

Correlation of *TERT* and Stem Cell Markers in the Context of Human Breast Cancer

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Abstract. *Background:* Telomerase reverse transcriptase (*TERT*) has a well-known role in carcinogenesis due to its functions in inducing cell immortality and preventing senescence. In this study, the relationships between *TERT* and a panel of known stem cell markers was examined in order to direct future enquiries into the role of ‘stem-ness’ in human breast cancer. *Materials and Methods:* Breast cancer tissues ($n=124$) and adjacent normal tissues ($n=30$) underwent reverse transcription and quantitative polymerase chain reaction. Transcript levels were analyzed for the correlation with that of *TERT*. *Results:* A significant direct correlation was found in cancerous tissue between *TERT* and *BM11* proto-oncogene polycomb ring finger 4 (*BM11*; $n=88$, $p<0.001$), *nestin* (*NES*; $n=88$, $p<0.001$), *POU domain, class 5, transcription factor 1* (*POU5F1*; $n=88$, $p<0.001$), *aldehyde dehydrogenase 1 family member A2* (*ALDH1A2*; $n=87$, $p=0.0298$), *cyclin-dependent kinase inhibitor 1A* (*CDKN1A*; $n=88$, $p<0.001$), *integrin subunit beta 1* (*ITGNB1*; $n=88$, $p<0.001$), *integrin subunit alpha 6* (*ITGA6*; $n=88$, $p<0.001$), *cluster of differentiation antigen 24* (*CD24*; $n=88$, $p=0.0114$), *MET* proto-oncogene (*MET*; $n=78$, $p<0.001$) and *noggin* (*NOG*; $n=88$, $p<0.001$). *Conclusion:* The evidence presented in this article of possible interactions between *TERT* and a discrete subset of known stem cell markers would significantly contribute to further enquiries regarding clonal dynamics in the context of human breast cancer.

Breast cancer continues to be a major cause of pathology in women. It is the most common cause of invasive cancer in females, with two million newly-diagnosed cases worldwide in 2018 (1). It is the second most common cause of cancer-related mortality for females in the UK, accounting for 15% of all cancer-related deaths in women in 2016 (2). However, it has to be acknowledged that survival has improved over the past several decades due to advances in treatment (3).

A greater understanding of the molecular pathways underlying breast cancer has been fundamental to many of these advances. The recognition of breast cancer as a diverse collection of diseases characterised by distinct molecular signatures has been an important recent development. This finding was the basis for the recent development of genomic assays for breast cancer, which have greatly improved prognostication and decision-making with regards to adjuvant chemotherapy (4). In addition, this highlights the potential role for tailored treatment of breast cancer on the basis of specific pathways which may be involved (5).

Human telomerase reverse transcriptase (*TERT*) is a molecule with a role in cell aging and immortality. By virtue of its function, it is expected to have a role in oncogenesis. This was borne out by the results of our previous study, in which we found the mRNA expression of *TERT* in breast cancer samples to be associated with poor prognosis (6).

Neoplastic lesions, by virtue of their high cellular turnover, have cells which acquire a degree of pluripotency reminiscent of stem cells (‘stem-ness’). According to the stem cell theory regarding oncogenesis, such cells may have role in maintaining cellular turnover, and may have a profound implication in the development of tailored and targeted treatments for breast cancer (7).

In this study, we aimed to examine the role of *TERT* in the context of cancer-related cellular pluripotency by studying the relations of *TERT* mRNA expression levels in breast cancer samples with a panel of 30 known stem cell marker molecules previously studied in the context of breast cancer (7-9).

