Prognostic Value of Telomere Length in Peripheral Blood Leucocytes in Breast Cancer patients

(South Egypt Cancer Institute Experience).

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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) in previous studies in breast cancer patients suggested the possibility of having 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) to evaluate the difference between their TL and control group also to correlate between TL and clinicopathological characteristics and survival of those patients

Patients and Methods: This is a prospective study which included 83 newly diagnosed breast cancer patients with Stage I to stage IIIC and 20 healthy individuals as control group, 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. ~~Also our results revealed that telomere shortening has inferior DFS and OS when compared to patients with long telomere length,~~ Patients with short TL had inferior DFS and OS than those with long TL. We found significant associations of short telomere length with advanced stages of disease, starting from stage IIB onwards ~~started from stage IIb, as it reflecting the telomere shortening was has significantly association with more~~ and with high lymph node involvement. Furthermore, our results showed a significant association with positive Her2 neu expression in tumor (P = 0.001). On the other hand, our results 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 patients.

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 sequences ~~repeats that can~~ that protects the chromosomes ends. In each cell division, shortening of telomeres occurs. 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~~ is independent of 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 TL in breast cancer patients by telomere specific fluorescence in situ hybridization (FISH) in peripheral blood leucocytes (PBL) to evaluate the difference between their TL and control group also to correlate between TL and clinicopathological characteristics and survival of those patients

2. PATIENTS AND METHODS

This study is a prospective case control study which included 83 patients newly diagnosed breast cancer and 20 healthy volunteers as control group. All patients were diagnosed in South Egypt Cancer Institute (SECI) from December 2014 to January 2016.

We included patients with non-metastatic AJCC 7th Edition stage I to stage IIIC ~~only~~ invasive ductal carcinoma. ~~was included in this study.~~ Patients 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 excepting tru-cut or excision biopsy from breast lump for tissue diagnosis and mastectomy or breast conserving surgery, never took a selective estrogen receptor modulator such as Tamoxifen , and did not receive any neoadjuvant chemotherapy prior to surgery.

All control volunteers who were recruited in the study (n=20) were female with comparable age to patients and all of them were subjected to breast ~~sonar~~ high resolution ultrasound and X ray mammography before blood sample withdrawal ~~of the sample~~ to be sure they are not have breast cancer. These volunteers were not 1st or 2nd degree relatives of the patients (clarification ???).

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 (pTNM) according to WHO Classification of Tumours of the Breast published in 2012. ~~WHO 2012~~ (clarification ???) [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~~ was approved by International Review Board (IRB) at SECI ethical committee (SECI-IRB IORG0006563 N0185/2014.). All study recruits signed an informed 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.

Heparinized whole blood samples were ~~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 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).

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, with only 20 (24%) patients having associated ductal carcinoma in situ (DCIS).

Analysis of these data revealed that, the number of cases ≤45 years was 26 cases (31.3%), while the number of cases with age >50 years was 57 cases (68.9%).

Regarding the grade, stages and tumors size, our results included 9 cases (10%) of grade I, 67 cases (80 %) of grade II and 13 cases (15 %) of grade III.

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. The mean size of breast masses was 4.8 ± 3.32cm.

~~With respect to lymph nodes affection~~; As for lymph node involvement, 31 cases (37.3%) showed no metastatic tumor deposits (pN0), 14 cases (16,9%) showed metastatic tumor deposits in 3 or less LNs (pN1), and 12 cases (14,5%) showed metastatic tumor deposits in 4 to 9 LNs (pN2) while 25 cases (30,1%) showed metastatic tumor deposits in more than 9 LNs (pN3).

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%).~~

Estrogen receptors were positive in 52 cases (62.7%) and ~~negative in 31 cases (36.3%), while the~~ progesterone receptors were 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 .

