Original Research Article

Value of Telomere Length in Peripheral Blood Leucocyte as a Prognostic Marker for Breast Cancer

Running title: Telomere length and breast cancer

Abbreviations: FISH, Fluorescence In Situ Hybridization, TL, Telomere Length ,PNA, Peptide Nucleic Acid, DCIS, Ductal Carcinoma In Situ , OS, Overall Survival, DFS, Disease Free Survival, PBLs ,Peripheral Blood Leucocytes, IRB, International Review Board. SECI, South Egypt Cancer Institute, FBS, Fetal Bovine Serum, ER, Estrogen Receptor, PR, Progesterone Receptor, NOT, Not Otherwise Specified, pT, pathologic Tumor Stage. Sig, significant. P value, Probability Value.

ABSTRACT

Background: Telomeres ensure genome integrity during replication. Loss of telomeric function leads to cell immortalization, accumulation of genetic alterations and tumorigenesis. Telomere length (TL) studies in breast cancer patients could be a valuable prognostic marker.

The goal of this study was to evaluate TL in breast cancer patients by telomere specific fluorescence in situ hybridization (FISH) in peripheral blood leucocytes (PBL), compare between their TL and control group, and correlate between TL and clinicopathological characteristics of these patients and DFS and OS

Patients and Methods: Prospective study was carried out on 83 newly diagnosed breast cancer patients with Stage 0-stage IIIc and 20 healthy individuals as control, for detection of peptide nucleic acid (PNA) by telomere specific FISH.

Results: Our results showed the mean length of telomere was 32 ± ,our results revealed that telomere shorting has inferior DFS and OS when compared to patients with long Telomere length , also we found significant associated with short telomere length and advanced stage started from stage IIb as it reflect that telomere shortening has significant association with tumor size and lymph node involvement. Moreover our results showed significant association with positive Her2 neu expression in tumor (P<0.001), However it didn't show any significant association t with hormonal receptors status.

Conclusion: TL assessment in PBL could be used as valuable prognostic marker in breast cancer.

Key words: telomere length, breast cancer, FISH, survival.

1. INTRODUCTION

Breast cancer is one of the most common causes of cancer related deaths in women [1], and according to the American Cancer Society, it accounts for 25% of all new cancer diagnoses in women globally [2].Telomeres are repetitive DNA sequences protecting the ends of chromosomes. Telomeres shorten with each cell division, which regulates the cellular lifespan in somatic cells and limits their renewal capacity. Cancer cells are often able to overcome this physiological barrier and become immortal with unlimited replicative capacity [3].

Telomere maintenance is an essential step in cancer progression [4]. Most cancer cells maintain the integrity of their telomeres by telomerase reactivation [5].

Alternative Lengthening of Telomeres (ALT) pathway is a telomerase-independent pathway for telomere maintenance [6].

Telomere abrasion during successive cell divisions induces chromosomal instability and contributes significantly to genomic rearrangements that can result in tumorigenesis [7]. Numerous observational studies of TL have been conducted among breast cancer patients in the last 20 years and it could be a valuable breast cancer prognostic marker [8].

The goal of this study was to evaluate leucocytes telomere length in breast cancer patients by telomere specific fluorescence in situ hybridization, to compare between telomere length in cancer breast patients and control group and correlate between telomere length and the clinicopathological characteristics of these patients.

2. PATIENTS AND METHODS

This study is a prospective case control study was carried out on 83 patients with newly diagnosed breast cancer and 20 healthy volunteer This study was done on breast cancer female patients diagnosed in South Egypt Cancer Institute (SECI) outpatient clinic from December 2014 to January 2016.

We included patients with stage 1 to stage III C and only invasive ductal carcinoma included was included in this study. Women were eligible if they were not older than 70 years, were not pregnant, had no previous diagnosis of cancer other than non-melanoma skin cancer, never had any breast surgery including breast reduction or implants, never took a selective estrogen receptor modulator such as Tamoxifen or Raloxifen, and did not receive any treatment prior to surgery.

All control volunteer who were recruited in the study (n=20) were female with comparable age to patients and all of them were subjected to breast sonar and mammogram before blood withdrawal of the sample to be sure they are not have breast cancer .

The follow up of the patients was ended in December 2018. And the follow up include physical examination ,chest X ray and abdominal US , every 6 month. C.T scan every year as a routine or if indicated if there suspicious

Whole blood samples was taken from all patients for cytogenetic , after mastectomy or conservative surgery the tissue removed patients subjected to pathological stage (pT) according to WHO 2012 (Lakhani S et al 2012) [9], regional lymph nodes status, vascular and lymphatic tumor invasion, presence of necrosis, ER (estrogen receptor), PR (progesterone receptor) and HER2 neu status. These parameters were evaluated in this study.

