Low expression of leptin and its association with breast cancer: A transcriptomic study

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Abstract. The incidence of breast cancer is alarmingly increasing worldwide and also among Saudi women. Obesity is linked with an increased cancer risk and studies have also revealed that leptin may be involved in breast tumorigenesis particularly among obese women. Numerous transcriptomic studies have been carried out worldwide; however, molecular studies among breast cancer patients of diverse ethnic groups from the Arabian Peninsula are scarce. In the present study, whole transcriptome analysis of 45 surgically resected breast tumors from Saudi Arabian female patients was carried out. Expression data were analyzed, and molecular networks and canonical pathways were identified. We identified 1,159 differentially expressed genes using p-value with a false discovery rate <0.05 and a fold-change >2 as a cut-off. Using ingenuity pathway analysis tool, we identified many canonical pathways that were implicated in breast cancer for the first time. Notably, along with other lipid metabolism molecules, leptin (*LEP*) was one of the most downregulated genes (fold cut-off, -7.03) with significant differences between the breast cancer and the control groups (p<0.0001) and was further confirmed in all the samples using qPCR. Transcriptomic profiling of breast cancer from a Saudi female population revealed downregulation of *LEP*. Molecular pathway analysis demonstrated the role of *LEP* and other associated molecules of the lipid metabolism pathway. Involvement of leptin and lipid metabolism in breast cancer was highlighted. The majority of cases presented were of late stage, stressing the need to educate individuals concerning early diagnostic testing and the life-style risk factors for breast cancer such as unhealthy diet and obesity.

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Abbreviations: BC, breast cancer; LEP, leptin

Key words: human breast cancer, gene expression, microarray, leptin, lipid metabolism, Saudi Arabia

Introduction

Breast cancer (BC) is the most commonly diagnosed female cancer (1). BC ranks first in Saudi females as well, accounting for 26% of all newly diagnosed female cancers as reported by the National Cancer Registry, Saudi Arabia (2). In the US and Western Europe, the median age at presentation is ~63 years in comparison to 48 years in Saudi Arabia (2). BC is clinically a heterogeneous disease depending on genetic variation between tumor and healthy tissue (3). Studies characterizing transcriptomics and molecular genetics of cancers including BC have been conducted on European or US patients. However, variability in the molecular signature of cancers from different ethnic groups has been reported, and clear differences in the patterns of p53 mutations in BC have been found among different ethnic groups (4,5).

In the recent decade, a high throughput transcriptomic genome-wide approach has had a major impact on BC research (6). Studies have been conducted to classify clinically distinct subclasses of tumors and treatment prediction (6-8). To date, gene expression profiling studies have been reported mainly from the Caucasian population, while less data are available for non-Caucasian and Arabian populations (9,10). Saudi society is different from Western countries and common BC risk factors such as nulliparity, low parity, first time pregnancy at late age, and no history of breast feeding, are usually not applicable in the present study, yet, the high incidence of BC is alarming among Saudi women (11,12).

LEP, formerly depicted as a circulating hormone, is predominantly produced by adipocytes along with other adipocytokines such as adiponectin, tumor necrosis factor-α (*TNF-α*), vascular endothelial growth factor (*VEGF*) and

interleukin-6 (*IL-6*). LEP activates the leptin receptor in different tissues and basically acts as a mitogen, survival factor and regulator of metabolism, feeding behavior and energy homeostasis. Research has demonstrated that the function of LEP goes far beyond metabolism and it has been postulated to be a key mediator in obesity-associated cancers such as that of breast, colorectal and prostrate cancer (13). Researchers have demonstrated the overexpression of LEP and its receptors in BC using immunohistochemistry or RT-PCR, and LEP has been proposed as a prospective molecular target for cancer prediction, prevention and therapeutics (14,15). In the present study, we examined the expression status of LEP in Arabian female BC patients and the possible LEP-mediated mechanism involved in BC cell proliferation.

