Thomas Jefferson University

Annual Progress Report: 2011 Formula Grant

Reporting Period

July 1, 2013 – June 30, 2014

Formula Grant Overview

Thomas Jefferson University received $2,899,793 in formula funds for the grant award period January 1, 2012 through December 31, 2015. Accomplishments for the reporting period are described below.

<u>Research Project 1: Project Title and Purpose</u>

FOXD3-Erbb3 Signaling as an Adaptive Response to RAF Inhibitors – Melanoma is the deadliest form of skin cancer and represents a paradigm for drug resistance. The serine/threonine kinase, B-RAF, is somatically mutated in 40-60% of melanomas. In phase 1-3 trials with mutant B-RAF melanoma patients, the RAF inhibitor, PLX4032/vemurafenib, has yielded promising results. However, some patients are intrinsically resistant to PLX4032 and initial responders relapse from acquired drug resistance. The mechanisms underlying resistance to RAF inhibitors are poorly characterized and must be elucidated in order to optimize future clinical trials. The purpose of this proposal is to understand mechanisms of resistance in melanoma in order to improve targeted therapeutic strategies.

Anticipated Duration of Project

1/1/2012 – 12/31/2015

Project Overview

Our working hypothesis is that up-regulation of a stemness transcription factor, FOXD3, and its target ERBB3/HER3 is an adaptive response that promotes resistance to PLX4032-induced apoptosis in mutant B-RAF melanoma cells. The experimental approaches are innovative in that we modulate B-RAF activity using clinical grade inhibitors and molecular approaches in physiologically relevant *in vitro* and *in vivo* models. At the completion of our experiments, we expect to have identified a novel resistance-promoting mechanism for RAF inhibitor-resistant melanoma cells.

<u>Specific Aim 1: Determine the role of FOXD3 in melanoma resistance to RAF inhibitors *in vivo*.</u> The hypothesis that FOXD3 promotes resistance to PLX4032/4720 *in vivo* will be tested. Initially, we will knockdown FOXD3 in mutant B-RAF melanoma cell intradermal xenografts, and determine effects on PLX4720-induced tumor regression and tumor resistance. We expect to identify FOXD3 as a factor contributing to RAF inhibitor resistance *in vivo*.

Specific Aim 2: To dissect the regions of ERK2 required for down-regulation of FOXD3 following B-RAF inhibition. To elucidate mechanisms underlying ERK2 regulation of FOXD3, erase and replace experiments to determine the ERK2 effector domains required for FOXD3 repression will be performed. We anticipate further elucidating mechanisms regulating FOXD3.

Specific Aim 3: Determine the role of ERBB3-ERBB2 signaling in resistance to RAF inhibitors. It is hypothesized that FOXD3 regulation of ERBB3 initiates the formation of an ERBB3-ERBB2 complex, which signals to promote resistance to PLX4032. We will test whether targeting ERBB2 promotes sensitivity to PLX4032 *in vitro* and orthotopic *in vivo* assays, and whether neuregulin-ERBB3 signaling is sufficient to promote resistance to PLX4032. We expect to identify activation of ERBB3-ERBB2 as a mechanism contributing to RAF inhibitor resistance.

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Expected Research Outcomes and Benefits

At the completion of the proposed study, we expect to have identified a novel resistance-promoting mechanism for RAF inhibitor-resistant melanoma cells. This is a key question since resistance is hampering the clinical benefit to RAF inhibitors in melanoma. Ultimately, this work could provide avenues to develop new therapeutic strategies for the treatment of melanoma. These avenues could be in combination with RAF inhibitors and may be particularly important in patients who develop resistance to initial treatments. Furthermore, the research may identify a marker that will serve to identify particularly resistant forms of melanoma.

Summary of Research Completed

We are continuing experiments to test our hypothesis that up-regulation of a stemness transcription factor, FOXD3, and its target, ERBB3/HER3, is an adaptive response that promotes resistance to vemurafenib-induced apoptosis in mutant B-RAF melanoma cells.

Specific Aim 1 is focused on determining the role of FOXD3 in melanoma resistance to RAF inhibitors *in vivo*. During this reporting period we generated a series of FOXD3 targeting shRNA constructs in a lentiviral delivery system and mutant BRAF melanoma cell lines that will express these shRNAs under inducible control. When we tested for efficiency of knockdown in these cell lines, there was insufficient knockdown of FOXD3 over the long time period (4-6 weeks)

required for in vivo PLX4720-induced tumor regression and tumor resistance studies. Thus, as a new approach, we have decided to utilize the CAS9/CRISPR gene editing system to selectively delete FOXD3 in melanoma cells. This approach provides greater stability and certainty of inactivation compared to shRNA/siRNA approaches. The targeting vectors for this approach have been designed and we are currently generating the isogenic cell lines to disrupt endogenous FOXD3.

FOXD3 induction is mediated by depletion of ERK2; however, the downstream effectors are unknown. The RAF-MEK-ERK cascade bifurcates at ERK with activation of kinases, and induction of intermediate early genes. In Specific Aim 2, we proposed to dissect the regions of ERK2 required for down-regulation of FOXD3 following B-RAF inhibition. Here, we utilized FMK and ISK42, two specific inhibitors to the ERK effector, p90RSK. The inhibitors were provided by Dr. Jack Taunton (HHMI, University of California San Francisco). We were able to rule out a role for p90RSK since inhibition of this kinase did not lead to up-regulation of FOXD3.

We also used establishing erase and replace systems to determine the ERK2 effector domains required for FOXD3 repression. We targeted two of the main effector binding sites that are present in ERK2: i) Y261 regulates effectors containing DEF domain sites and controls immediate early gene expression; ii) the common docking domain around D319 interacts with effector kinases: RSKs, MSK and MNK. We generated lentiviruses to express wild-type ERK2, ERK2-Y261A (which doesn't bind DEF domain containing effectors), and ERK2-D319A (which doesn't bind RSK/MSK/MNK). These mutants retain normal activation and nuclear translocation. We perform erase-and-replace experiments to knockdown endogenous ERK2 and re-express wild-type and mutant forms of ERK2 in mutant B-RAF melanoma cells. Measuring FOXD3 expression by Western blot did not provide evidence for either the Y261 or the D319 region in ERK2 regulation of FOXD3. These results have suggested that the DEF and docking domains are dispensable in ERK2 regulation of FOXD3.

