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INTRAOPERATIVE DETECTION OF LYMPH NODE MICROMETASTASIS USING A RAPID IMMUNOHISTOCHEMICAL STAINING METHOD IN NON-SMALL CELL LUNG CANCER

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Abstract
Nodal micrometastasis in non-small cell lung cancer (NSCLC) is associated with a poorer survival rate than node-negative disease. Furthermore, lymph node micrometastasis often cannot be detected using conventional hematoxylin and eosin staining of frozen sections ; detection requires additional time-consuming immunohistochemical (IHC) analysis of paraffin-embedded tissue. We previously developed and reported a device that enables us to complete IHC analyses within 11 minutes. In the present study, we used this rapid-IHC protocol with an anti-cytokeratin antibody and analyzed 205 mediastinal lymph nodes dissected during surgery for NSCLC. Although we modified the original rapid-IHC procedure to block endogenous peroxidase activity, which could potentially cause misdiagnosis, the staining was still completed within 19 min. On the basis of conventional histological examination, 7 lymph nodes from 3 patients were deemed positive for micrometastasis. By contrast using hematoxylin and eosin staining, 13 lymph nodes from 7 patients were diagnosed positive on the basis of cytokeratin-detection using routine-IHC, and the same 13 nodes were diagnosed positive on the basis of rapid-IHC. That is, all nodes deemed positive with the routine-IHC procedure were also positive with the rapid-IHC procedure. Assuming the results of the routine-IHC are correct, the sensitivity, specificity and accuracy of rapid-IHC are 100%, 100% and 100%, respectively. These findings demonstrate the utility of our rapid-IHC analysis for intraoperative diagnosis of micrometastasis. However, our findings are limited by the fact that we tested the method using a single antibody at a single institute. Further investigation in multicenter studies will be needed to confirm the utility of this method.

Key words : non-small cell lung cancer, lymph node metastasis, lymph node micrometastasis, immunohistochemical staining

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Introduction

Surgeons performing lung resections to treat non-small cell lung cancer (NSCLC) rely on timely intraoperative assessment of lymph node metastasis when deciding whether to increase the extent of the surgical treatment

(e.g., with mediastinoscopy or extended segmentectomy). Lymph node metastasis is commonly diagnosed intraoperatively based on an examination of frozen sections. However, micrometastases within lymph nodes sometimes cannot be detected in hematoxylin and eosin (HE)-stained frozen sections; detection must await additional time-consuming immunohistochemical (IHC) investigation of paraffin-embedded tissue. This is noteworthy, as nodal micrometastasis in NSCLC is reportedly associated with a poorer survival rate than node-negative disease[1,2]. Unfortunately, although the sensitivity of IHC analysis using an anti-cytokeratin (CK) antibody is sufficient to detect micrometastases, the protocol routinely requires 2-4 hours to complete. To solve this problem, several investigators have proposed rapid methods that enable IHC protocols to be completed within only 12-30 min[3-11]. None of these methods is problem free, however. For example, our use of flow cytometry was limited by the frequency of false-positives caused by the lack of morphological information. To overcome that limitation, we developed and reported on a device that enables us to complete IHC analyses within 11 min using an alternating current (AC) electric field[12]. With this device, we apply a high-voltage, low-frequency AC electric field to the sections while they are incubating with the primary antibody. The effect of the device is to reduce the time required for the antigen-antibody reaction to occur. In our earlier report, we focused on the novel method developed and provided no group data or statistical analysis. The aim of the present study, therefore, was to show the statistical details of the rapid detection of lymph node micrometastases using our rapid-IHC protocol.

Patients and Methods

Patients

Twenty-five patients with NSCLC were enrolled in the study between May 2011 and July 2012 after obtaining signed informed consent. Surgically resected specimens were used under approval of the Institutional Review Boards at Akita University School of Medicine and University Hospital. After preoperative evaluation, the patients were taken to an operating room, and the standard preparations were made for a thoracotomy, lung resection, and mediastinal lymph node dissection. Lymph nodes from each patient were used for this study.

Tissue preparation

Immediately after surgically resecting the mediastinal lymph nodes, the nodes were embedded in O.C.T. compound (Sakura Finetek Japan Co., Ltd., Tokyo, Japan), frozen for 30 s in liquid acetone at −80°C using a Histo-Tek Pino System (Sakura Finetek Japan Co., Ltd., Tokyo, Japan), and transferred to a cryostat (CM1900 Leica, Wetzlar, Germany).

