INTRODUCTION

Lung cancer remains the most common cause of cancer-related death in Europe and the United States (9, 10). Nonsmall-cell lung cancer (NSCLC) affects approximately 80% of all lung cancer patients (5, 9). Surgery remains the gold standard treatment for locoregional NSCLC, and pathological lymph node (pN) status has remained the strongest clinical prognostic characteristic in early stages of operable NSCLC (21, 23). However, even in early stages, the 5-year survival rate of N0 patients remains at only 60–70% after complete resection of the primary tumor (7, 17). This suggests that tumor cell dissemination occurs early and occult micrometastases or single disseminated tumor cells (DTC), which are not discovered by conventional histopathologic methods, may be present in the lymph nodes at the time of surgery (6, 16, 18, 22, 26). Detection of these cells might potentially improve clinical lymph node staging and help to identify patients who could benefit from adjuvant or neoadjuvant therapy.

DTC are defined as single tumor cells or small cell clusters that can be detected in regional lymph nodes, peripheral blood, or organs remote from the primary tumor (e.g., bone marrow). These cells can be identified by sensitive immunohistochemical and molecular techniques (25).
DETECTION METHODS AND FREQUENCIES OF DTC IN REGIONAL LYMPH NODES

Immunohistochemistry

DTC in regional lymph nodes can be reliably detected using sensitive immunohistochemical techniques with monoclonal antibodies (mAb) against epithelium-specific proteins. Our group used mAb Ber-Ep 4 directed against epithelial cell adhesion molecule (EpCAM) to detect micrometastatic tumor cells (12, 14). The antibody does not react with mesenchymal tissue, including lymphoid tissue (19). In order to compare the effectiveness of the immunohistochemical analyses directly with the conventional hematoxylin–eosin (HE) method, two additional sections consecutive to those displaying Ber-Ep 4-positive cells were either stained by the routine HE method or by Ber-Ep 4. Both sections were then compared with the original positive section by an experienced pathologist without knowledge of the initial results. As a control, consecutive sections from Ber-Ep 4-negative lymph nodes were stained under the same conditions. Repeated immunostaining resulted in the redetection of Ber-Ep 4-positive cells in a neighboring section in 93.3% of the nodes and in 90.9% of the patients, respectively. In contrast, repeated HE-staining and histopathologic examination did not reveal any tumor cells.

The incidence of DTC detected by immunohistochemical staining with Ber-Ep 4 was 6.2% (35/565) in lymph nodes, which were negative by routine histopathology, and in 27 (21.6%) of 125 patients with resectable NSCLC (12). These cells occurred either as isolated, single cells, or as cell clusters of up to three cells present in the sinuses (60%) and the lymphoid tissue of the node (40%). In most patients, DTC were found in more than one of the three lymph node sections (31%), or more than one lymph node (55%). By conventional histopathology, 70 of 125 patients were staged as having pN0 disease and 55 as having pN1–2 disease according to the International Union Against Cancer (UICC) TNM classification (20). In pN1–2 patients, immunohistochemical staining detected tumor cell dissemination in 16 cases (29.1%). This was clearly higher than in pN0 patients, who had Ber-Ep 4-positive cells in their lymph nodes in 11 cases (15.7%) (p = 0.019). Other pathological characteristics were not associated with an increased rate of DTC.

Other immunohistochemical staining methods have been applied in order to screen histopathologically negative lymph nodes for micrometastases. Cytokeratins (CK) are intermediate filaments of the cytoskeleton of epithelial cells, and they serve for the detection of DTC originating from solid epithelial tumors (25). At least 20 different types of CK have been identified on the basis of differences in molecular weight and pH. Previously, the anti-CK monoclonal antibody AE1/AE3 has been used for immunostaining of lymph node sections (36). Five lymph nodes from each of 20 NSCLC patients were cut into three pieces and analyzed by immunohistochemical staining, flow cytometry (for both AE1/AE3 was used), and by conventional HE-staining (8). HE-staining revealed 8/100 positive lymph nodes in 4 (20%) patients, whereas immunohistochemistry resulted in 33/100 positive
lymph nodes on 13 (65%) patients, and flow cytometry detected DTC in 38/100 lymph nodes in 14 (70%) patients. Ito et al. also found a correlation between flow cytometric and immunohistochemical detection of CK-positive cells within the lymph nodes, and the sensitivity of these two methods was greater than that of standard HE-staining. In a study performed by Yasumoto et al. (38), 34 of 216 (15.7%) stage I NSCLC patients had DTC in their hilar and mediastinal lymph nodes. They were detected immunocytochemically by the AE1/AE3 antibody as well.

