

Quantification of Steroid Receptors Gene Expression in Breast Cancer Patients: Possible Correlation with Serum Level of Adipocytokines

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Received September 1st, 2011; revised October 9th, 2011; accepted November 20th, 2011.

ABSTRACT

Background: While the role of adipocyte-derived hormones in breast cancer pathogenesis well studied, it is speculated that there is a crosstalk between adipocytokines and Esterogen receptor (ER) signaling. To test this hypothesis we evaluate the possible correlation between serum levels of adipocytokines with steroid hormone receptors gene expression in breast cancer patients. **Methods:** In this case-control study, 70 women with breast cancer participated with different grades of obesity (36 none obese, BMI < 25 kg/m² and 34 obese, BMI ≥ 25 kg/m²). Serum level of adiponectin, leptin, TNF α and IL6 determined by ELISA. Following quantitative expression of androgen receptor (AR), progesterone receptor (PR) and estrogen receptors (ER α and ER β) mRNA in tumor tissues evaluated by Real-time PCR. **Results:** We find a significant reverse correlation between serum level of Adiponectin and ER α and PR mRNA ($r = -0.229$, $n = 64$, $p = 0.035$ and $r = -0.220$, $n = 64$, $p = 0.041$) but no correlation was between adiponectin and, ER β and AR in samples ($P > 0.05$). In case of leptin, we observed a positive correlation between leptin and ER α and PR mRNA. ($r = 0.553$, $n = 64$, $p < 0.001$, $r = 0.359$, $n = 64$, $p = 0.002$ and a reverse significant correlation between leptin and ER β ($r = -0.506$, $n = 64$ and $p < 0.001$). We also observed a significant correlation between TNF α and IL6 with steroid receptors gene expression in samples ($P < 0.05$). **Conclusion:** As the presence of steroid receptors are used as the most common prognostic markers in breast cancer patients, so targeting adipocytokines as potential regulators of steroid receptors gene expression can be a new approach in treatment of obese breast cancer subjects.

Keywords: Obesity, Breast Cancer, Steroid Receptors, Adipocytokines

1. Introduction

Regarding that Obesity is a well-known risk factor for breast cancer [1], obese women are likely to have poor prognosis and metastatic breast cancer [2] when they are first diagnosed regardless of their menopausal status [3]. Although the exact mechanism remains to be determined [4], the hormonal changes associated with obesity are considered to be responsible for this relationship [5],

with particular emphasis being placed on the increased production of estrogen [4]. Estrogens act on target cells through two receptors, esterogen receptor α (ER α) and esterogen receptor (ER β) [6].The actions of ER α and ER β on gene transcription can be opposite; depending on cell context .Studies demonstrated that ER β is not necessary for proliferation and that ER β opposes the proliferative effects exerted by ER α [7].The ratio of ER α to ER β is a prognostic marker in breast tumors [8], such that

