hTERT mRNA expression correlates with telomerase activity in human breast cancer

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Aims: Telomerase is a ribonucleoprotein enzyme that synthesises telomeres after cell division and maintains chromosomal length and stability thus leading to cellular immortalisation. hTERT (human telomerase reverse transcriptase) gene seems to be the rate-limiting determinant of telomerase reactivation. hTERT mRNA expression was reported to correlate with telomerase activity in cell lines and some human tumours. However the correlation between telomerase activity and hTERT mRNA expression has not been previously examined in human breast cancer. The present study aims to quantitatively measure the expression of hTERT mRNA and telomerase activity in human breast cancer and examine the relationship between these parameters. Furthermore the associations with other parameters including estrogen receptor (ER) and progesterone receptor (PgR) status, DNA ploidy, and S-phase fraction (SPF) are also examined

Methods: RNA was extracted from 18 breast carcinomas and hTERT mRNA expressions were estimated by reverse transcriptase-PCR (RT-PCR) and Taqman methodology. These tumours had already been analysed for ER and PgR status using ligand-binding assays and had had their DNA ploidy and S-phase fractions measured by flow cytometry. Telomerase activity had already been determined by using a modified telomeric repeat and amplification protocol (TRAP) assay.

Results: The expression of hTERT mRNA in the breast tumours ranged between 1.3 and $2.7 \times 10^7$ copy numbers per µg of cellular RNA (the median value was $2.7 \times 10^5$ and the mean was $3.1 \times 10^6$). Telomerase activity was between 0 and 246 units of Total Protein Generated (TPG), where one unit of TPG was equal to 600 molecules, of telomerase substrate primers extended by at least three telomeric repeats. The median level of TPG was 60 units and the mean level was 81 units. Telomerase activity was found to significantly correlate with hTERT expression ($r_s = 0.51112$, $P = 0.0302$). There was no significant correlation between hTERT and other parameters.

Conclusion: hTERT mRNA expression significantly correlates with telomerase activity in human breast cancer. This is consistent with the hypothesis that hTERT is the catalytic and rate-limiting determinant subunit of the enzyme.

Key words: telomerase; hTERT; breast cancer.

INTRODUCTION

Telomerase is a multi-component ribonucleoprotein located within the nucleus, the function of which is to synthesise the repetitive nucleotide sequence forming the telomeres at the end of chromosomes. During cell division, DNA polymerase is unable to fully replicate the ends of linear DNA and genetic material is lost from chromosomal ends. Progressive shortening can result in chromosome instability and cellular senescence. Telomerase recognises the G-rich strand of the telomere repeat sequence and in the absence of a complementary DNA strand, synthesises a new copy of the repeat. Furthermore telomerase appears to somehow stabilise short telomeres, so that even if telomeric length is not increased, cellular proliferation can continue thus leading to cellular immortality. Telomerase is active in 70–90% of malignant tissues and many immortal cell lines. Most somatic cells have no detectable telomerase activity, with the exception of certain stem cells, lymphocytes and germline cells. Telomerase activation does not seem to cause carcinogenesis but it allows a cell to continue division and attain
immortality, a necessary achievement for a cancerous
cell to be successful. Although alternative methods of
maintaining telomere stability have been identified,5,6 the
majority of tumours in all types investigated appear to
employ telomerase activation as a means of maintaining
proliferation at a high rate without becoming senescent.

The fundamental components of telomerase have
been identified as the RNA template (hTR), the reverse
transcriptase (hTERT) and the telomerase associated
protein (TEP1).1,7–9 Of these hTR and TEP1 are expre-
ssed ubiquitously in both normal and cancerous
tissue,7 whereas hTERT is detectable in tumour cells
but not in normal somatic cells.8,9 The gene coding for
hTERT has been recently cloned and mapped to
5p15.3,9

Telomerase enzyme activity can be reconstituted in
fibroblasts by the ectopic expression of hTERT and
Relive telomerase has been shown in vitro to require the
presence of only the hTR ana hTERT components.10,11
These observations suggest that these two subunits
represent the core enzyme complex. However, the
levels of hTR (and TEP1) expression have been shown
to be unrelated to the level of telomerase activity.8,9,12,13
whereas induction of hTERT expression has been shown
to be essential for telomerase activation in cell-lines.8,9
Furthermore, when leukaemic cells were induced to
differentiate in vitro, hTERT mRNA levels (but not the
mRNA of the other sub-units) fell dramatically before a
reduction in telomerase activity.14 This reduction in
hTERT levels has been shown to be due to a reduction
in gene transcription.15 These observations suggest that
hTERT is the rate-limiting determinant of telomerase
enzyme activity.

