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ORIGINAL ARTICLE

Potential utility of telomere length assessment in breast cancer in a diagnostic histopathology setting

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Abstract

Introduction: Telomeres shorten with cell cycling but are restored above mortality threshold in many cancers making them potentially exploitable for differentiating malignant from benign tissues, and for cancer evaluation. *Materials and Methods:* We assessed telomeres in a diagnostic histopathology setting using quantitative fluorescence *in situ* hybridisation on 33 fibroadenoma (FA) and 73 invasive breast carcinoma of no special type (IBC-NST) (prototypes of benign and malignant breast tumours, respectively) with paired benign, non-lesional breast tissues (BNL). Telomere lengths were expressed as telomere/chromosome-2-centromere ratio (TCR). The telomere length cut-off for malignancy was also determined. *Results:* Mean TCR of IBC-NST was significantly shorter than FA and BNL ($p<0.001$). Mean TCR of FA was shorter than BNL but not significantly ($p>0.05$). TCR cut-off for IBC-NST based on FA was ≤0.29 (sensitivity=75.3%; specificity=78.8%), and ≤0.30 based on BNL (sensitivity=76.7%; specificity=89.0%). TCR of IBC-NST did not differ in relation to histological grade, nodal and hormonal status ($p>0.05$) but was significantly shorter in HER2-overexpressing cancers ($p<0.05$). *Conclusion:* We have demonstrated a first-step to the development of methodology-based cut-off values of mean telomere length for distinguishing benign from malignant breast tissues. Telomere length may not value-add to the standard prognostic and predictive parameters, but has potential in relation to HER2.

Keywords: Breast cancer, benign breast, Q-FISH, telomere length

INTRODUCTION

Telomeres comprise of non-coding hexameric DNA tandem repeats (telomeric DNA) and specialized proteins located at chromosomal ends, which prevent chromosomal end-to-end fusion and loss of genomic DNA.[1,2] In normal somatic cells, telomeres erode with each round of cell division as a result of incomplete DNA replication, until the cell reaches its Hayflick limit. At this point, telomeres are critically short and cell division is arrested. These cells are usually subjected to replicative senescence and eliminated by apoptosis.[3-5] Rare cells bypass both proliferative barriers and reconstruct their telomeres by telomerase activation, allowing them to undergo indefinite proliferation and achieve cellular immortalisation, which are hallmarks of malignant change. Thus, telomeres which are shortened during physiological aging can be substantially restored in malignancy.[6,7]

It is known that telomeres of breast cancer are shorter than those of non-malignant breast tissues.[8-17] This finding has been explained by telomeric loss due to the multiple cell cycles of malignant cells, although the telomere length is maintained above the mortality-inducing critical level by the activation of telomerase. This phenomenon has posed an attractive target for cancer research, as knocking tumour telomeric lengths below critical level, hence inducing cell mortality, would seem an achievable therapeutic option.[18,19] In the current clinical practice, breast cancer management is mainly based on several prognostic and predictive factors, i.e., histological grade, nodal status, and immunohistochemical (IHC) expression of three protein markers: estrogen (ER), progesterone (PR), human epidermal growth factor receptor 2 (HER2 or HER2/neu). Using ER, PR, and HER2 IHC expressions as surrogate markers

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of their genetic profiles, breast cancer is classically categorised into four main intrinsic molecular subtypes: luminal A (ER and/or PR-positive, HER2-negative), luminal B (ER and/or PR-positive, HER2-positive), HER2-enriched (ER and PR-negative, HER2-positive), and triple-negative (ER, PR, and HER2-negative). Molecular subtyping has impact on the treatment course for breast cancer patients as hormone-positive cancers tend to respond well to anti-hormonal treatment (such as tamoxifen), whereas cancers which overexpress HER2 show excellent response to trastuzumab, a humanised monoclonal antibody against HER2/neu. Till now, triple-negative breast cancer has limited targeted therapy options and thus is the subtype with the poorest outcome. Studies that have focused on the associations of telomere length and the prognostic and predictive factors of breast cancer showed contradictory observations and hence the potential utility of telomere length in breast cancer remains controversial.

On the other hand, benign breast disease (BBD) is another group of breast abnormalities that are non-cancerous and occurs more commonly than breast cancer. Nevertheless, BBD are relatively neglected by researchers because it is usually not life-threatening.[20-22] However, because some proliferative BBD can histologically mimic malignant lesions and vice versa[23,24], there always remains the challenge to differentiate benign from malignant breast lesions as an accurate diagnosis has obvious impact on clinical management of patients with breast disease. Our research group has an interest in this field.[15] Given that our group showed significantly shorter telomeres in invasive breast carcinoma of no special type (IBC-NST) than fibroadenoma (FA), the lesions being used as prototypes of malignant and benign tumours of the breast, testing for telomeres may be a useful ancillary to help to differentiate benign from malignant breast lesions. However, in our previous study, a Southern blot-based approach was used to assess telomere length in IBC-NST and FA. Like many others including those which used quantitative polymerase chain reaction, this study suffered from a lack of actual morphological identification of the cells included in the telomere length analysis with definite likelihood of inclusion of undesired cells (e.g., non-neoplastic cells) in the analysis.[25] Having extended our work to establish quantitative fluorescence *in situ* hybridisation (Q-FISH) as a method to measure telomere length in formalin-fixed, paraffin-embedded (FFPE) tissues[26], we embarked on employing this cytogenetic technique in our current study for the following reasons: (1) to re-affirm the earlier findings and (2) to assess a method that is friendly to a routine surgical pathology laboratory, if telomere length measurement is to be introduced into routine diagnostic practice. For these purposes, FA and IBC-NST were again chosen as prototypes of benign and malignant breast tumours. Main reasons for selecting Q-FISH over other telomere length measurement approaches were: (1) it enables the identification of specific cell types against preserved tissue morphology, (2) it does not require unfragmented (good quality) and large amounts of DNA which are not possible to obtain from FFPE tissues, and (3) it can be applied to FFPE tissues which is the material routinely used in surgical pathology departments for testing and archived FFPE tissues are easily available for innovative studies. Additionally, we also further studied telomere length in relation to the main prognostic and predictive factors in IBC-NST, to gauge its potential utility in breast cancer assessment.

