Immunocytochemical Detection of Bone Marrow Micrometastasis in Operable Non-Small Cell Lung Cancer


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ABSTRACT

Present diagnostic techniques do not allow the detection of early metastatic spread of tumor cells, although this spread largely determines the clinical course of patients with small primary cancers. By use of monoclonal antibody CK2 to the epithelial cytokeratin component number 18 (CK18), individual disseminated carcinoma cells present in bone marrow of cancer patients can now be identified (G. Schlimok, I. Funke, B. Holzmann, G. Göttlinger, G. Schmidt, H. Häuser, S. Swierkot, H. H. Warnecke, S. Zschender, H. Kropowski, and G. Riethmüller, Proc. Natl. Acad. Sci. USA, 84: 8672-8676, 1987; F. Lindemann, G. Schlimok, P. Dirschedl, J. Witte, and G. Riethmüller, Lancet, 340: 685-689, 1992). In the present study, we applied this approach to patients with operable non-small cell lung cancer. CK18 was expressed on 84 of 88 (95.5%) primary adenocarcinomas and squamous cell carcinomas. Irrespective of primary tumor histology, single aspirates of iliac bone marrow from 18 of 82 (21.9%) lung cancer patients exhibited between 1 and 531 ∴ K1X cells present in the bone marrow of patients with such cancer at the time of primary surgery. The specificity of our assay is underlined by the small rate of "false-positive" cells being observed in only 2 of 117 (1.7%) marrow samples from control patients with no evidence for an epithelial malignancy at the time of aspiration. Comparison with established risk factors demonstrated positive correlations (P < 0.05) between the size and histological grade of the primary carcinoma and cytokeratin positivity in iliac bone marrow. In contrast, the association with the metastatic involvement of regional lymph nodes was only weak (P = 0.09). Following a median observation period of 13 months, patients who displayed cytokeratin-positive cells present in bone marrow aspirates from patients with operable NSCLC. Our results show that the detection of such cells at primary surgery was associated with established risk factors and significantly linked to an increased rate of early relapse. Thus, the immunological assessment of micrometastasis might be of clinical value as a prognostic indicator of early relapse in these patients. In addition, this approach may contribute to the identification and monitoring of patients who might benefit from adjuvant therapy.

MATERIALS AND METHODS

Diagnostic Evaluation of Patients. During the period from November 1989 to September 1991 patients with primary NSCLC were staged according to the TNM classification. The size, histological type, and grade of the primary tumor were assessed by conventional histopathology. After informed consent, only patients in TNM stage M0 (i.e., no diagnostic signs for distant metastasis) with completely resected (R0) primary adenocarcinomas (n = 48) or squamous cell carcinomas (n = 43) were admitted to the study and investigated under identical conditions. This group included patients with nonepithelial neoplasias and inflammatory diseases.

Specimens. Primary tumors from patients with NSCLC were surgically excised and cut into halves for analysis by either conventional histopathology or immunohistochemistry. Three tumors were rejected from analysis because the nuclear antigen p120 or the tyrosine kinase receptor erbB2, exemplified the oncogenic capacity of CK18 micrometastatic cells. In conclusion, CK18* cells present in the bone marrow of patients with apparently operable non-small cell lung cancer exhibit the potential to form solid metastases. Therefore, the approach presented here may be used to determine the risk of early relapse in operable non-small cell lung cancer with potential consequences for adjuvant therapy.

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2 The abbreviations used are: NSCLC, non-small cell lung carcinomas; CK18, cytokeratin polypeptide number 18; mAb, monoclonal antibody(s).

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Every 6 months after primary surgery, questionnaires were sent out to the consulting physician of each patient in order to obtain the essential follow-up information, including the time and cause of relapse as well as the time and cause of death. A population bias could be excluded because the questionnaires of all examined patients were filled out completely by the consulting physician of each patient. If possible, a relapse was confirmed at the Central Hospital of Gauting, where most of the patients underwent additional therapy. Survival of less than 1 month after surgery was always caused by cardiopulmonary complications due to the extensive surgical intervention.

