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Spatial Assessment of Telomere DNA Content and Allelic Imbalance in Human Breast Tissues

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Introduction

Detailed mechanisms for the development and progression of breast carcinoma remain largely unknown. However, some key insights are known like the role of genomic instability, which is believed to be an initiating event in tumorigenesis.

The loss of telomere function is one mechanism of attaining genomic instability.[1] Human telomeres are nucleoprotein structures that support chromosome ends and protect them from being marked as DNA double strand breaks. They aid in preventing genomic errors that can result in chromosomal degradation, recombination, and genomic instability.[2] Unfortunately, telomeres can become eroded and therefore dysfunctional. This is a common occurrence in tumors and confers the ability to change gene**tic** and phenotypic profiles leading to malignant characteristics.[3] Previous research has demonstrated that telomere length and/or telomere DNA content is a predictor of disease progression in breast carcinoma and other cancers.[4]

In addition to telomere aberrations, another marker of genomic instability is allelic imbalance, which is a situation where one allele of a gene pair is lost or amplified. In this study allelic imbalance was assessed by looking at the ratio of peak heights of heterozygous loci using a multiplex PCR assay.[5] Both forms of genomic instability, allelic imbalance and telomere attrition, arise in breast tumors and in adjacent histologically normal tissue.[6,7] As a result, it has been hypothesized that within histologically "normal" breast tissues adjacent to tumors lie a store of pre-malignant, yet potentially tumorigenic cells.[5]

Heaphy *et al*. (from the Griffith/Bisoffi lab) investigated the relationship between genetic alterations in tumors and matched tumor-adjacent histologically normal (TA-HN)

tissues in two groups of breast cancer. One group included tumor and matched TA-HN tissue taken at 1cm and 5cm from tumor margins. It was found that the breast tumors demonstrated properties of the matched TA-HN breast tissue at 1 cm, and there was conservation of unbalanced alleles in these samples. The average telomere content (TC) in the TA-HN 5cm and TA-HN 1 cm tissues was 101% and 66% of TC in normal placental DNA standard, respectively. The average TC of breast tumors was 59%. TC of the TA-HN 5cm tissue was within the range of normal (non-tumor) tissue. However, TC of the TA-HN 1cm tissue was significantly less than the normal breast tissue and TA-HN 5cm tissue. Mean TC in tumor and TA-HN 1cm was nearly the same.

The degree of genomic instability in these breast cancer samples was further analyzed for allelic imbalance (AI) at 16 unlinked microsatellite loci. Results from the Heaphy *et al.* study showed that in one group of breast tumors the mean number of unbalanced loci in the TA-HN 1cm and tumor tissues was 0.42 and 1.25 loci per specimen, respectively. This was roughly 5 and 15 times higher than the incidence in the TA-HN 5cm tissues. In a second group of breast tumors the mean numbers of unbalanced loci in the TA-HN (undefined distance from tumor) tissues and matched tumors were 2.61 and 2.48 loci per specimen respectively, and they were significantly higher than normal breast tissue.

These were the first studies to investigate genomic instability at defined distances of 1cm and 5cm from surgical tumor margins. They found that genomic instability in these tissues was a function of distance from the tumor margin, and the extent of genomic instability decreased with increasing distance from the tumor margin. As a result, Heaphy *et al*. proposed a hypothesis that breast epithelial carcinogenesis occurs at higher

frequency in fields of cells with increased genomic instability. All together a strong case for field cancerization was made and supported by this studies.

The aim of this current project was to further explore the extent and spatial distribution of genomic instability in tissue adjacent to breast tumors based upon the results of recent Heaphy *et al*. work which looked at TA-HN tissue in only one direction at 1cm and 5cm from the margins of tumors. To accomplish this goal, two novel aspects of a proposed field of cancerization were introduced and investigated. These aspects were to evaluate genomic instability in more than one direction and at an additional point of 3cm from the margin of tumors. For the multidirectional aspect of the study one patient breast tissue sample was used. It consisted of breast tumor, TA-HN breast tissue taken at 1cm, 3 cm, and 5cm in two directions (perpendicular) away from the tumor margin, and contralateral normal breast tissue. To define a more precise relationship between genomic stability as a function of distance from a tumor we looked at six independent breast cancer tissue samples and their respective TA-HN tissue taken in one direction at 1, 3, and 5 cm from the margins of breast tumors. Here the novel component was the addition of a middle point taken in TA-HN tissue at 3cm from a tumor margin.

