A Pilot Trial of Platinum, Gemcitabine, or Pemetrexed Single- or Multi-Agent Therapy with Serial Tumor Specimen Collection in Patients with Advanced Non-Small-Cell Lung Cancer

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Study Summary

Recruitment: NSCLC patients scheduled to receive single- or multi- agent chemotherapy as front line, maintenance (after 4-6 cycles of induction therapy) or second or higher-line line therapy for advanced disease. Patients must have a PS 0-2 and a life expectancy of at least 3 months, a tumor lesion that can be safely biopsied and has not been radiated, and measurable disease.

Biopsy: From each patient, specimens will be collected at two different time points, prior to chemotherapy and prior to cycle #3. Collection of the pre-treatment specimens is mandatory. Histological specimens can be obtained from any accessible tumor lesion with any available technique. They must be preserved in 10% v/v neutral phosphate-buffered formalin. Biopsies obtained through FNA are permissible provided the specimen is processed as a cell block similar to histological material.

Treatment: Treatment selection is at the discretion of the treating physician and must contain cisplatin, carboplatin, gemcitabine, and/or pemetrexed as a single-agent or in any combination treatment at a dose deemed appropriate by the treating physician. Dose reductions for toxicities are as described per package insert and institutional guidelines.

Efficacy: The enrollment goal is 150 patients that initiate therapy. Best treatment response will be determined according to RECIST (version 1.1) after treatment cycle #2. Up to 10 separate tumor lesions will be followed. The greatest diameter of each lesion will be recorded (using one decimal point). The percentage of change of the sum of tumor diameters comparing the post-treatment with the pre-treatment measurements will be calculated using the formula 1-(SumCTpost/SumCTpre). All patients who receive at least one dose of chemotherapy will be considered evaluable for analysis. Expression levels of RRM1, TS, ERCC1other relevant molecules, and maximal disease response will be log-transformed to approximate normal distribution. Each biomarker's pre-treatment expression level will be used to correlate with drug response using Pearson correlation. Multiple linear regression will be used to assess the relationship of these expression levels to drug response.

Follow-up: Prior to each cycle of chemotherapy. Further follow-up intervals are at the discretion of the care provider. However, we recommend follow-up every 6-8 weeks until year one of treatment initiation with appropriate standard-of-care imaging studies.

Study Enrollment & Duration: The total duration of the protocol is 120 months from initiation of treatment of the first patient. A total of 150 patients will be enrolled over 108 months, with an estimated accrual rate of 1-2 patients per month (12-24- per year). Treatment duration is 6-8 weeks and patients will be followed up for 12 months after initiation of therapy.

Abbreviations

Abbreviations or Terms	Definitions
AE	Adverse Event
ALAT	Serum Alanine Aminotransferase
ANC	Absolute Neutrophil Count
ASAT	Serum Aspartate Aminotransferase
AQUA	Accurate Quantitative Analysis of Protein Expression
Bid	Twice a day
BSA	Body Surface Area
CALGB	Cancer and Leukemia Group B
CBC	Complete Blood Count
CI	Confidence Interval
CNS	Central Nervous System
CR	Complete Response
CRF	Case Report Form
CTCAE	Common Terminology Criteria for Adverse Events v4.0
D	Day of cycle (d1 is the first day of the treatment cycle)
D5W	5 percent dextrose in water
DDP	Cisplatin
DNA	Deoxyribonucleic Acid
ECG	Electrocardiogram
ECOG	Eastern Cooperative Oncology Group
FDA	Food & Drug Administration
FSR	Final Study Report
GCP	Good Clinical Practice
G-CSF	Granulocyte Colony Stimulating Factor
i.m.	Intramuscular
i.v.	Intravenous
ICH	International Conference on Harmonization
IEC	Independent Ethics Committee
IHC	Immunohistochemistry
IRB	Institutional Review Board
ITT	Intent-To-Treat
LCM	Laser Capture Microdissection
MoAb	Monoclonal Antibody
MRI	Magnetic Resonance Imaging
NCI	National Cancer Institute
NS	Normal Saline
NSCLC	Non-Small-Cell Lung Cancer
OS	Overall Survival Time
PD	Progressive Disease
PET	Positron Emission Tomography
PFS	Progression Free Survival Time
p.o.	*Per Os* (by mouth)
PR	Partial Response
PS	Performance Status
RECIST	Response Evaluation Criteria in Solid Tumors
RNA	Ribonucleic Acid

RR	Response Rate
RTPCR	Reverse Transcriptase Polymerase Chain Reaction
SAE	Serious Adverse Event
SAERF	Serious Adverse Event Report Form
SAP	Statistical Analysis Plan
SD	Stable Disease
SGOT	Serum Glutamic Oxalo-Acetic Transaminase
SGPT	Serum Glutamic Pyruvic Transaminase
SOP	Standard Operating Procedure
SWOG	Southwest Oncology Group
Tid	Three Times a Day
UNL	Upper Normal Limit for that institution
VEGF	Vascular Endothelial Growth Factor
Vs	Versus
WBC	White Blood Cell Count
WHO	World Health Organization

1.0 Introduction

1.1 Current treatment for first-line advanced stage NSCLC patients with good performance status

Lung cancer continues to be the leading cause of cancer-related death in the United States (159,480 deaths in 2013). Since 2008, it has also become the malignancy with the highest incidence in the U.S. (not counting non-melanoma skin cancer) with an estimated 228,190 new cases in 2013 despite a substantial decline in tobacco consumption since the 1960s.[1,2] Similarly, lung cancer accounted for 384,077 of all new cancer cases and 341,800 deaths in Europe in 2004.[3] Approximately 70% of all lung cancers are classified as non-small-cell lung cancers (NSCLC), which comprises adenocarcinoma, squamous cell carcinoma, large cell carcinoma, NSCLC not otherwise specified (NSCLC-NOS), and other rare subtypes. According to SEER statistics, 45.1% of patients present with advanced metastatic disease (stage IV, Appendix I), 16.7% with potentially surgically curable disease (stage I and II), 24.5% with locally advanced disease (stage III, with an approximately 15-20% cure rate by concomitant radiation and chemotherapy), and 13.7% with unstaged disease (http://seer.cancer.gov/data/). As a result, an estimated 70,000 - 100,000 patients are diagnosed annually in the U.S. with advanced and incurable NSCLC.

The use of unselected double-agent chemotherapy, the current standard of care according to NCCN guidelines (http://www.nccn.org/index.asp), has resulted in an approximately 50% improvement in overall median survival of patients with advanced NSCLC compared to best supportive care.[4] The only criteria currently used for selection of agents are histology,[5] toxicity profiles, and convenience of delivery. The principal chemotherapeutic agents used are platinum analogues, taxanes, gemcitabine, pemetrexed, vinorelbine, and etoposide. Response rates (RR) in patients with advanced stages to double-agent therapy are 17-37%; and the median overall survival (OS) is 7-12 months, the 1-year survival rate is 31-46%, and the 2-year survival rate is 9-21% in a Western population.[5-15]

Although platinum is considered by many an essential component of initial multi-agent therapy, in four randomized phase III trials that compared platinum-containing regimens to a non-platinum regimen for patients with advanced disease,[8,12,16,17] the non-platinum regimen was equivalent in terms of survival and had less toxicity. In a randomized phase II study of four regimens (gemcitabine/vinorelbine with or without carboplatin and gemcitabine/paclitaxel with or without carboplatin) all four regimens yielded comparable survival rates, while objective response rates were higher (34-42%) in the platinum-containing regimens as compared to the non-platinum regimens (both 29%).[18] Identical conclusions were reached in a meta-analysis of platinum-containing versus non-platinum-containing chemotherapy in advanced NSCLC.[19]

After patients fail initial therapy, response to further chemotherapy is 6.7 - 10.8% for the FDA-approved single agents docetaxel and pemetrexed.[20-22] Thus, resistance to systemic therapy does not appear to be an all-or-none phenomenon, but rather a function of molecular characteristics of individual tumors. In fact, specific molecular characteristics highly associated with tumor response to a class of therapeutic agents used in NSCLC, epidermal growth factor receptor tyrosine kinase inhibitors, has recently been reported.[23-25]

For the foreseeable future, systemic treatment with cytotoxic agents will remain the mainstay of therapy. Efforts at increasing chemotherapeutic efficacy through adding agents in unselected (or minimally selected) patient populations have been disappointing with small or no benefits observed.[5,15,26,27]

1.2 Using single-agent chemotherapy for treatment of advanced NSCLC

Because a high rate of toxicity has been observed with dual-agent therapy in patients with a poor performance status (PS 2), single-agent therapy has become an accepted standard. However, subgroup analyses of cooperative group trials have suggested improved survival with dual- versus single-agent therapy for this group of patients.[28,29] A subgroup analysis of a randomized phase III trial reported a median OS of 2.4 months (CALGB 9730) in 50 patients with PS 2 treated with single-agent paclitaxel.[28] This estimate is perhaps too low as evidenced by a single-arm trial of gemcitabine in a similar group of patients (4.8 months),[30] a PS 2 subgroup analysis of a randomized phase III Italian trial (3.5-4.2 months in 86 patients treated with single-agent vinorelbine or gemcitabine),[31] and data from a randomized phase II trial in PS 2 patients showing a median OS of 4.8 months for single-agent gemcitabine.[32] It is conceivable that the survival of patients enrolled in trials specifically designed for those with PS 2 may be better than the survival observed in PS 2 subgroup patients from a trial open to patients with PS 0-2 as a result of a biased interpretation of the PS classification by enrolling physicians. We had conducted a randomized phase III trial of gemcitabine versus gemcitabine/carboplatin in 170 previously untreated patients with PS 2 in community-based oncology practices. The median OS was 5.1 months for gemcitabine and 6.7 months for gemcitabine/carboplatin (p = .24) leading to the conclusion that single-agent chemotherapy remains the standard of care for patients with advanced NSCLC and poor performance status.[33] However, optimal treatment for patients with PS 2 continues to be an active area of investigation.[34]

Single-agent chemotherapy with gemcitabine, vinorelbine, or a taxane is considered an acceptable standard of care for patients ≥70 years of age. When compared to best supportive care alone, single-agent vinorelbine produces superior survival.[35] In a large Italian trial, single-agent gemcitabine was equally as efficacious as vinorelbine, and they were each not less efficacious and better tolerated than both combined.[36] A subset analysis of a randomized phase III cooperative group trial (ECOG 5592) of platinum-based therapy suggested longer survival for elderly patients treated with the combination compared to single-agent alone[37] and has led to the frequent use of paclitaxel/carboplatin in this group of patients; however, the superiority of this approach has never been convincingly established. In fact, a subgroup analysis of a randomized phase III trial of paclitaxel alone versus paclitaxel/carboplatin conducted by the Cancer and Leukemia Group B (CALGB 9730) did not demonstrate a statistically significant survival advantage for the combination in elderly patients.[28]

Superiority of single agent docetaxel over best supportive care as 2nd-line therapy was established by the Canadian Lung Cancer group[20] and confirmed by Fossella et al in the U.S.[21] In addition, equal efficacy and lower toxicity was demonstrated for pemetrexed when compared to docetaxel in a randomized phase III trial conducted by the Hoosier Oncology Group.[22] The trial demonstrated a response rate of approximately 9%, a median OS of 8 months, a 1-year survival of 30%, and a median PFS of 3 months. Of note, PS 2 patients were included in the trial, benefited equally from therapy, and had shorter survival times then PS 0-1 patients in both arms.

