National Surgical Adjuvant Breast and Bowel Project (NSABP) Foundation

Annual Progress Report: 2007 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 $1,306,064 in formula funds for the grant award period January 1, 2008 through December 31, 2011. Accomplishments for the reporting period are described below.

Research Project 1: Project Title and Purpose

Mutation Profiling of Breast and Colon Cancer - Cancers are caused by mutations of the genes that control important functional components within the cells. These gene mutations, therefore, govern the behavior of cancer cells. Studies have shown that within a certain cancer type such as breast, there are different sets of mutated genes. These differences (also called heterogeneity) of gene mutations result in different clinical behaviors of cancer cells, i.e., differential prognoses and responses to anti-cancer therapies. The goals of this project are to characterize gene mutations in breast and colon cancers and categorize them according to matched clinical behaviors so that the information can be used to optimize treatment for breast and colon cancers as well as to identify new targets for anti-cancer therapies.

Anticipated Duration of Project

1/1/2008 - 12/31/2011

Project Overview

It has been known for sometime now that human cancer is caused by the accumulation of mutations in oncogenes and tumor suppressor genes. A great deal of work has been done to identify a plethora of mutations in a variety of human cancers. However, until recently, the application of this information has had little impact on improving the treatment of cancer patients. The broad long-term goal of this project is to profile mutations in breast and colon cancer which can be used to improve treatment through identifying mutations that may pinpoint new targets for new drugs.

There is mounting evidence that the heterogeneity that exists within breast and colon cancers may be due to different mutation profiles between different cancer sub-types (i.e., in the breast, luminal A and B, Her2, normal and basal). However, until recently there were various technical and pragmatic barriers to carrying out any systematic study of mutation profiles. The mass

spectrometric genotyping technology based on the Sequenom platform is suitable for the execution of such studies even using formalin-fixed, paraffin-embedded tissue specimens. The successful application of this approach was described by Thomas et al. (2007) during the past year. In their study, using this technology, 1000 tumor samples were tested for 238 known oncogene point mutations in 17 human oncogenes. The overall distribution of oncogene mutations found was consistent with previous literature reports, but many low frequency events involving both rare and common neoplasms also were detected. These included an FGFR1 mutation and a BRAF mutation in 2 breast tumors (in addition to prevalent PIK3CA mutations). These results endorse the notion that potentially 'drug-able' oncogene mutations may exist at low to moderate frequencies in many tumor types, including breast cancer, and that they are detectable using this approach.

In the project proposed, breast cancer tumors from neoadjuvant trial NSABP B-27 and colon carcinoma tumors from NSABP trial C-07 will be used for mutation screening. In our previous studies involving patients enrolled in NSABP neoadjuvant trial B-27, we have identified patients who had a poor prognostic gene signature and did not respond to chemotherapy, and, therefore, they are at very high risk of relapse and are resistant to chemotherapy. Cancer (CAN)-gene screening in such a cohort may yield rich information as to new treatment strategies beyond chemotherapy. Colon cancer tumors will be included in our project with the same goals.

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Expected Research Outcomes and Benefits

In the proposed project, we will identify mutations in key genes that are involved in the development of breast and colon cancer.

The proposed project will yield the following information:
1) The frequency of gene mutations in breast and colon cancer. This will be an important finding because it defines what genes may be the most important targets for the development of new therapies.

2) The prognostic significance of gene mutations in breast and colon cancer. This is of obvious importance to the development of strategies for selecting appropriate patients with regard to chemotherapy.

3) The role of gene mutations in predicting response to specific anti-cancer therapies.

4) The identification of gene mutations that can be targets for new drug development.

Potential benefits will include the following:
1) Better prognostication of patients diagnosed with breast and colon cancer
2) Better prediction of response to anti-cancer therapies

Summary of Research Completed

Summary of Studies
We have completed mutation profiles from NSABP C-07 and two other NSABP clinical trials. NSABP C-07 is a trial conducted to test the worth of adding oxaliplatin to 5-FU + Leucovorin for the treatment of stage II and III colon cancer. The other two trials included NSABP C-08, a trial conducted to test the worth of adding bevacizumab to 5-FU + LV and bolus oxaliplatin, and B-31, a trial conducted to test the efficacy of Herceptin in the adjuvant setting of Her2-positive breast cancer.

