RESEARCH COMMUNICATION

Comparison of Telomere Length and Telomerase Activation between Breast Fibroadenoma and Infiltrating Ductal Carcinoma in Malaysian Women

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Abstract

A study was initiated to explore possible differences in handling telomere attrition in the most common malignant and benign tumours of the breast in Malaysian women. Infiltrating ductal carcinoma (IDC) and fibroadenoma (FA) represented the malignant and benign prototypes respectively. 29 IDC, 28 FA and 22 benign non-lesional control (BNL) breast tissue samples were analysed for telomerase activation using a Telomerase PCR ELISA kit (Boehringer Mannheim). In addition, 23 IDC, 12 FA and 14 BNL were subjected to telomere length determination with a TeloTAGGG Telomere Length Assay Kit (Roche Diagnostic GmbH, Germany), following digestion of genomic DNA by frequently cutting restriction enzymes RsaI and HinfI. Mean telomerase activity in IDC (A_{450nm}=0.3338), but not FA (A_{450nm}=0.0003) was significantly raised ($p<0.05$) compared with BNL (A_{450nm}=0.0031). Similarly IDC (1.2 kb), but not FA (2.2 kb), showed significant telomere shortening ($p<0.05$) relative to BNL (2.9 kb). The findings imply that telomere attrition and telomerase activation differ between malignant and benign tumours of the breast and may be important for targeted therapy.

Keywords: Telomerase - telomere length - breast - infiltrating ductal carcinoma - fibroadenoma

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Introduction

Telomeres are non-encoding tandem repeats of TTAGGG at the ends of chromosomes. Telomeres are shortened because of incomplete replication at every cell cycle (end replication problem) (Harley et al., 1990) or oxidative damage (von Zglinicki, 2007). Eventual loss of telomeres gives rise to a critical stage of replicative senescence with p53-induced cell cycle arrest. Should this self-regulatory mechanism be breached, cycling can continue with further telomere shortening. Chromosomes, unprotected by the shortened telomeres are unstable and prone to fusion, gene alteration, translocation etc, priming a second critical stage which usually results in death of most cells. A rare cell may activate telomerase, a specialized ribonucleoprotein catalytic complex (Kim et al., 1994) or more uncommonly engage mechanisms for alternative lengthening of telomere (ALT) to maintain telomere lengths at a new steady state (Shay and Wright, 2006), giving rise to a state of "immortality", the hallmark of neoplasia. Most studies have focused on studying telomere length and telomerase activation in malignant tumours with a lack by way of benign tumours. A study of the "new steady state" resulting from telomere shortening and telomerase activation in benign versus malignant tumours can provide important insight into differences between the two categories. Carcinoma of the breast is the most common malignancy amongst females in many parts of the world (Jemal et al., 2009). In Peninsular Malaysia, it is also the most common cancer amongst women accounting for 31% of all cancers in women (Lim et al., 2008). Fibroadenoma is the most common benign breast neoplasm (Giri, 2009). Breast carcinoma and fibroadenoma, the most frequently encountered malignant and benign breast tumours in women were studied for their telomerase activation and telomere lengths.

Materials and Methods

For purposes of this study, infiltrating ductal carcinoma (IDC) was selected to represent the malignant prototype of breast tumours and fibroadenoma (FA) the benign. Excisions and mastectomies of IDC and FA received by the Department of Pathology, University Malaya Medical Centre were prospectively recruited into the study. 30 IDC and 29 FA which were earlier diagnosed clinically, on biopsy or fine needle aspiration as IDC or FA were considered. Fresh tissues, approximately 0.5 cm3, were sampled in non-necrotic areas in the centre of the IDC and FA during the cut-up after adequate sampling for routine histopathological examination had been completed. Only tumours larger than 2.0x2.0x2.0cm were sampled. Breast

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tissue was also sampled fresh from the excision margin most removed from the tumour (minimum clearance of 5cm) of 22 mastectomy specimens after careful macroscopic examination and confirmation of absence of tumour at the margin (Hara et al., 2001). This constituted the benign, non-lesional control (BNL). All fresh tissues were snap-frozen and stored at -80˚C immediately after sampling until batch analysis for telomerase activity and telomere length. The routinely processed histopathology sections of each case of IDC and FA were reviewed to re-confirm the histopathological diagnoses and only re-confirmed IDC and FA were entered into the study. Before digestion and extraction of proteins and DNA from fresh tissue, one cryostat section stained with haematoxylin and eosin was made from each tissue sample of IDC, FA and BNL to confirm the presence and adequacy of tumour or non-lesional material. It was also carefully ensured that BNL did not show any atypical ductal or atypical lobular hyperplasia. Information on the age of patients was obtained from histopathology request forms accompanying the specimens.

