Patterns of Relative Telomere Length is Associated With *hTERT* Gene Expression in the Tissue of Patients With Breast Cancer

Karuvaje Thriveni,[1] Anisha Raju,[1] Rekha V. Kumar,[2] Swamyvelu Krishnamurthy,[3] Ramesh Chaluvarayaswamy[4]

Abstract

Leukocyte telomere length is one of the prognostic factors in breast cancer. In this study, we analyzed the telomere length from breast cancer tissue, which is relatively increased with hTERT mRNA expression, in 98 patients. The results showed shortened tissue telomere in early stages and elongated telomere in advanced stages, in contrast to the leukocyte telomere length pattern, indicating different dynamics in breast cancer tissue.

Background: Homeostasis of telomere in breast cancer might be altered as a result of cumulative effects of various factors causing genomic instability and affecting prognosis. This study aimed to compare the relative telomere length (RTL) and *hTERT* mRNA expression in the tissue of patients with breast cancer along with the clinicopathologic parameters. **Patients and Methods:** Frozen tumor tissues and adjacent normal breast tissue from 98 patients with invasive ductal breast cancer were used for the analysis. RTL and *hTERT* mRNA expression were measured using quantitative real time polymerase chain reaction. **Results:** Among the 98 cases, 51% had an early-stage carcinoma, 66% were tumor size < 5 cm, 30% were node-negative, and 20% were low-grade tumors. In this study, 63% of cases showed higher *hTERT* gene expression with an odds ratio of 2.77 ($P = .02$). The median RTL for elongated telomere was 3.49, and the value was significantly elevated when compared with the shorter telomere. Shortened RTL was present in 60% of early-stage cancer cases, 55% where the tumor size was < 5 cm, 72% of the lymph node-negative cases, and 68% of low-grade carcinoma. Significantly elongated RTL, with median 4.22, 3.19, 3.17, and 3.28 was observed ($P < .05$) in the advanced stage, larger tumor size, node-positive, and high-grade cases respectively. **Conclusion:** In this study, shortened telomere was observed in early-stage cancer, and elongated telomere was found in advanced diseases. However, 13% of patients with lower *hTERT* gene expression showed elongated telomeres, indicating relative telomere length measurement in tissue is different from blood leukocyte, showing the dynamic process of tumorigenesis in tissue.

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Introduction

Cancer is known to be a multifactorial disease resulting from genetic and environmental interactions. Breast cancer is the most common malignancy and the leading cause of cancer deaths in women. Telomeres are capping nucleoprotein structures presents at the end of each chromosome, comprising hexameric tandem repeats of noncoding $(TTAGGG)_n$ with a 5 to 15kb length with telomere binding proteins.[1] Normally, telomere shortens on each round of replication. Telomeres are essential to protect it from chromosome end-to-end fusion, nucleolytic decay, being attacked by DNA repair enzymes at double-stranded breaks, and aberrant recombination, thus maintaining chromosome integrity and genomic stability.[2] Telomeres are essential to prevent improper fusion of chromosomes, which otherwise may cause increasing mutation potential.[3] In addition, alteration in telomere length (TL) may prevent apoptosis and help cells to continuously divide, ultimately leading to cancer development.

TL is maintained by enzyme telomerase, which is overexpressed in germ cells and neoplastic tissue.[1,4] In most eukaryotic tissue,

[1]Department of Biochemistry
[2]Department of Pathology
[3]Department of Surgical Oncology
[4]Department of Biostatistics, Kidwai Cancer Institute, Bangalore, India

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Address for correspondence: Dr Karuvaje Thriveni, PhD, Associate Professor, Department of Biochemistry, Kidwai Cancer Institute, Dr M.H. Marigowda Road, Bangalore 560029, India
E-mail contact: thrivenibhat@yahoo.com

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Table 1 Primer Sequences Used in This Study