Methods and Materials

Breast Tissue Samples

As approved by the UNM Human Research Review Committee (HRRC #03-085), a set of human breast tissue was used in this study. The tissue came from seven women who underwent mastectomies for breast carcinoma and was provided by the UNM Health Sciences Center Pathology Laboratory (in collaboration with Nancy Joste, MD and Myra Zucker, PA). The breast tissue was donated as "remnant material" after pathological evaluation. Approximately 500mg of tissue was excised from the tumors and sites 1cm, 3cm, and 5cm from the visible tumor margins. Contralateral normal breast tissue was also excised from one individual for the multidirectional study. This patient tissue set also had tissues excised in two perpendicular directions from the margin of it's tumor. 10-12 μm sections of the breast tissue was prepared and stained with hematoxylin and eosin and examined microscopically to verify histologically normal and tumor tissue.

DNA Isolation and Quantification

DNA from breast tissue samples was isolated using Qiagen DNeasy Tissue kits as previously described[5]. The isolated DNA was quantified using Picogreen dsDNA quantification reagent, an ultra-sensitive nucleic acid stain, and a fluorometer.

Telomere DNA Content (TC) Assay

TC was measured using the slot blot titration assay as previously described.[9] Isolated DNA was denatured at 56°C in 0.05 M NaOH/1.5 M NaCl, neutralized in 0.5 M Tris/1.5 M NaCl, and applied and UV cross-linked to Tropilon-Plus blotting membranes (Applied Biosystems, Foster City, CA). A telomere-specific oligonucleotide, end-labeled with fluorescein, $(5'\text{-TTAGGG-}3')_4$-FAM, (IDT, Coralville, IA) was hybridized to the

genomic DNA, and the membranes were washed in wash buffers containing SSC and SDS of increasing stringency to remove non-hybridizing oligonucleotides. Hybridized oligonucleotides were detected using an alkaline phosphatase-conjugated anti-fluorescein antibody that produces light when incubated with the CDP-Star substrate (Applied Biosystems, Foster City, CA). Blots were exposed to Hyperfilm® for 1-2 min (Amersham Pharmacia Biotech, Buckinghamshire, UK) and digitized by scanning. The intensity of the telomere hybridization signal was measured from the digitized images using Nucleotech Gel Expert Software 4.0 (Nucleotech, San Mateo, CA). TC is expressed as a percentage of the average chemiluminescent signal of four replicate DNA samples compared to the same amount of a placental DNA standard.[5]

Allelic Imbalance (AI) Assay

DNA (approximately 1 ng) was amplified using the AmpFlSTR Identifiler PCR Amplification Kit (Applied Biosystems, Foster City, CA) using the manufacturer's protocol. Each multiplex PCR reaction amplifies 16 unlinked, genome-wide short tandem repeat (STR) microsatellite loci (Amelogenin, CSF1PO, D2S1338, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D19S433, D21S11, FGA, TH01, TPOX and vWA). Each of the PCR primers were labeled with one of four fluorescent dyes (6-FAM, PET, VIC and NED), each with a unique emission profile, allowing the simultaneous resolution of amplicons of 16 similar sizes. PCR products were resolved by capillary gel electrophoresis and detected using an ABI Prism 377 DNA Sequencer (Perkin Elmer, Foster City, CA). Data was analyzed using the ABI Prism GeneScan and Genotype Analysis software (Applied Biosystems, Foster City, CA). The ratios of peak heights from all were calculated. By convention, the allele with the greater

fluorescence intensity as the numerator was used, and thus the ratio was always ≥ 1.0, with 1.0 representing the theoretical ratio for normal alleles. Allelic pairs with a ratio of ≥ 1.61 were used as an indication of AI.[9]

Statistical Analysis

Statistical analysis was performed using the JMP® statistical package (SAS Institute, Cary, NC) using a significance level of 0.05. The non-parametric Wilcoxon/Kruskal-Wallis Log Rank test was used to determine the comparative distribution of TC and AI (markers of genomic instability) in breast tumor and histologically normal tissues located at 1cm, 3cm, and 5cm from the tumor margins.