Materials and methods

Patients and samples. A transcriptomic study was carried out on 45 female Saudi BC patients undergoing treatment at King Abdulaziz University Hospital (Jeddah, Saudi Arabia) during the years 2008-2010. All collected tissue specimens were immediately stored in RNAlater (Invitrogen, Life Technologies, Grand Island, NY, USA), and clinicopathological features, including, age, tumor grade and size, hormone receptor status, lymph node involvement and pathology reports were retrieved from the patient records. The average age of the patients in the present cohort was 48 years, with a median of 47 years (range, 27-80 years). To estimate the effect of obesity on BC, we also determined the mean body mass index (BMI). The present study was approved by the Ethics Committee (no. 08-CEGMR-02-ETH) of King Abbdulaziz University, and informed consent was provided by all the patients included in the present study.

RNA extraction and array processing. Total RNA was extracted from fresh breast tissue specimens according to the manufacturer's recommendations provided with the Qiagen RNeasy Mini kit (Qiagen, Hilden, Germany). RNA quality was verified on an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) with a cut-off of RNA integrity number (RIN) >5. RNA concentrations were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and 300 ng of each RNA sample was processed according to the manufacturer's recommendations for Human Gene 1.0 ST GeneChip arrays (Affymetrix, Santa Clara, CA, USA).

Gene expression analysis. Affymetrix CEL files were imported to Partek Genomics Suite (PGS version 6.6; Partek Inc., St. Louis, MO, USA) and the robust multi-array average (RMA) was used for data normalization. We performed principal component analysis to visualize high dimensional data and to assess overall variance in gene expression. Analysis of variance (ANOVA) was carried out on the complete dataset and a differentially expressed transcript list of genes was then conducted using an false discovery rate (FDR) (Benjamini-Hochberg) of 0.05 with a 2-fold change cut-off. The complete dataset and associated clinicopathological information were submitted to the NCBI Gene Expression Omnibus (GEO) which are accessible through accession no. GSE36295.

Real-time quantitative PCR assay. LEP expression levels were validated using the TaqMan® quantitative real-time PCR (qPCR) assay (ID Hs01084494_m1; Life Technologies, Carlsbad, CA, USA). We used 0.3 μg of template RNA and 50 ng/μl of random hexamer in a 5 μl mixture that was denatured at 70˚C for 5 min, and ice cooled for at least 1 min before use for cDNA conversion. $MgCl_2$ (1.5 mM), dNTP mix (0.5 mM), RNasin (1 U), reverse transcriptase (2.5 U) and RT buffer (1X) were used for cDNA conversion in the following steps: initial incubation of 10 min at 25˚C, 1 h incubation at 37˚C, 5 min heat inactivation at 95˚C and storage at 4˚C to obtain a cDNA concentration of ~650 ng/μl. PCR was performed using cDNA (100 ng), primer (1X) and Master Mix (1X) for 1 cycle (2 min at 50˚C), 1 cycle of denaturation (10 min at 94˚C), 40 cycles of denaturation, annealing and extension (30 sec at 97˚C; 45 sec at 59˚C and 1 min at 72˚C), and final extension of 10 min at 72˚C. Gene expression analyses were performed using the ΔCt value model (average Ct_{gene} - average Ct_{GAPDH}). Statistical significances between BCs vs. normal control were evaluated using the Student's unpaired t-test. A p-value of <0.01 was considered to indicate a statistically significant result.

Identification of functionally significant proteins interacting with LEP. We used Search Tool for the Retrieval of Interacting Genes/Proteins (STRING version 9.1) to identify significant proteins interacting with LEP. It is a biological database and web resource of known as well as predicted protein interactions derived from high-throughput experimental sources, text mining and co-expression (16,17).

Functional and pathway analysis. The identified differentially expressed transcripts with corresponding probesets ID, Entrez gene ID as clone identifier, gene symbol, p-value and fold-change values were uploaded into the Ingenuity Pathways Analysis (IPA) software (Ingenuity Systems, Redwood City, CA, USA). We used IPA to identify biological associations, and to perform interaction and functional analysis among the differentially regulated BC genes. The impact of the link between the expression data and canonical pathways were calculated by ratio and/or Fisher's exact test. To develop an assumed network of differentially expressed genes downstream of *LEP*, the network was grown downstream of LEP using the differentially regulated genes.

