. 3B).

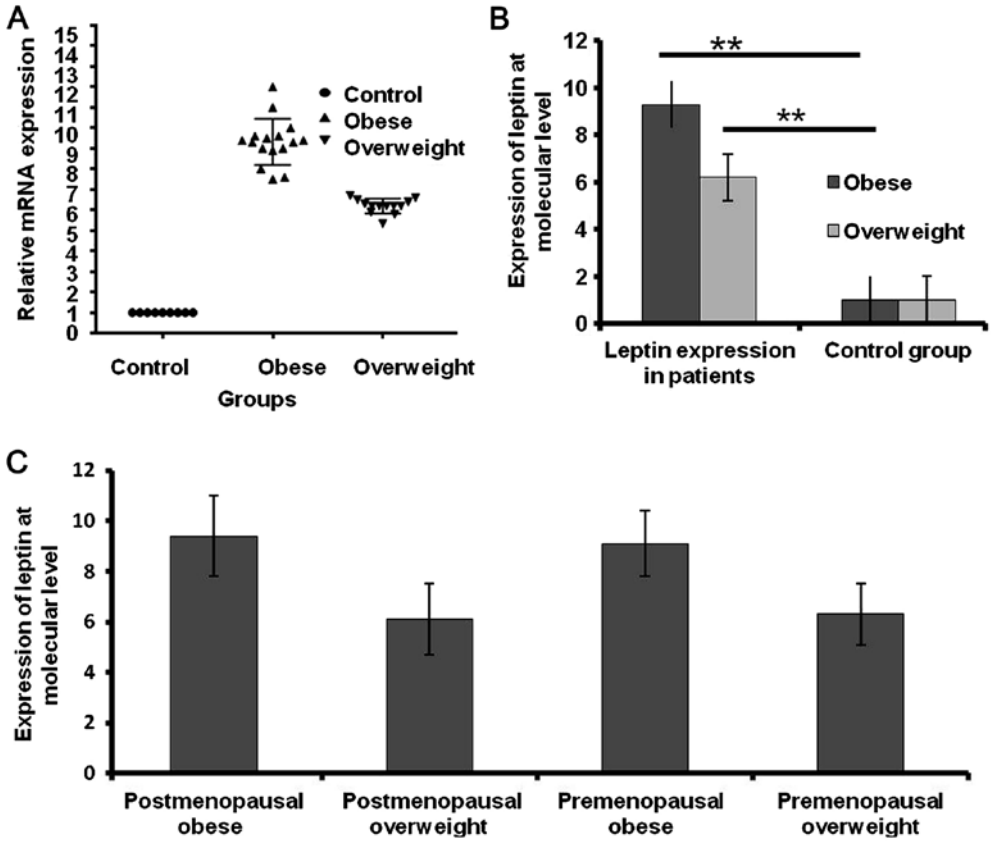

Figure 1. Expression of leptin gene in breast cancer tissue from obese and overweight patients. (A) Comparison between mRNA fold expression of leptin for each sample of obese and overweight patients compared with control samples. (B) Comparison between expression levels of leptin mRNA in obese patients compared with overweight patients and normal patients analysed using the Student's *t*-test. (C) Comparison between expression of leptin mRNA in post and pre-menopausal obese and overweight patients. Data are presented as mean ± standard deviation. **P<0.001.

