

Value of Telomere Length in Peripheral Blood Leucocytes as a Prognostic Marker of Breast Cancer in South Egypt Cancer Institute.

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ABSTRACT

Introduction and aim of the work: 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 with disease free survival (DFS) and overall survival (OS) evaluation.

Patients and Methods: Prospective study was carried out on 83 newly diagnosed breast cancer patients with Stage 1-stage IIIc and 20 healthy individuals as control, for detection of peptide nucleic acid (PNA) by telomere specific FISH.

Results: Our results showed that, the mean length of telomere was 32.26 ± 10.08 . It revealed that telomere shortening has inferior DFS and OS when compared to patients with long telomere length, also we found a significant association of short telomere length and advanced stage started from stage IIb, as it reflects that telomere shortening has significant association with tumor size and lymph node involvement. Moreover our results showed a significant association with positive Her2 neu expression in tumor ($P < 0.001$), however it didn't show any significant association with hormonal receptors status.

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

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 represents 25% of all new cancer cases in women [2]. Telomeres are repetitive DNA repeats that can protect the chromosomes ends. In each cell division, shortening of telomeres occurs .And this regulates the cellular lifespan in somatic cells and limits their ability to renew. Overcoming this physiological barrier can occur in cancer cells, to become immortal with unlimited replication [3].

Cancer progression in cells depends on telomere maintaining mechanisms [4], which can be obtained by telomerase enzyme reactivation [5]. Alternative Lengthening of Telomeres (ALT), another pathway for telomere maintenance, which can be activated by another mechanism which does not depend on telomerase [6].

Telomere erosion during repeated cell divisions, leads to chromosomal instability and also causes genomic rearrangements that can result in occurrence of tumors [7]. Many studies of TL have been done in the last 20 years on breast cancer patients and it may be an important prognostic marker of breast cancer [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 volunteers .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 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 , never had any breast surgery including breast reduction or implants, never took a selective estrogen receptor modulator such as Tamoxifen , 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. This follow up included: 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 were taken from all patients for cytogenetic study, after mastectomy or conservative surgery. The tissue removed from patients was subjected to pathological staging (pT) according to WHO 2012 [9]. The parameters evaluated in this study included, regional lymph nodes status, vascular and lymphatic tumor invasion, presence of necrosis, ER (estrogen receptor), PR (progesterone receptor) and HER2 neu status.

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 group, 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 was collected in heparin tube for each patient and control. Preparation of metaphase spreads from blood cultured cells for FISH analysis was carried out. 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 $63 \times$ 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, Altusheim, Germany).

The chromosomes separation was done by interactive separation and dislapping functions. Then, the chromosomes were transferred to the karyotype window. An automatic DAPI banding classifier was used in chromosome classification followed by interactive corrections. Measuring the telomere was done by applying two horizontal lines to each chromosome in the karyogram, which define the telomere measurement areas (for p- and q-arms) of each chromosome. The reference signal was measured by applying two horizontal lines on the respective chromosome (chromosome 2).

The calculation of telomere length was done by a software as a ratio between the fluorescence of each telomere (T) and the fluorescence of the centromere (C) of chromosome 2 (as T/C ratio), which used as the internal reference in each metaphase analyzed as it has a stable length [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 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 $P < 0.05$.

3. Results

This study included 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 ductal carcinoma in situ (DCIS).

Analysis of these data revealed that, the number of cases < 50 years was 30 cases (36.1%), while the number of cases with age ≥ 50 years was 53 cases (63.9%). The mean size of breast masses was 4.81 ± 3.32 cm.

The study included 9 cases (10%) of grade I, 67 cases (80 %) of grade II and 13 cases (15 %) of grade III. Regarding the stage, our results included, three cases had stage I (3.6%), 42 cases stage II (55.4%) and 38 cases (45 %) of stage III. The mean telomere length was 32.26 ± 10.08 .

As regarding lymph nodes affection; 31 cases (37.3%) showed no metastatic tumor deposits (N0), 14 cases (16,9%) showed metastatic tumor deposits in 3 or less LNs (N1), and 12 cases (14,5%) showed metastatic tumor deposits in 4 to 9 LNs (N2) while 25 cases (30,1%) showed metastatic tumor deposits in more than 9 LNs (N3).

