Lymph Node Metastasis from Non-Small Cell Lung Cancer

- Imaging, Resection, Enhanced Pathologic Detection and Survival Implications

By

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This work is dedicated to my deceased father, Dr. Cyril Ekenwa Nwogu, who instilled in me the discipline and delayed gratification required for any worthwhile endeavor.

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DISSERTATION FORMAT

This dissertation has been prepared in 'manuscript format' and discusses the research methods and results from related and overlapping studies on lymph node metastases from lung cancer. The introductory chapter is a review of the lung cancer epidemiology, staging, screening, diagnosis and treatment with an explanation of the indications for improvements in lymph node staging of lung cancer. Chapters two, three and four are formatted as three stand-alone manuscripts describing the background, methodology and results of each individual study in detail. Each of these three chapters also include individual results, tables and figures. Chapter five is the conclusion section which consists of a brief summary, strengths and limitations of the three studies and discusses the significance of the findings.

ABSTRACT

Background:

Lymph node staging is a critical prognostic factor in non-small cell lung cancer (NSCLC) patients. Many surgical patients have grossly inadequate lymph node (LN) sampling. A standard of care for lymph node sampling is essential. There are limitations in the ability to accurately identify all lymph node malignant disease in patients even after sufficient numbers of nodes are harvested. This results in understaging of patients. Radioguided selection of the most suspicious lymph nodes in a patient permits the use of advanced pathologic methods to detect micrometastases. Understanding the role of lymphangiogenic factors in the onset of lymphatic metastases may facilitate the application of novel therapies to improve NSCLC survival.

Methods:

We used the Surveillance, Epidemiology and End Results (SEER) database to perform multivariate cox proportional hazards assessment of the prognostic value of the number of resected LNs and LNR in over 25,000 stages I-III NSCLC patients in the 1988-2007 SEER database. A gamma probe was used in 100 stage I or II patients with resectable lung cancers to detect increased fluorodeoxyglucose (FDG) uptake within thoracic lymph nodes during pulmonary resection procedures. We compared the accuracy of detecting LN metastases using either positron emission tomography- computed tomography (PET-CT) or the gamma probe and quantified the ability of the gamma probe to up-stage patients using IHC and RT-PCR for epithelial markers. We also correlated detection of LN micrometastases with VEGF A, C, D and VEGF-R3 expression. Clinical follow-up to correlate LN micrometastasis with survival is ongoing.

Results:

Fewer nodes examined corresponded with a worse prognosis. Prognosis improved as more LNs were examined. Patients with low or moderate ratios of positive to total LNs had better prognoses than those with high ratios. Following radioguided LN selection, IHC and RT-PCR detected micrometastatic lymph node disease in 4% and 47% of patients, respectively. Using RT-PCR as the gold standard, the sensitivity and specificity of PET-CT for detection of lymph node metastasis were 11% and 98% respectively, in contrast to 38% and 50% respectively for the gamma probe. There was a high correlation between detection of micrometastases and VEGF-A/C/D or VEGF-receptor-3 expression levels in LNs.

Conclusions:

More LNs resected and lower ratios of positive LNs to total examined LNs are associated with better patient survival after NSCLC resection independent of age, sex, grade and stage of disease. The intra-operative hand held gamma probe is more sensitive but less specific than PET-CT in identifying lymph node harboring micrometastases from lung cancer, resulting in limited up-staging of patients. Micrometastases correlate with the expression of VEGF in LNs in NSCLC patients. This may reflect the role of lymphangiogenesis in promoting metastases.

I CHAPTER 1

INTRODUCTION

I.1 LUNG CANCER EPIDEMIOLOGY AND DIAGNOSIS

I.1.1 Lung Cancer Epidemiology

The public health significance of lung cancer is reflected by the fact that this disease is one of the most common cancers in the world and it has a high case fatality rate. In the span of a few decades, lung cancer has gone from being a rare disease to the most common cancer worldwide and the greatest cause of cancer death globally (1, 2). In 2008, lung cancer accounted for 13% (1.6 million) of the total cases and 18% (1.4 million) of the deaths, worldwide (2). In the United States, lung cancer is the second most common cancer in both sexes (3). It is only exceeded in frequency by breast cancer in women and prostate cancer in men (Figure 1). However it is the most common cause of cancer death by a wide margin. It actually causes more cancer deaths than prostate, breast and colon cancer combined (3). In the United States, it is estimated that lung cancer will account for 14% (226,160) of new cancer cases and will cause 28% (160,340) of cancer deaths in 2012 (3).

The risk factors for lung cancer have been well established over many years (4-6). The major etiologic agent is cigarette smoking (1, 5-7) and it is estimated to account for 90% of all lung cancers (6). This exposure can arise from personal tobacco use, second-hand smoking or tertiary exposure via inanimate objects (1, 8, 9). Per capita consumption of consumption of commercially produced cigarettes rose dramatically from 1920 to 1945 (10). The casual attitude towards cigarette smoking at that time is illustrated by the distribution of cigarette packs as standard supplies to US soldiers in World War II. In the 1960's, the tobacco industry embarked on efforts to expand their market by targeting women. An effective advertising campaign resulted in a marked rise in smoking by women in industrialized countries, which predictably resulted in a rapid increase in the incidence of lung cancer in women two decades later. As

increasing efforts have been made to limit the exposure of people in industrialized countries to tobacco smoke, the tobacco industry has increased its marketing efforts in developing countries. In recent years, extensive research has been published on the hazards of second hand smoking, also referred to as environmental tobacco smoke (ETS). One study estimated that at least 17% of lung cancers in nonsmokers are attributable to exposure to high levels of ETS during childhood and adolescence (11). An increased risk for lung cancer in nonsmoking women married to men who smoke has been shown (12, 13). One study quantified the impact of such exposure to nonsmoking women from their spouses as 30% excess risk for all types of lung cancer (14). A summary analysis of a large number of epidemiologic studies on the risk for lung cancer in nonsmokers found an excess risk for lung cancer of 24% in nonsmokers who lived with a smoker (15). This has induced a passage of laws in many states and countries to protect air quality in public places (16, 17). More recent reports have documented the retention of nicotine and other cigarette smoke constituents on inanimate objects resulting in tertiary tobacco exposure. This may be linked to increased lung cancer incidence (9, 18-21).

The International Agency for Research on Cancer (IARC) has identified arsenic, asbestos, beryllium, cadmium, chloromethyl ethers, chromium, nickel, radon, silica and vinyl chloride as carcinogens (1). In 2000, it was estimated that 10% of lung cancer deaths among men and 5% among women could be attributed to occupational lung carcinogens including diesel fumes (22-24). Asbestos is the most widely known and most common occupational cause of lung cancer (1). Workers in asbestos mining, textile production, brake lining, cement production, construction, insulation and shipyards may all experience increased asbestos exposure. The spouses and families of workers in such fields are often exposed also when these individuals carry the asbestos fibers on their clothes into their homes. Asbestos acts synergistically with

cigarette smoke in the causation of lung cancer. The relative risk for lung cancer with asbestos exposure alone is 6-fold, with cigarette smoking alone 11-fold, but with exposure to both asbestos and cigarette smoke, the increase may be as high as 59-fold (25). Radon is a common indoor air pollutant in homes and has been projected as the second leading cause of lung cancer after smoking although there is conflicting data on the magnitude of the risk in domestic situations (1). Radon (radon 222) is a naturally occurring product of radium 226, itself a decay product of uranium 238 (26). Uranium and radium are ubiquitous in soil and rock, although in variable concentration. Radon decay products emit alpha particles that can cause damage to respiratory epithelium following inhalation of these products (6). This occurs primarily due to increased radon levels in homes, which is dependent on the concentration of radium in the soil and rock beneath these homes. This recognition has necessitated the routine measurement of radon levels in homes before their sale. Radon exposure can also occur in mining, which is the oldest occupation associated with lung cancer (1).

The role of air pollution from exhaust fumes or wood burning has been investigated. In China, incomplete combustion of coal in homes has been linked with lung cancer (27). The IARC has classified indoor emissions from household coal combustion as a human carcinogen and emissions from biomass fuel primarily from wood as a probable human carcinogen. A European cohort showed an association of solid fuel use for heating and cooking with lung cancer risk; odds ratio (OR) in lifetime users of solid fuel was 1.80; switching to nonsolid fuels resulted in lower risk (28). Air pollution is on the rise globally and has been shown to increase the relative risk of lung cancer (29, 30).

The genetic predisposition to lung cancer has been studied in families. An increased incidence of this disease has been reported among first degree relatives of lung cancer patients. A

meta-analysis involving 32 studies showed a 2-fold increased risk for lung cancer in persons with a family history of lung cancer with an increased risk also present in nonsmokers (31). This has been associated with a region on chromosome 6q23-25 (146cM-164cM); the addition of smoking history to this inheritance was associated with a 3-fold increased risk of lung cancer (32). Only a minority of cigarette smokers develop lung cancer. It has been suggested that there are characteristics such as single nucleotide polymorphisms (SNPs) that make individuals more susceptible to the toxic effects of cigarette smoke. This may occur via abnormal activation or reduced detoxification of these carcinogenic tobacco products of combustion. Amos and colleagues performed a genome-wide association study (GWAS) and identified a susceptibility locus for lung cancer at chromosome 15q25.1, a region that contains the nicotinic acetylcholine receptor genes (33). Another GWAS in never smokers with lung cancer found that a SNP at chromosome 13q31.3 was associated with an increased risk of non-small cell lung cancer (34). This effect appeared to be mediated through a down regulation of the glypican 5 (GPC5) gene. As molecular epidemiology advances, it may become possible to target genetically high risk groups for preventive intervention. The influence of gender on the incidence of lung cancer is controversial. Different studies have reported a lower (35, 36) or higher (37) risk of lung cancer in women compared to men. Observed gender differences in susceptibility may be related to differences in nicotine metabolism and in metabolic activation or detoxification of lung carcinogens (1). Hormonal factors may also play a role in susceptibility. Estrogen replacement therapy especially in combination with cigarette smoking has been associated with an increased risk for adenocarcinoma (38). Lung cancer is more common in non-smoking women than non-smoking men (37). This may also be related to susceptibility to non-tobacco environmental carcinogens (1).

The role of race in the epidemiology of lung cancer is well documented. African Americans have the highest lung cancer incidence and mortality in the United States compared to all other racial/ethnic groups (39). Although differences in socioeconomic status, educational level, smoking prevalence and perhaps other environmental factors may partially explain these observations, it is likely there are genetic predispositions to lung cancer that vary by population (40). A large observational cohort study showed that African Americans and Native Hawaiians were most susceptible to lung cancer when fewer than 30 cigarettes per day were smoked; there was no difference among the ethnic groups when smoking exceeded 30 cigarettes per day (41). Another study by Menck (42) showed that the incidence of lung cancer is substantially higher among African Americans and Native Hawaiians and other Polynesians and lower among Japanese Americans and Hispanics than among Caucasians in the United States. The explanation for these observed racial or ethnic differences in risk for lung cancer is unknown (1).

A protective effect on lung cancer incidence has been attributed to a diet high in fruits and vegetables (43). However, efforts to identify the specific nutritional elements responsible for this have been unsuccessful. For instance, several large epidemiologic studies investigating the role of β-Carotene not only showed the lack of benefit but actually caused harm in the form of higher than expected incidence of lung cancer and mortality (44-47).

Non-malignant conditions such as chronic obstructive pulmonary disease and interstitial fibrosis have been associated with an increase in lung cancer risk (48, 49).

All this epidemiologic information is useful for primary, secondary and tertiary lung cancer prevention strategies.

I.1.2 Presentation

Most lung cancer patients present with nonspecific symptoms. These include cough, dyspnea, chest pain, fatigue, weight loss, etc. In early stage disease there are often no physical findings. As the disease becomes more advanced, clinical manifestations of pleural effusion, post-obstructive atelectasis and bulky mediastinal lymphadenopathy can be seen. Many patients undergo workup for suspicion of pneumonia before a mass is detected. Metastatic disease can produce symptoms based on location. These include bone pain, headaches, dizziness, gait disturbances or other neurologic problems. There are well-described syndromes that may result from aberrant production of certain hormones from lung cancers. These include Cushing's syndrome from small cell carcinomas, Syndrome of Inappropriate Antidiuretic Hormone secretion (SIADH) from adenocarcinomas or hypercalcemia from squamous cell carcinoma.

I.1.3 Histology

Lung cancer is a very heterogeneous disease. The WHO classifies lung cancer (50) into the following histologic types:

Small Cell Lung Cancer (SCLC)

Non-Small Cell Lung Cancer (NSCLC)

- Adenocarcinoma
- Squamous cell carcinoma
- Large cell carcinoma
- Adenosquamous carcinoma
- Sarcomatoid carcinoma
- Carcinoid Tumor

Salivary Gland Tumors

Small cell lung cancer (SCLC) comprises 15 percent of cases while Non-Small Cell Lung Cancer (NSCLC) makes up about 85 percent of cases. The most common Non-Small Cell Lung Cancer is Adenocarcinoma (38%) followed by Squamous Cell Carcinoma (20%) and Large Cell Carcinoma (5%). Due to remarkable advances in the understanding of lung adenocarcinoma since the 2004 WHO classification, an international multidisciplinary classification was sponsored by the International Association for the Study of Lung Cancer, American Thoracic Society and the European Respiratory Society (51). In the past the only clinically relevant distinction among these histologic types was between SCLC and NSCLC since almost all of the tumors in the latter category were treated similarly. However, it has been shown that adenocarcinomas behave quite differentially from squamous cell carcinomas. There are specific molecular characteristics such as mutations, deletions, rearrangements, etc. that have been identified in adenocarcinomas that have led to the use of more effective targeted therapies against these neoplasms (52). The most prominent discovery relates to the role of the epidermal growth factor receptor (EGFR) mutation in lung carcinogenesis and its prediction of response to EGFR tyrosine kinase inhibitors (TKIs) in adenocarcinoma patients (51).

