University of Pittsburgh

Annual Progress Report: 2012 Formula Grant

Reporting Period


Formula Grant Overview

The University of Pittsburgh received $7,752,646 in formula funds for the grant award period January 1, 2013 through December 31, 2016. Accomplishments for the reporting period are described below.

Research Project 1: Project Title and Purpose

Understanding the Role of Inflammation in Chronic Obstructive Pulmonary Disease – Chronic Obstructive Pulmonary Disease (COPD) exhibits significant variability in disease pattern and rate of progression. At this time, no clinical attribute or biomarker can determine disease prognosis in an individual patient. This project will offer the most robust analysis to date defining the natural progression of tobacco-related lung disease, which should lead to a reclassification of COPD into more meaningful biological phenotypes. We further aim to discover biomarkers that identify patients at greatest risk for disease progression. Discovery of new markers associated with unique subclasses of disease will lead to more effective personalized treatment algorithms and monitoring of a therapeutic response.

Anticipated Duration of Project

1/1/2013 – 12/31/2016

Project Overview

Chronic obstructive pulmonary disease (COPD) is the third leading cause of death in the United States. Clinical presentation and clinical progression can vary dramatically between individuals with respect to the traditional measure of impairment in forced expiration and the potentially independent attributes of parenchymal emphysema, airway narrowing, symptoms, exercise impairment or associated comorbidities. Remarkably, no clinical attribute or biomarker can predict progression rates of these parameters in an individual patient.

Our first aim is to classify COPD into subphenotypes representing unique disease pattern and prognosis using both cross-sectional baseline and 6-year longitudinal measures of change in physiologic, radiographic, and clinical/demographic features reflecting the activity of various independent pulmonary and comorbid conditions. Five hundred subjects from the Pittsburgh Specialized Center in Clinically Oriented Research (SCCOR) cohort will return for 6-year follow-up measures, including visual and quantitative CT analysis of airway remodeling and
parenchymal emphysema, physiologic measures of air flow obstruction and air trapping, incremental shuttle walk exercise, symptom/demographic and quality of life surveys, and measures of vascular and bone comorbidity. We will perform both supervised and unsupervised clustering approaches to accomplish the reclassification. Successful completion of this aim will offer the most robust analysis to date defining the natural progression of tobacco-related lung disease and allow significant progress toward reclassification of COPD into more meaningful biological phenotypes.

Our second aim will be to define peripheral blood molecular and cellular biomarkers that identify progression of independent clinically relevant measures of COPD progression as well as the unique clinical and prognostic phenotypes defined in Aim 1. We have identified nearly 30 novel blood molecular and cellular biomarkers, many of which have never been linked with lung pathogenesis, that associate with unique cross-sectional COPD subphenotypes and/or short-term longitudinal progression of these phenotypes.

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**Expected Research Outcomes and Benefits**

We have developed a longitudinal design wherein we will identify patterns of disease progression over time in patients with tobacco-related lung disease and further define blood markers that are predictive of these patterns. This analysis offers a significant advance beyond the single point cross-sectional methodology available in the published literature. We believe that, given the likelihood that disease activity varies within an individual in the course of chronic disease, cross-sectional characteristics may not always reflect the consequences of molecular and cellular events that drive disease progression. We therefore believe that biomarkers associated with disease activity represented by changes in radiographic and/or physiologic indices over the course of a 6-year study are more likely to reflect the pathogenic events most relevant to disease subclassification. Novel biomarkers that are predictive of more clearly elucidated subcategories of COPD disease activity, and which will emerge following successful completion of our aims, are desperately needed in the clinical and scientific arenas. Such markers will enable progress with personalized therapeutics that alter disease progression by targeting disease-specific pathways and will distinguish patients with active, progressive processes from those with stable, apparently quiescent, chronic processes that do not warrant expensive or potentially detrimental therapies.
Summary of Research Completed

**Aim 1:**

Study initiation: We have completed IRB modification and received approval. We have modified data collection forms and made final administrative arrangements for radiographic, physiologic, DEXA, and vascular study testing. We have initiated recruitment of subjects from the Pittsburgh SCCOR cohort consisting originally of former smokers (>10 pack-years), age >40, with no significant non-COPD related comorbidities. Patient accrual for the CV3 (six year +/- 6 mo. follow-up) cohort is proceeding as shown in Figure 1. Thus far, 22 subjects have consented, enrolled, and received testing. Chest computed tomographs have been analyzed using a semiquantitative visual scoring as well as quantitative density histogram analysis under Dr. Leader’s direction.

T-cell studies on these subjects have been completed in Dr. Duncan’s laboratory on the day of collection: Peripheral blood mononuclear cells (PBMNCs) were isolated from fresh venous phlebotomy specimens. PBMNCs were characterized for selected cell-surface receptors. We are finalizing logistics for the measurement of intracellular cytokine levels in the effluent of stimulated cells. Blood specimens have been processed and frozen in Dr. Zhang’s laboratory for use in future protein, RNA, and DNA genetic analyses.

Progression in subphenotypes over time: COPD not only varies cross-sectionally in phenotypic pattern but has varying levels of activity within an individual and between individuals, resulting in significant variation in progression of the various phenotypic attributes over time (Table 1 and Figures 2-4). The value of the extended longitudinal characterization is reflected in the early observations from our CV3 compared to 2-year follow-up (CV2). Of the 22 subjects completing baseline (CV1) and both CV2 and CV3 follow-up visits, including semiquantitative visual and quantitative CT parenchymal scoring of emphysema, five subjects demonstrated an increase in the visual emphysema score, suggesting progression of emphysema at CV2 whereas six patients demonstrated progression at CV3; only two patients had progression (defined by quantitative CT low attenuation unit increase of 0.5 percent) at CV2, whereas six patients demonstrated progression at CV3. In addition, seven patients demonstrated progression in airflow obstruction at CV2 (defined as a decrease in forced expiratory volume in one second [FEV1] percentage by 10 percent), whereas nine demonstrated a change at CV3. Notably, individual patients demonstrated progression in different sub-phenotypes defined by these three parameters, such that no subjects at either time point demonstrated progression in all sub-phenotypes.

Importantly, more patients with the longer follow-up (15/17) demonstrated progression in at least one phenotype at CV3, whereas only 9/17 demonstrated progression at the shorter CV2 follow up. This observation validates the importance of longer follow-up in improving our ability to determine molecular markers of disease activity and progression, a focus of this project.

While supervised and unsupervised clustering analyses on the longitudinal CV3 data will require a complete data set from patients collected over the several year duration of the project, we have continued to work with Drs. Tseng and Pererra on computational aspects of the CV1 and CV2 data sets. A peer reviewed manuscript is in press describing methodological issues inherent in classification of COPD patients. (Bon J, Liao S, Tseng G, Sciurba FC. Considerations and
Aim 2:

Peripheral protein marker determination
We determined the association of a putative regulatory single nucleotide polymorphism (SNP) rs7616177 of PTX3 with 2-year disease progression in order to further validate the proposed plasma biomarker pentraxin 3 (PTX3) as a plausible prognostic marker in the context of Aim 2. We have identified an association of plasma and tissue levels of PTX3 with quantitative emphysema. To extend our finding, we conducted a genotype analysis of a putative regulatory SNP, rs7616177, of the PTX3 on all subjects with 2-year change data available. The SNP rs7616177 is located at 2kb 5’ end of the transcription start site. Subjects with AA or GA genotypes (homozygous or heterozygous for the minor allele of the SNP) have a higher chance of clinical change in at least one subphenotype over two years compared to subjects with the GG genotype (47.0 percent compared to 34.1 percent) (Figure 5). This finding further strengthens our hypothesis that plasma biomarkers and genetic variants associated with the expression of these markers may be associated with the prediction of disease change over time.

T cell marker determination
Other ongoing studies have been initiated to identify mechanistically plausible markers to include in the analysis. These include T-cell expression of perforin, FoxP3, CD28, and CXCR5. The absence of CD28 (along with findings of perforin+ and FoxP3-) among circulating CD4 T-cells is a marker of repetitive antigen stimulation and lymphocyte end-differentiation and, thus, a plausible marker for pathogenesis of COPD.

