Telomere-Mediated Genomic Instability and the Clinico-Pathological Parameters in Breast Cancer

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A study was undertaken to correlate telomere dysfunction and genomic instability with the histopathological grades and the estrogen and progesterone receptor status in breast cancer. Sixty-one archived breast tissues (38 cancer tissues and 23 paired normal tissues) were used in the study. The breast tumor tissues showed significantly shorter telomeres (7.7 kb) compared with the paired adjacent tissues (9.0 kb) by Southern blot analysis. Moreover, telomere shortening was more significant in Grade III tumors than in the Grade II tumors ($P = 0.05$). Quantitative fluorescence in situ hybridization on paraffin tissue sections revealed a similar trend in telomere shortening. Telomere attrition was associated with telomere dysfunction as revealed by the presence of significantly higher anaphase bridges in tumor cells which was tumor grade dependent. Furthermore, estrogen receptive negative tumors displayed higher anaphase and internuclear bridges. Selected samples from each grade showed greater genomic imbalances in the higher grades than the lower grade tumors as detected by array-comparative genomic hybridization. Telomerase activity was found to be higher in the higher grades (Grade II and III) compared with the lower grade (Grade I). The average mRNA expression of *TRF1* and *POT1* was lower in the tumor tissues than in the normal tissues. *Tankyrase 1* mRNA expression showed a grade-dependent increase in tumor tissues and its expression was also high in estrogen and progesterone negative tumors. The data support the notion that telomere dysfunction might be of value as a marker of aggressiveness of the tumors in breast cancer patients.

INTRODUCTION

Breast cancer is the most common cause of cancer deaths among women worldwide (Chia et al., 2002). Suitable biomarkers are necessary for assessment of the tumors and proper clinical treatment decisions. Tumor/nodes/metastases stages have long been used as the most powerful indicators of breast cancer prognosis and recurrence (Hayes et al., 2001). In addition, a number of other markers like tumor type and grade, hormonal receptor status, markers of proliferation, cell death, micrometastasis, and neovascularization provide additional information about the prognosis, response to treatments, and recurrence (Hayes et al., 2001). Increasing number of markers is being developed to understand the disease prognosis and determine the treatment options.

Genomic instability is an important factor in cancer initiation and progression (Hanahan and Weinberg, 2000; Meeker and Argani, 2004). Telomeres and the associated telomeric proteins play significant roles in maintaining the integrity of the genome. Loss of telomere function is one of the mechanisms underlying genomic instability. Telomere loss or dysfunction results in complex types of genomic abnormalities, including loss of heterozygosity, gene amplifications, deletions, and aneuploidy (Lo et al., 2002; Murnane and Sabatier, 2004; Sabatier et al., 2005). Furthermore, the consequential genomic instability has also been shown to be associated with aggressiveness and poor prognosis of tumors (Kronenwett et al., 2006). In carcinomas, histomorphologically increasing gradation of dysplasia is associated with the aggressiveness of the tumors. Hence, it is important and interesting to understand the association of telomere dysfunction with the aggressiveness of the tumors.

The regulation of telomere length is a dynamic process involving telomerase and a highly complex

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system involving a number of telomere-associated proteins. Telomere repeat binding factor 1 (TRF1), protection of telomeres 1 (POT1), and Tankyrase 1 (TRF1-interacting ankyrin-related ADP ribose polymerase) are telomere-associated proteins that are part of telomere structure and have essential roles in telomere length regulation (Liu et al., 2004a). TRF1 binds to TTAGGG double stranded repeats of telomeres and has been identified as a negative regulator (Bilaud et al., 1996, 1997; Broccoli et al., 1997). Tankyrase 1 interacts with TRF1 and its over expression was shown to release TRF1 from the telomeres and induce telomere elongation. Tankyrase 1 has thus been shown as a positive regulator of telomere length (Smith et al., 1998). POT1 is recruited to the 3′ single-stranded portion of the telomeric DNA and it was found to employ telomerase for telomere elongation (Liu et al., 2004a,b; Smogorzewska and de Lange, 2004).

The aim of this study was to investigate the association of telomere dysfunction and genomic instability with the tumor prognostic factors like histopathological grades and hormonal receptor status. In addition, the mRNA expression of selected telomere related genes such as *TRF1*, Tankyrase 1, and *POT1* were also studied.

MATERIALS AND METHODS

Patient Samples

Archived human invasive ductal breast carcinoma with the paired adjacent tissues or DNA and RNA samples ($n = 61$; Tumor = 38; Paired normal = 23) were obtained from the Department of Pathology, National University Hospital, Singapore. This study was approved by National University of Singapore—Institutional Review Board (NUS-IRB; NUS-062). The samples were histopathologically graded based on the modified Scarff-Bloom-Richardson grading system. Estrogen receptor (ER) and progesterone receptor (PR) status of the tumor samples were also determined.

Telomere Restriction Fragment Length Analysis

Genomic DNA was isolated from the frozen tissues using QIAamp Tissue Kit (Qiagen, Valencia, CA). Telomere length was assessed by measuring the telomere restriction fragments using the Telo TTAGGG telomere length assay kit (Roche Diagnostics, Indianpolis, USA) by following manufacturer's instructions. After digestion of the genomic DNA with *Hin*fI and *RSA*I, they were separated on a 0.8% agarose gel. The DNA was then transferred onto the nylon membrane (Roche Diagnostics, Indianpolis, USA) by Southern blotting and the blotted DNA fragments were hybridized to digoxigenin-labeled probe specific for telomeric repeats and incubated with a digoxigenin-specific antibody covalently coupled to alkaline phosphate. Finally, the immobilized telomere probe was visualized by metabolizing CDP-Star, a highly sensitive chemiluminescence substrate. The average TRF length was determined by comparing the signals to a molecular weight standard using the Kodak Molecular Imaging Software (Kodak, Rochester, USA).

