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BRCA2 Expression as a Predictor of Response to Radiation Therapy

PRINCIPAL INVESTIGATOR:
Roy A. Jensen, M.D., Principle Investigator

CONTRACTING ORGANIZATION:
Vanderbilt University Medical Center
Nashville, Tennessee 37232-5310

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14. ABSTRACT Women with germline mutations in BRCA2 have a high likelihood of developing breast cancer. The loss of BRCA2 heterozygosity, observed in 20-67% of sporadic cases, suggests a role of BRCA2 in these tumors. We suggest that, in some cases of sporadic breast cancer, the level of BRCA2 expression might be an important determinant of the malignant phenotype. Therefore, the major objective of this proposal was to test the hypothesis that BRCA2 expression is decreased in a significant subset of sporadic breast cancers and that these tumors have a molecular pathogenesis related to that of hereditary breast cancers containing mutations in BRCA2. To test this hypothesis we proposed the following specific aims: 1) To develop assays for BRCA2 expression in fresh and archival tissue from sporadic breast cancers, To determine whether BRCA2 expression is altered in sporadic breast cancer, 3) To determine whether BRCA2 expression in tumors correlates with response to radiation and selected chemotherapeutic agents. Unfortunately, we were unable to develop BRCA2 antibodies of sufficient sensitivity and specificity to carry out the proposed research. To circumvent this problem we are attempting to measure BRCA2 levels via mass spectroscopy.					
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Table of Contents

Introduction.....	5
Body.....	5
Key Research Accomplishments.....	10
Reportable Outcomes.....	11
Conclusions.....	11
References.....	11
Appendices.....	NA

Introduction

Individuals who carry mutations in BRCA2, one of the genes involved in a familial syndrome of inherited breast cancer, have a very high likelihood of developing breast cancer. BRCA2 appears to be involved in repairing DNA mutated by radiation or DNA-damaging chemicals. If a cell carries a defective copy of BRCA2 (i.e., has inherited a mutant copy of BRCA2) and loses its normal copy, it will be unable to repair its DNA and over time will accumulate enough mutations to cause the development of a cancer.

Could mutations in BRCA2 help explain sporadic (non-inherited) cases of breast cancer as well? The initial studies would suggest not, because the vast majority of sporadic breast cancers do not contain BRCA2 mutations. However, these studies looked only for mutations in BRCA2. But what if there just isn't enough BRCA2 in a cell to do its job? This can happen even with a normal gene if the gene has been "turned off." In sporadic tumors, instead of a mutant BRCA2 causing the problem, tumor cells might not make enough of the normal protein to repair damage to DNA. There is some evidence to suggest that this may be true, but a direct test of this idea has not been done.

Body

Task 1: To develop assays for BRCA2 expression in fresh and archival tissue from breast cancers.

a. Construct BRCA2 probes for RNase protection assay. Probes were prepared for the N-terminus, C-terminus, and Exon 11 portions of BRCA2 to enable measurement of full length BRCA2 along with splice variants in RNase protection assays. In retrospect it is clear that a more accurate way to perform these measurements would be by quantitative PCR by light cycler methods or similar technology. These methods require significantly less RNA and would enable a significantly larger study to be performed that would be capable of achieving statistically significant results.

b. Microdissect breast tissues and prepare RNA. Invasive breast cancer cases were microdissected by two different methods. The first method was the standard Arcturus Laser Capture Microdissection method that utilizes focused laser energy to melt specially prepared plastic membranes over microscopically localized tumor

cells. The second method used the P.A.L.M. laser microdissector to laser ablate unwanted tissue surrounding the tumor cells and then catapult the tumor tissue into special caps for molecular analysis. This was done by heating the space beneath the tissue rapidly enough to produce a shock wave powerful enough to lift the tissue into the cap. Each of these methods was found to produce adequate material for molecular analysis and essentially our studies found that the quality and quantity of material harvested was equivalent.

c. Prepare and test polyclonal BRCA2 rabbit antibodies. The major downfall of this study was our inability to develop polyclonal antibody reagents that were sensitive and specific enough to measure BRCA2 protein in formalin fixed paraffin embedded tissue. We were not alone in our failure. Over the last ten years there has not been a single publication in the biomedical literature that describes an antibody with the characteristics necessary to carry out the study that we proposed. This situation is not unusual. For example, the analysis of estrogen receptor alpha was carried out via biochemical methods for over fifteen years until antibodies against estrogen receptor were developed that were capable of detecting estrogen receptor in formalin fixed paraffin embedded tissue. A major issue with BRCA2 is that an antigenicity plot of the protein reveals no region that is strongly or even moderately antigenic in terms of its ability to elicit an antibody response. This is a very difficult hill to climb especially if the intent is to utilize the reagent in formalin fixed paraffin embedded material. A number of antibodies have been developed that that are suitable for Western blotting, but these do not work in immunohistochemistry.