Telomerase activity has been detected in the majority
of human cancers and has been shown in some cases to
correlate with prognostic variables.1,16–21 We detected
telomerase activity in 74% of invasive breast cancers and
in none of benign or normal breast tissue specimens.
Furthermore we observed a correlation between
telomerase activity and tumour size, nodal status,
lymphovascular invasion and Ki-67 expression.17,18 This
indicates that measurement of telomerase activity may
be useful both in the diagnosis of malignancy and as a
prognostic indicator that may influence treatment.
However, telomerase detection by enzyme assay is
time-consuming to perform, and may be complicated by
the presence of enzyme inhibitors, proteases or
Rnases22 and this restricts its usefulness in the clinical
setting. Measurement of hTERT expression is simpler,
more sensitive and more rapid using RT-PCR with
the automated fluorescent systems such as TaqMan that
are now available and which circumvent the need for
post-reaction manipulation of specimens to obtain
results.23–25 Furthermore, these techniques are less
sensitive to RNases, because only a fragment of mRNA
needs to be present,22 and they provide a quantitative
measurement of mRNA levels. We have recently
reported that hTERT mRNA is much higher in breast
cancer specimens compared with adjacent non-
cancerous breast tissue.19

However, expression of hTERT mRNA may not
necessarily correlate with telomerase activity. Post-
transcriptional modification of RNA and the presence of
enzyme inhibitors may alter telomerase activity for a
given level of hTERT expression. Moreover, it has been
suggested that a threshold level of hTERT protein is
required in order for telomerase to become active, and
therefore low levels of hTERT mRNA may not indicate
active telomerase.23 In this respect hTERT protein as
detected by immunohistochemistry has not been found
to correlate with telomerase activity in pre-malignant
gastric lesions26 and colorectal tumours.27 Moreover
hTERT mRNA expression has been detected in lymph-
cytes regardless of telomerase activity28 and in some
normal telomerase negative tissues such as human brain,
prostate liver and ovary.29

Nevertheless, other investigators have found a good
level of correlation between telomerase and
hTERT mRNA expression in human cell-lines and haemo-
poietic cells.30,31 In human skin and skin tumours, the
presence of hTERT mRNA as detected by RT-PCR has
been found to correlate strongly with telomerase
activity measured using the TRAP assay.7 Similar obs-
evations were found in gastric carcinomas,32 hepato-
cellular carcinomas,13 renal-cell carcinoma,33 ovarian
carcinoma34,35 and both normal and malignant endo-
metrial tissue.36 To our knowledge, no study has been
reported concerning the levels of hTERT mRNA and
telomerase activity in breast carcinoma.

Our aim in this study is to investigate whether the
level of hTERT mRNA expression is correlated with
telomerase activity in human breast carcinomas.

MATERIALS AND METHODS

Specimens of human breast carcinoma tissue (n = 18)
were acquired from the MD Anderson Cancer Centre
(Texas, USA). Local ethics committee approval was
obtained. The specimens had been already analysed for
telomerase activity (using a variation on the TRAP assay)
by this unit as described previously.37

The specimens had originally been obtained from the
San Antonio Breast Cancer Specialized Program of
Research Excellence (SPORE) tissue resource. As such,
these tumours had already been analysed for oestrogen
receptor (ER, ER+ ≥ 3 fmol/mg protein), and pro-
gestosterone receptor (PgR, PgR+ ≥ 5 fmol/mg protein),
DNA ploidy status (diploid or aneuploid), S-phase
fraction (< 6% = low, 6–10% = intermediate, >10% =
high). The ER and PgR status was determined using
ligand-binding assays, whereas DNA ploidy and S-phase
fractions were measured by flow cytometry. The results
of the telomerase assay and other parameters were not known to us prior to the analysis of the data.

**RNA extraction**

Total cellular RNA was extracted from 10 to 20 mg of these tumours using the RNeasy Mini isolation kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Quantification of the RNA following treatment with DNase (Promega) was carried out in triplicate using the RiboGreen reagent (Molecular Probes Europe BV) according to the manufacturers protocol.

**RT-PCR**

TaqMan RT-PCR was performed in duplicate for each sample using the ABI PRISM 7700 Sequence Detector (Perkin-Elmer Applied Biosystems) and the TaqMan EZ RT-PCR Core Reagents Kit (Perkin-Elmer Applied Biosystems). The RT-PCR mix was set up in an extraction hood in a laboratory distant from the one in which RNA extraction was carried out. The RT-PCR was performed for hTERT using the forward and reverse oligonucleotide primers and TaqMan probes given in Table 1. These oligonucleotides were designed using Primer Express software (PE-Applied Biosystems, Warrington, UK) using gene sequences obtained from the GenBank database. Primers and probe were intron spanning to prevent amplification of genomic DNA. The primers and probe are in exons 3 and 4 of the hTERT gene (accession number AF128893.1) and define a 73 base pair amplicon.

Conditions for the reverse transcription step were 50°C for 2 min, 60°C for 10 minutes, and 92°C for 5 min. The polymerase chain reaction was carried out for 50 cycles of 20 s at 92°C and 25 s at 62°C. For the negative control RNase free water was used in the RT-PCR mix instead of RNA. For the positive control, the amplicon created for the standard curve was used. The standard curve for hTERT (Fig. 1) was constructed using serial dilutions of a single stranded sense oligonucleotide specifying the amplicon as previously described by Bustin et al. and this together with the known RNA concentration was used to quantify the mRNA copy number per microgram of RNA.

