Immunohistochemical Detection of Occult Lymph Node Metastases in Non-Small Cell Lung Cancer: Anatomical Pathology Results from Cancer and Leukemia Group B Trial 9761


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ABSTRACT

Purpose: Our purpose was to study the detection of occult metastases (OM) in regional lymph nodes using immunohistochemical stain for cytokeratin, and for this study we targeted clinical stage I patients with non-small cell lung cancer.

Received 1/30/03; revised 7/23/03; accepted 8/4/03.

Grant support: The research of Cancer and Leukemia Group B 9761 was supported in part by a grant from the National Cancer Institute (CA31946) to the Cancer and Leukemia Group B (Richard L. Schilsky, Chairman). The following institutions participated in the study: (a) University of North Carolina at Chapel Hill, Dr. Thomas C. Shea, supported by CA47559; (b) Dana Farber Cancer Institute (Boston, MA), Dr. George P. Canellos, supported by CA32291; (c) Dartmouth Medical School, Norris Cotton Cancer Center (Lebanon, NH), Dr. Marc S. Ernstoff, supported by CA04326; (d) Duke University Medical Center (Durham, NC), Dr. Jeffrey Crawford, supported by CA 47577; (e) State University of New York Upstate Medical University (Syracuse, NY), Dr. Stephen L. Graziano, supported by CA21060; (f) University of Iowa (Iowa City, IA), Dr. Gerald Clamon, supported by CA47642; (g) University of Maryland Cancer Center (Baltimore, MD), Dr. David Van Echo, supported by CA31983; (h) University of Minnesota (Minneapolis, MN), Dr. Bruce A. Peterson, supported by CA16450; (i) University of Missouri, Ellis Fischel Cancer (Columbia, MO), Dr. Michael C. Perry, supported by CA12046; and (j) Washington University (St. Louis, MO), Dr. Nancy Bartlett, supported by CA77440.

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Experimental Design: The study comprised the first 193 patients entered onto Cancer and Leukemia Group B protocol 9761. All had clinically staged T1–2 N0 M0 non-small cell lung cancer, and all underwent curative resections of their primary tumors. Samples of the primary tumor and lymph nodes were taken from lymph node stations 2–12 and shipped to a central laboratory, where each lymph node was histologically processed and stained with H&E as well as with immunohistochemical stain using antibodies to cytokeratin (AE1/3).

Results: Altogether, we examined 825 lymph nodes. Whereas routine H&E staining allowed us to detect 18 positive lymph nodes, immunohistochemical staining allowed us to detect 45 positive lymph nodes (P < 0.0001). There were 28 OM [i.e., those detectable only by immunohistochemistry (IHC)], and there was 1 metastasis detected only by H&E staining. The OM included 9 OM in N1 stations and 19 OM in N2 stations. Twelve patients with OM had skip metastases. Routine H&E staining upstaged six patients to N1, and IHC added another five. Routine H&E upstaged 9 patients to N2, and IHC added another 11. We also uncovered new details about the way in which H&E detection depends on metastatic tumor burden. Specifically, for the probability of detecting metastases by H&E to exceed 0.50, the maximum diameter of the metastasis must be greater than 0.23 mm.

Conclusions: IHC detects greater than twice as many positive regional lymph nodes as does H&E staining, and the foci of tumor it detects are significantly smaller than those detected by H&E staining.

INTRODUCTION

The most important prognostic variables in NSCLC are the clinical and pathological stages. However, neither clinical nor pathological stage provides complete information about outcomes. For example, after complete resection, approximately 60–80% with pathological stage T1 N0 M0 (IA) and 50% of those with pathological stage T2 N0 M0 (IB) survive for at least 5 years; whereas the remaining patients suffer earlier recurrence of their cancer and die (1). Thus, routine examination using H&E stains does not tell us who among these patients will have recurrent tumor. From prior studies, we know that some patients with either pathological stages IA or IB have occult lymph node metastases, which for this paper we define as those detectable
just by either immunohistochemical stains (2–14) or by molecular techniques (7, 9, 15), and in general those with OM had shorter disease-free intervals (3, 6, 8, 10, 12–14). Thus, OM may account for at least a proportion of T1–2N0M0 tumors that recur after curative surgical excision of the primary tumors.

