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A Pilot Study of Markers of Tumor Burden and Radiation Toxicity in the Blood, Urine, and Stool of Patients Receiving Radiotherapy for Gastrointestinal Malignancies

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PRECIS

Background:

- Gastrointestinal (GI) carcinomas represent one of the most commonly diagnosed malignancies in the United States[1].
- A sensitive and specific marker of tumor persistence or recurrence would permit a more accurate determination of the appropriateness of adjuvant therapy in patients with no clinical evidence of disease following curative resection and allow the diagnosis of recurrences at earlier stages that may be amenable to curative salvage therapies.
- A biomarker detectable shortly after treatment or in the early stages of chronic radiation toxicity may allow the identification of patients at risk and early intervention.

Objectives:

- Our primary objective is to determine if patient specific tumor markers in stool, urine, or serum can be reliably detected prior to treatment and followed after treatment to monitor the extent of residual disease.
- A second objective is to evaluate the predictive value of potential markers of chronic gastrointestinal injury after radiotherapy.

Eligibility

- Age ≥ 18 years
- Histologically confirmed carcinoma of the gastrointestinal tract (esophagus, stomach, pancreas, rectum)
- Planned to receive radiotherapy to the site of the gastrointestinal malignancy on an NCI protocol

Design:

- This protocol provides a means of acquiring tissue, serum, urine, and stool samples from patients who will receive radiation therapy as part of their treatment for gastrointestinal malignancies.
- Patients treated with radiation therapy on NCI treatment protocols will be asked to provide samples prior to any local or systemic therapy as well as before, during and after their radiation treatment.
- These samples will be tested for the presence of tumor specific DNA mutations and aberrant methylation patterns determined to be present in each patient's tumor by screening of initial biopsy or surgical material.
- Tumor markers specific to each patient, such as tumor specific DNA mutations or aberrant DNA methylation, may provide an individualized method to evaluate disease status and determine prognosis after therapy. Additionally, a number of stool and serum markers will be explored as early indicators of acute and eventual chronic gastrointestinal injury in patients receiving radiotherapy to the abdomen.

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1.0 INTRODUCTION

1.1 STUDY OBJECTIVES

1.1.1 Primary Objectives:

- To determine if tumor specific DNA mutations and aberrant DNA methylation found in the tumor of patients treated with definitive intent for gastrointestinal malignancies can be detected in the serum, urine, or stool at the time of diagnosis
- To determine if changes in mutational status at follow-up are associated with disease persistence, recurrence, or survival.
- To determine if pre-treatment, post-treatment, and follow-up measurements of TGFβ1 in the urine and plasma can be used to predict late gastrointestinal toxicity.

1.1.2 Secondary Objectives:

- To determine which biological fluid (serum, urine, or stool) is most highly associated with the detection of tumor specific DNA mutations and tumor specific DNA methylation.
- To determine which biological fluid (serum, urine, or stool) provides the best source of tumor specific DNA to measure at follow-up for association with disease persistence, recurrence, or survival.
- To determine if novel serum, urine, and stool biomarkers obtained at the time of treatment and shortly after treatment can be used to predict the likelihood of chronic gastrointestinal toxicity in patients receiving radiotherapy for gastrointestinal malignancies.
- To determine whether novel serum, urine, and stool markers obtained at the time of treatment and shortly after treatment are influenced by the radiation dose delivered to abdominal organs.

1.2 BACKGROUND AND RATIONALE

1.2.1 Gastrointestinal carcinoma

Gastrointestinal (GI) carcinomas represent one of the most commonly diagnosed malignancies in the United States[1]. Although significant improvements in local control and survival have been achieved in these disease sites over the past several years, locoregional and distant failure remain a significant concern. Radiation plays a prominent role in the definitive management of many GI malignancies, including esophageal cancer, gastric cancer, rectal cancer, and pancreatic cancer. Radiation and chemotherapy are often delivered as adjuvant therapy following resection, as neoadjuvant therapy prior to resection, or as definitive therapy for these malignancies.

Assessment of response to therapy and accurate determination of disease status in gastrointestinal malignancies are complicated by the internal location of the tumors, the resolution of current imaging modalities, and the anatomic distortion introduced with surgery and radiation. When radiation is used as a component of definitive therapy, residual scarring and radiographic abnormalities often persist, making the determination of clinical response difficult. In the case of radiation as a component

of adjuvant therapy after a total resection, treatment is delivered to patients at high risk of local recurrence, often without evidence that residual disease exists. A sensitive and specific marker of tumor persistence or recurrence would permit a more accurate determination of the appropriateness of adjuvant therapy in patients with no clinical evidence of disease following curative resection and allow the diagnosis of recurrences at earlier stages that may be amenable to curative salvage therapies.

Multiple serum markers of malignancy have been explored in the hopes of developing effective screening, assessing response to therapy, and developing prognostic markers. The best studied and clinically useful tumor markers are proteins elaborated in elevated amounts by the tumor tissue, such as Ca 19-9, CEA, AFP, beta-HCG, CA 125, and PSA[2-4]. None of these markers is completely specific to tumor tissue, and elevation in the serum levels of these markers may be associated with inflammatory states and other benign disease processes[3, 5-9].

1.2.2 Tumor derived nucleic acids

1.2.2.1 Nucleic acids as a tumor marker

While numerous serum, tissue, and biological fluid markers of cancer have been described, many of these markers are not specific to tumor cells. For example, proteins elaborated by tumor cells may be present in patients with no evidence of disease and in patients with non-cancerous diseases, leading to significant overlap in marker levels between healthy patients, patients with cancer, and patients with benign diseases. An optimal marker would be produced only in tumor cells, detectable with minimally invasive methods, and detectable in patients with a small burden of disease. The use of tumor nucleic acids as a marker is a promising method as tumors are typically associated with genetic mutations that are not present in the host cells. Additionally, the clonal nature of tumors supports the hypothesis that these mutations would be evident in all or most of the tumor cells if the screened mutation was an early event in tumorigenesis.

Tumor specific DNA mutations, promoter hyper- and hypo-methylation, and microsatellite alterations have been detected in the serum and biological fluids of patients with a variety of malignancies[10] including non-small cell lung cancer[11, 12], gastric cancer[13, 14], bladder cancer[15, 16], cervical cancer[17], renal cancer[18], and breast cancer[19]. While many studies have confirmed the ability to detect tumor DNA in patients with known malignancy and have correlated retreatment characteristics to prognosis[20-22], few have evaluated the utility of serial evaluations of these markers following therapy as a marker of disease status[19, 23].

Other biological specimens have also been evaluated for the presence of tumor specific mutations, most successfully with stool. The development of colon cancer is associated with a stepwise progression from pre-malignant to invasive tumors which is correlated with sequential DNA mutations in the colon cells destined to become malignant[24]. This stepwise progression through well-described mutations has been exploited in the search for a marker of colon cancer. Numerous reports in recent years have validated the detection of these genetic alterations in stool from shed luminal cells as a method to screen for both

malignant and pre-malignant lesions of the colon[25-27]. Other GI malignancies are potentially amenable to the approach of stool screening for DNA mutations as malignant cells are shed into the lumen and incorporated into the feces.

1.2.2.2 *Circulating and excreted nucleic acids as a marker of disease status*

Previous studies of DNA mutations and hyper- and hypo-methylation have screened for a variety of markers in the serum or urine of patients, however most have limited the analysis to a few markers in each study, resulting in a significant percentage of patients in whom none of the screened markers were detected. By screening the patient's tumor for mutations and aberrant methylation prior to treatment, it may be possible to select appropriate markers to monitor for each patient. This individualized approach of following markers known to be present may provide a sensitive tumor marker panel specific to each patient's tumor.

In protocol, we hope to evaluate whether we can reliably detect tumor specific mutations and tumor specific epigenetic alterations in the serum, urine, and stool of patients with GI cancers. In addition, we hope to determine if any of these biological specimens is superior for this purpose. Finally we hope to determine if the ability to detect these markers in the biological fluids of patients who have undergone curative therapy for cancer is predictive of failure.

The specific mutations and alterations in nucleic acids to be tested will vary with the primary site and histology of the tumor, based on published frequencies of mutations and aberrantly methylated sequences specific to each histology and tumor site (See Appendix IIA). Each of these mutations has been tested with positive and negative control cell lines in the laboratory of Dr. Citrin and have previously undergone primer and buffer optimization (Figure 1).

The ability to recover these mutations and aberrations from paraffin embedded tumor sections has also been demonstrated in Dr. Citrin's laboratory. Positive and negative controls have been identified and tested for each of these mutations and methylation patterns (see Appendix IIB). Additional candidate mutations and aberrant DNA methylation markers may be tested in the tumors and specimens of patients in whom none of these are found to be present.

