hTERT mRNA Expression Correlates with Matrix Metalloproteinase-1 and Vascular Endothelial Growth Factor Expression in Human Breast Cancer: A Correlative Study Using RT-PCR

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Abstract. Background: Telomerase activity has been significantly associated with nodal metastasis and cellular proliferation in human breast cancer, indicating that its degree of expression has some form of vital control over the invasive nature of the malignancy concerned. Of the telomerase subunits, the reverse transcriptase (hTERT) is the main determinant of enzyme activity. Vascular endothelial growth factors (VEGF)-C and (VEGF)-D, matrix metalloproteinase type 1 (MMP-1) and protease-activated receptors (PARs) have all been linked to promotion of tumour invasiveness and metastatic dissemination. This study aims to examine the association between hTERT transcription and that of VEGF-D, VEGF-C, MMP-1, PAR1a and PAR1b through a correlative analysis of the mRNA transcripts of these genes in human breast cancer. Materials and Methods: Breast cancer tissues (n=116) and normal tissues (n=31) were collected immediately after surgery and stored at –80°C until use. The level of hTERT transcripts from the prepared DNA from the above samples was determined using real time-quantitative PCR based on the Amplifluor technology. The levels of the transcript were generated from a standard that was simultaneously amplified with the samples. Normalisation against cytokeratin 19 (CK19) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was also carried out. Results: There was a positive correlation between hTERT mRNA expression (after CK19 normalisation) with both VEGF-D and MMP-1 in human breast cancer. PAR1 was seen to correlate with hTERT (after GAPDH normalisation) with a highly significant correlation with PAR1a alone. However there was no correlation between hTERT transcription and VEGF-C or with PAR1b alone. Conclusion: Our findings suggest that hTERT is a potential up-regulator of MMP-1, PAR1 and VEGF-D expression and this may explain its apparent control over the invasiveness and metastasis of the malignancy concerned.

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 (1). During cell division, DNA polymerase is unable to fully replicate the ends of linear DNA and genetic material is lost which eventually can result in chromosome instability and cellular senescence. Telomerase synthesizes a new copy of the telomere repeat (1) so that cellular proliferation can continue, leading to cellular immortality (2, 3). Telomerase is active in 70%-90% of malignant tissues and many immortal cell lines, but most somatic cells have no detectable telomerase activity. Telomerase activity has been shown to correlate with prognostic variables in some types of cancer (4, 5). 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 (6, 7).

The fundamental components of telomerase have been identified as the RNA template (hTR), the reverse transcriptase (hTERT) and telomerase-associated proteins, including (TEP1) (1, 8, 9). Of these hTR and TEP1 are expressed ubiquitously in both normal and cancerous tissue (8), whereas hTERT is detectable in tumour cells but not in normal somatic cells (9-11). Telomerase enzyme activity can be reconstituted in fibroblasts by the ectopic expression of hTERT (12) and induction of hTERT expression has been shown to be essential for telomerase activation in cell lines (9, 10). These observations suggest that hTERT is the rate-limiting determinant of telomerase enzyme activity. We recently reported that hTERT mRNA is higher in breast...
cancer specimens compared with adjacent non-cancerous breast tissue and this correlates with clinical outcome (13). Furthermore, we reported that telomerase activity is significantly correlated with hTERT mRNA expression (14).

Vascular endothelial growth factor (VEGF)-C and VEGF-D are potent lymphangiogenic factors produced by tumour and stromal cells and have been shown to be of a prognostic value in human cancer (15). Matrix metalloprotease type 1 (MMP-1) in the stromal-tumour microenvironment alters the behaviour of cancer cells through interaction with protease-activated receptor type 1 (PAR1) to generate PAR1-dependent Ca\textsuperscript{2+} signals and promote cell migration and invasion in breast cancer (16).

This study aims to examine the association between hTERT transcription and that of VEGF-D, VEGF-C, MMP-1, PAR1a and PAR1b through a correlative analysis of the mRNA transcripts of these genes in human breast cancer.

Materials and Methods

Materials. RNA extraction kits and reverse transcription kits were obtained from Sigma Ltd (St. Louis, MO, USA). PCR primers were designed using Beacon Designer (Palo Alto, CA, USA) and synthesized in-house. Custom-made hot-start Master mix for quantitative PCR was obtained from Abgene (Epsom, Surrey, UK).

