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Elevated TRF2 in advanced breast cancers with short telomeres

Malissa C. Diehl¹, Michael O. Idowu², Katherine Kimmelshue², Timothy P. York^{1,3}, Colleen Jackson-Cook^{1,2,5}, Kristi Turner², Shawn E. Holt^{1,2,4,5}, and Lynne W. Elmore^{2,5,✉}

¹Department of Human and Molecular Genetics, Virginia Commonwealth University, Richmond, Virginia 23298

²Department of Pathology, Virginia Commonwealth University, Richmond, Virginia 23298

³Virginia Institute for Psychiatric and Behavioral Genetics, Virginia Commonwealth University, Richmond, Virginia 23298

⁴Department of Pharmacology and Toxicology, Virginia Commonwealth University, Richmond, Virginia 23298

⁵Massey Cancer Center, Virginia Commonwealth University, Richmond, Virginia 23298

Abstract

Introduction—Telomere repeat binding factor 2 (TRF2) binds directly to telomeres and preserves the structural integrity of chromosome ends. *In vitro* models suggest that expression of TRF2 protein increases during mammary cancer progression. However, a recent study has reported that TRF2 mRNA levels tend to be lower in clinical specimens of malignant breast tissue. Here, we conduct the first large scale investigation to assess the levels and cellular localization of the TRF2 protein in normal, pre-malignant and malignant breast tissues.

Methods—Breast tissue arrays, containing normal, ductal carcinoma in situ (DCIS) and invasive carcinoma specimens, were used to assess the expression and localization of TRF2 protein. Telomere lengths were semi-quantitatively measured using a pantelomeric peptide nucleic acid probe. A mixed effects modeling approach was used to assess the relationship between TRF2 expression and telomeric signal scores across disease states or clinical staging.

Results—We demonstrate that TRF2 is exclusively nuclear with a trend towards lower expression with increased malignancy. More case-to-case variability of TRF2 immunostaining intensity was noted amongst the invasive carcinomas than the other disease groups. Invasive carcinomas also displayed variable telomere lengths while telomeres in normal mammary epithelium were generally longer. Statistical analyses revealed that increased TRF2 immunostaining intensity in invasive carcinomas is associated with shorter telomeres and shorter telomeres correlate with a higher TNM stage. All immortalized and cancer cell lines within the array displayed strong, nuclear TRF2 expression.

Conclusion—Our data indicate that elevated expression of TRF2 is not a frequent occurrence during the transformation of breast cancer cells *in vivo*, but higher levels of this telomere binding protein may be important for protecting advanced cancer cells with critically short telomeres. Our findings also reinforce the concept that serially propagated cancer cells, although tumor-derived, may not model all types of authentic tumors; especially those demonstrating genetic heterogeneity.

✉Correspondence: Department of Pathology, Virginia Commonwealth University, 1101 E. Marshall St, Richmond, VA 23298, USA; lelmore@mcvh-vcu.edu.

Keywords

TRF2; breast cancer; cancer progression; telomere length; DCIS; TNM

Introduction

Telomeres are specialized structures found at the ends of linear chromosomes that contain non-coding DNA distinguished by a 5'-TTAGGG-3' sequence [1]. The 'end replication problem' produces a stretch of unreplicated DNA between the final RNA priming event and the terminus. To counteract this problem, the processive ribonucleoprotein enzyme, telomerase, extends these continuously shortened ends. Telomerase maintains telomeres in the vast majority of cancer cells, providing for unlimited proliferative potential [2-3]. Despite high levels of telomerase activity in tumor cells, telomeres are generally maintained at a relatively short length and telomere length abnormalities are seen as early events in the initiation of epithelial carcinogenesis, including ductal carcinoma *in situ* (DCIS) of the breast [4-5].