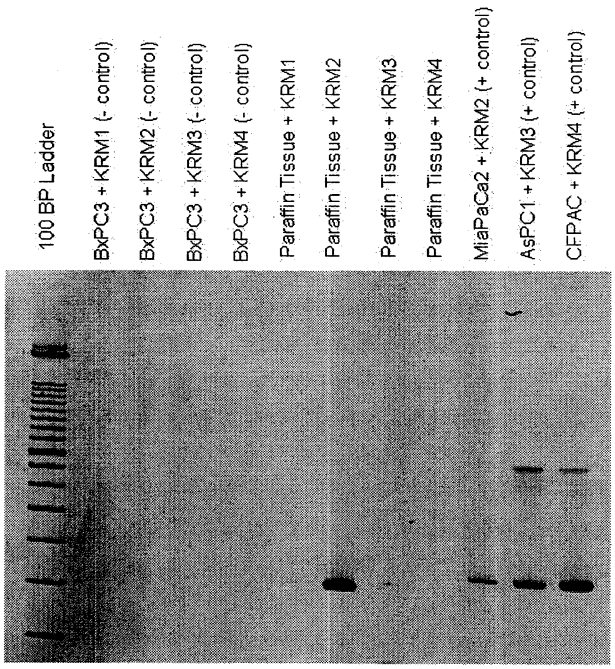

Figure 1. PCR products of positive and negative control cell lines and paraffin embedded tissue sections. BxPC3 is a pancreatic cell line known to have wild type ras. MiaPaCa-2 is a cell line known to have the KRM2 k-ras mutation. AsPC1 is a pancreatic cell line known to have the KRM3 k-ras mutation. CFPAC is a pancreatic cell line known to have the KRM4 k-ras mutation.

1.2.3 Gastrointestinal Radiation Injury

Radiation induced acute and chronic gastrointestinal toxicity is an important cause of morbidity following irradiation for malignancies of the abdomen and pelvis including gastrointestinal, genitourinary, and gynecologic cancers[28]. Delayed radiation enteropathy presents in most patients between 6 months and 3 years after radiation[29, 30]. A significant proportion of patients develop some form of late radiation intestinal injury that is likely underreported. Estimates in the frequency of radiation toxicity after abdominal radiation vary depending on the dose of radiation used, the location being treated, and the use of surgery and concurrent chemotherapies, however prior studies have estimated some level of intestinal dysfunction in 60-90% of patients followed longitudinally[30-32].

The small bowel, colon, gastric wall, and the rectum are frequently considered dose-limiting structures due to their lower radiation tolerance compared to surrounding organs. Long-term complications following radiotherapy can manifest as radiation enteropathy, fistulae, fibrosis, adhesions, perforations, and telengiectasias with hemorrhage[29, 33, 34]. While several factors place patients at increased risk of gastrointestinal toxicity, such as inflammatory bowel disease or prior abdominal or pelvic surgeries[35], it is difficult to predict which patients are destined to experience significant morbidity.

1.2.3.1 Clinical measures of acute and chronic gastrointestinal radiation injury

Radiation induced gastrointestinal toxicity can be effectively measured with toxicity scoring schemas. Acute gastrointestinal toxicity from radiotherapy manifests differently from chronic toxicity. The RTOG and EORTC have developed toxicity scoring schemas which are widely used as the gold standard for quantifying gastrointestinal toxicity in randomized trials designed by the RTOG including those designed to test the toxicity of various treatment regimens. These toxicity scoring schemas will be used as the standard for determining GI toxicity for the purposes of this study (Appendix IIIA and Appendix IIIB).

1.2.3.2 Markers of radiation-induced gastrointestinal toxicity in serum, plasma, urine, and stool

While clinical markers provide a measure of acute and chronic radiation toxicity, there is little ability to predict which patients may eventually experience chronic radiation toxicity. The ability to identify patients shortly after treatment destined to experience radiation toxicity may eventually allow early intervention. As the biologic events responsible for chronic radiation toxicity have become better understood, it has become clear that chronic radiation toxicity is a dynamic process that develops over the course of months to years after treatment. Many key processes in this ongoing injury are being defined, and agents are being developed with the intent of mitigating ongoing damage. Development of a biomarker that could accurately predict the likelihood of eventual radiation toxicity would allow selection of patients at high risk of toxicity for trials of these agents and provide candidate biomarkers of response to preventative therapy.

Several mechanisms of this ongoing injury in normal tissues have been described from evaluation of preclinical models. Depletion of stem cells in the bowel wall with resulting denudation has been implicated in acute intestinal injury, and it was long thought that late radiation toxicity of the bowel was a result of progressive damage as a consequence of this stem cell depletion[34]. Recently, inflammatory cytokines have been implicated in bowel and other tissues as possible mediators of chronic inflammation and fibrosis following radiation[36]. In addition, the expression of numerous collagens and matrix remodeling peptides have been shown to be altered early in preclinical models of late GI injury[37-39] and in the affected tissues of patients with late GI radiation toxicity[40]. It is possible that early detection of this ongoing inflammation and pro-fibrotic pathways may eventually allow early intervention to mitigate late toxicity.

Table 1: Candidate markers of toxicity to be evaluated on this protocol

Plasma	Serum	Urine	Stool
TGFβ1	Neutrophil elastase	TGFβ1	Neutrophil elastase
	Procollagen peptides	Procollagen peptides	Calprotectin
		Intestinal permeability	pH

▪ TGFβ1

Cytokines are known to play an important role in the acute and chronic phases of radiation injury in a variety of organs. One of the best studied cytokines in both pre-clinical and clinical studies of chronic radiation fibrosis is TGFβ1. TGFβ1 plays important roles in cell growth, apoptosis, immune responses, and the homeostasis of the extracellular matrix (reviewed in [41]). As all of these processes are implicated in radiation induced chronic intestinal injury, TGFβ1 is a candidate marker for ongoing gastrointestinal injury. In preclinical models of radiation enteropathy, TGF-β overexpression has been observed acutely and chronically in intestinal tissues[42, 43]. This overexpression was found to correlate with fibrosis and inflammatory cell infiltrates[42, 44, 45].

TGFβ1 as a marker of fibrosis and chronic injury has successfully been measured in the plasma of patients treated with radiation for lung cancer. In these studies, the kinetics of TGFβ1 levels correlated with radiation induced pneumonitis[46-48]. Since the expression of TGFβ1 in intestinal tissues is correlated with radiation injury, it may be possible to detect the molecule as a marker of ongoing intestinal inflammation. We hope to evaluate the ability to detect this marker in the urine and plasma of patients treated with radiation, to compare the levels detected with each method, to determine if one method is superior, and to determine if levels of TGFβ1 correlate with chronic gastrointestinal toxicity. TGFβ1 levels will be determined in platelet poor plasma.

▪ Leukocyte markers

Other possible markers of early and late gastrointestinal toxicity are markers of intestinal inflammation. Without biopsies of irradiated tissues, direct evidence of inflammation in the intestinal wall is unavailable; however numerous indirect measures of intestinal inflammation are available. Neutrophil elastase is known to be secreted from activated neutrophils and has been implicated in endothelial cell and epithelial cell apoptosis[49]. Importantly, endothelial cell apoptosis and microvascular dysfunction have been implicated in radiation induced clonogen depletion in the intestine[50]. In addition, neutrophil elastase has been implicated in epithelial injury[51] and inflammatory diseases of the intestines[52-54]. This acute inflammation may play a role in the initiation of ongoing bowel inflammation and injury that is the hallmark of late GI toxicity. In pre-clinical models, several markers of neutrophil transmigration have been found to correlate with histologic markers of acute and chronic radiation enteropathy[55]. Fecal lactoferrin has been shown to correlate with histologic evidence of bowel inflammation[56, 57]. We will determine the clinical utility of fecal and serum elastase, fecal calprotectin, and fecal lactoferrin as markers of neutrophil transmigration and surrogate markers of acute and chronic bowel injury.

▪ Extracellular matrix proteins

Evaluations of gene expression in chronic radiation enteropathy have reported an increase in transcripts related to extracellular matrix (ECM) remodeling and fibrosis [58-61], consistent with the pathologic findings of fibrosis described in clinical series[40]. Altered collagen subtype ratios have been reported in intestinal injury to be mediated by TGFβ, which is thought to play a major role in the pathogenesis of chronic bowel injury due to radiation[40].

Collagen type I and III are synthesized as procollagen precursors that must be cleaved to form collagen. Measurements of serum pro-peptide levels, such as the carboxy-terminal propeptide of procollagen type I (PICP) and the amino-terminal propeptide of collagen Type III (PIIINP) provide a measure of collagen deposition and have been evaluated in a variety of disease states linked to fibrosis[62-72]. In addition, the amount of cross-linked carboxy-terminal telopeptide (CITP) is proportional to the amount of collagen degradation and together with PICP and PIIINP may provide markers of the collagen turnover that occurs with fibrosis [73].

Preclinical models of radiation induced bowel fibrosis have confirmed increased deposition of Type I and Type III collagen in the bowel wall detected by immunohistochemistry[38]. In patients, altered collagen deposition has been evaluated in radiation injury of the skin[74-76] and in patients with radiation induced pulmonary fibrosis [77, 78]. The measurement of serum markers of collagen deposition and turnover through measurement of pro-collagen peptides provides a non-invasive method of evaluating ongoing extracellular matrix remodeling and fibrosis.

The utility of these markers of fibrosis in the setting of radiation toxicity will be evaluated in patients who undergo radiotherapy. The timing of any surgery or biopsies, if performed, will be recorded as this may be a factor that alters the utility of these measures as markers of for ongoing fibrosis. It is likely that the ongoing chronic inflammation and fibrosis characteristic of the late toxicity of radiation will have a different time course of expression of these molecules than that induced by surgery. While elevated procollagen peptides have been reported in the serum of patients with widespread metastatic bony disease[79-82], this should not be a confounding factor except in patients who develop this complication. Special attention will be paid to the symptoms or radiographic evidence of the development of bone metastases during the follow-up period.

