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Detection of Occult Bone Marrow Micrometastases in Patients with Operable Lung Carcinoma

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Objectives
A large proportion of patients with operable lung carcinoma (no evidence of systemic spread of tumor) develop metastatic disease after primary therapy. More sensitive and specific methods are needed to identify patients at highest risk for recurrence who may benefit most from adjuvant therapy, while sparing those patients who do not require such treatment.

Summary Background Data
Using epithelial-specific monoclonal antibodies, the authors have developed an immunocytochemical assay capable of detecting as few as 2 lung cancer cells in 1 million bone marrow cells.

Methods
The assay was used to test the bone marrow (from resected ribs) of 43 patients with primary non-small cell lung carcinoma who showed no clinical or pathologic evidence of systemic disease.

Results
Occult bone marrow micrometastases (BMMs) were detected in 40% of patients (17/43) with non-small cell lung cancer, including 29% (5/17) of patients with stage I or II disease and 46% of whom (12/26) had stage III disease. The median follow-up was 13.6 months. Patients with occult BMMs had significantly shorter times to disease recurrence compared with patients without BMMs (7.3 *vs.* >35.1 months, $p = 0.0009$). Furthermore, for patients with stage I or II disease, the presence of occult BMMs was significantly associated with a higher rate of recurrence ($p = 0.0004$).

Conclusions
The detection of occult BMMs identifies patients with operable non-small cell lung carcinoma who are at significantly increased risk for recurrence, independent of tumor stage, and may be useful in evaluating patients for adjuvant treatment protocols.

The incidence of cancer in the United States continues to rise as our population increases in size and age. A total of 1,252,000 new cancers, with an anticipated 547,000 deaths, is projected for 1995.[1] Although lung cancer is the third most common form of cancer, it is the leading cause of cancer deaths, with 157,400 deaths projected for 1995.[1] Once a tumor has developed, surgery (either alone or in combination with adjuvant therapy) represents the only potentially curative modality.[2] Of the four major histologic subtypes of lung cancer (squamous, adenocarcinoma, small cell, large cell), only small cell carcinoma is generally considered refractory to surgical therapy. However, small cell carcinoma accounts for only 22% of lung tumors overall,[3] thus 78% of lung carcinomas would be potentially curable by surgery if they were detected early enough. Approximately 50% of all patients with lung carcinoma are candidates for, and will undergo, definitive surgical resection.[2]

The use of the TNM staging system and the staging map of the mediastinal lymph nodes[4] has been very helpful in establishing treatment plans and prognosis for resectable lung cancer.[5-9] For non-small cell carcinomas, accurate staging of disease has greater prognostic significance than cell type. For a T1, N0, M0 (stage I) lung carcinoma treated with surgical excision, preferably lobectomy, patients have an anticipated 5-year survival rate of 60% to 85%.[5,9,10] For a larger carcinoma, such as T2, N0, M0 (stage I), patients have a 50% to 60% 5-year survival rate.[5,9,10] However, survival rates decrease dramatically with increasing stage of disease. In the case of stage III lung cancer, survival rates as low as 5% have been reported for patients with stage IIIb disease, despite the absence of clinically detectable systemic metastases at the time of surgery.[2]

The single most important determinant of prognosis and management of lung cancer is the absence or presence of micrometastatic dissemination of cancer at the time of initial presentation and treatment, because primary treatment failure is secondary to undetectable systemic spread of tumor. Current measures of disease extent are primitive; standard prognostic indexes, although providing reliable information about populations of patients, cannot predict which patients will experience disease progression after primary therapy. Furthermore, the success of adjuvant therapy is assumed to stem from its ability to eradicate microscopic metastases before they become clinically evident.[11]

Table 1. DETECTION OF LUNG CANCER MICROMETASTASES

Diagnosis	No. of Patients	No. of BMM+ (%)
Primary (stage I–III) lung carcinoma	43	17 (40)
Metastatic (stage IV) lung carcioma	4	4 (100)
Carcinoma metastatic to lung	8	4 (50)
Sarcoma metastatic to lung	5	0 (0)
Benign (infectious/hamartoma)	11	0 (0)
All patients	71	

BMM+ = bone marrow micrometastases detected.

The ability to detect the earliest systemic spread of lung cancer would identify several important groups of patients, including those with low-stage (stage I) disease who have evidence of occult tumor metastases and who may therefore benefit from adjuvant systemic treatment. In addition, patients with locally advanced (stage III) disease, who generally are not considered to be surgical candidates, may be identified and thus benefit from more aggressive local (surgical) control of their tumor.

