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Diagnosis of lymph node micrometastasis at the pN_0 stage of lung adenocarcinoma using a combination of markers

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ABSTRACT. This study aimed to detect micrometastatic tumor cells in the lymph nodes of patients with pN_0 lung adenocarcinoma using a combination of thyroid transcription factor-1 (TTF-1) expression and cytokeratin 7 (CK7) expression and to investigate the association of lymph node micrometastasis with the clinicopathological characteristics of patients with lung adenocarcinoma. A total of 54 patients with pN_0 lung adenocarcinoma and whose primary tumors were positive for both TTF-1 and CK7 expression were included in this study. In total, 893 lymph nodes were obtained from these 54 patients and were analyzed for micrometastasis by immunohistochemical staining with anti-CK7 and anti-TTF-1 antibodies. CK7- and TTF-1-positive cells were found in the lymph nodes of 9 (16.7%) of 54 patients, and 21 (2.4%) of 893 lymph nodes exhibited positivity for these factors. No cells positive for both CK7 and TTF-1 were detected in the 5 lymph nodes obtained from patients with benign lung tumors. Lymph node micrometastasis was found to be associated with the differentiation grade and primary tumor position ($P < 0.05$). The detection of lymph node micrometastasis by a combination of CK7 and TTF-1 immunohistochemical staining

provides a more accurate assessment of tumor staging for pN_0 lung adenocarcinoma.

Key words: Lung adenocarcinoma; Lymph node micrometastasis; Thyroid transcription factor-1; Cytokeratin 7

INTRODUCTION

Lung carcinoma is one of the most common malignancies worldwide, with incidence and death rates both ranking among the top malignancies. According to 2011 global cancer statistics, approximately 1,600,000 new cases of lung carcinoma occur in each year, and 1,400,000 patients die of the condition annually (Jemal et al., 2011). In China, the incidence of lung carcinoma is also rapidly increasing (Lin et al., 2008). According to the SEER database, the 5-year survival rate of patients with lung carcinoma in developing countries is only 8.9% (Parkin et al., 2005).

The TNM staging system has long been used as the guideline for the prognostic evaluation and clinical treatment of lung carcinoma. However, in clinical practice, even patients with stage I non-small cell lung cancer (NSCLC), who have undergone a radical operation [total tumor resection and systemic lymph node (LN) dissection] have a recurrence rate of 25-40% (Osaki et al., 2002; Maeda et al., 2006; Xi et al., 2006; Rena et al., 2007; Li et al., 2008; Melfi et al., 2008; Verhagen et al., 2010) and a 5-year survival rate of 57-85% (Maeda et al., 2006; Tezel et al., 2006; Xi et al., 2006; Rena et al., 2007; Borgia et al., 2009; Marchevsky et al., 2010; Qiu et al., 2010). These findings suggest that LN micrometastasis, which is difficult to be detected using routine pathological examination methods, may be correlated with postoperative recurrence and patient survival. However, effective and sensitive approaches that identify LN micrometastasis have not been developed to date (Ahrendt et al., 2002). In the present study, we detected the occurrence of micrometastasis in routine pathology-confirmed negative LNs of patients with lung adenocarcinoma after a radical operation using a combination of cytokeratin 7 (CK7) and thyroid transcription factor-1 (TTF-1) immunohistochemical staining. In addition, we investigated the association of LN micrometastasis with clinical characteristics.

MATERIAL AND METHODS

Patients

A total of 57 patients with pN_0 lung adenocarcinoma underwent radical surgery for the primary tumor with dissection of the hilar and mediastinal LNs in Jingdu Hospital between January 2008 and August 2010. A total of 54 patients were eligible for this study (3 patients were excluded because of TTF-1 negativity in their primary tumors). The eligibility criterion for entering this study was CK7 and TTF-1 positivity in the primary tumor as assessed by immunohistochemistry (IHC). Of the 54 patients, 33 were men and 21 were women, and their median age was 66.5 years (range: 46-81 years); 47 patients had peripheral-type cancer, whereas 7 had the central type. Additionally, 12, 21, and 21 patients had grade I, II, and III cancer, respectively. The primary tumor size was ≤2, 2.1-3, 3.1-5, 5.1-7,

and >7 cm in 14, 13, 20, 7, and 3 patients, respectively. A total of 893 hilar and mediastinal LNs were removed during surgery from these 54 patients, with 6-37 LNs retrieved from each patient. Ten homochronous patients with TTF-1 and CK7 positivity in the primary lung adenocarcinoma niduses were selected, and 2 metastatic LNs were chosen from each of these patients and used as positive control samples. Meanwhile, 5 hilar or mediastinal LNs from 5 patients with benign lung tumors were used as negative control samples. These 5 benign lung tumors were treated at Jingdu Hospital between August 2010 and May 2011. This study was conducted in accordance with the declaration of Helsinki and with the approval of the Ethics Committee of Jingdu Hospital. Written informed consent was obtained from all participants.