Results:

Allelic Imbalance Data: **One Multidirectional Breast Sample**

Allelic imbalance was measured in one multidirectional breast tissue sample set consisting of tumor, matched TA-HN taken at 1,3,5cm in two directions from the tumor margin, and normal contralateral breast tissue. The tumor contained 4 sites of AI. TA-HN tissues at all distances also contained sites of AI, although not as many as within the tumor. There were slightly more sites of AI at 3cm and 5cm in both directions when compared to 1cm. The contralateral breast tissue had 1 site of AI (Figure 1).

Sample	AI (A)	AI (B)
Tumor	4	
contralateral breast	1	0
Dimension 1, 1cm	1	2
Dimension 1, 3cm	2	2
Dimension 1, 5cm	2	2
Dimension 2, 1cm	0	2
Dimension 2, 3cm	2	2
Dimension 2, 5cm	2	2

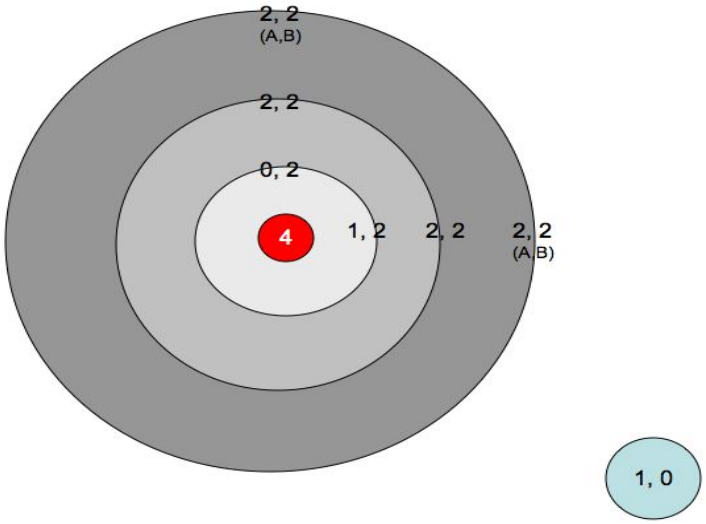

Figure 1: The table summarizes the number of sites with AI as a function of distance and direction. The letters A and B represent tissue taken from opposite ends of tissue excised

at each point (1,3,5cm) along the two dimensions from the tumor. The data is shown as a schematic below the table.

Allelic Imbalance Data: **Six Independent Unidirectional Breast Samples**

Allelic imbalance was measured in six independent sets of breast tumors and their TA-HN taken at 1,3,5 cm in one direction from the margins of the tumors. Mean number of AI for tumors was 3, while all TA-HN tissues were less than 1. There were statistically significant differences in the mean sites of AI between tumor and 1cm (p=0.0026), 3cm (p=0.0098) and 5cm (p=0.0079) TA-HN tissues. However, there was no statistically significant difference amongst the TA-HN tissues.

