Stratification Using hTERT and Stem Cell Markers Confers a Good Prognosis in Invasive Breast Cancer

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Abstract. Background/Aim: In this study, we aimed to investigate the prognostic role of a previously identified panel of 10 stem cell markers stratified against the catalytic subunit of telomerase (hTERT) in human breast cancer. Materials and Methods: The mRNA copy numbers of these genes were determined using real time quantitative PCR in 124 breast cancer tissues and adjacent non-cancerous tissues. Relations between mRNA levels and survival were analysed using Kaplan–Meier plots and Cox regression analysis. Results: Five genes (BMI1, NES, POU5F1, ALDH1A2 and CDKN1A) correlated with survival when stratified with hTERT and predicted overall (Wilcoxon: p=0.004; Cox: p=0.006) and disease-free (Wilcoxon: p<0.000; Cox: p=0.000) survival. Conclusion: This panel of genes stratified by hTERT could open new avenues for the development of new prognostic tools, as well as for the identification of new research directions regarding breast oncogenesis.

Breast cancer remains the commonest cancer among women and the second most common cause of cancer-related female mortality in the UK, accounting for 15% of all cancer-related death in women in 2016 (1). This is despite improvements in survival due to refinements in therapy and better understanding of the underlying pathology over the last several decades (2). A major step forward in understanding breast cancer has been the recognition of the disease as a diverse collection of molecular entities distinguished by specific genomic signatures. This has been the basis of the currently evolving genomic assays which have revolutionised decision-making in breast cancer treatment, and have highlighted the need for personalised cancer therapy (3, 4).

The study of stem cell markers in oncogenesis has been the focus of research in recent decades. The emergence of pluripotentiality is of interest in view of the high mitotic turnover in neoplasia. In addition, understanding of “stem-ness” has been especially important in the debate surrounding clonal proliferation within tumours, which has potential implications for therapeutic targeting and decision making regarding tumour resection in advanced disease (5, 6).

Recently, we have studied the relations between a panel of stem cell markers and human telomerase reverse transcriptase (hTERT) in the context of breast cancer (7). hTERT has an important role in cell immortality and senescence, and has been demonstrated to have a role in oncogenesis (8). Our study identified a subset of stem cell markers which have significant associations with hTERT in human breast cancer (7). These markers were B lymphoma Mo-MLV insertion region 1 homolog (mouse) (BMI1), nestin (NES), POU class 5 homeobox 1 (POU5F1), aldehyde dehydrogenase 1 family member A2 (ALDH1A2) and cyclin dependent kinase inhibitor 1A (CDKN1A).

In the current study, we aimed to examine the role of hTERT and the 5 stem cell markers identified above in human breast cancer by analysing the association of their mRNA expression with clinical outcomes and survival in breast cancer (7).

Materials and Methods

Tissues samples. Tissue samples (124 cancer tissues and 30 healthy breast tissues) were obtained according to the local institutional guidelines and stored at −80°C until mRNA analysis (8-10).

All the patients underwent breast cancer surgery and received adjuvant therapy according to local guidelines after multidisciplinary discussions. The clinical and pathological data (Table 1) were collected from the patient charts, and were collated in an encrypted database (5, 11).
RNA extraction and cDNA synthesis. The stem cell markers that were found to be significantly correlated with hTERT in our previous study were selected for the current project. These were: cluster of differentiation 24 (CD24), cluster of differentiation 29 (CD29), cluster of differentiation 44 (CD44), integrin subunit alpha 6 (ITGA6; also known as CD49F), POU5F1 (also known as OCT4), ALDH1A2, MET proto-oncogene (MET, also known as hepatocyte growth factor receptor), CDKN1A (also known as p21), and noggin (NOG). They are enumerated in Table II (7).

Approximately 10 mg of tissue were used for quantitative RNA extraction and cDNA synthesis using the relevant kits obtained from AbGene Limited in the UK according to the instructions of the manufacturer (5, 11).

