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JAK2, STAT3 and SOCS3 gene expression in women with and without breast cancer

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ABSTRACT

Introduction: Breast cancer is a disease that arises from the accumulation of alterations in the genome of cells that make up the mammary gland. The Janus kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3) signaling pathway has been reported to participate in the development of breast cancer and is activated by adipocytokines such as leptin, which are elevated in obesity. In contrast, alterations in suppressor of cytokine signaling 3 (SOCS3) gene expression have been found in patients with decreased breast cancer metastasis.
Objective: The current study sought to identify whether JAK2, STAT3 and SOCS3 gene expression is associated with body mass index (BMI) and breast cancer.
Methods: This was a cross-sectional prospective study. JAK2, STAT3 and SOCS3 gene expression levels were determined using RT-qPCR from the biopsies of 26 patients with breast cancer and 43 patients with benign breast lesions. We compared the expression of these genes, relative to the housekeeping genes, ACTB and GAPDH, against BMI, clinical stage and immunohistochemistry.
Results: STAT3 gene expression was increased in breast cancer patients ($p \leq 0.001$; AUC = 0.65; AUC 95% CI: 0.5–0.8), and SOCS3 expression was decreased in obese patients with benign breast lesions ($p \leq 0.001$; AUC = 0.51; AUC 95% CI: 0.36–0.65). With regard to the clinical stage, there were significant differences in STAT3 gene expression between stage II and III ($p \leq 0.011$) and stage II and IV ($p \leq 0.033$) breast cancers. Among all women, there was a positive correlation between JAK2 and STAT3 expression ($R = 0.493$, $p = 0.000$). In addition, breast cancers that were negative for HER2 were associated with JAK2 and SOCS3 ($R = 0.645$, $p = 0.003$).
Conclusion: High levels of STAT3 expression were associated with early stages of breast cancer development and patients in the control group with obesity showed higher expression of SOCS3 regarding overweight.

1. Introduction

Breast cancer is a disease that arises from the accumulation of alterations in the genome of cells that make up the mammary gland. Breast cancer is the most common type of cancer among women, with an estimated 1.38 million new cases of cancer diagnosed in 2008 (23% of all cancers), and the second most common type of cancer overall (10.9% of all cancers). In Mexico in 2008, there were 13,939 cases of breast cancer (27.2 per 100,000 individuals) and 5217 deaths as a result of breast cancer, resulting in a mortality of 10.1 per 100,000 individuals in the population (Del Socorro Romero-Figueroa et al., 2010; Knaul et al., 2009; WHO, 2008).

Although most studies of premenopausal women have not found a relationship between breast cancer and obesity, the prognosis for both pre- and postmenopausal breast cancer is substantially worse among obese compared to normal-weight individuals. Increasing evidence suggests that these associations may be mechanistically related to sex hormones, insulin and certain adipokines (Amadou et al., 2013). Obesity, defined as the presence of a body mass index (BMI) 30 kg/m^2, is another primary cause of mortality in Mexico, affecting 60–70% of the adult population. The incidence of both breast cancer and obesity has risen sharply over the past 20 years. An analysis of trends in BMI categories

Abbreviations: ACTB, beta actin; AUC, area under the curve; BMI, body mass index; CT, threshold cycle; EPO, erythropoietin; ER, estrogen receptor; ERK1/2, extracellular signal-related kinase 1/2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GH, growth hormone; GM-CSF, granulocyte macrophage colony-stimulating factor; HER2, human epidermal growth factor receptor 2; IL, interleukin; JAK2, Janus kinase 2; MAPK, mitogen-activated protein kinases; PI3K, phosphoinositol 3-kinase; PR, progesterone receptor; ROC, receiver–operator characteristic; SOCS3, suppressor of cytokine signaling 3; STAT3, signal transducer and activator of transcription 3; TPO, thrombopoietin; TYK2, tyrosine kinase 2.

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in women 20–49 years old developed in Mexico by the National Health Survey (ENSANUT) showed that, from 1988 to 2006, the prevalence of the overweight condition increased by 41.2% and that of obesity increased by 270.5%. While the trend of the overweight condition decreased 5.1% between 2006 and 2012, the prevalence of obesity increased by 2.9% during this time (ENSANUT, 2012; Villaseñor, 2011).

Obesity increases the risk of breast cancer development and progression, and several reports indicate that the adipokine leptin, whose synthesis and plasma levels increase with obesity, may play an important role in modulating the breast cancer cell phenotype (Giordano et al., 2013). Furthermore, the binding of leptin to its receptor (Ob-R) induces the activation of signaling cascades, including the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) (mainly JAK2/STAT3), extracellular signal-related kinase 1/2 (ERK1/2), mitogen-activated protein kinases (MAPK) and phosphoinositol 3-kinase (PI3K) pathways (Amitabha and Cleary, 2012).

