Molecular assessment of lymph nodes in patients with resected stage I non-small cell lung cancer: Preliminary results of a prospective study

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Objectives: Routine histologic examination of resected lymph nodes in patients with stage I non-small cell lung cancer may underestimate the incidence of advanced disease. The presence of occult lymph node metastases may predict a higher risk of recurrence after intended curative resection. The purpose of this study was to determine the prognostic significance of *TP53* and *K-ras* mutations in histologically determined negative lymph nodes from patients with stage I non-small cell lung cancer who underwent intended curative surgical resection.

Methods: Between July 1995 and March 1998, clinical data and tissue samples of primary tumors and lymph nodes were collected in a prospective fashion from 102 patients undergoing resection for non-small cell lung cancer (stage I, n = 55; stage II, n = 32; stage IIIA, n = 15). *TP53* and *K-ras* mutations were detected by direct sequencing. If molecular alterations were found in the primary tumor, the corresponding lymph nodes were examined for these same *TP53* (by oligonucleotide hybridization) and *K-ras* (by allele-specific ligation) mutations.

Results: *TP53* mutations were found in 47 of 94 primary tumors (50%), and *K-ras* mutations were present in 26 of 55 adenocarcinomas (47%). A total of 134 lymph nodes from 32 patients with stage I disease were analyzed. In 9 cases (28%) the same *TP53* or *K-ras* mutations were found in tumor and lymph node specimens, suggesting occult metastasis. On the basis of nodal location, 7 patients had their disease upstaged by a single stage and 2 patients by two stages. All 28 patients with stage II or III disease had pathologically determined positive nodes that were confirmed as positive by molecular analysis. Standard histopathologic assessment of regional lymph nodes failed to detect metastases at levels below 0.9% tumor-specific mutant *TP53* clones per node. No statistically significant difference in disease-specific or overall survival was observed between patients with stage I disease with and without molecular lymph node metastases.

Conclusions: Occult lymph node metastases are present in a significant percentage of patients with stage I non-small cell lung cancer. These data suggest that molecular analysis allows a more accurate assessment of staging. However, larger studies are needed to determine the clinical role of molecular staging.

Despite advances in preoperative techniques to determine lymph node status, about a third of all patients undergoing surgical resection for early stage non-small cell lung cancer (NSCLC) ultimately die of local recurrence.¹ The 5-year survival for localized NSCLC remains one of the lowest among all solid tumors. Multiple factors, including delay in diagnosis, the extent of pulmonary resection (lobectomy versus segmentectomy or wedge resection), the degree of lymph node sampling or dissection, and the presence of lymph node or bone marrow micrometastases not identified on histopathologic section, may contribute to this poor outcome.

Numerous prognostic factors identified in patients with NSCLC may enable stratification of patients into subsets according to risk of recurrence after complete resection. Pathologic stage, including the presence or absence of lymph node metastases, is the strongest prognostic factor in patients with lung cancer. Currently staging relies on histopathologic identification of malignant cells in regional lymph nodes. Routine pathologic analysis of resected lymph node usually consists of the preparation of one or two sections from the central area of the node, followed by staining with hematoxylin and eosin for microscopic examination. Unfortunately, this method is often inaccurate, because tissue sectioning for microscopy is random and the threshold for subjective confirmation of tumor presence would require at least 2% tumor replacement of the total tissue.² Because clinicians make important treatment decisions based on this limited examination, the number of patients with nodal metastases presumably is underestimated. Immunohistochemical study of regional lymph node in patients with NSCLC has demonstrated that 15% to 25% of patients with negative nodes have their disease understaged with routine pathologic assessment. However, it remains unclear whether the detection of micrometastatic disease in lymph nodes is clinically important.³⁻⁸

During the past decade the development and refinement of immunohistochemical staining techniques and of the polymerase chain reaction (PCR) to amplify genetic material have led to the use of molecular markers as a novel strategy to predict outcome. Molecular alterations in lung cancer include both induced expression of specific oncogenes (eg, *K-ras*), and loss of tumor suppressor genes (eg, *TP53*). Ras is a 21-kd G protein, with 20% to 40% of adenocarcinomas showing *K-ras* mutations.^{9,10} Mutations of *TP53*, with frequencies greater than 50% in NSCLC and 80% in small cell lung cancer, can lead to loss of tumor suppressor function, cellular proliferation, and inhibition of apoptosis.¹¹ These more common molecular abnormalities in lung cancer are currently used to develop diagnostic studies for detecting early disease and to discriminate targets for gene therapy.²

The purpose of this prospective study was to improve the ability to assign a pathologic stage in NSCLC by detecting the presence of micrometastatic disease in regional lymph nodes with common molecular techniques. In addition, the clinical significance of occult lymph node metastases was evaluated by determining the influence of micrometastases on survival.