Sample collection

All patients underwent pulmonary lobectomy and systematic LN dissection. We collected mediastinal LNs and anatomized intrapulmonary LNs intraoperatively.

A total of 893 lung hilar and mediastinal LN paraffin blocks were obtained from the 54 patients with lung adenocarcinoma. Three slices were then cut consecutively from each LN paraffin block with a thickness of 4 μm: 1 for routine pathologic examination, 1 for TTF-1 detection, and 1 for CK7 detection. All LNs were verified as negative by 2 pathologists via routine pathologic examination.

IHC

TTF-1 and CK7 expression in the regional LNs was detected by the 2-step EnVision method. The slides were placed in an oven overnight at a constant temperature of 56°C and then routinely hydrated using xylene dewaxing ethanol. The slides were then immersed in a 0.01 M citrate buffer solution and boiled in a high-pressure cooker for 2 min. The samples were then cooled to room temperature for antibody repair, treated with 3% hydrogen peroxide for 10 min, and then stored overnight in a refrigerator at 4°C after the addition of TTF-1 (code SDT24) and CK7 (code OV-TL12/30) antibodies (Maixin Biological Technology Development Company). Afterward, 100 μL secondary antibodies (Zhongshan Biotechnology Co. Ltd) was added. The slices were then incubated in water at 37°C for 20 min, stained with diaminobenzidine for 10-15 min, washed, restained with hematoxylin, dehydrated with ethanol, vitrified by xylene, and then sealed using a neutral gel for observation.

The presence of TTF-1- and CK7-positive cells, as indicated by brownish-yellow staining in the cell nucleus or cytoplasm of one or more cells, was accepted as evidence of micrometastasis. The results were confirmed by 2 pathologists.

Statistical analysis

The association of LN micrometastasis with clinicopathological characteristics was assessed using the chi-squared test. Alternatively, the Fisher exact test was used when the chi-squared test was violated. All statistical tests and P values were 2-tailed. P values <0.05 were considered to be significant. All analyses were performed using the SPSS 18.0 software.

RESULTS

IHC

LNs with brownish-yellow staining in the cell nucleus of one or more cells were considered to be TTF-1 positive (Figure 1A), whereas those that stained in a similar manner in the cytoplasm were considered to be CK7 positive (Figure 1B).

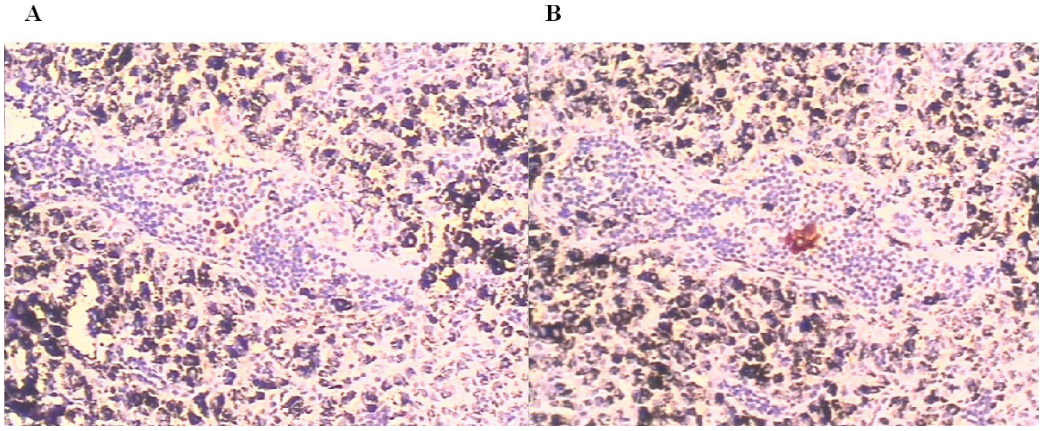

Figure 1. Lymph node staining. **A.** Expression of transcription factor-1 in the cell nucleus; **B.** Expression of cytokeratin 7 in the cytoplasm.

TTF-1- and CK7-positive cells were found in 94.7% (54/57) and 100% (57/57) of the primary tumors, respectively. All 893 LNs obtained were confirmed as negative by 2 pathologists via a routine hematoxylin-eosin (HE) staining examination.

We identified micrometastatic tumor cells in the pN_0 LNs in 9 (16.7%) of the 54 patients. In total, 21 of 893 LNs were confirmed to be positive for both TTF-1 and CK7 expression. TTF-1 and CK7 were expressed in all nodes of the positive control group. Conversely, none of the LNs in the negative control group exhibited TTF-1 or CK7 expression.

Micrometastasis

Restaging of the nodal status based on the combined TTF-1 and CK7 IHC staining was performed using the mTNM staging system. Of the patients with pN_0 disease as identified by conventional HE histopathological examination, 4 were restaged as N1, whereas 5 were restaged as N2. Two stage IA lesions were reclassified as stage IIA, 2 stage IA malignancies were upstaged to stage IIIA, 3 stage IB cancers were upstaged to stage IIA, 1 stage IIA lesion was reclassified as stage IIB, 1 stage IIA lesion was upstaged to stage IIIA, and 1 stage IIB cancer was reclassified as stage IIIA (Table 1).