MATERIALS AND METHODS

Ethics statement

The archived FFPE tissues (pathology repository) were accessed retrospectively, hence the use of tissue surplus for research does not interfere with patient management, e.g., diagnosis and treatment of the disease. Moreover, it was not possible to contact and seek consent from the patients a second time for the purpose of this research. Nevertheless, patient personal information and identity were anonymised, data confidentiality maintained, the study data handled only by the research team; and the study conducted in compliance with the Declaration of Helsinki. The University of Malaya Medical Centre Medical Research Ethics Committee/ Institutional Review Board had reviewed this study protocol and granted approval for this research (Reference number: 1182.2).

Sample cohort

33 FA and 122 IBC-NST cases from years 2013 to 2016 were identified from the archives of the Department of Pathology, Faculty of Medicine, University of Malaya**.** These were breast samples from patients of the University of Malaya Medical Centre. H&E-stained slides of the identified cases were retrieved and evaluated by the study pathologist (LML) to re-confirm the

diagnoses and determine the region of interest (ROI) for Q-FISH analysis. The relevant FFPE tissue blocks of the FA and IBC-NST cases were retrieved. For each IBC-NST case, non-neoplastic breast tissue sampled from the resected margin was also retrieved to serve as benign, non-lesional (BNL) control. Note that out of 122 IBC-NSTs, the BNL tissue of only 73 cases were included for telomere length measurement. The remaining 49 cases were excluded for the following reasons: (1) insufficient normal breast ducts for study, (2) BNL tissue not available (not surgically-removed/no BNL sampled from the resected specimen), (3) inherent tissue artefacts rendering the tissue unsuitable for current analysis. To determine whether telomere length has utility in the clinical assessment of breast cancer, we compared telomere length against pathological parameters with proven clinical utility (predictive and prognostic factors). For each study case of breast cancer, the following were retrieved from its histopathology report: (1) histological grade, routinely evaluated according to the modified Bloom and Richardson system, (2) nodal status, if available, based on histological detection of metastasis in axillary lymph nodes, and (3) molecular profile based on IHC expression of ER and PR proteins and HER2 oncoprotein. Histological grade and pathological stage (of which presence or absence of metastasis in axillary lymph nodes is a major factor) are standard prognostic factors in breast cancer. Molecular subtyping of breast cancer based on their ER, PR and HER2 expressions have predictive value in guiding choice of targeted therapy, which have consequently significantly impacted prognosis and outcome for breast cancer patients.

Normal testicular tissue of a 76-year-old patient in whom orchidectomy was performed as part of the treatment for prostatic carcinoma, which was fixed in formalin and embedded in paraffin, was utilised as a positive haploid control for the telomere Q-FISH technique. This positive control was chosen for the known distinctive and long telomeres in germ cells[27], and was included in every run of the Q-FISH test.

Q-FISH procedure

For each selected FFPE tissue block, a 4 μm thick microtomed section was cut on to a silanised histology glass slide. A diamond marking pen was used to mark the ROI on the non-tissue side of the slide by visual comparison with the corresponding H&E-stained slide. The tissue sections (on glass slides) were dried overnight in a 37°C incubator, and then baked at 60°C for 1 hour. Next, they were dewaxed in two 5 min changes of xylene. The sections were subjected to hydration in descending concentrations of ethanol (100%, 96%, 70%), followed by two changes of deionised water. The sections were then immersed in Tris-EDTA buffer (10 mM Tris base, 1 mM EDTA solution, 0.05% Tween-20, pH 9.0) at 97°C for 30 min and immediately allowed to cool down at room temperature for 20 min, and rinsed in two 3 min changes of deionised water. The tissue sections were then digested with 8 mg/ml pepsin (S3002, Dako, Denmark) in 0.01 N hydrochloric acid (pH 2.0) at 37°C, for 15 min. Subsequently, the sections were rinsed in two changes of deionised water, 3 min each, dehydrated in 96% ethanol, and allowed to dry at room temperature. The Telomere Peptide Nucleic Acid FISH Kit/FITC (K5325, Dako, Denmark) was utilised for telomere Q-FISH analysis. A 1:10 mixture of FITC-labeled telomere specific peptide nucleic acid probe (from the kit) and Cy3-labeled chromosome 2 centromere (CEN2) specific peptide nucleic acid probe (complementary gift from Dako) was prepared. 2 μl of the above probe mixture was applied to the tissue sections at the ROI. The ROI was then covered with a 10×10 mm cover slip and sealed with rubber cement. The respective telomere and CEN2 targets of the FA, IBC-NST and BNL and probes were allowed to denature at 80 °C for 5 min, and then hybridised at room temperature for at least 18 hours in a dark, humid chamber. Following that, the sections were immersed in 2×saline sodium citrate+0.1% Tween-20 buffer (pH 7.0–7.5) at room temperature until the cover slips dislodged, and the tissue sections were then washed in two changes of the same solution at 58 °C for 10 min each time. This was followed by another wash at room temperature for 1 min. Next, the sections were dehydrated in ascending concentrations of cold ethanol (70%, 85%, 96%) and air-dried. Vectashield fluorescence mounting medium (H-1200, Vector Laboratories, USA) containing 1.5 μg/ml DAPI nuclear counterstain was diluted with Vectashield fluorescence mounting medium (H-1000, Vector Laboratories, USA) to make up a working solution with 0.05 μg/ml DAPI. For each section, 5 μl of the working solution was applied on the ROI. The ROI was covered with a new cover slip, and then incubated in the dark for 15 min at room temperature.

