Investigation of Telomerase Activity in Inflammatory and Non-Inflammatory Breast Cancer

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Abstract

Background: Inflammatory breast cancer (IBC) is an aggressive type of breast cancer disease that has a high incidence in Egypt than western countries. It is characterized by rapid progression, involvement of dermal lymphatic emboli and extensive lymph node involvement. Basic and translational studies are needed to define IBC disease biology and identify specific biomarkers have been limited by the paucity of patient samples. Hence, the current study aimed to introduce the telomerase activity level as a novel diagnostic marker for breast cancer and specifically for IBC to be differentiated from non-IBC.

Methods: Breast cancer patients were enrolled from Ain Shams University hospitals in Cairo, divided into two groups: IBC (n=26) and non-IBC (n=27). Tissue samples were collected during modified radical mastectomy. TRAP (Telomerase repeat amplification protocol) assay was used to assess the telomerase activity in inflammatory and non inflammatory breast cancer tissue samples. Immunohistochemistry was used to investigate the expression of hTERT subunit of telomerase in paraffin embedded tissue samples of both types of patients.

Results: IBC showed Telomerase activity ranged from 12.2 to 367.1 units with a mean value of 78 and a median value of 43, while telomerase activity in non-IBC ranged from 6.1 to 109.34 units with a mean value of 41.1 and a median value of 24. On the other hand, normal tissues showed telomerase activity below 5 (P<0.001). Using immunohistochemistry, the hTERT expression was higher in IBC than non-IBC and no expression at all in normal tissues. Moreover, a positive mild correlation was found between the telomerase activity and the number of metastatic lymph nodes in both IBC (r=0.53) and non-IBC (r=0.54).

Conclusions: Telomerase could be a promising marker at the diagnostic and therapeutic levels in breast cancer and specifically in IBC.

Keywords: Breast cancer; Cancer death; Erythema; Telomerase

Introduction

Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death among females, accounting for 23% of the total cancer cases and 14% of the cancer deaths. Recent statistics revealed that approximately 207,090 new cases of invasive breast cancer and 40,230 deaths from disease were diagnosed in USA in 2010 [1].

In Egypt, breast cancer is number one among women constituting about 38.6% of female cancer cases (National cancer registry program of Egypt, 2010) and it was found that it is more prominent among young premenopausal Egyptian women characterized by poor prognosis and low survival rate [2].

Most of Egyptian patients are diagnosed at advanced stage (II and III) (Gharbiah population-based cancer registry, 2007 and National cancer registry program of Egypt, 2010) and most cases possess positive axillary lymph node metastasis about 70.6% at the time of diagnosis while only 29.4% are free of metastasis [3]. Besides, IBC occurs in approximately 1-6% of all breast cancers in the U.S [4,5], in Egypt it occurs in approximately 10% of all breast cancers [6], which reflects the invasive properties of breast cancer in Egyptian patients.

As a subtype, inflammatory breast cancer (IBC) is the most aggressive apparition of primary epithelial breast cancer and it belongs to the group of locally advanced breast cancer (LABC) characterized by a variety of clinical presentations including large tumors, extensive nodal involvement and direct involvement of the skin or chest wall. IBC is a clinicopathological entity characterized by the rapid onset of swelling and often enlargement of the breast. Although the overlying skin remains intact, it displays erythema often combined with 'peau d’orange', local tenderness, induration and warmth. All these changes can occur to a variable degree. Critical in this definition is the rapidity with which these occur [7-10]. According to the population-based cancer registry in Egypt, IBC cases represent around 11.1% of breast cancer cases between Egyptian patients and this percentage is on the rise [11].

The aggressive nature of IBC, in addition to the unique molecular and epidemiological characteristics supports the hypothesis that IBC has its own characters rather than being a subtype of locally advanced breast cancer [8,12,13]. For instance IBC can be distinguished from the LABC by the presence of numerous dermal tumor emboli in the papillary and reticular dermis of the skin overlying the breast [8,14-16].

The rapid onset, the misdiagnosis and lack of enough biological markers of IBC make it very critical to introduce new molecular and biological markers to accurately and early diagnose the disease.