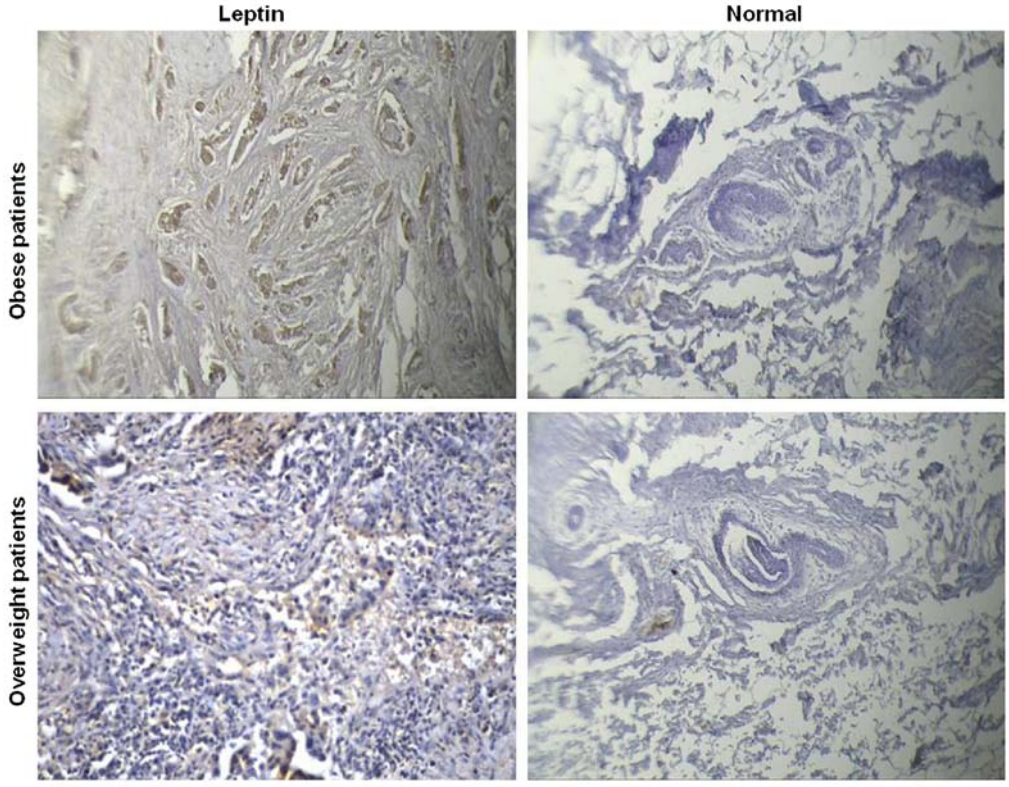

Figure 2. Expression of leptin protein in obese and overweight breast cancer tissue. Photomicrographs represent immunohistochemical staining using hematoxylin and eosin of leptin demonstrating high expression of leptin in breast tissue from an obese patient compared with normal tissue and high expression of leptin in breast tissue from overweight patients compared with normal tissue. Magnification, x100.

Table III. Association between leptin expression and prognostic factors in tissues from patients with breast cancer.

	Leptin (n=23)			
Variable	High expression (n=13)	Low expression (n=10)	Correlation coefficient	P-value
BMI, n (%)			0.916	<0.001[a]
Obese	12 (92.3)	0 (0)		
Overweight	1 (7)	10 (100)		
Menopausal, n (%)			0.373	0.08
Post	11 (84.6)	5 (50)		
Pre	2 (15.4)	5 (50)		
ER, n (%)			0.182	0.405
Positive	10 (76.92)	6 (60)		
Negative	3 (23.08)	4 (40)		
ER, n (%)			0.9	0.01
Obese	12 (92.3)	0 (0)		
Overweight	1 (7)	10 (100)	-0.346	0.297
PR, n (%)			0.182	0.405
Positive	10 (76.92)	6 (60)		
Negative	3 (23.08)	4 (40)		
HER-2, n (%)			0.171	0.435
Positive	3 (23.07)	1 (10)		
Negative	10 (76.93)	9 (90)		
Ki67, n (%)			0.238	0.273
Positive	7 (53.85)	3 (30)		
Negative	6 (46.15)	7 (70)		

[a]P<0.01. BMI, body mass index; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor-2; Ki67, Cell proliferating labelling index.