We have developed sensitive methods to detect the microscopic dissemination of tumor in the bone marrow of patients with cancer.[12-16] This technique is exquisitely sensitive and can detect as few as 2 cancer cells in 1 million (10^6) normal bone marrow cells.[15] We report here on the use of this technique to detect occult bone marrow micrometastases (BMMs) in patients with localized lung carcinoma who have no evidence of systemic metastases when routine clinical and pathologic methods are used. We have evaluated the early clinical follow-up of this group of patients and have found that the presence of BMMs is associated with a greater risk of early recurrence.

MATERIALS AND METHODS

Patient Population

A total of 71 patients were entered in this study. All patients had undergone thoracotomy at Beth Israel Hospital in New York between 1991 and 1993. The diagnoses for these patients are summarized in Table 1. Forty-three patients had primary non-small cell lung carcinoma without evidence of systemic metastases (stage I–III), 12 had metastatic carcinoma (including 4 with metastatic lung cancer), 5 had sarcomas metastatic to lung, and 11 had benign disease (e.g., infections, hamarto-

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mas). Of the patients with primary lung carcinoma, 15 had stage I, 2 had stage II, and 26 had stage III disease. Disease stage for all patients was determined according to the TNM system.[4]

All patients with primary lung cancer underwent bronchoscopy and mediastinoscopy before surgery. Surgery included careful mediastinal staging as well as surgical and pathologic TNM staging.

Bone Marrow Samples

Bone marrow samples were obtained at the time of primary surgery. Sections of rib removed routinely at the time of surgery were opened immediately and bone marrow curetted into heparinized media. This procedure results in a high-quality bone marrow sample yielding approximately 40 to 60 million mononuclear cells per patient.

Bone marrow samples were placed in an insulated container at room temperature and shipped overnight to the University of Southern California. Bone marrow aspirations can be stored for 24 to 48 hours without significant adverse effects on epithelial cell antigenicity.[15]

Two to four routine air-dried smears were made from the bone marrow specimen and stained with Wright-Giemsa stain for routine cytologic examination. The remainder of the specimen was layered onto a Ficoll-Hypaque density gradient (Pharmacia, Piscataway, NJ) and centrifuged at 400 × *g* for 20 minutes. The interface layer (which contains mononuclear cells and intact, viable epithelial cells[17]) was collected, and the mononuclear cells were counted with a hemocytometer counting chamber and brought to a final concentration of 10^7 cells/mL.

Charged glass slides (Probe On Plus; Fisher Scientific, Pittsburgh, PA) were used for cell plating. Exactly 0.1 mL (1 million mononuclear cells) of the mononuclear cell suspension was added and spread evenly onto each slide. Typically, 20 to 40 slides were prepared per patient. These slides were allowed to air-dry overnight (or for at least 3 hours) and then fixed in acetone for 5 minutes at room temperature and stored at −20 C. Slides can be stored for more than 2 years.[15,16]

Monoclonal Antibodies

Two mouse monoclonal antibodies, AE-1 (Hybritech, San Diego, CA) and CAM 5.2 (Becton-Dickinson, San Jose, CA), which are specific for cytokeratin intermediate filament antigens, were used in combination, each at a final concentration of 10 μg/mL. The specificity of these antibodies has been described previously.[18–21] AE-1 reacted with greater than 90% of lung carcinomas,[18,19] whereas CAM 5.2 reacted with nearly 100% of lung carcinomas.[20,21] These antibodies are epithelial specific and do not react with cells found normally in the bone marrow. They did not react with normal bone marrow cells in samples obtained from more than 65 patients without epithelial cancers.[15,16]

Immunocytochemical Staining Procedure

The immunocytochemical procedures have been described previously.[12,13,15,16] Briefly, specimen slides were brought to room temperature, washed, and incubated with suppressor serum (5.0% horse serum in phosphate buffered saline) for 30 minutes as a blocking step. The suppressor serum was aspirated, and the mouse monoclonal antibody cocktail was applied at appropriate dilution (100–200 μL/slide). The slides were incubated in a humid chamber for 30 minutes at room temperature and then incubated with biotinylated horse anti-mouse antibody (Vector Laboratories, Burlingame, CA) for 30 minutes at room temperature. The slides were again washed and then bathed in 2% H_2O_2/phosphate buffered saline/sodium azide for 20 minutes to quench the endogenous peroxidase activity of the bone marrow mononuclear cells. After an additional wash, the slides were incubated with Avidin DH biotinylated horseradish peroxidase H complex (Vector Laboratories) for 30 minutes at room temperature, washed, and bathed in a chromogenic substance (diaminobenzidine) for 10 to 15 minutes at room temperature. They were then rinsed, counterstained with hematoxylin for 1 to 3 minutes, dehydrated, and protected with a coverslip.

