National Surgical Adjuvant Breast and Bowel Project (NSABP) Foundation

Annual Progress Report: 2009 Formula Grant

Reporting Period

July 1, 2012 – June 30, 2013

Formula Grant Overview

The National Surgical Adjuvant Breast and Bowel Project (NSABP) Foundation, Inc. received $1,255,581 in formula funds for the grant award period January 1, 2010 through December 31, 2012. Accomplishments for the reporting period are described below.

Research Project 1: Project Title and Purpose

*Discovery and Validation of MicroRNAs as Biomarkers in Breast and Colon Cancer* - Messenger RNA (mRNA) expression profiling has yielded useful prognostic tests for breast cancer (e.g., Oncotype DX® and MammaPrint®), but has not been very useful in identifying definitive treatment targets. This is because mRNA expression profiling does not necessarily provide insight about which changes may be susceptible to therapies. For colon cancer, no clinically useful gene-expression-based prognostic tests exist yet.

MicroRNAs (miRNAs) are a subset of RNAs which have regulatory functions and, so, could be viewed as master switches for many coordinately regulated genes. This project’s purpose is to identify miRNAs that are prognostic or predictive of benefit from systemic therapies for breast and colon cancers, using archived tumor tissues from completed NSABP clinical trials. Identified markers may point us to new treatment strategies.

Anticipated Duration of Project

1/1/2010 - 12/31/2013

Project Overview

The long-term goal of this research project is to develop biomarkers that will improve the treatment of colon and breast cancers. To achieve this goal, we propose to examine the expression of microRNAs (miRNAs), which are small, non-coding RNAs that control translation of many mRNAs. miRNA expression analysis may not only allow for the identification of better biomarkers but may provide a greater understanding of current expression data.

One of the fundamental problems associated with the treatment of breast and colon cancers is clinical heterogeneity. Gene-expression mRNA signatures that distinguish several subtypes of
breast cancer have been identified, and, while no specific signatures currently exist for colon cancer, the molecular and clinical heterogeneity of colon tumors is known. The subtyping of breast cancers has vastly improved breast cancer treatment because different subtypes respond differently to different breast cancer therapies. Current molecular signatures that distinguish these subtypes of breast cancer involve the profiling of a large number of genes and have primarily been identified by whole genome mRNA expression analysis. Whole genome expression analyses are too costly and too complicated, and have too much background interference to be used as routine clinical tests.

miRNAs may be able to give prognostic and predictive information because they act as major switches that regulate the expression of many mRNAs. It may be possible to better characterize a tumor with a small number of miRNAs than is possible with mRNAs. Colon cancer lacks biomarkers for identifying tumors that have a high risk for recurring, and predicting response to chemotherapy is not possible. Given these considerations, we have adopted the following specific aims:

1) Discover and validate prognostic miRNA markers for breast cancer patients with resectable tumors and histologically positive axillary lymph node(s);
2) Discover and validate predictive and prognostic miRNA markers for colon cancer patients with stage II and III resectable colon cancer; and
3) Initiate the development of an integrated colon cancer molecular database using miRNA data as well as mRNA and mutation data from the NSABP clinical trial C-07 samples which will provide unique information for hypothesis generation.

Principal Investigator
Katherine L. Pogue-Geile, PhD
Assistant Director of Molecular Profiling
NSABP Foundation, Inc.
Four Allegheny Center, 5th Floor
Pittsburgh, PA 15212

Other Participating Researchers
Soonmyung Paik, MD; Patrick Gavin, BS; Chung Kim, MD – employed by NSABP Foundation, Inc.

Expected Research Outcomes and Benefits
In this project, we will examine the expression of miRNAs in breast and colon cancers.

The project has the following expected outcomes:
1) To identify the gene expression profile of miRNAs in a large number of colon and breast cancer samples;
2) To determine the prognostic significance of miRNAs in colon and breast cancers;
3) To determine the predictive significance of miRNAs in colon and breast tumor responses to specific anti-cancer therapies; and
4) To acquire new information for the development of a valuable integrated molecular database of colon tumors. The C-07 colon tumors that will be interrogated for miRNA expression have already been profiled for mRNA gene expression by Illumina® DASL arrays and profiled for critical cancer mutations in KRAS, NRAS, PIK3CA, BRAF and MET. This information along with standard histological and clinical information makes this a valuable resource for the discovery of the mechanisms that determine the success or failure of specific cancer treatments and to generate hypotheses regarding control of gene expression and its role in cancer development.