Necrosis was present in 43 cases (51.8%) and was absent in 40 cases (48.2%), vascular invasion was present in 54 cases (65.1%) and was absent in 29 cases (34.9%) and lymph infiltration was present in 61 cases (73.5%) and was absent in 22 cases (26.5%).

Regarding hormonal receptor status; the estrogen receptor was positive in 52 cases (62.7%) and negative in 31 cases (36.3%), while the progesterone receptor was positive in 30 cases (36.9%) and negative in 53 cases (63.9%). Fifty three patients (63.9%) presented with positive hormonal status (ER and /or PR positive) while 30 patients (36.1%) presented with negative ER and PR .Regarding Her2neu status, 29 patients (34.9%) presented with positive her2 neu status by Immunohistochemistry while 54 patients (65.1%) with negative Her2 neu status.

At the end of this study, the number of deaths was 19 cases (22.9%) and the number of still living patients was 64 cases (77.1%). The number of patients with disease recurrence was 35 cases (42.2%) and with absence of recurrence were in 48 cases (57.8%) (**Table 1**).

Table 1. Clinicopathological characteristics of the patients

Variable	Number of cases (%)
Age	
<50 years	30(36.1%)
≥50 years	53(63.9%)
Tumor size	
Mean ± SD	4.81±3.32
Necrosis	
Absent	40(48.2%)
present	43(51.8%)
Vascular invasion	
Absent	29(34.9%)
Present	54(65.1%)
Lymph infiltration	
Absent	22(26.5%)
Present	61(73.5%)
Lymph node metastasis	
N0	31(37.3%)
N1	14(16.9%)
N2	12(14.5%)
N3	25(30.1%)
Stages	
Stage Ib	3 (3.6%)
Stage IIa	30 (36.1%)
Stage IIb	12 (14.5%)
Stage IIIa	8 (9.5%)
Stage IIIb	5 (6.5%)
Satge IIIc	25 (30%)
Hormonal status	
ER and PR Negative	30(36.1%)
ER and /or PR positive	53(63.9%)
Her2 neu	
Negative	54(65.1%)
Positive	29(34.9%)
Classification of Breast Cancer according to Hormonal status & Her2Neu	
HR+ve & Her2neu -ve	35(42.3%)
HR-ve & Her2neu+ve	9(10.8%)
HR+ve & Her2-+ve	18(21.7%)
Triple negative	21(25.3%)
STATUS	
Living	64(77.1%)
Dead	19(22.9%)
Recurrence	
Absent	48(57.8%)
Present	35(42.2%)

ER; Estrogen receptor, **HR;** Hormonal status, **PR;** Progesterone receptor, **SD;** Standard deviation, *, significant.

3.1 Association of Telomere length and clinicopathological parameters:

T/C-FISH was performed by measuring the total fluorescence intensity of the signals of telomeres and of the centromere of chromosome 2. The fluorescence intensity is proportional to telomeric/centromeric ratio. We examined telomere intensities of the chromosomes that prepared from PBLs from all patients and controls. Twenty 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 was associated with stage IIB- stage IIIC (**Figure 2**) ($P<0.001$)(**Table 3**).. The presence of tumor necrosis, vascular and lymphatic invasion were associated with shortening of telomere length ($P<0.04$, $P<0.01$ and $P<0.03$, 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 Her2 neu expression ($P<0.001$) and Estrogen receptor negativity ($P=0.03$), but not with collective negative hormonal status (ER and PR negativity) ($P= 0.43$) (**Table 2**).