The mammalian family of JAKs is composed of JAK1, JAK2, JAK3 and tyrosine kinase 2 (TYK2), which selectively associate with the cytoplasmic domains of various cytokine receptors (O'Shea et al., 2013). In particular, JAK2 has been shown to be associated with breast cancer. JAK2 proteins are also associated with membrane receptors and possess enzymatic activity to phosphorylate tyrosine residues and participate in the signal transduction of erythropoietin (EPO), thrombopoietin (TPO), interleukin (IL)-3, IL-5, leptin, granulocyte macrophage colony-stimulating factor (GM-CSF), prolactin and growth hormone (GH). As a result, these proteins are involved in processes such as erythropoiesis, thrombopoiesis, mammary gland development, lactation and immunity. In various studies, constitutive activation and/or mutations of JAK2 have been linked to several pathologies, such as polycythemia vera, cancer and inflammatory diseases, and therefore represent novel therapeutic targets for treating such diseases (Haricharan and Li, 2013; Smirnova et al., 2007; Wagner and Hallgeri, 2008; Wagner and Schmidt, 2011).

The phosphorylated tyrosines on JAKs and their receptors recruit several signaling substrates, the most prominent of which are members of the STAT family. These proteins were numbered based on their order of discovery and include STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6 (Haricharan and Li, 2013; Wagner and Schmidt, 2011). Depending on the cell type and ligand receptor complex involved, each JAK activates one or more of the seven STAT proteins identified in mammalian cells.

Following JAK-mediated phosphorylation, STAT proteins dimerize and translocate to the nucleus, where they regulate gene expression (Constantinescu et al., 2007). Constitutive STAT3 (Pensa et al., 2009) and STAT5 activation has been associated with numerous cancers (O'Shea et al., 2013; Seavey and Dobrzansky, 2012), and disturbances to the state of equilibrium of these JAK/STAT pathways (in particular JAK2/STAT2 and JAK1/STAT3 signaling) lead to developmental defects and contribute to mammary carcinogenesis (Wagner and Schmidt, 2011).

Several studies have performed cell culture and immunohistochemistry and have shown that STAT3 and STAT5 proteins are found in greater abundance and are activated in different types of cancer; therefore, these proteins have been proposed to be associated with carcinogenesis. STAT3 is considered a proto-oncogene because a mutation that leads to a constitutive activation of STAT3 is sufficient for malignant cell transformation, activating diverse genes such as c-myc, cyclin D1, p21 waf1, c-jun, junB, erg-1 and Bcl-2, which are heavily involved in cell survival and proliferation (Cirillo et al., 2008; Walker et al., 2013).

The suppressors of cytokine signaling (SOCS) proteins are negative regulators of the JAK/STAT pathway and inhibit cytokine signaling. SOCS proteins are thought to attenuate this signaling by inhibiting JAK activity or by promoting protein degradation. Moreover, SOCS3 has been identified as an inducible suppressor of leptin signaling, and it has been proposed that prolonged Ob-Rb stimulation can attenuate leptin signaling via SOCS3 in human embryonic kidney cells and in the mouse hypothalamus. In breast cancer cells, SOCS3 overexpression has been shown to decrease proliferation and anchorage-independent growth (Palianopoulou et al., 2011). SOCS3 gene expression is stimulated by various cytokines, including GH, prolactin, ILs and insulin, but is inhibited by glucocorticoids. The growth and function of the mammary gland are regulated by cytokines and modulated by SOCS proteins. STAT3 and potentially STAT1 and STAT5 can stimulate SOCS3 gene transcription (Le Provost et al., 2005) and also possess negative regulatory effects on JAK signaling, and this suppressive action is known to block the signaling mediated by cytokine receptors in a classical feedback loop (Pijet et al., 2013). Thus, one potential mechanism for leptin resistance is the increased hypothalamic expression of SOCS3, a feedback inhibitor of the JAK-STAT pathway that prevents STAT3 activation. Ample studies have confirmed the important role of SOCS3 in leptin resistance and obesity. However, the degree to which SOCS3 participates in the regulation of energy homeostasis in non-obese conditions remains largely undetermined (Matarazzo et al., 2012).

The purpose of this study was to measure the expression of JAK2, STAT3 and SOCS3 in breast cancer patients and to determine the associations between the expression of these genes and BMI.

2. Materials and methods

2.1. Study population

This study was a cross-sectional prospective study conducted at the Maternal–Perinatal Hospital "MónicaPretelini" (HMPMP), Health Institute of the State of Mexico (ISEM), Toluca, Mexico, from January to December 2011. The identified women as potentially having a breast tumor via mammogram were referred to the Imagenology Service of the HMPMP for a Tru-Cut biopsy to determine whether their breast lesions were malignant or benign. Patients undergoing antineoplastic or hormonal therapy were excluded from this study.

