

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 phosphoinositide 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'-TCTCGATTCGGCAGCAAAC-3'
	Reverse	5'-TGACCATACGAACAAGGCCG-3'
MAPK	Forward	5'-GGGGCTGATTTTCTTGATAGC-3'
	Reverse	5'-ACCAACCTCTCGTACATCGG-3'
STAT-3	Forward	5'-CTGCTCCAGGTACCGTGTGT-3'
	Reverse	5'-CCTCTGCCGAGAAACAG-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 \pm standard deviation	51.66 \pm 1.506
Range	34-70
Menopausal state, n (%)	
Premenopausal	15 (34.1)
Postmenopausal	29 (65.9)
BMI, kg/m ² (%)	
\geq 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 (%)	
\leq 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 \pm standard deviation and correlations between categorical variables were assessed using Spearman correlations test and Student's *t*-test. $P \leq 0.01$ 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 \pm 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 \geq 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).

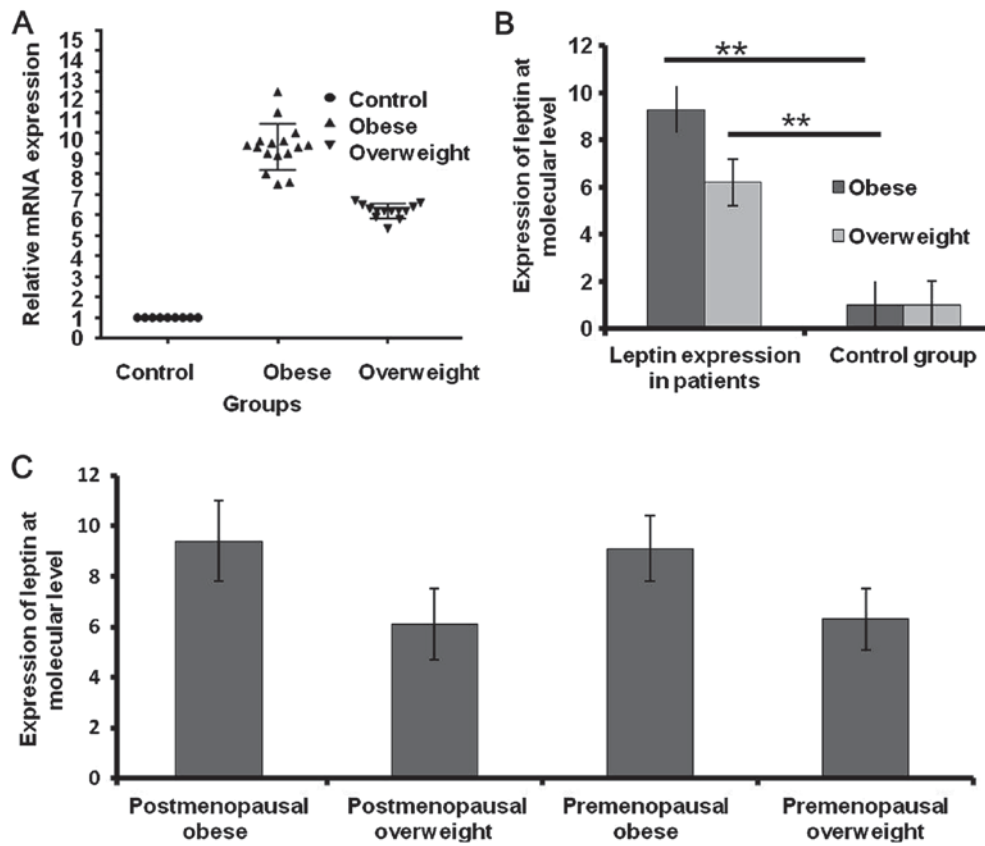


Figure 1. Expression of leptin gene in breast cancer tissue from obese and overweight patients. (A) Comparison between mRNA fold expression of leptin for each sample of obese and overweight patients compared with control samples. (B) Comparison between expression levels of leptin mRNA in obese patients 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 \pm standard deviation. ***P*<0.001.