In light of these abnormalities, a host of telomere binding proteins are critical for assuring that telomeres do not trigger a DNA damage response, since an unfolded telomere could be sensed as a double strand DNA break [6]. Shelterin is a complex consisting of six integral telomere binding proteins, TRF1, TRF2, TIN2, Rap1, TPP1, and POT1. This complex is abundant at chromosome ends and remains associated at the telomere throughout the cell cycle [7]. Two of the shelterin components, TRF1 and TRF2, are necessary for the proper formation of the cap structure. Both proteins contain DNA-binding domains and form homodimers and higher order oligomers [8]. TRF2 has emerged as a major protective factor at chromosome ends [9-10], and functional inactivation of TRF2 results in a high frequency of end-to-end fusions, which arise from the cells' inability to distinguish natural telomeric ends from broken DNA. In addition to these fusions, cells without a protective telomere cap are also vulnerable to chromosomal rearrangements and aneuploidy [11]. A cell, in response to loss or inactivation of TRF2, typically undergoes rapid senescence or death [10, 12].

Currently, very little is known about the role of TRF2 in mammary carcinogenesis. Cultures of immortally transformed and tumor-derived human breast cells have elevated levels of TRF2 protein compared to mortal mammary epithelial cells, suggesting involvement in mammary cancer progression [13]. For validation purposes, Nijjar et al., [13] included six breast tissue specimens in their immunostaining analysis, preliminarily concluding that TRF2 protein levels are elevated in invasive carcinomas compared to DCIS. TRF2 can act as a potent oncogene *in vivo* when combined with telomerase deficiency [14], further supporting potential involvement of TRF2 in carcinogenesis. If TRF2 is overexpressed in breast cancers and this telomere binding protein is mechanistically linked to malignant transformation, then targeting TRF2 in breast cancer cells, most of which have short telomeres, could prove to be a highly efficacious therapy. However, a recent investigation of the expression of multiple telomere-associated genes in clinical specimens has revealed that TRF2 mRNA levels tend to be lower in malignant breast tissues [15].

Here we conduct a comprehensive, large-scale study with breast tissue arrays to determine the relative levels of TRF2 protein during mammary cancer progression *in vivo*. Analysis of relative telomere lengths on the same case sets, together with correlations to TRF2 levels and tumor node metastasis (TNM) stage, has allowed us to better understand the role(s) of TRF2 in mammary carcinogenesis.

Materials and Methods

Western Blotting Analysis

Whole cell lysates were obtained according to a standard procedure [16] for the following panel of mammary epithelial cell strains and lines: HME-50, mortal human mammary epithelial cells from a patient with Li Fraumeni Syndrome [17]; HME 50-5E, a spontaneously immortal cell line derived from HME-50 [17]; MCF-10A, an immortal but non-tumorigenic cell line derived from a patient with fibrocystic disease [18]; MCF-7 and MDA-MB231 tumor-derived human breast cancer cells; and MCF-7/hTERT, which ectopically expresses the catalytic component of telomerase [19]. Fifty micrograms of each whole cell lysate were resolved by SDS-PAGE, immunoblotted with 1:2000 diluted TRF-2 antibody (clone 4A794; Millipore, Billerica, MA) and a monoclonal actin antibody (1:5000; Sigma, St. Louis, MO), and then processed according to a standard method [16]. TRF2 antibody (clone 4A794) was selected for use in this study because it has previously been siRNA/shRNA validated by Western Blotting [20-21].

Design of Tissue Microarrays (TMAs)

Breast tissue arrays from three individual case sets (#3, #5 and #7) were obtained from the National Cancer Institute's Cooperative Breast Cancer Tissue Resource (<http://cbctr.nci.nih.gov>). These second generation case sets were created from 679 breast tissue specimens divided into six non-overlapping sets. Each slide contains between 133-135 cores, including 79-80 invasive breast cancer cases, 23 normal, and 10-11 ductal carcinoma *in situ* (DCIS) breast specimens. Twenty-one control cores are embedded in each array, including several breast and non-breast tumor-derived cell lines. Clinico-pathological features (i.e., tumor size, number of positive lymph nodes, tumor-node-metastasis [TNM] stage, tumor grade, hormone status, age at diagnosis, and race) were provided for each anonymized specimen.

