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Journal of Cancer Biology & Research

Research Article

# Association of (hTERT) Gene Expression with Clinicopathological Parameters in Breast Carcinoma

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**Submitted:** 20 May 2022

**Accepted:** 22 June 2022

**Published:** 24 June 2022



**ISSN:** 2373-9436

OPEN ACCESS

**Keywords**
- Breast cancer
- hTERT
- Prognostic marker
- Telomerase

**Abstract**

**Introduction:** Telomerase a ribonucleoprotein enzyme maintains chromosomal stability by adding tandem DNA repeats to ends of chromosomes. Human telomerase reverse transcriptase (hTERT), the catalytical subunit of thetelomerase core enzyme, regulates enzyme activity. The present study aims at evaluating the expression of telomerase hTERT subunit in invasive ductal breast carcinoma with clinicopathological parameters like: age at diagnosis, menopausal status, clinical stage, histopathological grade, lymph node status, hormone receptor status(estrogen receptor and progesterone receptor) and HER2/neu status.

**Methods:** hTERT gene expression analysis was performed on tumor samples from 186 patients with primary invasive breast cancer using qPCR analysis and SYBR chemistry using Beta-2 microglobulin (B2M) a housekeeping gene as an internal control.

**Results:** hTERT tissue expression was found to be significantly higher in breast tumor tissues when compared tothe adjacent normal tissue ($p < 0.01$), with 73.98% cases positive for hTERT expression. We observed a significant association between hTERT expression and stage of disease ($p < 0.01$) and positive lymph node status ($p = 0.01$).However, there was no significant association with age, menopausal status, histopathological grade and hormone receptor status. This study showed that higher expression of hTERT in tumor tissue and its significant association with advanced stage of disease and metastasis may serve it as a molecular marker for adverse disease outcome and a predictor of high telomerase activity in tumor tissues.

## ABBREVIATIONS

hTERT: human Telomerase Reverse Transcriptase; qPCR: quantitative Polymerase Chain Reaction; HER-2: Human Epidermal Receptor-2

## INTRODUCTION

Breast cancer is the most common malignancy among females with an estimated 2.3 million new cases diagnosed globally in 2020 [1]. There is a significant increase in the incidence and cancer associated morbidity and mortality in Indian subcontinents mainly due to rapid urbanization, industrialization and population growth. Stage at diagnosis is an important determinant of the overall survival rate. Indian women predominantly present at advanced stage (III or IV) due to illiteracy, lack of awareness and financial constraints leads to late diagnosis which in turn increases mortality rate [2,3]. Breast cancer prognosis is mostly relied on the clinicopathological parameters like axillary lymph node status, tumor size, histological grade and molecular markers such as hormone receptor and human epidermal growth factor status (HER2) and Ki67 [4].

The prognostic markers for breast cancer indicate the aggressiveness, invasiveness and extend of metastasis. The use of biomarker helps in selection of optimal treatment for patients. Chemotherapeutic drugs arrest the cell cycle, damage DNA and slow the cells reproduction capability. Hormone receptor positive patients receive endocrine therapy in combination with systemic adjuvant therapy [5]. Advanced stage cancer cells undergo various clonal selections and have high proliferative capacity. At such stages, most cancer cells depend upon telomerase [6]. The DNA damaging effects as a result if systemic therapy can be reversed by high telomerase activity. Telomerase promote DNA damage repair, facilitate cell survival and hence induce resistance to anticancer drugs [7]. Numerous studies have reported high telomerase activity (about 70-90%) in various malignant tissues and many immortal cell lines, but no detectable telomerase activity is reported in most somatic cells [8].

Telomerase, a RNA dependant DNA polymerase, composed of three major components: the catalytical subunit hTERT (human telomerase reverse transcriptase); an RNA subunit hTR (human telomerase RNA) and a protein component hTEP1 (human telomerase associated protein 1) maintains the telomere length. Using hTR as a template, the hTERT subunit adds 5'-GGTTAG-3' repeats to the end of chromosomes [9]. Telomeres

are specialized structures composed of tandem DNA repeats and associated proteins that are present at the end of chromosomes and maintain genomic stability. Telomere length is not static and decreases with each cell division [10]. When telomere lengths reach a critical level, cells enter senescence or initiate apoptosis [11]. A dysregulation in the telomerase activity often leads to genomic instability, resulting in disruption of cell cycle control and making the cells immortal [12].