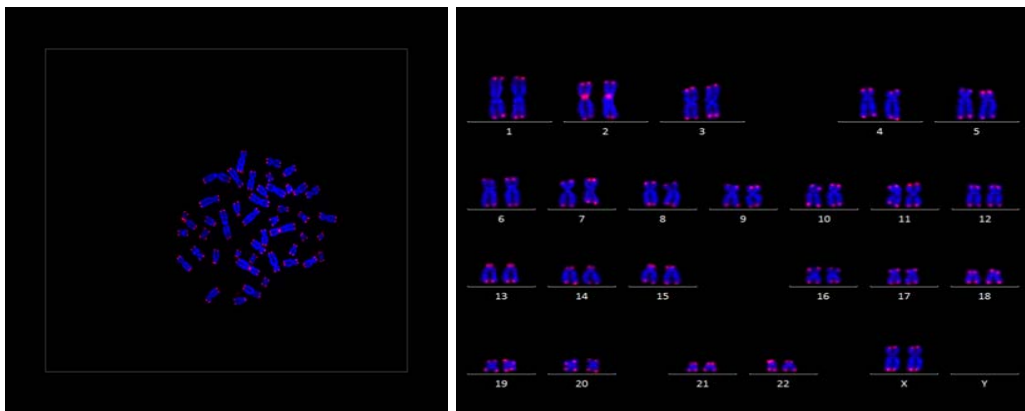


Figure 1. Telomeric signals in metaphase of peripheral blood leucocytes in a patient of early staged tumor (A) Telomeric signals in karyogram of peripheral blood leucocytes in a patient of early staged tumor (B).

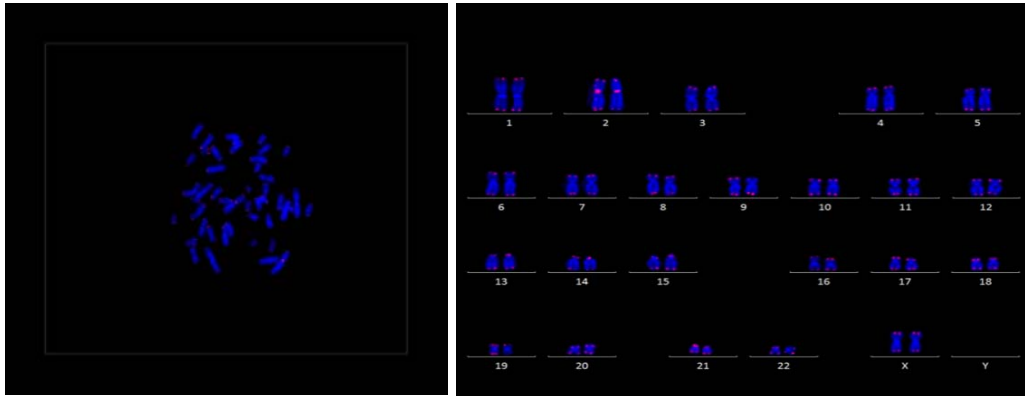


Figure 2. Telomeric signals in metaphase of peripheral blood leucocytes in a patient of late staged tumor (A)
 Telomeric signals in metaphase of peripheral blood leucocytes in a patient of late staged tumor(B).

Table 2. Association of Telomere length and clinicopathological parameters

Variable	Telomere Length Mean \pm SD	<i>P</i>
Age		
<50 years	33.8 \pm 10.9	0.28
\geq 50 years	31.3 \pm 9.6	
Necrosis		
Absent	38.14 \pm 11.55	0.04*
Present	26.78 \pm 3.27	
Lymph infiltration		
Absent	47.46 \pm 6.57	0.01*
Present	26.77 \pm 2.91	
Vascular invasion		
Absent	43.07 \pm 9.93	0.03*
Present	26.44 \pm 2.59	
Lymph node metastasis		
N0	41.72 \pm 10.58	< 0.001*
N1	27.25 \pm 1.37	
N2	24.68 \pm 1.80	
N3	26.57 \pm 1.92	
ER		
Positive	30.39 \pm 8.74	0.03*
Negative	25.39 \pm 11.47	
Hormonal status		
ER and PR Negative	29.52 \pm 6.32	0.43
ER and /or PR Positive	31.1 \pm 9.45	
Her2-neu		
Positive	24.97 \pm 1.91	< 0.001*
Negative	36.16 \pm 10.52	
Classification of breast		
HR+ve & Her2neu -ve	31.6 \pm 7.34	< 0.001*
HR-ve & Her2neu +ve	25.5 \pm 9.7	
HR+ve & Her2neu +ve	24.4 \pm 6.7	
Triple negative	30.9 \pm 7.5	

ER; Estrogen receptor, HR; Hormonal status, PR; Progesterone receptor, SD; Standard deviation *, significant.