Routine-IHC procedure

Frozen sections were cut at 5 μm and placed on slides, then air-dried for 30 s, fixed in acetone for 10 min at 4°C, air-dried for 15 s at room temperature, and subjected to the staining procedure. Details of the staining method are listed in Table 1. Briefly, the sections were first incubated for 60 min with an anti-pancytokeratin (CK) antibody cocktail (AE1/AE3; PROGEN Biotechnik GmbH, Heidelberg, Germany) at 1 : 200 dilution (5 μg/mL). They were then incubated for 30 min with EnVision™+ System/HRP Mouse (DAB+), developed using 3,3´diaminobenzidine substrate, counterstained with hematoxylin, dehydrated, and mounted on coverslips.

Rapid-IHC device and procedure

We have developed a device that reduces the time required for IHC analysis (Fig. 1). The device and its mechanism were described in detail previously[12]. With this device, we apply a high-voltage, low-frequency AC electric field to lymph node sections while they are incubating with the primary antibody. This markedly reduces the time required for the antigen-antibody reaction. For each specimen, frozen sections were cut at 5 μm and placed on slides, then air-dried for 30 s, fixed in acetone for 2 min at room temperature, air-dried for 15 s at room temperature, and subjected to the staining procedure. Details of the staining method are listed in Table 1. Briefly, the sections were incubated first for 5 min with AE1/AE3 at 1 : 200 dilution (5 μg/mL) and then for 5 min with EnVisionTM+ System/HRP Mouse (DAB+). Thereafter, the slides were developed with 3,3´diamino-

Table 1. Procedures used for routine-IHC and rapid-IHC

	Routine-IHC procedure	Rapid-IHC procedure
Acetone fixation	10 minutes 4°C	15 seconds
Blocking endogenous P.O. activity	5 minutes	15 seconds
Washing with PBS	5 minutes 3 times	30 seconds
Blocking nonspecific antibody binding with 5% BSA	15 minutes	5 minutes
Primary antibody	60 minutes	5 minutes
Washing with PBS	5 minutes 3 times	30 seconds
EnVision™+ System/HRP Mouse	30 minutes	5 minutes
Washing with PBS	5 minutes 3 times	30 seconds
3,3´ diaminobenzidine	2 minutes	2 minutes
Approximate time required	167 minutes	19 minutes

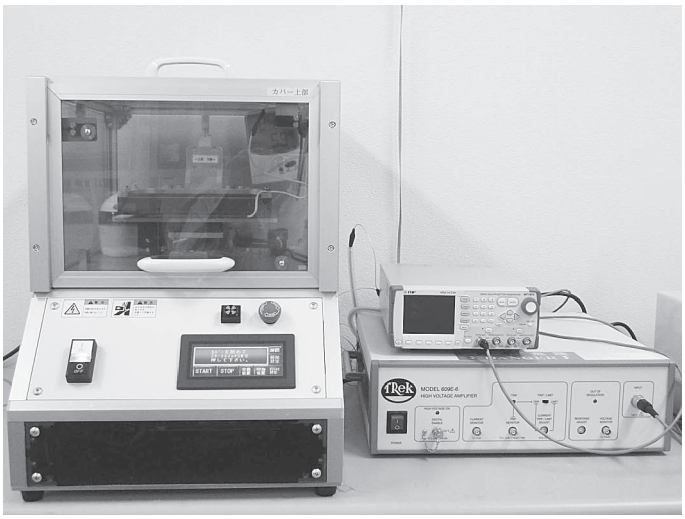

Fig. 1. Rapid-IHC device (prototype No. 1).

benzidine substrate, counterstained with hematoxylin, dehydrated, and mounted on coverslips. Using this method we can complete IHC analyses within 20 min.

Histopathological evaluation

Samples from all dissected lymph nodes were sectioned, conventionally stained with HE, and examined by a pathologist. Samples immunohistochemically labeled with anti-CK (AE1/AE3) antibody using the routine-IHC and rapid-IHC procedures were also examined by a pathologist. A result was considered positive if positive cell clusters or individual cells with the appropriate tumor cell morphology were recognized. As proposed by the new American Joint Committee on Cancer Staging Manual[11], isolated tumor cells (ITCs) were also considered positive in this study.

