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Intraoperative rapid immunohistochemistry with noncontact antibody mixing for undiagnosed pulmonary tumors

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Abstract

Knowledge of the histologic type and primary origin of pulmonary tumors is essential when preparing a surgical strategy. Intraoperative diagnosis of hematoxylin and eosin (H&E)-stained frozen sections is the gold standard, but reliable pathology requires time-consuming immunohistochemistry (IHC) to distinguish among histological types/organ origins and to analyze molecular status. The aim of this study was to evaluate the clinical reliability of a new rapid-IHC technique for intraoperative diagnosis of pulmonary tumors. In total, 169 patients with undiagnosed pulmonary tumors were enrolled in a multicenter prospective observational study. At three institutes, pulmonary tumor samples were collected through core needle biopsy and/or surgery to determine surgical strategies. Using a new device for rapid IHC, we applied a high-voltage, low-frequency alternating current (AC) field, which mixes the available antibody as the voltage is switched on/off. Rapid IHC can provide tumor histologic type/origin diagnoses within 20 min, as opposed to the 3–6 h required for conventional

Abbreviations: AC, alternating current; CK, cytokeratin; FFPE, formalin-fixed, paraffin-embedded; HER2, human epidermal growth factor receptor 2; IHC, immunohistochemistry; IRB, Institutional Review Board; NSCLC, non–small-cell lung cancer; PD-L1, programmed death ligand 1; SCLC, small-cell lung cancer; TTF-1, thyroid transcription factor-1; UMIN, University Hospital Medical Information Network.

IHC. No false diagnoses of malignancy were rendered in any of the cases when using simple H&E staining. With H&E staining alone, the overall definitive diagnosis rate, the rate of defined tumor origin, and the rate of determined histological type were 76.92%, 85.80%, and 90.53%, respectively. When rapid IHC was added, those rates were significantly improved to 88.76%, 94.67%, and 91.72%, respectively. By providing prompt and accurate intraoperative histological/molecular analysis, rapid IHC driven by AC mixing could serve as an effective clinical tool guiding the surgical strategy for undiagnosed pulmonary tumors.

KEYWORDS
alternating current field mixing, frozen section, immunohistochemistry, lung cancer, pulmonary metastasis, surgery

1 | INTRODUCTION

Determination of histologic type and primary origin of pulmonary tumors is essential for the selection of optimal oncological treatment and surgical procedures. However, the preoperative diagnosis of small pulmonary nodules through transbronchial or transthoracic biopsy can be difficult due to the nodule's small size and location. Intraoperative pathological examination of a frozen section is frequently used in clinical practice to establish the initial surgical strategy for primary lung cancer or pulmonary metastasis in these patients.[1–4] Whether lobectomy, sublobar resection or other surgical procedures are used often depends on the intraoperative pathological diagnosis.

Intraoperative frozen-section diagnosis using H&E staining has traditionally been used to assess indeterminate pulmonary lesions and guide surgical management.[1,4] However, a considerable limitation to this approach is that there is a 2%–13.1% false-negative rate and a 0%–0.2% false-positive rate among frozen-section diagnoses as compared with diagnoses made with post-FFPE tissue blocks.[1,5] This is because frozen sections of lung tissue can be difficult to accurately interpret due to severe distortion of the tissue architecture, ice-crystal formation, and collapse of the alveolar spaces during cryosection. The potential to increase detection of early-stage lung adenocarcinoma has stimulated interest in segmentectomy, which can preserve lung function, reduce perioperative morbidity, and potentially improve survival.[6,7] However, the small size of the lesion and lack of clinical information are pitfalls frequently encountered when making a frozen-section diagnosis in these cases. Nodules less than 1 cm in size can be very difficult to accurately diagnose with H&E-stained frozen sections alone, especially when the tumor is poorly differentiated.[8,9] By contrast, IHC is a reliable screening and molecular analysis method. Up to now, however, the use of IHC for intraoperative frozen-section diagnosis has not been possible because IHC involves time-consuming and skilled processing.

To overcome that limitation, we have been developing a rapid-IHC method that makes use of an AC electric field to facilitate the antigen–antibody reaction by stirring the diluted solution on the sections without a stirrer through recurrent transformation of the microdroplet's shape. (AC mixing).[10–15] The resultant AC mixing achieves more stable staining and accurate diagnosis/molecular analysis by increasing the opportunity for contact between the antigen and antibody, irrespective of the antibody type.[13,15] This rapid-IHC technique enables prompt, stable detection of target cells within frozen sections and can provide a surgeon with an intraoperative diagnosis within 20 min, as opposed to the 3–6 h required for conventional IHC. We previously reported its usefulness for detection of lung cancer metastasis, central nervous system tumors, and mammalian ova, as well as HER2 in breast cancer and harmonization across PD-L1 assays for lung cancer.[10–15] However, with respect to intraoperative frozen sections for undiagnosed pulmonary nodules including primary and metastatic lung cancer, the utility of rapid IHC with predeterminate antibodies for each organ cancer has not yet been evaluated. Therefore, we conducted a prospective observational study to evaluate the reliability in daily clinical practice.

