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ORIGINAL ARTICLE

Intraoperative diagnosis of lymph node metastasis during segmentectomy for non-small cell lung cancer by rapid immunohistochemistry using noncontact alternating current electric field mixing

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Keywords
Intraoperative diagnosis; lung cancer; lymph node metastasis; rapid immunohistochemistry; segmentectomy.

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Abstract

Background: Although lobectomy is considered the standard surgery for any non-small cell lung cancer (NSCLC), recent evidence indicates that for early NSCLCs segmentectomy may be equally effective. For segmentectomy to be oncologically safe, however, adequate intraoperative lymph node staging is essential. The aim of this study was to compare the results of a new rapid-IHC system to the HE analysis for intraoperative nodal diagnosis in lung cancer patients considered for segmentectomy.

Methods: This retrospective study analyzed the pathological reports from NSCLC resections over a six-year period between 2014 and 2020. Using a new device for rapid-IHC, we applied a high-voltage, low-frequency alternating current (AC) field, which mixes the antipancytokeratin antibody as the voltage is switched on/off. Rapid-IHC can provide a nodal diagnosis within 20 minutes.

Results: Frozen sections from 106 resected lymph nodes from 70 patients were intraoperatively evaluated for metastasis. Of those, five nodes were deemed positive based on both HE staining and rapid-IHC. In addition, rapid-IHC alone detected isolated tumor cells in one hilar lymph node. Three cStage IA patients with nodal metastasis detected with HE staining and rapid-IHC received complete lobectomies. Five-year relapse-free survival and overall survival among patients receiving segmentectomy with rapid-IHC were 88.77% and 88.79%, respectively.

Conclusions: Rapid-IHC driven by AC mixing is simple, highly accurate, and preserves nodal tissue for subsequent tests. This system can be used effectively for intraoperative nodal diagnosis.

Rapid immunohistochemistry based on alternating-current field mixing (completed within 20 minutes) is simple and highly accurate. This system will assist clinicians when making intraoperative diagnoses of lymph node metastasis and deciding upon the appropriate surgical procedure in segmentectomy for lung cancer.

Key points

Significant findings of the study

Rapid immunohistochemistry driven by alternating-current field mixing (completed within 20 minutes intraoperatively) is simple, highly accurate, and preserves lymph node tissue for subsequent pathological examination, including molecular assessments.

What this study adds:

Segmentectomy for lung cancer is oncologically safe, but only when there is adequate intraoperative node staging. Rapid immunohistochemistry will assist clinicians when making intraoperative nodal diagnoses.

Introduction

Lung cancer is the most common cause of cancer-related death globally. In cases of non-small cell lung cancer (NSCLC), surgery remains the best option for a potential cure. The Lung Cancer Study Group (LGSG) showed a preference for lobectomy over sublobar resection because it provides better local control, as demonstrated by a threefold decrease in local recurrence and improved survival for patients with pathological T1N0M0 NSCLC.[1] Although lobectomy and systematic lymph node dissection are the standard surgical procedures for any primary NSCLC, that is based on results from a single randomized trial published in 1995.[1] More recent evidence suggests that segmentectomy for small, stage I lung cancer can yield outcomes that are equivalent to lobectomy.[2–4] To date, two multicenter, prospective, randomized studies investigating the feasibility of sublobar resection for peripherally located clinical T1 lung cancer are underway (Cancer and Leukemia Group B (CALGB/Alliance) 140 503; The Japan Clinical Oncology Group (JCOG) 0802/West Japan Oncology Group (WJOG) 4607L).[5–7]

Although anatomic segmentectomy with hilar and mediastinal lymph node dissection was performed in the experimental treatment arm of the JCOG0802/WJOG4607L phase III trial, the surgical procedure must be converted to a lobectomy when regional lymph node metastasis is present or the resection margin is not cancer-free.[5, 7] This means that intraoperative lymph node exploration is necessary to avoid incomplete resection when treating with sublobar resection, as N1 or N2 disease may be present; 13%–17.9% of clinical stage I patients have been reported to have mediastinal lymph node involvement.[8–11] Segmentectomy for early small NSCLCs is an oncologically safe and reasonable alternative to lobectomy, but only when there is adequate intraoperative lymph node staging, as surgeons cannot complete segmental/subsegmental lymph node dissection for the remaining lung segment.

