

Thomas Jefferson University

Annual Progress Report: 2009 Formula Grant

Reporting Period

January 1, 2010 – June 30, 2010

Formula Grant Overview

Thomas Jefferson University received \$3,746,521 in formula funds for the grant award period January 1, 2010 through December 31, 2013. Accomplishments for the reporting period are described below.

Research Project 1: Project Title and Purpose

Genetic Targets of Breast Tumor-Initiating Cells - Breast cancer is the second most prevalent cancer-related death in women in the United States. A subpopulation of breast tumor cells have been identified and are referred to as “breast tumor-initiating cells” or cancer stem cells (CSCs). These self-renewing CSCs were first described in the hematopoietic system, and have now been putatively identified in breast, colon and brain tumors. It has been proposed that all tumor metastasis are caused by CSCs, and it is suspected that these cells contribute to therapy resistance and tumor recurrence. The focus of this project is to determine new genetic mechanisms governing breast CSC expansion and invasion, and to identify specific secreted factors regulated by gene deletion, to provide CSC-based therapies.

Anticipated Duration of Project

1/1/2010 - 12/31/2013

Project Overview

The goal of this project is to determine new genetic mechanisms governing CSC expansion and invasion, using transgenic mice. Over the last 15 years, this laboratory has generated mammary gland-targeted gene excision/inactivation mice, and crossed these mice to mammary gland-targeted oncogene mice. These mice and the genetic material and tissues previously derived from these animals, will be used to determine the role of six specific genes, in the expansion of CSC.

Specific Aim: Determine the role of genetic determinants in CSC expansion. The relative proportion of cells with characteristics of breast CSCs will be assessed in transgenic mice and tumor-derived cell lines by genetic deletion of Notch, Akt1, c-jun, NFκB, p21^{Cip1} and DACH1, using multiple assays (self renewal, self surface markers, Aldh1 production, lineage repopulation).

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Other Participating Researchers

None

Expected Research Outcomes and Benefits

Each of the six genes being examined in this project has been shown to regulate breast tumor proliferation and growth. Identification of specific secreted factors regulated by gene deletion may be used for CSC-based therapies. The transgenic mice and cell lines generated in this laboratory have the unique advantage of providing a normal immune system. Studies conducted in immune-deficient mice may ignore important effects of the immune system on tumor progression. This is particularly important in these studies, as the factors identified in breast CSCs that regulate expansion are also made by the normal immune system. This laboratory has established a subtractive proteomic approach and, based on the prior 15 years developing unique genetic mouse models; it is uniquely positioned to determine the importance of these key target genes in CSC expansion and the role of specific secreted factors in response to therapy.

This project will characterize a completely novel tumor and metastasis pathway if these functional analyses identify secreted factors and relevant antibodies which govern breast tumor proliferation and growth *in vivo*.

Summary of Research Completed

Specific Aim: Determine the role of genetic determinants in CSC expansion. The relative proportion of cells with characteristics of breast CSCs will be assessed in transgenic mice and tumor-derived cell lines by genetic deletion of Notch, Akt1, c-jun, NFκB, p21^{Cip1} and DACH1, using multiple assays (self renewal, self surface markers, Aldh1 production, lineage repopulation).

Our initial focus has been on the key regulator of progenitor cell expansion in several cell types-DACH1 *DACH1 expression is reduced in breast cancer cell lines enriched for cancer stem cells.* Recent studies have demonstrated the loss of DACH1 expression correlates with poor prognosis in human breast cancer and DACH1 inhibits MCF7 cell proliferation in tissue culture. In order to characterize further the expression of DACH1 in breast cancer cell types. Western blot analysis was conducted using a previously characterized polyclonal antibody (Fig. 1A). Quantitation of relative abundance from multiple experiments demonstrated a reduction of DACH1 abundance in the MDA-MB231 and HS578T cells. Immunoepitope staining for the breast cancer stem cell

markers CD44⁺/CD24⁻ demonstrated a relative increase in the proportion of CD44⁺/CD24⁻ cells in the MDA-MB231 and HS578T cells (Fig. 1B). Distinct subtypes of human breast cancer include the basal-like, luminal (A and B), Her2⁺ and normal breast like carcinomas with distinct prognostic significance. Basal-like breast carcinomas are of high grade with a distinctive proclivity to metastasize and express genes associated with the maintenance of the stem cell phenotype. Comparison between normal human breast epithelial cells, and basal-like vs non basal-like showed a significant reduction in mRNA expression (Fig. 1C) and in DACH1 abundance (Fig. 1D) in the basal-like tumors.

DACH1 expression inhibits the proportion of breast cancer cells expressing cancer stem cell markers in vivo. Given the association between low DACH1 expression and increased expression of cancer stem cells, we examined whether DACH1 expression could preferentially inhibit breast cancer stem cells. MET-1 cells were transduced with a DACH1 expression vector resulting in a ~2-fold increase in DACH1 expression by Western blot analysis (Fig. 2A). Immunohistochemistry demonstrated the presence of the DACH1-tagged Flag epitope throughout the cell population. The effect of DACH1 on mammary tumor growth *in vivo* was assessed by implantation in nude mice (Fig. 2B). DACH1 expression reduced the volume of tumors by ~80%. Tumor weight was reduced by ~90% (Fig. 2B). Serial transplantation experiments demonstrated a ~50% reduction in new tumor formation of DACH1 expressing Met 1 breast cancer cells (Fig. 2C).

DACH1 inhibits mammosphere formation and the CD44⁺/CD24⁻ phenotypes. Cancer stem cells can be enriched by sorting for CD24^{-low} cells. In order to determine whether DACH1 expression regulated the relative proportion of CD24^{-low} breast tumor cells *in vivo*, Met-1 cells transduced with either a DACH1 expression vector or a control vector were implanted into nude mice. Tumors were grown for 3 weeks in mice and subsequently analyzed for CD24^{-low} cells. Induction of DACH1 expression reduced the proportion of CD24^{-low} cells by ~50% (Fig. 2D).

As a complementary assay of the BTIC phenotype Aldefluor staining was conducted as previously described. The stem cell marker aldehyde dehydrogenase (ALDH) is thought to regulate stem cell differentiation through metabolism of retinal to retinoic acid. The fluorescent aldefluor assay measures ALDH activity and has been used to isolate cancer stem cells from brain tumors, multiple myeloma, acute myeloid leukemia and breast cancer. DACH1 expression reduced the proportion of Aldefluor-positive cells by ~60% (Fig. 2E). Expression of a DNA-binding defective mutant of DACH1 (Δ DS) was defective in reducing Aldefluor staining (Fig. 2E).

The cancer stem cell hypothesis suggests that many cancers are maintained in a hierarchal organization of cancer “stem cells” or tumor initiating cells rapidly dividing amplifying cells (early precursor cells) and differentiated tumor cells. . Cancer stem cells are thought to contribute to tumor progression, therapy resistance and recurrence and can be enriched by cell sorting for CD44^{high}/CD24^{-low} cells. A small number of primary breast cancer cells, tumor initiating cell (TIC) or cancer stem cells form secondary tumors. TICs form non-adherent mammospheres when cultured under specific conditions in the absence of serum. In order to examine further the role of DACH1 in TIC, mammosphere assays were conducted with the Met-

1 mammary tumor cell lines. Induction of DACH1 reduced mammosphere number by >60% in cell lines (Fig. 3A).

DACH1 expression in Met-1 cells reduced the relative proportion of CD44^{high}/CD24^{low} cells by ~80% (Fig. 3B, 15% vs 3%, n=6, P<0.003). In order to examine the biological significance of DACH1 mediated inhibition of the CD24 population, Met-1 cells transduced with DACH1 were grown in tissue culture and subjected to FACS analysis for the CD24^{high} vs CD24^{low} populations. Multipotentiality of the CD24^{high} and CD24^{low} populations was determined by their ability to form CD24^{high} and CD24^{low} populations and to form mammospheres as a surrogate measure of stem cell expansion (Fig. 3C). CD24^{low}/CD44⁺ cells and CD24^{high}/CD44⁺ cells were separated by FACS analysis and grown in cultures for 3 weeks. Restaining by FACS demonstrated CD24^{low}/CD44^{high} gave rise to both CD24^{high}/CD44^{high} and CD24^{low}/CD44^{high} whereas CD24^{high}/CD44^{high} gave rise to only the parental CD24^{high}/CD44^{high} population (Fig. 3D). The CD24^{low} and CD24^{high} Met-1 cells were next examined for mammosphere formation. The CD24^{low} cells gave a 4-fold greater yield of mammospheres (Fig. 3E). These studies suggest the CD24^{low} and CD24^{high} cells maintain multipotentiality. To determine the tumor growth characteristics of these two distinct Met-1 cell populations, tumor implantation analysis was conducted. The CD24^{low}/CD44^{high} grew ~ 4 times larger tumors that CD24^{high}/CD44^{high} Met-1 cells (Fig. 3F).