Results

The prime aim of the present study was to establish the transcriptomic profiles of BC from a Saudi female patient population. We profiled and compared 45 fresh BC tissue specimens with 8 normal breast tissues. The PCA results showed a clear difference between the BC and normal breast tissues. We also calculated the BMI of all enrolled patients and its average was found to be ~32.5 kg/m^2.

Identification of differentially expressed genes. Global genome-wide expression profiling of BC identified 1,159 differentially expressed genes: 544 upregulated and 615 downregulated (p<0.05; 2-fold change). Functional analysis of the BC-associated genes found an overexpression of genes associated with cell cycle progression, cell death, DNA repair, tumor

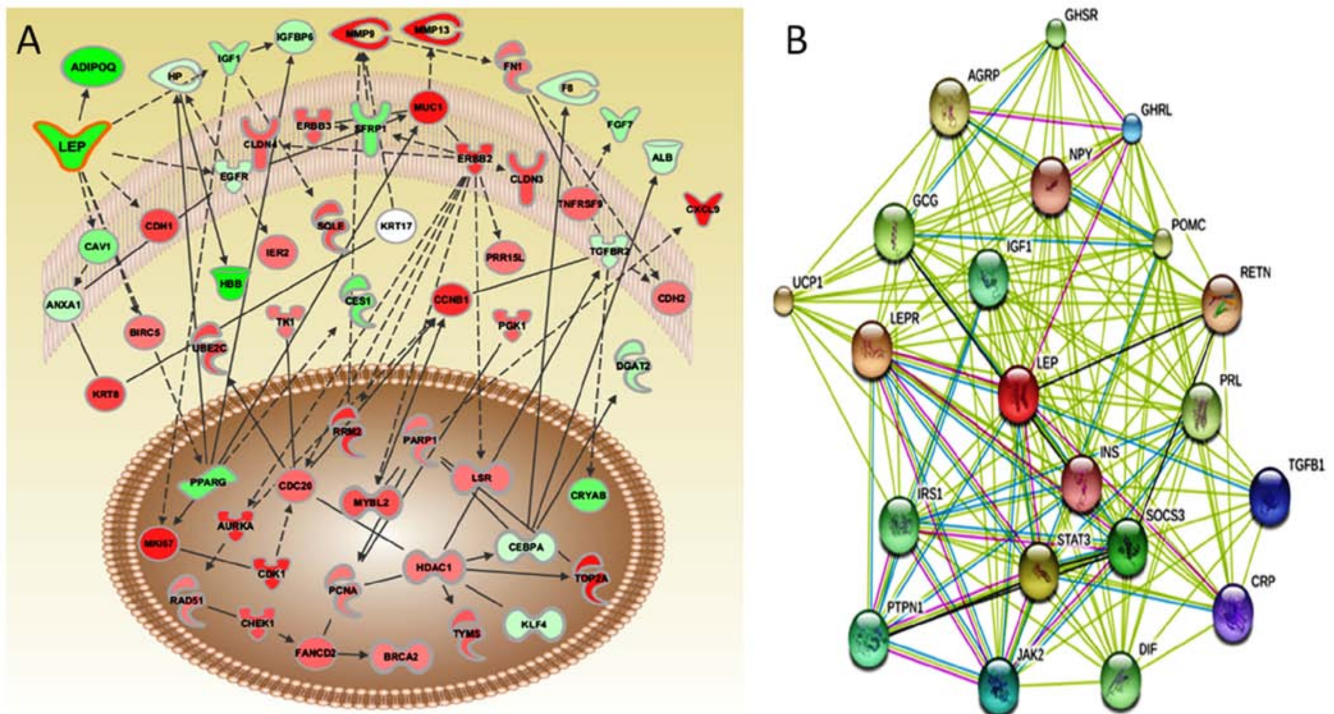

Figure 1. Putative network of differentially expressed genes downstream of leptin. (A) Using IPA, a biological network downstream of leptin was constructed based on existing literature as described in Materials and methods. Molecules colored with red denote upregulation and green denotes downregulation. (B) Interaction network of leptin protein with the 20 most significant interactions with predicted functional partners. Different line colors represent the types of evidence for the association. Green is from text mining, blue is from databases, magenta is from high throughput experimental data, and black is from co-expression studies.