Methods

Patients

Between July 1995 and March 1998, fresh tumor and lymph node samples were collected from 134 patients with clinical stage I NSCLC undergoing surgical resection at The Johns Hopkins Hospital and the Johns Hopkins Bayview Medical Center, Baltimore, Md. This study was approved by the institutional review boards at both institutions. Informed consent was obtained from all patients before the operation. Any patient who received preoperative therapy (neoadjuvant chemotherapy and radiation therapy) was not eligible to participate. Thirty-two patients were excluded because of a final histopathologic report showing more advanced disease (IIIB to IV tumors, *n* = 7), other non-NSCLC primary lung neoplasm (*n* = 8), no evidence of tumor (*n* = 5), pulmonary metastasis from another primary site (*n* = 5), or a positive bronchial or vascular margin (*n* = 7).

At the time of the primary operation all patients included in this study were treated with wedge or segmental resection, lobectomy, bilobectomy, or pneumonectomy according to the tumor location and the patient's pulmonary function. Complete resection was achieved in all cases. Lymph node sampling was performed from all accessible areas and labeled according to the mapping scheme of the American Thoracic Society.¹² TNM classification and tumor staging were performed according to the revised International System for Staging Lung Cancer.¹ Fifty-five patients had stage I tumors, 32 patients had stage II tumors, and 15 patients had stage IIIA tumors. Patient characteristics for the patients with stage I disease are shown in Table 1. Portions of the tumor and lymph node to be used for molecular analysis were immediately frozen at -80°C. DNA was prepared from all tissues in a separate laboratory to avoid any possibility of PCR contamination.

Clinical information was collected in a prospective fashion. These data included time of first recurrence, site of first recurrence, and time and cause of death. The molecular study investigators were blinded to all clinical and histopathologic information until completion of all molecular assays. Routine clinical follow-up was performed without the knowledge of the molecular data.

Histopathologic Examination

Lymph node specimens were embedded in optimum cold-temperature medium. The specimens were evenly planed with a cryostat until a smooth representative lymph node section was present. Two sections 7 µm thick were then obtained for hematoxylin and eosin staining. Thirty 14-µm sections were then taken for DNA extraction, followed by another two 7-µm sections for hematoxylin and eosin staining. An additional thirty 14-µm sections were also taken for DNA extraction, followed by a final two 7-µm sections for hematoxylin and eosin staining. The hematoxylin and

TABLE 1. Characteristics of 55 patients with stage I disease

Age (y)	
Mean	67
Range	40-87
Sex (No.)	
Male	31
Female	24
Race (No.)	
White	39
African American	14
Asian	1
Hispanic	1
Operative procedure (No.)	
Lobectomy	52
Bilobectomy	2
Pneumonectomy	1
Stage (No.)	
IA	27
IB	28
Cell type (No.)	
Adenocarcinoma	29
Squamous cell carcinoma	21
Large cell carcinoma	5

eosin-stained sections taken at three levels from each node were interpreted by a pathologist in blinded fashion as negative or positive for the presence of metastatic NSCLC.

Sequencing of the *TP53* Gene

A 1.8-kilobase fragment of the *TP53* gene (exons 5-9) was amplified from the fresh-frozen DNA in the primary tumor by PCR. A 500-ng sample of genomic DNA was used in a 25- μ L PCR reaction containing 16.6-mmol/L ammonium sulfate, 67-mmol/L tris(hydroxymethyl)aminomethane (Tris) hydrochloride (pH 8.8), 6.7-mmol/L magnesium chloride, 10-nmol/L β -mercaptoethanol, 1% dimethyl sulfoxide, 1.25 mmol/L of each deoxynucleotide triphosphate, 0.5 units of *Taq* DNA polymerase, and 150 ng of primers 5'-GTA GGA ATT CAC TTG TGC CCT GAC TT-3' and 5'-CAT CGA ATT CTG GAA ACT TTC CAC TTG AT-3'. PCR amplification was performed for 35 cycles of 95°C for 30 seconds, 58°C for 90 seconds, and 70°C for 90 seconds, with an extension at 70°C for 5 minutes during the final cycles. Before cycle sequencing the presence of an amplified product was confirmed by running 4 μ L of the 25- μ L reaction on a 1% agarose gel, followed by staining with ethidium bromide. The remaining 21 μ L was extracted with phenol and chloroform and was ethanol precipitated. The DNA pellet was resuspended in 15 μ L of LoTE (10-mmol/L Tris hydrochloride [pH 8.8], 0.05-mmol/L ethylene diamine tetraacetic acid), and 2 μ L was used for sequencing each exon of the *TP53* gene.