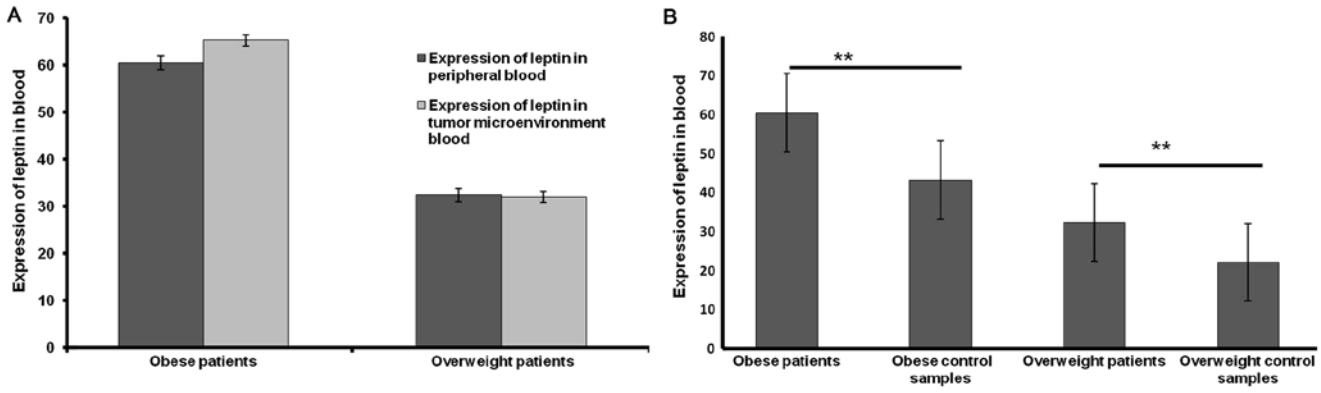

Figure 3. Leptin concentration in peripheral blood and tumor microenviroment measured by ELISA. (A) Comparison between the concentration of leptin in plasma from peripheral blood and tumor microenvironment blood in obese and overweight patients with breast cancer. (B) Comparison between the concentration of leptin in plasma from peripheral blood in obese and overweight patients compared with control samples. **P<0.001. Data are presented as mean ± standard deviation and were analyzed using Student's *t*-test.

In addition, a significant difference was observed between the concentration of leptin in the peripheral plasma samples of post- and pre-menopausal obese patients and control samples (P<0.001; Fig. 4). A significant increase was also observed between the concentration of leptin in plasma from peripheral blood between post- and premenopausal overweight patients and control samples (P<0.001; Fig. 4).

By contrast, no significant difference was observed between the concentration in leptin in plasma from peripheral blood among postmenopausal and premenopausal obese or overweight patients (Fig. 4).

Expression of leptin in obese and overweight patients with ER^+ and ER^- breast cancer. Patients were sub-grouped into

