University of Pittsburgh

Annual Progress Report: 2010 Formula Grant

Reporting Period

January 1, 2011 – June 30, 2011

Formula Grant Overview

The University of Pittsburgh received $8,236,620 in formula funds for the grant award period January 1, 2011 through December 31, 2014. Accomplishments for the reporting period are described below.

Research Project 1: Project Title and Purpose

Cellular Systems Biology in Cancer Drug Discovery - The project focuses on the development of a research program to discover and develop small molecule anticancer therapeutics. At the University of Pittsburgh, novel chemistries are combined with cellular systems biology and computational pharmacology approaches to drug discovery. The overriding goal of this project is to further enhance the broad scientific capabilities at the University of Pittsburgh in these research areas and to deploy them for the development of novel anticancer drug candidates and, ultimately, commercial drugs.

Anticipated Duration of Project

1/1/2011 - 12/31/2013

Project Overview

The pharmaceutical industry has been hampered in developing new drugs, including cancer drugs, by the complexity of biological systems that give rise to on- and off-target effects of lead compounds in living systems. It is now understood that life is based on integrated, interacting networks of genes, proteins, and metabolic processes that give rise to either normal or, in disease, abnormal functions. This complex "systems biology" must be addressed to create a safe and effective drug. The pharmaceutical industry has, instead, focused primarily on simplifying biological testing to speed up the process. The result has been a serious failure rate (>90%) in getting drug candidates safely through the drug discovery and development process. However, academic groups are ideally positioned to investigate cancer biology and potential off-target compound toxicity by directly studying this complexity with more sophisticated cell-based model systems and computational methods. The University of Pittsburgh Drug Discovery Institute (UPDDI) has been integrating its expertise in cancer biology, chemistry, medicinal chemistry, safety assessment, and computational molecular and systems biology to pursue novel cancer drug discovery. The goal of the present project is to advance anticancer drug discovery by integrating current activities with novel chemistry, systems biology, and computational

pharmacology approaches and applying the new methodology to developing three classes of targets: (1) improved modulators of microtubule dynamics, (2) transcription factors (e.g., STAT3 [signal transducer and activator of transcription]), and (3) modulators of the interaction between two critical proteins (e.g., Myc-Max) in an important cellular signaling pathway (Myc is also a transcription factor). Functional, cell-based models using human cell lines, primary cells, and ultimately validated stem cell-derived cells will be developed and investigated using medium-throughput and high-content light microscope imaging methods, as well as mechanistic probe molecules, to define the systemic response to both commercially available and novel drug candidates. Computational methods will be developed and applied to assess the protein-protein and protein-inhibitor interactions at the molecular structural level and to relate the cellular responses to known patterns of gene and protein responses, cellular pathways, and protein-protein interaction networks. Critical attention will be given to polypharmacology effects for early safety assessment and compound prioritization. Promising compounds will be further developed by lead optimization and pharmacokinetics studies, including animal models.

Principal Investigator

D. Lansing Taylor, PhD
Director, Drug Discovery Institute
University of Pittsburgh
10045 Biomedical Science Tower 3
3501 Fifth Avenue
Pittsburgh PA 15260

Other Participating Researchers

Albert Gough, PhD; Peter Wipf, PhD; Barry Gold, PhD; Larry Vernetti, PhD; Ivet Bahar, PhD – employed by University of Pittsburgh

Expected Research Outcomes and Benefits

This project will harness collaborations across the University of Pittsburgh from multiple departments and institutes to create novel compounds that can be progressed toward effective therapeutics. A key outcome will be to progress one or more compounds to the point at which there is significant interest by the pharmaceutical industry to either license the candidate drug or collaborate with UPDDI to develop the candidate further. The project will increase the University's research capacity by creating new opportunities in tissue engineering, chemical synthesis, systems biology, and computational biology and should yield new technologies that can be licensed to industry partners. The University of Pittsburgh has a strong presence in cancer biology with the University of Pittsburgh Cancer Institute (UPCI) and has significant expertise and federal funding that can facilitate the drug discovery process. Together with the Departments of Pharmaceutical Sciences, Chemistry, and Computational and Systems Biology, UPDDI is also a member of the Chemical Biology Consortium (CBC) of the National Cancer Institute, and the Department of Chemistry hosts one of five National Institutes of General Medical Sciences (NIGMS)-sponsored Centers for Chemical Methodologies and Library Development. These centers provide the researchers access to chemical libraries and broad

collaborations with other academic centers of excellence working on cancer therapies. The application of a cellular systems biology approach increases the potential of a more personalized medicine outcome for sub-populations when stem cells replace the human cell lines and primary cells in the future.

Summary of Research Completed

We have made progress toward our specific aims and met several milestones proposed for the first reporting period of this project.

Milestones for the period 1/1/2011 – 6/30/2011

Implement the protocols for growing and maintaining six selected cell lines from the National Cancer Institute (NCI)-60 human tumor cell line
Seven cells lines from the NCI set of human cancer cell lines (NCI-60) have been established for use in our laboratory, including CCRF-CEI, OVCR, K562, EKVX, H226, H28, and A549. HepG2 liver cell cultures and rat primary hepatocytes have also been established and are available as needed for early safety assessment.

Implement the cell systems biology profiling panel for the initial microtubule stabilizing leads
Since there has been a delay in the synthesis of the dictyostatin analogs, we chose to preferentially implement a panel profile for the heterogeneity of the transcription factors STAT3/STAT1 of pathway responses (Aim 2).

We have implemented a novel "hyperplexing" method. For the purpose of this report, we define hyperplexing as the effective use of six or more antibodies to interrogate pathway-specific and pathway-independent effects in a cell exposed to a bioactive stimulus. When coupled to organelle-specific and chemical live dyes, it is expected that 10 or more cell features can be independently measured within each cell with a high content screening (HCS) imager system. The hyperplexing of antibodies can be accomplished by a number of different methods, including chemical stripping; optical bleaching; use of antibody blocking agents; chemical bleaching; use of multiple fluorophores; deconvolution; and "clipping," or use of "nano-scissors." Presented here are our preliminary results showing the use of a chemical stripping method to remove the primary and secondary antibodies to visualize microtubules in A549 and HepG2 cells.

Implement the initial computational tools to overlay gene expression and pathway analysis with the cellular systems biology profiling data
We have focused on developing the tools required to identify subpopulations of cells based on multiparameter profiles. We have used a multiparameter data set from a human breast cancer cell line, MB231, to begin developing the subpopulation segmentation methods. We have developed a clustering approach using principal component analysis (PCA), which has been applied to the test data set. The profiles of these unique subpopulations will be overlaid on pathway maps along with gene expression data, using the R package for data processing and Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems) for pathway mapping.

Methods

Cell heterogeneity

A549 cells were cultured using standard cell culture methods, then treated by 25, 50, 100, or 200 ng/ml interleukin 6 (IL-6) or 15, 30, 60, or 120 ng/ml interferon gamma (INF-γ) for 15, 30, 60, and 120 minutes. The cells were formaldehyde-fixed and evaluated for nuclear intensity and phosphorylated STAT3 (pSTAT3) and pSTAT1 translocation. Images were collected at room temperature using an ArrayScan VTI HCS reader in the standard resolution mode with a 20 X /0.75 numerical aperture (NA) objective.

Chemical stripping

A549 and HepG2 cells were cultured by standard cell culture methods. The cells were formaldehyde-fixed and evaluated for microtubule staining by indirect immunofluorescence, and images were collected using the ArrayScan VTI HCS reader. The antibody probe was stripped from cells with a novel stripping reagent for 15 minutes, then probed again by indirect immunofluorescence for microtubule visualization.

Computational methods

The test dataset used contains 12 features measured by fluorescently-labeled MB231 cells treated with laulimalide, a microtubule stabilizer. The feature set includes measures of deoxyribonucleic acid (DNA) content, nuclear morphology, microtubule stability, and p53 activity. Each feature is measured in three ways; for example, nuclear morphology measurement includes size, length/width ratio, and perimeter to area ratio. The measurements are transformed using principal PCA and clustered using a K-means clustering function in R. The optimal number of clusters was selected by determining the mean square deviation within the cluster as a function of cluster size.

Results

The aim of this project is to study the heterogeneity of phenotypic and molecular responses of a population of cancer cells to an appropriate stimulus. We present our preliminary investigation into the heterogeneity of responses of the lung carcinoma cell line A549. The proteins of interest are active forms of the nuclear transcription factors STAT3, a proliferation inducer, and STAT1, an apoptosis inducer. The functional phenotype evaluated is cell cycle.

The concentration and time responses of IL-6-stimulated pStat3 and INF- γ-stimulated pStat1 in A549 lung carcinoma cells are shown in Fig 1. IL-6-dependent activation and cytoplasmic nuclear translocation of pSTAT3 can be imaged by indirect immunofluorescence probing (Figure 1 a, b). Unstimulated cells have no apparent staining evident in the cell nuclei (1a) compared to the increased levels in IL-6-treated cells (1b). The levels of pSTAT3 in the nuclear and cytoplasmic cellular compartments are processed using standard imaging modules provided on the Arrayscan VTI HCS reader to generate the dose and time curve (Figure 1c). The maximal effect is noted at 50 ng/ml after 15 minutes of treatment.

When A549 cells are stimulated with INF-γ, STAT1 is activated with translocation into the nucleus. This activation is shown by immunofluorescence (Fig 2a, b). No evidence of nuclear staining is noted in unstimulated cells reacted against pSTAT1 compared to cells treated with

INF-γ (2a,b) The Array Scan VTI HCS images are processed for dose and time curves, and the dose- and time-related increases are shown in Figure 2c. The maximal effect in this study for pSTAT1 is evident at 60 ng/mlINF-γ after two hours of treatment.

