

National Surgical Adjuvant Breast and Bowel Project (NSABP) Foundation

Annual Progress Report: 2011 Formula Grant

Reporting Period

July 1, 2013 – June 30, 2014

Formula Grant Overview

The NSABP Foundation received \$851,360 in formula funds for the grant award period January 1, 2012 through December 31, 2015. Accomplishments for the reporting period are described below.

Research Project 1: Project Title and Purpose

Markers and Mechanisms of Trastuzumab Resistance and Cardiotoxicity – The purpose of this project is to identify molecular changes associated with treatment failure in Her2-positive breast cancer patients treated with trastuzumab and chemotherapy. Specifically, specific molecular changes that have been implicated in preclinical models to be responsible for trastuzumab treatment failure will be investigated. Identification of molecular changes that are associated with treatment failure helps to identify those patients who may need additional treatment and may help to identify those pathways that are most critical to trastuzumab response and to understanding treatment success as well as failure.

Anticipated Duration of Project

1/1/2012 – 12/31/2015

Project Overview

The broad research objective is to improve treatment of breast cancer patients. We propose the following specific aims: 1) Identify DNA sequence alterations associated with resistance to trastuzumab chemotherapy in Her2-positive (+) breast cancer; 2) Identify DNA sequence alterations associated with trastuzumab cardiotoxicity; 3) Determine the molecular mechanism responsible for acquired trastuzumab resistance in node-positive, Her2 (+) breast cancer patients.

Several preclinical models implicate specific molecules and pathways as responsible for trastuzumab resistance. Resistance to trastuzumab can be inherent, meaning that the tumor does not respond to treatment, or it can be acquired, meaning that the tumor initially responds to treatment, and then later recurs. We propose to explore both types of resistance. To explore acquired resistance mechanism(s), we will examine molecular/genetic changes between primary and recurrent tumors in patients treated with trastuzumab and chemotherapy and in patients

given only chemotherapy. The changes to be investigated are ones that have been implicated in preclinical models as responsible for trastuzumab or other targeted antibody resistance. Changes that appear only in recurrent tumors from patients treated with trastuzumab and not in recurrent tumors from those given only chemotherapy would be likely candidates to be at least partly responsible for trastuzumab-acquired resistance. To explore inherent resistance mechanisms and to predict associated cardiotoxicity, we will examine white blood cells for single nucleotide polymorphisms (SNPs) for FC γ receptors and ERBB2 in all B-31 patient samples available for analysis, approximately 1400 cases. Previous reports indicated that certain polymorphisms in FC γ and ERBB2 receptors were associated with response to trastuzumab and to trastuzumab-associated cardiotoxicity, respectively. Our goal is to validate or dispute this initial finding by using all available cases in NSABP clinical trial B-31, which established the effectiveness of trastuzumab in early-stage breast cancer. If such results were conclusive, they would provide for a simple test to determine the responsiveness of breast cancer patients to trastuzumab before treatment is given and could suggest that other therapies be tried.

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Expected Research Outcomes and Benefits

The expected research outcomes will determine whether specific SNPs are associated with trastuzumab resistance or cardiotoxicity. This information may benefit patients with Her2-positive breast cancer by providing information that could improve the selection of therapy. These SNP tumor markers could identify patients who are likely to be resistant to trastuzumab or who may suffer serious cardiotoxicity. Patients with trastuzumab-resistant tumors could be treated with other Her2-targeted therapies, such as lapatinib. Patients who are likely to suffer from cardiotoxicity from the trastuzumab/anthracycline regimen, which is the most effective treatment, may opt for a trastuzumab/carbotaxol regimen, which is not associated with cardiotoxicity.

Other expected research outcomes will include answers to several important questions concerning trastuzumab response and resistance. On the basis of preclinical models, many mechanisms have been proposed to explain trastuzumab resistance but none of these proposed mechanisms have been shown to be relevant in women. DNA sequence and RNA expression analyses of the genes that have been proposed to be responsible for acquired resistance to trastuzumab may provide potential biomarkers to monitor the success or failure of trastuzumab treatment.

Summary of Research Completed

In this report period we have addressed specific aims 1) and 2), which are to identify DNA sequence alterations associated with resistance to trastuzumab chemotherapy in Her2-positive (+) breast cancer and to identify DNA sequence alterations associated with trastuzumab cardiotoxicity.

DNA Isolations. Blood collections from NSABP clinical trial B-31 were obtained from the Baylor College of Medicine. DNA was isolated from 1251 blood samples and from 323 formalin-fixed, paraffin-embedded (FFPE) lymph-node or normal tissue blocks. Two-hundred ul of blood was used for extraction using the Omega Bio-Tek Mag-Bind Blood DNA HDQ 96 Kit and the KingFisher Flex instrument, following the recommendations of the manufacturer. Four to six 5uM sections were cut for DNA extractions from lymph node or normal or tumor/normal FFPE tissue blocks. For blocks consisting of both normal and tumor tissue, 4 to 6 sections were placed onto slides and only tissue that was pathologically normal was scraped into a 1.5 ml tube. Tissue was deparaffinized with xylene, lysates were treated with protease, and DNA was isolated using the Omega Bio-Tek Mag-Bind FFPE DNA Kits and the KingFisher Flex instrument. DNAs were quantitated with PicoGreen.

