EASTERN COOPERATIVE ONCOLOGY GROUP

Laboratory/Clinical Correlative Studies in Non-Small Cell Lung Cancer

Ancillary Study to E3590
(RTOG 91-05; SWOG 9252; CALGB 9393; NCCTG 91-24-51; INT 0115)

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STUDY ACTIVATED
August, 1993

Addendum #1, 5/94: Added collection of EM-fixed and frozen samples; revised minimum requirement for paraffin samples (Schema, Sections 1.8, 2.3, 3.13, 3.23, 4.0, 5.0, 7.0, 8.1, Appendix II); added neuroendocrine differentiation (Sections 1.0, 1.5, 2.1, 6.1); routing of 1 paraffin block revised (Schema, Section 5.31); revised consent requirements (Sections 3.12, 5.11, 9.0); RTOG added as participant (Title, index, Section 4.3, 5.3, 8.0); PIN contact updated; renumbered sections and references as needed; revised pathology submission guidelines (Appendix II); eligibility checklist revised.

Addendum #2, 6/94: NCCTG added as participant (Title, Index, Sections 4.4, 5.3, 8.0); Form 310 replaced Form 596 (Section 4.0); SWOG submission of ECOG checklist added (Section 4.2, 8.2); renumbered sections as needed.

Addendum #3, 9/94: Submission of 1 paraffin block mandatory for ECOG institutions (Sec. 3.0); Intergroup Study numbers added (Sec. 3.11, 4.0); Retrospective study entry updated (Sec. 3.2); Patient Consent clarified (Sec. 9.0).

Addendum #4, 10/94: CALGB added as participant (Index, Sections 3.11, 3.21, 4.0, 4.5, 5.31, 8.0, path memo ); Intergroup study numbers added (Sec. 1.0, 3.1, 4.0); renumbered sections as needed.

Addendum #5, 6/95: Updated PIN Contact (Index); Clarify CALGB block submission (Section 5.3); Updated ECOG Coordinating Center and PCO address (Sections 4.1, 5.31-5.32)
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Appendix I Suggested Patient Consent
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1.0 INTRODUCTION

Lung cancer is the major cause of cancer-related death in the United States today, accounting for over 112,000 deaths per year (1). A number of recent developments in tumor and molecular biology have provided insights into the pathogenesis, progression, and natural history of this disease (2,3). These advances suggest that the mutational inactivation of tumor suppressor genes, such as p53 (4), or expression or activation of certain oncogenes, such as K-ras (5,6), are important in the pathogenesis of non-small cell lung cancer (NSCLC). In addition, expression of blood-group antigen A and the epidermal growth factor receptor (EGFR) (7) have been found to be a favorable prognostic factor in NSCLC (8). However, these studies have been done either on human lung cancer cell lines, or lung cancer tissue obtained retrospectively. These studies have also been done on different tumor samples obtained from different patients, and, therefore, the relationship of these markers to each other have not been determined in the same tumor samples. Markers of differentiation (nuclear proliferation, tumor vascularity, and neuroendocrine differentiation), which have also been found to correlate with prognosis in other tumor types, have not been studied in this disease (2,3). In order to determine the role these markers play in the pathogenesis of non-small cell lung cancer, or their importance as prognostic factors, these markers must be studied prospectively in a homogeneous group of lung cancer patients undergoing a defined treatment.

E3590, "Prospective Randomized Trial of Postoperative Adjuvant Therapy in Patients with Completely Resected Stage II and Stage IIIA Non-Small Cell Lung Cancer," is an Eastern Cooperative Oncology Group (ECOG) trial in which patients undergoing a thoracotomy will have a complete resection of their tumor and a complete intraoperative mediastinal node dissection or nodal sampling, followed by randomization to radiotherapy vs. chemotherapy plus radiotherapy. This high priority study (E3590, RTOG 91-05, SWOG 9252, CALGB 9393, NCCTG 91-24-51, INT 0115) offers a unique opportunity to prospectively determine the relevance of these molecular and cellular alterations to each other and to the outcome of patients with resectable NSCLC. The active and integral participation of thoracic surgeons on this trial, which is expected to accrue 465 patients over 3.5 years, provides a unique opportunity to obtain sufficient lung cancer tissue for therapeutic laboratory correlative studies. These correlates will ultimately lead to the development of new treatment strategies and identification of patients at high risk for relapse.

1.1 K-ras

Oncogenes of the ras family have been implicated in the pathogenesis of a wide variety of human and animal tumors (9). These genes (K-, H-, and N-) encode for membrane-bound 21-kDa proteins with GTP-binding activity and are postulated to have an important role in the cellular signal production pathway. The ras proteins acquire transforming potential when point mutations in the genes lead to amino acid substitutions in one of the 12, 13, 59 or 61 positions, presumably because alterations at these sites are associated with loss of the intrinsic GTPase activity (9,10). The frequency at which ras mutations in human cancer are found depends upon the organ of origin, being rare in squamous cell carcinomas and adenocarcinomas of the breast, stomach and ovary, and occurring in 30-50% of adenocarcinomas of the lung and colon (6,11-15) and in almost all pancreatic cancers (16).

The incidence and prognostic significance of K-ras mutations in lung cancer have been looked at in a number of retrospective series. In lung cancer, these mutations occur primarily in codon 12, and are found more often in adenocarcinomas obtained from smokers than nonsmokers (17). Presence of the K-ras mutation has been found to be a strong and negative prognostic factor, associated with early relapse and shortened survival (5,18-20).

These studies generally are retrospective, involve small numbers of tumors (typically less than 50) (17), or utilize frozen tumor specimens or cell lines obtained over a period of many years (15,18,19,21), or from patients who received a number of different, ill-defined treatments (14,5). Thus, these results need to be confirmed in a prospective trial involving a homogeneous group of patients to determine if K-ras mutations can be used to identify poor prognosis NSCLC patients who are candidates for adjuvant chemotherapy.

1.2 p53
p53 was first discovered as a 53 kDa nuclear phosphorylated protein which coprecipitates with the large T antigen of the SV40 virus (22). Although originally thought to be a classical oncogene, more recent findings suggest that the wild type p53 gene may function normally as a tumor suppressor gene, and that a mutant p53 gene could promote transformation by inactivating normal p53 function in a dominant negative fashion (23,24). A number of different point mutations have been observed in the highly conserved region of the open reading frame in a variety of common cancers, such as lung, colon, and breast cancer (25,26). Mutant p53 proteins have an increased half life that gives rise to high steady-state levels of the protein and is detected in many tumors (27).

