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ORIGINAL REPORT

Occult Metastases in Lymph Nodes Predict Survival in Resectable Non–Small-Cell Lung Cancer: Report of the ACOSOG Z0040 Trial

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ABSTRACT

Purpose
The survival of patients with non–small-cell lung cancer (NSCLC), even when resectable, remains poor. Several small studies suggest that occult metastases (OMs) in pleura, bone marrow (BM), or lymph nodes (LNs) are present in early-stage NSCLC and are associated with a poor outcome. We investigated the prevalence of OMs in resectable NSCLC and their relationship with survival.

Patients and Methods
Eligible patients had previously untreated, potentially resectable NSCLC. Saline lavage of the pleural space, performed before and after pulmonary resection, was examined cytologically. Rib BM and all histologically negative LNs (N0) were examined for OM, diagnosed by cytokeratin immunohistochemistry (IHC). Survival probabilities were estimated using the Kaplan-Meier method. The log-rank test and Cox proportional hazards regression model were used to compare survival of groups of patients. $P < .05$ was considered significant.

Results
From July 1999 to March 2004, 1,047 eligible patients (538 men and 509 women; median age, 67.2 years) were entered onto the study, of whom 50% had adenocarcinoma and 66% had stage I NSCLC. Pleural lavage was cytologically positive in only 29 patients. OMs were identified in 66 (8.0%) of 821 BM specimens and 130 (22.4%) of 580 LN specimens. In univariate and multivariable analyses OMs in LN but not BM were associated with significantly worse disease-free survival (hazard ratio [HR], 1.50; $P = .031$) and overall survival (HR, 1.58; $P = .009$).

Conclusion
In early-stage NSCLC, LN OMs detected by IHC identify patients with a worse prognosis. Future clinical trials should test the role of IHC in identifying patients for adjuvant therapy.

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INTRODUCTION

Lung cancer remains the most frequent cause of cancer-related deaths worldwide, with an estimated survival of only 15% at 5 years.[1] Even patients with stage I non–small-cell lung cancers (NSCLC) experience a 30% risk of recurrence after resection.[2] However, outcomes have recently improved for specific groups of patients with NSCLC. Several randomized controlled trials now show that patients with lymph node (LN) metastases experience a survival benefit from the addition of chemotherapy after resection.[3-5] Thus, it is increasingly important to identify patients with early-stage NSCLC who may benefit from combined-modality treatment.

Defining patients with early-stage cancer at increased risk for tumor progression is a major challenge. Efforts to detect the earliest dissemination of tumor before identification by routine pathologic or clinical methods, so-called occult metastases (OMs), led to studies in several cancers indicating that the detection of regional and/or hematogenous OMs can identify patients at increased risk for developing overt metastases.[6-10] Variable terminology and definitions have been used to describe such small metastatic foci, including micrometastases, disseminated tumor cells, isolated tumor cells, circulating tumor cells, minimal residual disease, and OM. For this trial, we chose the term occult metastases (OMs) because it globally describes metastases that are not

diagnosed by standard clinical and pathologic methods in all three compartments in the study.[11]

In NSCLC, several studies suggest that OMs identify patients who have a poor prognosis after resection. Sites of OM reported to have an adverse impact on survival in resectable NSCLC include the pleura (as diagnosed by lavage of the pleural space), the bone marrow (BM), and intrathoracic LNs.[2,4,12-23] However, previous studies were small and retrospective, used varying methodologies to identify OM, and did not examine these three potential sites of OM simultaneously. We undertook this prospective multicenter clinical trial to determine the prevalence of OM in the pleural space, BM, and intrathoracic LNs in patients undergoing resection of NSCLC and to determine whether the presence of OM was associated with a worse survival.

PATIENTS AND METHODS

Eligibility Criteria

Eligible patients had known or suspected, previously untreated, resectable stage I to IIIb NSCLC. Patients were excluded if they had had other malignancies or an ipsilateral thoracotomy or thoracoscopy within the previous 5 years. Computed tomography of the chest and upper abdomen was required before registration to the study. Additional imaging studies were performed at the discretion of the treating physician but were not required. This trial was approved by the institutional review boards of all participating institutions, and all patients gave informed consent.