▪ *Functional measures of intestinal injury*

Intestinal absorption of lipids, monosaccharides, and polysaccharides has been used to evaluate small intestine function in various disease states. Alterations in the bowel wall due to denudation, edema, barrier function, and fibrosis may affect intestinal absorption. Alterations in barrier saccharide absorption have been noted in radiation patients [28] but this has

not been correlated to clinical symptoms of radiation enteropathy. Acute radiotherapy induced acute intestinal malabsorption may correlate with the degree of bowel injury and eventually the likelihood of developing chronic intestinal toxicity. One test that has been used to evaluate changes in intestinal absorption during radiotherapy is a comparison of the transport of monosaccharides to disaccharides across the intestinal wall, which is commonly known as an intestinal permeability test [83]. This has yet to be evaluated as a correlate of late toxicity.

1.2.4 Conclusion

This study will aim to determine the feasibility of detecting tumor specific DNA mutations and DNA methylation abnormalities in biological fluids of patients with a variety of GI malignancies compared to tumor biopsies or resected tumor tissue. Additionally, we will compare the ability to detect these nucleic acids in serum, urine, and stool. A secondary endpoint will be to determine if detection of tumor specific nucleic acids can be used as markers of active disease. In addition, the study will aim to evaluate known markers of inflammation and fibrosis as possible markers of gastrointestinal toxicity in the serum, urine, and stool of patients receiving radiotherapy for gastrointestinal malignancies.

2.0 Eligibility Assessment and Enrollment

2.1 ELIGIBILITY CRITERIA

2.1.1 *Inclusion Criteria*

2.1.1.1 Age ≥ 18 years

2.1.1.2 Histologically confirmed carcinoma of the gastrointestinal tract (esophagus, stomach, pancreas, bile duct, rectum)

2.1.1.3 Treatment plan includes radiotherapy to the site of the gastrointestinal malignancy on an NCI protocol

2.1.1.4 Paraffin embedded tumor tissue from biopsy adequate in amount to perform PCR and methylation specific PCR or willingness to undergo rebiopsy

2.1.2 *Exclusion Criteria*

2.1.2.1 Inability to provide informed consent

2.1.2.2 Patients who have a history of prior therapeutic radiation

2.1.2.3 Patients with evidence of distant metastases on initial staging evaluation

2.1.2.4 Patients with other cancers excluding non-melanomatous skin cancers or carcinoma in situ

2.1.2.5 Patients who have undergone complete resection of the gastrointestinal malignancy prior to protocol enrollment

2.1.2.6 History of inflammatory bowel disease

2.1.2.7 History of collagen vascular disease or disease of altered collagen metabolism (end stage renal disease or hepatic fibrosis due to chronic hepatitis)

2.1.2.8 History of hypersensitivity to radiation or a history of a disease which results in mucosal or other hypersensitivity to radiation (Ataxia-Telengiectasia, Bloom's Syndrome, Human Immunodeficiency Virus, Fanconi anemia, nevoid basal cell carcinoma syndrome, Li-Fraumeni syndrome, and Nijmegen breakage syndrome)

2.1.2.9 Inability to return for followup visits

2.1.2.10 Patients who have previously received or are currently receiving MDX-101 (ipilimumab).

2.1.2.11 Diagnosis of HIV, Hepatits B, or Hepatits C

2.2 PRE-TREATMENT RESEARCH ELIGIBILITY EVALUATION

2.2.1 Clinical Evaluations

- History and Physical Examination within one month of entry: Specific items to document include:
  - Age
  - Primary tumor site
  - Primary tumor histology
  - Tumor stage
  - Baseline gastrointestinal function including RTOG acute toxicity scoring
  - Prior abdominal surgeries

- History of diseases known to affect systemic inflammatory states (ie. hypertension, liver function abnormalities, renal function abnormalities, cardiac disease, autoimmune disorders, collagen vascular disorders, and arthritis)

2.2.2 .Pathologic confirmation
Pathology report confirming carcinoma of the esophagus, rectum, or pancreas (outside report acceptable for study entry; pathology review by NIH to be performed if radiation therapy to be administered at NCI ROB).

2.3 PATIENT REGISTRATION
Authorized staff must register with the Central Registration Office an eligible candidate within 24 hours of signing the consent. A registration checklist from the Web site (http://camp.nci.nih.gov/dcs/rob/rob_index.html.) must be completed and faxed to 301-480-0757.

3.0 STUDY IMPLEMENTATION

3.1 STUDY DESIGN
3.1.1 Overall Trial Design (see Appendix IA)
Patients planned to receive radiotherapy for carcinoma of the esophagus, stomach, pancreas, rectum, or other biliary malignancies as part of their primary therapy will be potential candidates for enrollment on this protocol. This is not a study of an experimental therapy and will be administered in conjunction with protocol therapy for patients receiving radiation as part of the treatment of a gastrointestinal carcinoma. 120 patients will be included from the following sites: 30 patients with esophageal or GE junction cancer, 30 patients with locally advanced biliary malignancies (including pancreas and bile ducts only), 30 patients with resected biliary malignancies (including pancreas and bile ducts only), and 30 patients with rectal cancer.

Serum, plasma, urine, and stool samples will be collected prior to any therapy (surgery excluding biopsy, systemic therapy, or radiotherapy), prior to radiotherapy (if different from "prior to any therapy"), at completion of radiation (within the final five fractions), and at 1, 3, 6, 12, 24, and 36 months follow-up

The Intestinal Permeability Assessment will be performed following protocol sample collection (above) at the following time points: prior to any therapy, prior to radiotherapy (if different than "prior to therapy"), at 1 month follow-up, and at 3 months follow- up.

Tumor tissue obtained at biopsy or resection will be screened for a defined panel of mutations using PCR and abnormal methylation status will be determined by methylation specific PCR. Serum, urine, and stool samples obtained prior to therapeutic intervention will be screened for the same panel of markers. Serum, urine, and stool obtained during treatment and at follow-up will be evaluated for these

same DNA markers and correlated to evidence of gross residual disease on physical examination and follow-up imaging.

Potential serum, plasma, and stool markers of gastrointestinal toxicity will be correlated to validated measures of gastrointestinal toxicity.

3.2 PROTOCOL THERAPY ADMINISTRATION

3.2.1 Specimen Collection

3.2.1.1 Tumor tissue

3.2.1.1.1 Collection guidelines

- Biopsy material will be snap frozen in liquid nitrogen and transported to the laboratory of Dr. Deborah Citrin for storage at -80C.
- An NIH pathologist will be involved in selecting tumor tissue for experimental use if obtained at the time of resection to ensure adequate tissue is reserved for pathologic diagnosis and determination of margin status. Tumor tissue from resection will only be obtained if surgical therapy is the first form of therapy for the malignancy (i.e., no radiation or chemotherapy is delivered prior to resection). This tissue will be snap frozen in liquid nitrogen and transported to the laboratory of Dr. Deborah Citrin for storage at -80C.

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  Bldg 10, Rm 3B42
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- Patients in whom a biopsy would be considered more than minimal risk (i.e., pancreatic tumor biopsy not obtained at the time of surgery or already planned invasive procedure) are exempt from the biopsy requirement; however attempts will be made to obtain prior biopsy specimens from NIH or outside facilities if available.

3.2.1.1.2 Collection Schedule

Tumor tissue is obtained at biopsy or resection (pre-treatment only) at baseline

3.2.1.1.3 Specimen analysis

Tumor tissue will be screened for a panel of mutations with PCR and abnormal methylation with methylation specific PCR (see appendix IIA&B)

3.2.1.2 Blood, Urine, and Stool Specimens

3.2.1.2.1 Collection guidelines (see Appendix IA)

Nurses at the radiation oncology clinic will collect blood, urine, and stool samples before, during and following therapy.

- Blood –
  - 10 cc in one EDTA tube for plasma,; place only EDTA containing tube immediately on ice, EDTA containing tube must be collected first,

- 24cc total in serum separator tube (SST) for serum,
- Stool – at least 10 gm in a sterile collection cup.
- Urine – at least 15 cc in a sterile collection cup

3.2.1.2.2 Collection Schedule

Pre- treatment, pre-radiotherapy (if different than pre-treatment) at completion of radiotherapy and in follow- up at 1, 3, 6, 12, 24, and 36 months

3.2.1.2.3 Specimen Analysis (see Appendix IB)

- All samples will be sent to the laboratory of Deborah Citrin, M.D. for appropriate processing and storage
- Plasma samples will be processed and snap frozen at -80° C until the time of use for TGFβ1 assays.
- Serum, urine, and stool will be processed, aliquoted, snap frozen, and stored at -20° C (stool) or -80° C (urine, serum, and plasma) until use

3.2.1.3 Handling of Specimens collected for Research Purposes

- Tissue, serum, plasma, urine, and stool samples collected in the course of this research project may be banked and used in the future to investigate new scientific questions related to this study. However, this research may only be done if the risks of the new questions were covered in the consent document.
- No germline mutation testing will be performed on any of the samples collected.
- At the completion of the protocol, the investigatory will dispose of all specimens in accordance with the environmental protection laws, regulations and guidelines of the Federal Government and the State of Maryland.
- Any loss or unintentional destruction of the samples will be reported to the IRB.
- Any new use of the samples will require prospective IRB review and approval.

3.2.2 *Intestinal permeability test*

The Intestinal Permeability Assessment directly measures the ability of two non-metabolized sugar molecules, mannitol and lactulose, to permeate the intestinal mucosa. Mannitol is easily absorbed and serves as a marker of transcellular uptake, while lactulose is only slightly absorbed and serves as a marker for mucosal integrity. Intestinal permeability tests are commercially available. (see collection schedule in 3.3)

3.2.2.1 Collection guidelines (see Appendix IA):

- These assessments will be performed following protocol sample collection before, during and following therapy.
- The patent will fast over night for 8 hours and will then drink a "challenge" drink supplied by Genova Diagnostics composed of 5gm of lactulose and 1gm of mannitol. Patients are asked to avoid non-steroidal anti-inflammatory drugs for twenty-four hours prior to the test, unless medically required (ie low dose aspirin for cardiac disease).