To validate previous observations about the importance of OM in NSCLC and to gather further clinical and pathological information, the CALGB embarked on a prospective multi-institutional trial (CALGB 9761) of molecular and histochecmical staging of patients with clinically early-stage NSCLC. Accrual is now complete at 502 patients, and full analysis of outcomes awaits further follow-up as well as analysis of additional tissues. Herein we report details about the pathological findings in 825 lymph nodes from the first 193 consecutive patients with tumor who were entered on the study, because preliminary analyses demonstrated that these comprised a sufficient number of tissues and patients to learn about the pathology of OM.

MATERIALS AND METHODS
Our study population consisted of the first 193 patients of CALGB 9761 with primary tumors documented to stain for cytokeratin by IHC. All had clinical stage I disease (T1–2N0M0), and all underwent complete surgical resection of their tumors. To be eligible for CALGB 9761, patients were required to have either mediastinal nodes that measured <1 cm per thoracic CT exam or nodes sampled by mediastinoscopy and proven histologically to be negative. Positron emission tomography scanning was not required and was used preoperatively in only a small percentage of these patients. All patients gave their written, informed consent, and each participating institution’s review board also approved the study.

Before the study began, participating surgeons met in committee several times and agreed on a uniform surgical approach, which was then monitored by the senior author (M. A. M.). Briefly, immediately after thoracotomy incision, the operating surgeons harvested all accessible mediastinal lymph nodes (see D’Cunha et al., Ref. 15), and then the appropriate pulmonary resection was done, and intrapulmonary nodes were harvested. Portions of each patient’s primary tumor as well as lymph nodes from several stations were shipped to the Thoracic Oncology Laboratory at the University of Minnesota. We did not have access to or use routine pathology materials processed by local pathology laboratories. Nor did we use the information from their reports, because our goal in this pathology study was to confirm the diagnosis of NSCLC and that it was positive for AE1/AE3, and we found that all were positive. Then, each lymph node sample was handled in a uniform fashion so that H&E stains were matched to IHC slides. Specifically, three sections were cut, in order, from each block of tissue: the first for routine H&E staining; the second for cytokeratin by IHC; and the third as a control for the IHC. For IHC, we used the Dako prediluted AE1/AE3 product N1590 (Dako Corp., Carpinteria, CA), a 20-min pretreatment with protease 760-2018 (Ventana Medical Systems, Inc., Tucson, AZ), and 3,3′-diaminobenzidine detection kit 760-001 (Ventana Medical Systems, Inc.).

One pathologist (R. T. V.) evaluated all of the slides and decided the final status of the nodes. In addition, to study concordance in the interpretation of IHC, a second pathologist (N. Z. A.) evaluated a subset of 717 nodes, a number found sufficient for study of concordance. For each lymph node, we used the following sequence. First, the H&E slide was examined to determine the presence or absence of metastases. Next, the third or control slide was examined to make certain that no OM occurred at a deeper level. Finally, the IHC slide stained for AE1/3 was examined. Lymph nodes were considered positive for OM if both the first and third slides were found to be negative, and the IHC slide was found to be positive. If reexamination of the first or third slides demonstrated tumor, after it was first discovered by IHC, then the node was still counted as OM. Cells staining positive for AE1/3 were not counted as OM if they appeared to be contaminants on the surface or within artifactual clefts in the tissues. Nor were they counted if they appeared to be mesothelial cells, that is, a thin cell layer outside the nodal architecture. Finally, we measured the maximum diameter of the largest metastatic focus in each IHC-positive node using a calibrated eyepiece micrometer.