Study Interventions

At surgery, a tissue diagnosis of NSCLC was confirmed if this had not been done preoperatively. Patients went off study if they did not have NSCLC or had technically unresectable disease.

At thoracotomy or video-assisted thoracic surgery, a 3- to 4-cm long segment of rib was resected, placed into RPMI 1640 medium, and shipped at room temperature by overnight mail to the reference laboratory (University of Southern California [USC], Los Angeles, CA). After incision of the pleura, 250 mL of saline was instilled into the pleural cavity before manipulation of the lung, and the patient was gently rocked to ensure distribution throughout the pleural space. The lavage fluid was aspirated, and a 20-mL aliquot was added to each of two 50-mL tubes already containing 50% ethyl alcohol. A second lavage was performed after the pulmonary resection, and these specimens were shipped overnight to USC.

The primary tumor was removed via sublobar resection (wedge resection or segmentectomy), lobectomy, bilobectomy, or pneumonectomy, with or without bronchial sleeve resection and adjacent involved organs such as the chest wall or pericardium. A systematic LN sampling or dissection was required.[24] LNs were labeled according to the Mountain-Dresler modification of the American Thoracic Society lymph node map.[25] The primary tumor and the LNs were examined by the institutional pathologist using standard histologic techniques. Tumors were staged according to the sixth edition of the American Joint Commission on Cancer staging manual.[26] All LNs (both N1 and N2) from patients with no evidence of LN metastases by hematoxylin and eosin staining (N0) were sent to USC and examined for OMs.

Postoperatively, all patients were observed until death or for 5 years. Patients were seen every 4 months during the first year, every 6 months during the second and third years, and annually thereafter. A history, physical examination, and chest x-ray were required at follow-up visits. Additional studies were performed at the discretion of the treating physician to evaluate signs and symptoms of recurrent disease. Because adjuvant therapy was not known to alter survival at the time this study was designed, we did not collect data on the use of adjuvant chemotherapy or radiotherapy.

Data Quality Control

Operative summaries and pathology reports for all patients entered onto the study were reviewed by the study chair (V.W.R.) to verify patient eligibility,

Table 1. Patient Demographics and Surgical Procedure and Tumor Characteristics for Eligible Patients

Demographic or Characteristic	No. of Patients (N = 1,047)	%
Age, years		
Median	67.2	
Range	33.6-89.5	
Sex		
Male	538	51.4
Female	509	48.6
Race		
White	959	91.6
Hispanic/Latino	11	1
Black/African American	60	5.7
Asian	14	1.3
American Indian/Alaska Native	1	0.1
Other	2	0.2
Operation performed (pulmonary resection)		
Exploration, no resection	16	1.5
Wedge resection	24	2.3
Segmentectomy	79	7.5
Lobectomy	832	79.5
Bilobectomy	51	4.9
Pneumonectomy	71	6.8
Additional components of resection		
Chest wall resection ± reconstruction	33	3.2
Bronchial sleeve resection	33	3.2
Vascular sleeve resection/arterioplasty	13	1.2
Intrapericardial resection	20	1.9
Extent of resection		
R0	996	95.1
R1	31	3
R2	20	1.9
Tumor histology		
Adenocarcinoma	524	50
Squamous cell carcinoma	318	30.4
Large cell	54	5.2
Adenosquamous	14	1.3
NSCLC, other subtype or otherwise unspecified	137	13.1
Pathologic stage		
T stage		
Missing*	2	0.2
T1	384	36.7
T2	575	54.9
T3	55	5.3
T4	29	2.8
TX	2	0.2
N stage		
N0	739	70.6
N1	171	16.3
N2	129	12.3
NX	8	0.8
Overall stage		
IA	324	30.9
IB	367	35.1
IIA	32	3.1
IIB	158	15.1
IIIA	137	13.1
IIIB	29	2.8

Abbreviation: NSCLC, non–small-cell lung cancer.
*One was missing from the final operative and pathology reports, and one was missing as a result of lack of resection. Both patients had clinical T2 disease.

tumor histology, the operation performed, the completeness of resection, and the tumor TNM stage.