Potential benefits include the following:
1) Improved prognostication for patients with diagnoses of breast or colon cancer;
2) Improved prediction of response of patients with colon cancer to oxaliplatin; and
3) Generation of information for the development of integrated molecular databases. C-07 miRNA data will add to other molecular information on the same clinical samples, including data on whole genome mRNA expression and somatic cancer mutations. This database can be used for hypothesis-generation as it will provide a large database for correlating these molecular characteristics.

Summary of Research Completed

I. Presentation and publication of an association of mismatch repair (MMR) status with benefit from bevacizumab treatment

In the last annual report, we presented our rationale for narrowing the focus of this research project to the testing and analysis of NSABP C-08 samples relative to bevacizumab benefit.

As a prelude to the analysis of C-08 microRNA data, we conducted a routine examination of clinical covariates to determine their association with prognosis or with prediction of bevacizumab treatment. One of these covariates was mismatch repair (MMR) status, which refers to the stability of small repeat regions of the genome called microsatellites.

Approximately 15% of colon tumors show DNA instability resulting in an amplification or deletion of DNA in these regions of the genome. This instability or microsatellite instability (MSI) is due to deficient mismatch repair (dMMR) due to a loss or mutation in mismatch repair proteins. This is an important clinical variable because MMR status has been shown to be associated with prognosis and with genetic colon cancer syndromes. Surprisingly, we found that patients with tumors with dMMR received benefit from bevacizumab on the basis of their overall survival. In this report period we have presented the analysis and interpretation of this observation at an international meeting and in publication form. In October 2012, we presented an oral presentation and poster entitled "DNA mismatch repair deficiency and benefit from adjuvant bevacizumab in NSABP C-08: Molecular profiling results" at the Molecular Targets and Cancer Therapeutics conference in Hollywood, FL. We presented data showing that patients with mismatch repair defective (dMMR) tumors derived statistically significant survival benefit from the addition of bevacizumab (hazard ratio = 0.52) in contrast to no benefit in patients shown to have mismatch repair proficient tumors (pMMR) (hazard ratio = 1.03) p_{interaction} = 0.035) (Fig. 1). Although a post-hoc finding, these data suggest that a molecularly-defined subset of colon cancer patients may derive clinical benefit from anti-angiogenesis agents. This
observation remains to be validated in another clinical trial; however, to our knowledge, it is the only description of a biomarker defining a subset of early-stage colon cancer patients who received benefit from bevacizumab within a clinical trial. This is of particular importance since there was no bevacizumab benefit in the entire cohort of stage II and III patients in C-08. This observation, if validated, could have a significant impact on clinical practice. Identification of a subset in C-08 which did benefit from bevacizumab provides evidence that there is a subset of patients who received benefit from bevacizumab, and our analysis of microRNA may help us to better define this group.

We have proposed a testable hypothesis to explain the above observations: dMMR tumors are highly immunogenic due to the generation of mutated proteins produced as a result of mistakes made in mismatch repair. This strong immunogenic response must be at least in part responsible for the good prognosis associated with dMMR tumors. VEGF-A (the target of bevacizumab) is speculated to be one of the main tumor-derived soluble factors that act as chemo-attractants for immature myeloid cells from the marrow to the tumor site, suppressing dendritic cell maturation and creating an immune-suppressive microenvironment. Furthermore, VEGF-A directly induces regulatory T-cell (Treg) proliferation in tumor-bearing mice through VEGFR-2, and blocking VEGF-A alone was sufficient to inhibit Treg cell accumulation in tumor-bearing mice but not in tumor-naïve mice. In colon cancer patients, adding bevacizumab to chemotherapy resulted in a substantial reduction in the proportion of Treg cells in the peripheral blood. Thus, we hypothesize that bevacizumab may be particularly effective in dMMR patients because bevacizumab is able to block the immunosuppressive effect of VEGF-A.

II. Proposal for NCI-CTEP approval of C-08 analysis

Also, in the current reporting period, we have developed a written proposal for acquiring proper regulatory approval for the analysis of the C-08 molecular profiling data, which includes microRNA data. The National Cancer Institute (NCI) has recently mandated that a proposal be written and approved by the NCI for the use of any tissues from studies that were supported at any level with NCI funds. Thus, we have spent much of this report period developing a protocol entitled "Analysis of Molecular Profiling Data for Discovery of Models for Predicting Bevacizumab Benefit of Stage II and III Colon Cancer." This protocol was submitted originally to the Protocol Review Committee (PRC) of the Cancer Therapy Review Committee (CTEP) on February 15, 2012, which provided a written review on March 7, 2013. A teleconference with the PRC and relevant NSABP regulatory, biostatistical, and laboratory personnel took place on April 8, 2012; the discussion addressed all of the questions and concerns raised in the review. Based on the review and the teleconference, the revised protocol was re-submitted, along with a written response to all of their questions, on May 31, 2013. This proposal was approved on June 17, 2012. We are now approved to receive an anonymized data set for analysis of the discovery cohort of C-08 which will include the most recent current clinical follow-up information and maximizes the number of events and, thus, the power of the study.