As an alternative strategy, we started to investigate the involvement of GSK-3β which led us to test for a role of Axin1. Axin1forms a complex with GSK-3β that regulates beta-catenin, as well as other pathways. We observed that Axin1 knockdown mitigates the RAF inhibitor-induced up-regulation of FOXD3. We have shown this effect with multiple siRNAs to avoid concerns about off-target effects and in multiple mutant BRAF melanoma cell lines (Fig. 1).

Next, we tested whether the effects of Axin1 were mediated through β-catenin. Surprisingly, effective knockdown of β-catenin did not reverse the effects of Axin1 depletion on FOXD3 expression (Fig. 2). We are currently analyzing β-catenin-independent signaling pathways that are known to be regulated by Axin1, which may regulate FOXD3.

In Specific Aim 3, the goal is to determine the role of ERBB3-ERBB2 signaling in resistance to RAF inhibitors. ERBB3 is deficient in intrinsic kinase activity and relies upon other ERBB family members to phosphorylate it in response to ligand binding. We have shown that FOXD3 regulation of ERBB3 initiates the formation of an ERBB3-ERBB2 complex, which signals to promote resistance to vemurafenib. First, we showed that lapatinib (an ERBB2/EGFR inhibitor)

ablated cell viability promoted by NRG1β in the presence of vemurafenib (or the MEK inhibitor AZD6244) in multiple mutant BRAF melanoma cell lines in vitro. Our in vivo results showed that lapatinib enhances the efficacy of PLX4720 and impairs the re-growth of PLX4720-resistant tumors in vivo.

In the last year, we have extended these studies to use of anti-ERBB3 monoclonal antibodies. We have shown that an ERBB3 targeting monoclonal antibody, huHER3-8 (from Immunogen Inc., Waltham MA), inhibits neuregulin-1 (NRG1) activation of ERBB3 and downstream signaling to AKT in RAF-inhibited melanoma cells (Fig. 3). Targeting mutant BRAF in combination with huHER3-8 decreased proliferation and increased death *in vitro* and decreased tumor burden *in vivo* compared to targeting either mutant BRAF or ERBB3 alone (Fig. 4). These results support that ERBB3 neutralizing antibodies may enhance the effects of vemurafenib in mutant BRAF melanoma patients and delay or prevent tumor re-growth. We anticipate that these studies will form the pre-clinical basis for use of BRAF inhibitor/ERBB3 antibody combinations in mutant BRAF melanoma.

A manuscript outlining these data is under review. This describes the first use of ERBB3 neutralizing antibodies in melanoma systems and is a significant accomplishment with translational implications given that ERBB3 antibodies have a good safety profile. We plan to extend these studies by analyzing effects of ERBB3 neutralizing antibodies that have progressed into clinical trials in an *in vitro* system that incorporate aspects of the tumor microenvironment and *in vivo*.

Kugel, C.H., Hartsough, E.J., Davies, M.A. Setiady, Y.Y., and Aplin, A.E. (2014) Function-blocking ERBB3 antibody inhibits adaptive response to RAF inhibitor. Submitted.

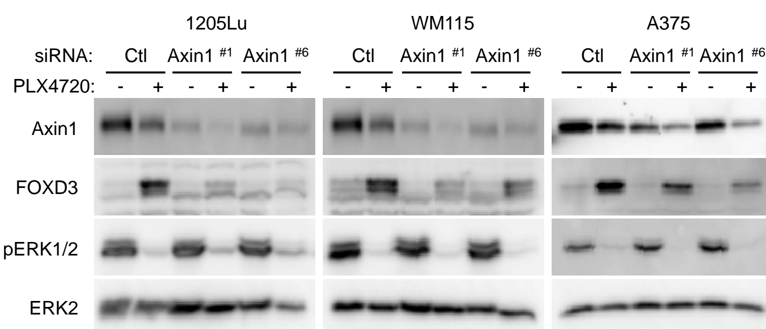

Figure 1: *Axin1 depletion impairs RAF inhibitor-induced up-regulation of FOXD3*. 1205Lu, WM115, and A375 melanoma cells were transfected with either control or Axin1 siRNAs (#1 and #6) for 72 hrs. Cells were then treated overnight with DMSO (-) or the RAF inhibitor, PLX4720 (+). Cell lysates were analyzed by Western blotting for Axin1, FOXD3, phosphoERK1/2 and total ERK2.

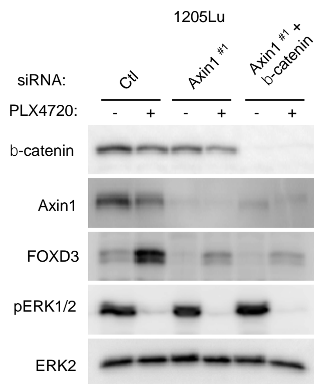

Figure 2: *Effects of Axin1 depletion are independent of β-catenin.*
1205Lu melanoma cells were transfected with either control, Axin1 siRNA alone, or Axin1 and β-catenin siRNA. Cells were then treated overnight with DMSO (-) or the RAF inhibitor, PLX4720 (+). Cell lysates were analyzed by Western blotting for β-catenin, Axin1, FOXD3, phosphoERK1/2 and total ERK2.