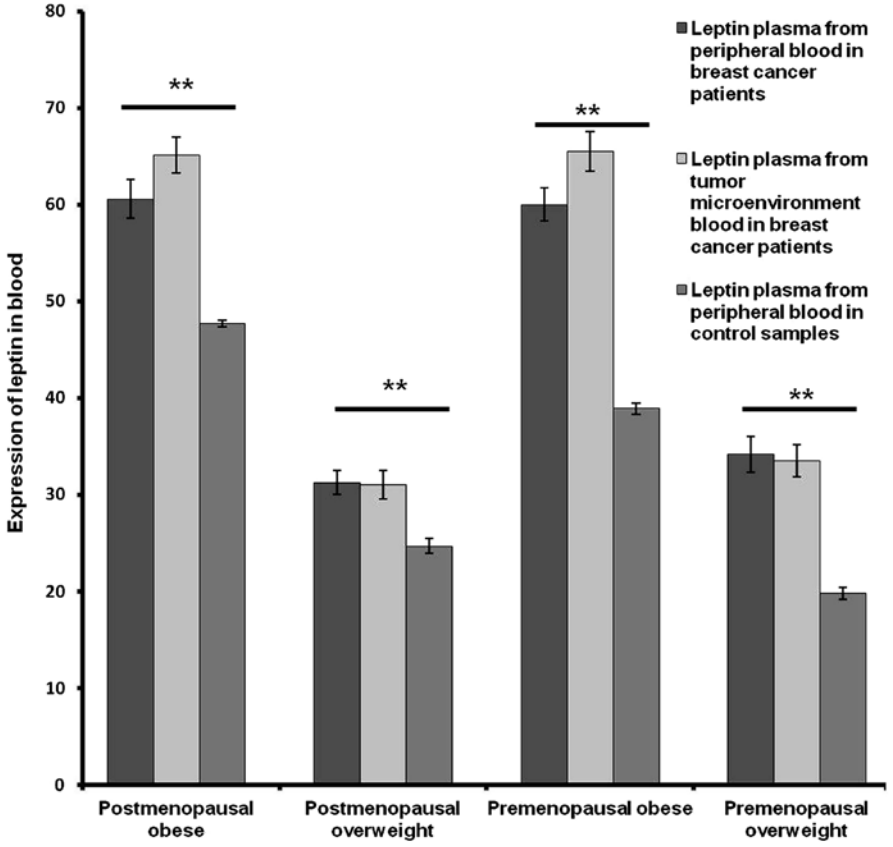

Figure 4. Association between leptin expression and menopausal status. Comparison between the concentration of leptin in plasma from peripheral and tumor microenvironment blood in postmenopausal obese, premenopausal obese, postmenopausal overweight and premenopausal overweight patients compared with control samples. **P<0.001. Data are presented as mean ± standard deviation and were analyzed using Student's *t*-test.

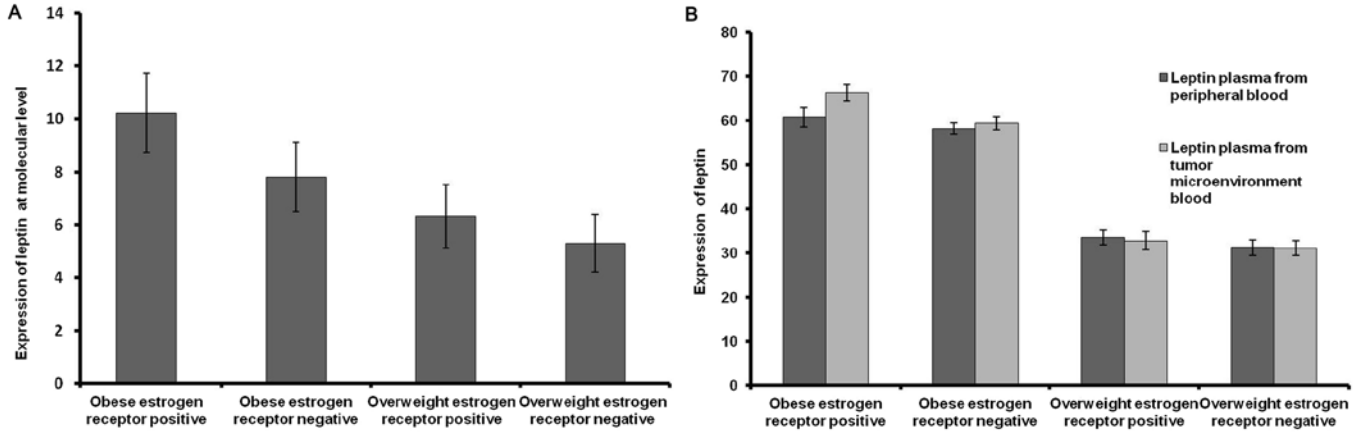

Figure 5. Association between leptin expression and estrogen receptor. (A) Molecular expression of leptin in obese and overweight patients with estrogen receptor positive and negative breast cancer. (B) Comparison between concentration of leptin in plasma from peripheral blood and tumor microenvironment blood in obese and overweight patients with estrogen receptor positive and negative breast cancer.

ER^+ and ER^- in both obese and overweight patients. Leptin expression increased by 2.42 fold more in obese ER^+ compared with obese ER^- breast cancer patients, while it increased by only one fold in overweight ER^+ compared with overweight ER^- breast cancer patients (Fig. 5A). Furthermore, leptin expression was higher in obese ER^+ than overweight ER^+ patients by 3.9-fold (Fig. 5A).

A markedly higher concentration of leptin was identified in plasma isolated from the tumor microenvironment blood compared with plasma from peripheral blood in ER^+ obese breast cancer patients (Fig. 5B).

Leptin stimulated expression levels of LEPR, aromatase, MAPK and STAT3 mRNA in tissues of obese patients with breast cancer. The mRNA expression level of leptin and LEPR, aromatase, MAPK and STAT3 in breast cancer patient tissues were assessed. Leptin, LEPR, aromatase, MAPK and STAT-3 were overexpressed in obese patients compared with overweight and normal tissues (Fig. 6). The mRNA expression

Table IV. Association between mRNA leptin expression and leptin receptor, aromatase, MAPK and STAT-3 expression in patients with breast cancer.

