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AWARD NUMBER: W81XWH-04-1-0468

TITLE: Center of Excellence for Individualization of Therapy for Breast Cancer

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CONTRACTING ORGANIZATION: Indiana University
Indianapolis, IN 46202

REPORT DATE: April 2009

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

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1. REPORT DATE
1 April 2009

2. REPORT TYPE
Annual

3. DATES COVERED
1 Apr 2008 – 31 Mar 2009

4. TITLE AND SUBTITLE

Center of Excellence for Individualization of Therapy for Breast Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-04-1-0468

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Indiana University
Indianapolis, IN 46202

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
No Abstract provided.

15. SUBJECT TERMS
No Subject Terms provided.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
U	U	U	UU	43	

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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COE Annual Report 08-09

Center of Excellence for Therapeutic Individualization for Breast Cancer

Annual Report: General Overview

George W. Sledge, Jr. M.D. Principal Investigator

Enclosed please find the "final" report for the DOD BCRP Center of Excellence for Therapeutic Individualization in Breast Cancer. Please note that we have asked for a no cost extension for this work, as we have substantial unexpended funds due to initial delays in clinical trials.

The Center of Excellence has been devoted to the development of novel strategies for determining therapeutic benefit with both standard and novel agents for the treatment of advanced breast cancer. The Center is based on a series of breast cancer populations with advanced disease receiving specific therapies, with associated annotated tumor and genomic tissue allowing genomic, proteomic, and pharmacogenomic analyses.

COE01, the centerpiece study for the COE, has continued to accrue patients for the evaluation of multiple therapies for advanced breast cancer, including the combination of doxorubicin and cyclophosphamide, as well as single agent capecitabine, vinorelbine, and gemcitabine. An amendment to this trial allowed us to add for analysis tissues collected by one of our investigators, Dr. Jenny Chang of Baylor, as part of a trial of patients receiving chemotherapy for locally advanced breast cancer.

COE02 was or planned analysis of the combination of paclitaxel and bevacizumab. This analysis was recently discontinued due to our belief that we would be unable to accrue sufficient patients for an appropriate statistical analysis in remaining time. We have therefore closed this trial.

COE03 involves the analysis of patients receiving the investigational agent lonafarnib, a farnesyl transferase inhibitor. This trial has recently opened for accrual, and is accruing at a rapid pace.

COE05 is our "retrospective prospective" analysis of the agents being evaluated in COE01. In this analysis, patients who received chemotherapeutic agents listed in COE01 (in particular, capecitabine, gemcitabine, and vinorelbine) and for whom response information and tumor blocks are available are analyzed for genomic, proteomic, and pharmacogenomic endpoints. The primary endpoint used for these patients is time to progression in the metastatic breast cancer setting. Accrual for this trial continues to proceed, and has been helped by the recent addition of sampled from colleagues in Poland.

As outlined in our initial proposal, tissues obtained in the various trials are processed by the University of Colorado group (led by Dr. Ann Thor), and then sent to other research sites for genomic, proteomic, and pharmacogenomic analysis. We have previously published on the processing of formalin-fixed, paraffin-embedded tissue for genomic analyses. More recently, in work presented at this year's American Society of Clinical Oncology meetings (Audet, R. et al., Abstract 11033), we have presented data suggesting that clinical benefit (in the form of time to progression) with capecitabine is associated with fluorescence in situ hybridization (FISH) measurements of thymidilate synthase in patient's formalin-fixed paraffin-embedded tumors. Further tumor tissues from patients receiving capecitabine are being analyzed to confirm this initial finding. If confirmed, this would offer a simple pathologic determination that would direct therapy for this widely used agent.

Processing and analysis of a large volume of accrued tissue samples are now ongoing. During our no-cost extension period we expect to complete trial accrual to our COE01, COE03, and COE05 trials, process tissues for pharmacogenomic, proteomic, and genomic analysis, and perform appropriate statistical analysis. We are grateful to the Breast Cancer Research Program for our opportunity to perform this important work.

Sincerely yours,

George W. Sledge, Jr. MD

Professor of Medicine and Pathology

COE update 4-22-09

Summary: We have been waiting for the group to accumulate at least 20 serum samples from each of the patient sets we decided would benefit from a proteomic analysis. We revised this approach during the March meeting, since the collection process has been going very slowly. During the meeting, we decided to begin a somewhat different series of analyses, in which we would look at the baseline proteomic signatures for all of those patients enrolled in each arm of COE01 and -02 and attempt to correlate these signatures with the response of these patients to treatment. We further proposed to perform a set of proteomic analyses using an affinity chromatography approach in which genomic and pharmacogenomic markers identified by Dr. Chang's and Dr. Leyland-Jones' laboratories would become the target of our screening approaches. The goal is to see whether we can identify these specific markers in serum and tissue specimens derived from these specific trials using antibodies to these proteins that had been immobilized onto SELDI chips. In addition, we proposed to use the metal binding chip surface (IMAC) to evaluate these specimens for the presence of specific phosphorylated proteins; including those associated with the signal transduction pathways utilized by the breast cancer cells within these tumor specimens.

While we were waiting for the appropriate minimum number of specimens to be collected for analysis, Ms. Dobrolecki, the technician we had trained to be the individual running these analyses moved with her husband to a new position in Houston. We have appointed a new research assistant, Ms. Brandy Snider, to replace Ms. Dobrolecki. Brandy is learning how to use the SELDI mass spectrometer at this time, and should be able to begin these studies within a few weeks.

Commander
U.S. Army Medical Research and Materiel Command
504 Scott Street
Fort Detrick, MD 21702-5012

Subject: Annual Report for Genomics Core

Introduction

Optimal systemic treatment after breast cancer is the most crucial factor in reducing mortality in women with breast cancer. Adjuvant chemotherapy and hormonal treatment both reduce the risk of death in breast cancer patients. However, while estrogen receptors status predicts for response to hormone treatments, there are no clinically useful predictive markers for chemotherapy responses. All eligible women are therefore treated in the same manner. Even denoval drug resistance will result in treatment failures in many breast cancer patients. Currently, there are no methods available to distinguish those patients who are likely to respond to specific chemotherapies, and given the accepted practice of prescribing adjuvant treatment to most parties, even if the average expected benefit is slow, the selection of appropriate patients represents a major advance in the clinical management of breast cancer today.

We therefore set out to identify gene expression patterns in breast cancer specimens that might predict response to taxenes. Chemotherapy allows for the sampling of the primary tumor for gene expression analysis and for direct assessment of response to chemotherapy by following changes in tumor size during the first few months of treatment. Hence, chemotherapy provides an idea platform to rapidly discover predictive markers of chemotherapy response.

In this present study, we hypothesize through high quantitation of gene expression, grade is possible to access thousands of genes simultaneously, and expression patterns in different breast cancers might correlate with and thereby predict response to treatment. The purpose of this study was to (1) demonstrate that sufficient RNA could be obtained from core biopsies to access gene expression, (2) to identify groups of genes that could be used to distinguish primary breast cancers to responsive or resistance to different chemotherapies, and (3) to identify gene pathways that could be important in a mechanism of action of these agents. Most of the gene expression experiments have been based on fresh frozen or recently fixed material. We have extended on these technologies to include the use of formalin-fixed, paraffin-embedded (FFPE) material for high throughput genomic analysis. This would enable easier access to tissue repositories that may enable us to discover predictive genes for therapy response.

Body of Research

We have been improving gene expression arrays obtained from small tissue samples, as technical development. We measured each core biopsy obtained from primary breast cancers as approximately 1 cm x 1 mm. As these core biopsies were too small for micro dissection, we ascertained the tumor cellularity of the pretreatment core biopsies. In general, the core biopsies showed good tumor cellularity with median tumor cellularity of 75% (range 40-100%). Each core biopsy yielded 3-6 mg of total RNA, which is more than sufficient to generate approximately 20 mg of label cRNA needed for hybridization with the Affymetrix U133A Genechip, using the manufacturer's standard protocols. We have also experimented with laser microdissection of tumors of lower tumor cellularity.

Previously, we had identified gene expression patterns that predicted response to neoadjuvant docetaxel. Other studies have validated that a high Recurrence Score (RS) by the 21-gene RT-PCR assay is predictive of worse prognosis but better response to chemotherapy. We investigated whether tumor expression of these 21 genes and other candidate genes can predict response to docetaxel. Core biopsies from 97 patients were obtained before treatment with neoadjuvant docetaxel (4 cycles, 100 mg/m2 q3 weeks). Three 10-micron FFPE sections were submitted for quantitative RT-PCR assays of 192 genes that were selected from our previous work and the literature. Of the 97 patients, 81 (84%) had sufficient invasive cancer, 80 (82%) had sufficient RNA for QRTPCR assay, and 72 (74%) had clinical response data. Mean age was 48.5 years, and the median tumor size was 6 cm. Clinical complete responses (CR) were observed in 12 (17%), partial responses in 41 (57%), stable disease in 17 (24%), and progressive disease in 2 patients (3%). A significant relationship ($p<0.05$) between gene expression and CR was observed for 14 genes, including CYBA. CR was associated with lower expression of the ER gene group and higher expression of the proliferation gene group from the 21 gene assay. Of note, CR was more likely with a high RS ($p=0.008$). We have established molecular profiles of sensitivity to docetaxel. RT-PCR technology provides a potential platform for a predictive test of chemosensitivity using small amounts of routinely processed material.

