RESEARCH ARTICLE

Effect of NUCKS-1 Overexpression on Cytokine Profiling in Obese Women with Breast Cancer

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Abstract

Background: Overweight and obesity are recognized as major drivers of cancers including breast cancer. Several cytokines, including interleukin-6 (IL-6), IL-10 and lipocalin 2 (LCN2), as well as dysregulated cell cycle proteins are implicated in breast carcinogenesis. The nuclear, casein kinase and cyclin-dependent kinase substrate-1 (NUCKS-1), is a nuclear DNA-binding protein that has been implicated in several human cancers, including breast cancer. Objectives: The present study was conducted to evaluate NUCKS-1 mRNA expression in breast tissue from obese patients with and without breast cancer and lean controls. NUCKS-1 expression was correlated to cytokine profiles as prognostic and monitoring tools for breast cancer, providing a molecular basis for a causal link between obesity and risk. Materials and Methods: This study included 39 females with breast cancer (G III) that was furtherly subdivided into two subgroups according to cancer grading (G IIIa and G IIIb) and 10 control obese females (G II) in addition to 10 age-matched healthy lean controls (G I). NUCKS-1 expression was studied in breast tissue biopsies by means of real-time PCR (RT-PCR). Serum cytokine profiles were determined by immunoassay. Lipid profiles and glycemic status as well as anthropometric measures were also recorded for all participants. Results: IL-6, IL-12 and LCN2 were significantly higher in control obese and breast cancer group than their relevant lean controls ($p<0.05$), while NUCKS-1 mRNA expression was significantly higher in the breast cancer group compared to the other groups ($p<0.05$). Significant higher levels of IL-6, IL-12, and LCN2 as well as NUCKS-1 mRNA levels were reported in G IIIb than G IIIa, and positively correlated with obesity markers in all obese patients. Conclusions: Evaluation of cytokine levels as well as related gene expression may provide a new tool for understanding interactions for three axes of carcinogenesis, innate immunity, inflammation and cell cycling, and hope for new strategies of management.

Keywords: Obesity - breast cancer - IL-6 - IL-12 - LCN_2 - NUCKS-1

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Introduction

Obesity is a growing health problem increasing worldwide, excess body weight has been linked to an increased risk of postmenopausal breast cancer, and growing evidence also suggests that obesity is associated with poor prognosis in women diagnosed with early-stage breast cancer (Ligibel, 2011). Hyperinsulinemia has been correlated with body mass index (BMI), waist circumference (WC), risk of recurrence and mortality in breast cancer (Goodwin et al., 2002). The role of lipids in cancer in maintenance of cell integrity is well documented, any alteration in the plasma lipid profile in breast cancer cases can increase its risk status and its measurement may be helpful in evaluation of prognostic and diagnostic importance of the disease (Cejas et al., 2004).

Lipid peroxidation is one of the most important factors that involves cell membrane integrity and causation of cancer (Kedzierska et al., 2010). Breast cancer is the most common lethal malignancy accounting for nearly 23% of all female cancers worldwide, with more than a million new cases each year (Parkin, 2006). The biology of breast cancer is complex, involving oncogenesis, evasion of host immune defense mechanisms, angiogenesis, invasion and metastasis (Xu et al., 2010). Recently, the contribution of interleukins, pleiotropic cytokines, to cancer progression has been demonstrated (Fernandez et al., 2013).

Lipocalin 2 (LCN2), interleukin-6 (IL-6) and IL-12 exert a variety of effects on the immune system, acute-phase responses and hematopoiesis. Macrophages, monocytes, adipocytes and lymphocytes as well as cancer cells have been documented to produce and secrete LCN2, IL-6, IL-12 and IL-10 (El-Kadre and Tinoco, 2013). These cytokines act in an autocrine or paracrine manner, inducing *in vitro* growth of ovarian cancer, cervical cancer, prostate cancer, lung cancer, kidney cancer and melanoma cells (Giri et al., 2001).

Furthermore, their contributions to the tumor angiogenesis have been reported (Salgado et al., 2002; Yang et al., 2013). Moreover, it has been shown that

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treatment effectiveness and prognosis of cancer are determined by disease stage and activity of the immune system modulated by different interleukins, e.g. LCN2, IL-6, IL-12, IL-8 and IL-10 (Bauer et al., 2008). Nuclear, Casein Kinase and Cyclin-dependent kinase Substrate-one (NUCKS-1) is a nuclear, DNA-binding and highly phosphorylated protein (Grundt et al., 2004). It belongs to a module of co-expressed genes located on chromosomal region 1q32.1, which is found to be amplified in breast cancer (Naylor et al., 2005), as well as in other cancers (Sargent et al., 2008).

NUCKS-1 is a substrate for casein kinase 2 (CK2), cyclin dependant kinase-1 (Cdk1) and DNA-activated Kinase *in vitro* and *in vivo*, therefore it appears to be important for cell cycle progression (Grundt et al., 2004; Wiśniewski et al., 2008).

