Telomere Length on Chromosome 17q Shortens More than Global Telomere Length in the Development of Breast Cancer

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Abstract

It is known that total telomere length is shorter in invasive breast cancer than in normal breast tissue but the status of individual telomere lengths has not been studied. Part of the difficulty is that usually telomere length in interphase cells is measured on all chromosomes together. In this study we compared normal breast epithelium, duct carcinoma in situ (DCIS), and invasive duct carcinoma (IDC) from 18 patients. Telomere length was specifically measured on chromosome 17q and was found to be shorter in DCIS and IDC than in normal breast epithelial cells, with more heterogeneity in telomere length in DCIS associated with IDC than in DCIS alone. More importantly, we found that the shortening of telomere on chromosome 17q is greater than the average shortening of all telomeres. This finding indicates that telomere shortening is not simply the result of the end replication problem; otherwise, all telomeres should be subjected to the same rate of telomere shortening. It seems there are mechanisms that preferentially erode some telomeres more than others or preferentially protect some chromosome ends. Our results suggest that the increased level of telomere shortening on 17q may be involved in chromosome instability and the progression of DCIS.

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Introduction

Breast cancer is one of the most common cancers afflicting North American women. Although the majority of early-stage breast cancers are not life threatening, a small proportion of cases will progress to metastatic breast cancer. Invasive duct carcinoma (IDC) is frequently observed to extend directly from ducts containing duct carcinoma in situ (DCIS). However, not all cases of DCIS develop into invasive tumors. Molecular markers hold the promise of becoming clinically useful diagnostic tools, particularly markers that can be studied in formalin-fixed paraffin-embedded (FFPE) tissue samples, as virtually all DCIS is processed in this way for routine pathologic diagnosis. Because telomeres are involved in maintenance of chromosomal stability, they represent one group of markers of particular interest.

The telomere is a specialized structure at the end of chromosomes consisting of a highly conserved repetitive DNA sequence, (TTAGGG).*1 Telomeres form caps on the ends of chromosomes that prevent fusion of chromosomal ends and provide genomic stability. In normal somatic cells, telomeres are progressively shortened with every cell division. This shortening in normal human cells limits the number of cell divisions. For human cells to proliferate beyond the senescence checkpoint, they need to stabilize telomere length. This is accomplished mainly by reactivation of telomerase [2]. Telomerase expression is under the control of many factors [3–10]. Expression of telomerase can lead to cell immortalization and is activated during tumorigenesis [11].

Using various approaches, it has been shown that telomere length in DCIS is generally shorter than in normal breast epithelial cells with inconsistent results with respect to associations between tumor telomere length and clinicopathologic features [12–15]. However, in other cancer types a correlation between longer telomeres and more aggressive behavior of cancer cells has been found [16,17]. Although these groups have measured the average length of pantelomeres in their studies, it has been reported that it may not be the average but rather the shortest telomeres that constitute telomere dysfunction and limit cellular survival in the absence of telomerase [18]. It has been suggested that loss of telomere function occurs preferentially on chromosomes with critically short telomeres [18]. Martens et al. [19] found that chromosome 17p has the shortest telomere among human chromosomes. However,
Perner et al. [20] showed that chromosome 17p telomere is among the shorter ones (but not the shortest), and its erosion occurs at the same rate as all others. Interestingly, using BJ fibroblast cells, Zou et al. identified chromosome 17q, not chromosome 17p, as one of the 10 shortest telomeres with a high frequency of signal-free ends [21].

Numerous studies have demonstrated genetic abnormalities on chromosome 17 in DCIS with rates approaching 60% to 80% [22,23], suggesting that oncogenes and tumor suppressor genes in these regions may be particularly important in the development of DCIS. A frequently amplified gene in IDC and DCIS is the human epidermal growth factor receptor-2 (HER2) oncogene located on chromosome 17q. HER2 is a member of a family of transmembrane receptor tyrosine kinases with no identified ligand. HER2 is overexpressed and amplified in 20% to 30% of IDC and up to 80% of DCIS. Many groups have reported a correlation between HER2 amplification and poor prognosis [24,25]. Despite much interest, the mechanism of HER2 amplification/overexpression in many cases is unclear. We hypothesized that telomere erosion on 17q could lead to instability on 17q and this may be associated with HER2 amplification/overexpression.