Fluorescence microscopy and image analysis
Fluorescence microscopy was carried out using a Zeiss Axio Imager Z2 epi-fluorescence microscope (Carl Zeiss, Germany) equipped with a CoolCube 1 charged-coupled device camera (Metasystems, Germany). Image acquisition and analysis were performed with the Metafer 4 MetaCyte Lite software version 3.11.3 (Metasystems, Germany). Fluorescence images of the ROI from the FITC, Cy3, and DAPI channels were captured using the ×100 oil objective. Nuclear identification and telomere length quantification were performed on merged fluorescence channels of FITC, Cy3 and DAPI. 30 non-overlapping, epithelial cell nuclei (luminal nuclei) with two CEN2 signals were selected in both FA and BNL for analysis. Nuclei of the luminal epithelial cells were targeted for analysis because the majority of the breast cancers resembled the cells of the luminal epithelium, thus luminal cells are believed to be more susceptible to neoplastic transformation. For IBC-NST tissue, 30 non-overlapping tumour nuclei were selected within the ROI for telomere length measurement for each case, regardless of the number of CEN2 signals present per nuclei. This is in recognition that due to aneuploidy or polyploidy occurring in malignant cells, abnormal numbers of CEN2 signals may occur in breast cancer cells under study.[28] For normal testicular tissue, 30 germ cell nuclei with only one CEN2 signal per nucleus were analysed as the positive haploid control.

For each selected nucleus, FITC-labeled telomere (green) and Cy3-labeled CEN2 (red) signals were identified by the software and the signals were expressed as fluorescence intensities. With CEN2 used for standardising the fluorescence staining, telomere length of each nucleus was quantified as the ratio of the fluorescence intensity of all the green FITC-labeled telomere signals to all the red Cy3-labeled CEN2 signals, i.e., telomere to CEN2 ratio (TCR), which represents the cumulative telomere lengths in the nucleus, as established previously.[26] The TCR was calculated by dividing the total telomere fluorescence intensities by the total CEN2 signal intensities. Finally, the telomere length for each case was represented by the mean TCR of 30 nuclei studied for each FA, IBC-NST and BNL tissue.

Statistical analysis
The paired T-test was used to compare TCRs of IBC-NST and paired BNL. The independent T-test was used to compare TCRs of FA and IBC-NST, TCRs of FA and BNL, as well as TCRs between respective histological grades, nodal statuses, hormone receptor statuses, HER2 statuses, and molecular subtypes. Fisher's exact test was used to determine associations of IBC-NSTs with higher TCR than paired BNL, IBC-NSTs with lower TCR than paired BNL, and the respective pathological variables of breast cancer (age, histological grade, nodal status). A p-value of <0.05 was considered significant for all statistical outcomes. The receiver operating characteristic (ROC) curve analysis was used to determine the experimental telomere length cut-off for IBC-NST. All statistical analyses were performed using the SPSS version 22.0 (IBM, USA).

RESULTS

Positive control
In all runs, the haploid testicular tissue was successfully positive for telomeres (Fig. 1) and analysis showed a mean TCR of the same case as 1.18 with a SD of 0.20 in the 22 runs.

Telomere length of FA, IBC-NST and BNL
33 FA and 73 IBC-NST cases with paired BNL were analysed for telomere length in this study. The average age of the patients with FA is 30.24±2.11 years (mean±SE) and 56.14±1.30 years (mean±SE) for IBC-NST patients. Fig. 2 illustrates the fluorescence intensities in FA, IBC-NST, and BNL. Telomere length (TCR) of the 33 FAs and 73 IBC-NSTs with their paired BNL are summarised in Table 1 and compared in Fig. 3. While telomere length of IBC-NST was significantly shorter than both BNL and

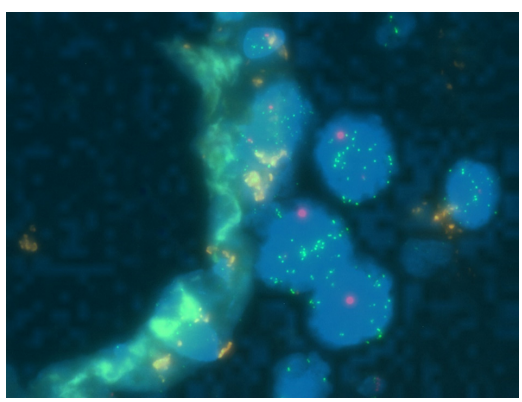

FIG. 1: Intense telomeric signals (green) signals of the testicular tissue positive control (magnification: ×1000).