In addition, five specimens collected in this study have been used in gene-function studies which are revealing important (and highly novel) biomarkers involved in the pathogenesis of smoking-associated COPD. We have discovered that certain HLA Class I Cw alleles are over-represented (notably Cw07) and others are under-represented (notably Cw05 and Cw12) among smokers who develop COPD. Cw alleles engage killer immunoglobulin-like receptors (KIRs) on the surface of NK cells, and these receptor-ligand interactions have distinct specificities. Some KIRs activate (or stimulate) NK cells (s-KIR), which then mediate cytotoxicity of the cells bearing these receptors, whereas others are NK-inhibiting (i-KIR). Hence, some Cw allele-KIR allele engagements induce cytotoxicity, whereas other permutations inhibit these functions. Epithelial cell cytotoxicity is believed to be an important component of COPD causality.

We have thus conducted functional studies using several specimens from the current study cohort. These assays demonstrate that NK cells have cytotoxic properties identical to those of COPD subjects during basal (unstimulated) conditions and after stimulation with IL-2 (a nonspecific NK cell activator), but markedly different behaviors after KIR ligation. The magnitude of KIR-stimulated cytotoxicity appears related to the severity of COPD. NK cell cytotoxicity after KIR-stimulation among PBMNCs from study subjects was correlated with their FEV1, a defining physiological parameter of expiratory airflow obstruction (e.g., COPD) (Figure 7). The proportion of NK cells among peripheral blood monocytes was examined and found to be increased among smokers with COPD (Figure 8). Thus, smokers
with COPD have genetic differences that enhance the cytotoxic potential of their NK cells, as well as having more of these immune effectors.

![Overall SCCOR Cohort Recruitment](image)

**Figure 1.** Recruitment of the CV3 extension relative to the overall cohort enrollment.

<table>
<thead>
<tr>
<th></th>
<th>Δ CV2</th>
<th>Δ CV3</th>
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<tbody>
<tr>
<td>FEV1 % Predicted</td>
<td>-4.01 ± 0.05</td>
<td>-8.04 ± 0.08</td>
</tr>
<tr>
<td>RV % Predicted</td>
<td>3.04 ± 0.1</td>
<td>10.35 ± 0.2</td>
</tr>
<tr>
<td>DLco % Predicted</td>
<td>-1.14 ± 0.1</td>
<td>-9.46 ± 0.1</td>
</tr>
<tr>
<td>SGRQ - Total</td>
<td>1.9 ± 6.0</td>
<td>3.3 ± 6.4</td>
</tr>
<tr>
<td>SGRQ - Activity</td>
<td>3.1 ± 9.5</td>
<td>4.3 ± 9.6</td>
</tr>
<tr>
<td>UCSD SOB Total</td>
<td>2.9 ± 7.0</td>
<td>5.3 ± 6.1</td>
</tr>
<tr>
<td>Shuttle Walk Distance (m)</td>
<td>-55 ± 51.6</td>
<td>-99 ± 58.4</td>
</tr>
<tr>
<td>MLVS</td>
<td>0.3 ± 0.4</td>
<td>0.4 ± 0.5</td>
</tr>
<tr>
<td>F950</td>
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<td>0.002 ± 0.01</td>
</tr>
<tr>
<td>Mean HU</td>
<td>-0.78 ± 20.2</td>
<td>-5.33 ± 19.6</td>
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**Table 1.** Changes from baseline at two years (CV2) and six years (CV3) of the 21 subjects enrolled.

FEV1 Percent Predicted - percentage of the predicted forced expiratory volume at one second;
RV Percent Predicted - percentage of the predicted residual volume representing air trapping at the end of forced expiration;
DLco Percent Predicted - percentage of the predicted diffusing capacity of the lung;
SGRQ – St. George Respiratory Questionnaire representing disease specific quality of life tool;
UCSD SOB - The University of California, San Diego shortness of breath questionnaire;
MLVS – Multi-Lobar Global Visual Emphysema Score representing a semiquantitative visual emphysema score;
F950 - fraction of lung voxels below -950 Hounsfield units as a ratio of lung volume representing quantitatively measured emphysema;
Mean HU - mean Hounsfield unit representing mean lung density.
Figure 2. Progression of airflow obstruction (FEV1), RV (trapped air), and SGRQ (quality of life) from baseline CV1, two year follow up (CV2) and six year follow up (CV3).

Figure 3. Distribution of change in outcome measure at CV3 visit compared to baseline. Note overall deterioration in each parameter over time, but great variation exists in individual subject progression.

Figure 4. The Venn diagram represents the number of subjects demonstrating significant disease progression within each subphenotype at CV2 and CV3. Notably, no subject demonstrated progression in all characteristics at either CV2 or CV3.
Figure 5. Subjects with the AA or GA genotypes have a higher chance of clinical change in at least one clinical subphenotype over two years compared to subjects with the GG genotype.

Figure 6. Variation in T-cell marker frequency within the subjects recruited at the CV3 visit.

Figure 7. NK cell cytotoxicity after KIR-stimulation among PBMNCs from study subjects was correlated with their FEV1.

Figure 8. Percentages of circulating NK cells among PBMNCs of smoking controls (SC, n=48) and COPD subjects (n=50). CD56 is a phenotypic marker of NK cells. The proportion of all CD56$^+$ cells was increased in COPD subjects.
Research Project 2: Project Title and Purpose

The Application of Adult Stem Cells and Native Tissue Matrices for Tissue Regeneration – Population incidence of degenerative joint diseases, including osteoarthritis, the leading cause of disability, is increasing, particularly in the elderly. The purpose of this research project is to develop novel tissue regenerative approaches to treat such diseases. We will investigate the regenerative activities of the recently identified induced mesenchymal progenitor cells (iMPCs), to use native tissue and cell matrices to engineer scaffolds with the optimal physical and biological microenvironment to support stem cell activities and differentiation, and to apply these novel technologies to enable point-of-care repair of joint defects and other musculoskeletal injuries in the future.

Anticipated Duration of Project

1/1/2013 – 12/31/2016

Project Overview

The increasing incidence of degenerative joint diseases such as osteoarthritis (OA) has contributed greatly to the burden of musculoskeletal diseases, particularly in the elderly. Cell-based therapy is a promising treatment approach for the engineering and regeneration of injured or diseased tissues. Adult stem cells, known as mesenchymal stem cells (MSCs), are able to differentiate into musculoskeletal cells and represent a promising cell type for cartilage tissue engineering. This project tests the utility of MSCs in tissue engineering applications in three different ways as addressed in the following specific aims:

Aim 1 – Use stem cell reprogramming techniques upon MSCs to create an inexhaustible reservoir of a patient’s MSCs suitable for the repair of large-scale, multi-tissue defects. The development of the induced pluripotent stem cell (iPSC)-based approach to generate induced multipotent cells (iMPCs) promises to overcome the paucity and limited expansion capacity of MSCs while retaining their pluripotency.

Aim 2 – Develop and characterize biomaterial scaffolds incorporating tissue matrix components for tissue regeneration with enhancement of tissue specific and stable differentiation of stem cells. Such bioactive matrices promise more effective long-term tissue repair by producing engineered tissues with anabolic/catabolic activities and mechanical properties more similar to those of native, healthy tissues; and

Aim 3 - Test the ability of engineered cartilage constructs produced with tissue-matrix enhanced scaffolds seeded with iMPCs or MSCs, and assembled in situ using a novel live-cell photopolymerization technique to repair cartilage lesions. Tissue repair will be tested in vivo using an articular cartilage degeneration model produced via supraphysiological impact in medium (rabbit) and large (goat) animals.