Concerning the 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 were 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 - pathological	
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).**

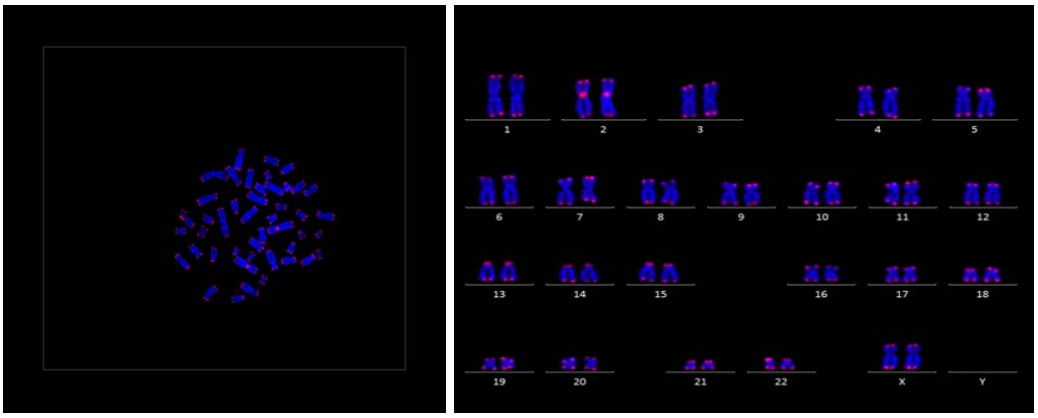


Figure 1. Telomeric signals in metaphase of peripheral blood leucocytes in a patient of early staged tumor (A) Telomeric signals in karyogram of peripheral blood leucocytes in a patient of early staged tumor (B).

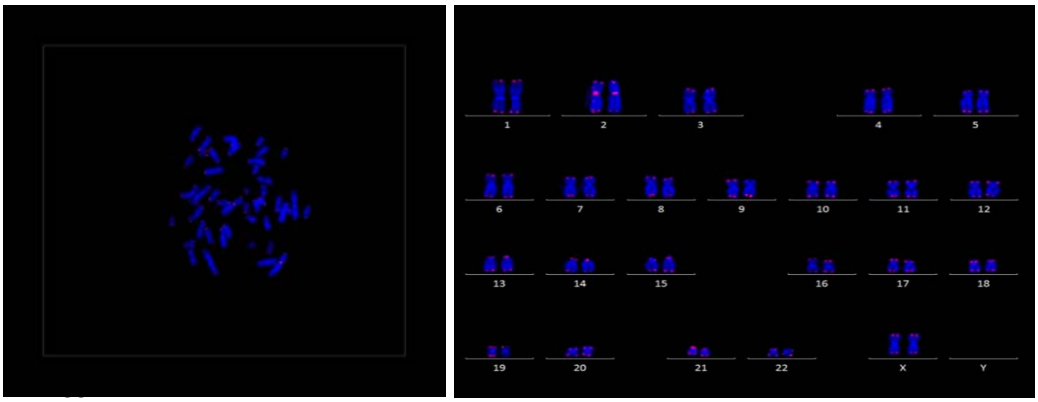


Figure 2. Telomeric signals in metaphase of peripheral blood leucocytes in a patient of late staged tumor **(A)** Telomeric signals in metaphase of peripheral blood leucocytes in a patient of late staged tumor(B).

Table 2. Association of Telomere length and clinicopatholgical parameters

Variable	Telomere Length Mean ± SD	*P*
Age		
<50 years	33.8 ± 10.9	**0.28**
≥50 years	31.3 ± 9.6	
Necrosis		
Absent	38.14 ± 11.55	**0.04***
Present	26.78 ± 3.27	
Lymph infiltration		
Absent	47.46 ± 6.57	**0.01***
Present	26.77 ± 2.91	
Vascular invasion		
Absent	43.07 ± 9.93	**0.03***
Present	26.44± 2.59	
Lymph node metastasis		
N0	41.72 ± 10.58	
N1	27.25 ± 1.37	**0.001***
N2	24.68 ± 1.80	

N3	26.57 ± 1.92	
ER		
Positive	30.39 ± 8.74	**0.03***
Negative	25.39 ± 11.47	
Hormonal status		
ER and PR Negative	29.5 2± 6.32	**0.43**
ER and /or PR Positive	31.1± 9.45	
Her2-neu		
Positive	24.97 ± 1.91	**0.001***
Negative	36.16 ± 10.52	
Classification of breast		
HR+ve & Her2neu -ve	31,6+ 7.34	
HR-ve & Her2neu +ve	25.5+97	**0.001***
HR+ve & Her2neu +ve	24.4+67	
Triple negative	30.9+75	

