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Markers of fibrosis and epithelial to mesenchymal transition demonstrate field cancerization in histologically normal tissue adjacent to breast tumors

Kristina A. Trujillo¹, Christopher M. Heaphy², Minh Mai¹, Keith M. Vargas¹, Anna C. Jones¹,
Phung Vo¹, Kimberly S. Butler¹, Nancy E. Joste³, Marco Bisoffi¹, and Jeffrey K Griffith¹

¹Department of Biochemistry and Molecular Biology, MSC08 4670 University of New Mexico, Albuquerque, NM

²Department of Pathology, Johns Hopkins School of Medicine, 411 N. Caroline St. Bond St. Building B301, Baltimore, MD

³Department of Pathology, MSC08 4640 University of New Mexico, Albuquerque, NM

Abstract

Previous studies have shown that a field of genetically altered but histologically normal tissue extends 1 cm or more from the margins of human breast tumors. The extent, composition and biological significance of this field are only partially understood, but the molecular alterations in affected cells could provide mechanisms for limitless replicative capacity, genomic instability and a microenvironment that supports tumor initiation and progression. We demonstrate by microarray, qRT-PCR and immunohistochemistry a signature of differential gene expression that discriminates between patient-matched, tumor-adjacent histologically normal breast tissues located 1 cm and 5 cm from the margins of breast adenocarcinomas (TAHN-1 and TAHN-5, respectively). The signature includes genes involved in extracellular matrix remodeling, wound healing, fibrosis and epithelial to mesenchymal transition (EMT). Myofibroblasts, which are mediators of wound healing and fibrosis, and intra-lobular fibroblasts expressing MMP2, SPARC, TGF- β 3, which are inducers of EMT, were both prevalent in TAHN-1 tissues, sparse in TAHN-5 tissues, and absent in normal tissues from reduction mammoplasty. Accordingly, EMT markers S100A4 and vimentin were elevated in both luminal and myoepithelial cells, and EMT markers α -smooth muscle actin and SNAIL were elevated in luminal epithelial cells of TAHN-1 tissues. These results identify cellular processes that are differentially activated between TAHN-1 and TAHN-5 breast tissues, implicate myofibroblasts as likely mediators of these processes, provide evidence that EMT is occurring in histologically normal tissues within the affected field and identify candidate biomarkers to investigate whether or how field cancerization contributes to the development of primary or recurrent breast tumors.

Keywords

field cancerization; wound healing; fibrosis; breast cancer; epithelial to mesenchymal transition

Abnormal tissues adjacent to tumors were first described in 1953 by Slaughter *et al.*,¹ who reported the presence of histologically abnormal tissue proximal to multi-focal cancerous lesions. The group called the process leading to this abnormal tissue “field cancerization.” Since this original work, investigations of field cancerization have changed from histological to molecular analyses of seemingly normal tissue directly adjacent to the tumors of the lung, skin, prostate and breast.² These investigations have focused on the histologically normal tissue that surrounds tumors, and thus are distinct from studies on tumor stroma, which is located within tumors.

We and others have previously demonstrated that histologically normal breast tissues proximal to tumors differ by several molecular criteria from normal tissues more distal to the tumor and from reduction mammoplasty (RM) (reviewed in Ref. 3). Although the extent, composition and biological significance of this field are only partially understood, the molecular alterations in affected cells could provide mechanisms for limitless replicative capacity, genomic instability, and a microenvironment that supports tumor initiation and progression. For example, telomere lengths in tumor-adjacent, histologically normal (TAHN) tissues located 1 cm from the tumor margin (TAHN-1) are typically 35–40% shorter than telomere lengths in patient-matched tissues 5 cm from the tumor (TAHN-5) or tissues from RM.⁴ Telomere attrition and resulting telomere dysfunction is a significant cause of genomic instability and an early event in breast tumorigenesis.^{5,6} Accordingly, allelic imbalance (a form of genomic instability which occurs in virtually all cancers) is 4–5 times more prevalent in TAHN-1 tissues than in patient-matched TAHN-5 tissues or RM.⁴ Furthermore, hTERT, the specialized reverse transcriptase that is expressed in approximately 90% of tumors and confers limitless replicative capacity,^{7,8} is also expressed in a sub-population of epithelial cells in TAHN tissues.⁹ Finally, the spectra of promoter methylation and gene expression differ between TAHN and RM tissues.^{10–14} For example, gene expression profiles in tumor adjacent tissues are suggestive of a wound healing microenvironment,¹² which is supportive of tumor initiation and progression.¹⁵ Together, these findings suggest that the field of genetically altered cells surrounding human breast tumors could influence tumor progression and/or represent the “fertile soil” from which primary or recurrent tumors develop. However, further understanding of cellular processes occurring in this field, the size of the field and informative biomarkers that define the field are needed to assess whether or how field cancerization contributes to the behavior and development of primary or recurrent breast tumors.

The objectives of our study were to identify markers that are over-expressed differentially between TAHN-1 and TAHN-5 tissues and to use these markers to delineate the biological processes occurring in the affected field. Microarray and qRT-PCR techniques identified genes that are up-regulated in TAHN-1 tissues relative to both patient-matched TAHN-5 tissues and RM tissues. Genes up-regulated in TAHN-1 tissues are involved in extracellular matrix remodeling, wound healing, fibrosis and epithelial to mesenchymal transition (EMT). Supporting these findings, immunohistochemical and immunofluorescent analyses demonstrated that myofibroblasts, important mediators of extracellular matrix remodeling, wound healing, and fibrosis, were prevalent in TAHN-1 tissues, but reduced or absent in TAHN-5 and RM tissues. Luminal and myoepithelial cells over-expressing several markers of EMT also were prevalent in TAHN-1 tissues but reduced or absent in TAHN-5 and RM tissues.

These results identify cellular processes that likely are occurring in TAHN-1, but neither TAHN-5 nor RM breast tissues, implicate myofibroblasts as likely mediators of these processes, provide evidence that EMT is occurring within the affected field and identify candidate biomarkers for future studies.