Intraoperative frozen section diagnosis using hematoxylin and eosin (HE) staining has traditionally been used to assess indeterminate lung lesions and guide surgical management. As a successful radical segmentectomy must be oncologically adequate, the accuracy of frozen section diagnosis is more important than ever.[12, 13] However, micrometastasis or isolated tumor cells (ITCs) within lymph nodes sometimes cannot be detected on the basis of HE staining alone. On the other hand, the sensitivity of immunohistochemistry (IHC) using an epithelial marker such as an anticytokeratin antibody is sufficient to detect micrometastasis or ITCs. Unfortunately, the standard protocol requires two to four hours to complete. To overcome that limitation, we have developed a rapid-IHC method that makes use of an alternating current (AC) electric field to facilitate the antigen-antibody reaction. With this device, we have applied a high-voltage, low-frequency AC electric field to the tissue sections.[14–16] The antibody is mixed within microdroplets as the voltage is switched on and off at specific intervals. The resultant coulomb force stirs the diluted solution on the sections, which increases the opportunity for contact between the antigen and antibody (AC mixing). This rapid-IHC method enables prompt detection of target cells within frozen sections and can provide a surgeon with an intraoperative diagnosis within 20 minutes, as opposed to the two to four hours required for conventional IHC.[14–18]

The aim of the present study was to compare the results of a new rapid-IHC system to the HE analysis for intraoperative nodal diagnosis in clinical stage I NSCLC patients considered for segmentectomy.

Methods

Patients

All experimental protocols were approved by the institutional review board at Akita University Hospital (approval number: 896, 929 & 2426 for the retrospective review), and all samples were collected under this IRB Protocol

No. 2426, which allows collection of tissue and medical record with consent or waiver of consent when no personalized health information is required, as was the case for this study. The medical records of 118 patients who underwent (or planned to undergo) pulmonary segmentectomy for clinical stage I NSCLC (UICC TNM Classification of Malignant Tumors eighth edition)[19] between January 2014 and February 2020 at our institute were retrospectively reviewed. Segmentectomy was performed in patients with clinical stage I or with computed tomography (CT) findings of ground-glass shadows, which may indicate an underlying minimally invasive adenocarcinoma. The other eligibility criteria were as follows: number of radiologically suspicious lung cancer lesions ≤3; main tumor located the outer third of the lung field on CT and no sign of nodal involvement; and lobectomy feasible. In 70 of these patients, intraoperative evaluation of lymph nodes for metastasis was performed using rapid-IHC with frozen sections. The patient characteristics are listed in Table 1.

Surgical procedure

All patients received standard pre- and intraoperative care. Segmentectomy was defined as resection of one segment or one segment and its adjacent segments. Because an adequate margin of healthy lung tissue was required, the pulmonary resection line could be placed on the segment adjacent to the affected one or portions of a few adjacent segments or subsegments extirpated. After completing the resection, frozen section (FS) examination confirmed that in all the analyzed patients the tumor was completely removed and that the lymph nodes removed were negative for involvement. If the resection was incomplete because of an inadequate surgical margin and anatomical difficulty, or intraoperative pathological analysis revealed the tumor to be greater than N0, the patient was converted to lobectomy. The frozen section slides for both HE staining and rapid IHC were prepared from the same lymph node sample at the same time intraoperatively. Our pathologists relied on intraoperative diagnosis of lymph node metastasis based solely on HE staining, but retained the optional diagnosis with rapid-IHC. The rapid IHC was only a guide in the present retrospective study.