Endogenous DACH1 inhibits the stem cell phenotype. These studies suggested that a modest induction of DACH1 expression was sufficient to inhibit mammosphere formation and the relative proportion of cells with features of breast cancer stem cells. In order to determine whether endogenous DACH1 functioned to inhibit cancer stem cells a lentivirus encoding DACH1 shRNA linked via an IRES to GFP was used to transduce Met-1 cells (Fig. 4A). Comparison was made to the control vector. Reduction of DACH1 abundance with DACH1 shRNA in multiplicate experiments increased the proportion of CD44⁺/CD24⁻ cells ~ 2.2-fold (Fig. 4B). The number of mammospheres reflects the relative proportion of progenitor cells, whereas the size of the mammosphere may also be affected in part by the proliferative capacity of the cells. Mammosphere volume was increased 3.5 fold by DACH1 ShRNA expression (Fig. 4B). The relative number of mammospheres was increased 350% by DACH1 ShRNA (Fig. 4C). c-Myc transduction of the immortal human MCF10A cells induced cells with contact-independent growth properties.; and increased the proportion of CD44⁺/CD24⁻ cells (Fig. 4D) from ~24% to 95%. Transduction of MCF10–c-Myc cells with DACH1 inhibited the proportion of breast cancer stem cells from 95% to ~40% (Fig. 4D). These findings suggest endogenous DACH1 is a key determinant of mammosphere number and therefore of BTIC.

MATERIALS AND METHODS

Mammosphere Formation and FACS Analysis of Stem Cell Surface Markers- Mammosphere formation assays were conducted as previously described. Aldefluor and immunostaining of cell surface markers by FACS analysis for breast cancer stem cells was based on prior publications. . Before labeling, the cells were blocked with normal mouse IgG in 1/100 dilution for 30 min and then incubated with PE labeled mouse anti-human CD24 (1/5) (clone ML5, BD Pharmingen, San Diego, California) and/or PE/Cy5 labeled rat anti human/mouse CD44 (1/200) (clone IM7, BioLegend, San Diego, CA) for 1 hour. All experiments were conducted at 4°C. Cell sorting was

performed on a FACSCalibur cell sorter (BD Bioscience, San Jose, California). The data were analyzed with FlowJo software (Tree Star, Inc., Ashland, Oregon).

Cell culture, plasmid construction, reporter genes, expression vectors, DNA transfection, and luciferase assays- Cell culture, DNA transfection, and luciferase assays using the Sox-2-Luc and Nanog-Luc reporter genes were performed as previously described. . Expression vectors encoding KLF4/c-Myc and Oct4/Sox 2 were from Addegene. The Met-1 cells were cultured in DMEM supplemented with 10% fetal calf serum, 1% penicillin, and 1% streptomycin. The MCF10A and MCF10A-Myc lines were previously described. . The expression plasmids encoding an N-terminal FLAG peptide linked to DACH1, DACH1 DS-domain alone (DS) or DACH1 DS-domain deleted (Δ DS) were previously described. Lentiviral DACH1 shRNA was from Open BioSystems. Transfection and infection were followed standard protocols. GFP positive were selected by FACS. Met-1 cells were plated at a density of 1×10^5 cells in a 24-well plate on the day prior to transfection with Superfect according to the manufacturer's protocol (Qiagen, Valencia, CA). A dose-response was determined in each experiment with 50 and 200 ng of expression vector and the promoter reporter plasmids (0.5 μ g). Luciferase activity was normalized for transfection efficiency using B-galactosidase reporters as an internal control. The fold effect of expression vector was determined with comparison to the effect of the empty expression vector cassette and statistical analyses were performed using the t- test.

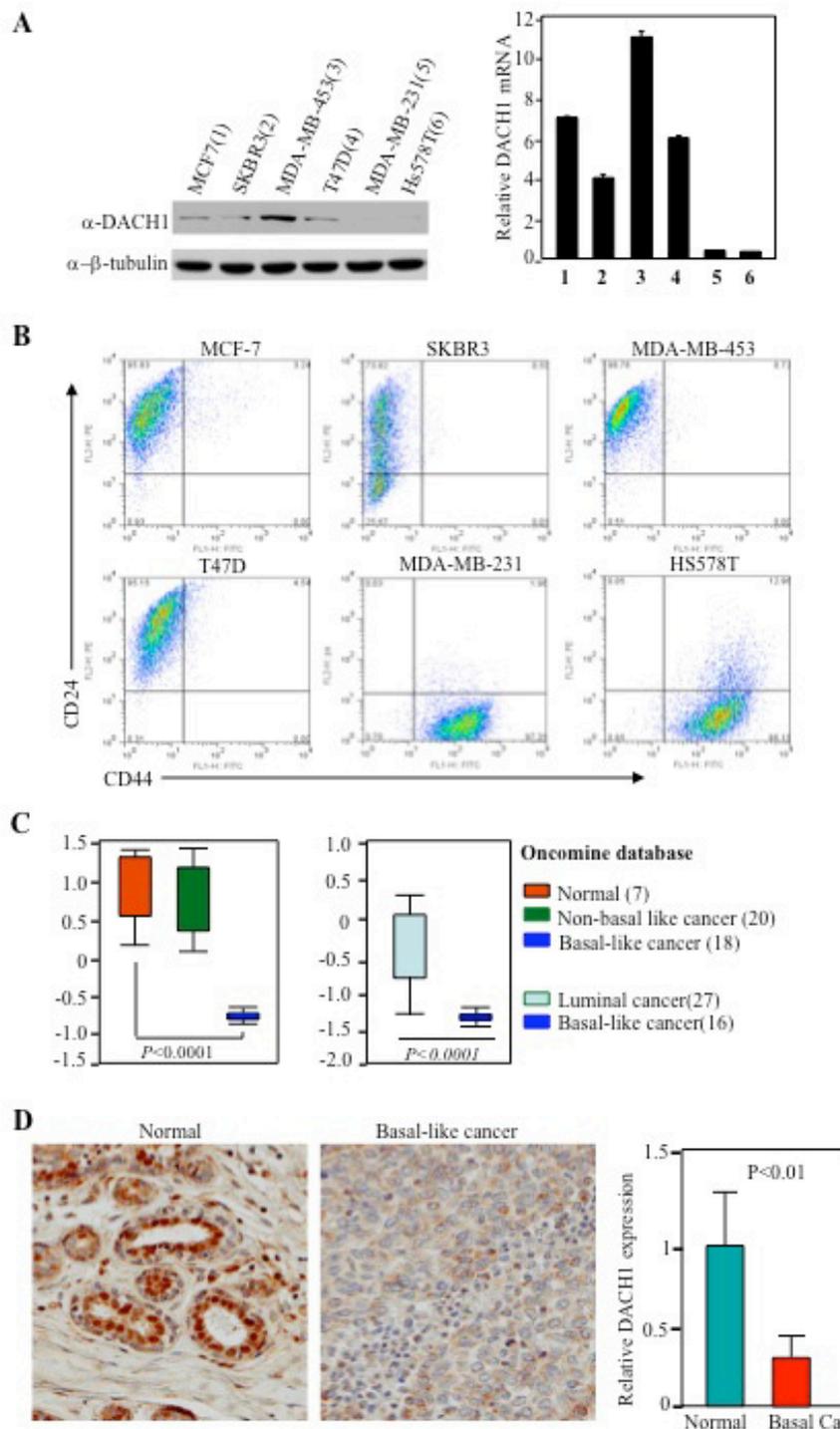


Figure 1. DACH1 expression in human breast cancer cell lines and breast carcinoma tissues. (A) Left panel: Western blot for DACH1 abundance of breast cancer cell lines, β -actin was used as a loading control. Right panel: normalized mRNA expression of DACH1 in breast cancer cell lines. (B) CD24/CD44 staining of breast cancer cell lines. (C) Normalized DACH1 expression from OncoPrint databases. (D) Representative image and semi-quantization of DACH1 expression in normal breast epithelium and triple negative invasive human breast cancer samples using DACH1 specific antibodies.