As "negative" controls, 117 patients without evidence of an epithelial neoplasm also were admitted to the study and investigated under identical conditions. This group included patients with nonepithelial neoplasias and inflammatory diseases.

Specimens. Primary tumors from patients with NSCLC were surgically excised and cut into halves for analysis by either conventional histopathology or immunohistochemistry. Three tumors were rejected from analysis because of the poor condition of the samples at arrival in our laboratory. Specimens for immunohistochemistry were snap-frozen in liquid nitrogen and stored at −80°C. Cryostat sections of 5-μm thickness were placed on glass slides, which had been precleaned with acetone and coated with 3-triethoxysilylpropylamine (Merck-Schuchardt, Hohenbrunn, Germany). Slides were air-dried overnight and subsequently stained.

INTRODUCTION

The "dark stage" of early metastasis remains largely undetected by conventional staging procedures (1), but it can be assessed by immunocytochemical analysis of bone marrow (for a review see Ref. 2). Using monoclonal antibodies to cytokeratin, as reliable markers for regional lymph nodes was only weak (P = 0.09). Following a median observation period of 13 months, patients who displayed cytokeratin-positive cells present in bone marrow aspirates from patients with operable NSCLC. Our results show that the detection of such cells at primary surgery was associated with established risk factors and significantly linked to an increased rate of early relapse. Thus, the immunological assessment of micrometastasis might be of clinical value as a prognostic indicator of early relapse in these patients. In addition, this approach may contribute to the identification and monitoring of patients who might benefit from adjuvant therapy.

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2 The abbreviations used are: NSCLC, non-small cell lung carcinomas; CK18, cytokeratin polypeptide number 18; mAb, monoclonal antibody(s).
During the period of our study, bone marrow was aspirated from all patients with apparently operable primary NSCLC in order to avoid a selection bias. At the time of primary surgery, one bone marrow sample/patient was aspirated from one site of the upper iliac crest. The aspiration volume varied from 0.5 to 5 ml, yielding between 1 x 10^6 and 5 x 10^7 mononuclear cells. After density centrifugation through Ficoll-Hypaque, interface cells were cytotoxicentrifuged at 1000 rpm on glass slides (8 x 10^4 cells/slide). Following overnight air-drying, slides were either stained immediately or stored at -80°C. For each marrow sample, five slides were examined, while one additional slide served as immunoglobulin isotype control.

Since bone marrow aspirates contain variable portions of sinusoidal blood, the quality of aspirates was controlled by Pappenheim staining in order to identify typical myeloid precursor cells. Nine aspirates were rejected from immunocytochemical analysis because they did not contain any morphologically identifiable myeloid precursor cells on the two smears analyzed by Pappenheim staining.

**Immunocytochemistry.** According to our previous work (3–6), mAb CK2 (kindly provided by Dr. M. Osborn, Max Planck Institut Göttingen, and later obtained from Dr. H. Bodenmüller, Boehringer Mannheim, Tübingen, Germany) directed to CK18 (14, 15) was used at 2.5 µg/ml as primary antibody for staining of both cryostat sections from primary tumors and cytospin preparations from autologous bone marrow aspirates. CK2 stains all "normal" (i.e., nonmalignant) cells of simple epithelial and tumors derived thereof as well as squamous or transitional cell carcinomas (14–17). Appropriate dilutions of mouse myeloma proteins served as IgG1 isotype control (MOPC21 purchased from Sigma, Deisenhofen, Germany).

The antibody reaction was developed with the alkaline phosphatase anti-alkaline phosphatase technique combined with the Neufuchsin method for visualizing antibody binding (18). Briefly, after incubation with mAb CK2, a polyvalent rabbit anti-mouse immunoglobulin antisera and preformed complexes of alkaline phosphatase and monoclonal anti-alkaline phosphatase antibodies were used at the dilutions recommended by the manufacturer (Dako-patts, Hamburg, Germany). To allow a fast screening for mAb-positive cells on the slides, no counterstaining was performed.