Noncontact alternating-current electric-field mixing

It is known that when an AC electric field is applied to a solution, electro-osmotic vortices are produced that induce noncontact mixing. We have been developing a rapid-IHC method that makes use of an AC electric field to facilitate the antigen-antibody reaction. The rapid-IHC device mounts with a temperature control unit and is equipped with a humidifier to prevent evaporation of the microdroplet. Microscope slides are placed between the electrodes, and a high voltage (4.0–4.5 kVp-p, offset 2.25 kV),

Table 1 Characteristics of patients

Characteristic		Characteristic		
Patients, n	70			
Age, years	68.3 ± 8.8	Tumor location, n		
Sex, n		Right	S1	4
Male	36		S2	9
Female	34		S3	6
			S6	8
Tumor size (mm)	Average 16.5		S7	1
Histology, n			S8	5
Adeno	62		S9	3
Squamous	7		S10	0
Other histology	1	Left	S1 + 2	15
p-stage			S3	5
0	4		S4	6
IA1	22		S5	1
IA2	30		S6	3
IA3	3		S8	2
IB	6		S9	1
IIA	1		S10	0
IIB	1		S8/S9	1
IIIA	3	Node status, n		
		pN0/N1/N2		66/1/3
		Diagnosed LNs, n		106

Adeno, adenocarcinoma; S, segment; Squamous, squamous cell carcinoma.

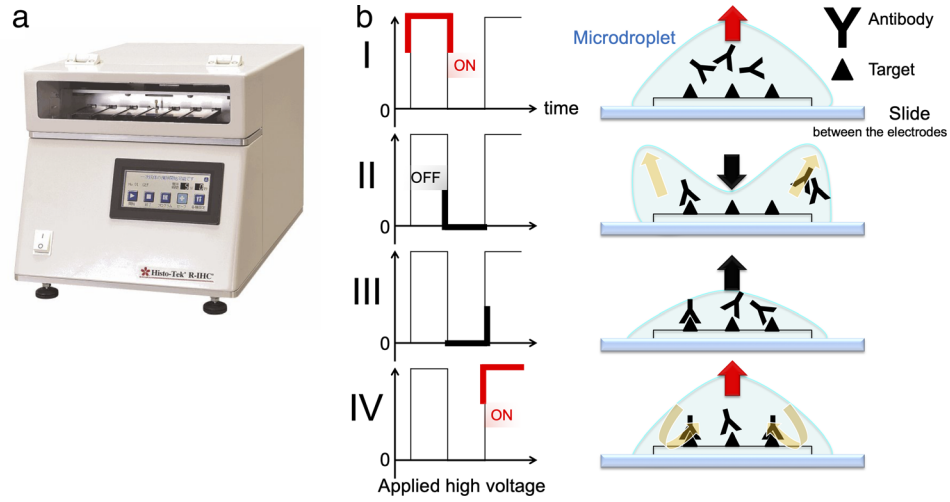

Figure 1 Rapid immunohistochemistry that makes use of an alternating current electric field to facilitate the antigen-antibody reaction. (**a**) Histo-Teq R-IHC. The device used to apply a high-voltage, low-frequency AC electric field can provide surgeons with an accurate intraoperative nodal diagnosis within 20 min. (**b**) Schema of the changes in the microdroplet as the voltage is switched on and off. The antibodies are mixed within the microdroplet as the voltage is switched on and off in a time series (I → II → III → IV). The resultant coulomb force stirs the antibody solution on the sections, and the microdroplet's shape is transformed. This increases the opportunity for contact between the antibody and antigen.

low-high frequency (5–90 Hz) AC electric field is applied. The device features an air insulation layer between the upper-electrode and the microdroplet. Relative permittivities of the air insulation and microdroplet (when the droplet is pure water) are $\varepsilon a = 1$ and $\varepsilon r = 80$, respectively. An attractive force mainly acts on the microdroplet and induces a positive electric field direction due to the difference between the relative permittivities ($\varepsilon a < \varepsilon r$). Even without a stirrer, the antibody is mixed within the microdroplet as the voltage is switched on and off in a time series (I → II → III → IV, Fig 1). The resultant coulomb force stirs the reagent solution on the sections, and the opportunity for contact between the antibody and antigen is increased as the voltage is turned on and off at regular intervals, transforming the shape of the microdroplet.