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 presence of breast cancer (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).**

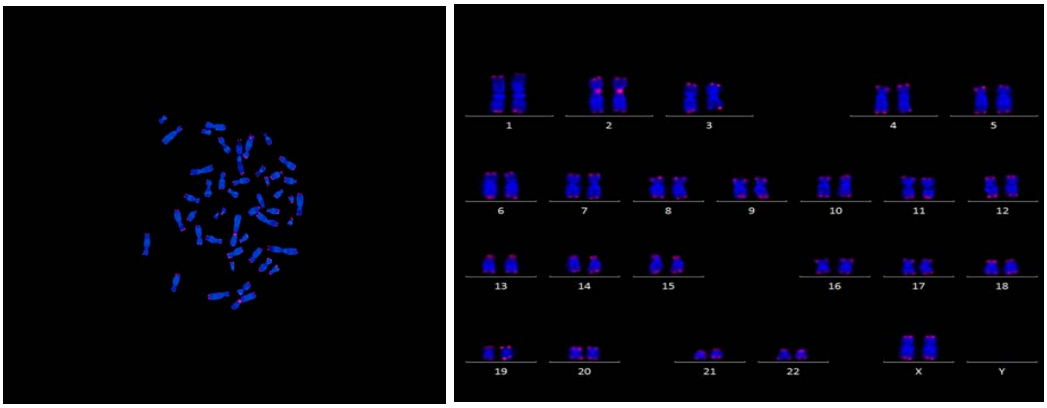


Figure 3: Telomeric signals in metaphase of peripheral blood leucocytes in control group (A). Telomeric signals in karyogram of peripheral blood leucocytes in control group (B).

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

	Number of cases	**Telomere Length (Mean ± SD)**	***P***

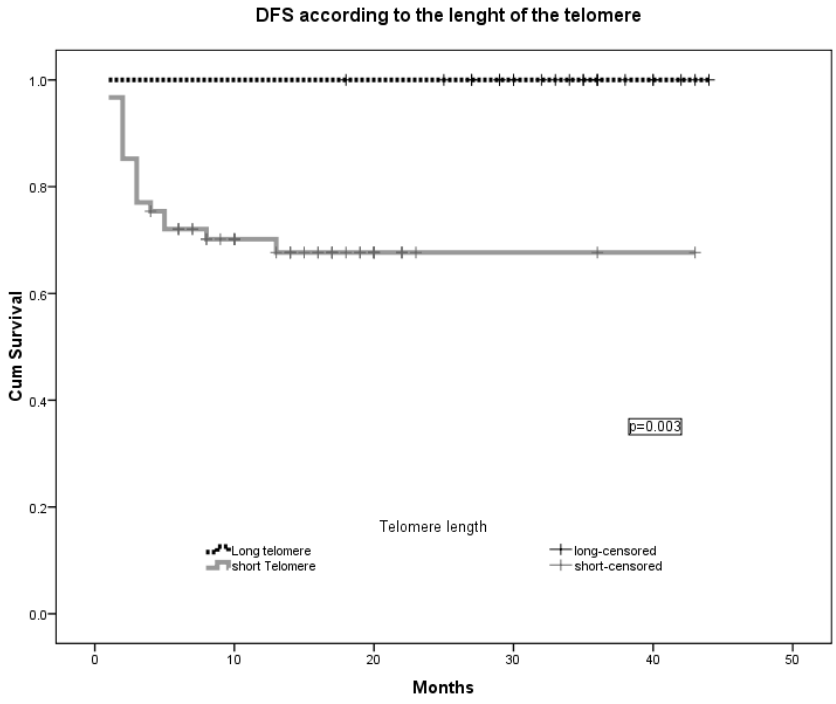
Control group	20	20.10 ± 0.91	
Breast cancer patients	83	32.26±10.08	**0.001***
Stage I & Stage IIA	22	48.43 ± 3.60	
Stage IIB –Stage IIIC	61	26.42 ± 1.96	