Peptide Nucleic Acid–Fluorescence In Situ Hybridization for Telomeres on Tissue Sections

Paraffin embedded tissue sections of 4-μm thickness were deparaffinized in 100% Xylene. After dehydrating with 100% ethanol, the slides were incubated in 1 M sodium thiocyanate at 80°C for 8 min. Slides were incubated in 1% pepsin in 0.01 M HCl for 2 min at 37°C and dipped through the ethanol series and air dried. The tissue sections were then hybridized with Cy3 conjugated PNA telomere probe (Applied Biosystems, Boston, MA) for 2 hr at room temperature after co-denaturation at 80°C for 3 min (Hande et al., 1999). Stringent washes were performed and the slides were counterstained with DAPI (0.1 μg/ml). Images were captured with Zeiss Axioplan 2 imaging microscope (Carl Zeiss, Gottingen, Germany) and the telomere signals were quantitated using the ISIS software (Metasystems, Altusseheim, Germany).

Telomerase Activity

The TRAPeze® XL Telomerase Detection Kit (Chemicon International, Temecula, CA) was used according to the manufacturer's instructions to measure the telomerase enzyme activity of breast cancer tissues and the paired adjacent tissues. The breast tissue extract was prepared after homogenizing the cells and resuspending in 100 μl of CHAPS XL Lysis Buffer (included in the telomerase detection kit) and incubating on ice for 30 min. The extract was pelleted (12,000*g* at 4°C), and the supernatant was frozen at −80°C until assayed for telomerase. The TRAPeze® XL Telomerase Detection Kit uses a modified TRAP (telomerase repeat amplification protocol) assay to detect telomerase activity through the amplification of telomeric repeats using fluorescence energy transfer primers (Amplifluor™) that produce measurable fluorescence only when incorporated into TRAP products. The fluorescence of each reaction was measured with a Magellan fluorescent plate reader.

Telomerase activity of each sample was determined by calculating the ratio of the increase in fluorescein absorbance (produced by the amplification of telomeric repeats) divided by the increase in sulforhodamine absorbance.

Anaphase Bridges and Internuclear Bridges

Anaphase bridges are chromosomal bridges that are not resolved during anaphase and are associated with telomere dysfunction and chromosome end fusions (Meeker and Argani, 2004; Jin et al., 2007; Stewenius et al., 2007). The frequency of anaphase bridges is commonly used as an indicator of telomere-mediated chromosomal instability. H&E stained paraffin sections were used to score the anaphase and internuclear bridges. Fifty mitotic cells were scored for anaphase bridges and 500 nuclei were scored for internuclear bridges.

Array Comparative Genomic Hybridization (Array-CGH) on Tissue Samples

Genomic DNA was extracted from the tissues by phenol-chloroform method. Normal female DNA (Promega, Madison, WI) was used as the control DNA. DNA was run on a 1.2% agarose gel to check for any degradation or RNA contamination and was quantitated by NanoDrop ND-1000 UV–vis spectrophotometer. Array-CGH (aCGH) was performed according to the protocol suggested by Agilent Technologies, Santa Clara, USA. Genomic DNA was amplified using the Qiagen REPLI-g mini kit (Qiagen, Valencia, CA). The amplified genomic DNA was digested by *Alu*I (Promega, Madison, WI) and *Rsa*I (Promega, Madison, WI) on a 37°C circulating water bath for 2 hr and transferred onto ice. The amplified digested genomic DNA was cleaned up using QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA). DNA was further quantitated using the NanoDrop ND-1000 UV–vis Spectrophotometer. It was then fluorescently labeled using random primers (supplied with Agilent Genomic DNA labeling kit PLUS) with Cyanine 3-dUTP (test) and Cyanine 5-dUTP (control). The labeled genomic DNA was cleaned using the Microcon YM-30 filters. Cy3-labeled and Cy5-labeled genomic DNA were mixed with human Cot-1 DNA (1 mg/μl) (Invitrogen, Carlsbad, USA), Agilent 10x blocking agent and Agilent 2x hybridization buffer. The hybridization sample mixture was applied to the gasket slide and then the active side of the microarray slide was placed on top of the gasket slide to form a "sandwich slide pair." The SureHyb chamber cover was placed onto the sandwiched slides and the slide clamp was assembled onto both pieces. The assembled slide was then placed in the rotator rack in a hybridization oven at 65°C to rotate at 20 rpm and left to hybridize at 65°C for 40 hr. The slides were disassembled in Oligo aCGH Wash Buffer 1 and washed in Oligo aCGH Wash Buffer 1 at room temperature for 5 min and in Oligo aCGH Wash Buffer 2 at 37°C for 1 min. The slides were scanned immediately to minimize the impact of environmental oxidants on signal intensities using Agilent scanner that was set to scan area (61×21.6 mm^2). Scan resolution was set to 10 using dye channel red and green. Green and red photomultiplier tubes were set to 100%. Data analysis was done using the CGH analytics software.

Quantitative Real-Time PCR

RNA was extracted from the frozen tissues by TRIZOL method and quantitated by the nanodrop spectrophotometer. The analyses of the *TRF1*, *Tankyrase 1*, and *POT1* mRNA expression were performed using the Light Cycler RNA Master SYBR Green Kit (Roche Diagnostics, Indianapolis) by real-time quantitative RT-PCR. The primer sequences for the genes are as follows: *TRF1* forward primer, 5′-gca aca ggg cag agg cta tta tt-3′; *TRF1* reverse primer, 5′-agg gct gat tcc aag ggt gta -3′, *Tankyrase 1* forward primer, 5′-atg ccc cca gag gcc tta-3′; *Tankyrase 1* reverse primer, 5′-ggt gga tgc tgg tga gat ca-3′ and *POT1* forward primer, 5′-tca gtc tgt taa ctt cat tgc cc -3′; *POT1* reverse primer, 5′-tgc acc atc ctg aaa aat tat atc-3′ (First base). The house keeping gene 18S rRNA was used to normalize the sample-to-sample variation in the amount of the input RNA, and also to evaluate the quality of the isolated RNA and RT efficiency. Amplification of the 18S rRNA was performed using the following primers: forward primer: 5′ gta acc cgt tga acc cca tt-3′and reverse primer: 5′ cca tcc aat cgg tag tag cg 3′. PCRs were performed in a LightCycler® instrument (Roche Diagnostics) as follows: reverse transcription at 61°C for 20 min, initial denaturation at 95°C for 2 min; amplification for 45 cycles of denaturation (95°C, 5 sec, ramp rate 2°C/sec), annealing (optimal temperature, 5 sec, ramp rate 2°C/sec) and extension (72°C, product length [bp]/25 sec, ramp rate 2°C/sec). The standard curve was constructed with 10-fold serial dilutions of the RNA which corresponded to the total RNA ranging from 0 to 500 ng. The PCR reaction for amplification was performed using 500 ng of the total RNA for the target genes and 5 ng for the house keeping gene. All of the measurements included the determination of the standards

