

National Surgical Adjuvant Breast and Bowel Project (NSABP) Foundation

Annual Progress Report: 2006 Formula Grant

Reporting Period

July 1, 2009 – June 30, 2010

Formula Grant Overview

The National Surgical Adjuvant Breast and Bowel Project (NSABP) Foundation, Inc. received \$1,286,019 in formula funds for the grant award period January 1, 2007 through December 31, 2010. Accomplishments for the reporting period are described below.

Research Project 1: Project Title and Purpose

Prediction of Response to Bevacizumab in Colon Cancer - To get oxygen and nutrition, tumor cells need blood vessels in the tumor to grow together. This process is called angiogenesis. Understanding of the molecular steps involved in angiogenesis has led to the development of an antibody drug, bevacizumab, that blocks angiogenesis. The NSABP Cooperative Group has conducted a large clinical trial with bevacizumab in colon cancer. The results should be available in the next 2 to 3 years, and if they are positive, bevacizumab will be widely used. However, the drug is not expected to benefit every patient. It is also very expensive, costing about \$100,000 per patient, and has significant side effects. Therefore, it is critical to develop a diagnostic test that can be used to predict which patients will gain the most benefit from bevacizumab and to spare those who do not need it from unnecessary, costly, and toxic therapy.

Anticipated Duration of Project

1/1/2007 - 12/31/2010

Project Overview

The aim of this project is to develop clinical tests that can be used to decide which patients with colon cancer should be treated with bevacizumab. Bevacizumab is a humanized antibody that blocks angiogenesis by binding to VEGF. In a study, AVF2107g, of 815 patients with advanced and untreated colorectal cancer, adding bevacizumab to chemotherapy resulted in a significant increase in response rate (44.9 vs. 34.7%), response duration (10.4 vs. 7.1 months), progression-free survival (10.6 vs. 6.2 months) and overall survival (20.3 vs. 15.6 months). The results of this trial led the U.S. FDA to approve the use of bevacizumab in combination with a 5-FU-based regimen for first-line treatment of patients with advanced colorectal cancer in February 2004. On the basis of the compelling evidence for clinical benefit in patients with advanced colorectal cancer, the NSABP C-08 trial was designed to study bevacizumab's utility for patients with colon cancer treated after surgery, with a goal of improving disease-free and overall survival.

More than 2700 patients were randomized by October 2006; the results of this trial are expected to be available within the next 2 to 3 years. Based on the strength of the effect observed in the advanced disease trial, it is expected that the NSABP C-08 trial will show the efficacy of bevacizumab for improving the clinical outcome of patients who have stage 3 colon cancer. However, bevacizumab is not without significant toxicity. In trial AVF2107g, the addition of bevacizumab to chemotherapy resulted in a significant increase in grade 3/4 hypertension (10.9 vs. 2.3%), grade 3/4 neutropenia (31 vs. 37%), diarrhea (25 vs. 33%), and vomiting (10.6 vs. 7.7%). Also, the cost of adding bevacizumab to chemotherapy is approximately \$100,000 per patient, so criticism regarding the societal costs involved in using this drug can be expected, even if the drug shows promising results. Ideally, treatment decisions should be based on assessment of both prognosis (base-line risk of recurrence when only chemotherapy is used) and prediction of benefit (expected degree of benefit from adding bevacizumab). The response rate of 44.9% in the advanced disease study suggests that not all patients will benefit from bevacizumab. Some patients might already have excellent prognoses based on treatment with chemotherapy only. Developing a prognostic and predictive assay for bevacizumab is critically needed. To achieve this, formalin-fixed, paraffin-embedded tumor tissue blocks from patients enrolled in C-08 will be used to profile expression levels of the whole genome using a proprietary method developed at the NSABP Foundation Division of Pathology.