I.1.4 Imaging and Diagnosis

Imaging is a critical component of the management of lung cancer. It is vital for the screening, diagnosis, staging and surveillance of lung cancer. The most impactful imaging study for lung cancer is the chest computed tomogram (CT). It provides detailed anatomic evaluation of the entire thorax including the lung parenchyma, lymph nodes, pleura and mediastinum.

Additionally it typically images the upper abdomen so that the liver and adrenal glands, which are common sites for lung cancer metastases, are adequately assessed.

Positron Emission Tomography-Computed Tomography (PET-CT) provides both functional and anatomic staging information. The radioisotope most frequently used clinically is ^{18}F-Fluorodeoxyglucose (FDG). Following intravenous injection, FDG is distributed to all cells but its uptake is proportional to the metabolic activity of the cells. Thus, there is greater concentration of FDG in malignant and inflamed tissues. PET-CT has been shown to improve staging compared to CT alone (53-55). It distinguishes scar or fibrosis from malignant tissue and can detect extra-thoracic malignant disease such as bone metastases. However, its limitations include its inability to differentiate inflammatory from malignant disease and its decreased ability to assess brain tissue because of marked glucose uptake in the brain. There is also a size threshold for PET-CT, so cancerous deposits in lymph nodes less than 6 mm in size can readily be missed by this imaging modality. Radioactivity from a pulmonary primary tumor may also obscure activity in adjacent lymph nodes due to the limited spatial resolution of this imaging modality (56, 57). Thus, there is a need to develop better radioisotopes and other functional imaging modalities.

Magnetic Resonance Imaging (MRI) is very useful for investigation of tumor extension to the spinal canal or for demonstration of brachial plexus involvement by superior sulcus (pancoast tumors). For decades, bone scans were commonly used to investigate possible bone metastases, but PET has reduced its use since PET provides information about the bone as well as other potential metastatic sites.

Screening for lung cancer has been studied for many years. Several screening studies for lung cancer using chest radiography showed no survival benefit (58-60). Observational cohort

lung cancer screening studies by the United States and International Early Lung Cancer Action Project (ELCAP and I-ELCAP) have demonstrated the ability of low dose CT Scans to detect early lung cancer (61, 62). However, because of the lack of a control group in these studies, the results cannot address the impact of screening on lung cancer specific or overall mortality. The recently reported prospective, randomized national lung cancer screening trial comparing low dose CT to chest radiography showed that the low dose CT decreased mortality for lung cancer by 20 percent (63). With this recent evidence for the benefit of lung cancer screening, a greater proportion of patients with high risk factors for lung cancer will likely be diagnosed at earlier disease stages resulting in improved survival from lung cancer. An actuarial analysis showed the cost-effectiveness of lung cancer screening for high-risk individuals who are at least 50 years old and have a smoking history of thirty pack-years or more (64). In this study, the cost per life saved was estimated to be below $19,000, an amount that compares favorably with screening for cervical, breast and colorectal cancers.

Prior to therapy, tissue diagnosis is typically confirmed. This tissue can be obtained via several approaches. These include CT or ultrasound guided percutaneous lung/lymph node biopsy, standard bronchoscopy with endobronchial biopsies/brushings or electromagnetic navigational bronchoscopy with fine needle aspiration. Tissue may also be obtained from thoracic lymph nodes via endobronchial ultra sound guided fine needle aspiration. In some clinical scenarios, mediastinoscopy, thoracoscopic resection or pleural biopsy might be the most feasible means of obtaining tissue diagnosis.

I.2 STAGING

The staging of lung cancer is critical for appropriate choice of therapy and for prognostication. This also permits stratification of patients for clinical trials and facilitates accurate comparison of investigational therapies. The Tumor-Node-Metastases (TNM) system with stage groupings is used (65, 66). Lymph node staging is the most significant prognostic factor for locoregional non-small cell lung cancer (72, 80, 81). Approaches to increase the accuracy of such staging are the major focus of this dissertation.

I.2.1 Clinical Staging

This is accomplished by clinical exam and imaging. It includes the information obtained from chest radiography, computed tomography, positron emission tomography, magnetic resonance imaging, bone scans, etc. It guides the initial approach to management but often requires histological confirmation to ensure accuracy.

I.2.2 Pathologic Staging

Pathologic staging is accomplished by histological examination of tissue specimens. Such specimens can be obtained by endobronchial ultrasound (EBUS), endoscopic ultrasound (EUS) guided fine needle aspiration (FNA), mediastinoscopy or by percutaneous image guided needle biopsy. However, the detailed information about tumor size, invasion of surrounding structures, nodal involvement and possible metastasis is determined following surgical resection. TNM staging can then be accomplished and used to categorize patients in a uniform and consistent manner.

I. 3 THERAPEUTIC OPTIONS

I.3.1 Surgery

Surgery is the mainstay of lung cancer treatment. If patients are medically fit and have adequate pulmonary reserve they usually undergo surgical resection for stages I and II lung cancer. A select group of patients with stage IIIA and occasionally stage IIIB lung cancer are also offered surgery, usually after neoadjuvant chemotherapy or chemoradiotherapy. A typical surgical resection involves lobectomy, bilobectomy or pneumonectomy depending on the anatomic location of the lesion. Some patients may be unable to tolerate a lobectomy, or the natural history of a specific neoplasm may warrant a lesser resection. Under such circumstances, a segmentectomy or wedge resection may be performed. Complete resection of lung cancer is usually accompanied by a lymphadenectomy to provide an adequate assessment of the extent of disease (67). This may also be therapeutic for some patients. The significance of the extent of lymphadenectomy during surgical resection of lung cancer has been very controversial for quite some time (67-73). Thus, a component of this dissertation will investigate the impact on survival of the number of resected lymph nodes in lung cancer patients.

I.3.2 Radiation

Radiation therapy is the typical alternative to surgical resection for patients with early stage lung cancer that have inadequate pulmonary reserve or have prohibitive medical comorbidities. It may also be used in the neoadjuvant setting for patients with stage IIIA disease in conjunction with chemotherapy prior to surgical resection. Patients with multi-station mediastinal nodal disease or contralateral nodal involvement (N3) usually are offered definitive chemoradiotherapy. Radiotherapy may be planned and delivered in a variety of ways. The

relatively recent technique of stereotactic body radiation therapy (SBRT) allows for the administration of higher doses of radiotherapy to a limited field with less associated toxicity (74). This has produced promising results so far (75).

I.3.3 Chemotherapy

Chemotherapy is the mainstay of the management of advanced stage lung cancer, specifically stages III and IV. It can be used in combination with radiotherapy in the neoadjuvant setting prior to surgical resection or as definitive therapy in patients who have regionally advanced disease. The large proportion of patients that present with metastatic disease (Stage IV) are treated primarily with chemotherapy alone. Chemotherapy usually consists of a Cisplatin-based regimen of two agents. In recent years, molecularly targeted therapy has been added to chemotherapy or used in its place for patients who have susceptible genetic mutations, primarily in the form of anti-EGFR (epidermal growth factor receptor) therapy (52).

I.3.4 Alternative Therapies

This includes radiofrequency ablation (RFA), cryotherapy and photodynamic therapy. Radiofrequency ablation refers to the localized destruction of a tumor using heat administered via an image guided catheter(s) placed into the tumor. Cryotherapy uses a similar principle of catheter based, image-guided therapy using cold instead of heat. Photodynamic therapy refers to the administration of a systemic (intravenous or oral) photosensitizer that is differentially concentrated in neoplastic tissue compared to normal tissue. Non-thermal laser energy or light delivered in the vicinity of a tumor will then activate the photosensitizer. The resulting photochemical reaction leads to the release of free oxygen radicals that destroy the tumor (76-

78). Some of these therapies are still under investigation but are utilized in special circumstances.

Section I.4 METASTATIC PATHWAYS

Mortality form lung cancer basically occurs through the metastatic spread of malignant cells to distant organs. Malignant cells metastasize from the primary tumor to other organs via either the lymphatic or vascular network (Figure 2). Indeed, tumor metastasis to regional lymph nodes often represents the first step of tumor dissemination and serves as a major prognostic indicator for the progression of human cancers (79).

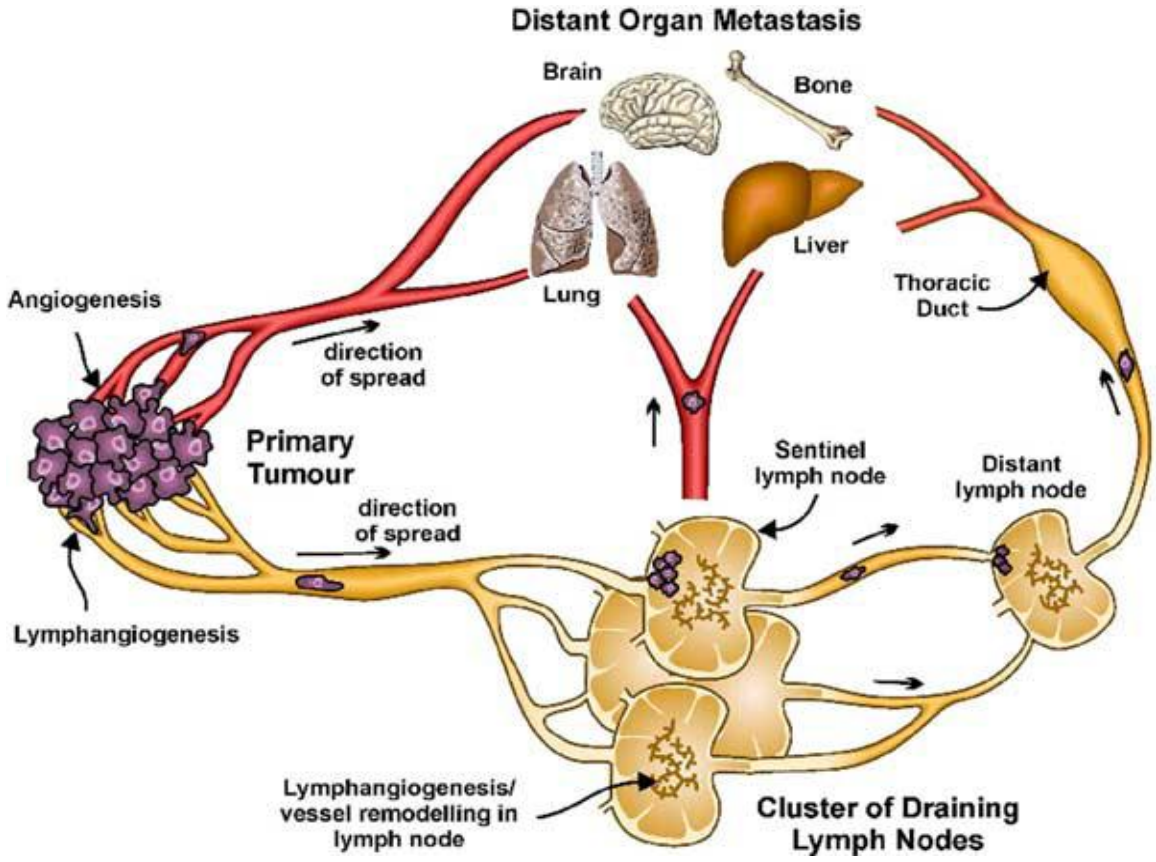


FIGURE 2. Schematic representation of potential routes of metastasis via the lymphatic vasculature (yellow), blood vessels (red) and lymph nodes. (Copied with permission from John Wiley and Sons, Publishers) (80)

I.4.1 Lymphatic Metastases

It is currently believed that lymphatics provide the major route of lung cancer metastases (80). The lymph node status of patients is the most significant prognostic factor for locoregional lung cancer (73, 81, 82). Tumor cells that become established and proliferate in the lymph nodes eventually can gain access to the systemic blood circulation because thoracic lymphatics drain into the thoracic duct which usually empties its contents into the left brachiocephalic vein or one of its tributaries. This can then result in distant hematogenous spread.

I.4.2 Hematogenous Metastases

This is the ultimate pathway for the spread of lung cancer to distant sites and is the primary cause of death from lung cancer. It is not clear why some tumors metastasize early either to the lymphatics or into the bloodstream while others remain localized for a prolonged period of time. The molecular profile of the primary tumor is thought to be responsible for this variability in biological behavior (52, 83, 84).

I.5 PATIENT OUTCOME

I.5.1 Survival

Patient outcome is primarily measured by disease free or overall survival. This in turn, is highly dependent on the stage at primary presentation (Table 1). Combined 5-year survival for all lung cancer patients is only about 16 percent and has not improved much in decades. This is remarkably lower than the 5-year survival rates for the other leading causes of cancer death in the United States, including cancers of the colon (66%), skin (melanoma 93%), breast (90%) and

prostate (near 100%) (39). This is due mainly to late presentation of lung cancer, lack of systematic screening and a preponderance of advanced stage disease. This deplorable statistic might be improved upon by mass surveillance of high risk patients.

I.5.2 Quality of Life

The quality of life of lung cancer patients is of primary importance. Because so many of them present with advanced disease, a therapeutic goal of cure is often unrealistic. Thus palliative therapy takes on great significance for these patients (85). This includes management of pain, dyspnea and fatigue amongst other symptoms.

I.6 STUDY RATIONALE

Lymph node staging is a critical prognostic factor in non-small cell lung cancer (NSCLC) patients. In the current lung cancer tumor-node-metastasis (TNM) staging system, the anatomic extent of lymph node metastases is the only factor used to define the N category of TNM (86). However, the TNM classification system for breast, esophageal, gastric and colorectal cancer has been updated from the traditional system to include number of metastatic lymph nodes (MLNs) in the N staging. In these cancers, the number of MLNs has been shown to be a more effective prognostic indicator than the anatomic location of MLNs (87). It has been suggested that the ratio of metastatic lymph nodes to total number of lymph nodes examined (lymph node ratio - LNR) in breast, bladder, gastric, colon and rectal cancers is a better prognostic indicator than the number of MLNs (88-92). For NSCLC, it has been reported that the number of MLNs can give a better N category prognosis than the anatomic location of metastatic lymph nodes, which is currently used (93). Therefore, we used the Surveillance, Epidemiology and End Results (SEER)

database to explore the prognostic value of the number of lymph nodes examined (LNE) and the ratio of metastatic lymph nodes to total number of lymph nodes examined (LNR). Our hypothesis was that a higher number of lymph nodes examined and a lower lymph node ratio would both be associated with better overall survival and disease specific survival in all stages of resectable NSCLC.