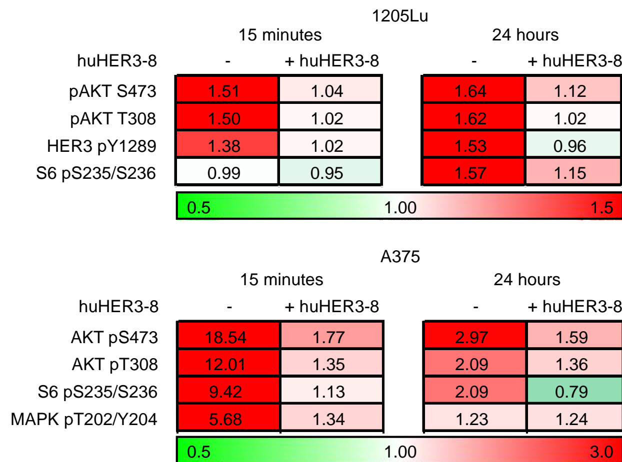

Figure 3. *NRG1-mediated ERBB3 signaling in vitro in mutant BRAF V600E melanoma cell lines is inhibited by huHER3-8.A)*
1205Lu and A375 cells were treated with 1 µM vemurafenib overnight. Cells were then treated with or without 10 µg/mL huHER3-8 for 45 minutes followed by treatment with 10 ng/mL NRG1 for 15 minutes or 24 hours, as indicated. Cells lysates were prepared for RPPA analysis. Linear RPPA scores were averaged and normalized to vemurafenib alone samples. Proteins in the 1205Lu samples which were regulated 1.5 fold or greater are shown. The four most up-regulated proteins from A375 samples are shown.

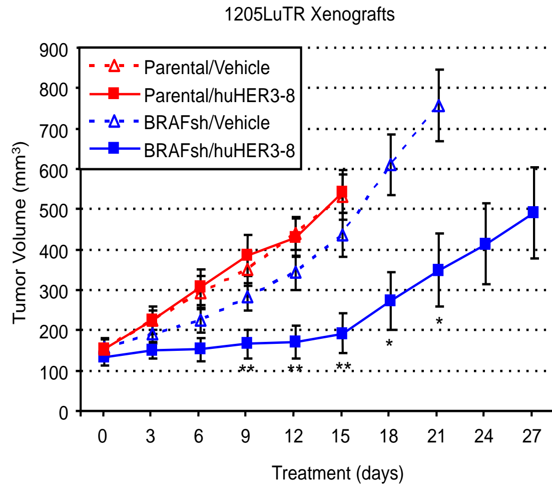

Figure 4. *huHER3-8 in combination with BRAF inhibition reduces tumor growth in vivo*. Parental 1205Lu or 1205LuTR cells harboring an inducible BRAF shRNA cells were injected intradermally into the backs of immune deficient mice. Tumors were allowed to form for approximately 2 weeks. Doxycycline was added, where appropriate, to the drinking water 3 days prior to the start of the experiment. As indicated, mice were treated with huHER3-8 (parental, n = 6; BRAFsh n=9) or vehicle (parental and BRAFsh both n = 8) via IP injection every 3 days. The graph indicates the mean tumor volumes following huHER3-8 treatment. Statistically significant comparisons are indicated by asterisks where * $p \leq 0.05$, and ** $p \leq 0.01$. Bars, SE.

Research Project 2: Project Title and Purpose

A Pilot Study of IL-2 and Rituximab Maintenance in High Risk B Cell Non-Hodgkin's Lymphoma (NHL) – The purpose of this project is to assess the safety and efficacy of combination immunotherapy with rituximab and interleukin-2 in patients with NHL, and investigate disease-free as well as overall survival rates.

Anticipated Duration of Project

1/1/2012 – 12/31/2015

Project Overview

This is an open-label uncontrolled single institution study for NHL that (1) have undergone standard chemotherapy but are at high risk of relapse, (2) have undergone salvage therapy after relapse, but are not candidates for stem cell transplant or, (3) have undergone autologous stem cell transplant and have achieved near complete response or complete response, but are at high risk of relapse. Patients will receive maintenance low dose IL-2 with rituximab for 12 months. IL-2 will be started at 3 MIU/m^2 given subcutaneously, twice weekly. Rituximab will be given at 375mg/m^2 IV weekly for 4 weeks, during weeks 5-8 and 25-28. One cycle of treatment will be a 4-week period.

The primary objectives are to investigate the potential efficacy of IL-2 with rituximab for high risk NHL by evaluating time to progression, and investigate the safety of IL-2 with rituximab as maintenance in patients with NHL. The secondary objective is to collect further evidence of efficacy such as impact on overall survival (OS) and event free survival (EFS).
Toxicity will be evaluated every two weeks for the first two cycles, and every four weeks thereafter. If patients experience Grade 2 constitutional symptoms including fever, rigors/chills and sweats, Grade 3 or greater other toxicity, or any other toxicity at the discretion of the physician, IL-2 will be temporarily discontinued. After resolution of toxicity to Grade 1 or less, IL-2 will be restarted at 2MIU/m^2 SQ twice a week. Patients with unacceptable side effects on IL-2 at 1MIU/m^2 will be removed from the study. The study treatment will be continued until disease progression or unacceptable side effects.

Patients will be evaluated every 4 weeks with clinical exam and laboratory data, and every 3 months with CT of chest, abdomen and pelvis and/or PET (if used for the baseline assessment) and bone marrow biopsy (if positive at baseline or clinically indicated).

Safety will be assessed from the start date of IL-2 through 28 days post the last dose of IL-2. Survival will be assessed throughout the study until death or 3 months after the last dose of IL-2 for the last patient. The study will require 12 months of treatment, with 2 years and 3 months of follow-up for survival analysis.

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Expected Research Outcomes and Benefits

Preclinical work suggests that IL-2 enhances rituximab efficacy by expanding and activating Fc receptor-bearing cells leading to enhanced antibody dependent cellular cytotoxicity. In a Phase I study using IL-2 and rituximab in relapsed or refractory NHL, two different treatment schedules were studied, each with increasing doses of IL-2. Interestingly, the lower dosing and interrupted schedule (4.5 MIU thrice weekly) resulted in a greater increase in natural killer cell (NK) counts than daily dosing, with improved response rates as well as decreased toxicity. These studies suggest that the use of lower doses of IL-2 would selectively up-regulate NK cells without concurrent up-regulation of regulatory T cells (Tregs), which we now know inhibit NK cell function. This may account for the lack of clinical benefit seen in other studies using much higher doses of IL-2 and rituximab in NHL.

We propose to combine a specific low dose of IL-2 given at 3 MIU/m^2 SQ twice weekly, with rituximab given weekly for 4 weeks, twice during treatment, in order to eradicate minimal residual disease in patients with high risk NHL, who receive primary or salvage therapy. It is hoped that this will decrease rate of disease relapse, and increase progression-free and overall survival.