3.2 Telomere length in breast cancer patients and control groups:

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

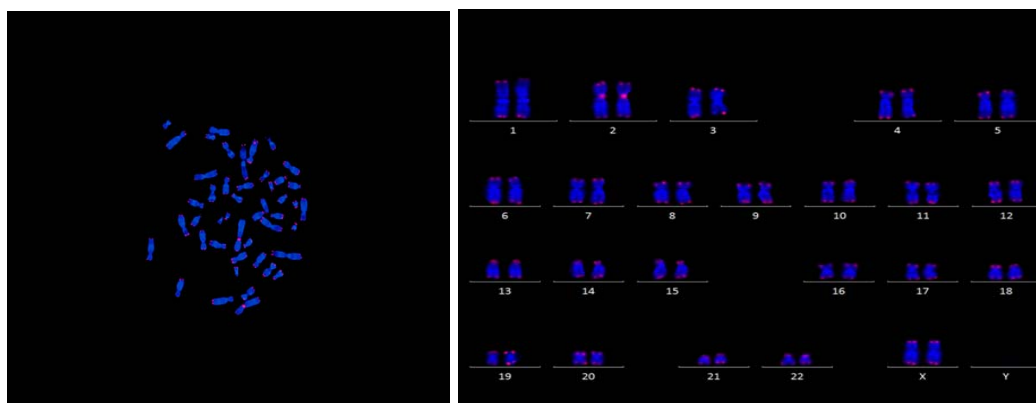


Figure 3. Telomeric signals in metaphase of peripheral blood leucocytes in control group (A). Telomeric signals in karyogram of peripheral blood leucocytes in control group (B).

Table 3. Association between Telomere length in breast cancer patients and control groups

	Number of cases	Telomere Length (Mean ± SD)	<i>P</i>
Control group	20	20.10 ± 0.91	
Breast cancer patients	83	32.26±10.08	<0.001*
Stage I & Stage IIa	22	48.43 ± 3.60	
Stage IIb –Stage IIIC	61	26.42 ± 1.96	

SD; Standard deviation *; significant

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 ($P<0.003$) and OS ($P<0.001$) (**Figure 4.a,b**). Despite telomere shortening showed a significant statistical association with negative estrogen expression ($P<0.05$) However, there was no 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)