Even though NUCKS-1 gene is ubiquitously expressed in all mammalian tissues and is highly expressed in multiple human cancers including the breast one (Naylor et al., 2005), yet its precise role in breast tumorgenesis relative to metabolic status and immunomodulatory mechanisms operating in breast cancer remain largely elusive. Here we checked NUCKS-1 mRNA expression levels in breast cancer tissues from obese patients and their allied controls as well as their circulating cytokines profiles of LCN2, IL-6 and IL-12, to investigate the role of NUCKS-1 overexpression in breast cancer relative to immunometabolic status and the impact of this on disease incidence and progression.

Materials and Methods

All patients have given their informed consent and the study was institutionally approved by the Research Ethical Committee of Faculty of Medicine, Tanta University, Egypt. This study included 59 postmenopausal women who were admitted to the surgical department of Tanta University Hospital for breast surgeries. Subjects were divided in to three age matched groups. Group I (n=10) women who underwent surgical plastic mastectomy, were recruited as controls non obese group, group II (n=10) obese women who underwent surgical plastic mastectomy, were recruited as controls obese group and group III obese age matched 39 female patients that had primary unilateral invasive breast cancer with no other primary cancer and they underwent modified radical mastectomy with no preoperative neoadjuvant treatment. According to histological typing, grade II (n=24) and grade III (n=15) were considered as G IIIa and G IIIb respectively. All patients and controls were age matched women employing the following exclusion criteria: Smokers, diseases of the gastrointestinal tract, pancreas, kidney or liver, acute infections and radio-or chemotherapy before surgery. During the surgical procedures, tissue samples were obtained, snap frozen and stored in liquid nitrogen or preserved in buffered paraformaldehyde (PFA) until furtherly processed.

Obesity assessment

Measurements of waist circumference (WC) in cm (were measured with a flexible, non stretching tape mid way between the last rib and anterior superior iliac spine) and calculation of body mass index (BMI) (weight in kilograms divided by the square of the height in meters (kg/m^2) were carried out to assess obesity especially abdominal obesity. These measurements were taken under proper condition of wearing light clothes and no shoes.

All subjects enrolled in this study were subjected to full clinical history, clinical examination of the breast, radiological and histopathological investigations.

All chemicals and solvents used unless otherwise described were purchased from Sigma (Sigma, Germany).

RNA extraction, cDNA synthesis and Real time PCR

Total RNA was extracted from breast tissues using Isogene (Nippon Gene, Toyama, Japan) according to manufacturer's instructions. The integrity of total RNA was checked by electrophoresis through 1% agarose gel. RNA samples were then stored at -80°C. cDNA synthesis was performed using the RevertAid H Minus First Strand cDNA Synthesis kit (#K1632,Thermo Scientific Fermentas, St. Leon-Ro, Germany) according to the manufacturer's instructions. Real-time PCR was carried out with single stranded cDNAs.

PCR reactions were performed using Power SYBR Green PCR Master Mix and 7500 Fast Real-Time PCR System (Applied Biosystems, CA, USA). Sequence specific primers were designed by Primer3 software: (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) as follows: NUCKS1 (No:NM_022731.4) GGGCAGTGAGGAAGAACAA and TTGATGCCTTTGAAGCTGTG.

β-actin (No:NM_001101.3) TGGCATTG CCGACAGGATGCAGAA, and CTCGTCA TACTCCTGCTTGCTGAT. β-actin primers were used as an internal control. Real-Time PCR was carried out, in duplicate, by 40 cycles of 95°C for 10 sec and 60°C for 1 min. Productions of the expected amplification fragments without unanticipated products and primers were confirmed by melting curve analysis. Comparative Ct (threshold cycle) method was used to determine relative amounts of the products, according to the Applied Biosystems instructions. Conventional PCR was performed with Conventional PCR was performed with the DreamTaq polymerase (#EP0701, Thermo Scientific Fermentas, St. Leon-Ro, Germany). All expression data were normalized by dividing the target amount by the amount of β-actin used as internal control for each sample.

Biochemical and immunoassays

An overnight fasting blood samples were obtained from each subject immediately before the induction of general anesthesia and centrifuged at 3,000 rpm for 10 min. The extracted plasma was aliquoted and stored at -80°C till the assay time. For LCN2 assay random urine samples were collected in sterile containers, centrifuged 20 minutes at 3000 rpm until no precipitate. The resultant supernatant was removed and stored at-80°C for further analysis. Fasting Blood Sugar (FBS) was measured by the oxidase method (Biodiagnostic., Egypt), total Lipid profile including total cholesterol (TC), triglycerides (TAG) and high density lipoprotein cholesterol (HDL-C)

were measured by enzymatic-colorimetric methods (Biodiagnostic., Egypt). Low density lipoprotein cholesterol (LDL-C) concentration was calculated according to Friedewald (Friedewald et al., 1972); LDL-C=TC-[(TG/5)+HDL- C] (mg/dl).