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Materials and Methods

Samples. Tissue samples were collected after informed consent with ethical approval as per contemporaneous institutional guidelines (Bro Taf Health Authority ethics approval numbers 01/4303 and 01/4046). Immediately after surgical excision, a tumour sample was taken from the tumour area. Another was taken from the background non-cancerous tissue within 2 cm of the tumour, without affecting the assessment of tumour margins in order to serve as controls for comparison with the cancerous tissue. Breast cancer tissues (n=124) and normal background tissues (n=33) were collected and stored at -80°C in liquid nitrogen until analysis. This cohort has been the subject of a number of completed and on-going studies (6, 10, 11).

All the patients were treated according to local guidelines, following discussions in multidisciplinary meetings (12). Patients undergoing breast-conserving surgery also underwent radiotherapy. Patients with hormone-sensitive disease were given tamoxifen. Hormone-insensitive cases, high-grade cancer, and node-positive cases were treated with adjuvant therapy. Clinicopathological data (Table I) were collected from the patient charts and collated in an encrypted database (7, 13).

Tissue processing, RNA extraction and cDNA synthesis. RNA extraction kits and reverse transcription kits were obtained from AbGene Ltd. (Epsom Surrey, UK). Custom made hot-start Master Mix for quantitative polymerase chain reaction (qPCR) was from AbGene Ltd (Epsom, Surrey, UK).

The literature was reviewed to identify known stem cell markers which were included in the panel of molecules studied for correlations with TERT: 30 were selected in all. They are enumerated in Table II (14-40).

Approximately 10 mg of cancerous tissue was homogenised. A larger amount of matched normal tissue (20-50 mg) was used as its high fat content made it difficult to obtain sufficient RNA for analysis. The concentration of RNA was determined using a UV spectrophotometer (Wolf Laboratories, York, UK) to ensure adequate amounts of RNA for analysis. Reverse transcription was carried out using a reverse transcription kit (AbGene) with an anchored olig (dT) primer using 1 mg of total RNA in a 96-well plate to produce cDNA. The quality of cDNA was verified using β -actin primers (primers 5'-ATGATATCGCCGCTCGTC-3' and 5'-CGCTCGGTGAGGATCTTCA-3') (7, 13).

Quantitative analysis. Transcripts of cDNA library were determined using real-time qPCR based on Amplifluor technology. The PCR primers were designed using Beacon Designer software (Premier Biosoft International Ltd., Palo Alto, CA, USA), but an additional sequence, known as the Z sequence (5'-ACTGAACCTGACCGTACA-3'), which is complementary to the universal Z probe (Intergen Inc., Oxford, UK) was added to the primer. The primers were synthesized by Invitrogen Ltd. (Paisley, UK).

The reaction was carried out 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 each transcript were generated from a standard that was simultaneously amplified within the samples. Levels of expressions of the molecules being studied were normalised against cytokeratin 19 (CK19).

With every PCR run, a negative and positive control was employed, using a known cDNA sequence (podoplanin) (7, 13).

Table I. Description of clinical cohort.

Parameter	Number of samples
Tissue type	
Background, normal	30
Tumour	124
Tumour grade	
1	24
2	42
3	58
Nottingham prognostic index	
Grade 1	68
Grade 2	38
Grade 3	16
Unknown	2
TNM Stage	
1	70
2	40
3	7
4	4
Unknown	3
Histology	
Ductal	94
Lobular	14
Other	16
Patient outcome	
Alive and well	85
Metastatic disease	7
Death from breast cancer	15
All poor outcomes (Metastatic disease and disease-related mortality)	27

TNM: Tumour, node and metastases staging (12).

The samples were selected out of a cohort of 124 samples. However, some samples had to be excluded due to spurious results caused by depletion of the sample or issues with pipetting or the apparatus.

Statistical analysis. Correlations between TERT and molecules on the stem cell panel was performed using the SigmaPlot 11 statistical software package (Systat Software Inc., Hounslow, UK). Correlations were studied using the Spearman rank correlation test. The transcript levels of stem-cell related molecules within the breast cancer specimens were compared to the mRNA expression of TERT. All samples in the cohort were included. However, some specimens had to be excluded due to spurious results caused by depletion of the sample or due to errors in the PCR readings due to pipetting errors.