DNA Anonymization. Before DNA assays could be done, it was necessary to further anonymize the samples as dictated by patients' consents. Therefore, 1574 DNA samples were normalized into 17 new 96-well plates so that each well contained a total of 20ul of DNA at a concentration of 5ng/ul. All plates but two were filled with samples or no template controls and were visually identical. In the two partially-filled plates not all of the wells were filled but both of these plates contained the same number of samples and in same wells so that these two plates were visually identical. No template controls were included in each of the 96-well plates. The normalized DNA plates were given new numbers by an honest broker associate who was not involved in the study. The anonymized samples were given a new ID based on their new plate number and well location. The link between the anonymized sample ID which was a plate number and a well number, and the original NON-anonymized plate and well number was maintained by the honest broker at the University of Pittsburgh Biostatistical Center. Laboratory personnel sent the link between the original plate location and Lab ID (in the case of the FFPE tissue [FFPET] DNAs) and the Baylor Repository ID (in the case of the blood DNAs) to the honest broker.

B31-SNP Molecular Profiling. The following SNPs in the following genes were investigated: rs1801274 (*FCGR2A*); rs396991 (*FCGR3A*); rs1136201 (*ERBB2*); rs8187710 (*ABCC2*); rs13058338 (*RAC2*); and rs1799945 (*HFE*). At least 1600 genotyping reactions, which included the 1574 sample DNAs and no template controls, were carried out for each of the 6 SNP assays. The *FCGR3A* SNP was problematic due at least in part to the fact that there is a *FCGR3B* pseudogene with extensive homology to the *FCGR3A* gene. For this reason we repeated this assay in all of the samples to check for reproducibility. We observed that a portion of the samples that we suspected were DNAs isolated from blood was quite concordant, but for DNAs we suspected were isolated from FFPET the concordance between runs was very poor (Figure 1). In addition, we observed that the automatic genotyping calls generated by the Typer software, Sequenom Inc., included many more "aggressive" and low probability calls, indicating that the frequency of the two alleles varied widely from the expected 50% for heterozygote alleles and

100% for homozygote alleles. The Typer software categorizes the genotyping calls as conservative, moderate, aggressive, or failed based on the area under the spectra curve for each allele, and again, this was particularly true for a portion of the samples that we suspected were FFPET samples.

The assay design was changed to improve the results of the *FCGR3A* genotyping calls. The original design included a pre-PCR amplification that amplified an 1187-base pair (bp) amplicon and then, in a second step, amplified a smaller amplicon; it was this amplicon that was used for the single base extension. We suspected our results with FFPET-isolated DNA were compromised due to a failure of the PCR reaction in the first pre-PCR step due to the large size of that amplicon. The degraded and chemically modified nature of the FFPET-isolated DNAs limits the size of DNA that can be amplified by PCR. Thus, the pre-PCR primers were redesigned to amplify a 348-bp fragment. Greater specificity was achieved by designing new primers. The homology between this reverse primer and the pseudogene (*FCGR3B*) contained 3 mismatches out of 5 bases in the 3-prime end of the primer. This primer now more uniquely targets the real SNP of interest in the *FCGR3A* gene and the smaller size of the pre-PCR amplicon should improve the robustness of the PCR. The 348-bp amplicon was used as a template to amplify a smaller fragment, which was used for the single base extension.

All suspected FFPET-isolated DNAs were assayed in duplicate using the newly designed assay *FCGR3A* (design_2). Figure 2 demonstrates that the reproducibility of run 1 and 2 is quite good as most of the points between run 1 and 2 fall on a straight line. However, the data from these FFPET samples still differed from what we expected and had seen when using the blood samples. The frequency of each allele should cluster around 50% or 100% and they do for the blood samples as shown in Figure 1 but there is no clustering of the frequencies of the G alleles for DNAs isolated from FFPET.

From the beginning, we appreciated that the FFPET-isolated DNA might prove to be a challenging source of DNA for genotype analysis. However, a significant number of blood samples were lost due to a flood that occurred at the storage site, and for this reason we chose to use FFPET rather than omit these cases completely. We anticipated that the FFPET samples would not yield results as robust as those from blood but thought that that we would be able to determine the genotype of some of these cases. Thus, to improve the quality of our data, we chose to use 3 quality filters: failure due to low rate of primer extension, low confidence based on Typer software, and lack of reproducibility.

As shown in Table 1 the assays that failed due to a failure to extend the primer (uepQC) is much higher in the FFPE samples than in the blood samples. There are 3 pools of assays (W1, W2, W3). The W1 pool contains the primers which amplify the SNPs in the following genes: rs1801274 (*FCGR2A*), rs1136201 (*ERBB2*), rs8187710, (*ABCC2*), rs13058338 (*RAC2*), and rs1799945 (*HFE*). The W2 and W3 assays contain a single pair of primers that are used to interrogate the rs396991 SNP in the *FCGR3A* gene with the original primers (W2) and with the design_2 primers (W3).

