

Early Lung Cancer Action Project Pathology Protocol

Madeline Vazquez MD¹, Douglas Flieder MD¹, William Travis MD²,
Darryl Carter MD³, David F. Yankelevitz, MD⁴,
Olli S. Miettinen MD PhD⁴, Claudia I. Henschke PhD MD⁴
for the Expert Pathology Panel and ELCAP Group

¹ Department of Pathology
Weill Medical College of Cornell University
New York, NY

² Armed Forces Institute of Pathology
Walter Reed Army Medical Center
Washington DC

³ Department of Pathology
Yale School of Medicine
New Haven, CN

⁴ Department of Radiology
Weill Medical College of Cornell University
New York, NY

The ELCAP Group:

Claudia I. Henschke, PhD, MD, Principal Investigator
Nasser K. Altorki, MD
Daniel Libby, MD
Dorothy McCauley, MD
Olli S. Miettinen, MD, PhD
Mark Pasmantier, MD
James P. Smith, MD
David F. Yankelevitz, MD

Expert Pathology Panel:

Darryl Carter, MD, Chair
Elizabeth Brambilla, MD
Adi Gazdar, MD
Masayuki Noguchi, MD
William Travis, MD

Corresponding author:

Claudia I. Henschke, PhD, MD
Department of Radiology
New York Presbyterian Hospital- Weill Cornell Medical Center
525 East 68th Street
New York, NY 10021
Tel: 212-746-2529
Fax: 212-746-2811
e-mail: chensch@med.cornell.edu

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Introduction

Lung cancer, more than any other cancer, calls for consideration of screening. Not only is the disease highly fatal – essentially incurable – when diagnosed on the prompting of symptoms and/or clinical signs, but its occurrence also is highly concentrated in identifiably high-risk persons. The results of the Early Lung Cancer Action Project (ELCAP) using CT screening for people at high-risk of lung cancer showed that most of the diagnoses are achieved not only in Stage I but when the tumor still is less than 10 mm in diameter (1, 2). In research on CT-based screening for lung cancer, the ELCAP has been followed by two collaborative efforts, New York ELCAP (NY-ELCAP) and International ELCAP (I-ELCAP) (3). In all of these efforts, the protocol is in principle the same, except that the original ELCAP protocol has been updated in various particulars other than pathology (4). The original ELCAP and the NY-ELCAP were conceived as projects, limited in their time horizons, while the I-ELCAP is a program of ongoing international collaboration in screening research on lung cancer.

In the screening regimen, CT identifies not only potentially malignant nodules but also allows selective assessment of the rate of growth in these. The diagnosis that leads to early intervention is invariably based on cytologic examination of a fine-needle aspiration biopsy. When the cytologic diagnosis is followed by surgical resection, that diagnosis is supplemented by one based on histologic examination of surgical specimens.

Given the critical role of pathology in the screening regimen, and a role that is quite separate from the ‘front-line’ role of radiology, the pathology protocol of the I-ELCAP was not embedded in the CT-centered protocol that already was published; it is presented separately here.

This pathology protocol is principally directed to quality-assurance for the diagnosis of presence/absence of malignancy. There is, however, a larger purpose as well as has been emphasized by the recent International Conferences on Screening for Lung Cancer (3). Pathologic characterization of the diagnosed early cancers will allow for further refinement of

the diagnostic, staging, and other prognostic criteria, and for more basic research in all these areas as well.

This protocol addresses cytologic and histologic aspects as separate topics; and under each of these topics, it addresses the research role of the pathologists in the participating institutions ('sites') for one and the central reading and interpretation for another. In no way does this protocol represent guidelines for diagnostic practices in the participating institutions, nor does the central, research-oriented diagnosis represent second opinion for patient care.

Cytology protocol

The cytologic specimen is a fine-needle aspirate obtained from a CT-detected abnormality either by percutaneous or bronchoscopic biopsy procedure. If more than one CT abnormality has been aspirated, each specimen is labeled as to its location.