Immunohistochemistry

A standard immunohistochemical staining procedure [22] was used to detect TRF2 in breast tissue arrays. This included use of a VectaStain ABC immunoperoxidase IgG detection kit (Vector Labs, Burlingame, CA), according to the manufacturer's recommendations, and diaminobenzidine (DAB; Vector Labs) as the substrate. Arrays were incubated overnight at 4°C with TRF2 antibody (clone 4A794; diluted 1:200). All images of immunostained tissue microarrays were magnified using a Nikon Eclipse E600 and captured by a Nikon Digital Camera DXM1200F, using the Nikon ACT-1 image editing program.

Fluorescence *in situ* hybridization (FISH)

Telomere lengths for the specimens on the TMA were semi-quantitatively assessed using a pantelomeric (C₃TA₂)₃, FITC-labeled peptide nucleic acid (PNA) probe (DAKO, Denmark). Following standard procedures for paraffin-embedded tissue [23], probe hybridization was achieved according to the manufacturer's (DAKO) recommendations.

Scoring of Tissue Arrays

For immunohistochemistry, mounted slides were semi-quantitatively scored by two independent pathologists [MOI and KK] in terms of reactivity (positive if >10% of the cells were immunostained), intensity based on an ordinal scale (negative = 0, weak = 1, moderate = 2, strong = 3), and cellular localization (nuclear or cytoplasmic). The telomere signal intensities were scored by certified cytogeneticists (CJC or KT), who also used an ordinal scale for their assessments of telomeric signal intensity (no fluorescent signal = 0; small number of weak signals = 1; moderate number and intensity of signals = 2; many signals

with bright intensity = 3). After characterizing the tissue morphology (normal versus tumor) using DAPI staining under low power, the nuclei were assessed (using single and triple band pass filters) (Figure 4). When present, both normal and tumor tissues were scored for all specimens, with successful hybridization in the normal adjacent tissue being required for the inclusion of a score of zero in a pathologic specimen (whether DCIS or invasive carcinoma).

Statistical Analyses

TRF2 immunostaining was performed for each individual in duplicate from cores on separate slides. Each slide was assessed by two pathologists for a total of four measures per subject. Thus the nested, repeated outcome measures were handled using a mixed modeling approach with terms included to account for this hierarchy (random effect) and possible biases due to the different pathologists (fixed effect), as previously reported by us for breast tissue arrays [22]. A mixed effects modeling approach was also used to assess mean differences in telomeric FISH signal across the three disease states (normal, DCIS and invasive carcinoma) and to assess the relationship between TRF2 protein expression and telomeric signal score or TNM stage. For TRF2 immunostaining scoring, inter-rater agreement was measured using a weighted kappa test. All statistical tests were performed using the R statistical package, version 2.9.1 [22].

Results

Western blotting confirms expression of TRF2 protein in human mammary epithelial cell cultures

Prior to the immunohistochemical analysis, western blotting was performed on a panel of human mammary epithelial cell lines. A 66 kDa band was evident in each sample corresponding to TRF2 (Figure 1), with a trend for reduced levels of TRF2 with increasing malignancy. A second smaller molecular weight band was detected in the mortal HME 50 cells and to a lesser extent in spontaneously immortal HME 50-5E cells. This TRF2 doublet, which has been sporadically observed in other cell types [25], was not detected in immortal MCF-10A, tumorigenic MCF-7, or highly invasive MDA-MB231 cells.

A trend for reduced TRF2 protein expression with increased malignancy in clinical breast specimens

We then immunohistochemically stained breast tissue arrays containing normal, DCIS, and invasive carcinoma cores. The cellular distribution of TRF2 showed localization to the nucleus in all specimens (Figure 2). We observed a trend for decreasing TRF2 intensity values with increasing malignancy ($p=0.0810$) (Table 1). Compared to normal and DCIS cores, invasive carcinomas exhibited greater case-to-case variability in TRF2 staining, ranging from very low intensity or negative immunoreactivity to strongly reactive (Figure 2f, g and Table 1). There was no evidence of DAB substrate in any of the PBS negative controls (Figure 2a, c, e).