Parameter	Primer Sequences	Size
Telo Forward	5'CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3'	39 bp
Telo Reverse	5'GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3'	39 bp
36B4 (single copy gene) Forward	5'-CAGCAAGTGGGAAGGTGTAATCC-3'	23 bp
36B4 Reverse	5'-CCCATTCTATCATCAACGGGTACAA-3'	25 bp
hTERT gene Forward	5'-GCACCCTCTTCAAGTGCTGT-3'	20 bp 20 bp
hTERT gene Reverse	5'-AAGTTCCTGCACTGGCTGAT-3'	20 bp
Beta-2-microglobulin-Forward	5'-GAGTATGCCTGCCGTGTG-3'	18 bp
Beta-2-microglobulin-Reverse	5'-AATCCAAATGCGGCATCT-3'	18 bp

telomerase expression may prevent telomere erosion. Human telomerase consists of 2 components; telomerase reverse transcriptase (hTERT) and telomere RNA component (hTR). The *hTR* and *TEP1* genes are ubiquitously expressed in both normal and tumor cells, whereas that of *hTERT* expression is physiologically confined to primary germline cells and tumor cells, indicating *hTERT* as the limiting factor for telomerase activity.[5,6] In normal replicating cells, progressive telomere attrition takes place in the absence of telomerase and alternative lengthening mechanism, leading to apoptosis.[7] Thus, measurement of TL gives an indication of the replicative potential of human cells in health and disease conditions.[8] In general, shorter TL is observed in cancer tissues than in adjacent tissues, as shown in patients with cancers of the lung and colorectal and breast cancer.[9] Telomere attrition is an early event in human breast cancer initiation and development because very short telomeres are more often found in premalignant breast lesions.[10]

Studies showed that samples with the shortest telomeres are preferably targeted by telomerase for elongation.[11] However, during replicative senescence in human somatic cells, in the absence of telomerase activity, telomeres eventually shorten and initiate cell cycle arrest at G1 phase.[12] Thus, telomere shortening limits the number of somatic cell divisions and provides an effective tumor suppressor mechanism. On the contrary, the presence of telomerase enzyme allows cells to divide indefinitely.[13] Most tumors express telomerase and maintain TL as a step of carcinogenesis.[14] Several reports have demonstrated that an alternative mechanism takes place for telomere maintenance including recombination processes.[15] The association between TL and the risk of cancer is ambiguous. The objective of the study was to measure the relative telomere length (RTL) in surgically resected tumor and adjacent normal tissue, to compare the relationship between TL and *hTERT* gene expression, along with prognostic factors of patients with breast cancer.

Patients and Methods

The Scientific Review Board and Medical Ethics Committee of the Institute approved this study. All individuals provided written informed consent before participating in the study. The informed consent was made as per Declaration of Helsinki. This cross-sectional study included patients with breast cancer who were registered in our Oncology Institute in between June 2015 and March 2016. We collected consecutive series of surgical fresh tissue samples from 110 cases of invasive ductal carcinoma prior to treatment and recorded the detailed clinical history from the case files. The tumor and adjacent grossly normal tissues were collected in duplicates in respective storage vials. The samples were stored at −80°C for genomic DNA (gDNA) isolation and RNA isolation in RNAlater (Sigma-Aldrich).

hTERT mRNA Analysis

Total RNA was isolated manually by the TRIzol (Sigma-Aldrich) method. Complementary DNA (cDNA) was synthesized by Roche kit as per the instruction manual. The resulting cDNA was subjected to gene expression analysis in terms of fold change by quantitative real-time polymerase chain reaction (PCR). Quantitative PCR was performed with SYBR Green PCR master mix (Thermo Fischer Scientific) reagents in a 10-μL reaction mixture containing 100 ng cDNA and 400 nM each of forward and reverse primers. Housekeeping gene *Beta2 microglobulin* served as the internal control for the expression levels of the target gene. hTERT gene expression was normalized based on the cycle threshold (C_t) value (18-24) of the housekeeping gene. Reactions with no templates were included as negative control. By setting the threshold at the level of the middle steady portion of the reaction cycles versus fluorescence curve, the C_t values of the target genes were calculated using the $2^{(-\Delta\Delta Ct)}$ method. The primer sequences are as mentioned in Table 1. All primers were standardized by conventional semi-quantitative PCR analysis before proceeding to the real-time quantitative analysis. The primers for *hTERT* gene expression were designed in a way to detect both α and β variants of the gene.