ER β expression is indicative of more benign tumors [9], whereas ER α indicates malignant, aggressive tumors [10]. Beside ER gene expression, the role of other steroid hormones such as Progesterone (PR) [11] and androgen Receptors (AR) in breast cancer development well studied [12]. Biochemical and immunohistochemical studies show that AR+ tumors are more frequent (70% - 90%) than ER α + and PR+ tumors (60% - 80% and 50% - 70%, respectively). Although ER α , PR and AR are frequently co-expressed in breast tumors approximately 10% of AR+ tumors and, perhaps more importantly, 25% of AR+ tumors metastases can be negative for ER α and PR [13]. Despite the well-recognized relationship between obesity and estrogenic activity, it is evident that this cannot fully explain the association between adiposity and breast cancer risk [14]. Indeed, the adipose tissue is increasingly viewed as not only a fat-storing tissue but also an endocrine organ secreting various adipocytokines [15]. Subsequently, both experimental and epidemiological data suggest the possible role of adipocytokines in the regulation of carcinogenesis as another link between obesity and breast cancer [1]. Leptin is a adipocyte derived hormone and obesity is associated with high circulating levels of leptin [16]. *In vitro*, leptin stimulates proliferation of multiple types of preneoplastic and neoplastic cells [17]. Leptin also functions as an inflammatory cytokine and appears to influence immune function, possibly by triggering release of interleukin-6 and other cytokines [16]. Adiponectin is an anti-inflammatory protein that is produced almost exclusively by mature adipocytes [18]. Plasma adiponectin concentrations are inversely correlated with the BMI [19]. A correlation between adiponectin and angiogenic adipokines suggests that adiponectin may be an effective suppressor of pathological neovascularization [16]. Many studies have shown that the clinical effects and biological actions of adiponectin are opposed of leptin [20]. Studies confirm a significant inverse correlation between serum adiponectin levels and poor-prognosis breast cancer [21]. The role of other adipocytokines such as tumor necrosis factor (TNF α) and interleukin-6 (IL6) is well recognized [22] and like as leptin, increased secretion of these adipocyte-derived hormones involve in breast cancer pathogenesis [23]. It is notable that these three adipocytokines can stimulate estrogen biosynthesis by the induction of aromatase activity [16]. As ER α is known to mediate tumor-promoting effects of 17- β estradiol [18], data suggest that adiponectin-triggered increase of ER β isoform expression might be one important molecular mechanism underlying the protective effects of this adipocytokine [24].

Also the relation between obesity and adipocytokines well studied but there is no information about the possi-

ble effect of steroid hormones on secretion of these adipocytokines in obese and none obese breast cancer subjects. So the aim of this study was to test the hypothesis that there is a correlation between steroid hormone receptors gene expression and serum level of ER regulated adipose tissue derived hormones in breast cancer patients with different grades of obesity.

2. Materials and Methods

2.1. Patients and Samples (Tissue and Serum)

This study was a descriptive—cross sectional study. All samples were obtained from 70 women who underwent biopsy or mastectomy surgical operation at Tabriz Emam Reza Hospital from July 2009 till May 2010. The whole project has been done in Tabriz Drug Applied Research Center. The samples were examined histologically for the presence of tumor cells by a Pathologist. The patients met the following criteria: primary unilateral non metastatic breast carcinoma for which complete clinical, histological and biological data were available; and no radiotherapy or chemotherapy before surgery.

2.2. Adipocytocine Analysis (Plasma Leptin, Adiponectin, TNF α and IL6 Levels)

Plasma concentrations of leptin and adiponectin were determined with the use of commercially available quantitative sandwich enzyme-linked immunosorbent assay (ELISA) kits (The mediagnost Leptin-ELISA E07 and Adiponectin-ELISA E09, Germany). The sensitivity of this assay was 0.2 ng/ml for leptin and <0.6 ng/ml for adiponectin. Masked split specimens included within each batch were used to calculate the coefficient of variation (CV) within and between batches: the intra- and inter-assay CVs of leptin were below 10% for both and below 6.7% and 4.7% for adiponectin, respectively. The BMS223/4 and BMS223/4TEN human TNF- α ELISA and the BMS213/2 and BMS213/2TEN human IL-6 ELISA have prepared by Benser MedSystems is an enzyme-linked immunosorbent assay for the quantitative detection of human TNF- α and IL-6, respectively. The sensitivity of this assay was 2.3 pg/ml for TNF- α and 0.92 pg/ml for IL-6, respectively. Masked split specimens included within each batch were used to calculate the coefficient of variation (CV) within and between batches: the intra- and inter-assay CVs of TNF- α were below 3.2% and 10.3%, and for IL-6 were 7.8% and 2.6%, respectively.

2.3. Preparation of Total RNA

Approximately 100 mg of tissue for RNA extraction by means of RNAX reagents previously described [25]. The quality of RNA samples was ensured by electrophoresis in an agarose gel followed by ethidium bromide staining,

where the 18 S and 28 S RNA bands could be visualized under UV light. Quantification of RNA was performed in duplicate by spectrophotometry at 260 nm.