Methods for Processing Pathology Specimens

Pleural lavage samples. At USC, pleural lavage samples were processed according to standard cytologic techniques and were read by a single cytopathologist (S.E.M.).

BM specimen processing. The BM was curetted into RPMI 1640, and cells were processed as previously described.[17] Briefly, after density gradient separation, 1 million cells were centrifuged onto ProbeOn (Fisher Scientific, Hampton, NH) glass slides, air dried and acetone fixed, and stored at −20°C. Four slides (approximately 4 million mononuclear cells) from each specimen were assessed by immunohistochemistry (IHC) for OM. When less than 4 million mononuclear cells were recovered, all available material was assessed.

Cytokeratin IHC. IHC was performed on BM and LN slides as previously described[17,27] using anticytokeratin antibodies CAM 5.2 (Becton Dickinson, San Jose, CA) and AE-1 (Signet Laboratories, Dedham, MA). LN slides were deparaffinized and subjected to antigen retrieval.[28] For both LN and BM slides, aviden-biotin IHC was performed, using as the chromogen Vector Red (Vector Laboratories, Burlingame, CA) for BM and AEC (Sigma-Aldrich, St Louis, MO) for LN. Positive controls for BM were cytospun breast cancer cell lines, and positive controls for LN were paraffin sections of NSCLC. In all cases, lymphoid cells served as internal controls.

Assessment of the BM and LN IHC. All IHC-stained BM and LN slides were reviewed for the presence of cytokeratin-positive cells. All cells with the appropriate color (red for BM and red-brown for LN) were identified and assessed for morphologic characteristics of malignancy (size, nuclear pleomorphism, and increased nuclear-to-cytoplasmic ratio). All LN and BM slides in which any candidate cells were detected were rereviewed by a second pathologist; in addition, more than 10% of negative slides were randomly selected to be rereviewed by the second pathologist. All BM slides containing IHC-positive and/or suspicious cells were sent to the National Institutes of Health (NIH) for rereview by a cytopathologist. In instances in which the USC laboratory did not agree with the NIH assessment, an additional blinded review by a third pathologist was performed. Only samples in which two or more reviewers agreed that tumor cells were present were finally assessed as positive. The total number of tumor cells present was recorded for all positive samples.