We extended on this work to discover markers of intrinsic chemoresistance. Current systemic therapies including endocrine therapy are initially effective in killing breast cancer cells and controlling tumor growth. Yet, nearly all patients with metastatic disease and a quarter of those with early disease relapse over time despite initial response. In part, this may be due to existing therapies which are aimed only at proliferative and apoptotic pathways resulting in temporary therapy-induced shrinkage of human breast cancers. Recent evidence supports the

existence of cancer stem cells, which are characterized by their relative quiescence but a capacity to self-renew and divide indefinitely._We hypothesized that breast cancer stem cells are resistant to chemotherapy, and that inhibition of stem cell self-renewal pathways will improve breast cancer patient outcome. We report that tumorigenic breast cancer cells were intrinsically chemoresistant ─ chemotherapy led to increased CD44^{+}/CD24$^{-/low}$ cells, increased self-renewal capacity on mammosphere formation efficiency (MSFE) assays. In matched human breast cancer biopsies (n=31 pairs) the relative proportion of CD44^{+}/CD24$^{-/low}$ cells increased with chemotherapy from a baseline mean of 4.7 % to 13.6% after 12 weeks of chemotherapy ($p<0.0001$) (Fig. 1). Consistent with the increase in the relative proportion of tumorigenic cells, mean MSFE was significantly increased after chemotherapy in matched pre- and post-chemotherapy samples ($p<0.0001$) (Fig. 2). Conversely, in patients with HER2 overexpressing tumors, lapatinib (EGFR/HER2 tyrosine kinase inhibitor) did not increase tumorigenic cells, but led to a statistically non-significant decrease in matched biopsies from a baseline mean of 10.6% to 4.7% after 6 weeks of lapatinib (Fig. 3). Consistent with its effect on tumorigenic cells, lapatinib treatment again did not show an increase as with chemotherapy, but led to a non-significant decrease in self-renewal capacity as measured by MSFE (Fig. 3). These studies provide the first clinical evidence for a subpopulation of chemotherapy-resistant breast cancer-initiating cells, and suggest that specific pathway inhibitors in combination with conventional chemotherapy may provide a therapeutic strategy for eliminating these cells in order to decrease recurrence and improve long-term survival.

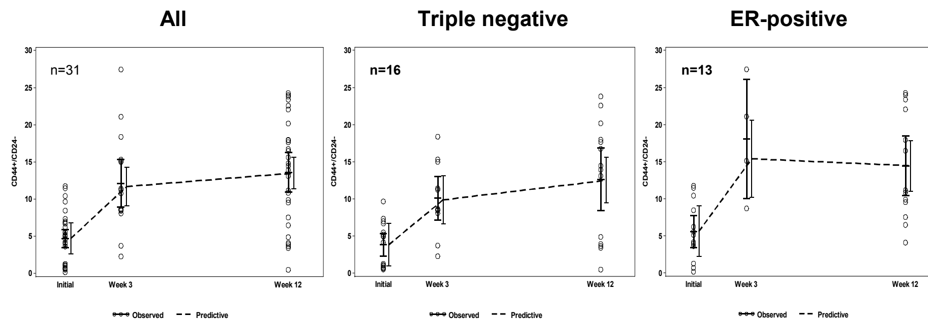

Fig 1. Effect of chemotherapy on mean % CD44+/CD24- cells before, during, and after treatment. Predictive values and their standard errors were estimated by linear mixed-effected models. Error bars represent two standard errors of the mean of experiments at baseline and each time point of follow-up.

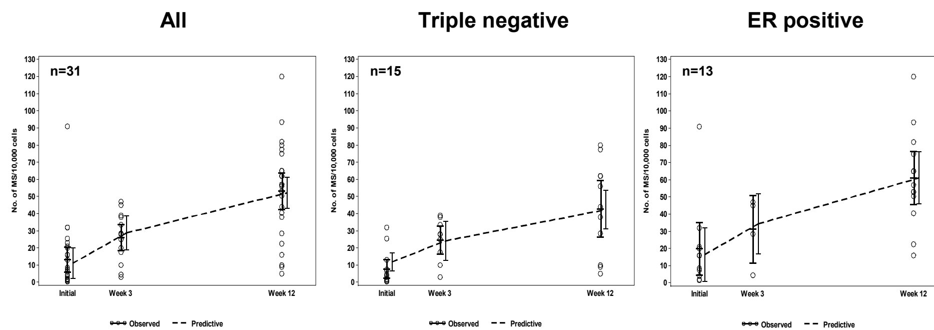

Fig 2 . Similar effect of chemotherapy on mean MSFE before, during, and after treatment in:
A). All patients
B). Triple negative patients
C). ER-positive patients

High throughput genomics offers a potential predictive test of sensitivity and resistance for different treatments, and may thereby allow selection of the most appropriate therapy for breast cancer patients, as we propose here. We are currently *prospectively identifying the AC and*

Taxotere molecular profile in a clinical randomized trial. To date, we have completed recruitment of the expected 230 patients who have been randomized to receive either neoadjuvant Taxotere or neoadjuvant AC. We have begun RNA extraction, in collaboration with the Pathology Core. Expression array analysis will begin in the near future. Results of this study show unique patterns for AC or Taxotere response. We anticipate completing these analyses within the next year, which will yield exciting data on patients who would respond to either AC or Taxotere chemotherapy.

Publications:

1. **J Chang**, A Makris, M Gutierrez, S Hilsenbeck, J Hackett, J Jeong, ML Liu, J Baker, K Clark-Langone, F Baehner, K Sexton, S Mohsin, T Gray, L Alvarez, G Chamness, C Osborne, S Shak. Gene expression patterns in formalin-fixed, paraffin-embedded core biopsies predict docetaxel chemosensitivity in breast cancer patients. *Breast Cancer Research and Treatment*, 2007 (in press)

2. **JC Chang**, A Makris, SG Hilsenbeck, JR Hackett, J Jeong, M Liu, J Baker, K Sexton, CK Osborne, S Shak. Gene expression profiles in formalin-fixed, paraffin-embedded (FFPE) core biopsies predict docetaxel chemosensitivity. Proceedings of the American Society of Clinical Oncology (ASCO) 42nd Annual Meeting 2006.

3. **JC Chang**, Xiaoxian Li, J Rosen, W Bu, H Wong, X Zhang, L Moreno, H Weiss, A Tsimelzon, , SG Hilsenbeck, AV Lee, CK Osborne, G Dontu, M Wicha and MT Lewis. Breast cancer stem cells are responsible for therapeutic resistance and residual disease. San Antonio Breast Cancer Symposium 2006.

4. Intrinsic Resistance of Tumorigenic Breast Cancer Cells to Chemotherapy. X Li, M Lewis, C Creighton, H Wong, X Zhang, H Pham, T Gray, MC Gutierrez, CK Osborne, M Wu, S Hilsenbeck, G Chamness, J Rosen and **JC Chang.** *Journal of National Cancer Institute* 2008; 100:672-9, 2008.

REPORT OF INVENTIONS AND SUBCONTRACTS

(Pursuant to "Patent Rights" Contract Clause) (See Instructions on Reverse Side)

Form Approved
OMB NO. 0704-0297
Expires: June 30, 1992

Public reporting burden is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and, completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0297), Washington, DC 20503.

1a. NAME OF CONTRACTOR/SUBCONTRACTOR	c. CONTRACT NUMBER	2a. NAME OF GOVERNMENT PRIME CONTRACTOR	c. CONTRACT NUMBER	3. TYPE OF REPORT (X one)
Hoosier Oncology Group, Inc.	W81XWH-04-1-0468			a. INTERIM [X] b. FINAL [ ]
b. ADDRESS (INCLUDE ZIP CODE)	d. AWARD DATE (YYMMDD)	b. ADDRESS (INCLUDE ZIP CODE)	d. AWARD DATE (YYMMDD)	4. REPORTING PERIOD (YYMMDD)
351 West 10th Street, Suite 330 Indianapolis, IN 46202	4/1/2004			a. FROM 4/1/2008 b. TO 3/31/2009

SECTION I - SUBJECT INVENTIONS

5. "SUBJECT INVENTIONS" REQUIRED TO BE REPORTED BY CONTRACTOR/SUBCONTRACTOR (If "None", so state)

a. NAME(S) OF INVENTOR(S) (LAST, FIRST, MI)	b. TITLE OF INVENTION(S)	c. DISCLOSURE NO. PATENT APPLICATION SERIAL NO. OR PATENT NO.	d. ELECTION TO FILE PATENT APPLICATIONS (1) UNITED STATES (a) YES	(1) UNITED STATES (b) NO	(2) FOREIGN (a) YES	(2) FOREIGN (b) NO	e. CONFIRMATORY INSTRUMENT OR ASSIGNMENT FORWARDED TO CONTRACTING OFFICER (1) YES	(2) NO
NONE								

f. EMPLOYER OF INVENTOR(S) NOT EMPLOYED BY CONTRACTOR/SUBCONTRACTOR		g. ELECTED FOREIGN COUNTRIES IN WHICH A PATENT APPLICATION WILL BE FILED	
(1)(a) Name of Inventor (Last, First, MI)	(2)(a) Name of Inventor (Last, First, MI)	(1) Title of Invention	(2) Foreign Countries of Patent Application
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SECTION II - SUBCONTRACTS (Containing a "Patent Rights" clause)