	Expression of leptin (n=29)			
Variable	High expression (n=14)	Low expression (n=15)	Correlation coefficient	P-value
BMI, n (%)				<0.001[a]
Obese	14 (100)	2 (13.33)	0.663	
Overweight	0 (0)	13 (86.67)		
Leptin receptor, n (%)				<0.001[a]
High expression	13 (92.86)	0 (0)	0.815	
Low expression	1 (7.14)	15 (100)		
Aromatase, n (%)				<0.001[a]
High expression	14 (100)	1 (60)	0.772	
Low expression	0 (0)	14 (40)		
MAPK, n (%)				<0.001[a]
High expression	14 (100)	1 (6.67)	0.771	
Low expression	0 (0)	14 (93.33)		
STAT-3, n (%)				<0.001[a]
High expression	11 (78.57)	0 (0)	0.679	
Low expression	3 (21.43)	15 (100)		

[a]P<0.01. BMI, body mass index; MAPK, mitogen activated protein kinase; STAT3, signal transducer and activator of transcription-3.

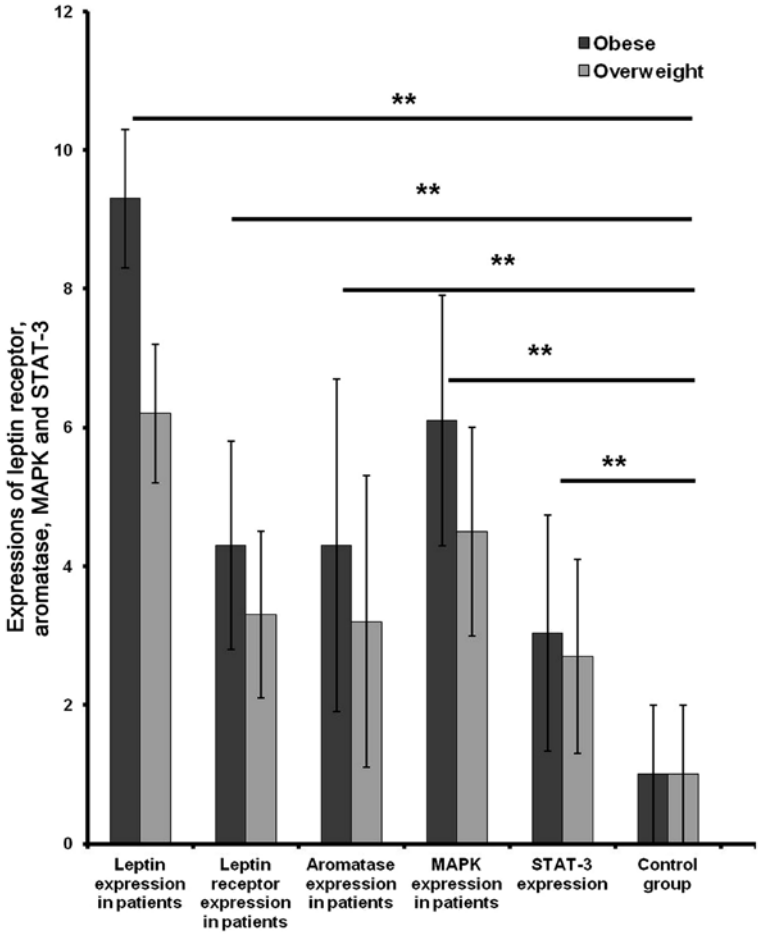

Figure 6. Association between leptin expression and associated pathways. Comparison between the expression level of leptin, leptin receptor, aromatase, MAPK and STAT3 in obese patients compared with overweight and normal control patients. **P<0.001. Data are presented as mean ± standard deviation and were analyzed using Student's *t*-test. MAPK, mitogen activated protein kinase; STAT3, Signal transducer and activator of transcription-3.

level of leptin and BMI in tissues from patients was assessed using the Spearman correlations test. Leptin in breast cancer tissue samples was significantly associated with the obesity status (r=0.663; Table IV). Positive correlations between leptin expression and that of LEPR, aromatase, MAPK and STAT3 were also identified (r=0.815, 0.772, 0.771 and 0.679 respectively; Table IV).