Sample collection. Institutional guidelines, including ethical approval and informed consent, were followed. Breast cancer tissues (n=116) and unrelated benign breast tissue (n=31) were collected immediately after surgery and stored at \(-80^\circ\text{C}\) until use. Patients were routinely followed up after surgery. The median follow-up period was 6 years. A consultant breast pathologist who examined H&E-stained frozen sections verified the presence of tumour cells in the collected tissues.

Details of histology were obtained from pathology reports. Follow-up data were recorded in a custom database. Table I shows the clinical data in regard to the tissues collected.

<table>
<thead>
<tr>
<th>Parameter Category</th>
<th>Number of patients</th>
</tr>
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<td>Node status</td>
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<tr>
<td>Node-positive</td>
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</tr>
<tr>
<td>Node-negative</td>
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<td>Tumour grade</td>
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<tr>
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<tr>
<td>2</td>
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<tr>
<td>3</td>
<td>53</td>
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<tr>
<td>Tumour type</td>
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<tr>
<td>Ductal</td>
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<tr>
<td>Lobular</td>
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<td>Tubular</td>
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<td>Mucinous</td>
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<td>4</td>
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</tr>
<tr>
<td>Clinical outcome</td>
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<tr>
<td>Disease-free</td>
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<tr>
<td>Alive with metastasis</td>
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</tr>
<tr>
<td>With local recurrence</td>
<td>5</td>
</tr>
<tr>
<td>Died of breast cancer</td>
<td>16</td>
</tr>
<tr>
<td>Died of unrelated disease</td>
<td>9</td>
</tr>
</tbody>
</table>

Tissue processing, RNA extraction and cDNA synthesis. Sections of tissue were cut at a thickness of 5-10 µm and were kept for routine histology. An additional 15-20 sections were mixed and homogenised, using a handheld homogeniser, in ice-cold RNA extraction solution. The concentration of RNA was determined using a UV spectrophotometer. Reverse transcription was carried out using a reverse transcription kit with an anchored oligo(dT) primer supplied by Abgene, using 1 µg of total RNA in a 96-well plate. The quality of cDNA was verified using β-actin.

Quantitative analysis of hTERT. The level of hTERT transcripts from the prepared DNA was determined using real-time quantitative PCR based on the Amplifluor technology, modified from a method reported elsewhere (17).

PCR primers were designed using Beacon Designer software (Palo Alto, CA, USA), but to the reverse primer an additional sequence, known as the Z sequence (5’-actgaacctgaccgtacagctt-3’), which is complementary to the universal Z probe (Intergen Inc, Oxford, UK) was added. The product expands one intron. The reaction was carried out using the following: Hot-start Q-master mix (Abgene), 10 pmol of specific forward primer, 1 pmol reverse primer which included the Z sequence, 10 pmol of FAM-tagged probe (Intergen Inc, Oxford, UK) and cDNA from 50 ng of RNA. The reaction was carried out using the IcyclerIQ (BioRad, Hercules, CA, USA) which is equipped with an optical unit that allows real-time detection of 96 reactions under the following conditions: 94°C for 12 min and 50 cycles of 94°C for 15 s, 55°C for 40 s and 72°C for 20 s. The levels of the transcript were generated from a standard that was simultaneously amplified with the samples. Normalisation against cytokeratin 19 (CK19) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was also carried out.


Statistical analysis. The data were analysed using parametric (t-test) and non-parametric (Mann-Whitney test) tests. Correlation between molecules was analysed using Spearman’s Rank Correlation. Survival curves were constructed using the Kaplan-Meier method.

Results

Table I summarises the clinical data in regard to the tissues utilised.

Figure 1 shows the overall survival curves using the Kaplan-Meier method. Cut-off point=0.20, p=0.0122, mean
survival time low (0, 0.20) = 139.7 (130.2-149.2, 95%CI) months, high (1, >0.20) = 105.4 (80.9-129.8) months.

There was a positive correlation between hTERT mRNA expression (after CK19 normalisation) and vascular endothelial growth factor (VEGF)-D in human breast cancer (correlation coefficient=0.272, p<0.01). However, there was no correlation between hTERT transcription and VEGF-C (Table II).