High levels of TRF2 protein in immortal and tumor-derived cancer cell line cores

In addition to breast tissue, numerous immortalized and cancer cell lines were embedded within the array, allowing for the simultaneous detection of TRF2 protein in breast-derived tissues and tumor-derived cell cultures. Unlike the variability observed in invasive carcinoma tissues, the nuclei from cell lines consistently exhibited intense TRF2 immunostaining (Figure 3). This was true whether the cell lines were immortal, tumorigenic, breast-derived, or originating from another solid tumor type. The vast majority of cell lines stained strongly ($73.4\% \pm 4.5\%$), while the remainder stained with a moderate intensity ($26.7\% \pm 4.5\%$).

Highly variable telomere lengths in invasive breast carcinomas; short telomeres were associated with elevated TRF2 protein

Since TRF2 has emerged as a major protective protein at the telomere [10], we tested whether TRF2 immunostaining intensity within breast carcinomas correlated with telomere length. The probe hybridization resulted in the presence of multiple, punctate, nuclear hybridization signals with the number and intensity of the signals being inferred to reflect length [26] (Figure 4). Normal breast tissue cores, which, on average had moderately strong telomeric signals (corresponding to longer telomeres), had significantly increased telomeric signal intensities when compared to the nuclei from DCIS specimens or invasive carcinomas tissues (Table 1), confirming previous findings of telomere attrition in pre-invasive (DCIS) and invasive breast cancer specimens [4-5]. Invasive breast carcinomas displayed variation in their telomeric signal intensities, ranging from cases having many bright telomeric signals (suggestive of long telomeres comparable to those seen in normal tissue), to cases having very weak signals (corresponding to short telomeres) (Figure 4). TRF2 immunostaining intensity in carcinoma cores was negatively associated with telomeric signal intensity (Table 2). Thus, greater TRF2 expression appeared to be present in nuclei having shorter telomeres in the invasive breast carcinomas evaluated ($p=0.049$). Interestingly, in the DCIS tissue type, we observed a similar relationship between TRF2 and FISH scores as seen in the invasive cores (Table 2). However, the power of FISH to serve as a predictor of TRF2 score did not reach statistical significance in DCIS ($p=0.549$) possibly due to the much lower number of samples as compared to the invasive tissue type.

Bright telomere signals in invasive breast carcinomas correlate with lower TNM stage

Intuitively, one would predict that cancers with short telomeres would be genomically more unstable, in part due to fewer telomeric repeats for telomere binding proteins to associate. This, in turn, could facilitate the development of a more advanced cancer. Therefore, we tested whether short telomeres in invasive carcinomas cores correlate with severity of disease, as reflected by TNM staging. We found that more intense telomeric signal (i.e., longer telomere) correlates with a lower TNM stage, indicative of a more indolent tumor ($p=0.025$, Table 2). In contrast, the TNM stage and TRF2 scores were not found to be associated ($p = 0.71$).

Discussion

Experimental data indicate that TRF2 is a critical protein for protecting chromosome ends from being recognized as DNA damage [9-10] and can act as an oncogene when paired with telomerase deficiency/short telomeres [14]. TRF2 protein expression has also been shown to increase with breast cancer progression [13]. However, prior to this study, only a very small sample set of breast tissues ($n=6$) had been assayed for TRF2 protein expression, with the investigators in that study concluding that this telomere binding protein was expressed at higher levels in breast carcinomas relative to DCIS [13]. Here, we have conducted the first large scale investigation of TRF2 protein expression in human breast tissue arrays and find that there is actually a trend for lower expression of TRF2 protein in invasive carcinomas without any significant differences in TRF2 staining intensity in invasive carcinomas relative to either normal or DCIS breast tissues. This same trend, as well as a lack of significant differences, has recently been reported for TRF2 mRNA in clinical breast specimens [15].