and no-template as a negative control, in which water was substituted for the RNA. Data for *TRF1* mRNA, *POT1* mRNA and *Tankyrase 1* mRNA were normalized to the data for 18 s RNA. The products were run on a 1% agarose gel to confirm the size of the final product for all of the genes.

Statistical Analyses

Statistical analyses of the data were done by unequal variance *t* test (Welch test) using Microsoft Excel 2007 (Microsoft Corporation, Redmond, USA) (Ruxton, 2006).

RESULTS

Significantly Greater Telomere Shortening in the Higher Grades of Breast Cancer Samples

Paired adjacent tissues were found to have longer TRFs (Mean ± SD and range: 9.0 ± 2 kb and 6.8–13.2 kb) than in the breast tumor tissues (Mean ± SD and range: 7.7 ± 0.63 kb and 6.7–10 kb). The difference in length was statistically significant ($P < 0.05$) (Fig. 1A). The average percentage decrease was more significant in Grade III tumors ($P < 0.05$) than in the Grade II tumors ($P = 0.05$) (Fig. 1A). To gain further insights into the telomere length changes, we have used in situ analysis of telomere length by quantitative-fluorescence in situ hybridization on paraffin tissue. Quantitative-fluorescence in situ hybridization analysis yielded similar reductions in telomere lengths in tumor tissues compared with the adjacent tissues (Fig. 1B).

Higher Telomerase Activity in Breast Cancer Samples than in the Adjacent Tissues

Telomere dysfunction results from critically short telomeres or the loss of stability of the telomere secondary structure. The telomere length is a balance between its loss due to the cell proliferation and the maintenance by telomerase. Hence, we decided to look at the status of telomerase in relation to the aggressiveness of the tumors. Telomerase activity was detected in all the cancerous tissues, and it was about 1.5 times more in the higher grades (Grade II and III) than the lower grade (Grade I) tumors (Fig. 1C). We have also detected low levels of telomerase activity in about 66% of the paired adjacent tissues; however, this was about 20 times lower than that detected in the cancerous tissues. There was no correlation between the telomerase activity and other clinicopathological parameters such as hormonal receptor status, lymph node status, or the HER2/neu status (Data not shown).

Telomere Attrition Associated with Elevated Chromosome Instability in the Higher Grades

To determine whether the telomere shortening observed in the higher grade tumors was associated with telomere dysfunction, the percentage of anaphase bridge formation was analyzed. Telomere shortening may compromise the integrity of chromosome ends and lead to the formation of dicentrics and rings. These abnormal structures may form bridges at anaphase that either break or initiate a series of breakage-fusion-bridge events. Thus, anaphase bridges are chromatin bridges in between separating chromosome masses during anaphase, which may result in gene amplification or loss when breaking (Lengauer, 2001). Anaphase bridge formation is a correlate of telomere dysfunction (Meeker and Argani, 2004; McPherson et al., 2006; Jin et al., 2007; Stewenius et al., 2007). There was a fivefold increase in the frequency of anaphase bridges in Grade III from Grade I ($P < 0.05$) in breast tumor samples (Fig. 2A). Internuclear bridges that are also indicative of chromosomal instability showed a threefold increase in frequency from Grade I to grade III ($P < 0.01$) and twofold from Grade I to Grade II ($P < 0.05$) (Fig. 2B) breast cancers. Thus, telomere shortening was correlated with telomere dysfunction which was concurrent with the histological grading. Telomere dysfunction in the form of anaphase bridges also correlated with the estrogen receptor status where the frequency was higher in ER-negative tumors (Table 1). ER status has also been associated with the prognosis of breast cancers with ER- negative tumors having worse prognosis than ER-positive tumors. However, the increased percentage of anaphase bridges in PR-negative compared to PR-positive tumors was not statistically significant.

Increased Genomic Imbalances in the Form of Amplifications or Deletions in Higher Grades of Breast Cancer by aCGH

Telomere-mediated chromosome rearrangements cause genomic imbalances, which were measured by aCGH. We used selected samples (six samples; two from each grade) to measure genomic imbalances by aCGH. Our results showed greater genomic imbalances in the form of deletions and amplifications in the higher grade tumors compared with the lower grade tumors (Figs. 3A