Statistical Methods. We used GEEs based on the binomial distribution and the logit link function to account for correlation among lymph node results within individual patients. Specifically, GEE models were used to examine the relationship between H&E and IHC staining results, as well as the distribution of metastases between N1 and N2 lymph node stations found by H&E and OM. The GEE model was also used to estimate the relationship between the maximum diameter of the largest focus of metastatic tumor and H&E results. A χ² test was used to compare the results from our study and prior studies relative to the prevalence of positive nodes assessed by IHC. λ statistics, which adjusted for within-patient nodal correlation, were used to assess the interobserver agreement between the two pathologists.

RESULTS
The median number of satisfactory nodes received from each patient was 4 (range, 1–8; in cases with fewer than 4 nodes, additional samples contained too little nodal tissue for complete study). Fig. 1 illustrates two nodes with OM (Fig. 1, B 9761 on approximately 60 cases, we observed that AE1/3 stained >95% of the primary tumors, whereas, cytokeratin antibody Cam5.2 stained just 74% of the primary tumors, and glycoprotein antibody BerEp 4 stained just 63% of the primary tumors. Therefore we relied on AE1/3.

First, we examined the primary tumor to confirm the diagnosis of NSCLC and that it was positive for AE1/AE3, and we found that all were positive. Then, each lymph node sample was handled in a uniform fashion so that H&E stains were matched to IHC slides. Specifically, three sections were cut, in order, from each block of tissue: the first for routine H&E staining; the second for cytokeratin by IHC; and the third as a control for the IHC. For IHC, we used the Dako prediluted AE1/AE3 product N1590 (Dako Corp., Carpinteria, CA), a 20-min pretreatment with protease 760-2018 (Ventana Medical Systems, Inc., Tucson, AZ), and 3,3′-diaminobenzidine detection kit 760-001 (Ventana Medical Systems, Inc.).
and D) and accompanying H&E stains (Fig. 1, A and C) for comparison. Because our primary observational units comprised lymph nodes, the main results are summarized in the top of Table 1. Routine H&E stain allowed us to detect 18 of 825 nodes as positive, and IHC allowed us to detect an additional 28 nodes as positive—a difference that was significant ($P < 0.0001$ by GEE). One node was detected just by H&E staining because the focus of tumor was too small to appear on the slide stained for IHC. Thus, the total number of positive nodes was 46 (i.e., 18 + 28). Of those 18 nodes detected by H&E, 8 were stage N1 (stations 10, 11, and 12), and the remaining 10 were stage N2 (stations 2–9). Of the 28 nodes with OM detected just by IHC, 9 were stage N1, and the remaining 19 were stage N2. The distribution of metastases in N1 versus N2 did not differ between those found by H&E staining versus those found by IHC ($P = 0.547$ by GEE). Whereas the prevalence of H&E-positive nodes was 2.2% of the total nodes, the prevalence of OM was 3.4%. Thus, our results suggest that IHC detected over twice as many foci of tumor as did routine H&E staining. The combined results of both stains on these study tissues indicated that among thoracic nodes either measuring <1 cm per CT or found to be negative by mediastinoscopy, approximately 6% (i.e., 46 of 825) contained tumor. Finally, if we subtract the lymph nodes with metastases detectable by H&E from the total, the rate of OM found in the remainder was 3.47% (i.e., 28 of 807).

To put these results into a patient perspective, the lower portion of Table 1 reexamines the results from the point of view of the patient rather than by tissue. By H&E stain, the 18 node metastases occurred in 15 patients. Among those 15 patients, 6 (3.1% of the total) had their N status increased from N0 to N1, and 9 (4.7% of the total) had their N status increased to N2. Eight of the 9 patients with N2 metastases had “skip” metastases, that is, their N1 nodes were negative by H&E. By immunohistochemical stain, 21 patients had OM. Among the 21 patients with OM, 7 (3.9% of the total patients) had their N stage increased from N0 to N1, and 14 (7.3% of the total) had their N stage increased to N2. Twelve of the patients with N2 nodes found positive by IHC had skip metastases, because their N1 nodes were not involved by either H&E or IHC. In some patients with OM, other nodes were positive by H&E, so that the IHC in such cases provided redundant information. Thus, the net number of patients increased to stage N1 by either H&E or IHC was 11, and the net number increased by either stain to stage N2 was 21. The total number of patients with nodal metastases found in these study tissues was 32, implying that approximately 17% of the patients had been clinically understaged (i.e., 32 of 193). The presence of lymph node metastasis, either by H&E or by IHC, was not associated with the number of nodes harvested ($P > 0.7$ by logistic regression).