Human telomerase is a ribonucleoprotein enzyme composed of catalytic component, telomerase reverse-transcriptase (TERT) [17,18] and RNA template TERC (or hTR), telomerase RNA component (or human telomerase RNA) [19]. Telomerase can elongate the G-rich 3’ telomere overhang by adding (TTAGGG)n repeats using its RNA as a template [20].

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In most human normal somatic cells, telomerase activity is undetectable [21]. Every type of human malignancy examined to the present time has evidence that >80% of overall malignancies have telomerase activation with different levels [22].

At carcinogenesis early stages, cancer cells do not require telomerase activity unless their telomere lengths become critically shortened, since carcinogenesis and cellular immortalization are independent processes. However, in advanced stages, most cancer cells have experienced several clonal selections, especially those in metastatic lesions since metastasis itself adds at least one clonal selection, and subsequently many cell divisions. At such stages, most cancer cells depend on telomerase to proceed in the proliferation rounds. Hence, activation of telomerase itself likely provides more malignant potential like what occurs in metastasis [22-24].

In a study conducted by Koskimaa et al. [25] they showed an up-regulation for telomerase activity in Cervical cancer. Similarly, telomerase activity was detected in bladder cancer as a diagnostic target [26]. Telomerase activity was investigated in bronchial epithelium for lung cancer detection and diagnostic purposes as well [27,28], and in colorectal cancer for prognostic significance value [29] and many other cancers either involved in telomerase studies or still under investigation.

Moreover, Shay and Bacchetti [22] reviewed that telomerase activity was 75% of breast carcinoma in situ lesions, 88% of ductal and lobular carcinomas, 5% of adjacent tissues and other more studies confirmed these results and did more investigation on different types and stages [30-32].

Besides, Telomerase inhibition should be effective and specific, while normal cells lacking telomerase should not be significantly affected by anti-telomerase therapy. Since telomerase expression in cancer cells can be used as a novel marker for screening, early detection, and prognosis, clinical trials are already in progress to assess this potential [33,34].

Thus, telomerase components and its associated proteins are becoming not only a diagnostic marker of cancer but also a promising molecular target of anticancer strategies and some of them are under clinical trials.

The purpose of this study was to investigate telomerase activity in inflammatory and non-inflammatory breast cancer and its correlation with the type and stage of cancer.

Materials and Methods

Study patients

The study was approved by the Institutional Review Board of Ain Shams University Ethic committee (IRB #.00006379). 26 IBC and 27 non-IBC patients were enrolled in the study, and the clinical and pathological information of these patients was collected from the hospital records and pathology reports (Table 1).

Inclusion criteria of breast cancer patients were dependent upon a combination of clinical, mammographic, ultrasound, and pathological diagnoses.

Clinical diagnosis of IBC is applied, according to the American Joint Committee on Cancer (AJCC) T4 d designation for IBC [8], when a patient presented with a diffuse erythema, peau d’orange and edema of the breast. For IBC patients, pathological confirmation of the clinical diagnosis was dependent upon examination of both skin and core biopsies (M.A.N.). In the absence of breast masses, diagnosis was depended upon pathological examination of skin biopsies that showed permeation of dermal lymphatics by carcinoma cells and the presence of dermal tumor emboli (M.A.N.) (Figure 1). Non-IBC patients of stage II-III were also included in our study as a comparison group.

Patients subjected to neo-adjuvant chemotherapy or those with viral hepatitis or autoimmune disease were excluded from our study. Tissue specimens were obtained from modified radical mastectomy (MRM) specimens. All tumor tissues preserved in 10% neutral formalin buffer for histopathological diagnosis and immunohistochemistry. Fresh tissues were available for all patients (26 IBC and 27 non-IBC) and 5 normal tissues obtained from healthy volunteers underwent mammaplastic (Breast reduction) surgery (Mean age 27.8 ± 5.9). Tissues were preserved in freezing media (70% RPMI 1640, 20% Fetal Bovine Serum (FBS) and 10% dimethyl sulfoxide (DMSO) and stored at -80°C until used.