Telomerase activity assay

The frozen samples were lysed to release crude cell protein. Quantitation of protein in cell lysates was performed using the DC protein assay kit (BioRad, USA). 50 µg of protein lysate per case was used for telomerase activity assay using the Telomerase PCR ELISA kit (Boehringer Mannheim) according to manufacturer's recommendations. The kit incorporated the Telomeric Repeat Amplification Protocol (TRAP) assay with photometric enzyme immunoassay detection of generated telomeric repeats. Controls were run with each batch and included an immortalized telomerase positive embryonic kidney cell line from the kit. The negative control consisted of a known telomerase positive breast cancer sample pretreated with 1ug/ul DNase free RNase. Blanks consisting of substitution of ultrapure distilled DNAse, RNAse free water for sample were also included in each run. Details of the assay methodology are as described in an earlier study (Cheah et al., 2002).

In principle, telomerase when present in the test samples would catalyse addition of TTAGGG repeats on the synthetic biotin-labelled primer using its intrinsic RNA as template. The extended telomeric products were PCR amplified (30 cycles) using an automated thermal cycler (Perkin Elmer DNA thermal cycler 480) and hybridized with a digoxigenin-labelled TTAGGG specific probe. The hybridized product was then immobilized via the biotin-labeled primer to a streptavidin-coated microtiter plate. Probe visualisation was via peroxidase-conjugated anti-digoxigenin-probe digoxigenin reaction and ensuing peroxidase action on 3,3',5,5'-tetramethyl benzidine (TMB). The optical density (OD) of the coloured product was measured using an ELISA reader as absorbance at 450 nm (A_{450nm}) against a blank (reference wavelength of 690 nm). Each batch of analysis was accepted as satisfactory when the positive control exhibited A_{450nm} of at least 1.50 and the negative of not more than 0.25. All tumours were initially tested with a sample protein concentration of 1µg/µl. In the event, telomerase activity was not raised; the case was re-tested at a protein concentration of 0.02 µg/µl. Whenever re-testing was conducted, the higher of the two sample absorbance values obtained was accepted if a difference existed between the values. The mean and standard deviation (SD) A_{450nm} values of BNL were calculated and the telomerase activation cut-off was arbitrarily taken as 2SD above the mean.

Telomere length analysis

Telomere length (TL) analysis was based on terminal restriction fragments cleaved from ends of chromosomes. Since TTAGGG sequences of the telomere are resistant to known endonucleases, they were cleaved together with a sub-telomeric region, after digestion of genomic DNA by frequently cutting restriction enzymes eg RsaI and HinfI which degrade non-telomeric sequences to low molecular weight fragments. TL were determined using the TeloTAGGG Telomere Length Assay Kit (Roche Diagnostic GmbH, Germany), a non-radioactive chemiluminescent assay. Genomic DNA (2μg) extracted from tissue samples was first completely digested by RsaI and HinfI. The generated DNA fragments were then electrophoresed in agarose gel. Size fractionated DNA fragments were subsequently transferred to a nylon membrane. Digoxigenin-labelled probes ($^{3'}$TTAGGG$^{5'}$) were hybridised to the immobilised DNA fragments on the nylon membrane. The resultant hybrids were detected with anti-digoxigenin coupled to alkaline phosphatase which metabolised CDP-Star, a sensitive chemiluminescence substrate. The chemiluminescent signals, appearing as a smear for each sample due to variation in restriction fragment lengths between chromosomes and cells within the sample, were then detected by autoradiography (Figure 1). The TL for each sample was determined by comparing the mean size of the smear relative to molecular weight standards supplied in the kit (DIG molecular