Discussion

The present study aimed to investigate the potential role of leptin in breast cancer progression in obese patients. Leptin is produced by adipose tissue, which constitutes the major breast tissue structure (13,15,16). Leptin serves a critical role in cell growth and differentiation in normal cells (32). Since its discovery in 1994, leptin has been identified to have an association with obesity (13,15,16). Reports of association between the expression of leptin and breast cancer are inconsistent. A number of studies indicate that leptin is associated with breast cancer development (33-35); while other studies demonstrate that leptin is not associated with breast cancer (36-38).

Obesity acts as a risk factor for a number of serious medical conditions (7). Obesity was identified to have an association with mortality in patients with breast cancer, with a higher mortality rate observed in obese patients compared with overweight patients (39). Obesity is associated with breast cancer through the secretion of growth factors and leptin by adipose stromal stem cells (ASCs) that, in turn, promote tumor growth (40,41). Furthermore, brown adipose tissue has been identified to activate breast cancer development through activation of the angiogenesis process in mice (42).

In the present study, leptin expression in tissue and plasma from both peripheral and tumor microenvironment blood of patients with breast cancer was assessed to investigate the association between leptin and breast cancer progression. To the best of our knowledge, this is the first investigation to assess the concentration of leptin in blood plasma collected from the breast tumor microenvironment of patients. The expression of leptin in the blood and tissues of patients with breast cancer was assessed as previous studies indicated that expression of leptin in blood was not associated with the expression of leptin in the tissue (23,43). The present study indicated that leptin was highly expressed in blood and tissue samples at molecular and proteomic levels in patients with breast cancer. Leptin expression was higher in obese ER^+ patients compared with obese ER-breast cancer patients by 2.42 fold. Additionally, leptin was overexpressed in obese ER^+ compared with overweight ER^+ breast cancer patients by 3.9 fold. The concentration of circulating leptin in the blood was markedly associated with mRNA expression of leptin in breast cancer patients, in agreement with a previous study (44).

A positive correlation was identified between the expression of leptin and estrogen receptor expression in obese patients. By contrast, a negative correlation was detected between the expression of leptin and estrogen receptor in overweight patients. A non-significant difference between the expression of leptin and progesterone receptor and human epidermal growth factor has been demonstrated (35,45,46). Previous results revealed a positive association between leptin expression and cell proliferating marker (ki67 labelling index) (35). Conversely, the present study revealed no significant association between leptin expression and ki67 labelling index, which is in accordance with a previous study by Garofalo *et al* (33).

The present results revealed that the level of leptin was higher in the plasma of obese and overweight breast cancer patients than those of healthy individuals, agreeing with previous studies (5,13,43-48). Obesity may be associated with breast cancer through stimulation of estrogen secretion, mediated by leptin in fat tissue during the postmenopausal period. This suggestion disagrees with the results of the present study as patients included both postmenopausal and premenopausal patients while previous studies included postmenopausal patients only. In addition, the enhancement of insulin and insulin growth factors by leptin was associated with metabolic disorders, and increased the production of adipokines including leptin, which are secreted by adipose tissue. This may lead to breast cancer progression (49). Also, leptin may stimulate tumor development in breast cancer cells by stimulating the CYP19A1 gene through activating MAPK and STAT3 pathways (17).

The plasma concentration of leptin higher in the tumor microenvironment blood than in peripheral blood of obese patients with ER^+ breast cancer. The latter results are concurrent with previous *in vitro* studies that identified higher levels of leptin in ASCs of the breast tumor microenvironment in breast cancer cells (50). ASCs produce growth factors that protect breast cancer cells from immune responses and stimulate breast cancer progression (41). The higher expression of leptin in breast tumor microenvironment may be attributed to the potential circulation of ASCs through blood to distant tumor regions where they differentiate into vascular pericytes or produce growth factors such as hepatocyte growth factor and insulin growth factor, which elevate leptin levels and anchor the tumor microenvironment (41,51). These growth factors are associated with breast cancer development (52). Other studies suggest that ASCs secrete proteases such as MMP2 and MMP9, and vascular pro-angiogenic factors such as VEGF that elevate leptin levels at the tumor site (53,54).