In resected lung cancers, point mutations of the p53 gene have been found in all histological types, including approximately 45% of resected NSCLC and even more frequently in SCLC (4,28,29). Seventy-four percent of NSCLC cell lines have a mutation of the p53 gene (30). In early stage, primary, resected NSCLC specimens, mutations are distributed between codons 132 to 283 (4). However, the p53 gene mutations in NSCLC cell lines are diverse in the location and nature of the mutations (30).

Similar to that observed with K-ras mutations, p53 mutations have been retrospectively correlated with clinical features. These studies have shown that p53 mutations are associated with younger age and squamous histology, but not sex, tumor stage, or nodal status (4). One study using NSCLC cell lines failed to identify an association with sex or histologic type (30). In addition, no association was observed with age, treatment intent, neuroendocrine differentiation, prior chemotherapy, or patient survival.

The interaction and cooperation of mutant p53 and K-ras has been postulated to be an important determinant of tumor progression. p53 and ras genes have been found to be frequently, but not always, mutated in the same lung carcinoma cell lines (31). However, the prevalence of p53 mutations in NSCLC cell lines with ras mutations did not differ from that in cell lines without ras mutations (30). Thus, additional data is needed to determine if multiple genetic changes, involving both proto-oncogenes and tumor suppressor genes, are needed for lung carcinogenesis.

1.3 Prognostic Value of Blood Group Antigen Expression in NSCLC

Altered expression of ABH blood group antigen has been observed in association with carcinogenesis and tumor progression (32,33). Recently, Lee et al. reported that loss of blood group antigen A (BGAA) expression in the primary tumor was an important prognostic factor among patients with NSCLC whose blood types were either A or AB (8). Although other studies had previously suggested that loss of blood group ABH or Le¹ antigen expression was associated with more aggressive tumor behavior in other types of cancer (34-42), the study done by Lee et al. was the first study demonstrating the definite evidence of such association. This finding is now independently confirmed by Dr. S.I. Hakomori and colleagues (43). They used an MIA-15-5 antibody recognizing the
H/Le\textsuperscript{a}/Le\textsuperscript{b} antigen, which is the predominant form of antigen present in blood group antigen A- or B-negative tumor cells (44). In their study, positive immunostaining of tumor cells was associated with poor prognosis in patients with NSCLC, most significantly in patients with type A or AB blood, confirming our original findings. Of further interest are the findings that this particular antibody had strong inhibitory effects on cell motility and the metastatic potential (45).

1.4 EGFR Expression in NSCLC

The EGFR is a 170-kD glycoprotein with intrinsic tyrosine kinase activity (46) that has a striking homology to the transforming protein of the v-erb-B oncogene of the avian erythroblastosis virus (47). In lung cancer, using an immunohistochemical technique, Cerny et al. reported positive EGFR expression in 36 of 42 squamous cell carcinomas and in 4 of 5 nonsquamous cell carcinomas, but in none of 15 small cell carcinomas (48). In the subsequent study, Veale et al. showed that 67% (27/40) of squamous cell carcinomas and 45% (9/20) of adenocarcinomas stained strongly or moderately strongly positive for EGFR (49). In another study using the same EGFR1 monoclonal antibody, Berger et al. reported EGFR immunostaining in 74% (29/39) of squamous cell carcinomas, 34% (12/35) of adenocarcinomas, and 33% (5/15) of large cell carcinomas, while zero out of 20 small cell carcinomas and carcinoid tumors stained positive (50).

Several lines of evidence suggest that the EGFR-related growth regulatory system is involved in both normal and neoplastic cellular proliferation (51). Enhanced EGFR levels have been reported to be associated with metastatic potential and poor prognosis in breast cancer (52-57), and with the degree of invasiveness in bladder cancer (58) and stomach cancer (59-61). In patients with NSCLC, enhanced EGFR expression has been suggested to be associated with advanced tumor stage (62) and possibly with shorter survival (63). Most of these studies were, however, undertaken using fresh frozen tissue sections, and little attempt was made to analyze whether EGFR status was an independent prognostic variable or a mere reflection of already known prognostic factors.

1.5 Neuroendocrine Differentiation

Up to 12% of large cell carcinomas may have neuroendocrine differentiation. In a recent report, carcinomas expressing neuroendocrine differentiation by immunohistochemistry or electron microscopy were more sensitive to chemotherapy than other NSCLC tumors, suggesting that this subgroup should be separately stratified (64).

Neuroendocrine differentiation in most tumors can be determined using markers of neuroendocrine function including: neuron-specific enolase, gastrin-releasing peptide (GRP), chromogranin A or the messenger RNA for chromogranin A, L-dopa decarboxylase and a brain isoenzyme of creatinine kinase (65-69). Since such markers are commonly expressed in small cell lung cancer, it would be desirable to know whether they are also expressed in large cell neuroendocrine tumors. However, tumors must be determined to be of neuroendocrine type before such studies should be performed (64). Thus, it is desirable to perform ultrastructural examination to subclassify the tumors prior to any immunocytochemical evaluation.

1.6 p105 Nuclear Antigen Levels

Since approximately 85% of carcinomas are aneuploid, information about aneuploidy of these tumors may not be useful in clinical cooperative trials (70, 71). However, since the proportion of tumors responding to chemotherapy and/or radiotherapy may be drastically
different for tumors expressing different percentages of growth fractions, it would be highly desirable to correlate indices of proliferation with DNA ploidy and tumor histologic type as determined ultrastructurally. Monoclonal antibodies have been developed that recognize nuclear antigens specific for proliferation (p105, cyclin, KI-67). These can be applied to tissues or cells and examined by immunocytochemical or flow cytometric techniques. The p105 assay utilizes a mouse monoclonal antibody (780-3) against p105 which is a proliferation-associated nuclear antigen and identifies cells in the G-1, S, and G-2M phases of the cell cycle but does not react with resting cells in G-0. The percentage of tumor cells positive for p105 is a measure of the growth fraction. Since this assay can be reliably performed on routinely processed paraffin-embedded tissue samples from tumors, it is easy to incorporate in a normal flow cytometric assay for DNA ploidy (72-76).