The aim of the present prospective, multicenter feasibility study was to evaluate the clinical reliability of this novel rapid IHC with AC mixing technique for intraoperative frozen-section diagnosis in patients with undiagnosed pulmonary nodules.

2 | MATERIALS AND METHODS

2.1 | Patients

All experimental protocols were approved by the IRB at Akita University Hospital (approval numbers: 896, 929, and 1632), Kobe University Hospital, and Iwate Medical University Hospital, and written informed consent was obtained from all patients. In total, 169 patients with undiagnosed pulmonary tumors were enrolled in this study between June 2017 and March 2022. At the three institutes, pulmonary tumor samples were collected through core needle biopsy and/or surgery to determine subsequent surgical procedures. This study was registered at the UMIN Clinical Trial Registry as UMIN000027922 (http://www.umin.ac.jp/ctr/index.htm). The patients' clinical characteristics are listed in Table 1. A diagram of the process by which cases were selected for study is shown in Figure 1.

TABLE 1 Characteristics of patients with pulmonary malignant tumors

Patients, *n*	169	Lung	
Median age, years (range)	69 (29–88)	Right	74
Sex, *n*		Left	89
Male	83	Multiple	6
Female	86	Tumor size	14.6 ± 8.72
		(Range, cm)	(1.5–50)
Primary tumor (duplicated case) in past history		Diagnostic method	
Lung	4 (4)	Needle biopsy	5
Colorectal	106	Wedge resection	110
Duodenum	1	Segmentectomy	25
Stomach	3 (5)	Lobectomy	29
Esophagus	6	Surgery	
Ear, nose, and throat	3	Wedge resection	96
Pancreas	1	Segmentectomy	30
Liver	1	Lobectomy	43
Kidney	9		
Uterus	7 (2)		
Prostate	1 (3)		
Breast	18		
Ovary	3		
Bladder	1 (4)		
Others	5 (3)		

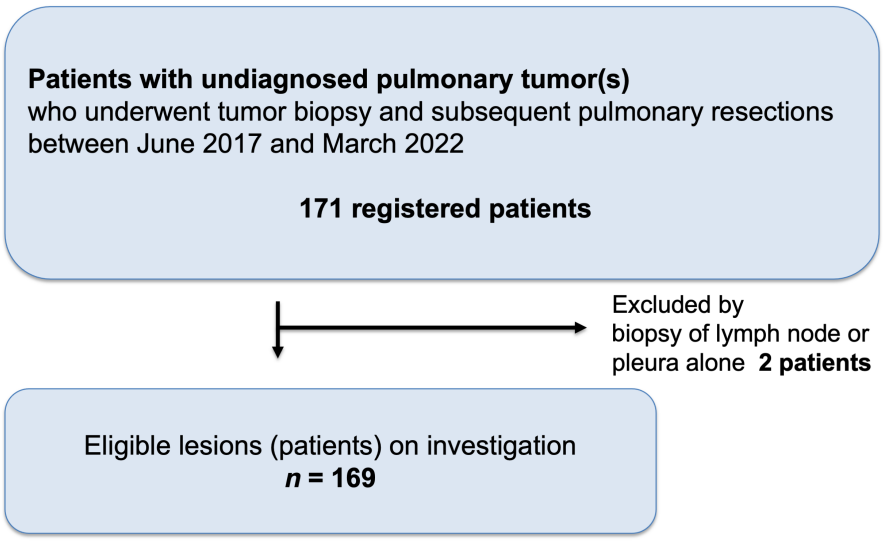

FIGURE 1 Flow chart illustrating the subject enrollment protocol.

2.2 | Surgical procedure

All patients received standard preoperative and intraoperative care. Sublobar resection was performed with a macroscopically greater safe margin than the tumor diameter. Energy devices and/or staplers were used in all pulmonary resections. Using resected lung tissue or a core needle biopsy specimen, frozen-section examination and rapid IHC were performed to assess the tumor histologic type/tumor organ origin in all the analyzed patients. If the resection was incomplete or intraoperative pathological analysis revealed a malignant-positive surgical margin, the surgeon added secondary pulmonary resection or converted segmentectomy/lobectomy at the discretion of the surgical team. Our pathologists made intraoperative diagnoses of undiagnosed pulmonary tumors

based solely on H&E staining, but retained the optional diagnosis made with rapid IHC. The rapid IHC was only a guide in the present prospective observational study.