Quantitative RT-qPCR. The cDNA transcripts were determined using real-time qPCR (Amplifluor technology). The PCR primers were designed using Beacon Designer software (Premier Biosoft International Ltd., Palo Alto, CA, USA), and were synthesized by Invitrogen Ltd. (Paisley, UK). The primers incorporated an additional sequence, known as the Z sequence (5'-AC TG AAC C TG AC C G TAC A-3'), which is complementary to the universal Z probe (Intergen Inc., Oxford, UK).

The PCR reaction was carried out as follows: One cycle at 94°C for 15 s, 20 cycles at 55°C for 40 s, and one cycle at 72°C for 20 s. The mRNA expression levels were normalised against cytokeratin 19 (CK19), a house-keeping gene. With every PCR cycle, a negative (PCR water) and positive control was employed, using a known cDNA sequence (podoplanin) (5, 11). Although all 124 samples were analysed, some samples had to be excluded due to spurious results caused by technical issues.

Statistical analysis. Correlations of stem cell panel molecules and clinicopathological parameters were performed using the SigmaPlot 11 statistical software package (Systat Software Inc). Survival analyses were conducted using Kaplan–Meier plots and Cox regression, and were performed using SPSS version 25 (IBM Corp., Armonk, NY, USA).

Results

The mRNA levels of the ten genes previously identified to be correlated with hTERT in the context of breast cancer were studied in relation to disease-free and overall survival. This was carried out using Kaplan–Meier plots plotted across a decade of follow-up. The cut off is the median value of expression in the cohort. The mRNA levels of BMI1, NES, POU5F1, ALDH1A2 and CDKN1A were found to correlate independently with both overall and disease-free survival (Figure 1A and B), whilst those of ITGA6, MET, NOG, CD24 & ITGB1 did not and were excluded from further analysis (Table II).

We discovered that when the mRNA expressions of these 5 genes were stratified using hTERT to divide the cohort, they predicted significant differences in both overall (Wilcoxon: p=0.004; Cox: p=0.006) and disease-free (Wilcoxon: p<0.000; Cox: p=0.000) survival (Figure 1C and D; Tables III and IV). Cox regression analysis showed that stratified mRNA expression of the aforementioned 5 molecules correlated with overall (p=0.003) and disease-free survival (p=0.007) within the cohort.

Discussion

The predominant opinion regarding cell proliferation within solid tumours has been that they consist of a body of dividing neoplastic cells which give rise to new clonal lineages marked with changes in aspects of cellular behaviour, such as migration, invasion and proliferation, as well as other genetic changes which contribute to post-treatment relapses and the emergence of therapeutic resistance (12).

More recently, there has been evidence suggesting a hierarchy amongst tumour lineages with some cell lines within the tumour behaving as stem cells, playing a central role in the proliferation and evolution of the disease. This has been termed the cancer stem-cell hypothesis, and was initially described by Bonnet & Dick in 1997 (13). This model has focussed interest in the role of stem cell markers, and cancer stem cells (CSC) in oncogenesis. CSCs have been
identified in melanomas (14), neoplasia of the brain (15), lung (16), prostate (17) and colon (18) cancers.

A major clinical implication of the cancer stem-cell hypothesis is pertaining therapeutic resistance. Conventional adjuvant therapy has been largely effective in the treatment of de novo disease. However, such non-specific treatments could select cancer cells which are resistant to therapy. This is believed to be the aetiology of therapeutic resistance and relapse of disease after adjuvant therapy (19).

However, if it is possible to achieve remissions by targeting a smaller subset of pluripotent cancer cells, this could potentially reduce the risk of relapse and therapeutic resistance, and may provide a discrete therapeutic target whose obliteration could potentially reverse the pathology (20).