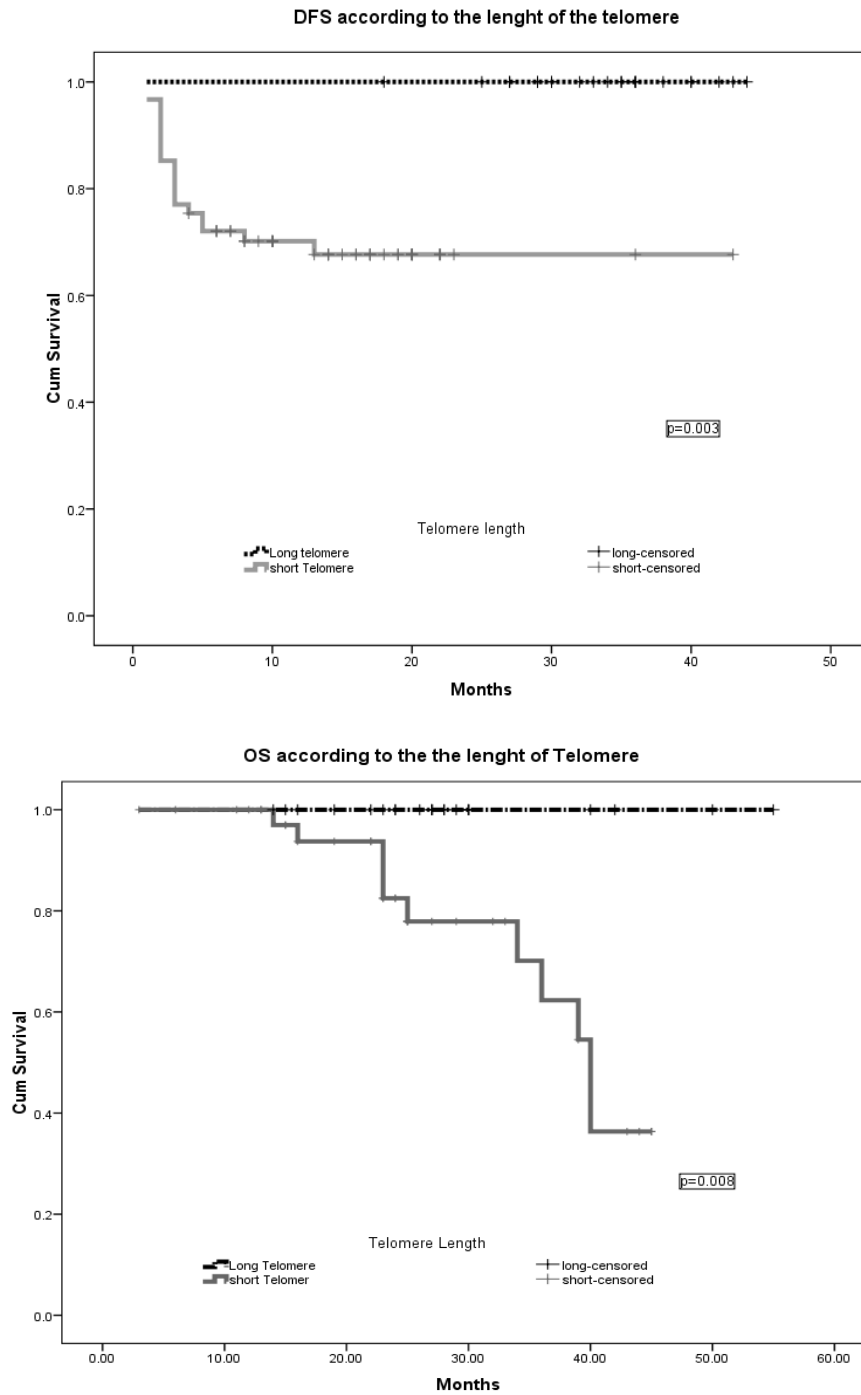


Figure 4. association between Telomere length with DFS (4a) and OS (4b)