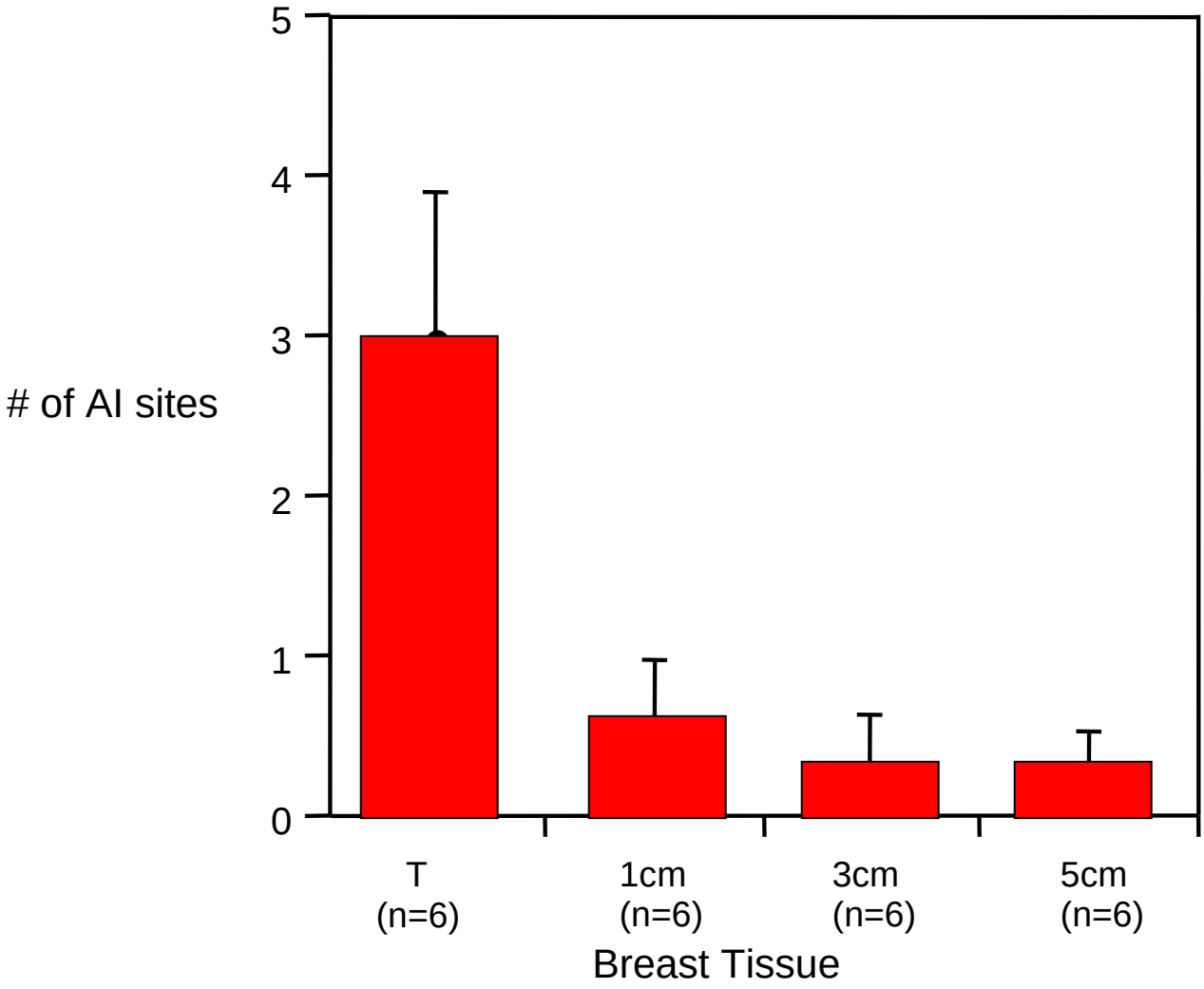

Figure 2: Number of AI sites in one directional samples. The tumors have more sites of AI than the TA-HN tissues. T = tumor. 1,3,5cm are TA-HN tissue taken at these distances. Standard error bars also shown.

Allelic Imbalance Data: **Six Independent Unidirectional Breast Samples**

Conservation of unbalanced alleles in matched tumor (T) and TA-HN breast tissues was measured in six independent breast tissue sets A-F. Two tissue sets (D and E) showed conservation of allelic imbalance in one locus between tumor and TA-HN tissue as seen in Figure 3.

