Breast Cancer: Basic and Clinical Research

ORIGINAL RESEARCH

Plasma Leptin, hTERT Gene Expression, and Anthropometric Measures in Obese and Non-Obese Women with Breast Cancer

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Abstract:

Introduction: Expression of human telomerase reverse transcriptase (hTERT) occurs in most cancers but its relation with obesity is unclear. This study explores the association between leptin levels and anthropometric indices with hTERT mRNA levels in breast cancer patients of different obesity grades.

Materials and methods: In this case-control study, 65 breast cancer patients participated. Expression of tissues hTERT mRNA was carried out by real-time reverse transcription polymerase chain reaction. Leptin concentrations were measured by enzyme-linked immunoassay.

Results: Twelve patients (18.46%) were hTERT negative and 53(81.54%) were positive. hTERT mRNA levels were associated with BMI but not with waist circumference (WC) ($r = 0.219$, $P = 0.22$) and waist to hip ratio (WHR) ($r = 0.212$, $P = 0.237$). Leptin level and hTERT mRNA levels ($r = 0.484$, $P = 0.008$) were correlated as well as BMI and hTERT expression.

Conclusions: This study has shown a correlation between leptin levels and hTERT expression. These findings may clarify the role of leptin in breast carcinogenesis, and hence obesity could be responsible for increased incidences in breast cancer as well as its progression via enhanced production of leptin.

Keywords: breast cancer, obesity, adipokienes

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Introduction

Breast cancer is the most common cancer for women with more than one million new cases detected each year.[1–3] Accumulating data suggest that lifestyle is implicated in the development of breast cancer.[4] In women with invasive breast cancer, a direct relation between high BMI (>25) and large tumor size and axillaries lymph node involvement (ALNI) has been reported.[5] It has also been shown that obesity in postmenopausal women is considered a risk factor for the progression of breast cancer.[6] Obesity is also associated with an increased mortality rate in women diagnosed with breast cancer.[7,8]

The exact molecular link(s) between obesity and breast cancer are not fully understood. However, adipokines are considered responsible for this connection. Leptin is an adipocyte derived hormone and obesity is associated with high circulating plasma levels of leptin.[9] It has been shown that leptin stimulates proliferation of various types of pre-neoplastic and neoplastic cells.[10] Leptin also acts as an inflammatory cytokine and by triggering interleukin-6 and other cytokines release, influences immune system function.[10]

Numerous studies have demonstrated that telomerase is activated in the vast majority of cancer types, including breast cancers. Telomerase activity has been detected in 80%–95% of breast tumor samples by the Telomeric Repeat Amplification Protocol assay,[11–13] and its up-regulation is observed as an early event during progression to malignancy of breast tumor.[14] Some other studies have shown conflicting results for traditional prognostic indicators, disease outcome, and telomerase activity with clinical and pathological parameters.[15–17] Zarghami et al have demonstrated that the difference in relative telomerase activity (RTA) between stages and grades is strong and statistically significant.[18] This suggests that telomerase activity may be influenced by parameters other than clinical/pathological features alone, such as telomerase inhibitors, tissue viability, and obesity status. It has been shown that enhanced expression of human telomerase reverse transcriptase (hTERT), the catalytic subunit of telomerase, is a direct determinant of telomerase activity in cancers.[19] Nonetheless, the relationship between telomerase and obesity in breast cancer is unclear.

Therefore, the objectives of this study were to evaluate hTERT mRNA expression in human breast cancer patients with different grades of obesity and to assess the relationship between mRNA levels of hTERT and clinicopathological variables, anthropometric indices and leptin as an adipocytokine.

Methods

Chemical compounds

Enzymes, molecular biology reagents, kits, and chemical reagents were obtained from the following sources: Tris-HCl, $MgCl_2$, (Fisher Scientific, Pittsburg, PA); RNase inhibitors (Promega, Madison, WI); PCR buffer, Taq HotStar polymerase (Qiagen, Hilden, Germany), RNX plus reagent (Cinnagene, Iran), DNase I RNase-free (Fermentas, USA), RevertAid™ First cDNA synthesis kit (Fermentas, USA), chloroform, ethanol, and isopropanol (Merck, Germany), Syber Green I master mix (Fermentas, USA).