Material and Methods

Tissue samples

Breast tissues from full mastectomy cases were obtained from the University of New Mexico Hospital (UNMH) Surgical Pathology Laboratory. Waste mastectomy tissues were obtained with IRB approval, which precluded collection of patient information. Since RM surgeries are not routinely performed at UNM hospital, RM samples were obtained from the Cooperative Human Tissue Network (CHTN) at Vanderbilt University Medical Center. RM tissue obtained from CHTN is indistinguishable from TAHN-5 tissues obtained from UNMH by the criteria of telomere length and allelic imbalance.⁴ Approximately 500 mg of tissue was excised from sites 1 cm (TAHN-1) and 5 cm (TAHN-5) from the visible tumor margins. After resection, the tissues were immediately frozen in liquid nitrogen and stored at -70°C until RNA isolation. Matched sets of TAHN-1 and TAHN-5 tissues were handled identically to assure that any observed differences between TAHN-1 and TAHN-5 tissues would not be due to differences in handling protocols. Formalin-fixed, paraffin-embedded sections (10–12 μm) were stained with hematoxylin and eosin (H&E) and analyzed microscopically to verify the absence of cancer (Supporting Information Figure 3).

RNA isolation

Frozen tissues were homogenized and RNA was extracted with phenol and chloroform followed by clean-up using the RNeasy Mini Kit (Qiagen, Valencia, CA). The RNA was analyzed for integrity using the Agilent Bioanalyzer 2100 (Agilent, Foster City, CA). RNA from disease-free breast tissue (pooled RNA derived from nine autopsies and one RM) served as control (Ambion, Austin, TX).

cDNA Synthesis and labeling

RNA was reversed transcribed into complementary DNA (cDNA). The control cDNA was made from the pooled RNA from disease-free breast tissues (see above) and labeled with Cy3. The experimental cDNAs were made from the RNA extracted from individual TAHN and RM tissues and labeled with Cy5. Labeling was achieved by synthesizing the cDNAs with amino allyl dUTP (Sigma-Aldrich, St. Louis, MO) followed by chemically coupling of either Cy3 or Cy5 mono-functional dye (Amersham-Pharmacia Biotech, Arlington Heights, IL) to the cDNA.

Microarray hybridization

Spotted arrays of the Human Genome Oligo Set Version 3.0 (Qiagen, Valencia, CA) were utilized. The design is fully based on the Ensembl (<http://www.ensembl.org/>) Human 13.31 Database and Human Genome Sequencing Project. Equal amounts of Cy3- and Cy5-dye labeled cDNAs (200 pmoles each) were combined and competitively hybridized to the microarray slides overnight.

Microarray data analysis

Following hybridization and washing, the slides were scanned at 635nm and 532nm on an Axon 4000A scanner (Axon Instruments, Union City, CA) and Axon GenePix Pro 5 Software was used for quantitation of the signals. The Cy3 and Cy5 signal intensities were determined for each feature (*i.e.*, oligonucleotide) and analyzed on Acuity 3.0 software (Molecular Devices, Sunnyvale, CA). Two thousand seven hundred and twelve features met standard quality calls (background removal, linear regression ratio >0.6 , signal to noise ratio >3.0) in at least four of the six samples of TAHN-1 tissue and at least four of the six TAHN-5 tissues. Four thousand one hundred and eighty-one features met standard quality calls in all five RM samples. Only data passing these quality filters were utilized in the

analysis. RNA expression levels for individual transcripts are reported as ratios of Cy3/Cy5 signals. To control for potential dye bias, resulting from either differential incorporation of Cy3 and Cy5 during cDNA synthesis, or differential hybridization of Cy3- and Cy5-labeled cDNAs, paired aliquots of the control cDNA were labeled with Cy3 and Cy5, combined in equal amounts, hybridized and analyzed as described above. The mean Cy3/Cy5 ratio was 1.91 ± 0.13 SD. These data were used to normalize the results shown in Figure 1. The average, normalized fold-change in expression relative to the normal control RNA was 1.26 (0.74 SD) for TAHN-1, 1.22 (0.65 SD) for TAHN-5 and 1.05 (0.75 SD) for RM.

Validation of microarray data by qRT-PCR

cDNA was synthesized using the Retroscript™ RT kit (Ambion, Austin, TX) according to manufacturer's instructions. Previously published primers were used for each of the three transcripts. For COL1A1, the forward primer was 5'-TACAGCGTCACTGTGCGATGGC-3' and the reverse primer was 5'-TCAATCACTGTCTTGCCCCAG-3'. For COL3A1, the forward primer was 5'-AATTTGGTGTGGACGTTGGC-3' and the reverse primer was 5'-TTGTCGGTCACTTGCACTGG-3'. For SPARC, the forward primer was 5'-GTGCAGAGGAAACCGAAGAG-3' and the reverse primer was 5'-AAGTGGCAGGAAGAGTCGAA-3'. Amplification of the genes of interest was normalized against the housekeeping gene, TATA Binding Protein (TBP). For TBP, the forward primer was 5'-CACGAACCAACGCGCACTGATT-3' and the reverse primer was 5'-TTTTCTTGCTGCCAGTCTGGAC-3'. PCR reactions were performed in quadruplicate on the LightCycler 480 real-time PCR system (Roche Applied Science). PCR arrays (Mesenchymal Stem Cell Array, SA Biosciences) were performed in 20 µL reactions according to manufacturer's instructions and analyzed using the online PCR array analysis tool (SA Biosciences).

Immunohistochemistry (IHC) and immunofluorescence (IF)

All IHC and IF protocols were performed according to antibodies manufacturer's instructions. The following mouse primary antibodies were used: α -smooth muscle actin (Sigma), vimentin (Dako), pan-keratin (Chemicon) and Ki-67 (Sigma). The following rabbit primary antibodies were used: MMP2 (Sigma), SPARC, SNAIL, TGF β 3 and S100A4 (All Abcam). For IHC, secondary antibodies used were goat anti rabbit-HRP and goat antimouse-HRP (Abcam). HRP activity was visualized using the Liquid DAB Plus Substrate Kit (Zymed) according to manufacturer's instructions. For IF, secondary antibodies used were goat antimouse-Alexa633 and chicken antirabbit-Alexa488 (Molecular Probes). IHC for was performed on all TAHN and RM samples used in the microarray experiments. IHC slides were visualized on a Zeiss Axiovert 25 microscope and photographed with a Zeiss AxioCam MRc camera. Fluorescence slides were viewed on a Zeiss Axioscope 2 MOT microscope and photographed with a Nuance camera (Cambridge Research Instrumentation). Fluorescence was quantified in at least three representative areas of the field shown in each figure using the Nuance camera software. Values are reported in scaled counts/sec. Masson's Trichrome stain was performed according to manufacturer's instructions (Sigma). All IHC and IF protocols were performed on five matched samples and four RM samples.