2.3 | Frozen sections

The frozen-section slides for both H&E staining and rapid IHC were prepared intraoperatively from the same pulmonary tumor sample at the same time. Pulmonary tissues were cut at optimal intervals, then immediately embedded in optimum cutting temperature compound, frozen for 30 s in liquid acetone at −80°C using a frozen specimen block preparation system, and transferred to a cryostat for sectioning.

2.4 | New rapid immunohistochemistry using noncontact alternating current electric field mixing (rapid IHC)

We used the Histo-Tek R-IHC™ device to apply an AC electric field (Sakura Finetek Japan Co, Ltd., Tokyo, Japan), which we have described in earlier reports.[10–12] The theory behind AC electric field mixing and the method for its application have been described previously in detail (Figure 2 and Doc S1).[10–15] Briefly, we use the device to apply a high-voltage (4–4.5 kV, offset 2.4 kV), low-frequency (15 [5–90] Hz) AC electric field to the sections. This causes the antibodies to be mixed within microdroplets as the voltage is switched on and off at regular intervals, changing the shape of the droplet. Table S1 summarizes the general procedure for the conventional IHC and new rapid IHC using AC mixing. Table S2 lists antibody used for IHC. The available antibodies and protocols are referred through the R-IHC Study Group website (http://www.rihc.jp).

2.5 | Pathological evaluation

Expert pathologists at each hospital evaluated the specimens for this study. All dissected tumors and surgical margins were sectioned and examined using H&E staining and conventional IHC with FFPE tissue blocks. The overall definitive diagnosis was determined when both the tumor organ origin and histological type were perfectly consistent with final pathological diagnosis with FFPE without doubt. For each stain, positive IHC staining was defined based on the WHO Classification of Tumors Series (https://tumourclassification.iarc.who.int/welcome/). Usually, the presence of more than 10% expression of the marker within tumor cells was considered positive. Samples that were negative for the most part, but contained small tumor areas in which nearly all cells stained IHC-positive, were classified as focal positive.

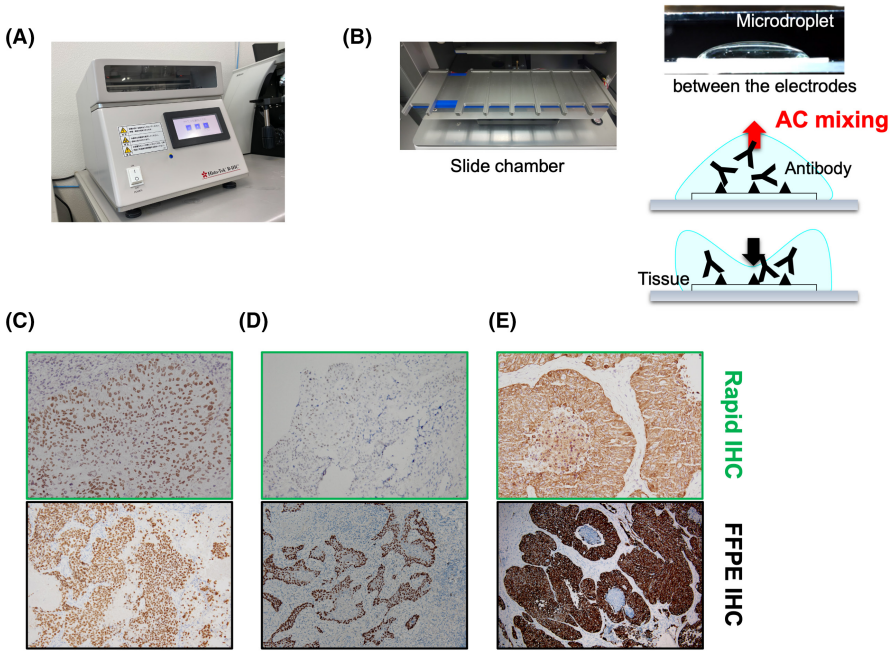

FIGURE 2 Rapid immunohistochemistry (IHC), which makes use of an alternating current (AC) electric field to facilitate the antigen–antibody reaction. (A) Histo-Teq R-IHC. The device is used to apply a high-voltage, low-frequency AC electric field and is able to provide surgeons with an accurate intraoperative diagnosis within 20 min. (B) Schema of the stir within a microdroplet as the voltage is switched on and off. Staining pattern obtained with rapid IHC. (C) Typical TTF-1-positive staining with rapid IHC or conventional post-formalin-fixed, paraffin-embedded (post-FFPE) IHC. (D) Typical p40-positive staining with rapid IHC or conventional post-FFPE IHC. (E) Typical CK7-positive staining with rapid IHC or conventional post-FFPE IHC.