Enzyme linked immunosorbent assay (ELISA) was used to detect plasma levels of Insulin (USCN Life Science Inc, Wuhan, China), IL-6 (R and D Systems Inc., Minneapolis, MN, USA) and IL-12 (AviBion, AniBiotech, Finland) and urinary LCN2 (Sunred Biological Technology Co. Ltd, Shanghai, China) according to manufacturers' instructions and read on microplate reader (Stat Fax®2100, Fisher Bioblock Scientific, France), at 450nm with correction wavelength set at 570nm. Insulin resistance was assessed by the homeostatic model assessment (HOMA-IR), calculated as: Fasting glycemia (mg/dl) * fasting insulinemia (μIU/mL) /405 (Duseja et al., 2007).

Histopathology

Breast tissues fixed in PFA (4%), dehydrated, and embedded in paraffin using standard procedures. 4μm thick slices were stained with Hematoxylin & Eosin (H&E) and microscopically examined (Leica Imaging System LTD., Cambridge, England). Tumors were graded according to the traditional system of grading used by the International Union against Cancer (UICC) (Robbins et al., 1995; Tavassoli 2003).

Statistical analysis

Results represented means± SD, multiple comparisons were performed by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test for multiple comparisons. Comparison between any two groups was analyzed by the unpaired Student's t-test using. Analysis was performed by Statistical Package for Social Sciences (SPSS), version 14.0 for windows (SPSS, Chicago, IL, USA). The difference was considered statistically significant when $p<0.05$. Correlations were analyzed using the Pearson test.

Results

Demographic and basic characteristics of the studied subjects

The demographic and clinical characteristics among all studied groups as well as the percentages of histopathological grading of breast cancer group are depicted in Table (1). There was no statistically significant difference in age between all subjects under study, however there were statistically significant differences in BMI (kg/m^2) and WC (cm) ($p<0.05$ for both). Using multiple comparisons test (Tukey's test), the values were markedly higher in breast cancer group (G III) and obese group (G II) when compared to lean control group (G I) with statistically higher level in breast cancer group ($p<0.05$).

Biochemical characteristics and cytokine profiling of the studied subjects

Table (2) revealed statistically significant differences in values of serum lipid profile, FBG, fasting insulin and HOMA-IR index among all the studied groups (p<0.05

Table 1. Demographic and Clinical Characteristics among all Studied Groups as well as the Percentages of Histopathological Grading of Breast Cancer Group

		G I Mean±SD	G II Mean±SD	G III Mean±SD	ANOVA test		Tukey's test		
		n=10	n=10	n=39	F	P	P1	P2	P3
Age (years)		46.1+1.35	45.5+2.93	48.2+5.3	0.526	0.258	0.526	0.336	0.114
BMI (kg/m^2)		22.36+2.7	38.6+4.8	41.9+5.7	3.336	0.024*	0.005*	0.006*	0.024*
WC (cm)		71.5+3.8	95.7+4.36	112.3+13.5	2.363	0.027*	0.023*	0.024*	0.030*
Grading (number)	Grade II (24)	-	-	61.30%	-	-	-	-	-
	Grade III (15)	-	-	38.50%	-	-	-	-	-

Data presented as means± SD. (S) statistically significant difference. P was calculated by one way ANOVA test followed by Tukey's post-hoc test. P was considered significant at <0.05.; *Significant; P1 comparison between group I and II; P2 comparison between group I and III; P3 comparison between group II and III

Table 2. Comparative Statistics of Different Biochemical and Molecular Findings among all the Studied Groups

	G I Mean±SD	G II Mean±SD	G III Mean±SD	ANOVA test		Tukey's test		
	n=10	n=10	n=39	F	P	P1	P2	P3
TC (mg/dl)	112.2+6.3	162.5+7.5	215.3+7.5	6.336	0.001*	0.001*	0.028*	0.001*
TAG(mg/dl)	123.5+8.3	172.6+8.96	233.9+15.3	6.335	0.002*	0.001*	0.024*	0.002*
HDL-C(mg/dl)	64.2+6.8	42.3+8.3	39.5+7.2	5.336	0.008*	0.005*	0.001*	0.085
LDL-C (mg/dl)	53.8+7.4	71.6+3.9	136.5+20.1	4.417	0.028*	0.037*	0.001*	0.01*
FBG (mg/dl)	76.3+9.6	185.3+11.9	188.6+9.6	8.336	0.009*	0.001*	0.001*	0.527
Fasting Insulin (μIU/ml)	4.6+1.5	11.9+2.4	21.6+3.8	5.336	0.002*	0.005*	0.001*	0.020*
HOMA-IR index	1.20+0.36	5.10+0.9	10.5+2.6	3.336	0.007*	0.001*	0.001*	0.003*
Serum IL-6 (pg/ml)	4.2+0.95	7.3+1.3	35.6+10.8	3.336	0.002*	0.049*	0.001*	0.001*
Serum IL-12 (pg/ml)	58.6+3.5	174.6+22.3	210+36.5	11.3	0.001*	0.001*	0.001*	0.001*
urinary LCN2 (pg/L)	52.3+13.5	64.2+6.8	85.6+4.27	2.36	0.011*	0.015*	0.024*	0.047*
Relative NUCKS-1 mRNA expression	0.42+0.06	0.49+0.10	0.60+0.2	3.685	0.024*	0.224	0.024*	0.039*

Data presented as means± SD. (S) statistically significant difference. P was calculated by one way ANOVA test followed by Tukey's post-hoc test. P was considered significant at <0.05.; *Significant; P1 comparison between group I and II; P2 comparison between group I and III; P3 comparison between group II and III

for all). Using multiple comparisons test (Tukey's test), the above mentioned values were markedly higher in G III and G II when compared to G I (Table 2). However, for HDL-C, and FBG there were statistically insignificant differences in G III versus G II.