Rapid immunohistochemistry (IHC)

The same frozen sections of lymph node were used for both HE staining and IHC intraoperatively. Resected lymph nodes were cut at 2 mm intervals, then immediately embedded in optimum cutting temperature compound, frozen for 30 seconds in liquid acetone at −80°C using a frozen specimen block preparation system, and transferred to a cryostat for sectioning. Anti-pancytokeratin (CK) antibody cocktail (AE1/AE3 approved as in vitro Diagnostics; IR053, Dako) in a 150 μL volume was applied to the sections under a high-voltage (4.0 kVp-p, offset 2.25 kV), low-frequency (5 Hz) AC electric field. Procedures and times for IHC are summarized in Table S1.

Patient follow-up

Within two months after surgery, patients were evaluated for follow-up based on a physical examination, chest x-ray, CT, and laboratory tests. Although the follow-up schedule after surgery varied, it usually entailed a chest CT every three to six months and brain magnetic resonance imaging, bone scintigraphy, and/or positron emission tomography/CT every 6–12 months for the first two years. This was followed by evaluations every 6–12 months. If recurrence was suspected, the follow-up schedule was tightened. Postoperative adjuvant chemotherapy was recommended if the pathological findings revealed a tumor diameter of 2 cm or greater, or if lymph node metastasis was present.

Statistical analysis

Group data are expressed as means ± standard deviation. Kaplan-Meier analysis was used to estimate five-year relapse-free survival (RFS) and five-year overall survival (OS). The log-rank test was used to assess the impact of rapid-IHC on PFS and OS. *P*-values were two-sided and considered significant if less than 0.05. Statistical analyses were performed using JMP IN 14.2.0 software (SAS Institute, Cary, NC, USA).

Results

Between January 2014 and February 2020, 70 clinical stage I NSCLC patients were deemed eligible for inclusion in this study. A diagram of the selection process is shown in

Table 2 Summarized outcomes of eight patients who were converted lobectomy from segmentectomy after intraoperative diagnosis

No. (Age/sex)	Location	Histology	cStage	pStage	FS of LNs	R-IHC of LNs	Reason of conversion	Outcome	Survival, years
59/M	Lt. S1 + 2	Adeno	IA2	IA2	#12u–	#12u–	Anatomical difficulty based on PA	A	5.71
74/F	Lt. S4	Adeno	IA2	IIIA	#6+, #7+, #12u+	#6+, #7+, #12u+	Positive LNs metastasis	A	5.24
55/F	Lt. S4	Adeno	IA3	IIB	#5–, #11+	#5–, #11+	Positive LN metastasis	R	4.95
71/M	Lt. S1 + 2	Squamous	IA3	IIIA	#11–, #12u–	#11–, #12u–	Hilar adhesion due to lymphadenopathy by IPF	D‡	1.23
68/M	Rt. S3	Adeno	IA1	IA1	#4–, #12u –, #13–	#4–, #12u –, #13–	Pleuritis	A	3.53
69/F	Rt. S1	Adeno	IA1	IA2	#4–, #12u–	#4–, #12u–	Tumor position	A	2.92
61/F	Lt. S3	Adeno	IA1	IIIA	#10 +†	#10 +†	Positive LN metastasis	A	0.30
62/F	Lt. S8	Adeno	IA1	IA2	#12l–	#12l–	Anatomic extent of VPI	A	0.04

†8 mm micrometastasis. ‡Death by acute exacerbation of interstitial pneumonia. –, negative metastasis; +, positive metastasis; A, alive without recurrence; D, death; FS, frozen sections; IPF, idiopathic pulmonary fibrosis; LN, lymph node; PA, pulmonary artery; PA, pulmonary artery; R, recurrence; VPI, visceral pleural invasion.