The FDA has approved pemetrexed as the first agent in patients with NSCLC for maintenance therapy after completion of four cycles of platinum-based dual-agent chemotherapy. The approval was based on a randomized phase III trial demonstrating better OS for patients that started single-agent pemetrexed immediately after completion of four cycles of platinum-based dual-agent therapy compared to patients that received placebo (median OS 13.4 vs 10.6 ms, p

= .01; median PFS 4.0 vs 2.0 ms, $p < .00001$). Three other cytotoxic agents have been tested in randomized trials as maintenance therapy after four cycles of combination chemotherapy, vinorelbine,[38] gemcitabine,[39] and docetaxel (Fidias et al, presented at ASCO 2007). Gemcitabine and docetaxel yielded a statistically significant improvement in PFS in the maintenance arm but failed to demonstrate a statistically significant OS advantage. Vinorelbine did not improve PFS or OS.

1.3 Using molecular tumor characteristics for selection of chemotherapy

Chemotherapeutic efficacy has been associated with distinct molecular features in genes associated with drug metabolism and mechanism of action, which, in the era of genome research, may provide an opportunity for optimal matching of molecular tumor characteristics with available drugs. A notable discovery has been the association of methotrexate resistance with amplification of the dihydrofolate reductase gene (DHFR) by Bertino and Schimke.[40,41] Other notable associations are between thymidylate synthase (TS) and 5-fluorouracil (5FU),[42] cytidine deaminase and cytosine-arabinoside, aldehyde dehydrogenase and cyclophosphamide, methyl-guanine-methyltransferase and temozolomide or carmustine, multidrug resistance (MDR) genes and anthracyclines, taxanes, and vinca alkaloids, nucleotide excision repair genes and platinum agents, and the regulatory subunit of ribonucleotide reductase (RRM1) and gemcitabine,[43,44] which has been the focus of our research efforts.

1.4 RRM1 mRNA and protein expression and gemcitabine efficacy in NSCLC

The antimetabolite gemcitabine (2',2'-difluorodeoxycytidine) is among the most efficacious and most frequently used drugs in the treatment of NSCLC.[5,7,45] Ribonucleotide reductase is the rate-limiting enzyme in DNA synthesis, and it is the only known enzyme that converts ribonucleotides to deoxyribonucleotides, which are required for DNA synthesis and repair. The ribonucleotide reductase holoenzyme consists of two dimerized subunits (RRM1 and RRM2a or RRM2b), the pairing of which is essential for deoxynucleotide synthesis. We had previously cloned the entire genomic region of RRM1, described its molecular organization and polymorphisms, and generated probes for molecular investigations.[46,47] Several publications from independent laboratories including ours have demonstrated that RRM1 is the major cellular determinant of sensitivity and resistance to this drug.[44,48-50]

Davidson et al generated *in vitro* resistance to gemcitabine through exposure of NSCLC lines to increasing doses of the drug.[48] Using oligonucleotide expression array-based profiling, they identified RRM1 as the gene with the most consistent and reproducible increase in expression. Nakahira et al reported identical results in pancreatic cancer cell lines.[50]

Using a mouse model, Bergman et al generated *in vivo* resistance to gemcitabine by serial subcutaneous transplantation of the tumor Colon 26 in BALB/c mice under repetitive intraperitoneal treatment with gemcitabine for one year. The authors found that RRM1 was the gene with the most striking increase in expression (25-fold) in the resistant tumor compared to the parent tumor in expression arrays, by RTPCR, and by immunoblotting.[49] Other genes involved in the intracellular metabolism of gemcitabine were only minimally changed (deoxycytidine kinase and deoxycytidine deaminase).

We had used knock-in and knock-down approaches to assess if RRM1 expression modulation in a human lung cancer cell line (H23) would directly impact gemcitabine cytotoxicity and apoptosis induction in clones of otherwise genetically identical background. The expression

levels of RRM1 and control genes were determined by real-time quantitative RTPCR and immunoblotting. The expression of RRM2a, the catalytic ribonucleotide reductase subunit, was unaffected. The sensitivity of these cell lines to gemcitabine, cisplatin, and carboplatin was compared to transfected control cell lines. Increased RRM1 expression resulted in an 8-fold increase in the gemcitabine IC_{50}, and reduced RRM1 expression increased sensitivity to gemcitabine 10-fold. There was a similar but much less pronounced relationship between RRM1 expression and cytotoxicity response to cisplatin and carboplatin. An increase in RRM1 resulted in 1.2-1.5-fold more resistant and a decrease in 1.1-1.3-fold more sensitivity to platinum analogues than the corresponding control cell lines. We also found an inverse relationship between drug-induced cell death and RRM1 expression.[44]

Two earlier and small exploratory retrospective datasets investigating this association in patients with stage IV NSCLC had provided evidence that RRM1 mRNA expression is a prognostic and possibly predictive marker of survival in patients treated with gemcitabine plus cisplatin.[51,52] Although these studies had limitations due to their retrospective nature and size, and the fact that no measurable impact on disease response was documented, the observed effects on survival suggested that testing of RRM1 mRNA expression levels could be used to predict benefit from gemcitabine/platinum-based chemotherapy.

To prospectively study if intratumoral RRM1 mRNA expression is predictive of response to gemcitabine/platinum-based chemotherapy, we conducted the first prospective clinical study in patients with NSCLC that directly addressed this question.[44] The study was supported by a grant from the NCI (R01-CA102736). Dedicated tumor sampling for determination of gene expression prior to therapy and at two time points after therapy, if feasible, was required in all patients. They were treated with two cycles of gemcitabine and carboplatin. Unidimensional tumor measurements were obtained before and after chemotherapy, and disease response was recorded as the percent change after treatment compared to before treatment and also categorized as complete remission (CR), partial remission (PR), stable disease (SD), and progressive disease (PD) according to RECIST.[53] In the 35 patients that had adequate tumor specimens for gene expression analysis, completed two cycles of therapy, and had disease response assessment, disease response ranged from a 9% increase to a 100% decrease in tumor diameters, RRM1 expression ranged from 0.18 to 129.3, and we found a significant (p = .002) inverse correlation (r = -0.50, Spearman correlation coefficient) between RRM1 expression and the magnitude of disease response (Figure 1). When grouping patients into those with response (CR/PR) and without response (SD), RRM1 expression was significantly (p = .03, Chi-square test) associated with response, where patients

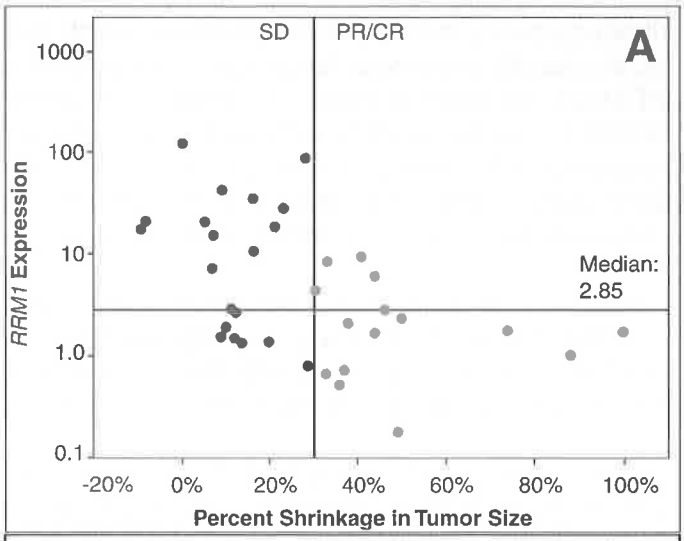

Figure 1: Scatter plot of *RRM1* mRNA expression in relation to the percent change in tumor size after two cycles of gemcitabine and carboplatin chemotherapy in 35 patients. Red dots indicate patients with less than 30% tumor shrinkage (SD), and green dots indicate patients with greater than 30% tumor shrinkage (PR/CR). The Spearman correlation coefficient (r) was -0.498 (p = .002).

with high tumoral expression of RRM1 were less likely to respond to gemcitabine/carboplatin than those with low levels of expression. There was no significant association between RRM1 mRNA levels and the number of tumor cells collected, patients' age or gender, tumor histopathology, tumor stage, and smoking status.

We have also generated the reagents and adapted technology, developed by Dr. Rimm at Yale University,[54] to assess *in situ* protein levels of RRM1 and other targets of interest for correlative investigations in formalin-fixed and paraffin-embedded (FFPE) specimens.[55,56] The technology allows for automated, accurate, and quantitative determination of target molecules using an algorithm that selects cells and cellular compartments of interest based on multi-color immunofluorescence (AQUA). We utilized this technology to assess prospectively if tumoral RRM1 protein levels are predictive of response to single-agent gemcitabine or gemcitabine/carboplatin in previously untreated patients with advanced NSCLC and a performance status (PS) of 2. This was a randomized phase III trial performed in community-based oncology practices across the U.S., conducted by US Oncology (USO-03012), and sponsored by Eli Lilly (B9E-US-S358). Tumor specimens were collected *a priori* and shipped to our laboratory for blinded determination of *in situ* RRM1 (and ERCC1) protein levels. One-hundred seventy patients were randomized, and median overall survival (OS) was 5.1 months for gemcitabine and 6.7 months for gemcitabine/carboplatin (p = .24). Routinely collected, histological or equivalent, diagnostic tumor specimens were received from 87 patients and 42 different study sites. Eight specimens had been stained, which resulted in 79 specimens potentially useful for analysis. Specimens from 10 patients were inadequate to produce results because the specimen had washed off during the procedure or because of absence of tumor cells. RRM1 data were obtained on 69 patients (ERCC1 data on 65). There were no significant differences between the groups of patients with and without biomarker data. Disease response was recorded as the best percent change during treatment compared to before treatment. RRM1 (range 5.3 – 105.6; median 34.1) values were significantly and inversely correlated with response (r = -0.41, p = .001) (Figure 2) (as were ERCC1 levels; r = -0.39, p = .003); i.e., response was better for patients with low levels of expression. A model for response prediction that included RRM1, ERCC1, and treatment arm, was highly predictive of the treatment response observed (p = .0005) [33]. The positive predictive value was 0.90, and the negative predictive value was 0.46.