6. SUBCONTRACTS AWARDED BY CONTRACTOR/SUBCONTRACTOR (If "None", so state)

a. NAME OF SUBCONTRACTOR(S)	b. ADDRESS (Include ZIP Code)	c. SUBCONTRACT NO.(S)	d. DFAR "PATENT RIGHTS" (1) CLAUSE NUMBER	(2) DATE (YYMM)	e. DESCRIPTION OF WORK TO BE PERFORMED UNDER SUBCONTRACT(S)	f. SUBCONTRACT DATES (YYMMDD) (1) AWARD	(2) ESTIMATED COMPLETION

SECTION III - CERTIFICATION

7. CERTIFICATION OF REPORT BY CONTRACTOR/SUBCONTRACTOR (Not required if [ ] Small Business or [X] Non-Profit organization.) (X appropriate box)

a. NAME OF AUTHORIZED CONTRACTOR/SUBCONTRACTOR OFFICIAL (Last,First, MI)	c. I certify that the reporting party has procedures for prompt identification and timely disclosure of "Subject Inventions," that such procedures have been followed and that all "Subject Inventions" have been reported.	
P. Quake Pletcher		
b. TITLE Executive Director	D. SIGNATURE [signature]	E. DATE SIGNED 09 APR 2009

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REPORT DOCUMENTATION PAGE

Form Approved
OMB NO. 0704-0188

Public Reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comment regarding this burden estimates or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188,) Washington, DC 20503.

1. AGENCY USE ONLY (Leave Blank)	2. REPORT DATE 09APR2009	3. REPORT TYPE AND DATES COVERED Interim 01 APR 2008 – 31 MAR 2009
4. TITLE AND SUBTITLE Center of Excellence for Individualization of Therapy for Breast Cancer		5. FUNDING NUMBERS C W81XWH-04-1-0468
6. AUTHOR(S) George Sledge, M.D.		
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Hoosier Oncology Group 351 W. 10th. Street, Suite330 Indianapolis, IN 46202		8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U. S. Army Research Office P.O. Box 12211 Research Triangle Park, NC 27709-2211		10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision, unless so designated by other documentation.

12 a. DISTRIBUTION / AVAILABILITY STATEMENT	12 b. DISTRIBUTION CODE
☐ Approved for public release; distribution unlimited. ☐ Distribution limited to U.S. Government agencies only; report contains proprietary information	

13. ABSTRACT (Maximum 200 words)

Statement of Work Task 1: Development of/Preparation for Metastatic Chemotherapy Parent Protocol
A total of 7 sites (6 US and 1 Peru) are currently open under the Protocol Amendment dated 12JAN2007.

An addendum was completed for the Main Protocol (1 site) to include samples from one of the core labs who had previously completed a protocol which obtained samples in treatments identified in the master protocol. These samples (199 in total) were obtained and closed one of the arms in the main protocol (arm A). An additional arm (arm E) was added since these samples included enough numbers for the analysis.

A minor amendment for clarification regarding the optional tumor tissue biopsy was completed and dated 25MAR2009. This amendment was reviewed and approved by the Department of Defense. It was sent out to sites for their submission to IRBs on 08APR2009.

2 sites closed to accrual due to lack of accrual at their site.

Monthly teleconferences conducted with George Sledge, M.D and Cores. Face-to-Face meetings conducted 19 and 20 March 2009.

4 sites (all US) are open under the second protocol dated 12JAN2007.

1 additional site (Peru) is in the regulatory process for opening the second trial. Dr. George Sledge is working to obtain the clinical trial material supply for this site in order for them to open.

There is a Retrospective / Prospective Research Plan currently collecting data and formalin-fixed paraffin embedded tissue samples. 101 samples have been retrieved to date. There will be approximately 200 samples to be added to this collection coming from a site in Poland.

Statement of Work Task 2: Performance of Metastatic Chemotherapy Trial/Tissue Collection/Patient Follow-up
57 patients enrolled to the trial in the US and Peru. 199 samples from the protocol addendum were collected. Tissue collection and submission to central laboratory conducted as planned.

Statement of Work Task 3: Analysis of Tissues by Laboratory Cores
10 patients enrolled to the trial in the US. Tissue collection and submission to central laboratory conducted as planned.

Statement of Work Task 4: Performance of Prospective Validation Trial
Awaiting completion of Task 1-3

Statement of Work Task 5: Performance of Investigational Agent Trials
The third protocol and informed consent has been reviewed and approved by the Department of Defense. Sites are in the process of determining their facility's ability to participate in this protocol. The clinical trial material for this trial will be available on June 2009.

14. SUBJECT TERMS			15. NUMBER OF PAGES 1 16. PRICE CODE
17. SECURITY CLASSIFICATION OR REPORT **UNCLASSIFIED**	18. SECURITY CLASSIFICATION ON THIS PAGE **UNCLASSIFIED**	19. SECURITY CLASSIFICATION OF ABSTRACT **UNCLASSIFIED**	20. LIMITATION OF ABSTRACT **UL**

REPORT DOCUMENTATION PAGE			Form Approved OMB No. 074-0188
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503			
1. Agency Use Only (Leave blank)	**2. Report Date** April 20, 2009	**3. Report Type and Period Covered (i.e., annual 1 Jun 00 - 31 May 01)** Annual	
4. Title and Subtitle Center of Excellence for individualization of Therapy for Breast Cancer. Pharmacodynamics/Pharmacogenomics Core			**5. Award Number** W81XWH-04-1-0468
6. Author(s) Dr. Brian Leyland-Jones			
7. Performing Organization Name (Include Name, City, State, Zip Code and Email for Principal Investigator) V M Institute of Research 2020 University Montreal, Quebec H3A 2A5 E-**Mail:** brian.leyland-jones@mcgill.ca			**8. Performing Organization Report Number (Leave Blank)**
9. Sponsoring/Monitoring Agency Name and Address U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			**10. Sponsoring/Monitoring Agency Report Number (Leave Blank)**
11. Supplementary Notes (i.e., report contains color photos, report contains appendix in non-print form, etc.)			
12a. Distribution/Availability Statement (check one) ☐ Approved for public release; distribution unlimited ☐ Distribution limited to U.S. Government agencies only - report contains proprietary information			**12b. Distribution Code** (Leave Blank)
13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*			
14. Subject Terms (keywords previously assigned to proposal abstract or terms which apply to this award)			**15. Number of Pages (count all pages including appendices)** **16. Price Code (Leave Blank)**
17. Security Classification of Report Unclassified	**18. Security Classification of this Page** Unclassified	**19. Security Classification of Abstract** Unclassified	**20. Limitation of Abstract** Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

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Introduction

The primary objective of the pharmacogenomics core is to develop user-friendly techniques readily available to clinicians for measuring a specific aspect of response and/or toxicity, which will lead to the individualization of therapy. For the moment our approach is exploratory in nature and will serve mainly to identify markers or signatures that could be used as a diagnostic tool after meticulous validation and definition of standards.

The quest for reliable predictive markers has been, and will certainly remain, a long and challenging enterprise. As we are gaining a better understanding of the weaknesses of the available methodologies, it is becoming increasingly evident that each step in the analysis process is critical with regard to accuracy, reproducibility and predictive value of new markers or signatures (Sauter et al., 2009). In order to minimize such inaccuracies, complementary techniques have been selected and will be used in parallel to assess the usefulness of theses markers to predict response of an individual patient to a specific therapy.

Two different strategies are used to identify markers or signatures for each chemotherapeutic agent used on the different arms of the Center of Excellence (COE) trials. Firstly, based on the concept that they have a better chance of being linked to a clinical response, a set of markers were identified as either targets of a particular chemotherapeutic agent or determinants of its metabolism. Secondly, distinct gene expression signatures linked to a good or bad prognosis for a particular chemotherapeutic agent will be characterized using an mRNA expression profiling microarray-based assay, DASL (cDNA-mediated annealing, selection extension and ligation) and confirmed by TaqMan quantitative real time polymerase chain reaction (qRT-PCR).

Planned analyses for the Center Of Excellence trials

Overview of analyses per protocol and treatment arm

Selected markers and the chosen methodologies to evaluate them are summarized in Table 1 for each of the COE trials and arms. The following sections will present interim results for RNA isolation as well as Fluorescent In Situ Hybridization (FISH) and qRT-PCR analysis for the capecitabine arm (with an asterisk in the table below) of COE-01 and COE-05.

Table 1: Overview of the planned analyses to be performed by the pharmacogenomics core on each arm of the COE trials.

Protocol	FISH			RNA	DASL	qRT-PCR
COE-01 and COE-05						
Arm A: AC	TOP2A	HER2		√	√	ALDH1A1, HER1, HER2, HER3, TOP2A,
Arm B: Capecitabine*	DHFR	TYMP	TYMS	√	√	DHFR, DPYD, TYMP, TYMS
Arm C: Vinorelbine				√	√	STMN1, TUBB3,
Arm D: Gemcitabine				√	√	CNT, DCK, ENT, RRM1
COE-01A						
AC & Taxotere	TOP2A	HER2		√	√	MAP4, MAPT, TUBB3, STMN1, TOP2A
COE-02						
Bevacizumab				√	√	HIF1A, PDGFR-B, VEGF-A
COE-03						
Lonafarnib				√	√	DNAJA1, HRAS, KRAS, CENP-E/F

Abbreviations: **AC**: **A**driamycin and **C**ytoxan, **COE**: Center of Excellence, **DASL**: c**D**NA-mediated-**A**nnealing, **S**election, extension and **L**igation, **DHFR**: official gene symbol of Dihydrofolate reductase, **DPYD**: Dihydropyrimidine dehydrogenase, **FISH**: Fluorescent In Situ Hybridization, **qRT-PCR**: quantitative real time polymerase chain reaction, **TYMS**: Thymidylate synthase, **TYMP**: Thymidine phosphorylase.