Subject

This case–control study was conducted from September 2009 to March 2010. The study involved 65 women diagnosed with breast cancer attending the Department of Surgery and Health examination clinics at Imam Reza Hospital, Tabriz, Iran. Patients confirmed in term of histopathological stages and grades were included in the study. Histopathological information revealed that there were 46 cases of invasive ductal carcinoma, 7 cases of invasive lobular carcinoma, and 12 cases of carcinoma in situ.

Body mass index (BMI)

According to the WHO Report 2000,[20] BMI was calculated as kg/m^2 using information from clinical notes at time of diagnosis. The subjects were divided into four categories (underweight with BMI $< 18.5\ kg/m^2$, normal weight with 18.5–24.99 kg/m^2, overweight with BMI 24.9–29.99 kg/m^2 and obese with BMI $> 29.99\ kg/m^2$).

Questionnaire collection

Written informed consent was obtained from all subjects after confirmation of their inclusion in the study. All participants filled out a questionnaire covering information on social demographic characteristics, menstrual and reproductive history, body mass index, fat distribution, age, menopausal status, lifestyle behaviours, education status and medical history. Data collectors were unaware of the study hypothesis. Data on height, weight, and circumferences of the

waist and hip by using standardized techniques were collected. BMI, subject's weight in kilograms divided by the square of their height in meters, was used as an indicator of generalized obesity. In addition, waist circumference (WC) and waist-to-hip ratio (WHR) represent an anthropometric measure of central adiposity. The influence of treatment on measurements was reduced by getting questionnaire data prior to surgery.

Sample preparation

Immediately after the interview, a 10 ml blood sample was drawn into coded EDTA-treated tubes. The sample was centrifuged at 3000 rpm for 10 minutes at room temperature within 1 hour of collection. Plasma, Buffy coat and red blood cells were separated and stored at −70 °C until subsequent analysis. In addition, during surgery tumor tissue samples were taken into liquid nitrogen in sterile tubes, then immediately aliquoted and stored at −70 °C until subsequent analysis. To avoid the influence of treatment on measurements, questionnaire data, blood specimens and tumor tissue samples were obtained prior to initiation of breast cancer surgery.

Preparation of total RNA

Approximately 100 mg of tissue was quickly frozen in liquid nitrogen. The sample was transferred to the laboratory and was manually pulverized with a hammer to a fine powder. The powder was collected and suspended in 1 ml of RNX plus reagent in a clean RNase-free tube and was incubated for 5 minutes at room temperature. After incubation, 200 µl chloroform was added, shaken rigorously for 15 seconds, and incubated for another 5 minutes. The mixture was centrifuged at 12000 g for 15 minutes. The aqueous phase was transferred to a clean RNase-free tube. The total RNA was precipitated by adding 0.5 ml isopropyl alcohol and incubating for 15 minutes at room temperature. The pellet including total RNA was washed using 75% ethanol and centrifuged at 7500 g for 8 minutes. After drying the ethanol, the RNA pellet was dissolved in TE buffer. The concentration and quantity of total RNA was calculated based on OD260/280 ratio measurements and agarose gel electrophoresis, respectively.

cDNA synthesis

RNA was converted to cDNA after treating with DNase I. Reverse transcription of RNA was performed in a final volume of 20 µl containing cDNA first strand synthesis buffer (Fermentas, USA) random hexamer primers and 1 µg of total RNA. According to manufacturer protocol, the samples were sequentially incubated at 65 °C for 10 min and 42 °C for 60 min. Reverse transcriptase was inactivated by heating at 70 °C for 5 min followed by cooling at 4 °C for 5 min.

Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

The cDNA samples served as template in real-time PCR. The beta-actin cDNA served as internal control for quantitation. After quantitation, results were analyzed by $2^{-\Delta\Delta Ct}$ method.

Measurement of hTERT mRNA

Levels of hTERT RNA molecules were determined by quantitative real-time RT-PCR technique using the Syber Green-I (Roche, Germany) by the Rotor-Gene™ 6000 system (Corbett Research, Australia) according to the manufacturer's instructions. After cDNA synthesis, specific primers were used to amplify hTERT mRNA (Table 1). Alternative spliced variants of hTERT mRNA were not measured because they do not reconstitute telomerase activity.[21,22] The quality of real-time PCR reactions was controlled by running standard samples as duplicate. 5-fold serial dilutions of cDNA obtained from the T47D breast cell line

Table 1. Sequences of primers for RT-PCR.