Direct Sequencing of *K-ras* Mutations

A 270-bp fragment containing exon 1 of the *K-ras* gene was amplified from the tumor DNA of patients with primary adeno-

carcinoma, adenosquamous carcinoma, and large cell carcinoma of the lung. These histologic cell types have been demonstrated previously to contain *K-ras* mutations, whereas squamous cell carcinomas only rarely contain these mutations. The genomic DNA was amplified in reactions similar to that described previously here for *TP53* with primers 5'-ATCGAATTCTGGTGGAG-TATTTGATAGTG-3' and 5'-ATCGAATTCCTCATGAAAATG-GTCAGAGAAAAC-3'. After purification of the PCR product, exon 1 of the *K-ras* gene was cycled-sequenced with the sequencing primer 5'-ATTCGTCCACAAAATGAT-3'. The products of the sequencing reactions were then separated by electrophoresis on denaturing gels, fixed, and exposed to film.

Molecular Probing of Regional Lymph Nodes

Patients found to have *TP53* or *K-ras* mutations in their primary tumors were selected to have their regional lymph nodes studied by molecular analysis with oligonucleotide hybridization and a modified allele-specific ligation assay, respectively.

DNA extracted from the lymph node were used to amplify exons 5 through 9 of the *TP53* gene as described previously here. These PCR products were next cloned into a phage λ vector and amplified further in *Escherichia coli* as described elsewhere.^{13,14} Between 1000 and 2000 clones were transferred to nylon membranes and hybridized with phosphorus 32-end-labeled oligonucleotide probes (17 or 18 bp) with sequences complementary to the mutant *TP53* sequence found during direct sequencing of the primary tumor. Thus these probes were unique and specific for the *TP53* mutations in each individual tumor. After hybridization the membranes were washed stringently at 54°C to 60°C to detect mutant-specific binding of the probes. The membranes were exposed to x-ray film; hybridizing plaques identified the presence of a mutant *TP53* gene.^{2,9} The percentage of mutant *TP53*-containing cells in each specimen was calculated by counting the number of labeled plaques and dividing this number by the total number of plaques that hybridized with the wild-type *TP53* probe.

For primary tumor samples with a *K-ras* mutation, a modified allele-specific ligation assay was used to analyze regional lymph nodes for metastatic spread.⁹ A 270-bp fragment containing exon 1 of this gene was amplified from the primary tumor and each regional lymph node. The PCR products were extracted as previously described here. The amplified DNA fragment was used as a template for the ligation assay, which detects all possible mutations at a given *K-ras* codon position (12a, 12b, 13a, or 13b). The presence and the nature of the mutations were determined according to the migration of ligation products formed in controlled experiments from templates with known *K-ras* mutations.

Statistical Analysis

Survival curves were estimated by the Kaplan-Meier method. Any differences were analyzed by the log-rank test.

Results

TP53 and *K-ras* Mutations in Primary NSCLC Tumors

One hundred two primary NSCLC tumors were analyzed for *TP53* and *K-ras* mutations. *TP53* mutations were detected in 47 of 94 (50%) of these primary tumors. This included 18 of 49 adenocarcinomas, 23 of 36 squamous cell tumors, 4 of 6

TABLE 2. Comparison of histopathologic assessment and molecular analysis of regional lymph nodes with the *TP53* plaque hybridization assay among patients (n = 39) with NSCLC with *TP53* mutation

Histopathologic assessment	Molecular assessment with <i>TP53</i> plaque assay	No. of lymph nodes
Negative	Negative	143
Negative	Positive	20
Positive	Negative	0
Positive	Positive	23

large cell carcinomas, and 2 of 3 poorly differentiated tumors of unspecified cell type. No *TP53* mutations were detected in adenosquamous tumors. Twenty-two of 50 stage I tumors (44%) contained a *TP53* mutation. The frequency of *TP53* mutations did not appear to correlate with tumor stage in this small group of patients (data not shown). In addition to missense mutations, 6 of the 46 mutations (13%) were frameshift, deletion, or nonsense (termination) mutations and would be predicted to encode a truncated *TP53* protein that immunohistochemical analysis usually fails to detect. G to T transversions were the most common mutations detected.