Mutation Profiling of C-07
In this report period, we established that the Sequenom platform and the ColoCarta panel comprised a *sensitive* method for mutation detection. For the sensitivity assay, MCF-7 (human breast carcinoma) and SK-BR-3 (human breast adenocarcinoma) cultured cells were mixed in various ratios. A PIK3CA mutation is present only in the MCF-7 cells and not in SK-BR-3. With this method, we were able to detect the PIK3CA mutation when it represented only 5% of the MCF7 cells and only 2.5 % of the mutant alleles (Figure 1). We reported these results, and other results that were enumerated in the 2009 progress report in *BMC Cancer* (Fumigalli and Gavin, et al, 10:101).

In this report period, the ColoCarta mutation data from the discovery cohort of C-07 (N=1059 with available assay data) was analyzed. Cox proportional hazard models were used to test whether these mutations were prognostic for tumor recurrence, overall survival (OS), and survival after recurrence (SAR), and predictive of response to oxaliplatin. Only *NRAS* was shown to be moderately prognostic for recurrence (HR= 1.919, CI 95% 1.098, 3.353, p=.0222) (Table 1). *BRAF* mutations were associated with a shorter OS (HR=1.414, P=0.0193) and this was at least in part due to their association with poor survival after recurrence (SAR) (HR=1.991, p=.0002) (Table 2). *MET* mutations were shown to be predictive of oxaliplatin benefit (Table 1).

To validate the findings in the discovery cohort, we profiled an additional 842 samples for mutations in *MET* and *BRAF*. *NRAS* was not investigated further since its prognostic effect was modest and it represented a small percentage of the cases. *MET* mutations were not confirmed in the validation cohort (Table 2). However, *BRAF* mutations were validated for their association with SAR (HR=2.694, P<.0001) (Table 1).

Since *BRAF* mutations occur in 24% of *KRAS* wt colon tumors and may confer resistance to EGFR-targeted agents, there is a strong rationale to develop new agents targeting molecular

abnormalities associated with mutations in *BRAF*. A manuscript has been prepared and is undergoing required internal approval processes.

Mutation Profiling of C-08

To further the goals of improving cancer treatment strategies, we attempted to identify colon cancer patients that would receive benefit from treatment with bevacizumab. NSABP clinical trial C-08 was a phase III trial designed to test the benefit of adding bevacizumab to 5-Fluorouracil (5-FU), Leucovorin, and Oxaliplatin (mFOLFOX6). C-08 did not meet its clinical endpoint of improving disease-free survival; however, it is possible that a subset of patients do receive benefit from bevacizumab. Mutation profiling has been done to determine whether mutations can be used to identify a subset of patients that benefit from bevacizumab treatment. The ColoCarta panel was used to profile a discovery cohort of 465 primary tumor samples from C-08. Mutation data has been sent to the NSABP Biostatistical Center for analysis.

Mutation Profiling of B-31

Another goal of this proposal was to identify mutations that might be of interest in breast cancer. We chose to profile PIK3CA mutations in the NSABP clinical trial B-31. We first surveyed the COSMIC data base for known mutations in breast cancer and found that the only gene that contained common, hot-spot mutations in breast cancer was *PIK3CA*. Although mutations in p53, PTEN, and CDH1 were common, the mutations were spread throughout the gene and, therefore, could not be effectively screened by the Mass Spec Type Plex technology. PIK3CA mutations were not only the most practical mutations to interrogate but they have also been shown to be associated with resistance to Herceptin® treatment in patients with Her2-positive breast cancer. Therefore, we profiled 563 breast cancer tumors from NSABP clinical trial B-31—a randomized trial that compared the safety and efficacy of adriamycin and cyclophosphamide followed by taxol (AC→T) to the same regimen plus Herceptin (AC→T + H) in node-positive and Her2-positive breast cancer patients. The frequency of PIK3CA mutations in tumor samples from NSABP B-31 are shown in Table 3. In our study, we did not find any association between PIK3CA mutations and response to Herceptin: Kaplan-Meier curves demonstrated a similar response to herceptin in PIK3CA wt and mutant tumors (Figure 2).