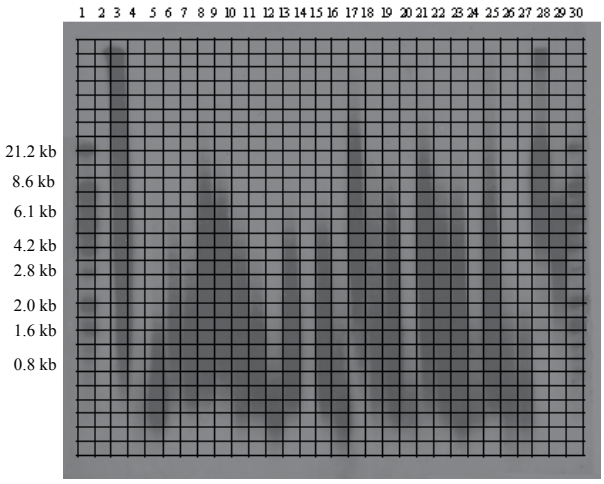

Figure 1. Chemiluminescent Terminal Restriction Fragments of TTAGGG Probed Signals Captured by Autoradiograph for Telomere Length Determination of Infiltrating Ductal Carcinoma and Fibroadenoma Cases. Lanes 1 and 30=DNA Molecular Weight Markers 0.8kb-21.2kb. Lane 28=10.2kb Control DNA, Lane 29=3.9kb Control DNA; Lanes 2-27 Cases of Fibroadenoma and Infiltrating Ductal Carcinoma Studied

weight markers) ranging from 0.8kb to 21.2kb following digitisation of the chemiluminescent signals and analysis by computerised software (Metamorph version 4.01; Universal Imaging Corp, US). Two control genomic DNA of immortal cell lines with known telomere lengths (3.9 kb and 10.2 kb) provided in the kit were included in each run as references.

Statistical analysis

Comparison between sample groups was analysed for statistical significance using the Student's t-test and Fisher's exact test.

Results

Of the 30 carcinomas considered, one was re-classified as metaplastic carcinoma on histological review and not included into the study, while 29 were histologically confirmed as IDC. In the FA group, one was re-categorised as benign phyllodes tumour and similarly excluded. Thus 29 IDC, 28 FA and 22 BNL were entered into the study. The patients' ages ranged between 29-79 years (mean=52 years) for IDC and 19-45 years (mean=29 years) for FA.

Telomerase activity

Twenty-nine IDC, 28 FA and 22 BNL were analysed for telomerase activity (Table 1). IDC (A450nm=0.3338) showed significantly higher ($p<0.05$) mean telomerase activity compared with FA (A_{450nm}=0.0003) and BNL (A_{450nm}=0.0031). No significant difference in mean telomerase activity was noted between FA and BNL ($p=0.43$).

Table 2 shows the number of IDC (17/29) and FA (2/28) with activated telomerase. A significantly increased number of IDC (58.6%) demonstrated A450nm value above the arbitrary cut-off value of A450nm=0.0217 compared with 7.1% FA ($p<0.05$).

Table 1. Mean Telomerase Activity of Infiltrating Ductal Carcinomas (IDC), Fibroadenomas (FA) and Benign, Non-Lesional Breast Controls (BNL)

Category	Mean Telomerase Activity (A_{450nm}) ± SD
IDC (n=29)	0.3338 ± 0.7428
FA (n=28)	0.0003 ± 0.0153
BNL (n=22)	0.0031 ± 0.0093

Table 2. Telomerase Activation in Infiltrating Ductal Carcinomas (IDC) and Fibroadenomas (FA)

Category	Telomerase activated cases
IDC (n=29)	17 (58.6%)
FA (n=28)	2 (7.1%)

Table 3. Mean Telomere Lengths of Infiltrating Ductal Carcinomas (IDC), Fibroadenomas (FA) and Benign, Non-Lesional Breast Controls (BNL)

Category	Mean TRF length (kb) + SD
IDC (n=23)	1.2 ± 1.1
FA (n=12)	2.2 ± 1.4
BNL (n=14)	2.9 ± 1.6

Telomere length analysis

Of the original fresh samples, there was sufficient material from 23 IDC, 12 FA and 14 BNL for TL analysis. The mean TL of IDC was 1.2 kb, FA 2.2 kb and BNL 2.9 kb respectively (Table 3). IDC demonstrated significantly shorter mean TL compared with FA and BNL ($p<0.05$). There was however no significant difference ($p=0.24$) in mean TL between FA and BNL.