In this study, we developed a technique to allow measurement of specific telomeres in routinely processed clinical samples. This allowed us to measure telomere length specifically on chromosome 17q in FFPE samples. We also investigated the relationship between HER2 expression and telomere shortening on chromosome 17q.

Materials and Methods

Sample Collection

Breast tissue was obtained from archival FFPE blocks that were stored in the Pathology Department of University Health Network and had been obtained initially for diagnosis. Institutional research ethics board approval was obtained for the study.

Quantitative Fluorescence In Situ Hybridization

To measure the relative changes in telomere length on chromosome 17q in breast epithelium, quantitative fluorescence in situ hybridization (Q-FISH) was used. Metaphase slides prepared from normal peripheral lymphocytes (Vysis Inc., Downers Grove, IL) and the breast cancer cell line MCF-7, colon cancer cell line HT-29, and prostate cancer cell line Du-145 were used to optimize Q-FISH conditions for two different probes: a PNA (CCCTAA)₃ pantelomere probe–fluorescein isothiocyanate (FITC; Applied Biosystems, Bedford, MA). Because the (CCCTAA)₃ PNA probe is not able to distinguish the parental telomeres from each other, all the telomere signals are the average of two homologous chromosomes in our report.

We also used a specific subtelomeric DNA probe for chromosome 17q (TelVysion 17q SpectrumOrange; Vysis) as a guide to locate chromosome 17q telomeres. Using these two probes, we were able to detect strong signals from telomeres and the chromosome 17q subtelomeric region simultaneously. The signals from these two probes were close enough to allow direct analysis of telomere length on the end of chromosome 17q in FFPE tissue samples.

Five-micrometer-thick FFPE tissue samples on positively charged glass slides were deparaffinized with xylene, washed with 100% ethanol, and air-dried followed by RNase A treatment. Slides were then placed in 1 mol/l NaSCN at 80°C and washed in water at room temperature. After pepsin digestion, slides were thoroughly washed with water followed by dehydration in serial ethanol dilutions (70%, 90%, and 100%) at room temperature and air-dried. The sections and the PNA pantelomere probe were then denatured at 80°C for 5 minutes in a Hybrite programmable heating block (Vysis) followed by incubation in a dark humidified chamber for 90 minutes at 25°C. Then slides were hybridized with a denatured specific chromosome 17q probe mixture and incubated overnight at 33°C. Slides were washed in 2 × SSC/0.3% NP-40 at 73°C, 2 × SSC/0.3% NP-40 and 2 × SSC at room temperature followed by dehydration in serial ethanol dilutions and air-dried. Finally, the slides were counterstained with 4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Inc., Burlingame, CA) and viewed with a fluorescence microscope (Leica DMRA2, Wetzlar, Germany).

Image Capturing and Analyzing

The normal breast epithelium, DCIS, and invasive areas in each tissue section were identified by hematoxylin and eosin staining (Figure 1A). Then, parallel sections were studied for the Q-FISH analysis (Figure 1B–D). In this study, we measured the length of telomere in normal breast epithelium (n = 8), DCIS (n = 14), and IDC (n = 12) from 18 patients. For each type of tissue (normal breast epithelium, DCIS, and IDC) a minimum of 20 nuclei in five different areas were scored for the following variables: the total telomere length (expressed as intensity), the telomere length at the end of chromosome 17q (expressed as intensity), and sub-telomeric chromosome 17q (expressed as intensity).

The TelVysion 17q SpectrumOrange probe contains a locus within 300 kb of the end of the chromosome (Vysis). This corresponds to a physical distance within 0.7 μm from chromosome 17q subtelomeric signals [26]. We searched for the telomere signals within 0.7-μm distance from the chromosome 17q signals. To ensure that we were not counting overlapping ends of other chromosomes we captured a series of Z stacked images at 0.7-μm intervals.