The present study aims at evaluating the expression of telomerase hTERT subunit in invasive ductal breast carcinoma with clinicopathological parameters like: age at diagnosis, menopausal status, clinical stage, histopathological grade, lymph node status, hormone receptor status (estrogen and progesterone receptor) and HER2/neu (Human Epidermal Receptor-2/neu) status. Our study is one of the few studies which involve Pan-Indian population (South East Asian population).

## MATERIALS AND METHODS PATIENTS

A total of 200 female patients diagnosed with primary breast invasive ductal carcinoma that were enrolled for treatment at Kidwai Memorial Institute of Oncology, a regional cancer centre. All the subjects underwent modified radical mastectomy. Adjacent normal grey white tissue was used as a control in each sample. Patients who received neo-adjuvant chemotherapy were excluded from the study.

The study was approved by the scientific review board and medical ethical committee of the Institute and written informed consent was collected from all the patients. Tumor tissue and grossly normal adjacent grey white tissue more than 5 cm from the tumor were collected in RNA Later™ (Sigma-Aldrich, USA) and stored at -80°C until analysis. Histopathologic typing, Ellis and Elston's modification of the Scarff-Bloom-Richardson (SBR) grading and measurement of estrogen receptor (ER) and progesterone (PR) receptor levels were performed by independent investigators.

### hTERT gene expression analysis

Total RNA was isolated from 20 mg of tissue samples stored in RNA Later using TRI Reagent (Sigma-Aldrich, USA) according to the manufacturer's instructions. The RNA obtained was treated with DNAse I (New England Biolabs, UK) to remove genomic DNA contamination. The integrity and purity of isolated RNA was electrophoretically examined on a 1% agarose gel and concentration was determined by OD measurement at a wavelength of 260 nm on Eppendorf Biospectrophotometer Kinetics™ (Eppendorf, Hamburg, Germany). Further steps were performed on the remaining 186 samples. 2 µg of DNAse-treated RNA was reverse transcribed into complementary DNA (cDNA) in a 20-µL reaction volume using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's protocol.

qPCR was performed to determine the fold change in the expression of hTERT gene using an Eppendorf Mastercycler® ep realplex (2S; Eppendorf, Hamburg, Germany). A 20-µL reaction volume consisting of 10 µL SYBR FAST master mix, 0.5 µL of forward and reverse primer each, 1 µL template cDNA and 8 µL double distilled water (ddH2O). KAPA SYBR FAST qPCR Master Mix served as the dye to bind to the amplified DNA and emit florescence during the reaction. Beta-2-microglobulin (B2M), housekeeping gene served as an internal control to measure the accuracy of RNA extraction and cDNA synthesis during the RT-PCR performance. The oligonucleotide sequences used for amplification of B2M and hTERT genes are listed in Table 2. The primers were designed in a way to detect both α and β variants of hTERT gene. Primers for the targets were synthesised using primer3 plus (v.0.4.0) and primer specificity was confirmed by running a primer blast.

The thermal cycling condition consisting of an initial incubation of 2 minutes at 95°C to activate the Taq polymerase followed by 40 cycles each of denaturation at 96˚C for 15 secs and annealing at 60˚C for 30 sec. A melting curve analysis was performed at a ramp speed of 1% to ensure the amplification of a single PCR product. Reactions with no template served as negative control. The Ct values of the target genes were calculated using Mastercycler ep realplex analysis software (Eppendorf) and the $2^{-\Delta\Delta CT}$ method.

### Statistical analysis

All experiments were performed in triplicate and values are expressed as fold change in mRNA expression. The Statistical analysis between the level of gene expression and clinicopathological parameters was done by Pearson's Chi-square and Fisher Exact test by using the Graphpad Prism (San Diego, CA, USA, Version 5.00). Mann Whitney U test was applied to find the median value and association with the categorical variables. Two-way contingency table was used to calculate the odds ratio with 95% CI. A p value of less than was considered statistically significant.