As for cytokine profiling, serum IL-6 and IL-12 as well as urinary LCN2 levels were significantly increased in obese women with and without cancer compared to their allied lean controls ($p<0.05$) Table (2), with statistically higher values in breast cancer group (Figure 1). The attained significant differences in levels of previous parameters reflect their intimate relation to breast cancer progression. Moreover, according to histological grading of breast cancer (Figure 3), Table (3) and (Figure 4a) showed that there were statistically higher significant differences in values of WC, TC, TAG, LDL-C, IL-6, IL-12 and LCN2 in Grade III patients (G IIIb) when compared to Grade II ones (G IIIa), ($p<0.05$). It is of note that no significant difference between both groups could be encountered for values of BMI, HDL-C, FBG, fasting insulin and HOMA-IR among the two subgroups; highlightening the role of cytokine profiling and WC in breast cancer progression.

NUCKS-1 mRNA expression in breast tissues

NUCKS-1 gene expression was significantly upregulated in breast tissues from obese women with cancer (by about 3 folds) as compared to obese and lean controls ($p=0.024$), (Figure 2). NUCKS-1 expression was noted to be higher in G II relative to lean G I, although that increase was not statistically significant ($p=0.224$), Table (2). According to histological grading (Figure 3), Table (3) and figure (4b) showed that NUCKS-1 gene expression was statistically higher in grade III (G IIIb) as compared

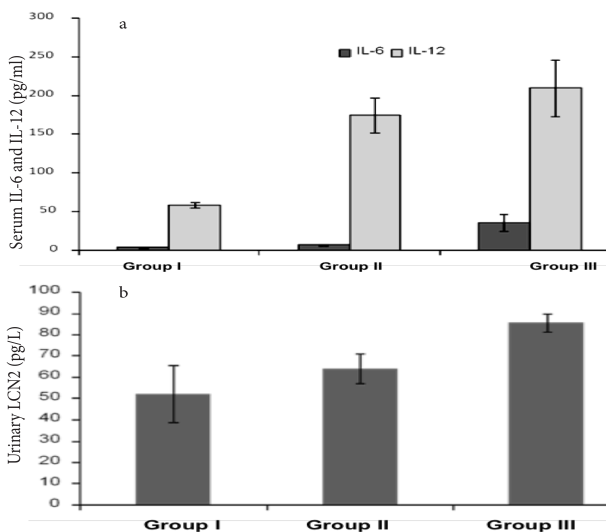

Figure 1. Cytokine Profiling among All the Studied Groups. a) Comparison of serum IL-6 and IL-12 among all the studied groups; **b)** Comparison of urinary LCN2 among all the studied groups. Data represents means; error bars indicate±SD, for significance and p values (Table 2)

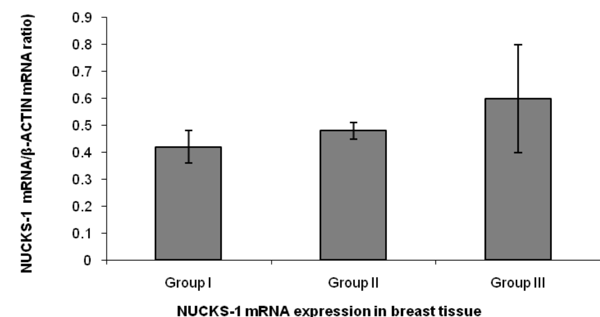

Figure 2. Relative NUCKS-1 Gene Expression in Breast Tissues. Comparison of Relative NUCKS-1 mRNA expression in breast tissues among all the studied groups. Data represents means; error bars indicate±SD, for significance and p values (Table 2)

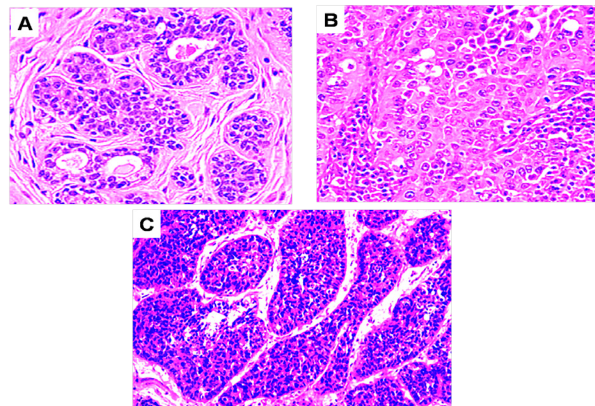

Figure 3. Histological Grading of Breast Tissues Used in This Study. Photomicrographs of breast tissues (A) healthy (B) grade II and (C) grade III. (H&E, X20)