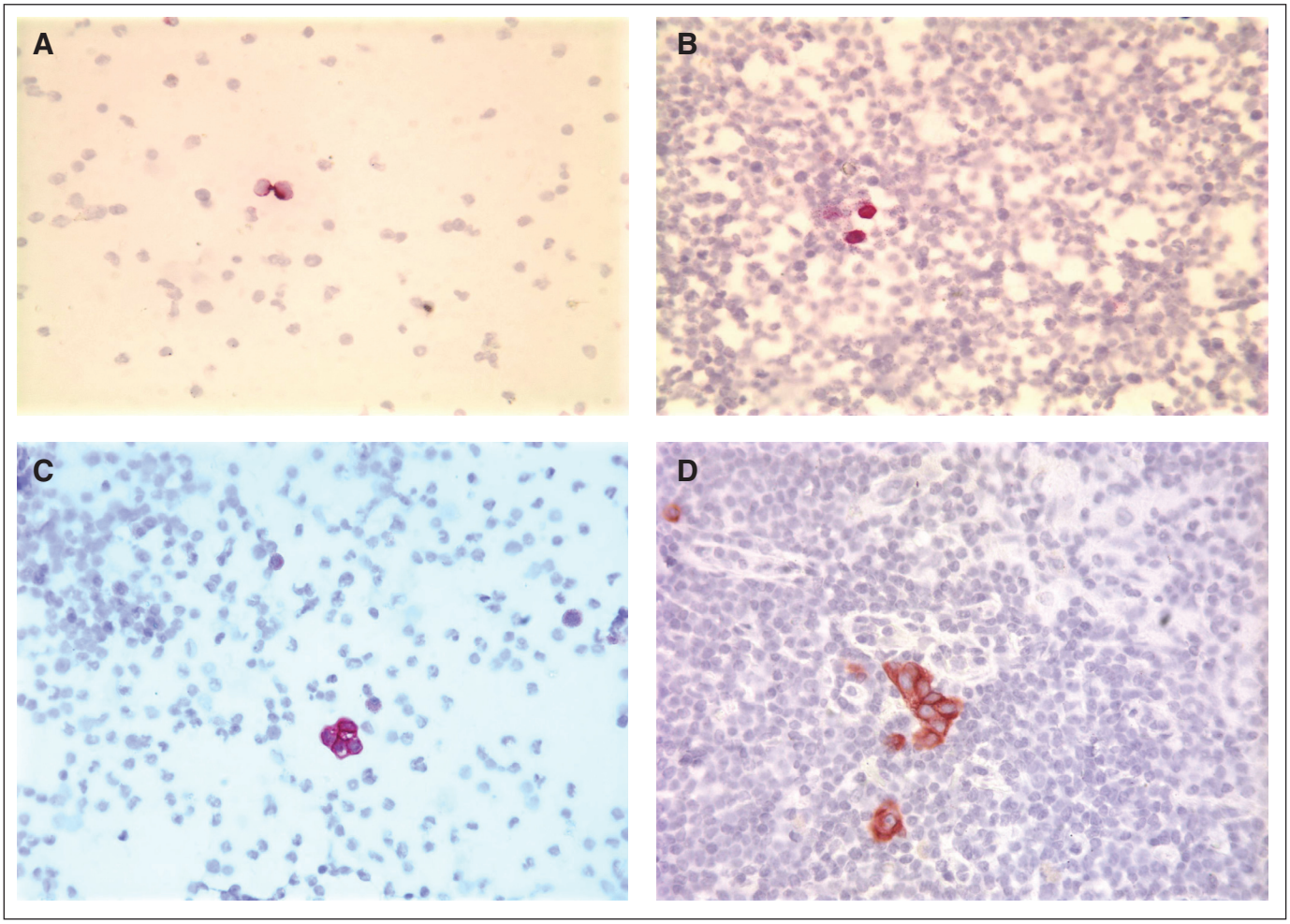

Fig 1. Representative examples of cytokeratin immunohistochemistry in bone marrow (BM) and lymph nodes (LNs). (A) Negative BM with false-positive result. This is an example of two hematopoietic cells showing false-positive staining (red). The cells lack the size and nuclear characteristics of tumor cells. Here the nuclear membranes are smooth and the chromatin is finely granular and evenly distributed. Cells such as these were seen in virtually all samples, as well as in negative controls, and are considered nonspecific background. (B) BM with suspicious cells not defined as cancer. The cells in question (red) are more suggestive of tumor cells than the cells in panel A but lack definitive features of malignancy. The cells are slightly smaller than would be expected for tumor cells, and the nuclear-to-cytoplasmic ratio and nuclear features cannot be clearly seen. (C) BM with occult metastases (OMs). Note the four cells showing strong immunoreactivity (red staining). These cells are larger than the surrounding cells and have a scant amount of cytoplasm with large, irregular nuclei; they were confirmed as OMs by multiple independent reviews. (D) LN with OMs. Note the cluster of about nine cells and the two individual cells with strong cytokeratin immunoreactivity as evidenced by brown-red staining. The cells have the morphologic features of cancer cells, including large size compared with surrounding lymphoid cells, high nuclear-to-cytoplasmic ratio, dense irregular nuclei, and prominent nucleoli, and were confirmed as OM by multiple independent reviews.

Concordance in interpretation was reached in all cases. Overall, the final interpretation agreed with the original interpretation from USC in 91% of samples; only 5% of samples showed major discordance (positive *v* negative) and all of these were resolved on further review.

For the LNs, more than 55% of slides underwent rereview. For slides in which there was not a consensus, the slides were rereviewed by both initial observers. In rare cases in which consensus was still not reached, a third pathologist served as the arbitrator. Only those slides in which two or more observers deemed OMs were present were assessed as positive. For all positive slides, the number of LNs containing OMs as well as OM number and location (capsular lymphatics, subcapsular sinus, medullary sinus, or parenchyma) were recorded and submitted to the American College of Surgeons Oncology Group Statistical Center. The pathologists at USC and at the NIH were blinded to all clinical information.