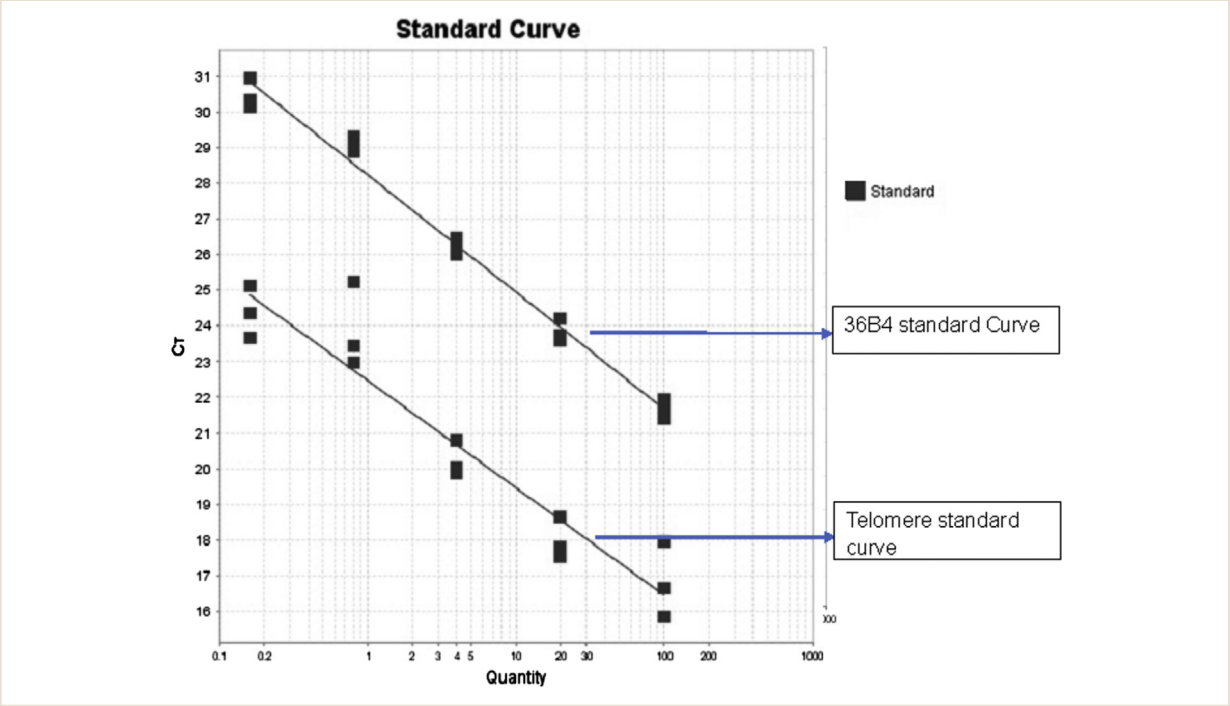
hTR mRNA Analysis

To synthesize primers, we used the site www.ncbi.nlm.nih.gov/tools/primer-blast/. The blast obtained for the primer synthesis was not good enough to carry the *hTR* mRNA gene expression procedure. Hence, analysis of *hTR* mRNA expression was excluded from the study.

TL Analysis

For RTL analysis, gDNA was extracted from tumor and adjacent tissue using QIAamp DNA kit (Qiagen) as per manufacturer's protocol by column method. Quantitative real-time PCR was used to measure RTL by following standard procedure[16,17] in 10-μL reaction mixture containing 1X SYBR Green PCR Master Mix (Thermo Fischer Scientific), 200 nM of each forward and reverse primers (Table 1), and 20 ng of gDNA. To produce a 5-point

Figure 1 Standard curve used to measure relative T/S ratio. Five DNA concentration over a 5-fold range was generated by a serial dilution and aliquoted to microtiter plate wells. The final concentration per well ranged from 0.16 ng/μL to 100 ng/ng/μL



standard curve, serial dilutions of a standard DNA sample were used as calibrator. The concentrations ranged from 0.16 ng to 100 ng/μL as shown in Figure 1. The gDNA of samples were run in triplicate with a series of standard and a reference sample. A single reference sample was used in all sets of the experiment. The values of the reference sample enabled us to find out inter-assay variability. The efficiency of the reaction was evaluated by performing experiments with a calibration curve. After properly setting the baseline and threshold, the slope of the calibration curve was translated into the efficiency value with the formula $E=10^{(-1/\text{slope})}-1$. The calibration curve for both single copy gene and telomere run parallel to each other, showing similar efficiency, as illustrated in Figure 1. The coefficient of variation for telomere measurement assay was 6.8% across all the batches.