Discussion

This study shows significant telomere shortening in IDC (mean=1.2kb) compared with BNL (mean=2.9kb). In addition, mean telomerase activity was significantly increased in IDC (A_{450nm}=0.3338) when contrasted with BNL (A_{450nm}=0.0031). That telomeres are shortened in IDC have also been noted by other workers (Odagiri et al., 1994; Meeker et al., 2004; Fordyce et al., 2006). Similarly, increased telomerase activity in IDC is well-documented (Kalogeraki et al., 2005; Murilo-Ortiz et al., 2006; Looi et al., 2007).

In contrast, FA (2.2kb) in this study did not show significant telomere shortening nor increased telomerase activity (A_{450nm}=0.0003) compared with BNL. There is a general paucity of studies in this area with Odagiri et al (1994) noting shorter telomeres in 8 FA studied compared with normal breast tissue while Hiyama et al (1996) reported that 15 FA in their study had telomeres within the normal range. Meeker et al (2004) demonstrated shortening of telomeres predominantly in the luminal cells in 1 of 4 FA. In this study, although the mean TL of the 12 FA studied was not significantly shortened when contrasted with the 14 BNL, there appears to be a trend towards shortened telomeres in FA which could become evident with larger number of cases. Telomerase activity of FA was not significantly raised compared with BNL although some studies have recorded telomerase activation in 7 to 60% of FA (Sugino et al., 1996; Poremba et al., 1998). Nevertheless, most report that telomerase activation in FA is weak (Hiyama et al., 1996; Wu et al., 1998; Poremba et al., 1999).

When compared with FA, IDC had significantly shortened mean telomere length. Not only was mean telomerase activity significantly higher in IDC in comparison with FA, the number of IDC (58.6%) with telomerase activation was also significantly increased to FA (7.1%). These findings support the notion that telomere attrition and telomerase activation, differ between malignant and benign neoplasms of the breast. This raises the possibility that IDC with the help of telomerase activation maintains the malignant cells in a "new steady state" of telomere length while FA, for reasons still unclear, maintains telomere length close to that of normal breast tissue and without telomerase intervention. Whether telomerase activation is a reflex consequence of telomere shortening or an inherent cellular characteristic of IDC, it appears that telomerase activation is an important difference between malignant and benign breast tumours and a possible target for therapy.

While the findings of this study generally agree with previous work in this area, it should be cautioned that

measurement of telomere length in this study was based on the Southern blot, which unlike in-situ methods does not accord localization of the shortened telomere. With current understanding that telomeres of luminal cells shorten more rapidly than those of myoepithelial cells and fibroblasts in normal breast (Meeker et al., 2004; Kurabayashi et al., 2008), it is pertinent to consider the possibility that stromal prominence could have contributed to the lack of shortened telomeres in FA compared with IDC. This will need further clarification perhaps through use of in-situ telomere detection methods and comparing infiltrating ductal carcinomas versus the less common breast epithelial adenomas instead of fibroadenomas. Nonetheless, studies of telomere lengths in adenomas and corresponding carcinomas at other sites have shown varying results. Maruyama et al., (1998) reported consistent telomere shortening in gastric adenomas compared with varying shortening in gastric carcinoma. Kammamori et al., (2000) showed that both follicular adenomas and thyroid cancers had shortened telomeres while Plentz et al., (2003) found telomere shortening in colorectal carcinoma but not in corresponding adenomas. Age of subjects has also been inversely correlated with telomere length (Hewakapuge et al., 2008; Ren et al., 2009). The mean age of IDC cases in this study was 52-years while FA was 29-years and should also be taken into account in the interpretation of the findings.

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References

Cheah PL, Looi LM, Ng MH, et al (2002). Telomerase activation and human papillomavirus infection in invasive cervical carcinoma in a set of Malaysian patients. *J Clin Pathol*, **55**, 22-6.

Fordyce CA, Heaphy CM, Bisoffi M, et al (2006). Telomere content correlates with stage and prognosis in breast cancer. *Breast Cancer Res Treat*, **99**, 193-202.

Giri D (2009). Recurrent challenges in the evaluation of fibroepithelial lesions. *Arch Pathol Lab Med*, **133**, 713-21

Hara Y, Iwase H, Toyama T, et al (2001). Telomerase activity levels for evaluating the surgical margin in breast-conserving surgery. *Surg Today*, **31**, 289-94.

