National Surgical Adjuvant Breast and Bowel Project (NSABP) Foundation

Annual Progress Report: 2006 Formula Grant

Reporting Period

July 1, 2010- Dec 31, 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.

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

Selection of Candidate Genes for Prediction of Bevacizumab Benefit

We reported previously that we had completed the gene expression platform profiling of 1500 cases on the NanoString platform. We have now completed the gene expression profiling for nearly all the cases for which we collected samples and had consent for use in clinical trail C-08. The entire data set for the discovery cohort and model building included 954 cases, and the validation cohort consisted of 921 cases. NanoString code sets included positive controls, which were included in all NanoString code sets. Data were normalized with a technical normalization factor, which was the (median (sum of spikes) / sum spikes), and a biological normalization factor, which was the (median (geometric mean of 5 housekeeping genes)) / geometric mean (geometric mean of 5 housekeeping genes). The housekeeping genes were MADD, NFYC, PDCD10, USP10, and RAB1A. These housekeeping genes were identified by two software programs, NormFinder and geNorm, using our colon data. These 5 genes were consistently ranked as the most promising normalization genes with both of these programs. Once the data were normalized, they were sent to the NSABP Biostatistical Center for model building and validation.

A predictive model for the benefit of bevacizumab was built with the NanoString data in the discovery cohort utilizing 9 genes. However, this model did not appear to be promising based on the fact that only 2 of the genes out of the 9 were seen as predictive in the original Agilent data (Table 1). Table 1 shows the p values for the 9 genes that were found to be predictive for bevacizumab benefit in the NanoString data. We compared the correlations from the 2 platforms for several of the genes in the code set and found them to be quite low (data not shown). On the basis of this lack of correlation between the Agilent data and NanoString data, we anticipated that the bevacizumab prediction model would not validate in the C-08 validation cohort, and it did not. Therefore, we have taken several steps to improve the selection of prognostic and predictive genes from whole-genome expression profiling data and are in the process of carrying out these steps.

A prognostic model was also built on the NanoString data. This model performed with statistical significance better than a model with clinical and pathologic covariates alone in the training set, but the magnitude of improvement over the model with covariates alone was not impressive, and, therefore, we did not pursue this model in the validation cohort. We think that a better

prognostic model would be built by applying the strategies described below to improve prognostic gene selection.

Improve identification of prognostic and bevacizumab (Bev)-predictive genes from whole-transcriptome expression profiling of C-08 cases in a discovery cohort

We took several steps to improve the selection of prognostic and predictive genes. These steps are detailed below.

1. Improve the whole-genome expression data

The lack of correlation between the Agilent whole-transcriptome expression data and the NanoString data may have indicated that the Agilent data were insufficient or inaccurate. To improve the selection of prognostic and predictive genes for C-08, we repeated expression profiling with the whole-genome DASL platform from Illumina, Inc. on more than 500 cases of C-08 within the expanded discovery cohort. It is, of course, unclear whether the DASL data will be on their own any better than the Agilent data but by comparing the data from the 2 platforms, we will be able to identify genes that are well correlated between the 2 platforms. A gene that shows good correlation between these 2 platforms will provide a means of prioritizing prognostic and predictive genes. Furthermore, a second whole-genome expression array data set, which included a greater number of cases (N=574), may provide a better data set for the selection of predictive genes, as these genes are more difficult to identify than prognostic genes. Evaluation of these data is in progress.

2. Minimize potential differences between genes selected on the whole-transcriptome profiling platform and the NanoString probes

Another possible scenario that could have affected the low correlation between the Agilent and NanoString probes could be differences in the region of the gene that the probes were interrogating. Thus, we designed a second code set (NanoString Colon Code set II) in which the NanoString probe design was based on the Agilent or Illumina *probe* names, rather than the gene names. In the NanoString Colon Code sets I and II, prognostic genes were identified with whole-transcriptome DASL arrays and predictive genes were identified with Agilent data. Agilent or Illumina probe names were submitted as identifiers for the development of the NanoString Colon Code set II. We found that the correlation coefficients between NanoString and DASL platforms were higher for the NanoString code set II that was designed on the basis of the probe names, shown in Figure 1 in green, rather than the gene names shown with the hatched marks. Therefore, we will use probe names as the identifiers for the NanoString probe design for future NanoString code sets.

3. Test NanoString data to ensure accuracy and reproducibility

Although the nCounter assays and the NanoString platform appeared to be an optimal platform for the development of a clinical test, this technology is relatively new and has not yet been used as a clinical test. In contrast, TaqMan® assays have already been used as a clinical test and are considered the gold standard for gene expression in the scientific community. Therefore, to

make sure that NanoString is as accurate as TaqMan, we designed TaqMan assays for most of the genes that were contained in NanoString code sets I and II.