2.1 Ethical Consideration

The research is approved by International Review Board (IRB) at SECI ethical committee. (SECI-IRB IORG0006563 N0185/2014.) All samples were collected from breast cancer patients visited the surgery outpatient clinic at SECI. All patients provided informed written consent.

2.2 Cytogenetic study

This test was done on all breast cancer patients in this study and 20 apparently healthy individuals as control, for detection of PNA by telomere specific FISH. We used PNA FISH Kit / Cy3 (code k5326, Dako Denmark A/S) containing a PNA probe for the telomeric sequence TTAGGG.

Whole blood collected in heparin tube for each patient and control. Preparation of metaphase spreads from blood cultured cells for FISH analysis was carried0 Heparinized blood was cultured in RPMI with 20% fetal bovine serum (FBS), glutamine, penicillin/streptomycin and phytohemagglutinine. Twenty metaphases of each patient and were captured at 63 × magnification with the Axioscope Imager M1 microscope (Carl Zeiss, Jena, German) with individual excitation filter sets for DAPI and Cy3, equipped with a CCD-camera. The telomere size was analyzed with ISIS software (MetaSystems, Altlussheim, Germany).

Separation of chromosomes was done by means of interactive separation and dislapping functions. Once separated, the chromosomes were transferred to the

karyotype window. Chromosome classification was performed using an automatic DAPI banding classifier followed by interactive corrections. The telomere measurement was done by applying two horizontal lines overlaid to each chromosome in the karyogram, which define the telomere measurement areas (for p- and q-arms) of each chromosome. The measurement area of the reference signal was displayed as two horizontal lines on the respective chromosome (chromosome 2).

The software calculates telomere lengths as the ratio between the fluorescence of each telomere (T) and the fluorescence of the centromere (C) of chromosome 2 (T/C), used as the internal reference in each metaphase analyzed. Centromere 2 sequence has a stable length to be used as internal reference [10].

The analysis was done for all metaphases and statistical parameters are automatically calculated and displayed: mean telomere intensity of the p- and q-arms of each chromosome, which expressed as T/C, standard deviations, median and the interquartile range. Mean telomere intensities are displayed as telomere length histogram.

2.3 Statistical analysis

Results were statistically analyzed using statistical package for the Social Sciences (SPSS version 21). Independent T test and one way ANOVA were used to detect the association between Telomere length and various clinicopathological data.

Kaplan - Meier survival test were used to analyze overall survival and disease free interval, and the significance of difference between the survival curves was evaluated by Log-rank test. All statistical analysis was two sided and the level of significance was defined as P=.05.

3. Results

This study includes 83 breast cancer female patients; all of them were invasive duct carcinoma, not otherwise specified (NOS) with different grades and stages while the other 20 (24%) patients have associated DCIS.

This study includes 4 cases (4%) of grade I, 51 cases (61.4%) of grade II and 8 cases (9.6%) of grade III. Regarding the stage, our results included two cases had stage I (2.4%), 46 cases stage II (55.4%) and 15 cases (18.1%) of stage III. The mean

telomere length was 32.26 ±10.08. 53 (63.9%) patients presented with positive hormonal status (ER and /or PR positive) while 30(36.1%) patients presented with negative ER and PR .Regarding Her2neu status ,29 (34.9%) patients presented with positive her2neu status by immunohistochemistry while 54(65.1%) patient with negative her2neu status **(Table 1).**

3.1 Association of Telomere length and clinicopatholgical parameters:

T/C-FISH was performed by measuring the total fluorescence intensity of the signals of telomeres and the centromere of chromosome 2. This integrated fluorescence intensity is proportional to telomeric and centromeric length. We examined telomere intensities of chromosome preparations in peripheral blood leucocytes (PBLs) from all patients and controls. 20 metaphases from each individual were examined and the mean of the T/C-FISH value was calculated.

Telomere lengthening was significantly associated with early stage (stage I and stage IIa) **(Figure 1)** and telomere shortening associated with stage IIb- stage IIIC **(Figure 2)** (P<0.001). The presence of tumor necrosis, vascular and lymphatic invasion were associated with shortening of telomere length (P=0.04, P=0.01 and P=0.028, respectively).The increased number of lymph node metastasis significantly associated with shortening of telomere length (P<0.001). Moreover short telomere length was associated with positive Her2neu expression p=0.001 and Estrogen receptor negativity p=0.03 but not with collective negative hormonal status (ER and PR negativity) p= 0.987 **(Table 2)**