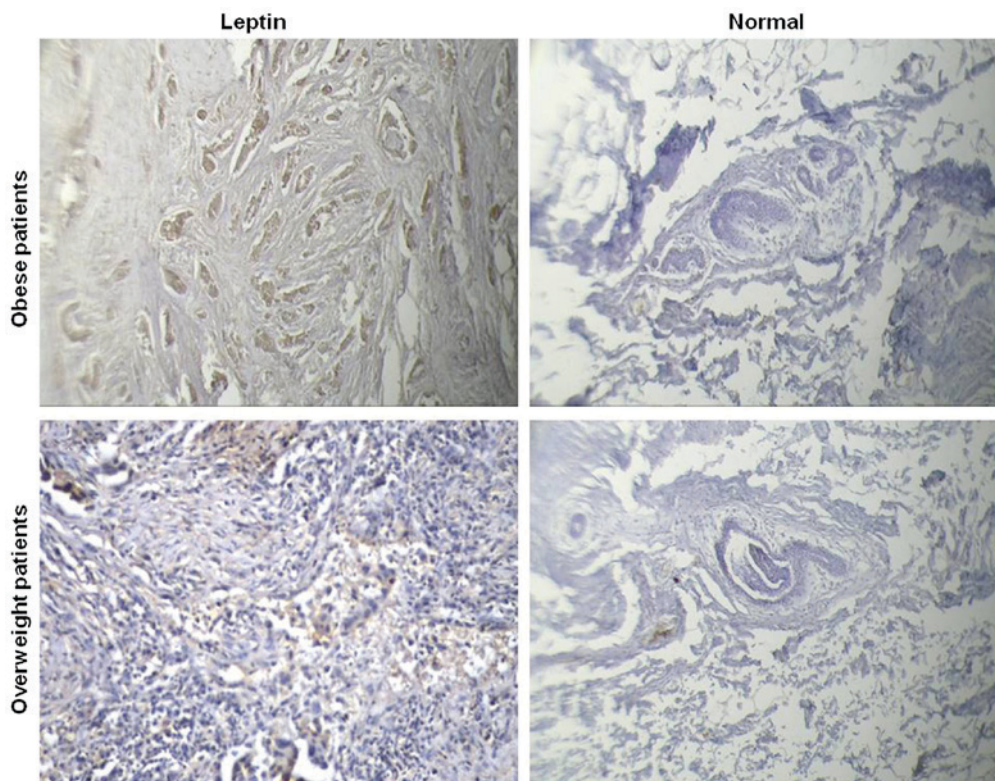


Figure 2. Expression of leptin protein in obese and overweight breast cancer tissue. Photomicrographs represent immunohistochemical staining using hematoxylin and eosin of leptin demonstrating high expression of leptin in breast tissue from an obese patient compared with normal tissue and high expression of leptin in breast tissue from overweight patients compared with normal tissue. Magnification, $\times 100$.

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

Variable	Leptin (n=23)		Correlation coefficient	P-value
	High expression (n=13)	Low expression (n=10)		
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)		

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

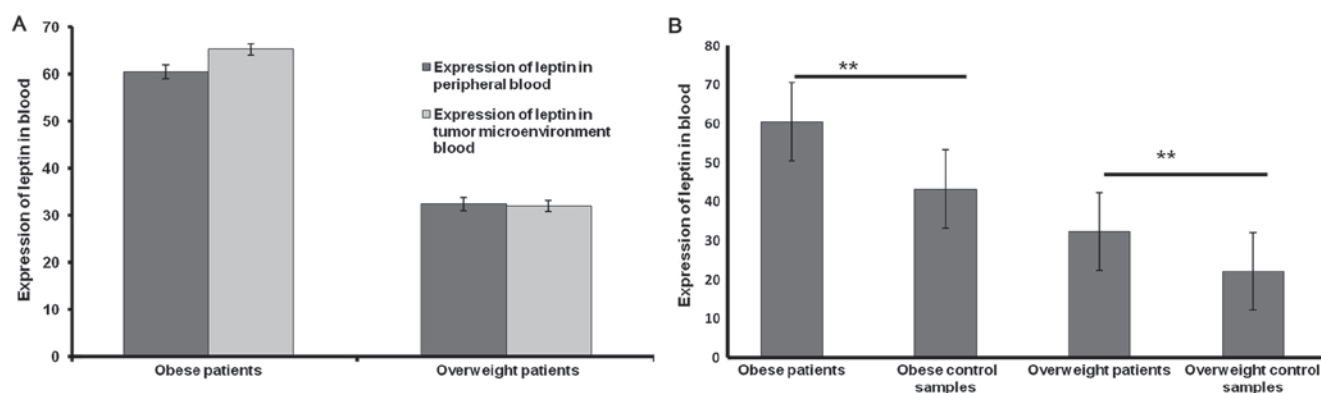


Figure 3. Leptin concentration in peripheral blood and tumor microenvironment measured by ELISA. (A) Comparison between the concentration of leptin in plasma from peripheral blood and tumor microenvironment blood in obese and overweight patients with breast cancer. (B) Comparison between the concentration of leptin in plasma from peripheral blood in obese and overweight patients compared with control samples. **P<0.001. Data are presented as mean \pm 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