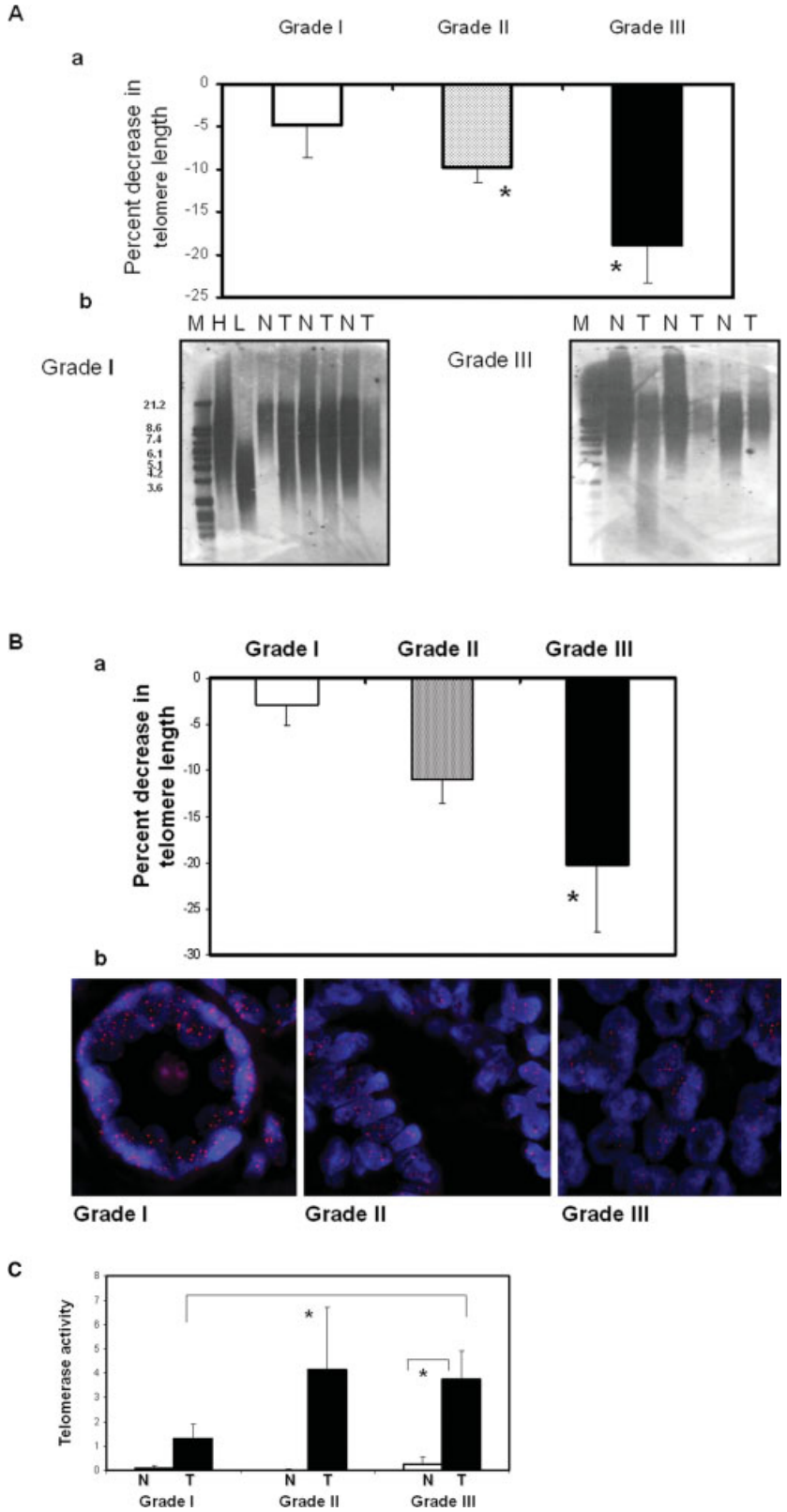

Figure 1. Average percent decrease in telomere length in different grades of tumors. A: (a) Telomere length measured by Southern blotting of the terminal restriction fragments. Grade I tumors showed 4.76% decrease, Grade II tumors showed 9.76% decrease, and Grade III tumors showed 18.86% decrease in telomere length on an average when compared with the paired adjacent tissues. *$P < 0.05$. (b) Representative samples of telomere length analysis by Southern blotting for Grade I and Grade III tumors are shown here. N, normal; T, tumor; L, control low; H, control high; M, marker. Bars indicate SE. B: (a) Telomere length measured on paraffin tissue sections by PNA FISH. Grade I tumors showed 2.8% decrease, Grade II tumors showed 9.9% decrease, and Grade III tumors showed 16.86% decrease in telomere length on an average when compared with the paired adjacent tissues. Bars indicate SE. (b) Representative pictures of tissue FISH in the paraffin sections of Grade I, II, and III tumors showing telomere signals using the telomere specific Cy-3 labeled PNA probe. *$P < 0.05$. C: Average telomerase activity (Δ Fluorescein/Δ Sulforhodamine) in the breast cancer tissues and the paired adjacent tissues. The solid bars are the cancer tissue samples and the white bars are the paired adjacent tissue samples. Bars indicate SE. *$P < 0.05$.