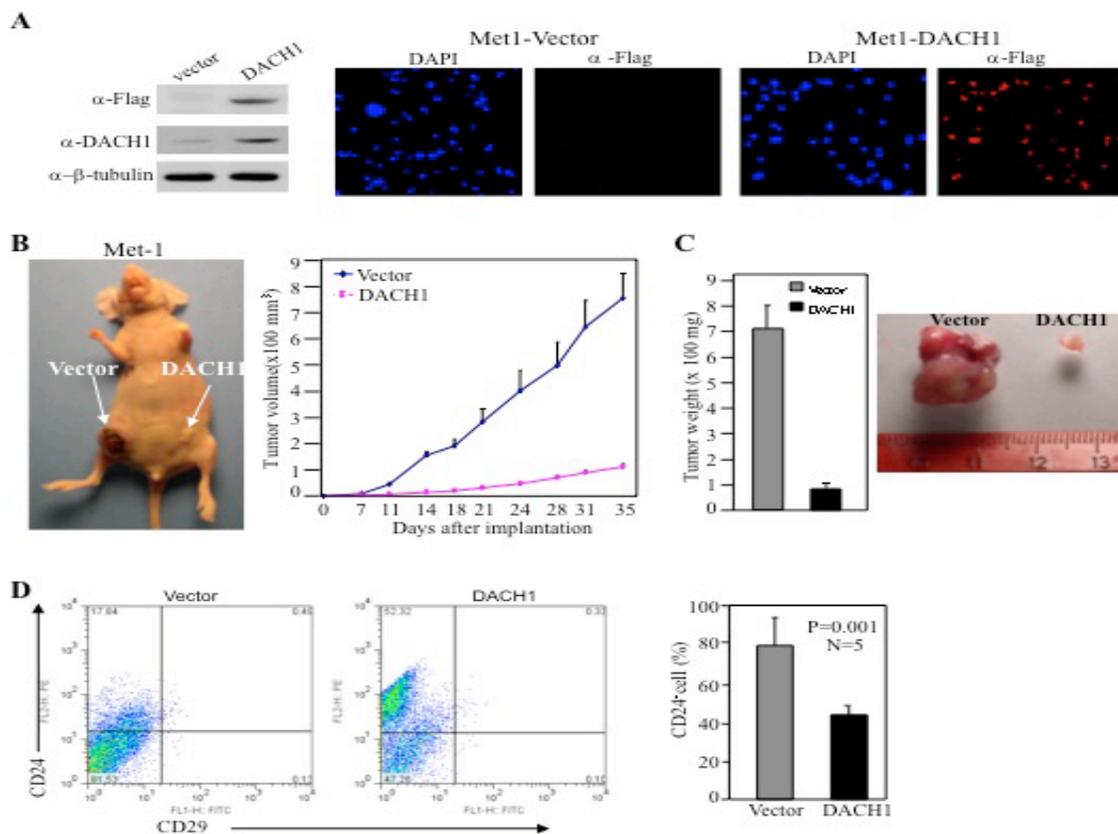


Figure 2. DACH1 blocks Met-1 tumor growth *in vivo* and inhibits properties associated with stem cell expansion. (A) Western blot and immunohistochemistry of Met-1 cells transduced with vector or DACH1 expression vector tagged with Flag. (B) Nude mice were injected with equal numbers of Met-1 breast cancer cells co-transduced with either control vector or DACH1 expression plasmid. The tumor volume and tumor weight of Met-1 cells implanted in nude mice. Analysis was conducted of N=5. Data are mean \pm SEM. (C) Serial implantation study of Met-1 cells transduced with either vector or expression plasmid. (D) FACS display of CD24/CD29 double staining of cells isolated from Met-1-GFP or Met-1-DACH1 tumors (Data are mean \pm SEM, N=5, P<0.001). (E) Aldefluor staining of Met-1 cells transduced either with DACH1 or a mutant of DACH1 which is defective in DNA binding (DACH Δ DS).

Fig. 3

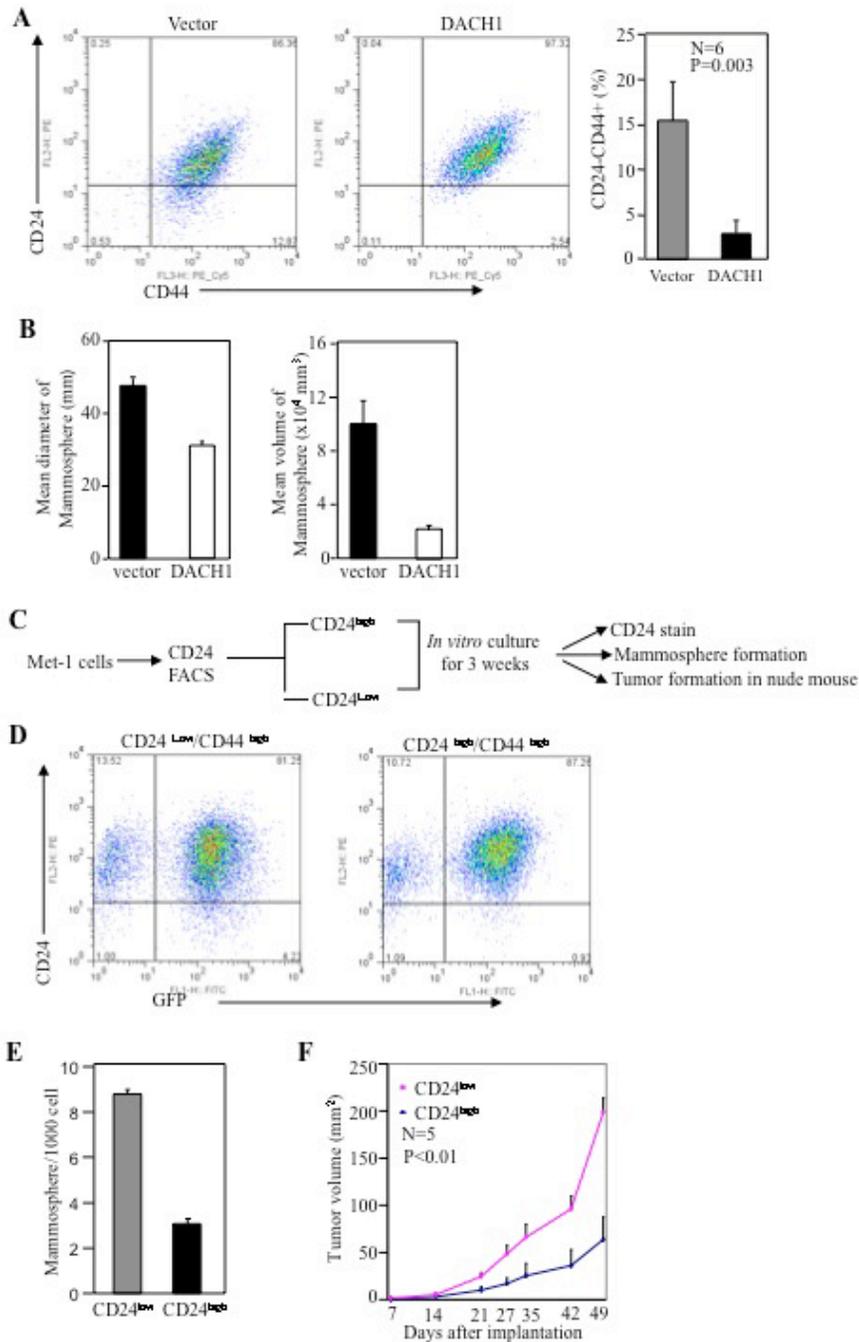


Figure 3. DACH1 reduces the proportion of CD24^{low}/CD44^{high} cells. (A) Mammosphere assays of Met-1 cells transduced with vector control or DACH1. (B) FACS based CD24/CD44 double staining of Met-1 cells *in vitro* expressing DACH1 or control vector. (C) The cellular potential of CD24^{low}/CD44^{high} vs CD24^{high}/CD44^{high} cells was determined. Schematic representation of methods for analysis of Met-1

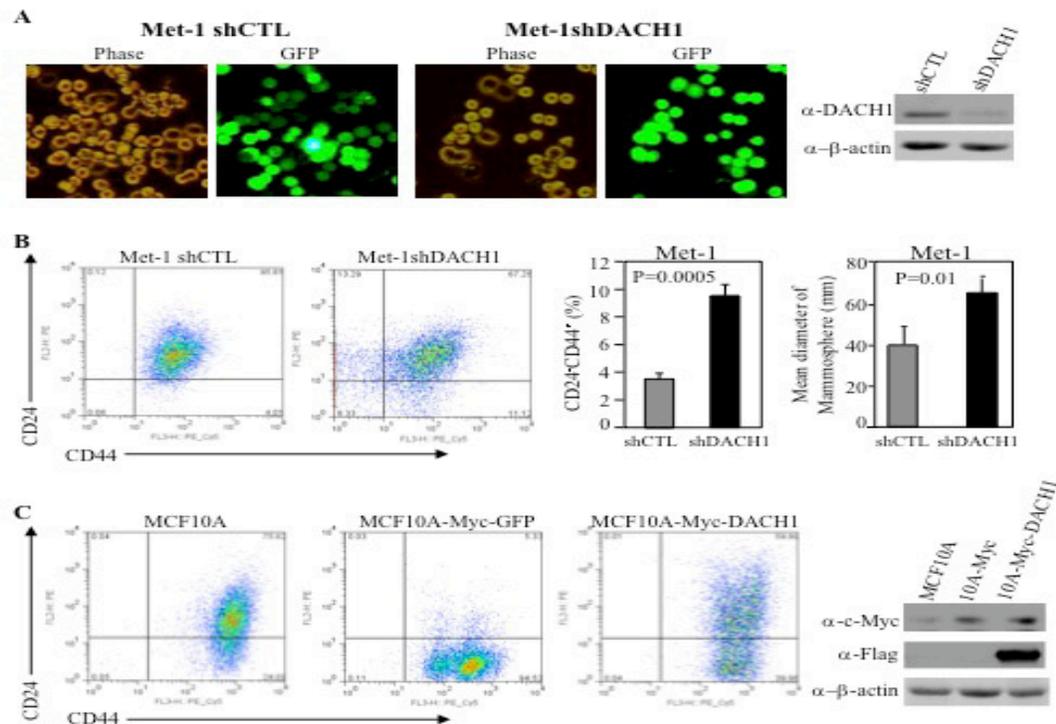


Figure 4. Endogenous DACH1 inhibits the stem cell phenotype. (A). Knocking down endogenous Dach1 expression by Lentivirus shRNA vector to Dach1. (B). FACS based CD24/CD44 double staining of Met-1 cells expressing shCTL or shDACH1. (C). Mammosphere number and size of Met-1 cells expressing shDACH1. Data are mean \pm SEM of 5 separate experiments. (D). FACS display of CD24/CD44 double staining of MCF10A and Myc transformed MCF10A with vector control or DACH1. Western blot of MCF10A transduced cells. Each cell type also expresses GFP from the vector as a marker.

