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13. ABSTRACT *(Maximum 200 Words)*

Breast cancers differ from benign breast epithelium through prominent expression of oncogenic pp32 gene family members. Whereas benign breast epithelium predominantly expresses pp32, a tumor suppressor, breast cancers express pp32r1 and pp32r2, which are oncogenic. The study purpose is to confirm and extend these preliminary results, to develop practical means to assay pp32 gene family members in clinical samples, and to determine the clinical significance of their presence in pre-invasive breast disease. The approved proposal encompassed four broad tasks: [1] characterization of the pp32 expression phenotype of a larger sample of 40 breast cancers; [2] development of a practical assay for altered pp32 transcripts in archival tissue; [3] determination of the association of specific pp32 variants in laser capture microdissected DCIS with invasion and with high-grade comedo DCIS; and [4] application of the results in a retrospective study (if [3] shows meaningful correlations) to a population of patients with follow-up. During the reporting period, we recognized that microheterogeneity of pp32 gene family expression in tissue would best be assessed by an in situ hybridization method that would have better resolution than laser capture. This technically formidable task is now nearly complete and will facilitate rapid completion of the remaining aims.

14. SUBJECT TERMS

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INTRODUCTION

Our previous work demonstrated that breast cancers differ from benign breast epithelium in their expression of oncogenic members of the pp32 gene family. Whereas benign breast epithelium solely expresses pp32, a tumor suppressor, breast cancers express pp32r1 and pp32r2, which are oncogenic. The approved proposal encompassed four technical objectives: [1] characterization of the pp32 expression phenotype of a larger sample of 40 breast cancers; [2] development of a practical assay for altered pp32 transcripts in archival tissue; [3] determination of the association of specific pp32 variants in laser capture microdissected DCIS with invasion and with high-grade comedo DCIS; and [4] application of the results in a retrospective study (if [3] shows meaningful correlations) to a population of patients with follow-up. Since submission and approval of the original proposal, several technical and scientific events have occurred that have positively impacted the strategy, efficiency with which the remaining aims can be accomplished, and dramatically increased the translational potential of the technology should the study establish analysis of the pp32 gene family as a clinically important assay of biologic potential in pre-invasive breast neoplasia.

BODY

Task 1. This task involves characterization of abnormal pp32 transcripts in frozen samples of human duct carcinoma compared to paired normal breast controls. Work under this approved task will entail the analysis of 40 frozen breast cancer specimens by RT-PCR, cloning, and sequencing of 10 to 20 clones of pp32 gene family members derived from the tumors to detect variant sequences. Additional features of the task involve analysis of the frequency and position of previously uncharacterized changes.

Progress: The following recent observations have significantly impacted this work:

1. Breast cancers, and indeed all other cancers examined to date, do express pp32r1 and/or pp32r2 when examined by a specific and quantitative RT-PCR method involving amplification of 250 – 300 bp amplicons. This method also shows that pp32 continues to be expressed in cancers although for reasons that are presently inapparent, it is less efficiently detected in the proposed assay. Indeed, the quantitative relationships suggest that pp32 is significantly more highly expressed than are pp32r1 and pp32r2. This raises the possibility that the cloning method could miss additional transcripts.

2. Evidence from studies performed under Task 2 suggest that individual cells may alternatively express either pp32, pp32r1, or pp32r2. Work carried out under Task 2 is on the verge of completing a sensitive and specific oligonucleotide-based *in situ* hybridization assay for pp32,

pp32r1, and pp32r2. While certain technical issues (v.i.) remain to be resolved, results so far suggest that while a tissue may express pp32, pp32r1, and pp32, individual cells in a normal structure or a lesion may express only one of the pp32 gene family members.

Based upon the foregoing observations, after some initial work, completion of this task has been deferred pending completion of Task 2. When Task 2 is completed, the following strategy will be applied, which will efficiently identify candidates for subsequent sequence analysis: [1] tumor sections will be *in situ* hybridized with a 298 bp consensus probe that hybridizes with all known pp32 gene family members; [2] adjacent sections will be *in situ* hybridized with probes absolutely specific for pp32, pp32r1, and pp32r2; [3] breast carcinomas or laser capture microdissected areas of breast carcinomas will be selected for further molecular analysis if they display a phenotype of pp32 consensus + and are negative by each of the highly specific *in situ* analyses for pp32, pp32r1, and pp32r2. This pattern would indicate the presumptive presence of novel members of the pp32 gene family. This modified approach is expected to increase the efficiency with which additional pp32 gene family members can be identified. Work on this approved Task is expected to resume shortly pending completion of the assay.

Task 2. This task involves development of a specific molecular assay compatible with use on archival tissue. The task approved in the proposal involved dissection or laser capture microdissection of tissue, RT-PCR amplification of pp32 gene family members, development of a restriction fragment length polymorphism analysis to distinguish pp32 gene family members from one another, and assay validation.

Progress: The restriction fragment length polymorphism analysis was complicated by unequal amplification of pp32 gene family members by consensus primers. Conditions for multiplex RT-PCR amplification of pp32, pp32r1, and pp32r2 were attempted, but significant differences in template concentrations from sample to sample led to irreproducible results.

For the above reasons, the proposed assay technology was set aside in favor of *in situ* hybridization. Oligonucleotide probes were developed that demonstrate absolute specificity for either pp32, pp32r1, or pp32r2, as shown below in Figure 1.