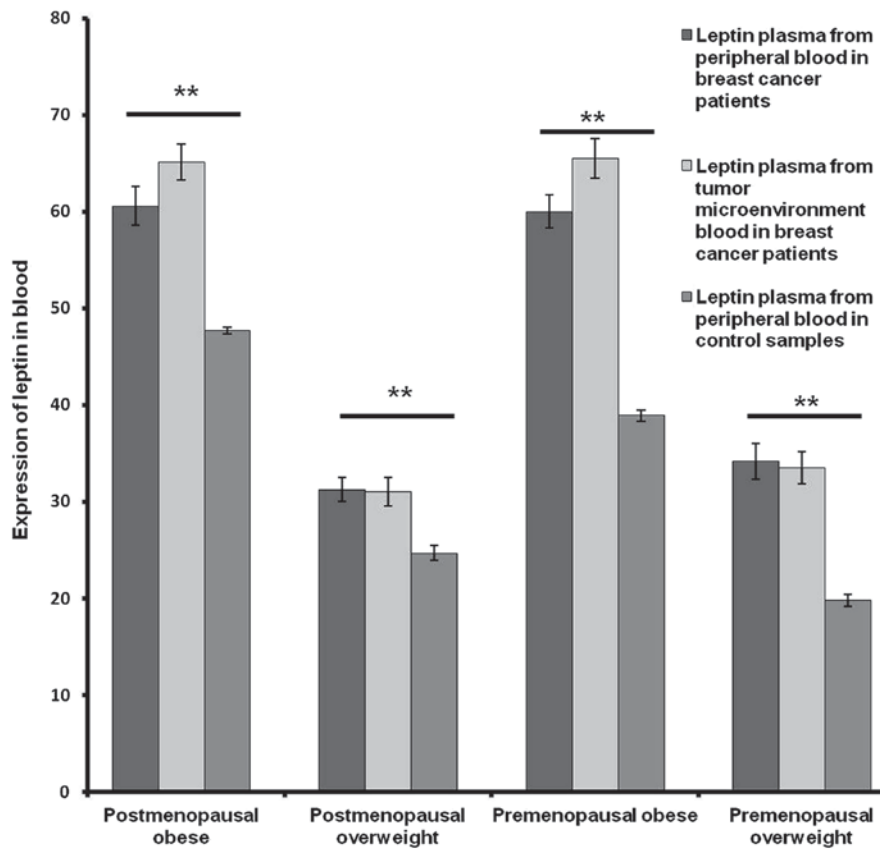


Figure 4. Association between leptin expression and menopausal status. Comparison between the concentration of leptin in plasma from peripheral and tumor microenvironment blood in postmenopausal obese, premenopausal obese, postmenopausal overweight and premenopausal overweight patients compared with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