2.6 | Assumed sample size and statistics

Referring to requirements for minimum sample size for sensitivity and specificity analysis,[16] a minimum sample size of 119 subjects (including 107 subjects having malignancy) will be required to achieve a minimum power of 80% (actual power = 82.0%) for detecting a change in the percentage value of sensitivity of a screening test from 0.80 to 0.90, based on a target significance level of 0.05 (actual $p = 0.040$). The primary endpoint of the study was to evaluate the overall definitive diagnostic rate including the tumor origin and histological type in patients with undiagnosed pulmonary tumors, when rapid IHC with AC mixing is added intraoperatively. Statistical analysis was performed using JMP IN 15.2.0 software (SAS Institute, Cary, NC, USA). The overall definitive diagnosing rate providing both histological type and organ origin was calculated by subtracting (excluded) cases that had suspicious diagnoses. Cohen's kappa-coefficient (κ), along with 95% confidence intervals were used to assess agreement of 4 ×2-contingency tables between protocols. Cohen's κ can be interpreted as: 0.81–1, almost perfect agreement; 0.61–0.80, substantial agreement; 0.41–0.60, moderate agreement; 0.20–0.40, fair agreement, and 0–0.20, slight agreement.[17]

3 | RESULTS

In total, 173 specimens (including two each from patients with multiple cancers) were collected at the abovementioned institutes from 169 patients with undiagnosed pulmonary tumors. Table S3 lists the numbers of the various neoplastic histological diagnoses encountered in the enrolled patients. No false diagnosis of malignancy was rendered in any case when using H&E staining alone.

In Table 2 the accuracy of the diagnoses of histological type from frozen sections of pulmonary nodules are compared between H&E staining and rapid IHC. When intraoperatively diagnosing frozen sections with H&E staining alone, the overall definitive diagnosis rate, the defining rate of tumor organ origin, and the rate of diagnosis of tumor histological type were 76.92%, 85.80%, and 90.53%, respectively. After adding rapid IHC the three rates improved to 88.76%, 94.67%, and 91.72%, respectively.

Table 3 summarizes the outcomes of 19 patients in whom there were discrepancies between the intraoperative diagnoses made with H&E staining or rapid IHC and the final pathology. In Case 42 (Figure 3A), who had a past history of colon cancer, there was a discrepancy between adenocarcinoma and colon cancer metastasis, which arose due to the TTF-1 negativity. In Case 71 (Figure 3B–D), SCLC was diagnosed as adenocarcinoma or SCLC due to the TTF-1 positivity and synaptophysin and chromogranin A negativity on rapid IHC alone. In Case 123 (Figure 3E), who had a history of ovarian cancer, and Case 144, who had a history of renal cell cancer, the discrepancy arose due to TTF-1 positivity on rapid IHC alone. Case 144 was diagnosed as having NSCLC precisely due being RCC marker negative on IHCs (Figure 3F). Figure 3 shows some typical IHCs illustrating discrepancies between H&E solo staining and rapid IHC.

In total, 39 patients did not receive a definitive diagnosis without suspicious findings through H&E staining of frozen sections alone. In contrast, only 19 patients did not receive a definitive diagnosis based on H&E staining and rapid IHC. Table 4 summarizes the outcomes of 10 patients for whom appropriate surgery or converted surgery was selected based on the combination of H&E staining and rapid IHC. In six cases, histology-appropriate wedge–wedge resection for pulmonary metastasis was decided and completed. In the other four patients, including Case 144, the surgical procedure was converted from wedge resection to lobectomy or segmentectomy.

We found an 82.25% (139/169) agreement between intraoperative frozen-section diagnoses obtained using H&E staining and rapid IHC. This fair-to-poor agreement (Cohen's κ = 0.391, 95% CI 0.222–0.560) reflects the finding that rapid IHC significantly improved diagnosis rates, including tumor histological type/primary origin, as compared with H&E staining alone.

4 | DISCUSSION

In the present prospective multicenter study, we demonstrated that rapid IHC with AC mixing on frozen sections improves accuracy when diagnosing histological type and/or organ origin of undiagnosed pulmonary tumors as compared with H&E staining alone. Notably, when rapid IHC was added, the overall definitive diagnosis rate (providing both histological type and organ origin) was 88.76%. Rapid IHC thus appears to be an effective and accurate method to guide the surgical strategy for undiagnosed pulmonary tumors.