Results

Differential gene expression between TAHN-1 and TAHN-5 tissues

Our previously published experiments demonstrating telomere attrition and allelic imbalance in TAHN tissues were performed with bulk tissue (*i.e.*, not micro-dissected⁴). Thus, we deduced that the altered cells (and therefore mRNA from the altered cells) must comprise a substantial fraction of the total and predicted that the spectrum of gene expression measured

in RNA purified from TAHN-1 tissue would differ from the spectrum measured in RNA purified from TAHN-5 tissue and disease-free breast tissue. Our strategy was predicated on the following assumptions. First, in contrast to the extensive and heterogeneous alterations in gene expression in tumors, there should be less variation between gene expression in TAHN-5 and TAHN-1 tissues from the same patient. Second, although the TAHN tissues contain a mixture of cell types, (*e.g.*, epithelial and stromal cells), histological evaluation of H&E stained tissue indicated similar tissue structure in patient matched TAHN-1 and TAHN-5 samples. Third, while gene expression may vary between individuals, consistent, reproducible differences between TAHN-1 tissues and patient-matched TAHN-5 tissues are not likely. Finally, although micrometastases that are not detectable by standard histopathological examination could potentially exist in TAHN tissues, these would represent an insignificant fraction of the total tissue and thus have a negligible effect on the observed spectrum of gene expression.

Gene expression profiles were compared in five normal breast tissues obtained from RM, one pair of unmatched TAHN breast tissues and five pairs of patient-matched TAHN breast tissues. Each patient-matched pair was obtained from an independent separate full mastectomy, and comprised of tissues excised from sites 1 cm (TAHN-1) and 5 cm (TAHN-5) from the visible tumor margins. Spotted oligonucleotide arrays representing 37,123 transcripts were used to compare gene expression between tissues. The fold-change in expression for each gene was calculated as the ratio of the median expression in the five individual TAHN-1, TAHN-5 and RM samples relative to the expression in pooled RNA from ten disease-free breast tissues. The mean fold-change in expression between TAHN-1 and TAHN-5 for the 2,712 transcripts that met the criteria for quality and reproducibility was 1.18 ($\pm 0.52SD$), consistent with the assumption that the vast majority of transcripts are expressed at similar levels in TAHN-1 and TAHN-5 tissues.

Stringent criteria were applied to identify genes over-expressed in TAHN-1 tissues relative to TAHN-5 and RM tissues. The TAHN-1/TAHN-5 ratios were rank-ordered and the top 2.5% (68 transcripts) were selected for further analysis. A high TAHN-1/TAHN-5 ratio could reflect either up-regulation of expression in TAHN-1 or down-regulation in TAHN-5 tissues. Transcripts in the latter category were eliminated by excluding all transcripts that were expressed < 2 -fold higher in TAHN-1 tissue than in either the pooled RNA from disease-free tissue or RM tissues. Unknown genes (*e.g.*, ORFs) were also excluded. The 15 transcripts that met these criteria (less than 1% of total) are listed in Figure 1a. Mean transcript levels for COL1A1, COL1A2, COL3A1, IGF1, VAT1, LAMB1 and FSTL1 differed significantly between TAHN-1 and TAHN-5 tissues ($p < 0.05$; Fig. 1b). A similar trend was evident for the other transcripts, but did not reach statistical significance. Similar differences were measured when transcript levels in TAHN-1 were compared to RM tissues (not shown). In contrast, there were no significant differences between transcript levels in TAHN-5 and RM tissues (not shown).

Microarray results were confirmed by two independent PCR methods. The levels of COL1A1, COL3A1 and SPARC were measured individually in RNA derived from the same TAHN tissues used for the microarray experiments. In each instance, mean transcript levels differed significantly between the TAHN-1 and TAHN-5 tissues ($p < 0.05$; Fig. 1b). The transcript levels were also compared in an independent validation set comprised of four additional pairs of matched TAHN-1 and TAHN-5 tissues, 6 paired TAHN-1 samples and one unpaired TAHN-1 sample. The transcript levels for COL1A1 and COL3A1 again differed significantly between the TAHN-1 and TAHN-5 tissues ($p < 0.05$; Fig. 1b). There was more overlap in SPARC transcript levels between the TAHN-1 and TAHN-5 tissues comprising the validation set and the difference was not significant.

In a second approach, a Mesenchymal Stem Cell PCR Array was used to measure and compare the levels of 84 transcripts in 6 TAHN-1 tissues to a control group comprised of six paired TAHN-5 tissues, two RM tissues and the pooled RNA from ten disease-free breast tissues. The array included 12 of the 2,712 transcripts used in the microarray analysis. The levels of six transcripts were significantly higher in TAHN-1 tissues than the control group: COL1A1, IGF1, Thy1, VWF, PDGFRB and TGF β 3. The first four transcripts were among the 15 initially identified by the microarray analysis, providing additional confirmation of their up-regulation in TAHN-1 tissues. The last two (Fig. 1c) were not among the 2,712 transcripts that met the quality standards for inclusion in the microarray analysis. However, as described subsequently, their differential up-regulation in TAHN-1 tissues was confirmed by immunohistochemical methods. No other significant differences in expression were detected in the remaining 77 transcripts, consistent with the observation that the vast majority of transcripts are expressed at similar levels in TAHN-1, TAHN-5 and RM tissues.