Five slides (i.e., 5 million bone marrow elements) from each patient were studied with use of the immunocytochemical assay. After the staining procedure, the slides were examined microscopically for the presence of antigen-positive cells. These cells were marked on the slide, counted, and usually photographically documented. All slides were screened without knowledge of patient diagnosis or stage of disease.

Statistical Analysis

Survival and time to the first recurrence of lung cancer were analyzed. Survival was calculated as the number of months from surgery until death or until last documented contact with the patient who was known to be alive. For patients with lung cancer recurrence, time to the first recurrence of lung cancer was calculated as the number of months from surgery to the date of first documented recurrence of disease. Patients who died before recurrence of disease were censored at the date of death; patients without recurrence were censored at the date last seen free of disease. Patients who were never disease free were not included in the analysis of time to recurrence.

Kaplan–Meier product limit estimates[22] of overall sur-

vival and recurrence-free survival were plotted. Standard errors for the probability of surviving or of not experiencing disease recurrence were based on Greenwood's formula[22] for the Kaplan–Meier estimates. The log-rank test was used to compare groups of patients. All probability values reported are two-sided.

RESULTS

Clinical Follow-up

Forty-three patients with non–small cell lung carcinoma without clinical evidence of systemic metastases were evaluated. The median follow-up was 13.6 months; 80% of patients (25/30) still alive at last follow-up were followed for more than 5.8 months. Nineteen patients developed systemic metastases; of these, 10 died. A total of 13 deaths occurred, including 3 of non–lung cancer causes.

Detection of Bone Marrow Micrometastases

Table 1 summarizes the rate of detection of BMMs in the entire cohort of 71 patients. Of note is that although BMMs were detected in 40% of the patients (17/43) with primary localized (stage I–III) lung cancer, in 100% of patients (4/4) with metastatic lung cancer, and in 50% of patients (4/8) with epithelial carcinoma metastatic to lung, no epithelial cells were detected in the bone marrow of patients with metastatic sarcomas (0/5) or in that of patients who had undergone operation for benign disease (0/11). These results further demonstrate the specificity of the immunocytochemical assay. Figure 1 shows examples of tumor cells detected by the immunocytochemical assay in the bone marrow of patients with stage I (Fig. 1A) and stage III (Fig. 1B) lung cancer.

Bone Marrow Micrometastases and Recurrence

For the 43 patients with primary lung carcinoma but no clinical evidence of systemic metastases, the presence of BMMs was related to stage of disease (Table 2). Twenty-nine percent of patients (5/17) with stage I or II lung cancer had detectable BMMs, compared with 46% of patients with stage III disease (12/26), but this was not statistically significant ($p = 0.27$). Thirteen of the 17 patients (77%) with BMMs experienced recurrence, compared with 6 of the 26 patients (23%) without evidence of BMMs.

The presence of BMMs was significantly associated with earlier time to recurrence ($p = 0.0009$) (Table 3, Fig. 2). The median time to recurrence for patients with no detectable BMMs was 35.1 months, compared with 7.3 months for patients with BMMs (Table 3). Similar results were seen in comparisons of bone marrow status to overall survival; of the 17 patients with BMMs, 7 died (41%), compared with 6 deaths (23%) among the 26 patients without BMMs. The median time to death in patients with no detectable BMMs was 35.1 months, compared with 18.6 months for patients with BMMs (Table 3, Fig. 3). However, results for overall survival were not statistically significant ($p = 0.072$).

Stage of Disease and Recurrence in Bone Marrow Micrometastases

We also determined whether bone marrow status could be used to stratify patients in conventional prognostic groups (Figs. 4 and 5). There was a highly significant difference in recurrence rate for patients with stage I and stage II lung cancer according to bone marrow status ($p = 0.0004$) (Fig. 4). Similar results were seen for patients with stage III lung cancer, although this did not reach statistical significance ($p = 0.11$) (Fig. 5). Of particular note is that when BMMs were not detected in patients with stage III disease, a substantial proportion (approximately 40%) of patients remained disease free at 2 years, despite the advanced stage of disease.