It has been demonstrated that obese adipose stromal stem cells (obASCs) from obese patients with breast cancer express higher leptin levels when compared with ASCs isolated from lean patients (50). Previous studies indicated that obesity may stimulate the production of ASCs within white adipose tissue that activates proliferation of breast cancer cells through an estrogen-induced response mediated by leptin (55).

It was suggested that the higher concentration of leptin in the breast tumor microenvironment in obese patients with ER^+ breast cancer may be due to their response to factors secreted by obASCs and not secreted by ASCs in lean patients (50). Additionally, pathways in ER-patients lack the estrogen receptor; and therefore, are unable to respond to factors synthesized by obASCs (50).

In addition, higher levels of leptin in breast tumor microenvironment of obese patients may attributed to the following: Adipose tissue of obese patients is characterized by chronic inflammation leading to stimulation of angiogenesis process (56). It consists of higher numbers of the macrophages and inflammatory cells that activate breast cancer progression compared with lean patients (57). Obese patients overexpress adipose triglyceride lipase, which is involved in breast tumor

progression (58), and they reduce pigment epithelium-derived factor expression, which is associated with aggressive metastatic risk for breast cancer (58). The present study hypothesized that cells in the breast tumor microenvironment of obese patients secrete higher levels of leptin due to activation by circulating levels of insulin and insulin-like growth factors, inflammatory cytokines and VEGF. Also, leptin stimulates the expression of MAPK and STAT3 activating aromatase that increases the synthesis of estrogen in obese ER^+ patients with breast cancer. Estrogen stimulates breast cancer progression through activation of numerous processes including cell division, angiogenesis and proliferations (59). The results of the present study indicate that cells of obese patients with ER^+ breast cancer secrete higher levels of leptin, which produces estrogen and activates breast cancer progression.

With respect to menopausal status, a positive association was identified between the expression of leptin in blood and obesity in breast cancer patients regardless of menopausal status, which was in accordance with previous results (35,43-47). By contrast, previous studies have indicated that breast cancer risk was associated with menopausal status (21,23,60) and that obesity may increase breast cancer progression in postmenopausal women by 30-50% (21).

Studies *in vitro* demonstrated that leptin is associated with breast cancer progression as it stimulates the JAK/STAT3, ERK1/2 and phoshphoinositide 3-kinase pathways leading to breast cancer cell proliferation and cell survival (21). Few studies measured the expression of LEPR and activation of cell proliferation signalling pathway (aromatase) in patients with breast cancer. Leptin initiates its actions through LEPR (14). Aromatase is expressed in adipose stromal cells and epithelial cancer cells (61). Leptin is able to crosstalk with estrogen through increasing the expression of aromatase enzyme and stimulating estrogen expression (61-64). MAPK is a protein kinase involved in breast cancer progression (65-67). STAT3 serves vital roles in cell growth, survival, transformation and development (68). STAT3 controls multiple genes including cyclinD1, B-cell lymphoma-2 (BCL2), BCL2-extra large and c-Myc that participate in proliferation and cell growth (69). STAT3 is able to enhance the proliferation of breast cancer (65-67).

The expression of potential genes regulated by leptin in progression mechanism of patients with breast cancer were assessed. The potential proliferation pathway(s) associated with leptin expression may be responsible for breast cancer progression. A positive association between the expression of leptin and expression of leptin receptor, aromatase, MAPK and STAT3 genes was identified in obese patients with breast cancer and these results are concurrent with previous *in vitro* studies (33,61,64,65,67,70). Accordingly, leptin may enhance breast cancer progression by stimulating the estrogen pathway through increasing aromatase expression, the ERK1/2 pathway via activating MAPK expression and the JAK/STAT3 pathway through enhancing STAT3 expression.

Inhibition of the leptin proliferation signalling pathway may be beneficial to identify novel therapeutic targets for breast cancer. Identifying the molecular mechanism of leptin in breast cancer progression may lead to novel targets for breast cancer treatment. To the best of our knowledge, this is the first investigation to determine the concentration of leptin in breast tumor microenvironment in patients.

In conclusion, the concentration of leptin was higher in plasma from tumor microenvironment blood than in plasma from peripheral blood samples of obese patients with ER^+ breast cancer. Leptin may enhance breast cancer progression by inducing the expression of JAK/STAT3, ERK1/2 and estrogen pathways in obese patients with breast cancer.

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