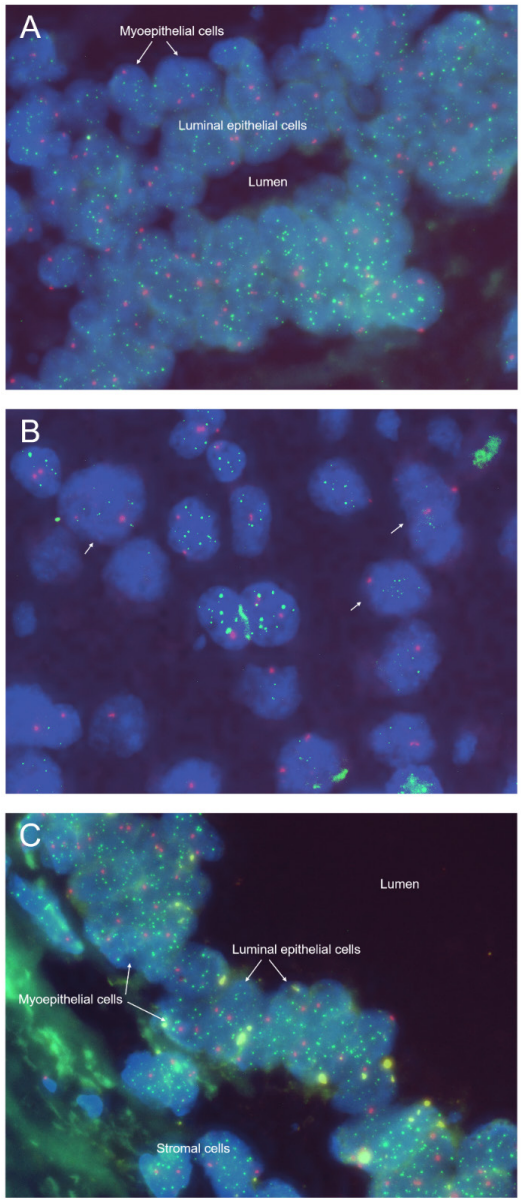

FIG. 2: Representative fluorescence images of the respective breast tissue types used for telomere Q-FISH analysis (magnification: ×1000). (A) Reduced telomeric signals (green) in the luminal epithelial cells of FA from a 26-year-old patient compared to those of BNL breast tissue in (C). (B) Remarkably diminished telomeric signals in tumour nuclei of IBC-NST tissue (large nuclei indicated by white arrows) of a 58-year-old patient compared to telomeres of FA (A) and paired BNL (C). (C) Bright telomeric signals were evident in the luminal epithelial cells of the paired BNL breast.

FA ($p<0.001$), no significant difference was observed between telomere length of FA and BNL ($p>0.05$). Nevertheless, 7 (9.6%) of the 73 IBC-NSTs, exhibited higher TCR (Table 2) than their paired normal breast. No significant difference was observed in age, histological grade, and lymph node involvement between IBC-NST with TCR higher than paired BNL and IBC-NST with TCR lower than paired BNL (Table 3).

Determination of IBC-NST experimental telomere length cut-off for IBC-NST

Based on the ROC curve analysis, the experimental TCR cut-off for IBC-NST based on FA was ≤0.29, with a sensitivity of 75.3% and specificity of 78.8%, and ≤0.30 based on BNL, with a sensitivity of 76.7% and specificity of 89.0% (Fig. 4).

Telomere length and prognostic and predictive factors of breast cancer

Mean TCR was described in Table 4 and compared in Fig. 5. No significant TCR difference was observed between Grade 1 and Grade 2 IBC-NSTs ($p>0.05$), between Grade 1 and Grade 3 IBC-NSTs ($p>0.05$), and between Grade 2 and Grade 3 IBC-NSTs ($p>0.05$). Similarly, no significant TCR differences was observed between node-positive and node-negative, ER-positive and ER-negative, and PR-positive and PR-negative IBC-NSTs ($p>0.05$). However, TCRs of HER2-positive IBC-NSTs were significantly lower than those of HER2-negative IBC-NSTs ($p<0.05$). Likewise, TCRs of luminal B and HER2-enriched IBC-NSTs ($p<0.05$) were significantly lower than luminal A. No significant TCR difference was observed for the following comparisons, i.e., luminal A and triple-negative IBC-NSTs, luminal B and HER2-enriched IBC-NSTs, luminal B and triple-negative IBC-NSTs, and HER2-enriched and triple-negative IBC-NSTs ($p>0.05$). Although not statistically significant, the TCR of triple-negative IBC-NSTs appeared to be about one-third higher than luminal B and HER2-enriched IBC-NSTs.

DISCUSSION

In this study, using the Q-FISH method, we were able to show a similar trend of findings as our previous study which used a Southern blot-based approach to measure telomere length.[15] As in the previous study, telomere length of

TABLE 1: Telomere length (TCR) of FA, IBC-NST and BNL

Breast tissue type	n	Mean TCR (Mean±SE)	95% CI	
			Lower	Upper
FA	33	0.37±0.02	0.33	0.40
IBC-NST	73	0.22±0.02	0.19	0.26
BNL	73	0.43±0.02	0.38	0.48

IBC-NST was significantly shorter than that of FA and BNL, while the telomere lengths of FA did not significantly differ from BNL. To the best of our knowledge, this is the first study which applies Q-FISH to assess telomere length on breast tissues both in Malaysia and Southeast Asia, although this method has been successfully used by others in similar scenario.[12] In spite of the similarity of findings of our two studies, we are not suggesting that Q-FISH is comparable to Southern blot as a method for determination of telomere length. Samples have not been concurrently analysed by both Q-FISH and Southern blot and differing intrinsic case and tissue factors, which were not controlled for, could have influenced telomere length determination in the two different cohorts. It was unfortunate that both methods could not be applied to the same cohort, as this would have shed more light on the comparability of the two methods. The archived tissues of the cases in the previous study had been stored for more than 10 years when the study was commenced, a period far exceeding the 2 years storage period caveat for Q-FISH analysis.[26] As this study was conducted retrospectively, no corresponding fresh tissue was available for Southern blot analysis of the cases. Although Southern blot can be conducted on FFPE tissue, we chose not to pursue this in the present study as it would not have mirrored the original analysis which conducted the Southern blot on fresh frozen tissue.