Statistical Considerations

The primary objective of the study was to evaluate the relationship between survival and markers of OM. The primary end point was overall survival, defined as the time period between patient registration and death.

Trial size computations are performed for a type I error probability of $P = .05$ (two-sided), an accrual rate of 300 patients per year for 4 years, and a follow-up interval of 5 years. The clinically consequential hazard ratio (HR; positive marrow death hazard rate over negative marrow death hazard rate) is considered to be in the range of 1.35.

For all patients, the estimated prevalence of an LN positive result by IHC is 20%. The estimated prevalence of a BM positive result is 30%. The estimated prevalence of a pleural fluid lavage positive result is expected to be between 20% and 30%. For a prevalence of 20% for OM with 900 patients enrolled, the study will have 90% power to detect an HR of 1.4.

Point estimates and 95% binomial CIs were calculated for each indicator of OM (LN, BM, and pre- and postpleural lavage). Cumulative survival probabilities were estimated using the Kaplan-Meier method, and a log-rank test was used to compare survival of groups of patients. Univariable and multivariable Cox proportional hazards regression models were used to compare groups and generate HRs and 95% CIs. Multivariable models were adjusted for age, sex, stage, size, and histology, as appropriate. In all cases, $P < .05$ was considered statistically significant.

RESULTS

Demographic Information

The study was activated on July 13, 1999, closed to accrual on March 14, 2004, and analyzed in June 2009 when all eligible patients could have been observed for at least 5 years. Of 1,310 patients registered, 263 were ultimately removed from the study for various reasons (eg, initially ineligible, final histology other than NSCLC), leaving 1,047 patients evaluable for study end points. A total of 114 patients were censored (lost to follow-up) before the final 5-year survival assessment (OM LN negative, n = 54; OM LN positive, n = 9). Table 1 and Appendix Table A1 (online only) show clinical and tumor characteristics. The median patient age was 67.2 years, and there were slightly more men (51.4%) than women. Lobectomy was the most common procedure (79.5%), and a complete resection (R0) was achieved in 95% of patients. The most common tumor histology was adenocarcinoma (50.0%), and the majority of patients (66%) had stage I tumors. All 29 tumors staged as IIIB were T4N0-2M0.

Prevalence of OM

Representative images of IHC staining on BM and LN are shown in Figure 1. Figure 2 summarizes the results of pleural lavage cytology and of IHC staining on BM and LN in relationship to pathologic tumor stage based on the total number of technically adequate specimens in each category. With respect to BM specimens, low yields during the initial part of the study were related to participating surgeons submitting curettings of the rib and were corrected by subsequent submission of the entire rib segment for processing at USC. Only 29 patients had cytologically positive pleural lavage samples before resection, and only 17 patients had positive pleural lavage after resection, making it impossible to correlate this site of OM with