The NCI proposal describes the analysis of the microRNA expression data and additional C-08 profiling data from the defined discovery cohort within C-08. Since this document is a 32-page document and only describes future work, we will not attempt to describe it in detail here. However, it is essential to realize that this document not only describes the types of analyses we
will do but also explains that we will submit a separate protocol, as mandated in the review, once we have selected a predictive or prognostic model. This provides an infrastructure for how our models will be validated using CTEP's infrastructure to provide algorithm lock-down before accessing any data from the separate validation cohort, a required and essential step for the development of a clinical signature.

Part of the NCI proposal required power calculations for the analyses of C-08 we proposed. Table 1 shows the results of the power calculations with power greater than 0.5 and \( \Delta < 1 \). For example, if 25% of patients are classified as the group benefiting from bevacizumab and if there is 7% reduction in recurrence in the benefit group [3% recurrence for treatment with infusional 5-Fluorouracil, Leucovorin, and Oxaliplatin (mFOLFOX6) plus bevacizumab (Bev) versus 10% recurrence for mFOLFOX6 treated patients] and no difference for the non-benefit group (16% recurrence for mFOLFOX6+Bev treated patients versus 14.4% recurrence for mFOLFOX6 treated patients), then the \( \Delta = 0.255 \) and the power is 0.64. If the reduction in the benefit group is increased to 8.3% (3% recurrence for mFOLFOX6+Bev treated patients versus 11.3% recurrence for mFOLFOX6 treated patients), and the difference in the non-benefit group remains negligible (16% recurrence for mFOLFOX6+Bev treated patients versus 13.9% recurrence for mFOLFOX6 treated patients), then the power is 0.75. Note, the C-08 data does not satisfy the exponential assumption of the power calculation method; therefore, all power calculations shown here are approximate. While these power calculations are not extremely strong, there is added evidence to indicate we should pursue this analysis. One is that we already have evidence that a subset of C-08 patients (patients with dMMR tumors) received benefit from bevacizumab. Secondly, we have already completed all of the microRNA profiling in both the discovery and validation cohorts, so an effort to use the data to define a subset of patients who benefited from bevacizumab is warranted.

Most of the questions requested by the PRC review did not require any additional experimental work with the exception of a question relating to our method used to determine MMR status and its analytical performance. Because we do not presently have immediate plans to use this method for a clinical test, we did not perform analytical validation of our process. Rather we chose to demonstrate in a general way that our use of an IHC staining process of the of two mismatch repair proteins (MLH1 and MSH2) worked. Other investigators have already shown that IHC staining of MLH1 and MSH2 provide a rapid, cost-effective, sensitive, and extremely specific method for detecting dMMR tumor. However, to demonstrate the proficiency of our staining procedure, we performed IHC on a few available colon samples. Below we demonstrate that we are able to distinguish positive and negative staining and a failed assay with the MLH1 and MSH2 antibodies in Figs. 2 and 3.

**Summary**

In a routine analysis of clinical covariates within C-08 we found that patients with dMMR tumors received significant benefit from the addition of bevacizumab to chemotherapy. The data provide evidence that a subset of stage II and III colon cancer patients did receive benefit from bevacizumab even though the entire cohort did not. MicroRNA analysis may better define such a cohort and now that we have received approval from CTEP, we can begin to build models to identify subsets of stage II and III colon cancer patients who received benefit from bevacizumab.
Table 1 Power Calculations for Analysis of C-08 Data