Association

The presence of micrometastatic tumor cells in pN_0 LNs was significantly associated

with the clinicopathological characteristics of patients based on the differentiation grade and primary tumor position. Micrometastasis was not significantly associated with age, gender, or pT staging (Table 2).

Table 1. pTNM and mTNM staging among 9 patients with micrometastases.

Tumor size (cm)	pT	pTNM	mTNM
2	T_{1a}	I_A	II_A
3	T_{1b}	I_A	II_A
3	T_{1b}	I_A	III_A
3	T_{1b}	I_A	III_A
5	T_{2a}	I_B	II_A
5.5	T_{2a}	I_B	II_A
6	T_{2b}	II_A	III_A
6	T_{2b}	II_A	II_B
7	T_3	II_B	III_A

Table 2. Association of micrometastasis with clinicopathological characteristics.

Characteristics	N	Micrometastasis (N, %)	P
Age			
<55	7	2 (28.6)	>0.05
≥55	47	7 (14.9)	
Gender			
Male	33	3 (9.09)	>0.05
Female	21	6 (28.6)	
Tumor position			
Peripheral	47	4 (8.51)	<0.05
Central	7	5 (71.4)	
T stage			
T1	27	4 (14.8)	>0.05
T2-T4	27	5 (18.5)	
Differentiation			
Grade III	21	8 (38.1)	<0.05
Grade I+II	33	1 (3.03)	

DISCUSSION

In this study, we demonstrated that LN micrometastasis occurs in a significant percentage of patients with pN_0 lung adenocarcinoma. These data suggest that combined CK7 and TTF-1 immunohistochemical analysis facilitates an accurate assessment of cancer staging.

LN metastasis significantly affects the prognosis of patients with NSCLC (Goya et al., 2005; Ramirez et al., 2012). The difference in 5-year survival between stages I and II is significant. Moreover, the 5-year survival rate significantly declines in accordance with pN upstaging (63.7% N_0 *vs* 47.3% N_1) (Pfannschmidt et al., 2007). Studies demonstrated that patients with LN micrometastasis have a higher recurrence rate and a poorer prognosis (Osaki et al., 2002; Xi et al., 2006; Mineo et al., 2007). The occult locoregional spread of cancer may be the leading cause of local recurrence after complete surgical resection. Accurate staging is therefore extremely important and can lead to either a more extensive primary therapy to reduce the risk of local recurrence or to systemic adjuvant therapy that eliminates these occult deposits. Conventional HE staining can accurately detect gross nodal metastases in patients with NSCLC, but cannot detect LN micrometastasis (Nwogu et al., 2013). Sensitive immu-

nocytochemical tests have been widely used to identify micrometastasis. These methods can detect 1 cancer cell in 1 x 10^4 to 1 x 10^5 normal cells (Ohta et al., 2001; Wu et al., 2001; Gu et al., 2002; Nosotti et al., 2005; Wang et al., 2005; Benlloch et al., 2009).

In our study, we identified the occurrence of LN micrometastasis in 9 (16.7%) of the 54 patients. The tumor stages of these 9 patients increase according to the mTNM stage. Therefore, these patients may be advised to receive postoperative adjuvant chemotherapy. The detection of LN micrometastasis provides an accurate assessment of tumor staging in lung adenocarcinoma and allows for the modification of therapy regimes.

In the analysis of the association between LN micrometastasis and clinicopathological characteristics, our study also demonstrated that the presence of micrometastatic tumor cells in the pN_0 LNs was significantly associated with the differentiation grade and primary tumor position. Micrometastasis was not significantly associated with age, sex, and pT staging. Primary tumors near the lung hilar or those with poor differentiation may be predisposed to metastasis.

Studies have revealed that CK7 is expressed in 100% of all patients with primary lung adenocarcinoma (Righi et al., 2011). This finding is consistent with our current results. TTF-1 is expressed in different types of lung cancer at varying intensities. Stronger TTF-1 expression is found in adenocarcinoma and small cell carcinoma (Santisteban and Bernal, 2005). TTF-1 was expressed in 94.7% (54/57) of the patients with primary lung adenocarcinoma in our study. The loss of TTF-1 expression in the remaining patients may be due to a TTF-1 mutation (Bai and Shen, 2008).

In conclusion, our study illustrates that combined CK7 and TTF-1 immunohistochemical staining can provide cogent evidence of LN micrometastasis and offer an accurate assessment of tumor staging for pN_0 lung adenocarcinoma. However, the inclusion of micrometastasis detection in routine clinical pathological examinations and its use as a reference for the subdivision of Union for International Cancer Control staging require appropriate attention and warrant further large-scale investigations.

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