Correlations with *p*-Values less than 0.05 were considered significant.

Results

Spearman rank correlation test showed that TERT had significant direct correlations with the expressions of BMI1 proto-oncogene polycomb ring finger 4 (BMI1; $r=0.581$, $n=88$, $p<0.001$), nestin (NES; $r=0.581$, $n=88$, $p<0.001$), POU domain, class 5, transcription factor 1 (POU5F1; $r=0.651$, $n=88$, $p<0.001$), aldehyde dehydrogenase 1 family member A2 (ALDH1A2;

Table II. Correlations of mRNA expression (normalised to that cytokeratin 19) of stem cell markers with that of telomerase reverse transcriptase (*TERT*) by the Spearman rank correlation test.

Gene symbol	Molecule encoded	Correlation coefficient (R)	p-Value	No. of samples	Citation
<i>CD24</i>	Cluster of differentiation 24	0.269	0.0114	88	(16)
<i>ITGB1</i>	Integrin subunit beta 1	0.476	0.0000034	88	(16)
<i>CD44</i>	Cluster of differentiation 44	-0.0185	0.861	91	(17)
<i>ITGA6</i>	Integrin subunit alpha 6	0.663	0.0000002	88	(16)
<i>BMI1</i>	BMI1 proto-oncogene polycomb ring finger 4	0.581	0.000000014	88	(18)
<i>NES</i>	Nestin	0.581	0.000000013	88	(19)
<i>POU5F1</i>	POU domain, class 5, transcription factor 1.	0.651	0.0000002	88	(20)
<i>ALDH1A2</i>	Aldehyde dehydrogenase 1 family member A2	0.233	0.0298	87	(21)
<i>MET</i>	MET proto-oncogene	0.591	0.000000016	76	(23)
<i>CDKN1A</i>	Cyclin-dependent kinase inhibitor 1A	0.611	0.0000002	88	(24)
<i>NOG</i>	Noggin.	0.421	0.0000311	93	(22)
<i>CD34</i>	Cluster of differentiation 34	-0.0093	0.931	88	(23)
<i>CDCP1</i>	CUB domain-containing protein 1.	0.119	0.268	88	(25)
<i>THY1</i>	Thymocyte differentiation antigen 1, or CD90.	0.143	0.206	80	(26)
<i>ALDH1A1</i>	Aldehyde dehydrogenase 1 family member A1.	0.0788	0.483	81	(21)
<i>ALDH1A3</i>	Aldehyde dehydrogenase 1 family member A3.	-0.0177	0.888	65	(21)
<i>ALDH1B1</i>	Aldehyde dehydrogenase 1 family member B1.	-0.0871	0.416	89	(27)
<i>LMNA/C</i>	Lamin A/C.	0.0884	0.396	94	(28)
<i>LMNB1</i>	Lamin B1.	-0.19	0.067	94	(28)
<i>NRP1</i>	Neuropilin 1	0.197	0.066	88	(29)
<i>NRP2</i>	Neuropilin 2.	-0.0796	0.46	88	(30)
<i>PMSA2</i>	Proteasome (prosome, macropain) subunit, alpha type, 2	0.0772	0.466	91	(31)
<i>FLT1</i>	Fms-related tyrosine kinase 1	0.0247	0.818	89	(32)
<i>KDR</i>	Kinase insert domain receptor	0.0173	0.872	89	(33)
<i>FLT4</i>	Fms-related tyrosine kinase 4	0.033	0.753	93	(34)
<i>SNAIL1</i>	Snail family transcriptional repressor 1	0.0996	0.381	79	(35)
<i>SNAIL2</i>	Snail family transcriptional repressor 2	0.0352	0.74	91	(35)
<i>TWIST1</i>	Twist family bHLH transcription factor 1.	-0.0641	0.548	90	(36)
<i>SELL</i>	Selectin L	0.0105	0.927	78	(37)
<i>SELE</i>	Selectin E	-0.158	0.165	79	(37)
<i>SELP</i>	Selectin P.	-0.0961	0.396	80	(37)
<i>NOTCH2</i>	Notch receptor 2	-0.0981	0.354	91	(38)
<i>NOTCH1</i>	Notch receptor 1	0.044	0.675	93	(38)
<i>PECAM1</i>	Platelet endothelial cell adhesion molecule 1.	0.0484	0.651	89	(39)
<i>STAT5</i>	Signal transducer and activator of transcription 5	-0.0296	0.782	90	(40)
<i>CD133</i>	Cluster of differentiation 133	0.149	0.172	86	(23)