Results

The clinical characteristics of the study participants are summarized in Table 2. Using the rapid-IHC procedure outlined in Table 1, IHC analyses were completed within 20 min, and CK-positive nodes were detected by the pathologist within about 30 min. Using the routine-

Table 2

Patients		25
Age (y)		68.5 ± 10.5
Sex	Male	13
	Female	12
Pathological stage	Stage IA	15
	Stage IB	6
	Stage IIA	0
	Stage IIB	1
	Stage IIIA	3
Histological type	Ad	21
	Sq	4
Tumor Location	RUL	7
	RML	0
	RLL	7
	LUL	7
	LLL	4
No. of tested LN	Total	205
	/patients	8.2 ± 4.8

Ad : adenocarcinoma, Sq : squamous cell carcinoma, RUL : right upper lobectomy, RML : right middle lobectomy, RLL : right lower lobectomy, LUL : left upper lobectomy, LLL : left lower lobectomy

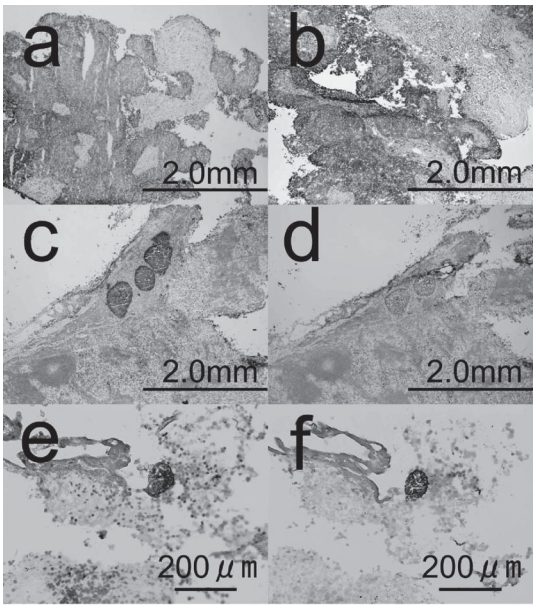

Fig. 2. Detection of micrometastasis using rapid-IHC
After labeling lymph nodes with an anti-CK antibody cocktail (AE1/AE3), the result was considered positive if individual cells or clusters of cells with the appropriate tumor cell morphology were recognized. As proposed by the new American Joint Committee on Cancer Staging Manual, isolated tumor cells were also diagnosed as metastasis. The photomicrographs in panels a, c and e show lymph node metastases stained using routine-IHC. Panels b, d and f show lymph node metastases stained using rapid-IHC. Panels a and b, c and d, and e and f show macrometastases, micrometastases, and isolated tumor cells, respectively.

IHC procedure, IHC analyses were completed within 167 min, and detection of CK-positive nodes required an additional 10 min, on average.

The purpose of intraoperative IHC analysis during surgery for NSCLC is detection of lymph node micrometastasis without misdiagnosis. This means that to apply the rapid-IHC procedure in practice, its ability to detect micrometastasis must be at least equal to that of the routine-IHC procedure. We therefore compared the abilities of the routine- and rapid-IHC procedures. Typical images of lymph node micrometastasis obtained using the routine- and rapid-IHC procedures are shown in Fig. 2. Note that the micrometastases detected using the routine-IHC procedure were always detectable using the rapid-IHC procedure.

Table 3 summarizes hisotological diagnosis of metastasis. We stained 205 mediastinal lymph nodes dissected as part of the surgical treatment for NSCLC. Among those, 7 lymph nodes from 3 patients were deemed positive for micrometastasis on the basis of conventional histological examination. By contrast, 13 lymph nodes from 7 patients were deemed positive on the basis of CK-detection using routine-IHC, and the same 13 nodes were deemed positive using rapid-IHC. In other words, all nodes deemed positive using the routine-IHC procedure were also positive using the rapid-IHC procedure. Assuming the results obtained using routine-IHC are correct, the sensitivity, specificity and accuracy of the rapid-IHC would be 100%, 100%, and 100%, respectively.