Gene interaction and ontology analysis of differential gene expression in TAHN-1 tissues

Gene Network analysis (Supporting Information Figure 1A) reveals that all but two of the 15 proteins identified by the microarray analysis as up-regulated in TAHN-1 tissues are known to be co-expressed with multiple other members of the group (Supporting Information Figure 1C), implying coordinate regulation. Eight of the 15 proteins physically interact with one another (Supporting Information Figure 1D). The analysis also shows that IGF1, COL1A1 and SPARC act to up-regulate the expression of other members of the group (Supporting Information Figure 1B). These results support the conclusion that coordinated physiological processes, not random differences in gene expression, differ between TAHN-1 and TAHN-5 tissues. Gene ontology categories related to extracellular matrix, vasculature development and platelet-derived growth factor binding were highly enriched in both the 15 transcripts identified by our stringent criteria and the initial, unfiltered list of transcripts based solely on the criteria of >2 fold up-regulated (Supporting Information Figure 2).

TAHN-1 tissues are enriched for myofibroblasts and abnormal myoepithelial cells

Myofibroblasts produce abundant levels of extracellular matrix (ECM), cytokines, and growth factors that foster tissue repair in sites of tissue damage. Several genes overexpressed in TAHN-1 tissues are among those expressed in myofibroblasts, suggesting that myofibroblasts are enriched in TAHN-1 tissues. To test this prediction, five independent pairs of TAHN-1 and TAHN-5 tissue and four specimens of RM tissue were stained with antibodies detecting: (1) vimentin: a marker of mesenchymal derived cells, (2) α -smooth muscle actin (α -SMA): a marker of myofibroblasts and myoepithelial cells, and (3) MMP2, SPARC and TGF- β 3: genes expressed in myofibroblasts. Furthermore, Masson's Trichrome stain was performed to identify relative levels and structure of collagen. Representative data from analysis of five specimens is shown in Figure 2. Increased numbers of intra-lobular fibroblasts staining positive with vimentin were apparent in TAHN-1 tissues and to a lesser degree in the TAHN-5 tissues compared to RM tissue (Fig. 2a). Myofibroblasts staining positive with α -SMA were also prevalent in TAHN-1 tissue, (Fig. 2a–2b), but neither TAHN-5 nor RM tissues. (Fig. 2a). In some instances, myofibroblasts were connected by extracellular networks of fibers that stained positive for α -SMA (Fig. 2a, 2b arrows), a characteristic of wound contraction.¹⁶ Intra-lobular fibroblasts expressing MMP2, SPARC and TGF- β 3 were also more prevalent in TAHN-1 (Fig. 2a–2b) than TAHN-5 tissues, and absent in RM tissues (Fig. 2a). This provides additional confirmation of their differential up-regulation in TAHN-1 tissues. Masson's Trichrome stain demonstrated an increase in the intensity of collagen staining in TAHN-1 tissue when compared to TAHN-5 or RM tissues (Fig. 2a). The structure of the collagen fibers appeared disorganized and dense in TAHN-1 tissues. Each of these results supports the conclusion that TAHN-1 tissues are enriched for myofibroblasts.

α -SMA staining also demonstrated myoepithelial cells with varying abnormal appearances in TAHN-1 but not TAHN-5 (Fig. 3a) or RM tissues (Supporting Information Figure 3). Some myoepithelial cells expressed the matrix-degrading protein, MMP2, and staining intensity was most prominent in the myoepithelial cells farthest from the duct (Fig. 3b). SPARC, another mediator of ECM degradation and cell migration, was occasionally seen in myoepithelial cells on one edge of lobules (Fig 3b). Proliferation in groups of myoepithelial cells in TAHN-1 tissue was demonstrated by KI-67 staining (Fig. 3b). In contrast, Ki-67 staining was rarely observed in myoepithelial cells in TAHN-5 and RM tissues. Although immunohistochemical staining reveals extensive morphological changes in the myoepithelial population, it is important to note that these changes are not evident by standard H&E staining, which was scored as “normal” by an experienced breast surgical pathologist (Supporting Information Figure 3).

Luminal and myoepithelial cells in TAHN-1 tissues express markers of an epithelial to mesenchymal transition

Recent research suggests that one source of myofibroblasts—cellular mediators of fibrosis—is through transdifferentiation from epithelial cells through an EMT (reviewed in Ref. 17). Several of the transcripts up-regulated in TAHN-1 tissues are inducers of EMT (TGF β 3, IGF1, MMP2 and SPARC, collagens),^{18–21} suggesting EMT may be occurring in TAHN-1 tissues. This possibility was investigated by double immunofluorescence staining with several pairs of markers of EMT. Unlike tumor tissue, TAHN tissue has normal structure, which is a distinct advantage for investigating EMT. Therefore, luminal and myoepithelial cells are easily identifiable by morphology. The presence of mesenchymal markers within luminal and myoepithelial cells in TAHN-1 tissues is described subsequently.

Expression of α -SMA, a marker of myofibroblasts and myoepithelial cells, and S100A4, a marker of mesenchymal cells, are increased approximately 3–5 fold and 2-fold, respectively, in luminal epithelial cells in TAHN-1 tissues relative to TAHN-5 and RM tissues (Fig. 4). Expression of S100A4 is also increased approximately 3-fold and 7-fold in myoepithelial cells in TAHN-1 tissues relative to TAHN-5 and RM tissues, respectively.

TAHN and RM tissues were also double-stained with S100A4 and keratin clone AE1 (Fig. 5a); AE1 is specific for luminal epithelial cells and the loss of luminal cytokeratins is indicative of EMT. S100A4 staining was approximately 4-fold higher in luminal and myoepithelial cells in TAHN-1 tissues than in TAHN-5 or RM tissues (Fig. 5b). In the cells that S100A4 was elevated, keratin staining was reduced 2–3 fold in the luminal cells in TAHN-1 tissues relative to TAHN-5 or RM tissues.

Two additional pairs of EMT-related markers were examined: 1) α -SMA and TGF- β 3, and 2) vimentin and SNAIL. TGF β 3, a mediator of EMT, is expressed in the fibroblast population (Fig. 2a); however, double immunofluorescence indicates it is also expressed in the luminal epithelial population within TAHN-1 tissue but not in TAHN-5 or RM (Fig. 6a). Vimentin, which stains cells of mesenchymal origin and is a commonly used marker of EMT²² is expressed in the luminal epithelial population in TAHN-1 tissues but not in TAHN-5 or RM tissues (Fig. 6b). Elevated levels of vimentin also were demonstrated in myoepithelial populations in both TAHN-1 and TAHN-5 tissues compared to RM tissue (Fig. 6b). Nuclear localization of SNAIL is closely linked with EMT, and is frequently used as an EMT marker, although EMT-independent functions have also been demonstrated (reviewed in Ref. 23). Therefore, SNAIL expression is likely necessary, but not sufficient, for EMT. Accordingly, nuclear localization of SNAIL was observed in both luminal and myoepithelial nuclei in RM, TAHN-5 and TAHN-1 tissues and was slightly elevated in luminal epithelial nuclei in TAHN-1 tissues (Fig. 6c).