Mutations at codons 12 and 13 of the *K-ras* gene were also detected by direct sequencing. *K-ras* mutations were detected in 26 of 55 adenocarcinomas of the lung (47%). *K-ras* mutations were not detected in patients with large cell, adenosquamous, or poorly differentiated carcinomas. Similar to *TP53*, the frequency of *K-ras* gene mutations did not change with increasing pathologic stage. Forty-three percent (12 of 28) of the stage I adenocarcinomas contained a *K-ras* mutation. The most common *K-ras* gene mutation was a cysteine mutation at codon 12. Seven of the 102 patients had mutations in both the *K-ras* and *TP53* genes. Therefore a total of 66 patients had either a *TP53* or a *K-ras* mutation (or both) that would permit examination of the lymph node for occult metastasis with one of the two molecular assays.

Lymph Node Micrometastases Detected by Molecular Techniques

Three hundred lymph nodes from 62 patients (4.8 lymph nodes/patient) were analyzed with molecular techniques. Molecular staging of lymph nodes was not possible in 4 cases because material harvested as node did not contain lymphoid tissue on histologic review or there was a failure to amplify DNA from lymph node. One hundred eighty-six lymph nodes from 39 patients (4.8 lymph nodes/patient) with *TP53* mutations were tested with the oligonucleotide plaque hybridization assay (Table 2). All 23 lymph nodes with microscopic evidence of metastatic cancer were positive according to molecular analysis. Twenty of the 163

Figure 1. Percentages of mutant *TP53* clones in histopathologically determined negative (black bars) and histopathologically determined positive (white bars) lymph nodes from patients with NSCLC. Only 1 of 11 lymph nodes with fewer than 4.35% mutant *TP53* clones (dashed line) was detected by standard light microscopy of hematoxylin and eosin-stained section.

(12%) histopathologically determined negative lymph nodes had tumor-specific mutant DNA detected with the plaque assay. Nine of the 90 (10%) histopathologically determined negative lymph nodes from the 21 patients with stage I disease (N0) contained tumor-specific *TP53* mutations. Seven of these 21 patients with stage I disease (33%) had the disease upstaged to stage II (n = 5) or stage III (n = 2) lung cancer because of the molecular analysis (Table 3). All 33 histopathologically determined negative lymph nodes from 6 patients with T3 N0 tumors were negative for tumor-specific *TP53* mutations. Eleven of 40 (28%) histopathologically determined negative lymph nodes from the 12 patients with either N1 or N2 disease (according to histopathologically diagnosed lymph node metastases in other lymph nodes) were positive for *TP53* mutant cells with the plaque assay.

The percentage of *TP53* mutant containing clones was quantitated in 25 lymph nodes (12 histopathologically determined to be negative and 13 histopathologically determined to be positive). Only 1 lymph node (0.9% mutant *TP53* clones/wild-type clones) containing fewer than 5% mutant *TP53* clones was detected by light microscopy. The detection threshold for detection of metastases by light microscopy of hematoxylin and eosin-stained frozen sections was between 1% and 4.35% (Figure 1).

One hundred fourteen lymph nodes from 23 patients whose tumors had *K-ras* mutations were analyzed with the mutation ligation assay. All 24 lymph nodes with histologic evidence of metastatic tumor contained mutant *K-ras* (Table 4). Tumor-specific *K-ras* mutations were detected in 2 of 44

TABLE 3. Comparison of histopathologic staging and molecular analysis in patients with stage I NSCLC with lymph node metastases according to molecular analysis

Patient	Histologic type	Lymph node mutation		Histopathologic staging		Molecular staging		Site of recurrence
		<i>TP53</i>	<i>K-ras</i>	TNM	Stage	TNM	Stage	
738	Adenocarcinoma	Neg	Pos	T1 N0	IA	T1 N1	IIA	Local
768	Squamous cell carcinoma	Pos	Neg	T1 N0	IA	T1 N2	IIIA	Local and distant (died 64 mo)
811	Squamous cell carcinoma	Pos	Neg	T1 N0	IA	T1 N2	IIIA	Local (died at 14 mo)
821	Adenocarcinoma	Pos	Neg	T1 N0	IA	T1 N1	IIA	None
901	Squamous cell carcinoma	Pos	Neg	T1 N0	IA	T1 N1	IIA	None
905	Large cell carcinoma	Pos	Neg	T2 N0	IB	T2 N1	IIB	Local
943	Squamous cell carcinoma	Pos	Neg	T2 N0	IB	T2 N1	IIB	Local (died at 8 mo)
1011	Large cell carcinoma	Pos	Neg	T2 N0	IB	T2 N1	IIB	None
1186	Adenocarcinoma	Neg	Pos	T2 N0	IB	T2 N1	IIB	None