Positive results from these proof-of-concept studies will yield a rational basis for the design of clinical trials using this cell- and natural-matrix-based technology. These studies will also contribute to developing a one-step tissue repair procedure in the operating theater.
Expected Research Outcomes and Benefits

The new discipline of regenerative medicine aims to restore form and function to the patient via promotion of the regenerative activities of cells and tissues. Cell-based therapy is one of the most promising approaches in regenerative medicine. Adult human mesenchymal stem cells (MSCs) are able to differentiate into multiple cell types and represent a highly promising cell type in cell-based therapy; however, their use is limited due to their paucity, finite expansion capacity, and loss of differentiation capacity with each passage. An integrated set of studies is described here aimed at biological repair of articular cartilage in degenerated joint diseases, such as osteoarthritis (OA). We will first characterize the differentiation capacities and trophic properties of the newly identified iPS-cell derived mesenchymal progenitor cells (iMPCs) for use in tissue engineering and regenerative medicine. This technology promises to provide an inexhaustible source of autologous stem cells. In the second subproject, we will extract and incorporate native tissue or cell matrix components into nanofibers and hydrogels to create native matrix enhanced (NME)-scaffolds with bioactive microniches to promote complete and functional differentiation of the encapsulated stem cells. In the third sub-project, we will develop live-cell photocrosslinking technologies to employ these matrices for in situ tissue repair. We will then test iMPCs encapsulated within the NME scaffolds in articular cartilage repair in vivo, using animal models of early post-traumatic OA. Findings from these studies will lead to the development of innovative clinical approaches using autologous stem cells and bioactive scaffolds to treat degenerative joint diseases such as OA, the primary cause of disability in the elderly.

Summary of Research Completed

This project has not yet begun. No funds have been spent on this project.

Research Project 3: Project Title and Purpose

Dissecting the Molecular Mechanisms of Stress-Response Proteins in Cancer – This project leverages modern biocomputation and experiments to study the molecular mechanisms of stress-response proteins in cancer. We have selected two key stress response proteins, Y box binding protein 1 (YB-1) and heat shock protein 27 (Hsp-27), that are not only highly conserved but also
involved in cancer drug resistance. Our approach will: (i) define, establish, and further study the YB-1 and Hsp27 interaction networks by understanding these networks’ composition and evolution and by determining the phenotypic consequences of their perturbations (Aim 1); and (ii) determine the physical principles of protein-protein and protein-ligand interactions in the Hsp27 and YB-1 networks (Aim 2).

**Anticipated Duration of Project**

1/1/2013 – 12/31/2016

**Project Overview**

Stress response proteins play a critical role in the establishment, progression, and metastasis of tumors. As such, they serve as important biomarkers for diagnostic strategies as well as targets for therapeutics. Y box binding protein 1 (YB-1) and heat shock protein 27 (Hsp27) are two highly conserved stress response proteins that function in critical aspects of cancer progression and multidrug resistance. YB-1 is a promising breast metastasis marker and is upregulated in prostate cancers. YB-1 also associates with Argonaute proteins, which are central to small RNA-mediated silencing events. Increased levels of Hsp27 expression often correlate with poor prognoses of many types of cancers, likely due to the cellular protective effects of Hsp27. This effect can be due to one or more of the many functions of Hsp27, which include protein chaperoning, redox homeostasis maintenance, and remodeling of the actin cytoskeleton. A more detailed examination of these important genes, their homologs, and their interaction networks will provide important insights into essential aspects of cancer biology.

This project will take a multi-pronged approach to examine the structural, network, and functional aspects of YB-1 and Hsp27 using both computations and experiments to achieve the following two aims:

**Aim 1:** To establish and characterize the molecular interaction networks of YB-1 and Hsp27

**Aim 2:** To structurally characterize YB-1 and Hsp27 macromolecular complexes.

Computational approaches such as efficient variants of molecular dynamics simulations, coarse-grained modeling, sequence analysis, network methods, and phylogenetic modeling will yield new insights into the structural determinants of these proteins as well as refining and revealing their interaction networks. *In vitro* and *in vivo* experimental methods, along with next-generation sequencing, will probe the mechanistic bases of YB-1 and Hsp27 function in a variety of settings. Together, these studies provide a comprehensive and integrated approach that will elucidate basic mechanisms underlying the functions of stress response proteins in cancer.

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Expected Research Outcomes and Benefits

The following outcomes are anticipated:

- Description of the functionally important residues in Hsp27 multimerization by structural and evolutionary techniques
- Identification and characterization of YB-1 macromolecular interactions by structural, evolutionary, and sequence-based methods
- Predictions of small-molecule lead-compounds that disrupt Hsp27 multimerization and YB-1 interactions with proteins and RNAs
- Identification and validation of novel short RNAs that bind to YB-1
- Functional characterization of the molecular mechanisms of Hsp27 in cell elimination processes during development.

Summary of Research Completed

We previously aimed to identify and characterize of YB-1 interactions with short RNAs, as well as infer the macromolecular interactions of YB-1 using sequence-structure analysis methods. We are pleased to report that these goals have been reached and we are preparing our results for publication. We also describe progress on our studies of the role of the Hsp27 protein in cell death and engulfment.

Identification of molecular interactions in the YB-1 pathway

In Aim 1a, we proposed to reveal key molecular interactions involved in the YB-1 pathway. We find that YB-1 interacts with a range of small RNAs that can be classified primarily into two distinct subpopulations. To investigate the presence of these short RNAs, the 20-100 length nucleotide fraction of RNAs in various cell types was immunoprecipitated with YB-1 and extracted and sequenced using the Illumina sequencing approach. Among the 257,907 genomic locations corresponding to distinct short (>40 nucleotides) Yb-1-associated RNAs (shyRNAs), approximately 62 percent of the locations generate Yb-1-associated small RNAs that considerably overlap (≥ 80 percent) their cognate short RNA sequences. The correlation between the abundance of shyRNAs and their small RNAs is also high (R=0.66). To investigate whether mRNA expression correlates with the corresponding shyRNAs and small RNAs, we quantified transcript levels of polyadenylated RNAs in the human prostate cancer cell line, PC-3, using direct RNA sequencing (DRS) as well as previously generated RNA-Seq data, available from the NIH sequence read archive (SRA) database. Negligible correlation is seen between mRNAs and the shyRNAs, as well as mRNAs and the small RNAs (R=0.03–0.2). These results suggest that shyRNA sequences generally serve as precursors to Yb-1-associated small RNAs.

To gain further insights into the YB-1 pathway interactions, we probed whether known RNA polymerase (RNAP) II binding sites preferentially overlap with well-transcribed shyRNA genomic locations (> five reads; 51,510 locations). Interrogation of global run-on sequencing
(GRO-seq) data of transcriptionally engaged RNA polymerases (RNAPI I-III) reveal that shyRNA locations across all tested five datasets are several-fold more enriched than both random and exon control (Figure 1A). The preferential association of polymerases at shyRNA locations at scales much higher than that of exon controls, as indicated by GRO-seq data of RNAPIII from multiple cell lines, supports the notion that shyRNAs represent independent transcriptional units. If this interpretation is correct, specific transcription factors (TFs) must also bind preferentially at shyRNA locations.

To test for TF binding, we downloaded ENCODE ChIP-seq binding sites for 148 TFs across 433 experimental conditions and analyzed TF binding patterns at shyRNA locations. Several TFs emerged as significantly (p<10^{-10}) overrepresented at shyRNA locations, at enrichment levels even higher than that of exon controls. Notably, CTCF (CTC binding factor protein), a well-known partner of YB-1, is overrepresented at shyRNA locations in the T47D cell line at levels higher than that of both random and exon controls (Figure 1A) and accounts for about 6 percent of the shyRNA locations (± 500 nucleotides). In contrast, the pattern is reversed in other cell lines where the CTCF occupancy pattern at shyRNA locations resembles that of random control locations (Figure 1A, johnlab.org/YB1). In addition to known master regulators such as CTCF and p300, binding patterns of a few additional TFs are noteworthy, particularly Znf274, KAP1, ZBTB33, Bcl3, and Bcl11a. The C_{2}H_{2} zinc finger transcription factor, Znf274, participates in transcriptional repression through its recruitment of the chromatin-modifying repressor and zinc binding protein KAP1. Remarkably, Znf274 is highly enriched at shyRNA locations in multiple cell lines including K562, a cell line in which KAP1/TRIM28 is also highly over-represented at shyRNA locations. While Znf274’s preference for shyRNAs is consistent across all five cell lines, the shyRNA binding patterns of its family members, Znf263 and Znf143, are similar to random controls across all cell lines, including K562. Furthermore, another zinc binding protein, ZBTB33/Kaiso, which is a CTCF partner, is also highly enriched at shyRNA locations in the K562 cell line. Interestingly, some related proteins, such as the B-cell lymphoma-related proteins Bc11a and Bcl3, are also overrepresented at shyRNA locations. Bcl3 strongly manifests this pattern in K562 cells, but not in Gm12878, an immature cell type. The identification of the aforementioned proteins that participate in the YB-1 pathway led us to identify a tightly regulated network consisting of these proteins and YB-1.