Discussion

TAHN tissue has many molecular alterations known to support tumorigenesis. Our previous investigations have shown that telomere attrition and allelic imbalance occur in histologically normal breast tissues at sites at least 1 cm, but not 5 cm from the tumors' margins.⁴ We have also shown that telomerase expression occurs in tumor-adjacent tissues, and other groups have demonstrated a wound healing gene expression profile in these tissues. These alterations provide mechanisms for genomic instability, limitless replicative capacity, and a microenvironment supportive of tumor initiation and progression. The biological significance of this field is poorly understood. However, two alterations found in field cancerization—telomere attrition and a wound healing gene expression profile—when assayed in tumor tissues are predictors of breast cancer-free survival following surgery.^{12,24,25} Thus, we sought to identify informative markers of the field and use these markers to delineate the biological processes occurring differentially in the affected field.

Two principal conclusions emerge from this study. First, a small number of genes (less than 1% of expressed total) are significantly over-expressed in TAHN-1 compared to TAHN-5 or RM tissues. Between microarray tissue sets and validation sets, gene expression data was performed on a total of 15 TAHN-1, 11-TAHN-5 and five RM tissues, ensuring that these genes can be consistently found within TAHN-1 tissues. Immunohistochemical staining for many of these proteins accurately discriminates TAHN-1 tissues from TAHN-5 and RM tissues, providing informative markers of the affected fields. Gene ontology analysis of the transcripts over-expressed in the TAHN-1 tissues implies involvement in ECM remodeling, wound healing and fibrosis, all processes that promote tumor initiation and progression (reviewed in Refs. 15,26). Increased ECM density is indicative of a reactive stroma which supports tumor growth and, in the absence of a pre-existing tumor promotes tumorigenesis in adjacent cells.^{27–29} In this context, mammographic density is a known risk factor for developing breast cancer³⁰ and areas within the breast that are the most dense are the most likely to develop ductal carcinoma *in situ* (DCIS).³¹ The cellular and molecular compositions of the areas of mammographic density have not been fully defined. However, some studies have demonstrated that the areas of increased mammographic density correspond to areas of increased collagen synthesis,^{32,33} consistent with the gene expression patterns and Masson's Trichrome staining patterns observed in TAHN-1 tissue, implying similar tumorigenic potential. Whether this tissue contributes to local recurrence if it remains after lumpectomy is not known, however this study identifies several markers that can be used to address this question.

The epidemiological studies on breast density,^{30,31} in concert with previous studies^{4,9,12} and the current findings indicate that the histologically normal tissue surrounding tumors is composed of cells with alterations that confer tumorigenic properties, along with a tumor promoting microenvironment. This evokes the provocative possibility that the field of abnormal tissue precedes and/or initiates tumorigenesis. Several studies have shown that tumors form at sites of chronic irritation and injury; (*i.e.*, areas with wound healing microenvironments, reviewed in Ref. 15). Furthermore, we have previously reported that a significant number of imbalanced chromosomal loci are conserved between TAHN-1 tissues and the patient-matched tumor, suggesting a clonal relationship between the tumor and the surrounding breast tissues.⁴ Since there is no histological evidence that tumor cells are present in TAHN-1 tissues, and micrometastases would not be detectable in the bulk tissue assays used to evaluate the imbalanced chromosomal loci, these results imply that the tumor arose clonally from the surrounding tissue. An alternative possibility is that the gene expression pattern observed in TAHN-1 tissue reflects the host's reaction to the tumor. It has been demonstrated that the host desmoplastic response in colorectal cancer correlates with increased survival³⁴ and the authors suggest that this response functions to restrict tumor

growth and invasion. However, there have been limited molecular studies on the desmoplastic response in the breast¹⁴ and the relationship with survival is not well defined.³⁵ A third possibility, suggested by Ronnov-Jessen and Bissel³⁶ and Ge *et al.*³⁷ is that the two alternatives are not mutually exclusive. For example, a dense ECM may contribute to the formation of the tumor, and once the tumor is formed, the surrounding tissue reacts to soluble factors and physical pressure being produced by the tumor.

The second principal finding of this investigation is that luminal and myoepithelial cells in TAHN-1 tissues express markers consistent with EMT. EMT is a process by which epithelial cells lose their epithelial characteristics and gain the traits and functions of mesenchymal cells. These include the loss of cell/cell contacts and increased motility. Although EMT has been proposed to induce a metastatic phenotype in tumor cells, its role in normal breast tissue is less clearly defined. However, EMT has been shown to alter cell polarity, and through this alteration, modify mammary tissue organization (reviewed in Ref. 38). These alterations in tissue architecture create a microenvironment that can promote tumor formation (reviewed in Ref. 39), making EMT not only a potential inducer of tumor progression, but also tumor initiation.

Several of the genes up-regulated in TAHN-1 tissues are either known inducers of, or associated with EMT. For example, collagen I and TGF β 3 are over-expressed approximately 9.0 and 2.2 fold, respectively, in TAHN-1 tissues. Collagen I has been reported to induce EMT by activating the TGF- β 3 signaling pathway,¹⁸ and colorectal carcinoma cells grown on collagen I express markers and display a phenotype consistent with EMT. Insulin-like growth factor 1 (IGF1), which is up-regulated approximately 3.9 fold in TAHN-1 tissue, has been shown to induce EMT in prostate cancer cells.¹⁹ MMP2 and collagen IV are over-expressed ~4.4 fold and 3.2 fold, respectively, in TAHN-1 tissue. Song *et al.*²⁰ have shown that turnover of collagen IV by degradation by MMP2 is a requirement for EMT in the embryonic heart. SPARC is up regulated approximately 3.6 fold in TAHN-1 tissue. SPARC acts at several stages of EMT such as induction of SNAIL and the switch from E-cadherin to N-cadherin (reviewed in Ref. 21). Finally, fibrotic microenvironments, characterized by excess deposition of ECM, induce EMT in organs such as the lung,⁴⁰ kidney,⁴¹ liver⁴² and eye.⁴³ The products of fibrosis-induced EMT are proposed to be myofibroblasts.¹⁷ Since the microenvironments of TAHN-1 and other fibrotic tissues closely resemble one another, it is possible that the increased numbers of myofibroblasts observed in TAHN-1 tissue are the products of EMT. However, further experimentation is needed to determine the fates of the cells expressing EMT markers in the breast and the origin of the excess myofibroblasts.