For double staining, we applied a protocol based on the combination of immunogold and immunoenzymatic techniques. A similar approach has been successfully applied to the detection of major histocompatibility complex class I molecules on disseminated CK18+ tumor cells (19). In the first step, cells were incubated with either one of the primary mAbs to Ki-67 (Dianova, Hamburg, Germany) for 45 to 60 min. After a thorough wash with phosphate-buffered saline, gold-conjugated goat a- mouse immunoglobulins were incubated for 45 to 60 min. Subsequently, the slides were washed and exposed to 2% glutaraldehyde diluted in phosphate-buffered saline for 5 min. The following immunoenzymatic step was similar to the single labeling method described above, except for the use of biotinylated mAb CK2 which was developed with preformed complexes of streptavidin and alkaline phosphatase. After rinsing in distilled water for at least 15 min, the freshly prepared silver enhancement mixture (Amersham, Braunschweig, Germany) was applied for about 20 min and the reaction was monitored every 5 min by placing the slides under the microscope. To abrogate the enhancement reaction, the slides were rinsed in distilled water.

All slides were examined by two independent observers in a double-blinded fashion.

**RESULTS**

**Cytokeratin Expression on Primary NSCLC and Autologous Bone Marrow.** In order to demonstrate the sensitivity of CK18 as a marker of tumor cells derived from NSCLC, cryostat sections of 88 primary lung carcinomas were stained with mAb CK2, using the alkaline phosphatase anti-alkaline phosphatase technique (Table 1). CK18 expression was observed on 84 tumors (95.5%) with a slight preference for adenocarcinomas over squamous cell carcinomas (100 versus 90%, respectively; Table 1). The majority of samples displayed a homogenous staining pattern in terms of the fraction of CK18+ tumor cells and the intensity of staining. However, there was slightly more variation among squamous cell carcinomas as compared to adenocarcinomas.

<table>
<thead>
<tr>
<th>Staining a</th>
<th>Adenocarcinoma b (n = 48)</th>
<th>Squamous cell carcinoma c (n = 40)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Heterogeneous, focal</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>Homogeneous, diffuse</td>
<td>48</td>
<td>24</td>
</tr>
</tbody>
</table>

a Cryostat sections were stained with mAb CK2 using the alkaline phosphatase anti-alkaline phosphatase technique. Specimens were analyzed by light microscopy and grouped as described in Ref. 16.

b Tumors were graded by histopathology as G1 (n = 5), G2 (n = 23), or G3 (n = 20).

c Tumors were graded by histopathology as G2 (n = 25) or G3 (n = 15).

At primary surgery, bone marrow was aspirated from 1 site of the upper iliac crest of 82 patients. All of these patients were staged M0 (by conventional staging procedures) and displayed completely resectable primary tumors as determined by routine pathological examination (R0 resection). Applying the same staining protocol used for analysis of primary carcinomas, single CK18+ cells were detected in 18 of 82 marrow samples (21.9%) with small differences between patients with adenocarcinomas or squamous cell carcinomas (Table 2). However, 15 of 18 CK18+ samples contained fewer than 10 CK18+ cells/4 x 10^5 nucleated cells with 2 of the remaining 3 samples containing more than 100 CK18+ cells. Clusters of CK+ cells are rare and were detected in only 4 out of 82 patients (4.9%). Thus far, all analyses were performed on cells obtained after density centrifugation with Ficoll-Hypaque. Thus tumor cell clusters may have been lost in the pellet. However, the examination of the pellets after density centrifugation did not reveal any CK+ clusters, suggesting aggregate formation is indeed a rare event.

As "negative" control for the specificity of cytokeratin labeling of tumor cells, iliac bone marrow from patients without evidence for an epithelial neoplasia ("noncancerous") patients was stained with mAb CK2, using identical sample sizes and staining conditions (Table 2). One single CK18+ cell was detected in each of only 2 of 117 samples (1.7%). Both of the two "false-positive" patients displayed a tubulovillous adenoma of the colon with a colon carcinoma being diagnosed 10 months later in one case.