Not surprisingly, we found that the threshold of metastatic tumor burden detected by IHC differed from that detected by H&E. Specifically, we found a significant relationship between detection of metastasis by H&E and the maximum diameter of

![Fig. 1](image-url) Two lymph nodes with tumor detected by IHC but not by routine H&E. The top panels illustrate the first lymph node stained for H&E (A) and cytokeratin (B), and the bottom panels illustrate the second lymph node stained for H&E (C) and cytokeratin (D).

### Table 1  
Comparison of metastases detected by H&E stain with OM detected by IHC for AE1/3

<table>
<thead>
<tr>
<th>Analysis by units of nodes</th>
<th>H&amp;E</th>
<th>OM$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. Positive Nodes (%)</td>
<td>18 (2.2)</td>
<td>28 (3.4)</td>
</tr>
<tr>
<td>Positive N1 nodes (%)</td>
<td>8 (44)</td>
<td>9 (32)</td>
</tr>
<tr>
<td>Positive N2 nodes (%)</td>
<td>10 (56)</td>
<td>19 (68)</td>
</tr>
<tr>
<td>Analysis by units of patients</td>
<td>No. Positive Patients (%)</td>
<td>15 (7.8)</td>
</tr>
<tr>
<td>Final N1 stage (%)</td>
<td>6 (3.1)</td>
<td>7 (3.9)</td>
</tr>
<tr>
<td>Final N2 stage (%)</td>
<td>9 (4.7)</td>
<td>14 (7.3)</td>
</tr>
<tr>
<td>Patients with skip metastases</td>
<td>8</td>
<td>12</td>
</tr>
</tbody>
</table>

$^a$Entries for the OM column include only nodes found to be negative by H&E.
with the largest focus of metastatic tumor present in the IHC slides ($P = 0.0002$ by GEE analysis). Furthermore, the fitted model suggested a continuous relationship between the natural logarithm of the maximum diameter of the metastatic tumor focus in millimeters and the probability of detection of tumor by H&E. This relationship is given in the following two equations and illustrated in Fig. 2.

\[
\text{Probability} = \frac{1}{1 + E} \\
\text{(Eq. 1)}
\]

\[
E = \exp(-5.49 - 3.78\log(\text{diameter})) \\
\text{(Eq. 2)}
\]

Here, diameter refers to the metastatic focus and is given in millimeters. In Fig. 2, the smooth line is a plot of Eqs. 1 and 2, and the points at the top and bottom are observed results for H&E detection (1 for detected and 0 for undetected). The logistic model suggests that when tumor foci are $>0.23$ mm, there is a $>50\%$ chance that they will be detected by H&E stain. The model also suggested that $<4\%$ of tumor foci measuring $0.1$ mm will be detectable by H&E, and in fact the smallest tumor focus we found by H&E was $0.15$ mm.

Finally, in an analysis of a subset of 717 lymph nodes, we found that there was a $3.8\%$ rate of discordance (i.e., 27 nodes) between two pathologists for the detection of metastases by IHC, with modest agreement as measured by $\kappa$ statistic ($\kappa = 0.19$; $P = 0.06$). All of the discordant cases were reviewed and found to be due to several circumstances. In a few cases, one observer detected a single positive staining cell; whereas the other did not. In the remaining cases, one observer counted the node positive when the other judged the staining to be surface contaminants, staining of mesothelial cells, or background staining of nonneoplastic cells or stroma. There was no discordance in the interpretation of the H&E slides.