Research Project 2: Project Title and Purpose

Phosphorylated Proteins in Breast Cancer Prognosis - Our overall objective is to develop techniques and protocols to measure quantitative changes in protein levels in the stromal and epithelial compartments of clinical samples to 1) identify new prognostic markers of response to therapy and outcome and 2) to understand the role of autocrine and paracrine signaling associated with the tumor microenvironment.

Anticipated Duration of Project

1/1/2010 - 6/30/2012

Project Overview

In this project, we aim to classify retrospectively collected tumor samples using phosphopeptide expression profiles and apply these profiles to classify tumor types. We hypothesize that the observed heterogeneity in clinical characteristics and therapeutic response in breast cancer populations is associated with differential expression of phosphopeptides. In order to investigate this hypothesis, we have defined the following objectives:

Specific Aim 1: Optimize protocols for the extraction of total proteins and phosphoproteins from formalin fixed tissue that is compatible with the iTRAQ reagents and with Multiple Reaction Monitoring (MRM) experiments.

Specific Aim 2: Optimize the methods for laser capture micro-dissection of formalin fixed tissue using slides that have been stained with Hematoxylin and two-color Immunohistochemistry that are compatible with mass spectrometry.

Specific Aim 3: Determine the differences in both total protein expression and phosphoprotein expression in the epithelium and stroma of normal breast tissue and in tumor tissue.

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Other Participating Researchers

None

Expected Research Outcomes and Benefits

Breast cancer is a heterogeneous disease in which tumors that are similarly classified by traditional diagnostic and prognostic indicators can have markedly different clinical outcomes. Although metrics such as lymph node status, histological grade, tumor size, hormonal receptor status and the presence of distant metastases are correlated with prognosis, these clinical parameters indicate little about the biology of the disease. In addition, the development and effectiveness of targeted therapies in defined patient populations in breast cancers (e.g. Tamoxifen, estrogen receptor-positive breast cancer; Trastuzumab, *ERBB2*-positive breast cancer) makes accurate sub-classification of tumors a priority.

Altered function of signaling pathways also plays a role in clinical response. For example, constitutive phosphorylation of *AKT* in the *PI3K* pathway mediated by loss of *PTEN* or mutation of *PIK3CA* has been identified as a mechanism of Trastuzumab resistance. Phosphopeptide expression profiling provides the means to catalog expression signatures of this and other similar mechanisms of drug action and resistance, and generate a tumor sub-classification system that more accurately predicts clinical outcome.

Our expectation is that examining changes in the protein levels associated with the loss or gain of particular gene products in human tumors will provide new insight into the signaling mechanism associated with disease development, progression and response to therapy that cannot be captured in either cell culture or animal models alone.

Summary of Research Completed

The three specific aims examine the use of proteomics to determine if changes in phosphorylated proteins in breast tumors and changes in total proteins in the epithelium and the stroma can be used to stratify patients to clinical response or identify new aberrant signaling pathways in the tumors.

For the reporting present reporting period through 6/30/2010, we have been working extensively on Specific Aims 2 and 3. For Specific Aim 2, we have optimized the extraction of proteins from formalin fixed paraffin embedded tissue, and were able to analyze the proteins after labeling with isobaric tags. We found that the number of proteins that we can identify is within 90% of the number from fresh frozen tissue. The protocol requires a two-step incubation to extract with a total time of 2.5 hours in a Tris-based buffer with 4% SDS (sodium dodecyl sulphate), to denature and solubilize the crosslinked proteins. The combination of Tris and SDS interfere with both enzymatic digestion of the extracted proteins, labeling of the peptides with isobaric tags, and the mass spectrometry. To solve this problem we utilized a buffer exchange using 30 kDa Molecular Weight Cutoff Filters (MWCO). First the SDS was removed by using 8 M Urea in triethylammonium bicarbonate (TEAB). The high content of Urea will effectively allow removal of the detergent micelles and the TEAB does not contain any free amines and is compatible with the iTRAQ labeling.

To test the effectiveness of the procedure, we compared the mass spectrometry results between two samples that were cut from the tissue where one was frozen, and the other was formalin

fixed and embedded in paraffin. Using the optimized protocol for protein retrieval, digestion and labeling, but a short mass set of mass spectrometry runs, we found that the frozen sample yielded 180 protein identifications compared to the formalin fixed sample which yielded approximately 90% of the identifications. In addition, the requirement to obtain a working amount of protein from these tissues is only two ten micron slides from a 1 cm tumor. The protocol requires lysis and heating in a buffer that is not compatible with the labeling so several buffer exchanges are performed into an amine free buffer. We have found that the labeling efficiency is greater than 99% and this processing does not negatively impact the results.

For Aim 3, we have been focusing our work on the identification of differentially expressed proteins in the stroma and epithelial cells in the tumor. The largest technical difficulty with the laser capture microdissection is the tissue sections are normally stained with hematoxylin and eosin to facilitate identification of the different cellular populations. However, there are several reports that occurrence of interference is due to eosin staining, and that the staining with hematoxylin will only minimally effect the mass spectrometry. To verify this, we took two tissue slides stained with Eosin and two slides that were unstained, isolated the proteins and analyzed the samples using mass spectrometry. Using a single reverse-phase liquid chromatography-mass spectrometry (LC-MS) run, we obtained approximately 70% of the protein identifications in the stained sample compared to the unstained sample. While this is acceptable, it would be better if the yield were higher. What is clear from the extracted ion chromatograms is that, the stained samples have a much higher background. This is likely due to residual eosin and we are currently exploring using ion exchange chromatography to remove the excess stain, and to determine if the eosin will interfere with the amine labeling of the isobaric tags.

We are currently examining eight breast cancer tumors, four of which stained positively for estrogen and progesterone receptors and are negative for Her2. The other four samples are the triple negatives and do not show positive staining for all three of the receptors. Laser Capture Microdissection is ongoing and should be completed shortly.

Research Project 3: Project Title and Purpose

The Role of Lipogenesis Pathway in Endometrial Cancer Progression - The number of women with newly diagnosed endometrial cancer (EC) increased 20% from 1987; however the number of deaths posted a 168% increase during the same time period. Estimated new cases and deaths from EC in the United States in 2009 are 42,160 and 7,780, respectively. As population size affected by this disease is expected to grow, particularly in developing countries, EC will continue to be a serious public health problem. Despite the fact that over-expression of lipogenic enzymes were observed in endometrial tumors, the role of the master regulator of lipogenesis, sterol regulatory element-binding protein 1 (SREBP1), is largely unknown. This project will determine the role of SREBP1 and DACH1, a newly identified transcriptional repressor, in endometrial tumorigenesis and the mechanisms by which lipogenic pathways regulate tumor progression.

Anticipated Duration of Project

1/1/2010 - 12/31/2011

Project Overview

The significant increase of lipogenesis is a metabolic hallmark of rapidly proliferating tumor cells. Although most normal cells acquire the bulk of their fatty acids from circulation, tumor cells synthesize more than 90% of required lipids de novo. Consistent with a great demand for lipid synthesis, diverse human cancer cells express high levels of lipogenic enzymes, such as fatty acid synthase (FASN). Although the clinical correlations between the over-expression of lipogenic enzymes and tumorigenesis are well documented, little is known about the molecular mechanisms by which the lipogenic pathway is enhanced in tumorigenesis. The sterol regulatory element-binding protein 1 (SREBP1) and peroxisome proliferator-activated receptor gamma (PPARgamma) are master regulators of lipogenesis in diverse organisms.

Previous studies have established that FASN, one of the major transcriptional targets of SREBP1, promotes synthesis of various fatty acids, which then serve as ligands for PPARgamma activation. However, whether SREBP1, FASN, and PPARgamma contribute to endometrial carcinogenesis has not been systematically investigated. Excitingly, the preliminary data of this project suggests that DACH1, a newly identified transcriptional repressor, may negatively regulate FASN expression. DACH1 is down-regulated in endometrial cancers, and the re-introduction of DACH1 reverts tumor-associated phenotypes. In this project, we hypothesize that increased SREBP1 and loss of DACH1 are associated with endometrial cancer progression. They may function collaboratively to promote endometrial tumorigenesis through enhanced lipogenesis.