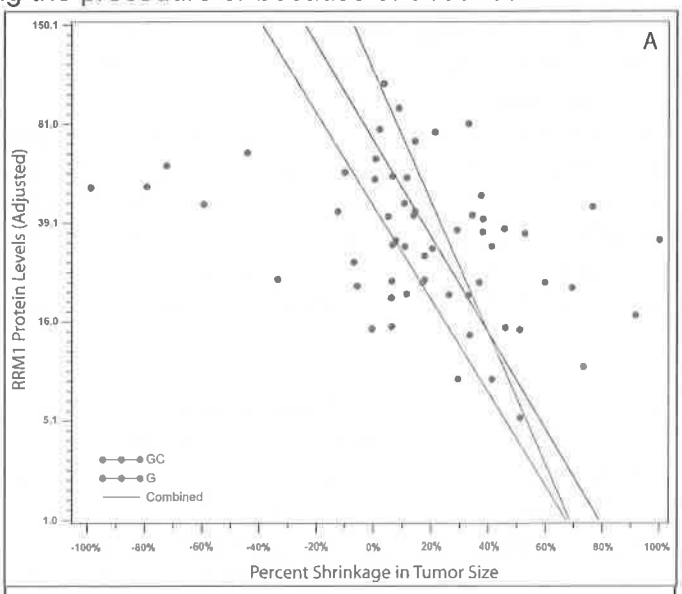

Figure 2: Scatter plot of RRM1 protein levels in relation to the percent change in tumor size after best response to gemcitabine or gemcitabine/carboplatin in 69 patients. The Spearman correlation coefficient (r) was -0.41 (p = .001).

1.5 RRM1 and TS expression and gemcitabine/pemetrexed efficacy

We also prospectively investigated if intratumoral RRM1 and TS mRNA expression is predictive of response to 4 biweekly cycles of neoadjuvant gemcitabine/pemetrexed in patients with

surgically resectable NSCLC (supported by a NCI grant R21-CA110487).[57] In this study, a total of 14 genes thought to be potentially associated with response to gemcitabine or pemetrexed were studied by real-time RTPCR. Gene expression levels and disease response were available on 35 patients. As before, RRM1 expression was inversely correlated with response ($r = -0.65$, $p < .001$). TS was also inversely correlated with response ($r = -0.45$, $p = .006$). None of the other 12 genes had meaningful associations with response (r between 0.28 and -0.08).

1.6 RRM1 gene polymorphisms and gemcitabine efficacy in NSCLC

A group of investigators from South Korea have recently reported that polymorphisms in the RRM1 promoter, which we had previously identified and demonstrated to impact RRM1 expression *in vitro*,[58] impact efficacy of gemcitabine-based chemotherapy. In this study, 97 patients with advanced NSCLC were treated with gemcitabine regimens as first-line treatment. RRM1 promoter allelotypes were determined by real-time PCR in genomic DNA from peripheral blood mononuclear cells. The two dominant allelotypes were 37CC/524TT in 58/97 and 37AC/524CT in 29/97 patients. The tumor response rate (RR) was significantly higher in the 37AC/524CT group (65.5%), the allelotype associated with low promoter activity *in vitro*, compared to all other groups (42.6%; $p = 0.039$). There was no significant difference in survival between these groups.[59]

1.7 RRM1 expression levels and gemcitabine efficacy in pancreatic cancer

Finally in collaboration with a group from Osaka, Japan, we investigated the impact of *in situ* RRM1 protein levels on therapeutic benefit from single-agent gemcitabine in 50 patients with recurrent pancreatic cancer after an initial complete surgical resection (23 received gemcitabine). The study showed that only patients with low levels (levels below the median of the entire cohort; RRM1 range 29.3 – 107.3; median 52.2 [these are values converted from software version 1.6 back to version 1.2 to allow for comparisons between various studies]) benefited from gemcitabine when compared to controls. In patients with RRM1 levels above the median, no survival benefit from gemcitabine was observed. There was a significant interaction between gemcitabine therapy and RRM1 expression for survival ($p = .011$).[60]

1.8 Validation of IALT-bio results using AQUA

We have completed and published a prognostic and predictive utility assessment of ERCC1 and RRM1 using AQUA technology on 784 retrospectively collected NSCLC specimens from the IALT trial.[61] We confirmed the predictive value of ERCC1 for benefit from adjuvant therapy with platinum-based therapy, showed that patients with high ERCC1 levels in the control group lived longer (prognostic value of ERCC1), and showed a non-significant trend towards benefit from adjuvant therapy in the group of patients with low RRM1 expression. We also uncovered substantial technical issues and provided recommendations for solutions in future trials. Most notably, the levels of ERCC1 and RRM1 differed significantly among centers and by specimen quality (rated as poor, medium, high), RRM1 levels were particularly sensitive to these parameters, and they were overall lower than expected from prior single-institution studies. We also found that RRM1 levels were higher in older patients, women, and adenocarcinomas, a result that may explain some of the differential efficacy of gemcitabine and platinum agents between adeno- and squamous cell carcinomas.

1.9 ERCC1 isoforms, and the monoclonal α-ERCC1 antibody 8F1

Investigators from France in collaboration with investigators from Canada, Austria, Germany, and the U.S. recently reported that traditional IHC evaluation (H-scoring system) for tumoral ERCC1 levels is not suitable as a predictive or prognostic biomarker because ERCC1 has at least 4 isoforms (201-204), and only one of these isoforms (202) appears to be involved in DNA damage repair.[62] The authors also performed epitope mapping of currently available commercial antibodies and found that none are specific for isoform 202. In addition, they were unable to reproduce their prior data[63] using the same dataset (IALT-bio) and reagents (monoclonal α-ERCC1 antibody 8F1), which they attributed to a batch-effect; i.e., the monoclonal antibody has changed its staining characteristics over time. This investigation was triggered by a validation effort of prior data using specimens from two other large adjuvant chemotherapy trials (JBR-10 and CALGB-9633).

1.10 CCTα is the second antigen detected by the α-ERCC1 antibody 8F1

We recently completed a collaboration with Dr. Laura Niedernhofer from the University of Pittsburgh and Scripps Clinic focused on ERCC1 and its role in platinum efficacy. We demonstrated that the commonly used α-ERCC1 monoclonal antibody 8F1 detects a second molecule of similar molecular weight as ERCC1, which may lead to erroneous data. Using mass spectrometry, this molecule was identified as cholinephosphate citidylyl transferase–α (CCTα), a phospholipid synthesis enzyme regulated by RAS. In 187 early-stage NSCLC samples, CCTα contributed strongly (rho = 0.38) to 8F1 immunoreactivity. In squamous cell carcinoma of the lung, CCTα was the dominant determinant of 8F1 immunoreactivity, while its contribution in other subtypes of lung cancer was negligible. High expression of CCTα, but not ERCC1 (as demonstrated by the ERCC1-specific antibodies FL297 and EP2143Y), was prognostic of longer disease-free (log-rank $p = 0.002$), and overall survival (log-rank $p = 0.056$). Similarly, in 60 head and neck squamous cell carcinomas (HNSCC), CCTα contributed very strongly to 8F1 immunoreactivity (rho = 0.74), and high CCTα expression was prognostic of survival (log-rank $p = 0.022$ for DFS and $p = 0.027$ for OS).[64]

1.11 Randomized Phase III Multicenter Trial of RRM1 & ERCC1 Directed Customized Chemotherapy versus Standard of Care for 1st Line Treatment of Patients with Advanced Non-Small-Cell Lung Cancer (NCT 00499109)

The randomized, multicenter, international, first-line, phase III trial for patients with advanced-stage NSCLC entitled *"Randomized Phase III Multicenter Trial of RRM1 & ERCC1 Directed Customized Chemotherapy versus Standard of Care for 1st Line Treatment of Patients with Advanced Non-Small-Cell Lung Cancer"* (NCT 00499109) was activated on 5/8/2007 and completed enrollment on 12/23/2010. A total of 4 NCI-designated comprehensive cancer centers (Karmanos, Moffitt, Fox Chase, Johns Hopkins), an academic medical center (Ponce School of Medicine, Puerto Rico) and 4 community centers (two in Florida, one in Nebraska, one in Germany) participate in the trial. The data lock was performed on 1/6/2012, and the manuscript has been published.[65] The trial randomized patients 2:1 to an experimental arm with treatment decision based on RRM1 and ERCC1 protein levels and a standard-of-care arm consisting of gemcitabine/carboplatin. Eligibility criteria and treatment in the experimental arm were identical to those established by us in a prior phase II trial.[66] Treatment continued to a maximum of 6 cycles if tolerated or until disease progression. No maintenance treatment was allowed. Treatment upon progression was at the discretion of the care provider. PFS was the primary endpoint, and 267 evaluable patients were required to achieve statistical significance for an expected 6-month PFS rate improvement of 32%. A planned interim analysis was

performed after 134 events (progression or death) had occurred, and the Data Safety and Monitoring Board decided to continue the trial. Results demonstrated that protein expression analysis for therapeutic decision making is feasible in newly diagnosed advanced-stage NSCLC patients, and that a tumor re-biopsy is safe, required in 17%, and acceptable to 89% (47/53) of patients. Unfortunately, the survival results observed appear to be false negative. We found no statistically significant differences between the experimental arm and the control arm in PFS (6.1 months vs. 6.9 months) or OS (11.0 months vs. 11.3 months). However, a subset analysis revealed that patients with low levels for both proteins who received the same treatment in both arms had a statistically better PFS (p = 0.02) in the control arm (8.1 months) compared to the experimental arm (5.0 months), which led us to the conclusion that the survival results are false negative. We also learned that non-random day-to-day variations in protein expression analyses occurred (Figure 3). We concluded that further assay development with special attention to reagent specificity, day-to-day assay conditions, and site-specific specimen processing is desirable before another trial is launched. In addition, we did not observe a statistically significant association between protein and mRNA levels for RRM1 or ERCC1.