The PCR procedure was carried out on a StepOnePlus Real-Time Applied Biosystem Analyzer, (Thermo Fisher Scientific), and version 2.0 software programs calculated the values. RTL was measured in a known quantity of gDNA as the ratio of telomere repeat copy number to the single copy gene (SCG) number based on the standard curve in the same plate. The number of real-time PCR cycles required to reach threshold fluorescence was recorded as C_t, and the results were recorded as relative T/S ratio. Initial copy number of telomere or SCG was inversely related to the C_t value. The T/S ratio indicated a number of copies of telomere in a diploid genome. Generally, the relative T/S ratio was normalized with reference DNA so that it enabled the comparisons of T/S ratio across the assays. The following formula was used to calculate relative T/S ratio. Based on the formula $2-(\Delta C_t)$, where $\Delta C_t = C_t$ telomere $- C_t$ SCG, the relative quantity of telomere versus albumin (T/S) was calculated

$$\Delta C_t(\text{Unknown sample}) - \Delta C_t(\text{Reference sample}) = \Delta\Delta C_t;\ \text{Relative T/S ratio} = 2^{(-\Delta\Delta C_t)}$$

Statistical Analysis

Statistical analysis was carried out by R software version 3.2.2 (R Foundation for Statistical Computing, Vienna, Austria). The Pearson χ^2 and Fischer exact probability tests were used to find the significance of association between different variables. RTL and *hTERT* mRNA expression was nonparametric continuous variables as it was not following Kolmogorov-Smirnov and Shapiro-Wilk tests. The Mann-Whitney *U* test was applied to find the median values with interquartile 1 and 3 (IQR) and association with categorical variables. Age-adjusted RTL logistic regression analysis did not improve an association between age and telomere attrition. Spearman rank correlation was used to analyze the r value between RTL and *hTERT* mRNA expression. The type 1 error rate was 5% throughout the analysis.

Results

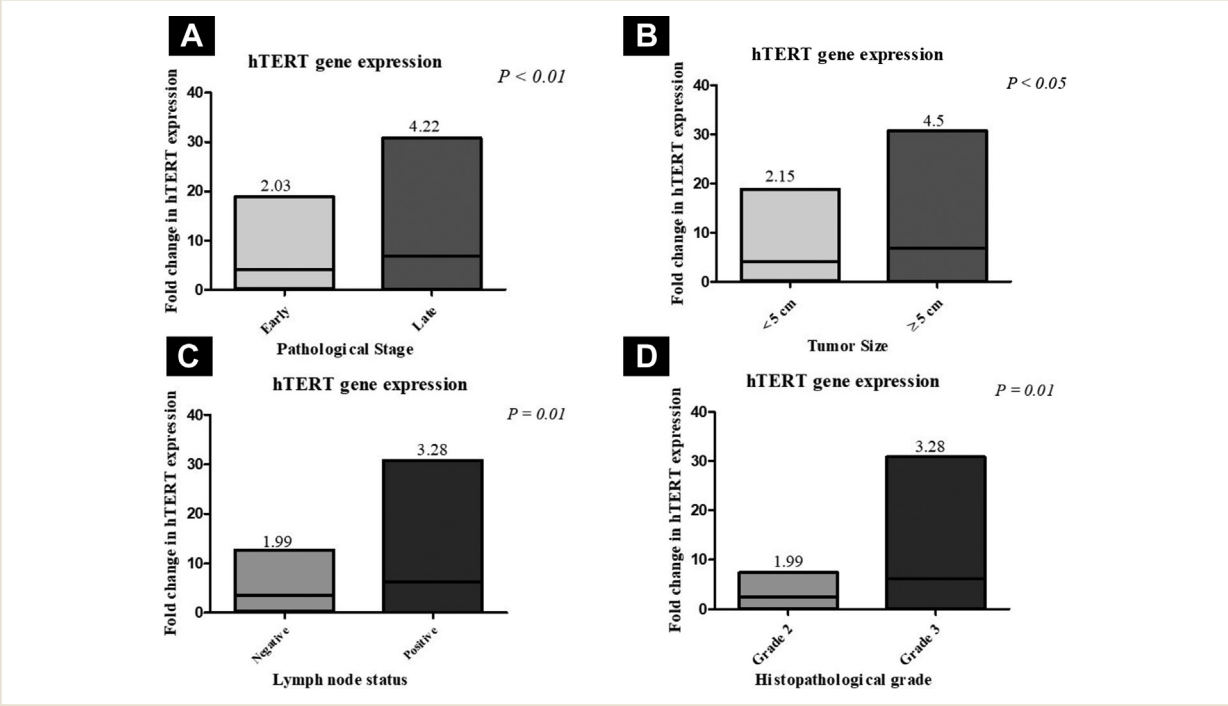
Among 110 samples, the results were available for 98 cases because gDNA and cDNA from 12 cases were not of good quality and were inadequate for quantification. The clinicopathologic features of patients with breast cancer are summarized in Table 2 as age at diagnosis, menopausal status, clinical stage, tumor size, lymph node status, the Ellis and Elston modification of the Scarff-Bloom-