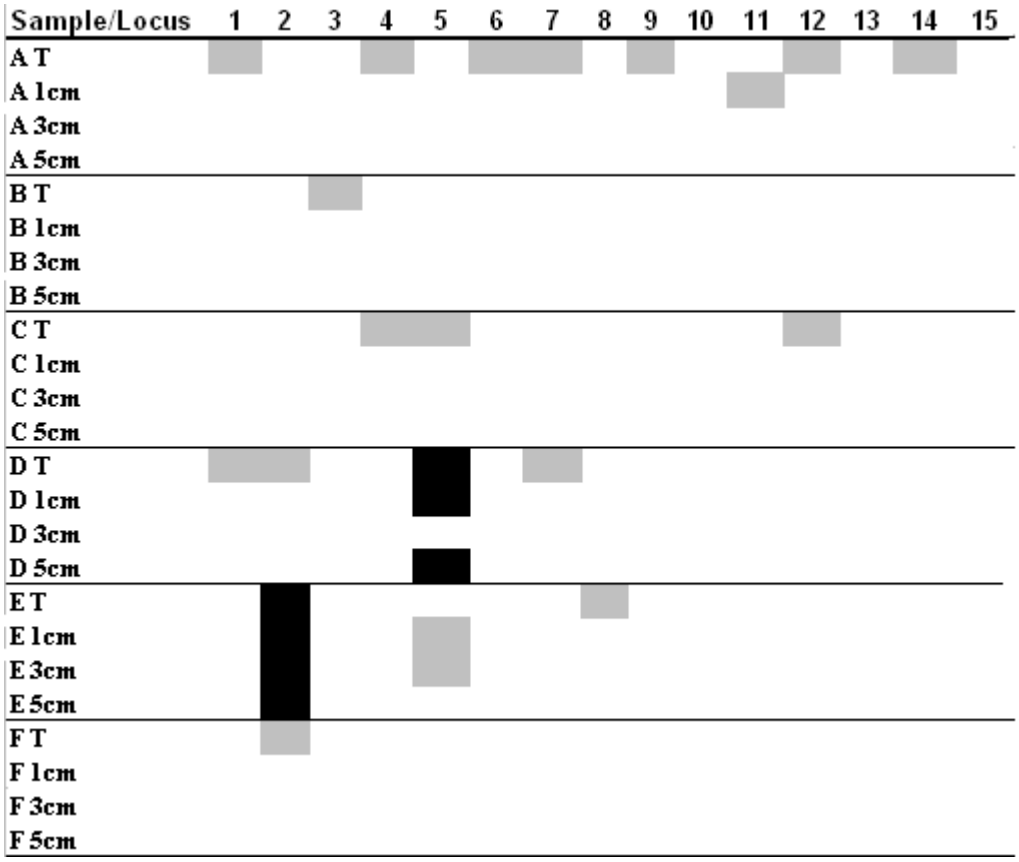

Figure 3: Conservation of unbalanced alleles in matched tumor (T) and tumor-adjacent histologically normal (TA-HN) breast tissues. There are six independent breast tissue sets A-F. Sites of allelic imbalances are indicated by gray boxes; sites of allelic imbalances conserved between tumor and TA-HN tissues are indicated by black boxes. The unlinked chromosomal loci are designated 1-15 and are as following (1) D8S1179, (2) D21S11, (3)

D7S820, (4) CSF1PO, (5) D3S1358, (6) TH01, (7) D13S317, (8) D16S539, (9) D2S1338, (10) D19S433, (11) vWA, (12) TPOX, (13) D18S51, (14) D5S818, (15) FGA. Note: homozygous amelogenin (all female samples) is not shown.

Telomere Content (TC) Data: **One Multidirectional Breast Sample**

TC was measured in one multidirectional breast tissue sample set consisting of tumor, matched TA-HN taken at 1,3,5cm in two directions from the tumor margin, and normal contralateral breast tissue. TC in all these locations is nearly equivalent as seen in Figure 4.

Sample	TC (A)	TC (B)
Tumor	1.09	
contralateral breast	1.14	1.04
Dimension 1, 1cm	1.15	1.06
Dimension 1, 3cm	1.14	1.14
Dimension 1, 5cm	1.19	1.15
Dimension 2, 1cm	1.15	1.02
Dimension 2, 3cm	1.15	1.05
Dimension 2, 5cm	1.18	1.15