The effect of IL-6 and INF-γ treatment on cell cycle in A549 cells at two hours is presented in Figure 3. The results suggest that IL-6 treatment induces cells to enter the cell cycle, as noted by the trending decrease in the percent of cells with DNA intensity of 2N cells (diploid). These preliminary results are consistent with the known biological behavior of IL-6 and are used here to demonstrate the potential application of HCS-based imaging for linking cell cycle effects to protein-specific states, other pathways of interest, and off-target effects. INF-γ in these experiments does not affect 2N cells but shows an apparent increase in apoptotic cells. These findings are consistent with other findings and represent the point at which detailed analysis can be conducted. Long cell cycling studies are planned.

A demonstration of the chemical stripping method is provided in Figures 4 and 5. A549 cells are fixed and reacted against an anti-α-tubulin antibody (Fig. 4a). Microtubules were imaged by the ArrayScan VTI HCS reader using secondary antibodies conjugated to fluorescein isothiocyanate (FITC) (ex/em 405/480), tetramethylrhodamine-5-(and 6)-isothiocyanate (TRITC) (ex/em 405/530), or cyanine 5 (Cy5) (ex 405/650). The primary and secondary antibodies were effectively removed by a novel treatment and reacted against the same primary and secondary antibodies to re-image the microtubules. Some loss of protein and background is evident (Fig 4b). Microtubules were probed, stripped, and re-probed and are shown in the same HepG2 cells (Figure 5).

Figure 6 illustrates the clustering of cellular subpopulations based on a 12-parameter feature set from MB231 cells treated with laulimalide, a microtubule stabilizer. For this data set, five subpopulations were identified, and a profile was generated for each subpopulation. A second data set containing untreated cells was clustered on the same feature set. We are currently analyzing the differential activity data from the treated and untreated cells. The IPA software was acquired for pathway mapping, and an initial training session has been completed.

Figures

Figure 1.

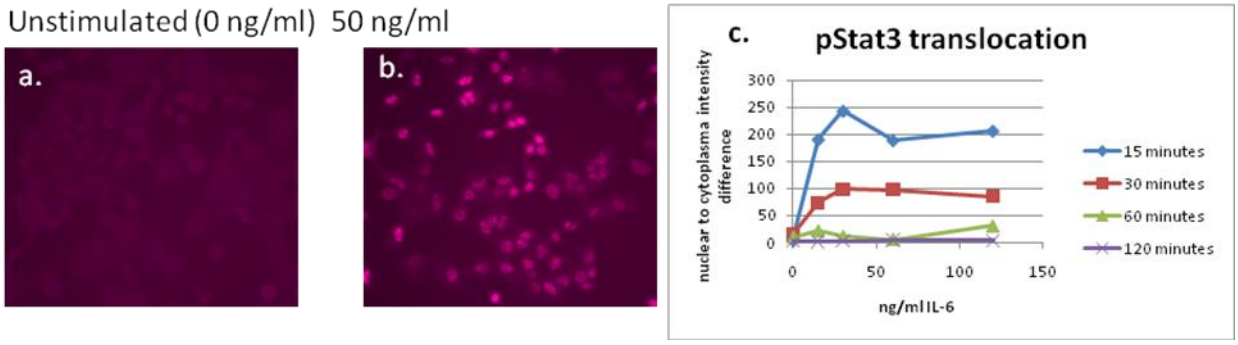


Figure 1. Effect of IL-6 treatment on pSTAT3 nuclear translocation in A459 cells. Normal, unstimulated cells with no nuclear pSTAT1 (a) compared to increased nuclear intensity of pSTAT3 following Il-6 treatment at 50 ng/ml for 15 minutes (b). Images collected and processed by ArrayScan VTI HCS reader and presented as dose and time response curves (c).

Figure 2.

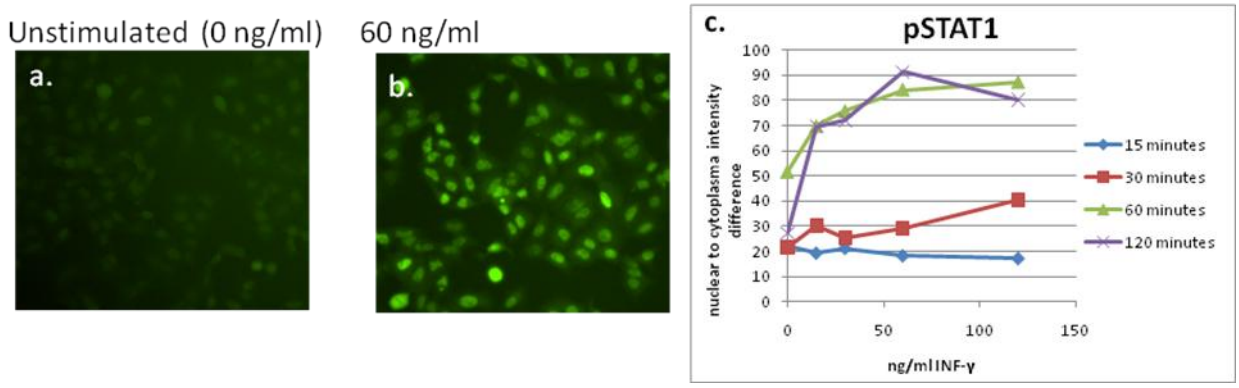


Figure 2. Effect of INF-λ treatment on pSTAT1 nuclear translocation in A459 cells. Normal, unstimulated cells with no nuclear pSTAT1 (a) compared to increased nuclear intensity of pSTAT1 following INF-λ treatment at 60 ng/ml for 120 minutes (b). Images collected and processed by ArrayScan VTI HCS reader and presented as dose and time response curves (c).

Figure 3.

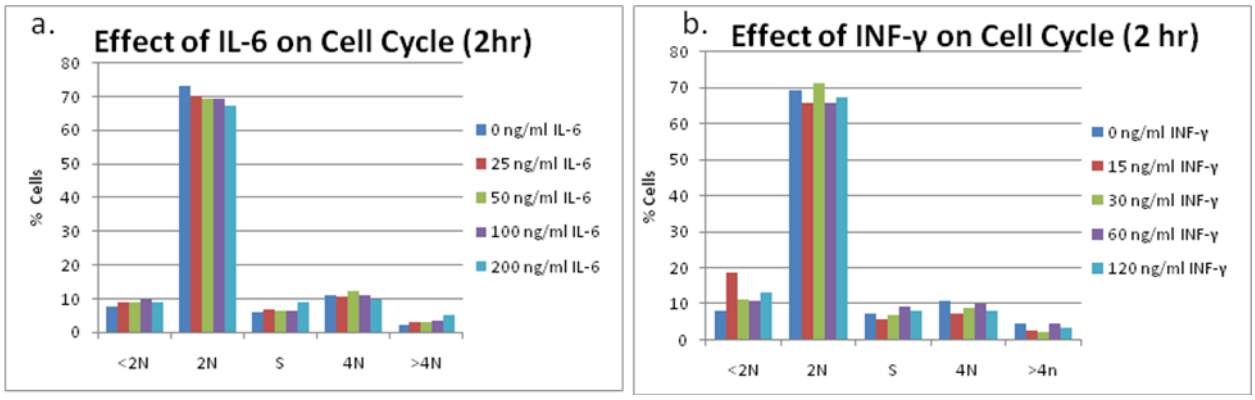


Figure 3. Effect of STAT 3 activator IL-6 and STAT 1 activator INF-γ on cell cycle. Short-term IL-6 treatment shows a dose-dependent effect on percentage of 2N (diploid) cells (a). Short-term INF-γ treatment is showing a slight treatment-related increase in apoptotic cells (b).

Figure 4.

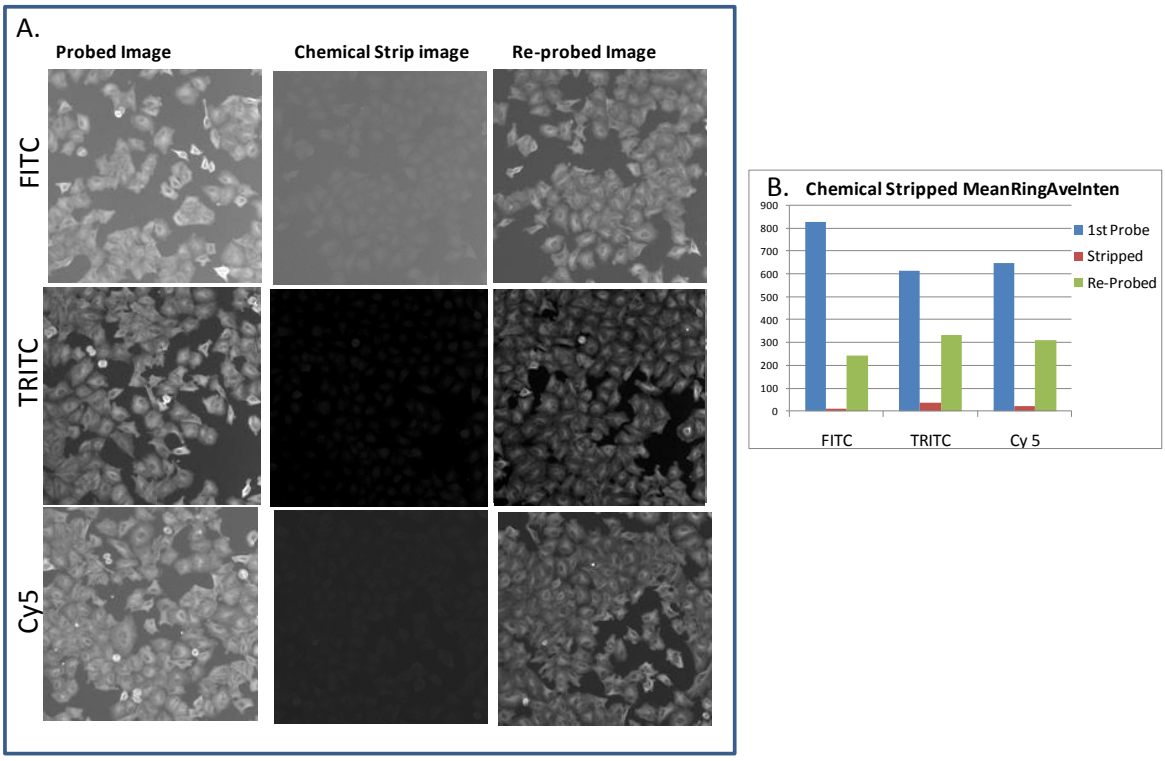


Figure 4. Demonstration of chemical stripping in A549 cells. Microtubules are reacted against anti-α-tubulin and visualized with one of three fluorophore labels. The primary and secondary antibodies can be removed with a chemical stripping agent and the cells re-probed (a). Some loss of protein and intensity is evident with chemical stripping (b).