Our western blotting analysis with breast epithelial cell strains and lines proved to be consistent with immunostaining results from clinical specimens that less, rather than more, TRF2 protein is associated with breast cancer progression. It is unfortunate that only 2 of the 6 cell lines we analyzed by immunoblotting were included on the tissue arrays as control

cores. Of note, we show that immortal but non-tumorigenic MCF-10A cells express relatively high levels of TRF2 protein by immunostaining and western blotting. Consistent with previous western blotting results [13], we report here that T47D breast cancer cells exhibit intense TRF2 immunostaining. We did observe an apparent discrepancy in the levels of TRF2 protein in MCF-7 breast cancer cells: immunostaining intensity of the duplicate MCF-7 cores was intense whereas the band on the western blot was weak relative to the non-tumorigenic cell strains and lines. Possible explanations for this inconsistency could be the limited quantitative power of western blotting, inter-laboratory differences with the “same” established cancer cell lines and/or very efficient antigen retrieval in cell pellets embedded in paraffin coupled with a sensitive avidin-biotin detection system. It was unanticipated that all cell lines within the array would consistently exhibit intense TRF2 immunostaining, a finding we have yet to explain. This was true for immortal, non-tumorigenic cells as well as cell lines with high malignant potential and independent of tissue origin. However, regardless of the reason *why* continuously cultured immortal/cancer cell lines uniformly exhibit intense TRF2 immunostaining, we clearly demonstrate that TRF2 levels are infrequently elevated in breast carcinomas *in vivo*. We therefore conclude that it is highly unlikely that upregulation of TRF2 in tumor-derived cells is mechanistically linked to mammary carcinogenesis but rather a consequence of serial passaging *in vitro* and/or the processing of formalin-fixed, paraffin-embedded cell pellets.

We also demonstrate that breast cancer tissues have shorter telomeres than normal tissues and that telomere length variability is evident in pre-malignant and malignant breast epithelial cells, findings that are highly consistent with previous reports [4-5, 27-29]. Observing variability in both TRF2 staining intensity and telomeric signal amongst invasive carcinomas naturally led us to test whether there was a connection between expression of this critical telomere binding protein and telomere length in breast cancer tissues. We found that there is a significant inverse relationship between high expression of TRF2 and short telomeres within invasive breast carcinomas. A recent study [25], using quantitative immunoblotting to determine the abundance and stoichiometry of the shelterin proteins in cell cultures, has come to a similar conclusion: human cells with short telomeres contain more shelterin (most notably more TRF2) than cells with long telomeres. Collectively, these findings suggest that TRF2 is upregulated in advanced breast cancer cells in an effort to protect critically short telomeres from being recognized as DNA damage. Without a protective cap, telomeres are vulnerable to end-associated chromosomal aberrations leading to growth arrest and possibly cell death [12]. Elevated levels of TRF2 in a subset of breast carcinomas could also interfere with the ability of telomerase to assess the telomere, theoretically contributing to stable, short telomere lengths despite high telomerase activity [4-5]. Since TRF2 has been implicated as an early mediator in interstitial DNA damage surveillance and repair pathways [30], upregulation of TRF2 in a subset of invasive carcinomas would also reflect elevated activity related to DNA repair (at telomeric and interstitial sites) in genomically unstable tumor cells.

Using an *in situ* approach, we have demonstrated a reduced telomeric signal intensity (inferred to reflect a shortened telomere length) in DCIS and invasive carcinoma specimens. In addition, we have shown in invasive carcinomas a significant correlation between telomeric signal intensity and TNM status, the latter of which is a cancer staging system that is revered as one of the most informative of current prognostic markers for breast cancer [31]. This finding is in agreement with the results of Fordyce et al. [32] who, using a quantitative slot blot assay relying on telomere DNA content as the end-point, also observed a correlation between reduced telomere signal and TNM stage in breast cancer. However, our findings are in contrast to the results of two other investigative teams who, using a TRF assay, failed to detect a significant correlation between telomere length and breast cancer stage [33-35]. One possible explanation for this apparent discrepancy between studies is that

the methodologies used to assess telomeric length varied. Our use of a FISH technique allowed for the collection of telomeric signal data in a cell-specific manner, with tumor cells being recognized and scored separately from normal cells. In contrast, the TRF assay, which is a southern-based technique, assesses telomere length by isolating genomic DNA from tissue containing cancer cells as well as stromal constituents to provide a global (or average) estimate of telomere length. Thus, the TRF reflects the telomeric lengths present in a sample comprised of an admixture of normal and tumor cells. As a result, it has the potential to mask differences that might be present between cell types.