Images were captured using Openlab 4.0.3d8 software (Improvision, Viscount Centre II, University of Warwick Science Park, Coventry, England, UK; http://www.improvision.com). Finally the images were analyzed with Image J software (developed at the Research Services Branch of the National Institute of Mental Health, National Institutes of Health; http://rsb.info.nih.gov/nih-image).

Immunohistochemical Staining of Paraffin Sections for HER2

Adjacent FFPE tissue sections from the same blocks were dewaxed in xylene and blocked for endogenous peroxidase
in 3% hydrogen peroxide for 10 minutes. After microwave antigen retrieval, the sections were blocked for endogenous biotin with the biotin blocking kit (Vector Laboratories). The sections were incubated with 1:300 diluted primary antibody HER2 rabbit polyclonal antibody (Dako, Carpinteria, CA) for 1 hour at room temperature and rinsed in PBS followed by incubation with biotin-conjugated anti–rabbit IgG (Signet Laboratories, Inc., Dedham, MA) for 30 minutes. Afterwards, the sections were incubated with streptavidin-labeled HRP for 30 minutes (Signet kit) and rinsing in PBS, the sections were incubated in NovaRed substrate (Vector Laboratories) for another 5 minutes. Finally, the nuclei were counterstained in Mayer’s hematoxylin for 10 seconds and the sections were dehydrated, cleared, and premounted for microscopic evaluation. Samples having more than 10% of cells with complete cell membrane staining of moderate to strong intensity were considered as HER2 positive.

Data Analysis
In this study, the following variables were used in the analysis: telomere length (TelGen) = average of the telomere length intensity (per patient, per type of tissue, over the minimum

Figure 1. FISH analysis of breast FFPE sections. (A) Comparable hematoxylin and eosin sections were used to determine regions of interest. (B–D) FISH with subtelomeric DNA probe to label chromosome 17q (orange signals) and pantelomeric PNA probe (green signals) on FFPE sections (original magnification, ×100). Representative images of normal breast epithelial tissue, DCIS, and IDC are shown in each row. DAPI was used as a counterstain. Identical images in black and white shown in (C–D) with nuclei outlined in gray and (C) telomere and (D) subtelomeric chromosome 17q signals represented by the dark spots (indicated by arrows). Note reduction in telomere signal number, intensity, and size in DCIS and IDC compared with normal tissue.
20 nuclei); normalized telomere length (N-TelGen) = average telomere length divided by the average chromosome 17q intensity; chromosome 17q telomere length (TelCh17q) = average of the telomere length intensity of the chromosome 17q PNA probe–FITC; normalized chromosome 17q telomere length (N-TelCh17q) = chromosome 17q telomere length divided by the average chromosome 17q intensity; and immunohistochemical percentage positivity for HER2 overexpression. The intensity of the subtelomeric chromosome 17q signals was used to normalize the values for telomere length. Cells without 17q signals were not scored.

Statistical Analysis

The exact Wilcoxon signed rank test and the exact Wilcoxon rank sum test were used to compare the telomere lengths between normal breast epithelium, DCIS, and IDC samples. Both tests are nonparametric. They are useful when the sample size is small or the assumption of normality does not hold.

Results

In this study, the variables (N-TelGen and N-TelCh17q) were compared between normal, DCIS, and IDC tissues. Using the exact Wilcoxon signed rank test, we found that total telomere length in DCIS was significantly shorter than that measured in normal breast epithelium ($P = .023$) (Figure 2A). Likewise, the telomere length on chromosome 17q was notably shorter than its counterpart in DCIS compared with normal tissues ($P = .078$) (Figure 2B). In contrast, the differences in the length of total telomere and telomere on chromosome 17q were minimal between DCIS versus IDC (Figure 2, A and B). Upon investigating the variables, N-TelGen and N-TelCh17q, among DCIS alone ($n = 6$) and DCIS + IDC ($n = 8$), no significant changes were apparent between these two groups (Figure 3, A and B).