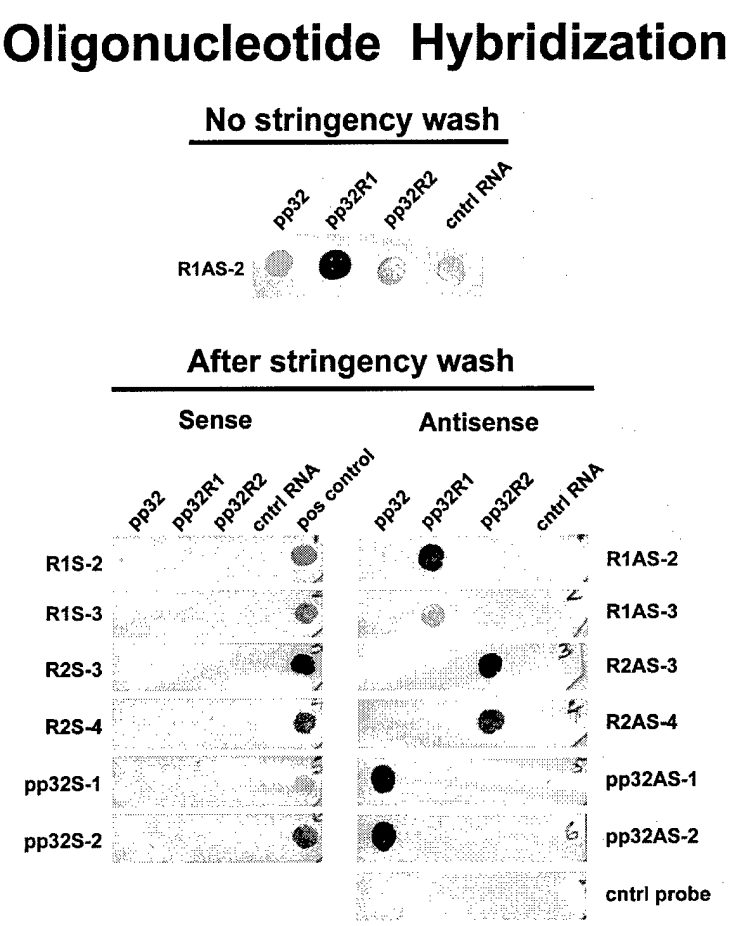

Figure 1. Oligonucleotide probe hybridization of pp32 gene family members. The figure simulates *in situ* hybridization through spotting of comparable amounts of *in vitro*-translated mRNA onto membranes that are subsequently probed with labeled oligonucleotide probes and developed with the same sample detection chemistry used to detect *in situ* hybridizations. Probes indicated on the left-hand side of the figure are sense controls that are negative as expected. The antisense probes, shown on the right, detect pp32, pp32r1, and pp32r2 with absolute specificity.

This assay is currently being applied to archival breast and breast cancer specimens with some success. Current problems are due largely to background from endogenous biotin or biotin-like substances that produce unacceptably high background. Before moving to valuable samples, it is important to guarantee the ability to obtain interpretable results through background reduction through on-slide isothermal RNA amplification as a means of optimizing the signal-to-noise characteristics.

The on-slide RNA amplification technique is in progress and promises to be successful over the next several weeks. In this method, isothermal RNA amplification prior to *in situ* hybridization is carried out in tissue sections by use of

modified specific primers such that the lower primer contains a T7 RNA polymerase promoter at its 5' end. Extension of this primer by reverse transcriptase at 41° creates an antisense cDNA copy, while the RNA in the hybrid is degraded by the RNAse H activity of the reverse transcriptase. The resultant cDNA then hybridizes with the upper primer that is extended by the reverse transcriptase to create a double-stranded cDNA with the T7 promoter at the 3' end. T7 polymerase then creates new antisense transcripts that re-initiate the isothermal amplification process. Two hours of amplification yield approximately 10^9 – fold amplification. Mild fixation in paraformaldehyde then minimizes amplicon diffusion. The amplified product is then hybridized to a labeled oligonucleotide probe.

This task is anticipated to be successfully completed in the next several weeks.

Task 3. Identification of pp32 variants preferentially expressed in DCIS with co-existing invasive duct carcinoma or in high-grade comedo DCIS. This task involves a comparison of the expression of pp32 variants in pre-invasive breast cancer using the presence of co-existing invasive cancer as a surrogate marker for increased risk. Studies will use the method developed in Task 2 to analyze tissue provided by Dr. David Page, with subsequent statistical analysis of the results for meaningful associations.

Progress: Preparations are in progress to commence this aim immediately upon the imminent completion of Task 2. The *in situ* hybridization method will create a number of advantages over the method initially proposed: [1] less tissue will be consumed; [2] the resolution of the technique is much higher, resolving differences existing between individual cells; [3] rare lesions such as atypical ductal hyperplasia can now be examined – in the original scheme, too little RNA would have been available; and [4] the method is far more suitable for translation to clinical settings.

Task 4. Determination of the significance of variant pp32 expression as a risk factor for subsequent development of invasive breast cancer (contingent upon Task 3 results).

Progress: Work on this task will commence upon completion of Task 3.

KEY RESEARCH ACCOMPLISHMENTS

A specific and sensitive *in situ* hybridization assay has been developed. This assay accomplishes the technically challenging task of distinguishing mRNA species that are ~90% identical from one another in tissue sections. This revised methodology offers a number of key advantages in the analysis of the clinical significance of pp32 gene family expression in pre-invasive breast neoplasia.

REPORTABLE OUTCOMES

Kadkol SS, Abou El Naga G, Brody JR, Bai J, Gusev Y, Dooley WC, and Pasternack, GR. Expression of pp32 gene family members in breast cancer. Breast Cancer Research and Treatment. *In Press*, 2001

CONCLUSIONS

New technology has been developed during the first reporting period that will greatly increase the potential impact of the studies to be carried during the remaining funding periods.

REFERENCES

N/A

APPENDICES

N/A