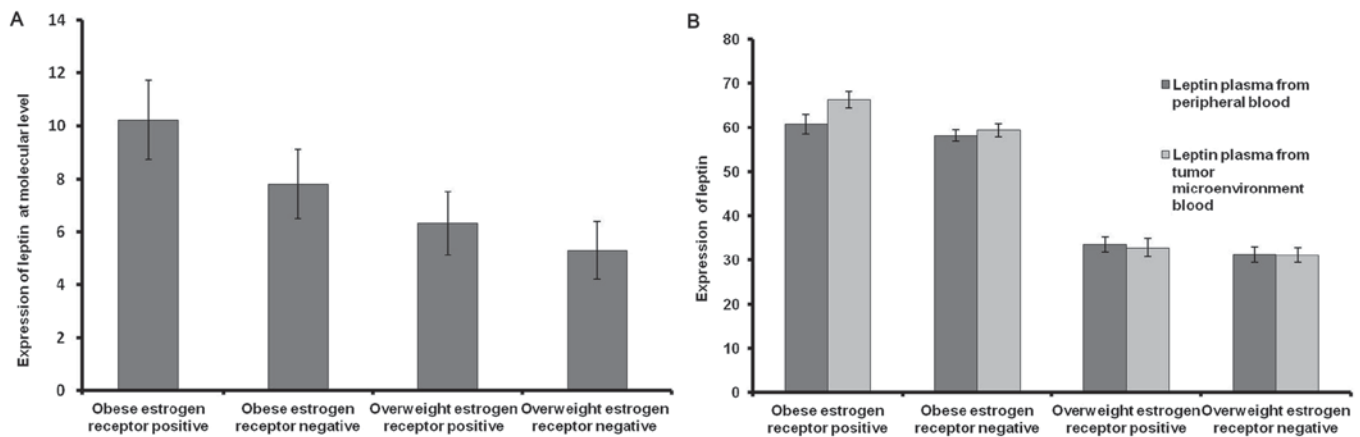


Figure 5. Association between leptin expression and estrogen receptor. (A) Molecular expression of leptin in obese and overweight patients with estrogen receptor positive and negative breast cancer. (B) Comparison between concentration of leptin in plasma from peripheral blood and tumor microenvironment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

Variable	Expression of leptin (n=29)		Correlation coefficient	P-value
	High expression (n=14)	Low expression (n=15)		
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)		

^aP<0.01. BMI, body mass index; MAPK, mitogen activated protein kinase; STAT3, signal transducer and activator of transcription-3.

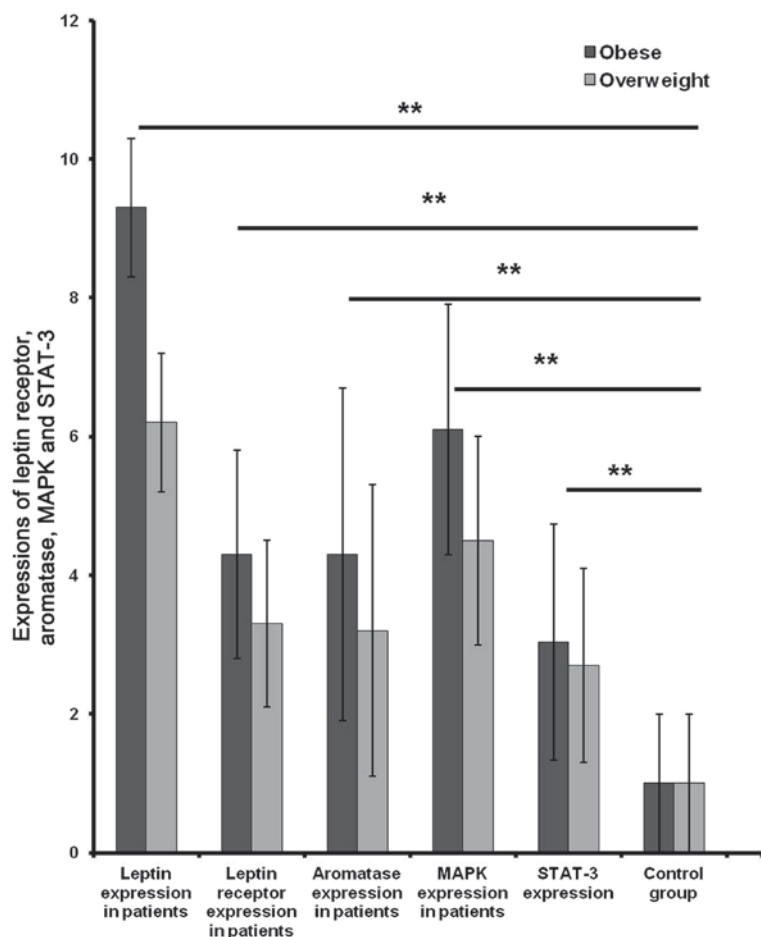


Figure 6. Association between leptin expression and associated pathways. Comparison between the expression level of leptin, leptin receptor, aromatase, MAPK and STAT3 in obese patients compared with overweight and normal control patients. **P<0.001. Data are presented as mean ± standard deviation and 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 phosphoinositide 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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References

1. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D and Bray F: Cancer incidence and mortality worldwide: Sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer* 136: E359-E386, 2015.
2. Bray F, Ren JS, Masuyer E and Ferlay J: Global estimates of cancer prevalence for 27 sites in the adult population in 2008. *Int J Cancer* 132: 1133-1145, 2013.
3. Ibrahim AS, Khaled HM, Mikhail NN, Baraka H and Kamel H: Cancer incidence in egypt: Results of the national population-based cancer registry program. *J Cancer Epidemiol* 2014: 437971, 2014.
4. Newell B, Proust K, Dyball R and McManus P: Seeing obesity as a systems problem. *N S W Public Health Bull* 18: 214-218, 2007.
5. Kaur T and Zhang ZF: Obesity, breast cancer and the role of adipocytokines. *Asian Pac J Cancer Prev* 6: 547-552, 2005.
6. Popkin BM: Understanding global nutrition dynamics as a step towards controlling cancer incidence. *Nat Rev Cancer* 7: 61-67, 2007.
7. Reeves GK, Pirie K, Beral V, Green J, Spencer E and Bull D; Million Women Study Collaboration: Cancer incidence and mortality in relation to body mass index in the Million Women Study: Cohort study. *BMJ* 335: 1134, 2007.
8. Danaei G, Vander Hoorn S, Lopez AD, Murray CJ and Ezzati M; Comparative Risk Assessment collaborating group (Cancers): Causes of cancer in the world: Comparative risk assessment of nine behavioural and environmental risk factors. *Lancet* 366: 1784-1793, 2005.
9. Frühbeck G, Gómez-Ambrosi J, Muruzábal FJ and Burrell MA: The adipocyte: A model for integration of endocrine and metabolic signaling in energy metabolism regulation. *Am J Physiol Endocrinol Metab* 280: E827-E847, 2001.
10. Porter GA, Inglis KM, Wood LA and Veugelers PJ: Effect of obesity on presentation of breast cancer. *Ann Surg Oncol* 13: 327-332, 2006.
11. Huber KE, Carey LA and Wazer DE: Breast cancer molecular subtypes in patients with locally advanced disease: Impact on prognosis, patterns of recurrence, and response to therapy. *Semin Radiat Oncol* 19: 204-210, 2009.
12. Diamond T, Vine J, Smart R and Butler P: Thyrotoxic bone disease in women: A potentially reversible disorder. *Ann Intern Med* 120: 8-11, 1994.
13. Zhang Y, Proenca R, Maffei M, Barone M, Leopold L and Friedman JM: Positional cloning of the mouse obese gene and its human homologue. *Nature* 372: 425-432, 1994.
14. Tartaglia LA: The leptin receptor. *J Biol Chem* 272: 6093-6096, 1997.