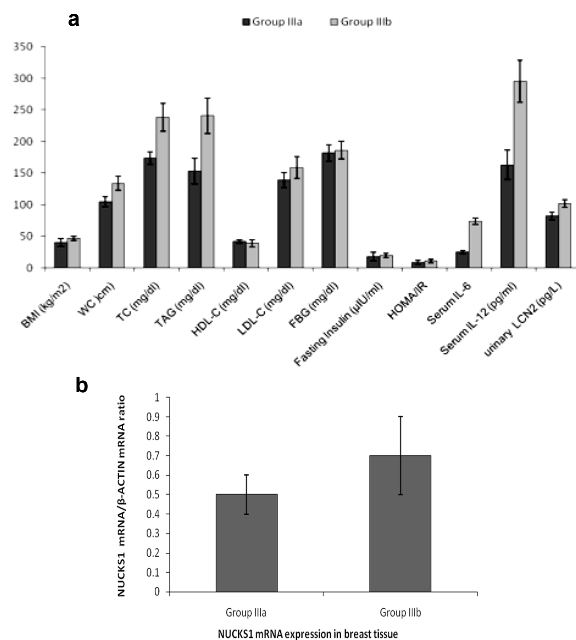

Figure 4. Comparison of Biochemical and Molecular Findings in Breast Cancer Subgroups. a) Comparison of BMI (kg/m^2) and WC (cm) as well as serum levels of TC (mg/dl), TAG (mg/dl) and HDL-C (mg/dl), LDL-C (mg/dl), FBG (mg/dl), fasting insulin (μIU/ml), HOMA-IR index, IL-6 (pg/ml) IL-12 (pg/ml) and urinary LCN2 (pg/L) between G IIIa and G IIIb; b) Comparison of relative NUCKS-1 mRNA expression in breast tissue between G IIIa and G IIIb. Data represents means; error bars indicate±SD, for significance and p values (Table 3)

with grade II (G IIIa) subgroups of breast cancer group (G III) using T-test reflecting their strong relation to breast cancer progression.

Among the entire cohort of breast cancer patients, NUCKS-1 expression correlated well with some obesity related risk factors including WC, Fasting insulin, HOMA-IR, lipid profile (except for HDL-C) as well as cytokines profile ($p<0.05$ for all), Tables (4, 5, 6), where a statistically significant positive correlation was found between NUCKS-1 mRNA expression with IL-6, IL-12 and LCN2, reflecting a strong relationship between NUCKS-1 mRNA expression and cytokine profile especially urinary LCN2 as a non invasive biomarker in this study of breast cancer.

Table 3. Comparative Statistics of BMI (kg/m^2) and WC (cm) as Well as Different Biochemical and Molecular Findings between among G IIIa and G IIIb

	G IIIa Mean±SD n=24	G IIIb Mean±SD n=15	T-test t	p
BMI (kg/m^2)	40.2±6.3	43.5±3.61	0.635	0.224
WC (cm)	105.6±8.3	134.5±11.8	5.336	0.009*
TC (mg/dl)	174.5±10.6	238.6±22.6	12.632	0.001*
TAG (mg/dl)	153.3±20.6	241.3±28.6	8.635	0.001*
HDL-C (mg/dl)	42.3±3.5	39.6±2.35	0.963	0.258
LDL-C (mg/dl)	139.5±12.9	159.8±17.6	3.159	0.020*
FBG (mg/dl)	182.6±13.5	186.6±14.3	0.635	0.241
Fasting Insulin (µIU/ml)	18.6±3.20	20.36±3.5	0.558	0.226
HOMA/IR	9.62±3.5	11.24±3.5	0.417	0.335
Serum IL-6(pg/ml)	25.71±3.5	74.25±5.6	12.336	0.001*
Serum IL-12 (pg/ml)	163.52±23.5	295.3±33.5	15.632	0.001*
urinary LCN2 (pg/L)	82.3±6.5	102.5±6.63	6.332	0.001*
Relative NUCKS-1 mRNA expression	0.5±0.1	0.7±0.2	3.225	0.047*

*Significant at p value<0.05

Table 4. Pearson Correlation between BMI and WC with Different Biochemical and Molecular Findings in Patient Group (G III)

	BMI r	BMI p	WC r	WC p
TAG (mg/dl)	0.532	0.001*	0.425	0.005*
TC (mg/dl)	0.495	0.006*	0.361	0.004*
LDL-C (mg/dl)	0.358	0.003*	0.5	0.002*
HDL-C (mg/dl)	-0.119	0.577	-0.429	0.008*
FBG (mg/dl)	0.136	0.856	0.528	0.006*
Fasting insulin (µIU/ml)	0.084	0.667	0.471	0.028*
HOMA-IR	0.102	0.447	0.663	0.001*
IL-6 (pg/ml)	0.256	0.020*	0.354	0.017*
IL-12 (pg/ml)	0.305	0.047*	0.429	0.006*
LCN2 (pg/L)	0.663	0.001*	0.447	0.005*
Relative NUCKS-1 mRNA expression	0.203	0.336	0.529	0.001*

