Development of a Novel One-Step Automated Rapid in situ Hybridization for Anaplastic Lymphoma Kinase Rearrangement Using Non-Contact Alternating-Current Electric-Field Mixing

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Keywords

Lung cancer · Anaplastic lymphoma kinase · In situ hybridization · Non-contact alternating-current electric-field mixing

Abstract

Echinoderm microtubule-associated protein-like 4 and anaplastic lymphoma kinase (*ALK*) fusion gene rearrangement is a key driver mutation in non-small cell lung cancer (NSCLC). Although Break-Apart *ALK* fluorescence in situ hybridization (FISH) is a reliable diagnostic method for detecting *ALK* gene rearrangement, it is also costly and time-consuming to use as a routine screening test. Our aim was to evaluate the clinical utility of a novel one-step, automated, rapid FISH (Auto-RaFISH) method developed to facilitate hybridization. This method takes advantage of the non-contact mixing effect of an alternating-current electric field. Ten representative specimens from 85 patients diagnosed at multiple centers with primary lung cancer with identified *ALK*-FISH status were collected. The specimens were all tested using FISH, RaFISH,

and Auto-RaFISH. With both RaFISH protocols, the *ALK* test was completed within 4.5 h, as compared to the 20 h needed for the standard protocol. We found 100% agreement between the standard FISH, RaFISH, and new Auto-RaFISH based on the *ALK* status, and all methods stained equally well. These findings suggest that Auto-RaFISH could potentially serve as an automated clinical tool for prompt determination of *ALK* status in NSCLC.

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Introduction

Several key genetic alterations drive non-small cell lung cancer (NSCLC). These include mutation of the epidermal growth factor receptor (*EGFR*) gene and rearrangement of the echinoderm microtubule-associated protein-like 4 and anaplastic lymphoma kinase (*EML4-ALK*) fusion gene [1–5]. The *EML4-ALK* fusion gene is one of the most desirable molecular targets for patients with *ALK*-rearranged NSCLC because there are several

specific tyrosine kinase inhibitors (TKIs) available for treatment [1], including crizotinib, ceritinib, alectinib, brigatinib, and lorlatinib [6, 7]. Moreover, *ALK* TKIs are well tolerated and induce rapid, durable responses in *ALK*-positive patients, and there is potential for ongoing benefit after initial disease progression in this population [6, 8]. It is therefore widely recognized that the detection of *ALK* gene rearrangements is a very important determinant when selecting an effective chemotherapy for patients with advanced and/or recurrent NSCLC.

Because *ALK* gene rearrangement involves large chromosomal inversion and translocation, fluorescence in situ hybridization (FISH) has become the method of choice for detecting all forms of *ALK* gene rearrangement, including the rare non-*EML4* fusions, and it was the assay used to detect this genetic aberration in the first clinical trials of *ALK* inhibitor therapy with crizotinib [8–11]. As many guidelines already recommend, Break-Apart *ALK* FISH is a reliable standard diagnostic method in surgical pathology because it is easily applicable to formalin-fixed, paraffin-embedded samples [5]. However, FISH is time-consuming and requires the use of expensive probes and a special fluorescence microscopy facility. This means that several obstacles to cost-effective and timely screening must be overcome before FISH is introduced as a routine test.

We have been developing a rapid immunohistochemistry (IHC) method that makes use of an alternating-current (AC) electric field to facilitate the antigen-antibody reaction. With this device, we apply a high-voltage, low- (or high-) frequency AC electric field to the tissue sections. 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. This rapid-IHC method enables prompt detection of target cells in frozen sections and can provide a surgeon with an intraoperative diagnosis within 30 min, as opposed to the 3–6 h required for conventional IHC [12–16]. In addition, we have shown that this technique is potentially applicable to multiple assays, for example in the hybridization step of FISH. We previously reported its usefulness for ISH-human *EGFR* 2 (*HER2*) in breast cancer and FISH-*ALK* rearrangement in lung cancer [17–19]. With rapid FISH using non-contact AC electric mixing (RaFISH), the *ALK* test was completed within 4.5 h, as compared to 20 h needed for the standard FISH. Moreover, there was 97.6% agreement between the standard FISH and RaFISH based on the *ALK* signal status (Cohen's kappa coefficient = 0.904, 95% con-

fidence interval: 0.802–1.000). Thus, while RaFISH produced results more promptly than standard FISH, staining and the accuracy of the *ALK* evaluation achieved with RaFISH was equal to the standard [18].