Unlike tumor tissue, TAHN tissue has normal structure, which is a distinct advantage for addressing some of the controversies relating to EMT in human tumor tissues.⁴⁴ First, the markers used for EMT are indicative, but not exclusive for the process. For example, α -smooth muscle actin also stains myoepithelial and vascular cells, and vimentin is a marker of all mesenchyme. Second, the lack of structure in tumor tissue makes histological evidence of cells undergoing EMT in tumor samples nearly impossible to find. In a recent review of EMT, Thompson and colleagues⁴⁵ wrote that if EMT is occurring in cancer, “the two major repositories available for sampling—primary tumor and metastatic disease—probably should lack evidence of EMT, and EMT may rather be harder to sample as it is likely to be a transient state.” Last, due to the genomic instability of the tumor cells, expression of any EMT marker could be a result of transcriptional dysregulation, rather than a true indicator of a lineage shift.

However, these confounding factors do not apply to TAHN tissue. Although the markers used to identify EMT also stain cells that are mesenchymal in origin, TAHN tissue retains its structure. Therefore, when EMT markers are found in the luminal epithelial lining of

ducts, as shown here, it is difficult to argue that they are mesenchymal in origin. Furthermore, since ducts can be identified in TANH tissue, immunohistochemical staining can uncover the transitional features that would not be identifiable within disorganized tumor tissue; *i.e.*, cells with both epithelial and mesenchymal characteristics (Figs. 4a, 5a). Finally, the findings that less than 1% of genes' expression differs between TANH-1 and TANH-5 tissues, and the differences are consistent between patients, imply a regulated, coordinated process, not random transcriptional dysregulation. Nonetheless, further experimentation is needed to determine whether EMT is indeed occurring within TANH-1 tissue and, if so, how it affects the adjacent tumor.

In summary, the current investigation provides new insights into the complex, reciprocal interactions between tumor and surrounding host tissues, particularly the processes of fibrosis and EMT. The results also pose significant questions that have important implications for the development, recurrence and treatment of breast cancer. A fundamental question is whether the field of abnormal tissue precedes, or is formed in response to the tumor. If the former, markers of the field may provide new tools for risk assessment and early detection. A related question is whether the field of abnormal TANH tissue promotes tumor initiation and progression. If so, markers of the field may provide improved means for assessing the adequacy of surgical margins. Finally, what is the molecular trigger that leads to changes in gene expression, telomere dysregulation and genomic instability? If TANH tissue promotes tumor initiation and progression, as we propose, then this trigger provides a potential target for preventive therapeutics.

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References

1. Slaughter DP, Southwick HW, Smejkal W. Field cancerization in oral stratified squamous epithelium; clinical implications of multicentric origin. *Cancer*. 1953; 6:963–8. [PubMed: 13094644]
2. Chai H, Brown RE. Field effect in cancer-an update. *Ann Clin Lab Sci*. 2009; 39:331–7. [PubMed: 19880759]
3. Heaphy CM, Griffith JK, Bisoffi M. Mammary field cancerization: molecular evidence and clinical importance. *Breast Cancer Res Treat*. 2009; 118:229–39. [PubMed: 19685287]
4. Heaphy CM, Bisoffi M, Fordyce CA, Haaland CM, Hines WC, Joste NE, Griffith JK. Telomere DNA content and allelic imbalance demonstrate field cancerization in histologically normal tissue adjacent to breast tumors. *Int J Cancer*. 2006; 119:108–16. [PubMed: 16450377]
5. Meeker AK, Argani P. Telomere shortening occurs early during breast tumorigenesis: a cause of chromosome destabilization underlying malignant transformation? *J Mammary Gland Biol Neoplasia*. 2004; 9:285–96. [PubMed: 15557801]