TABLE 4. Comparison of histopathologic assessment and molecular analysis of regional lymph nodes with the *K-ras* mutation ligation assay among patients (n = 23) with NSCLC with *K-ras* mutation

Histopathologic assessment	Molecular assessment with <i>K-ras</i> mutation ligation assay		No. of lymph nodes
Negative	Negative		87
Negative	Positive		3
Positive	Negative		0
Positive	Positive		24

lymph nodes (4.5%) from 11 patients with stage I tumors. Two of these 11 patients had the disease upstaged to stage II on the basis of the mutation ligation assay (Table 3). Overall 11 of 134 histopathologically determined negative lymph nodes from patients with stage I disease (8.2%) contained tumor-specific mutations of either *TP53* or *K-ras*. This finding resulted in an increase in stage in 9 of 32 patients with histopathologically determined stage I disease (28%).

Molecular Staging Analysis and Survival

Sixty-six patients had either a *TP53* or *K-ras* mutation in their primary tumor and were eligible for molecular staging and survival analysis. Six patients with T3 N0 tumors were excluded from the survival analysis. The remaining 60 patients were divided into three groups for analyses of overall and disease-specific survival. Median follow-up among surviving patients was 45 months.

In the first group 23 patients with stage I disease were found to be lymph node negative by both histologic and molecular techniques. Nine of these 23 patients died during follow-up. Four patients died of recurrent lung cancer, whereas 5 patients died of other causes. One of these

patients died in the immediate postoperative period (day 9) from a presumed pulmonary embolism. One patient died of a myocardial infarction 17 months after pulmonary resection. Three patients died of other nonlung malignancies (prostate, pancreatic, and esophageal cancer) and had no evidence of recurrent NSCLC at the time of death. Actuarial 1-, 3-, and 5-year survivals among patients with nodes found negative by both pathologic and molecular methods were 78%, 59%, and 59%, respectively. Actuarial 1-, 3-, and 5-year lung cancer-specific survivals among these patients were 85%, 78%, and 78%, respectively.

The second group consisted of 9 patients with stage I disease according to pathologic study who had micrometastases in regional lymph node detected by *TP53* oligonucleotide hybridization or the *K-ras* ligation assay. Seven of these patients had their disease upgraded to stage II with molecular analysis; the remaining 2 had their disease upstaged to IIIA. Their cases are summarized in Table 3. Lobectomy was performed in all these cases. Three of these patients have died of recurrent NSCLC at 8, 14, and 64 months after resection. Two patients had local recurrence of cancer at 8 and 48 months and were still alive at 5 years after adjuvant therapy. Both overall and lung cancer-specific survivals at 1, 3, and 5 years among these patients were 89%, 78%, and 78%, respectively.

The final group consisted of 28 patients with *TP53* or *K-ras* mutant tumors and microscopic N1 or N2 disease according to both pathologic and molecular analyses. Operations in this group consisted of 25 lobectomies, 1 bilobectomy, and 2 pneumonectomies. One patient with stage II squamous cell lung cancer died 14 months after lobectomy with metastatic colon cancer. Actuarial 1-, 3-, and 5-year survivals among these patients were 78%, 40%, and 40%, respectively. Actuarial 1-, 3-, and 5-year lung cancer-specific survivals among these patients were 78%, 42%, and 42%, respectively. Five-year disease-specific sur-

vival among patients with N0 disease according to both pathologic and molecular methods was significantly longer ($P < .03$) than that among patients with microscopic lymph node metastases according to both pathologic and molecular methods. No difference in disease-specific survival was present among patients with lymph node micrometastases detected by molecular but not pathologic means and the other two patient groups (Figure 2).

Discussion

Occult locoregional spread of cancer is probably the leading cause of local recurrence after complete surgical resection and is an early indicator of subclinical micrometastatic dissemination. Accurate staging is therefore extremely important and may result in either more extensive primary therapy to reduce the risk of local recurrence or systemic adjuvant therapy with the aim of eliminating these occult deposits. This study examined the prevalence of tumor-specific *TP53* and *K-ras* gene mutations in histologically determined negative regional lymph nodes from patients with resectable NSCLC. Overall, tumor-specific mutations were found in 8% of histologically determined negative nodes. These micrometastases led to an increase in pathologic stage for 28% of the evaluated patients with stage I disease. However, the presence of micrometastases did not negatively impact survival in this group of patients with stage I NSCLC.