Table 3 Comparison of intraoperative frozen section (hematoxylin and eosin stain) and final pathological diagnosis for detection of cancer cells among clinically dissected lymph nodes

	Final pathology, number of LNs		
	Positive	Negative	Total
FS (HE staining)			
Positive	5	0	5
Negative	0	101	101
Total	5	101	106

FS, frozen section; LN, lymph node.

Table 4 Comparison of intraoperative rapid-immunohistochemistry and final pathological diagnosis for detection of cancer cells among clinically dissected lymph nodes

	Final pathology, number of LNs		
	Positive	Negative	Total
Rapid-IHC			
Positive	5	1†	6
Negative	0	100	100
Total	5	101	106

†In one case isolated tumor cells were detected by intraoperative rapid-IHC alone.

Figure 1, and the clinical characteristics of the patients are summarized in Table 1. The median follow-up period was 3.70 years (range, 0.04 to 5.77 years). A total of 62 of these patients underwent segmentectomy, while the remaining eight patients were converted to lobectomy after intraoperative diagnosis. The causes of the conversions are summarized in Table 2.

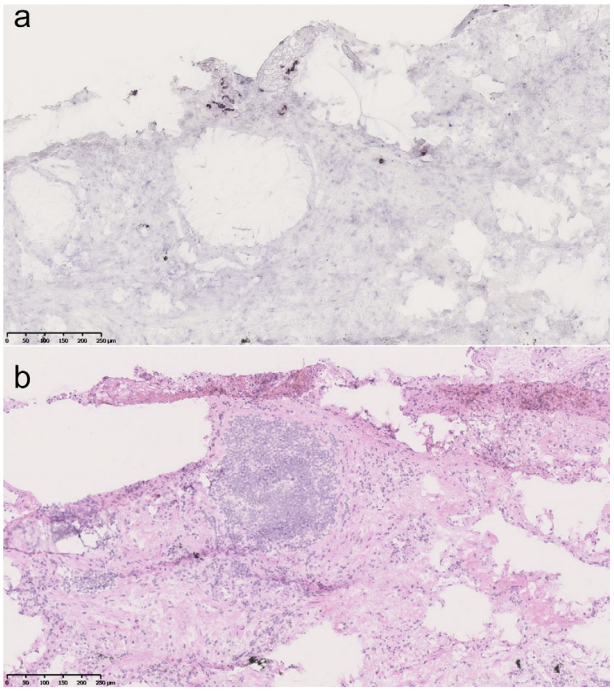

Figure 2 Isolated tumor cells of intrathoracic lymph node by rapid immunohistochemistry using noncontact alternating current electric field mixing. Isolated tumor cells detected within a resected intrathoracic lymph node with rapid immunohistochemistry (IHC) alone; these cells were not detected by intraoperative HE staining. Shown are images of positive staining for antipancytokeratin AE1/AE3 antibody with rapid-IHC (**a**) and HE staining (**b**). The patient was diagnosed as pN0 (i+).

A total of 106 resected lymph nodes were examined intraoperatively. Of those, 100 were judged to be negative based on HE staining, and this was confirmed by the rapid-IHC (Tables 3 and 4). On the other hand, five (including three mediastinal/hilar nodes originated from one patient) were deemed positive based on both conventional HE staining and rapid-IHC. In addition, rapid-IHC alone revealed the presence of ITCs in a hilar lymph node from one patient who received radical segmentectomy with systemic lymph node dissection (T2 [invades visceral pleura] N0M0, pathological stage IB) (Fig 2). Two years later, that patient had a recurrence with multiple metastases in mediastinal lymph nodes and pleural dissemination. Three patients were intraoperatively diagnosed with positive lymph node metastasis based on both HE staining and rapid-IHC and converted to lobectomy were still alive at the end of follow-up (Table 2).