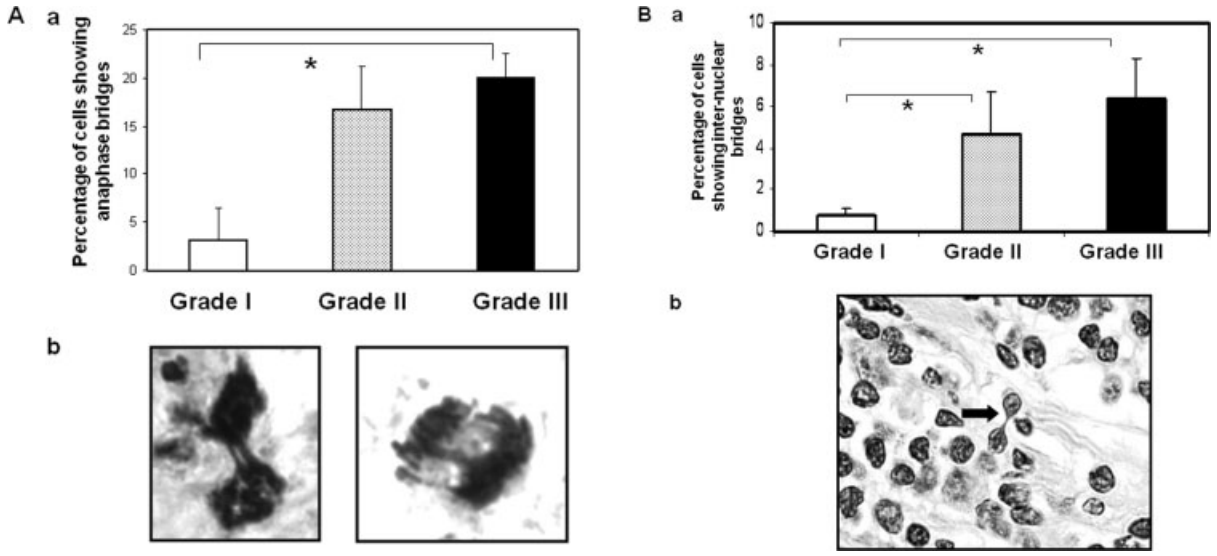

Figure 2. Anaphase bridges as an indicator of telomere dysfunction. A: (a) Average percentage of anaphase bridge formation in all of the three grades of tumors. Grade I tumors had 3.3%, Grade II 16.6%, and Grade III 20.7% of anaphase bridges. *$P < 0.05$. (b) Anaphase bridges in Haematoxylin and eosin (H&E) stained tumor paraffin sections. B: (a) Average percentage of internuclear bridges in all of the three grades of tumors. Grade I tumors had 0.7%, Grade II 4.75%, and Grade III 6.32% of internuclear bridges. *$P < 0.01$. (b) Internuclear bridge in Haematoxylin and eosin (H&E) stained tumor paraffin sections. Results are shown in percentage ± SE.

TABLE 1. Correlation of Telomere Length, Telomere Dysfunction and Expression of Telomere Related Genes with Clinicopathological Parameters

	Percent telomere shortening	Percent anaphase bridges	*TRF1* expression[a]	Tankyrase 1 expression[a]	*POT1* expression[a]
Histological grades					
Grade I	4.77	3.22	0.00011	1.8 E−088	0.46
Grade II	12.99[b]	16.67[c]	0.00018	3.1 E−08[c]	0.02
Grade III	19.19[c]	20.03[c]	0.00017	1.9 E−07	0.24
Oestrogen receptor (ER) status					
Negative	14.78	20.95[d]	0.00014[d]	2.4 E−07[d]	0.34
Positive	12.14	12.67	0.00032	3.1 E−08	0.12
Progesterone receptor (PR) status					
Negative	16.14	19.86	0.00022	2.2 E−07[e]	0.26
Positive	9.62	13.05	0.00021	3.1 E−08	0.15
Lymph node metastasis					
Absent	13.06	16.30	0.00021	9.8 E−08	0.33
Present	14.46	18.10	0.00024	2.1 E−07	0.29
Tumour size					
<2 cm	9.27	23.65	0.00048	3.1 E−08	0.30
≥2 cm	14.15	16.20	0.00019	1.5 E−07	0.31

[a]Normalized values for the expression of the target genes to the normal house keeping gene (18s rRNA) expression.
[b]$P = 0.05$ (compared with paired adjacent tissue samples).
[c]$P < 0.05$ (compared with paired adjacent tissue samples).
[d]$P < 0.05$ (compared with ER positive samples).
[e]$P < 0.05$ (compared with PR positive samples).

and 3B). Studies are in progress to determine the nature of these genes in breast tumors.

Differential Expression of mRNA for *TRF1*, *POT1*, and *TANKYRASE 1* in Relation to the Histological Grades of the Tumors

As mentioned earlier, in addition to telomerase, a number of telomere associated proteins have been shown to play a role in maintaining telomere integrity. We looked at the mRNA expression of some of the positive and negative regulators of telomere length. *TRF1* showed a heterogeneous pattern of expression in the samples. However, on average, the expression of *TRF1* mRNA was higher in adjacent tissues compared with the tumor tissues. On the other hand, when compared between