1.12 Summary and hypothesis

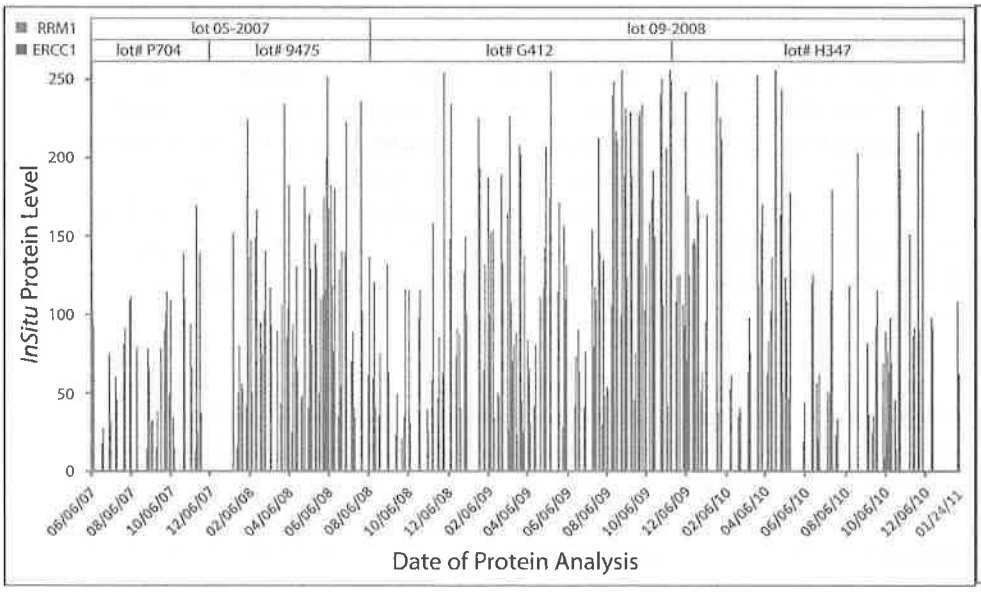

Figure 3: Trends in ERCC1 and RRM1 protein levels over time in the phase 3, randomized, international trial. Different batches of antibodies for ERCC1 and RRM1 quantification were used. Individual patient specimens were run real-time, each run included identical reference specimens as controls, and a standardized operating procedure, device, and image analysis application was used. Protein levels in the patient specimens are shown.

The **hypothesis** for this pilot trial, which builds on our prior experience, is that tumoral RRM1 levels, as determined by quantitative mass spectrometry, and those of TS, and ERCC1, , if determined by appropriate technology, are predictive of response to gemcitabine, pemetrexed, and platinum agents (cisplatin, carboplatin). In addition, we will test the hypothesis that these genes adapt to the selective pressure of the respective agents by increase in the levels.

2.0 Objectives

2.1 Primary objective

The *primary objective* is to describe the association between baseline gene expression levels at the protein and mRNA level and best treatment response after two cycles of single-agent or multi-agent chemotherapy. Best treatment response will be determined by CT scan, or other method as deemed appropriate by the treating physician, and recorded as a continuous variable. Studies will be performed prior to treatment and after completed of cycle #2 (approximately 6-8 weeks apart). The greatest diameter of each lesion will be recorded (using one decimal point). The percentage of change of the sum of tumor diameters comparing the post-treatment with the pre-treatment measurements will be calculated using the formula 1-(SumCTpost/SumCTpre).

Specific relationships to be investigated are:

RRM1 and gemcitabine
TS and pemetrexed
ERCC1 and platinum
Other molecules involved in drug metabolism or efficacy of gemcitabine, pemetrexed, or platinum

Specific questions to be addressed:

Are RRM1 protein levels as determined by quantitative MS predictive of change in tumor diameters after treatment with 2 cycles of gemcitabine.

2.2 Secondary objective

The *secondary objectives* are

2.2.1 to describe changes in protein and mRNA levels of RRM1, TS, and ERCC1 in serial biopsies obtained from patients being treated with gemcitabine, pemetrexed, and platinum.

2.2.2 to describe the association between changes in marker levels and changes in tumor diameters.

2.3 Tertiary objective

The *tertiary objectives* are

2.3.1 to explore the relationship between marker levels in circulating tumor cells and solid tumor specimens.

2.3.2 to explore the relationship between marker levels in viable peripheral blood mononuclear cells (PBMCs), circulating tumor cells, and tumor specimens.

2.3.3 should sufficient amounts and numbers of tumor specimens remain after these analyses, they will be used to assess if other genes implicated in NSCLC outcome and response to treatment might be useful as prognostic or predictive markers for patient outcome.

3.0 Study Design

3.1. Description of the design

This is a single-center, pilot, open label trial of established chemotherapeutic agents given as single agents or in combination according to best clinical practice principles. Treatment selection and dosing is at discretion of the treating physician.

3.2. Study centers

Patients will be evaluated, biopsied, treated, and followed-up at the Karmanos Cancer Institute. Tumor biopsies will be fixed in neutral phosphate-buffered formalin and processed in Dr. Bepler's laboratory. The central office for conduct of this trial will be the Clinical Protocol and Data Management Office at the Karmanos Cancer Institute in Detroit, MI.

4.0 Selection of Study Subjects

The study population will consist of patients with pathologically proven stage IV NSCLC. All patients will fall into at least one of the following three categories:

A) Patients with untreated advanced stage NSCLC who are candidates for single or multi-agent first line chemotherapy
B) Patients who are progressing after at least one type of chemotherapy for advanced stage NSCLC with PS 0-2
C) Patients that have completed four cycles of dual-agent platinum-based chemotherapy without progression, who will receive single-agent maintenance therapy.

Patients must fulfill all the inclusion/exclusion criteria to be eligible. Once eligibility is established, patients will be asked to sign the informed consent document at which time they will be considered as enrolled on the trial.

A core needle biopsy will then be obtained (unless it has already been performed for diagnostic purposes, sufficient material is available for analysis, and the patient did not receive chemotherapy after collection of the specimen), and gene expression analysis will be performed. In patients who had a surgical resection for lung cancer and no (neo)adjuvant chemotherapy within 12 months of enrollment on this trial, the surgical resection specimen may be used for gene expression determination. This tumor specimen will be classified as the pre-treatment specimen, and it is required for trial participation. All subsequent biopsies should be performed if feasible; however, inability or failure to obtain subsequent biopsies will not constitute a protocol violation.

Patients will receive single-agent or multi-agent treatment at the discretion of the treating physician at the doses and time intervals appropriate following institutional policies for administration of chemotherapy.

The time elapsed between the last radiographic study used to establish measurable or evaluable disease and first infusion of chemotherapy must be equal to or less than 28 days. If the last study is outside of this time-frame, a repeat study, preferably a CT scan or equivalent, is mandatory prior to treatment initiation.

4.1 Inclusion criteria

4.1.1 Patients with advanced stage NSCLC who are candidates for single or multi-agent first-line therapy.
4.1.2 Second-line or higher therapy for any patients with NSCLC with PS 0-2.
4.1.3 Maintenance therapy for patients after completion of four cycles of dual-agent platinum-based chemotherapy.
4.1.4 Stage IV, histologically or cytologically confirmed NSCLC. Confirmation may be obtained with the first protocol-specified tumor biopsy.
4.1.5 Adequate hematologic parameters: White blood cell count >3000/mm^3; Platelet count >100,000/mm^3; Hemoglobin >9.0 g/dl.
4.1.6 A tumor lesion that can be safely biopsied as judged by the treating oncologist and physician performing the procedure and has not been radiated.

4.1.7 At least one unidimensionally measurable tumor lesion ≥1 cm in longest diameter using spiral CT (≥2 cm in longest diameter by any other technique) that has not been radiated and is not located in a bone.
4.1.8 Age ≥18 years.
4.1.9 Performance status 0-2 by Eastern Cooperative Oncology Group criteria.
4.1.10 Life expectancy of ≥3 months.
4.1.11 Able to understand and sign the Informed Consent Document.

4.2 Exclusion criteria

4.2.1 Therapy that does not include cisplatin, carboplatin, gemcitabine, and/or pemetrexed.
4.2.2 Concomitant medical or psychiatric illness that is likely to interfere with a reasonably safe execution of the treatment plan.
4.2.3 Concomitant malignancy other than NSCLC that requires active therapy. Prior malignancies are allowed as long as the disease is controlled and does not require ongoing therapy of any kind. Prior therapy must have concluded at least 1 year before treatment initiation on this protocol. Exceptions are non-melanoma skin cancer, prostate cancer and PIN treated with local intervention and deemed cured, cervical cancer and CIS treated with local intervention and deemed cured, and laryngeal cancer and CIS treated with local intervention and deemed cured.
4.2.4 Carcinomatous meningitis.
4.2.5 Uncontrolled CNS disease.
4.2.6 The time interval between CNS radiation, whole brain radiation, spinal cord radiation, or radiosurgery, and initiation of protocol specified chemotherapy must be at least 1 week.
4.2.7 Malignant pleural, pericardial, or peritoneal effusion if it is the only site of disease activity; i.e., if no other measurable tumor lesions exist.
4.2.8 Coagulopathy or anticoagulation therapy that cannot be safely corrected or interrupted for tumor biopsy.
4.2.9 Significant hepatic dysfunction, renal dysfunction, or metabolic derangement that precludes full-dose chemotherapy at the specified starting doses.
4.2.10 Concomitant treatment with chemotherapeutic agents for diseases other than malignancy.
4.2.11 Pregnancy or lactation.

4.3 Post-enrollment guidelines

4.3.1 Systemic anticancer agents other than cisplatin, carboplatin, gemcitabine, and pemetrexed are permitted during study treatment as long as they are given in combination with the agents listed.
4.3.2 G-CSF may be used prophylactically for neutropenia in the first or subsequent cycles of treatment at the investigator's discretion.
4.3.3 Palliative radiation is allowed. However, if the radiated area is the only site of evaluable tumor, the patient must be withdrawn from the study.
4.3.4 All supportive measures consistent with optimal patient care will be given throughout the study.
4.3.5 The use of zolendronic acid (Zometa) or similar agents for bony metastases is permitted.

4.3.6 Patients will be treated with the selected agent for as many cycles as deemed appropriate by the treating physician if they have stable disease or responsive disease as defined by RECIST. Patients with progressive disease can remain on study if they are switched to one of the other single-agent regimens at the discretion of the primary care provider. Disease response will be coded as PD for the particular agent, and the patient will be treated similar to a new patient for the second regimen.

4

4.4 Criteria for withdrawal from study

4.4.1 Evidence for disease progression (the patient will be followed off protocol for OS)
4.4.2 Intercurrent illness that would, in the judgment of the investigator, affect assessments of clinical status to a significant degree or require discontinuation of study drugs.
4.4.3 Unacceptable toxicity. Patients will be followed until resolution or stabilization (see section 6.4)
4.4.4 Death
4.4.5 Patient withdraws consent
4.4.6 A treatment delay greater than three weeks for any toxicity
4.4.7 Concomitant treatment with a systemic anticancer drug that is not one of the study drugs (see 4.3.7)
4.4.8 Patients who do not have satisfactory compliance with study procedures
4.4.9 Major protocol violations, including, but not limited to:
failure to meet any of the inclusion/exclusion criteria
failure to complete full staging evaluation as required by protocol.

5.0 Study Plan

5.1. Study period

The total duration of the protocol is 120 months from initiation of treatment of the first patient. A total of 150 patients will be enrolled over 108 months, with an estimated accrual rate of 1-2 patients per month (12-24 per year). Treatment duration is 6-8 weeks and patients will be followed up for 12 months after initiation of therapy.

5.2. Detailed plan

5.2.1. Pretreatment evaluation

The procedures are summarized in the Study Diagram.