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 significant statistical association with negative estrogen expression (*P*=0.05) However, there was no statistical difference found between total positive hormonal status (ER and/or PR positive) versus both ER and PR negative (*P=0.43*)**, (Figure 5a)** on the other hand telomere shorting showed significant association with Positive Her2 neu expression (*P=0.000*). **(Figure 5b)**



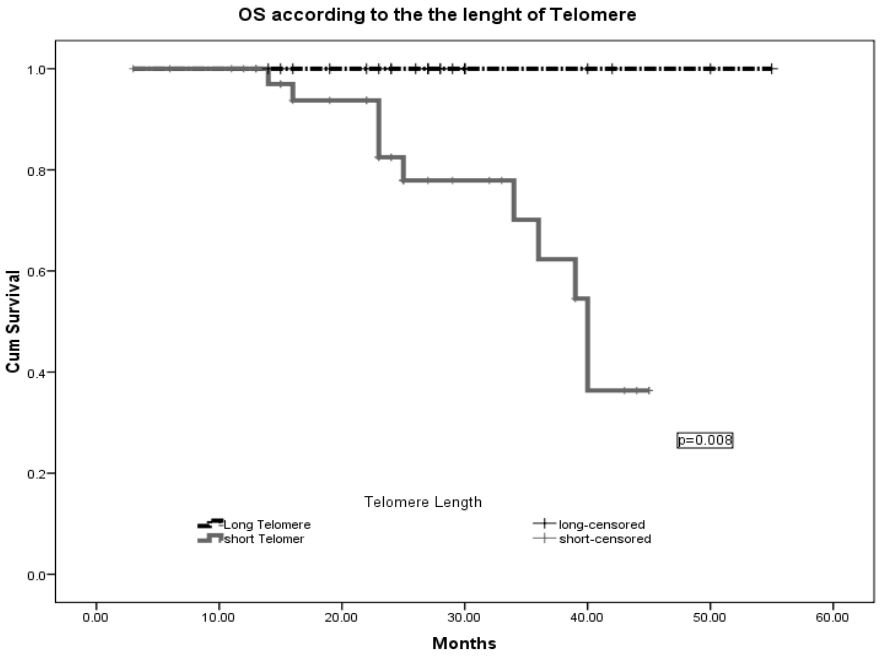
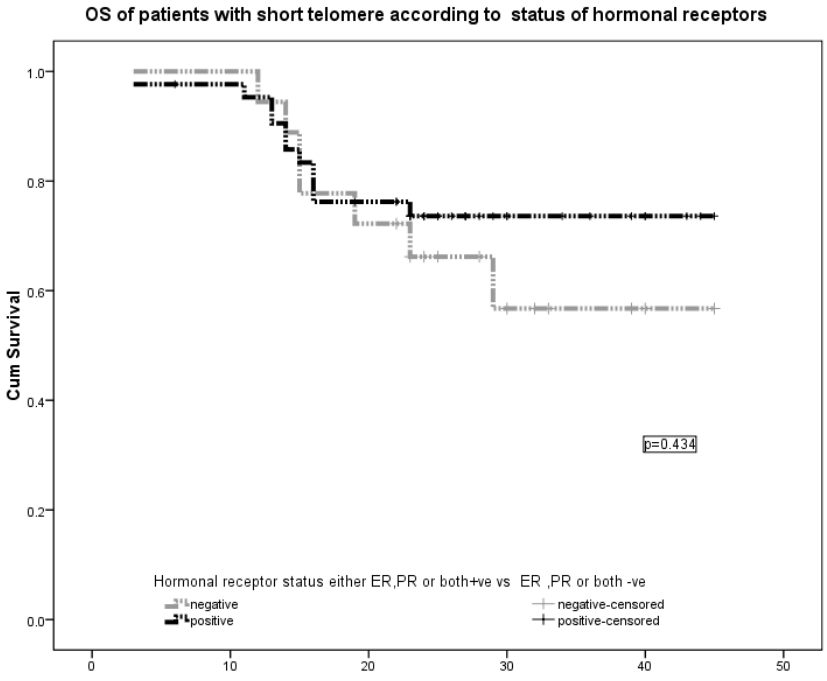