morphology and tissue developments. Specifically, genes that are known to be linked with BC, included H3 histone family, member A, histone 1, H3a (*HIST1H3A*), chemokine (*C-X-C* motif) ligand 10 (*CXCL10*), topoisomerase 2α (TOP2A), carcinoembryonic antigen-related cell adhesion molecule 1 (*CEACAM1*), cell division cycle 6 homolog (*S. cerevisiae*) (*CDC6*), ADAM-like decysin 1 (*ADAMDEC1*), actin-binding protein anillin (*ANLN*), centromere protein F (*CENPF*), mucin 1, cell surface-associated (*MUC1*), protease serine 8 (*PRSS8*), kinesin family member 23 (*KIF23*), interferon, γ-inducible protein 6 (*IFI6*), chemokine (C-X-C motif) ligand 9 (*CXCL9*), ubiquitin-conjugating enzyme E2T (*UBE2T*) and matrix metallopeptidase -9, -11 and -13 (*MMP-9*, *-11* and *-13*). Genes including *LEP* linked to lipid metabolism, and endocrine system development were significantly downregulated in the BC tissues.

Pathways and networks underlying BC. We examined molecular networks using IPA to comprehend the mechanisms by which the genes control a wide range of physiological processes including cancer. Transcriptomic signatures showed noteworthy disruption in the following signaling pathways: glycerolipid metabolism, ATM signaling, DNA damage and cell cycle, and ILK signaling involved in tumor initiation or progression. We were the first to report the role of the glycerolipid metabolism pathway in BC (18). The majority of the genes involved in glycerolipid metabolism were downregulated. These results further support the putative role of these pathways in rendering susceptibility to BC. Differentially expressed genes were imported into IPA to graphically represent all known relationships and potential interactions among them. IPA and gene ontology based network analysis suggested that *LEP* is significantly linked with the other differentially expressed genes, leading to BC progression (Fig. 1A).

Protein interaction study. Results of STRING showed a direct interaction and predicted a functional association between LEP and its interacting proteins. The following proteins showed prominent interactions with LEP: leptin receptor (LEPR); suppressor of cytokine signaling 3 (SOCS3); insulin-like growth factor 1 (IGF1), prolactin (PRL); ghrelin/obestatin prepropeptide (GHRL); insulin (INS); signal transducer and activator of transcription 3 (STAT3); protein tyrosine phosphatase, non-receptor type 1 (PTPN1); insulin receptor substrate 1 (IRS1); growth hormone secretagogue receptor (GHSR); resistin (RETN); glucagon (GCG); agouti-related protein homolog (mouse) (AGRP); proopiomelanocortin (POMC); C-reactive protein, pentraxin-related (CRP); neuropeptide Y (NPY); transforming growth factor, β1 (TGFB1); uncoupling protein 1 (mitochondrial, proton carrier) (UCP1); tumor necrosis factor precursor (TNF-α) (DIF); and Janus kinase 2 (JAK2). Based on the predicted result of STRING for LEP partners, we only used those likely candidates of protein interactions for a limited but focused hierarchical clustering (Fig. 1B).