To test this hypothesis, the correlation between cancer progression and lipogenic gene expression in human endometrial cancer samples will be determined. By using three independent endometrial tissue microarrays and frozen tissues together with experimental approaches including immunohistochemistry (IHC) staining, quantitative real-time PCR (qRT-PCR), and Western Blot, determination will be made of the correlation between 1) endometrial cancer (EC) progression and levels of the key lipogenic enzymes, such as SREBP1, FASN, and PPARgamma; 2) lipogenic gene expression and other known prognostic factors; and 3) evaluation of the diagnostic values of SREBP1 in predicting survival and metastasis in EC.

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Expected Research Outcomes and Benefits

Several reports have noted an enhanced lipogenic pathway in advanced stages of human cancer compared to normal tissue, including human endometrial cancer, in which the PPARgamma and SREBP1 targeted genes such as FASN and SCD1 are induced to high levels of expression. Interestingly, elevated expression of SREBP1 coincided with malignant transformation, cancer progression, and metastasis for several cancer types, particularly hormone-responsive tissues including breast and prostate cancers. The role of SREBP1 in endometrial cancer is largely unknown. Given the evidence of elevated expression of SREBP1 transcriptional target genes, one aim is to first determine the SREBP1 expression status in endometrial cancer. Increased nuclear staining for SREBP1 in higher grade (G2-G3) tumors from our pilot study suggests that enhanced SREBP1 transactivation may contribute to endometrial cancer progression through induction of lipogenic gene expression and lipogenesis.

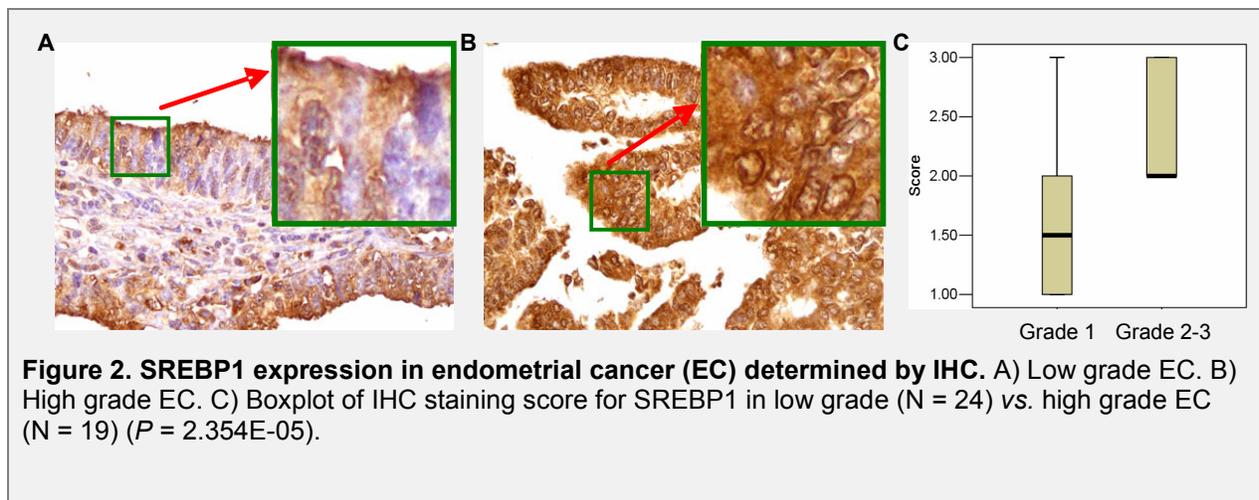
In this project, there will be expansion of analysis to determine whether increased expression and/or nuclear translocation of SREBP1 are the preferred mechanism for lipogenesis in human endometrial cancer. This project will allow identification of the molecular mechanisms underlying lipogenesis-dependent tumor growth in endometrial cancer. This is important because the data will provide new strategies for targeting endometrial cancer progression; according to this model, targeted SREBP1 inhibition may efficiently inhibit tumor growth. Furthermore, together with known prognostic factors, SREBP1, DACH1, and PPARgamma may serve as diagnostic biomarkers for human endometrial cancer and may be used to predict response to targeted therapies.

Summary of Research Completed

First six months of study has resulted in the following:

1. We have conducted initial screening for SREBPs expression in endometrial cell lines and showed that SREBP1a was highly expressed in poorly-differentiated AN3-CA cells compared to well-differentiated ECC-1 cells (Fig. 1).
2. Our data showed that by IHC staining SREBP1 is over-expressed in poorly-differentiated EC. Nuclear localization of SREBP1 was frequently seen in poorly-differentiated tumors (grade 2-3), but not in well-differentiated tumors (grade 1) (Fig. 2). The majority of SREBP1 was found in the cytoplasm of epithelial cells of normal endometrium and well-differentiated tumors, while nuclear SREBP1 was detected in high-grade from moderately differentiated to poorly differentiated tumors (Fig. 2). These observations strongly support the hypothesis that activated SREBP1 plays a role in tumorigenesis and cancer progression.

We have prepared the reagents and cell lines needed for these studies, and our preliminary data and published reports have confirmed the efficacy of these materials. We have successfully employed the tetracycline-inducible shRNA expression systems for knockdown experiments.



Research Project 4: Project Title and Purpose

Tailored Preference Intervention and Colon Cancer Screening in Primary Care - The purpose of this project is to determine the impact of a mailed, preference-based intervention on colorectal cancer screening among African Americans.

Anticipated Duration of Project

1/1/2010 - 6/30/2013

Project Overview

Colorectal cancer (CRC) screening can reduce CRC incidence and significantly lower CRC mortality by detecting and removing polyps at an early stage of disease. Unfortunately, screening is underused by African Americans. This project will be a cohort study ancillary to an ongoing American Cancer Society (ACS)-funded, IRB-approved investigation of African Americans who are 50 to 74 years of age.

This project will involve additional patients drawn from the same setting as that of the ACS study. We will assign 427 consenting participants to receive a mailed intervention that is based on the individual's preferred CRC screening test, ascertained from a baseline survey. This approach will be referred to as a "tailored preference intervention" (TPI). Participants in the TPI group who prefer stool blood testing, will be mailed a stool blood test kit; those who prefer colonoscopy will be mailed instructions for scheduling a colonoscopy; and those who have an equal preference for the two tests will be mailed both. A generic screening reminder will be sent to participants 45 days after randomization. An endpoint survey and endpoint chart audit will be completed six months following study group assignment. The primary aim of the project is to determine whether screening use is significantly greater in the TPI group than in the Standard Intervention (SI) group of the ACS study, who received a mailed stool blood test, along with instructions for colonoscopy screening.

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Expected Research Outcomes and Benefits

The primary goal is to determine if screening utilization in the Tailored Preference Intervention group over the six-month observation period is significantly greater than screening use in the Standard Intervention Group. This outcome will be a dichotomous variable based on data obtained from the endpoint chart audit and the endpoint survey. We will compute the proportion of participants who screened, and the corresponding 95% confidence interval for TPI group participants. Screening test use will encompass CRC screening tests (i.e., SBT, colonoscopy, flexible sigmoidoscopy, or barium enema x-ray) performed during the six-month observation period after randomization. A screening test occurrence will be counted if it is recorded or reported in either source, along with an associated procedure performance date that falls within the study's six-month follow-up period. Through this research, we expect to identify the intervention approach that achieves the highest level of participation.

Summary of Research Completed

The primary aim of the project is to determine if screening in the project tailored preference intervention (TPI) Group is higher than screening in the study groups in the original ACS-funded study.

Research methods

Intervention development

Intervention materials and procedures developed for use in this study include the following:

- A personalized study invitation letter
- Personalized letters from the participant's primary care practice that encourage screening. The letters are tailored to reflect the individual's preferred colorectal cancer screening test (colonoscopy or stool blood test).
- An informational brochure on CRC screening

Recruitment Procedures

The process used for recruiting the TPI group is the same as the one used in the ongoing ACS-funded study.

- Two samples are pulled using the IDX systems at Thomas Jefferson University (TJU) and Albert Einstein Healthcare Network (AEHN).
- Electronic medical records are reviewed and patients are excluded from the sample because of recent CRC screening, personal diagnosis with CRC or polyps and other conditions, such as family history of CRC.
- Study invitation letters are sent to the remaining sample of potential participants. The letter states that patients who do not want to participate can stop all further contacts related to the study. Potential participants are provided several methods to stop further contacts (or "opt out") (a) returning an enclosed opt out card in an enclosed pre-addressed, postage-paid envelope or (b) calling a toll-free telephone number, if they prefer.
- Professional telephone interviewers contact all potential participants who have not "opted out" by 14 days after the study invitation letters were mailed. The interviewers, following a script approved by the TJU and AEHN institutional review boards, obtain an oral informed consent and assess eligibility. They then conduct a baseline survey with consented eligible patients.
- Consented, eligible participants who complete the baseline survey are randomized into a study group.

Study accrual

To date, 31 participants have completed a baseline survey and have been assigned to the TPI group.

Intervention

The intervention given to those randomized to the TPI group is modified slightly from the ones used in the ongoing ACS study. The 31 participants randomly assigned to the TPI group have

received a mailed intervention that is based on the individual's preferred CRC screening test ascertained from the baseline survey. Of this number, 4 participants have been sent materials that encourage stool blood testing, and 27 have been sent materials that encourage colonoscopy.