2.2. Clinical measurements

We measured the weight (kg), height (m; Seca, GmbH, Germany) and waist circumference (cm) of all participants. BMI was calculated as weight (kg) divided by height (m) squared. Women were classified as (a) normal weight (BMI <24.9 kg/m^2), (b) overweight (24.9 kg/m^2 < BMI < 29.9 kg/m^2) or (c) obese (BMI >30 kg/m^2). The participating women were also categorized according to the Breast Imaging Reporting and Data System (BI-RADS) score and the St. Gallen criteria, the cutoffs/criteria used to determine whether women had cancerous tumors or benign lesions were 4 to 6. The biopsies were taken under anesthesia using the ultrasound-guided (Voluson E8, GE Healthcare,USA) Tru-Cut (Angiotech Pharmaceuticals, Inc., Canada) biopsy technique. One sample from each biopsy was stored frozen at −80 °C until processing, and a second sample was placed in saline solution for immediate histopathological analysis.

2.3. Pathology

Hematoxylin and eosin (HE) staining was performed on the paraffin-embedded biopsied tissue sections. Immunohistochemical analyses of the cancer tissues were conducted at the Pathology Service of the Oncological Cancer Center (COE), ISSEMYM, Toluca, Mexico. If the tissue samples were determined to be positive for cancer, an immunohistochemical analysis (VentanaBenchMark, Ventana Medical Systems Inc., Tucson, AZ) was performed. The tissue sections (4 μm) were formalin-fixed (fixation time 6–8 h), paraffin-embedded and analyzed for the presence of estrogen receptor (ER; anti-ER, 1DS clone), progesterone receptor (PR; anti-PgR, RBT22 clone) and HER-2/neu (c-erb-2 clone, her-2/neu).

2.4. Gene expression

Approximately 10 mg of each sample was suspended in 200 μL freshly prepared buffer and stored at −70 °C until mRNA extraction. The sample treatment was homogenized using Magnalyser Beads (Roche Diagnostics). mRNA extraction was performed using the MagNA pure LC RNA Isolation Kit III with the MagNA Pure LC 2.0 system (Roche diagnostics), according to the manufacturer's instructions. RNA was then isolated from the biopsied tissue using the MagNA Pure Compact RNA Isolation Kit (Roche Applied Science, Indianapolis, Illinois, USA). A total of 200–400 ng total mRNA was reverse transcribed to cDNA using a Transcriptor High Fidelity cDNA Synthesis Kit (Roche Applied Science). The amount of extracted RNA was quantified by measuring the absorbance at 260 nm. The purity of the RNA was assessed according to the ratio of the absorbance values at 260 and 280 nm; the purity ranged between 1.8 and 2.1, demonstrating a high quality of the RNA. The samples were measured with a NANOPhotometer (Implen), and the extracts were then adjusted to a concentration of 20 μg DNA L^{-1} for the PCR reaction. A real-time polymerase chain reaction (RT-qPCR) was performed using the primer sets and probes specific for the JAK2, STAT3 and SOCS3 genes. All reactions were performed with the 7500 Fast Real Time PCR System (Applied Biosystems, Applera UK, Cheshire, UK) using the TaqMan Universal PCR Master Mix and the following Assays on Demand (Applied Biosystems, Applera UK, Cheshire, UK): Hs 01078124_m1 (JAK2), Hs 01047580_m1 (STAT3) and Hs02330328_s1 (SOCS3). The expression levels of the three genes of interest and two selected reference genes, Hs99999903 m1 beta actin (ACTB) and Hs 99999905_m1 Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), were examined by placing 4 μL of the reverse transcription mix for each PCR reaction in a total volume of 20 μL. The thermal cycling conditions were as follows: 10 min at 95 °C followed by 45 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min. The comparative threshold cycle (C_T) method was to calculate the fold amplification, as specified by the manufacturer. Commercially available primers and probes for ACTB and GAPDH mRNA (Applied Biosystems, Applera UK, Cheshire, UK) were used for normalization based on previous publication of Ferreira and Lyng (Ferreira and Cronjé, 2012; Lyng et al., 2008). All samples were measured in duplicate. The fold change in JAK2, STAT3 and SOCS3 was normalized against the constitutively expressed reference genes and then compared to the untreated controls (calibration sample) as follows: $2^{-\Delta\Delta CT}$, where $\Delta\Delta C_T = (C_{T\text{-target}} - C_{T\text{-reference}})_{treated\text{-}sample} - (C_{T\text{-target}} - C_{T\text{-reference}})_{calibrator\text{-}sample}$. Calibrator-sample refers to the expression level (1×) of the target gene normalized to the constitutive gene. The calibrator was chosen from the group with benign breast disease and was given a relative expression value of 1 (Livak and Schmittgen, 2001; Pfaffl, 2001; Schmittgen and Livak, 2008).

2.5. Ethics

This study was approved by the ethics committees of the HMPMP (2010-12-156) and the Medical Sciences Research Center (CICMED), Autonomous University of the State of Mexico (UAEMex) (2010/02) and was performed according to the ethical standards of the Helsinki Declaration of 1964. Written informed consent was obtained from all patients.