Figure 5. Microtubules probed and re-probed

Figure 5. Demonstration of chemical stripping method in HepG2 cells. The same set of HepG2 cells are shown probed for microtubules, stripped, and then re-probed. All images are depicted with identical intensity and contrast.

Figure 6.

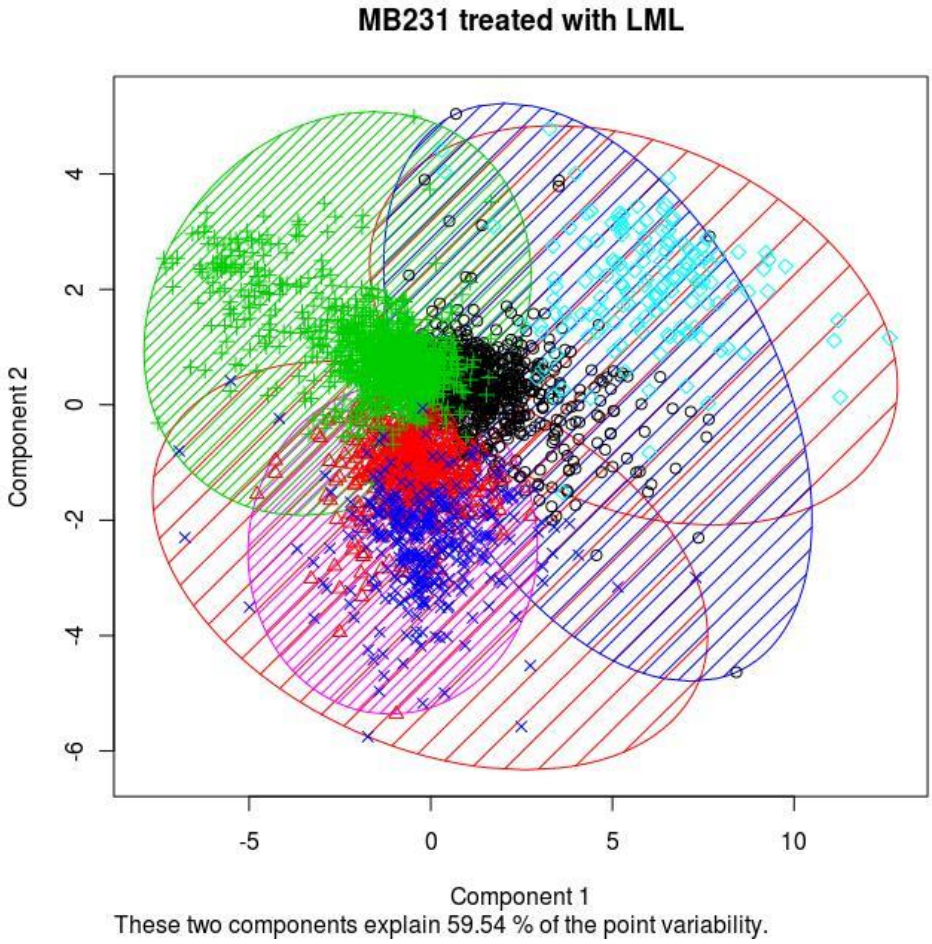


Figure 6. MB231 cellular subpopulations clustered on a 12-parameter feature set. The colored symbols represent five subpopulations identified in this clustering method.

Research Project 2: Project Title and Purpose

Complex Genetics of Congenital Heart Disease - This study will examine the complex genetics of congenital heart disease (CHD), specifically, the role of genetic variants of genes encoding ciliary proteins (the ciliome) in the pathogenesis of CHD. Previous studies have suggested an association between ciliary dysfunction and CHD with heterotaxy, a birth defect characterized by discordant cardiac, lung, and visceral organ situs resulting from aberrant embryonic left-right patterning. This study will use next-generation sequencing technologies to analyze whole exomes of CHD patients to identify disease-causing polymorphisms, specifically in the genes comprising the ciliome. These findings will likely shed light on the complex genetics of CHD and may lead to new diagnostic genetic tests that can help identify patients, specifically infants and children, at high risk for ciliary disorders.

Anticipated Duration of Project

1/1/2011 - 12/31/2014

Project Overview

Heterotaxy is a birth defect characterized by discordant cardiac, lung, and visceral organ situs resulting from aberrant embryonic left-right patterning. Patients with heterotaxy exhibit some of the most complex congenital heart diseases (CHD). Such patients usually require risky surgical repairs, with high rates of postsurgical morbidity, and often develop postsurgical respiratory complications, sometimes becoming ventilator dependent. As mucociliary clearance in the airway requires ciliary motion, respiratory complications and poor outcome could arise from undiagnosed ciliary dysfunction, such as that associated with primary ciliary dyskinesia (PCD). This possibility is particularly compelling for heterotaxy patients, as motile cilia are also required for the specification of laterality during embryonic development. To investigate the role of cilia defects in CHD, we recently initiated a clinical study that recruited patients with complex CHD associated with heterotaxy. From a cohort of 42 patients, 41 percent were observed to exhibit ciliary dysfunction. These results strongly suggest a role for the cilium in human CHD and heterotaxy. We hypothesize that sequence variants in genes required for cilia structure and function (i.e., genes of the ciliome) may play a central role in CHD pathogenesis. To test this hypothesis, we will use whole exome capture and massively parallel sequencing to scan for mutations in the coding exons of the human genome, with particular focus on the ciliome genes. The enriched genomic deoxyribonucleic acid (DNA) recovered from sequence capture will be amplified and sequenced with 100 X coverage using an AB SOLiD™ sequencing instrument. Of particular interest are nonsynonymous variants leading to missense or nonsense mutations and splicing defect variants or small insertions/deletions that may cause frameshifts. Mutations causing CHD are expected to be rare in the unaffected population, with allele frequencies < 1 percent. High priority single nucleotide polymorphisms (SNPs) identified from this analysis will be validated by direct sequencing of patient genomic DNA and further interrogated across datasets available in the public domain, such as from the 1,000 Human Genomes Project. The findings from this first patient cohort will be validated with similar analysis of a replication cohort of an additional 200 patients to be recruited from Children's Hospital of Pittsburgh of UPMC. Overall, these studies may yield novel insights into the complex genetics of CHD.

Results from this study may suggest changes in the standard of care that may improve the long-term prognosis of heterotaxy patients with complex CHD.

Principal Investigator

Cecilia Lo, PhD
Professor and Chair of Developmental Biology
University of Pittsburgh
8120 Rangos Research Center
530 45th St.
Pittsburgh, PA 15201

Other Participating Researchers

You Li, PhD; Jason Cooper; Ashok Srinivasan, PhD – employed by University of Pittsburgh

Expected Research Outcomes and Benefits

Our study seeks to demonstrate a link between ciliary dysfunction, mutations involving genes essential for cilia structure and function, and CHD. These findings may suggest a broad role for the cilium in a wide spectrum of CHD previously thought to have no unifying etiology. This hypothesis, if validated, may suggest changes in the standard of care that could improve the postsurgical outcomes and long-term prognosis of patients with complex CHD. In addition, results from our DNA analyses may provide the basis for the development of diagnostic genetic tests that can help identify patients at high risk for PCD among patients with complex CHD. These data could also suggest changes in the standard of care to include presurgical screening for PCD and instituting appropriate preoperative and postoperative pulmonary therapies, which could improve the long-term prognosis of heterotaxy patients with complex CHD. Overall, these studies may yield novel insights into the complex genetics of CHD and provide strategies for improving the long-term outcomes for our most vulnerable patient population—infants and young children with complex CHD.

Summary of Research Completed

To investigate the genetic etiology of CHD, we have been recruiting patients with CHD associated with heterotaxy (the randomization of left-right patterning of visceral organ asymmetry). As part of these studies, we are assessing each patient with two tests to determine functionality of the cilia in the respiratory airway epithelia. In addition, we obtain blood from each patient from which DNA is extracted for genetic analyses. These studies will test the hypothesis that ciliary dysfunction may play a role in the etiology of structural heart disease. We further hypothesize that respiratory airway ciliary dysfunction may contribute to more postsurgical respiratory complications and worse outcomes.

For the genetic analysis, we have developed a custom exome sequence capture array to interrogate the coding exon sequences of nearly 1,000 genes encoding proteins required for

ciliogenesis. This analysis includes the 13 genes known to cause PCD, as PCD patients also can have heterotaxy and CHD. Sequence analyses are carried out using large-scale next-generation sequencing with the SOLiD sequencing platform. In this first reporting period, we have developed the requisite pipeline to carry out the ciliome sequence capture and next generation SOLiD sequencing and have shown the efficacy of this approach. Thus far, we have completed sequencing analysis of 26 CHD patients with heterotaxy, 13 with ciliary dysfunction, and 13 without ciliary dysfunction. In addition, we sequenced three patients with PCD as disease controls.

