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TITLE: Identification of the Types, Properties, and Functional Characteristics of Telomerase Expressing Cells in Breast Cancer

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13. ABSTRACT (Maximum 200 Words)

The aims of this study are to identify the types and properties of telomerase producing cells within breast tumors, and further, to isolate these cells from breast tumors so that their biochemical and functional properties may be characterized. Through examining the role of telomerase in cancer, this project also fosters the education of the candidate through the interaction with several experts in breast cancer pathology, epidemiology, biostatistics, and clinical and basic research. The experiments involved require the interaction with professionals from several different fields of the biomedical sciences and the mastery of several challenging laboratory techniques. To date, all tasks; as outlined in the Statement of Work, are on schedule. The research is in progress.

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Real-Time RT-PCR, Telomerase, hTERT, in-situ hybridization (ISH), Immunohistochemistry (IHC), Fluorescence activated cell sorting (FACS)

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Hypothesis and Rationale

To understand the effects of hTERT on breast cancer cell immortalization and tumorigenesis, it is necessary to study telomerase expression at the level of the individual cell. The preliminary experiments and the evidence in the current literature suggest that: 1) the levels of hTERT expression vary within the cellular subpopulations of a tumor and/or 2) the fraction of cells expressing telomerase varies between tumors. Based on these considerations, ***I hypothesize that breast cancer cells with the highest expression of hTERT will have the most aggressive phenotype.*** I will evaluate this hypothesis by testing the predictions in two specific aims.

- **Specific Aim #1**
  To identify the types, numbers, and properties of breast tumor cells expressing hTERT, so as to define the variability in cellular expression of hTERT in human breast tumors.

- **Specific Aim #2**
  To characterize the biochemical and functional properties of breast tumor cells expressing elevated hTERT, so as to determine if these cells have the more aggressive phenotype that is correlated with a poor clinical outcome.

PERFORMANCE ACCOMPLISHMENTS

Specific Aim 1: (13 tasks)

Task 1 Month 1 **Complete**

- Identification of a sample breast tumor population consisting of low, intermediate, and high hTERT mRNA expressing tumors.

- The acquisition of human breast tissue is in progress as approved by our institution's Human Research and Review Committee (HRRC)

Task 2 Month 1-6 **Complete**

- DNA/RNA has been extracted from newly selected breast tumors.

Task 3 Month 1-6 **Complete**

- Quantitative RT-PCR analysis of hTERT mRNA on newly selected breast tumors

Task 4 Month 12-36 **Not initiated**

- Cryosectioning of breast tumors

Task 5 Month 6-12 **Complete**

- Optimize hTERT FISH assay
- The attached figure (appendix III) demonstrates typical ISH results:
  a) In Situ Hybridization negative control
  b) Anti EMA Immunohistochemistry labeling epithelial cells
  c) Anti Vimentin Immunohistochemistry labeling fibroblasts
  d,e) In Situ Hybridization using fluorescein labeled hTERT probe (1. merged DAPI and fluorescein channel 2) flurescein channel only)

Task 6 Months 12-18 **In Progress**

- perform hTERT FISH assay

Task 7 Months 12-18 **Not Successful**

- Optimize hTERT IHC assay:
- We have determined that the current commercial antibodies to telomerase did not produce satisfactory results for immunohistochemical detection within frozen breast sections.

Task 8 Months 12-18 **In Progress**

- Optimize cytokeratin-7 IHC assay

Task 9 Months 12-18 **In Progress**

- Optimize vimentin IHC assay

Task 10 Months 12-18 **In Progress**

- Optimize α-actin IHC assay

Task 11 Months 12-18 **Not Initiated**
- Optimize common acute lymphocytic leukemia antigen IHC assays

Task 12 Months 18-20 **Not Initiated**
- Optimize IHC triple-labeling experiments

PERFORMANCE ACCOMPLISHMENTS (contd)

Task 13 Months 21-36 **Not Initiated**
- Perform IHC triple-labeling assay

Specific Aim 2: (5 tasks)

Task 1 Month 12-36 **In Progress**
- Creation of primary cell cultures from fresh breast tumors.