Patients selection and demographics

Results described in this report all derive from patients accrued on the capecitabine arm of COE-01 and COE-05 for which we have a sufficient number of patients to run the analyses. Patients included in this study were all adult females over 18 years of age with pathologically confirmed breast cancer and locally advanced or metastatic disease treated with capecitabine (1000 mg/m^2 BID days 1-14 of a 21-day cycle).

FFPE blocks were obtained, sectioned and the resulting slices were either mounted on glass slides for FISH analysis or kept in RNase-free tubes for RNA extraction and further PCR-based analysis. Selected patients included in our present analyses were 49.5 ± 8.7 years old (mean ± s.d.), 31 were Caucasian, 1 African American and 3 unreported.

RNA extraction and quality control

Methodology

Formalin-fixed, paraffin-embedded (FFPE) archival tissues constitute a huge source of well-characterized clinical samples with long term follow-up that can be used to conduct biomarker investigations. Although this type of sample is easily accessible, the fixation process is at the source of RNA degradation that can limit their utilization for gene expression profiling or RT-PCR. Improvements to the RNA extraction procedures now make it possible to obtain RNA fragments of sufficient length and quality to perform gene expression profiling using DASL or qRT-PCR (Chung et al., 2006; Penland et al., 2007; Rupp and Locker, 1988).

Our group has determined that the RNA extraction method has an important effect on the reproducibility or data generated with the DASL assay and have conducted experiments to validate and standardize the RNA extraction process (Abramovitz et al., 2008). It was determined that the best protocol for RNA extraction from FFPE slices is as follows.

Briefly, total RNA should be extracted from at least three FFPE sections 5 µm thick placed in an RNAse-free low-binding plastic tube. Tubes should not contain more than 5 to 6 sections and if more sections are necessary, they should be processed in separate tubes and pooled at the end of the RNA extraction. Sections should be deparaffinized with 100% xylene for 3 minutes at 50°C and centrifuged, followed by two ethanol washes and then air dried. The deparaffinized tissue should be digested with Proteinase K at 50°C overnight. Most extraction kit manufacturers recommend shorter digestions in order to save time, but it was shown that longer incubation time significantly increases yield and improves overall quality (Abramovitz et al., 2008). RNA isolation was performed using Ambion's RecoverAll™ kits and the resulting RNA was quantified using a Nanodrop™ spectrophotometer. Quality was assessed by TaqMan qRT-PCR amplification of mRNA for a ribosomal protein, RPL13a. Samples should be considered usable if the concentration is >20 ng/µL, the A_{260}/A_{280} absorbance ratio should be >1.5 and RPL13a Ct ≤ 30.

Interim results

Using the protocol described above we isolated total RNA from five to six 5µm thick FFPE sections containing at least 70% tumor cells from 78 FFPE breast tumor samples coming from 68 patients treated with capecitabine. Total RNA yields ranged from 0.1 to 24.8 µg and, in the vast majority of

cases, at least 1 μg of total RNA was obtained with an average yield greater than 5 μg. RNA concentrations paralleled the total yield and ranged from 3 to 1166 ng/μl with an average of 289 ng/μl (Figure 1).

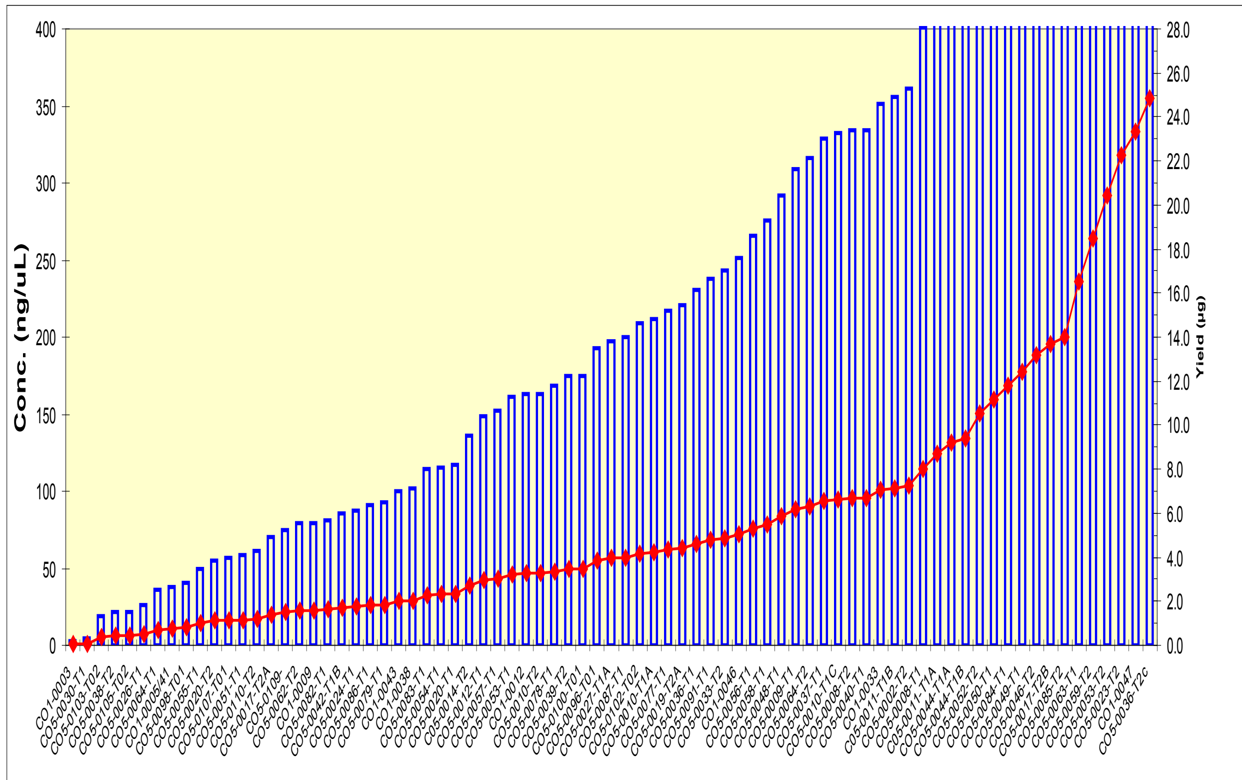

Figure 1: Concentration and total yield for RNA extracted in 78 FFPE breast tumor samples from 68 patients treated with capecitabine. Blue bars represent RNA concentration for each sample in μg/μl and red line represents yield in ng.

To test the quality of the RNA extracted from the FFPE samples, the classical 260/280 absorbance ratio was measured using a Nanodrop™ spectrophotometer. This 260/280 absorbance ratio was consistently near a value of 2 with an average of 2.03, which is considered excellent and demonstrates there is little if any contamination in our samples (data not shown).

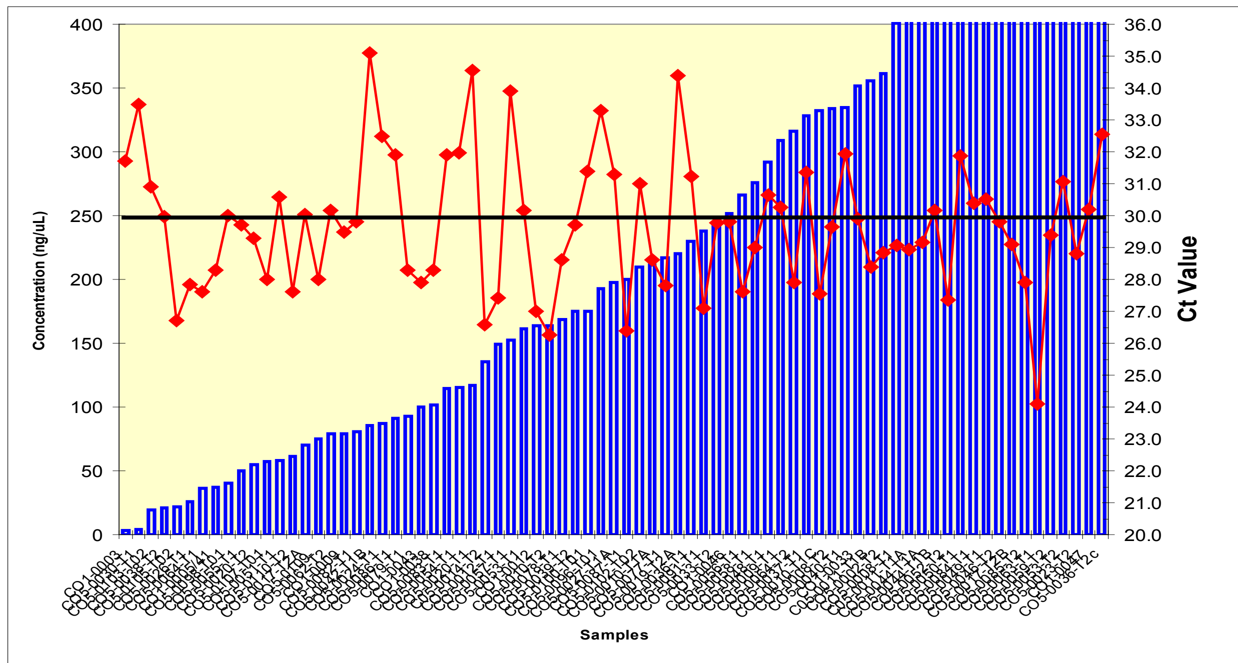

Figure 2: Concentration and quality control for RNA extracted from 78 FFPE breast tumor samples from 68 patients treated with capecitabine. Blue bars represent RNA concentration for each sample in ug/ul and red line represents Ct values for the amplification of the housekeeping gene RPL13a. The black line was set at a Ct value of 30 which is the upper limit usually used to consider RNA of sufficient quality for use.