A bioinformatics pipeline was developed to streamline and automate the processing and analysis of the next-generation sequencing data. The sequencing reads are first processed using Applied Biosystems AB Bioscope software, followed by analysis using CLC Bio Genomics Workbench software v4.0.3. Single nucleotide variants and small insertions and deletions (1-10bp) are identified at bases with quality scores >20 (99 percent accuracy), coverage >5x, and variant allele frequency >20 percent. Such sequence variants were further annotated and filtered against dbSNP131 and the 1,000 Genomes databases (http://www.1000genomes.org) to remove variants found in the general population. Novel coding variants (NCV) identified using our sequence analysis pipeline were annotated and validated with Sanger capillary sequencing. Several additional filtering strategies were used to identify potential disease-causing mutations in our patient cohorts. First, NCVs in all 13 known PCD genes were identified and sequence-validated, as those genes are known to cause ciliary dysfunction (CD) and situs abnormality. Second, we identified novel homozygous NCVs that fit the monogenic recessive disease model. Third, NCV within genes present in more than one CD patient, but not present in no-CD patients, were validated. We also used a candidate gene approach to selectively validate genes of interest.

Overall, 31 of 98 NCVs have been validated by Sanger sequencing. (Tables 1 and 2), and another 19 genes have sequence variants that are pending validation (Table 3). In the 13 known PCD genes, 18 NCVs were identified (Table 1), comprising 13 novel coding variants in seven of the 13 heterotaxy patients with CD versus five coding variants in only three of the 13 heterotaxy patients without CD (Table 1). While none of our patients had homozygous mutations in any of the PCD genes, we noted that two patients, 9004 and 9026, have heterozygous mutations in two different PCD genes. In one disease control, we observed a homozygous mutation in CCDC39 1072delA, which was previously known to cause PCD. Two other homozygous mutations were identified, NAT10 M566V (patient 9004) and PCM1 N1695S (patient 9003) (Red, Table 2). An additional 10 NCVs were identified in genes present in more than one CD patient (Black, Table 2). One heterozygous deletion, P845fs, was identified in candidate gene TTLL3 (green, Table 2).

To evaluate and biologically validate the function of the non-PCD genes identified with novel mutations in our sequencing analysis, we carried out gene knockdown with antisense morpholino injections in zebrafish embryos. Three genes have been evaluated, a motor dynein DNAH6 and two enzymes, NAT10 and TTLL3, which modify tubulin and can play an important role in ciliogenesis. These studies showed that morpholino knockdown of all three genes can cause heterotaxy, consistent with the phenotypes observed in our patients harboring mutations in these genes. Shown in Table 4 and Figure 1 are the results of experiments with

DNAH6 morpholino knockdown in zebrafish embryos. We observed a curly tail phenotype, which is typically seen when there is a disturbance of laterality, and the expected randomization of the direction of heart tube looping (Table 4). These findings confirm a role for these genes in the regulation of left-right patterning.

To further evaluate their gene function in human cells, we generated a lentivirus expression vector expressing these various genes. Studies underway with NAT10 small interfering ribonucleic acid (siRNA) showed that viral transduction into human-cultured cells blocked formation of nonmotile primary cilia. In addition, viral transduction of NAT10 siRNA into human airway epithelial cells inhibited ciliogenesis of motile cilia. Of significance, patient 9004, harboring a homozygous NAT10 mutation, did not have cilia in the airway epithelia. These same negative findings were observed in nasal scrapes conducted on three different occasions. These findings confirm an essential role for NAT10 in the human airway motile cilia and suggest that the mutations found in our heterotaxy patients may indeed contribute to the ciliary dysfunction in the airway. Taken together with the findings from the zebrafish morpholino experiments, our data suggest that the homozygous NAT10 mutations may also underlie heterotaxy and CHD. Experiments are currently underway to expand the sequence analyses to include more patients and to pursue whole exome sequence analysis. With whole exome sequencing, we will be able to expand our analyses to all coding exons rather than being limited to just the 1,000 genes currently thought to play a role in ciliogenesis. In addition, further biological validation is being pursued with experiments using both animal models and cell culture and primary tissue explant models to test gene function.

Table 1. Novel Variants in Known PCD Genes

Patient ID	CD	Gene	Nucleotide	Amino Acid	Prediction	Dosage
9002	CD	*DNAI1*	IVS1+2_3insT	Truncation	Damaging	het
9003	CD	*DNAH11*	4520A>C	Q1507P	Damaging	het
	CD	*DNAH11*	9397G>A	E3133K	Damaging	het
9004	CD	*TXNDC3*	1630G>A	A544T	Damaging	het
9006	CD	*CCDC39*	626C>G	A209G	Damaging	het
9008	CD		No-Calls			
9011	CD		No-Calls			
9015	CD	*LRRC50*	1294G>A	E432K	Tolerated	het
9017	CD		No-Calls			
9018	CD		No-Calls			
9026	CD	*DNAH11*	9203A>G	E3068G	Damaging	het
	CD	*DNAH5*	11140A>G	I3714V	Tolerated	het
	CD	*DNAH5*	638C>A	P213Q	Damaging	het
9027	CD		No-Calls			
9031	CD		No-Calls			
9037	CD	*DNAI1*	1579T>G	S527A	Damaging	het
7007	no		No-Calls			
9005	no		No-Calls			
9007	no		No-Calls			
9009	no		No-Calls			
9012	no		No-Calls			
9016	no		No-Calls			
9019	no	*DNAH5*	6710A>G	N2237S	Tolerated	het
9024	no		No-Calls			
9025	no	*DNAI1*	1795G>A	A599T	Damaging	het
	no	*DNAI1*	2054T>C	L685P	Damaging	het
9033	no	*CCDC39*	1865A>G	E622G	Damaging	het
	no	*DNAI1*	1177G>A	V393M	Tolerated	het
9035	no		No-Calls			
9040	no		No-Calls			
9068	no		No-Calls			
7016	PCD	*DNAH11*	11987C>T	P3996L	Damaging	Het
9028	PCD	*CCDC39*	1072delA	T358fs	Damaging	homo
9098	PCD	*CCDC39*	1072delA	T358fs	Damaging	homo

Table 2. Novel Variants in Non-PCD Ciliome Genes

ID	CD	Gene	Nucleotide	AminoAcid	Prediction	Dosage
7016	PCD	*ATP2B4*	1997C>T	A666V	Damaging	het
9028	PCD	*DNAH6*	10414A>G	M3472V	Tolerated	het
9098	PCD	*PRDM1*	820G>C	A274P	Tolerated	het
9002	CD	*DNAH6*	6182G>A	R2061Q	Tolerated	het
9003	CD	*PCM1*	5084A>G	N1695S	Tolerated	homo
9004	CD	*DCHS1*	9269A>T	Y3090F	Damaging	het
9004	CD	*NAT10*	1696A>G	M566V	Damaging	homo
9006	CD	*GOLGA4*	5390T>C	L1797P	Damaging	het
9015	CD	*ITGA4*	2053T>C	F685L	Damaging	het
9017	CD	*ABCB1*	1465C>T	R489C	Damaging	het
9018	CD	*TTLL3*	533delC	P845fs	Damaging	het
9027	CD	*DNAH6*	4451A>G	D1484G	Tolerated	het
9037	CD	*C21orf59*	674A>G	Q225R	Damaging	het

Table 3. Variants Pending Sanger Sequencing Validation

ID	CD	Chr	Position	Gene	Ref	Alt	Freq	Cov	Amino Acid
9004	CD	17	7756357	*KDM6B*	-	C	12.5	88	frameshift
9006	CD	2	166806191	*TTC21B*	A	G	68.8	16	Val22Ala
9006	CD	5	180046097	*FLT4*	A	G	83.3	6	Val925AlaAla
9006	CD	1	203677138	*ATP2B4*	G	A	46.9	49	Arg488HisHis
9011	CD	1	203708818	*ATP2B4*	-	G	17.5	57	frameshift
9011	CD	2	166797670	*TTC21B*	T	C	26.7	15	Asn193Asp
9011	CD	9	117139326	*AKNA*	A	G	25	12	Val254Ala
9011	CD	16	712743	*WDR90*	A	G	33.3	9	Ser1404Gly
9011	CD	3	37368986	*GOLGA4*	A	G	25	28	His1870Arg
9015	CD	3	37366388	*GOLGA4*	T	C	27.8	18	Leu1004Pro
9015	CD	11	6653975	*DCHS1*	G	A	69.6	23	Ser923Leu
9017	CD	22	29884976	*NEFH*	G	T	26.9	23	Lys449Asn
9017	CD	5	180057747	*FLT4*	T	G	63.6	11	Thr70ProPro
9018	CD	21	33982217	*C21orf59*	C	T	28.3	106	Gly80Ser
9026	CD	22	29886141	*NEFH*	C	G	29.2	24	Pro838Ala
9026	CD	22	29886230	*NEFH*	G	T	52.6	19	Lys867Asn
9028	PCD	6	106553257	*PRDM1*	G	C	29.0	31	Ala274Pro
9028	PCD	9	117139098	*AKNA*	G	A	35.7	14	Pro330Leu
9031	CD	16	705033	*WDR90*	T	C	23.5	34	Val481Ala

Table 4. *Dnah6* knockdown in zebrafish embryos causes left-right patterning defects

Curly Tail Phenotype

	Normal	Curly Tail
Control MO 10 ng	70	0
Dnah6 MO 7.5 ng	18	53
Dnah6 MO10 ng	1	46

Randomized Heart Looping

	Left Loop	Right Loop
Control MO 10 ng	68	1
Dnah6 MO 7.5 ng	60	22
Dnah6 MO10 ng	31	17

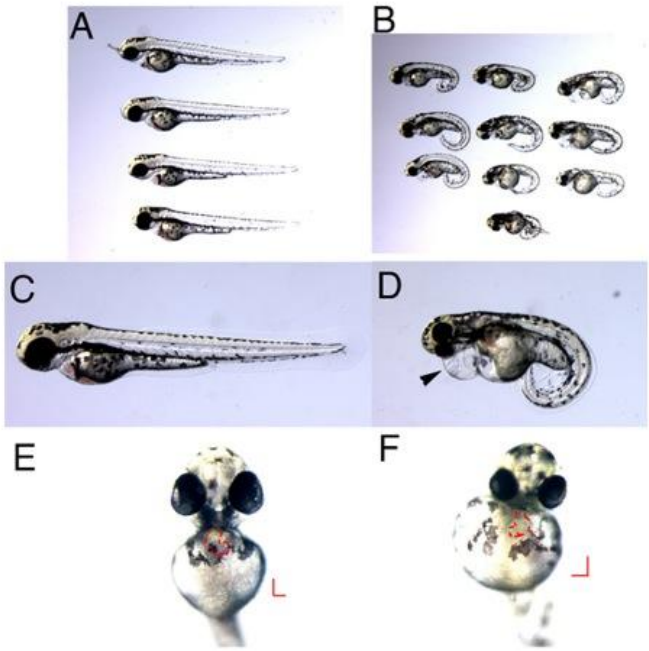


Figure 1. *Dnah6* morpholino knockdown confirms role for Dnah6 in left-right patterning.