RaFISH thus has the potential for use as a clinical tool enabling prompt determination of *ALK* status. However, a limitation of RaFISH is that it is subject to human error because the ISH steps are manually performed. To address this limitation, we developed a new automated RaFISH (Auto-RaFISH), which reduces the effort of researchers or technicians and stabilizes the quality of the *ALK* rearrangement assay. The aim of the present methods article was to evaluate the clinical utility and reliability of this new Auto-RaFISH technique.

Methods and Design

Patients and Specimens

A total of 10 representative samples were adequately collected from enrolled lung cancer patients described in our earlier report [18]. These included 4 of the 19 *EML4-ALK*-positive samples collected for IHC and FISH at the Akita University Hospital, Akita Red Cross Hospital, Omagari Kousei Medical Center, and Nakadori General Hospital between April 2008 and December 2016, plus 6 of the 66 negative/1+(2+) samples collected for study between January 2015 and December 2016 at Akita University Hospital. Using standard histological techniques, the specimens were fixed in 10% formalin, embedded in paraffin, cut at 3–4 μ m, transferred to slides, and stained using hematoxylin/eosin and IHC. In addition, the specimens were subjected to FISH and our Auto-RaFISH protocol.

Standard IHC

Anti-*ALK* IHC was performed using the iAEP method (*ALK* Detection Kit; Nichirei Bioscience, Tokyo, Japan) with an automatic staining protocol [20, 21]. To confirm *ALK* positivity, IHC (and/or FISH) was also performed and evaluated by a commercial clinical laboratory (SRL, Tokyo). *ALK* IHC results were classified using iScore for iAEP IHC [22, 23].

Standard FISH

Standard FISH was performed on unstained 4- μ m sections of 10% formalin-fixed, paraffin-embedded tumor tissue using an *ALK* Break-Apart probe set (Vysis *ALK* Break Apart FISH Probe kit; Abbott Molecular Inc., IL, USA) with a paraffin pretreatment reagent kit (Vysis Paraffin Pretreatment IV and Post-Hybridization Wash Buffer kit; Abbott Molecular Inc., IL, USA). Assays were performed following the manufacturer's instructions.

Non-Contact AC Electric-Field Mixing

It is known that when an AC electric field is applied to a solution, electro-osmotic vortices are produced to induce non-contact 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 the temperature control unit and equipped with a humidifier to prevent evaporation of

Table 1. Procedural details for standard FISH and RaFISH

Protocol	Standard	RaFISH	Auto-RaFISH
Dewaxing, activation, dehydration	1 h	1 h	1 h
Denaturation and hybridization	16–18 h (37°C)	3 h (37°C), AC	3 h (37°C), AC
Washing slides, etc.	10 min	10 min	(+ drop oil cover)
Microscopy after DAPI stain	10 min	10 min	10 min
Total required time	18–20 h	4.5 h	4.5 h

DAPI, 4',6-diamidino-2-phenylindole dihydrochloride; AC, alternating-current electric field (4.5 kv, 90 [or 15] Hz).

the microdroplet. The microscope slides can be put between the electrodes. A high voltage (4.5 kV_{p-p}, offset 2.25 kV), low-high frequency (15–90 Hz) AC electric field was applied to the sections. The device features an air insulation layer between the upper electrode and microdroplet. Relative permittivity of air insulation and microdroplet (when the droplet is pure water) is $\epsilon_a = 1$ and $\epsilon_r = 80$, respectively. An attractive force mainly acts on the microdroplet and induces positive electric-field direction by the difference between relative permittivity ($\epsilon_a < \epsilon_r$). Even if the stirrer is not in the droplet, the reagent (antibody or RNA/DNA probe) is mixed within the microdroplet as the voltage is switched on and off in a time series (I→II→III→IV, online suppl. Fig. 1; for all online suppl. material, see www.karger.com/doi/10.1159/000505631). The resultant Coulomb force stirs the reagent solution on the sections, and the opportunity for contact between the reagent and the gene is increased because as the voltage is turned on and off at regular intervals, the microdroplet's shape is transformed. If high-frequency AC electric field is applied, the shape is almost not transformed. However, a high-speed camera can observe the imperceptible transformation, and it can stir the reagents.