As a second and more reliable method to evaluate RNA integrity, we used qRT-PCR performed on a 90bp fragment of the highly abundant transcript of the ribosomal protein L13a (RPL13a). When performing TaqMan qRT-PCR, the fluorescent signal increases exponentially in direct relation with the cDNA copy number present initially and will eventually cross a determined threshold. The number of PCR cycles required for the fluorescent signal to cross this threshold is called the Ct value. For a particular gene product, a large number of cDNA copies will yield a low Ct value and conversely for a

lowly expressed gene. When qRT-PCR is performed on RNA prepared from FFPE sections using the RPL13a primer set, the Ct value that would reflect a good quality sample should be ≤ 30.

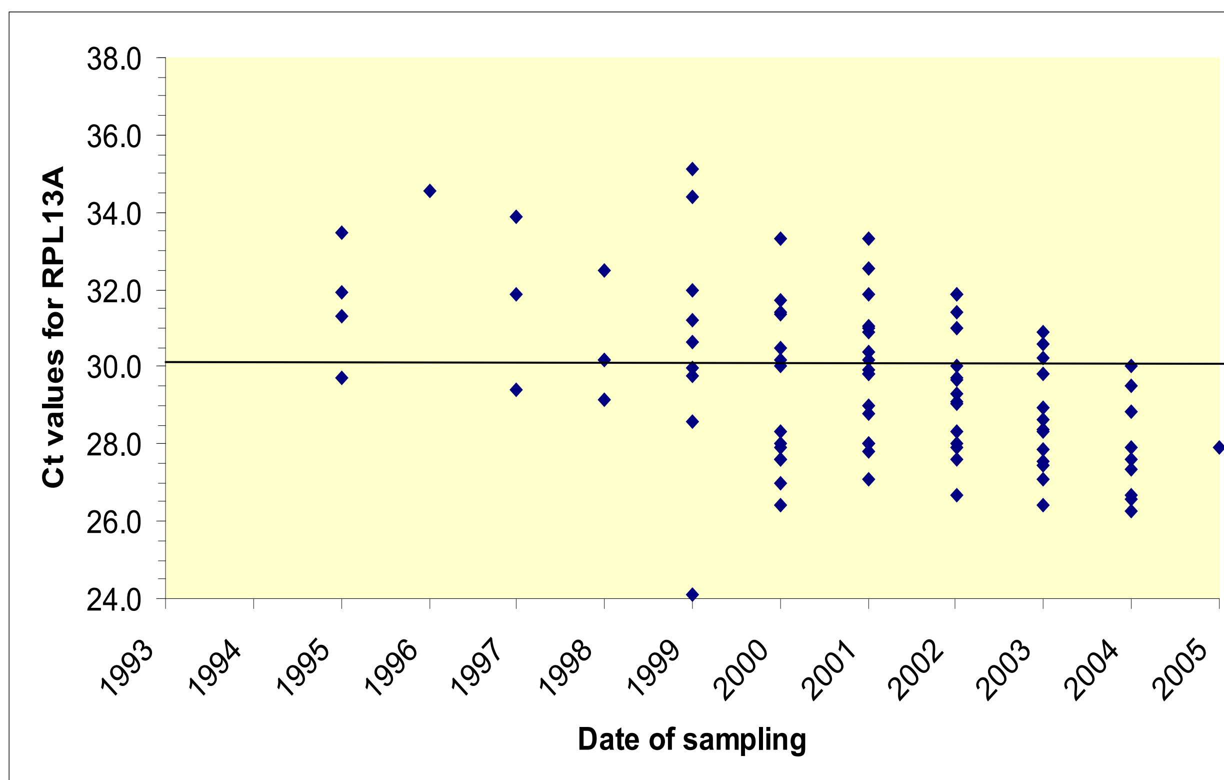

Figure 3: Relation between Ct values and date of sampling for RNA extracted from 78 FFPE breast tumor samples from 68 patients treated with capecitabine. Ct values are for the amplification of the reference gene RPL13a. The black line was set at a Ct value of 30 which is the upper limit usually used to consider RNA of sufficient quality for use.

Of the 78 samples evaluated 30 (38%) had Ct values for RPL13a above 30 (Figure 2 and 3) and in the 48 samples that passed QC, only 3 (6%) had yields or concentrations insufficiently high to perform the DASL analysis (data not shown). Moreover, the concentration of extracted RNA does not seem to

correlate with their respective Ct value, suggesting the integrity of the RNA has little relation with the amount or concentration extracted (Figure 2).

Interestingly, we found higher Ct values in many samples more than eight years old suggesting the RNA extracted from some of these older FFPE samples has been degraded to the point where it might compromise its use for PCR-based techniques (Figure 3).

These results demonstrate that RNA extracted from archival FFPE specimens can be used for PCR-based techniques and that some unusable samples should be expected when using older material emphasizing the need for quality control on each and every case.

Conclusions and projections

These interim results shed some light on the expected proportion of samples usable for PCR-based techniques; bearing in mind usable samples should decrease when using older FFPE blocks. Based on these preliminary results showing that from 68 patients we can derive 39 (57%) RNA samples with a concentration and yield high enough to be usable for PCR-based techniques, we can expect to be able to run qRT-PCR and DASL assays on roughly 60% of the samples received.

Markers selection rationale for capecitabine (Xeloda™)

Molecular pharmacology and mechanism of action

The fluoropyrimidine nucleoside analogue 5-fluorouracil (5-FU) was originally developed as a chemotherapeutic agent over 50 years ago and is the

standard treatment for a wide range of common solid tumors, including breast cancer. Attempts to increase the efficacy and tolerability of fluoropyrimidine treatment have led to the development of capecitabine (Xeloda™), a prodrug transformed into 5-FU preferentially in tumors (Figure 4). Capecitabine is now often used either alone or in combination with other drugs but, unfortunately, we still lack reliable methods for the selection of patients who will have the best chance to benefit from capecitabine-based treatments.

Capecitabine is activated at the tumor site by the enzyme thymidine phosphorylase (TP) (Miwa et al., 1998), which takes advantage of the fact that this enzyme is more highly expressed in tumor tissue (Takebayashi et al., 1996), including breast cancer (Kobayashi et al., 2005). Capecitabine and its intermediate metabolite, 5'-deoxy-5-fluorouridine (5'-DFUR), are not cytotoxic but become effective only after conversion to 5-fluorouracil (5-FU) by TP as well as further transformations into fluorodeoxyuridine monophosphate (FdUMP) and, fluorouridine triphosphate (FUTP) (Miwa et al., 1998). Inhibition of the enzyme thymidylate synthase (TS) by FdUMP is considered to be the main mechanism of action of fluoropyrimidine, including capecitabine (Walko and Lindley, 2005) (Figure 4).

TS is an important enzyme in pyrimidine metabolism which is crucial for *de novo* thymidine nucleotide synthesis used for DNA replication and cellular division (Peters et al., 1995). Inhibition of TS occurs as a result of the formation of an inactive ternary covalent complex between TS, FdUMP and 5-10 methylenetetrahydrofolate (CH_2FH_4). The stability of this ternary complex is highly dependent on the availability of CH_2FH_4 or one of its polylglutamates (Houghton et al., 1982; Houghton and Houghton, 1983). Dihydrofolate reductase (DHFR) is a key enzyme involved in folate metabolism and plays a role in the *de novo* pathway of pyrimidine biosynthesis that has been linked to

the modulation of fluoropyrimidine treatments (Capiaux et al., 2003; Will and Dolnick, 1989).