(A,C). Control randomized morpholino has no effects on development.

(B,D). *Dnah6* morpholino knockdown results in axial midline defects referred to as curly tail. This same phenotype is a classic feature of defects seen with knockdown of other genes required for left-right patterning.

(E,F). With *Dnah6* knockdown, we observed randomized heart tube looping. Thus, both normal left heart looping and reversed right heart looping are observed.

Research Project 3: Project Title and Purpose

Breaking Metabolic Symbiosis in Tumors: A New Cancer Treatment Paradigm - As cancer cells grow and evolve into tumors, they need specific nutrients and oxygen to fuel cellular metabolism, namely, through mitochondrial oxidative phosphorylation and glycolysis. This project will test the hypothesis that those cancer cells with the highest metabolic flexibility are most successful at developing tumors. In a tumor, the populations of cells farthest from an oxygen supply produce energy through glycolysis and create lactate, a waste product, which is used by the more oxygenated cell population to produce energy by oxidative phosphorylation. This project will examine how this metabolic symbiosis drives rapid tumor growth. Understanding how this interdependent metabolic sharing of carbon sources occurs in a growing tumor and learning how to break the cycle will enable improved therapeutic approaches to cancer.

Anticipated Duration of Project

1/1/2011 - 12/31/2012

Project Overview

Cancer cells require nutrients to grow. Finding out what they ingest and how they grow remains one of the frontiers of cancer biology. Over 70 years ago, Otto Warburg found that tumor cells enjoyed consuming large amounts of glucose through glycolysis, even when supplied an abundance of oxygen. He and his team surmised that the mitochondria of tumor cells were broken and, therefore, could not perform oxidative phosphorylation (OXPHOS). This idea was later proven incorrect, as many tumor cells do show significant respiration and mitochondrial function is vital for their growth. However, one of the most important technologies for identifying metastatic tumors, using positron emission tomography (PET) imaging of F^{18}-labeled deoxyglucose, clearly indicates that metastatic tumor cells have an increased glucose uptake. Furthermore, the identification of mitochondrial deoxyribonucleic acid (mtDNA) mutation accumulation in a large number of different types of tumors suggests that mitochondrial dysfunction does occur in tumor cells. This project will test the hypothesis that tumor cells display remarkable metabolic flexibility and, depending on environmental cues, can evolve into one of two populations of tumor cells: those that are highly glycolytic at the hypoxic tumor core or those with robust mitochondrial oxidative phosphorylation if well oxygenated through a vascular bed. Viewing tumor development and metastasis in this manner demands a two-pronged approach to kill both populations of cells by altering metabolism. This project will directly test this metabolic symbiotic model of tumor development through three interdependent aims. First, carbon source preference and cellular bioenergetics will be examined in several panels of breast, lung, and melanoma tumor cell lines. Specific inhibitors of these processes will be used to interrupt cellular bioenergetics and to examine subsequent effects on cell growth. Second, expression of key genes and proteins controlling glycolysis and OXPHOS will be correlated to the metabolic potential of these cells. The third aim will translate these *in vitro* experiments to human cell xenograft models and primary tumor analyses for direct proof of two populations of metabolically distinct tumor cells. This final aim will also test the hypothesis that mitochondrial mutations arise in the hypoxic glycolytic portion of the tumor.

Principal Investigator

Bennett Van Houten, PhD
Richard M. Cyert Professor of Molecular Oncology
Pharmacology and Chemical Biology, Director of the Molecular and Cellular Cancer Biology Program
University of Pittsburgh
Hillman Cancer Research Pavilion, Suite 140
5117 Centre Avenue
Pittsburgh, PA 15213-1863

Other Participating Researchers

None

Expected Research Outcomes and Benefits

Cancer killed more than 500,000 people in the United States last year and is the cause of death of about one in four Americans. In addition, Pennsylvania lost almost 29,000 citizens to cancer in 2009. Cancer is characterized by uncontrolled cell growth and lack of cell death, and tumor growth is fueled by cellular bioenergetics. Attacking this source of energy alone or in combination with other anti-cancer strategies will help to slow the growth of tumors. This project will directly test the hypothesis that tumor development is mediated by the production of two interdependent cell populations that arise from a single cell type. These two cell populations, pro-glycolytic and pro-OXPHOS, use metabolic symbiosis to share carbon sources to fuel their growth. The hypoxic tumor cell population, which is deprived of oxygen, uses glucose through glycolysis to generate adenosine triphosphate (ATP). The waste product from this reaction, lactate, is then used to fuel the oxygenated cell population to drive OXPHOS. This project will: (1) characterize carbon source preferences for cellular growth using both bioenergetic pathways; (2) show how specific inhibitors of carbon source utilization, glycolyis, or OXPHOS can limit ATP production and subsequent cell growth; (3) characterize and validate gene and protein markers for glycolysis and OXPHOS; (4) provide direct evidence of two cellular populations within a human xenograft model and primary human tumors; and (5) demonstrate that mtDNA mutations arise in the glycolytic, oxygen-poor regions of the tumor. This project will benefit the cancer biology field by providing validated markers of tumor metabolism and logical targets for inhibition to slow tumor growth. The long-term benefit of this project will allow new chemotherapeutic approaches that specifically attack cellular bioenergetics to be added to the arsenal of anti-tumor therapies in the treatment of human cancer.

Summary of Research Completed

During this first six-month period, we achieved several important goals:

Analysis of carbon source preference using the Biolog assay was performed on four human breast cancer cell lines (BT-20, MDA-MB-231, MCF-7, ZR-75-1) and one control cell line (HMEC), Figure 1.
Cancer cells use different carbon sources to grow. While many studies suggest that cancer cells use high amounts of glucose for growth, there has not yet been a systematic analysis of what other types of carbon sources help support cancer cell growth. As part of initiating Aim 1.1, we have analyzed the carbon preference of four breast cancer cell lines using a 96-well plate that contains 90 different carbon sources. Surprisingly, all cancer cell lines were able to grow in lactate (see blue box and arrow in Figure 1). BT-20, a triple-negative, aggressive cell line, was able to grow best using lactose as the sole carbon source. These data suggest that breast cancer cells are metabolically flexible and are capable of converting lactate to pyruvate by the action of lactate dehydrogenase. Pyruvate is then converted to acetyl-coenzyme A (coA) in the mitochondria and used in the tricarboxylic acid (TCA) cycle to generate ATP by OXPHOS. We are currently testing this hypothesis.

The effects of estrogen antagonists in breast cancer cell lines for OXPHOS and glycolysis by the Seahorse XF24 Flux analyzer (Aim 1.3)
We have recently found that antagonism of the estrogen receptor (ER) with either 4-hydroxy tamoxifen or ICI (an anti-estrogen) caused a decline in OXPHOS in ER positive, but not ER negative, breast cancer cell lines. Furthermore, we have found that estrogen stimulates both OXPHOS and glycolysis.

Western analysis of several metabolic enzymes in breast cancer cell lines (initiation of Aim 2.1).
We have begun to analyze some of the important metabolic enzymes in breast cancer cell lines using Western blotting and found an increasing pattern of lactate dehydrogenase (LDH) subunits in different types of breast cancer cell lines (Figure 2). Specifically, luminal cells (blue) and Her2 cell lines (purple) express high levels of LDH alpha, whereas triple-negative cells express high levels of LDH beta (red and grey). Furthermore, normal epithelial cell lines (in green) had about equal levels of the two enzymes. We also saw higher pyruvate dehydrogenase (PDH) levels in luminal, as compared to triple-negative basal or claudin-low, cell lines. Finally, despite the higher levels of OXPHOS and PDH in luminal and Her2 cell lines, we also saw an increase in an inhibitor of PDH, called PDH kinase (PDK), which phosphorylates and inactivates PDH (pPDH) (Figure 2). The precise roles of these enzymes on cellular bioenergetics will be investigated through pharmacological inhibition and genetic manipulation experiments.

Initial analysis of OXPHOS and glycolysis in several melanoma cell lines, including tumor biopsy material (initiation of Aim 3.1.1).
We have begun to analyze OXPHOS in melanoma cell lines and disaggregated metastatic melanoma cells from patients (Figures 3 and 4). To our knowledge, this is the first analysis of tumor cell bioenergetics from cells derived directly from a patient without further growth in culture. Furthermore, we found high levels of both glycolysis and OXPHOS in these metastatic tumor cells, suggesting that they maintain bioenergetic flexibility.

Initial work-up of mtDNA sequencing of cell lines (Aim 3.1.4).
We have adapted an Agilent Mitochip 2.0 for resequencing mtDNA and found that the MDA MB-231 cell line, which has low OXPHOS, has a higher number of mtDNA alterations

compared to MCF7 cells, which have higher levels of OXPHOS. These data are consistent with the hypothesis that triple-negative breast cancer cells accumulate mtDNA mutations.

