Original Article

High concordance of *ALK* rearrangement between primary tumor and paired metastatic lymph node in patients with lung adenocarcinoma

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Background: Lung cancer has heterogeneous features. It remains unclear whether *ALK* rearrangement was distributed heterogeneously in tumor from different anatomic sites. To address this issue, we investigate the concordance of *ALK* rearrangement between primary tumors and paired metastatic lymph nodes in pulmonary adenocarcinoma patients.

Methods: From Sep 2013 to May 2014, resectable lung adenocarcinoma patients with *EGFR* wildtype and paired metastatic lymph nodes from Tongji University affiliated Shanghai pulmonary hospital were selected into this study. An auto-mated Ventana *ALK* with clone D5F3 antibody immunohistochemistry (IHC) and reverse transcriptase-polymerase chain reaction (RT-PCR) were used to detected *ALK* rearrangement. Discordant cases between IHC and RT-PCR were further validated by fluorescence in situ hybridization (FISH).

Results: A total of 101 patients were enrolled into this study with a median age of 60 years old (range, 35–78 years). *ALK* rearrangement was found in 20 primary lesions, while in 18 paired metastatic lymph nodes. *ALK* rearrangement was more frequently happened in younger (P<0.001), Nonsmokers (P=0.012), high-stage disease (P=0.021) and predominantly solid growth pattern (P=0.024). The concordance rate between primary tumor and paired metastatic lymph nodes was 98%. Two patients with *ALK* rearrangement on primary tumor didn't show *ALK* gene fusion on paired metastatic lymph nodes. Sixty-eight cases had more than two stations of metastatic lymph nodes. *ALK* rearrangement in the different station of metastatic lymph nodes of the same patient was consistent.

Conclusions: High concordant rate of *ALK* rearrangement between primary tumors and paired metastatic lymph nodes were found in this study. The authors concluded that specimens from metastatic lesions and primary tumors are equally suitable for detection *ALK* rearrangement.

Keywords: *ALK* rearrangement; heterogeneity; lung adenocarcinoma; immunohistochemistry (IHC); reverse transcriptase-polymerase chain reaction (RT-PCR); fluorescence in situ hybridization (FISH)

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Introduction

Lung cancer remains the most prevalent malignant tumor and the leading cause of cancer related death world-widely (1). Non-small cell lung cancer (NSCLC) accounts for 85% of all lung cancer (2). Advances in the understanding of the molecular biology of lung cancer have made individual therapy into clinical practice for advanced NSCLC. As an example, patients with *EGFR* mutation or *ALK* rearrangement showed dramatic response to *EGFR* or *ALK* tyrosine kinase inhibitor (TKI) and have been recommended to receive *EGFR*-TKI or Crizotinib as first line setting (3-5). Thus, precise detection of these driver genes mutation will be urgently needed in clinical practice.

ALK rearrangement can be detected by different methods including immunohistochemistry (IHC), reverse transcriptase polymerase chain reaction (RT-PCR), and fluorescence in situ hybridization (FISH). Among them, FISH was approved as a gold standard by the Food and Drug Administration (FDA) with the Vysis *ALK* Break Apart FISH Probe Kit. *ALK* rearrangement could also detected by IHC, an auto-mated the Ventana *ALK* assay kit with D5F3 antibody (Ventana Medical Systems Inc., Tucson, Ariz) coupled with an ultrasensitive amplification and detection system have been verified in *ALK* protein detection (6,7). Besides that, RT-PCR is a more sensitive and specific method that can allows the detection of even a few molecules of chimeric *ALK* transcripts. But the accuracy of RT-PCR-based testing *ALK* rearrangement depends greatly on the RNA quality of specimens. Furthermore, some unknown fusion partners or breakpoint variants may be missed because the primers in the assay are specifically designed to evaluate these known alterations.