Milestones

Milestone(s) for 1/1/2010-6/30/2010: Recruit 125 participants and administer TPI.

Personnel changes at primary study site and unexpected delays in completing the development of intervention materials slowed the recruitment process. As these have been addressed, we expect that the pace of recruitment will increase.

Research Project 5: Project Title and Purpose

MicroRNA Genetic Variations as Predictors of Hepatocellular Carcinoma Risk - The project goal is to use a pathway-based polygenic approach to identify genetic variations in miRNA genes that can be used to predict the risk of hepatocellular carcinoma (HCC) in patients with chronic Hepatitis B virus (HBV) infection. The efforts will be essential for our ultimate aim to build up an HCC risk assessment model that can be applied in clinical settings.

Anticipated Duration of Project

1/1/2010 - 12/31/2011

Project Overview

Hepatocellular carcinoma (HCC) is the fifth most common solid malignancy and the third leading cause of cancer mortality worldwide. Chronic hepatitis B virus (HBV) infection is the most prominent established etiologic factor for HCC. Worldwide, there are over 400 million HBV patients, over 5% of the world's total population. Approximately 20% of these patients develop HCC. Previous studies have reported non-genetic predisposition factors for HCC. However, *bona fide* genetic determinants largely remain to be identified. This proposal builds on and seeks to further extend our previous studies on the molecular epidemiology of microRNA (miRNA) in cancer susceptibility. In the proposed study, our goal is to use a pathway-based polygenic approach to identify genetic variations in miRNA genes that can be used to predict the risk of HCC in patients with chronic HBV infection. The efforts will be essential for our ultimate aim to build up an HCC risk assessment model that can be applied in clinical settings.

The specific aims of the proposed project are:

- 1) To assess the genetic susceptibility of single nucleotide polymorphisms (SNPs) of miRNA genes in the development of HCC in chronic HBV patients. We will genotype 12 common SNPs located in precursor miRNA and mature miRNA regions in 800 chronic HBV patients (200 cases and 600 controls). Our hypothesis is that genetic polymorphisms in miRNA genes modulate HCC susceptibility through their influences on the production and functions of mature miRNAs.

- 2) To develop a multivariate quantitative risk prediction model for HCC in chronic HBV patients. We will develop a multivariate risk prediction model to identify the gene-gene and gene-environment interactions that modulate the progression from HBV infection to hepatocarcinogenesis. The prediction model will integrate epidemiologic, viral, clinical, and genetic data. We hypothesize that there are joint and interaction effects between these factors in the modulation of HCC susceptibility in chronic HBV patients.

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Expected Research Outcomes and Benefits

In this project, we expect to identify miRNA gene polymorphisms that contribute to the predisposition of HCC in HBV patients. Once validated, the biomarkers will become promising targets for downstream genetic fine-mapping, deep re-sequencing, and functional characterizations. Furthermore, we will incorporate findings with currently available risk factors, to develop an exploratory risk assessment model that stratifies chronic HBV patients, and identifies those with the highest potential for malignant progression. In addition, the further growth of our HBV patient cohorts will offer us the opportunity to develop a clinic-based prospective longitudinal study to evaluate the antiviral treatment efficacy and other factors that affect the risk of HCC in HBV patients.

In the United States, the incidence of new, acute, HBV infection has decreased in the past two decades, but the number of patients living with chronic HBV infection has been growing. This development is partly due to the trend of increasing immigration from countries with high HBV endemicity. Of the 41 million Americans born outside of the United States, approximately 1.5 million have HBV infection. The direct annual cost of HBV-related medical care increased from less than \$400 million to over 1.3 billion within 20 years. In addition, over 60% of current HBV patients in the United States are relatively young, with an age between 20 and 49 years. As these patients age, significant increases in HBV-induced HCC and corresponding medical costs are expected to occur. Continuous monitoring of these patients and identification of high-risk individuals among them for targeted intervention are important to the reduction of HBV-induced burden to the healthcare system of the United States. Therefore, the findings of this proposed project will be of considerable public health significance.

Summary of Research Completed

During the time period between January 1, 2010 and June 30, 2010, we have been working on the retrospective construction of the database for the chronic HBV (CHB) patients that have visited the Liver Disease Prevention Center, in the Division of Gastroenterology and Hepatology at Thomas Jefferson University. A trained clinical research specialist has been working on chart review and data entry. To ensure data accuracy and consistency, the Principal Investigator (PI) audited a sample of charts at regular intervals. As of June 2010, we have finished the charts of 1750 patients, among which 1501 patients had complete and validated data. A comprehensive collection of patient variables are identified for these patients. These variables included:

Epidemiological variables: The following HCC-related epidemiological data are identified from medical charts: age, gender, ethnicity, residence, family history of HBV and HCV infections, family history of HCC and other cancers, smoking status, drinking status, and cirrhosis. We do not have information on AFB1 exposure in the medical chart. However, AFB1 is not a major risk factor for HCC in Korean populations.

Viral status: From the medical chart, we have collected information on HBV viral characteristics, which have been reported to be potential HBV-HCC susceptibility factors. These data include viral DNA load, HBsAg status, and status of HBV e antigen (HBeAg) and antiHBe (HBeAb). All these variables have been associated with altered clinical outcome in CHB patients. Although we did not perform viral genotyping, nearly all HBV strains transmitted in Korean populations have genotype C. We also do not have information on HBV viral mutations in this population. The mutations in several key regions of the viral genome have been associated with the risk of HCC in CHB patients whereas the results were mixed.

Clinical variables: We have also collected clinical variables that are available from the medical charts including: serum levels of alanine transaminase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), albumin, total bilirubin, direct bilirubin, gamma-glutamyl transpeptidase (GGT), alpha-fetoprotein (AFP), ferritin, blood urea nitrogen (BUN)/creatinine, count of white blood cells and platelets, high-density lipoprotein (HDL), low-density lipoprotein (LDL), cholesterol, and triglycerides. The majority of these variables are available for the initial visit as well as many post-treatment follow-up visits. Fibrosis/cirrhosis was determined by clinical diagnosis and supported by magnetic resonance imaging (MRI) findings. MRI findings noted atrophy of the right lobe and/or medial segment of the left lobe, contour of the liver, presence of siderotic nodules, the nodularity of the liver parenchyma, signs of portal hypertension, including splenomegaly, presence of varices and other collaterals, and ascites. Size of the spleen, amount of ascites, and number of collaterals (including varices) were all graded by MRI assessment. Among these variables, ALT and presence with fibrosis/cirrhosis have been significantly associated with HBV-related HCC. All of these clinical variables will be tested in the multivariate risk assessment model.

Table 1 lists the basic characteristics of the 1501 study subjects that are currently in our database. Among the 1501 patients, 250 patients have HCC, whereas the rest are cancer-free HBV patients. The average age (standard deviation) of the 1501 patients is 45.3 (13.3). Approximately 65.5% patients are males. About 58.9% are never smokers and 53.9% are never drinkers.

Approximately 33.2% had family history of HCC and 17.1% had family history of cirrhosis. We further analyzed the distributions of these host characteristics in HCC and cancer-free HBV patients separately. It is apparent that there are statistically significant ($P < 0.001$) differences in the case-control distributions for the majority of these major host characteristics (Table 1). For example, cases are significantly older than controls (57.6 years vs. 42.9 years). There is significantly higher percentage of males in cases (80.8%) than in controls (62.4). Compared to controls, cases have higher percentages of ever smokers (60.4% vs. 37.3%) and ever drinkers (66.0% vs. 42.1). In addition, cases have a significantly higher percentage of patients with a positive family history of HCC (52.3%) than controls (27.9%). However, we did not find significant differences in the distribution of family history of cirrhosis between cases (16.9%) and controls (17.1%) (Table 1).

In addition to the host characteristics listed on [Table 1](#), we have also collected a wide spectrum of clinical variables. Because the majority of our patients had multiple visits to the clinic in our Institute, we are not able to list all the clinical variables in this summary. However, for each patient, we identified the earliest visit that have the most complete clinical data. We then conducted a preliminary analysis to determine the distribution of these clinical variables between cases and controls. We found significant differences between cases and controls on the distribution of total protein ($P = 0.03$), albumin ($P < 0.001$), total bilirubin ($P < 0.001$), ALP ($P < 0.001$), AST ($P < 0.001$), GGT ($P < 0.001$), count of platelets ($P < 0.001$), ferritin ($P < 0.001$), and BUN ($P = 0.02$). However, no significant difference was identified for the case-control distribution of direct bilirubin ($P = 0.70$), ALT ($P = 0.57$), count of white blood cells ($P = 0.08$), hemoglobin ($P = 0.24$), cholesterol ($P = 0.19$), HDL ($P = 0.94$), LDL ($P = 0.86$), triglycerides ($P = 0.09$), and creatinine ($P = 0.29$). Nonetheless, these results should be interpreted with caution since the rate of missing data is high (7.5%-76.4%).