Several techniques have been used to detect occult tumor cells in the regional lymph nodes and bone marrow of patients with NSCLC. Immunohistochemical techniques have been used to detect either epithelial-specific proteins (eg, cytokeratin) or abnormal *TP53* accumulation. With these techniques, cytokeratin-positive cells have been detected in 4% to 17% of the regional lymph nodes from patients with resected stage I disease, leading to a change in stage for 15% to 70% of patients.^{4,7,15} In several studies cytokeratin-positive cells in regional lymph nodes have been found to be associated with a decrease in disease-specific survival.^{5,7,15} Immunohistochemical methods have also been used to detect occult metastases in the bone marrow of patients with NSCLC. From 22% to 60% of patients with resectable NSCLC have cytokeratin-positive cells in the bone marrow at the time of resection.¹⁶⁻²⁰ The presence of occult bone marrow metastases does not correlate with the presence of occult lymph node metastases and has also been associated with a decrease in long-term survival.^{5,16-20} Immunohistochemical assay has several advantages, including applicability in all cases (the vast majority of epithelial tumors stain positively for cytokeratins), adequate sensitivity (1 in 10^5 cells), and easy adaptability to current pathologic analysis. However, immunohistochemical assay may be limited by variation in assay results according to the antibodies used, a low false-positive rate in bone marrow from

Figure 2. Disease-specific survivals for patients with *TP53* and/or *K-ras* mutations in NSCLC. Disease-specific survival in node-negative NSCLC according to both pathologic and molecular studies (dotted line) was significantly ($P < .03$) longer than in histopathologically determined node-positive (N1 or N2; positive by both histopathologic and molecular assays) NSCLC (heavy line). No difference in disease-specific survival was seen between patients with lymph node micrometastases according to molecular analysis alone (fine line) and patients with node-negative disease according to both pathologic and molecular studies.

control patients, and the presence of subjective interobserver variation.

Molecular techniques to date have relied on reverse transcriptase-PCR amplification of epithelium- or tumor-specific genes to detect occult tumor cells. Several problems have limited this approach. First, low-level expressions of both epithelial- and tumor-specific genes (eg, carcinoembryonic antigen, *MUC1*, *erb-b2*) are present in normal lymph nodes.^{21,22} In addition, pseudogenes similar in sequence homology to several cytokeratins may produce false-positive bands in normal lymph nodes, making interpretation difficult.²³

This study is the first analysis to use a DNA-based assay to detect tumor-specific changes in regional lymph nodes from patients with NSCLC. This approach has been used in squamous cell carcinoma of the head and neck to detect the presence of *TP53* mutations in the surgical margins and cervical lymph nodes that were histopathologically assessed as negative.² More importantly, patients with *TP53* mutation-positive margins were found to be at increased risk for local recurrence. In this study this technique and the *K-ras* mutation ligation assay identified tumor-specific mutations in 8.2% of histopathologically determined negative lymph nodes from patients with purported stage I disease, leading to a change in stage for 28% of these patients. These percentages are similar to numbers obtained with immunohistochemical methods.^{4-7,15} Long-term survival was not

diminished among the patients with micrometastases, although this may reflect the small sample size ($n = 9$). In addition, *TP53* and *K-ras* mutations in themselves may negatively affect survival, thereby limiting the ability to detect a further impact of molecular metastases on survival.^{10,24}

Our laboratory has focused on the use of molecular assays for the early detection and molecular lymph node staging of patients with NSCLC. One potential limitation of this approach is the relatively low prevalence of any single gene mutation or epigenetic alteration in most cancer types. With a larger panel of molecular alterations (*TP53* mutations, *K-ras* mutations, p16 methylation, and microsatellite markers), an evaluable marker was found in 86% of patients with NSCLC and used to detect tumor cells in bronchoalveolar lavage fluid taken at the time of surgical resection.²⁵ These techniques have advantages with respect to immunohistochemical staining, because they involve less operator variability and can quantitatively establish the presence or absence of specific molecular abnormalities at the DNA level. This technique also enables analysis of the entire lymph node in a single assay, whereas immunohistochemical assay requires visual or automated analysis of each individual section. Although step-sectioning of an individual lymph node can increase the yield of both standard histopathologic and immunohistochemical analyses, molecular techniques can still detect tumor-specific mutations in cytokeratin-negative lymph nodes.²⁶

There may be several explanations as to why a difference in survival was not seen between the group with negative nodes according to both pathologic and molecular analyses and those with micrometastasis determined by molecular assays only. The first is the small study population for both of these groups. The second is that the markers used in this study may themselves have a negative impact on survival, and thus a subset of patients with a worse prognosis may have been studied. The third is that resection of micrometastatic disease may translate into better survival than seen among those with incidental histologic or bulky N1 and N2 disease.