As we know, lung cancer is a heterogeneous tumor with diverse molecular and pathological characteristics. For example, several studies have reported the discrepancy of *EGFR* mutation status between primary tumor and metastatic lesions from the same patient (8,9). However, it remains unclear whether *ALK* rearrangement between primary and metastatic lesions showed a similar heterogeneous phenomenon as *EGFR* mutation.

To address this issue, we compare the *ALK* rearrangement status between primary lesions and paired metastatic lymph nodes in 101 resected patients with adenocarcinoma and EFGR wildtype through the methods of VENTANA IHC and RT-PCR. Discrepancy of *ALK* rearrangement between IHC and RT-PCR was further confirmed by FISH.

Methods

Clinical samples

The enrolled criteria included primary pulmonary adenocarcinoma patients with metastatic lymph node who received surgical resection in Tongji University affiliated Shanghai pulmonary hospital, the primary and paired metastatic lymphatic section containing more than 100 tumor cells and *EGFR* wild type. From September 2013 to May 2014, a total of 101 patients were collected. The histological type was confirmed according to the criteria of ERS/ATS/IASLC multidisciplinary classification of lung adenocarcinoma in our routine practice (10). All patients had signed an informed consent for further molecular analysis.

ALK protein expression

Representative formalin-fixed, paraffin-embedded blocks containing the most tumor cells from primary tumor and paired metastatic lymph node were chosen for this current study by two pathologists. Ten serial tissue sections of each block were prepared to detect *ALK* rearrangement (two tissue slides for IHC, one for FISH and the rest for RT-PCR). Immunohistochemical staining was performed on a VENTANA BenchMark XT automated slide-processing system at Ventana Medical Systems. Four-μm-thick FFPE tissue sections were deparaffinized with EZ Prep solution and antigen-retrieval with cell conditioning 1 (64 minutes at 100 ℃) was performed. Primary antibody (clone D5F3, VMSI) was incubated on tissue sections for 20 minutes. According to the manufacturer's protocol, OptiView DAB IHC Detection Kit (VMSI) and OptiView Amplification Kit (VMSI) were used. Slides were counterstained with Hematoxylin II and then dehydrated. Tissue slides were protected with coverslips. All immunohistochemical stains were evaluated independently by two pathologists. Specimens were scored positive if strong granular cytoplasmic brown staining in tumor cells (excluding nontumor cells: alveolar macrophages, cells of neural origin, glandular epithelial staining), the absence of strong granular cytoplasmic staining was classified a negative result as described previously (11).

ALK reverse transcriptase-polymerase chain reaction (RT-PCR) detection

The total RNA from tissue sections containing at least

Table 1 Clinicopathological features of the enrolled 101 patients

Characteristics	ADC cases (n=101)	*ALK* rearrangement	P
Primary site	101	20	
Paired metastatic lymph nodes	101	18	
Sex			0.122
Women	50	13	
Men	51	7	
Age (y)			<0.001
Median	60	52	
Range	35–78	35–69	
Smoking history[a]			0.012
Smokers	40	3	
Non-smokers	61	17	
Clinical stage			0.021
II	26	1	
III–IV	75	19	
Predominant pattern			0.024
Solid	22	9	
Acinar/papillary	76	11	
Micropapillary	3	2	

Note: [a], definition of smoking history: nonsmokers had smoked less than 100 cigarettes in their lifetime. The rest were categorized as smokers. ADC, Adenocarcinoma.

70% tumor cells was extracted and converted to first-strand cDNA according to the manufacturer's instructions (Amoy Diagnostics, Xiamen, China). This AmoyDx *EML4-ALK* Fusion Gene Detection kit is designed to qualitatively detect 21 *EML4-ALK* fusion transcripts in four reaction mixtures (including reference gene beta-actin) with a declared sensitivity of 1% mutant DNA. Real-time PCR was performed on in a 7500 real-time PCR system and analyzed by SDS 2.0 system software (Applied Biosystems, Foster City, CA). As defined by the manufacturer's instructions, cycle threshold (CT) values ≤30 of the samples detection was interpreted a positive result as described previously (11-13).