**Comparison with Established Risk Factors.** Comparison with established risk factors indicated that the detection rate of CK18+ cells in iliac bone marrow appeared to be associated with the size and histological grading of the primary carcinoma (Table 3). Our statistical analysis (x^2 test) demonstrated significant differences between patients with large T4+ primary tumors and those with small T1 primaries (30.8 versus 5.6%, respectively). Correspondingly, patients with dedifferentiated carcinomas (G3) exhibited a more frequent positive marrow finding than patients with moderately (G2) differentiated tumors (35.5 versus 15.6%, respectively).

It should be noted that only 1 of 23 patients with either small (T1) or well-differentiated (G1) primaries displayed cytokeratin positivity in iliac bone marrow.

**Table 1 Expression of CK18 on primary NSCLC**

**Table 2 Incidence of CK18+ cells in iliac bone marrow**

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were pooled from patients with adenocarcinoma and squamous cell carcinomas.

The remaining 26 patients met 1 of the following exclusion criteria:

(a) death within 30 days postsurgery due to cardiopulmonal compli-
(c) observation time was too short (less than 6 months).

Regarding the metastatic involvement of regional lymph nodes (N stage) determined by routine histopathology, we only observed a weak association (P = 0.09) with the finding derived from our assay (Table 3). In 6 of 42 (14.3%) patients staged “node-negative” (N₀), CK18+ cells were detected in iliac bone marrow samples, as compared to 12 of 40 cases (30%) staged “node-positive” (N^a).

**Follow-up.** We have obtained complete information on relapse and survival from a representative group of 56 patients (Table 4). The incidence of cytokeratin positivity in iliac bone marrow at the time of primary surgery was similar, comparing this subgroup of patients to the original group of 82 patients (26.8% versus 21.9%, respectively). The remaining 26 patients met 1 of the following exclusion criteria:

(a) death within 30 days postsurgery due to cardiopulmonal complications; (b) cause of death and/or status of relapse remained unclear; and (c) observation time was too short (less than 6 months).

Following a median observation period of 13 months (6 to 20 months), the total rate of relapse had reached 44.6% (25 of 56 cases) with an almost equal distribution between local and metastatic type of relapse (13 and 12 cases, respectively). Metastasis was diagnosed in the skeleton, central nervous system, liver, suprarenal, and contralateral lung.

However, separation of patients into two groups, depending on the outcome of our immunocytological assay, indicated differences in the rate and pattern of relapse. Ten out of 15 “cytokeratin-positive” patients (66.7%) showed either local or metastatic relapses (4 and 6 cases, respectively) with 4 of the 6 metastatic relapses involving the skeleton.

In contrast, the rate of total and metastatic relapse in patients with a “cytokeratin-negative” marrow finding at primary surgery was significantly lower. Fifteen of 41 “negative” patients (36.6%) relapsed within the same observation period. Furthermore, gross metastases were only detected in 6 patients (14.6%) with 1 case (2.4%) of skeleton involvement.

However, the rate of local relapses was similar in both groups of “positive” and “negative” patients (26.7 and 22.0%, respectively).

**Expression of Proliferation-associated Markers on Micrometastatic Cells.** To evaluate the growth potential of individual disseminated cells, a combined immunogold/immunoenzymatic double marker technique was applied to bone marrow samples from small subgroups of patients with NSCLC. As shown in Table 5, the proliferative fraction of CK18+ cells in marrow obtained at the time of primary surgery appears to be very small. None of the seven samples analyzed displayed CK18+ cells coexpressing the Ki-67 antigen known to be present in all phases of the cell cycle except G₀ and early G₁ (20, 21). Ki-67-positive “normal” marrow cells, however, were present in all specimens, serving as internal positive control for an appropriate staining. Nevertheless, native Ki-67 protein is known to be quite instable, which might lead to an underestimation of the actual proliferative fraction (22).