A multivariable analysis evaluating bone marrow status and controlling for stage of disease showed that the presence of BMMs is an independent predictor of disease recurrence ($p = 0.017$).

DISCUSSION

We have shown in this study that occult BMMs can be detected in a substantial proportion of patients with lung cancer who show no clinical evidence of systemic metastases, including patients with disease in its earliest stage (stage I). The presence of tumor cells in the bone marrow, detected by the immunocytochemical assay described here, is significantly associated with a higher recurrence rate for patients with primary, localized (stage I–III) non–small cell lung carcinoma. In addition, patients in conventional prognostic groups may be further stratified by their bone marrow status. Although the presence of BMMs is related to stage of disease, it is independent of stage regarding prediction of recurrence. The presence of BMMs appears to be a clinically important predictor of lung cancer recurrence.

Following the pioneering studies at the Ludwig Institute at Royal Marsden Hospital,[23] we have shown that the detection of BMMs with the methods described here is a significant predictor of recurrence for patients with early stage operable breast cancer.[13,16] Several other groups have also shown that the detection of BMMs is

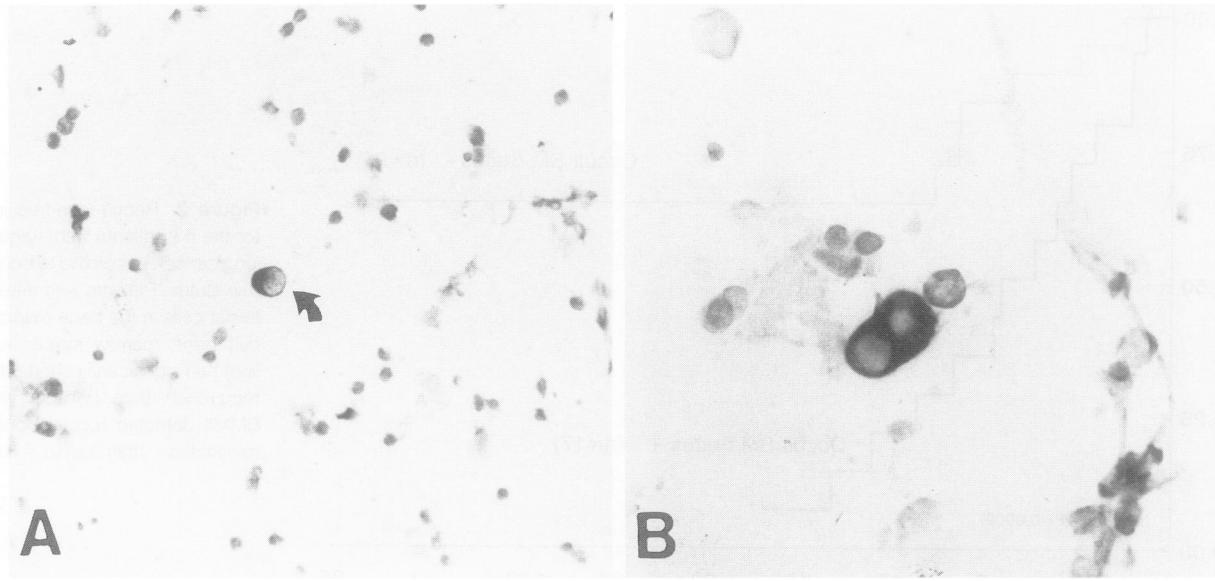

Figure 1. (A) Bone marrow from a patient with stage I lung cancer. A single tumor cell is identified by the immunocytochemical technique involving the use of the epithelial-specific antibody cocktail (arrow) (original magnification ×200). (B) Bone marrow from a patient with stage III lung cancer. A cluster of tumor cells is identified by the immunocytochemical technique (original magnification ×400). In both A and B, the surrounding bone marrow mononuclear cells show no immunoreactivity; only the nuclei (counterstained with hematoxylin) are observable.

significant in predicting early recurrence for patients with breast cancer[24–26] or colon cancer.[27,28] Methods for detection of BMMs in patients with epithelial cancers are based on the ability to distinguish extrinsic (epithelial) populations of tumor cells from normal bone marrow elements. This involves use of monoclonal antibodies, which react with antigens expressed by epithelial (carcinoma) cells but not by normal hematopoietic elements.[12,13] Studies have shown that occult BMMs (extrinsic, antigen-positive epithelial cells) can be detected in the bone marrow of patients with breast or colon carcinoma without other evidence of metastasis.[13,16,24–28] Furthermore, the presence of micrometastases identifies patients with early stage (operable) breast or colon carcinomas who are at significantly increased risk for recurrence.[13,16,24–28]