To determine the accuracy of the TaqMan and NanoString platforms, we profiled gene expression utilizing the custom TaqMan microfluidics cards and NanoString Colon Code set II. The samples for this experiment included universal human RNA (UHR) and human brain RNA. These samples were profiled alone and in various ratios. In Figure 2, the top panel shows a test of accuracy for the 2 platforms. The samples were mixed at a ratio of 75% UHR and 25% brain. Based on the results obtained for these samples when they were profiled alone, a predicted value for the gene expression of each gene was calculated. These predicted values were compared to the observed value. Both platforms performed well, with NanoString giving a slightly higher R^2 value of 0.967 compared to the value for TaqMan which was 0.931. Further indication that the NanoString platform performs as well or better than the TaqMan assays is the distribution of the % error shown in the middle panel in the Figure 2. The bottom panel shows that low expressing genes do no correlate well between the platforms—which is as expected for genes with low expression.

4. Can genes selected for prognostic or predictive value with whole-genome expression platforms (Agilent and Illumina) be used on the NanoString platform?

An essential feature of the development of our clinical test assumes that genes can be selected from data that is generated on a whole-transcriptome platform and then moved to another smaller, less complicated, gene expression platform. We used NanoString Colon Code set II and a TaqMan code set with the same genes and compared results from the same 96 samples across all the platforms of interest. In Figure 3 is a cross-platform comparison of 400 genes in the same 96 samples. The greatest correlations are seen between TaqMan (TM) and NanoString (NS). While many of the genes in this plot show poor correlations between the platforms, there are still many genes that are well correlated between Agilent and NanoString and between Illumina and NanoString. In fact, it appears that these correlations between the whole-genome platforms and the NanoString (green and light blue) platform are slightly better than those seen between the whole-transcriptome platforms and TaqMan (red and blue). This observation provides additional evidence that the NanoString platform is a better choice for the development of a clinical test. Taken together these data suggest that genes can be selected with whole-genome expression arrays and then moved to the NanoString platform.

5. Improve the analysis of prognostic and predictive genes for C-08

When the first analyses were done for the identification of Bev-predictive genes, we were overly optimistic and did not take sufficient factors into consideration. We have recently hired a Biostatistician, who is working closely with both the NSABP Division of Pathology laboratory and the NSABP Biostatistical Center to perform these analyses. We have written a detailed description of the analyses to be performed which has been reviewed by both biostatistics and pathology laboratory investigators.

6. Improve the selection of prognostic and predictive genes

We have shown that genes with a high dynamic range and higher intensity values show better correlation between Agilent and Illumina arrays (Figure 4). In Figure 4, the graph on the left demonstrates that the fraction of genes with higher between-platform correlations increases as a median cut off for the data is increased. In other words, as the low-level expressing genes are filtered out of the data set, the correlation between the platforms increases. The graph on the right demonstrates that as the data are filtered based on an increasing level of the standard deviation that there is an increase in the frequency of genes that show good between-platform correlations. A filter requiring a high dynamic range (standard deviation>1) shows a vast improvement in the correlations but eliminates nearly all the genes. These data demonstrate that the expression data should be filtered for genes with higher dynamic ranges and higher intensity values before the selection of prognostic and predictive genes. However, reasonable cut offs for these values must be determined empirically so that genes of interest can be identified.

Summary. In the last 6 months of this project, we have completed gene expression profiling on more than 1800 cases from NSABP C-08 using the NanoString platform. In addition, we have demonstrated that identification of genes by probe name/sequence is better than identification by gene name when building a NanoString code set. We have compared NanoString and TaqMan data from the same 96 samples with 400 genes that were shown to be prognostic or predictive for bevacizumab (Bev) or oxaliplatin benefit in Agilent or whole-genome DASL arrays. This comparison suggests that the NanoString platform will provide a good platform for the development of a clinical test. While the genes that were selected for Bev prediction failed to be validated on the NanoString platform, we have identified what needs to be done to improve the selection of prognostic and predictive genes. We did, however, identify Bev-predictive genes within the Agilent data. In conclusion, we have successfully completed the first step in the development of gene-expression signatures that can be used to predict patients who may respond to bevacizumab and/or to identify high- and low-risk colon cancer patients.