2.6. Statistical analysis

Statistical analysis was performed using SPSS 20.0 software. We used parametric or non-parametric tests as required by the variable distribution, the Student's *t*-test for normally distributed variables and the Mann–Whitney *U*-test for variables that were not normally distributed. The normality hypothesis was tested using the Kolmogorov–Smirnov test. The correlation coefficients (r) were determined using the Pearson and Spearman tests for normal and skewed variables, respectively. Finally, we performed receiver–operator characteristic (ROC) and area under the curve (AUC) analyses for JAK2, STAT3 and SOCS3 gene expression and BMI values.

3. Results

3.1. Study population

Of the 125 total patients admitted for Tru-Cut biopsy in the hospital during 2011, 69 were included in this study. Of these, 26 patients were confirmed to have cancer via immunohistochemistry, and 43 patients were confirmed to have benign breast lesions. Table 1 shows the recorded anthropometric values for the patients in the study. Compared with the control group, the cancer patients showed statistically significant differences in age ($p \leq 0.0001$) and BMI ($p \leq 0.024$), in line with the fact that this type of cancer occurs more frequently in elderly (postmenopause) and overweight/obese women.

3.2. Clinic pathological characteristics

Clinic pathological characteristics of the cohort, 26 patients with cancer were confirmed with hematoxylin and eosin further confirmed by immunohistochemistry as 25 ductal infiltrating, 1 lobular infiltrating type of tumor histological grade I, added the type of injuries was 16 with benign fibrocystic breast disease, 20 fibroadenoma, adenosis microglandular 3 and others 4. The sample (10 mg) was collected, as mentioned in the methods, directly from the tumor, i.e., the observable injury by ultrasound.

3.3. Gene expression

Upon analyzing the normalized C_T values, there were statistically significant differences in the expression of JAK2 ($p = 0.003$), STAT3 ($p = 0.001$) and SOSCS3 ($p \leq 0.000$) between cancer cases and controls. However, these differences were lost when either of the two housekeeping genes, ACTB or GAPDH, was used for adjustment (Table 1). To analyze whether JAK2, STAT3 or SOCS3 demonstrated a relationship with BMI in cancer patients, we performed additional analyses according to obesity status. Statistically significant differences in weight, waist circumference and BMI were observed between the obese and non-obese individuals, as expected (Table 2) (Fig. 1).

Interestingly, in the group of patients with benign breast lesions, the level of SOCS3 expression between the normal-weight and obese group was significantly different ($p \leq 0.001$) (Table 3). Of particular concern is the fact that, some of these patients showed SOCS3 expression near to

Table 1
Anthropometric characteristics and gene expression levels.

Variable	Cancer (n = 26) mean ± SD	Controls (n = 43) mean ± SD	p
Age (years)	53.8 ± 11.5	40.6 ± 13.04	≤0.001
Weight (kg)	66.9 ± 12.02	62.37 ± 12.2	0.135
Height (m)	1.55 ± 0.06	1.57 ± 0.07	0.096
Waist circumference (cm)	91.8 ± 10.5	87.7 ± 12.2	0.185
BMI (kg/m^2)	27.8 ± 4	25.1 ± 4.9	0.024
JAK2[a]	3.81 ± 1.9	5.12 ± 5.15	0.757[c]
STAT3[a]	5.9 ± 5.31	3.55 ± 3.6	0.100[c]
SOCS3[a]	3.17 ± 2.56	3.71 ± 4.77	0.556[c]
JAK2[b]	2.64 ± 1.73	3.80 ± 2.8	0.147[c]
STAT3[b]	3.25 ± 2.1	2.87 ± 2.11	0.753
SOCS3[b]	2.38 ± 2.55	3.82 ± 5.21	0.850[c]

BMI: body mass index; JAK2: Janus kinase; STAT3: signal transducer and activator of transcription; SOCS3: suppressor of cytokine signaling 3.
[a] $2^{-\Delta\Delta CT}$ relative expression using ACTB housekeeping gene.
[b] $2^{-\Delta\Delta CT}$ relative expression using GAPDH housekeeping gene.
[c] Mann–Whitney *U* test.

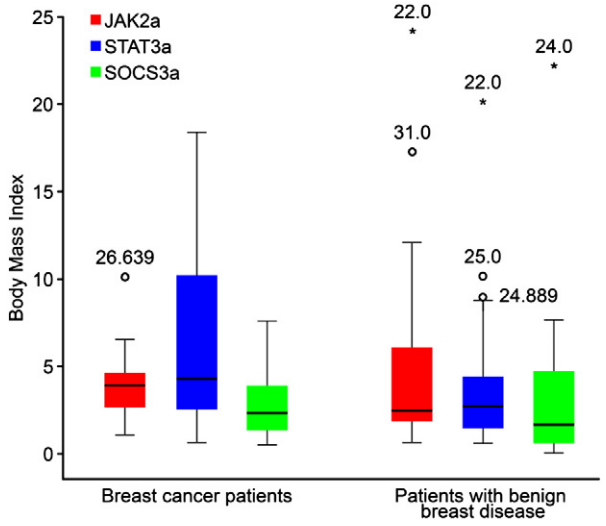

Fig. 1. JAK2, Janus kinase; STAT3, signal transducer and activator of transcription; SOCS3, suppressor of cytokine signaling 3.