- Urine is collected over six hours and refrigerated until the patient returns to the ROB clinic.

3.2.2.2 Collection Schedule

Pre- treatment and in follow- up at 1 and 3 months.

3.2.2.3 Specimen analysis

- The urine will be collected in a collapsible jug provided by Genova Diagnostics which will be stored in the clinic prior to use and provided to the patient prior to the ingestion of the challenge drink.
- The 6 hour urine will be brought to the ROB clinic by the patient.
- The specimen will then be transported to the laboratory of Dr. Deborah Citrin for shipment to Genova Diagnostics.
    - All personal identifiable information will be removed and the specimen will be labeled by a code based on the patient's enrollment number on the protocol
    - The urine will shipped to Genova Diagnostics for further testing. The jug is specially designed to serve as packaging as well and has a prepaid and preaddressed shipping label via DHL shipping. The address on this shipping label will be:

Genova Diagnostics
63 Zillicon Street
Asheville, NC 28801

3.2.3 Toxicity Assessment

3.2.4.1 Collection Guidelines

Radiation induced gastrointestinal toxicity will be measured with the RTOG toxicity scoring schemas (Appendices IIIA and IIIB)

3.2.4.2 Collection Schedule

Pre- treatment, at completion of treatment and in follow- up at 1, 3, 6, 12, 24, and 36 months

3.3 ON STUDY EVALUATION

3.3.1 Pre-Treatment (baseline)

- Informed consent obtained
- Clinical evaluation
    - See 2.2.1
    - Baseline acute RTOG toxicity assessment (appendix IIIA)
- Laboratory Evaluation
    - See 2.2.2
    - Hepatitis B
    - Hepatitis C
    - CBC, ALT, AST, total bilirubin, serum BUN, serum creatinine
- Research Specimen Collection:
    - Blood, urine, stool (see 3.2.1.2)
    - baseline intestinal permeability test, 6 hour urine (see 3.2.2)
- Pathologic confirmation and biopsy of tumor tissue (see 2.2.3)

3.3.2 Radiation Treatment Phase

- Patients will receive radiotherapy for carcinoma of the esophagus, stomach, pancreas, bile ducts, or rectum on an NCI protocol
- At completion of radiation (within the final five fractions)
    - Research Specimen Collection:
        - Blood, urine, stool (see 3.2.1.2)
    - Toxicity Evaluation: RTOG - Acute Radiation Morbidity Scoring Criteria (Appendix IIIA)

3.3.3 Post Active Treatment Evaluation (follow- up)

3.3.3.1 Patients will be seen for routine follow-up at 1, 3, 6 and 12, 24, and 36 months post-radiotherapy.

3.3.3.2 These follow- up visits will include:

- Clinical Evaluation
    - A complete history and physical exam detailing the patient's disease status, bowel function, and medication requirements related to gastrointestinal toxicity. All post-radiotherapy adjuvant therapy will be recorded.
- Toxicity Evaluation: Complete RTOG - Radiation Morbidity Scoring Criteria (Appendix IIIA, acute at 1 month and IIIB, late at 3, 6, and 12, 24, and 36 months follow-up.
- Laboratory Evaluation
    - As clinically indicated. The presence of accepted serum tumor markers (ie CA 19-9) in excess of defined maximum ranges will be recorded as evidence of disease recurrence.
    - CBC, ALT, AST, total bilirubin, serum BUN, serum creatinine
- Research Specimen Collection:
    - Blood, urine, stool (see 3.2.1)
    - intestinal permeability test, 6 hour urine (see 3.2.2) AT ONE AND THREE MONTHS ONLY
- Radiologic Evaluation
  As clinically indicated by primary site and primary protocol. For the purpose of radiographically evaluating disease status to correlate to laboratory findings the following radiographic evaluations may be included for determination of disease recurrence: CT, plain films, bone scans, MRI, US, and PET.
- Response Category Evaluation (as in 5.2)

3.4 CONCURRENT THERAPIES

This study allows blood and stool procurement before, during, and after patients receive radiation therapy. It is possible that patients may also be treated with neoadjuvant, concurrent, or adjuvant

chemotherapy. Patients may also receive surgery as part of definitive therapy. This is allowed but protocol enrollment and the initial sample collection must occur prior to initiation of chemotherapy or complete resection.

3.5 RADIATION THERAPY GUIDELINES

This is not a study of an experimental therapy. It is to be to be performed in conjunction with protocol therapy for patients receiving radiation as part of a primary treatment of carcinoma of the esophagus, stomach, bile ducts, pancreas, or rectum.

Radiation dose to various intraabdominal structures will be calculated and recorded. The organs contoured will vary for each site; however, typically this will include the stomach, small bowel, duodenum (contoured separately from the remainder of the small bowel), liver, colon, rectum, bladder, kidney, heart, lung, and esophagus. If any of these organs is included in any radiation field it will be contoured in its entirety on the CT simulation planning image set and the minimum, maximum, mean, and median dose to this organ will be recorded. In addition, the percentage and volume of this organ receiving above the accepted tolerance dose will be recorded.

3.6 OFF STUDY CRITERIA

3.6.1 Administrative

Patients may be taken off study for the following non-medical or administrative reasons:

- Patient refuses the procedure or further treatment
- It is deemed in the patient's best interest as determined by the PI.
- Serious protocol violation as determined by the PI.

3.6.2 *Development of a concurrent serious medical condition that precludes the completion of, radiation therapy or follow- up.*

3.6.3 Failure to begin radiation therapy or inability to complete a planned course of radiation therapy.

3.6.4 The completion of 3 years follow-up.

3.6.5 Death

3.8 POST-STUDY EVALUATION

- Additional blood and/or stool samples may be obtained if deemed necessary by the PI or study chairperson. In these cases, the collection will coincide with regularly scheduled clinic visits.

- The patient should be registered off- protocol by completing the Off Study/ Death Notification Form available from the web site

(http://camp.nci.nih.gov/dcs/rob/rob_index.html) and faxing it to the central registration office at 301-450-0757.

4.0 SUPPORTIVE CARE

No supportive care in excess of that required for routine radiation therapy should be required. If patients require supportive care, it will be delivered as per current standard of care. If any anti-fibrotic or immunosuppressive medications are delivered for the primary disease process, side effects, or other disease processes, these will be noted at followup as part of the interval history and physical examination as possible confounding factors.

5.0 DATA COLLECTION AND EVALUATION

5.1 Data Collection

- Research data and acquired samples will be recorded into the CCR database
- Clinical data collection will include: demographic information, pathologic diagnosis, primary tumor site, extent of resection (negative margin, microscopic positive margin, gross positive margin, or biopsy), gastrointestinal function, history including prior therapies, HIV status, sample collection date & time, radiographic evaluations (date & time), radiotherapy details (site, dose, schedule, irradiated volume), concomitant meds & treatments.
- Data from assays run at NIH will be collected in laboratory notebooks and in an electronic database that is shared with the ROB research office..

5.2 Response Criteria

This is not a treatment study and will be administered in conjunction with protocol therapy for patients receiving radiation as part of treatment of their gastrointestinal carcinoma. Response will be assessed according to the patient's primary treatment protocol, as clinically indicated based on their disease site. For consistency in addressing this protocol's objectives, we will use this response information to group patients into the following categories at their follow- up time points (1,3,6,12, 24 & 36 months):

5.2.1Complete response (CR) is defined as no clinical, radiographic or laboratory evidence of disease following treatment

5.2.2 All other classifications of the RECIST criteria (partial responders, stable disease, and progressive disease) will be considered not a CR.(as defined in 5.2.1). These patients will be considered not complete responders for the purposes of comparison to serum markers.

5.3 Toxicity Criteria

5.3.1 Acute toxicities will be evaluated using the Cancer Therapy and Evaluation Program Common Toxicity Criteria (CTC) Version 3.0 for toxicity and Adverse Event reporting. A copy of the CTC version 3.0 can be down loaded from the CTEP home page (http://ctep.info.nih.gov.).

5.3.2 Radiation induced gastrointestinal toxicity will be measured with the RTOG and EORTC scoring schemas in Appendices III A and IIIB. These toxicity scores will be correlated with proposed markers of active disease and markers of gastrointestinal toxicity in the serum, plasma, urine, and stool of patients receiving radiotherapy for gastrointestinal malignancies.

5.4 Statistical Section

The two primary objectives are to (i) to examine whether tumor-specific DNA mutations and aberrant DNA methylation at the time of diagnosis and during follow-up is associated with disease persistence, recurrence, or survival, and (ii) whether changes in TGFβ1 in the urine and plasma is associated with late gastrointestinal toxicity. We will discuss analysis and sample size considerations for each primary objective in turn.

5.4.1Association between tumor-specific DNA markers and subsequent disease.