Previous attempts to detect dissemination of lung carcinoma with such methods as skeletal surveys, radionuclide scans, and bone marrow and liver biopsies have been disappointing regarding patients with operable (non–small cell) lung carcinoma who were not suspected of having clinical metastases.[7,29–32] Recently, investigators have used immunocytochemical methods similar to those described here and have shown that occult BMMs can be detected in a substantial proportion of patients with operable non–small cell lung carcinoma.[33,34] Furthermore, these investigators have shown that the pres-

Table 2. INCIDENCE OF LUNG CANCER MICROMETASTASES: ASSOCIATION WITH STAGE OF DISEASE

	No. of Patients	No. of BMM+ Patients (%)
Stage I	15	5 (33)
Stage II	2	0 (0)
Stage III	26	12 (46)
All patients*	43	17 (40)

BMM+ = bone marrow micrometastases detected. p = 0.27.
* Patients with primary, operable non-small cell lung carcinoma.

Table 3. ASSOCIATION OF BMM WITH RECURRENCE AND DEATH IN PATIENTS WITH STAGE I–III LUNG CANCER

Occult BMM	No. of Patients	Median Time to Recurrence (mo)	Median Time to Death (mo)
Absent	26	35.1 (10.1–35.1)	35.1 (20.2–35.1)
Present	17	7.3 (3.3–9.3)	18.6 (8.6–18.6)

BMM = bone marrow micrometastases.
Values in parentheses are confidence intervals.

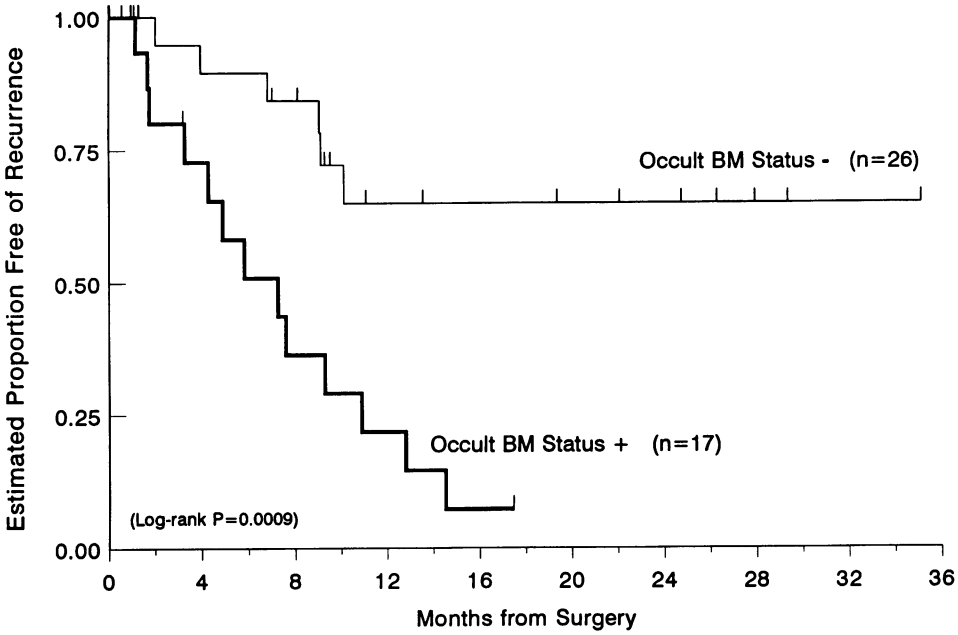

Figure 2. Recurrence-free interval for the 43 patients with stage I to III lung cancer, according to bone marrow status. Patients with detectable tumor cells in the bone marrow (occult bone marrow status +, dark line) had significantly shorter time to recurrence than patients with no BMMs detected (occult bone marrow status −, thin line) (p = 0.0009).

ence of BMMs was significantly associated with disease recurrence.[33,34] The results of the current study are consistent with these findings.

The immunocytochemical methods used in the current study are exquisitely sensitive and can detect as few as 2 tumor cells in a population of 1 million bone marrow cells.[15] We used a cocktail of epithelial-specific antibodies that recognize five distinct cytokeratin subtypes[18–21] to avoid overlooking extrinsic cells because of antigenic heterogeneity. Recently, molecular assays based on the polymerase chain reaction have been used to detect prostate cancer BMMs.[35,36] Such assays have not been established for other epithelial tumor types. It is unknown whether the use of assays based on the polymerase chain reaction for detection of BMMs is advantageous to the use of the immunocytochemical assays described here.