Genes and oligonucleotide	Location	Sequence	PCR product size (pb)
hTERT			
Forward primer	2165F	5′CCGCCTGAGCTGTACTTTGT3′	198
Reverse primer	2362R	5′CAGGTGAGCCACGAACTGT3′	
Beta-actin			
Forward primer	787F	5′TCCCTGGAGAAGAGCTACG3′	131
Reverse primer	917R	5′GTAGTTTCGTGGATGCCACA3′	

served as samples with strong expression of hTERT gene (Fig. 1). The beta-actin mRNA was measured as the internal control by specific primers (Table 1). The program for real-time PCR reaction was as follows: initial denaturation at 95 °C for 10 minutes, followed by 40 cycles of denaturation at 95 °C for 15 seconds, annealing at 60 °C for 30 seconds and extension at 72 °C for 30 seconds. Finally, amplicons were assessed by melting curve analysis of 70 °C to 95 °C.

Measurement of Plasma Leptin Level

Plasma leptin concentration was determined with the use of commercially available quantitative sandwich enzyme-linked immunosorbent assay (ELISA) with a detection limit of 0.2 ng/ml (mediagnost Leptin-ELISA E07, Germany). 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. All matched blood samples were handled identically and assayed in the same analytical run. The blood samples were labeled by number only and ordered randomly within each case–control pair.

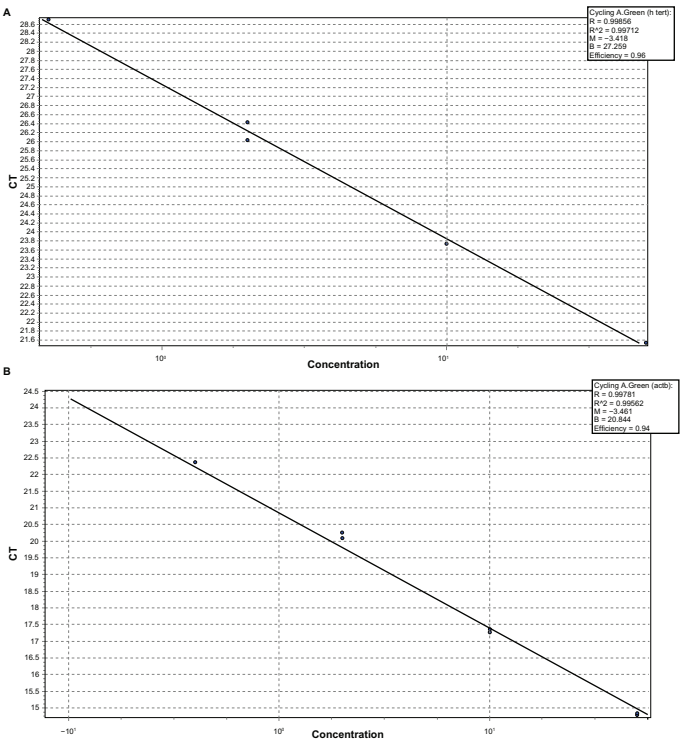

Figure 1. A) hTERT standard curve (R^2 = 0.99, M = –3.4, E = 0.96) and **B**) Beta actin standard curve (R^2 = 0.99, M = –3.46, E = 0.94), concentration unit in x-axis is nanogram per reaction.

Statistical Analysis

Differences between obese, overweight and non-obese groups in age at enrollment, age at menarche, age at menopause, and parity numbers were tested using the Student's t-test. In addition, the chi-square test was used to evaluate differences in categorical breast cancer risk factors between obese, overweight and non-obese. Linear regressions were used to examine relationships between hTERT expression and anthropometric measures of adiposity among subjects. All statistical analysis was conducted using SPSS statistical software (version 16). P values below the 0.05 ($P < 0.05$) were considered statistically significant.