1.7 Tumor Vascularity

Tumor vascularity has been shown in studies of breast carcinoma to correlate closely with metastatic potential (77). It has been observed by Yesner that small cell lung cancers with high mitotic rates have densely ramifying vessels (78). It is desirable to determine whether or not the density of vessels in non-small cell lung cancer would similarly correlate with metastatic potential. This can be easily measured in quantitative fashion using a mouse monoclonal antibody to Factor-8-related antigen (Von Willebrand factor) which is present on all the vascular endothelium (77). It is possible to quantify such proliferating vessels by counting them or by using morphometry or image analysis (79).

1.8 Study Rationale

The prognosis for patients with Stage II or III NSCLC as a group is poor. Recent studies, however, have suggested that adjuvant treatment may benefit these patients (80, 81). The endpoints that we are evaluating may identify a group of patients at high risk for developing recurrent disease who are more appropriate candidates for adjuvant therapy.

This study will allow us to: 1) prospectively correlate the expression of biological or molecular markers postulated to be relevant to human lung cancer with patient stage and outcome in a defined group of patients; 2) determine the relationship of these markers to each other within the same tumor sample; and 3) establish a central tissue repository that will allow us to study future parameters found to be relevant to NSCLC.

2.0 OBJECTIVES

2.1 Determine the incidence of K-ras and p53 mutations; assess Group A blood antigen and EGF receptor levels; evaluate neuroendocrine markers on keratin-negative, mucin-negative tumors by electron microscopy; and assess p105 and Factor 8 levels in patients with completely resected Stage II or IIIA NSCLC.

2.2 Correlate these results with patient histology, TNM stage, time to relapse, and survival.

2.21 To estimate the cut-point values for different markers that define risk groups.

2.3 Establish within ECOG a central tissue repository.
3.0 Eligibility Criteria

3.1 Eligibility for patients not currently randomized to E3590.

3.11 Eligible patients entering E3590 are eligible for this study.

ECOG INSTITUTIONS NOTE: ECOG patients must be registered to E4592 at the time they are registered to the parent protocol E3590. Submission of one (1) paraffin block of primary tumor or a portion of tumor tissue removed directly from the block (see Section 5.222) is mandatory.

SWOG/RTOG/NCCTG/CALGB NOTE: Registration to E4592 should be done at the time of randomization to E3590/SWOG 9252/RTOG 91-05/NCCTG 91-24-51/CALGB 9393/INT 0115. When entering patients on E3590, investigators will be given the opportunity to enter patients on this ancillary laboratory study.

3.12 Must give written informed consent (See Appendix I).

3.13 Must have minimum tissue requirements of one (1) paraffin block of primary tumor or a portion of tumor tissue removed directly from the block (see Section 5.222) and/or fresh tissue of primary tumor for submission of snap-frozen and EM-fixed specimens.

NOTE: The following are highly desirable but not mandatory (see Section 5.223):
- Two (rather than 1) paraffin blocks of primary tumor tissue.
- Two (rather than 1) paraffin blocks of normal lung tissue or normal lymph node.
- One, 2mm section each of tumor and normal lung tissue in EM fixative.
- At least three, 2mm sections snap frozen, of tumor and normal lung tissue or normal lymph node.

The institutional pathologist must be informed that paraffin blocks may be depleted. In addition, at completion of the study, unused portions of paraffin-embedded material will be retained at the ECOG Central Tissue Repository for use in future studies. This material will not be returned unless requested.

3.2 Eligibility for patients already receiving treatment on E3590:

3.21 SWOG, RTOG, NCCTG and CALGB patients already randomized to and participating in E3590 may be retrospectively entered on E4592 if adequate paraffin-embedded tissue is available (see Section 3.23).

ECOG patients randomized to and participating in E3590 prior to Addendum #3 may be retrospectively entered on E4592 if adequate paraffin-embedded tissue is available (see Section 3.23).

3.22 Patients entered retrospectively on this study will have previously given permission to enter parent therapeutic protocol E3590, which informs them that histopathologic material may be sent to a central office for review. It is at the discretion of the individual IRB whether additional consent must be obtained for these patients (see Section 9.0).

3.23 Must have minimum tissue requirements of one (1) paraffin block of primary tumor or a portion of tumor tissue removed directly from the block (see Section 5.222) and/or fresh tissue of primary tumor for submission of snap-frozen and EM-fixed specimens.
NOTE: One (1) paraffin block of normal lung tissue or normal lymph node are highly desirable, but not mandatory.

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The institutional pathologist must be informed that paraffin blocks may be depleted. In addition, at completion of the study, unused portions of paraffin-embedded material will be retained at the ECOG Central Tissue Repository for use in future studies. This material will not be returned unless requested.
4.0 REGISTRATION PROCEDURES

PATIENTS MUST BE RANDOMIZED TO THE PARENT PROTOCOL, E3590/SWOG 9252/RTOG 91-05/NCCTG 91-24-51/CALGB 9393/INT 0115, BEFORE THEY CAN BE REGISTERED TO E4592.

ALL APPROPRIATE TISSUE SAMPLES SHOULD BE COLLECTED AT THE TIME THE PATIENT UNDERGOES THORACOTOMY (SEE SECTION 5.0). SAMPLES SHOULD BE HELD AT THE INSTITUTION UNTIL THE PATIENT IS REGISTERED TO E4592. AT THAT POINT, SAMPLES SHOULD BE SUBMITTED ACCORDING TO SECTION 5.3.

4.1 ECOG Registration

A signed HHS 310 Form must be on file at the ECOG Operations Office before an ECOG institution may enter patients. The signed HHS 310 Form will be submitted to the following address:

ECOG Coordinating Center
Frontier Science
C/o Quality Control Office
303 Boylston Street
Brookline, MA 02146-7648

Patient samples should not be submitted prior to registration.