The primary responsibility of the cytologist during the aspiration procedure is to document the adequacy of the aspirate for diagnostic purposes. The cytologist smears part of the specimen by the Diff-Quik method (Dade AG, Dudingon, Switzerland) immediately on receiving the aspirate; this requires less than 30 seconds. The remainder of the aspirated material is preserved in alcohol for Papanicolaou staining; this is a 24-step staining process and requires up to 15 minutes. The blood clot is fixed in formalin and sent for histologic examination as a cell block. In order to evaluate the molecular biology of these tumors and correlate their biomarker expression prior to resection with the expression of biomarkers in the resected specimen, a cellular sample from the aspiration biopsy is expelled in an RNA preserving reagent. The DNA is extracted from the cell block or alcohol fixed material.

For study purposes, the routine diagnostic report and the entire set of slides pertaining to the case are sent to the Coordinating Center, the latter for the Expert Cytology Panel to examine and document.

The set of slides for each CT abnormality is read independently of the site diagnosis by two experts, at least one of whom is a member of the Expert Cytology Panel, and the descriptive

findings are recorded on the ELCAP Cytology Form. These two experts also translate the findings to diagnosis according to the World Health Organization categories (5). The other Expert Cytology panel members independently review the set of slides for the case and record their diagnoses. When there is a discrepancy among the members, a consensus diagnosis is produced. A record is kept of the diagnoses at the site, by the solo expert and each of the other expert panel members, as well as of the consensus diagnosis. All slides are be copied into a digital file before returning them to the site. At least one representative slide of each CT abnormality will be kept at the Coordinating Center for tissue banking purposes.

As the slides on the case are examined by two expert reviewers, the following findings are documented as input facts for the diagnosis, each on a five-point Likert scale: cell size (small to large), nuclear grade (low to high), isonucleosis (isonucleosis to anisonucleosis), chromatin pattern (fine to coarse), nucleoli (absent to prominent), mitotic activity (low to high), nuclear hyperchromasia (absent to prominent), nuclear/cytoplasm ratio (normal to increased), nuclear membrane (smooth to irregular) cell borders (distinct to indistinct), monomorphic/polymorphic cell population (monomorphic to polymorphic), nuclear crowding (absent to marked), polarity (present to absent), cellularity (sparse to abundant), and cohesion/dyshesion (cohesive to dyshesive). These findings, each documented by the Likert scale, represent the range from clearly benign (the lowest Likert value) to clearly malignant (the highest Likert value), and are used to classify the case as benign or malignant. The algorithm by which the diagnosis is reached is based on these findings (Figure 1), but has as yet not been explicitly defined. As a result of the evaluation by the Expert Panel, the data will be used to determine an explicit algorithm.

Cell size of malignant cells	-----Increasing malignant cytologic findings----->		
Small-cell			Small cell carcinoma
Non-small cell			
Adenocarcinoma (ADC)	BAC	Adenocarcinoma	Poorly differentiated ADC
Squamous cell (SCC)	Keratinized SCC		Non-keratinized SCC
Neuroendocrine carcinoma (NEC)	Carcinoid	Atypical Carcinoid	Large cell NEC

Figure 1. Schematic Diagram of Cytologic Diagnostic Classification

Additional features are also examined as they may allow for cytological subtyping; they also are used for assessment of the host inflammatory response to the neoplasm and for correlating with specific CT findings: background necrosis (absent to abundant), extracellular mucin (absent to abundant), histocytic infiltrate (absent to abundant), histocytic infiltrate (absent to abundant), giant cell infiltrate (absent to abundant), inflammation severity (insignificant to severe), intranuclear inclusions (absent to abundant), intranuclear cytoplasmic invaginations (absent to abundant), intracellular mucin (absent to abundant).

An acute or necrotizing granulomatous inflammation is recognized on the air-dried Diff-Quik stain and in this case the majority of the specimen is submitted in a sterile manner for culture and sensitivity analysis with some material, rinsed in Cyto-Lyt solution, being used to monolayer slides for acid-fast and fungal stains. If necrotic material alone is obtained, a second sample is to be taken since squamous cell carcinoma and metastatic tumors may show marked necrosis. If squamous cell carcinoma is then suspected, Papanicolaou stained smears are used to highlight keratin. If a metastatic tumor is favored, a cell block is used to perform immunostains utilizing standard controls to confirm the origin of the primary with greater accuracy. When there are multiple suspected malignant lung nodules, their pulmonary origin is confirmed by immuno-staining (e.g., with thyroid transcription factor (TTF-1)). Occasionally, a hamartoma is aspirated and the smears show varying amounts of fibrillary myxoid ground substance or rarely cartilage with benign cells, and these components are easily recognized in cell blocks.