Task 2 Month 22-36 **Not initiated**
- Growth analyses of primary cell cultures

Task 3 Month 12-15 **In Progress**
- Creation of GFP reporter plasmid (months 12-15)

Task 4 Month 15-36 **Not initiated**
- Optimization and flow separation of cells based on GFP levels

Task 5 Month 15-36 **Not initiated**
- Characterization of hTERT-rich cells by growth rate, anchorage independence, DNA flow cytometry, telomere content.

KEY TRAINING/EDUCATIONAL ACCOMPLISHMENTS:

Since the activation of this award the PhD candidate has had the opportunity to work and interact with oncologists, pathologists and other Ph.D. scientists who specialize in breast cancer. This has principally through tumor board meetings, journal clubs, special seminars and direct interaction within the laboratory. He has been trained by experts that oversee the Microscopy and Flow Cytometry core facilities at the UNM Health Sciences Center. During this time, the Ph.D. candidate's research was approved by his dissertation committee, a body comprised of three Ph.D. scientists with interests in breast cancer, and one M.D. that specializes in breast cancer pathology.

On an educational level, the candidate was a co-instructor for two upper-level courses: 1) Biochemical Laboratory Methods, and 2) Intensive Biochemistry II: Intermediary metabolism. The candidate has aspirations of continuing his research and remaining in academia and felt that this provided him with the opportunity to develop the essential teaching skills needed for furthering his career. In addition, he is working closely with the co-editor of *Clinical Studies in Medicinal Biochemistry*, Dr. Robert Glew, as a co-

author of the chapter: "Gaucher's Disease, a Sphingolipidosis." He is the co-author on a submitted manuscript and is currently in the process of preparing three more papers for publication.

In the spring of 2003, he was nominated for the University-wide "Teaching Assistant of the Year" Award. The candidate will be working with other senior faculty members as a co-instructor of Intensive Biochemistry I during the fall of 2003.

REPORTABLE OUTCOMES:

Presentations: (abstracts in Appendix)

AACR special conference: "The Role of Telomeres and Telomerase in Cancer" San Francisco, Dec 7-11, 2002. "Elevated Levels of hTERT mRNA Identify Breast Tumors with Multiple Poor Prognostic Markers." William C. Hines, Colleen A. Fordyce, Nancy E Joste, Jeffrey K. Griffith

ASBMB/Experimental Biology, April 11-15 2003. "The Telomeric Binding Proteins; TRF1 and TRF2, mRNAs are Elevated in Human Breast Tumors with Reduced Telomere Content and in Tumors Displaying Genomic Instability." Kimberly Butler, William Curtis Hines, Colleen A Fordyce, Jeffrey K Griffith.

APPENDIX I -

Elevated Levels of hTERT mRNA Identify Breast Tumors with Multiple Poor Prognostic Markers.

William Curtis Hines, Colleen A. Fordyce, Nancy E Joste, Jeffrey K. Griffith,

University of New Mexico Health Science Center

Background: There is large body of experimental evidence that support the role of telomeres and telomerase in facilitating carcinogenesis. As is widely known, nearly all tumors, in contrast to their corresponding normal tissues, express telomerase; the enzyme that lengthens telomeres. The purpose of these experiments was to investigate the mRNA levels of the catalytic subunit of telomerase, hTERT, and determine if these levels associated with any other known prognostic markers.

Methods: Thirty-seven tumors and their corresponding pathological information were collected. From these files, lymph node status, tumor size, DNA ploidy, and fraction of cells in S-phase were recorded. Frozen sections were stained with hematoxylin and eosin and analyzed for histopathological grade and fraction of tumor cells per section. Levels of hTERT mRNA were measured by real-time RT-PCR.