To register a patient, the investigator will telephone the Central Randomization Desk at the ECOG Coordinating Center, (617) 632-2022. The following information will be requested:

4.11 Protocol Number

4.12 Investigator Identification

4.121 Institution name and/or affiliate
4.122 Investigator’s name

4.13 Patient Identification

4.131 Patient’s name or initials and chart number
4.132 Patient’s Social Security number
4.133 Patient Demographics
4.1331 Sex
4.1332 Birthdate (MM/YY)
4.1333 Race
4.1334 Nine-digit zip code
4.1335 Method of payment
4.134 Patient sequence number for E3590

4.135 Type of fixative used for paraffin-embedded sample.

4.14 Eligibility Verification

Patients must meet all of the eligibility requirements listed in Section 3.0. An eligibility checklist has been appended to the protocol. The randomization specialist will verify eligibility by asking questions from the checklist.

A confirmation of registration will be forwarded by the ECOG Statistical Center Data Management Office.

4.2 SWOG Registration

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Note: A signed HHS 310 Form for this protocol must be on file at the SWOG Operations Office before any SWOG institution may enter a patient.

SWOG Investigators will telephone the SWOG Statistical Center at (206) 667-4623 between the hours of 6:30 a.m. and 2:00 p.m. Pacific time, Monday through Friday. The SWOG Statistical Center will obtain and confirm all eligibility criteria and information as per Section 4.11 - 4.13. The SWOG Statistical Center will then contact the ECOG Randomization Desk to enter the patient. The ECOG Statistical Center Data Management Office will forward a confirmation of registration and an ECOG sequence number to the SWOG Statistical Center for routing to the participating SWOG institution. SWOG institutions should submit the ECOG eligibility checklist directly to the ECOG Data Management Office.

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4.3 RTOG Registration

RTOG institutions will register a patient by faxing (215-928-0153) the completed current version of the Eligibility Checklist to RTOG Headquarters. This material must be transmitted between the hours of 8:30 a.m. and 4:00 p.m. Eastern Time, Monday through Friday. All items must be completed. RTOG will call the RTOG institution after RTOG obtains the group ID numbers from the ECOG Statistical Center Data Management Office. The ECOG Statistical Center Data Management Office will forward a confirmation of registration and an ECOG sequence number to RTOG for routing to the participating RTOG institution.

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4.4 NCCTG Registration

Note: A signed HHS 310 form for this protocol is to be on file at the NCCTG Randomization Center before any NCCTG institution may enter a patient. This study meets the criteria for expedited institutional review board procedure.

To register a patient, call the NCCTG Randomization Center (507-284-4130) 8 a.m. to 4 p.m. central standard time Monday through Friday. The NCCTG Randomization Center will verify eligibility by completing the eligibility checklist and will call the ECOG Statistical Center Data Management Office (617-632-2022) to enter the patient. The NCCTG Randomization Center will then contact the institution to verify patient entry.
ALL APPROPRIATE TISSUE SAMPLES SHOULD BE COLLECTED AT THE TIME THE PATIENT UNDERGOES THORACOTOMY. SAMPLES SHOULD BE HELD AT THE INSTITUTION UNTIL THE PATIENT IS REGISTERED TO E4592. AT THAT POINT, SAMPLES SHOULD BE SUBMITTED ACCORDING TO SECTION 5.3.
5.2 Sample Collection and Processing (see Appendix II for Pathology Submission Guidelines)

5.21 Collection of Tissue

This protocol is open to any institution participating in the parent protocol E3590. To minimize expenses, starter kits will be provided to the following institutions who are the biggest ECOG accruers to E3590:

- Albany Medical College
- Fox Chase Cancer Center
- H. Lee Moffitt Cancer Center
- Johns Hopkins University
- Marshfield CCOP
- Mayo Clinic
- New York University
- Rush-Pres-St. Luke's Medical Center
- University of Pennsylvania
- University of Pittsburgh
- University of Rochester
- University of Wisconsin
- Vanderbilt University

All other institutions may obtain a starter kit by contacting Karen Jankowski (608-256-1901 ext. 7818) at the University of Wisconsin.

5.22 Tissue Processing

The pathologist should divide both the tumor and normal lung tissue/normal lymph nodes into three sections:

5.221 The first section of each should go to the institution's pathology department for routine histology.

5.222 The second section of tumor and normal lung tissue/normal lymph node should be paraffin-embedded and sent to the ECOG PCO via regular mail (see Section 5.31 and Appendix II). Note: Minimum tissue requirement is at least one paraffin block of primary tumor. If there are institutional difficulties in obtaining the entire block, a piece of tumor tissue removed directly from the block will be accepted (i.e., the block may be cut in half and that portion submitted). The one paraffin block of normal lung tissue/normal lymph node is highly desirable but not required.
Questions regarding tissue preparation should be directed to Dr. Elizabeth Hammond (801-321-1029), Dr. Seena Aisner (301-328-5555), or Dr. Joan Schiller (608-263-8600).

Blocks of tumor tissue sent to the PCO should be preselected by a pathologist to insure that at least 15% of the tissue area is composed of tumor cells and that a 400 micron depth of tissue is available. The institution pathologist should review the corresponding hematoxylin and eosin (H&E) slide to insure that the block is representative and has minimal necrosis, hemorrhage, or fibrosis. In instances where blocks contain tumor only in specific areas, the technician can dissect out the indicated areas of the block to maximize recovery of tumor tissue. This selection technique is also useful for obtaining both normal and neoplastic tissue from the same block.

In addition to a block of the patient's tumor, it is strongly recommended that a normal tissue control be obtained. Ideally, this would be non-malignant tissue from the same patient. If non-malignant lung tissue is to be used as a control, it must have a cellular epithelial component. An uninvolved lymph node from the cancer dissection can also be a control tissue. If a lymph node is submitted, it should be uniformly well fixed. Poor fixation can adversely affect the quality of several types of assays (immunoperoxidase, DNA flow cytometry). If fixation is inconsistent or poor in the lymph node or any other block, an alternate block should be selected. Blocks should be clearly labeled as either tumor or normal lung tissue/normal lymph node.

If adequate tumor and normal tissue are available, the final section of each should be dissected into several (at least 4-6) 2mm sections.

5.223 EM Fixative

Requires one, 2mm section of tumor. One, 2mm section of normal lung tissue or lymph node is desirable.

Use the kits labeled EM fixative. These kits contain: a sample collection bag, 4% Buffered Formaldehyde, cotton, a styrofoam container, and instructions.