Figure 1. Carbon source utilization using a Biolog plate assay. Red indicates growth, and green indicates growth inhibition.

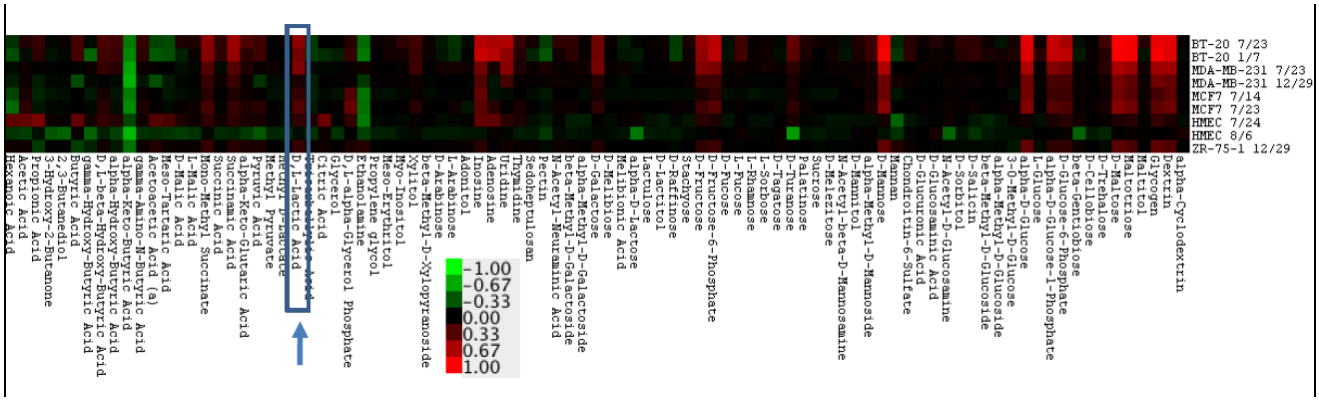


Figure 2. Western analysis of metabolic enzymes in breast cancer cells.

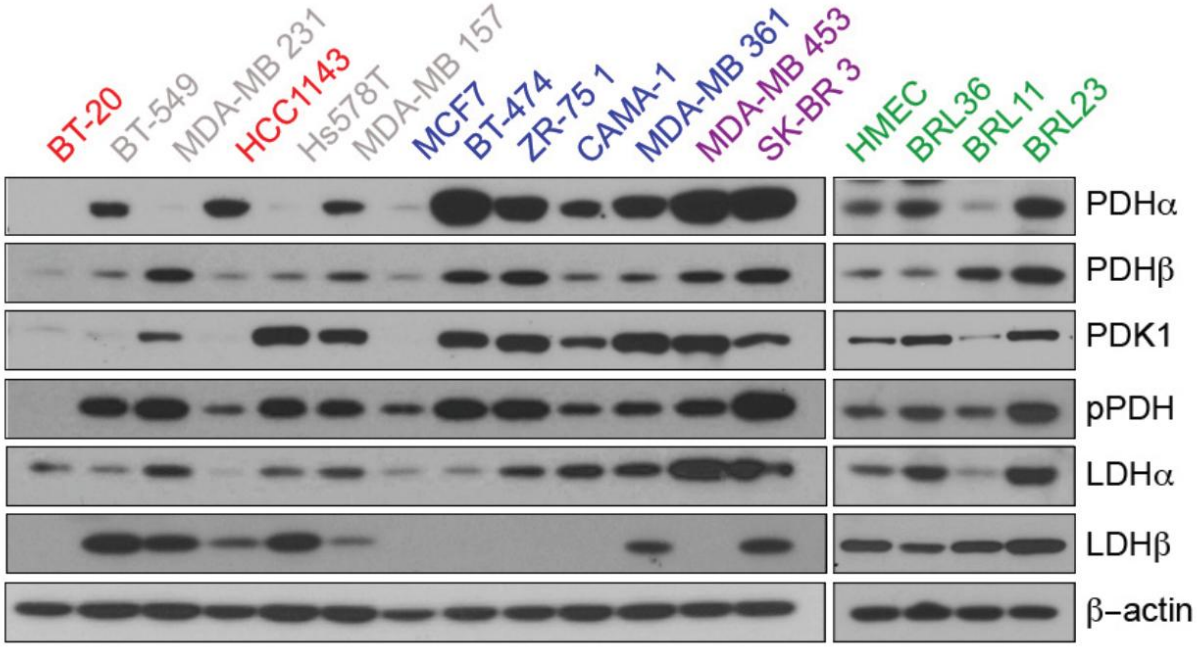


Figure 3. Analysis of cellular bioenergetics

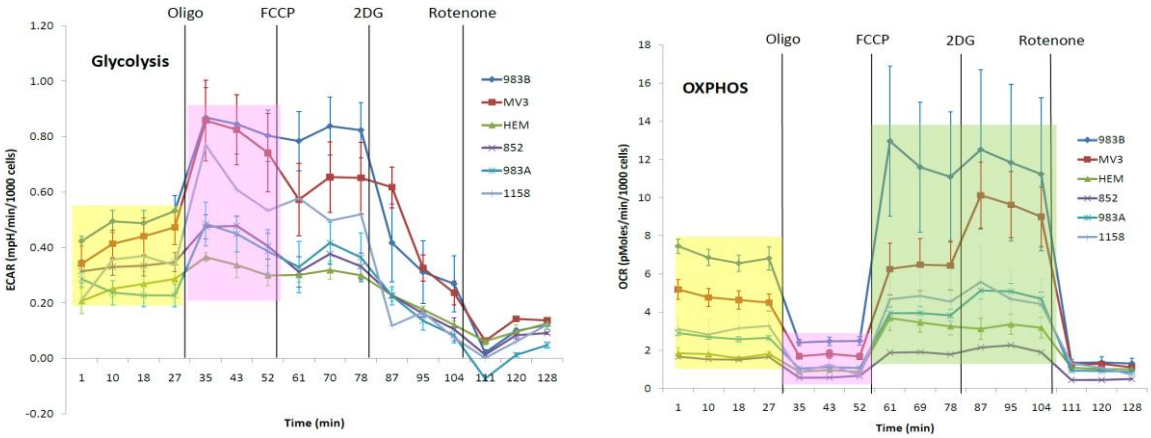
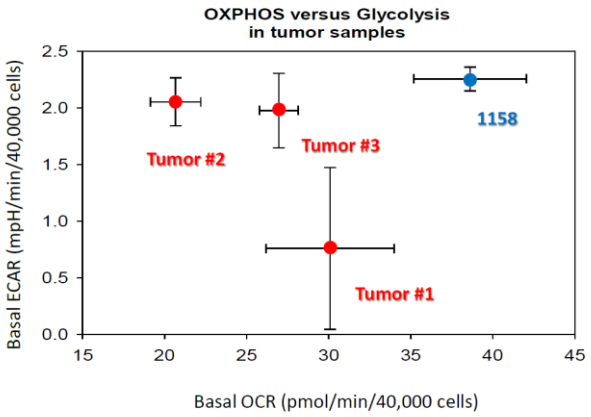


Figure 3. Analysis of cellular bioenergetics in malignant melanoma cells. The left panel indicates oxygen consumption rate (OCR), a measure of OXPHOS. The right panel indicates the extracellular acidification rate (ECAR), which represents glycolysis. In this figure, the yellow regions show the basal OCR and basal rate of glycolysis. Note that the WM983B and MV3 cell lines have a high basal rate of OXPHOS. WM983B also has the highest basal rate of glycolysis. The regions in red show the effect of oligomycin, revealing that cells respond to inhibition of OXPHOS with a concomitant increase in glycolysis. The green region shows the reserve OXPHOS capacity. Note that WM983B (highest) and MV3 have appreciable reserve OXPHOS capacity, while WM852 does not. This project directly tests the metabolic symbiosis model of metastatic melanoma development. This model predicts that tumor cells grown in culture will show high metabolic flexibility (i.e., high glycolytic rates when supplied glucose and high OXPHOS when supplied lactate). These same cells, when placed in a xenograft model, will show high rates of glucose consumption through glycolysis in the hypoxic tumor regions and high levels of lactate consumption and OXPHOS in the aerobic regions. It is interesting to note that WM983B is a metastatic melanoma from a primary melanoma WM93A, and is also BRAF V600E-mutated. WM983B shows robust bioenergetics using both high levels of OXPHOS and glycolysis. WM852 is wildtype for BRAF.

Figure 4. Analysis of cellular bioenergetics in tumor biopsies from patients with malignant melanoma.



Research Project 4: Project Title and Purpose

Prostate Cancer Vaccine Clinical Trials - This project encompasses investigator-initiated clinical trials that explore the safety and efficacy of novel therapeutic cancer vaccines, with the overarching goal of improving outcomes of patients with cancer. Aim 1 includes therapeutic trials of novel cancer vaccines based on polarized alpha dendritic cells (αDC1s) for the treatment of prostate cancer, and Aim 2 examines the efficacy of novel tumor peptide-based cancer vaccines for the treatment of prostate cancer.

Anticipated Duration of Project

1/1/2011 - 12/31/2012

Project Overview

The overall goal of this project is to examine the safety and efficacy of novel therapeutic cancer vaccines designed to improve patient outcomes. Aim 1 will examine αDC1-based vaccines loaded with an allogeneic prostate cell line (LNCap) in combination with androgen ablation (AA) in patients with recurrent prostate cancer who have failed local therapy and have no measurable metastasis but have a rising prostate specific antigen (PSA), with a doubling time of less than 12 months (UPCI 06-070). The primary efficacy objective is to evaluate the effect of the αDC1 vaccine on time to PSA progression compared to AA alone.