Results

Descriptive data for three groups (obese, overweight and non-obese) of patients at baseline are summarized in Table 2. The mean age (±SD) of obese, overweight and non-obese subjects were 43.41 ± 9.1, 47.88 ± 11.2 and 44.06 ± 10.17 years, respectively. There were no statistically significant differences between non-obese as control, overweight and obese cases in terms of age at marriage (20.91 ± 1.3 vs. 20.93 ± 2.8 and 17.47 ± 1.6 years, respectively), age at menarche (13.38 ± 0.184 vs. 13.5 ± 0.282 and 13.41 ± 0.309 years, respectively), menopausal status (33 premenopause vs. 32 postmenopause patients) and age at menopause (49 ± 0.9 vs. 48.3 ± 0.76 and 48.6 ± 0.83 years), family history of obesity, stress, blood pressure, university education and active cigarette consumption. By contrast, it was found that the family history of breast cancer was higher in non-obese patients (61.7% vs. 13.3% and 18.75%, respectively). There were statistically significant differences between three groups for anthropometric measures of adiposity and BMI (23.12 ± 1.3 vs. 27.34 ± 1.7 and 31.92 ± 2 kg/m^2). The average WC was significantly lower in non-obese and overweight than obese breast cancer patients (85.85 ± 8.3 vs. 87.76 ± 9.7 and 94.41 ± 5.5 cm, $P < 0.05$); but the average WHR was not statistically significant (0.84 ± 0.07 vs. 84 ± 0.11 and 0.86 ± 0.05, $P < 0.05$, respectively). As shown in Figure 2, there was a weak relationship between hTERT mRNA levels and BMI. The average hTERT mRNA level in non-obese group was 2.32, in overweight group was 2.65 and in obese group was

Table 2. Characteristics of obese and non-obese breast cancer patients with reference to BMI.

Baseline characteristics		Normal (n = 34) Mean ± SD	Overweight (n = 15) Mean ± SD	Obese (n = 16) Mean ± SD	*P* value
Age at enrollment (yrs)		44.06 ± 10.17	47.88 ± 11.2	43.41 ± 9.1	0.351
Age at menarche (yrs)		13.38 ± 0.184	13.5 ± 0.282	13.41 ± 0.309	0.982
Age at marriage (yrs)		20.91 ± 1.3	20.93 ± 2.8	17.47 ± 1.6	0.291
Age at menopause (yrs)		49 ± 0.91	48.3 ± 0.76	48.6 ± 0.83	0.781
Height (cm)		163.08 ± 6.9	161.5 ± 5.8	157.6 ± 3.4	0.019
Weight (kg)		61.4 ± 5.3	71.66 ± 6.3	79.11 ± 5.4	0.01
BMI (kg/m^2)		23.12 ± 1.3	27.34 ± 1.7	31.92 ± 2	0.000
WC (cm)		85.85 ± 8.3	87.76 ± 9.7	94.41 ± 5.5	0.002
HC (cm)		101.5 ± 8.1	105.4 ± 4.3	109.1 ± 4.8	0.001
WHR		0.84 ± 0.07	0.84 ± 0.11	0.86 ± 0.05	0.767
Body fat %		34.2 ± 6	37.23 ± 4.6	42.82 ± 3.8	0.009
		Normal (n = 34) No. (%)	**Overweight (n = 15) No. (%)**	**Obese (n = 16) No. (%)**	***P* value**
Family history of breast cancer	Yes	21 (61.7)	2 (13.3)	3 (18.75)	0.003
	No	13 (38.3)	13 (86.7)	13 (81.25)	
Family history of obesity	Yes	24 (70.59)	10 (66.6)	15 (93.75)	0.09
	No	10 (29.41)	5 (33.4)	1 (6.25)	
Stress	Yes	33 (50.77)	16 (24.61)	17 (26.15)	0.61
	No	1 (50)	1 (50)	0 (0)	
Blood pressure	Yes	9 (47.4)	5 (26.3)	5 (26.3)	0.97
	No	25 (51)	12 (24.48)	12 (24.48)	
University education	Yes	28 (47.4)	15 (25.4)	17 (27.1)	0.11
	No	6 (66.6)	3 933.3)	0 (0)	
Active cigarette	Yes	1 (33.3)	2 (66.6)	0 (0)	0.22
	No	33 (50)	15 (22.7)	17 (27.8)	
Menopause status	Premenopausal	19 (57.57)	5 (15.15)	9 (27.27)	0.09
	Postmenopausal	15 (46.87)	10 (31.25)	7 (21.87)	

2.98 nanogram. In contrast, any direct contribution of abdominal obesity on hTERT gene expression was not found (Fig. 3).

hTERT mRNA Levels in Breast Tumor Tissues

Twelve (18.46%) samples were scored hTERT negative (hTERT Ct $>$ 35) whilst 53 (81.54%) samples scored hTERT positive (hTERT Ct $\leq$ 35) as determined by $2^{-\Delta\Delta Ct}$ value (Fig. 4). In this run, three normal breast tissues are tested as hTERT negative sample and they did not amplified. Among these hTERT-positive tumors, the differences of $2^{-\Delta\Delta Ct}$ values were observed ranging from 1.0 to 17. Figure 5 shows hTERT mRNA levels in breast tumor tissues by a real-time qRT-PCR assay was developed on human breast tissues. hTERT mRNA was express in relative unites after normalized to beta actin mRNA by using of q-gene software.