Besides detecting systemic (bone marrow) spread of

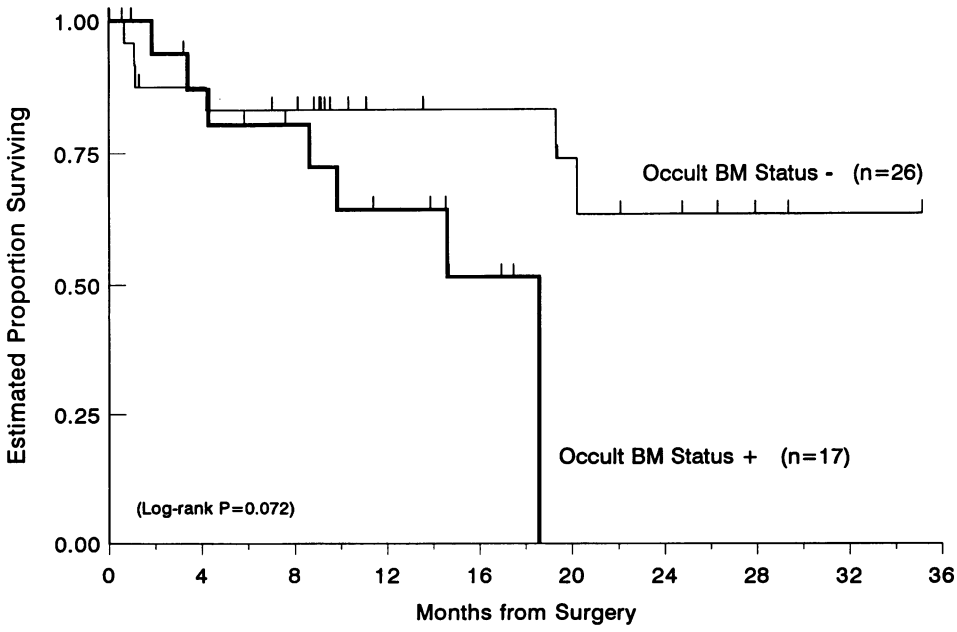

Figure 3. Survival of the 43 patients with stage I to III lung cancer according to bone marrow status. Patients with BMMs detected (occult bone marrow status +, dark line) died sooner on average than patients with no BMMs detected (occult bone marrow status −, thin line). This did not reach statistical significance (p = 0.072).