It is challenging to accurately identify all lymph node disease in patients. Nearly 40% of node-negative patients will develop recurrent disease and die within 2 years (94). This is believed to be due to understaging of lung cancer patients i.e. under-recognition of micrometastases by standard hematoxylin and eosin (H&E) staining of lymph nodes (79, 95-98). Intensive pathologic techniques such as serial sectioning, immunohistochemistry and reverse transcriptase polymerase chain reaction (RT-PCR) are more sensitive in detecting these micrometastases (99, 100, 101, 102). These techniques are labor intensive and expensive and can practically be applied only to a limited number of lymph nodes in each patient. Radioguided lymph node mapping in lung cancer patients can be used to identify the node(s) most likely to harbor micrometastases, so that these pathologic techniques can be applied in a cost-effective manner. Improvement in the staging of lung cancer will facilitate the selection of patients for novel therapeutic approaches in either the neoadjuvant or adjuvant setting. This may ultimately result in improved patient survival.

Improved therapy for lung cancer also requires better fundamental understanding of the molecular mechanisms leading to lymphatic metastasis. As a means of exploring the role of lymphangiogenesis in the occurrence of nodal metastases, we sought to correlate the presence of micrometastases with VEGF A, C, D and VEGF receptor-3 expression in LNs. The VEGF-C/VEGF-D/VEGFR-3 axis is the best validated signaling system for promoting

lymphangiogenesis associated with solid tumors and the metastatic spread of tumor cells to lymph nodes (80). Modifying these lymphangiogenic factors may be therapeutically useful.

I.7 STUDY OBJECTIVES

I.7.1 Study 1 Objective

Investigate the impact of the number of lymph nodes (LNs) resected and the ratio of positive LNs to total examined LNs on the overall survival of non-small cell lung cancer.

I.7.2 Study 2 Objective

Determine if gamma emission detection using an intra-operative hand held probe following intravenous 18F-fluorodeoxyglucose (FDG) injection would select lymph nodes (LNs) containing micrometastases more effectively than PET-CT.

I.7.3 Study 3 Objective

Determine if the presence of micrometastases positively correlates with VEGF-A/C/D and VEGF-receptor-3 expression in LNs.

I.8 CHAPTER I FIGURES

Figure 1: Ten Leading Cancer Types for the Estimated New Cancer Cases and Deaths by Sex, United States, 2012(3)**.**

Estimated New Cases*

Males				Females		
Prostate	241,740	29%		Breast	226,870	29%
Lung & bronchus	116,470	14%		Lung & bronchus	109,690	14%
Colon & rectum	73,420	9%		Colon & rectum	70,040	9%
Urinary bladder	55,600	7%		Uterine corpus	47,130	6%
Melanoma of the skin	44,250	5%		Thyroid	43,210	5%
Kidney & renal pelvis	40,250	5%		Melanoma of the skin	32,000	4%
Non-Hodgkin lymphoma	38,160	4%		Non-Hodgkin lymphoma	31,970	4%
Oral cavity & pharynx	28,540	3%		Kidney & renal pelvis	24,520	3%
Leukemia	26,830	3%		Ovary	22,280	3%
Pancreas	22,090	3%		Pancreas	21,830	3%
All Sites	**848,170**	**100%**		**All Sites**	**790,740**	**100%**

Estimated Deaths

Males				Females		
Lung & bronchus	87,750	29%		Lung & bronchus	72,590	26%
Prostate	28,170	9%		Breast	39,510	14%
Colon & rectum	26,470	9%		Colon & rectum	25,220	9%
Pancreas	18,850	6%		Pancreas	18,540	7%
Liver & intrahepatic bile duct	13,980	5%		Ovary	15,500	6%
Leukemia	13,500	4%		Leukemia	10,040	4%
Esophagus	12,040	4%		Non-Hodgkin lymphoma	8,620	3%
Urinary bladder	10,510	3%		Uterine Corpus	8,010	3%
Non-Hodgkin lymphoma	10,320	3%		Liver & intrahepatic bile duct	6,570	2%
Kidney & renal pelvis	8,650	3%		Brain & other nervous system	5,980	2%
All Sites	**301,820**	**100%**		**All Sites**	**275,370**	**100%**

Table 1: Overall Survival expressed as median survival time (MST) and 5-year survival by pathologic stage using the International Association for the Study of Lung Cancer Data (65)

Stage	**MST (months)**	**5-Year Survival (%)**
IA	119	73
IB	81	58
IIA	49	46
IIB	31	36
IIIA	22	24
IIIB	13	9
IV	17	13

II CHAPTER 2

NUMBER OF RESECTED LYMPH NODES AND METASTATIC LYMPH NODE RATIO ARE ASSOCIATED WITH SURVIVAL IN LUNG CANCER

II.1 ABSTRACT

Background:

The non-small cell lung cancer (NSCLC) TNM classification system uses only the anatomic extent of lymph node (LN) metastases to define the N category. The number of LNs resected and the ratio of positive LNs to total examined LNs are prognostic in other solid tumors. We used the Surveillance, Epidemiology and End Results (SEER) database to investigate the impact of these parameters on the overall survival of non-small cell lung cancer.

Methods:

All patients with NSCLC in the SEER database from 1988-2007 who had curative resections and had at least one lymph node examined were included. The prognostic value of age, race, sex, histologic grade, number of examined LNs and the ratio of positive LNs to total examined nodes was assessed using a multivariate Cox proportional hazards model for overall survival. The number of nodes examined was categorized into four levels. The percent LN positive was stratified into three levels.

Results:

Among patients with localized disease, fewer nodes examined corresponded with a worse prognosis. Prognosis improved as more LNs were examined. For patients with regional disease, the differences were significant only at the extremes. Older patients, males and those with higher grade tumors did worse. Patients with low or moderate ratios of positive to total LNs had better prognoses than those with high ratios.

Conclusions:

More LNs resected and lower ratios of positive LNs to total examined LNs are associated with better patient survival after NSCLC resection independent of age, sex, grade and stage of disease.

II.2 INTRODUCTION

Lung cancer is the leading cause of cancer death in the United States accounting for 157,000 deaths annually (103). Non-small cell lung cancer (NSCLC) comprises 80% of all cases. Unfortunately, only 20% of patients present with potentially surgically curable loco-regional disease (104). For these patients, lymph node metastasis is the most important prognostic factor. Survival is also influenced by age, sex, socioeconomic status, tumor size, histology, tumor grade and type of treatment (105).

In the current lung cancer tumor-node-metastasis (TNM) staging system, the anatomic extent of lymph node metastases is the only factor used to define the N category of TNM(86). However, the TNM classification system for breast, gastric, and colorectal cancer has been updated from the traditional system to include number of metastatic lymph nodes (MLNs) in the N staging. In these cancers, the number of MLNs has been shown to be a more effective prognostic indicator than the anatomic location of MLNs (87). It has been suggested that the ratio of metastatic lymph nodes to total number of lymph nodes examined (lymph node ratio - LNR) in breast, bladder, gastric, colon and rectal cancers is a better prognostic indicator than the number of MLNs (88-92). For NSCLC, it has been reported that the number of MLNs can give a better N category prognosis than the anatomic location of metastatic lymph nodes, which is currently used (93).

Therefore, we used the Surveillance, Epidemiology and End Results (SEER) database to explore the prognostic value of the number of lymph nodes examined (LNE) and the ratio of metastatic lymph nodes to total number of lymph nodes examined (LNR). Our hypothesis was that a higher number of lymph nodes examined and a lower lymph node ratio would both be

associated with better overall survival and disease specific survival in all stages of resectable NSCLC.

II.3 METHODS

Population-based data were obtained from the SEER program. Data on resected lung cancer cases were obtained from the SEER 9 registry for the years 1988-1992 and from the SEER 13 registry for the years 1993-2007. 1988 was selected because the extent of lymph node evaluation was not uniformly available in this database until then. The details about the data collection and database are provided in the National Cancer Institute SEER Cancer Statistics Review. Because we used existing data without individual subject identification, informed consent by the study participants was not necessary. The lung cancers included ICD codes C33.0 through C34.9 and C39.0 through C 39.9. Small cell lung cancers were excluded. The study sample was restricted to patients undergoing curative resections (lobectomy, bilobectomy and pneumonectomy) who had at least one lymph node examined. This included patients with both localized and regional disease (Stages I, II and III). American Joint Committee on Cancer (AJCC) tumor-node-metastasis (TNM) staging information was available for patients diagnosed in 2004 or later. Patients who received radiation therapy were excluded because such treatment may have been indicated by incomplete resections.

Based on the distribution of patients in our cohort, the number of lymph nodes examined was categorized into four: 1-3, 4-6, 7-9 and 10 or more. The percent LN positive was stratified into three levels: Low: 0.01% to 24%, Moderate: 25% to 49% and High: 50% or higher. They are also similar to the groups used in other reported studies (68-70, 82, 106).

The variables for the multivariate model were selected based on the literature. Prognostic value of a given variable (either the number of LN examined or the percentage of LN that were positive) was assessed using the associated hazard ratio and 95% confidence interval from a multivariate Cox proportional hazards model for overall and disease specific survival. Potential confounding variables associated with survival were included in the model to demonstrate that the prognostic effect persists after accounting for the effect of these variables. These included age, race, sex, and histologic grade of the tumor. Proportional hazard results were supplemented with Kaplan Meier survival curves.

II.4 RESULTS

Twenty-five thousand eight hundred eighty-seven (25,887) patients met the eligibility criteria; 15,978 had localized disease while 9,909 had regional disease. The former group had disease limited to the lung parenchyma while the latter group had disease involving the lymph nodes, chest wall, diaphragm or mediastinum. Demographic, surgical treatment and histopathologic characteristics of the entire cohort are listed in Table 1. The median follow-up time for the entire cohort was 48 months. A small proportion of the whole cohort (3,568 patients) had TNM staging information and their median follow-up time was 20 months.

The number of lymph nodes examined (LNE) had greater prognostic value for disease specific and overall survival in patients with localized disease than in those with regional disease (Tables 2 and 3). Fewer lymph nodes examined corresponded with a worse prognosis. The median number of nodes examined was six. Prognosis improved as more lymph nodes were examined. In patients with regional disease, the ratio of metastatic lymph nodes to total number of lymph nodes examined (LNR) was associated with disease specific and overall survival.

Patients with low (0.01% to 24%) or moderate (25% to 49%) ratios of positive to total LNs had better prognoses than those with high (50% or higher) ratios (Figures 1 and 2).

In the subset of patients with AJCC nodal staging information, the number of lymph nodes examined was not prognostic, but LNR was prognostic for both disease specific and overall survival in patients with N1 and N2 disease (Table 4).

The odds of having at least one malignant LN increased with the number of lymph nodes examined (LNE). Compared to patients with 1-3 LNE, the odds ratio for 4-6, 7-9 and 10 or more LNE were 1.57 (1.42, 1.73), 2.02 (1.82, 2.23), and 2.81 (2.57, 3.07), respectively. The p-values were all <0.001.

Younger age, lower grade disease and female sex were associated with better disease specific and overall survival (Tables 2, 3 and 4). Race was not a consistent independent predictor of survival.

II.5 DISCUSSION

In this study, we analyzed data from the Surveillance, Epidemiology and End Results (SEER) database to determine the influence of number of lymph nodes examined and the ratio of metastatic to total resected lymph nodes on the survival of all patients with resectable NSCLC. The case ascertainment rate of the SEER registries has been reported to be 97.5% and it is felt to accurately represent the entire American population (71). SEER currently collects and publishes cancer incidence and survival data from population-based cancer registries covering approximately 28 percent of the US population (107). It has been shown that the number of lymph nodes evaluated following resection for Stage I NSCLC is associated with patient survival (69, 71, 108). This study examined this association in both localized and regional disease (Stages

I to III). We also sought to corroborate the findings of others about the prognostic value of lymph node ratio in resectable NSCLC patients (93).

The extent of lymphadenectomy has remained controversial for quite some time, but at a minimum, systematic lymph node sampling is considered vital for adequate staging (67, 71). Unfortunately, a large number of patients are inadequately staged (108, 109). This may negatively impact survival by depriving under-staged patients of the potential benefits of adjuvant therapy. The National Comprehensive Cancer Network® Guidelines™ Version 2.2012 for treatment of Non-small Cell Lung cancer includes recommendations for the sampling of at least three N2 stations or complete mediastinal lymph node dissection. Formal ipsilateral mediastinal lymph node dissection for patients undergoing resection for stage IIIA (N2) disease is also recommended (110).

Our study results support the hypothesis that a lower number of lymph nodes examined and a higher ratio of metastatic to total lymph nodes is associated with poorer overall survival from non-small cell lung cancer. There are several possible explanations for our findings. Firstly, stage migration can certainly occur as more lymph nodes are harvested and pathologically examined, resulting in improved staging accuracy. Some patients who would have otherwise been erroneously included among stage I patients are upstaged (68).The patients that remain in stage I would then have better survival figures. Conversely, patients that migrate to stages II and III with less burden of disease improve the survival for those stages. Secondly, there is the possibility that a more robust immunologic response in the regional lymph nodes may result in both greater ease of identification/examination of these nodes and improved survival of such patients (111). Thirdly, there is the potential for therapeutic benefit of systematic

lymphadenectomy in a subset of patients that have minimal disease in the lymph nodes without systemic disease.

