A Pragmatic Approach to the Diagnosis of Nodal Micrometastases in Early Stage Non-small Cell Lung Cancer

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Introduction: This study was designed to develop a both sensitive and efficient algorithm for detection of lymph node micrometastases and to determine its prognostic impact in patients with early stage non-small cell lung cancer (NSCLC).

Methods: One hundred seventy patients with NSCLC p stage I and II were included in this study, of which $n = 5299$ lymph nodes were obtained and submitted to histopathologic analysis. From each patient, a median number of 31 lymph nodes was received (N-1 position: median $n = 16$; N-2 position: median $n = 15$). Immunohistochemistry was performed to detect micrometastases unobvious by conventional microscopy using antibodies against cytokeratins (CK) (pan-CK: KL-1, CK 5/6, CK 7) and the epithelial marker Ber-EP4.

Results: In 82 patients (48.2%), micrometastases were revealed in immunohistochemistry staining. KL-1 detected micrometastases in 201 (99.5%) of 202 positive lymph nodes. Subsequently, this resulted in an upstaging in 39 patients (20.5%). Detection of micrometastases in otherwise tumor-free N2-lymph nodes had a significant prognostic impact (mean disease-free survival 21.4 versus 45.3 months, $p = 0.022$), affecting 4.7% of patients. Survival differences between patients who were upstaged into stage II (N0>N1) and those remaining in stage I were not statistically significant ($p = 0.537$).

Conclusion: Extended workup of N2-lymph nodes using one broad-spectrum keratin marker in otherwise N2-negative lymph nodes may represent a both efficient and sensitive approach to the identification of micrometastases in dissected lymph nodes of patients with early stage NSCLC.

Key Words: Micrometastases, Lymph node, Lung cancer, Immunohistochemistry, Prognostic impact.

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Complete surgical resection remains the current standard therapy for operable patients with non-small cell lung cancer (NSCLC) in the stages I or II. However, there is a strong rationale for an additional adjuvant systemic therapy even in patients with early stage disease because distant relapse continues to be the dominant form of relapse after curative surgical resection of NSCLC.[1,2] Treating all patients after (or before) complete resection of NSCLC with adjuvant chemotherapy will clearly not benefit each one. Patients surviving 5 years after surgery without relapse, which accounts for ~60% of patients with stage I NSCLC would not have done any better by having received additional chemotherapy.[3] Thus, the identification of those patients with a poor prognosis that may benefit from additional therapy after surgery continues to be of high importance.

We hypothesized that occult micrometastases in lymph nodes may be associated with poor prognosis after complete resection in patients with early stage NSCLC. Micrometastasis as defined by Union International contre le Cancer (UICC)/The American Joint Committee (AJCC)[4,5] is a small tumor deposit 0.2 to 2 μm in diameter, usually detected by routine hematoxylin and eosin (HE) staining, and typically mitoses and invasion are seen.[6] In contrast, isolated tumor cells (ITC) are small clumps of tumor cells, typically without mitoses or vascular or lymphatic invasion within nodes (or distant sites). Micrometastases can be detected by standard histopathology, albeit with lower sensitivity. Therefore, to date, immunohistochemistry (IHC) remains the gold standard for the detection of micrometastases. Although a prognostic impact of occult lymph node micrometastases in lung cancer has been discussed by many authors, IHC for detection of lymph node micrometastases has not been implemented in routine diagnostic procedures mainly due to high costs. This study was designed to develop both a sensitive and inexpensive algorithm of IHC for the detection of occult lymph node micrometastases and to determine their prognostic impact in a large number of patients with early stage NSCLC.

PATIENTS AND METHODS

Patients

A total number of 170 completely resected patients with NSCLC with pathologic stages I and II (February 2003 until August 2005) was included in this trial. During preop-

erative staging, enlarged (>1 cm) mediastinal lymph nodes on computed tomography (CT) scan were diagnosed in 19 patients and resulted in clinical N2 classifications; $n = 16$ of these patients had single station minimal cN2 disease and the interdisciplinary tumor board decided on primary surgery. In $n = 3$ patients with multilevel cN2, mediastinoscopy was performed and revealed negative mediastinal nodes. Positron emission tomography scan was not routinely available during the study period and was performed in none of the patients. No induction treatments were performed. All patients underwent primary surgery and finally turned out to be N2 negative by pathologic staging after complete lymph node dissection. The standard surgical procedure was anterolateral thoracotomy, entering in the fourth or fifth intercostal space. Radical mediastinal and hilar lymphadenectomy were performed concurrently with all procedures, including four compartments in the right-sided thoracotomy (paratracheal, infracarinal, inferior mediastinal, and hilar) and four compartments in the left-sided thoracotomy (aortic, infracarinal, inferior mediastinal, and hilar).[7,8] Standard postoperative histopathologic investigation (HE staining) was performed according to the sixth edition of the TNM classification of the International Union Against Cancer.[9] Each participant in this study was informed and written consent to participation was given before entering the trial. The study was formally approved by the Ethics Committee of the University of Heidelberg (No: 270/2001).