To assess potentially relevant growth factor receptors, we further typed cytokeratin-positive cells for coexpression of the erbB2 protein. The erbB2 protein is a Mr 185,000 transmembranous tyrosine kinase with structural homology to the epidermal growth factor receptor. Interestingly, we detected erbB2 expression on CK18+ cells in five out of six marrow samples analyzed with one patient developing a solid metastasis at the site of previous marrow aspiration.

**DISCUSSION**

Several groups (including ours) have recently demonstrated that immunocytochemical methods are powerful tools to detect micrometastatic cells in early-stage cancer (1-7). By use of mAb to epithelial cytokeratins, carcinoma cells present in mesenchymal organs, such as bone marrow, can now be identified at the single cell level. In view of the lack of information on micrometastasis in NSCLC, we applied mAb CK2 to CK18 in order to reveal individual NSCLC cells in bone marrow aspirates obtained from patients with operable primary adenocarcinomas and squamous cell carcinomas.

The sensitivity of CK18 as a marker for NSCLC-derived cells was demonstrated by its expression on more than 90% of all primary tumors analyzed (Table 1). Originally, CK18 was thought to be expressed exclusively on “normal” (i.e., nonmalignant) simple epithelium and tumors derived thereof (14, 15, 25). Indeed, expression of CK18 was detected on all of the 48 lung adenocarcinomas analyzed in this study. However, squamous cell carcinomas also expressed CK18

### Table 3 Correlation between established risk factors and cytokeratin positivity in bone marrow

<table>
<thead>
<tr>
<th>Risk factor</th>
<th>No. of patients/group</th>
<th>No. of patients with 21 CK18+ cell/µl sample% (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor size</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T₁</td>
<td>18</td>
<td>1 (5.6)</td>
</tr>
<tr>
<td>T₂</td>
<td>38</td>
<td>8 (21.1)</td>
</tr>
<tr>
<td>T₃₋₄</td>
<td>26</td>
<td>8 (30.8)*</td>
</tr>
<tr>
<td>Histological grade</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G₁</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>G₂</td>
<td>45</td>
<td>7 (15.6)</td>
</tr>
<tr>
<td>G₃</td>
<td>31</td>
<td>11 (35.5)*</td>
</tr>
<tr>
<td>Lymph node involvement</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N₀</td>
<td>42</td>
<td>6 (14.3)</td>
</tr>
<tr>
<td>N₁₋₃</td>
<td>40</td>
<td>12 (30.0)*</td>
</tr>
</tbody>
</table>

* Samples of 4 × 10^5 nucleated cells/aspirate from the upper iliac crest were stained with mAb CK2 using the alkaline phosphatase anti-alkaline phosphatase technique; data were pooled from patients with adenocarcinoma and squamous cell carcinomas.

P < 0.05 (X² test) as compared to T₁

P < 0.05 (X² test) as compared to G₂

P = 0.09 (X² test) as compared to N₀

**Table 4 Correlation between cytokeratin positivity in bone marrow at primary surgery and early relapse (median observation time, 13 mo)**

<table>
<thead>
<tr>
<th>Cytokeratin staining of bone marrow</th>
<th>No. of patients/ group</th>
<th>Total</th>
<th>Local</th>
<th>Metastatic</th>
<th>Skeleton (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>56</td>
<td>25 (44.6%)</td>
<td>13 (23.2%)</td>
<td>12 (21.2%)</td>
<td>5 (8.9)</td>
</tr>
<tr>
<td>Positive</td>
<td>15</td>
<td>10 (66.7%)*</td>
<td>4 (26.7%)</td>
<td>6 (40.0%)</td>
<td>4 (26.7%)*</td>
</tr>
<tr>
<td>Negative</td>
<td>41</td>
<td>15 (36.6%)</td>
<td>9 (22.0%)</td>
<td>6 (14.6%)</td>
<td>2 (4.8)</td>
</tr>
</tbody>
</table>

* Samples of 4 × 10^5 nucleated cells/aspirate from the upper iliac crest were stained with mAb CK2 using the alkaline phosphatase anti-alkaline phosphatase technique; data were pooled from patients with adenomatous and squamous cell carcinomas.