Five-year RFS and OS among all the clinical stage I patients who completed radical segmentectomy and received an optional diagnosis using rapid-IHC (n = 62) were 88.77% (95% CI: 76.88–94.94) and 88.79% (95% CI: 75.46–95.33), respectively (Fig 3). There was no significant difference between complete and incomplete segmentectomy for RFS or OS (log-rank P = 0.1542 and 0.6106, respectively). The rate of distant metastasis and local recurrence, including regional lymph node metastasis, in patients who completed radical segmentectomy with rapid-IHC were 1.6% (1/61 patients, excluding one patient with ITCs) and 3.3% (2/60 patients, excluding one patient with ITCs and one patient with bilateral lung cancer), respectively.

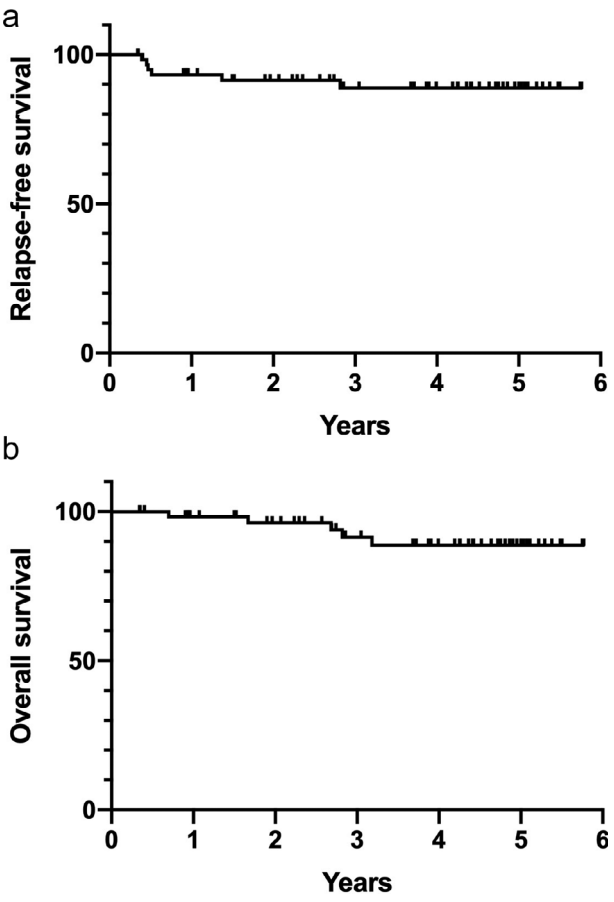

Figure 3 Relapse-free survival (**a**) and overall survival (**b**) of clinical stage I eligible patients receiving completed segmentectomy.

Discussion

In the present study, we demonstrated that rapid-IHC using anti-CK antibodies (AE1/AE3) and AC mixing can be used to intraoperatively diagnose intrathoracic lymph node metastasis within 20 minutes during segmentectomy for patients with NSCLC. The five-year OS after radical segmentectomy was 88.79%, and segmentectomy with intraoperative lymph node diagnosis with rapid-IHC should be considered an effective and accurate method to guide resection strategy for small peripheral NSCLCs.

Micrometastases or ITCs detected by IHC alone in patients with HE-negative lymph node metastasis has been reported to be controversial with survival.[20,21] The prognostic role of micrometastasis or ITCs within intrathoracic lymph nodes in primary patients with lung cancer is also controversial, and it is still unclear how their presence should be used for pathological staging and treatment planning. Two meta-analyses of 13 studies reported no significant association between micrometastases and survival, and that NSCLC patients probably should not be upstaged due to the presence of ITCs and micrometastases.[22,23] In addition, these studies provided no data suggesting that stage II/III NSCLC patients with pN(i+) or pN(mi) benefit from adjuvant chemotherapy and/or radiation therapy.[22] Thus, the significance of nodal micrometastases and ITCs in lung cancer remains a topic of shifting opinion.