4% Buffered Formaldehyde will be supplied to the institution. It can be stored at room temperature. The tissue must be minced into 1 mm fragments by the pathologist and placed in the 4% Buffered Formaldehyde. Samples should be sealed in a whirl-pak plastic bag (provided with the kit) and sent via regular mail to the ECOG Pathology Coordinating Office (PCO) (See Section 5.32)
5.2232 Snap-Frozen

Requires at least three to five 2mm sections of tumor. Three to five 2mm sections of normal lung tissue or lymph node are desirable.

Use the kit labeled "frozen." This kit contains: a sample collection bag, cotton, styrofoam container, instructions, and a preprinted Federal Express form.

The lung tumor and normal tissue should be dissected into at least three to five 2mm sections. After the fresh specimen is dissected, it is frozen, either in liquid nitrogen, or at -70°C, and stored in a -70°C, or lower, freezer until shipment. Alternatively, the specimen can be placed in a cryostat at -20/-30°C for a short holding period prior to storage in a -70°C freezer. Ideally, the tissue should be frozen in the operating room. However, it is acceptable to freeze the tissue in the Pathology Lab.

The three to five 2mm sections should be frozen and sent via Federal Express to the E3590 Lung Tissue Repository located at the UWCCC (see Section 5.33).

NOTE: All frozen specimens should be shipped to the laboratory in a styrofoam container on dry ice and promptly placed into a -70°C, or lower, freezer until ready for assay. Storing at 4°C or -20°C is not sufficient. Because cell membrane integrity is generally compromised in specimens frozen in this manner, these samples are suitable only for nuclear analyses.

5.3 Tissue Distribution

The Patient Data Form (Appendix II) and a copy of the institutional pathology report should be sent with each specimen to the location indicated below. Samples should be clearly labeled as either tumor or normal lung tissue/normal lymph node.

At completion of the study, unused portions of paraffin-embedded material will be retained at the ECOG Central Tissue Repository for use in future studies. This material will not be returned unless requested.

NOTE: SWOG, RTOG and NCCTG institutions should also submit materials as indicated below.

NOTE: RTOG institutions must also submit copies of the Patient Data Form and the Pathology Department Report to RTOG Headquarters.
5.31 **Paraffin Blocks**

Paraffin-embedded tissue should be sent to the ECOG Pathology Coordinating Office (PCO) for handling:

Pathology Coordinating Office  
ECOG Operations Office  
AMC Cancer Research Center  
1600 Pierce Street  
Denver, CO  80214

Unless the blocks will be exposed to intense heat, ambient temperature is usually satisfactory for shipping and storing.

**NOTE:** CALGB participants should submit paraffin-embedded tissue to the CALGB Pathology Office for handling. Blocks will be forwarded to the ECOG Pathology Coordinating Office (PCO).

Maurice Barcos, M.D., Ph.D.  
Pathology Coordinating Office  
Roswell Park Memorial Institute  
666 Elm Street  
Buffalo, NY 14263  
Tel: 716-845-4443, Fax: 716-845-8077  
The blocks will be distributed from the PCO as follows:

5.311 One set of blocks will be sent to Dr. Lee's and Dr. Ro's lab at M.D. Anderson for analysis of blood group antigens and EGF receptors.

5.312 The other set of blocks will be shipped to Dr. Schiller's lab at the University of Wisconsin for K-ras and p53 analysis.

5.313 Remaining tissue will be returned to the PCO, and will be forwarded to Dr. Hammond at the University of Utah for p105 and Factor 8 analysis.

If there is any tissue left over, it will be sent to the Tissue Repository at the ECOG PCO for storage, unless the institution requests return.

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The 2mm sections of tumor and normal lung tissue/normal lymph node (prepared according to Section 5.2231) in EM fixative should be mailed via regular mail to:

Pathology Coordinating Office  
ECOG Operations Office  
AMC Cancer Research Center  
1600 Pierce Street  
Denver, CO  80214

The PCO will then send the block to Dr. Hammond at the University of Utah for evaluation of neuroendocrine markers.
5.33 Snap-Frozen

The three to five 2mm \textit{frozen} sections (prepared according to Section 5.2232) should be sent via Federal Express to the E3590 Lung Tissue Repository via Dr. Joan Schiller's laboratory:

Joan H. Schiller, M.D.
University of Wisconsin
Comprehensive Cancer Center
K4/666 Clinical Science Center
600 Highland Avenue
Madison, WI 53792

Labels and prepaid, typed Federal Express forms are provided by the Comprehensive Cancer Center.

Before sending, please contact Karen Jankowski at the University of Wisconsin Comprehensive Cancer Center (608-256-1901, ext. 7818) to indicate you are shipping a lung cancer specimen, and to provide her with the Federal Express airbill number. Do not ship on Fridays or the day before a holiday.

6.1 Neuroendocrine, Aneuploidy, and Tumor Vascularity Studies

These studies will be conducted by Dr. Elizabeth Hammond at the University of Utah at the LDS Hospital.

6.11 Neuroendocrine Markers

Tissue to be examined ultrastructurally will include viable tumor determined by the pathologist at the examining institution. This tumor will be fixed in 4% Buffered Formaldehyde at the institution where the thoracotomy was performed.
Upon receipt at LDS hospital, one portion of the sample will be fixed in osmium tetroxide, dehydrated through graded acetones, and embedded in EMBED 812. The other portion of the sample will not be osmicated, but will be embedded in LR white resin for possible immuno gold labeling studies after the regular ultrastructural evaluation is performed. One-micron-thick sections of blocks will be examined at LDS Hospital and compared to the available H&E stained section of the tumor. Representative samples (at least two) will be thin-sectioned at 700 angstroms, stained with lead citrate and uranyl acetate, and examined in a JEOL 100 SX electron microscope. Representative electron micrographs will be taken by Dr. Hammond, who will also then render a pathological diagnosis on the ultrastructural features of the tumor.

6.12  **p105 Nuclear Antigen Levels**

p105 nuclear antigen levels will be examined by flow cytometry.

6.13  **Tumor Vascularity Studies**

For assessments of vascularity, 4-micron-thick sections of paraffinized tissue, from the same block used for DNA analysis, will be stained by standard immunoperoxidase techniques using a codon immunoperoxidase machine and mouse monoclonal antibodies against Factor-8-related antigen. Diaminobenzidine will be used as a chromogen, and hematoxylin will be used as a counter stain. A control slide run with each batch of cells to demonstrate reproducibility of the degree of staining will include a hemangioma similarly processed and stained. Slides prepared in this way will be evaluated using the Q2 image analysis system.