Correlation Between hTERT mRNA Levels and Clinical Staging and Pathological Grading

Data analysis for assessing any possible correlation between expression of hTERT mRNA and tumor staging and grading in all patients showed that most positive samples were in stage II and III and grade 1 and 2 (Table 3). There was a significant difference between obese and non-obese patients in stage III and grade 2 in terms of hTERT mRNA presence.

Association Between Plasma Leptin Levels and Tumor Tissue hTERT mRNA Levels

Data analysis revealed a significant direct correlation between levels of plasma leptin and levels of hTERT mRNA in breast tumor tissue in the subjects ($r = 0.484$, $P = 0.008$) (Fig. 6).

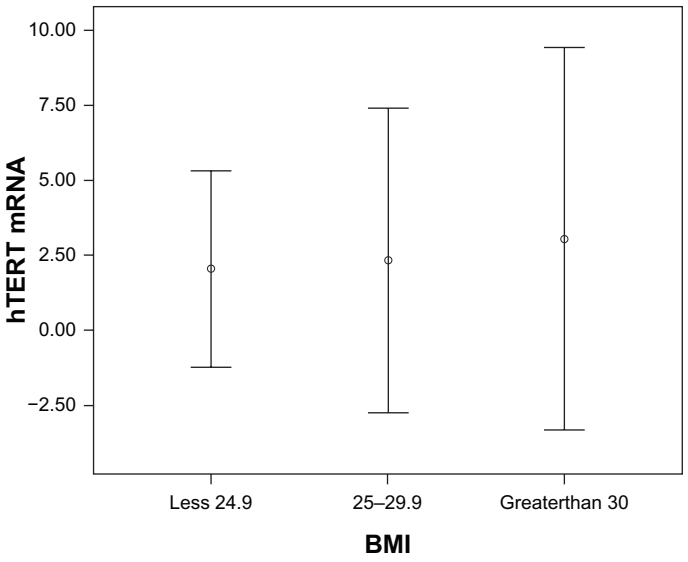

Figure 2. Relationships of hTERT gene expression with body mass in obese and non-obese breast cancer patients.

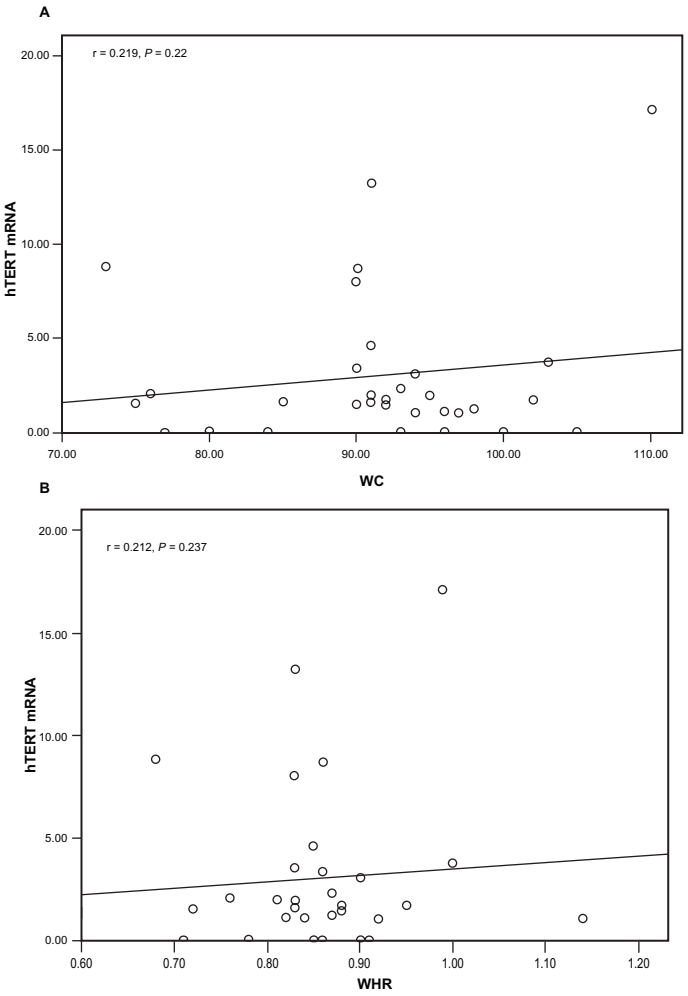

Figure 3. Correlation between hTERT gene expression with WC and WHR in **A**) obese and **B**) non-obese patients.

