National Surgical Adjuvant Breast and Bowel Project (NSABP) Foundation

Annual Progress Report: 2009 Formula Grant

Reporting Period

January 1, 2010 – June 30, 2010

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

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;
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

The goal of this project is to identify miRNAs that can be used for prognostic or predictive information to improve the treatment of breast and colon cancer. To initiate these studies we have examined 3 different miRNA platforms. When this grant was written in December 2009, the miRNA TaqMan® Megaplex and TILDA cards from ABI (Life Technologies) appeared to be the best platform. However, recently NanoString and Fluidigm® have launched assays for miRNA expression analysis and needed to be examined before the purchase of equipment.

The Fluidigm (South San Francisco, CA) platform is the same chemistry as TaqMan but the reactions are miniaturized in a microfluidics system in which tens of thousands of fluid control valves and interconnected channels are fabricated within miniature devices. These devices constitute integrated fluidic circuits of discrete pathways and contain intermediate switches to control the flow and mixing of primers and DNAs into discrete nanowells. This allows for rapid interrogation of expression of 96 DNAs with 96 genes in a single experimental set up. Potentially, this procedure would provide considerable cost savings because only nanoliter amounts of reagents are needed. However, in practice these savings are not yet realized because Fluidigm manufactures only the instrumentation. The miRNA TaqMan assays must be purchased from another manufacturer. The list price for individual assays for the entire miRNA library of nearly 800 was cost prohibitive. Initially, we considered running a pilot study using this technology and talked to the field representatives from Fluidigm, but the cost of reagents and the time was more costly than the other 2 technologies.

NanoString released the nCounter miRNA Expression Assay Kits in April 2010. The nCounter miRNA is a pool of multiplexed assays designed to profile the human miRNA transcriptome in a single tube. The assay is able to perform highly multiplexed, direct digital detection and counting of miRNAs at single-base resolution, and with no PCR amplification. NanoString's

Human miRNA Expression Assay Kit profiles more than 700 human and human-associated viral miRNAs with specificity and sensitivity comparable to qPCR at a fraction of the cost.

TaqMan from ABI (Applied Biosystems Inc., now Life Technologies) has long been considered the gold standard for mRNA analysis. However, the small size of miRNAs make standard TaqMan analysis impossible. miRNAs have an average size of 22 nucleotides—much too small to employ the use of two 20-base primers for amplification and a 30-base probe for detection. Some of the miRNAs exist as highly homologous family members that differ by only one or a few bases, making the specific detection of any particular miRNA difficult. To make the RNAs long enough for TaqMan analysis, the Megaplex procedure elongates the miRNA by using many different stem loop primers in the RT reaction which effectively primes 384 different miRNAs in a single reaction. These cDNAs are then amplified with specific primers and detected in real time with a TaqMan probes. Thus, this procedure uses 4 different and specific oligonucleotides to define a single miRNA.

To compare data obtained from the Megaplex and TILDA Cards with the miRNA kits from NanoString, we performed pilot projects with both platforms. While all of the instrumentation was available for performing the NanoString assays, none of the instrumentation was available to test the Megaplex TaqMan assays. Therefore, we contacted Life Technologies and the company agreed to send us all of the required instrumentation without purchase. For this pilot study, we ran 3 different RNA samples that had been isolated from NSABP from archival formalin-fixed, paraffin embedded tissues. Replicate or triplicate samples were run on 3 different samples.

Table 1 shows the reproducibility of the ABI and NanoString platforms by analyzing results from duplicate samples for their r^2 values. The full library of miRNAs with the ABI platform is contained in 2 separate TILDA cards, A and B. The reproducibility of the ABI platform was almost always better than that seen with the NanoString platform. We also used 2 different cycling conditions for amplification: slow and fast. The slow conditions are the current standard reaction conditions, which take approximately 2 hours to run a single card on a single sample. Because each sample would need to be run on 2 cards, a single sample would require 4 hours of run time on the instrument. We thought this was too slow, so ABI provided us with faster cycling conditions which enabled a card to be processed in one hour. We found that the fast condition generally gave higher r^2 values than the slow condition and were much better suited to the sort of throughput that we would require. Using the same samples, 455 and 540, we found that the ABI platform was more reproducible.

One of the advantages of the NanoString analysis for messenger RNA is that there are no enzymatic reactions and no PCR amplification. However, for the NanoString assay of miRNAs, 2 enzymatic steps are required. While the methodology is fairly simple it required more personnel time and is not as simple as the ABI procedure. While we thought that the NanoString platform provided a very cost-effective and promising technology for the future for miRNA expression profiling, we were concerned that the technology had not yet been fully tested. Also given that our own results showed the ABI technology to be more reproducible, we chose to purchase the ABI platform.

In conclusion, we have completed pilot studies comparing the ABI and NanoString platforms for miRNA expression profiling. We began the process of ordering all the instrumentation and software required to run one Real Time 7900 instrument and an autoloader that allows the instrument to run continuously for 72 hours. In addition, Life Technologies has provided us with a second 7900 and autoloader which we will be allowed to use for the duration of this project. This should allow us to process almost 24 samples per day.

	Table 1 R2 values for Real Time 7900 and NanoString					
	ABI Ct-A	ABI delta Ct	ABI Ct-B	ABI delta Ct-B	NanoString Sample 1 vs 2	NanoString Sample 2 vs 3
455 fast	0.972				0.833	
540-100 ng fast	0.961	0.96	0.937		0.897	0.861
2268- fast	0.972	0.972				
1418					0.882	
455 100 vs 200ng	0.927					
455 50 vs 100ng	0.917					
540-100ng- slow	0.927			0.88		