An additional issue was that we were not able to identify a breast cancer sample that had cleared arisen in a patient with a germline BRCA2 mutation that would result in a truncated protein to be a suitable negative control. Ultimately, the combination of a failure to develop robust BRCA2 antibody reagents and the lack of an appropriate negative control would prove to be fatal flaws in the grant.

d. Prepare and test single chain BRCA2 antibodies.

At the time this grant was prepared an exciting and emerging new technology was the production of single chain antibodies via the screening of phagemid libraries. The advantage of this technology is that there is potentially unlimited diversity in the phagemid library and the screening methods are reasonably straightforward. In addition, Dr. Ray Mernaugh, a widely acknowledged expert in this field had just been recruited to Vanderbilt from industry to establish a shared resource facility to develop such reagents. Unfortunately as this technology has evolved it has become apparent that the major utility of the antibodies developed by this technique was for ELISA assays which do not have as stringent a requirement for high affinity binding antibodies. Because by definition these antibodies exist only as single chains they often are a log-fold lower in affinity. As a result, the ability to utilize single chain phagemid antibodies in immunohistochemistry assays is very limited. The single chain antibodies that we developed were not of high enough affinity to demonstrate any promising results in formalin fixed paraffin embedded tissues or in Western blotting.

Task 2: To determine whether BRCA2 expression is altered in sporadic breast cancer.

a. Perform RNase protection assays for BRCA2 in breast cancer samples.

Our rate of accrual for frozen paired tumor and normal breast cancer specimens that would be necessary to carry out an analysis of BRCA2 expression and response to radiotherapy was not adequate to meet statistical significance as outlined in aim 3 of the grant. When this became apparent it was obvious that the best that we could hope for in analyzing the frozen material was a sufficient number of cases that we could show a trend in this regard. As a result, we focused our efforts on developing an analysis of the formalin fixed archival material. When it became apparent that we were not having success in identifying suitable BRCA2 antibody reagents, we decided to try and by-pass this roadblock by developing mass spectroscopy methods for analysis of BRCA2 using the frozen tissues. So in essence we made a strategic decision to utilize the tissues collected for this aim in an attempt to circumvent our BRCA2 antibody reagent deficiency.

b. Identify and pull archival tissue specimens.
We reviewed all breast cancer cases in the surgical pathology archives of Vanderbilt University Hospital from 1983 to 1993 and randomly selected 221 examples of invasive breast cancer that had at least two formalin fixed paraffin-embedded blocks that contained tumor tissue and for which we had adequate follow up information. Patient age, race, sex, date of diagnosis, last date of follow up, status, surgery type, treatment with chemotherapy and type, treatment with radiation, tumor type, tumor size, ER/PR status, histologic grade, p53 mutation status, lymph node status, and family history of breast cancer were recorded. BRCA1 and BRCA2 mutation status was unknown. One block from each case was archived along with a copy of their surgical pathology report.

c. Perform immunohistochemistry for BRCA2 expression.
We were never able to develop or identify BRCA2 antibodies that were of sufficient sensitivity and specificity to enable us to carry out this task.

d. Analyze data to determine correlates of BRCA2 expression.
Obviously, because this aim is completely dependent upon our ability to perform Task 2c, there is nothing to analyze.

Task 3: To determine whether BRCA2 expression in individual sporadic breast cancer correlates with tumor response to radiation therapy.

a. Pull medical records and obtain outcome information regarding radiotherapy.
As described above in Task 2b we have obtained this information for each patient in regards their utilization of radiotherapy and their outcome. Analysis will await development of an antibody that can assess expression of BRCA2 in formalin fixed paraffin embedded tissues.

Key Research Accomplishments

-Laser capture microdissection (LCM) and isolation of cells from frozen normal and cancerous breast tissues.

-Established conditions for mass spectroscopic assay of cells attached to LCM membranes.

-Successfully established unique cohort of 221 breast biopsy patients with follow up for specific aims two and three.

Reportable Outcomes

-Preliminary data utilized to develop project for the funded Vanderbilt-Meharry Specialized Program of Research Excellence in Breast Cancer.

-Abstract presentation at the DOD Era of Hope Breast Cancer Meeting Sept 2002.

Conclusions

This research project was not successful because we were unable to develop antibody reagents that had sufficient sensitivity and specificity to detect BRCA2 protein in an immunohistochemical assay.

References

Xu BJ, Sanders ME, Jensen RA, and Caprioli RM: Direct Analysis of Laser Capture Microdissected Cells by MALDI Mass Spectrometry. Journal of the American Society of Mass Spectrometry, 13: 1292-1297, 2002.

Appendices

None