Values are Pearson correlation coefficients; *Significance at p<0.05

Correlations of obesity with different biochemical and molecular findings in breast cancer

Table (4) showed person correlation between BMI and WC with different biochemical and molecular findings in breast cancer patients where, statistically significant positive correlation ($p<0.05$) was found between BMI and WC with all parameters of lipid profile, except HDL-C that showed significant negative correlation ($p<0.05$) with WC and insignificant negative correlation with BMI. FBG, fasting insulin and HOMA-IR showed insignificant positive correlation with BMI and significant positive correlation ($p<0.05$) with WC which may reflect that BMI may not always provide accurate information about the variation in body fat and body composition that is associated with morbidity, moreover WC may be used as a tool for identification and assessment of obesity. Meanwhile, IL-6, IL-12 and LCN2 showed statistically significant positive correlation ($p<0.05$) with BMI and WC.

The attained results reflect a strong relationship of the studied parameters to obesity and breast cancer incidence and progression.

Discussion

Obesity is a known risk factor for breast cancer. It is generally accepted that obese women have an increased risk for postmenopausal, but not premenopausal, breast cancer (Gaudet et al., 2013).

In this current study high values of BMI, WC, FBG, fasting blood insulin as well as HOMA-IR index were detected in obese patients than control ones. Moreover, higher levels were observed in obese patients with breast cancer than other groups, this in agreement with prior studies of (Moschos and Mantzoros 2002; Parker and Folsom, 2003), indicating the development of insulin resistance with the incidence of breast cancer. Additionally, Huffman and Barzilai (2009) reported that

Table 6. Pearson Correlation between NUCKS-1 mRNA Expression and Cytokine Profile in Patient Group (G III)

		Serum IL-6 (pg/ml)	Serum IL-12 (pg/ml)	urinary LCN2 (pg/L)
Relative NUCKS-1 mRNA expression	r	0.441	0.526	0.228
	p	0.008*	0.001*	0.030*

Values are Pearson correlation coefficients; *Significance at p<0.05

Table 5. Pearson Correlation between Different Biochemical and Molecular Findings in Patient Group (G III)

		TAG (mg/dl)	TC (mg/dl)	LDL-C (mg/dl)	HDL-C (mg/dl)	FBG (mg/dl)	Fasting Insulin (µIU/ml)	HOMA-IR
IL-6 (pg/ml)	r	0.352	0.352	0.441	-0.142	0.211	0.421	0.274
	p	0.025*	0.036*	0.004*	0.225	0.102	0.042*	0.024*
IL-12 (pg/ml)	r	0.358	0.258	0.418	-0.107	0.14	0.522	0.424
	p	0.024*	0.041*	0.022*	0.635	0.369	0.033*	0.001*
LCN2 (pg/L)	r	0.523	0.532	0.337	-0.088	0.095	0.369	0.522
	p	0.001*	0.001*	0.028*	0.635	0.756	0.018*	0.001*
Relative NUCKS-1 mRNA expression	r	0.417	0.258	0.339	-0.147	0.064	0.574	0.366
	p	0.001*	0.018*	0.007*	0.756	0.224	0.020*	0.003*

Values are Pearson correlation coefficients; *Significance at p<0.05.

accumulation of visceral fat represents a greater risk for the development of increase secretion of inflammatory adipokines, insulin resistance and dyslipidemia.

In the present study, obese patients with breast cancer had manifest dyslipidaemia in line with this Hsu et al., 2007; Abdelsalam et al., 2012, showed that alterations in lipid profile levels showed a significant correlation with breast cancer risk, disease status and treatment outcome. Abdelsalam et al., 2012 reported that lipids might be associated with cancers because they play a key role in maintenance of cell integrity, cholesterol synthesis may also produce various tumorigenic compounds and acts as a precursor for the synthesis of many sex hormones linked to increased risk of various cancers such as breast cancer.

Our current data revealed that increased serum level of IL-6 was significantly higher in G III and G II as compared to their allied controls. Interestingly higher levels were noted in patients with advanced grading of breast cancer (G IIIb) as reported by Gonullu et al., 2005 who suggested a possible contribution of endogenous IL-6 and hyperinsulinemia associated obesity to the development of breast cancer in overweight or obese patients reflecting the role insulin resistance in disease activity and prognosis.

Barton and Murphy 2001; Hussein et al., 2004 reported that IL-6 may also stimulate cancer cells growth and contribute to locoregional relapse as well as metastasis. In harmony with our results, Smyth et al., 2004 showed that permanent synthesis and release of this cytokines lead to augmentation of their serum level that might be utilized as a marker of immunity status and immune system activation in obesity and obesity associated with breast cancer for prognosis and monitoring of the course of cancer associated obesity.