* Including regional lymph node metastases.

* Distant metastases of the skeleton, central nervous system, liver, suprarenal, and contralateral lung.

P < 0.05 (X² test) as compared to CK18+ marrow.

P < 0.005 (X² test) as compared to CK18- marrow.
in 36 of 40 cases (90.0%). Recently, Broers et al. (16) suggested that expression of CK18 on squamous cell carcinomas of the lung might be a sign of dedifferentiation which is confirmed by the results of our study, since all of the squamous cell carcinomas analyzed were moderately (G2) and poorly (G1) differentiated. The application of mAb to additional markers for squamous carcinoma cells might therefore further increase the sensitivity of the immunocytological bone marrow assay, especially in patients with negative or heterogeneous focal staining of the primary tumor (Table 1). However, our recent evaluation of mAb to carcinoma-associated surface proteins has revealed a substantial degree of unspecific binding to "normal" marrow cells (6).

To prove the specificity of mAb CK2 for detection of disseminated CK18+ tumor cells in bone marrow, patients with no evidence for an epithelial neoplasia were used as "negative" controls (Table 2). Although previous investigations suggested that cytokeratins might be expressed in normal bone marrow cells (26-28), we detected single CK18+ cells in only 2 of 117 "control" aspirates obtained from the upper iliac crest (Table 2). Furthermore, colon cancer was diagnosed 10 months later in one of these two "false-positive" control patients, suggesting that tumor cell dissemination had already occurred before the primary tumor was detected. It is noteworthy that possible contamination with epidermal cells would not cause a false-positive finding because CK18 is not expressed in stratified normal epithelium. Moreover, the fact that our analysis is solely based on the outcome of immunostaining without including observer-dependent morphological criteria should support the applicability of our assay.

In any case, the incidence of CK18+ cells in control patients is very low as compared to that obtained in iliac bone marrow samples from patients with resectable NSCLC in TNM stage M0. CK18+ cells were revealed in 18 out of 82 cases (21.9%) which is consistent with the rate of micrometastasis detected in bone marrow biopsies or skeleton autopsies by an extensive histopathological evaluation (29, 30). No clear correlation between a particular tumor histology and the presence of CK18+ cells in marrow was found (Table 2), suggesting that the "seed" of cells derived from either adenocarcinomas or squamous cell carcinomas appears to be quite similar. This is consistent with previous reports demonstrating no apparent differences in the initial site or time of tumor recurrence for both types of lung tumors (10, 31).

A pilot study on 25 patients with NSCLC furthermore revealed that patients with NSCLC evaluated before and after primary surgery displayed almost identical incidences of CK18+ cells in marrow aspirates (data not shown). Thus, we did not find any evidence that surgical manipulation on the primary tumor induces tumor cell dissemination into the bone marrow, which had been a justified concern.

The individual CK18+ cells detected in our present study did not always show the typical morphology of tumor cells. This is consistent with our recent observation in colorectal cancer, demonstrating that prognostically relevant CK18+ cells were sometimes morphologically indistinguishable from hematopoietic cells (4). In view of this well known heterogeneity of tumor cells, the morphology of an individual cell is not a reliable parameter to discriminate between normal and malignant.

A routine histological examination on the bone marrow specimens was not performed because we had already demonstrated in a previous study that dispersed epithelial tumor cells are rarely detected by conventional cyto- or histopathology (3). Only 1 of 82 marrow samples (1.2%) contained hundreds of CK18+ cells. Nuclear counterstaining of this specimen revealed the typical tumor-associated morphology, suggesting that these cells may have been identified as tumor cells by routine cytopathological staining. Although immunostaining of biopsies can provide additional information, it is less sensitive than the presented cytological evaluation because the number of cells on a biopsect section is considerably smaller than that on a smear or a cytopsin preparation (see also our previous publication, Ref. 3).

