National Surgical Adjuvant Breast and Bowel Project (NSABP) Foundation

Annual Progress Report: 2009 Formula Grant

Reporting Period

July 1, 2010 – June 30, 2011

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., Onco*type* 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 - 12/31/2012

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.

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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

A C-08 discovery cohort of 1003 colon cancer tumors which was representative of the entire C-08 cohort was profiled for miRNA expression. In this report period, we have completed expression profiling of 760 miRNAs (mirs) of approximately 1000 C-08 samples of this discovery cohort. Global mir expression profiling was done by using TaqMan® Micro Fluidic Cards, Life Technologies. This procedure involves the reverse transcription, pre-amplification, and amplification in 2 mega-plexed reactions. Each case was interrogated with these 2 mega-plexed reactions, each reaction containing 384 assays, and then was profiled on the A and B TaqMan micro fluidic cards. These 2 cards provided comprehensive coverage of the Sanger miRBase v14 database content for human mirs. U6 snRNA, considered to be a control mir, was repeated 4 times on each card.

In addition, we have also profiled 20 normal tissue samples from C-08 for which we have the paired tumor samples. The purpose of this investigation was to identify differentially expressed mirs in colon cancer.

Detailed Methods

Isolation of RNA. Initially, we used total RNA preparations, which we had prepared for another project. The first 450 RNAs that we interrogated were prepared with a modified version Ambion® kit using a low-throughput robotics instrument, the KingFisher mL robotics instrument. When it was time to prepare additional RNAs, we prepared them using Omega Bio-Tek kits and utilized a high-throughput robotics instrument, the KingFisher® Flex. We found that these changes to our total RNA procedure improved the quality and quantity of total RNA based on RiboGreen® quantification and UV 260/280 results, respectively. Furthermore, use of the KingFisher Flex instrument increased the throughput by more than 9-fold. However, when we compared the expression of mirs in RNAs isolated with the Ambion procedure to the Omega

Bio-Tek procedure, we found that mir expression was less robust from RNAs isolated using the Omega Bio-Tek procedure. However, we could not rigorously conclude that the difference detected in expression values was due to the type of preparation since the same samples were not used with both isolations. Therefore, we isolated RNA from 10 samples with both the Omega Bio-Tek kits and the modified version of the Ambion procedure. The results are shown in Table 1. The percentage of mirs that were expressed, meaning the % of mirs that had a Ct value less than 37, was greater in all of the RNAs isolated with the Ambion procedure. The Ct value for U6 snRNA was also lower, i.e., greater expression in all of the samples isolated with the Ambion procedure. Therefore, RNAs were isolated using a modified version of the Ambion (now part of Life Technologies) kit, which we formatted into a custom kit and ordered from Life Technologies.

Profiling with TaqMan Micro Fluidic cards. RNAs were profiled for mir expression with TaqMan Micro Fluidic cards from Life Technologies through these specific steps:

1. Reverse transcription of RNA to cDNA;
2. Amplification of cDNA
3. Preparation and loading of samples into TaqMan fluidic cards both A and B;
4. Running samples on two, 7900 real-time instruments using the automation accessory that allowed for continuous loading of cards into the instrument; and
5. Export of data from the Oracle Database into an Excel spread sheet.

All reagents for these processes were obtained from Life Technologies.

Reverse transcription. Fifty (50) to 100 ng of RNA was reverse-transcribed in a reaction containing 1X RT primers either A or B, 1X RT buffer, 2.7 mM dNTPs, 2.8mM $MgCl_2$, and 10 u/ul MultiScribe™ Reverse Transcriptase. Reactions were cycled at 16°C for 2 minutes, 42°C for 1 minute, and 50°C for 1 second for 40 cycles, and the reaction was stopped by incubation at 85°C for 5 minutes.