1) Signed written informed consent before any study procedure.
2) Complete medical history including dates and description of initial diagnosis of NSCLC and documentation of any cancer therapies.
3) Any on-going adverse events resulting from prior cancer therapies (radiotherapy, surgery, etc.) will be recorded using the NCI-Common Terminology Criteria for Adverse Events (CTCAE) v4.0.
4) Any tumor-related signs and symptoms (within 14 days prior to starting treatment) will be recorded using CTCAE v4.0.
5) Complete physical examination including, but not limited to, vital signs, height, weight, and ECOG performance status using the ECOG scale (within 14 days prior to starting treatment).
6) Clinical laboratory testing: CBC with differential and platelet count, total serum bilirubin, alkaline phosphatase, AST (SGOT), ALT (SGPT), serum creatinine, serum electrolytes, serum calcium and serum albumin, and a biopsy for molecular assessment. Creatinine clearance will be calculated with the Cockroft-Gault formula or directly measured if clinically indicated (within 14 days prior to starting treatment).
7) Pregnancy test for female patients of childbearing potential (within 7 days prior to starting treatment).
8) Tumor evaluation: The appropriate clinical testing will be used to evaluate all malignant lesions. CT of the chest and upper abdomen including the complete liver and adrenal glands is mandatory. If clinically indicated, CT or MRI of the brain; ultrasound; or radionuclide scans of the bones; and/or other radiographic studies should be performed within 28 days prior to starting treatment. To ensure comparability, the baseline and subsequent radiographic studies used to assess disease response must be performed using identical techniques.

5.3 Collection, processing, and analysis of tumor and peripheral blood specimens

5.3.1 Tumor biopsy procedure: Tumor specimens will be collected prior to first chemotherapy, and after chemotherapy cycle #2, for a total of two serial specimens. Only the first specimen is mandatory for study participation. If technically feasible, the primary lung malignancy will be biopsied. After informed consent is obtained, the patient will have baseline laboratory tests to assess for bleeding risk. Specifically, PT and APTT will be checked. If they are normal and there is no previous history of bleeding, the patient will be taken to the CT suite (or other appropriate location) and cleaned and draped in a supine, prone, or lateral (or other best suited) position. A mild sedative in the form of midazolam (Versed) 5 to 10 mg in combination with meperidine (Demerol) 50 to 100mg will be administered as pre-medication. Under CT (or other appropriate) guidance a 19 gauge (0.93 mm diameter) guiding needle will be inserted to the target lesion and core biopsies (20 gauge, 0.81 mm diameter) will be performed through this needle. Multiple core biopsies will be obtained, which poses minimal additional risk over obtaining a single biopsy and substantially increases the chance of obtaining sufficient material for diagnosis and molecular analysis. The core biopsy will be handed to the P.I. or designee for appropriate processing. The guiding needle will then be withdrawn and hemostasis obtained by pressure to the local area. The patients will be monitored for three hours with serial vital sign measurements every 15-30 minutes. The patients will be discharged after a chest X-ray (or other appropriate study) shows no pneumothorax (or other appropriate procedure-related complication). The risk of a pneumothorax is between 15% and 35% depending on the location of the target lesion and the degree of underlying lung disease. The risk of a clinically significant pneumothorax, for which a chest tube insertion and hospitalization is required, is between 2% and 5%. A published review of 5,444 trans-thoracic biopsies performed in the United Kingdom reports a 20.5% pneumothorax rate, 3.1% rate of pneumothorax requiring chest tube placement, 5.3% rate of bleeding, and 0.15% rate of death.[67] In this report, the authors found no difference in the rate of pneumothorax as a result of fine-needle aspiration versus core needle biopsy. These risks are higher in patients with obstructive lung disease and lower in those with normal airway physiology.

Alternatively, if tumor is easily accessible from a distant site, it will be biopsied instead of the primary lung lesion. Common peripheral sites for NSCLC metastases are the subcutaneous tissue, lymph nodes from the supraclavicular or other superficial areas, brain metastases for surgical resection, bone, adrenal gland, and liver.

In our previous phase II study, 75 patients underwent the required biopsy. Of these, 48 had CT-guided lung biopsies, 7 had bronchoscopy-guided lung biopsies, and 20 had biopsies from a variety of other organs. A complication was noted in one instance of a CT-guided lung biopsy; it resulted in a small pneumothorax that spontaneously resolved.[66]

5.3.2 Peripheral venous blood specimen collection: Peripheral venous blood will be collected prior to first chemotherapy, and prior to chemotherapy cycles #3 and #5, for a total of three serial blood specimens. Each collection will occur during routine phlebotomy procedures. Up to 30 ml of blood will be collected in three separate and sequential 10 ml tubes (the tubes may be Cell Preparation Tubes; Becton-Dickinson, or similar tubes suitable for the proposed studies). These tubes will be kept refrigerated until processing. Processing will occur within 2 hours of collection. They are centrifuged at 1,600 to 1,800 g for 20 to 25 minutes. Plasma is separated, aliquoted into 500 ul portions, and stored frozen at -80°C. Peripheral blood

mononuclear cells (PBMCs) are collected from the plasma/ficol interface, washed, and stored as viable cells in 10% DMSO/FBS in liquid nitrogen in 250 ul aliquots. Other procedures for blood processing may be used as required to optimize the analytical procedures.

5.3.3 Tumor specimen processing, shipping, and gene expression analysis: The obtained core needle biopsies will be immediately fixed in neutral phosphate-buffered formalin and one will be embedded in paraffin wax (for pathological confirmation of NSCLC and specimen quality assessment). All further processing will be done in the reference laboratory (Dr. Bepler's laboratory).

Target gene determination at the protein level by quantitative mass spectrometry:
MS allows for the identification and quantitation of proteins without the use of antibodies, and it can accurately quantitate low abundance proteins through the use of stable, isotope-labeled peptides in fresh and, under controlled conditions, in formalin-fixed samples. Sample preparation can be achieved without the need for electrophoretic separation by in-solution digestion, which is comprised of breaking formalin-inducted, covalent disulfide bonds through reduction and alkylation followed by protein digestion into peptide fragments. The sample complexity can be reduced using HPLC prior to MS is required. During MS analysis, peptides are ionized and their mass-to-charge ration (m/z) is measured to yield a precursor spectrum. Selected ions are then fragmented by collision-induced dissociation, and the individual fragment spectra are assigned peptide sequences based on database comparisons. In principle, the quantitative assay development requires the selection of a suitable tryptic RRM1-specific fragment, synthesis of the peptide containing a stable isotope-labeled residue (for instance a carbon-13 labeled lysine), and evaluation of the peptide by MS/MS to select product ions of good intensity that allow unambiguous peptide identification. The peptide concentration, as determined by extracted ion chromatography, is then correlated to the intensity of its peak. To determine the RRM1 level in a formalin-fixed tumor specimen, a known amount of the isotope-labeled peptide is added to the tumor specimen solution, the entire sample is digested, and analyzed by MS/MS. A comparison between the peak areas of the endogenous and labeled peptides then allows for calculation of the absolute RRM1 protein amount in a given tumor specimen.

We have generated full-length RRM1 protein with and without a His-tag and obtained purities in access of 95%. We have selected 3 optimal tryptic peptides corresponding to RRM1, and we are in the process of optimizing the LC-MS/MS protocol to resolve and monitor the 3 selected peptides. This optimization process includes the parameters retention time, transition, and collision energy for synthetic RRM1, RRM1 in cell line lysates, and RRM1 in tissue samples (we are using human NSCLC SCID mouse xenografts). In parallel, we are evaluating and optimizing methods for extraction of protein from formalin-fixed samples. After completion of these tasks, we will synthesize 3 heavy isotope-labeled peptides to be used as internal standards for absolute RRM1 quantification.

Starting with formalin-fixation at concentrations up to 10% of synthetic, full-length RRM1, we were able to identified 3 optimal peptides with a best peptide identification probability of at least 95% and total spectral counts ranging from 1-14. Analysis of genetically modified cell lines with differential levels of RRM1 protein revealed similar relative quantities of RRM1 when determined by MS (Figure 4).

Multiple methods for recovery of proteins from 10% formalin-fixed specimens have been described in the literature. We have evaluated multiple methods, including commercially

available kits, and our present data suggest that a modified RIPA buffer containing 2% SDS and 200 mM DTT provides acceptable results.

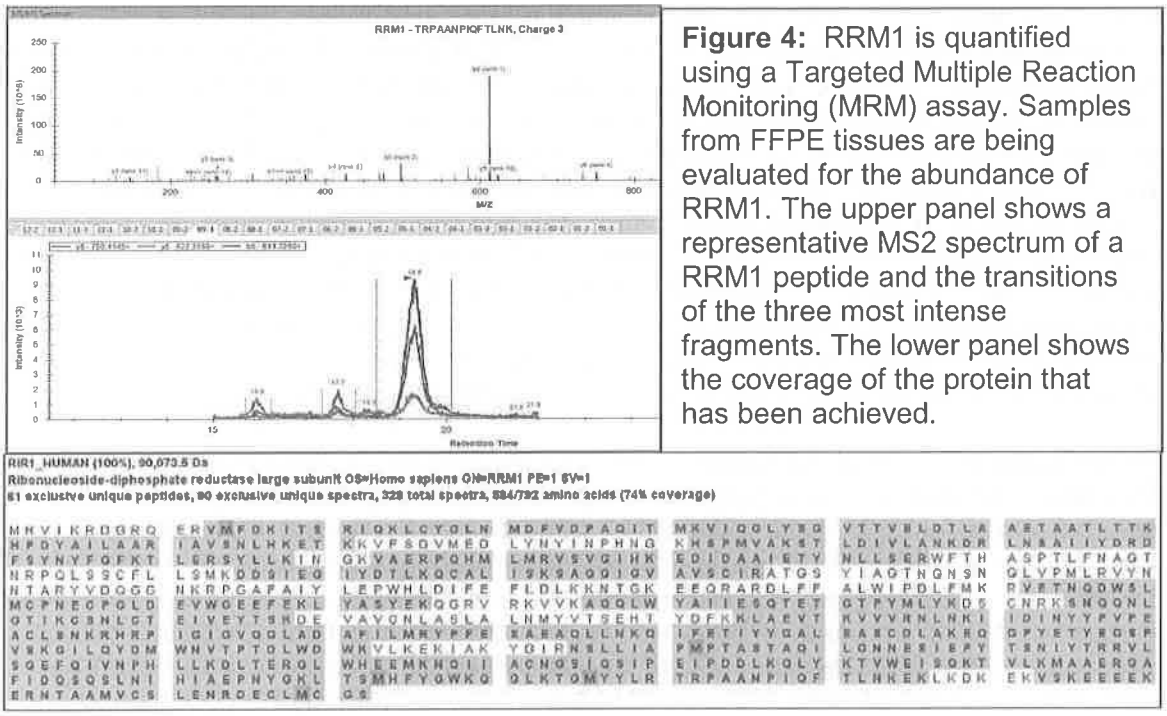

Figure 4: RRM1 is quantified using a Targeted Multiple Reaction Monitoring (MRM) assay. Samples from FFPE tissues are being evaluated for the abundance of RRM1. The upper panel shows a representative MS2 spectrum of a RRM1 peptide and the transitions of the three most intense fragments. The lower panel shows the coverage of the protein that has been achieved.