In this study, we also attempted to work out a possible cut-off threshold telomere length that can help differentiate benign from malignant breast lesions using ROC curve analysis.[29,30] Our findings suggest both proposed cut-off values can potentially differentiate benign from malignant breast tissues given that the AUC of each cut-off value (AUC based on FA=0.80, AUC based on

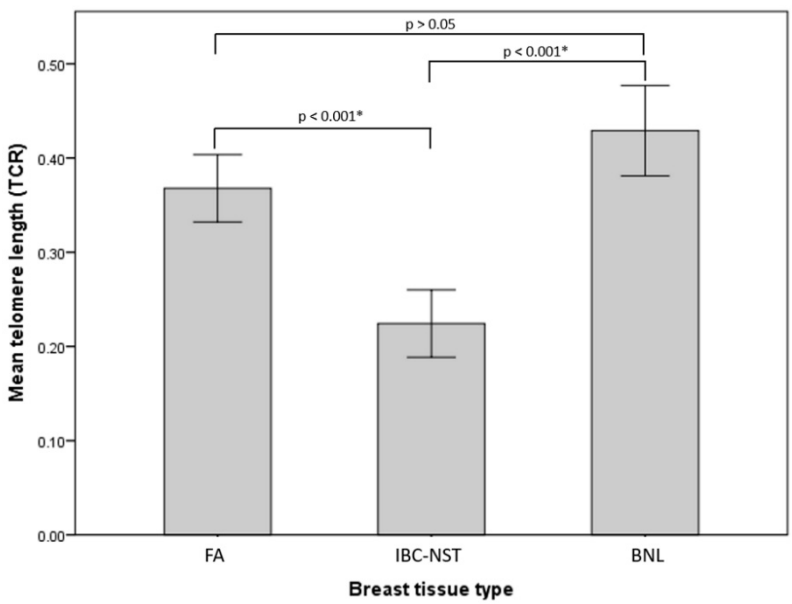

FIG. 3: Comparison chart of mean telomere length (TCR) for each breast tissue type. In general, TCRs of IBC-NSTs (n=73) are remarkably shorter than FAs (n=33) ($p=1.36\times10^{-7}$) and paired BNL (n=33) ($p=5.74\times10^{-11}$). TCRs of FAs (n=33) appear shorter than BNL (n=73) although not statistically significant (p=0.11). *indicates statistical significance. Error bars: 95% CI.

TABLE 2: Telomere length (TCR) of the 7 IBC-NST cases with higher TCR value in tumour tissue than respective paired BNL

IBC-NST case	Patient age	Telomere length (TCR)	
		Tumour	Paired BNL
1	57	0.55	0.48
2	54	0.59	0.39
3	57	0.63	0.35
4	53	0.43	0.22
5	44	0.38	0.30
6	65	0.42	0.36
7	57	0.34	0.29

BNL=0.84) are within the good range (0.8–0.9), and significantly differs from 0.5. Although the cut-off telomere length for IBC-NST worked using FA (≤0.29) and BNL (≤0.30) was almost equivalent, we feel that it is necessary to choose the appropriate cut-off as determined against FA (BBD) or BNL (non-lesional breast) based on the specific disease condition, for the following reasons. Firstly, there is an overall difference in telomere lengths of FA and BNL, although not statistically significant, i.e., telomere length of FA is not the same as BNL (Table 1), which has been also observed by another study.[8] Individuals with BBD history should use the cut-off as determined against the relevant BBD because the cancer might be a progression from BBD, whereas individuals without any BBD history should use the cut-off as determined based on BNL. It should be kept in view that telomeres of BNL obtained from surgical breast specimens may have been affected by the disease. Therefore, care must be taken when developing cut-off values against BNL such as to select non-neoplastic tissue as far away from the cancer as possible.

While Q-FISH seems a feasible method for telomere length analysis for use in a routine surgical pathology laboratory, it does require skilled identification of cellular morphology by a trained surgical pathologist. That this technique can be used on FFPE tissues is a massive advantage as formalin-fixation and paraffin-embedding is the routine form of processing of almost all tissues in diagnostic surgical pathology laboratories. This distinctive advantage makes Q-FISH easy to introduce into the routine diagnostic armamentarium of a surgical pathology laboratory. Another important factor for using Q-FISH on FFPE would be that retention and freezing of fresh tissue is not required. It is noteworthy that a small proportion of breast cancers (9.6%) demonstrated longer telomeres than non-neoplastic breast, and this phenomenon could not be ascribed to other usual variables such as age, histological grade,

TABLE 3: Comparison of pathological variables of IBC-NSTs with longer telomeres and IBC-NSTs without longer telomeres, respectively