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Expected Research Outcomes and Benefits

Cancer is the second leading cause of death in Pennsylvania, and colorectal cancer is the third most common cancer in women and men, accounting for 12% of all cancers. Each day colorectal cancer is expected to be diagnosed in 22 Pennsylvanians and 8 of them will die. Although there has been tremendous development of so-called molecular targeted therapies, such as those targeting blood vessel growth in tumors (thereby starving cancer cells), these therapies can be very expensive and have significant side effects. For example, one such drug, bevacizumab, is extremely costly and is known to cause high blood pressure and other side effects. Bevacizumab is expected to be very effective, but its use on all colon cancer patients will put economic pressure on the healthcare system. Clinical studies show that, among patients, the behaviors and responses of colon cancers are very different, and that not all patients benefit from bevacizumab. However, there is not a predictive test available to identify patients who will benefit from bevacizumab and patients who will not. Gene expression profiling has been used successfully to identify a set of genes that can help predict cancer recurrence or death for patients who have

colon cancer. Use of gene expression profiling may also lead to finding a set of genes that can be used to predict the degree of benefit from bevacizumab therapy. Gene expression profiling used to require specially processed, freshly frozen tumor tissue. Recently, the NSABP Foundation Division of Pathology developed a new method using routinely processed (formalin-fixed, paraffin-embedded) diagnostic tumor tissue for gene expression profiling. The goal of this project is to use the gene expression profiling method on routinely processed tumor tissues collected from a large clinical trial that tested bevacizumab in colon cancer and to develop a predictive clinical test for the use of bevacizumab for colon cancer. Such a predictive test will improve use and access to this important drug while saving healthcare costs by selecting those patients who will benefit from bevacizumab and sparing others from receiving unnecessary, toxic therapy.

Summary of Research Completed

As reported last year bevacizumab did not add statistically significant improvement over standard adjuvant chemotherapy in the treatment of stages II and III colon cancer. This is disappointing, but there was a clear temporary benefit from bevacizumab during the first year of follow up. The fact that there was no statistically significant benefit from bevacizumab actually makes this study even more interesting and clinically meaningful since we may be able to identify a subset of patients who benefit from bevacizumab.

Selection of Candidate Genes for Prediction of Bevacizumab Benefit We have used the whole genome expression data that was generated in 2009 by profiling 468 available cases from C-08 using Agilent arrays and a customized methodology that we developed for profiling degraded RNAs isolated from formalin-fixed, paraffin-embedded tissues. To identify genes that were predictive of bevacizumab benefit, Cox models were used to compute the interaction p-values for each of the 41,000 probes on the Agilent array. Fifty genes with an interaction p values less than 0.001 were included as candidate genes for further examination with a platform more appropriate for clinical testing.

We concluded that whole genome expression analysis with Agilent arrays is too expensive, too labor intensive, and too complex to be used as a clinical test. Therefore, in order to validate genes of interest for the prediction of benefit from adjuvant therapies, we sought a more suitable technology. The nCounter® system from NanoString Technologies (Seattle, WA) provided an ideal technology to validate candidate genes that were identified on the Agilent arrays: We have used the nCounter system in other projects and found it to be reproducible and to yield biologically meaningful results.

To understand the nature of the work: The nCounter analysis system is a new and simple technology that profiles gene expression of as many as 500 genes in one experiment. It utilizes a novel digital technology that is based on direct multiplexed measurement of gene expression and offers high levels of precision and sensitivity (<1 copy per cell). The technology uses molecular "barcodes" and single molecule imaging to detect and count hundreds of unique transcripts in a single reaction (Figure 1). Each color-coded barcode is attached to a single target-specific probe corresponding to a gene of interest, and mixed together with controls, they form a multiplexed code set. NanoString's technology employs two probes, a reporter probe and a capture probe, of

approximately 50 nucleotides per mRNA which hybridize in solution. The reporter probe carries the signal; the capture probe allows the complex to be immobilized for data collection. Sample cartridges are placed in the Digital Analyzer for data collection. Color codes on the surface of the cartridge are counted and tabulated for each target molecule.

To validate the NanoString platform for our work, we are repeating the gene-expression profiles of an expanded discovery cohort using a custom, NanoString Colon CodeSet. This code set includes 50 genes that had shown bevacizumab interaction p values below 0.01 based on the Agilent data. Currently, we have completed profiling the gene-expression profiles with the NanoString CodeSet on approximately 1500 cases from NSABP C-08. These cases include both the discovery and validation cohorts.