There were some unexpected findings from our study. The number of lymph nodes examined (LNE) was more prognostic in patients with localized disease than in those with regional disease. This suggests that the differences in survival may be more attributable to stage migration rather than to a therapeutic effect. As more lymph nodes are examined in patients with localized disease, the staging accuracy improves, but once metastatic lymph nodes are detected (regional disease), the benefit of examining more nodes could be diminished. Lymph node ratio (LNR) was consistently prognostic for overall and disease-specific survival, even in the small subset of patients with TNM staging information. LNR may thus be more attractive as a variable to potentially include in the next revision of the staging system. Only 14% of the cohort had TNM staging information because this was not required for SEER database entry until 2004. The number of lymph nodes examined (LNE) was not prognostic in this group of patients. However, the median follow up for these patients was only 20 months. Sufficient maturity of the data with longer follow-up may be necessary to permit adequate assessment of the value of LNE.

The favorable impact of younger age, lower grade disease and female sex is consistent with established knowledge (3). A major strength of SEER data is that the large sample size allows the detection of moderate associations and permits complex multivariate analysis (13). It is also more generalizable to the community but it lacks granular detail such as smoking history and the use of chemotherapy.

The American College of Surgeons Oncology Group (ACOSOG) conducted a large, prospective randomized multicenter study of N0 and nonhilar N1 resectable lung cancer patients (Z0030) (24). One group had systematic lymph node sampling while the other had complete

lymphadenectomy. For this subset of patients, there was no difference in survival between the two groups. However, it is must be emphasized that systematic lymph node sampling entails rigorous identification, resection and examination of a several nodes from a combination of hilar and mediastinal lymph node stations. This ACOSOG study cannot be used as justification to remove an insufficient number of lymph nodes, which would compromise accurate staging of the disease.

As the International Association for the Study of Lung Cancer (IASLC) continues to collect prospective data beyond the set used for the recent update in the Lung cancer staging system (25), it may become apparent that the addition of number of resected lymph nodes or the ratio of metastatic to total lymph nodes will improve our prognostication for NSCLC patients. The IASLC database includes patients from several countries and provides more lung cancer specific detail than the SEER database. Thus, it can serve as a great resource to study these lymph node variables further.

In summary, our study shows that a lower number of lymph nodes examined and higher metastatic lymph node ratio are both associated with poorer disease specific and overall survival. Even a slight change in practice patterns arising from dissemination of this and other reports in the broader surgical and pathologic communities, can make a major impact on thousands of patients.

II.6 CHAPTER 2 TABLES

Table 1: Clinical and Pathologic Patient Characteristics

	Variables	No. of Patients (%)
Overall Cohort	N	25,887 (100)
Age	≤ 70	16,080 (62.1)
	>70	9,807 (37.9)
Race	White	22,210 (85.8)
	Black	2,029 (7.8)
	Other	1,648 (6.4)
Gender	Male	13,883 (53.6)
	Female	12,004 (46.4)
Stage	Localized	15,978 (61.7)
	Regional	9,909 (38.3)
Grade	I	3,335 (12.9)
	II	10,359 (40.0)
	III	10,759 (41.6)
	IV	1,434 (5.5)
Histology	Squamous Cell Carcinoma	7,701 (29.8)
	Bronchiolo-alveolar Carcinoma	2,596 (10.1)
	Adenocarcinoma	11,254 (43.6)
	Other	4,252 (16.5)
Surgery	Lobectomy	24,521 (95.1)
	Pneumonectomy	1,277 (4.9)

	Variables	No. of Patients (%)
Nodal stage (where available)	N0	2,891 (81.6)
	N1	531 (15.0)
	N2	120 (3.4)
Nodes Examined	1-3	6,764 (26.1)
	4-6	7,144 (27.6)
	7-9	4,782 (18.5)
	10=<	7,197 (27.8)
Survival Status	Alive	10,661 (41.2)
	Dead	15,226 (58.8)
Follow-up (months)	Median (Min/Max)	48.00 (0.00/239.00)

Table 2: Cox proportional hazards model for overall survival

	Localized Disease (n=15,978) HR (95% CI)	*p-value*	*Regional Disease (n=9,909) HR (95% CI)*	*p-value*
Nodes Examined				
1-3	1.20 (1.13, 1.27)	p= <.001	1.08 (1.01, 1.15)	p= 0.03
4-6	1.09 (1.03, 1.16)	p= 0.004	1.06 (0.99, 1.13)	p= 0.08
7-9	1.06 (1.00, 1.14)	p= 0.07	1.03 (0.96, 1.10)	p= 0.43
10 +	1.0		1.0	
% Nodes Positive				
0.01-24%			0.51 (0.46, 0.55)	p= <.001
25-49%			0.68 (0.63, 0.75)	p= <.001
50-100%			1.0	
Age at Diagnosis (per year)	1.04 (1.04, 1.04)	p= <.001	1.03 (1.02, 1.03)	p= <.001
Race				
White	1.0		1.0	

Black	1.13 (1.05, 1.23)	p= 0.002	1.04 (0.95, 1.14)	p= 0.39
Other	0.77 (0.70, 0.85)	p= <.001	0.95 (0.86, 1.05)	p= 0.29
Sex (Female vs. Male)	0.70 (0.67, 0.73)	p= <.001	0.74 (0.71, 0.78)	p= <.001
Histologic Grade				
Grade I	1.0		1.0	
Grade II	1.32 (1.23, 1.42)	p= <.001	1.28 (1.16, 1.41)	p= <.001
Grade III	1.57 (1.46, 1.68)	p= <.001	1.49 (1.36, 1.64)	p= <.001
Grade IV	1.58 (1.42, 1.76)	p= <.001	1.57 (1.39, 1.79)	p= <.001

HR = Hazard ratio

CI = Confidence Interval

Table 3: Cox proportional hazards model for disease specific survival

	Localized Disease *HR (95% CI)*	*p-value*	*Regional Disease* *(n=9,909)* *HR (95% CI)*	*p-value*
Nodes Examined				
1-3	1.25 (1.16, 1.35)	p= <.001	1.02 (0.94, 1.10)	p= 0.65
4-6	1.11 (1.03, 1.20)	p= 0.01	1 *(n=15,978)*	p= 0.10
7-9	1.10 (1.00, 1.20)	p= 0.04	.06 (0.99, 1.15)	p= 0.57
10 +	1.0		1.02 (0.94, 1.11)	
			1.0	
% Nodes Positive				
0.01-24%			0.47 (0.43, 0.52)	p= <.001
25-49%			0.67 (0.61, 0.74)	p= <.001
50-100%			1.0	
Age at Diagnosis (per year)	1.02 (1.02, 1.03)	p= <.001	1.02 (1.01, 1.02)	p= <.001
Race				
White	1.0		1.0	

Black	1.21 (1.10, 1.34)	p= <.001	1.03 (0.92, 1.14)	p= 0.64
Other	0.77 (0.68, 0.88)	p= <.001	0.96 (0.85, 1.07	p= 0.45
Sex (Female vs. Male)	0.77 (0.73, 0.82)	p= <.001	0.82 (0.77, 0.87)	p= <.001
Histologic Grade				
Grade I	1.0		1.0	
Grade II	1.37 (1.24, 1.51)	p= <.001	1.30 (1.16, 1.46)	p= <.001
Grade III	1.72 (1.56, 1.89)	p= <.001	1.62 (1.44, 1.82)	p= <.001
Grade IV	1.82 (1.58, 2.09)	p= <.001	1.57 (1.39, 1.79)	p= <.001

HR = Hazard ratio

CI = Confidence Interval

Table 4: Cox proportional hazards model for disease specific and overall survival in patients with AJCC nodal staging data

	HR (95% CI) for Disease Specific Survival (n=3,568)	*p-value*	*HR (95% CI) for Overall Survival (n=3,568)*	*p-value*
Nodes Examined				
1-3	0.94 (0.74, 1.18)	p= 0.577	1.02 (0.83, 1.25)	p= 0.838
4-6	0.85 (0.66, 1.08)	p= 0.181	0.91 (0.73, 1.12)	p= 0.364
7-9	0.79 (0.60, 1.05)	p= 0.104	0.83 (0.65, 1.05)	p= 0.125
10 +	1.0		1.0	
% Nodes Positive				
0.01-24%	0.38 (0.26, 0.56)	p= <.001	0.42 (0.29, 0.60)	p= <.001
25-49%	0.56 (0.38, 0.82)	p= 0.003	0.58 (0.41, 0.82)	p= 0.002
50-100%	1.0		1.0	
Age at Diagnosis (per year)	1.03 (1.02, 1.03)	p= <.001	1.03 (1.02, 1.04)	p= <.001
Race				

White	1.0		1.0	
Black	1.05 (0.74, 1.50)	p= 0.780	1.01 (0.74, 1.36)	p= 0.973
Other	0.91 (0.61, 1.37)	p= 0.657	0.80 (0.55, 1.14)	p= 0.215
Sex (Female vs. Male)	0.69 (0.57, 0.83)	p= <.001	0.66 (0.56, 0.77)	p= <.001
Histologic Grade				
Grade I	1.0		1.0	
Grade II	1.18 (0.87, 1.60)	p= 0.284	1.18 (0.92, 1.52)	p= 0.192
Grade III	1.83 (1.36, 2.46)	p= <.001	1.69 (1.32, 2.17)	p= <.001
Grade IV	1.59 (0.97, 2.62)	p= 0.066	1.78 (1.19, 2.65)	p= 0.005

III CHAPTER 3

RADIOGUIDED DETECTION OF LYMPH NODE METASTASIS IN NON-SMALL CELL LUNG CANCER

III.1 ABSTRACT

Background:

Lymph node staging provides the most important prognostic information in patients with loco-regional non-small cell lung cancer. We hypothesized that local detection of gamma radiation using an intra-operative hand held gamma probe following intravenous 18F-fluorodeoxyglucose (FDG) injection would identify lymph nodes containing metastases in a much more sensitive manner than standard pathological practices. This study was designed to compare the accuracy of detecting thoracic lymph node metastases using positron emission tomography- computed tomography (PET-CT) versus the gamma probe and to determine the ability of the gamma probe to detect lymph node micrometastases, resulting in up-staging of lung cancer patients.

Methods:

One hundred (100) patients with resectable lung cancers were enrolled in this study. Every patient had pre-operative positron emission tomography-computed tomography (PET-CT) and mediastinoscopy. Patients had 10 mCi of F18-Fluorodeoxyglucose (FDG) injected on the day of surgery, within 4 hours of the planned surgical procedure. A handheld device (NodeSeeker™ probe) detected increased FDG uptake (gamma emission) within thoracic lymph nodes during pulmonary resection procedures. The lymph nodes that demonstrated increased FDG uptake, but were non-malignant by conventional hematoxylin & eosin staining underwent further serial sectioning, immunohistochemistry (IHC) and RT-PCR (reverse transcriptase – polymerase chain reaction). Sensitivity and specificity for lymph node metastasis detection by

PET-CT and the gamma probe were calculated. Receiver Operating Characteristic (ROC) curves were generated for the gamma probe.

Results:

Three patients had metastatic lymphadenopathy detected at mediastinoscopy, so their procedures were aborted, while the others proceeded to lung resection and complete lymphadenectomy. Fifteen (15) additional patients had lymph node involvement on routine pathologic analysis. IHC and RT-PCR detected micrometastatic lymph node disease in 4 and 29 patients, respectively. Using RT-PCR as the gold standard, the sensitivity and specificity of PET-CT for detection of lymph node metastasis were 11% and 98% respectively, in contrast to 38% and 50% respectively for the gamma probe.

Conclusion:

The intra-operative hand held gamma probe is more sensitive but less specific than PET-CT in detecting lymph node metastasis from lung cancer. Its overall accuracy was low, resulting in limited up-staging of patients. RT-PCR analysis of FDG-avid lymph nodes for epithelial markers increased the clinical utility of this probe in detecting micrometastasis. Such up-staged patients could derive a survival benefit from adjuvant chemotherapy.

III.2 INTRODUCTION

Lung cancer is the most frequent cause of cancer death in both men and women in the United States and will account for about 27% of all estimated cancer deaths in 2012(3). Small cell lung cancer (SCLC) comprises 15 percent of cases while Non-Small Cell Lung Cancer (NSCLC) makes up about 85 percent of cases. The staging of lung cancer plays a critical role in efforts to combat this disease. Lymph node metastasis is the most important prognostic factor in locoregional non-small cell lung cancer. However, there has been limited progress in the ability to accurately identify all lymph node disease in patients. This is reflected in the modest 5-year survival (73%) reported by the International Association for the Study of Lung Cancer (IASLC) for the earliest stage (stage IA) of NSCLC (65). Nearly 40% of node-negative patients will develop recurrent disease and die within 2 years (112). This is believed to be due to understaging of lung cancer patients. Current, standard methods of evaluating thoracic lymph nodes (hematoxylin-eosin staining) can miss micrometastases (78, 96-98, 112, 113). Thus, better staging methods are necessary to stratify patients, make therapeutic choices and evaluate effectiveness of various treatment modalities. Intensive pathologic techniques such as serial sectioning, immunohistochemistry and RT-PCR are more sensitive in detecting these micrometastases (102, 113-115). However, these techniques are labor intensive and expensive. Thus, they can practically be applied only to a limited number of lymph nodes in each patient. Multiple approaches to the use of sentinel lymph node mapping have been studied as a means of selecting a few lymph nodes per patient for detailed pathologic analysis (116-119). We hypothesized that local detection of gamma radiation using an intra-operative hand held gamma probe following intravenous 18F-fluorodeoxyglucose (FDG) injection would identify lymph

nodes containing metastases in a much more sensitive manner. A pilot study demonstrated the feasibility of using such a gamma probe to detect occult metastases in lymph nodes (56). It is known that intravenously injected 18F-fluorodeoxyglucose (FDG) accumulates in thoracic lymph nodes when they harbor metastatic carcinoma cells; this underlies the utility of positron emission tomography (PET) in clinical staging of lung cancer patients (120). This study was designed to evaluate the sensitivity, specificity and clinical utility of such a device.

III.3 METHODS

Our Institutional Review Board (IRB) approved this pilot study in September 2007 and individual patient consent was obtained.