Recognizing the critical role that TRF2 plays in maintaining a functional cap structure at the telomere and the rapid induction of cell death or senescence in breast cancer cells when TRF2 is removed from chromosome ends, it was our hope that levels of TRF2 protein would be elevated in the vast majority of breast cancer tissues, as observed by us and others [13] for many tumor-derived mammary cell lines. Such a scenario would support TRF2 as a possible therapeutic target for breast cancer. Instead, we found that TRF2 proteins levels are relatively constant in normal, DCIS and the majority of invasive carcinoma tissues. However, by employing *in situ* techniques to assess TRF2 protein and telomere length, we discovered that TRF2 may have a unique role in a subset of invasive breast carcinomas, namely those with short telomeres. It is presently unclear whether upregulation of TRF2 may contribute to enhanced telomeric protection, cell growth, DNA repair, and/or chemotherapeutic resistance, as this telomere binding protein has been associated with all of these properties [10, 30, 35]. We now, with awareness of these caveats, plan to utilize breast cancer cell cultures to directly test these possibilities.

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Fig. 1. An *in vitro* trend towards reduced TRF2 protein expression during breast cancer progression

Western blotting was performed on whole cell lysates of mortal (HME 50-5), immortal (HME 50-5E and MCF-10A), and tumor-derived (MCF-7, MCF-7/hTERT and MDA-MB231) breast epithelial cells to determine relative protein levels of TRF2. Actin served as a loading control. While TRF-2 (66kDa) was observed in all samples, expression levels appeared to be lower in the tumor-derived breast cancer cell lines (MCF2, MCF-7hTERT, and MDA-MB231).

Fig. 2. TRF2 expression in normal, DCIS, and invasive carcinoma cores of breast tissue arrays Moderately intense nuclear TRF2 immunostaining was typically observed in normal (b) and DCIS (d) cores while more variable expression levels were seen in the invasive carcinoma cores (f, g). The upper panels (a, c, e) show tissue in different microscopic fields following Hematoxylin staining without antibody (negative control). All images are shown at a 40x magnification level. Overall weighted kappa value: 0.5495.

Fig. 3. Established cell lines embedded within the tissue arrays consistently exhibit intense TRF2 immunoreactivity

In addition to breast tissues, the TMAs contained numerous cell lines. Shown are representative images of MCF10A (b) and breast cancer cell lines, MCF7 (d) and T47D (f), immunostained for TRF2. The upper panels (a, c and e) represent the respective no primary antibody controls. All images are shown at a 40x magnification level. Overall weighted kappa value: 0.5495.

Fig. 4. Invasive carcinomas exhibit variability of telomeric signals

The probe signals observed varied between invasive carcinoma tissues, with specimens demonstrating strong (a), moderate (b), or weak (c) intensities. DAPI-stained nuclei were viewed under high power (1000x magnification) with single and triple band pass filters to assess the number and intensity of the telomeric probe signals.

Table 1

Average TRF2 immunostaining and Telomeric FISH scores

	Observed means (s.d.)			p-value*
	Normal	DCIS	Invasive	
TRF2	1.92 (0.36), n=66	1.78 (0.22) n=30	1.8 (0.40) n=235	0.081
Telomeric FISH	2.20 (0.82) n=67	1.27 (0.66) n=31	1.36 (0.82) n=219	<0.001

*p<0.05 are significant and derived from a test of whether there is any difference in mean values across the three tissue types.

Table 2

Relationship between TRF2 score or TNM and telomeric FISH scores

Predictor	Tissue Type	Coefficient	p-value *
TRF2	Invasive Carcinoma n=217	-0.046	0.049
TRF2	DCIS n=30	-0.036	0.549
TNM	Invasive Carcinoma n=211	-0.184	0.025

*
p<0.05 are significant