For prompt intraoperative diagnoses, H&E staining of frozen sections represents the gold standard when evaluating lung lesions for the purpose of tumor tissue sampling or lymph node assessment. An important drawback is that, currently, we can use only H&E staining for intraoperative diagnosis, despite the fact that IHC following FFPE tissue processing is required to distinguish the histological types and organ origins for an accurate pathological diagnosis. The novel rapid-IHC technique, which can be completed within 20 min, resolves that problem.[10–12,14] Moreover, rapid IHC also has a potential advantage

TABLE 2 Comparison of the accuracy of the diagnoses of histological type in frozen sections of pulmonary nodules between H&E staining and rapid IHC with AC mixing ($n = 169$)

H&E staining alone (on frozen section), %			Rapid IHC, %		
Definitive[a]	Organ	Histology	Definitive[a]	Organ	Histology
76.92	85.80	90.53	88.76	94.67	91.72

[a] Excludes cases that had a suspicious diagnosis.

TABLE 3 Summarized outcomes of 19 patients with a discrepancy between the diagnosis with H&E staining or rapid IHC with AC mixing and the final pathology

Case	Age	Sex	Primary (PH)	Size, mm	Side	FS by H&E	R-IHC	Final	Biopsy	Surgery
24	77	M	Colon	12	R	NSCLC	NSCLC	SCC	Lo	Lo
26	79	M	Colon and stomach	19	L	Colon met	Gastric > colon mets	Gastric met	W	W
42	74	F	Colon	21	R	Lung adeno	Adeno[a]	Lung adeno	W	Lo
49	64	F	Breast	14	R	Por adeno	Adeno	Breast met	W	W
52	66	M	Colon	18	L	Colon met > lung adeno	Colon Met > lung adeno	Colon met	W	W
71	76	M	NSCLC	13	L	SCLC	Adeno[b]	SCLC	N	Lo
74	70	M	Colon	19	L	NSCLC	NSCLC[a]	Lung adeno[a]	S	S
91	71	M	Bladder	30	L	Cancer	Cancer	SCC unknown origin	W	W
100	58	F	ACC	4	L	Cancer	Cancer except lung origin	ACC met	W	W
123	60	F	Ovary	5	L	Adeno	Lung adeno	Ovarian met	Lo	Lo
128	60	F	Ovary	7	R	Ovarian met	adeno	Ovarian met (adeno)	S	S
130	29	F	Breast	22	L	Cancer	Cancer	Breast Met	Lo	Lo
134	72	M	Colon and ENT	15	R	Cancer	Cancer	ENT Met	W	W
137	58	M	Pancreas	15	R	Adeno	Met > lung adeno	Pancreas Met	W	Lo
140	83	M	Colon and stomach	19	R	Adeno	Adeno	Lung adeno	W	W
144	73	F	Kidney	26	L	Renal met	NSCLC	Lung adeno	W	S
145	76	M	Colon	10	L	NSCLC	NSCLC	Lung adeno	S	S
147	78	M	Peritoneal GCT	10	R	GCT met > carcinoid	Denied carcinoid	GCT Met	W	W
148	50	F	Uterus[c]	11	L	Lung carcinoid	Undeterminable	Lung NETs	W	Lo

Abbreviations: ACC, adenoid cystic tumor; adeno, adenocarcinoma; ENT, ear, nose, and throat; F, female; GCT, granular cell tumor; L, left; Lo, lobectomy; M, male; Met, metastasis; N, needle biopsy; NSCLC, non–small-cell lung cancer; Por, poorly differentiated; R, right; S, segmentectomy; SCC, squamous cell carcinoma; W, wedge resection.

[a]TTF-1 negative.

[b]Synaptophysin and chromogranin A negative on R-IHC alone.

[c]Endometrial cancer.