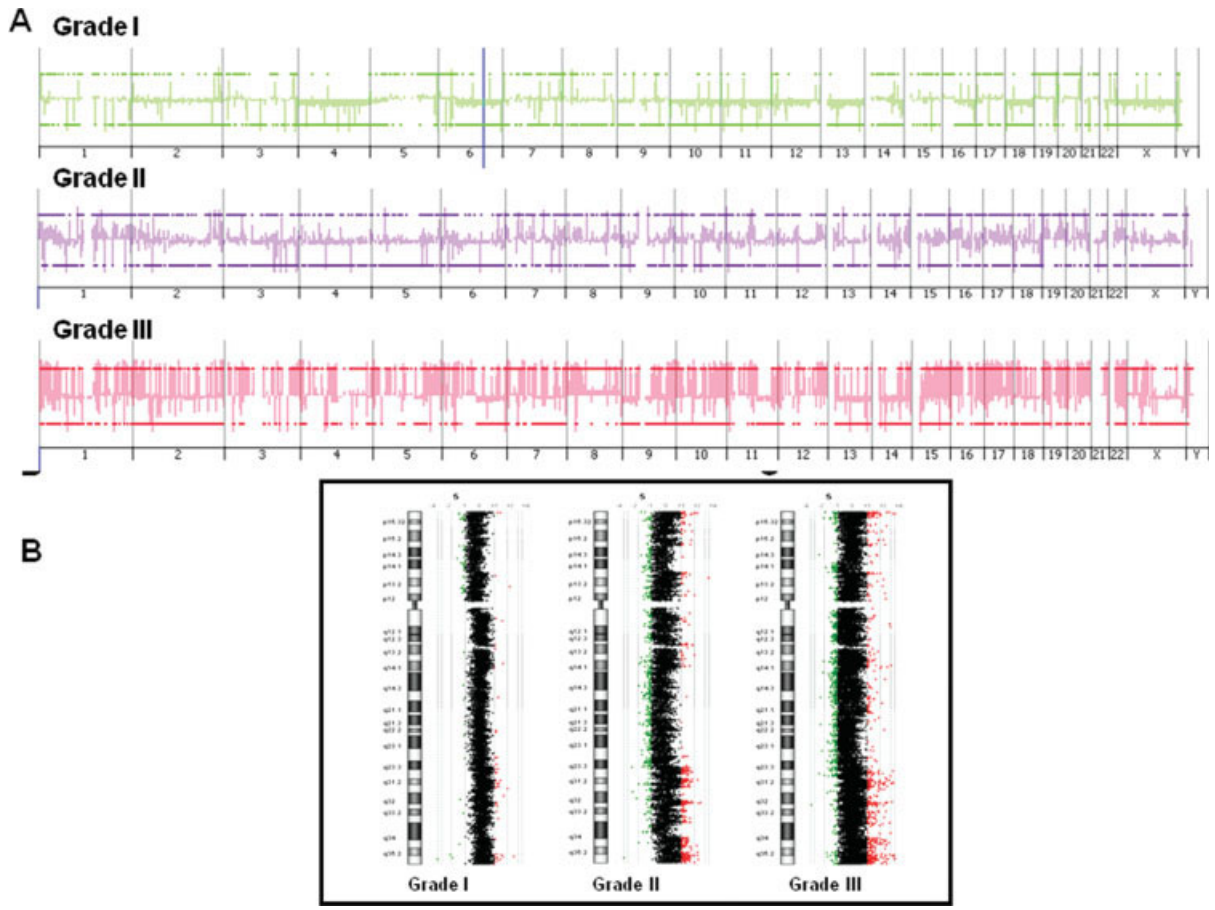

Figure 3. Array-CGH analysis in breast tumor samples. A: Complete genome view by array-CGH showing greater genomic instability in the higher grade than the lower grade of tumors. Log_2 +1.0 horizontal scale bars (above which is considered amplification) and Log_2 −1.0 horizontal scale bars (below which is considered deletions) are included for reference. B: Chromosome 5 used as a representative chromosome shows greater genomic imbalances in the higher grades than the lower grades. Green dots represent deletions and red dots represent amplifications.

grades the *TRF1* expression did not show a significant trend, but was higher in the lower grades than the higher grades (Fig. 4A and Table 1). Average expression of *POT1* mRNA appears to be down-regulated in cancer tissues. The expression was observed to be higher, but not statistically significant $P > 0.05$), in the early grade (Grade I) compared with the latter grades (Grade II and III) (Fig. 4B). *Tankyrase 1* mRNA expression was significantly higher in cancerous tissues compared with the adjacent tissues. There was also an increase in the mRNA expression from lower grades to higher grades (Fig. 4C and Table 1). Its expression was also high in ER- and PR-negative tumors (Table 1).

DISCUSSION

Breast cancer patients are often classified into those with favorable (well-differentiated tumors) or less favorable (poorly-differentiated tumors) outcome using prognostic markers. Histopathological grading is widely accepted and a good prognostic indicator of breast cancer and prospectively used in the clinical treatment decisions (Dalton et al., 2000; Ellsworth et al., 2008). The cells in Grade I tumors resemble normal cells and appear to grow and multiply slowly, whereas higher grade tumors grow rapidly and are poorly differentiated. Genetically unstable tumors have been shown to be larger in size and to be at higher grade compared with the stable tumors (Christov et al., 1989; Kronenwett et al., 2006). Genomic instability contributes to a major problem in management of breast cancer phenotypes because of their more aggressive phenotype and chemo-resistance (Loeb, 2001; Chin et al., 2004).

Telomere dysfunction may be one of the molecular causes of genomic instability. We have reported the telomere dysfunction phenotype in BRCA1-deficient cells suggesting the fact that loss