Pathological variables		IBC-NSTs with TCR higher than paired BNL (n=7)	IBC-NSTs with TCR lower than paired BNL (n=66)	p-value
Age (years)	Mean±SE	59.86±1.95	55.74±1.41	0.10
Histological grade*	1 (n = 8) 2 (n = 31) 3 (n = 33)	0 (0%) 3 (43%) 4 (57%)	8 (12%) 28 (43%) 29 (45%)	0.87
Nodal status*	Positive (n=32)	4 (57%)	28 (43%)	0.70
	Negative (n=40)	3 (43%)	37 (57%)	

*one case without traceable characteristic

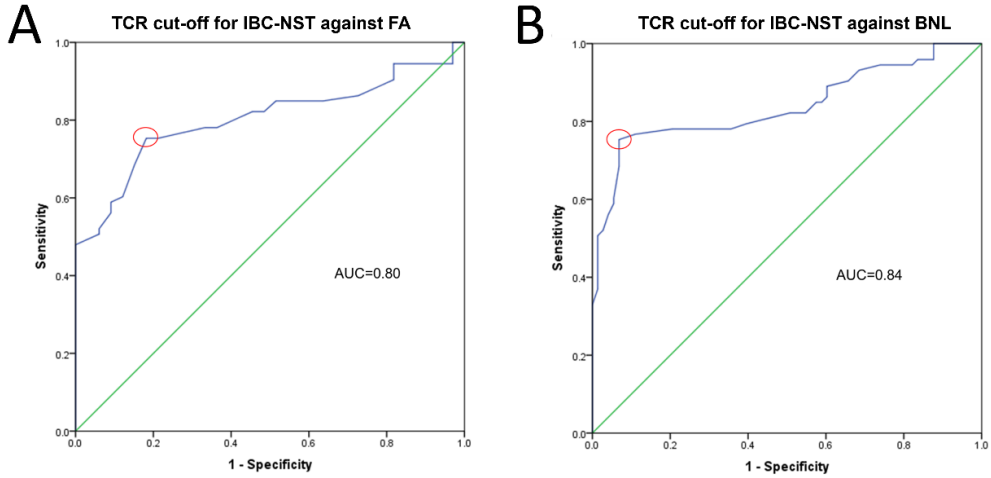

FIG. 4: ROC curves of telomere length (TCR) used to determine IBC-NST from FA and BNL, respectively. Red circle indicates the optimal sensitivity and specificity used to determine the TCR cut-off for IBC-NST. Area Under the Curve (AUC) is the probability of a case to be classified as IBC-NST or non-malignant breast.

and lymph node status (Table 3). A plausible explanation for this finding may be related to alternative lengthening of telomere (ALT) pathways which have been observed by other workers to result in longer telomeres in malignant breast cells.[31]

Our data showed no telomere length differences between the respective histological grades, between nodal statuses, as well as between the respective hormone receptor statuses. This could be possibly due to the high heterogeneity of telomere length within our IBC-NST cohort. The high heterogeneity of telomere length (range: 1.07 kb–9.97 kb) within a IBC-NST cohort comprising of 120 cases has been reported by others[32], which possibly also explains the contradictory observations in studied pathological variables between studies.[8,16,17,33-37] Given the high telomere length heterogeneity within breast cancer, even within

TABLE 4: Telomere length (TCR) of the prognostic and predictive factors of breast cancer

Prognostic and predictive factors		n	Mean age (Mean±SE)	Mean TCR (Mean±SE)	95% CI	
					Lower	Upper
Histological grade (n=121)	Grade 1	14	58.57±3.15	0.23±0.04	0.15	0.31
	Grade 2	57	57.05±1.57	0.23±0.02	0.19	0.27
	Grade 3	50	57.16±1.56	0.26±0.03	0.20	0.32
Lymph node status (n=121)	Positive	60	57.92±1.46	0.24±0.02	0.20	0.28
	Negative	61	56.56±1.50	0.25±0.03	0.20	0.30
ER status (n=103)	Positive	64	54.63±1.39	0.26±0.02	0.21	0.30
	Negative	39	58.33±1.86	0.23±0.03	0.17	0.28
PR status (n=103)	Positive	42	52.86±1.70	0.26±0.03	0.20	0.32
	Negative	61	58.21±1.44	0.23±0.02	0.19	0.28
HER2 status (n=103)	Positive	29	55.66±2.05	0.16±0.03	0.11	0.22
	Negative	74	56.18±1.35	0.28±0.02	0.24	0.32
Molecular subtypes (n=103)	Luminal A	51	54.94±1.61	0.28±0.03	0.23	0.33
	Luminal B	13	53.38±2.67	0.16±0.04	0.07	0.24
	HER2-enriched	16	57.50±3.01	0.17±0.04	0.09	0.25
	Triple-negative	23	58.91±2.41	0.27±0.04	0.19	0.34