Identification and validation of short RNAs that bind to YB-1
Since the next-generation sequencing of RNAs associated with YB-1 resulted in thousands of RNAs, we sought to validate the association of these non-coding RNAs with YB-1. We noticed that well-characterized cytoplasmic small/short non-coding RNAs, such as mirtrons, and vault RNAs are also present among the Yb-1-associated RNAs, including the recently discovered MALAT1-associated small (about 60 nucleotides) cytoplasmic RNA, and we have identified that mascRNA generates a novel small RNA that is processed precisely from its 5’ end. Similarly, 77 percent (10/13) of the most well-characterized cytoplasmic human mirtrons were detected. Three of the four cytoplasmic vault RNAs overlap with both shyRNAs and their terminal small RNAs. Unlike snoRNAs, which manifest a clear preference toward YB-1 in both the shyRNA and small RNA fractions, we cannot rule out the possibility that some of the annotated RNAs are within the shyRNA population simply because of the multifunctional nature of YB-1 as well as artifacts associated with library preparation and sequencing.
To exclude the latter possibility, we used qPCR on size-fractionated RNAs to probe the observed association of 26 RNAs with YB-1. The qPCR results reconfirm that nearly all 26 RNAs are highly enriched in the cytoplasm (> five fold) in YB-1 fractions at either the shyRNA or the small RNA levels (A-C). The sequencing and validation of these known cytoplasmically-localized RNAs support the notion that the shyRNAs and their small RNAs are cytoplasmically localized.

To investigate whether any of the shyRNA has a functional role in PC-3 cells, we used locked nucleic acid (LNA) oligomers that are complementary to a small/short RNA (Shad1) that we discovered, as well as an additional oligomer that is complementary to Shad1 shyRNA but not to the small RNA (Figure 4). Transfections of Shad1 LNA result in an increase in metabolic activity (MTT assay) compared to the control LNA oligomer. Cytotoxicity assays of the transfections showed no increased cell death in the transfection reactions compared to negative (no LNA) and positive (fully lysed cells) controls. Cell counts with trypan blue exclusion also showed no cell death, with increased proliferation in the cells transfected with LNA oligomers that are complementary to Shad1. These results reveal that Yb-1-associated Shad1 limits the proliferation of PC-3 cells. Taken together with the observation that shyRNAs include many short RNAs such as Y-RNAs, pre-miRNAs, and snoRNAs which have regulatory roles, these findings support the notion that shyRNAs represent a group of RNAs that share a common pathway but have evolutionarily acquired diverse cellular roles.

pHMA as a tool to detect cell death and cell engulfment events in Drosophila cell culture
In Aim 1b, we proposed to characterize and validate the functions and predicted interactions of Hsp27. We have examined the role of Hsp27 in artificially-induced cell death using a Drosophila cell-based assay developed by Fishilevich and colleagues, which uses an S2R+ cell line (S2R+pHMA) that stably expresses Ubi-pHMA (ubiquitin promoter-driven pHluorin fused to moesin’s actin) binding domain. pHluorin is a pH-sensitive ratiometric derivative of green fluorescent protein (GFP). In acidic environments, the green fluorescence emitted is higher when stimulated with 470 nm light than with 410 nm light; in neutral environments, the emission spectra are reversed. Thus, comparing the ratio of emission using 410 and 470 nm light (R_{470/410}) gives a measure of the local pH, which allows one to detect the engulfment of cells in culture (cells engulfed in acidic compartments have a higher R_{470/410}). In healthy cells, pHMA localizes to the cortex, where it binds cortical actin, whereas in dying cells, pHMA diffuses into the cytoplasm. This change in localization provides a readout for cell death in culture.

Hsp27 is not necessary for artificially induced cell death in Drosophila S2R+ cells
To determine whether Hsp27 is required for artificially induced cell death in Drosophila cell culture, S2R+pHMA cells were treated with dsRNA targeting Hsp27 or dronc (Drosophila caspase 9), or were left untreated for 48 hours. Cells were observed at 48 hours and all appeared healthy (data not shown). Actinomycin D (AD; 0.1 μM) was added to the cell media to induce apoptosis and the cells were incubated for an additional 24 hours and imaged using phase-contrast microscopy. Control cells that were not incubated with dsRNA prior to addition of AD detached from the coverslip and exhibited appreciable amounts of cell death and fragmentation (Figure 5A). The cells also rounded up and lost their cortical localization of pHMA (Figure 5D). In contrast, cells that were treated with dronc dsRNA remained adherent to the coverslip, maintained pHMA at their cortices, and appeared healthy (Figure 5C, F). Cells treated with
*Hsp27* dsRNA were indistinguishable from untreated cells in that they rounded up, lost cortical localization of pHMA, and fragmented (Figure 5B, E), suggesting that Hsp27 is not necessary for artificially induced cell death in *Drosophila* cell culture.

**Hsp27 is not necessary for S2R+ cell engulfment by mbn2 cells following induced cell death**

To examine the role of Hsp27 in cell engulfment, we treated S2R+pHMA cells with dsRNA and AD as above and then added mbn2 cells to engulf the dying S2R+pHMA cells (8, 9). Mbn2 cells are derived from a larval malignant blood neoplasm cell line and become highly phagocytic when stimulated with low levels of the hormone ecdysone. S2R+pHMA or mbn2 cells were treated with dsRNA targeting *Hsp27*, *Dronc* (caspase 9), *drpr* (*CED-1* ortholog, which is a receptor on phagocytes) or *thread* (inhibitor of apoptosis protein). Mbn2 cells were then treated with 1 µM ecdysone, and S2R+pHMA were treated with 0.1 µM AD on the following day. One day later the cells were mixed at approximately 1:1 and imaged on a widefield fluorescence scope. The $R_{470/410}$ was calculated and plotted for each dsRNA at zero, four, six, and eight hours. The maximum level of engulfment was consistently seen at four hours, so the difference between the initial level of engulfment (T=0) and four hours was graphed to provide a readout of the maximal amount of engulfment.

Knockdown of *Hsp27* in S2R+ cells did not appear to prevent engulfment of dying S2R+ cells by mbn2 cells (Figure 6A, C). RNAi of *thread* promotes cell death of the S2R+pHMA cells, encouraging engulfment by mbn2 cells. RNAi of *dronc* inhibits cell death, preventing engulfment of S2R+pHMA cells by mbn2 cells (Figure 6A, C). Knockdown of *Hsp27* in mbn2 cells does not prevent engulfment of dying S2R+ cells by mbn2 cells (Figure 6B, D). RNAi of *thread* promotes apoptosis in mbn2 cells, decreasing the number of mbn2 cells capable of cell engulfment, thereby decreasing the overall amount of engulfment. RNAi of *dronc* inhibits cell death of mbn2 cells, increasing the number of mbn2 cells capable of engulfment (Figure 6B,D). Therefore, Hsp27 does not appear to play a necessary role in cell engulfment following artificially induced cell death in *Drosophila* cell culture.
Figure 1: Specific transcription factor and histone modifications mark shyRNA loci in a cell-type dependent manner. A) shyRNA 5’ locations are frequently located proximally (position 0) to protein binding sites in a context-dependent manner. The curves correspond to the distance distribution between the location of each feature (blue: shyRNAs; red: random exons; green: random genomic positions) and the nearest histone mark. Transcriptionally engaged polymerase locations (GRO-seq, left) are consistently more enriched toward shyRNAs in all cell lines analyzed. In contrast, regulatory factors such as CTCF manifest a cell-type dependent enrichment profile (e.g., T47D vs. H1hesc). Note that in embryonic stem cells, CTCF binding patterns at shyRNA locations are close to those random locations, while this pattern is strikingly changed in T47D, with enrichment levels surpassing that of exons. B) Examples of well-expressed shyRNA locations that precisely overlap with known binding sites of multiple TFs. Both locations reside within very low abundance genes (ANKRD30BL, UNC45B) as revealed by the lack of reads from both DRS and RNA-Seq of PC3 cells.
Figure 2: A putative network of molecular interactions and interaction partners involved in the YB-1 (YBX-1) pathway. The links represent known interactions that either have been identified as physical (e.g., YB-1 vs. CTCF) or inferred interactions (e.g., co-expression, shared protein domain). The network is constructed using the TFs that we identified as associated with the YB-1 pathway, and their neighboring interacting partners, detected by the GeneMania algorithm.