<table>
<thead>
<tr>
<th>% benefit group</th>
<th>$\lambda_{11}$</th>
<th>$\lambda_{21}$</th>
<th>$\lambda_{12}$</th>
<th>$\lambda_{22}$</th>
<th>$R_{11}$ (%)</th>
<th>$R_{21}$ (%)</th>
<th>$R_{12}$ (%)</th>
<th>$R_{22}$ (%)</th>
<th>$\Delta_1$</th>
<th>$\Delta_2$</th>
<th>$\Delta$</th>
<th>Power</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>0.01</td>
<td>0.03</td>
<td>0.058</td>
<td>0.054</td>
<td>3</td>
<td>8.6</td>
<td>16</td>
<td>15</td>
<td>0.333</td>
<td>1.084</td>
<td>0.308</td>
<td>0.51</td>
</tr>
<tr>
<td></td>
<td>0.02</td>
<td>0.045</td>
<td>0.055</td>
<td>0.049</td>
<td>5.8</td>
<td>12.6</td>
<td>15.2</td>
<td>13.7</td>
<td>0.444</td>
<td>1.126</td>
<td>0.395</td>
<td>0.52</td>
</tr>
<tr>
<td></td>
<td>0.015</td>
<td>0.04</td>
<td>0.057</td>
<td>0.05</td>
<td>4.4</td>
<td>11.3</td>
<td>15.7</td>
<td>13.9</td>
<td>0.375</td>
<td>1.122</td>
<td>0.334</td>
<td>0.58</td>
</tr>
<tr>
<td>0.01</td>
<td>0.035</td>
<td>0.058</td>
<td>0.052</td>
<td>3</td>
<td>10</td>
<td>16</td>
<td>14.4</td>
<td>0.286</td>
<td>1.118</td>
<td>0.255</td>
<td>0.64</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.015</td>
<td>0.04</td>
<td>0.057</td>
<td>0.049</td>
<td>4.4</td>
<td>12.6</td>
<td>15.7</td>
<td>13.7</td>
<td>0.333</td>
<td>1.161</td>
<td>0.287</td>
<td>0.7</td>
</tr>
<tr>
<td>0.01</td>
<td>0.04</td>
<td>0.058</td>
<td>0.05</td>
<td>3</td>
<td>11.3</td>
<td>16</td>
<td>13.9</td>
<td>0.25</td>
<td>1.155</td>
<td>0.216</td>
<td>0.75</td>
<td></td>
</tr>
<tr>
<td>0.01</td>
<td>0.045</td>
<td>0.058</td>
<td>0.049</td>
<td>3</td>
<td>12.6</td>
<td>16</td>
<td>13.7</td>
<td>0.222</td>
<td>1.195</td>
<td>0.186</td>
<td>0.83</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>0.02</td>
<td>0.035</td>
<td>0.072</td>
<td>0.061</td>
<td>5.8</td>
<td>10</td>
<td>19.4</td>
<td>16.7</td>
<td>0.571</td>
<td>1.194</td>
<td>0.479</td>
<td>0.51</td>
</tr>
<tr>
<td></td>
<td>0.015</td>
<td>0.03</td>
<td>0.077</td>
<td>0.066</td>
<td>4.4</td>
<td>8.6</td>
<td>20.6</td>
<td>18</td>
<td>0.5</td>
<td>1.179</td>
<td>0.424</td>
<td>0.57</td>
</tr>
<tr>
<td>0.01</td>
<td>0.025</td>
<td>0.082</td>
<td>0.071</td>
<td>3</td>
<td>7.2</td>
<td>21.8</td>
<td>19.2</td>
<td>0.4</td>
<td>1.166</td>
<td>0.343</td>
<td>0.65</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.025</td>
<td>0.045</td>
<td>0.067</td>
<td>0.051</td>
<td>7.2</td>
<td>12.6</td>
<td>18.2</td>
<td>14.2</td>
<td>0.556</td>
<td>1.331</td>
<td>0.417</td>
<td>0.7</td>
</tr>
<tr>
<td>0.02</td>
<td>0.04</td>
<td>0.072</td>
<td>0.056</td>
<td>5.8</td>
<td>11.3</td>
<td>19.4</td>
<td>15.5</td>
<td>0.5</td>
<td>1.301</td>
<td>0.384</td>
<td>0.74</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.015</td>
<td>0.035</td>
<td>0.077</td>
<td>0.061</td>
<td>4.4</td>
<td>10</td>
<td>20.6</td>
<td>16.7</td>
<td>0.429</td>
<td>1.276</td>
<td>0.336</td>
<td>0.79</td>
</tr>
<tr>
<td>0.01</td>
<td>0.03</td>
<td>0.082</td>
<td>0.066</td>
<td>3</td>
<td>8.6</td>
<td>21.8</td>
<td>18</td>
<td>0.333</td>