ALK fluorescence in situ hybridization (FISH) analysis

When *ALK* rearrangement was discordant between IHC and RT-PCR, FISH evaluation was further performed according to the guidelines of the LSI *ALK* Dual Color, Break-apart Rearrangement Probe kit (Abbott Molecular, Abbott Park, Illinois), Briefly, for FISH, tissue sections were baked at 60 ℃ and deparaffinized, and then pretreated with protease solution at 37 ℃ for 20 min. A thermoBrite (Abbott Molecular, IL, USA) was used for the denaturation step at 73 ℃ followed by hybridization at 37 ℃ overnight for 18 hours. After stringency wash steps, nuclear counterstaining with DAPI was performed. Sections were observed under a ×100 objective with a fluorescence microscope (Leica DM6000, Wetzlar, Germany) equipped with "Applied Imaging 4.0" analysis software (Genetix, England, UK) independently by two experienced pathologists. The positive tumor cells show split signals (at least two signals diameters apart) or a single red signal (deleted green signal) in addition to fused and/or split signals. If the average percentage of positive cells was ≥15% (≥15/100), the sample was considered to be positive.

Statistical analysis

Differences in demographic characteristics including age, gender, smoking history, histological subtype and TNM stage between *ALK*+ and *ALK*– patients were analyzed using pearson' chi-square test or Fisher's exact test. Two-sided P values <0.05 was considered statistically significant. Statistical calculations were performed using SPSS17.0 software (SPSS Inc., Chicago, IL, USA).

Results

Clinicopathological features of the enrolled patients

One hundred and one patients were enrolled into this study with a median age of 60 years old (range, 35–78 years). Among them, 50 (49.5%) were females, 61 (60.4%) were non-smokers and 20 patients (20/101, 19.8%) were found to have *ALK* fusion in either of the paired samples. The patients with *ALK* fusion were more likely in younger (P<0.001), Nonsmokers (P=0.012), high-stage disease (P=0.021) and predominantly solid tumor growth pattern (P=0.024). The clinicopathological characteristics are summarized in *Table 1*.

The concordance rate of ALK rearrangement between primary tumor and paired metastatic lymph nodes by immunohistochemistry (IHC) and reverse transcriptase-polymerase chain reaction (RT-PCR)

A total of 101 paired specimens (primary tumor site and

Table 2 *ALK* rearrangement concordance between primary tumor and paired metastatic lymph nodes by IHC and RT-PCR (99 of 101 cases)

Methods	Primary site (No.)	Paired metastatic lymph nodes (No.)	*ALK* rearrangement
IHC–	81	81	Negative
IHC+	18	18	Positive
RT-PCR–	81	81	Negative
RT-PCR+	18	18	Positive

IHC, immunohistochemistry; RT-PCR, reverse transcriptase-polymerase chain reaction.