Detailed Methods

The method for DNA extraction has been improved in the last year by automating the extraction of DNA from lysates. DNAs prepared from the C-08 discovery cohort, the B-31 discovery cohort, and the C-07 validation cohort were prepared from lysates prepared in the same manner as the in the previous report period but only 3 unstained 5-µm sections were used. DNA was isolated with the E.Z.N.A.® FFPE DNA Isolation Kit from Omega Bio-Tek (Norcross, GA) utilizing the KingFisher Flex instrument (ThermoFisher; Burlington, ON). DNA was measured with fluorescence, using the Quant-iT ™ PicoGreen® dsDNA Assay Kit (Invitrogen; Carlsbad, CA) and the Infinite®F200 fluorometer (Tecan; Männedorf, Switzerland).

The mutation panel used to interrogate the C-07 validation set consisted of only one primer pool, which profiled two *MET* mutations, and 2 assays that were used to interrogate *BRAF-V600E* and *BRAF-D594V/G*. All primer sequences were identical to the primers described in the OncoCarta kit.

Statistical Analysis

Cox proportional hazard models were used to calculate hazard ratios for each mutation or any mutation. Analyses were controlled for treatment, nodes (0, 1-3, 4+), age (<65, ≥=65), and gender. The endpoints for these analyses were recurrence (even if preceded by a second primary cancer), overall survival (OS), and survival after recurrence (SAR). The mutation-by-treatment interaction was tested in the Cox model using the cross-product term of indicator variables for oxaliplatin treatment and mutation status. Gene mutation status was parameterized as a dichotomous variable. Time to event analyses were based on 1035 patients in the discovery data cohort and 813 patients in the validation cohort that were eligible for the parent C-07 study and had follow-up information.

Current State of Expected Research Outcomes and Benefits

Progress during this reporting period for each of the project objectives is noted below.

1) The frequency of gene mutations in breast and colon cancer.

In this report period, we have determined the frequency of all common PIK3CA mutations in Her2-positive breast cancer samples. The frequency of PIK3CA mutations in breast cancer were profiled in 563 breast cancer samples from NSABP clinical trial B-31. The frequency of these mutations breast cancer is shown in Table 3.

2) The prognostic significance of gene mutations in breast and colon cancer.

We have examined the prognostic value of all of the common hot-spot mutations in colon cancer and have found that only BRAF mutations were associated with poor overall survival in the discovery cohort which is at least in part due to its effect on survival after recurrence.

3) The role of gene mutations in predicting response to specific anti-cancer therapies.

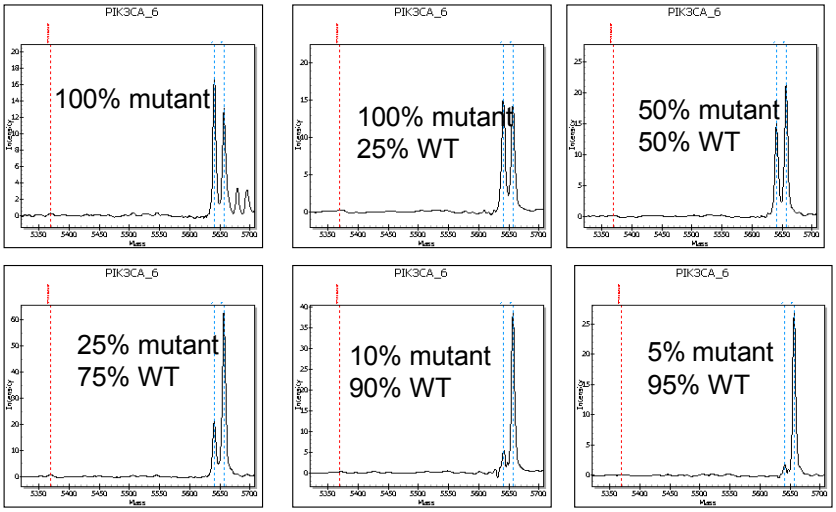
We did find that MET mutations were predictive of oxaliplatin benefit for colon cancer in the discovery cohort but this was not confirmed in the validation cohort and the association seen in the discovery cohort was probably a chance finding.