In an attempt to unravel the prognostic significance of our immunocytological assay, we evaluated whether the incidence of cytokeratin positivity in bone marrow was associated with established risk factors (9, 10). Indeed, positive correlations to both the size and the histological grade of the primary carcinoma were observed (Table 3). Patients with locally restricted (T1) or well differentiated (G1) primaries rarely exhibited CK18+ cells in iliac bone marrow, which is consistent with the relatively rare occurrence of manifest metastasis in these patients (11, 31).

On the other hand, the association with regional lymph node involvement as determined by routine histopathological staging appears to be weaker. Six of 42 node-negative patients were found to be positive in our assay (Table 3). This supports the view that hematogenous spread of lung carcinoma cells may be uncoupled from metastatic involvement of regional lymph nodes (31).

The presence of CK18+ cells in bone marrow at the time of primary surgery recently has been associated with an unfavorable prognosis in breast, gastric, and colorectal cancer (3-7). In the present investigation, a similar association could be observed in NSCLC following a median observation period of only 13 months (Table 4). Almost 50% of our patients relapsed within this short period of time, which is consistent with the poor prognosis reported in the literature (9-13). As expected, cytokeratin positivity in iliac bone marrow at primary surgery was positively correlated to metastatic relapse with a preference for the skeleton but not to the local outgrowth of residual tumor cells. However, the prognostic significance of the presented assay remains to be substantiated by logistic regression analysis. Being at an early stage of our work it is evident that only after another 3 to 4 years we can provide meaningful clinical data.

Our data suggest that single CK18+ cells in bone marrow inherit principally the potential to form gross metastases. This assumption was further supported by coexpression of the proliferation-associated erbB2 oncoprotein on CK18+ cells in bone marrow from five out of six cancer patients, suggesting that erbB2 might be involved in the outgrowth of metastatic cells. Immunoenzymatic detection of erbB2 is closely linked to overexpression of the oncoprotein which is caused by amplification of the normal erbB2 gene (32). Such amplification and overexpression occurs frequently in several types of carcinomas including NSCLC (33) and it has been shown to be associated with tumor cell growth in vitro as well as a more aggressive disease behavior in vivo (34). However, the fraction of cycling cells at the time of primary surgery appears to be rather small, as indicated by the quite infrequent coexpression of the nucleolar antigens Ki-67 and p120 on CK18+ cells in marrow. Nevertheless, a caveat must be added since the number of carcinoma cells analyzed per patient was rather small.

Consistent with previous reports (23, 33, 35), the proliferation-associated markers analyzed were heterogeneously expressed in the corresponding primary tumors with the number of specimens being too small to obtain any meaningful correlations between the expression on primary and micrometastatic tumor cells. However, it may be noteworthy that the expression of erbB2 on CK18+ bone marrow

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### Table 5 Coexpression of proliferation-associated markers on CK18+ cells in bone marrow of patients with NSCLC

<table>
<thead>
<tr>
<th>Target antigens</th>
<th>No. of patients analyzed</th>
<th>No. of patients with target antigen &quot;CK18&quot;+ cells in bone marrow*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ki-67</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>p120</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>p185erbB2</td>
<td>6</td>
<td>5</td>
</tr>
</tbody>
</table>

*Samples of 4 x 10^5 nucleated cells per aspirate were stained with mAb CK2 and mAb to either Ki-67, p120 (FB2), or p185erbB2 (9G6) using a combined immunogold/immunoenzymatic double-labeling technique.
micrometastases was frequently observed in breast cancer patients and positively correlated to tumor progression (data not shown).

Taken together, the present findings support the significance of our immunocytological assay as a clinically relevant tool to identify patients with operable NSCLC, who are at high risk to develop manifest metastasis. Furthermore, the bone marrow is an easily accessible site, where samples can be obtained repeatedly after removal of the primary tumor. In the future, this might enable us to detect failure of adjuvant therapy before the manifestation of gross metastases signals incurability.

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