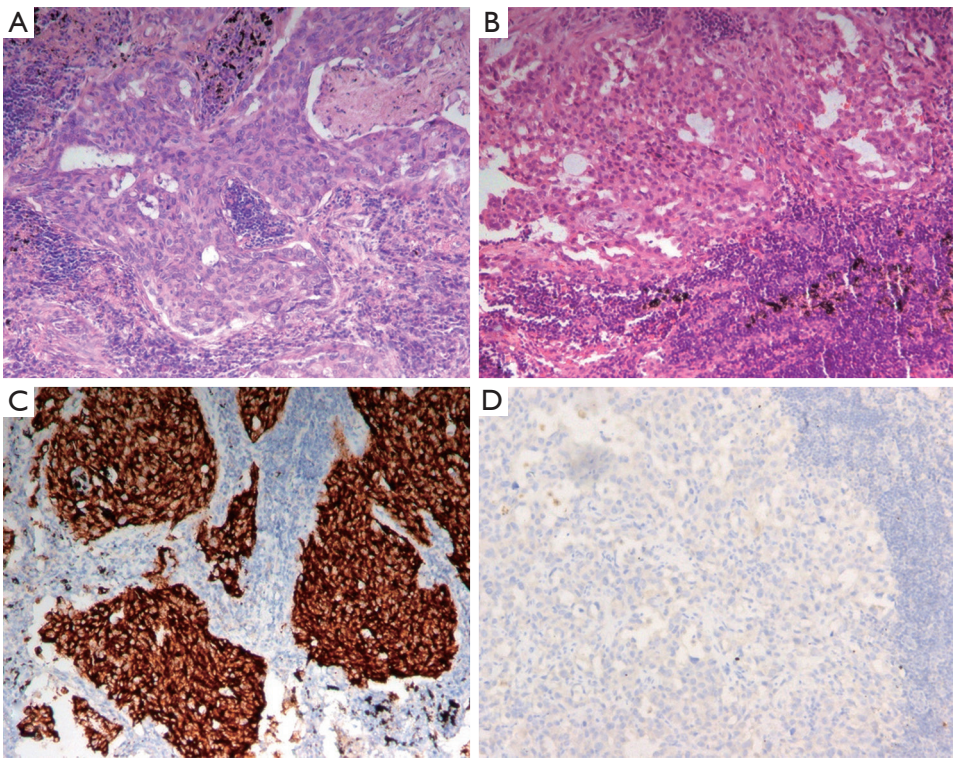

Figure 1 HE staining and *ALK* VENTANA D5F3 for detecting *ALK* rearrangement on primary tumor and paired metastatic lymph nodes. (A,B) HE staining on primary tumor and paired metastatic lymph node (100×); (C,D) *ALK* positive on primary tumor and negative on paired metastatic lymph node by *ALK* VENTANA D5F3 (100×).

paired metastatic lymph nodes) have to be confirmed as *ALK* rearranged. Of 101 paired specimens detected by IHC and RT-PCR, 81 paired specimens were both IHC and RT-PCR negative, 18 paired specimens were both IHC and RT-PCR positive (as shown in *Table 2*). Sixty-eight cases showed more than two stations of metastatic lymph nodes. *ALK* rearrangement was totally concordant between different stations of metastatic lymph nodes in the same patient (*Table S1*). The concordance rate of *ALK* rearrangement between primary tumors and paired metastatic lymph nodes was 98.0%.

The discordance of ALK rearrangement between primary tumor and metastatic lymph nodes may derive from the intra-tumoral heterogeneity of primary lesions

Two patients were confirmed with discordance of *ALK* rearrangement between primary tumors and paired metastatic lymph nodes requiring verification by a third methodology, FISH. One patient (Case #15) was IHC, RT-PCR and FISH positive in primary tumor, triples negative in paired metastatic lymph nodes (*Figures 1,2*). The other (Case #21) who was IHC and FISH positive in both primary