**RESULTS AND STATISTICAL ANALYSIS**

The expression of hTERT mRNA in the breast tumours ranged between 1.3 and $2.7 \times 10^7$ copy numbers per μg of cellular RNA; the median value was $2.7 \times 10^5$ and the mean was $3.1 \times 10^6$. Telomerase activity was between 0 and 246 units of Total Protein Generated (TPG), where one unit of TPG was equal to 600 molecules, of telomerase substrate primers extended by at least three telomeric repeats. The median level of TPG was 60 units and mean level was 81 units. Four tumours expressed less than 100 copy numbers of hTERT mRNA per μg of RNA and these expressed either no, or very little, telomerase activity, indeed the lowest level of hTERT

![Table 1](image)

<table>
<thead>
<tr>
<th>Forward</th>
<th>Reverse</th>
<th>Probe (5' FAM 3' TAMRA dual labelled)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hTERT</td>
<td>GCA AGT TGC AAA ACC TCT GCT TCC AGC TGC ACC CTC</td>
<td>GCA TTG GA GAC AGC TC TTC AAG TGC TGT</td>
</tr>
</tbody>
</table>

Figure 1  Standard curve for hTERT.
mRNA where significant amounts of telomerase were detected was 1 \times 10^4 copy numbers. Statistical analysis was performed using the SAS system (Version 6.11, SAS Institute, Cary, NC, USA). The hTERT expression, ER, PgR, and telomerase activity were considered as continuous variables and the relationship between them was investigated using Spearman’s non-parametric correlation coefficient.

No relationship was found between hTERT mRNA levels and tumour ER (r_s \approx 0.364, P \approx 0.13) or PgR (r_s = 0.076, P = 0.76). Telomerase activity was found to correlate with hTERT expression (r_s = 0.51112, P = 0.0302). Figure 2 illustrates the variation of hTERT expression and telomerase activity. Analysis of hTERT expression as a continuous variable using the Kruskal–Wallis test gave evidence for a trend towards correlation for hTERT mRNA levels and tumour S-phase fraction (P = 0.103).

DISCUSSION
We have shown a significant correlation between hTERT mRNA expression and telomerase activity in human breast carcinomas. The correlation coefficient in our results is 0.5, giving a coefficient of determination (R^2) of 25%. The interpretation of this is that 25% of the variation in telomerase activity in our study can be accounted for by the variation in hTERT mRNA expression. This relationship, whilst encouraging, is not close enough to allow telomerase activity to be established through the measurement of hTERT mRNA. Moreover, the number of samples in this study is small, and our findings need to be corroborated with further research.

The fact that the correlation is not stronger may partly be the result of the complex expression of hTERT with post-transcriptional modification and the formation of splice variants. A number of these variants have been detected and at least two involve deletions of exons and are therefore likely to alter protein activity. One of these splice variants (β), with a deletion in exon 6, appears to be an inhibitor of telomerase activity when over-expressed in vitro. Another (α) has been shown to encode a truncated protein lacking vital reverse transcription motifs that in vitro is unable to reconstitute active telomerase enzyme. Both of these splice variants will have been detected by the primers used in our study. Furthermore, Ulaner et al. found that in normal ovarian tissue there was no telomerase activity despite the absence of either the α or the β variants, suggesting the presence of other significant variants and/or post-translational modification or enzyme inhibition. It has also been suggested that a lack of strong correlation may reflect the lower sensitivity of the TRAP assay, although this may have some effect it would not explain those situations in which hTERT mRNA levels are very high without functional telomerase. Further work to fully characterise the implications of the splice variants will allow primers to be designed that only amplify functional hTERT mRNA and this is likely to have a closer correlation with telomerase activity. This might allow the use of RT-PCR as a rapid screening tool in breast malignancy diagnosis in the future.

Our results are supportive of the hypothesis that a threshold level of hTERT is required for telomerase to be active, since a considerable level of telomerase activity was only detected in tumours expressing 10 000 or more copy numbers of hTERT mRNA per microgram.
of RNA. It will be of interest to further investigate this with a larger sample size in order to clarify the level of hTERT expression at which enzyme activity becomes detectable. Our study did not account for the effects of contaminant tissue such as lymphocytes, however such cells express very low levels of both hTERT and telomerase,\(^{19}\) and therefore they are unlikely to influence our findings. We also found a trend towards a significant association between hTERT mRNA and tumour S-phase fraction (which is a marker of proliferation and a negative prognostic indicator). This was not statistically significant but in a sample of this size significance at the desired 5% level is difficult to achieve, and further work in this area is warranted.

**REFERENCES**

2. Blackburn E. Switching and signaling at the telomere. **Cell** 2001; 106: 661–73.


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