Furthermore, AE1/AE3 antibody was used in combination with an anti-p53 protein antibody DO-1 by Gu et al. In total, 22 of 49 (44.9%) stage I (= pN0) NSCLC patients had to be upgraded (7). Patients with DTC displayed a poorer prognosis than those whose lymph nodes were free of DTC. Maruyama et al. (18) applied another monoclonal anti-CK antibody (CAM-5.2) to investigate 973 regional lymph nodes from 44 NSCLC patients. 70.5% patients had to be upstaged N1 and N2 as 91 lymph nodes showed CK-positive cells. Similar findings were reported by Chen et al. (2) in a recent retrospective study, in which 17% of the lymph nodes and 63% of the patients were considered as DTC-positive.

We conclude that serial sectioning and immunohistochemical staining improve the sensitivity of detecting DTC (24). The incidence of immunohistochemically positive lymph node specimens varies between 30 and 70% depending on the immunohistochemical staining and the primary monoclonal antibody used for DTC detection. However, immunohistochemistry is laborious, time consuming, and skilled observers are required for an objective evaluation, but a great advantage lies in the fact that IHC offers the possibility to further characterize DTC by molecular and biochemical methods (25).

**Molecular Methods**

Reverse transcriptase polymerase chain reaction (RT-PCR)-based tumor cell detection is in principle an observer-independent and rapid method for detecting DTC. The theory is that minute amounts of vital cancer cells can be detected in clinical samples by the amplification of specific messengerRNA (mRNA) transcripts (“markers”), selectively expressed in the cancer cells of interest but not in normal tissues. The major problem associated with RT-PCR is illegitimate transcription, which is transcription of marker genes at a minimal basic level in normal tissues without necessarily being translated into detectable amounts of protein (39). Quantitative RT-PCR (qRT-PCR) has the potential to solve this problem by setting a cut-off value using control samples to differentiate illegitimate marker gene transcription from cancer-specific expression. However, the marker transcript might be downregulated in cancer cells (e.g., CK19) and it might therefore be difficult to find an appropriate cut-off level suitable for a broad range of samples from different cancer patients with heterogeneous primary tumors.

A study performed by Salerno and coworkers (32) used a RT-PCR assay to detect occult tumor cells in lymph nodes of 28 patients with NSCLC. A total of 88 pathohistologically tumor-free nodes were examined for the expression of mRNA
transcripts for mucin-1 (MUC1). MUC1 mRNA was detected in 33 (37.5%) of 88 nodes of lung cancer patients, and based on this result 16 (70%) of 23 patients had to be restaged. However, subsequent studies revealed the ubiquitous presence of MUC1 mRNA in lymph nodes and other tissues (1), diminishing its value as a tumor marker in RT-PCR studies.

A recent study applied carcinoembryonic antigen (CEA) as a marker for DTC detection (3). CEA is a 200 kDa cell-surface glycoprotein involved in cell-to-cell adhesion. Twenty-three control lymph nodes from six patients without malignancy were tested and CEA mRNA was not detected in any of the control lymph nodes analyzed. In contrast, Bostick et al. (1) found CEA mRNA in normal lymph nodes. The technology of automated quantitative real-time RT-PCR (qRT-PCR) allows rapid processing of many samples. The qRT-PCR assay for CEA mRNA was performed in such an automated real-time PCR machine, allowing quantitation of DTC in lymph nodes. The median cell number was 7,190 tumor cells per lymph node station. No statistical difference was observed between adenocarcinomas (median 7,425 tumor cells), squamous cell carcinomas (median 11,165 tumor cells), and undifferentiated tumors (median 7,190 tumor cells). Furthermore, of 232 apparently tumor-free lymph nodes from 53 stage I NSCLC patients analyzed for CEA mRNA by qRT-PCR, 59 (25.4%) were positive, revealing DTC in 30 (56.6%) patients. Similarly, Maeda and coworkers (16) observed DTC by qRT-PCR for CEA mRNA in 25% of histopathologically negative lymph nodes (52/211) and 64% of node-negative NSCLC patients (14/22).

Another study carried out by Nosotti et al. (22) evaluated the detection of DTC in histologically tumor-free lymph nodes by CEA mRNA through qRT-PCR as well as its prognostic value. In 44 NSCLC patients classified stage I, all primary tumors were positive for CEA mRNA. Of 261 analyzed lymph nodes, 35 (13.4%) showed elevated CEA mRNA levels compared with control lymph nodes and 16 (36.4%) patients were subsequently considered to have “micrometastatic” nodes.