<td>1.255</td>
<td>0.266</td>
<td>0.84</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.02</td>
<td>0.045</td>
<td>0.072</td>
<td>0.051</td>
<td>5.8</td>
<td>12.6</td>
<td>19.4</td>
<td>14.2</td>
<td>0.444</td>
<td>1.43</td>
<td>0.311</td>
<td>0.89</td>
</tr>
<tr>
<td></td>
<td>0.015</td>
<td>0.04</td>
<td>0.077</td>
<td>0.056</td>
<td>4.4</td>
<td>11.3</td>
<td>20.6</td>
<td>15.5</td>
<td>0.375</td>
<td>1.391</td>
<td>0.27</td>
<td>0.91</td>
</tr>
<tr>
<td>0.01</td>
<td>0.035</td>
<td>0.082</td>
<td>0.061</td>
<td>3</td>
<td>10</td>
<td>21.8</td>
<td>16.7</td>
<td>0.286</td>
<td>1.359</td>
<td>0.21</td>
<td>0.94</td>
<td></td>
</tr>
<tr>
<td>0.75</td>
<td>0.01</td>
<td>0.02</td>
<td>0.155</td>
<td>0.131</td>
<td>3</td>
<td>5.8</td>
<td>37.2</td>
<td>32.5</td>
<td>0.5</td>
<td>1.179</td>
<td>0.424</td>
<td>0.56</td>
</tr>
<tr>
<td></td>
<td>0.025</td>
<td>0.04</td>
<td>0.11</td>
<td>0.071</td>
<td>7.2</td>
<td>11.3</td>
<td>28.1</td>
<td>19.2</td>
<td>0.625</td>
<td>1.54</td>
<td>0.406</td>
<td>0.74</td>
</tr>
<tr>
<td></td>
<td>0.03</td>
<td>0.045</td>
<td>0.095</td>
<td>0.056</td>
<td>8.6</td>
<td>12.6</td>
<td>24.8</td>
<td>15.5</td>
<td>0.667</td>
<td>1.685</td>
<td>0.396</td>
<td>0.74</td>
</tr>
<tr>
<td>0.02</td>
<td>0.035</td>
<td>0.125</td>
<td>0.086</td>
<td>5.8</td>
<td>10</td>
<td>31.3</td>
<td>22.7</td>
<td>0.571</td>
<td>1.446</td>
<td>0.395</td>
<td>0.75</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.015</td>
<td>0.03</td>
<td>0.14</td>
<td>0.101</td>
<td>4.4</td>
<td>8.6</td>
<td>34.3</td>
<td>26.1</td>
<td>0.5</td>
<td>1.38</td>
<td>0.362</td>
<td>0.79</td>
</tr>
<tr>
<td>0.01</td>
<td>0.025</td>
<td>0.155</td>
<td>0.116</td>
<td>3</td>
<td>7.2</td>
<td>37.2</td>
<td>29.4</td>
<td>0.4</td>
<td>1.331</td>
<td>0.3</td>
<td>0.85</td>
<td></td>
</tr>
<tr>
<td>0.02</td>
<td>0.04</td>
<td>0.125</td>
<td>0.071</td>
<td>5.8</td>
<td>11.3</td>
<td>31.3</td>
<td>19.2</td>
<td>0.5</td>
<td>1.751</td>
<td>0.286</td>
<td>0.94</td>
<td></td>
</tr>
<tr>
<td>0.025</td>
<td>0.045</td>
<td>0.11</td>
<td>0.056</td>
<td>7.2</td>
<td>12.6</td>
<td>28.1</td>
<td>15.5</td>
<td>0.556</td>
<td>1.952</td>
<td>0.285</td>
<td>0.94</td>
<td></td>
</tr>
</tbody>
</table>
Survival Benefit from Bevacizumab in Patients with dMMR Tumors

Fig. 1

**A**

- mFF8, 128 Pts, 31 Deaths
- mFF8+Bev, 124 Pts, 18 Deaths

HR = 0.52, 95% CI (0.29-0.94)
P = 0.028

**B**

- mFF8, 873 Pts, 172 Deaths
- mFF8+Bev, 868 Pts, 177 Deaths

HR = 1.03, 95% CI (0.84-1.27)
P = 0.78

No. at risk

<table>
<thead>
<tr>
<th>Years from randomization</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>mFF8</td>
<td>118</td>
<td>111</td>
<td>105</td>
<td>95</td>
<td>85</td>
<td>85</td>
<td>28</td>
</tr>
<tr>
<td>mFF8+Bev</td>
<td>119</td>
<td>112</td>
<td>107</td>
<td>106</td>
<td>90</td>
<td>49</td>
<td></td>
</tr>
</tbody>
</table>

**MMR-Treatment Interaction P = 0.035**
Fig. 2

Results of MLH1 Staining

pos

neg

Failed assay

Fig. 3

Results of Staining for MSH2

pos

neg

Failed assay