2.4. cDNA Synthesis

RNA was converted to cDNA after treating with DNase I. Reverse transcription of RNA was done in a final volume of 20 µl by using of cDNA first strand synthesis kit (Fermentase) random hexamere and 1 µg of total RNA. The samples were incubated at 65°C for 10 min and 42°C for 60 min, and reverse transcriptase was inactivated by heating at 70°C for 5 min and cooling at 4°C for 5 min.

2.5. Real-Time RT- PCR

Principle: Reactions are characterized by detection of cycling amplification of the PCR product, rather than the amount of PCR product accumulated after a fixed number of cycles. If the amount of the target molecule was larger, the earlier a significant increase in fluorescence is observed. The parameter *C_t* (threshold cycle) is defined as the cycle number at which the fluorescence generated by cleavage of the probe passes a fixed threshold above baseline. The $\Delta\Delta C_T$ method used for determination of relative ERs gene expression, The *C_t* of each target gene compared to the *C_t* of its internal control (beta actin gene). Final results, expressed as N-fold differences in tested gene expression (obese) relative to control gene (none obese), termed “N fold changes” were determined as follows:

$$N \text{ fold changes} = 2^{(\Delta C_{\text{test}} - \Delta C_{\text{control}})}$$

where ΔC_t values of the sample and control are determined by subtracting the average *C_t* value of the target gene from the average *C_t* value of the beta actin gene. The qRT-PCR assay of steroid receptors in details have been described in our previous work [25]. Primer sequences for qRT-PCR summarized in **Table 1**.

2.6. Statistical Analysis

Differences between obese and non-obese groups in age at enrollment, age at menarche, age at menopause were tested using the chi-square test. In addition, Student’s t-test

was used to evaluate differences in categorical breast cancer risk factors between obese and non-obese. Pearson correlation coefficients were used to examine cross-sectional relationships between steroid hormone receptors expression and adipocytokines among subjects. All statistical analysis was conducted using SPSS statistical software (version 16). P values below the conventional level of statistical significance ($P < 0.05$) were considered statistically significant.

3. Results

In this case-control study, 70 women with breast cancer participated with different grades of obesity (36 none obese, BMI < 25 kg/m² and 34 obese, BMI ≥ 25 kg/m²). The mean age of Participants was 44.53 ± 1.79 yr (ranged from 21 - 70 years old). Patients with previous history of radiotherapy or chemotherapy Excluded from this study. The baseline characteristics are summarized in **Table 2**.

3.1. Serum Level of Adipocytokines in Blood Samples

We analyzed serum level of leptin, adiponectin, TNF α and IL6 in 70 breast cancer subjects. The mean serum level of adipocytokines summarized in **Table 3**. The mean serum level of leptin, TNF α and IL6 were higher in obese cases ($P = 0.04$ and $P = 0.00$ and $P = 0.04$ respectively) while in contrast mean serum level of adiponectin was lower in obese ones ($P = 0.011$). As a result, there was a significant positive correlation between leptin and TNF α and IL6 with BMI ($r = 0.619$, $n = 64$, $P < 0.001$ and $r = 0.728$, $n = 64$, $P = 0.001$ and $r = 0.413$, $n = 64$ and $P < 0.001$) and also there was a negative reverse correlation between adiponectin and BMI in breast cancer cases ($r = -0.27$, $n = 64$, $P = 0.030$). Spearman correlation show that there was a significant negative correlation between serum level of adiponectin and leptin ($r = -0.29$, $n = 64$, $P = 0.017$). We find no correlation between serum level of adiponectin and other two adipocytokines ($P > 0.05$) but we observed a positive significant correlation between leptin and TNF α and IL6 serum level ($r = 0.506$, $n = 64$, $P < 0.001$ and $r = 0.49$, $p < 0.001$ respectively).

Table 1. Primer sequences for RT-real Time PCR.