In addition to the above, a positive correlation was also identified between hTERT mRNA expression (after CK19 normalisation) and MMP-1 (correlation coefficient=0.321, p<0.01). PAR1 was seen to correlate with hTERT (after GAPDH normalisation) (correlation coefficient=0.237, p<0.05) with a highly significant positive correlation between the two and hTERT demonstrated in this study. PARs are a unique class of G-protein-coupled receptors that play critical roles in thrombosis, inflammation, and vascular biology. PAR1 is proposed to be involved in the invasive and metastatic processes of various cancers (16). The most abundant class of nonserine proteases present in invasive and metastatic tumours are the zinc-dependent matrix metalloproteases (MMPs). MMPs are used by invasive cancer cells to hydrolyse the structural proteins that compromise the extracellular matrix (26). In a study by Boire et al. (16) in 2005, the matrix metalloprotease, MMP-1, was identified as a novel protease agonist that cleaves PAR1 at the appropriate site for receptor activation thus generating PAR1-dependent Ca\textsuperscript{2+} signals promoting cell migration and invasion. This MMP-1 was shown not to be produced by the breast cancer cells but instead was secreted by fibroblasts.

PAR1 expression has been directly correlated with the degree of invasiveness in both primary breast tissue specimens and established cancer cell lines from humans (27, 28). Similarly, MMP-1 has been shown to be a marker of poor prognosis in breast, colorectal, and oesophageal tumours (29, 30).

In summary, from this study we have demonstrated a highly significant positive correlation between hTERT mRNA expression, MMP-1, PAR1, and VEGF-D expression in human breast cancer. As mentioned previously, there are clear indications that the degree of telomerase expression has some form of vital control over the invasive nature of the malignancy concerned. The mechanism by which this occurs can be explained by the potential action it may have on MMP-1, PAR1 and VEGF-D, all of which have been demonstrated to result in a higher degree of invasiveness of the malignancy concerned, leading to an increased capability for tumour spread and a higher potential for metastatic dissemination.

**Discussion**

Angiogenesis in carcinomas, in particular breast carcinoma, is an important development in maintaining tumour growth and metastatic dissemination (18). The proliferation and migration of vascular endothelial cells is vital to the promotion of angiogenesis. It has been noted that this process is stimulated by the cytokine, VEGF (19). A number of structurally related peptides have now been identified, named VEGF-B, VGEF-C, VGEF-D and PIGF, with VEGF now being renamed VEGF-A.15 (20). Higher levels of VEGF protein are associated with increased microvascular density and disease relapse (21, 22).

In this study we demonstrated that there was a significant positive correlation between hTERT mRNA expression and VEGF-D in human breast cancer. This observation suggests that hTERT may act as an up-regulator of VEGF-D thus facilitating a higher degree of invasiveness of the malignancy concerned and leading to an increased capability for tumour spread and a higher potential for metastatic dissemination.

In addition, hTERT seems to have a similar relationship with MMP-1 and its receptor PAR1, as evidenced by the highly significant positive correlation between the two and hTERT demonstrated in this study. PARs are a unique class of G-protein-coupled receptors that play critical roles in thrombosis, inflammation, and vascular biology. PAR1 is proposed to be involved in the invasive and metastatic processes of various cancers (16). The most abundant class of nonserine proteases present in invasive and metastatic tumours are the zinc-dependent matrix metalloproteases (MMPs). MMPs are used by invasive cancer cells to hydrolyse the structural proteins that compromise the extracellular matrix (26). In a study by Boire et al. (16) in 2005, the matrix metalloprotease, MMP-1, was identified as a novel protease agonist that cleaves PAR1 at the appropriate site for receptor activation thus generating PAR1-dependent Ca\textsuperscript{2+} signals promoting cell migration and invasion. This MMP-1 was shown not to be produced by the breast cancer cells but instead was secreted by fibroblasts.

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**Table II. Pearson’s correlation between molecules studied.**

<table>
<thead>
<tr>
<th></th>
<th>hTERT</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAR 1a</td>
<td>0.535</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PAR 1b</td>
<td>0.008</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>VEGF-D</td>
<td>0.276</td>
<td>&lt;0.01</td>
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<tr>
<td>VEGF-C</td>
<td>-0.037</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>MMP-1</td>
<td>0.383</td>
<td>&lt;0.01</td>
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**Figure 1. Overall survival using the Kaplan-Meier method. Mean survival time: low (0, <0.20) = 139.7 (130.2-149.2, 95%CI) months; high (1, >0.20) = 105.4 (80.9-129.8) months.**
dissemination. Further research using breast cancer cell lines is required in order to investigate this hypothesis.

Acknowledgements

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References