Table 3
Gene expression levels classified by BMI in the control group.

Gene	Normal weight (n = 20) mean ± SD (median)	Overweight (n = 17) mean ± SD (median)	Obesity (n = 6) mean ± SD (median)	p[d]	p[e]
JAK2[a]	5.3 ± 5.7	4.5 ± 3.9	5.75 ± 6.5	0.687	0.762
STAT3[a]	4.2 ± 4.7	3.0 ± 2.4	2.6 ± 0.81	0.098	0.083
SOCS3[a]	3.5 ± 5.0	4.7 ± 5.2	2.0 ± 2.41	0.798[c]	0.506
JAK2[b]	4.06 ± 2.9	4.1 ± 3.08	2.33 ± 1.16	0.556	0.074
STAT3[b]	3.39 ± 2.55	2.2 ± 1.55	2.93 ± 1.47	0.037	0.123
SOCS3[b]	2.4 ± 2.8	2.7 ± 3.7	10.38 ± 8.7	0.284	0.001

BMI: body mass index; JAK2: Janus kinase; STAT3: signal transducer and activator of transcription; SOCS3: suppressor of cytokine signaling 3.
[a] $2^{-\Delta\Delta CT}$ relative expression using ACTB housekeeping gene.
[b] $2^{-\Delta\Delta CT}$ relative expression using GAPDH housekeeping gene.
[c] Mann–Whitney *U* test.
[d] Between normal weight and obesity.
[e] Between overweight and obesity.

the levels of the endogenous control, using normalized values by the $2^{-\Delta\Delta CT}$ method.

Table 4 shows the expression levels of JAK2, STAT3, and SOCS3, normalized for GAPDH and ACTB, in comparison to the clinical stage and immunohistochemistry results of the tumors. In some cancer patients, there was a wide range of variability; however, ACTB appeared to be expressed to a greater extent in this group. When analyzing the immunohistochemistry reports and the data for JAK2, STAT3 and SOCS3 expression in each clinical stage, there were significant differences between stages II and III ($p \leq 0.011$) and II and IV ($p \leq 0.033$) in STAT3 gene expression (Table 5).

According to the results of the ROC curve, the variable with the greatest AUC was STAT3 gene expression, which corresponded to postmenopausal women and obesity (Fig. 2).

Among all women, there was a positive correlation between JAK2 and STAT3 ($R = 0.493$, $p = 0.000$) and between JAK2 and SOCS3 ($R = 0.334$, $p = 0.015$). Among all women with breast cancer, the correlation between JAK2 and SOCS3 ($R = 0.555$, $p = 0.007$) was positive, and there were also significant positive correlations ($R = 0.593$, $p = 0.033$) between JAK2 and SOCS3 among overweight or normal-weight women. Furthermore, in the subgroup of women with breast cancer and obesity, the correlation between SOCS3 and STAT3 ($R = 0.920$, $p \leq 0.001$) was significant.

Regarding the patients with stage IV breast cancer, there was a positive correlation between STAT3 or SOCS3 (only one correlation coefficient and p-value are presented; thus, only one gene was compared) and the housekeeping genes ACTB ($R = 0.734$, $p = 0.060$) and GAPDH ($R = 0.809$, $p = 0.028$). In patients with positive ER status, the correlation between JAK2 and SOCS3 ($R = 0.537$, $p = 0.018$) was positive. For patients with positive PR status, the Pearson correlation between JAK2 and SOCS3 ($R = 0.644$, $p = 0.004$) was positive. Using the data from Table 4 for patients with negative HER2 expression, the correlation between JAK2 and SOCS3 ($R = 0.645$, $p = 0.003$) was positive.

Using the data from Table 1 the correlations shown here were obtained. The analysis was conducted in SPSS software using Pearson correlation. Among women with benign breast disease, the correlation between JAK2 and STAT3 ($R = 0.761$, $p = 0.000$) was positive. In the group with a normal weight and no cancer, a positive correlation was observed between JAK2 and STAT3 ($R = 0.920$, $p \leq 0.001$), and this was also found for the overweight group without cancer ($R = 0.654$, $p = 0.021$). In obese patients with benign breast lesions, there was

Table 2
Anthropometric characteristics and gene expression levels classified by BMI in women with breast cancer.