Pathological diagnosis was examined for each patient, including tumor size, tumor grade, disease stage, lymph node status and presence or absence of lymphovascular invasion and dermal lymphatic emboli. According to the modified Bloom Richardson- Elston histological system, tumors divided into I, II and III grades as well as breast cancer is staged into four main stages from I to IV based on TNM classification [35].

TRAP (Telomerase Repeat Amplification Protocol) Assay

TRAP assay is a sensitive PCR-based telomerase activity detection method that could detect telomerase activity in human tissue and cells. The assay, which was originally described by Kim et al. [36], is a one buffer with two enzymes system utilizing the polymerase chain
reaction (PCR). In the first step of the reaction, telomerase adds a number of telomeric repeats (GGTTAGG) onto the 3' end of a substrate oligonucleotide (TS). In the second step, the extended products are amplified by PCR using the TS and RP (reverse) primers, generating a ladder of products with 6 base increments starting at 50 nucleotides: 50, 56, 62, 68, etc. Hence, if there is telomerase activity, a ladder of bands will be detected starting from 50 bp and if not, only the 36 bp band will be detected.

TRAP-eze Telomerase Detection kit (catalog number #S7700) was purchased from Chemicon International, Inc (Millipore, USA). Bradford reagent (catalog number R1271), Ribolock RNAase inhibitor (catalog number E00381), Dream taq DNA polymerase (catalog number Ep0702) and Maxima Hot start taq DNA polymerase (catalog number Ep0601) were purchased from Fermentas (R 1271, Fermentas, Burlington, ON, Canada). SYBR green I (catalog number S9430) was purchased from SIGMA (St. Louis, MO).

Tissue extract was prepared using 1X CHAPS lysis buffer (10 mM Tris-HCl, pH 7.5, 1 mM MgCl2, 1 mM EGTA, 0.1 mM Benzanidine, 5 mM β-mercaptoethanol, 0.5% CHAPS, 10% Glycerol) supplied with the TRAP-eze telomerase detection kit. RNase inhibitor was added to the CHAPS Lysis Buffer prior to the extraction for a final concentration of 100-200 units/mL.

Extracts containing 5 µg protein were added to the TRAP reaction mixture containing 2.5 µl of 10X TRAP reaction buffer (200 mM Tris-HCl, pH 8.3, 15 mM MgCl2, 630 mM KCl, 0.5% Tween 20, 10 mM EGTA), 0.5 µl of 50X dNTP mix (2.5 mM each dATP, dTTP, dGTP, dCTP), 0.5 µl TS primer, 0.5 µl TRAP primer mix (RP primer, K1 primer, TSK1 template), 0.3 µl of Hot start Taq polymerase (5 units/μl), and±18.8 μl of PCR grade water.

Reaction tubes were incubated in thermocycler at 30°C for 30 min. This step of the TRAP assay allows telomerase of cell lysates to add hexamer repeats of telomeric sequence (TTAGGG) onto the 3’-end of the included primer. The reaction mixtures were then subjected to conventional polymerase chain reaction consisted of 34 PCR cycles of 94°C for 30 sec, 56°C for 30 sec and 72°C for 1min followed by a final extension step for at 72°C for 5 min.

To re-amplify the PCR product for better visualization, a second PCR run took place by adding 5 μl of the 1st PCR product to 20 μl of the master mix.

The PCR products were analyzed by electrophoresis at 100 V for about 1.5 h on a 15% polyacrylamide gel and the gel was stained with SYBR Green I nucleic acid gel stain.

Telomerase activity was assessed by measuring the intensity of bands in the whole lane of each sample compared to the TSR8 quantitation control (Q) using Image software according to the following formula as described by Gümüş-Akay et al. [37].

Telomerase activity (TA) = ([X/C] / [r/Cr]) × 50

Where X is the intensity of the telomerase ladder of the test sample, C is the intensity of the internal standard (two primers in each reaction mixture for amplification of a 36 bp standard) in the test sample, r is the intensity of the TSR8 quantitation control (a telomerase product-like ladder for comparison with the test sample), and Cr is the intensity of the internal standard in TSR8 quantitation control.

TSR8 quantitation control (Q) is an oligonucleotide, provided with the kit, with a sequence identical to the TS primer extended with 8 telomeric repeats AG(GGTTAG). This control serves as a standard for estimating the amount of TS primers with telomeric repeats extended by telomerase in a given extract.