Summary of Research Completed

This is an open-label uncontrolled single institution study for NHL for patients that (1) have undergone standard chemotherapy but are at high risk of relapse, (2) have undergone salvage therapy after relapse, but are not candidates for stem cell transplant or, (3) have undergone autologous stem cell transplant and have achieved near complete response or complete response, but are at high risk of relapse. Patients will receive maintenance low dose IL-2 with rituximab for 7 months. IL-2 will be started at 3 MIU/m^2 given subcutaneously, twice weekly. Rituximab will be given at 375mg/m^2 IV weekly for 4 weeks, during weeks 5-8 and 25-28. One cycle of treatment will be a 4-week period.

The primary objectives are to investigate the potential efficacy of IL-2 with rituximab for high risk NHL by evaluating time to progression, and investigate the safety of IL-2 with rituximab as maintenance in patients with NHL. The secondary objective is to collect further evidence of efficacy such as impact on overall survival (OS) and event free survival (EFS).

<u>Changes to study:</u>
The study was closed to accrual in May 2014. The decision to close the trial was made by Dr. Carabasi and the heme oncology multidisciplinary group based on:

1. Loss of ability to obtain drug without charge for patients whose insurance would not cover participation
2. Slow rate of accrual
3. Availability of new antibodies that may be better suited to this strategy.

<u>Activity:</u>
A total of 12 patients have been enrolled in the trial. Since the trial was opened on 2/5/2009, there were 62 subjects who were screened for the trial. Barriers to enrollment included duration of therapy and need for high IPI score in patients treated after induction therapy. Only 25% of patients will have an IPI score of 3 or greater at diagnosis; despite the presence of several lymphoma thought leaders on campus, we were unable to recruit many patients from outside the campus.

There has been no one enrolled since the last report.

3 expired (all had PD post treatment)
1 withdrew consent (had PD post treatment)
7 in follow up (1 with PD post treatment)
1 never received treatment
0 patients currently receiving treatment

Please find the detailed patient information below:

#001 – Patient completed one year of therapy, remains in remission
#002 – Patient completed one year of therapy, has since progressed
#003 – Patient never started on treatment. His IPI score was only 2, making him ineligible
#004 – Patient completed 5 cycles of treatment, then progression showed on PET
#005 – Patient completed 1 cycle of treatment, then withdrew consent, patient expired one year after enrollment
#006 – Patient completed 5 cycles of treatment, then progression, patient expired one year after enrollment
#007 – Patient completed one year of treatment, remains in remission
#008 – Patient completed 7 months of treatment (protocol has since been changed from 12 to 7 months to enhance recruitment), remains in remission
#009 – Patient completed 7 months of treatment, then progressed during follow-up
#010 – Patient completed 7 months of treatment, remains in remission
#011 – Patient completed 1 cycle, was taken off due to persistence low counts, remains in remission
#012 – Patient completed 7 cycles of treatment remains in remission There are no lab correlates for study and there have been no publications.

Research Project 3: Project Title and Purpose

Transcription Independent Regulation of Liver Repair – The purpose of this project is to delineate the regulation of liver progenitor cells that influence the development of chronic liver injury, facilitate repair and impact the extent of liver fibrosis. The two aims will investigate mechanisms by which inhibition of target gene expression by notch receptor-mediated inhibition of b-catenin activity (Aim 1) and by miRNA mediated inhibition of target gene expression (Aim 2) regulate progenitor cell expansion and maturation during chronic liver injury. Understanding the biology of hepatic progenitor cells will have a wide-ranging impact on the development of therapeutics for acute liver failure, chronic cirrhotic liver disease and hepatocellular carcinoma.

Duration of Project

1/1/2012 – 6/30/2013

Summary of Research Completed

This project ended during a prior state fiscal year. For additional information, please refer to the Commonwealth Universal Research Enhancement C.U.R.E. Annual Reports on the Department's Tobacco Settlement/Act 77 web page at http://www.health.state.pa.us/cure.

Research Project 4: Project Title and Purpose

Role of Retinoblastoma Tumor Suppressor in Estrogen Receptor Negative Breast Cancer – Breast cancer is a major health concern, with over 200,000 new diagnoses rendered each year in the United States. Approximately 1 in 8 women will develop breast cancer; thus, substantial

effort has been directed at defining the basis of tumor development and progression. Breast cancer is represented by multiple disease-subtypes which are distinguished by differential markers, prognoses, and treatment regimens. In general, estrogen receptor (ER) negative disease is faster progressing and more difficult to treat. Genetic analyses established the paradigm that specific tumor suppressor pathways are differentially disrupted in ER-negative disease, but the specific relevance of these events for tumor behavior or therapeutic response remains unclear. Here, we will delineate the role of retinoblastoma tumor suppressor (RB) in the progression to ER-negative disease and the treatment of such tumors based on rational drug delivery.

Duration of Project

1/1/2012 – 12/31/2012

Summary of Research Completed

This project ended during a prior state fiscal year. For additional information, please refer to the Commonwealth Universal Research Enhancement C.U.R.E. Annual Reports on the Department's Tobacco Settlement/Act 77 web page at http://www.health.state.pa.us/cure.

Research Project 5: Project Title and Purpose

Chromosomal Instability (CIN) in Tumorigenesis – Chromosomal instability (CIN) is a characteristic of human solid tumors. Genomic instability is defined as a persistently high rate of loss and gain of whole chromosomes and may be observed as an elevated rate of gain or loss of whole chromosomes (aneuploidy) and/or as structural chromosomal aberrations (translocations, deletion, inclusions). The molecular mechanisms underlying CIN and the relative contribution of CIN to tumor progression, invasion and metastasis is poorly understood. Using engineered mice models and reconstitution assays we will determine the mechanism by which cyclin D1 regulates CIN in cells and *in vivo*.

Duration of Project

1/1/2012 – 6/30/2013

Summary of Research Completed

This project ended during a prior state fiscal year. For additional information, please refer to the Commonwealth Universal Research Enhancement C.U.R.E. Annual Reports on the Department's Tobacco Settlement/Act 77 web page at http://www.health.state.pa.us/cure.