6. Meeker AK, Hicks JL, Iacobuzio-Donahue CA, Montgomery EA, Westra WH, Chan TY, Ronnett BM, De Marzo AM. Telomere length abnormalities occur early in the initiation of epithelial carcinogenesis. *Clin Cancer Res.* 2004; 10:3317–26. [PubMed: 15161685]
7. Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PL, Coviello GM, Wright WE, Weinrich SL, Shay JW. Specific association of human telomerase activity with immortal cells and cancer. *Science.* 1994; 266:2011–5. [PubMed: 7605428]
8. Yang G, Rosen DG, Mercado-Urbe I, Colacino JA, Mills GB, Bast RC Jr, Zhou C, Liu J. Knockdown of p53 combined with expression of the catalytic subunit of telomerase is sufficient to immortalize primary human ovarian surface epithelial cells. *Carcinogenesis.* 2007; 28:174–82. [PubMed: 16829690]
9. Hines WC, Fajardo AM, Joste NE, Bisoffi M, Griffith JK. Quantitative and spatial measurements of telomerase reverse transcriptase expression within normal and malignant human breast tissues. *Mol Cancer Res.* 2005; 3:503–9. [PubMed: 16179497]
10. Lewis CM, Cler LR, Bu DW, Zochbauer-Muller S, Milchgrub S, Naftalis EZ, Leitch AM, Minna JD, Euhus DM. Promoter hypermethylation in benign breast epithelium in relation to predicted breast cancer risk. *Clin Cancer Res.* 2005; 11:166–72. [PubMed: 15671542]
11. Tripathi A, King C, de la Morenas A, Perry VK, Burke B, Antoine GA, Hirsch EF, Kavanah M, Mendez J, Stone M, Gerry NP, Lenburg ME, et al. Gene expression abnormalities in histologically normal breast epithelium of breast cancer patients. *Int J Cancer.* 2008; 122:1557–66. [PubMed: 18058819]
12. Troester MA, Lee MH, Carter M, Fan C, Cowan DW, Perez ER, Pirone JR, Perou CM, Jerry DJ, Schneider SS. Activation of host wound responses in breast cancer microenvironment. *Clin Cancer Res.* 2009; 15:7020–8. [PubMed: 19887484]
13. Montel V, Mose ES, Tarin D. Tumor-stromal interactions reciprocally modulate gene expression patterns during carcinogenesis and metastasis. *Int J Cancer.* 2006; 119:251–63. [PubMed: 16482564]
14. Iacobuzio-Donahue CA, Argani P, Hempen PM, Jones J, Kern SE. The desmoplastic response to infiltrating breast carcinoma: gene expression at the site of primary invasion and implications for comparisons between tumor types. *Cancer Res.* 2002; 62:5351–7. [PubMed: 12235006]
15. Schafer M, Werner S. Cancer as an overhealing wound: an old hypothesis revisited. *Nat Rev Mol Cell Biol.* 2008; 9:628–38. [PubMed: 18628784]
16. Hinz B, Gabbiani G. Mechanisms of force generation and transmission by myofibroblasts. *Curr Opin Biotechnol.* 2003; 14:538–46. [PubMed: 14580586]
17. Kalluri R, Neilson EG. Epithelial-mesenchymal transition and its implications for fibrosis. *J Clin Invest.* 2003; 112:1776–84. [PubMed: 14679171]
18. Shintani Y, Maeda M, Chaika N, Johnson KR, Wheelock MJ. Collagen I promotes epithelial-to-mesenchymal transition in lung cancer cells via transforming growth factor-beta signaling. *Am J Respir Cell Mol Biol.* 2008; 38:95–104. [PubMed: 17673689]
19. Graham TR, Zhou HE, Otero-Marrah VA, Osunkoya AO, Kimbro KS, Tighiouart M, Liu T, Simons JW, O'Regan RM. Insulin-like growth factor-I-dependent up-regulation of ZEB1 drives epithelial-to-mesenchymal transition in human prostate cancer cells. *Cancer Res.* 2008; 68:2479–88. [PubMed: 18381457]
20. Song W, Jackson K, McGuire PG. Degradation of type IV collagen by matrix metalloproteinases is an important step in the epithelial-mesenchymal transformation of the endocardial cushions. *Dev Biol.* 2000; 227:606–17. [PubMed: 11071778]
21. Podhajcer OL, Benedetti LG, Girotti MR, Prada F, Salvatierra E, Llera AS. The role of the matricellular protein SPARC in the dynamic interaction between the tumor and the host. *Cancer Metastasis Rev.* 2008; 27:691–705. [PubMed: 18542844]
22. Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. *J Clin Invest.* 2009; 119:1420–8. [PubMed: 19487818]
23. Barrallo-Gimeno A, Nieto MA. The Snail genes as inducers of cell movement and survival: implications in development and cancer. *Development.* 2005; 132:3151–61. [PubMed: 15983400]

24. Heaphy CM, Baumgartner KB, Bisoffi M, Baumgartner RN, Griffith JK. Telomere DNA content predicts breast cancer-free survival interval. *Clin Cancer Res.* 2007; 13:7037–43. [PubMed: 18056180]
25. Fordyce CA, Heaphy CM, Bisoffi M, Wyaco JL, Joste NE, Mangalik A, Baumgartner KB, Baumgartner RN, Hunt WC, Griffith JK. Telomere content correlates with stage and prognosis in breast cancer. *Breast Cancer Res Treat.* 2006; 99:193–202. [PubMed: 16752076]
26. Dvorak HF. Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *N Engl J Med.* 1986; 315:1650–9. [PubMed: 3537791]
27. Shekhar MP, Pauley R, Heppner G. Host microenvironment in breast cancer development: extracellular matrix-stromal cell contribution to neoplastic phenotype of epithelial cells in the breast. *Breast Cancer Res.* 2003; 5:130–5. [PubMed: 12793893]
28. Provenzano PP, Inman DR, Eliceiri KW, Keely PJ. Matrix density-induced mechanoregulation of breast cell phenotype, signaling and gene expression through a FAK-ERK linkage. *Oncogene.* 2009; 28:4326–43. [PubMed: 19826415]
29. Provenzano PP, Inman DR, Eliceiri KW, Knittel JG, Yan L, Rueden CT, White JG, Keely PJ. Collagen density promotes mammary tumor initiation and progression. *BMC Med.* 2008; 6:11. [PubMed: 18442412]
30. Boyd NF, Lockwood GA, Martin LJ, Knight JA, Byng JW, Yaffe MJ, Tritchler DL. Mammographic densities and breast cancer risk. *Breast Dis.* 1998; 10:113–26. [PubMed: 15687568]
31. Ursin G, Hovanessian-Larsen L, Parisky YR, Pike MC, Wu AH. Greatly increased occurrence of breast cancers in areas of mammographically dense tissue. *Breast Cancer Res.* 2005; 7:R605–8. [PubMed: 16168104]
32. Alowami S, Troup S, Al-Haddad S, Kirkpatrick I, Watson PH. Mammographic density is related to stroma and stromal proteoglycan expression. *Breast Cancer Res.* 2003; 5:R129–35. [PubMed: 12927043]
33. Guo YP, Martin LJ, Hanna W, Banerjee D, Miller N, Fishell E, Khokha R, Boyd NF. Growth factors and stromal matrix proteins associated with mammographic densities. *Cancer Epidemiol Biomarkers Prev.* 2001; 10:243–8. [PubMed: 11303594]
34. Caporale A, Vestri AR, Benvenuto E, Mariotti M, Cosenza UM, Scarpini M, Giuliani A, Mingazzini P, Angelico F. Is desmoplasia a protective factor for survival in patients with colorectal carcinoma? *Clin Gastroenterol Hepatol.* 2005; 3:370–5. [PubMed: 15822042]
35. Walker RA. The complexities of breast cancer desmoplasia. *Breast Cancer Res.* 2001; 3:143–5. [PubMed: 11305947]
36. Ronnov-Jessen L, Bissell MJ. Breast cancer by proxy: can the microenvironment be both the cause and consequence? *Trends Mol Med.* 2009; 15:5–13. [PubMed: 19091631]
37. Ge L, Meng W, Zhou H, Bhowmick N. Could stroma contribute to field cancerization? *Med Hypotheses.* 2010; 75:26–31. [PubMed: 20149555]
38. Godde NJ, Galea RC, Elsum IA, Humbert PO. Cell polarity in motion: redefining mammary tissue organization through EMT and cell polarity transitions. *J Mammary Gland Biol Neoplasia.* 2010; 15:149–68. [PubMed: 20461450]
39. Barcellos-Hoff MH. It takes a tissue to make a tumor: epigenetics, cancer and the microenvironment. *J Mammary Gland Biol Neoplasia.* 2001; 6:213–21. [PubMed: 11501581]
40. Kim KK, Kugler MC, Wolters PJ, Robillard L, Galvez MG, Brumwell AN, Sheppard D, Chapman HA. Alveolar epithelial cell mesenchymal transition develops in vivo during pulmonary fibrosis and is regulated by the extracellular matrix. *Proc Natl Acad Sci USA.* 2006; 103:13180–5. [PubMed: 16924102]
41. Rastaldi MP, Ferrario F, Giardino L, Dell'Antonio G, Grillo C, Grillo P, Strutz F, Muller GA, Colasanti G, D'Amico G. Epithelial-mesenchymal transition of tubular epithelial cells in human renal biopsies. *Kidney Int.* 2002; 62:137–46. [PubMed: 12081572]
42. Zeisberg M, Yang C, Martino M, Duncan MB, Rieder F, Tanjore H, Kalluri R. Fibroblasts derive from hepatocytes in liver fibrosis via epithelial to mesenchymal transition. *J Biol Chem.* 2007; 282:23337–47. [PubMed: 17562716]