Immunohistochemistry (IHC) for telomerase enzyme in tissue blocks

Super Sensitive™ Polymer-HRP IHC Detection System/DAB (catalog number QD420-YIKE™) and universal blocking reagent (10X) (catalog number HK085-5K) were purchased from Biogenex (Fremont CA U.S.A.). Rabbit monoclonal antibody anti human telomerase (catalog number EST21-A) was purchased from Alpha Diagnostic Int. Co., (San Antonio, TX, USA). Unless otherwise stated all other reagents were from Sigma (St. Louis, MO).

Tissue sections were prepared from paraffin blocks and stained with hematoxylin and eosin to select tissue sections for immunostaining and scoring. IHC staining was performed on 5 µm thick tissue sections. Tissue sections were first deparaffinized and rehydrated followed by antigen retrieval by heating with pressure cooker for 5-7 mins. Blocking was performed using peroxidase blocking reagent then serum blocking.

Tissue sections were incubated overnight with the primary antibody prepared in Dako Antibody diluent with reduced background components at 5 µg concentration polyclonal anti hTERT.

Detection was then completed by incubating tissue sections with 50 µl of super in hancrer for 20 mins and 50 µl of polymer – HRP for 30 mins. Then staining was performed by adding 50 µl of DAB for 5 mins. Nuclei were counterstained with hematoxylin and specimens were rinsed in alkaline water and mounted using Permount® for microscopic examination.

Statistical analysis

Results analysis was performed using statistical package for the social sciences software (SPSS, Chicago, IL), version 16.0. Qualitative data were expressed as frequencies and percentages while quantitative data were expressed as mean ± SE.

The analysis of telomerase activity between populations was tested using Kruskal–Wallis one-way analysis of variance (Chi-square) [38] for testing equality of population medians among groups.
The correlation between telomerase activity and number of metastatic lymph nodes was assessed using Pearson product-moment correlation coefficient (PPMCC) to measure of the strength of linear dependence between the two variables.

**Results**

**Clinical and pathological characterization of IBC versus non-IBC patients**

Clinical and pathological characterization of the IBC (n=26) and non-IBC patients (n=7) used in this study is indicated in table 1. Age of IBC patients ranged from 29-72 years (mean age of 51 ± 12.3), whereas the age of non-IBC patients ranged from 37-78 years (median age of 55 ± 10.5).

For IBC patients, 8% of them exhibited tumor masses less than 2 cm and 92% had a tumor mass more than 2 cm. Non-IBC patients had tumor with 10% having tumor sizes less than 2 cm and 90% having tumor sizes greater than or equal to 2 cm. Tumor grading revealed that 81% of IBC patients were tumor grade I or II and 19% were tumor grade III. In non-IBC patients 86% were diagnosed as tumor grade I or II, and 14% were diagnosed as tumor grade III.

We assessed the number of axillary lymph nodes that were positive for metastases in IBC versus non-IBC patients. All IBC patients who underwent surgery had positive metastatic lymph nodes: 24% had less than 4 positive metastatic lymph nodes, 76% had more than or equal 4 positive metastatic lymph nodes. Among non-IBC patients, 61% had less than 4 metastatic lymph nodes (2 patients in this category had no metastatic lymph nodes) and 39% had more than or equal 4 metastatic lymph nodes. In addition, the difference between the number of positive metastatic lymph nodes in IBC versus non-IBC patients was determined to be statistically significant (P<0.001).

Positive staining for ER, PR and HER-2 was detected in 45%, 55% and 18% of the IBC patients, respectively. In non-IBC patients, positive staining for ER, PR and HER-2 was 37%, 37% and 21%, respectively.

**Telomerase activity in IBC, non IBC and normal tissues**

To compare relative amounts of telomerase activity between samples, the TRAP assay signals of the telomerase ladder (50 bp+6 bp increment-ladders according to the activity) were normalized to that of the internal standard (36 bp) (Figure 2).