Primary lung tumors are best identified on the air-dried Diff-Quik smears. The aspirated material is high in cellularity and occasionally has background mucin. The malignant cell population is first analyzed as to cell size with special emphasis in distinguishing small cell from non-small-cell carcinoma. The presence of intracellular or extracellular mucin permits a definitive diagnosis of adenocarcinoma; the presence of keratin, squamous cell carcinoma. The various malignant cellular parameters of the tumor are specifically denoted so as to determine if a more specific diagnosis can be made according to the World Health Organization categories.

We have recognized a new category of lesions of uncertain malignant potential which frequently present as sub-solid nodules (7). Sub-solid nodules are 2 subtypes: non-solid and part-solid. Aspirates of non-solid nodules tend to yield a population of atypical bronchioloalveolar cells with subtle malignant parameters that show intranuclear inclusions, cytoplasmic invaginations (grooves), and are frequently accompanied by a histiocytic infiltrate. On excision, the non-solid nodules most often prove to be bronchioloalveolar carcinoma of the non-mucinous type.

Gross pathology and histology protocol

Gross specimens derive from thoracotomy or thoracoscopy, core specimens from CT- or bronchoscopy-guided biopsy procedures.

The site pathologist immediately fixes the core biopsy specimen in formalin for 24 hours. Then, 4-micron (or 6-micron, if routine) thick sections are cut for histologic examination.

The site pathologist measures the wedge specimen in three dimensions, removes the staples and fixes it in formalin for at least 24 hours, with the container with the specimen been shaken occasionally to prevent specimen atelectasis. The wedge specimen is then cut at 5-mm sections for gross inspection and tissue blocks are prepared and cut at 4-micro (or 6-micron if routine) for histologic examination. When the nodule diameter is 10 mm or larger, a 1-2 mm thick section is kept frozen (-80 degrees centigrade) before the fixation process.

The site pathologist also measures the lobectomy/pneumonectomy specimen in three dimensions. When no wedge specimen was previously obtained and the nodule diameter is 10 mm or larger, a 1-2 mm thick section is preserved frozen (at -80 degrees centigrade). The remainder is then inflated with formalin through the main bronchus or bronchi, and allowed to fix for at least 24 hours. Trans-axial sections are then cut at 5-mm intervals.

The site pathologist documents and photographs the gross findings on the specimen. Each lesion is described as to location, size in three dimensions, shape, consistency, necrosis, hemorrhage, relation to pleura, airways, bronchial, vascular and stapled resection margins. Normal lung is described as to color, consistency, enlargement of airspaces, airway lesions and peritumoral changes. The cartilage-bearing airways are described as to diameter, mucosal color (pink, red, dark red), consistency (soft, firm) and intraluminal lesions or mucus plugs. Specimen lymph nodes are described as to location (levels 1-12), size in three dimensions, color (pink, red, anthracotic), and consistency (soft, firm). The pleura is described as to color (pink, red, with or without black pigments), finish (shiny, dull), thickness, presence/absence of adhesions and lesions.

After completion of the gross specimen examination, the entire tumor (if 10 mm or less or non-solid) or *at least ten* sections of tumor, all additional lesions, the bronchovascular resection margin, all specimen lymph nodes as well as *at least ten sections* of normal lung are processed in a routine manner resulting in paraffin-embedded tissue and hematoxylin and eosin or hematoxylin phloxin and saffron-stained to provide 4-micron (6-micro, if routine) tissue sections suitable for microscopic examination.

The gross description is narrated and submitted to the Coordinating Center together with the entire set of slides pertaining to the case and one representative tissue block of tumor, for examination and documentation by the Expert Pathology Panel.

The set of slides for each case is read independently of the site diagnosis by two experts, at least one of whom is a member of the Expert Pathology Panel and the descriptive findings are recorded on the ELCAP Histology Form. These two experts also translate the findings to

diagnosis according to the World Health Organization categories (5). The other panel members independently review the set of slides for the case and record their diagnoses. When there is a discrepancy among the expert diagnoses, a consensus diagnosis is produced. A record is kept of the diagnosis at the site, by the solo expert and each of the other members, as well as of the consensus diagnosis. All slides are copied into a digital file and a limited set of slides of the tumor (one representative slide from each block) and of normal tissue (one representative slide from each block) will be kept at the Coordinating Center for tissue banking purposes.