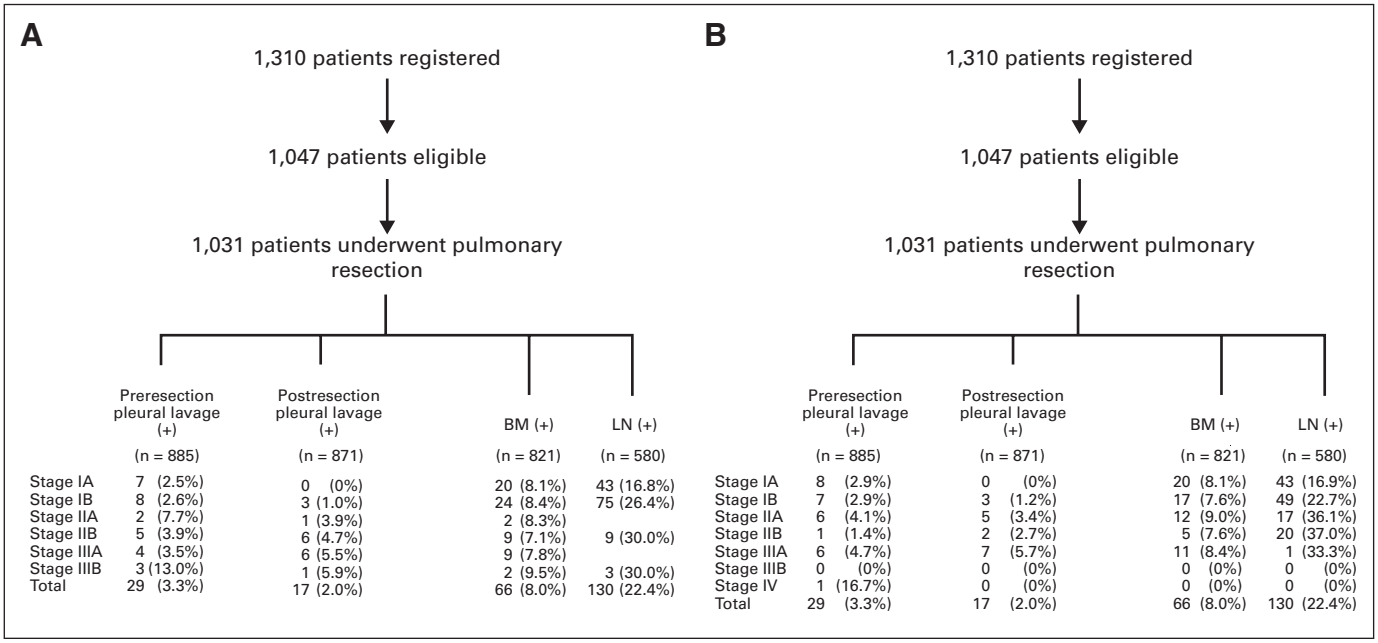

Fig 2. Schema summarizing the results of pleural fluid lavage cytology (before and after resection) and of immunostaining on bone marrow (BM) and lymph node (LN) samples in relationship to pathologic tumor stage according to the (A) sixth and (B) seventh editions of the American Joint Committee on Cancer lung cancer staging system.

survival. BM samples were positive for OMs in 66 (8.0%; 95% CI, 6.3% to 10.1%) of 821 patients with technically satisfactory specimens. LN OMs were found in 130 (22.4%; 95% CI, 19.1% to 26.0%) of 580 patients with histologic N0 disease.

The correlation of cytokeratin IHC staining results in BM and LN samples analyzed by pathologic tumor stage is shown in Appendix Table A2 (online only). Of 458 patients who had IHC performed on both BM and LN, only 10 patients (2.2%) had OM in both BM and LN. This number is too small to allow statistically valid comparison of this group to the patients who had OM in either site alone.

Survival Analysis

Currently, 663 patients (63.3%) remain alive, with a median follow-up time of 5 years (range, 0 to 5 years). Five-year overall survival by final pathologic stage (Appendix Fig A1, online only) ranged from 80% for stage IA tumors to 25% for stage III tumors.[2]

No significant difference in disease-free or overall survival was seen between patients who had OM in BM versus patients who did not (Appendix Fig A2, online only). However, a significant difference in both disease-free (HR, 1.63; 95% CI, 1.13 to 2.36; $P = .009$) and overall survival (HR, 1.59; 95% CI, 1.13 to 2.23; $P = .007$) was found in N0 patients who had OM in LN as opposed to patients who did not (Figs 3A and 3B). Neither the total number of OM-positive LNs (HR, 0.99; 95% CI, 0.98 to 1.0; $P = .054$) nor the location of IHC positivity within the nodes (HR, 1.03; 95% CI, 0.8 to 1.33; $P = .825$) seemed to be associated with a significantly worse overall survival. A statistically significant survival difference (Appendix Table A1) was observed when comparing stage IB patients with OM-positive LNs versus OM-negative LNs (HR, 1.82; 95% CI, 1.17 to 2.85; $P = .01$). No such difference was observed among patients with stage IA tumors. This is likely because the sample size of LN-positive tumors was almost twice as larger in the stage IB than the IA group (75 *v* 43 LN-positive tumors). It should be noted that this study was not designed or powered to detect a difference in these subgroups. By multivariable analysis (Appendix Table A3, online only), adjusted for patient age and sex and tumor histology, the presence of OM in LN had a significant adverse impact on disease-free survival (HR, 1.51; 95% CI, 1.04 to 2.19; $P = .031$) and overall survival (HR, 1.58; 95% CI, 1.13 to 2.22; $P = .008$).