It is thought that 15 to 30 % of circulating IL-6 levels derives from adipose tissue production in the absence of an acute inflammation (Goyal et al., 2012). Moreover, Goyal et al., 2012 reported that IL-6 is a potent pleiotropic inflammatory cytokine that is considered a key growth-promoting and antiapoptotic factor.

Haura et al., 2005 stated that most IL-6 target genes are involved in cell cycle progression and suppression of apoptosis, which underscores the importance of IL-6 in tumorigenesis. This indicates that IL-6 acts in an autocrine fashion to mediate oncogene-induced senescence. On the other hand, the pool of IL-6 secreted to the extracellular compartment by the senescent cells had promitogenic activity, as it enhanced proliferation of tumor cells in a paracrine fashion (Minamino et al., 2003).

We also found positive correlations between IL6 levels and BMI, WC, fasting insulin and HOMA-IR index as well as also lipid profile except for HDL-C in obese patients with breast cancer as reported in previous studies of Dandona et al., 2004; Goyal et al., 2012.

Interleukin-12 (IL-12) is a heterodimeric class-I helical cytokine that is mainly produced by dentritic cells and macrophages and influences differentiation of T helper 1 (Th1) immune cells, promoting in this way a linkage between innate response and adaptive immunity (Trinchieri, 2003). IL-12 has been recently suggested to participate during development of obesity-related insulin resistance in rodents, since it clearly increases in both the epididymal adipose tissue and adipose tissue-associated proinflammatory macrophages in high-fat-diet-fed mice (Strissel et al., 2010) thus; levels of IL-12 are increased in overweight and obese individuals and show a strong relationship with markers of low-grade inflammation and obesity.

Recent experimental evidence from high-fat-diet-fed mice suggests that IL-12 could have an additional role in the systemic low-grade inflammation and the concomitant advent of obesity-related disorders, such as insulin resistance (Silswal et al., 2005), these results run hand in hand with our data where serum IL-12 levels were higher in obese patients with breast cancer particularly advanced one (G IIIb).

For this reason, it is of much relevance to study the systemic levels of IL-12 in humans that show high metabolic risk, such as obese individuals. In this sense, it has been previously reported that circulating concentrations of IL-12 are significantly increased in subjects with metabolic syndrome as reported by Surendar et al. (2011).

Furthermore, peripheral blood mononuclear cells (PBMCs) from type 2 diabetes (T2D) patients are able to produce higher levels of IL-12 in response to lipopolysaccharide stimulation than those cells from healthy subjects (Wu et al., 2010).

Concomitantly, a recent study in a rural population of Mexican women suggests that the risk of having elevated serum IL-12 diminishes in women with high plasma concentrations of zinc, a micronutrient that has been related with a reduced risk for being obese (Zavala et al., 2012). In this sense, our results reveal that circulating concentrations of IL-12 increase parallel with all parameters of obesity, including BMI, WC, fasting insulin and HOMA-IR index as well as all parameters of lipid profile except HDL-C levels in patient groups as reported by Derin et al. (2007); Sharabiani et al. (2011); Suárez-Álvarez et al. (2013) who stated that interleukins are known to play a fundamental role in cancer as they investigated the serum levels of IL-8 and IL-12, in breast cancer patients, and proved their relationship with the prognostic parameters and therapy. In harmony with our results, Silswal et al. (2005) reported that serum levels of IL-12 are highly elevated in breast cancer patients and correlate with tumor progression. Assays for serum levels of IL-12 can be used as predictive non-invasive tests for tumor progression in breast cancer patients, it might also promote tumor development and this can explains its higher serum level in advanced breast cancer cohort.

In the present study urinary levels of LCN2 were significantly higher in G III and G II in comparison to controls G I with higher levels in obese patients with advanced breast cancer (G IIIb). Our data suggest that LCN2 may be added to the growing list of secreted molecules that adipocytes use to modulate glucose homeostasis and insulin resistance as a subsequent complication of obesity for examples breast cancer.

In harmony with our results Wang et al. (2007); Auguet et al. (2011) reported that LCN2 levels were elevated in both circulation and adipose tissue of obese people moreover; circulating LCN2 concentration was positively

correlated with insulin resistance index and inflammatory markers.

The results of the present study revealed that urinary concentration of LCN2 increase at the same time that with parameters of obesity, including BMI, WC, and fasting insulin levels as well as HOMA-IR and all parameters of lipid profile except HDL-C levels in patients group as reported by previous studies of Liu et al. (2011).

LCN2 has been associated with breast cancer. LCN2 gene is among the genes most highly associated with estrogen receptor (ER) negative breast tumors, it is also one of the genes that is most increased in the luminal epithelial cells compared with myoepithelial cells (Jones et al., 2004), a significant finding because the majority of breast carcinomas are thought to arise from the luminal epithelial cells (Bauer et al., 2008). Taken together, these data suggested that LCN2 may actively participate in breast cancer progression so its levels were higher in G IIIb than G IIIa of breast cancer patients.

Roy et al. (2004), considered the possibility that LCN2 levels might be elevated in the urine of women with breast cancer, its increase in the urine of women with metastatic breast cancer, suggesting that LCN2 may have potential as a noninvasive biomarker for advanced breast cancer.