15. Halaas JL, Gajiwala KS, Maffei M, Cohen SL, Chait BT, Rabinowitz D, Lallone RL, Burley SK and Friedman JM: Weight-reducing effects of the plasma protein encoded by the obese gene. *Science* 269: 543-546, 1995.
16. Bray GA: The underlying basis for obesity: Relationship to cancer. *J Nutr* 132 (11 Suppl): S3451-S3455, 2002.
17. Andò S and Catalano S: The multifactorial role of leptin in driving the breast cancer microenvironment. *Nat Rev Endocrinol* 8: 263-275, 2011.
18. Ahima RS and Flier JS: Adipose tissue as an endocrine organ. *Trends Endocrinol Metab* 11: 327-332, 2000.
19. Bonnet M, Delavaud C, Laud K, Gourdou I, Leroux C, Djiane J and Chilliard Y: Mammary leptin synthesis, milk leptin and their putative physiological roles. *Reprod Nutr Dev* 42: 399-413, 2002.
20. Snoussi K, Strosberg AD, Bouaouina N, Ben Ahmed S, Helal AN and Chouchane L: Leptin and leptin receptor polymorphisms are associated with increased risk and poor prognosis of breast carcinoma. *BMC Cancer* 6: 38, 2006.
21. Garofalo C and Surmacz E: Leptin and cancer. *J Cell Physiol* 207: 12-22, 2006.
22. Purohit A, Newman SP and Reed MJ: The role of cytokines in regulating estrogen synthesis: Implications for the etiology of breast cancer. *Breast Cancer Res* 4: 65-69, 2002.
23. Vona-Davis L and Rose DP: Adipokines as endocrine, paracrine, and autocrine factors in breast cancer risk and progression. *Endocr Relat Cancer* 14: 189-206, 2007.
24. Mauro L, Catalano S, Bossi G, Pellegrino M, Barone I, Morales S, Giordano C, Bartella V, Casaburi I and Andò S: Evidences that leptin Up-regulates E-cadherin expression in breast cancer: Effects on tumor growth and progression. *Cancer Res* 67: 3412-3421, 2007.
25. Park HY, Kwon HM, Lim HJ, Hong BK, Lee JY, Park BE, Jang Y, Cho SY and Kim HS: Potential role of leptin in angiogenesis: Leptin induces endothelial cell proliferation and expression of matrix metalloproteinases in vivo and in vitro. *Exp Mol Med* 33: 95-102, 2001.
26. Frankenberry KA, Skinner H, Somasundar P, McFadden DW and Vona-Davis LC: Leptin receptor expression and cell signaling in breast cancer. *Int J Oncol* 28: 985-993, 2006.
27. Gonzalez RR, Cherfils S, Escobar M, Yoo JH, Carino C, Styer AK, Sullivan BT, Sakamoto H, Olawaiye A, Serikawa T, *et al*: Leptin signaling promotes the growth of mammary tumors and increases the expression of vascular endothelial growth factor (VEGF) and its receptor type two (VEGF-R2). *J Biol Chem* 281: 26320-26328, 2006.
28. Rene Gonzalez R, Watters A, Xu Y, Singh UP, Mann DR, Rueda BR and Penichet ML: Leptin-signaling inhibition results in efficient anti-tumor activity in estrogen receptor positive or negative breast cancer. *Breast Cancer Res* 11: R36, 2009.
29. Perera CN, Chin HG, Duru N and Camarillo IG: Leptin-regulated gene expression in MCF-7 breast cancer cells: Mechanistic insights into leptin-regulated mammary tumor growth and progression. *J Endocrinol* 199: 221-233, 2008.
30. El-Shinawi M, Abdelwahab SF, Sobhy M, Nouh MA, Sloane BF and Mohamed MM: Capturing and characterizing immune cells from breast tumor microenvironment: An innovative surgical approach. *Ann Surg Oncol* 17: 2677-2684, 2010.
31. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25: 402-408, 2001.
32. Hwang CS, Loftus TM, Mandrup S and Lane MD: Adipocyte differentiation and leptin expression. *Annu Rev Cell Dev Biol* 13: 231-259, 1997.
33. Garofalo C, Koda M, Cascio S, Sulkowska M, Kanczuga-Koda L, Golaszewska J, Russo A, Sulkowski S and Surmacz E: Increased expression of leptin and the leptin receptor as a marker of breast cancer progression: Possible role of obesity-related stimuli. *Clin Cancer Res* 12: 1447-1453, 2006.
34. Nyante SJ, Gammon MD, Kaufman JS, Bensen JT, Lin DY, Barnholtz-Sloan JS, Hu Y, He Q, Luo J and Millikan RC: Common genetic variation in adiponectin, leptin, and leptin receptor and association with breast cancer subtypes. *Breast Cancer Res Treat* 129: 593-606, 2011.
35. Jeong YJ, Bong JG, Park SH, Choi JH and Oh HK: Expression of leptin, leptin receptor, adiponectin, and adiponectin receptor in ductal carcinoma in situ and invasive breast cancer. *J Breast Cancer* 14: 96-103, 2011.
36. Stattin P, Söderberg S, Biessy C, Lenner P, Hallmans G, Kaaks R and Olsson T: Plasma leptin and breast cancer risk: A prospective study in northern Sweden. *Breast Cancer Res Treat* 86: 191-196, 2004.