Variable	Normal weight (n = 4) mean ± SD (median)	Overweight (n = 16) mean ± SD (median)	Obesity (n = 6) mean ± SD (median)	p[c]	p[d]
Age (years)	58.75 ± 21.6 (53)	55.06 ± 9.1 (55)	49.7 ± 9.1 (49.5)	0.508	0.379
Weight (kg)	54.3 ± 5.96 (56)	63.9 ± 6.6 (66)	83.3 ± 11 (79.5)	0.012	0.001
Height (m)	1.5 ± 0.07 (1.58)	1.5 ± 0.06 (1.6)	1.57 ± 0.05 (1.56)	0.876	0.453
Waist circumference (cm)	81.4 ± 9.4 (84)	91.6 ± 5.8 (91.5)	103.5 ± 14.27 (101.5)	0.013	0.041
BMI (kg/m^2)	22.7 ± 0.48 (23)	26.8 ± 1.4 (27.1)	33.62 ± 3.1 (32.3)	≤0.001	≤0.001
JAK2[a]	3.95 ± 2.0	3.8 ± 2.3	4.15 ± 2.22	0.924	0.825
STAT3[a]	4.7 ± 4.3	6.54 ± 5.91	4.16 ± 1.9	0.574	0.971
SOCS3[a]	4.95 ± 4.74	4.7 ± 8.3	3.0 ± 1.71	0.863	0.402
JAK2[b]	2.6 ± 0.23	2.0 ± 1.34	4.15 ± 2.22	0.481	0.286
STAT3[b]	3.15 ± 1.83	2.82 ± 2.21	4.17 ± 1.9	0.789	0.426
SOCS3[b]	3.96 ± 3.97	2.46 ± 3.78	3.04 ± 1.7	0.154	0.625

BMI: body mass index; JAK2: Janus kinase; STAT3: signal transducer and activator of transcription; SOCS3: suppressor of cytokine signaling 3.
[a] $2^{-\Delta\Delta CT}$ relative expression using ACTB housekeeping gene.
[b] $2^{-\Delta\Delta CT}$ relative expression using GAPDH housekeeping gene.
[c] Between normal weight and obesity.
[d] Between overweight and obesity.

Table 4
Comparison of gene expression levels according to the clinical stage of the breast cancer tumor.

Classification	Patients with cancer N (%)	JAK2[a] Median (range)	STAT3[a] Median (range)	SOCS3[a] Median (range)	JAK2[b] Median (range)	STAT3[b] Median (range)	SOCS3[b] Median (range)
Clinical stage (n = 26)							
I	3 (11.53)	4.59 (1.7–4.2)	16.2 (0.58–18.4)	1.86 (1.05–3.88)	1.37 (1.14–1.58)	4.46 (0.34–7.05)	0.81 (0.62–0.94)
II	8 (30.77)	2.26 (1.65–4.94)	2.44 (0.62–2.89)	1.43 (0.52–4.47)	3.18 (1.18–7.63)	2.89 (0.39–4.18)	2.3 (0.47–9.71)
III	8 (30.77)	4.16 (1.11–4.67)	8.13 (1.8–15.42)	2.76 (0.60–11.49)	1.97 (0.55–2.75)	5.22 (0.94–6.66)	1.96 (0.30–9.7)
IV	7 (26.92)	3.62 (1.91–10.1)	4.72 (0.84–11.0)	3.53 (1.28–7.6)	1.85 (0.56–5.58)	2.43 (0.67–5.57)	1.0 (0.94–3.49)
Inmunohistochemistry (n = 26)							
ER +	23 (88.46)	3.7 (0.57–1.11)	3.22 (0.58–18.4)	2.34 (0.52–11.49)	2.4 (0.55–7.63)	3.55 (0.34–7.05)	1.83 (0.30–9.7)
ER–	3 (11.54)	3.6 (3.5–5.54)	4.72 (2.53–11.0)	3.53 (1.87–5.40)	1.85 (0.96–2.8)	2.43 (0.67–5.57)	0.97 (0.94–2.73)
PR +	22 (84.6)	3.7 (1.1–10.1)	3.22 (0.58–18.4)	2.34 (0.52–11.5)	2.4 (0.55–7.6)	3.55 (0.34–7.0)	1.83 (0.3–9.7)
PR–	3 (11.54)	3.6 (1.9–3.7)	4.7 (2.5–11.02)	3.53 (1.87–5.4)	1.85 (0.96–2.80)	2.43 (0.67–5.57)	0.97 (0.94–2.73)
HER–2 positive	3 (11.54)	4.2 (3.6–4.4)	8.6 (4.7–10.2)	3.1 (1.87–7.6)	1.85 (0.56–2.75)	2.4 (1.38–5.36)	1.0 (0.97–1.95)
Triple negative	2 (7.69)	2.86 (1.97–3.74)	4.57 (3.61–5.54)	6.78 (2.53–11.02)	1.9 (0.97–2.8)	3.1 (0.67–5.57)	1.8 (0.94–2.73)
ER + EP +	23 (79.3)	3.2 (1.1–10.12)	2.9 (0.58–18.4)	2.1 (0.52–11.49)	2.5 (0.55–7.63)	3.6 (0.34.7.05)	1.95 (0.30–9.7)
ER − EP −	3 (11.54)	3.6 (1.93–3.74)	4.71 (2.53–11)	3.5 (1.87–5.4)	1.85 (0.96–2.8)	2.43 (0.67–5.57)	0.96 (0.94–2.7)