For the first primary objective, tumor-specific DNA mutation markers will be collected at baseline (before any therapy and before radiation begins) at completion of radiation therapy and at 1, 3, 6, 12, 24, and 36 months of follow-up. Of interest is examining whether changes in the average mutation frequency (of the disease-specific mutations given in Appendix IIA) effects response to therapy, overall survival, and disease-free survival among complete responders to all therapy. Various analyses will be done to address with objective. These analyses will be done separately by tumor type as well as combined over tumor type (assuming that the relationship between marker changes and disease outcome do not vary across tumor type). First, we will examine the relationship between changes in mutational status over time and survival with Cox proportional hazards models. The relationship between changes in mutational status over time and complete response will be estimated using logistic regression analysis. One key primary analysis will be to examine whether having all tumor-specific markers being negative at 3 months after radiation therapy ends is associated with long-term survival. Note that based on the tumor marker prevalences in the literature (see Appendix IIC) and an assumption that marker prevalence is independent across the various markers, the probability of having at least one mutation which is positive before treatment is very high . Our sample size calculations assume that 50% of patients will have all tumor-specific markers negative 3 months after radiation therapy ends. Based on conducting a two-sided log-rank test at the 0.05 significance level with a total enrollment of 120 patients, we would have 89% power to detect a difference between 35% overall 2-year survival in the positive marker group and 65% overall 2-year survival in the negative marker group. We will have power only to detect large differences for the tumor-specific analyses. With 30 patients in each group (15 patients in the positive and negative groups, respectively), we would have 83% power to detect a difference in 2 year survival of between 80% in the marker negative group and 20% in the marker positive group using a log-rank test at the 0.05 significance level. The above sample size calculation depends heavily on the assumption that 50% of patients will have all tumor-specific markers negative at 3 months. The power may be severely diminished if this proportion deviates too much from 50%. Thus, alternative similar analyses will also be conducted. For example, we will compare survival by whether the change in tumor-specific marker frequency from

baseline to 3 months after radiation therapy ends (i.e., difference between proportion of positive markers at 3 months after radiation and proportion at baseline) is above or below the median value. The above sample size calculation also applies to this analysis (e.g., we will have 89% power to detect a difference between 35% overall 2-year survival in the above the median change group and 65% in the below the median change group).

5.4.2 Association between longitudinal measures of TGFβ1 and late gastrointestinal toxicity

We will examine whether levels of TGFβ1 measured in urine and plasma at various timepoints during and after therapy are associated with late gastrointestinal toxicity. Of particular interest will be examining whether changes in TGFβ1 measured over time affect the probability of RTOG≥2 toxicity at 12 months after the completion of radiation therapy. The analysis will be done by relating change in TGFβ1, evaluated by estimating a slope using least-squares regression on each patients repeated follow-up measurements, and the probability of RTOG≥2 at 12 months after completion of therapy using logistic regression. These analyses will be done with and without adjustment for radiation dose to non-target organs (radiation dosimetry). Interest will focus on whether a positive change in *TGFβ1* over time (positive slope) is associated with an RTOG≥2 toxicity at 12 months post-therapy. We expect that, on average, approximately 60% of patients will have a RTOG≥ 2 at 12 months. We also assume that at a minimum of 76 patients of 120 accrued to the study will be alive at 12 months (there is a 95% chance that there will be 76 or more patients will be alive and evaluable at 12 months if the one-year survival/evaluable rate is 70%). Assuming a that 76 patients will be evaluated for toxicity at 12 months and that 50% of estimated slopes are positive, we will have 93% power to detect a difference in the proportion of RTOG≥2 at 12 months of 80% for a positive slope as compared with 40% for a negative or zero slope with a Chi-square test at the 0.05 significance level. A logistic regression analysis treating the estimated slope as a continuous variable should have more power than the Chi-square test.

5.4.3
Analysis of secondary objectives

We will compare the accuracy of DNA mutations and DNA methylation tests in serum, urine, and stool (relative to tumor biopsy) by comparing estimated sensitivities and specificities of the various tests.

We will evaluate which biological fluid provides the best source of tumor specific DNA for association with subsequent disease using logistic regression and Cox proportional hazards models with DNA tests from the different fluids being independent variables in the models.

We will relate novel serum, urine, and stool markers measured during and shortly after treatment to toxicity using logistic regression analysis similar to 5.3.2.

We will examine the relationship between radiation dose and serum, urine, and stool biomarkers measured longitudinal during and after treatment using linear mixed models.

5.4.4 Accrual

We anticipate accruing 120 patients within 3 years.

5.5 Data Safety and Monitoring Plan

5.5.1 Plan for monitoring the progress of the trial and the safety of participants

- As this is not an experimental therapeutic trial, and the risks and discomforts are expected to be small (see section 6.2), the PI will assume primary responsibility for monitoring the progress of the trial and the safety of participants.
- As part of the NCI CCR, this trial may be selected at random to be monitored by the staff of Harris Technical Services Corporation
- Data will also be submitted to the NCI IRB annually for continuing review and at the completion of the study.
- In addition, at monthly research/ protocol meetings, senior staff of the NCI ROB will review protocol progress and provide feedback to the PI.

5.5.2 Plan for assuring the compliance with the requirements for reporting adverse events

- Adverse events will be reported as in section 5.3 and 7.3

5.5.3 Plan for assuring that any action resulting in suspension of the trial is reported to the IRB

- As this is an intramural NCI CCR protocol without external sponsors, the NCI IRB would be responsible for issuing the suspension and would therefore necessarily be aware.

5.5.4 Plan for assuring data accuracy and protocol compliance

- Data will be collected as in section 5.1
- Research data and acquired samples will be recorded in the NCI CCR C3D database.
- Data integrity and protocol adherence are assured by regular data verification and protocol compliance checks performed by the research team (PI, research nurse, data manager and clinic nurse).

6.0 HUMAN SUBJECTS PROTECTION

6.1 RATIONALE FOR SUBJECT SELECTION:

6.1.1 The racial ethnic and gender makeup of the study population is that of the population of the patients seen in the Radiation Oncology Branch, NCI. No racial or ethnic group is excluded. Adults who are cognitively impaired prior to study entry must have already assigned a DPA or will not be eligible for study entry because they cannot give informed consent.

6.2 *Participation of children:* Children younger than 18 will not be included.

6.3 EVALUATION OF RISKS/DISCOMFORTS:

6.3.1 This is not an experimental therapeutic trial, and so risks and discomforts are expected to be small and related only to the risks of obtaining the blood samples, biopsies, and the intestinal permeability assessment. Venipuncture is a minimal risk procedure, which may include pain or bruising around the site of the needle puncture, and a small risk of fainting or of infection of the site.

6.3.2 Any side effects from the radiotherapy will be outlined in separate radiation oncology branch consent for radiation form.

6.3.3 *Biopsy*

The risks of endoscopic biopsies and percutaneous biopsies are low, and are procedures that are performed routinely by many of the associate investigators for staging or diagnostic purposes for this patient population. These potential risks and benefits will be carefully discussed with the patient at the time consent is obtained. Consent will be obtained separately for the biopsy procedure if one is required.

6.3.4 Intestinal Permeability Assessment

The intestinal permeability assessment may cause in some individuals intestinal cramping or diarrhea. These symptoms are uncommon and typically resolve within 24 hours of the test.

6.4 *Risk/Benefit Analysis*

The patients will derive no direct benefit from the procurement of biopsy tissue, blood, urine, and/or stool, and that will be clearly stated in the protocol consent document. In the course of the investigation, should any information from the blood, urine, or stool testing emerge that might benefit patient treatment for the primary disease process or any toxicity of treatment; we would attempt to inform the patient, after discussion with Medical Ethics consultation and the IRB. The risks to the patients participating in this trial are anticipated to be small, and are primarily the risks associated with appropriate staging procedures, biopsies (ie gi endoscopies), and subsequent multidisciplinary therapy for their malignancies. Specifically, the risks of endoscopic biopsies and percutaneous biopsies are exceedingly low, and are procedures that are performed routinely by many of the associate investigators for staging or diagnostic purposes for this patient population. These potential risks and benefits will be carefully discussed with the patient at the time consent is obtained. If a biopsy is considered to be more than minimal risk based on the location of the tumor (ie pancreatic tumors with no invasive procedures or surgical procedures planned) than biopsy may be omitted.

6.5 CONSENT AND ASSENT PROCESSES:

The investigational nature, the research objectives of this trial, and the blood and/or urine collection procedure will be carefully explained to the patients. Attendant risks and

discomforts will be described. The patients will be asked to read the consent and will be encouraged to ask questions. Patients will be enrolled after eligibility criteria have been reviewed, and after the consent document has been signed. Children will not be enrolled onto this study.

6.6 PATIENT ADVOCATE

The patient's rights representative is available to patients on this protocol at (301) 496-2626 in Building 10, Room 1C132, NIH. Patients may ask any questions about the study and may withdraw their consent at any time without compromising their medical care.

7.0 DATA REPORTING

7.1 PATIENT REGISTRATION FORM

Demographic information and results of pretreatment studies should be entered and reported to Central Registration office at the time of patient entry onto the trial (see section 2.3).

7.2 DATA SUBMISSION

- Summary information will be submitted to the IRB annually for continuing review and at the completion of the study.

- Data may be reported in laboratory publications as derived from this study. Patients will not be indicated by name.

7.3 SAFETY REPORTING

7.3.1 As this is intended as a companion study to standard radiotherapy, adverse events occurring while on study should be reported in the patient's primary experimental protocol.

7.3.2 For toxicites related to the procedures associated with this protocol

- Toxicity will be graded as indicated in Section 5.3

- Expected acute side effects from radiotherapy treatment and/ or progression of disease will not be reported as adverse events, except grades 4 or 5.

- For all other adverse events, occurring during the active treatment phase and for 30 days thereafter, the NCI-IRB Adverse Event Reporting Requirements will apply. The PI will report:

    - All serious events as defined by the FDA that are unexpected and related to the research;
    - All deaths (Grade 5: CTCAE) expected and unexpected, related and unrelated to the research; and
    - All Grade 3 and 4 (CTCAE), unexpected, and related to the research with attributions of possibly, probably, or definitely,

within 7 calendar days.