Using qRT-PCR for creating a cut-off value to differentiate illegitimate marker gene transcription from cancer–specific expression also allowed to use cytokeratin 19 (CK19) as a marker for the detection of DTC in lymph nodes of NSCLC-patients with resectable tumors (35). CK19 is a specific cytoskeletal structure of simple epithelia, including bronchial epithelial cells. CK19 is an abundantly expressed polypeptide of epithelial cells but it can be downregulated in solid tumors. On the other hand, a low level transcription of CK19 was observed in lymph nodes of patients without malignancy (1, 35). Using qRT-PCR, out of 94 tumor-free lymph nodes, staged by the pathologist, CK19 transcripts were detected in 26 (28%) nodes, resulting in 13 (56.5%) patients that were considered as DTC-positive.

Saintigny et al. (31) tested 84 histologically negative lymph nodes of 19 patients for CK7 and CK19 mRNA by real-time RT-PCR. In the event of two (10.5%) patients (and six lymph nodes), the staging had to be changed as lymph nodes were positive for at least one marker. In another study, histopathology, immunohistochemistry,
and RT-PCR were compared for 254 mediastinal lymph nodes of 49 patients suffering from NSCLC (15). Of 225 nontumoral lymph nodes based on histopathological screening, 32 (14.2%) were positive for CK19 mRNA by RT-PCR, and 16 (32.7%) patients were upstaged. Seventeen patients remained pN0 (negative by RT-PCR and HE) and 16 were classified pN2 on histopathology. IHC did not provide significant additional information.

Another study included 40 lung cancer patients (261 lymph nodes) and compared the standard HE-staining to RT-PCR for CK19 (6). In 18 patients, regional lymph node metastases were found by both HE and RT-PCR, whereas in the other 22 patients who were pathologically lymph node-negative, DTC were detected by RT-PCR in six (27%) cases.

In summary, the reported rates of DTC in lymph nodes of patients with apparently tumor-free lymph nodes range from 10 to 70%. The range of the reported rates might depend on tumor characteristics and could be influenced by specificity and sensitivity of the different applied RT-PCR methods. Compared with conventional HE-staining, RT-PCR facilitates a more sensitive assessment of the “micrometastatic status” of lymph nodes in lung cancer patients, regardless of the marker used. Moreover, the frequencies of DTC detected by qRT-PCR and those reported in immunohistochemical studies were similar. Applying molecular methods, 10.5–64.0% of the patients (i.e., 7.1–28.0% lymph nodes were DTC-positive) had to be upstaged, and 21.6–70.5% of the patients by IHC (i.e., in 6.2–33.0% of lymph nodes DTC were found), respectively. There are only a few studies directly comparing the clinical value of both technical approaches. At present, it can be concluded that both immunohistochemical and molecular detection of DTC in lymph nodes may serve as a supplement for current tumor staging in lung carcinoma. However, larger multicenter trials are needed to establish these tests in clinical practice.

**PROGNOSTIC RELEVANCE OF DTC IN REGIONAL LYMPH NODES**

The detection of DTC in lymph nodes by IHC is associated with a poor prognosis in patients with resectable lung cancer. Our own study on NSCLC patients revealed that after an observation time of 64 months, patients with immunohistochemically proven DTC in regional lymph nodes had a significantly reduced overall survival \( p = 0.0001 \) (12). Correspondingly, patients with DTC experienced a higher rate of disease relapse than patients without such cells \( p = 0.0001 \). Because of the elevated frequency of Ber-Ep 4-positive cells in higher pN-stages, stratification for pN-stage was done. In pN0 disease, patients with DTC had a significant overall survival disadvantage compared with those without DTC \( p = 0.010 \). In pN1–2 disease, the overall survival rate was also definitely reduced in the presence of DTC and the impact of minimal tumor cell spread on overall survival was comparably strong \( p = 0.027 \). A Cox regression model was applied to analyze the influence of lymphatic DTC, pT-stage, pN-stage, and age on overall survival. The multivariate analysis showed a 2.5-times increased risk of shorter survival and a 2.7-times increased risk of tumor relapse in patients with DTC vs. patients without such cells.
Pathological N-stage had a prognostic value for reduced survival in the same range (relative risk 2.3).