The proposed mechanisms by which LCN2 promotes growth and metastasis of breast cancer cells are multiple. Association of LCN2 with matrix metalloproteinase-9 (MMP-9) was shown to induce allosteric activation of the enzymatic activity of MMP-9 and to protect MMP-9 from auto degradation (Cramer et al., 2012). LCN2 has also been inferred as a direct regulator of expression of pro-oncogenic factors necessary for mesenchymal transition (Yang et al., 2009).

In this sense LCN2, a newly identified biomarker and a potential therapeutic target for breast cancer, and the possible mechanisms underlying its role in tumorigenesis and metastasis as reported by Xiaohong et al. (2011); Yang et al. (2013).

The results of the present study showed that increased urinary level of LCN2 was associated with high levels of serum IL-6 and IL-12 reflects that LCN2 is associated with pro-inflammatory markers suggesting its potential involvement in the low-grade chronic inflammation accompanying obesity and its subsequent complications as reported by previous studies of Law et al. (2010); Auguet et al. (2011).

Gene expression profiling of breast tumors is a novel tool in the effort to classify tumor subtypes, predict risk of relapse, identify genes that mediate disease progression and select optimal therapeutic options (Nguyen and Massagué, 2007).

NUCKS-1 protein seems to be phosphorylated at more than one site in all phases of the cell cycle, by CK-2, which is implicated in cell growth and proliferation, and the cyclin-dependent kinases (Cdk1, 2, 4, and 6) all of which play important roles in regulation of the cell cycle, in addition, NUCKS-1 is an *in vitro* substrate for DNA-activated protein kinase, which is involved in DNA repair (Wiśniewski et al., 2008).

NUCKS-1 from proliferating cells exhibits higher affinity for DNA when compared to NUCKS-1 from non-proliferating cells, NUCKS proteins are known and as the protein is highly expressed throughout the mammalian kingdom and serves as a substrate for Cdk1 *in vivo* (Ostvold et al., 2001).

Our obtained results show that there is an increased NUCKS-1 gene expression in breast cancer from obese patients compared to normal tissues, with a statistically significant increase in its expression in G IIIb than G IIIa this is in line with Liu et al. (2007), who reported that NUCKS-1 has been found to correlate with the invasive gene signature (IGS) which consists of 186 genes, overexpressed in breast cancer stem cells, compared to normal breast epithelial cells.

The NUCKS-1 gene is located on chromosome 1q32.1. There are several scientific reports utilizing array-based Comparative Genomic Hybridization (CGH) techniques, which show that there is a high frequency (59-74%) of gain of chromosome region 1q.32 in breast cancer and this gain is an early event in the cancer progression (Nyante et al., 2004). In harmony with our results Drosos et al. (2009) reported in his study that the quantitative, real-time RT-PCR analysis of primary culture cells derived from benign lesions and breast carcinomas revealed higher NUCKS-1 gene expression compared to normal and fibroadenoma culture cells.

Moreover, there are a number of scientific data indicating that NUCKS-1 could play a role in the DNA damage response (Wiese et al., 2007). Furthermore, NUCKS-1 is a substrate for ATM (ataxia telangiectasia mutated) and ATR (ATM and Rad3-related) kinases (Matsuoka et al., 2007), implying that NUCKS-1 is a cell cycle related protein involved in DNA damage response. Since the activation of the DNA damage checkpoint is an early event in carcinogenesis (Gorgoulis et al., 2005).

In this sense, our results reveal that NUCKS-1 mRNA expression increase at the same time with parameters of obesity, including WC, fasting insulin and HOMA-IR index as well as all parameters of lipid profile except HDL-C in patient group.

To our knowledge there was no studies showed an evaluation of NUCKS-1 protein expression in association with cytokine profiling in obese breast cancer patients that give a new linkage between cytokines milieu and the cell cycle protein expression, important components in this linkage are the cytokines produced by activated innate immune cells that stimulate tumor growth and progression as well as cell cycle regulation.

In summary, there is still much work to be done on elucidating the mechanisms by which NUCKS-1 contributes to oncogenesis so that in the long term this protein could serve as a potential prognostic marker for breast cancer progression.

In conclusion, the risk of postmenopausal breast cancer is significantly increased by obesity. Further, low grade chronic inflammation, a hallmark of obesity, can contribute to detrimental health effects including high cancer incidence. Our goal is to understand the molecular basis for obesity-breast cancer interactions. Elevated IL-6 and IL-12 serum concentrations and urinary LCN2 level are strongly associated with breast cancer and they also correlate with clinical stage of disease. This can be

possibly used to diagnose obese women with breast cancer and to identify patients with a poor prognosis who may benefit from more aggressive management. The fact that NUCKS-1 belongs to IGS demonstrates its important role in breast cancer complicated by obesity but further investigation is needed in order to elucidate whether the observed overexpression in breast cancer is correlated with oncogenic progression and poor prognosis or reflect a preventive response to cancer-related genomic instability.

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