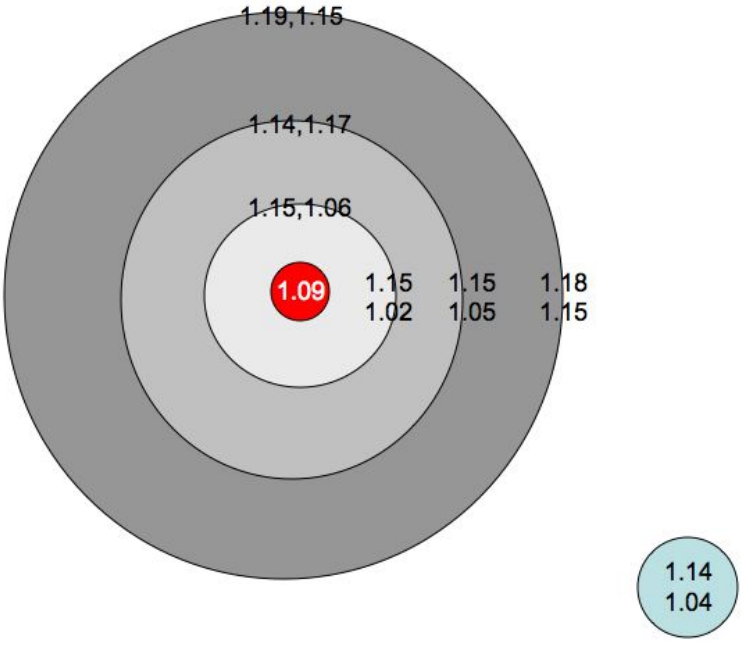

Figure 4: The table summarizes the TC as a function of distance and direction. The center red is the tumor. The letters A and B represent TA-HN tissue taken from opposite ends of tissue excised at each point (1,3,5cm) along the two dimensions from the tumor The smaller outer circle is TC values for the normal tissue contralateral breast. The data is shown as a schematic below the table.

Telomere Content Data: **Six Independent Unidirectional Breast Sample**

Telomere content was measured in six independent sets of breast tumors and their TA-HN taken at 1,3,5 cm in one direction from the margins of the tumors. The distribution for TC for all tissues significantly overlapped and the p values between all were greater than 0.05 as seen in Figure 5. Yet, the p-values are not shown in Figure 5.

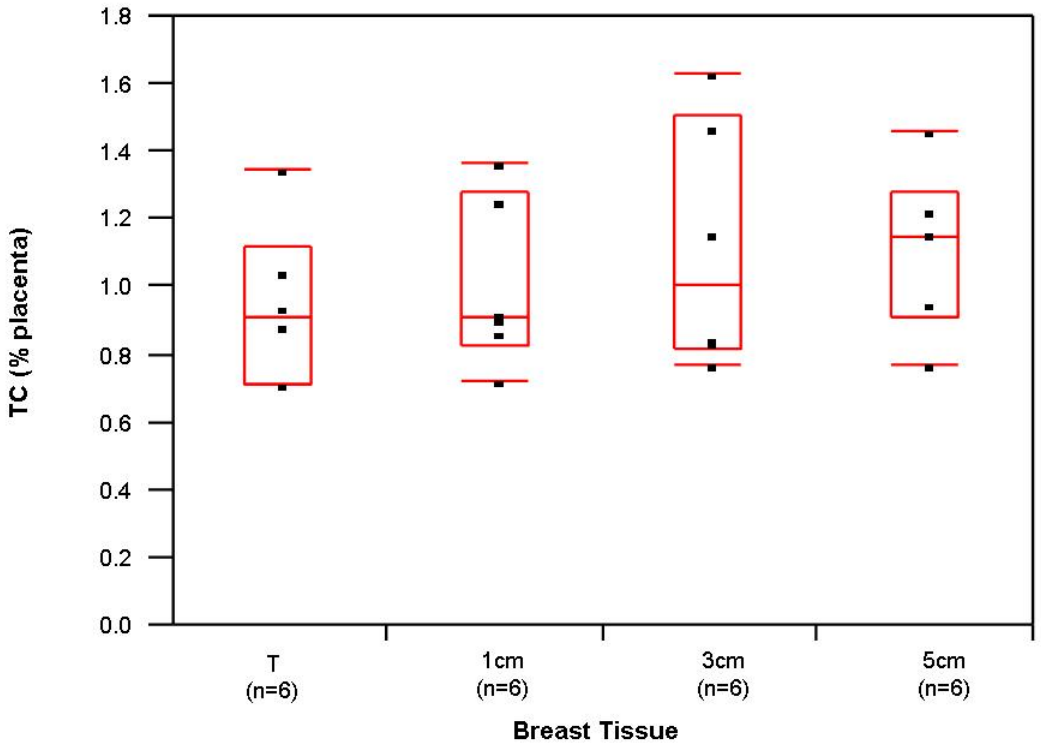

Figure 5: Distribution of telomere DNA content (TC) in six independent breast tissue sets. N= 6 for T(tumor) and TA-HN at 1,3,5cm from tumor. TC is expressed as percentage of TC in placental control. The boxes represent group median (line across

middle) and quartiles (25th and 75th percentiles) at its ends. Lines below and above boxes indicate 10th and 90th percentiles.