<i>mRNA</i>	<i>Primer sequence</i>	<i>Product size</i>	<i>source</i>
ERα	TGA TGA AAG GTG GGA TAC GA AAG GTT GGC AGC TCT CAT GT	125 bp	Pfeiler <i>et al.</i> , 2007 [18]
ERβ	GCT TAG TGG AGC TCA GCC TG AGGATCATGGCCTTGACACAGA	262 bp	Pffafel <i>et al.</i> , 2001 [30]
PR	GAACCAGATGTGATCTATGCAGGA CGAAAACCTGGCAATGATTTAGAC	122 bp	Cremoux <i>et al.</i> , 2002 [11]
AR	CCTGGCTTCCGCAACTTACAC GGACTTGTGCATGCGGTACTCA	168 bp	Bieche <i>et al.</i> , 2001 [31]
Beta actin	TCCTGGAGAAGAGCTACG GTAGTTTCGTGGATGCCACA	134 bp	Genebank accession: NM_0011101

Table 2. Characteristics of obese and non-obese breast cancer patient.

Baseline characteristics	None obese (n = 36) Mean ± SE	Obese (n = 34) Mean ± SE	P value
Age at enrollment (yrs)	43.82 ± 1.78	45.42 ± 2.27	0.45
BMI (kg/m ²)	23.23 ± 0.23	29.80 ± 0.63	0.000
Family history of Breast cancer	No. (%)	No. (%)	
Yes	18 (54.5)	6 (22.2)	
No	15 (45.5)	21 (77.8)	0.01
Family history of obesity	No. (%)	No. (%)	
Yes	23 (69.7)	22 (81.5)	
No	10 (30.3)	5 (18.5)	0.22
Menopause status			
Premenopausal	21 (63.6)	12 (46.2)	
Postmenopausal	12 (36.4)	14 (53.8)	0.14
Age at first live birth (yr)			
17>	12 (37.5)	12 (48.0)	
18-23	11 (34.4)	10 (40)	0.32
24<	9 (28.1)	3 (12)	
Age at menarche(yr)			
12>	10 (30.3)	6 (23.1)	
13	5 (15.2)	10 (38.5)	0.12
14<	18 (54.5)	10 (38.5)	

Table 3. Serum level of adipocytokines in obese and none obese breast cancer patients.

Adipocytokine	None Obese (n = 34) Mean ± SE	Obese (n = 36) Mean ± SE	p
Adiponectin(ng/ml)	10.61 ± 0.66	8.33 ± 0.48	0.011
Leptin(ng/ml)	9.57 ± 1.08	31.68 ± 4.49	0.004
TNF α (pg/ml)	3.53 ± 0.12	8.44 ± 2.21	0.000
IL6(pg/ml)	3.72 ± 0.28	7.17 ± 0.62	0.004

3.2. Correlation between Steroid Receptors and Serum Level of Adipocytokines in Breast Cancer Patients

One tailed spearman coefficient test show that there was a significant reverse correlation between serum level of Adiponectin and ER α and PR mRNA ($r = -0.229$, $n = 64$, $P = 0.035$ and $r = -0.220$, $n = 64$, $P = 0.041$) but no correlation was between adiponectin and, ER β and AR in

samples ($P > 0.05$). In case of leptin, we observed a positive correlation between leptin and ER α and PR mRNA. ($r = 0.553$, $n = 64$, $P < 0.001$, $r = 0.359$, $n = 64$, $P = 0.002$ and a reverse significant correlation between leptin and ER β ($r = -0.506$, $n = 64$ and $P < 0.001$). In case of TNF α , we observed a positive correlation between TNF α and ER α and PR mRNA. ($r = 0.497$, $n = 64$, $P < 0.001$, $r = 0.361$, $n = 64$, $P = 0.002$) and a reverse significant correlation between TNF α and ER β ($r = -0.483$, $n = 64$ and

$P < 0.001$). There was a positive correlation between IL6 and ER α and PR mRNA. ($r = 0.371$, $n = 64$, $P = 0.001$, $r = 0.227$, $n = 64$, $P = 0.036$ and a reverse significant correlation between IL6 and ER β ($r = -0.503$, $n = 64$ and $P < 0.001$). We find no correlation between AR mRNA and none of adipocytokine (see **Table 4**).