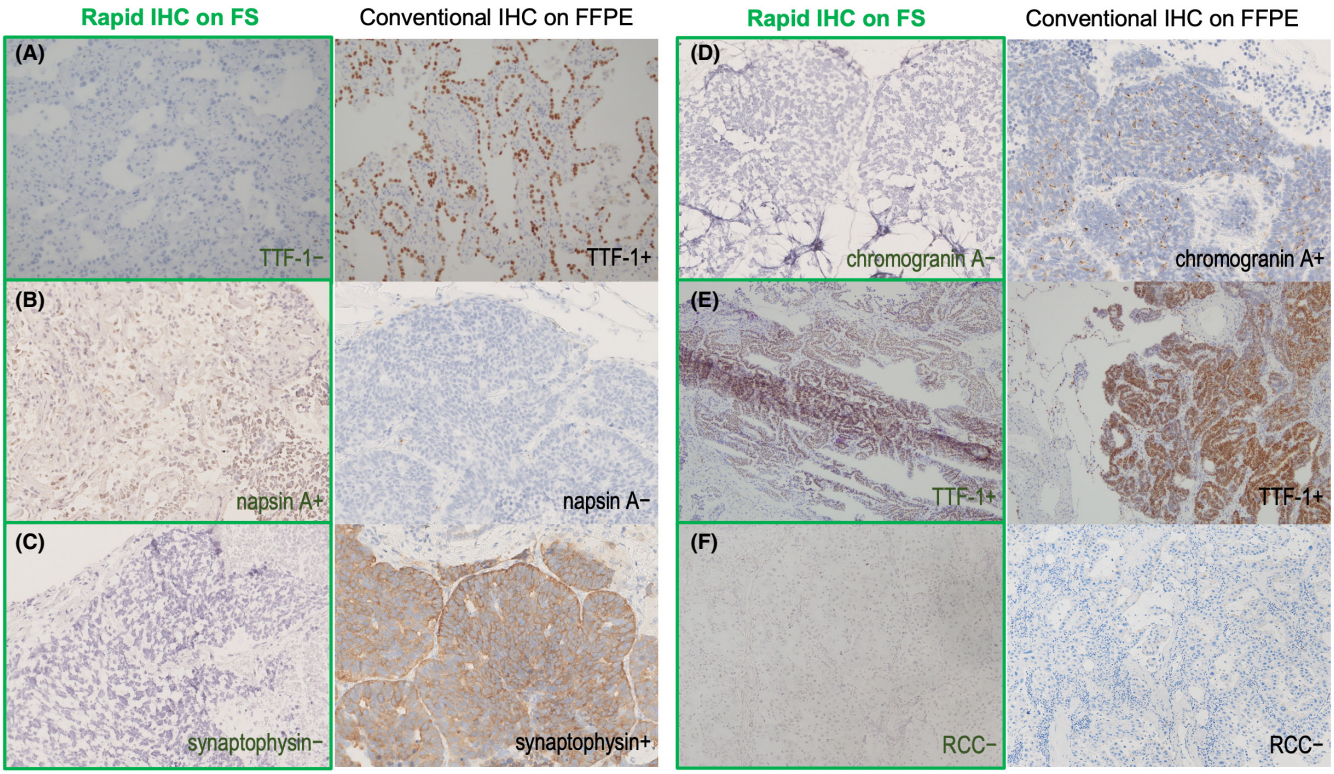

FIGURE 3 Comparison of the discrepant staining/diagnostic patterns obtained with rapid immunohistochemistry (IHC) and post-formalin-fixed, paraffin-embedded (FFPE) tissue block IHC. Case 42, who had a history of resectable colon cancer, was TTF-1-negative on rapid IHC (CK7-positive) but was diagnosed with lung adenocarcinoma intraoperatively/ultimately based on TTF-1 (−/+) (rapid IHC/FFPE final pathology) (A), napsin A (N/+), CK7 (+/+) CK20 (−/−), CDX2 (−/−), and SATB2 (−/−). Case 71 was diagnosed with adenocarcinoma based on intraoperative rapid IHC, but was corrected to small-cell lung cancer (SCLC) based on TTF-1 (+/+), napsin A (+/−, B), synaptophysin (−/+, C), chromogranin A (−/+, D). Case 123, who had a history of ovarian cancer, was intraoperatively diagnosed with adenocarcinoma (unknown origin) based on H&E solo staining and with primary lung cancer based on rapid IHC, but this was corrected to TTF-1-positive ovarian cancer metastasis based on TTF-1 (+/+, E) and CK7 (+/+). In addition, Case 144, who had a history of renal cell carcinoma, showed mismatching of primary or nonprimary cancer on rapid IHC but was ultimately corrected to primary lung cancer based on TTF-1 (+/+), napsin A (−/−), CD10 (−/−), PAX8 (−/−) and RCC (−/−) (F). Typical discrepant IHCs. (A) TTF-1, negative/positive staining on rapid IHC/FFPE IHC in Case 42, (B) Napsin A-positive/negative, (C) synaptophysin negative/positive, and (D) chromogranin A negative/positive staining in Case 71 (TTF-1-positive SCLC). Misleading IHCs. (E) TTF-1 positive/positive ovarian cancer metastasis in Case 123. Precise diagnostic IHCs. (F) RCC negative/negative in Case 144, who had a history of renal cell carcinoma.

for evaluating the molecular status of samples for biomarkers such as HER2 and PD-L1, among many others.[13,15]