Figure 3: Validation of the observed preference of YB-1 to associate with different classes of RNAs. YB-1 associates with known miRNAs including let-7g precursor (A), snoRNAs (B), and other noncoding RNAs (C) such as Y RNAs, vault RNAs (VTRNAs) and their analog miR-886, and mirtrons (miR-1224, miR-1226).
**Figure 4:** Knockdown of Shad1 shRNA increases cell proliferation in PC-3 cells  A) MTT assay of cells 48 hours post-transfection. Percent cell viability is based on non-transfected cells plated at the same initial density. To reconfirm the anti-proliferative role of Shad1, an additional LNA oligomer (-50 nucleotides upstream of Shad1 small RNA) targeting an alternate location of Shad1 shRNA was used (* denotes p-value<0.05; n=4). B) Cell counts of transfected cells at 24 and 48 hours post-transfection (* denotes p-value<0.05; ** denotes p-value<0.001).

**Figure 5.** Hsp27 is not necessary for artificially induced cell death in *Drosophila* S2R+ cells.
**Figure 6.** Hsp27 is not necessary for cell engulfment in *Drosophila* S2R+ and mbn2 cells following artificially induced cell death. S2R+ (A, C) or mbn2 (B, D) were treated with dsRNA on day 1, mbn2 cells were treated with ecdysone on day 2, S2R+ cells were treated with Actinomycin D on day 3, and cells were mixed and imaged on day 4. **A, C** Knockdown of Hsp27 in S2R+ cells does not prevent engulfment of dying S2R+ cells by mbn2 cells. RNAi of Thread promotes cell death of S2R+ cells, encouraging engulfment by mbn2 cells. RNAi of Dronc inhibits cell death of S2R+ cells, preventing engulfment by mbn2 cells. **B, D** Knockdown of Hsp27 in mbn2 cells does not prevent engulfment of dying S2R+ cells by mbn2 cells. RNAi of Thread promotes apoptosis in mbn2 cells, decreasing the number of mbn2 cells capable of cell engulfment. RNAi of Dronc inhibits cell death, increasing the number of mbn2 cells capable of engulfment.

**Research Project 4: Project Title and Purpose**

*Mitochondrial Biology in Cancer: A New Therapeutic Target for Killing Tumor Cells* – The purpose of this study is to develop novel strategies that target mitochondrial biology to kill tumor
cells. Two complementary approaches will be examined. We have recently discovered that inhibition of mitochondrial fatty acid oxidation (FAO) causes diminished oxidative phosphorylation, loss of adenosine triphosphate (ATP), and cell growth inhibition. FAO inhibitors alone and in combination will be examined for tumor cell growth inhibition, and novel in vivo imaging will be used to monitor fatty acid uptake into tumors. The second aim of this project is to inhibit mitochondrial dynamics in combination with cisplatin to initiate apoptotic cell death. Both in vitro and in vivo models will be examined.

**Anticipated Duration of Project**

1/1/2013 – 12/31/2014

**Project Overview**

Tumor cells have increased energy demands and alterations in their cellular metabolism in order to undergo rapid proliferation. Mitochondria are important organelles that provide energy in the form of ATP and help to convert key intermediates into cellular building blocks such as nucleotides for DNA, fatty acids for lipids, and amino acids for proteins. These organelles also have the potential to kill the cell through the release of pro-apoptotic factors. This project investigates mitochondria as a therapeutic target for the treatment of several types of cancer, including breast, ovarian, melanoma, and head and neck, among others. Since mitochondria are shared by all cells in the body, the therapeutic window of inhibiting this organelle’s function might be very small, and monotherapy will have to be augmented with combinations of chemotherapies. This project will develop strategies to combine mitochondrial poisons with standard anticancer drugs to achieve efficient killing of tumor cells.

**Specific Aim 1:** We will test the novel hypothesis that tumor cells generate a large portion of energy through the process of fatty acid beta-oxidation. This aim will develop innovative approaches using fluorescence microscopy and positron emission tomography (PET) to visualize fatty acid beta-oxidation in living tumor cells. This aim will also test the idea that inhibitors of fatty acid beta-oxidation alone and in combination with a standard chemotherapy (cisplatin) will inhibit tumor cell growth.

**Specific Aim 2:** We will exploit a novel finding from our laboratory, that inhibition of an important mitochondrial protein, Drp1, in combination with administration of cisplatin causes rapid initiation of apoptosis. We will explore the effects of combination treatment with mdivi-1, a novel thio-quinazolinone derivative that targets Drp1, and cisplatin in a panel of cell lines representing multiple cancer types. We will also explore structure-function relationships of this novel inhibitor to increase treatment efficacy and examine efficacy and toxicity in a xenograft model. Finally, we will analyze the effectiveness of this treatment against ovarian cancer cells isolated from patients.
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Expected Research Outcomes and Benefits

This year, one in four Americans who die will die of cancer. This project will investigate new ways to kill tumor cells. Specifically, this study will target the mitochondria of tumor cells, which provide energy and important metabolites that support cell growth. One limitation of current treatment strategies is that cancer cells have inherent or acquired resistance to the killing effects of specific chemotherapeutic agents. This project seeks to develop new strategies to overcome drug resistance by combining novel mitochondrial toxins with highly used anticancer agents such as cisplatin. Our first approach involves eliminating the ability of tumor cells to burn fatty acids in the mitochondria. Under this aim, we will visualize cellular uptake of fatty acids using fluorescent and PET tracers; and, for the first time, we will be able to track fatty acid uptake into tumor cells. We will also ask whether inhibition of fatty acid beta-oxidation in combination with standard anti-cancer therapy (cisplatin) will inhibit tumor cell growth. Our second approach is to inhibit normal mitochondrial dynamics in combination with cisplatin. We have found and patented that mdivi-1, a novel thio-quinazoline derivative that targets a key enzyme controlling mitochondrial fission (Drp1), given in combination with cisplatin, activates apoptotic death in cells that are inherently resistant to cisplatin. This aim will test the hypothesis that cisplatin plus mdivi-1will increase the effectiveness of cisplatin and overcome both inherent and acquired resistance to cisplatin. Cisplatin resistance in ovarian cancer is a common problem that limits patient outcomes. This aim will also explore novel structure-function relationships of the parent compound (mdivi-1) to improve delivery and efficacy. Finally, this aim will include pre-clinical xenograft studies that will generate information essential to the conduct of a future phase I clinical trial. Completion of this project is expected to result in new therapeutic targets for the treatment of breast, ovarian, melanoma, and head and neck tumors.

Summary of Research Completed

We have made good progress on each of the five benchmarks proposed in the first six months of this project, as outlined below.