Harley CB, Fisher AB, Greider CW (1990). Telomeres shorten during ageing of human fibroblasts. *Nature*, **345**, 458-60.

Hewakapuge S, van Oorschot RA, Lewandowski P, et al (2008). Investigation of telomere lengths measurement by quantitative real-time PCR to predict age. *Leg Med (Tokyo)*, **10**, 236-42.

Hiyama E, Gollahon L, Kataoka T, et al (1996). Telomerase activity in human breast tumors. *J Natl Cancer Inst*, **88**, 116-22.

Jemal A, Siegel R, Ward E, et al (2009). Cancer Statistics. *CA Cancer J Clin*, **59**, 225-49.

Kalogeraki A, Kafousi M, Ieromonachou P, et al (2005). Telomerase activity as a marker of invasive ductal breast carcinomas on FNABs and relationship to other prognostic variables. *Anticancer Res*, **25**, 1927-30.

Kammori M, Takubo K, Nakamura K, et al (2000). Telomerase activity and telomere length in benign and malignant human thyroid tissues. *Cancer Lett*, **159**, 175-81.

Kim NW, Piatyszek MA, Prowse KR, et al (1994). Specific association of human telomerase activity with immortal cells and cancer. *Science*, **266**, 2011-5.

Kurabayashi R, Takubo K, Aida J, et al (2008). Luminar and cancer cells in the breast show more rapad telomere shortening than myoepithelial cells and fibroblasts. *Human Pathol*, **39**, 1647-55.

Lim GCC, Rampal S, Halimah Y (2008). Cancer Incidence in Peninsular Malaysia 2003-2005. The Third Report of the National Cancer Registry. National Cancer Registry, Ministry of Health Malaysia. Kuala Lumpur.

Looi LM, Ng MH, Cheah PL (2007). Telomerase activation in neoplastic cell immortalization and tumour progression. *Malays J Pathol*, **29**, 33-5.

Maruyama Y, Hanai H, Kaneko E (1998). Telomere length and telomerase activity in intestinal metaplasma, adenoma and well differentiated adenocarcinoma of the stomach. *Nippon Rinsho*, **56**, 1186-9.

Meeker AK, Hicks JL, Gabrielson E, et al (2004). Telomere shortening occurs in subsets of normal breast epithelium as well as in situ and invasive carcinoma. *Am J Pathol*, **164**, 925-35.

Murillo-Ortiz B, Astudillo-De la Vega H, Castillo-Medina S, et al (2006). Telomerase activity, estrogen receptors (alpha, beta), Bcl-2 expression in human breast cancer and treatment response. *BMC Cancer*, **6**, 206.

Odagiri E, Kanada N, Jibiki K, et al (1994). Reduction of telomeric length and c-erbB-2 gene amplification in human breast cancer, firboadenoma and gynecomastia. Relationship to histologic grade and clinical parameters. *Cancer*, **73**, 2978-84.

Plentz RR, Wiemann SU, Flemming P, et al (2003). Telomere shortening of epithelial cells characterises the adenoma-carcinoma transition of human colorectal cancer. *Gut*, **52**, 1304-7.

Poremba C, Bocker W, Willenbring H, et al (1998). Telomerase activity in human proliferative breast lesions. *Int J Oncol*, **12**, 641-8.

Poremba C, Shrover KR, Frost M, et al (1999). Telomerase is a highly sensitive and specific molecular marker in fine-needle aspirates of breast lesions. *J Clin Oncol*, **17**, 2020-6.

Ren F, Li C, Xi H, et al (2009). Estimationof human age according to telomere shortening in peripheral blood leukocytes of Tibetan. *Am J Forensic Med Pathol*, **30**, 252-5.

Shay JW, Wright WE (2006). Senescence and immortalization: role of telomeres and telomerase. *Carcinogenesis*, **26**, 867-74.

Sugino T, Yoshida K, Bolodeoku J, et al (1996). Telomerase activity in human breast cancer and benign breast lesions: diagnostic applications in clinical specimens, including fine needle aspirates. *Int J Cancer*, **69**, 301-6.

Von Zglinicki T (2002). Oxidative stress shortens telomeres. *Trends Biochem Sci*, **27**, 339-44.

Wu S, Liu Z, Sun H (1998). Telomerase activity in infiltration ductal carcinoma of breast. *Zhonghua Yi Xue Za Zhi*, **78**, 515-6.