New RaFISH and One-Step Auto-RaFISH

We have developed a prototype for Auto-RaFISH (Fig. 1). The theory behind the technique for AC electric-field mixing and its protocol were also described in detail previously [17–19]. Ten microliters of Vysis LSI ALK Dual Color Break-Apart FISH Probe and 40 μ L of sterile distilled water were applied to each slide, after which 40 μ L of HAIKORU-K140N (KANEDA, Japan), a very low-viscosity liquid paraffin (4.0–5.5 mm²/s), were added as an oil cover to prevent probe evaporation. The slide was then placed between electrode plates, and a high-voltage (4.5 kV_{p-p}, offset 2.25 kV), high-frequency (90 Hz) AC was applied after denaturation for 3 min at 73 °C. There was a distance of 3.8–4.0 mm between the slide and electrode plates, and the current was applied for 3 h at 37 °C. After washing the slide, 10 μ L of DAPI (4',6-diamidino-2-phenylindole dihydrochloride) counterstain were applied. Signals were analyzed using an Olympus BX51 fluorescence microscope (Olympus, Tokyo, Japan). A minimum of 50 nuclei from two separate areas of the tumor were independently scored by two technologists. The run protocol for Auto-RaFISH was established so that the entire assay procedure, consisting of pretreatment, hybridization, stringency wash, and signal detection, was completed as a one-step automated assay. Only the oil cover step was performed manually. Table 1 summarizes each procedure and the time required for conventional FISH, RaFISH, and Auto-RaFISH.

Fig. 1. One-step Auto-RaFISH device used to apply a high-voltage, low-frequency, AC electric field. The new device automatically performs the denaturation, hybridization, and slide-washing steps.

Statistical Analysis

Statistical analysis was performed using JMP IN 11.0.0 software (SAS Institute, Cary, NC, USA). Cohen's kappa coefficient was used to assess agreement of 4 × 2-contingency tables between protocols. A kappa value <0.4 indicated fair-to-poor agreement, 0.4–0.8 indicated moderate-to-good agreement, and >0.8 indicated excellent agreement.

Detection of ALK Rearrangement Using New Auto-RaFISH

Using the procedures outlined in Table 1, the new Auto-RaFISH enabled detection of ALK rearrangement within 4.5 h, as compared to 18–20 h needed for standard FISH. In ALK-positive patients, the performances of the new Auto-RaFISH protocol, RaFISH, and standard FISH were equal (Cases 1–4, Fig. 2). Online supplementary Figure 2 shows typical ALK rearrangement-positive and -negative cells detected by Auto-RaFISH. All 10 samples were well stained and accurately evaluated. Table 2 shows the ALK statuses determined from the ALK signal for all samples. We found 100% agreement between conventional FISH, RaFISH, and Auto-RaFISH based on the status of the ALK signals in the 10 representative samples.

Fig. 2. Detection of *ALK* rearrangement using new Auto-RaFISH. **a** Positivity demonstrated using conventional FISH. **b** Positivity demonstrated using RaFISH. **c** Positivity demonstrated using Auto-RaFISH. **d** Negativity demonstrated using conventional FISH. **e** Negativity demonstrated using RaFISH. **f** Negativity demonstrated using Auto-RaFISH. Samples were classified as positive for

ALK rearrangement when $\geq 15\%$ of the tumor nuclei showed split signals (red and green signals were separated by ≥ 2 signal diameters, white arrows) or single red signals ($3'$ *ALK*) were observed in FISH or RaFISH. Yellowish cytoplasmic staining in the tumor cells shows tissue collagen.

Table 2. Positive *ALK* rearrangement determined using standard FISH, RaFISH, and one-step Auto-RaFISH

Sample	Standard FISH	RaFISH	Auto-RaFISH
1	+	+	+
2	+	+	+
3	+	+	+
4	+	+	+
5	–	–	–
6	–	–	–
7*	–	–	–
8	–	–	–
9	–	–	–
10	–	–	–

–, no stain; +, rearrangement. Results were evaluated by two independent pathologists in accordance with the new guideline recommendations. * Sample from another tumor location in the same patient from whom sample 6 was collected. Auto-RaFISH, automated FISH with AC mixing.

Discussion

In the present methods article, we demonstrated that our new Auto RaFISH, performed with a high-voltage, high-frequency AC electric field, can be used to detect *ALK* rearrangement within 4.5 h and can be completed as a one-step automated assay. The Auto-RaFISH and standard FISH stained nearly equally well in patients with *ALK*-positive lung cancer.