Molecular diagnosis of nodal metastasis has been attempted for various types of cancer.[24–31] In cases of NSCLC, nodal involvement has been tested using qRT-PCR[29,30] and one-step nucleic acid amplification (OSNA),[28] was developed as a quick (30–40 minutes) and semiautomatic procedure for detection of lymph node metastasis by targeting CK19 mRNA. However, because OSNA requires tissue homogenization, it is limited by the frequency of false-positives caused by the inability to make morphological observations. Moreover, allocation bias is suspected to be the reason for the discrepancy between OSNA and histopathological diagnoses, as these two methods were applied to different lymph node slices in some clinical studies.[28, 31] That is also one of our limitations, although rapid-IHC offers advantages over OSNA. First, rapid-IHC is a simple procedure employing antigen-antibody reactions and preserves the ability for morphological observation. Second,

the same frozen sections of lymph node can be both used for both HE staining and IHC; consequently, with rapid-IHC it is not necessary to replace conventional histopathological diagnoses.

After standard surgery, the rate of recurrence among patients with stage I NSCLC ranges from 6% to 10% per person-year during the first four years, with 5% to 10% of patients eventually developing local recurrence and 10% to 20% developing distant metastasis within the first four years.[1, 32, 33] In the present study, the rates of distant metastasis and local recurrence in patients receiving complete radical segmentectomy and intraoperative diagnosis with rapid-IHC were 1.6% and 3.3%, despite segmentectomy being a fundamentally more limited parenchymal resection. However, intraoperative diagnosis of lymph nodes metastasis using rapid-IHC with anti-CK antibody could potentially lead to false positive and overdiagnosis because the prognostic impact of lymph node micrometastasis/ITCs in lung cancer is unclear, while having the possibility of better patient outcomes. Further investigation will be needed to evaluate the intraoperative rapid IHC for lymph node metastasis.

That said, rapid-IHC driven by AC mixing has several limitations. First, because pores created when an electric current is applied to the cell membrane could cause false positives and over-diagnosis, and it is important to consider the effect of electroporation when using the AC mixing device for IHC. In our earlier report, we showed that the electric current does not flow through the inside of microdroplets during AC mixing.[16] This is because the stirred microdroplet is separated from the electrode by both glass and air, which act as insulators. As a result, no pores are created. Second, rapid-IHC is subject to human error because the IHC steps are performed manually. To address this limitation, we are developing a new one-step fully automated rapid-IHC device, which reduces the effort of researchers and technicians and stabilizes the quality of any IHC stain. The important third limitation of this study is its small sample size and possible selection and allocation bias, which are the main pitfalls of histological tissue comparison studies. The analysis of survival data has provided interesting results, but these results should be interpreted cautiously. There were only a small number of patients who required conversion to lobectomy. To complete this new diagnostic system, future analysis of a larger number of patients undergoing converted lobectomy will be required.

In summary, our rapid-IHC system could potentially serve as a novel and effective procedure for intraoperative diagnosis of lymph node metastasis during segmentectomy in NSCLC patients. The advantages of this procedure are its simplicity, high accuracy, cost-effectiveness, and preservation of lymph node tissue for subsequent pathological examination including molecular assessments. Rapid-IHC will help pathologists and surgeons when making intraoperative diagnoses during radical lung cancer surgery.

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Disclosure

The authors have no conflict of interest.

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Supporting Information

Additional Supporting Informationmay be found in the online version of this article at the publisher's website:

Table S1. Procedures and times for immunohistochemistry (IHC).

Figure S1. Supplementary Figure.