This study was designed when the sixth edition of the American Joint Committee on Cancer lung cancer staging system was in effect. To determine whether our results were still valid within the current (seventh edition) staging system,[29] all pathology reports were reviewed and tumors restaged accordingly (Fig 2B, Appendix Fig A1B, and Appendix Table A1). Exploratory analyses showed that the survival differences for OM-positive LN versus OM-negative LN tumors were still present (Table 2; Appendix Tables A4 and A5, online only).

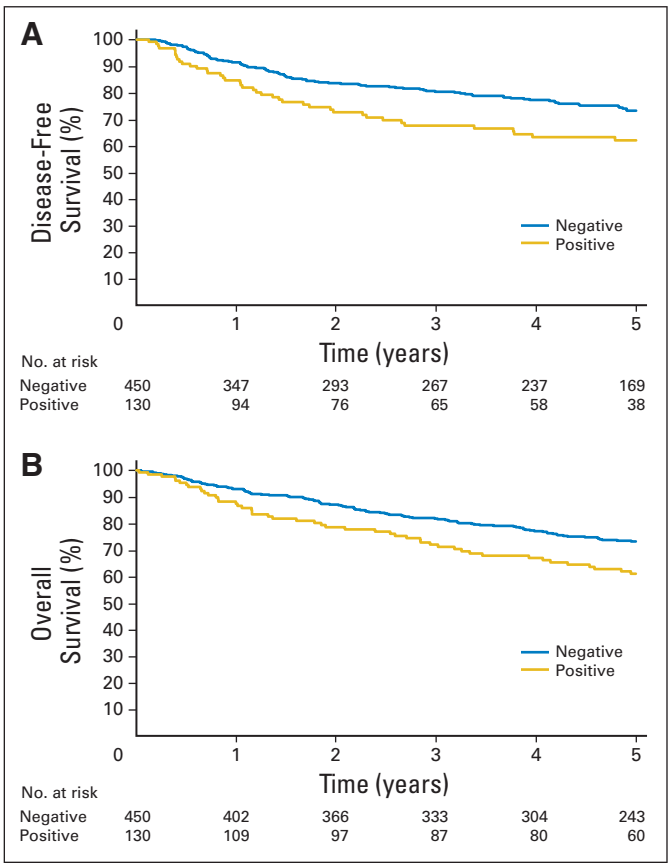

Fig 3. (A) Disease-free survival ($P = .009$) and (B) overall survival ($P = .007$) for patients with histologic N0 non–small-cell lung cancer who had occult metastases in lymph nodes by immunohistochemistry versus patients who did not.

DISCUSSION

Using techniques widely available to pathologists, this study shows that OMs in LN identify a group of patients with NSCLC at high risk for relapse and death after resection. Such patients may benefit from postoperative chemotherapy, which has been shown to improve the overall survival of patients with NSCLC with LN metastases.[4,12]

Cytologic detection of OM in pleural lavage has been investigated for more than 50 years with mixed results.[13,14] A recent meta-analysis

Table 2. Multivariate Analysis of Overall and Disease-Free Survival for Presence or Absence of OM in LN, Controlling for Patient Age, Sex, Tumor Stage,* and Histology