Although our results demonstrate that intraoperative rapid IHC is a promising method for identifying histological types in lung cancer, there is still room for improvement. TTF-1 is a homeodomain-containing nuclear transcription factor and a member of the NKX2 gene family,[18] which has been used as a specific marker of lung adenocarcinomas.[19,20] However, TTF-1 is also expressed in pulmonary/extrapulmonary neuroendocrine cancers.[21,22] TTF-1 positivity is predictive of a poor prognosis and is detected in 80%–97% of SCLCs but also 50%–75% of large cell neuroendocrine carcinomas.[21,23,24] In the present study, one patient (Case 71, who also showed stain-mismatched napsin A false positivity for adenocarcinoma and synaptophysin and chromogranin A false negativity for SCLC) showed a discrepancy between adenocarcinoma on frozen section and SCLC in the final FFPE pathology, due to misinterpretation of TTF-1 positivity detected by the rapid IHC. Conversely, rapid IHC rarely showed TTF-1 negativity in pulmonary adenocarcinomas (Cases 42, 74, and 140). TTF-1 is reportedly detected in ~60%–80% of lung adenocarcinomas.[25] Although these examples illustrate the difficulties that can occur in distinguishing pulmonary adenocarcinoma and other NSCLCs, these difficulties are not likely to lead to a difference in intraoperative surgical management of patients. This is because intraoperative examination of H&E-stained frozen sections is, itself, a reliable method for diagnosing histological types in lung cancer. Addition of rapid IHC will help pathologists intraoperatively and adds a potential advantage by promptly providing tumor molecular status/information.

The well established immunohistochemical marker napsin A has emerged as a potential highly specific marker for pulmonary adenocarcinoma that reliably distinguishes between adenocarcinoma and squamous cell carcinoma.[26–28] However, napsin A stained poorly with rapid IHC in frozen sections. We found two false-positive cases (Cases 71 and 156 diagnosed with colon cancer metastasis) and four discrepant (negative or equivocal) cases along with seven napsin A-positive pulmonary adenocarcinomas among a total of 107 cases

TABLE 4 Summarized outcomes of 10 patients in whom initial surgery or a converted[a] surgical procedure was completed based on the combination of H&E staining and rapid IHC with AC mixing

Case	Age	Sex	Primary (PH)	Size, mm	Side	FS by H&E	R-IHC	Final	Biopsy	Surgery
59	66	F	Esophagus	6	R	Por cancer	Esophageal SCC	Esophageal SCC	W	W
80	69	M	Colon	7 (X2)	L	Undiagnosable	Colon met and NSCLC	Colon met and NSCLC	W	S[a]
90	69	F	Uterus	6	R	Adeno (origin unknown)	Uterus met	Uterus met	W	W
97	63	F	Colon	6	L	Adeno (origin unknown)	Colon met	Colon met	W	W
116	83	M	Colon	6	L	Adenoma with atypia	Colon met	Colon met	W	W
126	63	M	HCC	7	R	Cancer (origin unknown)	HCC met	HCC met	W	W
144	70	M	Kidney	26	L	Renal mets	NSCLC	Lung adeno	W	S[a]
147	78	F	Peritoneal GCT	10	R	GCT met or carcinoid	denied carcinoid	Peritoneal GCT met	W	W
150	56	M	Colon	16	L	Adeno (origin unknown)	Colon met	Colon met	N	S[a]
164	73	M	Duodenum	15	L	Cancer (origin unknown)	SCC	Lung SCC	W	Lo[a]

Abbreviations: adeno, adenocarcinoma; F, female; GCT, granular cell tumor; L, left; Lo, lobectomy; M, male; Met, metastasis; N, needle biopsy; NSCLC, non–small-cell lung cancer; Por, poorly differentiated; R, right; S, segmentectomy; SCC, squamous cell carcinoma; W, wedge resection.

[a]Converted surgery.

in which napsin A was evaluated. Several other studies have noted a similarly low sensitivity of napsin A (33%–69%) but equally high specificity.[19,26,29,30] Napsin A is highly specific but unfortunately loses its diagnostic power in relation to other established markers in the unclear adenocarcinoma or squamous cell carcinoma morphology of NSCLCs.[19,27] We recommend inclusion of napsin A with rapid IHC because positive staining may be helpful when TTF-1 staining is equivocal. However, these cases highlight the need to pay close attention to the false-negative interpretation of napsin A staining, especially when based on rapid IHC alone.

p40, p63 and cytokeratin 5/6 are the characteristic phenotypic panel specific for most squamous cell carcinomas.[20] Of those, p40 is a squamous/basal-type biomarker corresponding to the nontransactivating isoform of p63 gene (delta Np63).[31–33] The human p63 gene, a homologue of the p53 tumor suppressor gene, is located on chromosome 3q27-29 and is a nuclear antigen found in basal epithelial cells.[31] Squamous cell carcinoma samples from 14 patients with NSCLCs probed using an anti-deltaNp63 rabbit polyclonal antibody stained equally well and showed a 92.9% agreement between rapid IHC and IHC following FFPE processing. This suggests that rapid IHC with AC mixing may be especially useful for staining nuclear antigen.