- The NCI-IRB requires a summary report of adverse events that have occurred on the protocol since the previous continuing review. The method of presentation should provide the NCI-IRB with the information necessary to clearly identify risks to participants and to make a risk:benefit determination. The summary report is based on the following guidance:

  Any unexpected severity and/or unexpected frequency of expected events needs to be reported and interpreted in relation to the risk:benefit of study participants in the narrative.

  1. Grade 1 events are not required.
  2. Grade 2 unexpected related to the research events is required.
  3. Grade 3 and 4 expected and unexpected events related to the research are required.
  4. All Serious Events regardless of attribution.
  5. Grade 5 (all) events are included regardless of attribution.

- Based on protocol-associated risks to participants, the NCI-IRB retains the authority to establish more frequent Continuing Review periods more frequently than the customary annual review period.

- After 30 days, only unexpected, late- radiation toxicities will be reported, with the exception of deaths on study, which will continue to be reported, regardless of cause, in order to provide survival data.

7.3.3 Serious Adverse Events

- **Serious Adverse Event (SAE) or Serious Adverse Drug Reaction (Serious ADR)**
  Any untoward medical occurrence that at any dose:
  - results in death,
  - is life-threatening,
  - requires inpatient hospitalization or prolongation of existing hospitalization,
  - results in persistent or significant disability/incapacity,
  or
  - is a congenital anomaly/birth defect

- All serious adverse events must be reported to Dr Deborah Citrin, Principal Investigator of this study and to NCI IRB **within 7 calendar days in writing** using the NCI AE form.

Deborah Citin, MD, PI
NCI, ROB
Bldg 10CRC/ Rm B23500

NCI IRB
c/o Linda Adams
Bldg 82 (Bloch)/ Rm 115

Phone: 301-496-5457
Fax: 301-480-5439

9030 Old Georgetown Rd
Phone: 301-496-6375
Fax: 301-480-0106

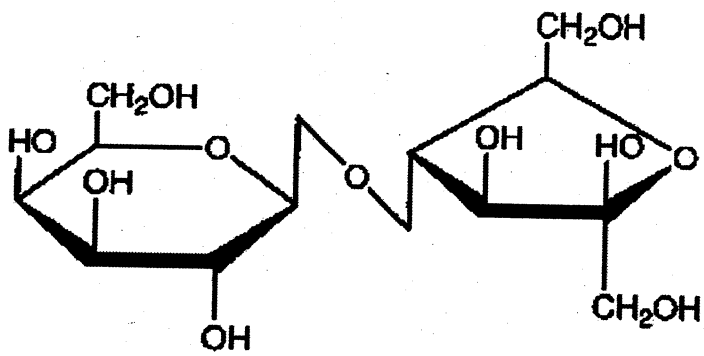
- In addition, the following reactions occurring during or within 30 days of active protocol treatment should be within 7 calendar days to Dr Deborah Citrin, MD the Principal Investigator, and to the NCI IRB
  - All life-threatening events (grade 4, Common Toxicity Criteria, version 2.0) which may be due to protocol therapy administration
  - All fatal events (grade 5, CTC version 2.0) while on study (or within 30 days of active treatment)
  - First occurrence of any previously unknown clinical event (regardless of grade)

8. PHARMACEUTICAL INFORMATION

Intestinal permeability tests are commercially available and contain lactulose and mannitol. The patent will fast over night for 8 hours and will then drink a "challenge" drink supplied by Genova Diagnostics composed of 5gm of lactulose and 1gm of mannitol.

8.1 Lactulose

- Chemical Information
  Chemical name: 4-O-beta-D-galactopyranosyl-D-fructofuranose
  Molecular Formula: $C_{12}H_{22}O_{11}$
  Molecular weight: 342.30
  Chemical structure:

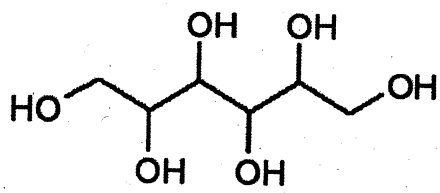


- Supplied by : commercially available from Genova Diagnostics
- Dosage and Administration: The lactulose and mannitol test is to be administered after at least a six hour fast. A glass of water is drunk just prior to ingesting the lactulose and mannitol and each hour for the first two hours. Urine is collected for six hours.
- Drug-related side effects: Diarrhea, gas, vomiting, cramping if ingested in significant quantities. Hypernatremia and hypokalemia after repeated dosing.

- Drug interactions: Lactulose is minimally absorbed and is primarily excreted in the feces or metabolized by enteric bacteria.
  - Acenocoumarol (moderate, probable)
  - Dicumarol (moderate, probable)
  - Droperidol (major, theoretical)
  - Levomethadyl (major, theoretical)
  - Licorice (major, theoretical)
  - Phenprocoumon (moderate, probable)
  - Warfarin (moderate, probable)
- Pregnancy category: B

8.2 Mannitol

- Chemical Information
  Chemical name: hexan-1,2,3,4,5,6-hexol
  Molecular Formula: $C_6H_{14}O_6$
  Molecular weight: 182.172
  Chemical structure:



- Supplied by : commercially available from Genova Diagnostics
- Dosage and Administration: The lactulose and mannitol test is to be administered after at least a six hour fast. A glass of water is drunk just prior to ingesting the lactulose and mannitol and each hour for the first two hours. Urine is collected for six hours.
- Drug-related side effects: typically with intravenous administration.
  - Cardiovascular: Chest pain, Hypotension, Palpitations, Tachyarrhythmia
  - Endocrine metabolic: Disorder of fluid AND/OR electrolyte, Hypernatremia, hyponatremia, hyperkalemia, acidosis
  - Gastrointestinal: Diarrhea (typically after greater than 20gm oral load), Nausea, Vomiting, Xerostomia
  - Neurologic: Headache, seizure
  - Respiratory: Rhinitis, pulmonary edema (rare)
  - Renal: Renal failure (rare), Urinary retention
- Drug interactions: Mannitol is a common food additive in candies for people with diabetes.
  - Arsenic Trioxide (major, theoretical)
  - Droperidol (major, theoretical)
  - Levomethadyl (major, theoretical)
  - Licorice (moderate, probable)
  - Sotalol (major, probable)
- Pregnancy category: C

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Appendis IA
Protocol #06-C-
Specimen Collection Flowsheet

Patient Name:_____ Tumor site:_____ XRT start date:_____

Gender: (M/F) Tumor histology: _____ XRT completion date:_____

Age (yrs):_____ Tumor stage:_____ HIV date:_____ Result: (+ / -)

Baseline GI fxn: _____ Date resection/ biopsy:_____ Hepatitis B/ C date:_____Result: (+ / -)

Other therapies/ abd surg/ dates:_____

Date										
Time										
XRT Target		**Baseline**[1]	**Prexrt**[1] **(0)**	**COT**	**1-month F/U**	**3-month F/U**	**6-month F/U**	**12-month F/U**	**24 month F/U**	**36 month F/U**
Blood										
ResearchEDTA*	10 cc	❑	❑	❑	❑	❑	❑	❑	❑	❑
Research SST*	24 cc (3 x 8cc)	❑	❑	❑	❑	❑	❑	❑	❑	❑
Clinical			2		2	2	2	2	2	2
Other										
Total blood (cc)										
Urine										
Research- cup*	45cc	❑	❑	❑	❑	❑	❑	❑	❑	❑
Other										
Stool	10gm	❑	❑	❑	❑	❑	❑	❑	❑	❑
Intestinal Permeability			❑		❑	❑				
(6hour urine)			❑		❑	❑				
PE		❑	❑	❑	❑	❑	❑	❑	❑	❑
RTOG		❑acute	❑acute	❑ acute	❑ acute	❑	❑	❑	❑	❑

[1]Baseline and pre-xrt may be the same if xrt is the initial treatment

[2] CBC, ALT, AST, total bilirubin, serum BUN, serum creatinine

*Deliver to Citrin lab
Bldg 10, Rm 3B42
PH301.496.5457 BP102-13475

Appendix IB. Specimen processing and storage

Urine

- **Note: Urine will remain on ice or at 4° Celsius until processed. Time to processing will be minimized.**
- Urine will be collected in a sterile collection cup in the ROB clinic (with the exception of urine collected for the intestinal permeability test which will be brought to the clinic by the patient in the previously provided collection jug).
- Urine will be transported on ice immediately to the laboratory of Deborah Citrin, M.D. Building 10, 3B42 for further processing
- Urine collected for the intestinal permeability assessment will be shipped by Dr Citrin's lab via federal express to Genova diagnostics without further processing.
- Urine for further analyses at NIH will be centrifuged at 1500 x g for 10 minutes at 4° Celsius
- The supernatant will be aliquoted into five 1 mL fractions in 1.5 mL tubes and two 5 mL aliquots and stored at -80° Celsius until use.
- The pellet of urine sediment will be resuspended in 100 uL of sterile PBS and stored at -80° Celsius.