ER: estrogen receptor; HER2: human epidermal growth factor receptor 2; JAK2: Janus kinase; PR: progesterone receptor; SOCS3: suppressor of cytokine signaling 3, STAT3: signal transducer and activator of transcription.
[a] $2^{-\Delta\Delta CT}$ relative expression using ACTB housekeeping gene.
[b] $2^{-\Delta\Delta CT}$ relative expression using GAPDH housekeeping gene.

also a positive correlation between JAK2 and SOCS3 (R = 0.984, p = 0.002).

4. Discussion

Cancer is now recognized as a disease in which abnormalities in the genome and epigenome accumulate, enabling cells to escape from normal regulatory controls as a result of exposure to endogenous and exogenous damaging agents (Korkola and Gray, 2010). Alterations to the JAK/STAT pathway are particularly recognized in breast cancer (Baudot et al., 2010).

In addition to being conventionally activated by JAKs in response to cytokine signaling, STAT3 is also a target of oncogenic tyrosine kinases such as Brk (Gao et al., 2012). In this study, STAT3 gene expression was increased in breast cancer patients, suggesting a mechanism for transcription of this gene in cancer, this was only observed using non-normalized C_T values. Although this mechanism is beyond the scope of the current study, some hypotheses have been proposed (Wang et al., 2012). Although, the role of STAT3 in breast cancer has yet to be properly delineated, our data suggest correlation to breast cancer development, being clear from this study, the necessity to keep in mind the possible role of STAT3 as predisposing factor in this neoplasia. Chen et al. recently showed that STAT3 could serve as a biomarker of poor survival due to its potential association with lymph node metastasis (Chen et al., 2013), and another study found that overexpression of the pro-metastatic chromatin modifier protein MTA1 promotes STAT3

Table 5
Associations between gene expression levels and clinicopathological characteristics breast cancer patients.

Classification	Breast cancer patients N (%)	JAK2[a] p	STAT3[a] p	SOCS3[a] p	JAK2[b] p	STAT3[b] p	SOCS3[b] p
Clinical stage (n = 26)							
II	8 (30.77)	0.418	0.011	0.215	0.054	0.147	0.576
III	8 (30.77)						
II	8 (30.77)	0.192	0.033	0.051	0.174	0.777	0.265
IV	7 (26.92)						
Inmunohistochemistry (n = 26)							
ER+	23 (88.46)	0.686	0.975	0.766	0.423	0.759	0.556
ER−	3 (11.54)						
PR+	22 (84.6)	0.641	0.654	0.229	0.171	0.456	0.420
PR−	3 (11.54)						
HER-2 positive	3 (11.54)	0.808	0.529	0.465	0.337	0.867	0.452
HER-2 negative	23 (88.46)						
ER + PR+	23 (79.3)	0.680	0.928	0.647	0.364	0.712	0.535
ER − PR−	3 (11.54)						

ER: estrogen receptor; HER2: human epidermal growth factor receptor 2; JAK2: Janus kinase; PR: progesterone receptor; SOCS3: suppressor of cytokine signaling 3, STAT3: signal transducer and activator of transcription.
[a] $2^{-\Delta\Delta CT}$ relative expression using ACTB housekeeping gene.
[b] $2^{-\Delta\Delta CT}$ relative expression using GAPDH housekeeping gene.

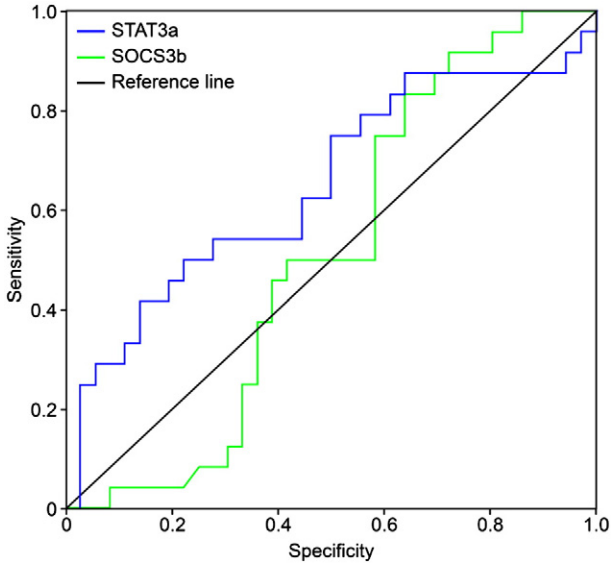

Fig. 2. ROC analyses in cases and controls of STAT3 (AUC, 0.65 (95% CI, 0.5–0.8)) and SOCS3 (AUC, 0.51 (95% CI, 0.36–0.65)). STAT3, signal transducer and activator of transcription; SOCS3, suppressor of cytokine signaling 3; ROC, receiver–operator characteristic.