We also segregated the data such that alleles considered by the Typer software to be conservative or moderate as passing a second quality control (Table 2). As expected the % of samples that

passed quality control (qc) was much higher in blood-isolated DNAs than in FFPET DNAs. Importantly, the % of samples that passed this quality filter was much higher among the FFPET samples when design_2 was used than when the original design was used for SNP rs396991. This is additional evidence that design_2 was a better design than the original design for genotyping the re396991 SNP.

The different assays were also analyzed for their concordance between replicate runs (Table 3). Again, this analysis showed that all blood samples performed better than FFPET samples and that design_2 performed better than the original design. There were many fewer discordances in blood than in FFPET-isolated DNAs as well as in design_2 (18/530) versus the original design (110/530) (Table 3). Among the blood samples, there were 0 discordances out of 306 tested for 4 of the assays, 2 discordances for assay rs1801274, and 6 out of 1772 for rs396991.

Table 1 Measurements which failed or passed QC based on primer extension rate

Count – MEASUREMENTS		uepQC		Total Result
assay.pool	suspected.tissue.source	fail	pass	
W1	blood	180	6800	6980
	ffpe	35	1785	1820
W2	blood	62	2231	2293
	ffpe	25	702	727
W3	blood	2		2
	ffpe	21	697	718
Total Result		325	12215	12540

Table 2 Measurements which passed quality control based on Typer confidence calls

Count – MEASUREMENTS		softwareQC		Total Result	fail	pass
Assay.Id	suspected.tissue.source	fail	pass			
rs1136201	blood	42	1354	1396	3.0%	97.0%
	ffpe	56	308	364	15.4%	84.6%
rs13058338	blood	51	1345	1396	3.7%	96.3%
	ffpe	37	327	364	10.2%	89.8%
rs1799945	blood	37	1359	1396	2.7%	97.3%
	ffpe	30	334	364	8.2%	91.8%
rs1801274	blood	44	1352	1396	3.2%	96.8%
	ffpe	61	303	364	16.8%	83.2%
rs396991	blood	239	2054	2293	10.4%	89.6%
	ffpe	269	458	727	37.0%	63.0%
rs396991_design2	blood		2	2	0.0%	100.0%
	ffpe	121	597	718	16.9%	83.1%
rs8187710	blood	87	1309	1396	6.2%	93.8%
	ffpe	43	321	364	11.8%	88.2%
Total Result		1117	11423	12540	8.9%	91.1%

Table 3. Concordance between replicates.

Count – MEASUREMENTS		concordance QC				
Assay.Id	suspected.tissue.source	fail	NA	pass	Total Result	discordances
rs1136201	blood		1090	306	1396	0/306
	ffpe		364		364	0/0
rs13058338	blood		1098	298	1396	0/298
	ffpe		364		364	0/0
rs1799945	blood		1088	308	1396	0/308
	ffpe		364		364	0/0
rs1801274	blood	2	1096	298	1396	2/300
	ffpe		364		364	0/0
rs396991	blood	6	521	6	2293	6/1772
	ffpe	110	419	198	727	110/308
rs396991_design2	blood		2		2	0/0
	ffpe	18	188	512	718	18/530
rs8187710	blood		1106	290	1396	0/290
	ffpe		364		364	0/0
Total Result		136	8428	397	12540	

Figure 1.

Concordance of T Allele Frequency for SNP in *FCG3A*

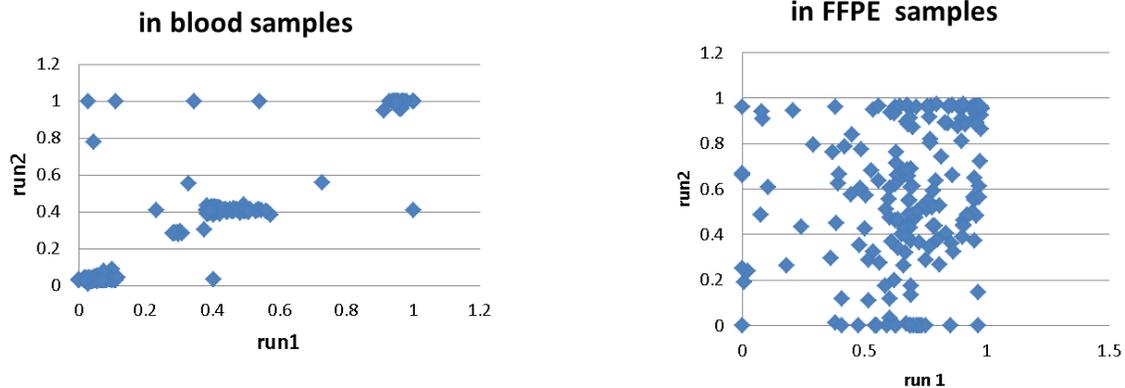


Figure 2

**Concordance of G allele frequency in
FFPE with Primer Design 2 for SNP in
*FCG3A***