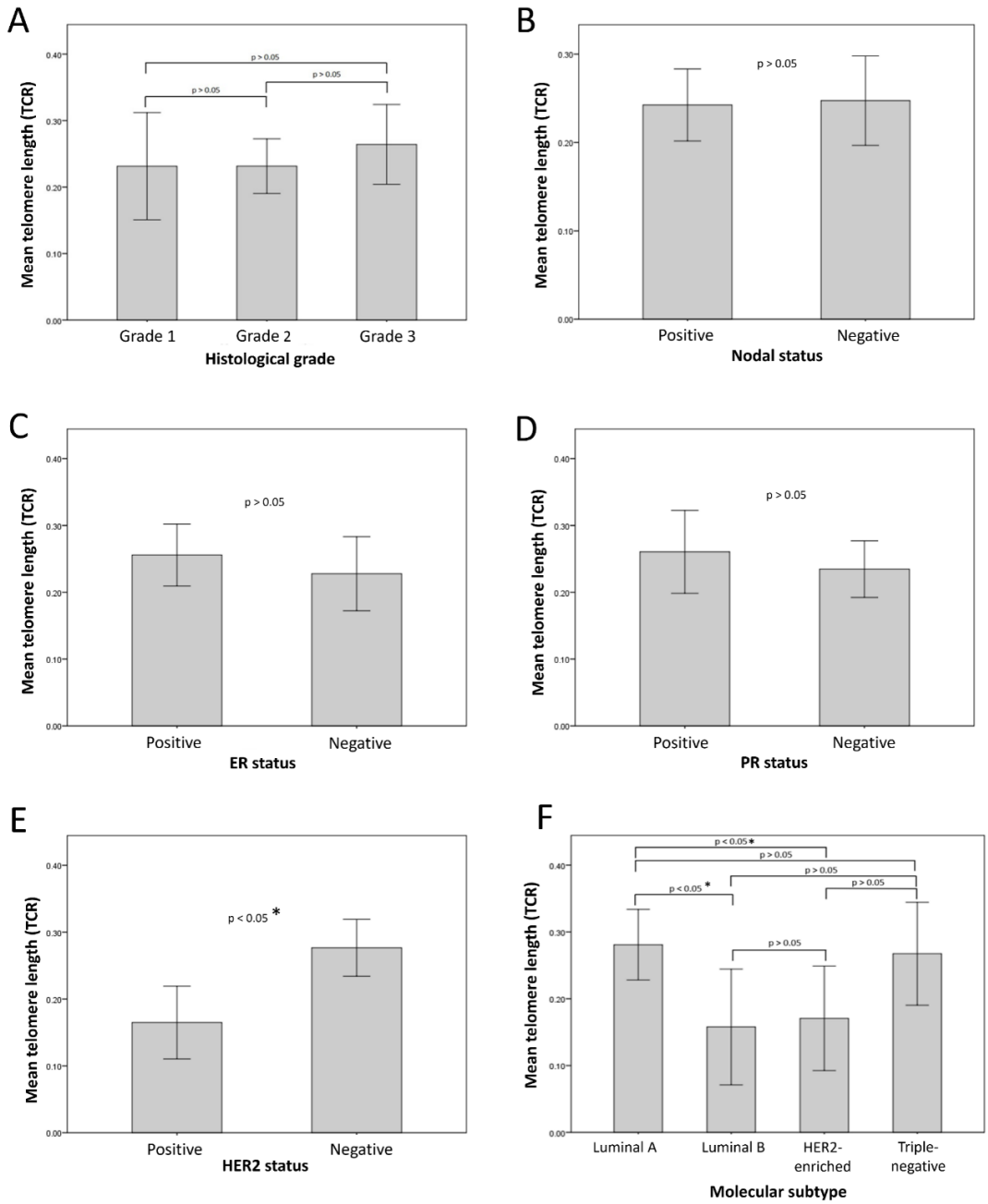

FIG. 5: Bar graphs illustrating comparisons of telomere length (TCR) and the predictive and prognostic factors of breast cancer, respectively. (A) Grade 1 (n=14) vs. Grade 2 (n=57) (p=1.00), Grade 1 (n=14) vs. Grade 3 (n=50) (p=0.59), Grade 2 (n=57) vs. Grade 3 (n=50) (p=0.36), (B) node-positive (n=60) vs. node-negative (n=61) (p=0.88). (C) ER-positive (n=64) vs. ER-negative (n=39) (p=0.45). (D) PR-positive (n=42) vs. PR-negative (n=61) (p=0.48). (E) HER2-positive (n=29) vs. HER2-negative (n=74) ($p = 4.00 \times 10^{-3}$). (F) Luminal A (n=51) vs. luminal B (n=13) (p=0.032), luminal A (n=51) vs. HER2-enriched (n=16) (p = 0.036), luminal A (n=51) vs. triple-negative (n=23) (p=0.77), luminal B (n=13) vs. HER2-enriched (n=16) (p=0.81), luminal B (n=13) vs. triple-negative (n=23) (p=0.07), HER2-enriched (n=16) vs. triple-negative (n=23) (p=0.08). * indicates statistical significance. Error bars: 95% CI.

the same histological subtype, we suggest that mean telomere length measurement may not provide any added value to histological grade, nodal status, and hormone receptor status which are well-established prognostic and predictive factors. Lu *et al*. had previously evaluated the prognostic significance of telomere length in a cohort of breast cancer, and no correlation was demonstrated between telomere length and any of the clinicopathological features (e.g., tumour grade, nodal status, hormone receptor status) of the disease.[35] Consistent with Lu *et al*.'s finding, Simpson *et al*. also demonstrated median telomere length according to histological grade, lymph node status, and hormone receptor status does not have prognostic significance. It has been suggested that it is actually the shortest telomeres that identifies a subset of breast cancer patients with poor prognosis.[32] Nevertheless, our study is a relatively small one in terms of sample size, and whether telomere length has clinical utility in relation to histological grade, lymph node involvement, and hormone receptor statuses in breast cancer should be explored in further depth with a bigger sample size incorporating prognostic, treatment outcome, and survival information.