Table 1. NanoString Predictive Genes and p-Values in Agilent Data

NanoString Bev Predictive Genes	p values for Interaction in Agilent
BHLHB3	0.003703936
C14orf129	0.265004614
COL19A1	0.719067219
GPR110	0.358286293
HTR3D	0.000220874
KCNK10	0.237973666
RHOQ	0.111223153
SOX4	0.323176978
ZNF718	0.324062379

Figure 1. Correlation Coefficients Between NanoString Code Sets I and II and DASL

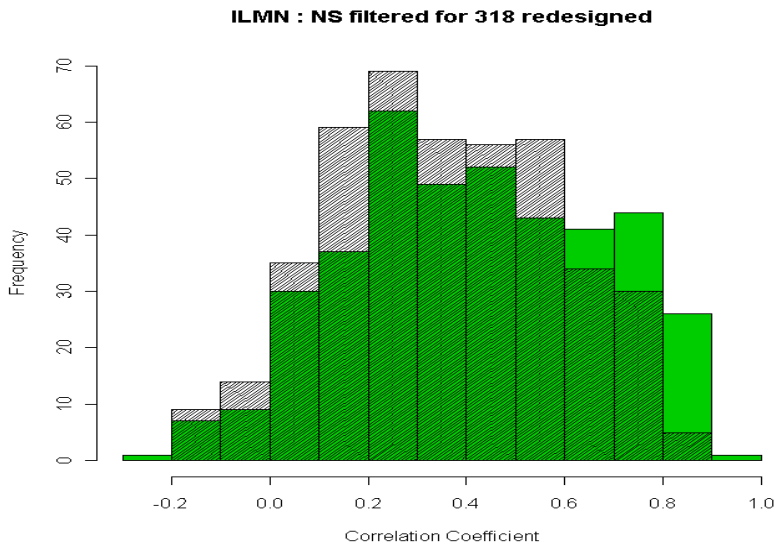

Figure 2. Comparison of NanoString and TaqMan Data

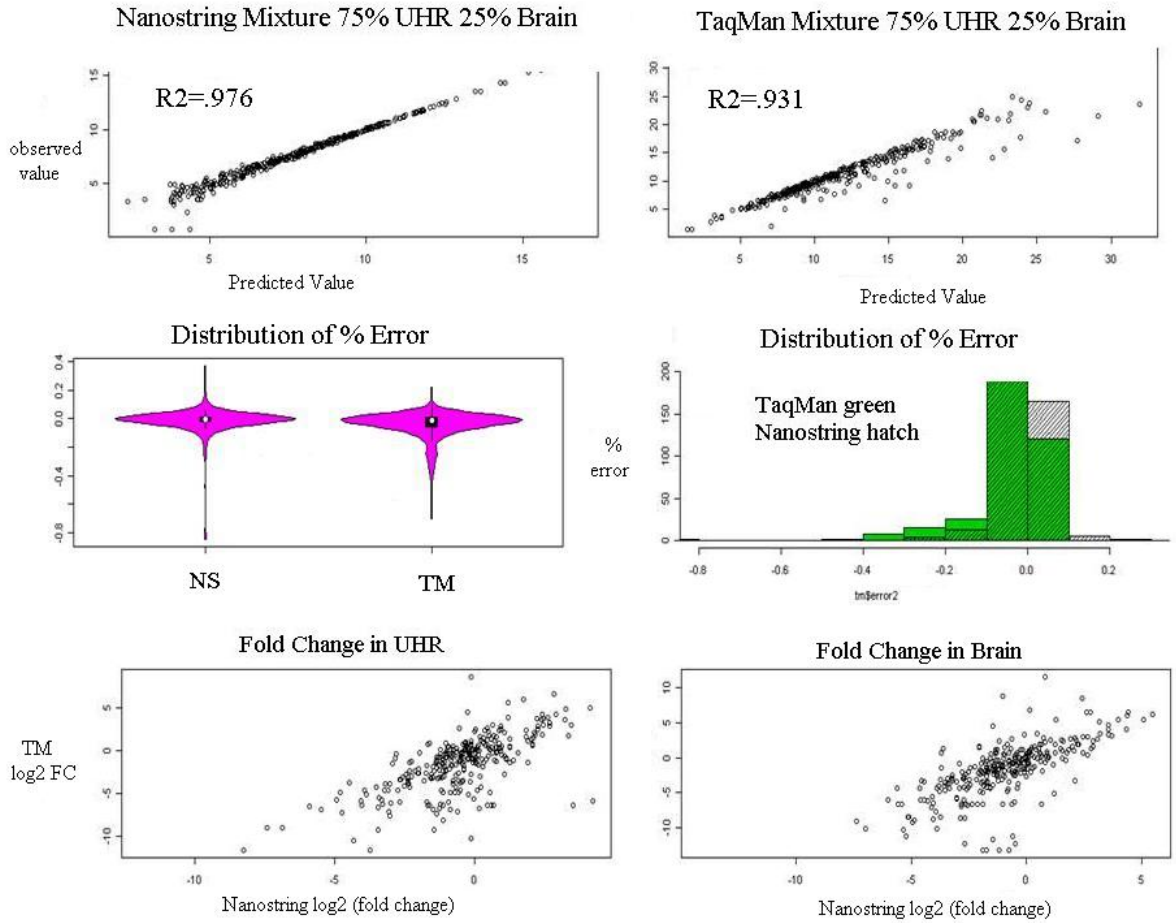

Figure 3. Agilent and Illumina versus TaqMan and NanoString

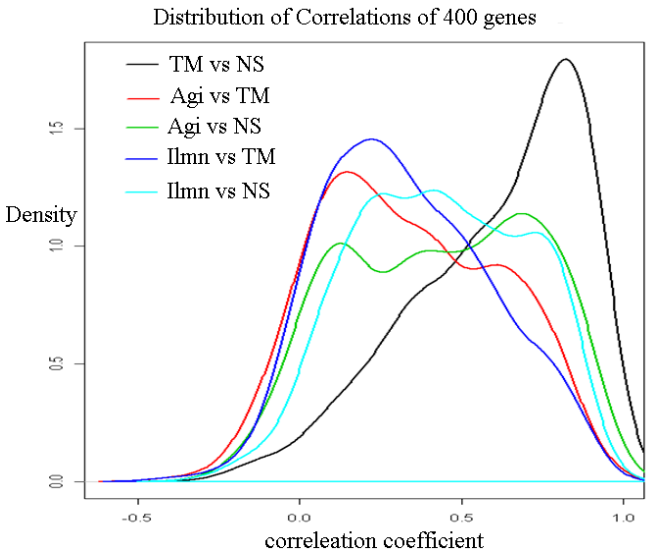
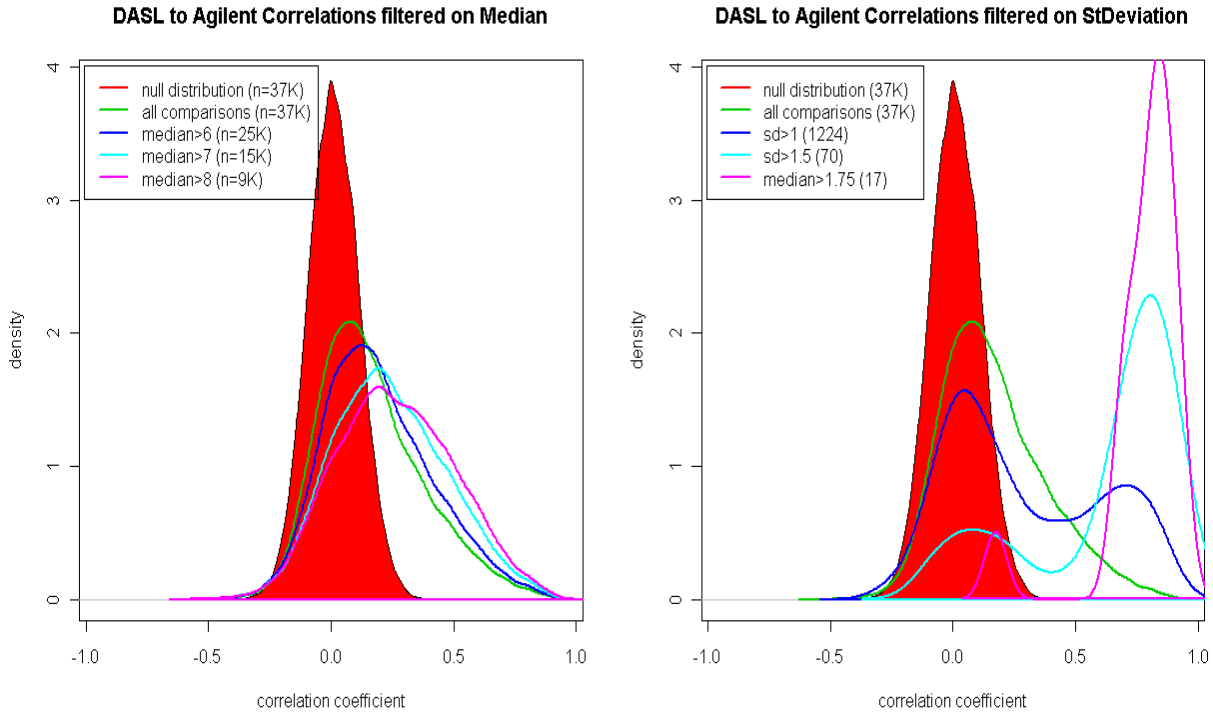
Figure 4. Data Filtering Improves Correlations Between Platforms