**DISCUSSION**

Our study is unique in several respects. It comprises the largest number of patients with NSCLC analyzed for OM in regional lymph nodes, and it comes from multiple institutions. Its results validate the observations of some prior studies, but not others. Finally, it provides new details on the subject of OM in NSCLC.

Table 2 compares our rate of nodes with OM with the rates of positive nodes reported by nine other studies, and it includes data only for patients with negative nodes as determined by routine H&E staining. Our observed rate of $3.47\%$ nodes with OM was close to three previously published rates of $3.84\%$ (3), $5.02\%$ (10), and $3.41\%$ (12). However, some authors have reported rates of positive nodes less than $1\%$ (5, 8), and others have reported rates greater than $10\%$ (2, 9). In fact, a test for equality of proportions among these 10 studies showed that their differences in the rate of OM were significant, that is, the differences were unlikely to be due to random effects alone ($P < 0.00001$). Such differences are important and may translate into different rates of clinical outcomes for patients with OM. The different reported rates are likely due to several factors. For example, Chen et al. (2) collected data retrospectively from surgical pathology records; whereas other studies like ours were done prospectively on patients identified by surgeons. Chen et al. (2) used data collected in the 1980s; whereas most of the other studies including ours used data largely collected in the 1990s, when more modern practices for staging and sampling of N2 nodes were in place (16). Of all of the 1990s studies, ours is the only one specifying the requirement that the N2 nodes be either $<1$ cm per CT or negative by mediastinoscopy. Another potential explanation for the differences in the rates of OM may be due to the different IHC antibody-antigen systems used. Five studies of Table 2 including ours used the AE1/3 mixture for cytokeratin (8, 10, 12, 13); whereas others used different antibodies such as BerEp 4 (3), A575 (2), Cam5.2 (9, 14), or MNF116 (5). Because in preliminary and unpublished work on CALGB 9761 we found that AE1/3 stained more of the primary tumors, we believe that the best results for detecting OM in NSCLC are obtained with AE1/3.

Different reported rates of OM are also likely due to variation in the practice among surgical pathologists. Clearly, interpretation of IHC staining is subjective, and we have docu-

**Table 2** Rates of positive nodes with OM in NSCLC

<table>
<thead>
<tr>
<th>Author (ref. no.)</th>
<th>No. of patients $^a$</th>
<th>Total lymph nodes</th>
<th>% Nodes with OM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chen et al. (2)</td>
<td>60</td>
<td>588</td>
<td>17.3</td>
</tr>
<tr>
<td>Passlick et al. (3)</td>
<td>72</td>
<td>391</td>
<td>3.84</td>
</tr>
<tr>
<td>Nicholson et al. (5)</td>
<td>49</td>
<td>1447</td>
<td>0.276</td>
</tr>
<tr>
<td>Goldstein et al. (8)</td>
<td>80</td>
<td>573</td>
<td>0.523</td>
</tr>
<tr>
<td>Hashimoto et al. (9)</td>
<td>22</td>
<td>170</td>
<td>27.6</td>
</tr>
<tr>
<td>Ohta et al. (10)</td>
<td>122</td>
<td>2030</td>
<td>5.02</td>
</tr>
<tr>
<td>Wu et al. (12)</td>
<td>103</td>
<td>1438</td>
<td>3.41</td>
</tr>
<tr>
<td>Gu et al. (13)</td>
<td>49</td>
<td>474</td>
<td>7.38</td>
</tr>
<tr>
<td>Maruyama et al. (14)</td>
<td>44</td>
<td>973</td>
<td>9.35</td>
</tr>
<tr>
<td>This study</td>
<td>178</td>
<td>807</td>
<td>3.47</td>
</tr>
</tbody>
</table>