Patient Selection

One hundred (100) patients with resectable, confirmed or suspected clinical stage I or II non-small cell lung cancer were selected for enrolment onto this study. Adequate pulmonary and cardiac function was ascertained pre-operatively. Patients with tumors which were not FDG-avid on PET-CT scan were excluded. Routine pre-operative staging included positron emission tomography- computed tomography (PET-CT) and mediastinoscopy.

Operative procedure

On the day of surgery (within 4 hours of the planned surgical procedure), each patient had intravenous injection of 10 mCi of F18-Fluorodeoxyglucose (FDG) by our nuclear medicine technicians. The lymph nodes that were harvested by mediastinoscopy underwent frozen section pathologic analysis. If mediastinal lymph node metastasis was detected, the primary tumor resection was aborted. Standard anatomic lung resection (lobectomy, bilobectomy or pneumonectomy) via thoracoscopy or thoracotomy, as appropriate for the individual patient's

tumor, was followed by complete thoracic lymphadenectomy. The lymph nodes were labeled using the ATS/Naruke lymph node map (112). The surgical and lymph node mapping procedures were performed by six different surgeons, all following a standardized protocol with a research nurse present in the operating room to collect the detailed data.

All harvested lymph nodes were scanned with a handheld gamma probe (Node Seeker™ - IntraMedical Imaging LLC, Los Angeles CA) outside the thoracic cavity to measure the gamma radiation resulting from any accumulated FDG in individual nodes. The resected tumor was similarly scanned outside the thorax. These ex-vivo measurements were found to be much more reliable than those taken within the thorax (in-vivo counts) during our previous pilot study (56).

We compared the radioactive signals from the lymph nodes to each other. The FDG avid (hot) nodes had more than twice the signal intensity of the coldest lymph node in the entire thoracic field for that particular patient. Such 'gamma probe positive' lymph nodes were labeled for the pathologist and subjected to ultra-staging. In addition, an equal number of non-FDG avid (cold) nodes (gamma probe negative) were randomly selected from similar nodal stations for identical pathological ultra-staging. If the probe detected no hot nodes, the surgeon randomly selected one cold node for detailed pathological evaluation.

Lymph Node Ultra-staging

All surgically removed lymph nodes were bisected and examined by routine H&E. The gamma probe-detected FDG-avid lymph nodes that were malignant on H&E staining required no further pathologic analysis. However, the FDG-avid lymph nodes that were not malignant on H&E staining were subjected to ultra-staging with multiple step sections and immunohistochemistry (IHC) and RT-PCR for CK-7, CEACAM5 and PLUNC (epithelial markers).

These nodes were processed according to a standard protocol. After formalin fixation and embedding in paraffin, step sections of each lymph node were taken at 30-40 micron intervals. The sections were stained with H&E and an average of ten serial sections were evaluated. IHC was performed with a standard cytokeratin cocktail - CK AE1/AE3. IHC was considered positive if it demonstrated positive cell clusters or individual cells with the appropriate tumor cell morphology.

RNA extraction was performed on fresh primary tumors and an equal number of FDG avid and non-avid lymph nodes. Human mRNAs for beta actin (ACTB), keratin 7 (CK7), carcinoembryonic antigen-related cell adhesion molecule 5 (CEACAM5), and palate, lung and nasal epithelium associated protein (PLUNC) were quantified by RT-PCR-based TaqMan™ Gene Expression Assays (Applied Biosystems, Foster City, CA). The average quantification cycle (CQ) values of triplicate PCR reactions were used for analysis. CQ values for the mRNAs of interest were normalized by subtracting the value for beta actin mRNA from them. Beta actin is a highly conserved gene frequently used as a loading control in PCR assays.

Clinical Follow-up

Patients were seen or scheduled to be seen post-operatively in the thoracic surgery clinic every six months for two years, then annually for three additional years. Specific data to be collected on all patients include:

History: The presence of symptoms suggestive of lung cancer metastases – neurologic symptoms, bone pain or weight loss.

Physical Examination: The presence of enlarged cervical, axillary or supraclavicular lymph nodes; occurrence of incisional or cutaneous nodules or abdominal organomegaly.

Radiologic Imaging: Chest and Upper abdominal computed tomography (CT) (including the liver and adrenal glands. Brain magnetic resonance imaging (MRI) or CT will be performed in patients with neurologic symptoms.

Histologic confirmation of recurrent disease will be obtained when clinically feasible.

Statistical Analysis

Descriptive statistics such as frequencies and relative frequencies were computed for categorical variables. Numeric variables were summarized using simple descriptive statistics such as the mean, standard deviation, median, range, etc. The accuracy of PET-CT and the gamma probe for detection of lymph node metastases were compared by calculating sensitivity and specificity using two by two (2 X 2) tables.

The lymph node pathologic status was determined by using either:

1. A combination of H&E and IHC findings.
2. RT-PCR levels of epithelial markers (CK-7, CEACAM5 or PLUNC)

To find the positive threshold based on the Combination of H&E and IHC or RT-PCR as gold standard, the Receiver Operating Characteristic (ROC) curves were generated using the fitted logistic regression models with the lymph node radioactivity count, lymph node weight, time from injection and tumor radioactivity count as the independent predictors. The threshold was determined by optimizing the positive predictive value from the ROC curve (121). The area under the curve (AUC) is a measure of the predictive power of the model and a measure of the diagnostic accuracy of each test in terms of prediction of outcome. 95% Confidence Interval of the AUCs was computed using bootstrap methodologies.

III.4 RESULTS

One hundred nine (109) patients were enrolled into this prospective clinical trial which was started in September 2008. Nine patients were ineligible and had to be replaced in order to complete accrual in June 2012. The demographic, clinical and histologic information from the patients is summarized in Table 1. There were no adverse reactions to FDG injection on the day of surgery. The serious adverse events following the surgical procedures are listed in Table 2. None of these events was attributable to the use of FDG. Three patients had malignant mediastinal lymph nodes detected by pathologic frozen section so lung resection was aborted. The median amount of additional operative time for the lymph node mapping was 5 minutes (range = 0 – 20 minutes).

PET-CT detected FDG avid lymph nodes in only 6 patients while the gamma probe detected at least one FDG avid lymph node in 86 patients. The median number of lymph nodes harvested per patient was 10 (range = 1-21), while the median number of FDG avid lymph nodes per patient was 2 (range = 0 – 5). All patients had a minimum of one lymph node subjected to immunohistochemical analysis, while a subset of 62 patients had at least one lymph node analyzed by RT-PCR.

Eighteen (18) patients had malignant lymph nodes detected by routine pathologic evaluation (H&E). Immunohistochemistry (IHC) detected metastatic disease in lymph nodes that appeared normal on H&E staining in only four (4%) patients. One such case is illustrated in Figure 1. However, RT-PCR was positive for epithelial markers in 29 of 62 (47%) patients.

A total of 179 lymph nodes that were designated as FDG avid and 180 lymph nodes designated as FDG non-avid 'control' lymph nodes underwent H&E and immunohistochemical analysis. From this group, a subset of 205 lymph nodes had RNA extraction for detection of epithelial markers by RT-PCR. The sensitivity and specificity of PET-CT for detection of lymph node metastases by H&E/IHC were 30% and 99%, respectively (Table 3A). When RT-PCR was used as the standard, the sensitivity and specificity of PET-CT were 11% and 98% (Table 3B).

Sensitivity and specificity of the gamma probe for detection of lymph node metastases by H&E/IHC were 74% and 52% (Table 4A). This resulted in positive and negative predictive values of 11% and 96%, respectively. When RT-PCR was used as the standard, the sensitivity and specificity of the gamma probe were 38% and 50% (Table 4B). This resulted in positive and negative predictive values of 14% and 78%, respectively.

Receiver operating characteristic (ROC) curves were constructed to assess the performance of the gamma probe for the detection of lymph node metastases by a combination of H&E and immunohistochemistry (IHC). When the curve was constructed as a function of only the lymph node radioactivity counts, the radioactivity threshold was 27 counts per second (CPS) and the area under the curve (AUC) was only 0.573 (95% CI: 0.497, 0.69) (Figure 2A). This indicates poor discrimination between malignant and benign lymph nodes. When the curve was constructed as a function of the lymph node radioactivity count, lymph node weight, duration of time from FDG injection and the primary tumor radioactivity count, the radioactivity threshold was 18 counts per second (CPS) and the area under the curve (AUC) increased to 0.692 (95% CI: 0.609, 0.809) (Figure 2B). Table 5 demonstrates improvement of the AUC with stepwise addition of more variables to the ROC curve model.

The median follow-up of patients is only 14.5 months currently, so few disease recurrence events have occurred. Only two deaths from disease and five recurrences have been documented so far. Survival analysis will be performed when a median follow-up of 24 months is reached.

III.5 DISCUSSION

Clinical and even pathologic understaging of surgical patients is a persistent challenge in the management of NSCLC. The benefits of accurate documentation of extent of disease are well established. These include the prognostication for individual patients and appropriate stratification of patients for clinical trials of adjuvant therapy. Sentinel lymph node mapping has improved lymph node staging in other solid tumors such as melanoma and breast cancer. The technique has shown some promise in lung cancer but has not been widely embraced. Some of the reasons for this include perceived difficulty of the technique, limited delay after intra-operative radioisotope injection (due to migration time), debatable clinical benefit and negligible morbidity of lymphadenectomy for lung cancer compared to breast cancer or melanoma (114).

PET-CT has become a routine staging modality for NSCLC patients. Its ability to detect nodal disease is limited by a size threshold 5 to 6mm. Any FDG-avid lymph nodes in the vicinity of the primary tumor would be difficult to detect due to spatial resolution challenges. In our study, only four patients had positive nodes on their pre-operative PET-CT. The simplicity of FDG administration, the extensive experience with its clinical use and the value of PET in NSCLC staging prompted our investigation of its utility for lymph node mapping in NSCLC patients.

Several different dyes and radiotracers have been used for sentinel lymph node mapping in lung cancer. These include isosulphan blue, Patent V, methylene blue, indocyanine green, technetium sulfur colloid, nanocolloid, tin colloid, phytate and ferromagnetic particles (113). Various levels of success in identifying sentinel lymph nodes have been achieved. However, accurate staging of NSCLC requires evaluation of all the sites of potential thoracic lymph node metastasis. This requires assessment of more than the first echelon of draining lymph nodes. Detection of lymph node involvement beyond the first echelon can increase the patient's tumor stage from II to III and has major prognostic implications. It has been shown that the total number of involved lymph nodes and the ratio of malignant to benign lymph nodes influence survival. The presence of occult mediastinal lymph node involvement may indicate the need for adjuvant radiotherapy. A clinical technique to screen all the thoracic lymph nodes for micrometastases is not currently available. This prompted our investigation of FDG as a radioisotope for intraoperative lymph node mapping. FDG is safe, readily available even in small communities and has a prolonged half-life. However, it also has significant limitations. It is unable to differentiate malignant from inflamed nodes. The signal from the heart and great vessels also interferes with in-vivo (intrathoracic) lymph node radioactivity measurements. The development of newer radioisotopes may eliminate these technical limitations.

Surgical resection is the mainstay of treatment for locoregional non-small cell lung cancer (NSCLC). Thoracic lymph node sampling or complete dissection is a recommended part of the standard anatomic resection (67). Routine assessment of lymph nodes harvested during lung cancer resections consists of bisecting individual nodes and examination of a single section after hematoxylin and eosin (H&E) staining. It is well documented that this often results in the non-recognition of small malignant deposits within lymph nodes (95, 98-100, 114). This may

negatively impact survival by depriving understaged patients of the potential benefits of adjuvant therapy. Improved pathologic techniques such as immunohistochemistry (IHC) or reverse transcriptase – polymerase chain reaction (RT-PCR) enhance the ability to detect micrometastatic lymph node disease (102, 113-115). These techniques can only be applied cost-effectively to a few lymph nodes in each patient, so research efforts to develop ideal methods to select these nodes have been ongoing for over a decade (115, 116, 122-124). This study was designed to evaluate the utility of a handheld intra-operative gamma probe for this purpose.

Following a promising 10-patient pilot study (56), we accrued 100 patients to this study over four years. Some logistic amendments to the study protocol were made after the first quarter of the study. These changes included the abandonment of intrathoracic (in-vivo) lymph node radioactivity measurement because even thick shielding of the gamma probe head could not eliminate interference from emissions from the heart and great vessels after intravenous FDG injection. The extra-thoracic counts had, de novo, been identified as the relevant measurement for identifying FDG-avid nodes. Also, ultra rapid IHC (125) was initially performed on the lymph nodes harvested via mediastinoscopy. Given the fact that this was technically difficult, yielded no positive results to warrant immediate intervention and IHC was being performed on the selected lymph nodes post-operatively, it was eliminated from the protocol. RT-PCR for the epithelial markers (101, 102, 126) was incorporated into the study because the yield of micrometastatic lymph node disease by IHC was low and additional funding for these studies was obtained.

Ninety Percent (90%) of our patients presented with clinical stage I disease and the median tumor size was 2.45cm. This probably contributed to the low rate of detection of lymph node micrometastasis by IHC in our study. In our pilot study, we selected patients likely to have

micrometastatic disease in some of their thoracic lymph nodes. This was based upon the identification of lung masses greater than 3 cm in size or the presence of enlarged thoracic lymph nodes on computed tomogram (CT). The ACOSOG Z0040 study reported a 22.4% prevalence of lymph node occult metastases in patients with histologic N0 disease by using IHC for anticytokeratin antibodies for CAM 5.2 and AE-1 (114). However, the study cohort included patients with stage I to IIIB NSCLC. It is well-known that RT-PCR is more sensitive for detection of lymph node micrometastasis (101, 115, 126), but concerns have been raised about potential false positive results from mesothelial or endothelial cells within lymph nodes.