As the slides on the case are examined by the two expert reviewers, the following findings are reported as input facts for the diagnosis: size in three dimensions, cell type, degree of differentiation, nuclear grade, fibrosis, necrosis, granulomatous response, presence/ absence of each of: stromal invasion, pleural invasion, angiolymphatic invasion, bronchial invasion, satellite lesions (with complete descriptions of these lesions) and precursor lesions (atypical adenomatous hyperplasia, squamous metaplasia and/or dysplasia and pneumocyte proliferations of uncertain malignant potential). The acellular mucinous component of mucinous tumors and cystic component of partially cystic tumors are included in size measurements. Tumor disruption by previous diagnostic procedures (i.e., FNA needle tract(s)) are also noted. The non-neoplastic lung is carefully studied for the presence of satellite tumors (site, size, type) and all other findings including evidence of previous diagnostic procedures, i.e., fine needle aspirate needle tract, tobacco-related diseases such as emphysema and respiratory bronchiolitis are noted. Pre-invasive lesions (squamous cell dysplasia, atypical adenomatous hyperplasia, and neuroendocrine tumorlets) are similarly documented. The consensus WHO tumor category is recorded.

Commonly, lung cancers show heterogeneous histology. Classification into squamous cell carcinoma means that keratinization and/or intercellular bridges must be identified, adenocarcinoma must have glands or mucin with various patterns of growth (eg.; acinar, papillary, bronchioloalveolar, solid, colloid, signet ring, clear cell), and small cell carcinoma is composed of small cells with scant cytoplasm, ill-defined cell borders, fine granular chromatin,

and high mitotic activity. Other non-small carcinomas with neuroendocrine features (eg.; cells in organoid nests, trabecular or rosette-like formations) are classified by the amount of necrosis and mitoses in the tumor; typical carcinoid, without necrosis and with less than 2 mitoses per 10 high power fields (HPF); atypical carcinoid, with punctate necrosis and with 2 to 10 mitoses per 10 HPF; large cell neuroendocrine carcinoma with large zones of necrosis and more than 10 (11 or more) mitoses per 10 high powered field (HPF) (2 mm²). The diagnosis of large-cell neuroendocrine carcinoma requires the recognition of at least one of the specific neuroendocrine markers as positive (chromogranin A, synaptophysin, NCAM). Histological type and subtypes recorded as final diagnoses refer to the criteria defined in the 1999 WHO classification (5).

A major change in the classification of adenocarcinoma in the most recent WHO proposal included the strict definition of bronchioloalveolar carcinomas to non-invasive adenocarcinomas with lepidic spread. These commonly present as stage I solitary tumors, mostly non-solid, on screening CT studies. Extensive sampling of tumors with bronchioloalveolar growth pattern is required to make sure that any foci of invasion are identified; if invasion is identified the tumor is classified as adenocarcinoma, mixed type with bronchioloalveolar features according to the new WHO histologic classification. The stained section of the frozen material should always be available for this purpose. The ELCAP pathology protocol further analyzes these lesions by requiring a measurement for the invasive component as well as for the entire tumor.

Quality Assurance

Even though the collaboration in the I-ELCAP is motivated by concerns for collaborative research in addition to providing presumably useful care, the quality assurance built into it is no more ambitious than what befits good practice. Quality assurance begins with the qualifications of the key personnel, including their continuing education. These qualifications are provided for all personnel, other than the pathologist, in a previous document (6). Qualifications of the site pathologist consist of 1) board-certification or board eligibility in pathology, if possible, with

subspecialization in chest pathology. These qualifications are supplemented by on-site training at a center experienced in the pathology readings of malignancies obtained in the context of the I-ELCAP protocol and that center's recognition of readiness to prepare the cytology and histology slides according to the protocol. The required continuing education consists of review of I-ELCAP teaching files (electronically available by the web-based management system) and participation in the International Conferences on Screening for Lung Cancer. Quality assurance is provided by comparisons of the site readings with those of the Expert Cytology Panel and Expert Pathology Panel.

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