Aim 2 will examine trials of novel tumor peptide-based vaccines, including safety and efficacy studies of: (1) a vaccine targeted against mucin-1 (MUC-1), given in conjunction with poly-ICLC (polyinosinic-polycytidylic acid stabilized with polylysine and carboxymethylcellulose) in patients with recurrent and/or advanced prostate cancer (UPCI 05-086). The vaccine approaches described in this project offer immunotherapeutic potential to reduce the risk of tumor recurrence, which may translate into improved patient survival.

Principal Investigator

Gurkamal S. Chatta, MD
Associate Professor of Medicine
University of Pittsburgh
UPMC Cancer Pavilion
5150 Centre Avenue
Suite 564
Pittsburgh, PA 15232

Other Participating Researchers

None

Expected Research Outcomes and Benefits

Aim 1:
UPCI 06-070: This study aims to develop a novel vaccine to treat men with recurrent prostate cancer who have relapsed following surgery and/or radiation and now have a rising PSA, with a PSA doubling time of less than 12 months. This target patient population is ideal for an immunologic intervention, given that: (1) this is the largest sub-population of men with prostate cancer in the United States, (2) there is no standard treatment for this sub-group, and (3) this sub-group has minimal systemic disease burden. Therefore, there is great potential to improve patient outcomes in this group through the development of novel therapies.

Aim 2:
UPCI 05-086: The goal of this study is to develop a safe and effective peptide-based vaccine for men with advanced prostate cancer, which could potentially prolong survival with minimal side effects.

Summary of Research Completed

UPCI 06-070:
To date, nine of 16 patients have been accrued to this study, and we anticipate completing accrual by December 2012.

UPCI 05-086:
Results from the Phase I portion of this trial were acquired in our 2008 formula project, titled "Therapeutic Vaccine Trials in Recurrent Prostate Cancer," which ended in December 2010. We are continuing these studies in the current project and presented the Phase I results at the June 2011 American Society of Clinical Oncology (ASCO) meeting in Chicago, Illinois. We have modified the Phase II portion of the protocol and are currently awaiting approval from the University of Pittsburgh Institutional Review Board to begin accruing Phase II participants.

Research Project 5: Project Title and Purpose

Melanoma Vaccine Clinical Trial - This project is an investigator-initiated clinical trial that explores the safety and efficacy of novel therapeutic cancer vaccines based on polarized alpha dendritic cells (αDC1s) for the treatment of melanoma.

Anticipated Duration of Project

1/1/2011 – 12/31/2012

Project Overview

The overall goal of this project is to examine the safety and efficacy of novel therapeutic cancer vaccines designed to improve patient outcomes. We will examine multi-epitope anti-melanoma vaccines based on polarized alpha dendritic cells (αDC1s), comparing αDC1s to standard DCs in patients with advanced (stage III/IV) melanoma (UPCI 03-118). This study was designed to evaluate the ability of both types of DCs to induce different sets of chemokine receptors on tumor-specific $CD8^+$ T cells.

Principal Investigator

John M. Kirkwood, MD
Director, Melanoma Center
University of Pittsburgh
Hillman Cancer Center Research Pavilion
5117 Centre Avenue
Suite 1.32
Pittsburgh, PA 15213

Other Participating Researchers

Pawel Kalinski, MD, PhD – employed by University of Pittsburgh

Expected Research Outcomes and Benefits

The planned studies represent the first human application of prolonged infusion of antigen-loaded DCs. The success of these studies will allow the reduction of the DC dose used in similar applications in patients with melanoma and other types of cancer, thereby reducing treatment costs and increasing its effectiveness.

Summary of Research Completed

We are currently in the accrual and early testing phase of this project. To date, 20 of 28 patients with stage III/IV melanoma have been or are currently being treated. Patients were vaccinated with either peptide-loaded αDC1 vaccines or vaccines based on standard DCs. We examined immunological data of several patients in the initial cohort and found that they exhibited

antitumor effects and autoimmune antipigmentary immune responses—a finding that we interpret as early proof-of-concept. However, full accrual of the remaining eight patients will be required to assess the appropriate dose of DCs as we move forward with our specific aims.

Research Project 6: Project Title and Purpose

Glioma Vaccine Clinical Trials - The University of Pittsburgh Cancer Institute's Clinical Research Service (CRS) provides valuable resources for researchers seeking to improve patient care standards and treatment efficacy. This project encompasses investigator-initiated clinical trials that explore the safety and efficacy of novel tumor peptide-based cancer vaccines for the treatment of low-grade glioma (LGG).

Anticipated Duration of Project

1/1/2011 – 12/31/2012

Project Overview

We will examine trials of novel tumor peptide-based vaccines, including safety and efficacy studies of: vaccinations with glioma-associated antigen (GAA) peptides emulsified in Montanide ISA-51 and in combination with poly-ICLC (polyinosinic-polycytidylic acid stabilized with polylysine and carboxymethylcellulose) in patients with either newly diagnosed low-grade glioma with high risk factors for recurrence (UPCI 07-057) or in patients with recurrent LGG (UPCI 08-135). The vaccine approaches described in this project offer immunotherapeutic potential to reduce the risk of tumor recurrence, which may translate into improved patient survival.

Principal Investigator

Hideho Okada, MD, PhD
Associate Professor of Medicine
University of Pittsburgh School of Medicine
Hillman Cancer Center Research Pavilion
5117 Centre Avenue
Suite G12.a
Pittsburgh, PA 15213

Other Participating Researchers

None

Expected Research Outcomes and Benefits

Adults with supratentorial LGG have a significant risk of tumor progression following treatment with surgery or surgery followed by radiation therapy (RT), and most LGG patients eventually die of the disease. The vaccine approach described in these trials offers both immunotherapeutic

and immunoprophylactic potential to reduce the risk of tumor recurrence, which could translate into improved survival. The slower growth rate of LGG (in contrast to malignant gliomas) should allow sufficient time to administer multiple immunizations, potentially leading to the induction of high levels of glioma-associated antigen-specific immunity.

Summary of Research Completed

UPCI 07-057

Objectives: Our primary objectives are to determine both immunological activity and safety of the regimen to decide whether a larger follow-up study is warranted. To assess induction of GAA-specific T-cell responses, we determine the response rate and magnitude of immune responses in postvaccine peripheral blood mononuclear cells (PBMC) against GAA peptides in response to this form of vaccine, using interferon gamma (IFNγ)-enzyme-linked immuno-spot (ELISPOT) and tetramer assays. For safety, the incidence and severity of adverse events (AE) associated with the vaccine regime will be assessed, with an early stopping rule based on the frequency of regimen-limiting toxicity (RLT). Other exploratory objectives include: (1) clinical response, (2) radiological response, (3) two-year, progression-free survival (PFS), and (4) evaluation of tumor tissues for biological correlates.

Methods: Eligible participants are human leukocyte antigen (HLA)-A2+ patients (age ≥18 years old) with histologically diagnosed supratentorial World Health Organization (WHO)-grade II astrocytoma or oligoastrocytoma with "high-risk" factors, defined as: (1) age ≥ 40 with any extent of resection; (2) age 18-39 with incomplete resection (postoperative magnetic resonance imaging [MRI] showing >1 cm residual disease based on the maximum dimension of residual T2 or fluid-attenuated inversion-recovery [FLAIR] abnormality from the edge of the surgical cavity laterally, antero-posteriorally, or supero-inferiorally); or (3) tumor size ≥ 4 cm (any age).

Eligible patients are stratified based on whether they have undergone prior RT. Cohort 1 includes patients who have undergone surgery or biopsy alone (no postoperative RT or chemotherapy) and have a baseline MRI scan (within four weeks of the first vaccine) that shows stable disease or regression (no progression from the initial surgery/biopsy). Cohort 2 includes patients who received surgery or biopsy and RT completed ≥ six months prior to enrollment and have a stable MRI scan (no progression after RT). Prior chemotherapy excludes patients from both cohorts. The sample size was originally nine patients/cohort; however, the sample size for Cohort 1 has been expanded to a maximum of 18 because of the high accrual rate in this cohort.

All participants must have discontinued dexamethasone or similar corticosteroid medications at least four weeks before administration of the first vaccine. Participants are treated with subcutaneous (s.c.) injections of GAA/TT (tetanus toxoid)-vaccines on an outpatient basis in weeks 0, 3, 6, 9, 12, 15, 18, and 21; poly-ICLC is administered (20 mg/kg intramuscularly [i.m.]) on the day of vaccination and on day 4 after each vaccine. Table 1 and schema 1 list the antigens and treatment schedule, respectively. Participants are evaluated for any possible AE, RLT, and clinical response by clinic visits and MRI. PBMC samples are drawn at pre- and postvaccine time points to evaluate immune responses.

Results: As of June 2011, 10 patients had received at least one vaccine in cohort 1. Four of them have completed the initial course of eight vaccines. Among these, two were taken off the regimen due to radiologic (one) or symptomatic (one) progression. One patient progressed after four vaccines—the only patient to date to progress before completion of the eight initial vaccines. Most patients experienced mild and transient CTCAE (common terminology criteria for adverse events)-grade 1 or 2 flu-like symptoms, including low-grade fever, chills, myalgia, headache, and fatigue. One patient demonstrated a grade 3 fever, a dose-limiting toxicity (DLT), after seven vaccines and was removed from the study. This patient was the only participant who demonstrated DLT. In Cohort 2 (patients with prior RT), only one patient has been enrolled thus far. This patient completed the initial eight vaccines with stable disease and is currently receiving booster vaccines with a stable disease condition.

Immune response analyses are performed after the completion of the regimen treatment for each patient. As most patients remain on the study, we have performed immunological analyses in only three. All three participants demonstrated positive immune responses against the vaccinated antigens. In particular, participant #3 demonstrated remarkable and sustained immune response (Figure 1). Approximately 6 percent of his circulating CD8+ T cells were reactive to the WT1 epitope and expressed multiple effector molecules (i.e., at least two of IFN-γ, tumor necrosis factor (TNF)-α, or CD107a), indicating effective induction of polyfunctional, antigen-specific CD8+ T cells).