4. Discussion

Obesity has been associated with increased mortality from cancers of the colon, breast (in postmenopausal women) [5], endometrium, kidneys (renal cell), esophagus (adenocarcinoma), gastric cardia, pancreas, prostate, gallbladder, and liver [19]. The mechanisms and pathways involved in obesity-related carcinogenicity are multifaceted and difficult to extricate [1]. For example, increased energy intake, decreased energy output and increased adipose tissue mass could be causes of cancer [26]. Increased adipose tissue as a potential source of metabolic and endocrine modulators of oncogenic cascades will be cause cancer [3]. Studies have shown that the common denominators between obesity and cancer include Adipocyte derived hormones (leptin ,resistin, adiponectin, TNF α , IL-6, ...)neurochemicals; hormones such as insulin like growth factor 1 (IGF-1), insulin, leptin; sex steroids such as estrogen; adiposity; insulin resistance; and inflammation [19]. Like many other researcher our study indicate for the undeniable effects of adipocytokines in breast cancer [27], in our study there was a negative correlation between leptin, TNF α and IL6 and a reverse correlation between adiponectin and BMI as predicted. Also the connection between obesity and Adipocytokines well studied [1]; there is evidence show that secretion of some of these adipocytokines can affect pattern of estrogen receptors status [28]. Trecek *et al.* postulated that

there is a crosstalk between adiponectin and ER signaling. In their study In MCF-10A cells, adiponectin triggered a significant increase of ER β 2 and ER β 5 mRNA levels [24]. ER β is the dominant estrogen receptor in normal breast tissue [10], but its expression declines during tumorigenesis, but at the other hand expression of ER α that can exert mitogenic effects—increases during tumorigenesis [29]. ER β has anti mitogeneic activites and that regulate expression of ER α expression [6]. In our study we investigated only the total ER β mRNA, while far now at least five isoforms for this esterogen receptor have been described and that the most anti mitigenic activites that can inhibit ER α expression through hetrodimerization can attributed to ER β 2 isoforms [10]. Maybe it's a reason that why our finding not confirmed findings of what treck and his colleagues that there may be a possible synergic crosstalk between adiponectin and some ER β isoforms. Also in our study we find no correlation between serum level of ER β mRNA and adiponectin but those that have lower adiponectin exhibit a high expression of ER α and PR. Moreover we find a negative correlation between serum level of leptin and ER β . In case of two other bad adipocytokines like as TNF α and IL6—the results was like as leptin. These three bad adipocyte derived hormones that their secretion increases during mammary tumorigenesis specially in obese subjects was in a close positive correlation with expression of bad steroid hormones (ER α and PR) but in a reverse correlation with expression of ER β that act as a tumor suppressor gene and in this case is comparable with adiponectin. While ER β and adiponectin both have similar effects, they both exert anti tumor effects and decreased production of both is in relation with poor prognosis in breast cancer.

Table 4. Correlation between steroid hormone receptors mRNA and adipocytokines level.

Adipocytokine	Steroid hormone				
		ER α	ER β	PR	AR
Adiponectin (ng/ml)	r_s	-0.229	0.072	-0.220	-0.084
	P	0.035	0.285	0.041	0.256
Leptin (ng/ml)	r_s	0.553	-0.506	0.359	0.023
	P	0.000	0.000	0.002	0.428
TNF α (pg/ml)	r_s	0.497	-0.483	0.361	0.107
	P	0.000	0.000	0.002	0.200
IL6 (pg/ml)	r_s	0.371	-0.503	0.227	0.188
	P	0.001	0.000	0.036	0.068
(n = Number of cases)		64	64	64	64

5. Conclusions

In brief, as the presence of steroid receptors are used as the most common prognostic value in breast cancer patients and adipocytokines can play a significant role in steroid hormone receptors gene expression so targeting and controlling the serum level of these adipocyte—derived hormones with emphasizing on losing weight can improve ER positiveness in obese subjects, while losing weight can decrease serum level of bad adipocytokines such as leptin and at the other hand increases the serum level of good adipocytokines such as adiponectin and this in turn may lead to achieving better prognosis and better response to endocrine therapy in obese breast cancer patients .

6. Acknowledgements

This work financially supported by Iranian National Science Foundation (INSF) with the ref code of 88000604 (April2/2010). We appreciate all individuals who participated in this study.

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