1. Analyze the effects of etomoxir on the growth of melanoma cell lines (WM-983A and WM-983B) using CyQuant (Aim 1). Etomoxir is an inhibitor of carnitine palmitate transporter 1 (CPT1) and thus blocks fatty acid (FA) utilization by tumor cells. We had previously shown that
metastatic melanoma uses both oxidative phosphorylation and glycolysis to generate energy, and we were interested in determining the extent to which melanoma tumor cells used fatty acids for the production of energy. For this study, we used two melanoma cell lines derived from the same patient, a primary melanoma (WM-983A) or a metastatic site (WM-983B). Cells were seeded in a 96-well plate at a density of 2x10^3 (983A) or 1x10^3 (983B) cells per well. Twenty-four hours later, cells were treated with etomoxir, and cell numbers were determined using the CyQUANT assay. The IC50 (inhibitory concentration at which the response is reduced by half) for etomoxir was determined 72 hours after treatment using the GraphPad Prism program. The IC50s were 86.5 and 72.7 μM for WM-983A and WM-983B, respectively. The reduction in cell numbers in the etomoxir-treated groups compared to controls indicates that melanoma tumor cells derive some of their growth potential from fatty acid beta-oxidation (data not shown).

2. Examine the effect of etomoxir on levels of oxidative phosphorylation in the melanoma cells (Aim 1). Having established growth inhibitory doses of etomoxir, we next measured the effect of etomoxir on the oxygen consumption rate (OCR), a measure of oxidative phosphorylation. The bioenergetic profiles of malignant melanoma cells WM983A and WM983B were obtained using the Seahorse XF24-3 Extracellular Flux Analyzer (Figure 1). After baseline measurements, etomoxir (eto; 100 μM) was added to the cells twice to determine the effect of blocking FA β-oxidation on OCR and extracellular acidification rate (ECAR). This was followed by trifluorocarbonylcyanide phenylhydrazone (FCCP) injection (300 nM). Both melanoma cell lines were sensitive to etomoxir, but the effect was higher in the metastatic cells (WM983B), which showed a 75% reduction in oxygen consumption in response to etomoxir. These data indicate that melanoma cells use FA β-oxidation to drive a major percentage of oxidative phosphorylation and clearly indicate an essential role of mitochondrial function in melanoma cell growth.

3. Quantify palmitate uptake and utilization in the melanoma cells lines (Aim 1). In collaboration with Dr. Eric Goetzman, we measured the uptake and utilization of 3H-palmitate in the two melanoma cell lines. Cells were incubated with 3H-palmitate for one hour, and the amount of palmitate uptake was measured by the amount of tritium in the organic extraction phase. Both cell lines took up 30-40 nmoles/mg/hr of palmitate in either phosphate buffered saline (PBS), or more surprisingly, Dulbecco's Modified Eagle Medium (DMEM). Additional plates of cells were incubated in the presence or absence of etomoxir for two additional hours, and the amount of palmitate metabolized to water through FA β-oxidation was measured by the amount of 3H-water produced. Both cell lines used about 1 nmol/mg/hr of palmitate in PBS; palmitate metabolism was inhibited by over 70% with the addition of 100 μM etomoxir. Cells treated in complete media (DMEM) showed about 0.35 nmol/mg/hr of palmitate utilization, of which about 70% was inhibited by etomoxir.

4. Prepare fluorescent thia-oleate and examine its uptake in head and neck cancer cells (OSC19, Cal33) in vitro (Aim 1). This work continues, but due to the potential development of intellectual property, cannot be released at this time.

5. Survey several cell lines for their sensitivity to the combination of cisplatin and mdivi-1 (Aim 2). Mitochondrial division inhibitor-1 (mdivi-1) is a thioquinazolinone that has been shown to inhibit mitochondrial dynamics by blocking the effect of Drp1, a mitochondrial fission protein. We have been studying the effects of mdivi-1 alone and in combination with cisplatin. We have
made the remarkable discovery that, while cancer cells are blocked at the G2/M phase of the cell cycle by mdivi-1, normal human fibroblasts are unaffected by this compound. These data suggest that something in the transformation process makes cells sensitive to the growth inhibitor effects of mdivi-1, and we are actively seeking the mode of action. To elucidate the synergistic effects of mdivi-1 and cisplatin, we analyzed apoptosis in several tumor cell lines. In this assay, cells (10^3 viable per well in a 384-well plate) are treated with various concentrations of mdivi-1 (0-50 μM) and cisplatin (0-50 μM) for 20 hrs, and the amount of caspase 3/7 activity is determined using a highly sensitive bioluminescence assay (Promega). As shown in Figure 2, we have found that a wide range of tumor cells are sensitive to the combination treatment of mdivi-1 and cisplatin. These include H1299 (colon cancer), LN-428 (glioblastoma), 2008 (cisplatin-sensitive ovarian cancer), 2008/C (ovarian cancer that is 8-fold resistant to cisplatin), and patient ovarian cancer cells derived from ascites fluid. Note that this patient had not yet been treated with cisplatin, but the tumor cells showed strong resistance to cisplatin.

Related PI publications supported by PA Department of Health CURE funds (1/1/13-6/30/13):
Figure 1. Effect of etomoxir on cellular bioenergetics. Melanoma cell lines were analyzed using a Seahorse Flux analyzer for both the oxygen consumption rate (OCR) and the extracellular acidification rate (ECAR), measures of oxidative phosphorylation and glycolysis, respectively. Note that the injection of etomoxir (ETO) causes a rapid decline in oxygen consumption and a concomitant increase in glycolysis. Trifluorocarbonylcyanide phenylhydrazone (FCCP) injection shows that cells still have the capacity to respond to uncoupling by increasing the oxygen consumption rates.
Research Project 5: Project Title and Purpose

A Pilot Study Evaluation of Sulforaphane in Atypical Nevi – This pilot trial is designed to determine the tolerance and pharmacokinetics of broccoli sprout extract-derived sulforaphane as a melanoma preventive agent in patients with multiple atypical nevi and a history of prior melanoma.

Anticipated Duration of Project

1/1/2013 – 12/31/2014

Figure 2. Synergistic induction of apoptosis by mdivi-1 and cisplatin. Apoptosis was assessed in human tumor cells 20 hours after treatment with the indicated concentrations of mdivi-1 and cisplatin. H1299 (colon cancer), A; LN-428 (glioblastoma), B; 2008 (ovarian cancer), D; 2008/C (ovarian cancer that is 8-fold resistant to cisplatin), E; and patient ovarian cancer cells derived from ascites fluid, C and F.
Project Overview

Sulforaphane is a bioactive compound derived from precursor molecules found in high concentration in Brassica vegetables like broccoli and broccoli sprouts. In relation to melanoma, application of broccoli sprout extract rich in sulforaphane (BSE-SFN) decreased ultraviolet radiation (UVR) erythema response in human skin and may thus protect against the DNA-damaging effects of UVR. We are conducting a pilot trial of daily oral BSE-SFN as a non-toxic, natural chemopreventive agent for melanoma, which will be administered in groups of 6 patients at 3 dosages of 50 mcg/d, 100 mcg/d, and 200 mcg/d for 28 days each, with the goal of evaluating two objectives:

1. The primary objective is to determine the safety and clinical as well as pathological effects of BSE-SFN for patients with multiple atypical nevi and a history of melanoma.

2. The secondary objective is to document the pharmacokinetics and pharmacodynamics of BSE-SFN and the ability of oral BSE-SFN to modulate the level of signaling molecules in tissue biopsies of atypical nevi and skin in patients with multiple atypical nevi and a history of melanoma.

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Expected Research Outcomes and Benefits

The safety and tolerability of sulforaphane at the three dosages to be tested is anticipated to be proven, and the pharmacological features of this agent administered orally in cohorts of 6 patients over a period of 28 days is expected to be determined. The laboratory corollary studies proposed will determine the pharmacokinetics in blood and skin as well as atypical nevi, which are to be biopsied prior to and following the 28-day treatment interval. Evaluation of the impact of sulforaphane on clinical, pathological and molecular features will inform future phase II cooperative group studies of this compound as a chemopreventive for melanoma.

Summary of Research Completed

As of June 2013, three subjects have been accrued to the trial, with two screen failures and three pending enrollment. During this reporting period, we have standardized the technique for
immunohistochemistry (IHC) digitization and semi-quantitative evaluation of the staining process using new software (Aperio). Samples have been validated and standard reagents have been formulated, and the laboratory is now prepared to commence analysis of patient samples. Based on our experience with the new software and the technical demands of sample analysis, we have decided to batch assays to minimize data variability. The pharmacokinetics and pharmacodynamics (PK/PD) laboratory has run pilots of the assays that they plan to employ and is prepared to run PK/PD analysis once the first half of patients have been accrued (expected to be completed in fall 2013). The study to date has shown no safety signals that would alter our plan to accrue 6 patients at each of the three dosages selected in the original protocol.