Conclusion/Discussion:

Genomic Instability is an important event involved in the development and progression of breast carcinoma. In this current study two quantitative markers of genomic instability were assessed, telomere content (TC) and allelic imbalance (AI). Both markers of genomic instability (AI and TC) occur in breast tumors and in adjacent histologically normal tissue. Therefore, it has been hypothesized that fields of cancerization or areas of tumorigenic potential exist around tumors.

Heaphy *et al.* were the first group to investigate genomic instability in TA-HN breast tissue at defined distances of 1cm and 5cm from surgical tumor margins and hence within the field affected by cancerization. They concluded that genomic instability in these tissues was a function of distance from the tumor margin, and the extent of genomic instability decreased with increasing distance from the tumor margin. However, in their study only TA-HN breast tissue taken in one direction from a tumor's margin was explored. Therefore, the purpose of this current study was to further investigate the extent and spatial distribution of genomic instability in tumors and their matched TA-HN tissues by looking in more than one direction and at an additional distance point of 3cm from a tumor margin.

Originally, I had hypothesized that telomere DNA content (TC) in tumor adjacent-histologically normal (TA-HN) breast tissues at 1cm, 3cm, and 5cm in more than one direction from a tumor margin would define a field of increasing TC with

increasing distance from the tumor margin. Similarly, for allelic imbalance (AI) I had hypothesized that TA-HN 5cm tissue would be significantly different (low extent of AI) when compared to the 1cm, 3cm, and tumor tissues.

In this study we were able to procure one multidirectional breast tissue sample set that showed four sites of AI in the tumor TA-HN tissues at all distances (1,3,and 5cm) also had sites of AI, although not as many as the tumor. This data is consistent with the fact that there is increased inherent genomic instability within tumors. For this patient contralateral breast tissue was available and had one site of AI. Overall, this data is not entirely consistent with what Heaphy *et al.* had found in previous studies as they showed AI similarities between tumor and 1cm TA-HN tissues. The telomere content (TC) in the one multidirectional breast tissue sample set was nearly equivalent in the tumor and matched TA-HN tissues at all distances (1,3,5cm) from the tumor margin. In addition we measured in this study was AI in six independent sets of breast tumors and their TA-HN taken at 1,3,5 cm in one direction from the margins of the tumors. Data from this study showed that on average tumors had significantly more sites of AI when compared to their matched TA-HN tissues at any distance from the tumor margin. With regard to the 1cm distance from the tumor margin, this data does not correlate with previous studies, which showed AI similarities between tumor and TA-HN at 1 cm. In addition, data from this current analysis showed no difference in AI in TA-HN tissues at 1,3,and 5cm.

Telomere Content was measured in one multidirectional sample set. There was no difference in TC between tumor, TA-HN, or contralateral tissues. The same was true for the six unidirectional breast tumors and TA-HN tissues analyzed in this study. There

was no statistical difference in TC for these tissues. As with AI, these data are not in agreement with previous studies by Heaphy *et al.*

There were some major limitations in this study. First, for the multidirectional study our lab was able to obtain only one breast tissue set from a single patient. As a result, an accurate comparative analysis of genomic instability in a multidirectional field around breast tumors was limited and not amenable to statistical assessment. Also, in the investigation of the six unidirectional breast tissue sets there was considerable variability in TC values in all samples. In addition, I had to use high standard deviation cutoffs to include data in this TC analysis study. While this variability could be due to experimental errors, the overall agreement with our previously reported data was low.

However, despite the limitations of this study, the overall concept of the project is still worthy of further investigation. There is potentially practical and clinically important information to be gained. For example, from a surgical perspective it would be useful to have excision margins that were truly free of any cancerous potential. Genetically aberrant areas around breast tumors may leave a risk for local recurrence, which occurs in up to 22% of patients receiving conservative treatment for small invasive and non-invasive breast cancer.[10] The concept of this project aims to complement standard histology with molecular testing for genomic instability for field cancerized breast tissue. Consequently, a better understanding as well as detection of areas of genomic instability in TA-HN breast tissue may allow for better diagnosis, treatments, and overall survival for patients with breast carcinoma.

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