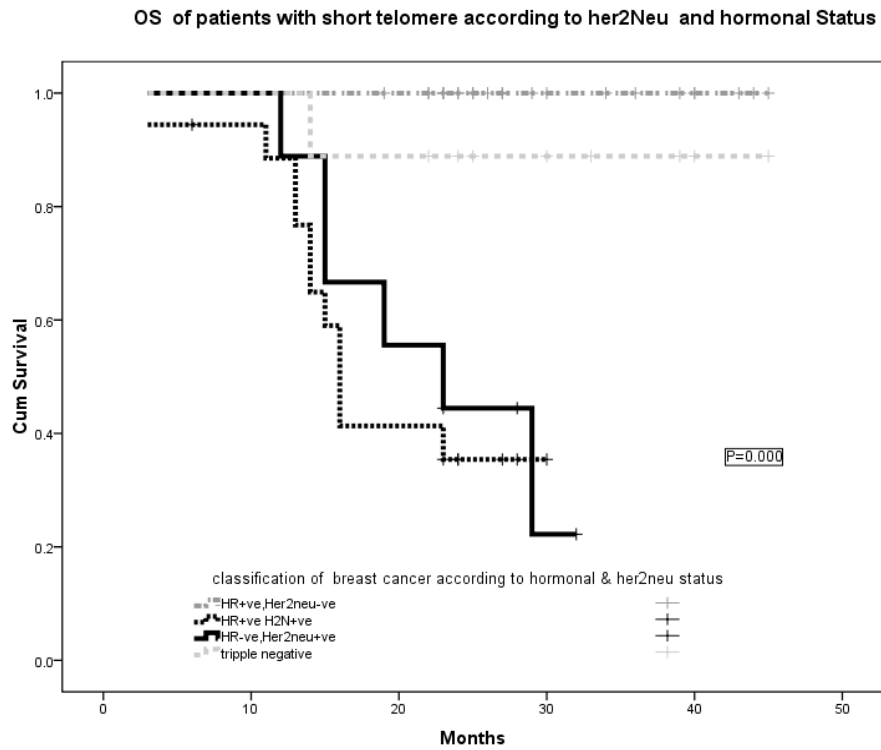
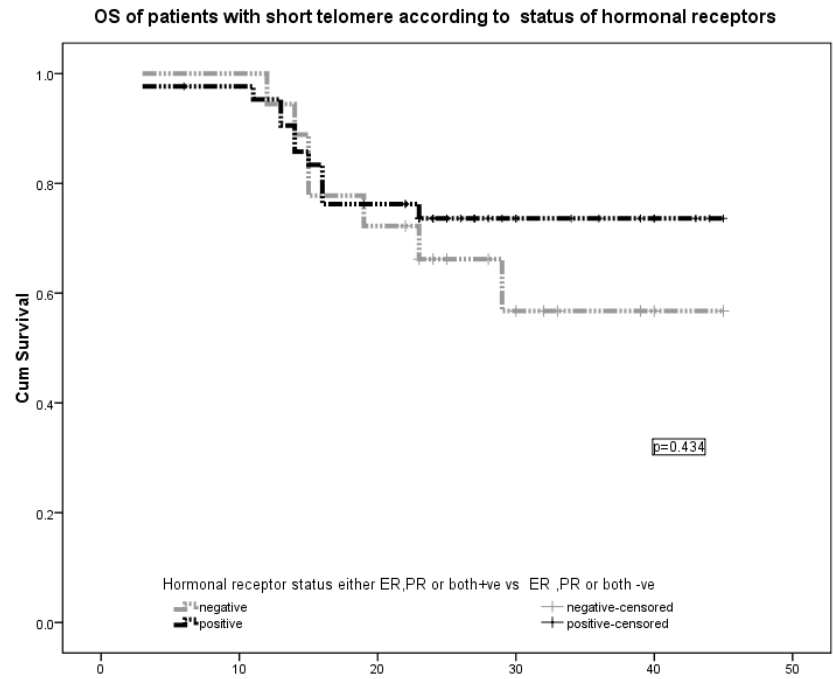


Figure 5.(a) Association between short telomere length and Hormonal status.

(b) Classification of breast cancer according to Hormonal status and Her2neu.

4. Discussion

Breast cancer is considered the major cause of cancer-related deaths due to cancer in women globally [11]. Telomeres presents at chromosomal ends with repeated sequence TTAGGG [12].they have an important role in protecting the chromosomal ends, preventing the DNA damage response and preserving genomic stability [4]. Also, telomeres have a vital role in cancer development mechanisms [13]. Cancer cells have unlimited capability for division through maintenance of telomeres by increased telomerase activity or by an alternative lengthening of telomeres (ALT) mechanism [14], and these mechanisms lead to presence of abnormal clones of cells which become genomically-unstable during carcinogenesis [15].

In our study, we found that telomere lengthening in blood leucocytes was significantly associated with early stages in 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 between breast cancer patients and age adjusted normal controls. However, this may be due to ethnic variation as there is a 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 lengthening 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 staged tumor presented with Her 2 neu negative disease. This could affect the convulsion of results and could also explain the contradiction of other study by 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, that might show associated 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 significantly associated with positive estrogen expression but it was not strong association; this finding was matched with Ennour-Idrissi et al [21]. Association of longer telomeres with increased breast cancer risk may also be due to an estrogen effect, as increased exposure to estrogen is a well-known risk factor for development of breast cancer. Estrogen affects telomere length directly through the activation of the promoter of human telomerase enzyme [22], as well as by post transcriptional regulation of telomerase [23]. However, there was 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 tumor characteristics like age of the patients, tumor size and grade, we did not find any association between telomere length and above characteristics.

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 association with tumor 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, the observation of Barczak et al [20] contradict our results as they stated that there was an increased telomere length among cases of Her2 neu + breast cancer cases. Such contradiction in different trial results may suggest that there are other

mechanisms or associations with 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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