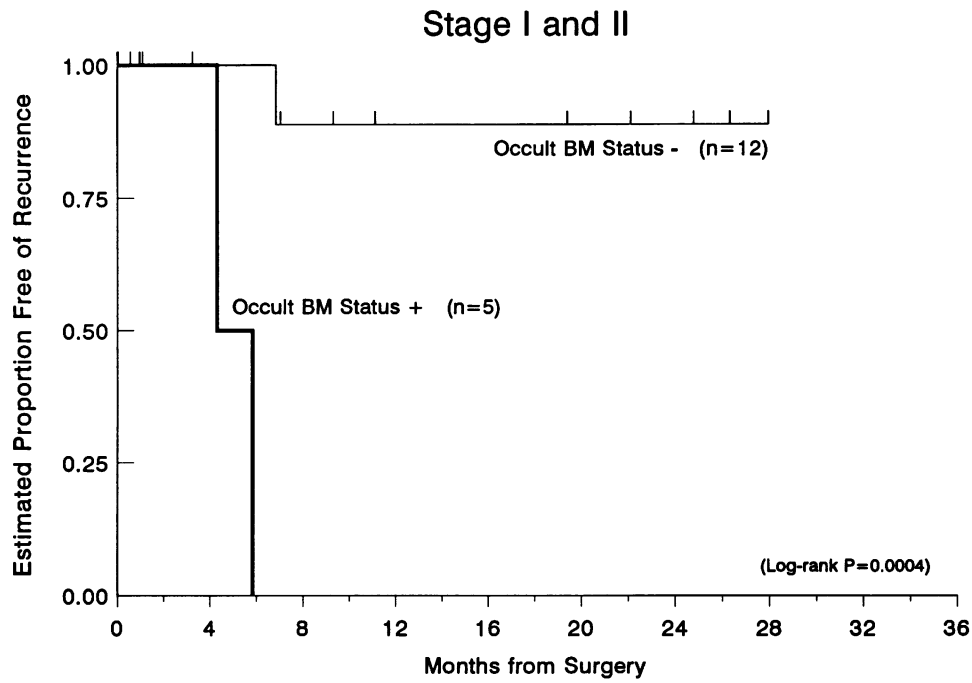

Figure 4. Recurrence-free interval for the 17 patients with stage I or II lung cancer according to bone marrow status. Patients with BMMs detected (occult bone marrow status +, dark line) had significantly shorter time to recurrence than patients with no BMMs detected (occult bone marrow status −, thin line) (p = 0.0004).

tumor, immunocytochemical assays can help identify regional spread of tumor to lymph nodes, which cannot be observed by routine histopathologic examination. Several studies have shown that occult lymph node micrometastases (LNMs) can be detected in the regional lymph nodes of a substantial proportion of patients with stage I or II lung cancer and that the presence of occult LNMs is associated with increased recurrence.[37,38] It will be interesting to determine whether the detection of occult LNMs and BMMs in the same patient confers additional prognostic information concerning patients with stage I or II lung carcinoma.

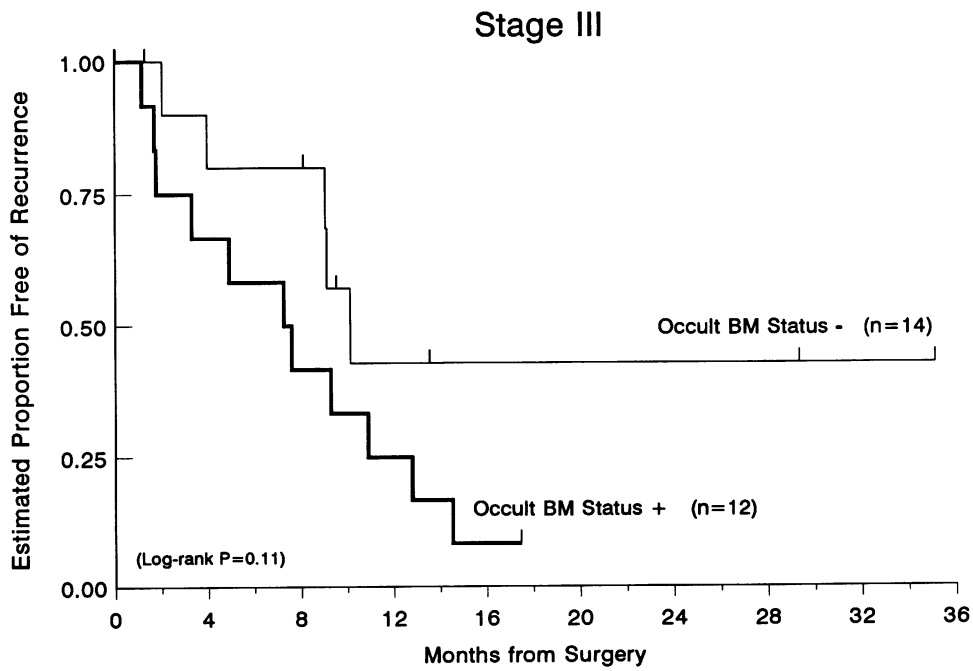

Figure 5. Recurrence-free interval for the 26 patients with locally advanced (stage III) lung cancer according to bone marrow status. Patients with BMMs detected (occult bone marrow status +, dark line) had shorter time to recurrence than patients with no BMMs detected (occult bone marrow status −, thin line). This did not reach statistical significance (p = 0.11).

The presence of occult BMMs (and LNMs) may define not only patients who are at higher risk for recurrence and death at worse prognosis, but may also identify biologically distinct mechanisms of tumor spread (e.g., lymphatic *vs.* vascular dissemination). Use of these techniques may also allow us to identify biologically important populations of cells, that is, those cells constituting the earliest metastatic population of tumor cells. Thus, techniques that identify occult metastases may be valuable in furthering our understanding of the events regulating tumor dissemination.