$r=0.233$, $n=87$, $p=0.0298$), Cyclin dependent kinase inhibitor 1A (*CDKN1A*; $r=0.611$, $n=88$, $p<0.001$), integrin subunit beta 1 (*ITGNB1*; $r=0.476$, $n=88$, $p<0.001$), Integrin subunit alpha 6 (*ITGNA6*; $r=0.663$, $n=88$, $p<0.001$), cluster of differentiation antigen 24 (*CD24*; $r=0.269$, $n=88$, $p=0.0114$), *MET* proto-oncogene (*MET*; $r=0.591$, $n=78$, $p<0.001$) and *NOGGIN* (*NOG*; $r=0.421$, $n=88$, $p<0.001$) (Table II).

Discussion

Telomeres are the straight portions at the end of a chromosome and are composed of repeating segments of nucleotides. During mitosis, DNA polymerase is unable to replicate this portion of the genome, which leads to genomic

instability and senescence. The telomeres are repaired by a specialised enzyme referred to as *TERT*, which is a multimeric molecule resident in the nucleus (41). *TERT* enables the cell to continue to proliferate without undergoing senescence, thereby achieving cell immortality (42).

Cell immortality represents an important gain of function for neoplastic cells. Indeed 70-90% of neoplastic lesions and cancer cell lines surveyed in one study were found to have increased activity of *TERT* (43). In another study, Elkak *et al.* found high mRNA expression of *TERT* to be associated with poorer outcomes in human breast cancer (6). Telomere-lengthening, with consequent replicative immortality has been characterised as a prerequisite for oncogenesis, with *TERT*-mediated telomere lengthening being identified as a

factor in 85-90% of cancers types, with alternative pathways accounting for the remainder (43). However, recent evidence has suggested that in addition to telomere lengthening, TERT may have a role in endothelial–mesenchymal transformation, as well as in the induction of stemness (44, 45).

Classically, it was believed that tumours consisted of uniformly neoplastic cells capable of proliferating independently, and giving rise to new clonal lineages which may contribute to the emergence of therapeutic resistance or to relapse of the disease after the end of adjuvant therapy. This has been referred to as the clonal evolution model, and was initially elucidated in 1976 by Nowell (46). The high degree of allelic heterogeneity seen in cancerous cells within solid tumours and *in situ* lesions is believed to be due to clonal neoplastic expansion, as would be predicted by the clonal evolutionary model (47, 48).

However, over the past three decades, evidence has been cited in favour of a tumour progression model which posits a hierarchy among the clonal lineages, privileging a small sub-population of pluripotential cells with known stem cell markers. This has been labelled as the cancer stem-cell (CSC) hypothesis. This was initially described by Bonnet and Dick in 1997 in the context of acute myelogenous leukaemia (49). In 2003, Al Hajj *et al.* showed that all cells within a solid breast tumour were not capable of generating a tumour when injected into nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice, and identified cells with a CD44⁺/CD24⁻ phenotype to be capable of pluripotential differentiation (50). Subsequently, CSCs were identified in melanoma (51), and neoplasias of the brain (52), lung (53), prostate (54) and colon (55). Other markers, most significantly acetaldehyde dehydrogenase 1 (ALDH1), have also been identified (56).