Research Project 6: Project Title and Purpose

A Phase III Study of Adjuvant Ipilimumab vs. High-Dose Interferon α-2b for Resected High-Risk Melanoma – In this clinical trial, patients with stage III or stage IV melanoma that has been completely resected and who are at high risk for recurrence and death after surgery will be randomly assigned to receive adjuvant (post-surgical) treatment with either ipilimumab (investigational agent) or high-dose interferon α-2b (HDI, the current standard of care). This study has the potential to change the management of patients with high-risk resected melanoma, if ipilimumab is shown to be superior to HDI as hypothesized.

Anticipated Duration of Project

1/1/2013 – 12/31/2014

Project Overview

This randomized phase III trial will test adjuvant therapy with the anti-CTLA4 monoclonal antibody (mAb) ipilimumab at high-dose (hiIpi) and standard dose (siIpi) versus the standard high dose interferon (IFN)-α2b regimen (HDI) in patients with surgically resected stage IIIB/C and M1a/b melanoma at high risk for recurrence and mortality. This ongoing trial plans to enroll 1600 patients nationally with up to 60 patients expected to be enrolled at the University of Pittsburgh Cancer Institute (UPCI).

Specific aims:
(1) Primary Endpoints:
1. To evaluate recurrence-free survival (RFS) between melanoma patients randomized to receive post-operative adjuvant ipilimumab given at either 10 mg/kg (high dose ipilimumab; HIP) or 3 mg/kg (low dose ipilimumab; LIP) versus those randomized to receive HDI using a hierarchical design assessing HIP versus HDI first and LIP versus HDI second, if the first comparison is significant.
2. To evaluate overall survival (OS) between melanoma patients randomized to receive post-operative adjuvant ipilimumab given at either 10 mg/kg (HIP) or 3 mg/kg (LIP) versus those randomized to receive HDI using a hierarchical design assessing HIP versus HDI first and LIP versus HDI second (if the first comparison is significant).

(2) Secondary Endpoints:
1. To evaluate safety and tolerability of post-operative adjuvant ipilimumab therapy given at either 10 mg/kg (HIP) or 3 mg/kg (LIP).
2. Among patients enrolled by Community Clinical Oncology Programs (CCOPs), to compare the global quality-of-life (QOL) between the ipilimumab arms versus HDI and to evaluate the effect of treatment-related side effects that may affect the health-related domains of QOL.

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Expected Research Outcomes and Benefits

The current standard of care for high-risk melanoma, high dose IFN-α2b (HDI), has limited efficacy as an adjuvant therapy. Ipilimumab offers a potentially superior adjuvant therapy, and its superiority compared to HDI has already been proven in the treatment of more advanced inoperable melanoma leading to regulatory approval. In addition, this study is unique in its aggressive and systematic investment in the banking of tumor and blood specimens for use in future predictive biomarker studies. Being able to predict which patients will benefit would radically improve our approach to adjuvant therapy and allow us to avoid toxicity in the absence of expected benefit.

Summary of Research Completed

This study has been accruing very well both nationally and at our site at the University of Pittsburgh Cancer Institute. Locally, a total of 39 subjects have been screened for the study, of whom 18 are currently active or in follow-up. Nationally, 957 subjects have been accrued as of June 14, 2013.

This trial has a quality-of-life component that includes a tissue banking corollary for the tumor blocks, serial blood serum, and mononuclear cells in preparation for the evaluation of blood, genetic, chemokine, and immunological therapeutic predictive biomarkers that may serve as predictors of benefit for ipilimumab as well as interferon alpha (IFNα). The ultimate goal of such biomarker studies is to minimize the number of patients to be treated by targeting those who are likely to benefit from therapy while saving others from potential toxicities and cost.
Research Project 7: Project Title and Purpose

A Phase I Study of ABT-888 in Combination with Carboplatin and Paclitaxel in Advanced Solid Tumors – The overall purpose of this trial is to determine the dose limiting toxicities and recommended phase II dose of the combination of weekly administered carboplatin, paclitaxel, and the poly (ADP-ribose) polymerase (PARP) inhibitor, veliparib (ABT-888). This proposed portion of the trial is a mandatory biopsy expansion at the recommended phase II dose in patients with triple negative breast cancer, which is postulated to have particular efficacy to this combination. Pre- and post-treatment biopsies will be analyzed for a molecular signature of faulty DNA repair mechanisms that may underlie sensitivity to this treatment.

Anticipated Duration of Project

1/1/2013 – 12/31/2014

Project Overview

The treatment of metastatic, triple negative breast cancer (TNBC) remains a challenge for clinicians. Although there is much molecular heterogeneity within TNBC, at least one specific type of triple negative disease, basal-like breast cancer, appears to be influenced by faulty DNA repair mechanisms. Based on the observations of increased poly (ADP-ribose) polymerase (PARP) levels and additional similarities between BRCA-mutation related and TNBC, clinical trials like this one have been studying PARP inhibitors in TNBC.

Specific Aim 1: Clinical trial. This trial is a phase I dose escalation trial to determine the dose-limiting toxicities and recommended phase II dose of weekly administered paclitaxel, carboplatin, and the PARP inhibitor, veliparib (ABT-888). This trial builds on the previous work with this compound at the University of Pittsburgh Cancer Institute (UPCI). Thus far, more than 20 patients have been enrolled and the recommended phase II dose has been determined.

Specific Aim 2: Evaluate biopsy samples for proof-of-principle (PAR and gamma-H2AX measurement). A pre-planned, mandatory biopsy cohort will now commence in patients with TNBC. Subjects will undergo a biopsy of a metastatic site prior to the start of therapy and after one cycle of therapy. The primary objective of this portion of the trial is to obtain additional data as to the efficacy of this combination in TNBC specifically and to obtain preliminary pharmacodynamic and molecular data examining why this combination may or may not be active in TNBC.

Specific Aim 3: Determination of preliminary predictors of response as defined by analysis of “BRCA-ness” (BRCA promoter methylation, Fanconi anemia pathway), and transcriptome/microarray analysis to determine gene signatures of DNA repair. A specific goal is to determine a molecular DNA response signature to assess which TNBCs have faulty DNA repair pathways similar to those seen in BRCA-mutation-related cancers, which may then explain why this combination therapy is more active in those patients.
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Expected Research Outcomes and Benefits

The expected outcome from this portion of the trial is the enrollment of 12 patients who will have pre- and post-cycle 1 biopsies of a site of metastatic disease. The correlatives planned with these biopsies include proof of principle (PAR and gamma-H2AX measurement), analysis of “BRCA-ness” (BRCA promoter methylation, Fanconi anemia pathway), and transcriptome/microarray analysis to determine gene signatures of DNA repair. The use of mandatory biopsies will provide unprecedented information about specific mechanisms of sensitivity and resistance that have yet to be performed on any published PARP inhibitor trial. Evaluation of the 12 patient samples from the mandatory biopsy expansion cohort in this trial will provide powerful preliminary data as to the validity of the gene signatures in patient samples and allow for the optimization of laboratory techniques on the small amount of tissue obtained from biopsy of metastatic sites.

Summary of Research Completed

This project has not yet begun. No funds have been spent on this project.

Research Project 8: Project Title and Purpose

*Phase II Study of Azacitidine and Entinostat (SNDX-275) in Patients with Advanced Breast Cancer* – The purpose of this trial is to determine the objective response rate, safety, tolerability, progression-free survival, overall survival, and clinical benefit rate of the combination of the DNA methyltransferase inhibitor azacitidine (5-AZA) and the histone deacetylase inhibitor entinostat in women with advanced breast cancer (triple-negative and hormone-refractory). In addition, an optional continuation stage will evaluate whether the addition of hormonal therapy to 5-AZA and entinostat provides benefit after progression while receiving 5-AZA and entinostat.