Table II. Correlations of mRNA expression (normalised to CK19) of stem cell markers with that of TERT by Spearman rank correlation test.

| Gene symbol | Molecule encoded | Correlation coefficient (R) | p-Value | Number of samples |
|-------------|------------------|-----------------------------|---------|------------------|
| CD24 | Cluster of differentiation 24 (CD24), also known as small cell lung carcinoma cluster 4 antigen | 0.269 | 0.0114 | 88 |
| ITGB1 | Integrin subunit beta 1, also known as CD29 | 0.476 | <0.001 | 88 |
| ITGA6 | Integrin subunit alpha 6, Also known as CD49F | 0.663 | <0.001 | 88 |
| BMI1 | BM1 proto-oncogene or polycomb ring finger 4, previously known as B lymphoma Mo-MLV insertion region 1 homolog (mouse) (BM11) | 0.581 | <0.001 | 88 |
| NES | Nestin, Previously known as neuroectodermal stem cell marker | 0.581 | <0.001 | 88 |
| POU5F1 | POU domain, class 5, transcription factor 1, also known as OCT4: (octamer-binding transcription factor 4) | 0.651 | <0.001 | 88 |
| ALDH1A2 | Aldehyde dehydrogenase 1 family member A2 | 0.233 | 0.0298 | 88 |
| MET | MET proto-oncogene. Also known as hepatocyte growth factor receptor | 0.591 | <0.001 | 76 |
| CDKN1A | Cyclin dependent kinase inhibitor 1A, also known as p21: (protein of 21 kDa atomic weight) | 0.611 | <0.001 | 88 |
| NOG | Noggin | 0.421 | <0.001 | 93 |

Table III. Cox regression model for disease-free survival.

| Variables in the equation | Beta | Standard error | Wald test | df | Significance (p) | Hazard ratio |
|---------------------------|------|----------------|-----------|----|-----------------|--------------|
| Exp(B) | | | | | | |
| Nottingham Prognostic Index | 0.789 | 0.354 | 4.978 | 1 | 0.026 | 2.202 |
| Tumour grade | 0.295 | 0.260 | 1.284 | 1 | 0.257 | 1.343 |
| TNM stage | 0.473 | 0.261 | 6.810 | 1 | 0.009 | 1.604 |
| High mRNA expression of 5-gene panel stratified by TERT | −0.341 | 0.114 | 8.928 | 1 | 0.003 | 0.711 |

Table IV. Cox regression model for overall survival.

| Variables in the equation | Beta | Standard error | Wald test | df | Significance (p) | Hazard ratio |
|---------------------------|------|----------------|-----------|----|-----------------|--------------|
| Exp(B) | | | | | | |
| Nottingham Prognostic Index | 0.884 | 0.339 | 6.806 | 1 | 0.009 | 2.420 |
| Tumour grade | 0.208 | 0.313 | 0.440 | 1 | 0.507 | 1.231 |
| TNM stage | 0.212 | 0.248 | 0.733 | 1 | 0.392 | 1.237 |
| High mRNA expression of 5-gene panel stratified by TERT | −0.248 | 0.092 | 7.363 | 1 | 0.007 | 0.780 |
This has been the raison d’etre for much of the research into the role of stem cell markers in carcinogenesis. Whilst there have been some issues regarding reproducibility of results, as well as concerns regarding the suitability of the techniques and materials used (19), significant evidence has been accumulated regarding the role of known stem cell markers in human oncogenesis. For instance, 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 the so-called “stem-ness” is likely to have a role in solid tumours (21, 22).
In our previous study, we studied the mRNA expression of hTERT, a regulator of cell aging, and that of a panel of 30 known stem cell markers in breast cancer tissue samples from a cohort of 124 patients (7). Previous studies had already established hTERT as a marker of poor prognosis in human breast cancer (8). Of the markers studied, we found 10 which directly correlated with hTERT. In our current project, we used this subset as a starting point for studying the relation of stem cell markers with clinical outcomes. We identified five that had significant implications as prognostic markers for poor clinical outcomes. Specifically, genetic modification of the expression of the target molecules could yield important information regarding their effects on cell behaviour, such as invasion, proliferation and migration as well insights into the cellular mechanisms affected.

Conclusion

Our current study identified 5 stem cell markers that once stratified using hTERT are significantly related to clinical outcome in human breast cancer. Our findings will help future in vitro and mechanistic studies on the role of stem cells in human breast cancers, which hopefully would provide us with a better understanding of the micro-environment within human breast cancer and development of new therapeutic modalities.

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 & MWO performed the literature review and drafted the manuscript. KM & MWO proof-read the manuscript.

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