Finally, the choice to use a lymph node sampling technique rather than dissection may fail to pick up micrometastases, particularly in nodes not resected. This could account for the cancer-related deaths in the group with N0 disease according to both methods. Although the topic of lymph node sampling versus dissection remains controversial with standard lung resections, there is recent evidence to show that there may be a survival advantage in doing this for certain stages (II and IIIA),^{27,28} although no study has shown a benefit in N0 disease. In addition, only 4.8 lymph nodes/patient were examined in this report. In other studies that showed a negative survival impact when micrometastases were detected by immunostaining, lymph node tissue was more available (22.1 lymph nodes/patient in the study of

Maruyama and colleagues³ and 10.2 lymph nodes/patient in the study of Dobashi and associates⁷). A more appropriate application of these molecular techniques may be directed toward detection of the sentinel nodes,²⁹ which may further increase the yield and decrease the cost of these assays.

Recent progress in translational research has defined numerous molecular and biologic markers that may serve as predictors of survival and may also be used for more accurate postoperative disease staging. These molecular indicators not only will help to predict those at risk for recurrence of disease but will also further define the roles of primary and adjuvant therapy. With these and other promising biologic markers, a more precise molecular classification may someday provide a firm basis for therapeutic decision in the clinical setting. Further investigation and large multi-institutional trials are necessary to confirm the use of these micrometastatic markers as useful primary staging tools.

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Discussion

Dr Thomas A. D'Amico (Durham, NC). The use of molecular techniques in the staging of malignancy, termed *molecular biologic substaging*, holds the potential to improve the assessment of prognosis and the assignment of therapy for patients with NSCLC. The current TNM staging system for lung cancer tends to under-stage, and clinical staging is even less accurate than pathologic staging. Molecular biologic substaging may be applied to the pri-

mary tumor to assess prognosis or chemoresistance and to lymph nodes, serum, or bone marrow to assess occult metastases.

Ahrendt and colleagues' study used molecular techniques to detect occult micrometastases in the lymph nodes of patients with completely resected NSCLC and attempted to correlate the presence of micrometastatic disease with prognosis. This study was well designed and carefully analyzed, and it provides a significant contribution to the mounting evidence that molecular biologic substaging will become a critical component in the evaluation and management of patients with early-stage NSCLC. Unlike many studies in the literature, this study is particularly valuable because the analysis was necessarily limited to patients with stage I disease by the construct of evaluating patients who had histologically determined N0 disease, emphasizing the relative importance of biologic staging.

The assessment of occult metastases with molecular markers requires the detection of factors with a high prevalence in the primary tumor, either genetic mutations or protein expressions. The prognostic value of the expression or overexpression of these factors in the primary tumor is irrelevant. The marker is chosen for its ability to be detected with a greater sensitivity than routine histopathologic analysis. Thus the higher its prevalence in the primary tumor, the greater is a marker's utility in detecting micrometastases.

First, Dr Yang, you chose two markers, *K-ras*, present in 40% of patients with only adenocarcinoma, and *TP53*, present in 52% of patients. In this study only 10% of patients had their disease upstaged on the basis of *TP53* and only 3% on the basis of *K-ras*. Have you considered using genetic mutations or other markers with a greater prevalence, such as epidermal growth factor receptor, present in 60% to 80% of patients, or using other techniques to measure cytokeratins, which are present in more than 70% of patients?

Second, this study assessed micrometastases in lymph nodes, which represent locally advanced disease and are only a surrogate marker for distant metastatic disease. Our laboratory has applied your techniques to the detection of occult metastases. Have you attempted to detect micrometastases in either the bone marrow or the serum?

Third, other groups have demonstrated that the presence of occult micrometastatic disease in lymph nodes is associated with significant differences in recurrence and cancer-specific survival. This study did not demonstrate risk stratification according to the presence of micrometastatic disease. The 5-year cancer-specific survival for patients with stage I disease with and without micrometastases was 78%, compared with 42% among patients with histologically determined positive nodes. Although the sample sizes and other factors that you mentioned may be important, are other factors perhaps the real cause, such as the lack of sensitivity of your markers and the insistence on using molecular techniques rather than other techniques that may take advantage of markers with greater sensitivity?

In the article you suggest that molecular staging may alter the use of adjuvant therapy for patients with early-stage disease. How do you propose to test this hypothesis, given the absence of risk stratification in your study?