43. Saika S, Kono-Saika S, Ohnishi Y, Sato M, Muragaki Y, Ooshima A, Flanders KC, Yoo J, Anzano M, Liu CY, Kao WW, Roberts AB. Smad3 signaling is required for epithelial-mesenchymal transition of lens epithelium after injury. *Am J Pathol.* 2004; 164:651–63. [PubMed: 14742269]
44. Tarin D, Thompson EW, Newgreen DF. The fallacy of epithelial mesenchymal transition in neoplasia. *Cancer Res.* 2005; 65:5996–6000. discussion 6000–6001. [PubMed: 16024596]
45. Hugo H, Ackland ML, Blick T, Lawrence MG, Clements JA, Williams ED, Thompson EW. Epithelial--mesenchymal and mesenchymal--epithelial transitions in carcinoma progression. *J Cell Physiol.* 2007; 213:374–83. [PubMed: 17680632]

Figure 1. Differential gene expression in TAHN-1 tissues. (a) Listing of genes over-expressed in TAHN-1 tissues. (b) Transcript levels measured by microarray in RNA from individual TAHN-1, TAHN-5 and RM samples measured by microarray (m) or qRT-PCR (p). Asterisk denotes statistically significant ($p < 0.05$) differences between TAHN-1 and TAHN-5 tissues. (c) Transcript levels of additional up-regulated genes identified by PCR array. Asterisk denotes statistically significant ($p < 0.05$) differences between TAHN-1 and TAHN-5 tissues. All Y axes denote fold expression over control RNA from disease-free breast tissues.

Figure 2.

Immunohistochemical staining of fibroblast populations demonstrates the presence of myofibroblasts within TAHN-1 tissue. (a) Immunohistochemical (IHC) staining for vimentin, α -smooth muscle actin (α -SMA), TGF- β 3, MMP2, SPARC and Masson's Trichrome Stain for collagen in TAHN-1, TAHN-5 and RM tissues. Areas within boxes in α -SMA, TGF- β 3 and MMP2 figures are shown at a higher magnification in (b). IHC staining was performed using a diaminobenzadine stain (brown) and nuclei were counterstained with hematoxylin (blue). Since α -SMA stains both myoepithelial cells and myofibroblasts, these populations are identified by morphology and indicated by arrows in the α -SMA panels. "Fib" indicates fibroblasts and "Myo-epi" indicates myoepithelial cells. In Masson's Trichrome stained tissues, nuclei appear maroon/black and collagen appears blue.

Figure 3. Immunohistochemical staining demonstrates abnormal myoepithelial cells within TAHN-1 tissues. (a) Staining for the myoepithelial marker, α -smooth muscle actin (α -SMA) in TAHN-1 and TAHN-5 issues. Since α -SMA stains both myoepithelial cells and myofibroblasts, myoepithelial populations are identified by morphology and indicated by arrows. (b) Staining for MMP2, SPARC in TAHN-1 and TAHN-5 tissues. (c) Staining for the proliferation marker, Ki67, in TAHN-1 and RM tissues. Immunohistochemical staining was performed as in Figure 2. Arrows marked "Myo-epi" indicate myoepithelial cells.

Figure 4.

Double immunofluorescence demonstrates increased expression of S100A4 and α -SMA, common markers of EMT, in both luminal and myoepithelial populations. (a) Double immunofluorescence for S100A4 (green) and α -SMA (red) in RM, TAHN-5 and TAHN-1 tissues. Nuclei are stained with DAPI (blue). (b) Quantitation of α -SMA in luminal epithelial population and S100A4 in luminal and myoepithelial populations. See methods for additional details.

Figure 5. Double immunofluorescence demonstrates loss of cytokeratin in luminal epithelial cells highly expressing S100A4. (a) Double immunofluorescence for S100A4 (green) and pan-cytokeratin (red) in RM TAHN-5 and TAHN-1. Nuclei are stained with DAPI (blue). (b) Quantitation of cytokeratin in luminal epithelial population and S100A4 in luminal and myoepithelial populations. See methods for additional details.

Figure 6. Double immunofluorescence demonstrates additional markers of EMT in TAHN-1 tissues. (a) Double immunofluorescence for TGFβ (green) and α-SMA (red), (b) vimentin (red) and (c) vimentin (red) and SNAIL (green). Nuclei are stained with DAPI (blue). Quantitation of each marker is shown for luminal and myoepithelial populations on the right of each panel.