3.2 Telomere length in breast cancer patients and control groups:

In our study, Telomere lengthening was significantly associated with breast cancer (mean±SD length =32.26±10.08) patients in comparison to telomere length in control group (mean±SD 20.10 ± 0.91) (P<0.001). **(Figure 3) ((Table 3)**

3.3 **Telomere length and survival**

At the end of this study only 64 patients were still alive (77.1%) .The number of patients with disease relapse occurred in 35 cases (42.2%)

Telomere shortening is associated with poor DFS and OS **(Figure 4.a,b)** (P<0.001) Telomere shorting showed statistical significant association with negative estrogen expression (P<0.05) However, there is statistical difference between total positive hormonal status (ER and/or PR positive) versus both ER and PR negative p= 0.434 **,(Figure 5a)** on the other hand telomere shorting showed significant association with Positive Her2 neu expression (P=0.000). **(Figure 5b)**

4. Discussion

Breast cancer is the principal leading cause of cancer-related death among women worldwide [11]. Telomeres are composed of series of tandem repeats of TTAGGG nucleotides localized to chromosome ends [12].An important role of telomeres is to protect chromosome ends preventing the activation of DNA damage response and preserving genomic stability [4].Telomeres play a critical role in tumorigenesis [13]. Cancer cells characteristically acquire infinite capability to divide through maintenance of telomeres by sustained expression of telomerase or by an alternative lengthening of telomeres (ALT) mechanism [14], and this may be permissive and required for clonal evolution of genomically-unstable cells during progression to malignancy [15].

In our study, we found that telomere lengthen was significantly associated with early stages breast cancer patients in comparison to telomere length in control group and this finding was matched with Svenson U et al and Gramatges M et al [16,17], On the other hand our results contradict the findings observed by Barwell J et al [18] who reported that there was no significant difference in telomere length in blood leucocytes between breast cancer patients and age adjusted normal controls , hower this may be due to ethnic variation as there is at trial conducted by Qu S et al [19] from china carried on more than 600 patients and equal number of age adjusted individual as control group and they found the telomere lighting in control group associated with increased susceptibility of breast cancer

In the current study, we demonstrated that the telomere length was significantly longer in leucocytes from individuals diagnosed with the early stage of breast cancer up to stage II a than in leucocytes in the advanced stages (stage IIa-stage IIIC and this results was in line with the study done by Barczak W et al [20] who explained that

by telomere length is significantly associated with lymph node metastases so patients with negative LN involvement have long telomere length and longer overall survival , however in our study most of early stage presented with her 2 neu negative disease this could affect the convulsion of results and could also explain the contradiction of others study Ennour-Idrissi et al, who reported that no association was observed for telomere length with stage ,that may be because the sub-analysis of their patients criteria in early stages might show assciated poor prognostic criteria or because due to using different method of measurement of the telomere length Barczak W et al 2016, Ennour Idrissi [20,8].

Telomere lengthening was statistically significantly associated with positive estrogen expression but not strong association; this finding was matched with Ennour-Idrissi et al [21]. Longer telomeres associated with increased breast cancer risk may also be attributed to estrogen effect, as increased estrogen exposure is a well-known risk factor for breast cancer development. Estrogen affects telomere length directly through the activation of the human telomerase reverse transcriptase promoter [22], as well as by post transcriptional human telomerase reverse transcriptase regulation [23].However there is no association between telomere length and PR expression that was in agreement with Jones et al [24] also it didn't show any significant association with positive hormonal receptors (either ER and /or PR positivity) and that match with Ennour-Idrissi et al [21]

Regarding others patients and tumors characteristics like age of patients , tumors size and grade , we didn.t find any association between telomere length and above characteristic.

Concerning the age, our results was matched with Shen et al[25] and Pavanello et al [26], who stated in their studies that the effect of age on telomere length was undetectable or negligible, due to the complex alternation in telomere maintenance mechanisms associated with carcinogenic process.

Regarding the grade and tumor size, our results found negative association between tumor grade and tumor size and telomere length, that was matched Barczak et al[20] who reported negative assciation with tumour grade also Ennour-Idrissi et al reported

that no association was observed for telomere length with tumor size, which was in agreement with our results for breast cancer patients [8].

Telomere shorting in our study was significantly associated with positive Her2 neu expression, and this was in concordance with previous studies done by Shen et al.[25] However Barczak et al [20] his observation contradict our results as they stated that increased telomere length among cases of HER2+ breast cancer. Such contradiction in different trial results may suggest might be there is other mechanism or association p53 deletion which could be responsible for this extreme difference between different studies .

5. Conclusion

In conclusion, Analysis of telomere length by FISH may serve as a prognostic tool reflecting changes of telomere length in leucocytes in different breast cancer stages. However further study with large number is recommended to give more accurate results.

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