In conclusion, Ahrendt and colleagues are to be commended for this important study. Molecular biologic substaging of patients with early-stage NSCLC should be investigated to improve the

accuracy of the TNM staging system in assessing prognosis and to discriminate patients who may benefit from adjuvant therapy.

Dr Jack A. Roth (*Houston, Tex*). I congratulate Ahrendt and colleagues for one of the most comprehensive studies of molecular markers that has been performed to date. My question is really about the clinical utility of these studies. Dr Yang, how do you think this will fit into the practice of thoracic oncologic surgery in the future? For example, if we knew patients had occult metastases at the time of operation, we would still go ahead with a resection. Similarly, if we knew it afterward, we probably would still not consider adjuvant therapies because these have not been of proven value. Have you applied these techniques earlier in staging, for example at the time of mediastinoscopy, or are they applicable for fine-needle aspiration of primary tumors?

Dr Raphael Bueno (*Boston, Mass*). My questions addresses the term *micrometastatic disease* and whether you can truly call this micrometastatic given that you did not find cancer cells in the lymph nodes, nor did you find genetic mutations that are related to cancer or predisposition toward cancer. Specifically, did you look at a single lymph node or more than one lymph node per patient? At which levels of lymph nodes did you look? Did you look at a tumor itself for the same *K-ras* and *TP53* markers or normal adjacent lung tissue to dissect out whether you are truly looking at tumor cells, as we believe you are, or just at the predisposition of the tissue toward metastasis? Perhaps these should be called *prognostic markers* rather than *micrometastatic disease*.

Dr W. Roy Smythe (*Houston, Tex*). What we know about the clonality of tumor cells suggests that the cells that leave the primary tumor and end up in the lymph nodes and peripheral sites as metastases often have a different genotype than that of the tumor itself. Also, this technique may offer no greater sensitivity or specificity than the more prevalent markers, such as cytokeratins. Have you considered looking with a wider array of markers to determine whether the metastatic lesions in the lymph nodes may have a different genotype than that of the tumor and whether that actually might be more predictive of prognosis than finding these mutated sequences alone?

Dr Yang. We have not used other agents such as endothelial growth factor receptor or cytokeratins. It has not been the thrust or the focus in the laboratory with Dr Sidransky. Have we used other surrogate markers for occult metastases in bone marrow and serum? We have used the same markers, microsatellite markers that were discovered for bladder and for head and neck tumors off of chro-

mosomes 3p and 3q, and we are starting to look at p16 methylation, not only in the tumors and lymph nodes but also in the serum. Our early results, although I cannot give you the exact numbers, indicate that there is a higher incidence of p16 methylation in the serum.

Why did we not demonstrate a significant survival difference? I still think that it is because we chose lymph node sampling rather than dissection. The average number of lymph nodes that we analyzed per patient was 4.8. If you look at some of the immunohistochemical papers from the Japanese group,^{3,7} they used upward of 10 to 22 lymph nodes per patient, and they were able to find a survival difference. I think that this lower sampling rate probably is the cause for our lack of a survival difference.

We would like to use this in the future as a test for adjuvant therapy. To take the devil's advocate position, we know that adjuvant therapy probably does not help for N1 disease; however, this may be a subset of patients with N1 disease that it can help. In reverse, it probably provides more support for the son of blot trial, giving it in the neoadjuvant phase rather than after the operation.

As to how this fits into clinical practice, I think that if we go back to the survey from The American Association for Thoracic Surgery or Society of Thoracic Surgeons about 8 years ago, 80% of surgeons still did not do lymph node sampling. Unfortunately I still see a number of patients who have recurrence who were treated by outside physicians and whose lymph nodes have not been sampled. I think that this provides more of an argument for surgeons who do lung cancer surgery that they must at least sample lymph nodes, if not move on to dissection, depending on what the American College of Surgeons Oncology Group trial shows. We have not used this for fine-needle aspiration or microscopy yet. We have thought about that.

Whatever available resected lymph nodes were taken were analyzed. In some cases we did look for pockets of nodes in the subcarinal and paratracheal areas. We did not go through a dissection, but if we did not find any nodes those were not sampled. We first looked for the *K-ras* and *TP53* mutations, and we were looking for the same type of mutations in the lymph nodes. So we did not have to go back and look at the tumor again. Early on we checked for the same mutations in normal tissue. In the first 10 cases the results were all negative.

I do agree that there probably are differences in clonality between bone metastases and serum and local recurrence, although right now we do not have any data to show that there is a difference from the mutation sampling.