Evaluation of the impact of specimen fixation time and subsequent processing is crucial for the success of this aim since pathology laboratories use standardized but not necessarily identical procedures across the country. To develop an accurate and reproducible quantification method for tumoral RRM1 protein levels, it is important to understand to putative impact of fixation times as well as storage and processing conditions on quantitative MS-based RRM1 determination. To this end, we have initiated and are planning to continue experiments that utilize human NSCLC SCID mouse xenograft with the goal to mimic the actual clinical environment. Xenografts are biopsied once they reach a diameter of 1-2 cm, biopsy specimens are placed in 10% buffered formalin, and they are fixed for periods ranging from 4 – 72 hours. In addition, parallel biopsy samples are washed after fixation in PBS or ethanol. An additional parameter is temperature, which we are addressing by using storage at RT versus 4°C. Specimens are then shipped via courier to our laboratory and processed for MS analysis. This approach does not require that specimens be paraffin-embedded, sectioned, and mounted on glass slides. It is our opinion, that a practical clinical assay for accurate quantification of RRM1 in a tumor specimen is more practicable if it directly starts from the fixed biopsy specimen rather than from specimens mounted on slides. However, the methodology being developed can easily be adapted to specimens mounted on slides as long as the method of fixation (10% buffered formalin and time in formalin) are within parameters established.

Target gene determination at the mRNA level by real-time RTPCR:

Formalin-fixed tissue samples, embedded in paraffin wax, will be cut in 5 to 7 µm sections and placed on uncharged glass slides. Tumor cells will be collected by LCM using the Arcturus system (60 mW, 1.5 milliseconds, intensity 100, spot size ~20 µm), and total RNA will be extracted using a commercially available method. Complementary DNA will be generated with Superscript II and a mixture of oligo-dT and random primers. Real-time quantitative RTPCR gene analysis will be performed in triplicate per sample and gene in 96-well plates. Each plate

will contain a serial dilution of reference cDNA for standard curve determination and negative controls without template. We have designed and validated the primers and probes as previously described.[56,68,69] Commercially available primers and probes will be used for expression analysis of other genes and of the housekeeping gene 18S*rRNA*, which will be used as internal reference standard. The relative amount of target mRNA in a sample will be determined by comparing the threshold cycle with the standard curve, and the standardized amount will then be determined by dividing the target amount by the 18S*rRNA* amount. The mean of triplicate values will be calculated and entered into a study-specific database. All gene expression analyses for the target genes RRM1, TS, and ERCC1 will be done as soon as specimens are received in the laboratory, even though these results are not required for therapeutic decision making. The rationale for this approach over batch analysis of samples is that future real-time gene expression-based therapeutic decision making will require short turn-around time, which does not allow for sample batching This approach will thus produce data that resemble a care environment applicable to the majority of patients.

Target gene determination at the protein level by *in situ* accurate quantitative analysis (AQUA): AQUA is an immunofluorescence-based technique combined with automated quantitative analysis that allows for rapid, automated, and quantitative analysis of *in situ* proteins,[54] thus reducing the human variability occurring with IHC scoring. AQUA automatically measures protein expression in subcellular compartments (i.e., nuclear versus cytoplasmic), providing a continuous score in an accurate, reliable, and reproducible way. Tumor specimens blocks prepared in the tissue procurement core will be transferred to our laboratory for biomarker analysis. Full specimen sections of 4 µm thickness will be cut from paraffin blocks and mounted on adhesive coated glass slides. The microtome sections will be microwaved in 10 mM Tris-EDTA, pH 9.0 or 10 mM Tris-HCl, pH 12.0 for 10 min, cooled to room temperature, and rinsed with PBS. Endogenous peroxidase will be inactivated with 3% hydrogen peroxide for 20 min. Slides are then incubated overnight at 4°C in appropriately diluted primary antiserum or antibody. Cells of epithelial origin, consisting mostly of malignant cells, will be identified by cytokeratin staining. The primary antibody is then visualized with different fluorochrome-labeled secondary antibodies (Envision® labeled polymer-HRP anti-rabbit or anti-mouse for primary antibody detection; Alexa 555 goat anti-mouse or goat anti-rabbit for cytokeratin detection). Fluorescence of the target signals is amplified with Cy5-tyramide. DAPI (4',6-diamidine-2'-phenylindole, strongly binds to DNA minor groove) added to the cover slip mounting solution, is used for identification of nuclei. Since, one full specimen section will be analyzed for each target protein, random spots (spot diameter 0.6 mm) ranging in number from no less than 5 to no more than 50 will be scanned with SpotGrabber, and image data will be analyzed (PM-2000, HistoRx, New Haven, Connecticut). Software version 1.6 or higher will be used with an optimal exposure time. The maximal range of the AQUA scores with the latest software versions is 0 to 33,333, and they are automatically corrected for exposure times. The specific parameters to be used for each target protein are summarized in table 5.3.3. Optimal conditions for targets not listed will be established using a customized tissue microarray that is comprised of a variety of normal human tissues, lung cancer cell lines processed as cell pellets, and a representative number of lung cancers. Antibodies will be validated by Western blotting and confocal microscopy using appropriate cell lines with and without target knock-down by RNA interference.

Table 5.3.3: Reagents and conditions for target protein analysis by IHC or AQUA

Antibodies (source)	Company	Antigen retrieval	Washing/dilution buffer	Dilution	Incubation
RRM1-6b (rabbit polyclonal)	Produced by our laboratory	10mM Tris-EDTA pH 9.0	1x PBS	1:150	O/N @ 4^{0}C
R1-E4138-C42 (rabbit moAb)	Produced by our laboratory	10mM Tris-EDTA pH 9.0	1x PBS	Batch dependent	O/N @ 4^{0}C
ERCC1 (mouse moAb clone 8F1, also detects CCTα)	Novus Cat# NB500-704 Lot# G412	10mM Tris-EDTA pH 9.0	1 x PBS	1:20	O/N @ 4^{0}C
ERCC1 (rabbit polyclonal FL297)	Santa Cruz Cat sc-10758 Lot# various	10mM Sodium Citrate pH 6.0 Vector	1 x PBS	1:200	O/N @ 4^{0}C
TS (mouse moAb clone TS 106)	abcam Cat# ab3145 Lot# 353377	10mM Sodium Citrate pH 6.0	1x PBS	1:200	O/N @ RT
Ki-67 (rabbit polyclonal)	Abcam Cat# ab15467 Lot# 688239	10mM Tris-EDTA pH 9.0, High Pressure Cooker 15 min	1x PBS	1:5	O/N @ 4C
MCM2 (mouse moAb clone N-19)	Santa Cruz Cat# sc-9839 Lot# I1907)	Pending	pending	Pending	pending
TTF1 (mouse moAb clone 8G7G3/1)	Dako Cat# M3575 Lot# 10021405	10mM Tris-EDTA pH 9.0, High Pressure Cooker 15mins	1 xPBS	1:250	O/N @ 4C

5.4 Treatment assignment

Treatment will be selected at the discretion of the primary oncologist. Treatment will continue indefinitely if tolerated or until disease progression. Treatment upon progression is at the discretion of the care provider.

5.5 The chemotherapy regimens

Any single or multi-agent regimen containing the drugs cisplatin, carboplatin, gemcitabine, and/or pemetrexed is acceptable. The dosing and administration of these agents either

alone or in combination is at the discretion of the treating physician following institutional guidelines.

Antiemetics and appropriate steroid premedication will be given according to best practice criteria.

5.6 Evaluations and procedures during study

5.6.1 Before each cycle

Laboratory assessment
Hematology: CBC with differential and platelet count
Serum chemistries: electrolytes, BUN, creatinine (creatinine clearance will be calculated for each treatment course containing carboplatin), glucose, alkaline phosphatase, total serum bilirubin, SGOT, SGPT. Serum albumin and calcium if clinically indicated.
Vital signs and weight
Interim medical history (including documentation of concomitant medications, and solicitation of adverse event (AE) information if not volunteered by the patient, specifically inquiring as to the known AEs attributed in the past to any of the study drugs).
Physical examination (including: estimation of ECOG performance status score by the treating physician and recording of measurements of clinically evident malignant lesions).
Any other clinically indicated procedure(s).

During any study drug infusion, patients must be carefully checked so that immediate intervention can be initiated should an adverse event (i.e. hypersensitivity) occur.

5.6.2 Procedures during each cycle

CBC with differential and platelet count (before each infusion)
Serum chemistries including creatinine and electrolytes

5.6.3 Evaluations after cycle #2 (at the end of this pilot trial)

Tumor assessments: appropriate imaging procedures to assess the tumor response will be done after the second cycle of treatment (approximately 6-8 weeks after treatment initiation). The investigator, subinvestigator, treating physician, or experienced radiologist will review the scans to assess tumor response. The patient will continue on treatment as long as the disease is at least stable or in remission according to RECIST at the discretion of the treating physician.

5.7 Provisions for dose reductions

Dose reductions or delays are at the discretion of the treating physician following institutional guidelines.

5.8 Concomitant therapy

The use of concomitant medications is at the discretion of the treating physician and may include (but not limited to) drugs necessary for pain control, patient comfort, diarrhea, emesis, or life-threatening events.

5.9 Follow-up requirements after 2 cycles of treatment

All patients will be followed up at intervals at the discretion of the treating physicians for 12 months after treatment initiation.

Adverse event information related to the treatment administered will not be collected. Only adverse events found to be related, including possibly or probably related, to the performance of tumor biopsies required by this protocol treatment will be followed until resolution.

The above information may be collected through telephone contact with the patient and by obtaining medical records.