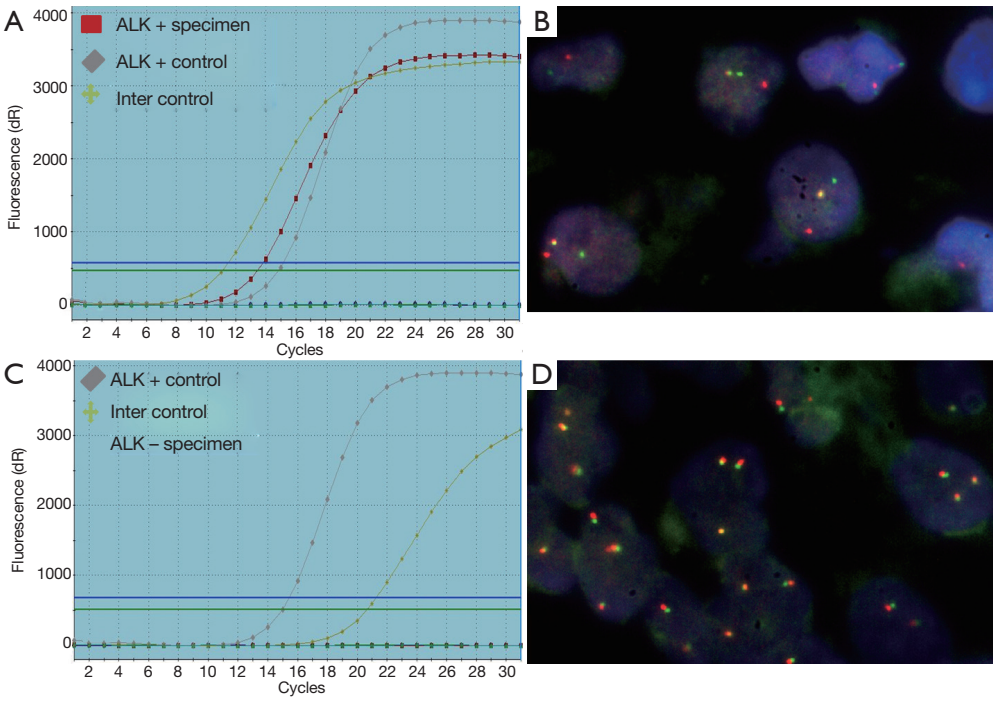

Figure 2 RT-PCR and FISH for detecting *ALK* rearrangement on primary tumor and paired metastatic lymph nodes in the same patients. (A,B) *ALK* positive on primary tumor by RT-PCR and FISH, respectively; (C,D) *ALK* negative on paired metastatic lymph node by RT-PCR and FISH in the same patient, respectively. RT-PCR, reverse transcriptase-polymerase chain reaction; FISH, fluorescence in situ hybridization.

tumor and paired metastatic lymph nodes was RT-PCR positive in primary tumor and negative in paired metastatic lymph nodes (*Table S2*). By IHC analysis of discordant Cases, We found that most of tumor cells were stained with strong granular cytoplasm in some areas of primary tumor section. In other areas of the same section, some tumor cells were negative in anti-ALK (D5F3) staining. *ALK* rearrangement showed intra-tumoral heterogeneity in different areas of the same tissue section (*Figure 3*). For case #21, RT-PCR was enough sensitive to detect the *ALK* rearrangement in the fewer fraction of tumor cells in metastatic lymph nodes, in which most of the metastatic tumor cells didn't contain *ALK* rearrangement by IHC and FISH. Two patients were both female, non-smokers and were diagnosed as adenocarcinoma with predominant solid growth pattern (as shown in *Table 3*).

Discussion

This study demonstrates a high concordance rate (98%) in the *ALK* rearrangement between primary tumors and paired metastatic lymph nodes. Two patients with *ALK* rearrangement on primary tumor didn't show *ALK* gene fusion on paired metastatic lymph nodes. In addition, *ALK* rearrangement was totally 100% concordant between different stations of metastatic lymph nodes in the same patient by IHC, RT-PCR or FISH.

EML4-ALK rearrangement was reported as a relatively low frequent rearrangement ranging from 1.5% to 6.7% among unselected Caucasian NSCLC patients (14-17). It seems to have higher frequency in Asian population, and was reported as high as 5.1–10% (18-21). Part of the explanation was that in those studies, patients were selected from *EGFR* wild population. Our previous study has shown *ALK* rearrangement could be as high as 32.3% among patients who have never smoked, and had adenocarcinoma harboring wild-type *EGFR* (13). In line with these results, we found the incidence of *ALK* rearrangement was 19.8% in patients with wild-type *EGFR*. The subgroup analysis showed that *ALK* rearrangement was more likely to occur in patients who were younger, never smokers and with adenocarcinoma histology, all of which were consistent with previous studies (22-24).