Since H&E staining alone clearly underestimates the micrometastatic disease burden in lymph nodes, we chose to use one of two 'standards' for the sensitivity and specificity testing of PET-CT and the gamma probe. We calculated these values using both RT-PCR and a combination of H&E and IHC. For both imaging modalities, the sensitivity was lower when RT-PCR was used compared to when H&E/IHC was used (Tables 2 and 3), but the specificity was similar. The sensitivity of PET-CT for detection of low metastatic disease burden in the lymph nodes was low (22% for H&E/IHC and 11% for RT-PCR), but the specificity was high (>97%). This has been demonstrated by others (127, 128) and has led to ongoing efforts to identify better radioisotopes than fluorodeoxyglucose (FDG) (55, 129, 130). The handheld gamma probe showed improvement in the sensitivity (74%) for lymph node micrometastatic disease detection when H&E/IHC was the 'standard' but when RT-PCR was used, it only demonstrated a sensitivity of 37%. These low sensitivities can be explained by the fact that RT-PCR detects such a low burden of metastatic cells that it they would be unlikely to generate enough FDG uptake to be detected reliably by either PET-CT or the gamma probe. The gamma probe also had low specificity (50 – 52%) for lymph node metastatic disease detection regardless of whether

H&E/IHC or RT-PCR was used as the 'standard'. This is probably due to the inability of FDG to distinguish between inflammatory and malignant tissue (56, 127). Many lung cancer patients harbor inflamed intrathoracic nodes from chronic bronchitis, obstructive pneumonitis and other reactive pulmonary conditions. A radioisotope that is safe, cheap, and readily available for clinical use with both high sensitivity and specificity remains an elusive ideal. This gamma probe had low positive predictive value (11% and 14% when IHC/H&E or RT-PCR respectively were used as the gold standard for detection of lymph node disease). The ROC curves in Figure 2 illustrate the diagnostic limitations of the gamma probe. When the radioactivity count of the lymph nodes was taken in isolation, the threshold for malignancy was 11 counts per second (CPS). The AUC of 0.59 indicated that the discriminatory capacity of the probe between malignant and benign lymph nodes was modest. The addition of the weight of the lymph nodes, the time interval between FDG injection and radioactivity measurement in the nodes and the FDG avidity of the primary tumor to the ROC model improved the ability of the gamma probe to discriminate between malignant and benign lymph nodes. However, the AUC remained modest at 0.692. Thus, in practice, the accuracy of this probe for the selection of lymph nodes likely to harbor metastatic disease was inadequate to make it clinically relevant. Thus, the search for an effective tool for this purpose continues.

The selective use of IHC and RT-PCR resulted in upstaging four of 100 (4%) and 29 of 62 (47%) patients, respectively. With more mature follow-up, we plan to compare the survival of patients with and without occult lymph node malignant disease. If this adds to the evidence for a negative impact of such occult disease on survival, it would be appropriate to design a study to investigate the use of adjuvant chemotherapy in such patients.

Conclusion

The intra-operative hand held gamma probe is more sensitive but less specific than PET-CT in identifying lymph nodes harboring micrometastases from lung cancer, resulting in limited up-staging of patients. RT-PCR analysis of FDG-avid lymph nodes for epithelial markers increases the ability of this probe to detect micrometastasis compared to IHC alone. Such up-staged patients could derive a survival benefit from adjuvant chemotherapy. Better radioisotopes or alternative imaging tools for selection of the lymph nodes to undergo pathologic ultra-staging are required.

III.6 CHAPTER 3 TABLES AND FIGURES

Table 1: Patient Demographic, Clinical and Histologic Characteristics

Patient Characteristic	**No, of patients (N=100)**	**%**
Age (years)		
Median	66	
Range	40 - 88	
Sex		
Male	30	30.00
Female	70	70.00
Clinical Stage		
Stage I	90	90.00
Stage II	10	10.00
SUV_{max}		
Median	8.5	
Range	1.7 - 29.9	
Operation performed		
Lobectomy	96	96.00
Bilobectomy	3	3.00
Pneumonectomy	1	1.00
Tumor size (cm)		
Median	2.45	
Range	0.53 – 4.8	
Tumor histology		
Adenocarcinoma	69	69.00
Squamous cell carcinoma	24	24.00
Adenosquamous carcinoma	2	2.00
Large Cell Carcinoma	2	2.00
Malignant carcinoid	1	1.00
NSCLC, NOS	2	2.00

Table 2: Serious adverse events amongst study patients

SAEs	Grade	Intervention	Attribution to FDG	Attribution to surgical procedure
Diverticulitis	3	Colectomy/Colostomy	Unrelated	Unrelated
Recurrent left lung atelectasis	3	Repeated bronchoscopies, minitracheostomy	Unrelated	Definitely related
Aspiration pneumonia, multiple organ failure	5	Intubation, critical care	Unrelated	Definitely related
Persistent air leak	3	Re-operation, pleural tent	Unrelated	Definitely related
Colitis/Ileus	3	Total parenteral nutrition, antibiotics	Unrelated	Unrelated
Pulmonary Embolism	5	Expired at a rehabilitation facility	Unrelated	Probably related
Complete lung atelectasis	3	Bronchoscopy, VATS re-exploration	Unrelated	Definitely related

Figure 1: Immunohistochemical detection of a cluster of few carcinoma cells using CK AE1/AE3

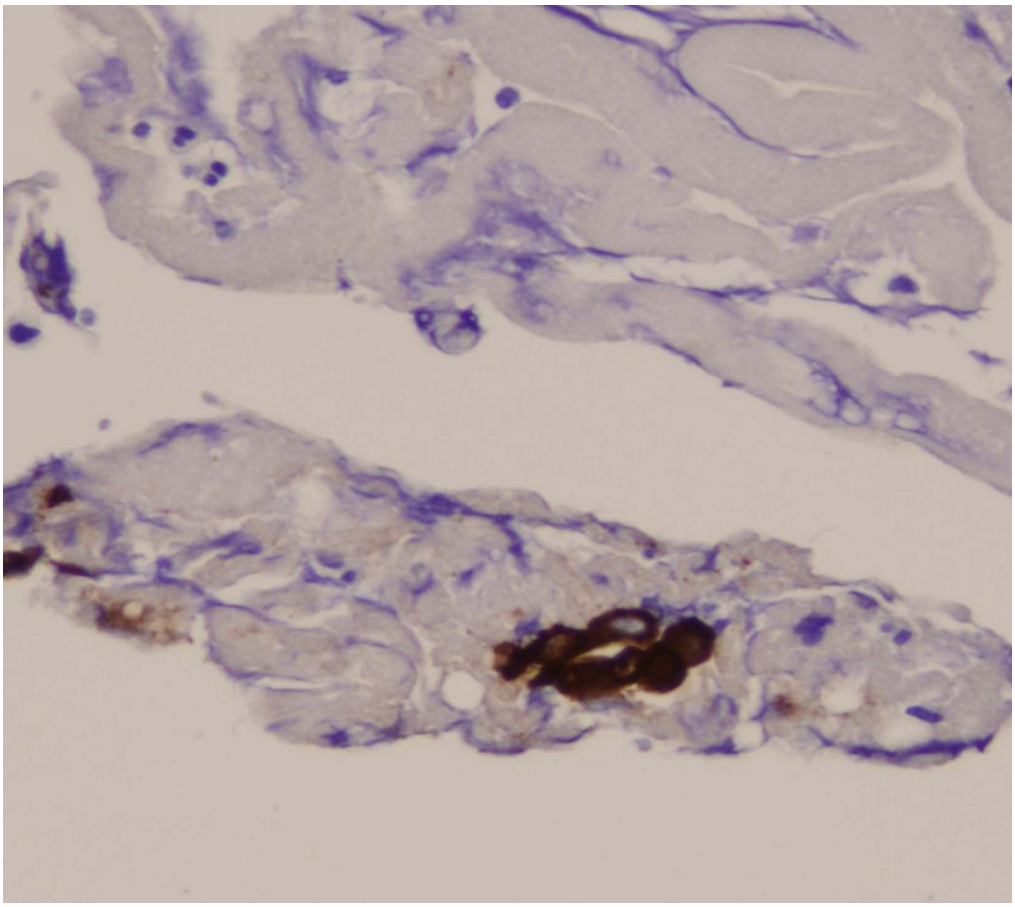


Table 3: Sensitivity and specificity of PET-CT for detection of lymph node metastases by

A) H&E/IHC

PET-CT LN Status	H&E/IHC positive LNs	H&E/IHC negative LNs
Positive	6	2
Negative	21	336

Sensitivity = 22% (95%CI=18.6%, 42.3%) Specificity = 99% (95%CI=97.9%, 99.93%)

B) RT-PCR

PET-CT LN Status	RT-PCR positive LNs	RT-PCR negative LNs
Positive	4	4
Negative	34	164

Sensitivity = 11% (95%CI=2.94%, 24.80%) Specificity = 98% (95%CI=94.05%, 99.35%)

Table 4: Sensitivity and specificity of the gamma probe for detection of lymph node metastases by

A) H&E/IHC

Gamma Probe LN Status	H&E/IHC positive LNs	H&E/IHC negative LNs
Positive (Hot)	20	162
Negative (Cold)	7	176

Sensitivity = 74% (95%CI=53.72%, 88.89%) Specificity = 52% (95%CI=46.60%, 57.51%)

Positive Predictive Value = 11% Negative Predictive Value = 96%

B) RT-PCR

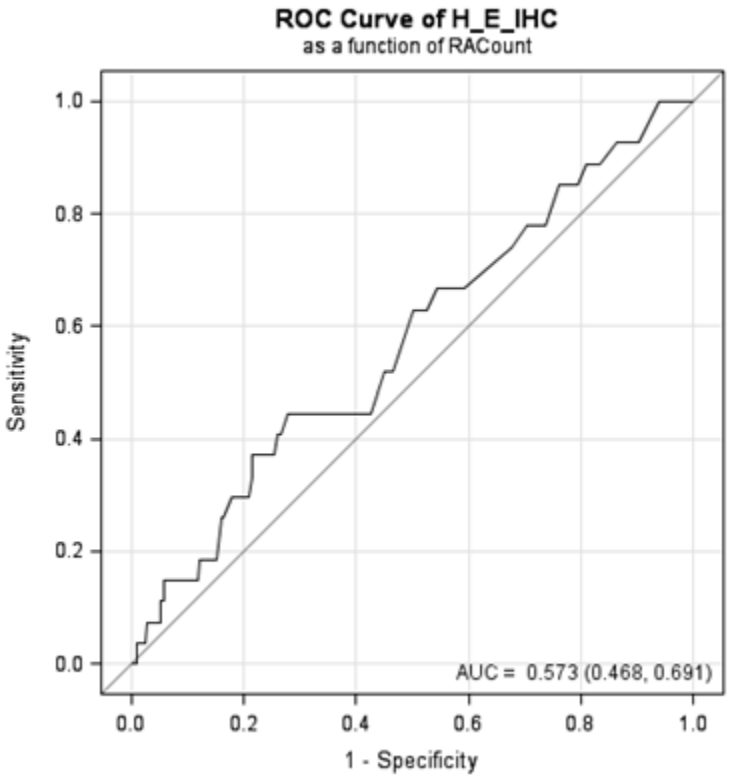
Gamma Probe LN Status	RT-PCR positive LNs	RT-PCR negative LNs
Positive	14	85
Negative	24	84

Sensitivity = 37% (95%CI=21.81%, 57.48%) Specificity = 50% (95%CI=41.93%, 57.48%)

Positive Predictive Value = 14% Negative Predictive Value = 78%

Figure 2: Receiver Operating Characteristic Curves of the Gamma Probe

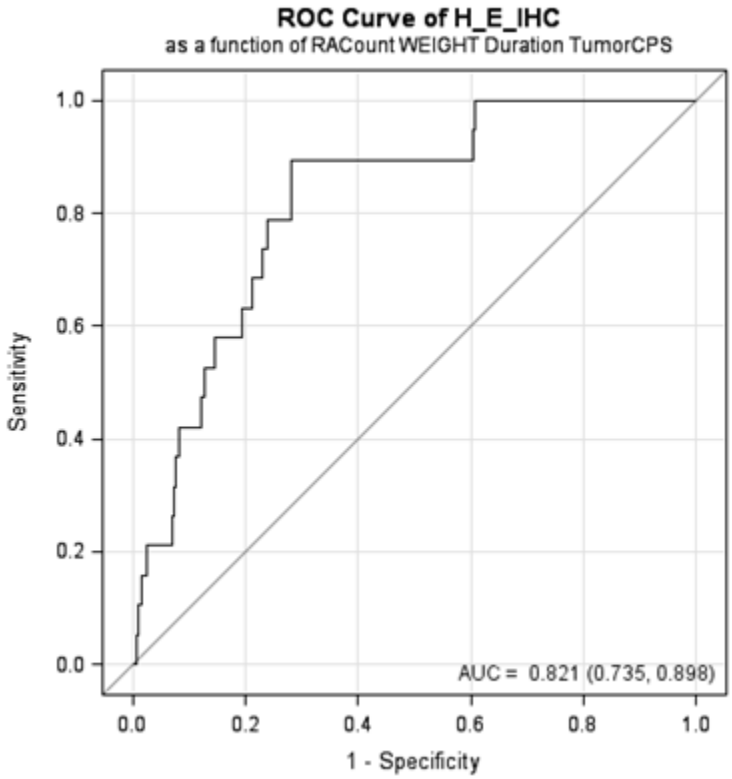
A) As a function of only the lymph node radioactivity count



Threshold:

RACount	_PROB_	_sens_	_spec_
27	0.074294	0.444	0.722

B) As a function of the lymph node radioactivity count, lymph node weight, duration of time from FDG injection and primary tumor radioactivity count



Threshold:

RACount	WEIGHT	Duration	TumorCPS	_PROB_	_sens_	_spec_
18	0.41	262	697	0.059550	0.895	0.719

Table 5: Model Selection

Model	AUC
H_E_IHC=RACount	0.569
H_E_IHC=WEIGHT	0.403
H_E_IHC=Duration	0.636
H_E_IHC=TumorCPS	0.604
H_E_IHC=RACount WEIGHT	0.576
H_E_IHC=RACount Duration	0.681
H_E_IHC=RACount TumorCPS	0.625
H_E_IHC=Duration TumorCPS	0.792
H_E_IHC=RACount Duration TumorCPS	0.813
H_E_IHC=RACount WEIGHT Duration TumorCPS	0.820

IV CHAPTER 4

LUNG CANCER LYMPH NODE MICROMETASTASIS DETECTION USING RT-PCR – CORRELATION WITH VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) EXPRESSION

1V.1 ABSTRACT

Objectives:

Lymph node (LN) staging provides critical information in non-small cell lung cancer (NSCLC) patients. Lymphangiogenesis may be an important contributor to the pathophysiology of lymphatic metastases. We hypothesized that the presence of lymph node micrometastases positively correlates with VEGF-A/C/D and VEGF-receptor-3 (lymphangiogenic factors) expression in lymph nodes.