Research Project 6: Project Title and Purpose

Involvement of Hedgehog Signaling in Muscle Wasting of Cancer Cachexia – Cachexia with its progressive wasting of fat and skeletal muscle is a devastating complication of cancer. There are no approved, effective treatments and the molecular pathways linking cancer to muscle wasting are ill-defined. The sonic hedgehog (Shh) and related ligands mediate embryogenesis, including

specification of skeletal muscle. This project will define the links among the hedgehog pathway, inflammation and muscle wasting in cancer. Ultimately these studies will provide a rationale for screening patients for hedgehog pathway activation in cachexia as well as for new therapeutic approaches for muscle preservation, including hedgehog pathway inhibitors already in clinical trials for anti-tumor therapy.

Duration of Project

1/1/2012 – 6/30/2013

Summary of Research Completed

This project ended during a prior state fiscal year. For additional information, please refer to the Commonwealth Universal Research Enhancement C.U.R.E. Annual Reports on the Department's Tobacco Settlement/Act 77 web page at http://www.health.state.pa.us/cure.

Research Project 7: Project Title and Purpose

Role of Dietary Cholesterol in Cancer – The hypothesis of this project is that plasma cholesterol levels play a crucial role in the regulation of mammary tumor onset and progression. During mammary tumor development, tumor cholesterol requirements are very high compared to other tissues, since tumor cells divide more rapidly than other cell types. Therefore, the project will examine the i) regulation of plasma cholesterol (in the form of lipoproteins) and ii) the role of a key cellular protein involved in lipoprotein metabolism (Caveolin-1) during mammary tumor formation and lung metastasis.

Duration of Project

1/1/2012 – 9/01/2012

Summary of Research Completed

This project ended during a prior state fiscal year. For additional information, please refer to the Commonwealth Universal Research Enhancement C.U.R.E. Annual Reports on the Department's Tobacco Settlement/Act 77 web page at http://www.health.state.pa.us/cure.

Research Project 8: Project Title and Purpose

New Methods of Detection of Residual AML Cells in Patients in Remission after Allogeneic HSCT – The high incidence of relapse in Acute myelogenous leukemia (AML) observed after induction of complete remission (CR) and allogeneic hematopoietic stem cell transplantation (HSCT), is thought to be due to the persistence in a protective niche in the bone marrow (BM) of a rare population of cryptic AML stem cells. New prospective therapies, such as those aiming at mobilizing these AML stem cells from the patient's bone marrow, are conditioned by the capacity to detect and quantify these rare cells in blood and bone marrow samples. The objective of this project is to develop new high-sensitivity methods based on next-generation sequencing,

to detect both recipient-specific and AML-specific genetic markers that will allow detection of rare AML cells.

Anticipated Duration of Project

1/1/2012 – 12/31/2015

Project Overview

A large fraction of patients with AML will relapse even after chemotherapy-induced complete remission (CR), and consolidation by HSCT. Relapse is thought to be related to the presence of cryptic AML stem cells (AML-SC) in the bone marrow that could therapeutically be mobilized by CXCR4 antagonists. The overall hypothesis of this project is that this occult population of AML-SC can be detected and quantified, using the applied genomics approaches that include targeted next generation sequencing (NGS), to identify specific mutations, and deep next NGS of amplicons containing these mutations, respectively. Mutations present in the original population of leukemic myeloblasts will be detected by targeted NGS, and subsequently used as genetic markers of biological relapse.

In addition, single nucleotide polymorphisms (SNPs) that are informative in discriminating between the recipient of the stem cell allograft and the donor will be determined by Sanger sequencing, and then used in NGS of amplicons to determine the extent of mixed chimerism with high sensitivity. This approach will provide a mechanism to quantify leukemic myeloblasts and to distinguish between recipient (i.e., the patient with AML) and donor. The latter should detect the return of recipient hematopoietic elements, which represent leukemic relapse. Bone marrow and blood samples from AML patients in CR after chemotherapy allogeneic SCT, will be obtained and the presence of occult populations of leukemic myeloblasts, which include AML-SC, will be monitored by deep NGS of amplicons.

Specific Aim 1. NGS of exon-capture products will be used to identify recurrent mutations in AML that will represent the specific molecular signature of AML cells in that patient. Sanger sequencing of HSC donor and recipient DNA will be used to detect informative SNPs that discriminate between the recipient genotype and the donor genotype.

Specific Aim 2. The identification and quantification of leukemic myeloblasts present during the hematologic remission following allogeneic HSCT will be determined by deep sequencing of polymerase chain reaction (PCR) amplicons containing the informative mutations and SNPs defined in Specific Aim 1.

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Expected Research Outcomes and Benefits

The survival of patients with AML is severely compromised by the high rate of relapse. Clinical relapse is believed to be related to the presence of a small number of occult AML stem cells (AML-SC) that survive in the patient's bone marrow even during complete remission induced by chemotherapy, and consolidation by heterologous HSCT. The benefit of prospective new therapies, such as the mobilization of these AML-SC by antagonists of CXCR4, the chemokine receptor involved in homing to the BM, is conditioned by the capacity to detect these rare cells in BM and blood samples. Multiparametric flow cytometry is the current method of choice for the detection and quantification of rare hematological events, but is hampered by the inconsistency of the phenotype of AML stem cells. Molecular techniques offer a different approach, and the quantitative study of mixed chimerism after HSCT is currently used as a measure of biological relapse, using a set of 16 polymorphic markers (loci of short tandem repeats) to discriminate donor and recipient. However, the sensitivity of this Powerplex16 assay is limited to about 1-2%. The project proposes to use the rapidly developing technology of NGS to develop innovative assays for the detection of rare residual AML cells, in patients in CR following HSCT. Genetic markers specific for the AML cells, as well as markers discriminating the donor and the recipient, will be first identified by exon capture and NGS or Sanger sequencing, respectively. The same markers will then be used in deep sequencing of amplicons by NGS, to monitor with high sensitivity the resurgence of cells carrying the recipient's and AML markers, as an early indication of biological relapse.