Real-time qPCR validation of LEP. The microarray results showed downregulation of *LEP* and most of its interacting molecules in the BC tissues (Fig. 2A). To evaluate the expression level of *LEP* in the BC tissues, we validated it using qPCR

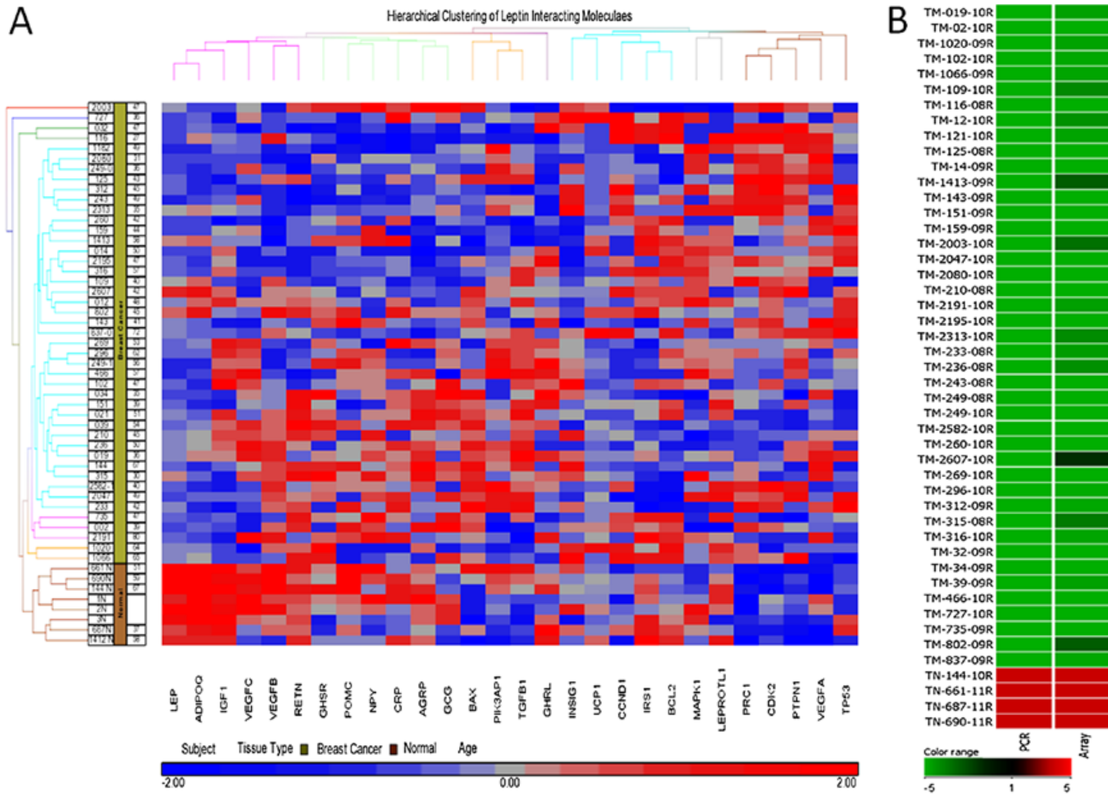

Figure 2. Comparisons of the microarray and quantitative real-time RT-PCR. (A) Dendrogram shows the change in expression levels of selected genes in breast cancer compared to normal control tissues. Samples from each tissue were hybridized to Affymetrix Human ST 1.0 array and agglomerative average-linkage hierarchical clustering for genes (y-axis) and tissue type (x-axis) were obtained using Partek GS 6.6 software. Each row is a single experiment from each subject and each column is a single gene. The cluster color represents the normalized expression level of a given gene in a particular tissue type or histopathological condition given below and is colored according to the color bar at the bottom. Red denotes upregulation and blue denotes downregulation according the color scale. (B) Results of the qRT-PCR analysis of relative gene expression for leptin. The relative levels of gene expression were normalized to the housekeeping gene (GAPDH). The expression levels detected by qRT-PCR were consistent and comparable to the microarray expression.