To study if the telomere shortening for chromosome 17q and total telomeres occurs at the same rate, we compared the telomere shortening of chromosome 17q in DCIS and normal tissue with total telomere shortening in the same tissues. For this purpose, we first calculated the fold

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**Figure 2.** Comparison of the normalized intensities of telomere length between normal breast epithelial tissue, DCIS, and IDC: (A) normalized total telomere length, (B) normalized chromosome 17q telomere length. Note significant reduction in telomere intensity in DCIS and IDC in comparison with normal breast epithelial tissue.

**Figure 3.** Distribution of telomere length between DCIS and DCIS associated with IDC: (A) normalized total telomere lengths, (B) normalized chromosome 17q telomere lengths. There are no significant differences in telomere length between DCIS and DCIS associated with IDC.
decrease of the N-TelCh17q compared with N-TelGen for both DCIS and normal tissue in each sample. Then we defined the telomere shortening as the fold decreases in DCIS compared with normal tissue for both chromosome 17q and for total telomeres in each sample. We found that the median telomere length on chromosome 17q in DCIS decreased by 89.8% compared with normal tissue, whereas the median of telomere length on all the rest of the chromosomes decreased by 49% (Table 1).

To study the relationship between telomere shortening on chromosome 17q and HER2 expression, we compared the intensities for TelGen, N-TelGen, TelCh17q, and N-TelCh17q between HER2-negative samples (n = 18) and HER2-positive samples (n = 16). Using the exact Wilcoxon rank sum test, we found that total telomere length in the HER2-positive group is not significantly different from telomere length in the HER2-negative group (Table 2). Likewise, no significant differences were found between the telomere length on chromosome 17q in both HER2 positive and negative (Table 2), indicating that the expression of HER2 is independent from telomere shortening for both total chromosomes and chromosome 17q.

**Discussion**

Invasive breast cancer is frequently observed to extend directly from ducts containing DCIS; however, not all cases of DCIS develop into invasive tumors. The challenge is to identify molecular markers that will allow prediction of which cases of DCIS will progress to IDC. Telomere length is reduced in IDC. Hence, we chose to investigate telomere length as a potential marker.

It has been reported that it may not be the average telomere length but rather the shortest telomeres that constitute telomere dysfunction as loss of telomere function has been shown to occur preferentially on chromosomes with critically short telomeres [18]. However, although the p arm of chromosome 17 (chr17p) has been reported to be the shortest telomere [19], it is the q arm of chromosome 17 (chr17q), not chromosome 17p, that shows a signal-free end with high frequency in senescent cells [21]. This means that telomere shortening does not occur at the same rate for all telomeres; otherwise, chromosome 17p should be one of the signal-free end chromosomes. In agreement with these findings, our data show that telomere shortening on chromosome 17q is significantly greater than the average erosion of total telomeres in DCIS compared to normal breast epithelium, supporting the idea that the erosion of telomeres can be different for different chromosomes.

Although we observed this event in malignant cells, other groups have shown similar results in different cell types [19,21]. Using BJ fibroblasts, Zou et al. [21] found that there were no signal-free ends in young BJ cells, whereas in near-senescent BJ cells chromosome 17q exhibited one of the highest frequencies of signal-free ends. Their report showed that chromosome 17p has no signal-free ends (0%), although this arm of chromosome 17 has been reported to have the shortest telomere among human chromosomes. Similarly, Martens et al. [19] showed that out of 13 samples from 10 different donors, 3 of them had significantly short chromosome 17q telomere lengths compared to the median for each individual donor (Figure 4 in Ref. [19]). All these three samples were from hematopoietic bone marrow cells, which have a high proliferation capacity. Thus, it appears that cells with higher proliferation rates show greater telomere shortening on chromosome 17q compared with chromosome 17p and the average of total telomeres. Based on these observations, we speculate that telomere shortening is not simply the result of the end replication problem. These data indicate that there are mechanisms that preferentially erode some telomeres more than others or preferentially protect some chromosome ends. Based on these findings, we suggest that telomere length on chromosome 17q plays a role in the progression of DCIS, possibly through chromosome instability. However, further studies are required to validate the mechanism behind the differential telomere shortening for different chromosomes as well as the role of this mechanism in chromosome instability and cancer.

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References