The role of adjuvant chemotherapy in treating patients with stage I or II lung cancer is controversial. However, identification of those patients at greatest risk for recurrence (who, presumably, will benefit most from such adjuvant approaches) may provide more rational grounds for making treatment decisions. For patients with locally advanced (stage III) disease, the role of surgery can be controversial. Regarding this group of patients, by identifying those at lowest risk for recurrence, we may be able to determine which patients will benefit most from local (surgical) control of tumors. Bone marrow micrometastases (and LNMs) may be important new prognostic factors in lung cancer that can greatly affect the selection of patients who may benefit most from adjuvant therapy and the selection of patients with advanced disease who may benefit from surgery. This approach confers great improvement in staging accuracy and therefore allows for the formulation of truly homogeneous treatment groups in future clinical trials.

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Discussion

DR. TOM R. DEMEESTER (Los Angeles, California): Dr. Beattie's paper and Dr. Giuliano's paper that we heard earlier this morning provide us with some objective evidence for what we always imagined to be true, that is, there are unrecognized micrometastases in the node removed or left behind after a resection.

In the past, this has been difficult to measure and as a consequence, not much was done about it. Now, with the advent of the epithelial-specific clonal antibody, the assessment is possible and will cause us to rethink our surgical approach to malignant disease in the future.

The question, of course, is, are these stain specific? If you do detect cells, are they viable, can they grow, and does the finding affect survival?

It appears that Dr. Beattie has given us good evidence that the cells, when detected, have a dramatic effect on survival. In fact, he has shown that the observation may account for inaccuracies in staging, at least in early disease.

What would be important to know is whether the finding is a sign of systemic disease. Does he have information as to where the recurrences occurred? Were they systemic or local?

I would also be interested to know whether he has had an opportunity to study lymph nodes, as reported by Dr. Giuliano, and how many patients had histologically normal but histochemically positive lymph nodes with histochemically normal bone marrow.

That would be an interesting group of patients, because if indeed they do exist, it could be argued that our surgical resection for cancer should be more extensive rather than the current emphasis on limited resections. It may be that the survival difference observed between limited and extensive resection for node-negative patients may be explained by the removal of nodes histochemically positive with the more extensive surgery.

Now that he has developed this technique, it would be interesting to know how he might use this information in patient care. Would he encourage the searching for histochemical evidence of metastases preoperatively and then subjecting these patients to adjuvant therapy? Or would he suggest adjuvant therapy if the nodes were found in the surgical specimen? Does he have any evidence that micrometastases detected by histochemical techniques are more susceptible to chemotherapy? I think these types of studies will certainly challenge our thoughts concerning surgical oncology in the future.

DR. JOHN R. BENFIELD (Sacramento, California): Dr. Beattie's concept of extending the TNM nomenclature to TNM small "m" is heading in just the right direction, and I hope that he intended the small "m" to stand for micrometastases and not for marrow. I thought I might share with you some data that we presented just 2 months ago before The Society of Thoracic Surgeons, heading in somewhat the same direction in a different way (Johnson JR, et al. Successful xenotransplantation of human lung cancer correlates with the metastatic phenotype. Ann Thorac Surg 1995; 60:32–37).

We studied 81 patients who had lung cancer in various stages whose lung cancers were then propagated in xenotransplantation in nude mice. The mean follow-up was 22.5 (2–61) months. Twenty-one xenotransplants successfully took and seven metastasized in the nude mice. Neither the predominant cell type nor the incidence of lymph node metastases correlated with the results of xenotransplantation. Of 21 patients whose non—small-cell lung cancer (NSCLC) took in transplant, 13 (61.9%) developed metastases, and 9 (42.8%) died from their cancers. Among 57 patients whose NSCLC did not take, 14 (24.5%) developed metastases and 9 (15.7%) have died from their cancers. The higher incidence of metastases in association with xenotransplantation take was significant ($p = 0.0032$). We concluded that patients whose NSCLC take in transplant are at high risk for metastases, and we surmised that this method of propagating human lung cancers is a step toward facilitating precise cellular biologic definition of the metastatic propensity of such neoplasms.

I suggest that an extension of Dr. Beattie's approach to finding occult human lung cancers propagated in xenotransplantation might give us some insight as to the mechanisms that are involved and perhaps another tool to identify patients with systemic disease before the usual approach of staging.

We concluded that patients whose lung cancers take in our transplantation model are at high risk for metastases. We believe that this method of propagating human lung cancers will facilitate carrying out precise cellular biologic studies aimed at defining the metastatic potential of such neoplasms before metastases become apparent. The search for evidence of micrometastases, as described by Dr. Beattie, plus cellular biologic markers of metastatic propensity should eventually permit us to identify patients whose apparently early stage lung cancers should nonetheless receive adjuvant systemic treatment.