Detailed Methods

We have improved our method of RNA extraction this year by automating the extraction process. RNAs from approximately 1600 additional C-08 cases have been prepared by using lysates prepared from 3 slides at a section width of 5 μm . Lysates were de-paraffinized and digested with protease and then loaded into a 96-well plate with the Tecan EVO robot, and processed with the KingFisher Flex 96 instrument (ThermoFisher; Burlington, ON) utilizing reagents provided by the E.Z.N.A.® FFPE RNA Isolation Kit from Omega Bio-Tek (Norcross, GA). RNAs were quantified with fluorescence, using the Quant-iT™ RiboGreen® Assay Kit (Invitrogen; Carlsbad, CA) and the Infinite® 200 fluorometer (Tecan; Männedorf, Switzerland).

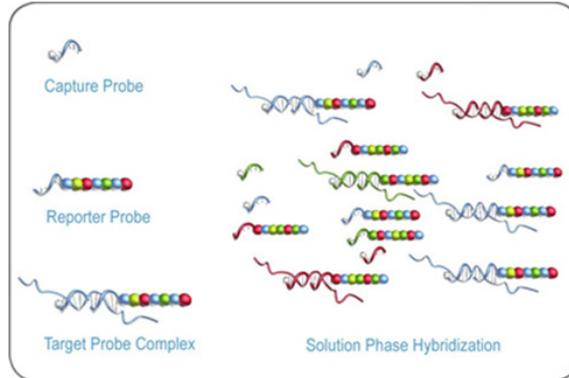
The Tecan EVO robot was used to combine the NanoString capture and reporter probes and the RNA (200 to 400 ng). Samples were hybridized for 16 to 24 hours at 65°C. Samples were loaded into a prep station where excess probes were removed and complexes were immobilized on the surface of a cartridge, which contains wells for 12 samples. Cartridges are then loaded into the scanner.

Milestones Accomplished

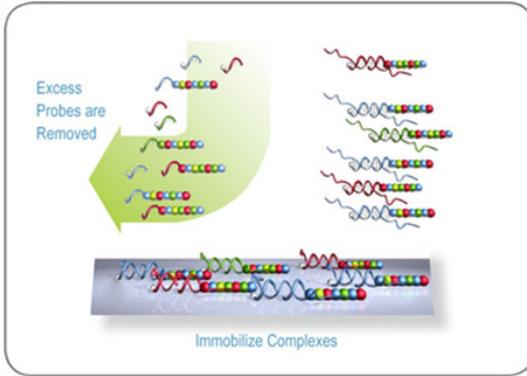
1. Identified 50 candidate bevacizumab-predictive genes and 50 housekeeping genes.
2. Identified, purchased, and set up the nCounter system from NanoString as a cost-effective and simple technology to be used for platform validation and for algorithm validation.
3. Completed gene-expression profiling with the custom NanoString Colon CodeSet on 1500 cases, which include about 900 cases of the 1000 samples that make up the expanded discovery cohort.

Figure 1

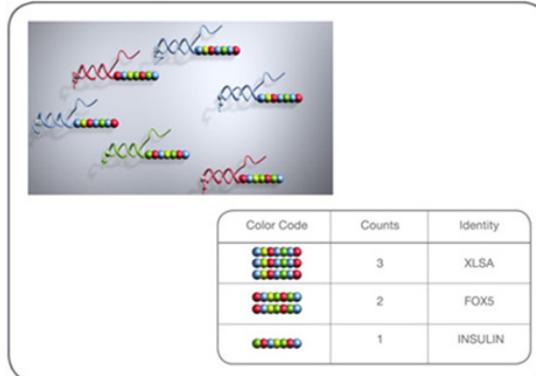
1. Mix RNA with capture and reporter probes . Samples are hybridized for 16 to 24 hours.



2. Load hybridized samples into prep station



3. Load samples into scanner and collect data



Pictures taken from Nanostring web site <http://www.nanostring.com/applications/technology/>