Moreover, we have also collected the data for another three clinical and viral variables that have been reported to be significantly associated with the incidence of HCC in HBV patients, including AFP, status of HBeAg/Ab, and viral DNA load. Based on previous reports, we grouped the continuous data in AFP and viral DNA load into categories. For AFP, the categories are < 20 (ng/ml), 20-99, 100-999, 1000-9999, > 9999 . For viral DNA load, the categories are < 300 (copies/ml), 300-9999, 10000-99999, 100000-999999, > 999999 . For HBeAg/Ab, the categories are +/-, -/+, +/+, -/-. The further analysis found that the case-control distribution of all the three variables are statistically significant with a P value of < 0.001 , < 0.001 , and 0.006 for AFP, HBeAg/Ab, and viral DNA load, respectively.

Table 1. Overall host characteristics and their case-control distributions for the currently available 1501 HBV patients in our database

Characteristics	Total patients (%) N=1501	Cases (%) N=250	Controls (%) N=1251	P value
Age (\pm SD*) (years)	45.3 (13.3)	57.6 (10.8)	42.9 (12.3)	<0.001
Gender				
<i>Male</i>	983 (65.5)	202 (80.8)	781 (62.4)	<0.001
<i>Female</i>	518 (34.5)	48 (19.2)	470 (37.6)	
Smoking status				
<i>Ever smoker</i>	617 (41.1)	151 (60.4)	466 (37.3)	<0.001
<i>Never smoker</i>	884 (58.9)	99 (39.6)	785 (62.8)	
Drinking status				
<i>Ever drinker</i>	692 (46.1)	165 (66.0)	527 (42.1)	<0.001
<i>Never drinker</i>	809 (53.9)	85 (34.0)	724 (57.9)	
Family history of HCC				
<i>Yes</i>	499 (33.2)	171 (52.3)	328 (27.9)	<0.001
<i>No</i>	944 (62.9)	151 (46.2)	793 (67.5)	
<i>Missing</i>	58 (3.9)	5 (1.5)	53 (5.6)	
Family history of Cirrhosis				
<i>Yes</i>	256 (17.1)	43 (16.9)	213 (17.1)	0.70
<i>No</i>	1163 (77.5)	201 (78.8)	962 (77.2)	
<i>Missing</i>	82 (5.4)	11 (4.3)	71 (5.7)	

*SD, standard deviation

Research Project 6: Project Title and Purpose

Development of a Decision Aid for Hepatitis C Testing in High Risk Populations - More than 3 million Americans are infected with Hepatitis C virus (HCV); many have no symptoms and are unaware of their HCV status. HCV infection is a leading cause of chronic liver disease and the most common reason for liver transplantation. Specific populations that are at increased risk for HCV infection have been defined, and a reliable diagnostic test to determine HCV status exists. However, there is disagreement among experts about the utility of routine testing for high-risk individuals. In situations like this, patients may benefit from additional information to assist them in deciding to be tested. This project lays the groundwork for a larger study that applies a technique called “**mediated-decision counseling**” to assist patients in making an informed decision about HCV testing.

Anticipated Duration of Project

1/1/2010 - 6/30/2012

Project Overview

Medical experts disagree about the necessity of routine HCV testing for those at high-risk of infection. Decision counseling assists patients in making decisions regarding which courses of action are reasonable and acceptable. In this project, a trained professional will explain to patients the risks and benefits, as well as the expected outcomes and consequences, of HCV testing. This will enable the patient to comprehend their choices regarding whether to participate in testing, and will allow shared-decision making with a physician.

The primary objective for this project is to develop and pilot test a “decision aid” for high-risk individuals considering HCV testing. All materials will be pre-tested with representatives of the target audience prior to use in the pilot test, and key processes and outcomes will be measured. Existing materials related to HCV testing will be reviewed and altered if necessary, to fit the needs of this project. For the pilot test, individuals who present for a health care visit at Jefferson Family Medicine Associates (JFMA), will be assessed of their HCV risk using a short questionnaire. Those who are found to be at increased risk will be eligible to participate in the research project.

The Research Assistant will provide an explanation of the project to participants, and obtain informed consent prior to their scheduled appointment. The physician-patient encounter will be audio taped to assess baseline rates of informed decision making by the patient. After the physician visit, the patient will complete a brief baseline survey, and then be guided through decision counseling by a research staff member to clarify their preference for HCV testing. Within seven to ten days, patients will complete an end-point survey; roughly 90 days after the office visit, a chart review will assess HCV test performance.

There are multiple aims for this project. The first is to develop the baseline and end-point surveys, and the HCV testing decision aid for the counseling sessions. The second is to recruit high-risk individuals to participate in the pilot test of the decision counseling protocol. The third is to measure baseline rates of informed decision making during physician-patient encounters. The fourth is to determine which factors in decision-making predict HCV testing among high-risk individuals.

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Expected Research Outcomes and Benefits

Expected outcomes include the development of materials and procedures that can lay the groundwork for future projects of a larger scale. Individuals who are involved in the pilot test will benefit from knowing their HCV risk. Those who are found to be at increased risk of HCV may opt to take preventive measures, while those who are found to be infected with HCV as a

result of testing will have the opportunity to begin treatment early, which could prevent long-lasting infection, liver damage and possibly death.

Summary of Research Completed

During the first 6 months of the study, from January 1, 2010 through June 30, 2010, substantial progress was made and is detailed below.

Specific Aim 1: Develop Study Materials

The following materials were developed for use in this study:

1) Participant Eligibility Screener: To be eligible to participate in the study, a person must be at increased risk of hepatitis C. To determine the most common risk factors, a comprehensive review was conducted of studies in medical journals, and information from expert organizations and government agencies. It was determined that a participant must identify with having at least one of the following eight risk factors: donating blood or receiving an organ transplant prior to 1992; a tattoo or body piercing (not including ear piercing); a needle stick injury; kidney dialysis; a spouse or partner with hepatitis C; incarceration in prison for more than 24 hours; a history of intravenous drug use.

Due to the sensitive nature of these risk factors, it was important to have a method that provided confidentiality. We worked with experts in computer-automated research, to design a custom program that would provide ease and anonymity for participants, when answering the eligibility questions. The finished product was loaded onto a laptop (a netbook), with an audio version and earphones available for participants with limited literacy, vision or lack of computer skills. Only a composite eligibility result (*increased risk=eligible, low risk=not eligible*) is displayed for the research assistant.

2) Study Questionnaires

After eligible participants have completed written consent, they are given a paper-based, self-administered, baseline questionnaire. The purpose of the baseline questionnaire is to assess participants' specific risk factors for infection, knowledge about hepatitis C, attitudes toward testing, confidence and support in making healthcare decisions, frequency of related health behaviors, and demographic information. Items in the baseline survey were taken from previously used questionnaires related to hepatitis C and decision counseling.

Approximately ten days after completing study related activities, participants are contacted via telephone, to complete an end-point questionnaire. This contact is made by a research team member who was not involved in the participant's baseline session, to decrease the chance of response bias. Changes in measures collected during the baseline questionnaire, as well as self-reported testing status, and an evaluation of the decision counseling session and use of the netbook for eligibility screening are assessed. Items in the end-point questionnaire are compatible with those in the baseline questionnaire, to assess temporal changes where appropriate.

3) Educational Booklet

We conducted an assessment of currently available educational materials related to testing for hepatitis C, to develop a short pamphlet that could be used during the decision counseling session. However, none of these met the needs of this study. Therefore, the research staff created an 8-page booklet, with the assistance of a graphic artist, designed to our study specifications and patient population. This includes information about the risk of hepatitis C, the consequences of infection, the risks and benefits of testing, an explanation of test results, and treatment availability.

Specific Aim 2: Pre-test Study Materials

The materials from Specific Aim 1 were pre-tested for readability and sensitivity, using two groups of Thomas Jefferson University (TJU) patients. One group consisted of three patients who had undergone treatment for hepatitis C. These patients reviewed the educational booklet. All of the patients felt that the booklet was informative, written in language that was understandable, and designed in a way that was appealing to others. The patients felt it would have been valuable to have had such an educational pamphlet available when they were learning about hepatitis C.

The educational booklet was also pre-tested with seven patients from Jefferson Family Medicine Associates (JFMA), the location of study recruitment. These patients completed an Educational Materials Review form, which was developed by staff in Medical Oncology. Four of the patients noted the easy readability, and three patients appreciated the diversity of the people featured in the booklet's pictures. Four patients reported that the format was easy to follow and was in a logical order. The results from the pre-testing assisted the study team in making minor changes to ensure readability, likeability, and cultural sensitivity for the educational booklet.

The educational screener and the questionnaires were pre-tested with two patients who had received treatment for hepatitis C. Additionally, TJU researchers with expertise in research questionnaire design were consulted on the study's final surveys. The patients were comfortable taking the eligibility screener and had little to no difficulties answering the questionnaires. Based on comments from the patients, a few items on the questionnaires were re-worded to improve readability. The research experts offered improvements to the order of the risk factor questions in the eligibility screener, so as to not begin with the most sensitive questions. The experts also suggested additional questions for the questionnaire, based on similar studies in the past.