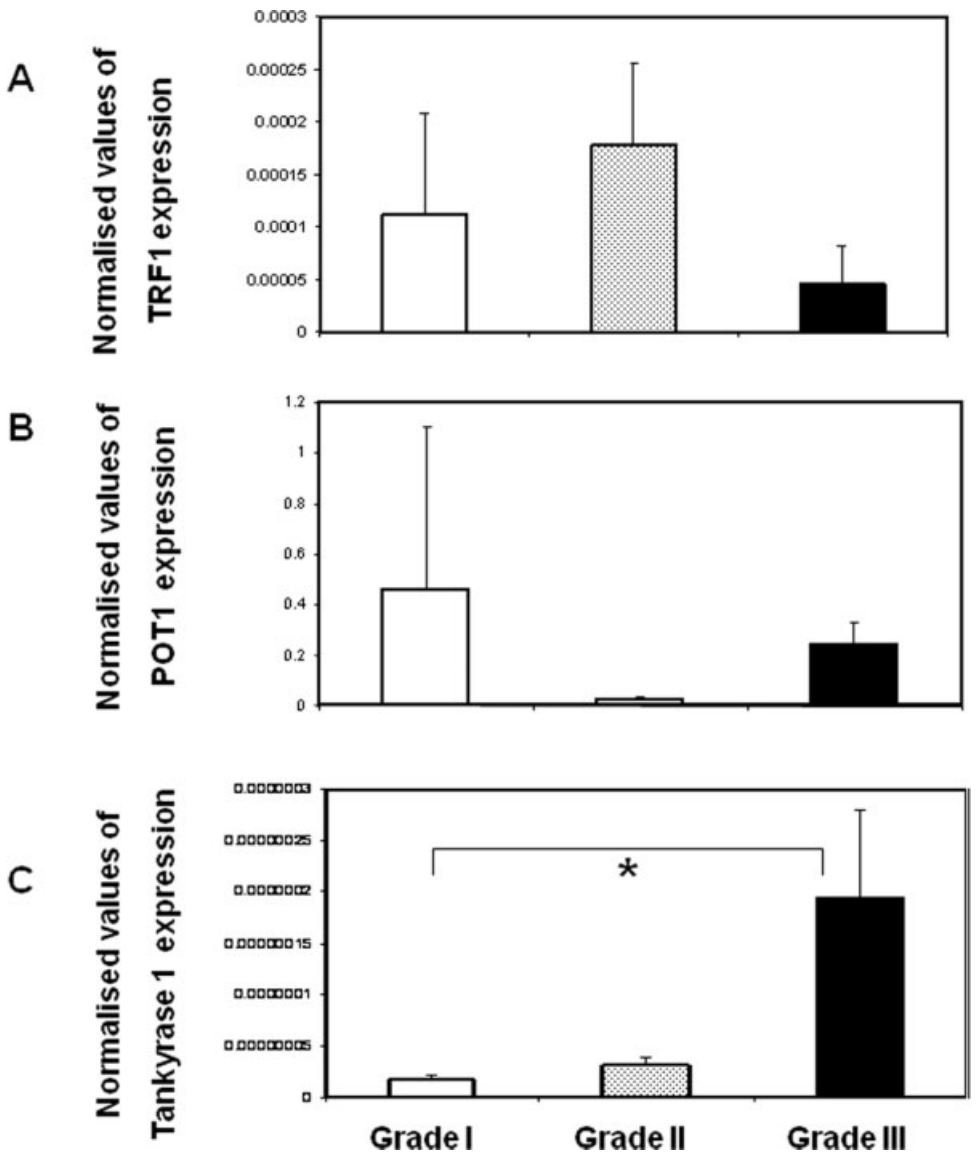

Figure 4. Differential mRNA expression of telomere associated proteins by real time RT-PCR. The values on the *y*-axis are normalized to the 18 s rRNA expression. A: *TRF1* mRNA expression in comparison between grades of tumor tissues. B: *POT1* mRNA expression in comparison between grades of tumor tissues. C: *Tankyrase 1* mRNA expression in comparison between grades of tumor tissues. $^*P < 0.01$.

of telomere integrity might contribute to chromosome end dysfunction and permit the formation of potentially oncogenic translocations (McPherson et al., 2006). Breast cancer patients exhibited significant chromosome instability and telomere loss following radiotherapy, which suggests that there is marked increase in telomere dysfunction in breast cancer patients (Banerjee et al., 2008). Recent studies in bladder cancer (Jin et al., 2007) and Wilms' tumor (Stewenius et al., 2007) demonstrated telomere shortening and telomere dysfunction in higher grade tumors. Evidence from our study support the hypothesis that telomere dysfunction impairs chromosomal stability and is associated with the aggressiveness of the tumors. The breast cancer cells have infinite replicative potential and they replicate at a much faster rate than the normal human cells (Bignold, 2007). Therefore, it is reasonable to assume that the rate of telomere loss is higher in the breast cancer cells compared with normal human cells, thereby leading to more rapid telomere shortening in the breast cancer cells.

Earlier studies examined the telomere length in breast cancer by terminal restriction fragment analysis in Southern blotting (Odagiri et al., 1994;

Rha et al., 1999). But these studies showed inconclusive results regarding associations with clinicopathological features like grading, hormonal receptor status, etc. It is also reported that telomere shortening in breast carcinomas are associated with histological grading (Odagiri et al., 1994) and increased genomic instability and metastasis (Griffith et al., 1999). Telomere shortening was also correlated with poor prognosis in several types of cancer (Griffith et al., 1999). O'Hagan et al., (2002) demonstrated that telomere dysfunction is a major cause of chromosome instability using aCGH. They examined chromosomal gains and losses in $mTerc^{-/-}$ $trp53^{+/-}$ mice and found that telomere dysfunction results in segmental gains and losses that drive epithelial carcinogenesis in the mouse model (O'Hagan et al., 2002) similar to that ones seen in human epithelial tumors. This study supports the hypothesis that telomere-based crisis and associated breakage-fusion-bridge cycles drive chromosomal instability in cancer cells (Raptis and Bapat, 2006).

Breast cancer is assumed to progress as a linear model with poorly-differentiated (Grade III) breast tumors would developing from moderately- (Grade II) and well-differentiated (Grade I) tumors. Earlier studies have shown that allelic imbalances were greater in the higher grades compared to the lower grades (Ellsworth et al., 2008). Our data by aCGH supports this observation. However, the majority of the observed genetic alterations were not identical among different grades with only few common genetic modifications.

Genetically unstable breast cancers have also been associated more frequently with ER- and PR-receptor negative breast cancers (Kronenwett et al., 2006). Breast cancer patients with tumors that are ER-positive and/or PR-positive have lower risks of mortality and better prognosis after their diagnosis compared with women with ER-negative and/or PR-negative disease (Dunnwald et al., 2007). In the present study, higher telomere dysfunction was observed in ER-negative tumors supporting the complex nature of such tumors (Table 1).

It is known that telomerase is activated by tumor cells to ensure survival of the cancer cells with genomic instability (Campisi et al., 2001). Some studies have shown the association of telomerase activity with tumor type, nodal metastasis (Roos et al., 1998; Mokbel et al., 1999), high cellular proliferation (Mokbel et al., 1999), large tumor size, and lymphovascular invasion in invasive breast cancer (Bednarek et al., 1997; Roos et al., 1998; Mokbel et al., 2000). For the first time, as far as we are aware of, the present study has shown greater telomerase activity in the higher grades (Grades II and III) of breast cancers than the lower grade (Grade I). One earlier study has shown that higher grades of tumors have more telomerase positive cells detected in the form of increased hTERT expression (Kirkpatrick et al., 2003a). Greater telomere shortening in the higher grades could be an effect of rapid proliferation compared with the slowly proliferating lower grade tumors. Higher telomere loss in the higher grades may probably initiate a feedback mechanism to increase the telomerase activity compared with the lower grades. Accordingly, the cells in the higher grades showed increased telomerase activity than the lower grades in our study. Moreover, there may be a selection pressure for telomerase-positive cells in the higher grades allowing the telomerase positive cells bypass the restriction of proliferation common in cells with critically short telomeres (Clark et al., 1997). Additionally, we found detectable telomerase activity in the adjacent apparently normal tissues indicating that they are not completely non-cancerous. In earlier studies, hTERT expression was seen in the adjacent tissues (Kirkpatrick et al., 2003b; Hines et al., 2005). Adjacent tissues could be in the initial stages of malignant transformation since telomerase reactivation has been shown as an early event in breast carcinogenesis (Shpitz et al., 1999). This proposition is supported by our data that the telomerase activity in the adjacent tissues also followed a similar trend as the cancerous tissues. The tissues adjacent to the aggressive Grade III tumors showed greater telomerase activity than the less aggressive Grade I tumors. The aggressive cells in higher grades probably have greater tissue invasion and infiltration resulting in spill over of cancerous cells into the adjacent tissues and hence the detectable telomerase activity. On the other hand, it may also indicate initiation of tumorigenesis in these tissues.