6.0 Table of Studies

	Pre-Study	Prior to cycle #2 (≤7 days or day of)		After cycle #2 (and before cycle 3 is given)	Long Term Follow-up
History	X[5]	X		X	
Physical Examination[1]	X[5]	X		X	
Weight, Performance Status	X[5]	X		X	
CBC/Differential	X[5]	X		X	
Platelet Count	X[5]	X		X	
CMP[2], Calculated Creatinine Clearance[3]	X[5]	X		X	
Tumor Measurements	X[6]			X[8]	
Pregnancy Test	X[7]				
Disease Status & Survival[4]					for 1 year[10]
Concomitant Medication	X	X		X	
Adverse Events		X		X[9]	
Collection of histological tumor specimen	X			X[11]	
Collection of research blood	X			X[8]	

1) Including the vital signs blood pressure, heart rate, respiration rate, temperature
2) CMP, which includes SGOT, SGPT, alkaline phosphatase, bilirubin, albumin, total protein, creatinine, electrolytes
3) Creatinine clearance will be calculated for each treatment course containing carboplatin
4) To occur at approximately 6-8-week intervals for 12 months following study treatment initiation.
5) To be obtained within 14 days prior to treatment
6) To be obtained within 28 days prior to treatment
7) Pregnancy test to be obtained within 7 days prior to treatment
8) To be obtained prior to Cycle #3 of treatment
9) Adverse event information related to the study-specified tumor biopsies will be collected. Adverse event information related to single or multi-agent chemotherapy will not be collected.
10) Patient follow-up is 12 months from treatment initiation.
11) Second biopsy should be obtained as close as possible to disease assessment but prior to Cycle #3

7.0 Assessment of Efficacy

7.1.1 Response assessment

Best treatment response will be determined according to RECIST. The anticipated RR (number of patients with a partial and complete remission divided by the number of patients that started therapy) is approximately 10%. Best treatment response will be determined by CT scan or other methods as deemed appropriate by the treating physician and recorded as a continuous variable. Studies will be performed prior to treatment initiation and after cycle #2 (no earlier than 14 days after day 1 of cycle #2). For most patients, images will be obtained with intravenous contrast on a multichannel helical CT scanner at 3 mm intervals. Measurements will be performed on a picture-archive communication system workstation (Siemens, Munich, Germany). Up to 10 separate tumor lesions will be followed. The greatest diameter of each lesion will be recorded (using one decimal point). The percentage of change of the sum of tumor diameters comparing the post-treatment with the pre-treatment measurements will be calculated using the formula 1-(SumCTpost/SumCTpre). The percentage of change will have a positive value if the tumor shrinks and a negative value if the tumor increases in size. For new lesions, a prior diameter value of zero will be assumed. Non-measurable lesion will not be included in the continuous disease response parameter; however, they will be included in the RECIST defined response categories.

7.1.2 A **secondary endpoint** is PFS (determined from the date of protocol-specified treatment initiation). Patients will be followed by history and physical examination at least every month (+/- 7 days) from the day of treatment initiation. Radiographic studies in form of a CT scan, MRI scan, or other method as deemed appropriate by the treating physician will be used at a frequency of at least every 2 months (+/- 7 days) for patients not receiving chemotherapy or at the latest on the day of every uneven number of chemotherapy cycle following cycle #2 (3^{rd}, 5^{th}). The data of first documented disease progression or death, as defined by RECIST, will be recorded, and the time interval from treatment initiation to that date will be calculated for each patient and used to generate Kaplan-Meier survival estimates and to calculate PFS. Patients without an event (progression or death) will be censored as of the date of last documented encounter.

7.1.3 Another **secondary endpoint** is OS (determined from the date of protocol-specified treatment initiation). OS will be determined as described under 7.1.2 using death as the only event variable. The date of death will be verified as entered into a patient's chart, by telephone verification of the date with the treating physician and/or family member, and/or by query of vital statistics records such as obituaries and the social security death index.

7.2 Definition of measurable and non-measurable lesions according to RECIST

At baseline, tumor lesions will be categorized as:

measurable: lesions that can be accurately measured in at least one dimension (longest diameter to be recorded except lymph nodes) as ≥ 20 mm with conventional techniques or as ≥ 10 mm with spiral CT scan. For lymph nodes, the shortest diameter will be reported if the diameter is >15 mm.

non-measurable: all other lesions, including small lesions (longest diameter < 20 mm with conventional techniques or < 10 mm with spiral CT scan) and truly non-measurable lesions.

The term "evaluable" in reference to measurability is not recommended and will not be used because it does not provide additional meaning or accuracy.
All measurements should be recorded in metric notation, using a ruler or calipers. All baseline evaluations should be performed as close as possible to the treatment start and never more than 4 weeks before the beginning of the treatment.
Lesions that are considered as truly non-measurable include the following:
bone lesions
leptomeningeal disease
ascites
pleural / pericardial effusion
inflammatory breast disease
lymphangitis cutis / pulmonis
abdominal masses that are not confirmed and followed by imaging techniques
cystic lesions

7.3 Tumor response evaluation according to RECIST

7.3.1 Assessment of overall burden and measurable disease

To assess objective response, it is necessary to estimate the overall tumor burden at baseline and use this as a comparator for subsequent measurements. Only patients with measurable disease at baseline should be included in this protocol. Measurable disease is defined by the presence of at least one measurable lesion. If the measurable disease is restricted to a solitary lesion, its neoplastic nature could be confirmed by cytology/histology at the discretion of the investigator, subinvestigator, or treating physician.

7.3.2 Baseline documentation of "Target" and "Non-Target" lesions

All measurable lesions up to a maximum of 10 lesions representative of all involved organs should be identified as target lesions and will be recorded and measured at baseline. Target lesions should be selected on the basis of their size (lesions with the longest diameter) and their suitability for accurate repetitive measurements (either by imaging techniques or clinically). A sum of the longest diameter (LD) for all target lesions will be calculated and reported as the baseline sum LD. The baseline sum LD will be used as reference to further characterize the objective tumor response of the measurable dimension of the disease.

All other lesions (or sites of disease) should be identified as non-target lesions and should also be recorded at baseline. Measurements are not required and these lesions should be followed as "present" or "absent".

7.3.3 Evaluation of target lesions

Complete Response (CR): disappearance of all target lesions.

Partial Response (PR): at least a 30% decrease in the sum of LD of target lesions taking as reference the baseline sum LD.

Progression (PD): at least a 20% increase in the sum of LD of target lesions taking as references the smallest sum LD recorded since the treatment started or the appearance of one or more new lesions.

Stable Disease (SD): neither sufficient shrinkage to qualify for PR nor sufficient increase to qualify for PD taking as references the smallest sum LD since the treatment started.

7.3.4 Evaluation of non-target lesions

Complete Response (CR): disappearance of all non-target lesions.

Non-Complete Response (non-CR) / Non-Progression (non-PD): persistence of one or more non-target lesion or/and maintenance of tumor marker level above the normal limits.

Progression (PD): appearance of one or more new lesions. Unequivocal progression of existing non-target lesions.

7.3.5 Evaluation of best overall response

The best overall response is the best response recorded from the start of the treatment until disease progression/recurrence (taking as reference for progressive disease the smallest measurements recorded since the treatment started). In general the patient's best response assignment will depend on the achievement of both measurement and confirmation criteria.

Target Lesions	Non-Target Lesions	New Lesions	Overall Response
CR[1]	CR	No	CR
CR	Non-CR/Non-PD	No	PR
PR[2]	Non-PD	No	PR
SD[3]	Non-PD	No	SD
PD[4]	Any	Yes or No	PD
Any	PD	Yes or No	PD
Any	Any	Yes	PD

[1] Complete Response, [2] Partial Response, [3] Stable Disease, [4] Progressive Disease

Note: Patients with a global deterioration of health status requiring discontinuation of treatment without objective evidence of disease progression at that time should be reported as "symptomatic deterioration". Every effort should be made to document the objective progression even after discontinuation of treatment.
In some circumstances it may be difficult to distinguish residual disease from normal tissue. When the evaluation of complete response depends upon this determination, it is recommended that the residual lesion be investigated (fine needle aspirate/biopsy) before confirming the complete response status.

7.4 Confirmatory measurement and duration of response

7.4.1 Confirmation

A confirmation of the achieved best response by a second scan is not required.

7.4.2 Duration of overall response

The duration of overall response is measured from the time that measurement criteria are met for complete response or partial response (whichever status is recorded first) until the first date that recurrent or progressive disease is objectively documented (taking as reference for progressive disease the smallest measurements recorded since the treatment started). The duration of overall complete response is measured from the time measurement criteria are first met for complete response until the first date that recurrent disease is objectively documented.

7.4.3 Duration of stable disease

Stable disease is measured from the start of the treatment until the criteria for disease progression is met (taking as reference the smallest measurements recorded since the treatment started).

8.0 Toxicity and Adverse Event Assessment

8.1 Safety and toxicity analysis

Safety and toxicity are well described for the single and multi-agent regimens.

Safety and toxicity data for the treatments given will not be tabulated for the purpose this study.

Only safety and toxicity data related to the protocol-required tumor biopsies will be recorded and tabulated. CTCAE version 4.0 (http://ctep.cancer.gov) will be used.

8.2 Adverse events

An adverse event is any unintended or undesirable experience as a result of the study-specified tumor biopsies. At each evaluation patients should be interviewed in a non-directed manner to elicit potential adverse reactions from the patient. The occurrence of an adverse event will be based on changes in the patient's physical examination, laboratory results, and/or signs and symptoms.

All adverse events (except grade 1 and 2 laboratory abnormalities that do not require an intervention), regardless of causal relationship, will be recorded in the case report form and source documentation. The investigator, subinvestigator, or treating physician must determine the intensity of any adverse events according to the NCI Common Terminology Criteria for Adverse Events v4.0 and their causal relationship using the guidelines in Appendix III.

Adverse events will be followed until resolution while the patient remains on-study. Once the patients is removed from study, events thought to be related to the study medication will be followed until resolution or until the patient starts a new treatment regimen.

8.3 Serious adverse events (SAE)

An adverse event occurring while on study, within 30 days of the intervention:

1) Death.
2) A life-threatening adverse drug experience.
3) A persistent or significant disability/incapacity.
4) Inpatient hospitalization or prolongation of existing hospitalization. For the purposes of this study inpatient hospitalization or prolongation of existing hospitalization for the following reasons are not considered to be events for reporting: study drug administration, transfusion support, disease staging/re-staging procedures, concomitant radiotherapy, placement of indwelling catheter, or other elective procedures associated with conventional medical practice, unless associated with other serious adverse events.
5) A congenital anomaly/birth defect.
6) Important medical events that may not result in death, are life-threatening, or require hospitalization may be considered a serious adverse drug experience when, based upon appropriate medical judgment, they may jeopardize the patient or subject and may require medical or surgical intervention to prevent one of the outcomes listed above. Examples of such medical events include allergic bronchospasm requiring intensive treatment in an emergency room or at home, blood dyscrasias or convulsions that do not result in inpatient hospitalization, or the development of drug dependency or drug abuse.

8.3.1 Reporting of SAEs

All serious, related adverse events (and unanticipated) will be reported and documented on forms as required by institutional guidelines and forwarded directly to the IRB in electronic version within 2-5 business days of becoming aware of the event (https://irb.research.usf.edu/ae/login.asp).

9.0 Statistics

9.1 Data collection, conventions, early stopping rule, and statistical analysis

The enrollment goal is 150 patients that initiate therapy on one of the three single-agent regimens. We anticipate a near equal distribution of patients to these regimens. The total duration of the protocol is 120 months from initiation of treatment of the first patient. A total of 150 patients will be enrolled over 108 months, with an estimated accrual rate of 1-2 patients per month (12-24 per year). Treatment duration is 6-8 weeks and patients will be followed up for 12 months after initiation of therapy. We anticipate an approximately equal selection of patients to three specified therapeutic regimens. The thoracic MDT sees 590 new lung cancer patients per year, and it has 5,480 total patient visits per year. The majority of patients seen (approx. 80%; i.e., 472), have advanced stage disease, and essentially all of these patients will eventually be eligible for a single or multi-agent chemotherapy regimen. In the past, the thoracic MDT has enrolled approximately 55 patients on therapeutic trials annually. The current protocol has few exclusions for patient enrollment since it seeks to identify molecular parameters predictive of therapeutic benefit from the most commonly used agents.