Table 2 Clinicopathologic Variables in Association With *hTERT* mRNA Expression and Telomere Length in 98 Patients With Breast Cancer

Variables	*hTERT* mRNA Expression		Telomere Length	
	Low (n = 36), n (%)	High (n = 62), n (%)	Short (n = 47), n (%)	Long (n = 51), n (%)
Telomere length				
Short (n = 47)	23 (23)	24 (25)	_	_
Long (n = 51)	13 (13)	38 (39)	_	_
P value	.02[a]			
OR (95% CI)	2.77 (1.1-7.2)			
Age, y				
≤ 40 (n = 33)	13 (13)	20 (20	12 (12)	21 (21)
> 40 (n = 65)	23 (23)	42 (42)	35 (36)	30 (31)
P value	.83		.14	
Menopausal status				
Pre (n = 45)	19 (19)	26 (27)	19 (19)	26 (27)
Post (n = 53)	17 (17)	36 (37)	28 (29)	25 (25)
P value	.40		.32	
Stage				
Early (n = 50)	24 (24)	26 (27)	30 (31)	20 (20)
Late (n = 48)	12 (12)	36 (37)	17 (17)	31 (32)
P value	.02[a]		.01[a]	
OR (95% CI)	2.74 (1.08-7.19)	2.7 (1.12-6.73)		
Tumor size, cm				
< 5 (n = 65)	28 (29)	34 (35)	36 (37)	29 (30)
≥ 5 (n = 33)	8 (8)	25 (25)	11 (11)	22 (22)
P value	.049[a]		.05	
OR (95% CI)	2.54 (0.93-7.6)		2.46 (0.95-6.61)	
Nodal status				
Negative (n = 29)	16 (16)	13 (13)	21 (21)	8 (8)
Positive (n = 69)	20 (20)	49 (50)	26 (27)	43 (44)
P value	.02[a]		.002[a]	
OR (95% CI)	2.97 (1.17-8.15)		4.27 (1.54-12.88)	
Tumor grade				
Low (n = 19)	10 (10)	9 (9)	13 (13)	6 (6)
High (n = 79)	26 (27)	53 (54)	34 (35)	45 (46)
P value	.12		.07	
ER status				
Negative (n = 37)	16 (16)	21 (21)	21 (21)	16 (16)
Positive (n = 61)	20 (20)	41 (43)	26 (27)	35 (36)
P value	.38		.21	
PR status				
Negative (n = 36)	17 (17)	19 (19)	21 (21)	15 (15)
Positive (n = 62)	19 (19)	43 (45)	26 (27)	36 (37)
P value	.13		.14	
HER-2/neu status				
Negative (n = 56)	16 (16)	40 (41)	29 (30)	27 (28)
Positive (n = 42)	20 (20)	22 (23)	18 (18)	24 (24)
P value	.06		.41	
Triple-negative (n = 19)	9 (9)	10 (10)	13 (13)	6 (6)
Others (n = 79)	27 (28)	52 (53)	34 (35)	45 (46)
P value	.30		.07	