Results: Thirty-six of 37 tumors expressed detectable levels of hTERT mRNA. These levels ranged from 0.03 to 22.42 units and were greater in tumors that were large ($p=0.012$), mitotically active ($p=0.024$) and high grade ($p=0.033$) as determined by the wilcoxon rank sum test. There was no apparent association between and hTERT mRNA levels and the fraction of tumor cells per section. The hTERT mRNA levels were statistically higher ($p=0.003$) in the subset of tumors that were large, mitotically active, node positive, aneuploid and high grade.

Conclusion: Increased levels of hTERT mRNA define a subset of tumors with the most aggressive phenotypes, suggesting a potential use of hTERT mRNA as a prognostic marker or a therapeutic target in cancer. This work was supported by Army Pre-doctoral Breast Cancer Traineeship and Breast Cancer Concept Awards.

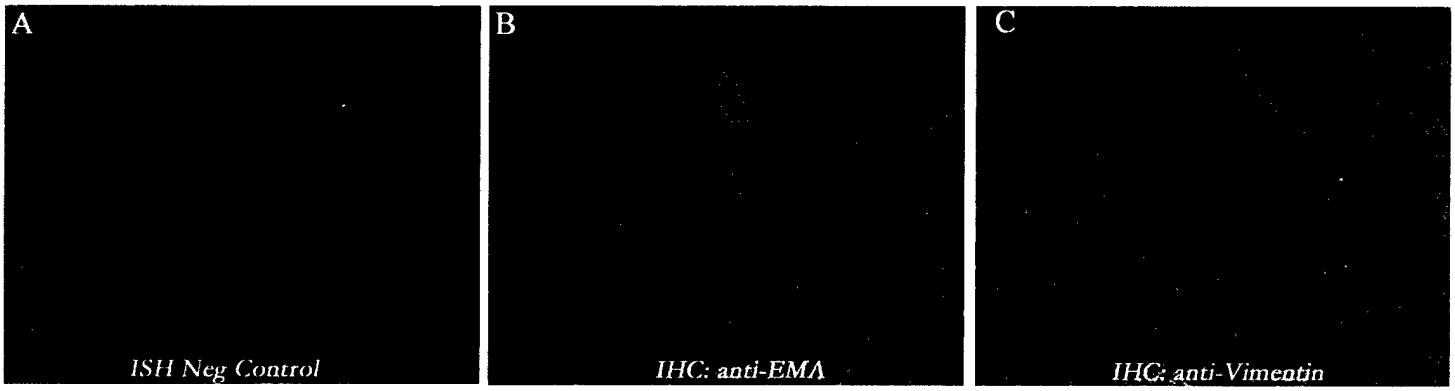
APPENDIX II

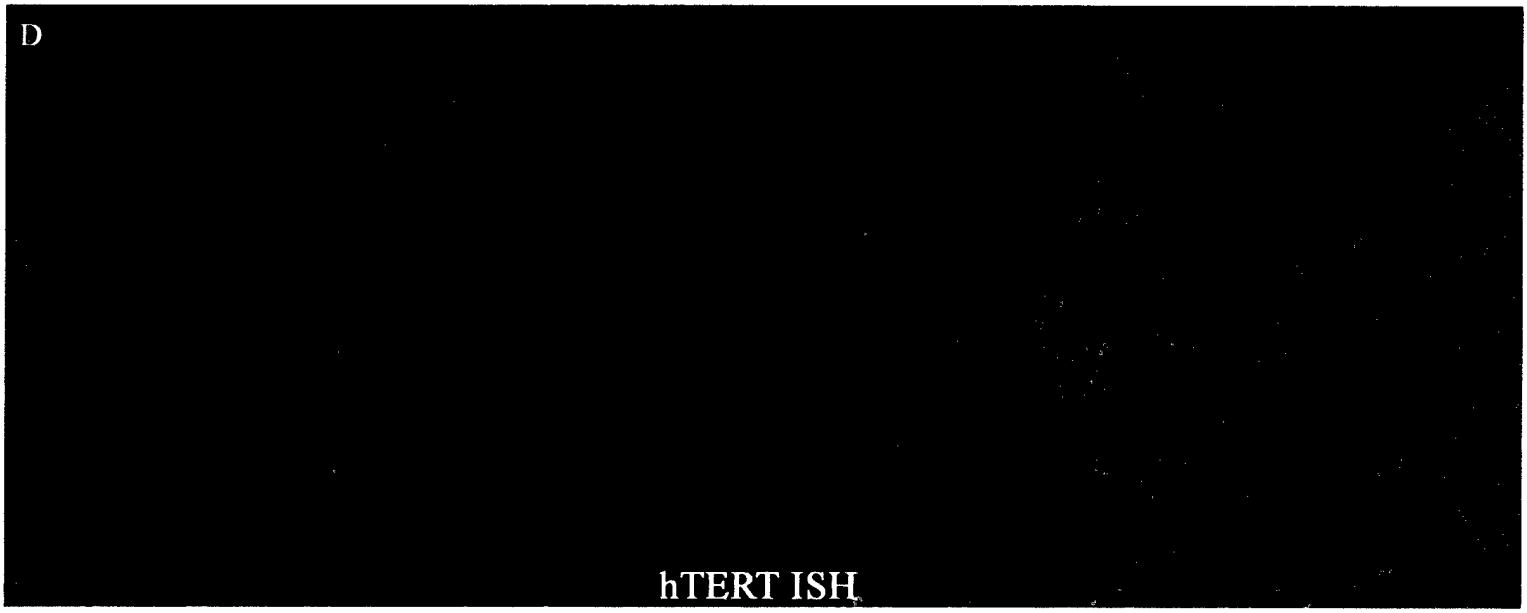
The Telomeric Binding Proteins; TRF1 and TRF2, mRNAs are Elevated in Human Breast Tumors with Reduced Telomere Content and in Tumors Displaying Genomic Instability

Kimberly Butler, William Curtis Hines, Colleen A Fordyce, Jeffrey K Griffith. Biochemistry and Molecular Biology, University of New Mexico Health Science Center, 915 Camino de Salud NE, CRTC B91, Albuquerque, NM 87131