The lung is the organ that is most frequently involved in metastatic malignancies, with an incidence of 20%–54% of nonpulmonary solid malignancies.[34,35] When patients have an extrapulmonary malignancy and undiagnosed lung nodules, a question is raised as to the originating organ of the tumor. The combination of CK7 and CK20 is useful for distinguishing, for example, ovarian, pulmonary, breast, colon, urothelial, and renal/prostatic carcinomas.[35] Earlier reports have shown that CK stained very well and accurately in rapid IHC.[10,14] In the present study, the discrepancy rate for CK7, CK20, or CK5/6 between rapid and conventional IHC was only 7.0% (18 of 257 IHC samples). Although unusual IHC profiles may lead to an incorrect diagnosis, information on the expression patterns of rapid-IHC markers facilitates histopathological diagnostics for undiagnosed primary and metastatic pulmonary tumors, especially if the patient has a history of treatment for other cancers.

The rapid-IHC technique with AC mixing has several potential limitations. First, rapid IHC entails universal use of antibodies. Consequently, before their clinical use we must determine whether all scheduled antibodies are adaptable to each molecular target. Although the rapid-IHC method has not yet been sufficiently tested in other organs or with other detection methods, we anticipate that this technique will be applicable in multiple settings, for example, for speedier in situ hybridization or enzyme-linked immunosorbent assays. A second limitation of rapid IHC is its poor staining of napsin A, which is a useful marker for distinguishing primary from metastatic adenocarcinomas in the lung. Further development of an appropriate protocol and specialized napsin A antibody for the rapid-IHC device will be needed. A third limitation is that there may be concerns that rapid IHC may mislead surgical management. In the present study, there were 19 discrepancies between the diagnoses made with H&E staining and rapid IHC as compared with the final FFPE pathology. In particular, the surgical strategy should be needed to change when

there are discrepancies due to the negativity of TTF-1 or neuroendocrine markers (for example in Cases 42 and 71). IHC plays a crucial role in the distinction of malignant neoplasms with similar morphology, but can also be misleading. However, this is the essential problem of applying immunohistochemistry in surgical pathology including the selection of antibodies and staining quality. By contrast, 10 patients received appropriate surgery (or converted) for pulmonary tumors through the addition of rapid IHC. Although more challenging cases may need additional studies, intraoperative rapid IHC appears to be both feasible and valuable. An important fourth limitation is possible selection, allocation bias, and the issue of heterogeneity by sampling bias, which are the main pitfalls of histological tissue comparison studies. IHC status may show intratumoral and intertumoral heterogeneity, and it is important to understand the variation in target protein expression among different sample sites to assess their suitability for testing. To complete this new diagnostic technique and system, future research will be needed to provide additional data and IHC profiling from patients with other organ cancers.

In summary, we have shown that rapid IHC with AC mixing can serve as an effective diagnosing procedure for intraoperative evaluation of undiagnosed pulmonary tumors. Especially in the patient who has a past history of cancer treatment, the determination of tumor organ origin through addition of rapid IHC can provide important and useful information when planning the surgical strategy intraoperatively. The advantages of this procedure are its simplicity, high accuracy, and preservation of surgical tissue for subsequent pathology, including molecular assessments.

AUTHOR CONTRIBUTIONS

K.I., H.N., and Y.Minamiya performed study concept and design; K.I. performed development of methodology and writing, review, and revision of the paper; W.S., T.S., T.I., Y.Maniwa, S.T., H.S., N.Y., Y.T., T.D., and Y.H. provided acquisition, analysis, and interpretation of data; K.N. suggested the statistics. M.T. and S.T. supervised technical/material and analyzed support. All authors read and approved the final paper.

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DISCLOSURES

The authors have no conflict of interest.

CLINICAL TRIAL REGISTRY NUMBER

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ETHICS STATEMENT

This study was approved 22 November 2016, by the Institutional Review Board at Akita University Hospital (approval numbers 896, 929, and 1632).

INFORMED CONSENT

Written informed content was obtained from all patients.

REGISTRY AND THE REGISTRATION

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ANIMAL STUDIES

None.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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