Abbreviations: CI = Confidence interval; ER = estrogen receptor; HER2 = human epidermal growth factor receptor 2; OR = odds ratio; PR = progesterone receptor
[a] *P* value < .05

Figure 2 Gene expression analysis of *hTERT* by real-time polymerase chain reaction. Expression was measured in terms of fold change. *hTERT* expression showed significant association with late pathologic stage (A); tumor size (B); histopathologic grade 3 (C); and positive lymph node status (D). The corresponding median values are represented above the bars



Richardson grade, hormone receptor status, and human epidermal growth factor receptor 2 (HER-2/neu) status. The median age of the patients was 49 years, and the age range was 26 to 78 years. Early-stage breast cancer as per TNM classification included women diagnosed with stage I and stage IIA and IIB without lymph node metastasis; late-stage breast cancer included stage III and stage IV diseases. Based on tumor size, patients were categorized into tumors < 5 cm and ≥ 5 cm. Patients with grade 1 and grade 2 were grouped as low-grade, and grade 3 pathologic tumors were considered under a high-grade classification. The estrogen receptor (ER) and progesterone receptor (PR) nuclear localization was scored using the Allred scoring system, considering the proportion of positive cells with the staining intensity. According to American Society of Clinical Oncology/College of American Pathologists guidelines, $\geq 1\%$ positive cells have been considered as a positive test in invasive breast carcinoma tumor cells. Immunohistochemistry 3+ was considered positive for HER-2/neu status based on circumferential membrane staining.

hTERT Gene Expression

The general consensus for measuring gene expression was a 2-fold cutoff when compared with adjacent border normal breast tissue. *hTERT* mRNA expression was grouped into low and high expressions based on the presence of a 2-fold increase in gene expression in tumor tissue (Table 2). In this study, 63% (62 of 98) of cases showed higher *hTERT* gene expression in tumor area than adjacent normal area. Among them, 61% (38 of 62) of cases with high *hTERT* mRNA expression showed elongated telomere, illustrating a significant association ($P = .02$). However, 36% (13 of 36) of patients with lower *hTERT* mRNA expression showed elongated RTL. TL was shortened in 64% (23 of 36) of patients with low *hTERT*.

Gene expression was compared with prognostic factors. *hTERT* mRNA expression was significantly associated with TL, clinical stage, tumor ≥ 5 cm, and positive lymph node with an odds ratio of 2.77, 2.74, 2.54, and 2.97, respectively. Figure 2 shows that the median *hTERT* expression in the advanced stage was 4.22 (IQR, 2.28-8.69), tumor size ≥ 5 cm was 4.50 (IQR, 2.51-8.69), positive lymph node was 3.28 (IQR, 1.78-8.69), and high histopathologic grade was 3.28 (IQR, 1.60-8.69). The gene expression was found higher in late stage, larger tumor size, lymph node-positive cases, and high-grade tumors ($P = .01$). A positive correlation was observed between TL and *hTERT* gene expression.

TL Analysis

We further analyzed the association between TL and clinicopathologic characteristics of breast cancer. Breast cancer cases were grouped by TL into 2 types, shortened TL ($n = 47$) and elongated TL ($n = 51$). This classification is based on individual characteristics of a paired sample as shown in Table 2. Each tumor sample was compared with adjacent border normal breast tissue. Table 3 showed shortened TL had a significantly ($P < .001$) low median value of 1.96 when compared with the elongated TL median value of 3.49.

Table 3 Relative Telomere Length of Tumor Tissue in Association With Prognostic Factors

Parameters	Subtype	Median Telomere Length	IQR	*P* Value
Group	Short telomere	1.96	0.53-3.12	.001[a]
	Long telomere	3.49	2.13-8.37	
Menopausal status	Pre	2.41	1.00-5.52	.836
	Post	2.65	1.02-3.66	
Age, y	<40	2.58	1.56-6.75	.69
	>40	3.26	1.52-7.65	
Stage	Early	2.03	0.82-6.52	.005[a]
	Late	4.22	2.22-8.69	
Tumor size, cm	< 5	2.25	0.73-3.5	.015[a]
	≥ 5	3.19	1.96-8.37	
Node	Negative	0.97	0.40-2.52	< .001[a]
	Positive	3.17	1.94-6.25	
Tumor grade	Low	1.99	0.7-3.15	.01[a]
	High	3.28	1.6-8.69	
ER	Negative	2.50	1.44-3.50	.246
	Positive	3.28	1.25-8.05	
PR	Positive	2.24	0.97-3.49	.193
	Negative	3.30	1.23-8.05	
HER-2/neu	Negative	2.62	1.07-4.8	.875
	Positive	2.30	0.85-4.23	
Triple-negative	TNBC	3.16	1.49-7.7	.35
	Others	2.30	0.85-4.2	
hTERT mRNA expression	Low	2.65	1.21-4.59	.56
	High	2.13	0.65-4.23	