UPCI 08-135:

Objectives: This is a pilot vaccine study in adults with recurrent WHO-grade II glioma. The overall objective of this study is to collect immunological and safety data that will be used to decide whether a larger study of clinical efficacy is warranted in these patients. All patients will be followed for a minimum of two years so that the actual two-year overall survival (OS) and six-month and two-year PFS rates can be determined in an exploratory manner. The detailed methods for evaluation of the primary and exploratory endpoints are the same as in the UPCI 07-057 study.

Methods: Eligible participants are HLA-A2+ patients (age ≥18 years old) with histologically diagnosed supratentorial WHO-grade II glioma with recurrence. Patients must be steroid-free for four weeks before initiation of vaccines and have a lymphocyte count of 400/µL or more. The sample size for this study is nine patients. Participants are treated with s.c. injections of GAA/TT-vaccines on an outpatient basis in weeks 0, 3, 6, 9, 12, 15, 18, and 21; poly-ICLC is administered (20 mg/kg i.m.) on the day of vaccination and on day 4 after each vaccine. Table 1 and Schema 2 list the antigens and treatment schedule, respectively. Participants are evaluated for any possible AE, RLT, and clinical response by clinic visits and MRI. PBMC samples are drawn at pre- and postvaccine time points to evaluate immune responses.

Results: As of June 2011, eight patients have received at least one vaccine. Of those, three have completed the initial eight vaccines, despite the fact that all patients had recurrent LGG at the time of entry into this study. Disease recurred after two booster vaccines in one of the three participants, and the other two participants are receiving booster vaccines. One patient had to be removed from the study after four vaccines due to rapid tumor progression. The toxicity profile has been very similar to that of UPCI 07-057. There were no RLTs. To date, comprehensive

immune response assessments have been completed in one patient (the same patient who completed the initial eight vaccines but whose disease recurred after two booster vaccines). This patient demonstrated positive IFN-γ responses in ELISPOT assays (data not shown). One more patient will need to be accrued for completion of this study.

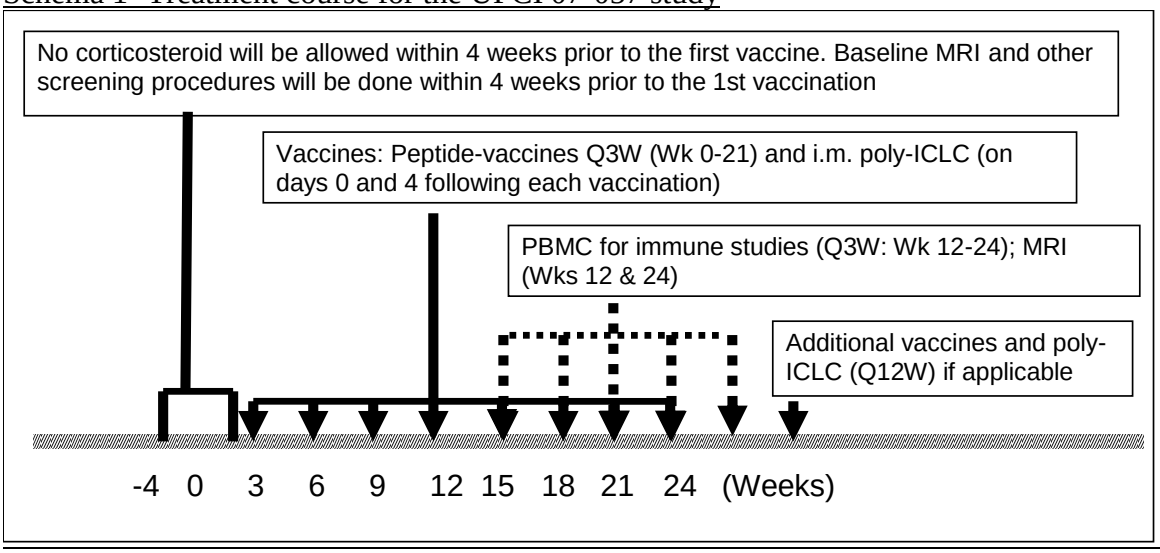
In summary, accrual for these trials has been going quite well, and the regimen has been well-tolerated in both studies. Although further analyses await completion of treatments, the data available to date clearly indicate the immunological potency of the vaccine regimen used in both trials.

Table 1. List of antigen peptides used in UPCI 07-057 and UPCI 08-135 studies

Antigen Peptide	Presented By:	Prevalence in HG / GIIA
IL-13Rα2 $_{345\text{-}353:1A9V}$	HLA-A2	>80% / low
EphA2 $_{883\text{-}891}$	HLA-A2	75-80%/ 50%
Survivin $_{96\text{–}104:M2}$	HLA-A2	All astrocytoma (GII-IV)
WT1 $_{126\text{-}134:Y1}$	HLA-A2	All astrocytoma (GII-IV)
Tetanus Toxoid (Tet$_{A830}$)	Pan-DR	(heterologous antigen)

HG; high grade (grade III-IV) glioma, GIIA; grade II astrocytoma

Schema 1- Treatment course for the UPCI 07-057 study



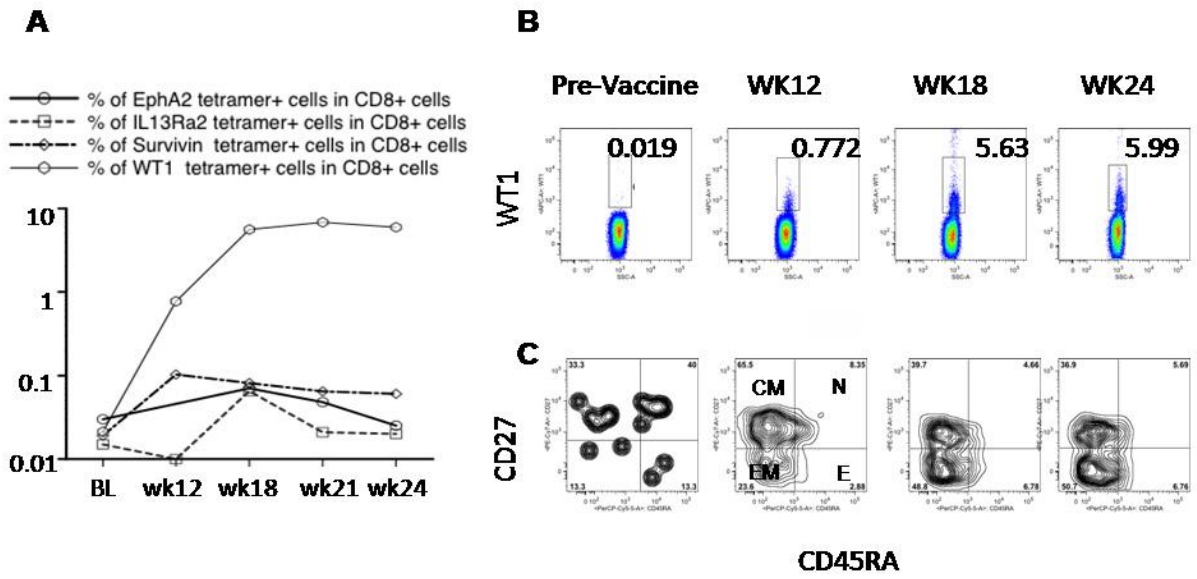
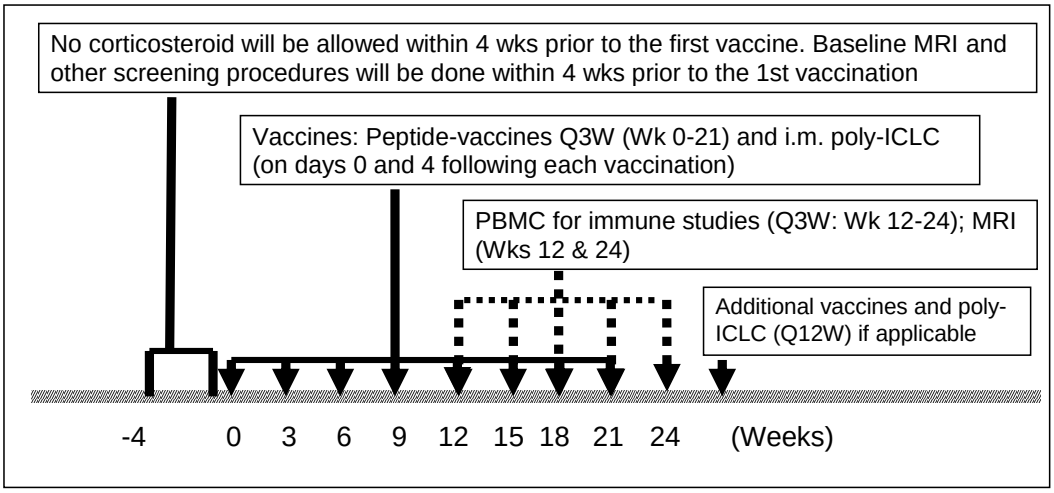


Figure 1. Robust expansion of CD8+ T cells specific for HLA-A*0201 GAA epitopes. (A), Representative *ex vivo* tetramer staining of CD8+ T cells specific for HLA-A*0201 WT1, EphA2, IL13Ra2, or survivin from PBMC taken at baseline (BL), 12, 18, 21, and 24 weeks. (B), Frequency of tetramer positive cells in log scale, indicating phenotypic composition of tetramer positive cells during the course of vaccination from BL to 24 weeks postvaccination. (C), Subsequent CD45RA vs. CD27 plots were gated on the tetramer+ cells, and numbers in each quadrant indicate the frequencies of corresponding subsets. Corresponding CD8+ T cell maturation stages, Naïve (N), Central Memory (CM), Effector Memory (EM), and Effector (E), are also depicted.

Schema 2 – Treatment course for the UPCI 08-135 study