Figure 4. association between Telomere length with DFS (4a) and OS (4b)



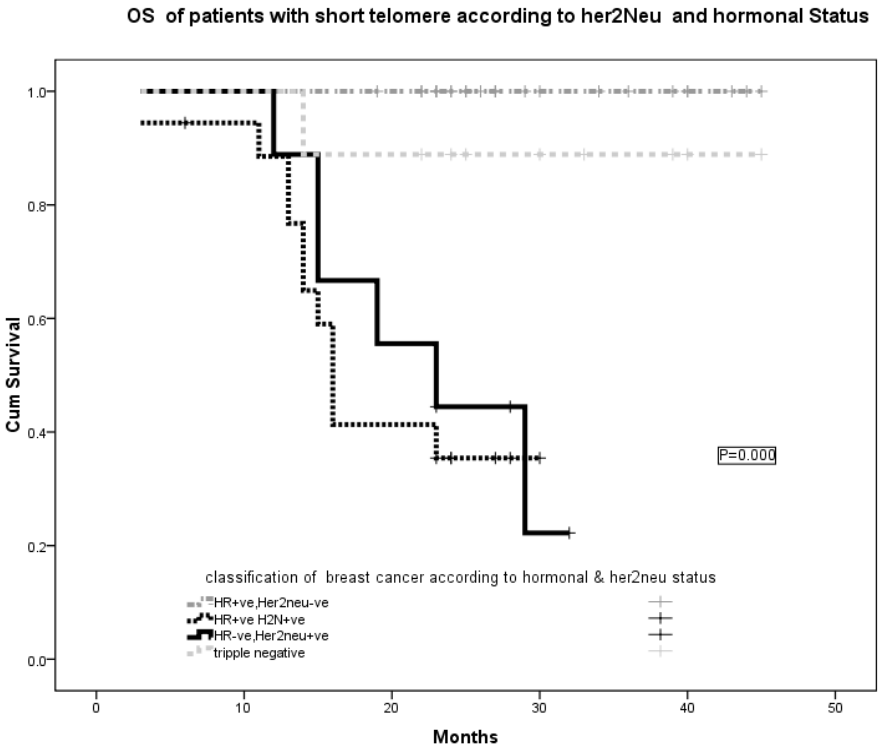


Figure 5.(a) Association between short telomere length and Hormonal status.

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

4. Discussion

Breast cancer is considered the major cause of cancer-related deaths 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 Hamatges 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 IIA, than in leucocytes in the advanced stages (stage IIB to stage IIIC) and this ~~results was~~ results were in line with the study done by Barczak W et al [20] who explained that ~~by~~ short or long ??? telomere length is significantly associated with lymph node metastases. So patients with negative LN involvement have long telomere length and longer overall survival . Moreover, in our study most of early staged tumor presented with Her 2 neu negative disease. This could explain our results, also could also clarify the contradiction of other study by Ennour-Idrissi et al, who reported that no association was observed for telomere length with stage ,that might be explained by bad prognostic criteria was found in their early staged patients or because due to using different method of measurement of the telomere length [8,20].

Telomere lengthening showed marginal statistical significant association with positive estrogen expression, ~~this~~ finding of which 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].

On the other hand, there was no association between telomere length and PR expression that was in agreement with Jones et al [24]. Telomere length didn't show

any significant association with positive hormonal receptors (either ER and /or PR positivity) and that matched with Ennour-Idrissi et al [21].

We did not find any statistically significant association of telomere length with age, tumor size and grade.

~~Regarding the correlation others patients characteristics like age, tumor size and grade, we did not find any statistical association between them and telomere length~~

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 in their study that no association was observed for telomere length and tumor size, which was in agreement with our results [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] in opposition, 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 to reflect changes of telomere length in leucocytes in different breast cancer stages and with presence bad prognostic markers. However further study with large number is recommended to give more accurate results.

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