Maruyama et al. (18) reported that relapse-free survival was significantly shorter in patients with immunohistochemically identifiable DTC in lymph nodes than in those with DTC-negative nodes \( (p = 0.004) \), mainly due to distant relapse. In another Japanese study (4), paraffin-embedded specimens from 315 lymph nodes of 31 pN0 patients with completely resected (R0) NSCLC, whose primary tumors were positive for the tumor-suppressor gene product p53, were reexamined immunohistochemically using a monoclonal anti-p53 antibody. Cells positive for p53 were detected in 26 (8%) of 315 lymph nodes from 14 (45%) of the 31 patients. Once again, the finding of occult nodal tumor cells had a significant impact on survival \( (p = 0.0001) \).

Le Pimpec-Barthes et al. (15) used a nonquantitative CK19 RT-PCR. In their study, patients with molecularly detected DTC in lymph node had significantly reduced survival. The 2-year cancer-related death survival of the pN0 patients (100%) and the upstaged patients (64.5%) due to DTC in their lymph nodes was significantly different \( (p = 0.04) \). The relative risk of recurrence in the patients with DTC detected by RT-PCR compared to the pN0 patients, evaluated by the Cox model multivariate analysis, was 5.61 \( (p = 0.02) \). In a comparative analysis of apparently tumor-free lymph nodes between conventional HE-staining and again RT-PCR for CK19, the median observation time was 26 months (range 4–60 months) (6). Patients with DTC in the lymph nodes showed significant shorter disease-free survival duration than node-negative patients (log-rank test, \( p = 0.001 \)). Finding of CK19 mRNA also correlated to tumor size and tumor grading. The results diagnosed by HE had no significant effect on prognoses \( (p = 0.455) \). A survival analysis of patients with DTC detected by an elevated CEA mRNA level through qRT-PCR revealed that patients with DTC in lymph nodes suffered from more recurrences than patients without CEA mRNA-positive lymph nodes (22).

In addition, Yasumoto et al. (38) also found a poor prognosis for those stage I NSCLC patients with immunocytochemically detected DTC in the lymph nodes by univariate \( (p = 0.004) \) and multivariate \( (p = 0.018) \) analysis. Taken together, the majority of studies investigating DTC in lymph nodes demonstrated a prognostic impact (22). This information might be used for stratification of patients in future clinical trials investigating new forms of adjuvant therapy.

**Tumor Biology of DTC in Regional Lymph Nodes**

The biology of early micrometastatic spread to lymph nodes is largely unknown. In esophageal cancer, a permanent cell line was established from a DTC-positive lymph node classified as tumor-free by routine histopathology. The cells of this cell line had characteristic features of malignant epithelial tumor cells, and they were tumorigenic and micrometastatic in vivo when transplanted in mice with severe combined immunodeficiency (33).
Genetic heterogeneity of the primary tumor is a well-known phenomenon. So far, it remains unclear which genes might be responsible for the early release of DTC into the blood or lymphatic system. In breast cancer, we found specific signatures associated with hematogenous or lymphatic spread of tumor cells (37). These signatures showed little overlap, suggesting different mechanisms leading to these two routes of dissemination. In lung cancer, similar studies are ongoing. Using array-CGH (comparative genomic hybridization) for genetic screening, our pilot study indicates the existence of certain genomic aberrations linked to micrometastatic spread to BM and some overlap with the aberrations associated to lymph node metastasis was observed (11).

Another approach is to directly characterize DTC using single cell technologies (25, 27). So far little is known about the molecular features of DTC in lymph nodes, but several groups, including ours, have used such techniques to assess DTC in bone marrow and blood. A summary of these interesting data is far beyond the scope of this review but one of the hallmarks of DTC is their dormant (i.e., non-proliferating) state (28). This “tumor cell dormancy” is characteristic for the latency period between the time from tumor cell dissemination and the development of clinically overt metastases, and the underlying mechanisms are poorly understood.

The nonproliferating state of DTC favors immunotherapeutic or other targeted therapies over S-phase-specific chemotherapeutic agents. In this context, the EpCAM antigen used for detection of DTC appears also to be an interesting therapeutic target. EpCAM is a cell adhesion molecule expressed on the cell membrane of different epithelial tumor cells, including disseminated NSCLC cells (29). Other targets for immunotherapeutic interventions belong to the class of MAGE (melanoma-associated antigen) antigens (13, 30, 34), which are the most specifically expressed tumor antigens known so far. MAGE antigens are also expressed on NSCLC cells including DTC (13). First clinical trials using vaccination strategies against MAGE antigens are ongoing.

In conclusion, the detection and characterization of DTC may lead to (a) an improved tumor staging, (b) the discovery of new therapeutic targets to eradicate minimal residual disease, and (c) novel insights into the biology of early tumor cell dissemination in cancer patients.

REFERENCES
27. Pantel and Panabière (2006) accepted by Nature Clinical Practice Oncology