Pre-amplification of cDNA. Samples were "pre-amplified" because this step was reported to be an unbiased amplification step that would significantly increase the concentration of miRNAs in the sample, for maximum sensitivity and detection using real-time PCR. For each sample, a portion (2.5) ul of the reverse transcription reaction was added to 22.5 ul of a pre-amplification reaction mix, which consisted of 2.5 ul of the Applied Biosystems® Megaplex™ PreAmp Primers either A or B (depending on the primers used in the reverse transcription reaction) in 1X TaqMan PreAmp Master Mix. Reactions were incubated at 95°C for 10 minutes, 55°C for 2 minutes, and 72°C for 2 minutes and then were cycled at 95°C for 15 seconds and 60° C for 4 minutes for 12 cycles. Reactions were stopped by incubation at 99.9° C for 10 minutes and then mixtures were held at 4°C for up to 18 hours. After completion of the amplification, 75 ul of 0.1X TE at pH 8.0 was added and samples were mixed and stored at -20°C or were immediately processed for TaqMan analysis.

Preparation, loading, and running of samples. Nine (9) ul of diluted pre-amplified reaction was diluted to a final volume of 900 ul in 1X TaqMan Universal PCR Master Mix, No AmpErase® UNG and was mixed. One hundred (100) ul of this mix was added into each of 8 ports on the micro fluidic cards. Cards were sealed and centrifuged, and real-time PCR was run on the

Applied Biosystems® 7900HT instrument. Conditions for this reaction for real-time amplification were fast real-time PCR conditions: 94.5°C for 10 minutes followed afterward by 37 cycles of 97°C for 5 seconds and 59.7°C for 45 seconds.

This system was supposed to offer fully automated robotic loading but, as mentioned below, we encountered several problems associated with the continuous loading of the instrument.

From the outset of this project, we appreciated that it would be necessary to have two 7900 real-time instruments running continuously, 24 hours per day, 7 days per week in order to complete the mir expression profiling in a timely manner. While budgetary constraints prevented us from being able to afford two 7900 instruments, Life Technologies agreed to lend us a second instrument for the duration of our project.

The preparation of the samples for mir expression profiling was straightforward and simple to perform. However, we had many problems with all stages of the work beyond sample preparation. We had one malfunctioning robot arm that fed plates into the 7900 and several problems associated with one of the 7900 real-time machines—which necessitated its replacement with a new machine. As a result of these various problems, the server was replaced once and all three work stations were replaced at least once. All of these replacements were done in an effort to correct some problem; by far the most problematic issues with running these instruments were software errors and bugs. The SDS Enterprise software that was responsible for importing the data into an Oracle Database routinely produced vague errors that could not be explained, and the Oracle Database itself was initially unstable and had to be rebuilt more than once.

Statistical analysis of C-08 mir expression data. We have hired a full-time biostatistician to help us both plan and execute analysis of all our correlative science projects, including this one. We are currently writing a detailed protocol for the analysis of this large data set.

In summary, we have completed the mir expression profiling of a large discovery cohort consisting of 1000 cases from NSABP clinical trial C-08 and are currently working on the analysis of the data.

Table 1. Comparison of RNAs Isolated Using Ambion and Omega Bio-Tek Procedures

	AMBION EXTRACTION		OMEGA EXTRACTIONS	
Sample	Percentage mirs expressed below c_t37	average U6 snRNA Ct	Percentage mirs expressed below c_t37	average U6 snRNA Ct
C08-0693R	65.9%	16.8	56.3%	17.1
C08-0710R	62.0%	17.1	56.0%	17.2
C08-0711R	66.1%	16.5	55.7%	18.1
C08-0723R	62.0%	16.7	53.4%	17.2
C08-0726R	62.2%	16.9	49.5%	19.2
C08-0733R	55.7%	17.3	43.0%	19.3
C08-0742R	62.8%	16.7	41.1%	22.6
C08-0744R	62.2%	16.6	47.4%	18.4
C08-0762R	61.7%	17.1	54.4%	17.1
C08-0764R	58.6%	16.7	49.0%	17.5