We found that telomere lengths of HER2-positive IBC-NSTs were significantly shorter than HER2-negative IBC-NSTs. Similarly, analyses of telomere length and molecular subtypes revealed significantly shorter telomeres in subtypes that are HER2-positive, i.e., Luminal B, HER2-enriched. This has also been observed in several other studies.[8,17,34,36,38] Short telomeres are usually indicative of telomere dysfunction commonly associated with genomic instability. Short dysfunctional telomeres have been identified in a variety of tumours, including breast cancer. It is known that HER2-positive breast cancers harbour high levels of HER2 gene amplification, which is suggestive of a greater degree of genomic instability and aberration, leading to the overexpression of its gene product – HER2 protein.[39,40] Triggered by the presence of a large number of HER2 proteins on the cell surface membrane, cells of HER2 overexpressed breast cancers are more aggressive such that they proliferate more rapidly.[41,42] Reasonably, the remarkable telomere shortening in HER2-positive IBC-NSTs in the present study may be due to more rapid proliferation of cells of HER2 overexpressed breast cancers. Telomeres of fast proliferating cells were expected to be much shorter because these cells undergo more cycles of cell division, hence they lose their telomeres more rapidly. It would appear that the contribution to telomere shortening by HER2 overexpression supersedes other pathological variables in IBC-NST. This implies that very short telomeres in breast cancer may have clinical utility in indicating breast cancer aggressiveness, i.e., a marker of poor prognosis. Furthermore, our reasoning and findings concur with a previous study that demonstrated a proportion of breast cancer patients with very short telomeres presenting an extremely poor prognosis, i.e., median survival of less than 12 months.[32]

We had expected telomeres to be shortest in the molecular subtype that has the worse outcome – triple-negative, as has been demonstrated in a previous study.[34] Intriguingly, triple-negative IBC-NSTs of our cohort exhibited longer telomeres than those of the less aggressive luminal B and HER2-enriched subtypes, and near equivalent to those of the least aggressive subtype – luminal A. It should however be noted that not only can shortened telomeres lead to telomere dysfunction, but extraordinarily long telomeres may also do so, and it is one of the hallmarks of the ALT pathway. The ALT pathway is a telomere lengthening mechanism which have been observed to increase overall telomere length in the absence of telomerase in some immortalised mammalian cell lines and tumours.[43] Longer mean telomere length (about 20 kb) have been demonstrated in human cell lines and cancers with the ALT phenotype[44-48] as compared to human immortalised cell lines or cancers with telomerase activity (about 10 kb).[44,49] Moreover, a previous study had identified a small subset of aggressive breast cancer with the ALT phenotype, although they were all HER2-positive (3 out of 21 HER2-positive cases) but none were triple-negative. ALT-positive cancers are rare, and since the sample size was relatively small in that study, it is possible it did not capture any triple-negative breast cancers which utilised the ALT pathway. Notwithstanding, the authors demonstrated that breast cancers with the ALT phenotype are aggressive, i.e., 2 out of the 3 breast cancers with the ALT phenotype showed rapid tumour progression.[31] Given that triple-negative breast cancers are usually aggressive, it is possible that some triple-negative IBC-NSTs utilise the ALT pathway to maintain their telomeres, and this may be a plausible explanation for the longer telomeres observed in our triple-negative cohort.

Apart from being potential predictors of

aggressive breast cancer which may have importance in the determination of patient prognosis, very short and extraordinarily long telomeres which were observed in HER2 overexpressed and triple negative IBC-NST subtypes indicate that telomere length has potential in stratifying patients for therapy. Since higher telomerase levels and shorter telomeres have been observed in most IBC-NSTs,[15] and that extraordinarily long telomeres may indicate the ALT phenotype, our findings suggest that telomere length may have utility to identify patients suitable for the relevant therapy, e.g., telomerase inhibitors for treating patients with very short telomeres, therapies targeting the ALT pathway for treating patients with extraordinarily long telomeres. In addition, continued telomere length measurements during and after the relevant treatment can be used to monitor therapeutic response and predict clinical outcome based on telomere length.

In essence, it has to be noted that conclusions in this study are based on analyses of IBC-NST and FA. These are the most clinically applicable choices since IBC-NST is the most common cancer and FA the most common benign tumour of the breast. However certain limitations must be recognised. In the determination of TCR in FA, only the epithelial component was analysed although FA is a biphasic tumour with proliferation of both epithelial and mesenchymal components. Hence the mesenchymal component has not been taken into account. This aspect needs further study. Furthermore, it is too premature to apply the TCR cut-offs of IBC-NST determined based against FA and BNL on other histological subtypes of malignant and benign breast lesions as there would be several biological variables to consider across the spectrum of breast lesions, as well as variations in test performance across patient groups.[50] Therefore, determining the telomere length cut-offs for other histological types of benign and malignant breast tumours will need to be addressed by further studies for those specific tumour types. Nevertheless, using IBC-NST and FA as prototypes of malignant and benign breast neoplasms respectively, we have found encouraging results that indicated cut-off values of telomere length has potential to be exploited as an additional parameter to distinguish malignant from benign breast tissues. Our study revealed that mean telomere lengths of HER2 overexpressing subtypes (i.e., luminal B and HER2-enriched) were significantly shorter ($p<0.05$) than those that did not overexpress HER2. The contribution of HER2 amplification/overexpression to telomere shortening or loss warrants further scientific investigation. Whether shorter telomeres may have an impact on the response of HER2-positive breast cancers to anti-HER2 therapies (e.g., trastuzumab) would also be an interesting subject for future studies. An unexpected finding was that mean telomere lengths of triple-negative breast cancer were longer than Luminal B and HER2-enriched cancers, although this did not achieve statistical significance. The contribution of ALT mechanisms in telomere dysfunction including long telomeres in triple-negative breast cancer warrants further scientific investigation.

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