Summary of Research Completed

<u>*Specific Aim 1.*</u>
As a new tool for analyzing both AML-specific mutations and SNPs in HSCT patients, we have developed a custom amplicon-based NGS panel for sequencing targeted regions of 55 AML-associated genes. Libraries are generated by amplifying the selected DNA segments from patient samples, followed by NGS on the Illumina MiSeq platform. The panel allows sequencing of 1322 regions, each ~175-bp in length. In genes where most AML-associated mutations are found in short hotspots, only hotspots are sequenced, while for genes where AML-associated mutations are found throughout the gene, the entire coding sequence is sequenced. In addition to AML-associated mutations, the panel also covers 299 SNPs that are present in >5% of the population, and consequently can be used for detecting recipient DNA in HSCT patient specimens. Thus, this panel can detect both disease-specific mutations and recipient-specific SNPs.

To test the performance of this AML panel, we have used it to sequence 41 AML patient samples, many of which contained mutations that had been identified previously. Preparation of multiplexed libraries and sequencing were performed according to Illumina recommendations, and data were analyzed using Illumina's bioinformatics pipeline, followed by analysis for insertions in FLT3 using Pindel. We determined that Pindel analysis is necessary for detecting large (>~24 bp) ITDs (internal tandem duplication) in FLT3, which are recurrent mutations in

AML. Results were viewed using in-house reporting software. SNPs (that were likely present in the patient germline) were identified and distinguished from pathogenic somatic mutations using the dbSNP database.

In runs containing 10-12 multiplexed samples, median coverage depth was 1800x, with 91.7% of amplicons having median coverage >200x and 6.9% having coverage >5000x (Figure 1). To assess the usefulness of the panel for identifying AML mutations, we asked to what extent previously-reported cancer-associated mutations (from the COSMIC database) were present in DNA regions that were sequenced by our panel with sufficient depth of coverage for successful analysis (>200x). For 43 genes in the panel, including commonly mutated genes FLT3, NPM1, KIT, DNMT3A, IDH1, IDH2, TET2, and TP53, DNA regions containing >90% of previously-reported cancer mutations were sequenced successfully. However, <30% of COSMIC mutations were successfully covered in CDKN2A, CEBPA, MPL, SRSF2, and U2AF1, while for the other 7 genes, the panel successfully covered 44-89% of COSMIC mutations. Among the 41 different patients, we identified 139 mutations in 31 genes (Figure 2), with a range of 0 to 6 mutations per patient, which is consistent with published reports of the number of mutations found in AML patients. Several of the sequenced specimens had been sequenced previously using a different NGS panel (TruSeq Amplicon Cancer Panel, Illumina) or had been analyzed clinically for mutations in FLT3, NPM1, KIT, and/or CEBPA. Concordance between AML panel results and prior results was 100%, with the exception of CEBPA, which could not be analyzed using our panel due to low coverage. Combined, these results indicate that this panel can successfully detect most of the common mutations found in AML patients.

To test the limit of detection of this panel for low frequency variants, we performed reproducibility and sensitivity experiments. In both inter- and intra-run reproducibility experiments where the same specimen was analyzed multiple times, we found that variants detected at >5% frequency were 100% reproducible. The bioinformatics pipeline also reported putative variants at lower frequencies, but most of these were present at <2.0% and were not reproducible, suggesting that they represented background noise in the sequence data. FLT3 ITDs could be identified at levels below 1% (see below), since background noise does not include large insertions. We also performed a sensitivity experiment in which we serially diluted one patient-derived DNA sample containing known variants into a second control sample. The variants in the diluted sample included 7 sample-specific SNPs, 2 pathogenic mutations that were present at ~50% in the original sample, and a FLT3-ITD mutation present at ~4.5%. This experiment showed that 8 of the 9 major variants were detected at close to the expected frequencies of 8%, 6.5%, 5%, and 3.5% in both duplicates of the dilutions (Figure 3). One SNP (in CSF3R) was not detected in the 3.5% samples and showed greater variability in the other samples, likely because of low coverage of the corresponding amplicon (~400x vs. ~1200x-4500x for the other 8 amplicons). Remarkably, the FLT3 ITD was also detected in 7 of 8 diluted samples, at frequencies as low as 0.25%. Together, these results indicate that this panel can accurately and reproducibly identify unknown mutations present at frequencies >5%, and as low as ~1% for FLT3-ITDs. This is of particular clinical relevance because the presence of FLT3-ITDs has been associated with a poor prognosis, and these patients merit close monitoring. As detailed below, it may be possible to detect previously identified mutations, or a group of previously identified mutations, at lower frequencies.

Specific Aim 2.

To test the potential utility of our AML panel for early detection of relapse in HSCT patients, we next sequenced DNA from 6 sets of paired bone marrow specimens, where one specimen was taken from an HSCT patient 1-5 months before relapse and the other after relapse. To identify pathogenic mutations and recipient-specific SNPs, we compared variants in the relapse specimen, which included SNPS and/or pathogenic mutations from both donor and recipient, to variants in the pre-relapse specimen, which included only donor SNPs at high levels. We identified 2-6 pathogenic mutations and 4-11 recipient-specific SNPs per patient. When only variants with coverage >1000x were considered, we detected 1-4 mutations and 1-8 recipient-specific SNPs per patient.

We next analyzed levels of AML-associated mutations and recipient-specific SNPs in the pre-relapse samples. In all 6 samples, all recipient-specific variants were detected in <1.5% of total reads, with most <0.7%. At this level, it is possible that background noise in the sequence data is making a substantial contribution to the numbers of variant reads. Consequently, in order to interpret these results, it was important to analyze the background carefully. In MiSeq NGS data, the spectrum of background noise is different at each position. Some nucleotide positions consistently show higher levels of one incorrect nucleotide, while the adjacent nucleotide may show a very different spectrum of noise. Taking this into account, we analyzed background noise at each position corresponding to an informative mutation or SNP, using other samples in the same run that did not contain the variant. Background noise was defined as the fraction of reads with the specified variant (i.e. only C> T at the designated position) in samples not containing the variant in their genome. So far this has been done for two patient samples. Only variants showing >1000x coverage in the pre-relapse sample were considered. In the sample from Patient 1, which was taken ~5 months prior to relapse, 7 of 8 variants were detected at >2 standard deviations above the average background, while one variant in ASXL1, which was the only pathogenic mutation among these, was not detected in the pre-relapse sample (Figure 4). However, to see the expected fraction of the ASXL1 variant, only 5 variant reads would have been expected, making it possible that the absence of this variant was due to sampling error. The combined increased levels of the 7 other variants suggests that recipient DNA is being detected in this sample, and that the frequency is ~0.4%. In the sample from Patient 2, which was taken 3 months prior to relapse, eight of eleven variants were detected at >1 standard deviation above background, including two pathogenic mutations (KDM6A and WT1), while three variants were below this level, including two other mutations (NRAS and BCOR) (Figure 4). Even among variants that appeared to be present above background, the difference between the signal and the background was only ~0.2%. A preliminary conclusion that can be drawn from these data is that it is unlikely that that it will be possible to detect variants present at <~0.5% with confidence using this method. However, as detection of variants between 0.5% and 2.0% would be an advance in detection of recipient DNA, this approach merits further investigation and may have clinical utility in management of patients with AML.