Plasma

- Collect plasma in EDTA containing tubes and place on ice immediately
- Plasma will then be transferred to the laboratory of Deborah Citrin, M.D. Building 10, 3B42 for further processing
- Centrifuge at 1000 x g at 4° Celsius for 15 minutes **within 30 minutes of collection**, followed immediately by centrifugation at 10,000 x g at 4° Celsius for 10 minutes
- Aliquot immediately into at least four 500 uL aliquots and store at -80° Celsius

Serum

- Collect blood in serum separator tubes and allow sample to clot at room temperature for 30 minutes
- Serum will then be transferred to the laboratory of Deborah Citrin, M.D. Building 10, 3B42 for further processing
- Centrifuge at 1000 x g for 15 minutes
- Remove serum immediately, divide into 1 mL aliquots, and store at -80° Celsius

Stool

- Transport stool immediately to the laboratory of Dr. Deborah Citrin, M.D. Building 10, 3B42 for further processing
- Stool will immediately be divided into 180-220 mg aliquots, pH tested, and stored at -20° C until further use.

Appendix IIA: Specific Tumor Markers To Be Screened In Tumor By Disease Site. Positive Markers Will Then Be Screened In Serum, Urine, And Stool.

Site	Mutation	Aberrant methylation
Esophageal squamous cell carcinoma	**p53** (exons 5, 6, 7, 8, 9) [82]	**p16**[83] **MGMT**[84] **E-cadherin**[84]
Gastric and esophageal adenocarcinoma	**p53** (exons 5, 6, 7, 8, 9)[85]	**APC**[14, 86, 87] **E-cadherin**[13, 14, 86, 88] **hMLH1**[14] **TIMP3**[14] **p16**[13, 88]
Hepatobiliary (non-pancreas)	**K-ras** (KRM1, KRM2, KRM3, KRM4, RFLP)[89, 90] **p53** (exons 6, 7, 8)[89]	**p16**[91] **p14**[92] **TMS1/ASC**[93]
Pancreatic adenocarcinoma	**K-ras** (KRM1, KRM2, KRM3, KRM4)[94] **p53** (exons 5, 6, 7, 8)[95] **DPC4/smad4** (exons 8, 9, 10, 11) [96]	**p16**[91] **ppENK**[97]
Rectal adenocarcinoma	**p53** (exons 4, 5, 6, 7, 8)[98] **APC** (exon 15 cluster region)[98, 99] **K-ras** (KRM1, KRM2, KRM3, KRM4, RFLP)[98, 99]	**p16**[100, 101] **p14**[101] **vimentin exon 1** [102]

Appendix IIB Tumor Marker Prevalence

Pancreatic adenocarcinoma
K-ras – 95% [1] 75-90% [2]
p53 - 50-75% [1] 50-75% [2]
DPC4/smad4 - 55% [1] 45-55% [2]
p16 methylation – 15-20%[3] 52% [2]
ppENK methylation – 80-90% [4] [3]

Rectal adenocarcinoma
p53 – 30-40% [5] [6]
APC – 30-40% [5] [6]
K-ras – 30-40% [5] [6]
(One of K-ras, p53, APC – 75% [6])
p16 methylation – 25% [7], 34% in serrated adenoma [8]
p14 methylation – 83% in serrated adenoma [8] 13 % in colorectal ca [9]
APC methylation – 45%[9]
vimentin methylation – 53-83% in colon ca [10]

Esophageal squamous cell carcinoma
p53 - 10-85% [11]
p16 methylation – 56% [12]
MGMT methylation – 39% [12]
E-cadherin methylation – 70% [12]

Gastric and esophageal adenocarcinoma
p53 – 95% in EAC [11]
APC methylation – 70% in EAC [12]
E-cadherin methylation – 70% in EAC [12]
hMLH1 methylation – 12% in EAC [12]
TIMP3 methylation – 56% in EAC [12]
p16 methylation – 50% in EAC [11] 45% in EAC [12]

Hepatobiliary (non-pancreas)
K-ras - <10% in HCC [13]; 21-100% in cholangio [13]; 50% in cholangio [14]
p53 – 30-60% in HCC [13] ; 31-91% LOH or mutations in cholangio [13]; 30-37% in cholangio [15]
p16 methylation and **p14 methylation** – 83% in cholangio [16]
p16 methylation - 30-60% in HCC [17]
TMS1/ASC methylation – 36% in cholangio [18]

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APENDIX IIIA: RTOG - Acute Radiation Morbidity Scoring Criteria

Date: Patient Name: ID#

(circle grade)

Organ/tissue	0	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5
Esophagus	No change over baseline	Mild dysphagia or odynophagia/ may require topical anesthetic or non-narcotic analgesics/ may require soft diet	Moderate dysphagia or odynophagia/ may require narcotic analgesics/ may require puree or liquid diet	Severe dysphagia or odynophagia with dehydration or weight loss(>15% from pre-treatment baseline) requiring N-G feeding tube, I.V. fluids or hyperalimentation	Complete obstruction, ulceration, perforation, fistula	Death due directly to treatment
Upper GI	No change	Anorexia with <=5% weight loss from pretreatment baseline/ nausea not requiring antiemetics/ abdominal discomfort not requiring parasympathol ytic drugs or analgesics	Anorexia with <=15% weight loss from pretreatment baseline/nausea &/ or vomiting requiring antiemetics/ abdominal pain requiring analgesics	Anorexia with >15% weight loss from pretreatment baseline or requiring N-G tube or parenteral support. Nausea &/or vomiting requiring tube or parenteral support/abdominal pain, severe despite medication/hematemes is or melena/ abdominal distention (flat plate radiograph demonstrates distended bowel loops	Ileus, subacute or acute obstruction, perfomation, GI bleeding requiring transfusion/abdo minal pain requiring tube decompression or bowel diversion	
Lower GI including pelvis	No change	Increased frequency or change in quality of bowel habits not requiring medication/ rectal discomfort not requiring analgesics	Diarrhea requiring parasympatholy tic drugs (e.g., Lomotil)/ mucous discharge not necessitating sanitary pads/ rectal or abdominal pain requiring analgesics	Diarrhea requiring parenteral support/ severe mucous or blood discharge necessitating sanitary pags/abdominal distention (flat plate radiograph demonstrates distended bowel loops)	Acute or subacute obstruction, fistula or perforation; GI bleeding requiring transfusion; abdominal pain or tenesmus requiring tube decompression or bowel diversion	

GUIDELINES: The acute morbidity criteria are used to score/grade toxicity from radiation therapy. The critera are relevant from day 1, the commencement of therapy, through day 90. Therafter, the EORTC/RTOG Criteria of Late Effects are to be utilized.

The evaluator must attempt to discriminate between disease- and treatment-related signs and symptoms.

An accurate baseline evaluation prior to commencement of therapy is necessary.

All toxicities Grade 3, 4 or 5* must be verified by the Principal Investigator.

PHYSICIAN _____ DATE_____

APENDIX IIIB: RTOG/EORTC Late Radiation Morbidity Scoring Schema

Date: Patient Name: ID#

(circle grade)

Organ/tissue	0	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5
Esophagus	None	Mild fibrosis Slight difficulty in swallowing solids No pain on swallowing	Unable to take solid food normally Swallowing semi-solid food Dilatation may be indicated	Severe fibrosis Able to swallow only liquids May have pain on swallowing Dilation required	Necrosis/ Perforation Fistula	Death due directly to treatment
Intestine	None	Mild diarrhea Mild cramping Bowel movement 5 times daily Slight rectal discharge or bleeding	Moderate diarrhea and colic Bowel movement >5 times daily Excessive rectal mucus or intermittent bleeding	Obstruction or bleeding requiring surgery	Necrosis/ Perforation Fistula	
Liver	None	Mild lassitude Nausea, dyspepsia Slightly abnormal liver function	Moderate symptoms Some abnormal liver function tests Serum albumin normal	Disabling hepatitic insufficiency Liver function tests grossly abnormal Low albumin Edema or ascites	Necrosis/ Hepatic coma or encephalopathy	

PHYSICIAN _____ DATE_____

INSTITUTE: National Institute of Health

STUDY NUMBER: 07-C-0111 PRINCIPAL INVESTIGATOR: Deborah Citrin, M.D.

STUDY TITLE: A Pilot Study of Markers of Tumor Burden and Radiation Toxicity in the Blood, Urine, and Stool of Patients Receiving Radiotherapy for Gastrointestinal Malignacies

Latest IRB Review: Initial Review 1/8/07
Latest Amendment Approved: N/A Date Posted to Web: 2/23/07
Standard

INTRODUCTION

We invite you to take part in a research study at the National Institutes of Health (NIH).

First, we want you to know that:

Taking part in NIH research is entirely voluntary.

You may choose not to take part, or you may withdraw from the study at any time. In either case, you will not lose any benefits to which you are otherwise entitled. However, to receive care at the NIH, you must be taking part in a study or be under evaluation for study participation.

You may receive no benefit from taking part. The research may give us knowledge that may help people in the future.

Second, some people have personal, religious or ethical beliefs that may limit the kinds of medical or research treatments they would want to receive (such as blood transfusions). If you have such beliefs, please discuss them with your NIH doctors or research team before you agree to the study.

Now we will describe this research study. Before you decide to take part, please take as much time as you need to ask any questions and discuss this study with anyone at NIH, or with family, friends or your personal physician or other health professional.