6.2  **K-ras and p53 Analysis**

These studies will be conducted by Dr. Joan Schiller at the University of Wisconsin Comprehensive Cancer Center.

6.21  **Polymerase Chain Reaction (PCR) Amplification of K-ras and p53 Specific Sequences**

6.211  **Isolation of DNA**

DNA will be amplified directly by PCR reactions.

6.212  **K-ras**

Amplification of ras-specific sequences of DNA from paraffin embedded tumor samples by PCR, followed by oligonucleotide hybridization, has been commonly used to screen and detect K-ras mutations in large numbers of clinical samples (6,14-15,17-18). We plan to analyze tumor samples, and, when available, corresponding normal lung tissue, for mutations at codons 12, 13, and 61 using this technique.
6.213 **p53**

To analyze p53 mutations, we will amplify the following three fragments: a 408 base pair p53 gene fragment containing exons 5 and 6; a 139 base pair p53 fragment containing exon 7; or a 330 base pair p53 gene fragment containing exons 8 and 9. These three fragments span the highly conserved domains of p53 in which the majority of mutations (98% of 280 base substitution mutations) associated with various human carcinomas have been localized (26).

6.22 **Differential Allele-Specific Oligonucleotide Hybridization for K-ras Analysis**

With this technique, point mutations are detected by differential hybridization with $^{32}$P-labeled oligonucleotide probes specific for mutant or “wild-type” sequences of the K-ras gene spanning codons 12, 13, and 61.

6.23 **Single Strand Conformation Polymorphism (SSCP) Analysis of p53 Mutations**

SSCP takes advantage of the fact that a point mutation in an amplified fragment of DNA causes a significant conformational change and that this change can be detected by the difference in mobility in non-denaturing acrylamide gels. This method has been shown to detect about 90% of p53 mutations in NSCLC by nucleotide sequence analysis of duplicate samples (82).

6.24 **Nucleic Acid Sequencing**

Nucleic acid sequencing will be conducted on tumors that have p53 mutations by SSCP in order to identify the mutation.

6.25 **Immunohistochemistry Analysis of Mutant p53**

The monoclonal antibody PAab1801 (Ab-2; Oncogene Science, Manhasset, NY) has been used to determine the expression of p53 in mesothelioma and endometrial cancer (83). This antibody recognizes a linear epitope in human p53 located between amino acids 32 and 79 (83). A second antibody to an epitope near the carboxyl terminus (PAab 122; 14091A; Pharmingen, Inc., San Diego, CA) will also be used. A monoclonal antibody to SV40 large T-antigen will serve as an isotype matched negative control (PAab 416, Ab-2; Oncogene Science).

6.3 **Group A Blood Group Antigens and EGF Receptor**

These studies will be done by Drs. Jin Soo Lee and Jae Y. Ro at M.D. Anderson. Paraffin-embedded tumor tissue sections will be analyzed for the expression of blood group antigens A, B, H, and EGFR.

6.31 **Immunostaining Procedure for Blood Group Antigens**

Formalin-fixed, paraffin-embedded specimens will be cut into 4-µm sections, mounted on silane-coated glass slides, deparaffinized in a xylene-ethanol series, rehydrated through graded ethanol, and washed in PBS. The sections will then be incubated for 15 minutes in 1% methanolic hydrogen peroxide to inactivate endogenous peroxidase activity, and blocked by incubation with normal horse or goat serum for 30 min. at room temperature. After being washed with PBS, sections will be incubated with primary antibodies (e.g.,
monoclonal antibodies for blood group A, B, and H antigens) in a moist chamber at
37°C for 1 hour. After another wash, sections will be incubated with biotinylated
second antibody (biotinylated horse anti-mouse IgG or biotinylated goat anti-mouse
immunoglobulin M [IgM]; Vector Laboratories, Burlingame, CA) for 1 hour at room
temperature. After another wash, sections will be incubated with avidin-biotinylated
peroxidase, and color will be developed with 50 mM Tris-HCl buffer (pH 7.6)
containing 0.05% 3,3'-diaminobenzidine (Sigma Chemical Co., St. Louis, MO) and
0.03% hydrogen peroxidase for 5-10 min. After a final wash, sections will be
counterstained with hematoxylin, dehydrated in a graded ethanol series, put through
xylene, and mounted in suitable mounting medium.

6.32 Immunostaining Procedure for EGFR:
The staining procedure will be the same as the one outlined for blood group antigen
staining with modification in two areas. First, the tissue sections will be treated with
0.1% pepsin at 37°C for 5 minutes before the blocking with nonimmune serum.
Second, the incubation period with the primary antibody (anti-EGFR antibody,
BioGenex, San Ramon, CA) will be 2 hours.

6.33 Evaluation of Immunostaining Results
The results of blood group antigen staining will be expressed semiquantitatively for
the proportion of tumor cells stained positive. The results of EGFR expression will be
expressed for both intensity of the staining (using an arbitrary scale of 0, 1+, 2+, 3+)
and the proportion of tumor cells stained positive. Positive and negative control slides
will be included in each batch of staining for EGFR. Control slides will not be included
for blood group antigen staining because red blood cells and endothelial lining cells
provide a good internal positive control for these antigens.

7.0 STATISTICAL CONSIDERATIONS
The objective of the proposal is to investigate whether:

1) K-ras and p53 mutation,
2) blood group antigen A and epidermal growth factor receptor,
3) neuroendocrine differentiation, and
4) p105 antigen and factor 8 levels

are predictive of patient survival and local recurrence in patients undergoing defined therapy
postoperatively for completely resected Stage II and Stage IIIA NSCLC, while controlling for other
known prognostic factors.