Variable	Hazard Ratio	95% CI	*P*
Overall survival			
LN OM (negative *v* positive)	1.58	1.13 to 2.22	.008
Age	1.03	1.02 to 1.05	< .001
Sex	0.66	0.48 to 0.91	.013
Stage	1.48	1.29 to 1.70	< .001
Histology (squamous *v* adenocarcinoma *v* other)			.31
Adenocarcinoma	0.77	0.54 to 1.12	
Other	1.0	0.66 to 1.51	
Disease-free survival			
LN OM (negative *v* positive)	1.51	1.04 to 2.19	.031
Age	1.0	0.99 to 1.02	.85
Sex	0.71	0.50 to 1.02	.060
Stage	1.47	1.27 to 1.70	< .001
Histology (squamous *v* adenocarcinoma *v* other)			.86
Adenocarcinoma	0.91	0.60 to 1.37	
Other	1.01	0.63 to 1.62	

Abbreviations: LN, lymph node; OM, occult metastases.
*According to the sixth edition of the American Joint Committee on Cancer staging system.

of 8,736 patients found that 511 patients (5.8%) had cytologically positive pleural lavage and that this independently predicted a poor survival. However, lavage technique was not standardized, and clinical follow-up in most institutions was less than 3 years.[30] Our study allows a definitive estimate of the prevalence of positive pleural fluid cytology and shows that it is uncommon and thus cannot be correlated with survival.

OMs in BM are reported in several tumor types, especially early-stage breast cancer,[6-8] where they occur in 15% to 60% of patients and are associated with a shorter time to recurrence. The impact on overall survival is not well established because previous studies are limited by small numbers of patients, short follow-up, and methodologic differences. Our results indicate that BM OMs in resectable NSCLC occur less frequently than previously reported, are unrelated to OMs in LN, and are not associated with a worse survival. Issues that may limit a general conclusion include the lower incidence of BM OMs in this study compared with others. Thus, it is possible that further work may show that BM OM is a predictor of lung cancer progression, as earlier studies (including those from our group) suggest.

LN OMs are reported to predict outcome in patients with breast, colon, prostate, and other cancers.[8-10,27] Detection of OMs by IHC has been shown to be superior to rereview of multiple serial histologic sections.[27] LN OMs examined by a variety of techniques have previously been reported in early-stage NSCLC[15,16,19-23,31] at frequencies ranging from 4% to 70%, reflecting methodologic differences, case selection, and the fact that studies have been small and retrospective. We did not seek to repeat studies comparing IHC to serial hematoxylin and eosin–stained sections of LN. However, using rigorous pathologic criteria, the current study shows that LN OMs are frequently detected by IHC in tumors deemed N0 by standard histology and are associated with a significant decrease in survival, with the greatest impact in patients with stage IB disease, patients for whom it has been difficult to demonstrate the benefit of adjuvant chemotherapy using standard TNM staging.[32,33]

Consistent with our prior studies,[17,27] we used the combination of the anticytokeratin antibodies AE-1 and CAM 5.2 because they recognize a spectrum of cytokeratins ubiquitously and highly expressed by virtually all tumor cells of simple epithelial origin, including NSCLC; facilitate detection of small numbers of tumor cells; and do not react with the normal constituents of BM or LN. Although reverse transcriptase polymerase chain reaction has been used to detect OM, this technique does not allow simultaneous morphologic evaluation of specimens and has been associated with false-positive results.[34] A recent study showed that site-specific methylation of tumor-associated genes in LN identified patients with NSCLC at increased risk for progression.[35] However, unlike IHC, these molecular assays are not yet available in routine clinical practice. Our results indicate that the IHC detection of OM in BM and LN is reproducible among different laboratories.

The three potential metastatic sites examined in this study differ widely from one another.[36,37] The heterogeneity of individual tumor biology makes it unlikely that metastases would occur in one or more of these sites simultaneously, and our results confirm this. Although BM OMs were not associated with survival in this trial, recent improvements in techniques for OM detection[38,39] warrant further work and could provide a better measure of the prognostic significance of BM OMs than was possible in the current study. The reasons why LN OMs, but not BM OMs, were associated with outcome in this study are unclear but may, in part, be a result of differences in incidence for these findings.

The association between LN OMs and survival has direct clinical implications. Our results clearly suggest that IHC should be routinely used to evaluate histologically negative LNs and to select patients with early-stage NSCLC for future trials of adjuvant chemotherapy.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

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Manuscript writing: All authors
Final approval of manuscript: All authors

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