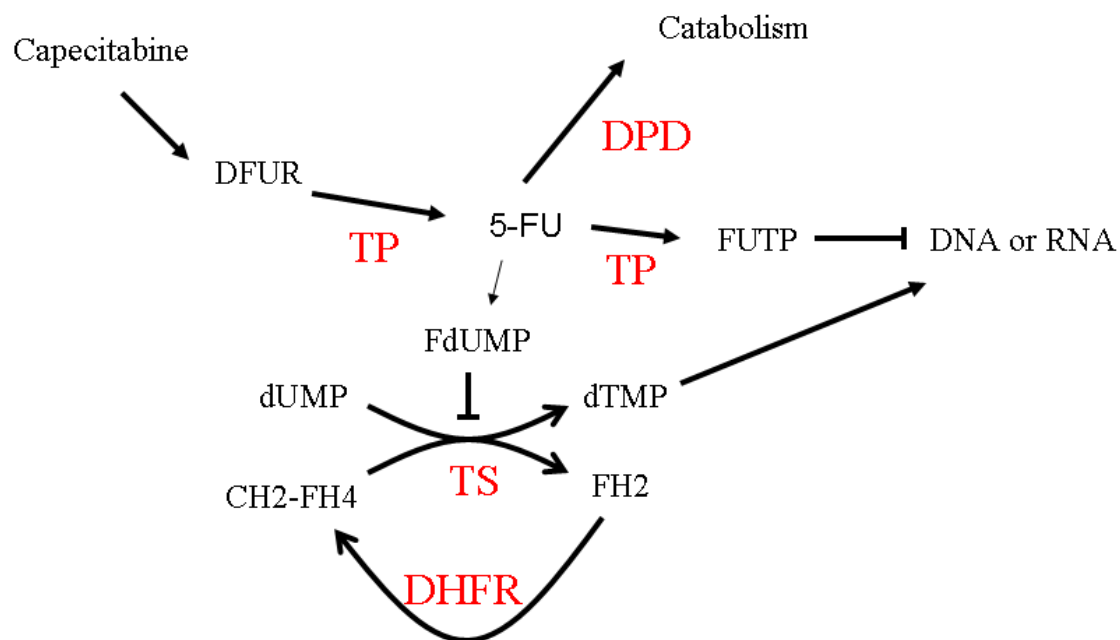

Figure 4: Pathway of capecitabine metabolism and catabolism. Abbreviations: CH2-FH4, 5-10 methylenetetrahydrofolate; DFUR, 5'-deoxy-5-fluorouridine; DHFR, dihydrofolate reductase (GeneID: 1719; EC 1.5.1.3); DPD, dihydropyrimidine dehydrogenase (GeneID: 1806; EC 1.3.1.2); dTMP, deoxythymidine-5'-monophosphate; dUMP, deoxyuridine-5'-monophosphate; FdUMP, 5-flurodeoxyuridine-5'-monophosphate; FH2, dihydrofolate; 5-FU, 5-fluorouracil; FUTP, Fluorouridine triphosphate; TP, Thymidine phosphorylase (GeneID: 1890; EC 2.4.2.4); TS, Thymidylate synthase (GeneID: 7298; EC 2.1.1.45).

Dehydropyrimidine dehydrogenase (DPD) is the enzyme responsible for the first and rate limiting step in the catabolic conversion of 5-FU to inactive metabolites and decreases 5-FU levels within cells (Johnson et al., 1997; Lee et al., 2004; Lu et al., 1993). Several studies have underlined the role of DPD deficiency in the development of severe 5-FU toxicity and conversely DPD overexpression is associated with resistance to these therapies (Kornmann et al., 2003). Both elevated DPD gene copy number and mRNA expression

were linked to increased resistance to capecitabine and other 5-FU-based treatments in several human cells lines, including breast (Kobunai et al., 2007).

Since DPD is rate limiting for the catabolic pathway and TP is key to the production of active capecitabine metabolites, the TP/DPD ratio has been frequently used to correlate with capecitabine or 5-FU efficacy. It was first shown that a high TP to DPD ratio correlated with a high capecitabine efficacy and conversely a low TP/DPD ratio was linked to resistance in a large number of xenograft models, including breast (Ishikawa et al., 1998). Recent immunohistochemical (IHC) data has shown that a higher TP/DPD ratio correlates with a better clinical response in a small cohort of breast cancer patients treated with capecitabine monotherapy (Honda et al., 2008).

Similarly, RT-PCR analysis of tumors from 22 breast cancer patients revealed that the group of patients expressing high levels of TS and DPD were resistant to 5-FU, whereas those patients expressing low levels of TS and DPD were sensitive to 5-FU (Kakimoto et al., 2005). Using IHC, it was shown that high levels of TP expression in tumors was a significant prognostic indicator of 5'-DFUR efficacy in breast cancer patients (Tominaga et al., 2002).

Therefore, the fluoropyrimidine pathway enzymes, TP, TS, DPD and DHFR, were selected as potential candidate biomarkers that could be used to predict tumor response to capecitabine. Efforts have been made to select assays that would be easily accessible to clinicians in order to correlate gene copy number and gene expression profiles with disease state, therapy and drug response.

Interim results for capecitabine

Gene copy number using FISH

Newly developed FISH probes (Dako, Glostrup, Denmark) have been used on 5 µm FFPE tissue slices to investigate TS, DHFR and TP gene copy number. Hybridization signals have been evaluated using the ratio of red signals for TS, DHFR or TP to green signals for a reference sequence on the same chromosome in at least 60 morphologically intact and non-overlapping nuclei (Figures 5 and 6).

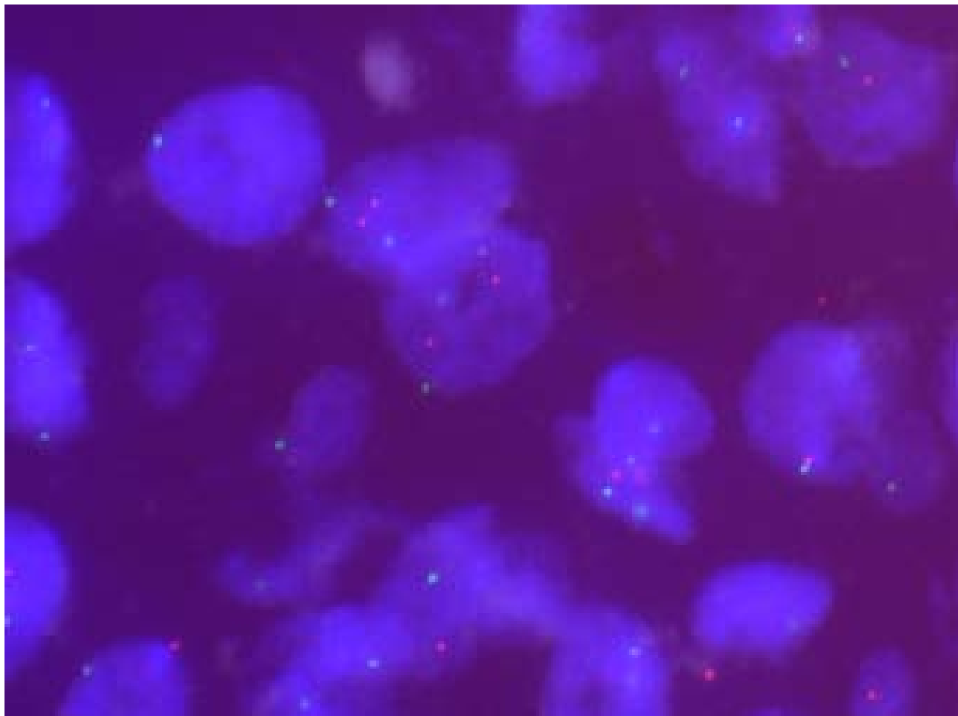

Figure 5: Representative photograph of cells stained with TYMS FISH probes gene, with reference signals for the centromere of chromosome 18 (green dots) and signals for TYMS (red dots) in nuclei (blue). It should be noted that only one plane of focus is shown; whereas the scoring of green and red dots used to calculate the ratio was performed in all available focus planes and in 60 morphologically intact and non-overlapping nuclei. 100X magnification.

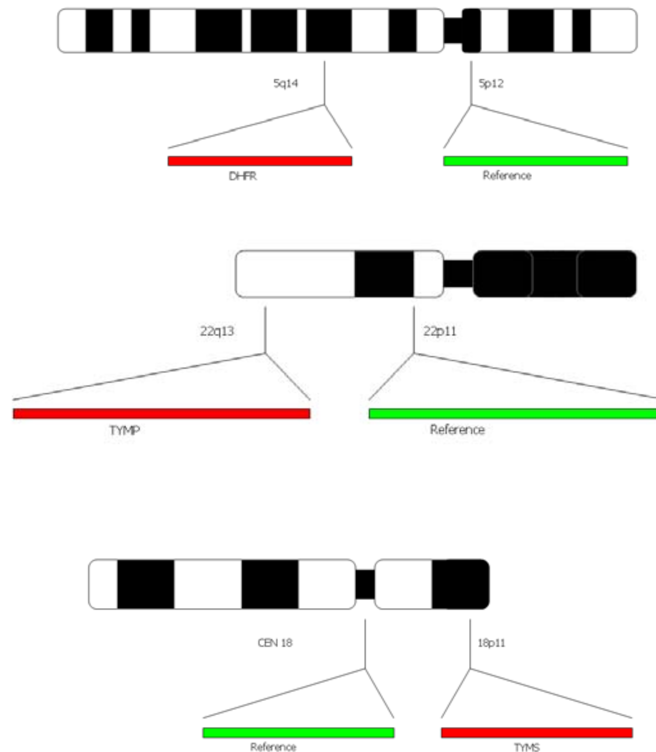

Figure 6: Maps of each of the DHFR, TYMP and TYMS FISH probe sets along with their respective reference sequences. The ratio of the gene to reference signals (red/green) was scored in 60 morphologically intact and non-overlapping nuclei and used to correlate with clinical data.