Intratumor heterogeneity has been described recently

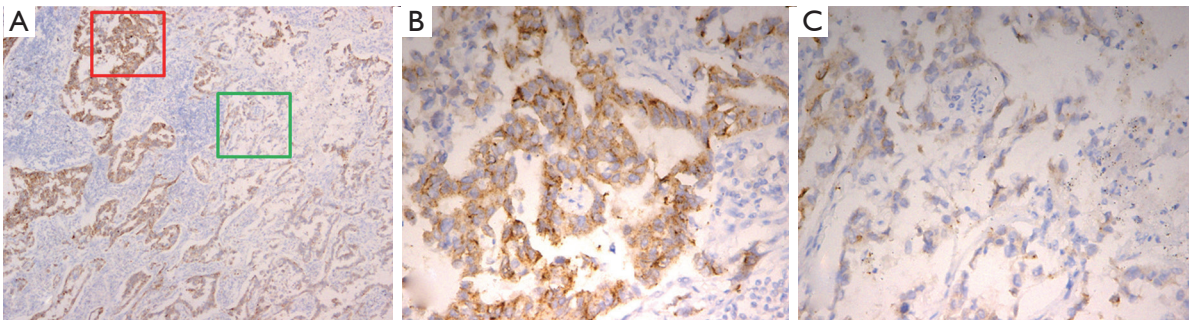

Figure 3 The heterogeneous strong granular cytoplasmic staining in some tumor cells of different primary tumor areas by IHC. (A) Heterogeneous granular cytoplasmic staining pattern throughout the section (40×); (B) strong granular cytoplasmic staining in most tumor cells (red square area's magnification in *Figure 3A*, 200×); (C) most tumor cells without strong granular cytoplasmic staining pattern (green square area's magnification in *Figure 3A*, 200×). IHC, immunohistochemistry.

Table 3 Characteristics of patients with discordance between *ALK* rearrangement results in primary tumor and paired metastatic lymph nodes

Patients (No.)	IHC	RT-PCR	FISH	*ALK* fusion	Age (years)	Gender	Smoking history	Predominant pattern
No. 15					50	Female	No	Solid
PS	+	+	+	Positive				
PMLN	–	–	–	Negative				
No. 21					48	Female	No	Solid
PS	+	+	+	Positive				
PMLN	–	–	–	Negative				

PS, primary tumor; PMLN, paired metastatic lymph node. IHC, immunohistochemistry; RT-PCR, reverse transcriptase-polymerase chain reaction; FISH, fluorescence in situ hybridization.

and poses a significant challenge to personalized cancer medicine. It was reported that activating *EGFR* mutations were found to display intratumor heterogeneity and spatial discordance in 6.3–30% of cases in lung cancer (25,26). However, intratumor heterogeneity of *ALK* rearrangement was rarely evaluated. Rossi *et al*. firstly reported that the discrepancy of *ALK* rearrangement in a heavy smoking 54-year-old man with pulmonary adenocarcinoma. This patient showed *ALK* rearrangement negative on primary lesion while harboring *ALK* fusion in metastatic peritoneal biopsy specimen 8 month later (27). In the present study, the primary and paired samples were collected simultaneously after surgical resection. Unlike *EGFR* mutation, our result showed a high concordance between primary tumors and paired metastatic lymph nodes. What's more, Two patients with discordant *ALK* rearrangement showed an *ALK* fusion positive in primary tumor while not in paired metastatic lymph nodes. Recent study reported that *ALK* rearrangement was discordant among spatially separated tumor areas in the same primary tumor of *ALK* positive patient by RT-PCR. It therefore seems reasonable to infer that metastatic tumor cells may come from the *ALK* negative tumor cells in primary lesion because of the genetic intratumoral heterogeneity (28). Different clones and innate tumor heterogeneity of samples may attribute to the discrepancy (29-31).