Short telomere: relative telomere length is lesser in tumor tissue when compared with adjacent grey-white tissue; long telomere: relative telomere length is higher in tumor tissue.
Abbreviations: ER = Estrogen receptor; HER2 = human epidermal growth factor receptor 2; IQR = interquartile range; PR = progesterone receptor; TNBC = triple-negative breast cancer
[a] *P* value < .05

Relative T/S ratios for these patients are depicted in Table 3. Telomere was shortened in tumor tissue region of early cancer cases with low *hTERT* gene expression. However, 13% of overall tumor tissue showed elongated telomere in spite of having lower *hTERT* expression. It had been observed that shortened telomeres were present in early breast carcinoma, smaller tumor size, lymph node-negative, and low-grade carcinoma.

Among the 98 cases, 51% cases were with early-stage carcinoma, 30% were node-negative, and 20% were low-grade tumors. RTL was shortened in 60% (30 of 50) of early-stage cancer cases, 55% (36 of 65) of cases with tumor size < 5 cm, 72% (21 of 29) of lymph node-negative cases, and 68% (13 of 19) of low-grade carcinoma. Elongated RTL was observed in 65% (31 of 48) of advanced breast carcinoma, 67% (22 of 33) of tumor size ≥ 5 cm, 62% (43 of 69) of node-positive cases, and 57% (45 of 79) of high-grade breast carcinoma. Table 3 shows the significant median values of RTL as 4.22, 3.19, 3.17, and 3.28 for advanced stage, tumor size ≥ 5 cm, node-positive, and high-grade cases, respectively, with quartile 1 and quartile 3 as range of values. There was no association observed in age at diagnosis, menopausal status, hormone receptor status, HER-2/neu receptor status, or triple-negative cases. This study showed a correlation between RTL and *hTERT* mRNA expression as r = 0.243 with *P*-value = .01, which was calculated by the Spearman rank correlation.

Discussion

Telomere attrition has been shown to be associated with multiple aging-related diseases, such as cancer.[8] When the telomeres became too short, the enzyme telomerase prevented it from getting shorter and entering apoptosis. In the event of tumorigenesis and progressiveness of cancer, maintenance of TL is essential to sustain cancer cells. The association between TL and breast cancer risk is conflicting. In this study, we measured RTL and *hTERT* mRNA expression in order to find the TL patterns in the tissue of human breast carcinoma. Gene *hTERT* expression was considered as a measure of telomerase activity as it is strongly correlated with telomerase activity in breast cancer.[18,19]

In most of the previous studies, TL analysis was carried out either on leukocyte DNA or blood-based assays. Solid tumors are highly heterogeneous and localized, and telomere attritions with associated mechanisms are confined to the tumor nuclear DNA during cancer initiation. In this study, 39% of cases with high *hTERT* expression showed elongated telomere in contrast to 23% patients with low *hTERT* expression and shorter TL. Cancer cells maintain telomere stability primarily by producing telomerase enzyme, which counterbalances the telomere attrition processes during the trajectory of cancer development. Telomere shortening and telomerase activation in malignant tumor tissue were considered as risk factors for cancer initiation.[20] In our study, 25% of cases showed lesser TL even though *hTERT* gene expression was high. Probably, in the dynamic

processes of tumor development, the lengthening of telomere is yet to start in spite of having higher telomerase expression. Scientifically, it is fathomable that telomerase expression subsequently initiated the elongation of TL.