The *ALK* Break-Apart FISH Probe Kit has become the gold standard diagnostic tool for detecting *ALK* rearrangements to determine the appropriateness of targeted therapy with *ALK* inhibitors in NSCLC [5]. However, the FISH protocol has several well-known disadvantages: it is time-consuming, technically demanding, costly, and may delay final determination of *ALK* status. The standard FISH protocol requires approximately 20 h to complete, as hybridization usually takes 16–18 h. By contrast, RaFISH enabled the hybridization step to be completed in less than 3 h. However, because all steps in the RaFISH protocol except hybridization are performed manually, the method is subject to human error and needed im-

provement. For this series, therefore, we developed a one-step Auto-RaFISH device, which stabilizes test quality.

We previously reported the usefulness of AC mixing for detection of lung cancer metastasis, breast cancer metastasis, central nervous system tumors, and mammalian eggs, as well as for ISH-*HER2* and ISH-*EML4-ALK* rearrangement in lung cancer [12–19]. New ISH with AC mixing is summarized in Table 3. The AC mixing device can also be applied to rapid ISH [17, 18] and reagent-saving ISH [19]. Auto-RaFISH of *ALK* in the present study did not have the costly advantage because the same amount of Vysis LSI *ALK* Dual Color Break-Apart FISH Probe was used in both new RaFISHs and the standard FISH. Importantly, in our earlier report [19], ISH with AC mixing can be used with 1:2 saving ISH to accurately diagnose *HER2* status and achieve stable *HER2* gene hybridization in breast cancer samples, even when reagent concentrations vary. Although ISH with AC mixing produces results more promptly while using smaller amounts of probe, staining and the accuracy of the target gene status evaluation with ISH with AC mixing may be equal to or greater than with standard ISH. These results suggest that new ISH with AC mixing could be used as a clinical tool to promptly determine target gene status.

However, AC mixing ISH has some limitations. First, ISH usually requires a warm temperature (37 °C for hybridization overnight and 80 °C for denaturation) and a longer processing time than the rapid IHC method. Consequently, evaporation could be a problem. We therefore developed a very-low-viscosity liquid paraffin to be used as an oil cover with the Ra-FISH device. Second, because pores created when an electric current is applied to the cell membrane could cause false positives and overdiagnosis, it was important to consider the effect of electroporation when using the AC mixing device for IHC or ISH. In our earlier report, we showed that the electric current does not flow through the inside of microdroplets during AC electric-field mixing [14]. This is because the stirred microdroplet is separated from the electrode by both glass and air, which act as insulators. Consequently, no pores are created. Third, the new Auto-RaFISH device is currently still in development. In particular, the automatic washing step did not work in some cases because the washing pressure was compensated for. The important fourth limitation of this study includes the small sample size and possible selection bias, as the prototype under development was used in the present study. Future research is needed to provide the additional data required to complete this product. Fifth, specimens in the present study sometimes could not be evaluated using the RaFISH protocol because

Table 3. New ISH with AC mixing

First author [ref], year	Target gene	Probe dilution	Protocol time	Kappa value
Saito [17], 2016	<i>HER2</i>	1:1	6 h	0.960
Fujishima [18], 2017	<i>ALK</i>	1:1	4.5 h	0.904
Kurihara [19], 2019	<i>HER2</i>	1:2*	19–21 h	0.912

ISH with AC mixing could be used as a clinical tool for prompt and stable determination of target gene status, even when reagent concentrations vary. * Practical application of AC mixing reagent-saving ISH.

of poor *ALK* hybridization, which was caused by there being too much collagen tissue on the specimens. However, it is a common problem for *ALK*-FISH.

Conclusion

In summary, we have presented a new one-step automated FISH (Auto-RaFISH) technology that could potentially serve as a clinical tool for prompt determination of *ALK* status in lung cancer samples. The clinical benefit of time-effective Auto-RaFISH is that *ALK*-positive NSCLC patients can receive specific TKIs therapy more promptly, if the patients have the result of Break-Apart *ALK*-FISH at the same time of diagnostic biopsy. Although the present study is preliminary, with only a small sample size and without an optimized *ALK* RaFISH protocol, our findings indicate that this approach warrants further investigation.

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Statement of Ethics

All experimental protocols were approved by the institutional review board at Akita University Hospital (Permit No. 1408), and written informed consent was obtained from all patients. The methods used in this study were carried out in accordance with approved guidelines.

Disclosure Statement

The authors have no conflicts of interest to disclose.

Author Contributions

K.I. analyzed the data and wrote the paper. S.T., S.F., T.M., S.W., K.T., A.W., Y.S., and S.M. helped collect the data. H.N. made pathological diagnoses and supported this research. R.N. and Y.A. contributed to the development of the device enabling application of a high-voltage, low-frequency AC electric field. Y.M. designed and supervised the research. All authors read and approved the final manuscript.

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