We did not find that PIK3CA mutations were associated with resistance to herceptin response in breast cancer. This is an important finding as early reports have suggested that PIK3CA mutations were associated for herceptin response in breast cancer.

4) The identification of gene mutations that can be targets for new drug development.

BRAF mutations may be an important target for new drug development.

Fig. 1 Spectra of Mixing Cell Experiment



Spectra of MCF-7 cells (mutant) alone or mixed with SKBR3 cells (WT) are shown. % are based on the ng amounts of DNA. This assay detects a E545K mutation in PIK3CA.

Figure 2

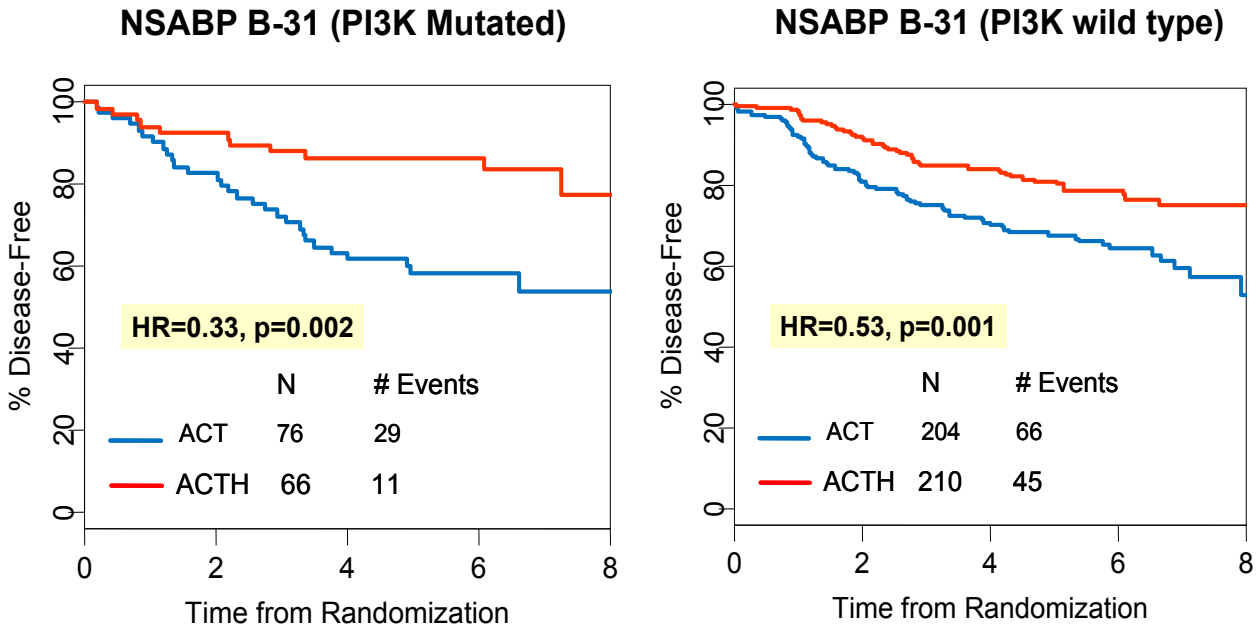


Table 1: Mutations and their Association with Recurrence, Overall Survival, and Survival after Recurrence in C-07 Tumors

