National Surgical Adjuvant Breast and Bowel Project (NSABP) Foundation

Annual Progress Report: 2011 Formula Grant

Reporting Period


Formula Grant Overview

The NSABP Foundation received $851,360 in formula funds for the grant award period January 1, 2012 through December 31, 2014. 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 – 12/31/2014

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 FCγ receptors and ERBB2 in all B-31 patient samples available for analysis, approximately 1400 cases. Previous reports indicated that certain polymorphisms in FCγ 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

The research work began on April 20, 2012 when grant funds were received. In this report period of 3 months, we have developed the assays to address the following specific aims:

1. Identify DNA sequence alterations associated with resistance to trastuzumab chemotherapy in Her2-positive breast cancer; and
2. Identify DNA sequence alterations associated with trastuzumab cardiotoxicity.

To address specific aim 1, we have proposed to determine the association of SNPs in the FCGR2A (Fc fragment of IgG, low affinity II a receptor) and FCGR3A (Fc fragment of IgG, low affinity III a receptor [CD16A]) genes with trastuzumab response using DNAs from patients who participated in the NSABP clinical trial B-31. However before we can use DNA from B-31, we must first demonstrate that we are able to successfully assay these mutations in DNAs from sources other than from patients in the B-31 trial because material collected in B-31 is more appropriately used for validation of assays that have already been shown to work, rather than for development of assay methods. Therefore, we have used commercial preparations of DNA from cell lines to test our assays.

**SNP Analysis of FcγRIIa and FcγRIIIa Polymorphisms Using the Sequenom® Platform.** We have developed an assay for the relevant SNP in the FCGR3A gene (rs396991). This G/T SNP results in an amino acid change at position 158. The G allele codes for a valine and the T for a phenylalanine. Because there is extensive homology between FCGR3A and FCGR3B, it was necessary to do a nested PCR amplification. In the first round of amplification the forward and reverse primers were GCTGCAGGGCCAGACCCAG and CACTCCGTGGCCACCGTCAC, respectively. Specificity for the FCGR3A gene is due to the reverse primer, which has 3 bases unique to the FCGR3A gene. These PCR products were used in a second round of amplification using the following PCR primers: ACGTTGGATGTTCAGGTCAAAGACAC, ACGTTGGATGTCCAGGTACACAGCT and extension primer GACACATTTTACTCCAA. All Sequenom PCR primers contain the same 10 bases on the 5-prime ends to enhance the specificity of the PCR reaction. The cluster plot of the extension products from 12 different cell lines and 'no template' controls is shown in Figure 1. The heights of the 2 allele peaks on the X and Y axes are plotted. Two cell lines had the TT genotype and are seen in Figure 1 as orange dots along the vertical axis; eight cell lines had a TG genotype and are seen as green dots in the center of the plot; and two cell lines have the GG genotype seen as blue triangles along the horizontal axis of the plot. The no template controls (NTC) are the red dots at the bottom left of the plot. These results demonstrate that this assay can detect all 3 genotypes at this locus.

The polymorphism in the FCGR2A gene is a C/T polymorphism at amino acid position 131 and is also known as SNP rs1801274. The (C) allele encodes arginine (R) and the (T) allele encodes histidine (H). The (H) isoform has a high-binding affinity to IgG2 and IgG3, while the (R) isoform is considered to be low-binding. Genomic DNAs from cell lines were amplified with ACGTTGGATGTGCTGACTGACTTGTGTGCTGCT and ACGTTGGATGTGCTTCCAGAATGGAATTACCC and extended with AGAAGGTGGATCCAAA. Figure 2 shows that seven cell lines are homozygous for the T
allele, (orange dots), four are homozygous for the C allele (blue triangles), and one contains both the C and T alleles (green dot). No amplification was seen with the NTC, red dot.

To address specific aim 2, a Sequenom assay for the ERBB2-Val655Ise SNP (rs1136201) was developed. This SNP has previously been described as being associated with trastuzumab cardiotoxicity.

Commercially available genomic DNAs were amplified with ACGTTGGATGAGCAGAATGCCAACCACCG and ACGTTGGATGACTAGCCCTCAATCCCTGAC and extended with GCCAACCACCGCAGAGA. Figure 3 shows a cluster plot from the results from genotype analysis from 11 different cell lines and a no template control (NTC). Seven cell lines are homozygous for the A allele (orange circles), two cell lines are homozygous for the G allele, (blue triangles) and two cell lines have a GA genotype (green dots). The NTC sample is seen at the lower left. This demonstrates that these assays are able to detect all 3 genotypes.

**Other Detailed Methods**

Cell line DNAs for genotype studies were SK-BR-3, HCC1395, NCI-HCI1299, MCF7, NCI-H358, NCI-H1395, A2058, MDA-MB-231, HT-29, HS578T, UACC893 and HL-60. Conditions for amplification, inactivation of unincorporated dNTPs, and the one base pair extension have been described with minor modifications. The iPLEX® Pro kit (Sequenom®) has replaced the discontinued Typlex® reagent kit. Removal of salt and other contaminants from the reactions will be removed via a cation exchange resin and loaded onto SpectroCHIP® II Arrays. The height of the separable peaks allows for the quantification of each oligonucleotide—which allows for detection of each allele even when it represents only 2.5 % of the alleles for some of the assays, as we have previously shown.

**Summary**

In conclusion we have developed all of the proposed SNP assays which can be used to interrogate the DNAs from patients in the B-31 trial—which will allow us to do the proposed SNP analyses.
Fig. 2 Cluster Plot of FCGR2A-Val131His SNP

rs1801274

- No Call (2)
- C (4)
- CT (1)
- T (7)
- Other (2)

High Mass Height vs. Low Mass Height
Fig. 3 Cluster Plot of ERBB2-ile655Val SNP

rs1136201

- No Call (3)
- G (2)
- GA (2)
- A (7)
- Other (3)