Specific Aim 3: Pilot Test a "Decision Aid" for High-Risk Individuals Considering Hepatitis C Testing.

Our goal was to maximize patient participation, while minimizing the burden to the patients and providers at JFMA. We recruit patients from the JFMA waiting area, and recognize the importance of not disrupting patients who are waiting for their scheduled appointments. The Research Assistant approaches patients to assess their interest in learning about hepatitis C and knowing their risk of infection. Those who are interested use the netbook to determine if they are eligible to participate. If they are eligible and wish to participate, the patient completes

informed consent and sees their doctor as scheduled. Subsequent to the appointment, the patient completes the baseline questionnaire and receives an education and decision counseling session delivered by the Research Assistant. The educational booklet is used during the session to guide the order of information and the discussion. Approximately 7-10 days later, the Research Assistant administers the follow-up questionnaire via telephone and determines the patient's hepatitis C testing status through the electronic medical records system at JFMA.

Patient recruitment for the pilot test started on May 10, 2010. To date, a total of 15 patients have been enrolled from the waiting area at JFMA. One hundred and ninety-five people have been approached to assess their hepatitis C risk status (via the participant eligibility screener). Fifty-six patients completed the eligibility screener on the netbook, while an additional 8 patients were unable to complete the screener before their pre-scheduled appointment. Of the 48 who completed the screener, 23 were found to be at increased risk of hepatitis C and were eligible for the study. Out of the 23 that were eligible, 15 agreed to participate. Table 1 summarizes this data.

Table 1: Summary of recruitment activities

Number Approached	Number Screened	Screen Completed	Screen Incomplete	Eligible	Enrolled
195	56	48	8	23	15

Research Project 7: Project Title and Purpose

CAV3, Myo-Epithelial Barrier Function and Breast Cancer - Advanced medical technologies and therapeutic strategies are necessary for the successful detection, diagnosis, and treatment of human breast cancer. This project will identify new paracrine signaling mechanism(s) that contribute to breast cancer pathogenesis, using a new mouse model, the Cav-3 knock-out (KO) mice. These mice develop pre-malignant mammary lesions including mammary ductal thickening, increased primary ductal branching, and lobulo-alveolar hyperplasia. In addition, the mice will be crossed into the polyomavirus middle T-antigen transgenic mice (MMTV-PyMT) genetic background to determine the role of Cav-3 in mammary tumor formation and metastasis. This will allow the elucidation of the role of Cav-3 and myo-epithelial barrier function in ductal carcinoma *in situ* (DCIS) and invasive ductal carcinoma (IDC). The Cav-3 knock-out mice will also be valuable for testing new therapies for breast cancers.

Anticipated Duration of Project

1/1/2010 - 12/31/2010

Project Overview

Caveolin-3 (Cav-3) is a muscle-specific gene that is primarily expressed in muscle cell types, including myo-epithelial cells. Caveolins function as broad-spectrum kinase inhibitors that can induce cell cycle arrest in the G0/G1 phase of the cell cycle. In this project, the role of Cav-3 in the pathogenesis of human breast cancer will be investigated, with a focus on myo-epithelial cell functioning. Myo-epithelial cells play a tumor suppressor role as the gatekeepers of luminal

mammary epithelial cells. During progression from DCIS to IDC, the protective role of the myo-epithelial cell layer is compromised.

A genetic approach will be used to study the protective role of myo-epithelial cells in this transition process using Cav-3 (-/-) null mice, which lack caveolin-3 protein expression in their myo-epithelial cell layer. These mice are predicted to develop myo-epithelial cell dysfunctions such as hyper-proliferation and defects in terminal differentiation—since Cav-3 normally functions in mediating cell-cycle arrest. As such, loss of Cav-3 may also increase the mammary stem/progenitor cell population, predisposing the mice to the development of invasive mammary lesions. Thus, the role of Cav-3 will be assessed in the tumor micro environment using Cav-3 null mice as a novel preclinical model.

The two Specific Aims of the project are:

1. Determine the role of Cav-3 and Myo-Epithelial Cells in Mammary Cell Hyperplasia, Dysplasia, and Ductal Carcinoma *In Situ* (DCIS).
2. Determine the role of Cav-3 and Myo-Epithelial Cells in Mammary Tumor Onset, Progression, and Metastasis.

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Expected Research Outcomes and Benefits

Breast cancer is a major cause of morbidity and mortality worldwide. New diagnostic and therapeutic strategies are urgently needed. The current research project will elucidate novel signaling pathways that directly contribute to the onset and progression of human breast cancers.

Recently, a novel pre-clinical mouse model was generated by this laboratory, to determine the functional role of Cav-3 and the myo-epithelial cell layer in the onset and development of mammary tumorigenesis and metastasis. The results indicate that this novel mouse model (Cav-3 KO mice) shows the onset of mammary hyperplasia, which can be considered a pre-malignant mammary abnormality.

Finally, Cav-3 KO mice will be examined to determine if they are more susceptible to the development of mammary tumors and lung metastases, by crossing these mice with a well-established mouse model of mammary tumorigenesis, namely MMTV-PyMT mice. This project will then examine if a loss of Cav-3 and abnormal myo-epithelial cell functioning exacerbates the development of DCIS and full-blown mammary tumors.

This novel mouse model could also be used for testing the efficacy of newly developed breast cancer therapies.

Summary of Research Completed

Results for the first six months of the project (January 1, 2010 to June 30, 2010)

Cav-3 may function either as a tumor suppressor or tumor promoter, depending on the signaling pathway which is activated during mammary tumorigenesis. Our current results may also explain the lethality of pregnancy-associated breast cancers (which are likely to be Wnt-related), as compared to other types of human breast cancers. Given that our results suggested that a loss of Cav-3 suppressed mammary tumorigenesis by inducing a more differentiated phenotype via enhanced Wnt-signaling (data not shown) in MMTV-PyMT mice, we next chose to cross Cav-3 +/- mice with MMTV-Wnt1 mice. We predicted that under these conditions, a loss of Cav-3 would promote tumor growth, as it would synergize and lead to over-activation the Wnt-pathway. Figure 1 shows that our results were exactly as predicted. A loss of Cav-3 in the context of MMTV-Wnt1 mice increased tumor burden by 3.6-fold.

Wnt Mammary Tumor Studies. Wnt transgenic mice in the FVB/N background were purchased from Jackson Laboratories. Matings were performed with Wnt male hemizygous mice. Wnt/Cav-3 (+/+) and Wnt/Cav-3 (+/-) male mice were interbred with Cav-3 (+/+) or Cav-3 (-/-) female mice to generate a cohort of Cav-3 (+/+), Cav-3 (+/-), or Cav-3 (-/-) virgin female mice, all hemi-zygous for the Wnt transgene. None of the Wnt transgene-negative control mice developed tumors. At 16 weeks, virgin female mice were sacrificed, and all mammary tumors were carefully excised and weighed.

Primary Tumors: MMTV-Wnt1

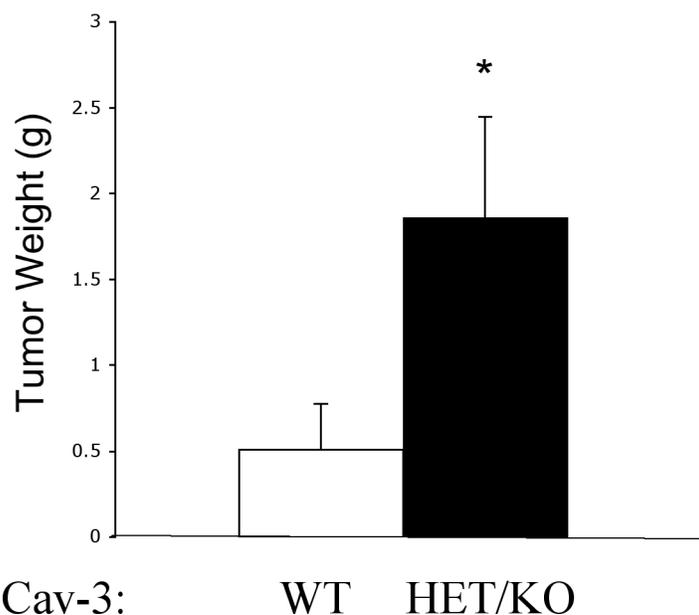


Figure 1. Genetic ablation of Cav-3 promotes mammary tumor formation in MMTV-Wnt1 mice. Wnt virgin female transgenic mice were sacrificed at 16 weeks of age, and all tumors were carefully excised and weighed. As Wnt/Cav-3 (+/-) and Wnt/Cav-3 (-/-) mice showed similar results, the two groups were combined together. Note that Wnt/Cav-3 (+/-) and Wnt/Cav-3 (-/-) mice show a ~3.6-fold increase in tumor mass, as compared with Wnt/Cav-3 (+/+) mice. **WT**, Wnt/Cav-3 (+/+); **HET**, Wnt/Cav-3 (+/-); **KO**, Wnt/Cav-3 (-/-). N=14 mice for the WT group. N=18 mice for the HET/KO groups * p < 0.05.