In our study, telomerase activity was detected in all the breast cancer tissues either inflammatory or non inflammatory but the level of activity was variable. In IBC tissue samples, telomerase activity ranged from 12.2 to 367.1 and the mean value were 78 with a standard deviation of 87.4. In NIBC tissue samples, telomerase activity ranged from 6.1 to 109.34 and the mean value was 41.1 with a standard deviation of 30.7. In normal tissues, all the values were equal or below 5 (Figure 3).

These values represent the relative telomerase activity with a constant protein concentration for all the samples.

We compared telomerase activity between IBC, non-IBC and normal samples using Kruskal–Wallis one-way analysis of variance (Chi-square) test and the result showed statistical significance (P<0.001).

Moreover, post Hoc tests by multiple comparisons were made comparing groups in pairs as follows; the difference in telomerase
activity between IBC and non-IBC samples was determined to be statistically significant (P < 0.05). The difference in telomerase activity between IBC and normal samples was determined to be statistically extremely significant (P<0.001). The difference in telomerase activity between non-IBC and normal samples was determined to be statistically significant (P<0.01).

The correlation between telomerase activity and the number of positive metastatic lymph nodes in IBC and non IBC patients

In IBC, the correlation between the telomerase activity and the lymph node metastasis was mild positive (r=0.53) and the P value was 0.004. In NIBC, the correlation was mild positive (r=0.54) and the P value was 0.01.

The correlation was made for 25 of 26 IBC and 22 of 27 patients whose clinical data was available (Table 1 and Figure 4); some patients’ samples had the same telomerase activity and so they were presented with the same dot on the scatter plot.

Over expression of hTERT by carcinoma cells in paraffin tissue sections of IBC versus non-IBC

In IBC samples, hTERT expression was detected in IBC carcinoma cells and observed mainly in the nucleus and a faint signal in the cytoplasm. Also, IBC samples presented tumor emboli showed very dense nuclear and cytoplasmic staining (Figure 5) and the specimens without tumor emboli showed dense staining as well (Figure 6B). In non-IBC samples, a weaker signal than IBC samples was observed in the nucleus and also in the cytoplasm (Figure 6A). Interestingly, normal tissues didn’t exhibit hTERT expression signal at all with the same antibody dilution and condition (Figure 7).

Discussion

In this study, we investigated telomerase activity in inflammatory and non inflammatory breast cancer tissue samples. The clinical and pathological features are very important in diagnosis of breast cancer cases and some of them are critical in the differentiation between inflammatory and non inflammatory breast cancer (Table 1) [39-41].

In our study, we found that the number of axillary lymph nodes in IBC was significantly higher than those involved in non-IBC (P<0.001)
Telomerase activity was observed in previous studies to be correlated to the grade and stage of breast cancer and it was shown that the activity of the enzyme is higher in metastatic stages than non metastatic ones in breast cancer [48-51] and hence IBC is a highly aggressive and metastatic disease, telomerase activity may play a considerable role in the metastasis process. Tumor emboli, which is one of the hallmarks of IBC, is found to have tumor cells with stem cell characters (cancer stem cells) [52] and being highly proliferative. Stem cells need the telomerase enzyme to survive the continual division [53,54] and to escape the mortality stages M1 and M2 [55-57].

Recently, the role of telomerase in metastasis was investigated by several studies which found a positive correlation between the Akt activation, which is a play maker in tumor progression, vascular invasion, and distant metastasis [58,59] and the level of telomerase activity [60-62]. Moreover, epidermal growth factor receptor (EGFR) and telomerase was found to be functionally interplay besides p53 activation of stromal fibroblasts induces tumor development, invasion, and differentiation [63] taking into consideration the role of EGFR in breast cancer in several processes like angiogenesis [64], cell migration and invasion [65].

No strong correlation between number of axillary lymph nodes and telomerase activity was found when we compared the telomerase activity of each patient to its metastatic lymph nodes' number and as we noticed that some patients showed a very high telomerase activity with a high number of metastatic lymph nodes while in some patients this relation was not typically observed.

In conclusion, this work is a novel attempt to compare the telomerase expression between inflammatory and non inflammatory breast cancer and it could be a new way to more research on this issue that can be used for breast cancer diagnosis and IBC in specific depending on the significant difference in expression between those subtypes.

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