## RESULTS

The mean age of patients with primary invasive ductal carcinoma breast at diagnosis was 50 ± 11 years with a range of 26-83. 14 out of 200 samples did not yield quality RNA and hence were excluded from the study. The complete clinicopathological data was available for only 176 patients. Table 1 summarizes the demographic details of the patients. 79% (139/176) of the patients were above 40 years of age at diagnosis and 59% of the patients were reported to be post-menopausal. The pathological review shows that most of the cases were of grade 3 (75%) and lymph node positive (64%). The Table 1 shows 44% ER positive, 47% PR positive and 36% HER- 2/neu cases.

### hTERT analysis

The RT-PCR analysis revealed upregulated expression of hTERT gene in tumor tissues in comparison to the adjacent normal tissue ($p < 0.01$) as shown in Figure 1. 73.98% of the patients demonstrated high hTERT expression which also significantly associated with advanced stage disease ($p < 0.001$) positive lymph node status ($p < 0.01$). However, no association could be drawn between hTERT expression and age, menopausal status, histopathological grade, hormone receptor status and HER2/neu receptor status (Figure 2). Table 3 shows the association between hTERT expression and clinicopathological parameters with an odd's ratio of 2.48 and 2.38 for lymph node positive and late pathological stage respectively.

**Table 1:** Demographic details of the patients.

| CHARACTERISTICS | N (%) |
|---|---|
| ALL PATIENTS | 176 (100) |
| AGE OF THE PATIENTS | |
| ≤ 40 years | 37 (21.02) |
| > 40 years | 139 (78.98) |
| MENOPAUSAL STATUS | |
| Pre-menopausal | 72 (40.91) |
| Post-menopausal | 104 (59.09) |
| PATHOLOGICAL STAGE | |
| IIA | 53 (30.11) |
| IIB | 46 (26.13) |
| IIIA | 35 (19.88) |
| IIIB | 25 (14.20) |
| IIIC | 17 (9.65) |
| NODAL STATUS | |
| N-negative | 64 (36.36) |
| N-positive | 112 (63.64) |
| HISTOPATHOLOGICAL GRADE | |
| Grade 2 | 45 (25.57) |
| Grade 3 | 131 (74.43) |
| ESTROGEN RECEPTOR STATUS | |
| Positive | 77 (43.75) |
| Negative | 99 (56.25) |
| PROGESTRONE RECEPTOR STATUS | |
| Positive | 84 (47.72) |
| Negative | 92 (52.28) |
| HER2/Neu RECEPTOR STATUS | |
| Positive | 63 (35.80) |
| Negative | 113 (64.20) |
| hTERT EXPRESSION | |
| Low | 78 (44.32) |
| High | 98 (55.68) |

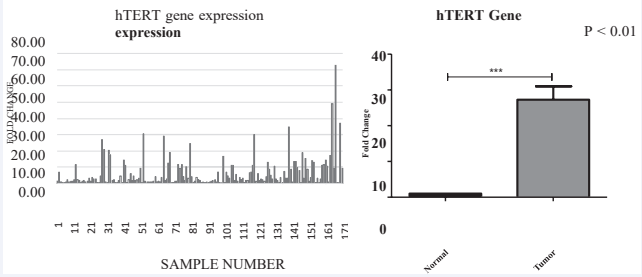


**Figure 1** a) Fold change in hTERT gene expression across patients by RT-PCR analysis; b) hTERT expression is significantly higher in tumor tissues compared to normal tissue sample.

### hTR analysis

The primer sequences for hTR were also synthesised using primer3 plus and blast was run for the same. The blast results obtained was not satisfactory for hTR gene expression analysis and hence was excluded from the study.

## DISCUSSION

In the present study, we investigated the tissue gene expression of hTERT (the protein subunit of the telomerase enzyme) in invasive ductal carcinoma breast patients. For the cancer cells to grow and sustain, telomere length maintenance is essential for cancer cell immortalization. The hTR and TEP1 genes are ubiquitously expressed in both normal and tumor cells, while that of hTERT remains exclusive in tumor cells. Hence, telomerase activity is essentially rate-limited by hTERT expression [13]. hTERT transcriptional and post-transcriptional modification is complex, and to date, more than 20 splice variants have been identified [14].