Previous studies reported that IHC assay was a valuable tool for the screening of patients with *ALK* rearrangement in clinical practice (7,32,33), IHC with new antibody and modified protocols will extend its serviceable range for detecting *ALK* rearrangement (34). Although FISH was approved by Food and Drug Administration as a companion diagnostics, FISH required specialized equipment and highly trained and skilled readers for interpretation because of variability between inter-observers. However, surgical samples were only available in minority patients

with NSCLC. Several studies investigated the feasibility of cytological sample obtaining from minimally invasive procedures like EBUS-TBNA or CT-guided fine needle biopsy for molecular testing (35-40). A substantial part of patients had insufficient tumor cell number for FISH testing after successful differential diagnosis for NSCLC subtype by IHC marker. RT-PCR is a sensitive method for detecting *ALK* rearrangement and had an advantage to utility of cytological samples. Thus, RT-PCR is also regarded as an acceptable method to detect *ALK* rearrangement by Chinese FDA (41,42). Our study also found that FISH, Ventana IHC and RT-PCR have good concordance though one case showed different results. The efficacy of *ALK* inhibitors detected by RT-PCR need be further evaluated in clinical trials or clinical practice (43).

Several limitations have to be mentioned in this study. Firstly, all the enrolled patients were resected, thus the finding from this study may not represent the whole population. Secondly, most of the patients are still not received the treatment of *ALK* inhibitor, thus we could not observe the response to *ALK* inhibitor from the patients who showed a discordant result on primary and metastatic lymph nodes. Thirdly, the samples of this study are still small though enrolling more than 100 patients. Similar results were observed in previous study which showed *ALK* arrangement was heterogeneous in 67 paired NSCLC specimens between primary tumors and their corresponding metastatic lesions by FISH (44), Thus, large scale study with the data of efficacy to *ALK* inhibitor is warranted to further support the findings in this study.

In conclusion, we have demonstrated that high concordant rate of *ALK* rearrangement between primary tumors and paired metastatic lymph nodes through three different detection methods. Though *ALK* rearrangement was found positive in the primary tumor while not in metastatic lymph nodes in the two discordant patients, primary tumor and metastatic lesions were equally suitable to perform *ALK* rearrangement testing for therapeutic strategies in patients with advanced NSCLC.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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Supplementary

Table S1 *ALK* rearrangement concordance between different stations of metastatic lymph nodes by IHC and RT-PCR (68 of 101 cases)

Methods	Cases with N1 (No.)	Cases with N2 (No.)	*ALK* rearrangement
IHC–	66*	58	Negative
IHC+	2	2	Positive
RT-PCR–	66*	58	Negative
RT-PCR+	2	2	Positive

Note: *, 66 cases were diagnosed with metastatic lymph nodes, including 58 cases with N1 and N2 metastatic lymph nodes, 8 cases with 2 stations of metastatic lymph nodes in N1. IHC, immunohistochemistry; RT-PCR, reverse transcriptase-polymerase chain reaction.

Table S2 Average CT value of *ALK* positive paired tumors of 20 cases using RT-PCR method

Patient number	Average CT value (FAM)	
	Primary tumor site	Metastatic lymph nodes
Case #01	18.3	19.5
Case #9	12.8	16.6
Case #11	23.3	24.1
Case #17	13.1	13.7
Case #20	21.6	22.3
Case #29	17.8	18.2
Case #31	23.5	23.9
Case #35	17.3	18.6
Case #41	18.8	20.4
Case #45	21.2	22.6
Case #47	16.4	18.8
Case #48	23.6	24.6
Case #53	20.5	20.6
Case #61	15.1	16.8
Case #77	23.6	24.1
Case #89	18.8	20.6
Case #90	23.2	24.3
Case #93	21.4	22.7
Case #15	20.1	0
Case #21	19.5	27.8

RT-PCR, reverse transcriptase-polymerase chain reaction; FAM, Reporter Dye.