Discussion

In the present study we demonstrated and confirmed

Table 3. Diagnosis of micrometastasis using rapid-IHC, routine-IHC and HE

Methods	No. of metastatic nodes			N^+ nodes	N^+ patients
	macromets	micromets	ITC		
Rapid-IHC	7/205	3/205	3/205	13/205 (6.3%)	7/25 (28%)
Routine-IHC	7/205	3/205	3/205	13/205 (6.3%)	7/25 (28%)
HE	7/205	0/205	0/205	7/205 (3.4%)	3/25 (12%)

Macromets : macrometastasis, micromets : micrometastasis, ITC : isolated tumor cells

that lymph node micrometastasis can be diagnosed within 30 min using the rapid-IHC protocol developed at our institute. Conventional analysis of frozen tissue sections using HE staining enables diagnosis within 20 min at most institutions[10], but the ability of this method to detect micrometastasis is comparatively low. The sensitivity of IHC analysis using an anti-CK antibody is sufficient to detect micrometastasis, but the standard IHC procedure commonly requires 2-4 hours to complete. To address this issue, several investigators have proposed rapid methods that enable the IHC procedure to be accomplished within as little as 12-30 min[5-10,13-15]. These methods are not problem-free, however. For example, the new EnVision™ system has been described as a very sensitive and rapid IHC-based detection system[9,10], which enabled Kämmerer *et al*.[7] and Mönig *et al*.[8] to reduce the time needed to immunostain frozen sections to less than 13 min. To do this, however, they applied a higher concentration of primary antibody than is routinely used for IHC. Notably, our rapid-IHC procedure required about the same amount of time as the EnVision system, and we were able to use the same concentration of primary antibody used in the routine IHC procedure. Given the expense of primary antibodies, we believe this represents a significant advantage over the EnVision system. It is also known that microwave irradiation shortens IHC times. For example, Hatta *et al*.[16] reported that IHC analyses could be completed within 15 min using intermittent microwave irradiation in combination with a prepared immune complex consisting of the primary antibody, a secondary antibody and EnVision™-solution. The utility of our rapid-IHC method is comparable to this microwave approach.

Although the main purpose of the rapid-IHC is the diagnosis of sentinel node micrometastasis during surgery, avoiding axillary lymph node dissection based on the results of sentinel node biopsy is now controversial in breast cancer. Recently, the American College of Surgeons Oncology Group Z0011 trial demonstrated that, among patients with limited sentinel node metastatic breast cancer, the use of sentinel node dissection alone did not result in inferior survival, as compared to axillary lymph node dissection[17]. This result indicates that intraoperative diagnosis of sentinel node micrometastasis may not be required, at least in breast cancer, and we speculate that it reflects the efficacy of radiation, chemotherapy, and endocrine therapy in breast cancer. Unfortunately, radiation and chemotherapy are not particularly effective in NSCLC. In addition, Ou and Zell demonstrated that the number of lymph nodes removed at surgery in patients without apparent lymph node metastasis is a significant prognostic factor in NSCLC[18], and mediastinal lymph node dissection is still the standard surgical procedure for patients with NSCLC. Therefore, lymph node micrometastasis should not be ignored when applying sentinel node mapping to NSCLC.

The mechanism by which our method promotes the antigen-antibody reaction is not fully understood. In this study we applied a high-voltage, low frequency AC electric field to specimens while they were incubating with the primary antibody[12]. We previously showed that an interaction force affecting a droplet can be generated as a coulomb force applied from an AC electric field[19]. The generated force reflects the difference between the dielectric constants and the electric conductivity. In particular, when a droplet is attracted to the upper electrode by applying an electric field with a positive polarity, a wave is generated and a stirring phenomenon is caused by changes in the surface of this wave. We speculate that this stirring promotes the antigen-anti-

body reaction.

In our earlier report, we did not block endogenous peroxidase activity, which enabled us to complete the staining protocol within only 11 min. We were then able to discern CK-positive cancer cell nests based on their morphology. However, staining due to endogenous peroxidase could potentially lead to misdiagnosis. We therefore added blocking of endogenous peroxidase to the rapid-IHC protocol used in the present study (Table 1). This lengthened the staining protocol to 19 min, but we were still able to make a diagnosis of micrometastasis within 30 min, and the quality of the staining was much better.

Our findings demonstrate the utility of our rapid-IHC analysis for intraoperative diagnosis of micrometastasis. However, our findings are limited by the fact that we tested the method using a single antibody at a single institute. Further investigation in multicenter studies will be needed to confirm the utility of this method.

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