In our study, the hTERT mRNA expression was found to be significantly higher in patients with advanced disease stage and positive lymph node status. Our results are in concordant with the previous study of C Porembo et al. on tissue microarray from a total of 611 breast cancer samples a significant association of hTERT protein expression with positive lymph node status [15]. Findings from Hiyama et al. and Clark et al. also revealed a relation between the prognostic factors such as clinical stage, tumor size, lymph node status and telomerase activity [16,17]. hTERT being an integral part of telomerase enzyme, its expression correlates with cellular differentiation and neoplastic transformation and can directly determine telomerase associated risk factor in breast cancer patients. The current adjuvant systemic treatment protocol remains same for patients with node-positive disease or node-negative disease with tumor size greater than 1 cm. Lymph node status are primarily used as a predictor of treatment outcome [18].

hTERT in combination with two other oncogenes (SV40 large T-antigen and oncogenic allele of H-ras) has direct effect on tumorigenic conversion of normal human epithelial and fibroblastic cells as reported in a previous study by Hahn et al., [19]. This result indicates the relation of hTERT expression on cancer development. With the use of hTERT as a prognostic marker to differentiate aggressive tumors with potential lymphatic spreading form locally limited tumors treatment plans can be made to incorporate anti-telomerase based therapy. High hTERT expression was reported in malignant tumors and cancer cell lines but not in normal tissues or telomerase-negative cell lines and hTERT expression strongly correlated with telomerase activity in breast cancer [20].

In a study conducted by Lu L et al., to evaluate the effect of telomerase expression on adjuvant therapy in breast cancer, it was observed that patients had worse survival outcome with high telomerase expression and large tumor size. The enzyme may facilitate malignant transformation and render cells with resistance to apoptosis [21]. A progressive elevation in the telomerase activity was detected in DCIS lesions and infiltrative breast carcinoma [22]. In contract to our results, Salhab M et al. did not find any association between telomerase and disease stage although high telomerase expression was found in high grade tumors [23]. Adjuvant chemotherapy along with endocrine therapy is a preferred treatment pattern for patients with hormone-receptor positive status. A number of invitro studies have shown that tamoxifen exerts antagonistic effects on telomerase expression in several breast cancer cell lines [24] our study did not show any correlation with ER and PR status, unlike a study done by Ivan B et al., where the hTERT