The clinical data will be collected by a dedicated and experienced coordinator. They will be entered into Oncore™ a clinical studies management system adopted Cancer Center wide. This clinical studies management system is a comprehensive solution for managing all aspects of clinical research, including study setup and activation, scientific reviews, subject registration, compliance tracking, visit tracking, data collection, data and safety monitoring, financial management, data extraction, regulatory reporting, and outreach. Oncore™ enables the staff to track all protocols from initial scientific review through IRB approval and activation, and provides data for ongoing monitoring until final closure. The database also provides a mechanism for electronic data capture and reporting. Serial measurements of tumor lesions will be performed by dedicated thoracic radiologists, recorded in the official patients' records, and they will serve as the source documents. A spreadsheet describing the number and location of all target lesions will be maintained for each patient, and the interpreting radiologist will be alerted to specifically address each lesion on subsequent scans.

Computing:
The current version of SAS® available at the time of data analysis will be used to perform all analyses. All data, including tumor response, OS, PFS, safety/toxicity, histopathology, demographics, and molecular parameters will be summarized in tables.

Analysis Population:
All patients who receive at least one dose of therapy will be considered evaluable for analysis.

Baseline:
Baseline is defined as the last sampling time prior to treatment administration. All within-group analyses will compare subsequent data to baseline data.

P-values:
P-values and confidence intervals for the primary parameters of gene expression levels and disease response (both continuous variables) will be two-sided, and p-values ≤ 0.05 will be considered statistically significant. The primary research questions are: Is there a correlation between RRM1, TS, and ERCC1 levels with the change in the sum of tumor diameters comparing the sum on the CT scan following cycle #2 to the baseline sum in patients treated with gemcitabine, pemetrexed, and docetaxel, respectively.

Rounding Conventions:
Totals and ranges will carry the same number of significant digits as the data on which they are based (one digit for gene expression levels and no digits for tumor size changes). Means, medians, standard deviations, variances and standard errors will carry one additional digit. All p-values will carry three digits. If the last digit in the decimal to be rounded is <5 the number will be rounded down. If the last digit in the decimal is ≥5 the number will be rounded up.

Early Stopping Rule:
We will monitor the number of clinically significant pneumothoraces for the initial trans-thoracic core biopsies. If this number reaches four during the enrollment of the first 25 patients, we will terminate the trial for reasons of patient safety. This will happen with a probability of 3% if the true rate of pneumothoraces is 5% and with a probability of 90% if that rate is 25%.

Primary Objective:
The primary analysis is to evaluate the role of baseline marker measurement in predicting the treatment outcome. A univariable regression of continuous disease response on baseline marker value will be performed. A multivariable regression model adjusted for clinical covariates will be evaluated as well. Expression levels of RRM1, TS, ERCC1, and other molecules and disease response after cycle #2 will be log-transformed. Log transformation of zero is undefined, because, in our biomarker analyses, zero values are not encountered. All our analyses detected at least an infinitely small amount. Zero values in our biomarker analyses denote a failed quality check.

Power calculation
Assuming that 50 patients will receive one of the specified agents, with a 20% loss of sample size due to attrition or non-evaluable cases, a sample size of 40 achieves 80% power to detect a change in the slope from 0 under the null hypothesis to 0.21 under the alternative hypothesis with a standard deviation of the marker of 1, treatment outcome of 0.5, and a two-sided significance level of 0.05.

Secondary objectives
The secondary analysis is to evaluate the difference (before and after treatment) of each biomarker's expression levels associated with drug response using Pearson correlation. If data are not normally distributed after log transformation, a non-parametric method (e.g., Spearman correlation) will be used.
Tertiary objectives
Descriptive analyses for the tertiary objectives will be performed for patients who received at least one dose of therapy.

Other Analyses:
In order to assess the relationship between expression levels of RRM1, TS, ERCC1, and other molecules and demographic and disease variables, we will use the Wilcoxon rank sum test or Kruskal-Wallis test for dichotomous or polychotomous categorical variables (such as sex, and histology) and the Spearman correlation coefficient for continuous ordinal variables (such as age or stage). We prefer these non-parametric methods over their parametric analogues (t test, one way ANOVA, and Pearson correlation coefficient) because our experience with these data indicates that they are not normally distributed. Detailed examples for our previous biomarker analyses in a cohort of similar patients have been published.[33,65,70-72]

10.0 Quality Control and Quality Assurance Procedures

10.1 Monitoring of the study and regulatory compliance

The Principal Investigator and the Clinical Research Coordinator assigned to the trial will be primarily responsible for maintaining all study related documents including the clinical research forms. All electronic CRF entries will be verified with source documentation. The review of medical records will be done in a manner to assure that patient confidentiality is maintained.

This study will be monitored according to the Karmanos Cancer Institute's Data Safety Monitoring Plan which is available at:
https://research1.karmanos.org/Research/Home2/ClinicalTrials2/DataSafetyMonitoringPlan.aspx

In brief, scheduled meetings will occur monthly or more frequently depending on the activity of the protocol. These meetings will include the Principal Investigator (PI) and research staff involved with the conduct of the protocol. In addition the PI will meet with the co-investigators on a regular basis (frequency to be based on the rate of enrollment) and review the clinical outcomes of the patients enrolled on the study.

During these meetings the following items will be reviewed and discussed:

1. Safety of protocol participants (AE reporting).
2. Validity and integrity of the data (data completeness on case report forms and complete source documentation).
3. Enrollment rate relative to expectations and the characteristics of participants; (Eligible and Ineligible patients).
4. Retention of participants and adherence to the protocol (potential or real protocol violations).
5. Completeness of collected data.
6. Protocol amendments.

Data and Safety Monitoring Reports (DSMR) (appendix X) of investigator meetings will be completed by the Study Coordinator and submitted to the Data and Safety Monitoring Committee monthly for quarterly review.

The Barbara Ann Karmanos Cancer Institute's Data and Safety Monitoring Committee (DSMC) provides the primary oversight of data and safety monitoring for KCI Investigator-initiated trials.

10.2 Radiology review

A central radiology review for verification of response will not be performed.

10.3 Protocol modifications

No modifications will be made to the protocol without the agreement of the investigators. Changes that significantly affect the safety of the patients, the scope of the investigation, or the scientific quality of the study will require Institutional Review Board approval prior to implementation, except where the modification is necessary to eliminate apparent immediate hazard to human subjects. Any departures from the protocol must be fully documented in the case report form and the source documentation.

10.4 Patient privacy

In order to maintain patient confidentiality, all case report forms, study reports and communications relating to the study will identify patients by initials and assigned patient numbers. The US Food and Drug Administration (FDA) may also request access to all study records, including source documentation for inspection.

10.5 Publication policy

The investigators plan to publish and present the information obtained from the study.

11.0 Ethical Considerations

11.1 Informed Consent

The investigator will obtain written informed consent from all participating patients or their authorized representatives. The form must be signed, witnessed, and dated. The consent will contain all the Essential Elements of Informed Consent set forth in Title 21, Code of Federal Regulations, Part 50. Copies of the signed document will be given to the patient and filed in the Investigator's study file, as well as the patient's medical record if in conformance with the institution's Standard Operating Procedures.

11.2 Institutional Review Board

The trial will not be initiated without approval of the appropriate Institutional Review Board (IRB). All administrative requirements of the governing body of the institution will be fully complied with. This protocol, consent procedures, and any amendments must be approved by the IRB in compliance with current regulations of the Food and Drug Administration. A letter of approval will be sent to the institution(s) funding the study prior to initiation of the study and when any subsequent modifications are made. The IRB will be kept informed by the investigator as to the progress of the study as well as to any serious or unusual adverse events.

12.0 Data Handling and Record Keeping

12.1 Data recording

The Research Data Specialist, Clinical Research Coordinator, and Investigator will be responsible for the recording of all data into an electronic data capture system.

12.2 Record retention

Federal law requires that an Investigator maintain all study records for two years after the investigation is discontinued.

13.0 Recruitment Procedure

All patients referred to or seeking treatment for advanced stage NSCLC will be offered this trial.

Women and men will be recruited, and are anticipated to be equally represented in the trial.

Persons equal to or over the age of 18 are eligible for trial participation, thus by NIH criteria, children are eligible for trial participation. However, the median age of persons with NSCLC is 71 years, and thus the vast majority of participants will be above the age of 21.

Minority participation will be especially encouraged. Past experience at the Karmanos Cancer Institute suggests that approximately 25% of patients enrolled will be African American.

Cognitively impaired patients will not be allowed to enroll on the trial owing to the need for an invasive procedure, which will need the co-operation of the individual.

14.0 Description of Study Medications

The single and multi-agent treatment regimens will be given at the discretion of the treating physician following established institutional guidelines. A specific description of the potential therapeutic regimens is not required. The regimens are part of the care plans established and approved by the Thoracic Oncology Multidisciplinary Team and the Pharmacy & Therapeutics Committee of the Karmanos Cancer Institute.

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Appendix I

International Staging System (AJCC Cancer Staging, version 7, 2010)

TNM Definitions: T = Primary Tumor
N = Regional Lymph Nodes
M = Distant Metastasis

Occult Carcinoma	TX	N0	M0
Stage 0	T*is*	Carcinoma in situ	
Stage IA	T1a-b	N0	M0
Stage IB	T2a	N0	M0
Stage IIA	T2a T2b	N1 N0	M0 M0
Stage IIB	T2 T3	N1 N0	M0 M0
Stage IIIA	T3 T1-3	N1 N2	M0 M0
Stage IIIB	Any T T4	N3 Any N	M0 M0
Stage IV	T4 (pleural effusion) Any T Any T	Any N Any N Any N	Any M M1a (contralateral lung) M1b (distant)

Appendix II

ECOG Performance Status

Point	Description
0	Fully active, able to carry on all pre-disease performance without restriction
1	Restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature, e.g., light house work, office work
2	Ambulatory and capable of all selfcare but unable to carry out any work activities. Up and about more than 50% of waking hours
3	Capable of only limited self-care, confined to bed or chair more than 50 % of waking hours.
4	Completely disabled. Cannot carry on any self-care. Totally confined to bed or chair.

Appendix III

Common Terminology Criteria for Adverse Events (CTCAE) v4.0

This study will utilize the Common Terminology Criteria for Adverse Events v4.0 (CTCAE) for grading of toxicity and adverse event reporting. It can be downloaded from the CTEP home page (http://ctep.cancer.gov).

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All appropriate treatment areas will have access to a copy of the CTCAE v4.0.