TRF1 is known to protect telomeres and inhibit telomere replication. Thus, the decreased TRF1 probably facilitates the access of telomerase to telomeres. There was nonsignificant decrease in the levels of TRF1 in the malignant tissues in our study which is in agreement with earlier studies (Saito et al., 2002; Salhab et al., 2008). Furthermore, the decreased *TRF1* mRNA expression in the higher grades accompanied by telomere shortening suggests a possible transcriptional suppression of TRF1 to release telomerase from negative regulation. It has been shown that nuclear over-

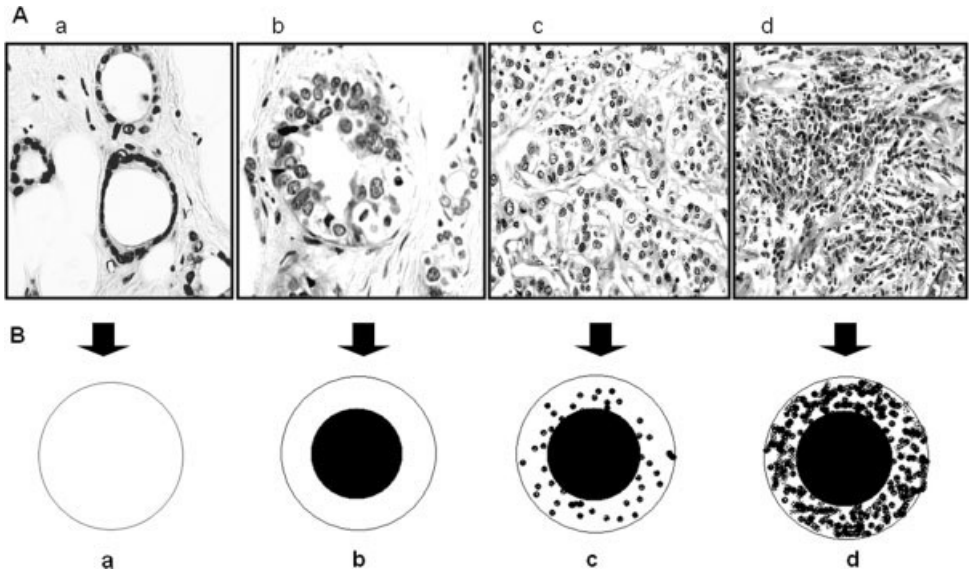

Figure 5. A: Haematoxylin and eosin stained paraffin of normal and tumor tissues showing different histological grades. (a) Normal, (b) Grade I, (c) Grade II, (d) Grade III. B: Schematic representation of tumor (solid black) with surgical cut margins and the normal area (white) with possible infiltration of cancer cells into the adjacent normal tissues in Grade I, Grade II, and Grade III tumors.

expression of Tankyrase 1 leads to poly(ADP-ribosyl) ation of TRF1 and loss of TRF1 with consequential increased telomerase access and telomere elongation (Donigian and de Lange, 2007). Consistent with the reports by Gelmini et al. (2004), we observed significantly higher levels of Tankyrase 1 in advanced tumors. Furthermore, for the first time we report a correlation of Tankyrase 1 expression to the histopathological grades where there is a grade dependent increase in Tankyrase 1. The increased expression of Tankyrase 1 and decreased expression of TRF1 in higher grades act in synergism at the telomeres and have a prominent role in tumor progression and aggressiveness.

Presence of telomerase activity and grade dependent expression of telomere-related genes (data not shown) in the tissues adjacent to the malignant tissues reiterates our proposition that the apparently normal tissues next to the malignant tissues may not be completely normal. We speculate that surgical resection margins, which may appear histologically normal might harbor potentially neoplastic cells, possibly through infiltration of tumor cells into the adjacent tissues beyond the surgical cut margin (Fig. 5). There are many incidences of recurrence of cancer after lumpectomy in the adjacent tissues previously thought to be histologically normal. Adjacent mammary epithelium might not have been completely removed during lumpectomy for invasive carcinoma or ductal carcinoma in situ and the adjacent cells may subsequently progress to form another carcinoma. It is therefore essential to find prognostic indicators for recurrence in the morphologically normal epithelium. Earlier studies have revealed that patients with loss of heterozygosity in their normal tissues are more likely to have a tumor recurrence than patients whose normal tissues are not genetically aberrant (Deng et al., 1996). In depth analysis of our aCGH data are underway to determine the gene loss or gains in breast tumors. Hence, it is necessary to identify patients who would be at risk of recurrence in the adjacent tissues, so as to benefit from the more aggressive local therapy or counseling about their higher risk for local failure. Therefore, in addition to histopathological analysis, other molecular targets such as telomere dysfunction and expression of telomere related genes should be used as an integrated strategy to classify the tissues adjacent to tumor tissues as normal.

Although histopathological grading is an important factor for the risk assessment in patients with breast cancer, it could be subjective and a large proportion of tumors are characterized as intermediate-grade tumors, making the best treatment modality inaccurate. An association was observed between the telomere dysfunction and the grade of differentiation of breast cancer in this study. The data presented in this study show that telomere dysfunction when used as an adjunct to conventional histopathological assessment might provide clinically relevant information with respect to

the degree of malignancy. Therefore, telomere dysfunction could also be used as a marker for the aggressiveness of the tumors.

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