Conversely, a critically short telomere in tumor cells also undergo an alternative lengthening telomeres (ALT) mechanism in the deprivation of telomerase activity.[21,22] In the current study, 13% of overall cases showed elongated telomere despite having lower *hTERT* expression. ALT mechanisms might have occurred in these cases. Excessive telomere shortening causes chromosomal instability, providing an easy access for fusion. This may be one of the reasons for having elongated telomere in the tumor region when compared with adjacent border normal tissue even though *hTERT* expression is low. Generally, around 15% to 25% of breast cancer cases showed ALT mechanism, where the TL is higher even though *hTERT* expression is low.[23] This may be owing to homologous DNA recombination or end-end fusion of chromosomes.

Blood leukocytes and breast epithelial tissue are 2 different compartments with independent telomere dynamics.[24] In breast cancer, upon mitogenic stimulation, it is mainly the breast epithelial cells that produce telomerase, but hematopoietic cells rarely do.[25] Accordingly, unlike other studies, performing RTL analysis on tissue to understand the patterns is more relevant rather than blood leukocytes. Further, the ALT mechanism is more evident in tissue samples compared with blood leukocytes. hTERT targeted therapeutics may favor the existence of ALT-dependent cancer cell survival and proliferation causing new therapeutic challenges. Hence, we aimed to show RTL in DNA derived from tumor and adjacent grossly normal breast tissues to distinguish different patterns.

The meta-analysis study including 51 publications on blood leukocyte TL showed that shortened telomeres were associated with increased risk of gastrointestinal tumors and head and neck cancers.[26] In another meta-analysis, Zhang et al[27] showed that a shorter telomere had a significant association with poor cancer survival. In an 11.2-year follow-up study, researchers found that shorter telomere had an increased breast cancer risk and mortality and that a change in blood leukocyte TL could act as a biomarker of prognosis.[28] Pooley et al[29] studied TL in both retrospective and prospective cohorts and observed that telomere attrition begins once the cancer initiates and not during cancer development nor before cancer diagnosis. A strong association between shorter blood leukocyte telomere and breast cancer risk was observed in the retrospective study. Studies demonstrated that shorter telomere on chromosome 9p was associated with increased breast cancer risk.[30] In a similar fashion, the studies carried out on breast tumor tissue by researchers did not show any association with TL, but patients with high telomerase expression had poor response to chemotherapy and overall survival.[31]

Cawthon reported that the RTL measurement is comparable with the Southern blot techniques. However, the real-time PCR based method is less tedious and more economical, allowing the measurement of RTL in gDNA derived from the tissue. Southern blot assays measure absolute TL using restriction digestion enzymes. However, RTL correlates well with telomere restriction fragment lengths produced by restriction enzymes. The sub-telomeric region of DNA contributes up to 2 kb base variation between individuals as measured by the Southern blot technique along with telomere region. The real-time PCR method provides RTL measurement in a biologically innovative way with good correlation with Southern blot techniques.

This study was a cross-sectional study with limitations. In invasive ductal carcinoma of breast epithelial tissue, both fibroblasts and lymphocytic cells were present. Quantity of gDNA may vary from case to case and also in the heterogeneous population of cells. Further, aneuploidy in tumor may have varied the quality and quantity of gDNA. However, the aneuploidy rate is significantly less frequent in early-stage, lymph node-negative, and low-grade tumors when compared with advanced diseases of breast cancer, and TL was not drastically altered in these cells.[32]

In conclusion, our results on tissue samples showed that shortened telomeres were found in early-stage, low-grade, and lymph node-negative breast carcinoma, and elongated telomeres were present in advanced carcinoma. Studies with a large cohort are needed to confirm the findings. Measurement of TL as a biological marker may carry valuable information for future treatment strategies and developing inhibitors of telomerase, hTERT promoter-based therapy, telomere-driven approaches, and telomerase vaccines. Thus, measuring RTL and *hTERT* mRNA expression may serve as prognostic and predictive markers in patients with breast cancer.

Clinical Practice Points

- Measurement of RTL in tissue gives a localized tumor picture, and the tumor region could always be compared with adjacent normal breast tissue of the same patient.
- The TL should also be considered when drugs such as telomerase inhibitors are given based on *hTERT* gene expression.
- This study shows an insight into measurement of RTL in tissue, although blood leukocyte telomere measurement was studied and considered as prognostic biomarker in various cancers.

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Disclosure

The authors have stated that they have no conflicts of interest.

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