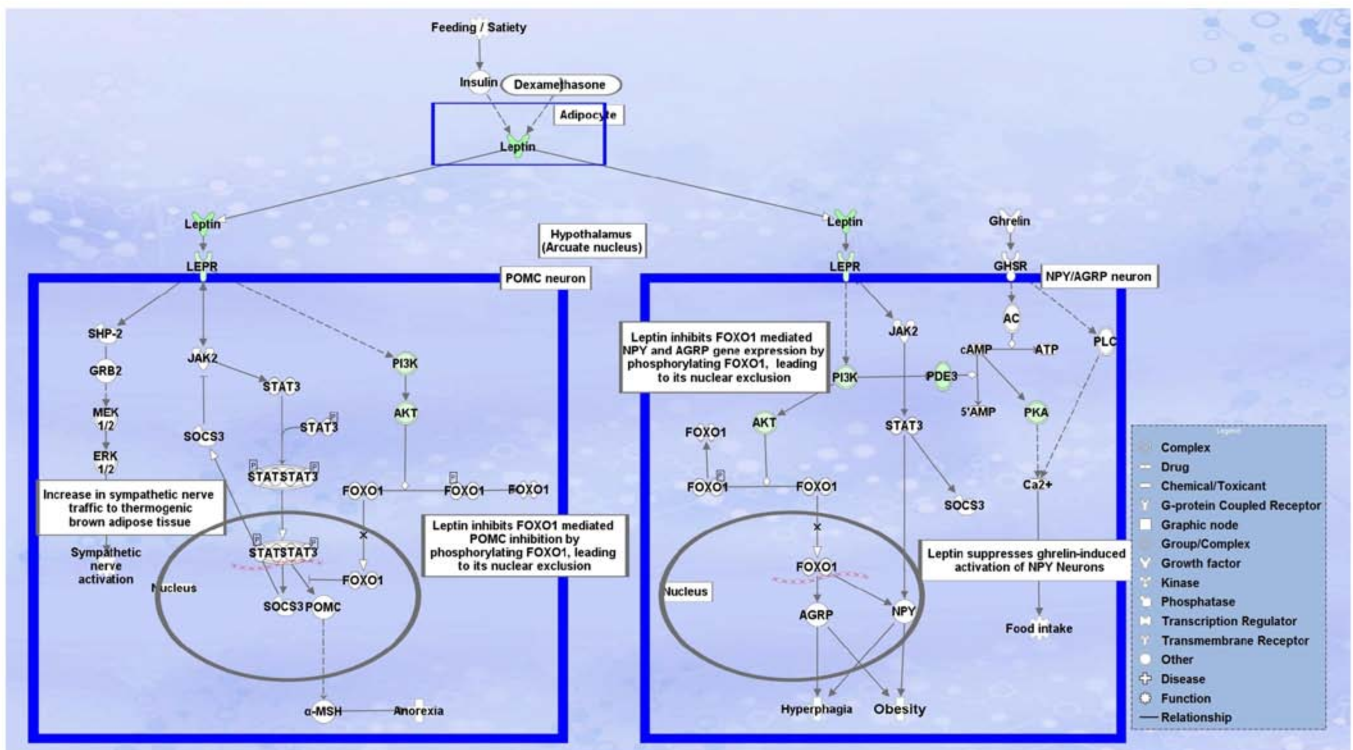

Figure 3. Leptin signaling in obesity. Using IPA, differentially expressed genes were overlaid on to canonical pathways for leptin signaling in obesity. Molecules colored with green denote downregulation and white denotes no change in expression as compared to the controls.

in 43 BC and 4 normal tissues (Fig. 2B). Our results further confirmed LEP downregulation in all the 43 BC samples with significant differences between the BC and the control groups ($p<0.0001$).

Discussion

The therapeutic strategies currently employed for breast cancer (BC) are generally based on histopathological characterization, tumor size, grade and axillary lymph node and receptor status (1). However, patients when diagnosed with similar conditions and when treated with similar agents can present with extensive differences in the development and relapse of BC. Studies have demonstrated that women who have had a full-term pregnancy at an age younger than 30; and who have had three full-term pregnancies, and three or more years of breast feeding, were significantly protected against BC (12). However, the incidence of BC is high among women from Saudi Arabia. BC risk factors, including nulliparity or low parity, first full-term pregnancy at a late age, no history of breast feeding, are not common in the Saudi female population (11). These factors combined with the early onset of BC among this ethnic population prompted us to study the molecular mechanisms underlying this malignancy.