Background: TRF1 and TRF2 are novel telomere-binding proteins that form and stabilize telomere complexes. These two proteins have been shown, in vitro, to regulate telomere length. The loss of telomeric repeats destabilizes the telomeric complex; leading to chromosomal abnormalities and gross genomic instability, a recognized facilitator of carcinogenesis. These experiments examine the associations between the levels of TRF1 and TRF2 mRNA, Telomere content, and genomic stability. Methods: TRF1 and TRF2 mRNA levels were measured by real-time RT-PCR and telomere content (TC) was measured by slot blot assay in 37 breast tumors. Results: TRF1 and TRF2 mRNA was detected in 35/37 and 37/37 tumors, respectively. The mRNA level of TRF1 and TRF2 were positively associated with each other ($p=0.042$). A linear correlation between TRF1 and TC exists ($p=0.023$). When the tumors are dichotomized by DNA content (ploidy), the aneuploid tumors display elevated levels of both TRF1 and TRF2 mRNA ($p=0.054$ and $p=0.073$). Conclusion: The association between genomic stability and tumor initiation and progression are well established. Our studies demonstrate a link between telomere binding proteins, telomere maintenance and genomic stability in human breast tumors. Supported by Army Pre-doctoral Breast Cancer Traineeship and Breast Cancer Concept Awards.

Breast Tumor *in situ* analyses





in situ detectection of telomerase reverse transcriptase (hTERT) mRNA. The above images display the identification of epithelial cells via IHC detection using an antibody directed against Epithelial Membrane Antigen (EMA), B. Identification of fibroblasts via IHC using an antibody directed against Vimentin, C. *In situ* detection of hTERT: DAPI and hTERT; D, hTERT only, E. ISH negative control, A.