The parent protocol, E3590, was activated in April, 1991, and as of July 6, 1993, there were 142
patients registered. The accrual goal of this protocol is 465 patients, and the accrual rate in the last
6 month period has been 7.0 patients per month. Under the assumption that 75% of all future cases
entering E3590 will contribute adequate paraffin blocks, we can expect approximately 233
cases for subsequent correlative analyses.
It is suggested in the literature that approximately 22% of cases have K-ras mutation in codon 12. The survival information available in some of the articles is very scanty, especially for Stage II and IIIA disease, which is the study population for the parent protocol, E3590. To obtain some sense of the degree of sensitivity this study will yield, let us assume that 233 cases will be accrued over an approximately 3 year period, that 10% of cases will be excluded further from the study due to cancellation, ineligibility, etc., and that 25% of the cases will be positive for K-ras mutation. Under the exponential survival distribution, the following table gives the power of the two-sided level .05 logrank test (84) for various combinations of the differences in median survival from 12 months for those with K-ras mutation and the additional follow-up duration (85, 86).

<table>
<thead>
<tr>
<th>Difference in</th>
<th>Follow-up Durations in Months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median Survival</td>
<td>12</td>
</tr>
<tr>
<td>6 Months</td>
<td>0.598</td>
</tr>
<tr>
<td>9 Months</td>
<td>0.853</td>
</tr>
<tr>
<td>12 Months</td>
<td>0.957</td>
</tr>
</tbody>
</table>

Therefore, with 24 months of additional follow-up, there is approximately 90% chance of detecting the 9-month increase in median survival for those without K-ras mutation, from 12 months for those cases with K-ras mutation, but only about 66% chance of detecting the 6-month increase in median survival.

Due to the paucity of baseline information on survival and local recurrence, however, the nature of this study will be exploratory, and, as a consequence, statistical analyses will focus mainly on modeling the relationship of outcome to the different laboratory data as well as other well known prognostic factors such as age, histology, etc. Multiple logistic regression (87) and Cox proportional hazards regression models (88) will be employed to examine the relationship between the laboratory data and survival and local recurrence, while adjusting for other concomitant variables to be identified as prognostic factors in this patient subpopulation.

These analyses will enable us to determine if the laboratory values are prognostic. By including other prognostic factors in the model we can test whether the laboratory values add independent prognostic information, thus enabling us to define the poor risk and the good risk patient more precisely.

Laboratory data from the three participating laboratories will be sent to the ECOG Statistical Center, either via electronic mail or via magnetic medium, and will be entered into and merged with the clinical database for the parent therapeutic protocol, E3590, for correlative analyses. The data should be recorded in such a way that they can be easily identified with the clinical data from E3590 and merged for subsequent statistical data analyses.
8.0 RECORDS TO BE KEPT

8.1 Patient Data Form: Submit both items with each specimen (paraffin Pathology Report: blocks, EM-fixed, snap-frozen) to the location indicated in Section 5.3.

Rev. 6/94 NOTE: SWOG, RTOG and NCCTG participants should follow ECOG guidelines regarding Pathology and Data Form submission. RTOG participants must also submit copies of these two forms, clearly identified with both RTOG and ECOG study and case numbers, to RTOG headquarters. SWOG Institutions should submit the ECOG eligibility checklist directly to the ECOG Data Management Office.

Rev. 10/94 NOTE: CALGB participants should submit the Pathology Report and the Patient Data Form to the CALGB Data Management Center. These forms will be forwarded to the ECOG Pathology Coordinating Office (PCO).

CALGB Data Management Center
First Union Plaza, Suite 340
2200 West Main Street
Durham, NC 27705

9.0 PATIENT CONSENT AND PEER JUDGMENT

Rev. 5/94 Rev. 9/94 This study conforms to a study eligible for "Expedited Review". This means that the local IRB chairperson can waive the need for full IRB review or the need to obtain informed consent if the patient is already randomized to and participating on E3590 and is being retrospectively entered on E4592. Otherwise informed consent must be obtained from living patients. Patients will have previously given permission to enter an ECOG therapeutic protocol, and no further data will be collected from them or their survivors. Further analysis will be performed on tissue that was collected earlier, and no new biopsies will be performed. Patient privacy will be protected in the standard manner, and no patient can or will be individually identified.

10.0 REFERENCES


[Missing 16-23]


Laboratory/Clinical Correlative Studies in 
Non-Small Cell Lung Cancer

APPENDIX I

Suggested Patient Consent Form

Name __________________________________________ History # ___________________

Introduction

I have been invited to participate in a treatment study, EST 3590, for patients with non-small cell lung cancer. I have also been invited to participate in this laboratory companion study E4592. At the time of my surgery, all of my tumor was removed. As usual, part of the tumor went to the pathology department at the hospital for routine diagnosis. I am being asked for permission to use the remainder of the tumor and surrounding normal tissue for research purposes. Since the tissue was removed at the time of my surgery, the use of my tissue will not involve any additional procedures to me.

Purpose of the Study

Doctors will be looking at cells from my lung tumor to see if they can find special "markers" that can help them predict how a patient might respond to treatment for non-small cell lung cancer. An important part of this laboratory study is comparing the lab results with how I respond to treatment on EST 3590. Therefore, if after my surgery it is determined that I will not be treated on the study EST 3590, my tissue will not be used as part of this study.

What are the Likely Benefits?

The results of this laboratory study will not directly affect me or my treatment plan. Doctors hope to use these results in the future to show them which patients will respond the best to additional therapy following surgery for lung cancer.

What are the Risks?

There is no additional risk to me if I agree to participate. The lung tumor and surrounding normal tissue were removed at the time of my surgery.

What are the Alternatives?

Participation in this study is entirely voluntary. I may choose not to participate. If I choose not to participate, my doctor will continue to give me the best care possible. I understand that I may ask questions and take as much time as needed to make my decision.

Contact Person

For more information concerning this laboratory study, I may contact the investigator in charge, ____________________________, at ____________________________ or the study chair, Dr. Joan Schiller, at 608-263-8600.
Confidentiality

I understand that a record of my progress while on the study will be kept in a confidential form at _________ (institution) _________ and also in a computer file at the Statistical Office of the Eastern Cooperative Oncology Group. The confidentiality of the central computer record is carefully guarded. During their required reviews, representatives of the Food and Drug Administration (FDA), the National Cancer Institute (NCI) and sponsoring agencies may have access to medical records that contain my identity. However, no information by which I can be identified will be released or published.

Authorization

I have read all of the above, asked questions, received answers concerning areas I did not understand and willingly give my consent to participate in the study described above. My signature indicates that I have received a copy of the consent form.