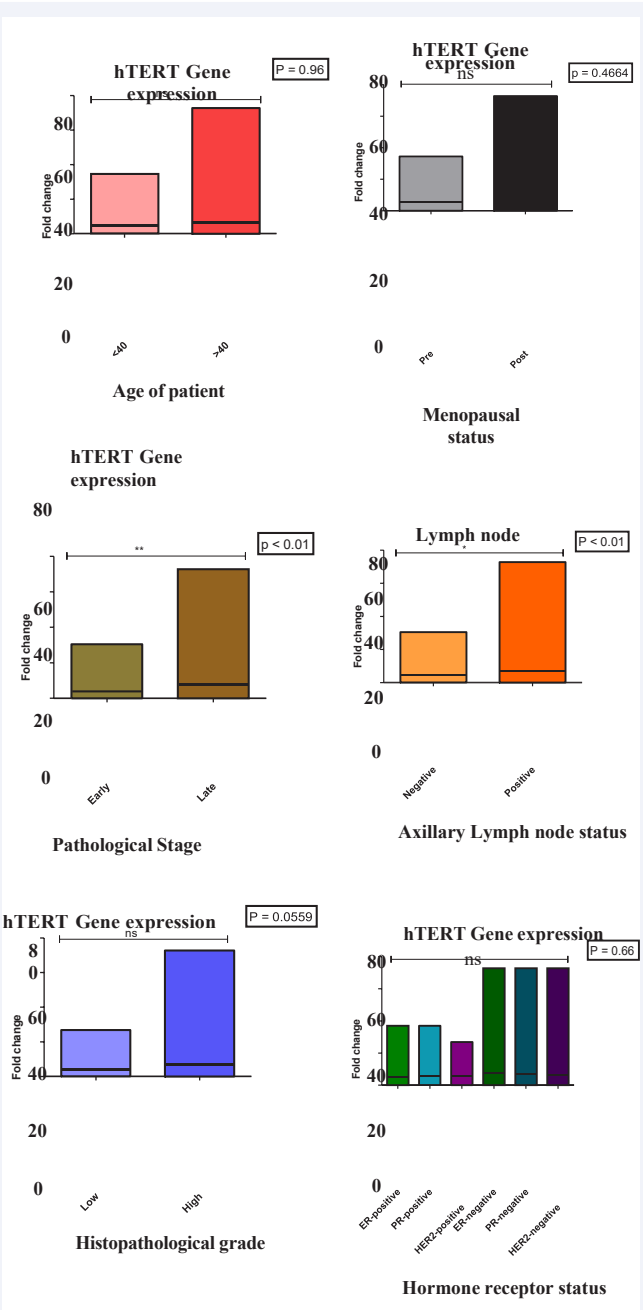


**Figure 2** Gene expression analysis of hTERT by RT-PCR. Expression was measured in terms of fold change. The association of hTERT expression with clinicopathological parameters a) age, b) menopausal status, c) Pathological stage, d) axillary lymph node status e) Histopathological grade, f) Hormone receptor status.

mRNA expression was significantly correlated with the negative ER and PR status [25]. Yuan X et al., reviewed and summarized that hTERT overexpression/hyperactivity due to promoter mutation, cause structural alterations at TERT locus and this application can be used to study cancer initiation, progression and therapy. Gay-Bellile M et al., observed that upregulation of TERT was associated with worse prognosis in breast cancer, lung adenocarcinoma and thyroid carcinoma [26]. A review study by Dratwa M et al., highlighted the main roles of hTERT in various mechanisms of cancer development and regulation. The study concluded hTERT expression can be a useful prognosis marker in various cancers and a new therapy approach [27].

**Table 2:** Primer sequence for gene expression analysis.

| Gene | Primer sequence | Size |
|---|---|---|
| Human telomerase reverse transcriptase (hTERT) | FP 5'-GCACCCTCTTCAAGTGCTGT-3' | 20 bp |
| | RP 5'-AAGTTCCTGCACTGGCTGAT-3' | 20 bp |
| Beta-2-microglobulin (B2M) | FP 5'-GAGTATGCCTGCCGTGTG-3' | 18 bp |
| | RP 5'-AATCCAAATGCGGCATCT-3' | 18 bp |

Footnote: FP = Forward Primer; RP = Reverse Primer

**Table 3:** Association between hTERT and clinicopathological parameters.

| Factor | Group | hTERT Expression | | P value | OR (95% CI) |
|---|---|---|---|---|---|
| | | Low | High | | |
| Age | ≤ 40 | 16 | 23 | 0.76 | 0.84 (0.37-1.83) |
| | > 40 | 62 | 75 | | |
| Menopausal Status | Pre | 30 | 44 | 0.443 | 0.76 (0.39-1.46) |
| | Post | 48 | 54 | | |
| Lymph Node | Negative | 37 | 26 | 0.004* | 2.48 (1.26-4.93) |
| | Positive | 41 | 72 | | |
| Pathological Stage | Early | 47 | 38 | 0.006* | 2.38 (1.24- 4.60) |
| | Late | 31 | 60 | | |
| Luminal Type | A | 34 | 38 | 0.342 | 1.77 (0.62-5.44) |
| | B | 8 | 16 | | |
| | | | | | |
| Histopathological Grade | 2 | 25 | 20 | 0.085 | 1.83 (0.87-3.87) |
| | 3 | 53 | 78 | | |

(*Significant P < 0 .05)
Luminal Type A: ER-positive, PR-positive, Her2/neu-negative
Luminal Type B: ER-positive, PR-positive, Her2/neu-positive/negative with high Ki67 levels

We conclude that the higher expression of hTERT and its significant association with advanced disease stage and metastasis may act a predictor of high telomerase activity and serve as a molecular marker for adverse disease outcome. Breast cancer patients with high hTERT expression can be followed up and the therapeutic decision may be improved by measuring the residual telomerase activity as well as measuring the serum hTERT level.

## ACKNOWLEDGEMENT

The work was financially supported by the Advanced Research Wing, Rajiv Gandhi University of Health Sciences, Bangalore, India. We are grateful for all the participants for their corporation. We thank Dr. Shilpa V and Dr. Bhaskari J for their valuable inputs.

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**Cite this article**

*Raju A, Thriveni K, Bharatnur SS, Arjunan R (2022) Association of (hTERT) Gene Expression with Clinicopathological Parameters in Breast Carcinoma. J Cancer Biol Res 9(1): 1132.*