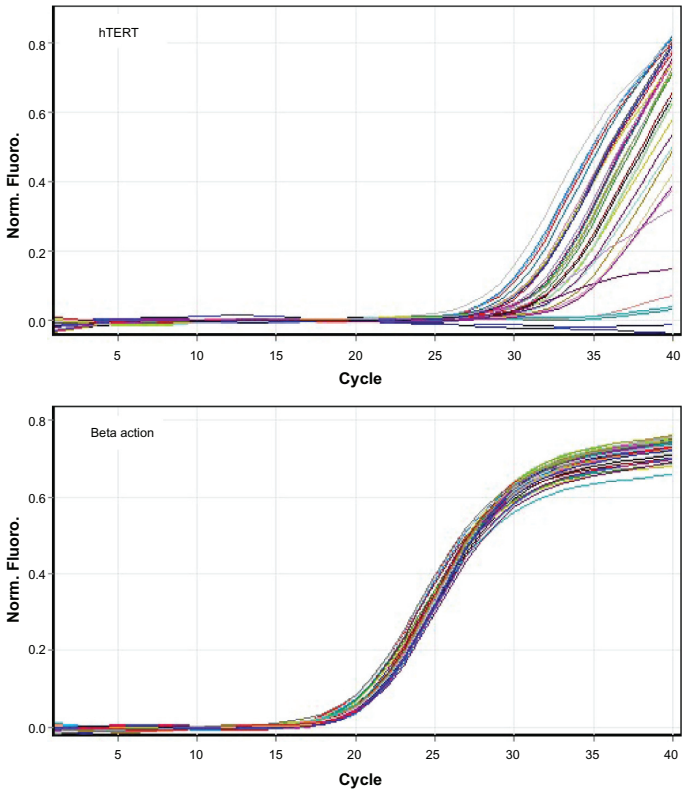

Figure 4. hTERT and beta actin mRNA quantitation data for cycling A. Green by real-time RT-PCR in the obese breast tumor samples and the calibrators. Three normal breast tissues are tested as hTERT negative control.

Discussion

Obesity or Adiposity is one of the major risk factors for development and progression of breast cancer in the postmenopausal women.[23] It seems that the possible biological mechanism which causes excess adiposity and breast cancer risk is that adiposity increases circulating levels of estrogens through peripheral conversion of androgens to estrogens by aromatase

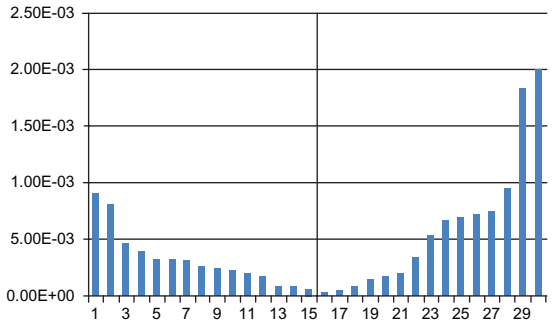

Figure 5. hTERT mRNA levels in breast tumor tissues. A real-time qRT-PCR assay was developed on human breast tissues. hTERT mRNA was normalized to beta actin mRNA, and express in relative unites, samples 1–15 were non-obese and 16–30 were obese by using of q-gene software.

Table 3. Detection of breast tumor hTERT mRNA in relation to tumor staging and grading.