37. Miyoshi Y, Funahashi T, Tanaka S, Taguchi T, Tamaki Y, Shimomura I and Noguchi S: High expression of leptin receptor mRNA in breast cancer tissue predicts poor prognosis for patients with high, but not low, serum leptin levels. *Int J Cancer* 118: 1414-1419, 2006.
38. Harris HR, Tworoger SS, Hankinson SE, Rosner BA and Michels KB: Plasma leptin levels and risk of breast cancer in premenopausal women. *Cancer Prev Res (Phila)* 4: 1449-1456, 2011.
39. Whiteman MK, Hillis SD, Curtis KM, McDonald JA, Wingo PA and Marchbanks PA: Body mass and mortality after breast cancer diagnosis. *Cancer Epidemiol Biomarkers Prev* 14: 2009-2014, 2005.
40. Grossmann ME, Ray A, Nkhata KJ, Malakhov DA, Rogozina OP, Dogan S and Cleary MP: Obesity and breast cancer: Status of leptin and adiponectin in pathological processes. *Cancer Metastasis Rev* 29: 641-653, 2010.
41. Razmkhah M, Jaberipour M, Hosseini A, Safaei A, Khalatbari B and Ghaderi A: Expression profile of IL-8 and growth factors in breast cancer cells and adipose-derived stem cells (ASCs) isolated from breast carcinoma. *Cell Immunol* 265: 80-85, 2010.
42. Lim S, Honek J, Xue Y, Seki T, Cao Z, Andersson P, Yang X, Hosaka K and Cao Y: Cold-induced activation of brown adipose tissue and adipose angiogenesis in mice. *Nat Protoc* 7: 606-615, 2012.
43. Arita Y, Kihara S, Ouchi N, Takahashi M, Maeda K, Miyagawa J, Hotta K, Shimomura I, Nakamura T, Miyaoka K, *et al*: Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. *Biochem Biophys Res Commun* 257: 79-83, 1999.
44. Tessitore L, Vizio B, Jenkins O, De Stefano I, Ritossa C, Argiles JM, Benedetto C and Mussa A: Leptin expression in colorectal and breast cancer patients. *Int J Mol Med* 5: 421-426, 2000.
45. Ostlund RE Jr, Yang JW, Klein S and Gingerich R: Relation between plasma leptin concentration and body fat, gender, diet, age, and metabolic covariates. *J Clin Endocrinol Metab* 81: 3909-3913, 1996.
46. Hou WK, Xu YX, Yu T, Zhang L, Zhang WW, Fu CL, Sun Y, Wu Q and Chen L: Adipocytokines and breast cancer risk. *Chin Med J (Engl)* 120: 1592-1596, 2007.
47. Al Awadhi SA, Al Khaldi RM, Al Rammah T, Kapila K and Mojiminiyi OA: Associations of adipokines & insulin resistance with sex steroids in patients with breast cancer. *Indian J Med Res* 135: 500-505, 2012.
48. Mohammadzadeh G, Ghaffari MA, Bafandeh A and Hosseini SM: Association of serum soluble leptin receptor and leptin levels with breast cancer. *J Res Med Sci* 19: 433-438, 2014.
49. Lorincz AM and Sukumar S: Molecular links between obesity and breast cancer. *Endocr Relat Cancer* 13: 279-292, 2006.
50. Strong AL, Ohlstein JF, Biagas BA, Rhodes LV, Pei DT, Tucker HA, Llamas C, Bowles AC, Dutreil MF, Zhang S, *et al*: Leptin produced by obese adipose stromal/stem cells enhances proliferation and metastasis of estrogen receptor positive breast cancers. *Breast Cancer Res* 17: 112, 2015.
51. Zhang Y, Daquinag A, Traktuev DO, Amaya-Manzanares F, Simmons PJ, March KL, Pasqualini R, Arap W and Kolonin MG: White adipose tissue cells are recruited by experimental tumors and promote cancer progression in mouse models. *Cancer Res* 69: 5259-5266, 2009.
52. Leek RD: The prognostic role of angiogenesis in breast cancer. *Anticancer Res* 21: 4325-4331, 2001.
53. Somiari SB, Shriver CD, Heckman C, Olsen C, Hu H, Jordan R, Arciero C, Russell S, Garguilo G, Hooke J and Somiari RI: Plasma concentration and activity of matrix metalloproteinase 2 and 9 in patients with breast disease, breast cancer and at risk of developing breast cancer. *Cancer Lett* 233: 98-107, 2006.
54. Linderholm BK, Hellborg H, Johansson U, Elmberger G, Skoog L, Lehtiö J and Lewensohn R: Significantly higher levels of vascular endothelial growth factor (VEGF) and shorter survival times for patients with primary operable triple-negative breast cancer. *Ann Oncol* 20: 1639-1646, 2009.
55. Strong AL, Strong TA, Rhodes LV, Semon JA, Zhang X, Shi Z, Zhang S, Gimble JM, Burrow ME and Bunnell BA: Obesity associated alterations in the biology of adipose stem cells mediate enhanced tumorigenesis by estrogen dependent pathways. *Breast Cancer Res* 15: R102, 2013.
56. Nieman KM, Romero IL, Van Houten B and Lengyel E: Adipose tissue and adipocytes support tumorigenesis and metastasis. *Biochim Biophys Acta* 1831: 1533-1541, 2013.

57. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL and Ferrante AW Jr: Obesity is associated with macrophage accumulation in adipose tissue. *J Clin Invest* 112: 1796-1808, 2003.
58. Gnerlich JL, Yao KA, Fitchev PS, Goldschmidt RA, Bond MC, Cornwell M and Crawford SE: Peritumoral expression of adipokines and fatty acids in breast cancer. *Ann Surg Oncol* 20 (Suppl 3): S731-S738, 2013.
59. Dehdashti F, Mortimer JE, Trinkaus K, Naughton MJ, Ellis M, Katzenellenbogen JA, Welch MJ and Siegel BA: PET-based estradiol challenge as a predictive biomarker of response to endocrine therapy in women with estrogen-receptor-positive breast cancer. *Breast Cancer Res Treat* 113: 509-517, 2009.
60. Calle EE, Rodriguez C, Walker-Thurmond K and Thun MJ: Overweight, obesity, and mortality from cancer in a prospectively studied cohort of U.S. adults. *N Engl J Med* 348: 1625-1638, 2003.
61. Catalano S, Marsico S, Giordano C, Mauro L, Rizza P, Panno ML and Andò S: Leptin enhances, via AP-1, expression of aromatase in the MCF-7 cell line. *J Biol Chem* 278: 28668-28676, 2003.
62. Garofalo C, Sisci D and Surmacz E: Leptin interferes with the effects of the antiestrogen ICI 182,780 in MCF-7 breast cancer cells. *Clin Cancer Res* 10: 6466-6475, 2004.
63. Dubois V, Jardé T, Delort L, Billard H, Bernard-Gallon D, Berger E, Geloën A, Vasson MP and Caldefie-Chezet F: Leptin induces a proliferative response in breast cancer cells but not in normal breast cells. *Nutr Cancer* 66: 645-655, 2014.
64. Khanal T, Kim HG, Do MT, Choi JH, Won SS, Kang W, Chung YC, Jeong TC and Jeong HG: Leptin induces CYP1B1 expression in MCF-7 cells through ligand-independent activation of the ER α pathway. *Toxicol Appl Pharmacol* 277: 39-48, 2014.
65. Alshaker H, Krell J, Frampton AE, Waxman J, Blyuss O, Zaikin A, Winkler M, Stebbing J, Yagüe E and Pchejetski D: Leptin induces upregulation of sphingosine kinase 1 in oestrogen receptor-negative breast cancer via Src family kinase-mediated, janus kinase 2-independent pathway. *Breast Cancer Res* 16: 426, 2014.
66. Qian Y, Shi D, Qiu J, Zhu F, Qian J, He S, Shu Y, Yin Y and Chen X: ObRb downregulation increases breast cancer cell sensitivity to tamoxifen. *Tumour Biol* 36: 6813-6821, 2015.
67. Wang L, Tang C, Cao H, Li K, Pang X, Zhong L, Dang W, Tang H, Huang Y, Wei L, *et al*: Activation of IL-8 via PI3K/Akt-dependent pathway is involved in leptin-mediated epithelial-mesenchymal transition in human breast cancer cells. *Cancer Biol Ther* 16: 1220-1230, 2015.
68. Takeda K, Noguchi K, Shi W, Tanaka T, Matsumoto M, Yoshida N, Kishimoto T and Akira S: Targeted disruption of the mouse Stat3 gene leads to early embryonic lethality. *Proc Natl Acad Sci USA* 94: 3801-3804, 1997.
69. Bromberg JF, Wrzeszczynska MH, Devgan G, Zhao Y, Pestell RG, Albanese C and Darnell JE Jr: Stat3 as an oncogene. *Cell* 98: 295-303, 1999.
70. Giordano C, Chemi F, Panza S, Barone I, Bonofiglio D, Lanzino M, Cordella A, Campana A, Hashim A, Rizza P, *et al*: Leptin as a mediator of tumor-stromal interactions promotes breast cancer stem cell activity. *Oncotarget* 7: 1262-1275, 2015.