The FISH gene to reference ratio was determined on 21 patients for TYMS and on 23 patients for TYMP and correlated with time-to progression (TTP) and progression-free survival (PFS) using Cox proportional hazard models. These correlative studies revealed that a higher TYMS gene copy number was significantly associated with a decrease in PFS (HR 1.46, 95% confidence interval 1.08 to 1.96, p= 0.014) and TTP (HR 1.49, 95% confidence interval 1.05 to 2.13, p= 0.028) (Table 2 and 3). As shown in Figure 6, PFS was inferior in patients with the higher TYMS to reference ratio

(highest third versus lowest two thirds). TP gene copy number was not significantly associated with either PFS or TTP (Table 2 and 3).

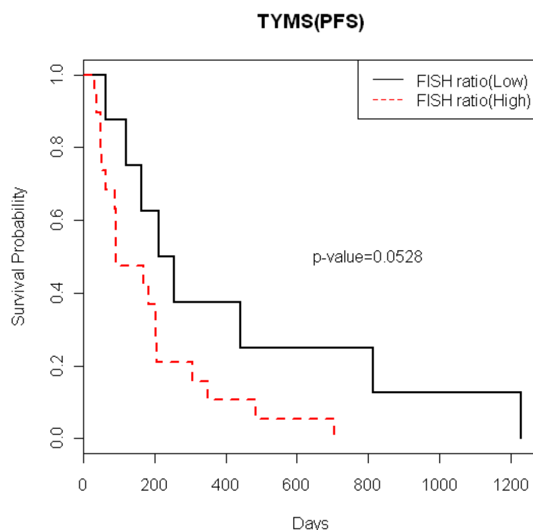

Figure 7: Kaplan-Meier plot of progression free survival based on thymidylate synthase (TYMS) gene copy number to reference signal ratio determined by fluorescent in situ hybridization (FISH). (- - -) high TYMS to reference signal FISH ratio (highest one third); (—) low TYMS to reference signal FISH ratio (lowest two thirds). n=21

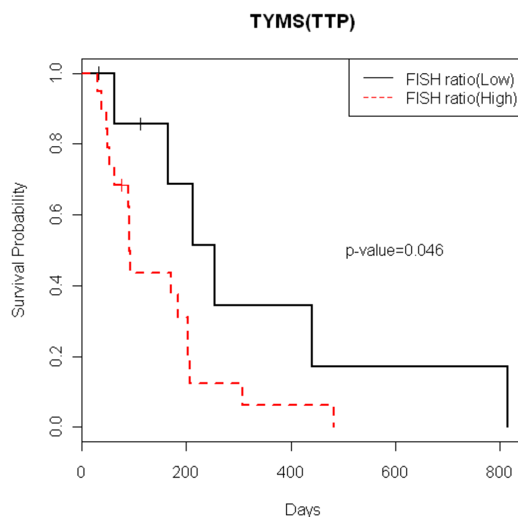

Figure 8: Kaplan-Meier plot of time-to progression based on thymidylate synthase (TYMS) gene copy number to reference signal ratio determined by fluorescent in situ hybridization (FISH). (- - -) high TYMS to reference signal FISH ratio (highest one third); (—) low TYMS to reference signal FISH ratio (lowest two thirds). n=23

Table 2: Correlation between FISH TYMS and TYMP gene to reference signal ratio and time-to progression (TTP)

Gene	Hazard Ratio	p-value	95% C.I.
TYMP	1.07*	0.4129	[0.91 1.26]
TYMS	1.49	0.0276	[1.05 2.13]

*For every 0.1 unit increase in FISH signal ratio, the hazard ratio is 1.07.

Table 3: Correlation between FISH TYMS and TYMP gene to reference signal ratio and progression-free survival (PFS)

Gene	Hazard Ratio	p-value	95% C.I.
TYMP	0.99	0.8982	[0.83 1.18]
TYMS	1.46	0.0135	[1.08 1.96]

FISH assays are readily available to clinicians and the identification of markers able to help predict response to fluoropyrimidine treatment would represent a major advance for patient care. These data indicate that TS gene copy number, assessed by FISH with proper standardization, might be a useful and easily accessible marker for capecitabine sensitivity in human breast cancer and warrants further investigation.

Gene expression using qRT-PCR

The RNA extracted from FFPE slices, as described in previous sections, was amplified in qRT-PCR assays using TaqMan probes for DPYD, DHFR, TYMP, TYMS, and GUSB as a control.

The association between TS RNA levels (expressed as Ct values) and PFS (HR 0.82, 95% CI 0.61 to 1.11, p=0.198) or TTP (HR 0.74, 95% CI 0.49 to 1.14, p=0.172) failed to reach statistical significance (Table 4 and 5). Similarly, DPD, DHFR or TP gene expression was not associated with PFS or TTP (Tables 4 and 5).

Table 4: Correlation between time-to progression (TTP) and gene expression using TaqMan qRT-PCR assays for DPYD, DHFR TYMP and TYMS. n=24

Gene	Hazard Ratio	p-value	95% C.I.
DPYD	1.137	0.6464	[0.657 1.966]
DHFR	0.630	0.3244	[0.251 1.580]
TYMP	1.029	0.9263	[0.565 1.873]
TYMS	0.744	0.1720	[0.487 1.137]

Table 5: Correlation between time-to progression (TTP) and gene expression using qRT-PCR TaqMan assays for DPYD, DHFR TYMP and TYMS. n=24

Gene	Hazard Ratio	p-value	95% C.I.
DPYD	1.170	0.4643	[0.768 1.782]
DHFR	0.678	0.2391	[0.355 1.295]
TYMP	0.925	0.7502	[0.573 1.494]
TYMS	0.822	0.1984	[0.609 1.108]

These gene expression data from qRT-PCR assays were also compared to Affymetrix results generated in Dr. Jenny Chang's genomics lab using the GeneChip Human X3P array. This array is designed specifically for whole-genome expression profiling of FFPE samples. The target sequences on the X3P Arrays are identical to those of used for designing the human genome U133 Array with a total of 47,000 transcripts. Transcripts of all four genes

analyzed with our TaqMan qRT-PCR assa ys were co mpared with their corresponding transcripts on the Affymetrix array.

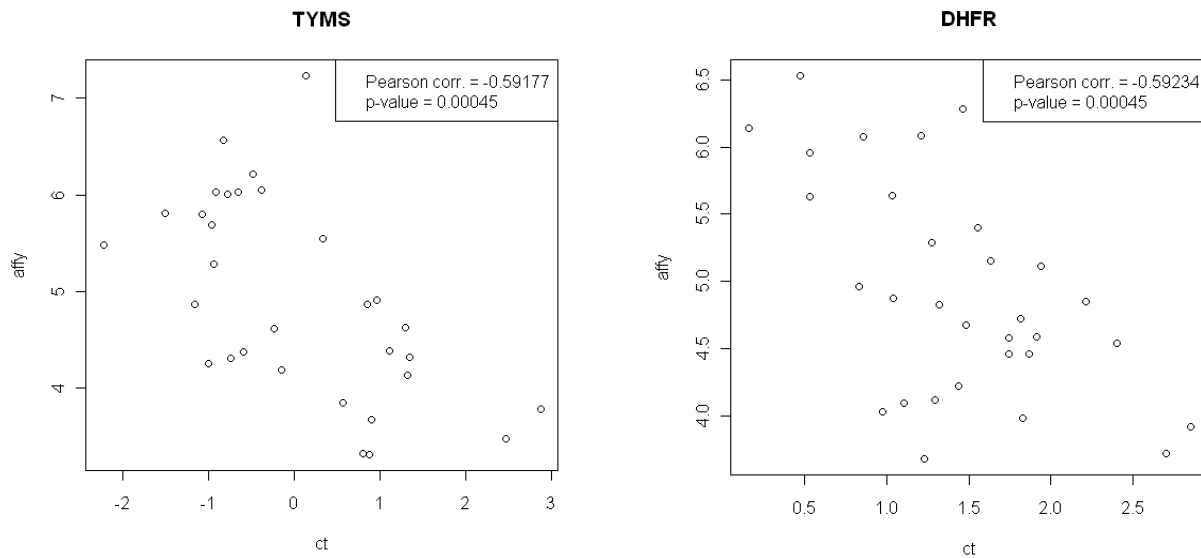

Figure 9: Scatter plots comparing Affymetrix data and qRT-PCR for TYMS and DHFR. n=31

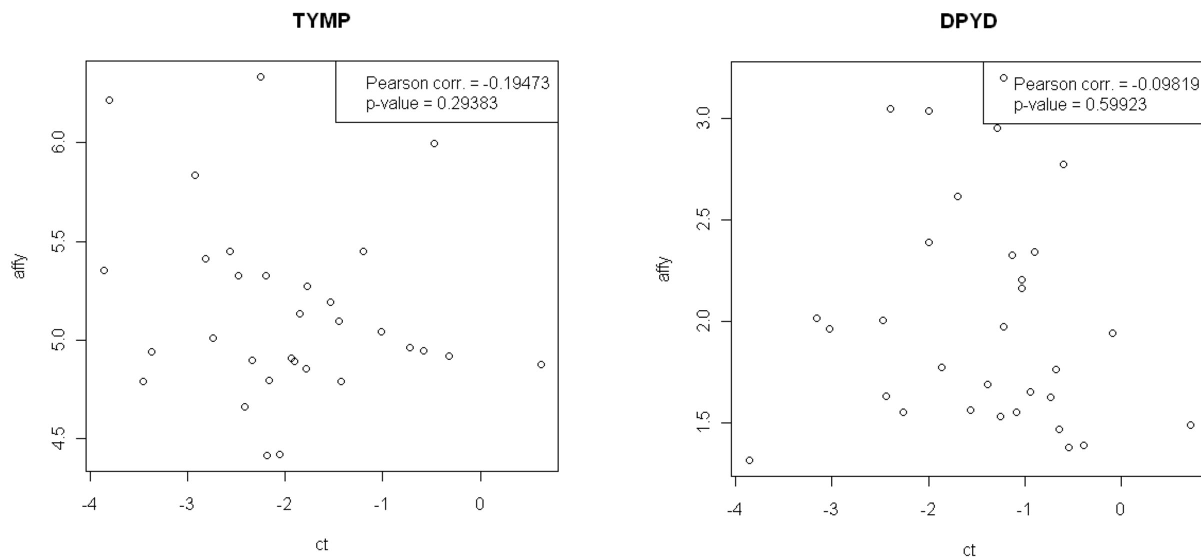

Figure 10: Scatter plots comparing Affymetrix data and qRT-PCR for TYMP and DPYD. n=31

As shown in Figures 9 and 10, mRNA levels determined by Affymetrix, were significantly correlated with qRT-PCR for TYMS and DHFR, but were not in the case of TYMP and DPYD. Again, the number of samples compared is very limited; it is reassuring to note a 50% concordance between the two methodologies but more samples will have to be analyzed to properly validate these results.