$^a$ In this table, only those patients (and nodes) with lymph nodes found to be negative by H&E stain are included.
mented a discordance rate of nearly 4% even after agreed upon rules. The diligence used to search the IHC stained tissues for metastases must vary between individuals. Whereas many routinely use the 4× microscope objective to screen lymph nodes for tumor, some may use the 10× objective. In our study, we treated these nodes the same as routine clinical specimens and relied on the 4× objective for screening. Furthermore, the decision of whether staining is due to a tumor cell or some other phenomena is subjective. IHC for cytokeratin is inherently more difficult to interpret for the presence of cytologic atypia than H&E because the cytoplasmic staining is often so intense that it masks nuclear detail—detail required by pathologists to reliably diagnose malignancy. This is especially true when trying to evaluate singly arranged cells positive for IHC. Additionally, in our study we encountered occasional nodes that were found first to be positive by IHC, but which had tumor foci found in retrospective evaluation of the H&E. Fig. 1 shows two such nodes. We counted these as OM because the H&E staining had not initially allowed us to detect the tumor. Others may have excluded such nodes as OM. We excluded cells appearing as mesothelial cells, whereas most other studies have not commented on this and may have achieved a higher rate of OM by including mesothelial cells. Thus, there are a variety of human and subjective issues involved in the detection of OM.

The differences in reported rates of OM detailed in Table 2 and discussed above undoubtedly cause differences in reported outcomes. For example, Fig. 3 compares the overall survival reported at 4 years after surgery for six studies, which were the ones with most updated data in Table 2 and which provided Kaplan-Meier curves for those with OM versus those without OM (6, 9, 10, 12–14). In Fig. 3, the vertical axis gives the reported observed percentage surviving 4 years in those with OM, and the horizontal axis gives the percentage surviving 4 years in those without OM. The line shows where the points would be if the survival curves were the same. Whereas all six studies found that the presence of OM shortened survival time (that is, the points fell below the line), the scatter of the points demonstrates that the observed survival times for those with or without OM varied greatly. The drop in percentage surviving to 4 years for patients with OM ranged from 13% to 48% (median, 18%). This degree of variation must be due to differences in the patients studied or to the methods used for detecting OM. Such differences make it difficult to develop treatments for OM, and our hope is that our large prospective, multi-institutional study will narrow this range in expected outcomes for patients with OM. Survival analysis in our study, however, must wait until the follow-up of our patients is longer.

Our results have made quantitative the conjecture that the threshold of tumor burden detected by routine H&E differs from that detected by IHC. The logistic regression model predicts that routine H&E has a >50% chance of detecting metastases measuring over 0.23 mm in maximum diameter, >85% chance of detecting those over 0.4 mm, and nearly 100% chance of detecting those over 1 mm. On the other hand, H&E stain allows us to detect <4% of tumor foci measuring 0.1 mm and virtually none of single cells positive for IHC. Thus there is a different threshold of tumor burden for detection of metastasis by H&E versus by IHC, and our results provide significant detail about this difference. The tumor burden threshold for detecting tumor by IHC in comparison with molecular techniques such as quantitative PCR for mRNA of carcinoembryonic antigen (15) is not known, but subsequent follow-up studies of CALGB 9761 may help establish this.

Before either IHC or quantitative PCR can be adopted to detect OM, we must first demonstrate that the diagnosis of OM can be matched by effective treatment and is cost effective. What must be demonstrated is that there is a treatment that can improve survival in those approximately 18% of patients with OM who suffer a shortened survival while not adversely affecting the quality of life of the others with OM who do not have shortened survival. It is possible that the measured size of the IHC-detected metastasis will help decide who can benefit from adjuvant treatment, but such questions require further study as well as randomized clinical trials. Finally, detecting OM with IHC is expensive. If we assume a technical cost of $30.00 (United States) and a professional cost of $75.00 per IHC stain, then our study suggests that the cost of detecting each OM by IHC is over $3000.00. The pathology costs for detecting OM with IHC could be reduced if the IHC were restricted to just sentinel nodes, which have recently been described in NSCLC (17); however, our results suggest that skip metastases are common, so that one would need to prove that sentinel technology detects skip metastases. Altogether, these cost considerations suggest that prospective trials designed to optimize the best treatment for patients with OM will require many patients and be expensive to conduct.

ACKNOWLEDGMENTS

We are grateful to Debra L. Herzan for assistance with data management.
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