Anticipated Duration of Project

1/1/2013 – 12/31/2014
Project Overview

In preclinical breast cancer models, epigenetic modifiers lead to re-expression of aberrantly silenced genes and proteins that represent important therapeutic targets (e.g., estrogen receptor alpha, or ER). Combination therapy with a DNA methyltransferase inhibitor (DNMTI) and a histone deacetylase inhibitor (HDACI) yields superior ER re-expression and greater restoration of tamoxifen responsiveness than either agent alone. The proposed trial is a multicenter, phase II clinical trial to evaluate the efficacy, safety and surrogate biomarkers of response to the DNMTI 5-azacitidine (5-AZA) and the HDACI entinostat in women with advanced breast cancer. Women with advanced triple-negative (ER/progesterone receptor [PR]/HER2-negative, Cohort A) or hormone-resistant (Cohort B) breast cancer receive 5-AZA at 40 mg/m² subcutaneously (days 1-5, 8-10) and entinostat at 7 mg orally (days 3, 10) every 28 days. The primary endpoint is objective response rate (ORR) in each cohort. Secondary endpoints include safety, tolerability, survival, and clinical benefit rate. Exploratory endpoints include pharmacokinetics, pharmacogenetics, and change from baseline of candidate gene re-expression/methylation in circulating DNA and mandatory tumor samples. Because of the potential for restoration of hormone sensitivity, patients are offered continuation of study therapy at progression with addition of hormonal therapy (an optional continuation phase). This trial has a Simon two-stage design, with interim analysis being performed after 13 patients are enrolled per cohort (1st stage). If ≥1 response is observed, patient accrual will continue for a total of 27 per cohort (2nd stage). The null hypothesis is that ORR is at most 5%, against the alternative hypothesis that ORR is at least 20%, with type I error of 4% and power of 90%.

Specific Aim 1: Clinical trial
Accrual is ongoing at our institution for this phase II study of 5-AZA and entinostat in patients with advanced breast cancer.

Specific Aim 2: Evaluation of biopsy samples
Exploratory endpoints for this aim include pharmacokinetics, pharmacogenetics, and change from baseline of candidate gene re-expression/methylation in circulating DNA and mandatory tumor samples.

Specific Aim 3: Methylation analysis of primary tumors
Perform methylation analysis on primary tumors and determine whether the primary and metastatic lesions have a similar profile.

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Expected Research Outcomes and Benefits

The expected outcome of this trial is to establish the objective response rate (ORR) in each cohort of patients with advanced breast cancer who are treated with 5-AZA and entinostat. The secondary endpoints will be to examine the safety, tolerability, survival, and clinical benefit rate of the combined therapy. The unique feature of this trial is the mandatory biopsies of metastatic sites at baseline and after cycle 2, which will allow for exploratory scientific endpoints of change from baseline of candidate gene re-expression/methylation in the tumor samples. By taking advantage of the synergy between HDAC and DNMT inhibitors, we hypothesize that clinically tolerable doses may translate not only into changes in gene methylation, but also disease responses.

Summary of Research Completed

In this multicenter Phase II clinical trial to evaluate the efficacy, safety, and surrogate biomarkers of response to the DNA methyltransferase inhibitor (DNMTI) 5-azacitidine (5-AZA) and the histone deacetylase inhibitor (HDACI) entinostat, women with advanced triple-negative (estrogen receptor [ER]/progesterone receptor [PR]/HER2-negative, Cohort A) or hormone-resistant (Cohort B) breast cancer receive 5-AZA 40 mg/m² subcutaneously (days 1-5, 8-10) and entinostat 7 mg orally (days 3,10) every 28 days. The primary endpoint is the objective response rate (ORR) in each cohort. Secondary endpoints include the safety, tolerability, survival, and clinical benefit rate. Exploratory endpoints include pharmacokinetics, pharmacogenetics, change from baseline of candidate gene re-expression/methylation in circulating deoxyribonucleic acid (DNA), and mandatory tumor samples. Because of the potential for restoration of hormone sensitivity, patients are offered continuation of study therapy at progression with the addition of hormonal therapy (optional continuation phase).

In the fall 2012 (prior to the start of this funding), 13 patients (Eastern Cooperative Oncology Group [ECOG] 0-1) were enrolled in the first stage of the triple negative Cohort A (median age=47 [range 31-67], median number of prior chemotherapies=3 [range 1-5], 77% white/33% black, 77% visceral disease, median cycles received=2 [range 1-4]). Treatment, analysis, and follow-up of these patients continued into the current reporting period. The combination treatment was well tolerated in this group, with the most common grade 3/4 treatment-related adverse events being leucopenia and neutropenia (23% each). No partial/complete responses were observed following the first stage, and this cohort was closed. A median of 1.5 additional cycles were completed in the optional continuation phase (received by four patients), with no responses to date (one patient remains on therapy). These results were presented as an abstract at the 2013 American Association for Cancer Research (AACR) Meeting.

The hormone-resistant Cohort B has now proceeded to the second stage, as one partial response was observed in the first stage. The trial has now completed enrollment, with the last patient of this cohort consenting to begin therapy as of July 2013.

Research Project 9: Project Title and Purpose

Bevacizumab, Metronomic Chemotherapy, Diet and Exercise after Preoperative Chemotherapy
for Breast Cancer – The purpose of this phase II trial is to explore the role of anti-angiogenic therapy using bevacizumab combined with metronomic chemotherapy in patients with residual disease after neoadjuvant chemotherapy to determine the efficacy of these treatments. All patients will also be randomized to one of two lifestyle interventions to explore the impact of changes in diet and exercise on relevant biomarkers in a breast cancer survivor population. Given the lack of standard options in this clinical setting, it is hoped that the proposed novel biologic therapy will improve breast cancer outcomes in this high-risk patient population.

Anticipated Duration of Project

1/1/2013 – 12/31/2014

Project Overview

Patients with residual breast cancer after neoadjuvant chemotherapy have an increased likelihood of tumor recurrence. Currently, no standard systemic treatments exist for these high-risk patients who likely have chemotherapy-refractory disease. The proposed phase II trial will randomize patients with residual invasive breast cancer after neoadjuvant treatment with an anthracycline and/or a taxane to one of four interventions: 1) anti-angiogenesis inhibitor therapy with bevacizumab and metronomic chemotherapy with a dietary change, 2) bevacizumab and metronomic chemotherapy with a dietary and exercise change, 3) a dietary change without anti-angiogenesis inhibitor therapy, or 4) a dietary and exercise change without anti-angiogenesis inhibitor therapy.

Specific Aims:
1) Primary objective of the study is to compare the recurrence-free survival (RFS) in patients who are randomized to post-preoperative angiogenesis inhibitor therapy compared to those who are randomized not to receive the angiogenesis inhibitor therapy.
2) Secondary objectives are to: a) evaluate the feasibility and characterize the side effects and tolerability of bevacizumab and metronomic chemotherapy administration in this adjuvant setting; b) compare overall survival between the treatment groups; c) compare the impact of a lifestyle intervention incorporating diet and exercise modifications versus an intervention including only dietary modification on levels of fasting insulin and insulin-like growth factor-1 (IGF-1), change in body weight, and levels of biomarkers associated with breast cancer risk and prognosis; and d) broadly characterize the cardiovascular effects of exposure to three years of angiogenesis inhibitor therapy. Given the lack of standard options in this clinical setting, it is hoped that the proposed novel biologic therapy will improve breast cancer outcomes in this high-risk patient population.

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Expected Research Outcomes and Benefits

This phase II trial will provide insight into the clinical utility of a novel biologic therapy for the treatment of recurrent breast cancer. In addition, this study will evaluate the impact of lifestyle interventions that modulate physical activity, weight, and diet on breast cancer prognosis, which may also lead to improved outcomes for this patient population.

Summary of Research Completed

Unfortunately, this trial was closed due to slow accrual nationwide. As of 6/20/13, it was terminated by the University of Pittsburgh Institutional Review Board. No work was done; no CURE funds were spent on this project.