Conclusions and projections

Our results showing a significant correlation of TYMS gene copy number and clinical outcome are extremely encouraging, but since the number of patients used in the above analyses is still small, the resulting data should be interpreted with caution. Our ongoing efforts to add more patients to this study will undoubtedly provide the necessary power in order to answer to this question. Nevertheless, these interim results bring hope that some new and valuable diagnostic tests could be available in the near future. Knowledge gained in the past few years regarding the benefit derived from Herceptin treatment of HER2 positive tumors can serve as an example of the clinical influence adequate diagnostic tools can provide (Sauter et al., 2009).

It should be noted that an abstract detailing the FISH and qRT-PCR results on capecitabine treated patients has been accepted as a poster discussion at the annual meeting of the American Society of Clinical Oncology in Orlando, Florida on May 31st 2009. The discussant will be Dr. David R. Spriggs from the Memorial Sloan-Kettering Cancer Center.

Objectives for 2009-2010

COE-01 and COE-05 trials

Priorities

- Capecitabine arm:
    - Complete TYMS and TYMP FISH analyses
    - Include DHFR FISH analysis
    - Complete qRT-PCR assays on DHFR, DPYD, TYMS and TYMP
    - Include DASL analysis

Work pending accrual

- Vinorelbine arm:
    - DASL assay
    - qRT-PCR on STMN1 and TUBB3

- Gemcitabine arm:
    - DASL assay
    - qRT-PCR on CNT, DCK, ENT and RRM1

COE-01A

- AC and Taxotere
    - TOP2A and HER2 FISH analysis
    - qRT-PCR assays on ALDH1A1, HER1, HER2, HER3, TOP2A
    - DASL analyses

General conclusions

Critical determinants that govern individual responsiveness to routinely used chemotherapeutic agents will be identified as specific "signatures" and, with proper validation, could be used by clinicians to individualize therapy. These results will provide invaluable insight into monitoring inter-individual variations in efficacy and toxicity of capecitabine and these observations could be used to help select appropriate drug and dosage regimens for each patient.

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Pathology Core progress report for 08-09

Overview:

The activities of the pathology core, located at the University of Colorado Denver, include biobanking, processing and coordination of tissue acquisition and distribution. We receive frozen human serum, frozen human tissue and formalin fixed paraffin embedded tissue blocks from submitting institutions. Frozen serum and tissue sections are inventoried and placed in a -80°C freezer upon arrival. Paraffin blocks are stored by protocol and specimen number in block storage files. Data is entered into the HOG electronic data capture system on the day that the specimens are received. Data entered into the system includes: specimen number, date received, condition, and storage location. Paper records, without any protected health information, are stored (submission forms and pathology reports) in a locked file cabinet.

Processing protocols for tissues and samples is determined by protocol. In all cases, we section frozen and formalin fixed paraffin embedded tissues, followed by a hematoxylin and eosin stain for quality control purposes. The goal is to determine whether sufficient tumor exists in the block that was submitted. This allows us to request additional materials and give feedback to the submitting institution. For cases with small amounts of tumor, in relation to the total amount of tissue present, the hematoxylin and eosin slide will be annotated so that tissue prepared for RNA analyses can be enriched for tumor.

We communicate with other investigators and Dr. Sledge through monthly conference calls, e mails and annual in person meetings. We have had on site visits by HOG administrative staff. The core mission is to provide outstanding technical and professional biobanking services and to be responsive to the needs of the reference laboratories and submitting institutions.

CO1 Protocol
Through March 2009, samples from a total of 49 patients enrolled on clinical trial CO1 have been received. Of these, 6 patients have enrolled on more than one arm or study. Among the 49 total patients, 13 were accrued to Arm A (Cytoxan/Adriamycin), 14 have been accrued to Arm B (Capecitabine), 4 were accrued to Arm C (Vinorelbine) and 18 were accrued to Arm D (Gemcitabine). During the 2008-2009 reporting period we have cut and distributed ten to twelve six micron thick sections from 11 samples from Arm A, 7 samples from Arm B, 4 samples (3 patients) from Arm C and 8 samples from Arm D to the Pharmacogenomics Core, who have prepared the RNA for themselves and the Genomics core. We have also distributed 8-five micron thick slides from 6 samples from arm B to the Pharmacogenomics Core for fluorescence in situ hybridization (FISH) staining.

CO1A Protocol
Through March 2009 we have received a total of 232 frozen tissue specimens from 199 patients from Baylor College of Medicine from a prior study. Among these 199 patients, 124 specimens from 107 patients were accrued to Arm A and 108 specimens from 92

patients were accrued to arm E (Taxane). We have sectioned 231 frozen specimens to determine whether > 70% of the specimen consists of tumor in these frozen tissues which will allow us to return the specimens to the Genomics Core for RNA preparation without tumor enhancement. A total of 46 specimens have been returned with an additional 12 specimens in preparation for return. The remaining specimens are divided into specimens without any tumor tissue (45), specimens which will require laser capture microscopy (LCM) for tumor enrichment (36), and specimens, which are under review by the core director Thor (94). Once these 94 have been reviewed, they will be reclassified into insufficient tumor for further study or specimens requiring LCM for tumor enrichment.

CO2 Protocol

Through March 2009, a total of 8 patients have been accrued to this trial. One specimen from a patient also accrued to the CO1 trial has been sent to the Pharmacogenomics Core for RNA preparation. Once additional specimens have been received, we will begin distributing serum and tissue specimens in accordance with the approved SOPs. Distribution is being held until 10 patient specimens have been accrued.

CO5 Protocol

Through March 2009, formalin fixed paraffin embedded archival specimens from 102 patients have been received at the Pathology Core. Four specimens have been returned to the submitting institutions without tumor in the submitted block and additional material has been requested. We have received material from 45 patients treated on arm A (Adriamycin/Cytoxan), 74 patients treated on arm B (Capecitabine), 41 patients treated on arm C (Vinorelbine) and 36 patients treated on arm D (Gemcitabine). Most patients have been treated on multiple arms. During the 2008-2009 year we have distributed 8-five micron sections from 16 patients, all treated with Capecitabine) to the Pharmacogenomics Core for fluorescence in situ hybridization (FISH) analysis. Ten to twelve, 6 micron thick sections (from 52 blocks) were also sent to the same lab, for RNA extraction. RNA will be used by both the Pharmacogenomics Core and the Genomics Core.

Statistical Summary

• Publications

1. **Shen, C.**, Sheng, Q., Dai, J., Li, Yi., Zeng, R., and Tang, H. On the estimation of false positives in peptide identifications using decoy search strategy. *Proteomics* 2009; 9: 194-204.

2. **Shen, C.,** Liu, Y., Wang, G. Nephew, K., Huang, T., and Li, L. Estrogen regulatory pathway and the impact of selective estrogen receptor modulator. *Genome Research* (submitted).

3. Jeong, J., Liu, Y., Li, L., Nephew, K., Balch, C., Huang, T., and **Shen, C.** An integration model to correlate gene expression and methylation in breast cancer cell lines (being prepared).

• Abstracts

1. Audet, R., **Shen, C.**, Breen, T., Edgerton, S., Vang Nielsen, K., Lay, L., Thor, A., Chang, J., Miller, K., Sledge, G., Leyland-Jones, B. Thymidilate synthase (TS), Thymidine phosphorylase (TP), Dihydropyrimidine dehydrogenase (DPD) and Dihydrofolate Reductase (DHFR) as predictive markers of Capecitabine Efficacy in Breast Cancer Patients. 2009, 45th Annual Meeting of American Society of Clinical Oncology.

• Development

1. R program developments for standard analysis of microarray data (Affymetrix and DASL platform)
2. Statistical methodology and R-program development for new methods to analyze microarray data
3. Efforts in standardizing naming conventions of samples for the merge of different type of data
4. Communications between data management staff and statisticians on data transfer, storage and manipulation issues.

• Plans for 2009

1. Complete the implementation of standard data analysis tools for genomics and proteomics data analysis in R.
2. Complete the implementation of new statistical methods for microarray data analysis
3. Implement Bayesian network analysis in R
4. Complete the data transfer and management planning and implementation