______________________________            _______________________
Patient Signature                      Date

______________________________            _______________________
Witness Signature                     Date

______________________________            _______________________
Physician Signature                   Date
Laboratory/Clinical Correlative Studies in Non-Small Cell Lung Cancer

APPENDIX II

Pathology Submission Guidelines

Rev. 5/94

The following items are included in Appendix II:

1. Guidelines for Submission of Pathology Materials (instructional sheet for data managers)

2. Instructional memo to submitting pathologists

Rev. 5/94

3. List of Requested Materials for E4592

4. Patient Data Form
GUIDELINES FOR SUBMISSION OF PATHOLOGY MATERIALS

The following items are needed for the submission of pathology materials for E4592:

- Instructional memorandum to the submitting pathologist
- List of Requested Material
- Patient Information Form

Instructions:

1. Complete the top portion of the Patient Information Form.

2. Forward the pathologist's instructional memo, the List of Requested Material, and the Patient Information Form to the appropriate pathologist.

   The pathologist should return to you the required pathologic samples and reports, along with the completed Patient Information Form.

3. Make sure the pathologist has retained a copy of the Patient Information Form for his/her records.

4. Double check that ALL required pathology materials have been submitted (see appropriate List of Requested Material).

5. Mail paraffin and EM-fixed samples to: (Must be submitted within 1 month of registration to study)

   Pathology Coordinating Office
   ECOG Operations Office
   AMC Cancer Research Center
   1600 Pierce Street
   Denver, CO 80214

   Pathology specimens submitted for a patient WILL NOT be processed by the Pathology Coordinating Office until all necessary items are received.

   The institutional pathologist must be informed that the blocks may be depleted. In addition, at completion of the study, unused portions of paraffin-embedded material will be retained at the ECOG Central Tissue Repository for use in future studies. This material will not be returned unless requested.

   Mail frozen tissue (prepared according to Sections 5.2232 and 5.33) to:

   Joan H. Schiller, M.D.
   University of Wisconsin
   Comprehensive Cancer Center
   K4/666 Clinical Science Center
   600 Highland Avenue
   Madison, WI 53792

   If you have any questions concerning the above instructions, contact the Pathology Coordinator at the ECOG Operations Office (303-239-3500).
MEMORANDUM

TO: Submitting Pathologists

FROM: Barbara C. Wolf, M.D.
Executive Director
ECOG Pathology Coordinating Office

SUBJECT: Submission of Pathology Materials for E4592/SWOG 9323/RTOG 94-09/NCCTG 93-24-52/CALGB 9466

The patient named on the attached Patient Information Form has been entered onto an ECOG protocol requiring pathology review.

Please complete appropriate sections of the Patient Information Form. Keep a copy for your own records, and return the completed Form, the surgical pathology report(s), the slides and/or blocks, and any other required material (see attached List of Requested Material) to the Data Manager. The
Data Manager will forward all required pathology material to the ECOG Pathology Coordinating Office or Dr. Joan Schiller's office at the University of Wisconsin.

Unused portions of paraffin-embedded material will be retained at the ECOG Central Tissue Repository for future studies. These items will not be returned unless requested. Frozen tissue will be retained at the Central Tissue Repository at the University of Wisconsin.

If you have any questions regarding this request, please feel free to contact the Pathology Coordinating Office at 303-239-3500.

Thank you.
LIST OF REQUESTED MATERIAL

E4592  Laboratory/Clinical Correlative Studies in Non-Small Cell Lung Cancer: Ancillary Study to E3590.

---

1. Patient Data Form.

2. Surgical Pathology Report.

Rev. 5/94

3. Minimum tissue requirements:

- One (1) paraffin block of primary tumor or a portion of tumor tissue removed directly from the block (see Section 5.222).

NOTE:  The following are highly desirable but not mandatory (see Section 5.223):

- Two (rather than 1) paraffin blocks of primary tumor tissue.
- Two (rather than 1) paraffin blocks of normal lung tissue or normal lymph node.
- One, 2mm section each of tumor and normal lung tissue in EM fixative.
- At least three, 2mm sections snap frozen, of tumor and normal lung tissue or normal lymph node. The institutional pathologist must be informed that paraffin blocks may be depleted. In addition, at completion of the study, unused portions of paraffin-embedded material will be retained at the ECOG Central Tissue Repository for use in future studies. This material will not be returned unless requested.

See "Guidelines for Submission of Pathology Materials" for mailing instructions.
# Patient Data Form

Date of Surgery: __/__/____

Patient Name: 

MI Last

ECOG Pt. Sequence No. for E3590: ______

ECOG Pt. Sequence No. for E4592: ______

Other Group Protocol No.: ________________

Other Group Patient No.: ________________

Institution: ____________________________ Affiliate (if appropriate): ____________

Patient's Hospital Number: ____________________________

Type of Tissue: (check those that apply)

**Source:** ___ Surgical biopsy or ___ Surgical resection

<table>
<thead>
<tr>
<th>Type</th>
<th>No. of Blocks Submitted</th>
<th>Surgical Path. ID Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paraffin-embedded</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-malignant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EM-Fixed</td>
<td></td>
<td>N/A</td>
</tr>
<tr>
<td>Primary</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-malignant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Snap-Frozen</td>
<td></td>
<td>N/A</td>
</tr>
<tr>
<td>Primary</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-malignant</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Surgeon: 

____

____

____
5.12 Patients already randomized to and participating in E3590 may retrospectively be placed on E4592 if adequate paraffin-embedded tissue is available (see Section 3.23). Patients entered retrospectively on this study will have previously given permission to enter parent therapeutic protocol, E3590, which informs them that histopathologic material may be sent to a central office for review. No further data will be collected from them. Further analysis will be performed on the paraffin tissue samples collected earlier, and no new biopsies will be performed. Patient privacy will be protected in the standard manner and no patient will be individually identified. Therefore, it is at the discretion of the individual IRB whether additional consent must be obtained for these patients. **Payment for shipment of pathology materials will be awarded.**
6.0 RESEARCH METHODS

Rev. 5/94 Since limited tissue may be available for any given patient, the prioritization of the studies is as follows:

1. **Paraffin Blocks:**
   a. K-ras and p53
   b. Blood group antigen and EGF receptor
   c. p105 and Factor

2. **EM-Fixed:** neuroendocrine markers

3. **Snap-Frozen:** tissue repository