	Tumor staging					Tumor grading			
	0	I	II	III	IV	0	1	2	3
Non-obese cases (%)	6 (17.6)	2 (5.9)	16 (47.1)	9 (26.5)	1 (2.9)	6 (8.6)	12 (17.1)	12 (17.1)	4 (5.7)
Obese cases (%)	1 (2.9)	0 (0)	22 (61.1)	13 (36.1)	0 (0)	1 (1.4)	10 (14.3)	19 (27.1)	6 (8.6)

in adipose tissue among obese women.[24] In obese women, the adipose tissue caused secretion of more biologically active estrogen to stimulate mammary epithelial cell mitosis and development of tumor.[25]

Recent studies have aimed to better our understanding of the influence of adipocyte-derived adipocytokines on tumorigenesis in breast tissue. The discovery of leptin and the demonstration that its circulating concentrations in serum positively correlates with BMI, have been followed by attempts to link serum leptin levels to breast cancer risk.[26] Leptin has been implicated as a link between obesity and breast cancer.[27,28] Some studies show that breast cancer patients have higher serum levels of leptin and leptin mRNA expression than control subjects, while other studies found no correlation in plasma leptin levels and breast cancer.[29,30] Our study by focusing on the mechanism of obesity on hTERT mRNA expression in breast tumor tissues indicates that, circulating levels of leptin increased in obese breast cancer women and was in correlation with hTERT mRNA expression. On the other hand, in vitro studies on MCF-7 cell line and HepG2 cell line have shown that leptin upregulated the expression of hTERT at mRNA and protein levels.[31,32] It showed the mechanism of the proliferative effect of leptin on breast cancer cells and provides a new explanation of obesity-related breast cancer.

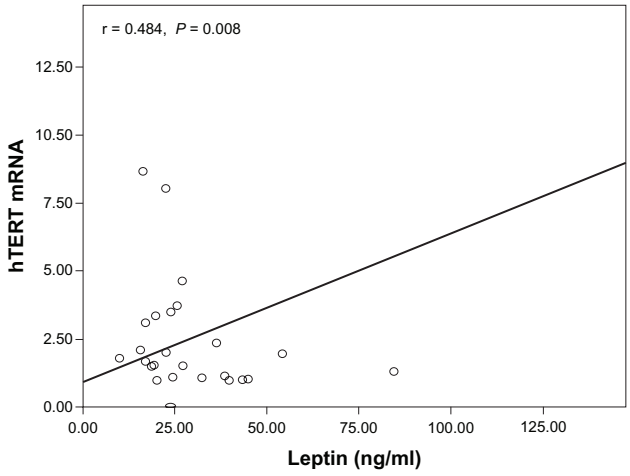

Figure 6. Association of plasma leptin levels and tumor tissue hTERT mRNA levels.

Recently studies demonstrated that women with breast cancer appeared to have high BMI, WC and WHR than control subjects, these results suggest that general obesity assessed by BMI and central obesity measured by WC may both have contributory effects on breast cancer risk.[33] In the current study, we showed positive effects of BMI, WC and WHR on breast cancer but the effect WHR was not statistically significant. Also, we demonstrated that WC and WHR have positive non-significant effects on hTERT expression level and hTERT level increased in obese patients, especially those with central obesity, it seems leptin or some other adipocytokines could be the link. We have not found any studies regarding a correlation between WC and WHR with hTERT expression.

Some researches deduced the lack of correlation between hTERT protein expression and tumor size and grade.[34] However, others previously reported that telomerase activity correlated with these clinicopathological parameters.[15,16,18] Although it has been shown hTERT expression is associated with malignancy, it does not seem to correlate with tumor stage.[17] On the other hand, in high stages a positive correlation between hTERT mRNA expression and cancer stage has been found in other cancers which is completely consistent with our present study.[35] Elkak et al demonstrated that hTERT mRNA expression is higher, although not statistically significant in breast cancer tissues and increased with tumor grade progression, which is not completely consistent with our study,[36] The present study showed high hTERT mRNA expression occurred in high grades and stages. This could be explained by considering that probably high hTERT expression by factors such leptin caused tumor progression and affect clinicopathological variables.

In summary, we conclude that high hTERT mRNA levels are associated with a poor clinical outcome in obese breast cancer patients and could be included

as a prognostic marker in future studies. Our study revealed a correlation between high serum levels of leptin and hTERT expression. These findings may help to elucidate the mechanisms of hormonal control of hTERT expression, clarify the key roles of leptin in breast carcinogenesis, and show how obesity could increase breast cancer incidence and progression by leptin production. Meanwhile, in order to best understand hormonal control of hTERT expression, the correlation between hTERT expression level and other adipocytokines must be further studied.

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Disclosure

This manuscript has been read and approved by all authors. This paper is unique and is not under consideration by any other publication and has not been published elsewhere. The authors and peer reviewers of this paper report no conflicts of interest. The authors confirm that they have permission to reproduce any copyrighted material.

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