Recurrence Discovery

Variable	No Mutation* Recurs/N	Mutation Recurs/N	*P*-value**	Hazard Ratio** (95% CI)	Oxali-Gene Interaction *P*-value***
BRAF	242/879	46/153	0.6800	1.070(0.775, 1.478)	0.7725
KRAS	171/632	116/400	0.4059	1.105(0.873, 1.400)	0.2833
KRAS G12V	258/947	31/88	0.0900	1.381(0.951, 2.005)	0.5440
MET	278/1002	11/33	0.4355	1.272(0.695, 2.328)	0.0131
NRAS	274/1001	13/29	0.0222	1.919(1.098, 3.353)	0.8257
PIK3CA	234/817	55/213	0.9603	1.008(0.750, 1.354)	0.9867

Overall Survival Discovery

Variable	No Mutation* Deaths/N	Mutation Deaths/N	*P*-value**	Hazard Ratio* (95% CI)	Test of Trt-Gene Interaction *P*-value**
BRAF	237/879	61/153	0.0193	1.414(1.058, 1.890)	0.1852
KRAS	179/632	117/400	0.5136	1.081(0.856, 1.366)	0.0622
KRAS G12V	272/947	27/88	0.6072	1.109(0.747, 1.649)	0.1519
MET	287/1002	12/33	0.4231	1.268(0.710, 2.265)	0.1763
NRAS	285/1001	12/29	0.1381	1.549(0.869, 2.764)	0.7516
PIK3CA	244/817	55/213	0.8629	0.974(0.726, 1.308)	0.4806

Survival After Recurrence Discovery

Variable	No Mutation* Deaths/N	Mutation Deaths/N	*P*-value**	Hazard Ratio* (95% CI)	Test of *Trt*-Gene Interaction *P*-value***
BRAF	186/237	40/43	0.0002	1.991(1.386, 2.862)	0.1141
KRAS	130/166	95/113	0.2376	1.177(0.898, 1.542)	0.8539
KRAS G12V	204/250	23/31	0.1350	0.707(0.449, 1.114)	0.1683
MET	218/270	9/11	0.6228	0.840(0.420, 1.682)	0.2926
NRAS	215/266	10/13	0.9502	0.980(0.514, 1.866)	0.7888
PIK3CA	188/229	39/52	0.9483	0.989(0.697, 1.402)	0.9574

Validation Set *BRAF*				
Endpoint	No Mutation Events/N*	Mutation Events/N	*P*-value**	Hazard Ratio*** (95% CI)
Recurrence	183/672	33/102	0.3678	1.193(0.813, 1.751)
Overall Survival	171/672	35/102	0.1605	1.311(0.898, 1.915)
Survival After Recurrence	137/180	31/32	<.0001	2.694(1.746, 4.157)

N=Available clinical data

Table 2: Oxaliplatin Hazard Ratio for Recurrence by MET Mutation Status

	Discovery			**Validation**		
	Hazard Ratio**	95% CI	*P*-Value	Hazard Ratio**	95% CI	*P*-Value
WT* *MET*	0.82	0.64 - 1.03	0.0898	0.83	0.63 - 1.09	0.1798
MET Mutation	0.03	0.003 - 0.38	0.0065	0.92	0.18 - 4.86	0.9246
Overall	0.76	0.60 - 0.96	0.0197	0.84	0.64 - 1.09	0.185
	MET-Oxaliplatin Interaction p=0.0131			*MET*-Oxaliplatin Interaction p=0.84		

*wt refers to the wt sequence corresponding to amino acid positions 970 and 992 in the short form of MET.
**Model controls for pos. nodes (0, 1-3, 4+), age (<65, ≥65), and gender

Table 3: PIK3CA Mutations in Breast Samples from NSABP B-31

	Mutations found in B31	% Mutated in B31		Mutations found in COSMIC	%Mutated in COSMIC
PIK3CA-C420R	3	0.5%		15	0.3%
PIK3CA-E542K	19	3.4%		134	2.9%
PIK3CA-E545K	27	4.8%		252	5.4%
PIK3CA-H1047L	9	1.6%		65	1.4%
PIK3CA-H1047R	78	13.9%		660	14.1%
PIK3CA-N345K	7	1.2%		24	0.5%
PIK3CA MUTATED?	143	25.4%		1150	24.5%****

****This value reflects the percentage mutated restricted to the base positions we assayed. By sequencing the mutation rate is 26.1%