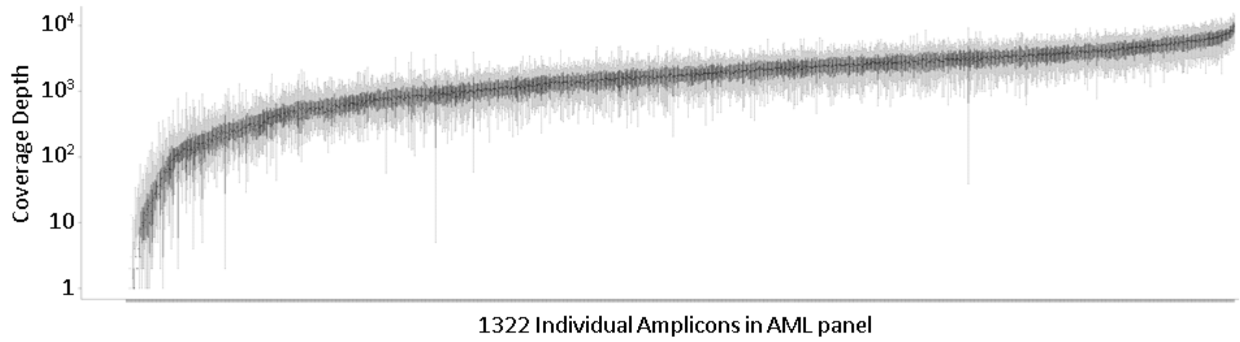

Figure 1. Average coverage for each of the 1322 amplicons across all samples in 6 runs. Data are shown as a box representing the 25% to 75% percentile with a line for the mean. Error bars show the minimum and maximum.

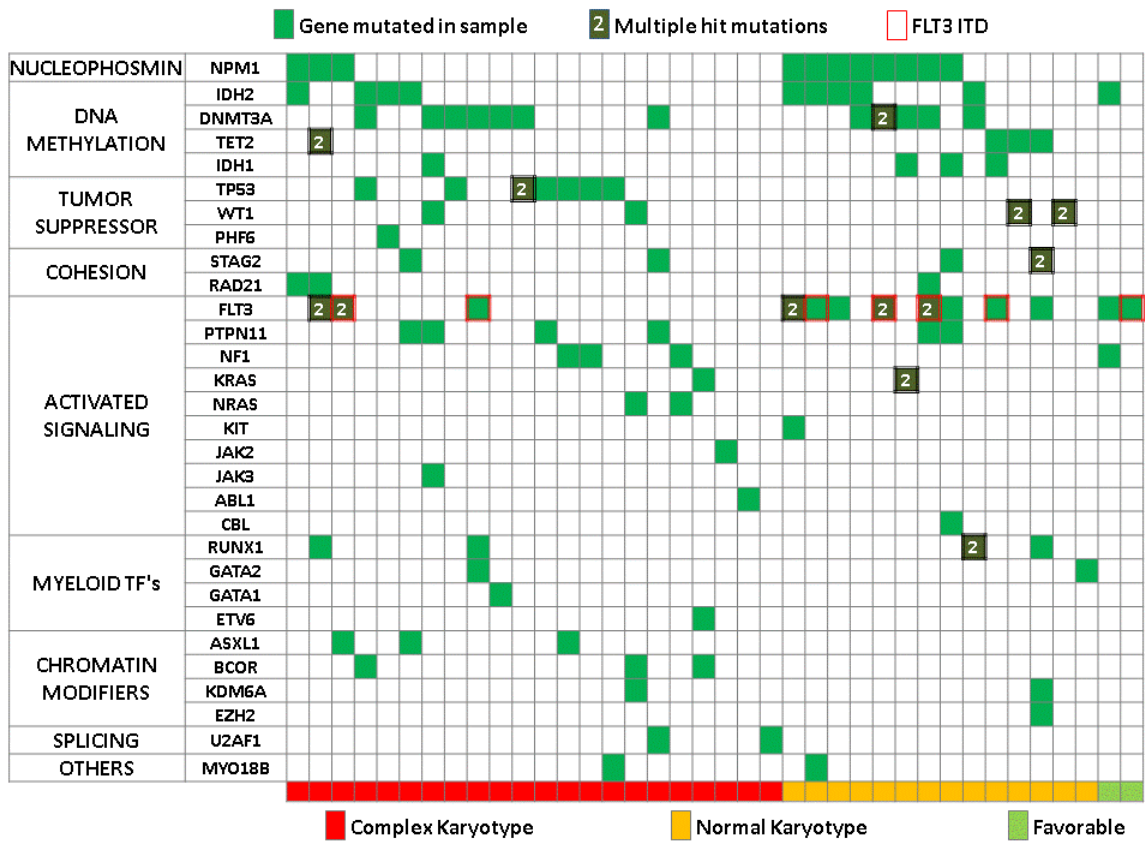

Figure 2. Distribution of mutations in 38 patient specimens in which mutations were identified using the AML panel. Each column represents an individual patient.