In the present study, we characterized the role of the specific gene *LEP* from identified transcriptomic signature in BC tissues from Saudi Arabian patients that were associated with clinical and histological parameters. Further pathway analysis of the differentially regulated transcripts provided new hypotheses underlying metastatic BC progression as reported in our previous publications (18,19).

The prevalence of obesity is high in Saudia Arabia with an incidence of 33.9% (20). The mean body mass index (BMI) of the BC subjects in the present study was 32.5 kg/m^2. One study demonstrated that native Hawaiian women with a BMI 30 kg/m^2 or greater, or obese women, had an 82% higher risk of BC, compared to those who had a BMI of 20-24.9 kg/m^2 (21). The strong association between increased BMI and postmenopausal BC was also found specifically in Asia-Pacific populations from 34 studies of a total sample size of 2,559,829 (22).

LEP, a product of the obesity gene and an adipose derived hormone is mainly secreted by adipose tissue that regulates energy intake and energy expenditure, including metabolism (23,24). Disruption of lipid metabolism has been implicated in breast tumorigenesis in several studies (25-29). Studies have reported that increased adiposity (body fat mass) is associated with higher circulating levels of *LEP* (30,31). Studies have also shown that increased expression of *LEP* in epithelial mammary cells may promote tumorigenesis via cell proliferation, angiogenesis, apoptosis, cell cycle regulation and cell survival mechanisms (32). Furthermore, clinical reports have also shown a strong correlation between BC risk and blood *LEP* levels (33). Similarly, Saxena *et al* showed the existence of bidirectional crosstalk between *LEP* and insulin-like growth factor 1 (*IGF1*) upregulation in triple-negative breast cancer (TNBC) cell lines (34). Additionally, increased *LEP* and decreased *ADIPOQ* levels interrupt cellular signaling networks that are linked to cell survival, angiogenesis, proliferation and cell cycle regulation (35), and the *LEP-ADIPOQ* axis has been well implicated in BC tumorigenesis (35). However, most of the lipid metabolism genes including *LEP*, *ADIPOQ* and *IGF-1* were found to be downregulated in our analysis, even though the mean BMI of the BC subjects in the present study was 32.5 kg/m^2. The effect of the downregulation of *LEP* is somewhat unclear. The LEP signaling pathway clearly shows that *LEP* is at the top of the pathway and almost all downstream genes were found to be downregulated, suggesting the downregulation of *LEP;* however, regulation of LEP is still unclear. Studies have shown that decreased levels of insulin (36), glucosamine (37), glucocorticoids (38), cyclic AMP (39) and stimulation of adipose tissue β-andrenergic receptor (40) inhibit LEP expression (41). Thus, we overlaid the differentially regulated genes onto the canonical pathway for LEP signaling in obesity and our analysis showed that most of the proteins present in the pathway to the downstream of LEP were also downregulated (Fig. 3).

Obesity is perhaps related to LEP resistance not deficiency, as an elevated level of LEP is found in many obese persons. Whether elevated LEP concentrations are responsible for an increased cancer risk is not yet certain. The variable reports on circulating LEP and its level in cancer may be a consequence of different analytic methods, or difference in sample preparation where a number of influencing aspects, such as food intake and circadian rhythm, were inappropriately controlled. Understanding the underlying genetics and complex interactions of lifestyle, diet and other known risk factors will remain a key area of research.

In conclusion, the present study described genome-wide profiling of BC from Saudi ethnic females. We identified differential expression signatures, biological functions and pathways that were significantly altered in BC and may serve as targets for novel therapeutics. In our analysis, genes associated with lipid metabolism and small-molecule biochemistry were significantly downregulated in the BC tissues. LEP was one of the most downregulated genes, and biological network analysis revealed a strong connection between LEP and other molecules of the lipid metabolism pathway. Further studies are needed to determine the relationship between dysregulation of the lipid metabolism and the mechanisms underlying BC.

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