transcription and pulmonary metastasis in breast cancer (Pakala et al., 2013). Contrary to this finding, another study supported the role of phospho-STAT3 as a marker of positive outcome in breast cancer patients treated with adjuvant chemotherapy (Sonnenblick et al., 2013). Whether SOCS3 could be useful as a biomarker is still a matter of debate (Ferreira and Cronjé, 2012; Lyng et al., 2008); but probably its decrement might open the door to neoplastic pathways, in support of this notion is the finding that the highest levels were in control cases with obesity, as shown in Fig. 2, whereas in the cancer group, there were no significant differences between SOCS3 expression and either of the analyzed reference genes (Ferreira and Cronjé, 2012; Lyng et al., 2008); this finding is similar to that reported by Nakagawa in 2008 (Ferreira and Cronjé, 2012; Lyng et al., 2008; Nakagawa et al., 2008), who detected decreased SOCS3 expression in patients with metastatic breast cancer. Moreover, the activation of STAT3/SOCS3 has been associated with the development of other cancers, such as pancreatic cancer, through the activity of IL-6 (Barclay et al., 2009; Lesina et al., 2011).

According to Palianopoulou in 2011, the effect of leptin on the antiapoptotic gene survivin is limited by the inhibitory role of SOCS3 in the leptin-activated JAK2/STAT3 signaling pathway in MCF-7 breast cancer cells. In our study, patients with benign breast lesions and obesity was correlated with the expression of JAK2 and SOCS3, which was similar to the result reported by Palianopoulou, and these levels were most likely elevated by leptin (Palianopoulou et al., 2011).

The above results indicate that the increased expression of SOCS3 may be directly associated with the regulation of breast cancer development when patients are positive for the ER (Matthews et al., 2005) and PR and negative for HER2. Until now, there is scarce information explaining the role of SOCS3 in relation to the hormone receptors (progesterone and estrogen). More studies to afford this question are mandatory. GAPDH is a commonly used housekeeping gene for gene expression comparisons in biopsies of patients with breast lesions, although this gene is also involved in metabolic pathways (Schulze and Harris, 2012). ACTB is not as valuable for this purpose because its gene variation can be associated with breast cancer cell proliferation. Thus, GAPDH has been used as a control gene in numerous studies and has shown satisfactory results (Kastl et al., 2010). In contrast, we failed to observe such consistency, and some patients had to be excluded from the study because JAK2, STAT3 and SOCS3 were amplified earlier than the housekeeping gene. GAPDH is typically the preferred choice as an internal control for gene expression studies because it demonstrates a reduced potential for the formation of isoforms and pseudo-gene cross-reactivity. However, in our study, optimal control gene expression was observed for ACTB.

SOCS3 can inhibit the tyrosine-kinase activity of JAK proteins through their kinase-inhibitory region, which binds to the surface of the kinase domain and induces a conformational change to inhibit the transfer of phosphates from ATP to the substrate peptide. In this study, patients in the control group with obesity showed higher expression of SOCS3 regarding overweight $p = 0.001$ (Sasi et al., 2010; Yoshimura, 2013).

In addition, the function of JAK2, STAT3 and SOCS3 in breast neoplastic cells indicates that these genes are potent therapeutic targets, and future clinical approaches utilizing these genes may result in better patient prognoses (Behera et al., 2010; Garofalo and Surmacz, 2006; Palianopoulou et al., 2011). Together, these results highlight the important role of mutations leading to JAK2 activation in neoplastic cells via the STAT3 pathway. To our knowledge, this is the first clinical study to report JAK2 expression in mammary gland biopsies.

Several authors have shown the activity of the STAT3 protein in breast cancer biopsies using techniques such as immunohistochemistry. Furthermore, nuclear Phospho-Stat3 (Tyr705) is independent of all other commonly used prognostic markers and pathological parameters, except for tumor size (Dolled-Filhart et al., 2003). On the other hand, Sato et al. indicates that STAT3 is frequently activated in breast cancer, suggesting, based on multiple lines of evidence, that that Stat3 promotes tumor progression (Sato et al., 2011). It is important to note that more members of the STAT family have been evaluated in breast cancer (Yamashita et al., 2006).

In conclusion, the current study revealed an interaction between the JAK2 and STAT3 signaling pathways in obese breast cancer patients. However, other signaling pathways should be analyzed, such as phosphoinositide 3-kinase (PI3K/Akt), mitogen-activated protein kinase (MAPK) and the mammalian target of rapamycin (mTOR) that may also be active (Chen, 2011). Moreover, SOCS3 expression and its role in breast cancer development may be a result of obesity due to increased cytokine expression, although whether this is protective or an indicator of good prognosis is beyond the scope of this study.

Conflict of interest statement

The authors declare no conflict of interest.

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