Tissue Preparation and Standard Histopathologic Analysis

For routine histopathologic analysis, lymph nodes were embedded in paraffin and further processed for routine HE staining. The standard histopathologic analysis included one section through the middle in those lymph nodes in which the shortest axis fell short of 5 mm, and two sections in those lymph nodes in which the shortest axis exceeded 5 mm.

Immunohistochemical Analysis

For IHC of the lymph nodes, four additional sections of 2 μm thickness with a distance of ~150 μm in between were performed. The slides were deparaffinized and pretreated in an antigen retrieval buffer (DAKO, Glostrup, Denmark) to block unspecific bindings. Subsequent steps were performed in an immunostaining device (DAKO, Techmate 500 plus). The immunostaining protocol was based on the avidin-biotin-peroxidase principle, which used amino-9-ethylcarbazole as chromogen, as well as hematoxylin for the counter stain. The tissue sections were covered with glycerine gelatin. After each incubation period, the capillary slides were rinsed and dried with blotting pads. Immunohistochemical staining was performed with antibodies raised against pan-cytokeratins (CK) (KL-1); 1:50 (Immunotech, Marseille, France), CK 5/6; 1:100 (DAKO, Glostrup, Denmark), CK 7; 1:100 (DAKO, Glostrup, Denmark), and BerEp-4; 1:200 (DAKO, Glostrup, Denmark). For negative controls, the primary antibodies were omitted. As positive controls, normal colon mucosa was used for Ber-EP4 and known positive tumor samples of NSCLC were used for CK 7-Ab, CK 5/6-Ab, and KL-1. In the IHC analysis of the lymph nodes, small tumor deposits (as defined by the UICC/AJCC) were defined as micrometastasis; solitary tumor cells or clump of cells without mitoses or vascular invasion were not defined as micrometastases.

Follow-Up

Eleven of the 170 patients received postoperative adjuvant chemotherapy (stage I: $n = 4$), stage II: $n = 7$). None of the patients received pre- or postoperative radiotherapy. All patients were followed-up (including physical examination, chest roentgenogram, chest CT, and abdominal ultrasonography) every 3 months over the first 2 years and in 6 months intervals thereafter. End point of follow-up was disease-free survival (DFS).

Statistical Analysis

The results were evaluated by SPSS (Statistical Package for Social Sciences) Windows 15.0 software for statistical analysis. The Wilcoxon signed rank test was used for comparing qualitative data. The analysis of the DFS time was performed by Kaplan-Meier analysis. The results were evaluated with a 95% confidence interval. A p value ≤ 0.05 was regarded as statistically significant.

RESULTS

One hundred seventy patients with a mean age of 65.4 years were included in this study. A synopsis of the clinical data and the histopathologic classification of the tumors is shown in Table 1. A total of 5299 lymph nodes underwent histopathologic analysis (N-1 position: $n = 2722$; N-2 position: $n = 2577$); a median number of 31 $\pm$ 13 lymph nodes was obtained from each patient (N-1 position: median 16 $\pm$ 9; N-2 position: median 15 $\pm$ 8). Subsequently, a total of 21,196 histologic sections were analyzed by four different immunohistochemical stainings.

By immunostaining, micrometastases were detected in 375 sections corresponding to a total of 202 lymph nodes. The pan-CK antibody KL-1 detected micrometastases in 201 lymph nodes (99.5%) and CK7-Ab in 107 lymph nodes (52.9%). The CK5/6 antibody was less sensitive and detected micrometastases only in 15 lymph nodes (7.4%). The epithelial marker BerEp4 revealed micrometastases in 52 lymph nodes (25.7%). KL-1 failed to detect micrometastases in only one patient in which the presence of the single CK marker CK7 solely proofed micrometastases (Table 2).

Lymph nodes of 82 patients (48.2%) revealed micrometastases by immunohistochemical staining. Subsequently, 39 patients (20.5%) were immunohistochemically upstaged. Among patients with N0 status in standard histopathology, 35 patients (29.6%) were immunohistochemically upstaged (N0→N1: $n = 31$; N0→N2: $n = 4$). In addition, four patients initially classified as pN1 in standard histopathology (8.4%) were immunohistochemically upstaged due to the detection of micrometastases in N2-lymph nodes. In 43 patients presenting with metastases in N1 lymph nodes by HE staining, additional tumor cell clusters defined as micrometastases were found by IHC. Of course, this finding did not result in an upstaging of the tumor disease. The reclassification of the patients according to the lymph node status after IHC is shown in Table 3. On the basis of the clinical staging, none of the CT-based clinical N2-positive patients had postoperative pathologic N2 disease by conventional workup. However, in mediastinal nodes of 2 of