Whilst both of these hypotheses have broad similarities, they have very varied and mutually exclusive clinical implications, particularly with regards to treatment of relapsing disease and therapeutic resistance. Most current treatment modalities target proliferating cells indiscriminately. Such an approach would be expected to select clonal lineages more likely to be resistant to treatment, and potentially result in relapse in the disease after adjuvant treatment (57). However, if a progenitor population of pluripotential cells is positively identified, it would provide a discrete therapeutic target whose obliteration could potentially reverse the pathology (58).

However, issues have arisen with regards to the purported role of CSCs. There has been a failure in consistent replication of findings. There have been queries whether immunodeficient mice provide a suitable microenvironment to replicate *in vivo* conditions of human cancer (57). Furthermore, the use of proteolytic enzymes in the processing of tumours before sorting by flow cytometry has been cited as a destructive process which may affect the quality and reliability of the samples studied (57, 59).

Being that as it may, the mRNA expression levels of stem cell markers, such as *CD44* and *ALDH1*, have been found to be predictive of poor prognosis in breast carcinoma, suggesting that so-called ‘stem-ness’ is likely to have a role in solid tumours (56, 60).

More recently, it has been suggested that a viable model of tumour progression would be midway between clonal evolution and stem cell-based clonal hierarchy, likely with certain clonal lineages acquiring stem cell-like qualities under certain conditions (15).

By examining the association between known stem cell markers and TERT, we hope to link two highly influential concepts regarding our understanding of oncogenesis. By stratifying the multitude of stem cell markers by their correlation with TERT, an oncogenic molecule for which the evidence base has attained a degree of maturity, we hope to have provided an addition avenue of enquiry for research in breast tumour heterogeneity and clonal dynamics. This knowledge would hopefully enable more tailored treatments for recurrent and relapsing disease.

Several of the molecules identified in our study have been shown to have roles in oncogenesis. Prominent among these is *POU5F1* (better known as OCT4), which has been identified as an essential transcription factor mediating stem-ness. Shen and colleagues studied the effects of overexpression of *POU5F1* in MDA-MB-231 and 4T1 breast cancer cell lines, and found that the overexpression of *POU5F1* suppressed migration and invasion in these usually aggressive cell types (20).

NES has been identified as a marker of poor prognosis in ovarian (61), breast (62) and pancreatic (63) neoplasia. CD24, CD29 and CD44 have been studied as markers for colonic CSCs (64). BMI1 has been shown to have a role in mediating bone metastasis in breast cancer (18, 65). Similarly, evidence of a role in cancer has been suggested in the literature for the molecules identified as interacting with TERT in this study (8, 9).

However, certain limitations have to be acknowledged in our study. We were limited to mRNA expression data for a cohort for which we do not have information regarding protein expression. Furthermore, *in vitro* studies in transfected cell lines would be required to better characterise the effects of knocked-down and ectopic expression of these molecules, as well as to be able to delineate the interactions which may mediate their effects. By highlighting this subset of stem cell markers and their relation with TERT, we believe we have identified a potentially important direction of enquiry in understanding the role of stem-ness in breast cancer.

Conclusion

CSCs continue to be an important if challenging research question. An understanding of their role in tumour progression would go a long way in enabling more tailored treatments of

human breast cancer. We believe that the evidence we presented in this article of possible interactions between *TERT* and a discrete subset of known stem cell markers would significantly contribute to further enquiries regarding clonal dynamics in the context of human breast cancer.

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Conflicts of Interest

The Authors have no conflicts of interest to report.

Authors’ Contributions

The study was initiated and designed by KM and WGJ. TAM & WGJ conducted the qPCR assays. WGJ curated the database and performed the data analysis. UW performed the literature review and drafted the manuscript. KM & MWO proof-read the manuscript. MWO constructed the tables.

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