Methods:

Forty NSCLC patients had pre-operative PET-CT and mediastinoscopy. RT-PCR assays for mRNA expression of epithelial markers (CK-7, CEACAM-5 and PLUNC) were performed in selected fluorodeoxyglucose (FDG)-avid lymph nodes. VEGF-A/C/D and VEGF-receptor-3 expression levels were measured in primary tumors and lymph nodes. Wilcoxon rank sum test was run for the association between the RT-PCR epithelial marker levels and VEGF expression levels in the LNs.

Results:

RT-PCR for CK-7, CEACAM5 or PLUNC indicated lymph node micrometastatic disease in 19 of 35 patients (54%). There was a high correlation between detection of micrometastases and VEGF-A/C/D or VEGF-receptor-3 expression levels in lymph nodes. Median follow-up was 12.6 months.

Conclusions:

RT-PCR analysis of FDG-avid lymph nodes results in up-staging of patients. Micrometastases correlate with the expression of VEGF in lymph nodes in NSCLC patients. This may reflect the role of lymphangiogenesis in promoting metastases.

IV.2 INTRODUCTION

Lung cancer is the most frequent cause of cancer death in both men and women in the United States and will account for about 27% of all estimated cancer deaths in 2012 (3). Most lung cancer patients present at an advanced stage and it is the metastatic burden of disease which typically leads to their demise. It is currently difficult to predict which primary tumors will metastasize early. Some patients present with small tumors that metastasize early, while others have large, locally invasive tumors which remain completely localized. This latter group of patients is much easier to treat. Non-small cell lung cancer consists of a heterogeneous collection of tumors with diverse molecular characteristics and variable metastatic potential. Understanding the exact molecular differences between such groups will facilitate the development of novel and specific treatment strategies to improve the survival from this lethal disease.

Malignant cells metastasize from the primary tumor to other organs via either the lymphatic or vascular network (Figure 1). Indeed, tumor metastasis to regional lymph nodes often represents the first step of tumor dissemination and serves as a major prognostic indicator for the progression of human cancers (79). As a means of exploring the role of lymphangiogenesis in the occurrence of nodal metastases, we sought to correlate the presence of micrometastases with VEGF A, C, D and VEGF receptor-3 expression in LNs. The VEGF-C/VEGF-D/VEGFR-3 axis is the best validated signaling system for promoting lymphangiogenesis associated with solid tumors and the metastatic spread of tumor cells to lymph nodes (80). VEGF-A has also been shown to influence lymphangiogenesis (131) although its primary effect is the promotion of tumor angiogenesis.

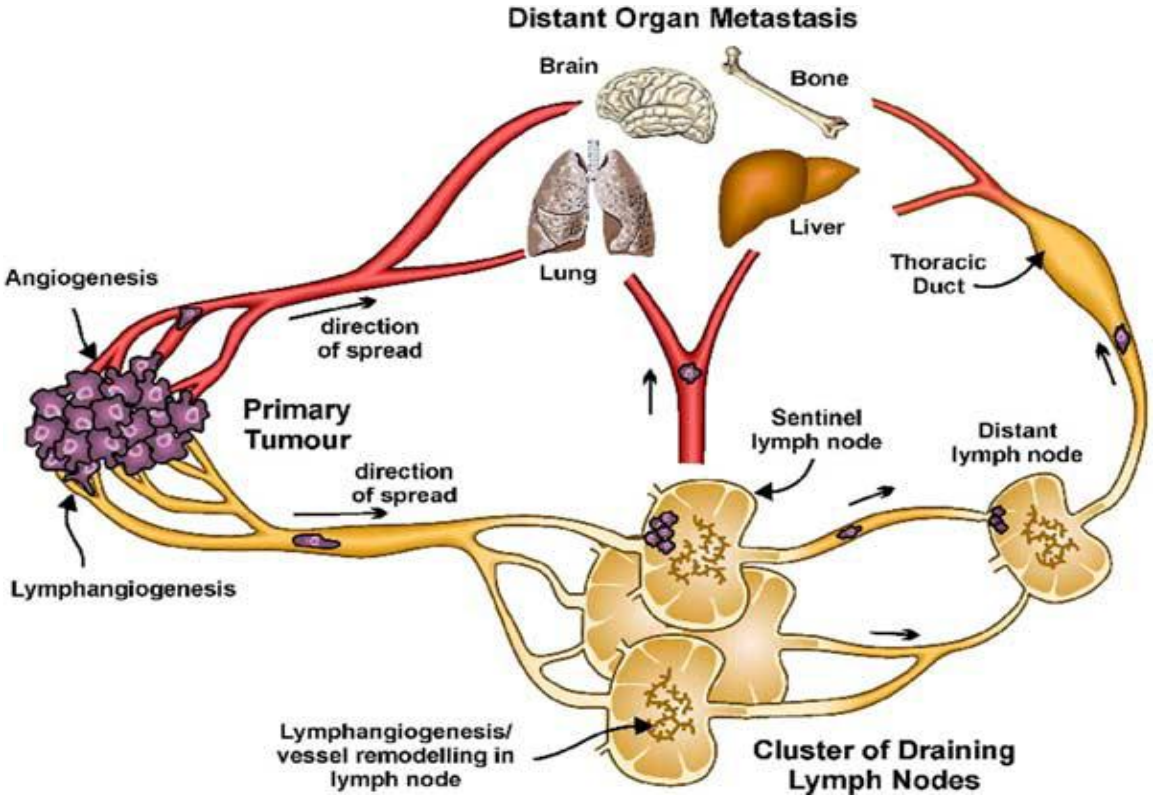


FIGURE 1. Schematic representation of potential routes of metastasis via the lymphatic vasculature (yellow), blood vessels (red) and lymph nodes. (Copied with permission from John Wiley and Sons, Publishers) (80)

IV.3 METHODS

Our Institutional Review Board (IRB) approved a radioguided lymph node mapping study on September 6, 2007 for 100 patients with resectable, clinical Stage I or II non-small cell lung cancer. Individual patient consent was obtained. Ancillary studies using molecular markers were written into the protocol. An exploratory subset of 40 patients was selected for correlation of lymph node epithelial marker expression with VEGF A, C, D and VEGF receptor-3 expression in the same lymph nodes using reverse transcriptase – polymerase chain reaction (RT-PCR) techniques.

A handheld gamma probe was used to select the lymph nodes for these assays as reported previously (56). On the day of surgery, each patient had intravenous injection of 10 mCi of F18-

Fluorodeoxyglucose (FDG) followed by mediastinoscopy and anatomic lung resection if all the sampled mediastinal lymph nodes were benign on frozen section analysis. Standard thoracoscopic or open lung resection, as appropriate for the individual patient's tumor, was followed by complete thoracic lymphadenectomy. The lymph nodes were labeled using the ATS/Naruke lymph node map (112). All harvested lymph nodes were scanned with the gamma probe outside the thoracic cavity to measure the gamma radiation resulting from any accumulated FDG in individual nodes. Intrathoracic radioactivity measurements were abandoned early in the study because of their unreliability due to interfering signal from the heart and great vessels. The resected tumor was similarly scanned outside the thorax. We compared the radioactive signals from the lymph nodes to each other. The FDG avid (hot) nodes had more than twice the signal intensity of the coldest lymph node in the entire thoracic field for that particular patient. An equal number of FDG avid (hot) and non- FDG avid (cold) nodes were selected for detailed pathologic analysis.

All surgically removed lymph nodes were bisected and examined by routine H&E. The selected lymph nodes that were malignant on H&E staining required no further pathologic analysis. However, the selected lymph nodes that were not malignant on H&E staining were subjected to ultra-staging with multiple step sections, immunohistochemistry (IHC) using cytokeratin AE1/AE3 and RT-PCR for CK-7, CEACAM5 and PLUNC (epithelial markers). These nodes were processed according to a standard protocol. After formalin fixation and embedding in paraffin, step sections of each lymph node were taken at 30-40 micron intervals. The sections were stained with H&E and an average of ten serial sections were evaluated. IHC was performed with standard monoclonal mouse anti-human cytokeratin antibody clones AE1/AE3 (Dako Inc, Carpinteria, CA). Formalin fixed, paraffin embedded tissue was pretreated

with Proteinase K for 5 minutes. The primary antibody was diluted 1:100 and then incubated on the slides for 30 minutes. All staining steps were performed on a Dako Autostainer machine. Detection was done using the Mouse Envision + system (also from Dako). The testing was performed in a CLIA certified clinical laboratory using prostate tissue as positive controls.

RNA extraction was performed on fresh primary tumors and an equal number of FDG avid and non-avid lymph nodes. Tissues were homogenized with Trizol reagent (Invitrogen Carlsbad, CA). RNA was then precipitated from the aqueous phase using isopropanol. For quality control, 260/280 ratios were examined to confirm preparation purity and an RNA aliquot was run on an Agilent 2100 bioanalyzer to confirm RNA integrity by generating an RNA Integrity Number (RIN) value. Human mRNAs for beta actin *(ACTB),* keratin 7 *(CK7),* vascular endothelial growth factor A *(VEGFA),* C (*VEGFC),* D (*VEGFD),* VEGF receptor 3 *(VEGFR3* or *FLT4*), carcinoembryonic antigen-related cell adhesion molecule 5 (*CEACAM5),* and palate, lung and nasal epithelium associated protein (*PLUNC)* were quantified by RT-PCR-based TaqMan™ Gene Expression Assays (Applied Biosystems, Foster City, CA). The assay IDs were respectively, Hs99999903_m1, Hs00559840_m1, Hs00900055_m1, Hs1099203_m1, Hs01128659_m1, Hs01047677_m1, Hs00944025_m1, and Hs00213177_m1. Briefly, random primers and reagents provided with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) were used to reverse transcribe 2µg total RNA from primary lung tumors and lymph nodes. The cDNA were used as template in 44 cycle-PCR reactions on a 7900HT real-time PCR machine (Applied Biosystems). For each reaction, the quantification cycle (Cq) value, approximately inversely proportional to log2 value of the concentration of the analyte RNA, was obtained with SDS™ software (Applied Biosystems; version 2.3). The average of Cq values of triplicate PCR reactions was used for analysis. Cq values for the mRNAs of interest were

normalized by subtracting the value for beta actin mRNA from them. Beta actin is a highly conserved gene frequently used as a loading control in PCR assays.

Descriptive statistics such as frequencies and relative frequencies were computed for categorical variables. Numeric variables were summarized using simple descriptive statistics such as the mean, standard deviation, median, range, etc. The Wilcoxon rank sum test was used to correlate the lymph node micrometastatic status to normalized VEGF numeric variables. Box plots were also provided to show the differences in VEGF expression according to lymph node micrometastatic status. A 0.05 nominal significance level was used in all testing. The expression of epithelial markers in lymph nodes was used to upstage individual patients. All statistical analyses were done using SAS (version 9.3).

IV.4 RESULTS

The demographic characteristics of the forty patients in this study are shown in table 1. The nodal stage distribution of patients by routine H&E, immunohistochemistry (IHC) and quantitative real-time polymerase chain reaction (RT-PCR) are shown in table 2. Immunohistochemistry and RT-PCR resulted in up-staging of patients, culminating in positive N1 and N2 lymph nodes in 45% and 15% of patients respectively by RT-PCR.

In the pathological examination of the excised nodes: 5 patients had proven metastatic disease in the studied LNs on H&E, while IHC identified LN disease in 2 of the 35 patients without H&E evidence for metastatic disease. The RT-PCR analysis suggested additional metastatic disease in 19 of 35 patients (54%).

189 lymph nodes were evaluated by RT-PCR from the 40 patients. There was a highly positive correlation between RT-PCR detection of micrometastases and VEGF A, C, D or VEGF-receptor 3 expression levels in LNs (Table 4). Box plots are also provided to show the differences in location and scale of VEGF expression between malignant (positive) and non-malignant (negative) lymph node groups.

There has been one death from disease and two other recurrences among patients who had micrometastatic disease detected in their lymph nodes by RT-PCR. In the group of patients without such disease detected in their lymph nodes, there has been only one recurrence so far. The difference in the overall and recurrence free survival between these two groups has not reached statistical significance during our limited follow-up period. This will be reassessed when the median follow-up reaches 24 months.

IV.5 DISCUSSION

Increased understanding of the complex biology of lung cancer has led to advances in management of the disease. Accurate staging of individual patients remains a critical need. It guides the choice of therapy and stratifies patients appropriately for clinical trial of novel interventions. It also facilitates the comparison of treatment outcomes. Standard methods of evaluating thoracic lymph nodes (hematoxylin-eosin staining) can miss micrometastases (79, 97-99, 114, 115). Intensive pathologic techniques such as serial sectioning, immunohistochemistry and RT-PCR are more sensitive in detecting these micrometastases (102, 102, 113-115). However, these techniques are labor intensive and expensive. Thus, they can practically be applied only to a limited number of lymph no

des in each patient. We used a handheld gamma probe to select lymph nodes for measurement of the expression of mRNA for epithelial markers by RT-PCR.