Description of Research Study

You are being asked to take part in this study because you have a cancer in you gastrointestinal system that will be treated with radiation therapy. The Radiation Oncology Branch and its collaborators are conducting a variety of research studies that require obtaining tumor, stool, blood and urine samples from patients with your diagnosis who are receiving radiation therapy. The purpose of obtaining these samples is to perform studies that might allow us to develop a way to predict how cancer like yours will respond to radiation therapy or if people like you will develop side effects from radiation treatment. We currently do not have the ability to accurately predict these things. We wish to study the changes in your stool, blood and urine before, during and after the course of your radiation treatment . You will also be asked several questions regarding any possible side effects you may be having at these times. This will provide valuable information about the way different cancers respond to radiation and the risk of developing side effects. This information may help us

develop new tests to determine which patients are likely to develop side effects or which patients may need more or less aggressive therapy. Examples of planned studies include testing for fragments of tumor cells in the stool, urine, or blood and looking for changes in hormones and protein levels in your stool, blood, or urine after radiation. A total of 120 subjects will be enrolled in this study, which will be conducted at this site.

First, we will do an evaluation to see if you are eligible to participate in this study. This will include a physical examination, and routine blood laboratory studies. To be eligible for this study we must evaluate whether you require radiation therapy at this time. We must also be certain we have complete information about any past cancer treatments, if any.

There are several samples that will be collected during the course of this research study. They include the following, which are further explained in the paragraphs below.

1) **Tumor biopsy** – before any treatment or at the time of surgery if it is the first treatment
2) **Urine collection** – before, during, and after treatment and at followup visits.
3) **Stool (feces) collection** – before, during, and after treatment and at followup visits.
4) **Blood collection** – before, during, and after therapy and at followup visits.
5) **Intestinal permeability assessment** – Before any treatment, before radiation (if radiation is not the first treatment), one month after radiation is completed, and one three months after radiation is completed.

After entrance into this protocol, you may need to undergo a **biopsy** of your tumor, if enough tissue cannot be gathered from an already existing biopsy. This will not be performed if you are at high risk of complications from the biopsy. If there is not enough tumor tissue available from an already existing biopsy of your tumor to complete the tests for this study, another biopsy will be performed. The possible risks of this procedure will depend on the location of your tumor, but generally the risks include the possibility of bleeding from the biopsy site, infection at the biopsy site, or pain at the biopsy site. If any of these problems develop, you will receive treatment for any complication at the National Cancer Institute. Usually the biopsy is performed after giving you sedation or a numbing injection to prevent pain. The biopsy is usually performed with a needle to remove a small amount of tumor.

You will also be asked to give **samples of urine, stool, and blood** before, during and after your radiation treatment. At each time when blood is sampled, approximately 3 tablespoons of blood may be withdrawn from your veins. The procedure to draw blood may be uncomfortable though it is no different than having blood drawn for any other blood test. The amount of blood is relatively small in volume and represents about 5-10% of the volume of blood that is used in a normal blood donation. Urine and stool will be collected in sterile cups that will be provided to you. At different times during and after your treatment, physicians may again request that you give a urine, stool or a blood sample. Ideally, we would like to obtain stool, blood and urine before, at the completion of, and during routine follow up visits after your radiation treatment. At no time will blood be drawn from your veins if it is felt that this would jeopardize your health in any way.

As part of this study, you will be asked to complete **intestinal permeability tests**. This test determines how your intestines are working to absorb sugar and may give us more information about side effects from radiation treatments. The test will be performed at three or four times during this study depending on how your therapy is sequenced: prior to any treatment, prior to radiation (if radiation is not the first treatment), at your one month follow up visit after radiation treatments, and at your three month follow up visit after radiation treatments. This test is used frequently for patients with digestive diseases, but has not been tested in patients receiving radiation treatments. To perform this test, you will be asked to eat and drink nothing after midnight and then drink a small glass of provided sugars. For six hours you will

be asked to collect your urine in a contained that will be provided to you which will then be brought to the radiation oncology department. Rarely, patients may experience mild diarrhea, bloating, or crampy discomfort after taking this test. If that happens, we will provide you with medication to treat these symptoms.

Radiation therapy is required to be part of this study. However, the guidelines for radiation therapy are dictated by your primary research study or the current standard of care for your condition. Planned radiation therapy will not be altered by participation in this study.

Stool, blood and urine samples collected may be stored and used in the future to study scientific questions related to this protocol. If there are any risks to you/your child or your family associated with these scientific studies, which are not covered in this consent form, your consent will be obtained before such studies are performed.

Alternative Approaches or Treatments

Another option is not to participate in this study. If you decide not to go on this study, it will not alter your planned treatment.

Risks or Discomforts of Participation

There risks and discomforts on this study are related to the specific tests that are required. The risks and discomforts of **blood draws** for this research protocol are the same as for routine blood drawing. These include pain, swelling, or bruising at the needle puncture site. These are expected and temporary. In addition, there is a very small risk of fainting or of infection at the needle entry site.

Occasionally, patients who take the **intestinal permeability assessment** will develop mild bloating, cramping, or diarrhea which lasts less than one day.

The risks and discomforts of **biopsy** depend on the site of your tumor, but usually include the possibility of infection or bleeding. A biopsy will not be performed if it is determined that it would place you at significant risk.

It is possible that you may experience some, all, or none of the side effects described above. It is also possible that you may experience some side effects that we cannot anticipate. For that reason, you will be watched closely so that we can treat any side effects early.

Potential Benefits of Participation

The information we collect during the course of this study will not be used for decisions about the treatment of your disease. Therefore, there is no direct benefit to you. The information obtained from stool, blood and urine samples will be known and may be published without any reference to your identity. If any information that is gathered during the course of this study were later determined to have a potential benefit on your treatment, we would attempt to contact you to discuss this.

Research Subject's Rights

Participation in this research study is voluntary and you can withdraw at any time. We encourage you to ask questions so you can make the most informed decisions during your participation in this study. Refusal to participate will not result in penalty or loss of benefits to which you are otherwise entitled

Every attempt will be made to protect your identity while we work with your samples in the laboratory. The samples will be identified by a code that can be linked back to you by the investigators, but this information will not be available to those not involved in you care at the National Cancer Institute unless specifically agreed to by you.

Besides the tests described above, other research tests may be done on your blood and/ or biological samples if related to this protocol. Blood and tissue obtained during participation in this study will be stored for future studies related to this protocol. If any tests are planned that are not related to this protocol, you will be asked to provide consent and you will have the right to refuse. The investigators conducting this study do not plan to routinely provide you with the results of research tests if the significance of these results is not clear. If information is obtained during the initial evaluation in this study that may be important for your health, you will be informed. By agreeing to participate in this study, you do not waive any rights that you may have regarding access and disclosure of information contained in your records.

The costs of the studies done at the Clinical Center National Institutes of Health (NIH) in relation to this protocol will be done at no charge to you if you choose to participate in the is study. The NIH cannot reimburse you for the costs of medical care delivered outside the NIH. Similarly, you cannot be reimbursed f you choose to have diagnostic radiographic tests performed outside of the NIH, even if they are done as a consequence of participation in this study.

It is important to stress that being in this protocol does not promise long-term medical care hear at the NIH Clinical Center. If there is no further research study that is suitable for you and your state of disease, or if you are not currently on another research study, you will be returned to the care of your referring doctor or institution, or alternative sources of care closer to your home. If you have any questions about your treatment at NIH, you can contact the Principal Investigator, Dr Deborah Citrin (301-496-5457) or the patient care representative (301-496-2626).

OTHER PERTINENT INFORMATION

1. Confidentiality. When results of an NIH research study are reported in medical journals or at scientific meetings, the people who take part are not named and identified. In most cases, the NIH will not release any information about your research involvement without your written permission. However, if you sign a release of information form, for example, for an insurance company, the NIH will give the insurance company information from your medical record. This information might affect (either favorably or unfavorably) the willingness of the insurance company to sell you insurance.

The Federal Privacy Act protects the confidentiality of your NIH medical records. However, you should know that the Act allows release of some information from your medical record without your permission, for example, if it is required by the Food and Drug Administration (FDA), members of Congress, law enforcement officials, or other authorized people.

2. Policy Regarding Research-Related Injuries. The Clinical Center will provide short-term medical care for any injury resulting from your participation in research here. In general, no long-term medical care or financial compensation for research-related injuries will be provided by the National Institutes of Health, the Clinical Center, or the Federal Government. However, you have the right to pursue legal remedy if you believe that your injury justifies such action.

3. Payments. The amount of payment to research volunteers is guided by the National Institutes of Health policies. In general, patients are not paid for taking part in research studies at the National Institutes of Health.

4. Problems or Questions. If you have any problems or questions about this study, or about your rights as a research participant, or about any research-related injury, contact the Principal Investigator: Dr. Deborah Citrin, Building 10, CRC, Room B2-3500, Telephone: (301) 496-5457.

You may also call the Clinical Center Patient Representative at 301-496-2626.

5. Consent Document. Please keep a copy of this document in case you want to read it again.

COMPLETE APPROPRIATE ITEM(S) BELOW:

A. Adult Patient's Consent
I have read the explanation about this study and have been given the opportunity to discuss it and to ask questions. I hereby consent to take part in this study.

Signature of Adult Patient/Legal Representative _____ Date _____

B. Parent's Permission for Minor Patient.
I have read the explanation about this study and have been given the opportunity to discuss it and to ask questions. I hereby give permission for my child to take part in this study.
(Attach NIH 2514-2, Minor's Assent, if applicable.)

Signature of Parent(s)/Guardian _____ Date _____

C. Child's Verbal Assent (If Applicable)
The information in the above consent was described to my child and my child agrees to participate in the study.

Signature of Parent(s)/Guardian _____ Date _____

THIS CONSENT DOCUMENT HAS BEEN APPROVED FOR ENROLLMENT OF PARTICIPANTS FROM JANUARY 8, 2007 THROUGH JANUARY 7, 2008.

Signature of Investigator _____ Date _____ Signature of Witness _____ Date _____