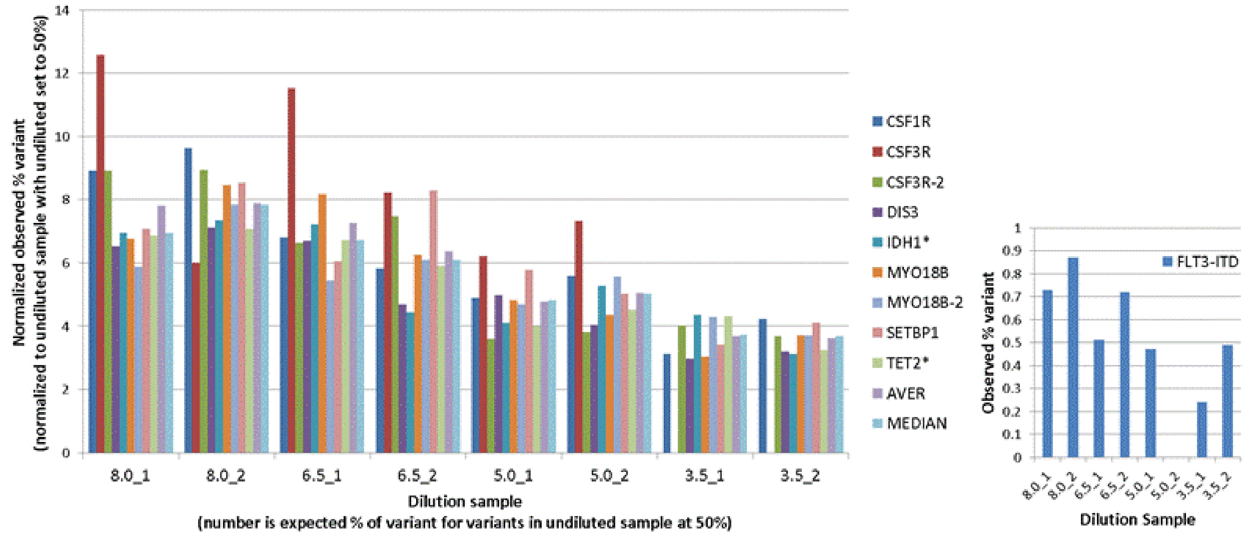

Figure 3. Variant detection in diluted samples. Left, SNPs and AML-associated mutations (asterisks) present in original sample at ~50% were diluted to expected frequencies of 8.0, 6.5, 5.0, or 3.5. Variants are designated by gene name. Observed frequencies detected by NGS are shown (normalized to undiluted sample set at 50%). Right, observed frequencies of FLT3-ITD present in original sample at 4.5%.

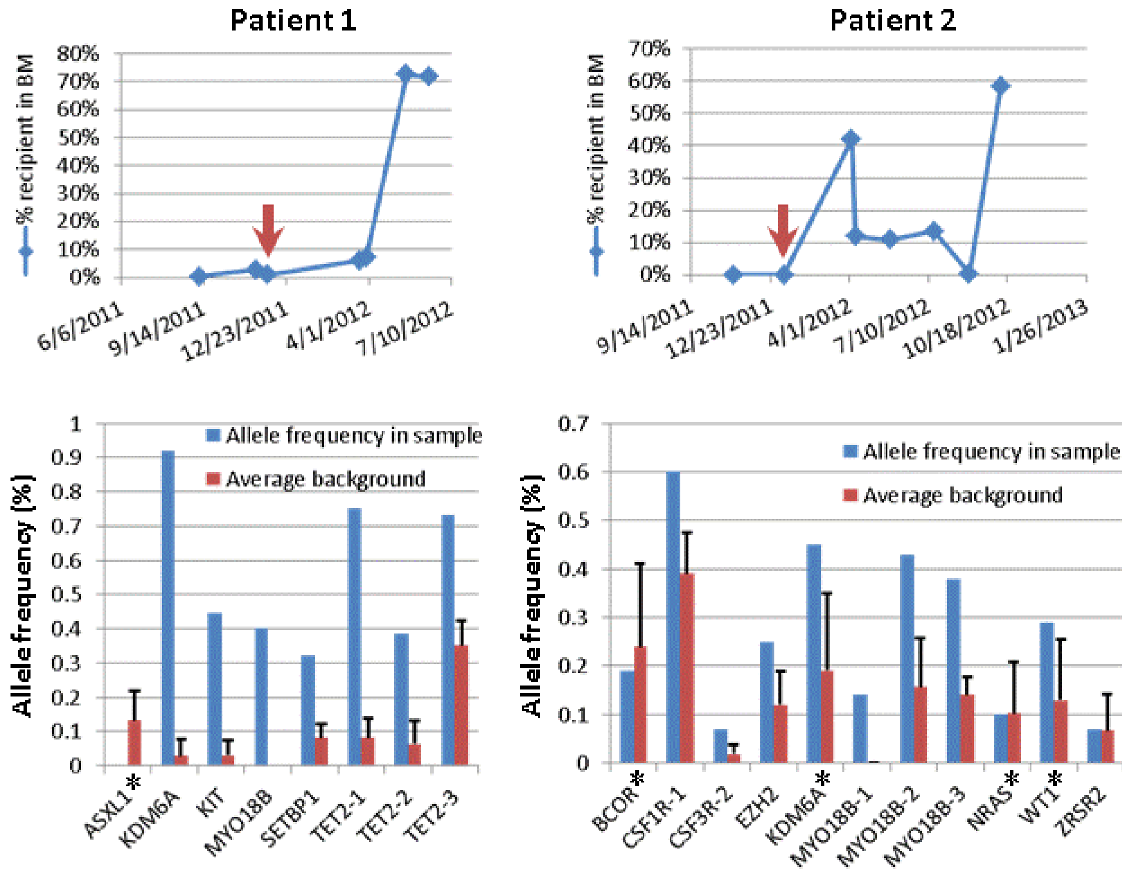

Figure 4. Analysis of frequency of recipient-specific alleles in two pre-relapse specimens. Left panels, Patient 1; right panels, Patient 2. Top panels, % recipient DNA in specimens taken on different dates was measured by current bone marrow engraftment assay. Red arrows indicate pre-relapse specimens used for NGS. Bottom panels, results from NGS. Blue bars indicate observed frequency of recipient-specific variants in pre-relapse sample. Red bars indicate average background of the same variant in samples that do not contain the variant. Error bars represent standard deviation. Variants are designated by gene name. Asterisks designate pathogenic mutations. Other variants are recipient-specific SNPs.