Due to the greater sensitivity of RT-PCR for micrometastatic lymph node disease detection, we upstaged 19 of 35 patients (54%) using this technique compared to routine pathology (H&E). This is consistent with other RT-PCR based studies (97, 101, 126). The data was reported on a "per patient" basis and not a "per node" basis. This is because decisions on adjuvant therapy would be based on the presence or absence of any lymph node metastases regardless of the number of lymph nodes involved. We also wish to assess survival based on the presence or absence of any micrometastatic disease in the lymph nodes (i.e. two groups).

Concerns have been expressed that RT-PCR may be overly sensitive and may include false positives from mesothelial or endothelial cells within lymph nodes. The prognostic significance of RT-PCR detection of tumor-specific molecular markers has been shown by others (102, 126, 132). We selected CK-7, CEACAM 5 and PLUNC as the epithelial markers of

interest based on literature review (101, 102, 126). The positive threshold for the expression levels of these markers in the lymph nodes was based on the minimal expression of the same markers in primary tumors. RT-PCR is an imperfect 'gold standard' for the presence of lymph node micrometastasis. Since, there is no readily available method to verify its accuracy, we have to depend on the recurrence free survival of the two groups defined by the presence or absence of RT-PCR detected nodal disease. Our median follow-up is still relatively short (12.6 months). Thus, it seems early to assess the survival impact of molecularly detected lymph node micrometastasis in our patient cohort. If the prognostic value of molecularly detected micrometastases is proven, it would be appropriate to run clinical trials to assess the benefit of adjuvant therapy in such patients. Chemotherapy is the logical choice but the value of innovative intraoperative interventions, radiotherapy and targeted agents in such patients could be investigated.

Malignant cells metastasize from the primary tumor to other organs via either the lymphatic or vascular network. Indeed, tumor metastasis to regional lymph nodes often represents the first step of tumor dissemination and serves as a major prognostic indicator for the progression of human cancers (79). It is currently believed that lymphatics provide the major route of lung cancer metastases. However, the exact molecular mechanisms remain unclear. There is experimental evidence that tumors can induce the formation of new lymphatic vessels (lymphangiogenesis) even before they metastasize to lymph nodes, and that metastastic tumor cells continue to induce lymphatic vessel growth within sentinel lymph nodes, possibly promoting their further metastatic dissemination (80, 133). The VEGF-C/VEGF-D/VEGFR-3 axis is the best validated signaling system for promoting lymphangiogenesis associated with solid tumors and the metastatic spread of tumor cells to lymph nodes (80). The secreted

glycoproteins VEGF-C or VEGF-D activate VEGFR-3, a cell surface receptor tyrosine kinase on lymphatic endothelium, leading to growth of lymphatic vessels (134). Over-expression of VEGF-C and/or VEGF-D by tumor cells increases peritumoral and/or intratumoral lymphangiogenesis, promotes metastasis to local lymph nodes and may facilitate distant organ metastasis. The role of VEGF-A in angiogenesis via activation of its receptors, VEGFR-1 and VEGFR-2, has been extensively documented, but it has also been shown to influence lymphangiogenesis (131). As a means of exploring the role of lymphangiogenesis in the occurrence of nodal micrometastases, we measured VEGF A, C, D and VEGF receptor-3 expression in LNs. The quantification cycle (Cq) values for the mRNAs of interest were normalized by subtracting the value for beta actin mRNA from them (Table 4). Beta-actin is a highly conserved gene frequently used as a loading control in PCR assays. Actins are proteins that are involved in cell motility, structure and integrity. Thus, all the lymph nodes were expected to express beta-actin. Note that lower Cq values reflect higher mRNA expression levels. The correlation analysis was performed with the VEGF Cq values as continuous variables and the lymph node status as categorical values, either positive (malignant) or negative (non-malignant). Our study showed a highly positive correlation between the expression of VEGF A, C, D and VEGF receptor-3 in lymph nodes and the presence of micrometastases in those same nodes. This is consistent with the lymphangiogenesis literature. It would be worthwhile to investigate whether anti-lymphangiogenic treatment can prevent lymphatic and distant metastasis of NSCLC.

Limitations of our study include the fact that not all lymph nodes had IHC and RT-PCR. Performing such analysis on every single node would be too laborious and expensive. Also, our short clinical follow-up precludes survival analysis at this time.

Conclusion

IHC and RT-PCR for epithelial markers can be used to identify non-small cell lung cancer patients with lymph node micrometastatic disease. The presence of micrometastases was associated with higher VEGF A, C, D and VEGF receptor-3 expression in LNs. The impact of these findings on survival will be determined with further follow-up.

IV.6 CHAPTER 4 TABLES AND FIGURES

Table 1: Patient Demographic, Surgical and Pathologic Characteristics

Demographic or Characteristic	No. of Patients (N=40)	%
Age (years)		
Median	71	
Range	52 - 84	
Sex		
Male	7	17.5
Female	33	82.5
Clinical follow-up (months)		
Median	12.6	
Range	3.7 - 31.6	
Clinical Stage		
I	36	90
II	4	10
Sex		
Male	7	17.5
Female	33	82.5
Operation performed		
Lobectomy	39	97.5
Pneumonectomy	1	2.5
Tumor histology		
Adenocarcinoma	29	72.5
Squamous cell carcinoma	9	22.5
Adenosquamous carcinoma	1	2.5
Large Cell Carcinoma	1	2.5

Table 2: Lymph Nodal Staging Distribution by Various Modalities

Staging Modality	**No. of Patients in N Categories**		
	N0	**N1**	**N2**
Routine H & E	35	4	1
Immunohistochemistry	33	6	1
Quantitative RT-PCR	16	18	6

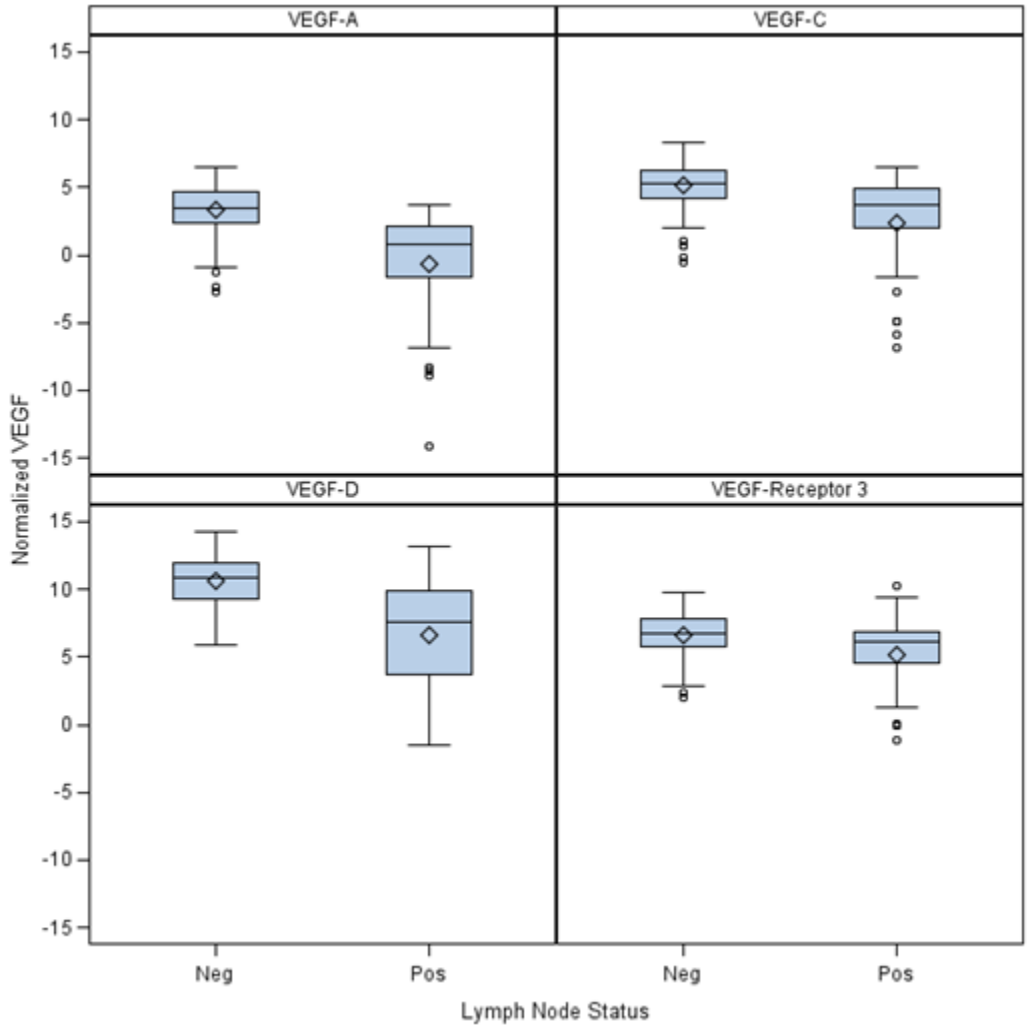
H & E = Hematoxylin & Eosin; RT-PCR = reverse transcriptase-polymerase chain reaction

Table 3: Association between Lymph Node micrometastatic status and normalized VEGF RT-PCR quantification cycles (Cq levels) in LNs

Normalized Variable	Statistic	Lymph Node micrometastatic status			
		Negative	Positive	Overall	P-value
VEGF-A	Mean (SD) / N Median (Range)	3.4 (1.7) / 148 3.5 (-2.7, 6.5)	-0.7 (4.1) / 41 0.8 (-14.1, 3.7)	2.5 (2.9) / 189 2.9 (-14.1, 6.5)	<.0001
VEGF-C	Mean (SD) / N Median (Range)	5.1 (1.7) / 148 5.3 (-0.5, 8.3)	2.3 (3.6) / 41 3.7 (-6.9, 6.5)	4.5 (2.5) / 189 5 (-6.9, 8.3)	<.0001
VEGF-D	Mean (SD) / N Median (Range)	10.6 (1.8) / 148 10.9 (5.9, 14.3)	6.7 (4.1) / 41 7.6 (-1.5, 13.2)	9.7 (3) / 189 10.5 (-1.5, 14.3)	<.0001
VEGF-Receptor 3	Mean (SD) / N Median (Range)	6.7 (1.5) / 148 6.8 (2.1, 9.8)	5.2 (2.8) / 41 6.1 (-1.1, 10.3)	6.4 (2) / 189 6.6 (-1.1, 10.3)	<.0025

VEGF = Vascular Endothelial Growth Factor; SD = Standard Deviation; N = Sample Number
Note: Lower Cq values reflect higher mRNA expression levels

Figure 1: Box plots of VEGF expression (measured as RT-PCR Cq levels) grouped by lymph node micrometastatic status.



Note: Lower Cq values reflect higher mRNA expression levels

V CHAPTER 5

CONCLUSION

V.1 SUMMARY

Non-small cell lung cancer (NSCLC) is a major global health challenge. Its epidemiology indicates that tobacco control is the major preventive strategy in the fight against this disease. Early stage lung cancer is treated primarily by surgical resection. The extent of lymphadenectomy during such resections is currently quite variable. Using the U.S. SEER database, we have demonstrated that the extent of lymphadenectomy has a prognostic impact on patients. There is a vital need to establish a global standard of care for this aspect of surgical treatment.

Accurate staging of NSCLC requires more detailed pathologic analysis of thoracic lymph nodes than is provided by hematoxylin and eosin (H&E) staining. Labor intensive and expensive pathologic analysis such as immunohistochemistry (IHC) and RT-PCR have been demonstrated to improve lymph node staging. Research efforts to identify a cost-effective tool for the selection of a few lymph nodes in each patient that should undergo such ultra-staging have been ongoing for many years. The ideal tool remains elusive. A hand-held probe to detect gamma emission after intravenous FDG injection was not accurate enough to make a major clinical impact in NSCLC staging.

The underlying biologic molecular mechanisms for lymph node metastasis of NSCLC remain unclear. Lymphangiogenesis may play an important role. The VEGF-C/VEGF-D/VEGFR-3 axis is the best validated signaling system for promoting lymphangiogenesis associated with solid tumors. As a means of exploring the role of lymphangiogenesis in the occurrence of nodal micrometastases, we measured VEGF A, C, D and VEGF receptor-3 expression in LNs. We demonstrated that there is a high correlation between detection of micrometastases and VEGF-A/C/D or VEGF-receptor-3 expression levels in LNs. Experimental

use of anti-lymphangiogenic factors in preclinical models will guide the potential use of these agents to treat NSCLC.

V.2 STRENGTHS AND LIMITATIONS

The strengths of this work include the investigation of multiple aspects of NSCLC lymph node metastasis. Population-based, clinical and basic science techniques were employed. A major strength of SEER data is that the large sample size allows the detection of moderate associations and permits complex multivariate analysis (13). It is also more generalizable to the community. This work involved the execution of a detailed prospective clinical trial. Our findings provide abundant avenues for further research.

Limitations include the fact that the SEER database lacks granular detail such as smoking history and the use of chemotherapy. IHC and RT-PCR analysis were performed only on selected lymph nodes. Ideally, the application of these techniques to all the lymph nodes would have provided useful information but the cost was prohibitive. The inability of fluorodeoxyglucose (FDG) to distinguish between malignant and inflamed tissue was a major limitation. There is no accurate means to verify RT-PCR results in lymph nodes thus it can only be used as a research gold standard. Our study cannot determine a direct causal relationship between lymphangiogenesis and the occurrence of lymph node metastases.

V.3 CONCLUSIONS

There is a need to improve the lymph node staging of lung cancer by sampling an adequate number of lymph nodes during surgical resections. Changes in practice patterns in both community and academic thoracic surgical practices can make a major impact on therapy for a large number of patients. Further studies may lead to modifications in the lung cancer staging system. The selective use of advanced pathologic techniques can improve the detection of lymph node micrometastases which may portend a worse prognosis for patients. The ideal tool for the selection of a few lymph nodes in each patient for these advanced pathologic techniques remains elusive. Lymphangiogenesis plays a role in the pathogenesis of lymphatic metastasis. The exploitation of this molecular process for therapeutic benefit requires further translational research.

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