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Original Article

Diagnostic value of a novel real-time reverse transcription-polymerase chain reaction for detection of ALK rearrangement in lung adenocarcinoma

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Abstract: The aim of the present study was to evaluate the diagnostic value of real-time reverse transcription-polymerase chain reaction (RT-PCR) for detection of anaplastic lymphoma kinase (ALK) fusion in lung adenocarcinoma patients. We examined 96 lung adenocarcinoma by fluorescence in situ hybridization (FISH) and real-time RT-PCR. Among 96 cases tested, 8 (8.33%) and 10 (10.42%) cases were ALK positive by FISH and real time RT-PCR, respectively. All the FISH-positive cases were real time RT-PCR positive. Two RT-PCR-positive cases showed FISH negative, but their ALK rearrangement was confirmed by direct sequencing. The total accordance rate between ALK real time RT-PCR and FISH was 97.96%. These results suggest that real time RT-PCR is a reliable screening tool in routine pathologic laboratories for identification of patients with ALK rearrangement for targeted therapy in lung adenocarcinoma.

Keywords: Lung adenocarcinoma, ALK, fluorescence in situ hybridization, real time RT-PCR, targeted therapy

Introduction

Lung cancer is the leading cause of cancer-related death in Europe and USA with metastasis responsible for >70% of deaths. The majority of late stage lung cancer patients die within 18-months of diagnosis [1]. The currently adopted treatment regimen for advanced non-small cell lung cancer (NSCLC) patients is first-line platinum-based drug chemotherapy combined with third generation agents (i.e. paclitaxel, gemcitabine, vinorelbine or docetaxel). Second line therapies clinically approved include erlotinib and pemetrexed. Five-year survival for advanced NSCLC patients with these therapies is currently less than 5% [2]. Further progress has been made more recently with improved understanding of the molecular mechanisms involved in oncogenesis [3]. These advances enabled the development of drugs that target cancer cell specific gene alterations. These targeted drugs significantly improved response rates and progression-free survival (PFS) in patients with specific genetic alterations [4]. Recent studies indicated inhibition of anaplastic lymphoma kinase (ALK) showed similarly promising response in patients with ALK fusion-bearing NSCLC [5-7].

Soda M originally identified the echinoderm microtubule-associated protein-like 4-ALK (EML4-ALK) fusion in NSCLC [7]. This fusion results from a small inversion within chromosome 2p, resulting in many fusion partner such as EML4, KIF5B [8], KLC1 [9] and TFG [10]. The incidence of ALK gene rearrangement appears to range from 2% to 7%, and most EML4-ALK fusion carcinomas are wild type for EGFR, KRAS [7, 11].

Clinical trials showed that an ALK inhibitor, Crizotinib, demonstrated promising results in patients with ALK positive locally advanced or metastatic NSCLC [6]. However, lung cancer with the ALK fusion constitutes only a small fraction of lung cancers, therefore, accurate identification of EML4-ALK lung adenocarcinomas is essential for the selection of appropriate therapy.

EML4-ALK fusion carcinomas can be identified by immunohistochemistry (IHC), fluorescence in situ hybridization (FISH), or reverse transcription polymerase chain reaction (RT-PCR) [12-15]. Each analytical method has its advantages and disadvantages, but no agreement has yet been reached on the optimal technique for clinical testing. In this study, we introduced a real time RT-PCR to determine ALK status, together with another reported FISH assay in 96 patients with primary lung cancer. We studied the concordance between FISH and real time RT-PCR to determine the optimal methodology for EML4-ALK testing in a reference laboratory setting.

Patients and methods

Patients

Ninety-six patients had received curative surgery at the First Affiliated Hospital of Sun Yat-sen University, Guangzhou, China, between December, 2012 and August, 2014. All these tumor samples were fixed in 10% neutral buffered formalin for 24-48 h and embedded in paraffin and routinely diagnosed as primary lung adenocarcinoma. The use of identified human tissue for this study was approved by the medical ethics committee of Sun Yat-sen University.

Fluorescence in situ hybridization

The FISH analysis was performed on formalin-fixed, paraffin-embedded tumor tissue using the LSI ALK Dual Color, Break-apart Rearrangement Probe (Abbott Molecular, Abbott Park, Illinois) according to the manufacturer's instructions. Cell line H2228 (positive for EML4-ALK variant 3a/b) and an ALK rearranged anaplastic large cell lymphoma were used as positive controls. Fused (yellow) or adjacent, touching, orange and green signals were classified as negative for ALK rearrangement. ALK rearrangement was concluded if the orange and green signals were not touching (split). Splits less than 2 signal distances apart were classified as rearranged, given the nature of the 2p23 inversions. The reference range for ALK rearrangement was established in normal lung tissue and indicated a cut off of ≥15% split signals, using the criteria defined above. Three viewers independently scored 100 tumor nuclei from each tissue to determine interobserver agreement.

Real-time RT-PCR

The EML4-ALK fusion mRNA was readily detected by PCR using AmoyDx EML4-ALK Fusion Gene Detection Kit (Amoy Diagnostics, Xiamen, China) according to manufacturer's instruction. Generally, total RNA was extracted with AmoyDx FFPE RNA Kit (Spin Column) from 5-10 μm thick FFPE sections with at least 30% tumor cells. For each sample, 100-500 ng of extracted RNA was used for reverse transcription into cDNA at 42°C for 1 h. Real-time PCR was then carried out in each of the four reactions of the EML4-ALK Fusion Gene Detection Kit according to the manufacturer's protocol. Reaction amplifies EML4-ALK variants 1, 2, 3a/b, 5', and 5a/b. All assays were performed on a Roche LightCycler 480 QPCR instrument. The following PCR procedure was used: an initial denaturation at 95°C for 5 min, followed by 15 cycles of 95°C for 25 s, 64°C for 20 s, 72°C for 20 s to ensure the specificity; and 31 cycles of 95°C for 25 s, 60°C for 35 s, 72°C for 20 s to perform the data collection. The qualitative judgment is according to the fusion fluorescence signal. Assay reactions achieving Ct values of <24 cycles were considered positive for one of the variants detected by that reaction mixture. Housekeeping gene (beta-actin) was used to control the integrity of RNA. The amplified PCR products from some samples were subjected to direct sequencing, using AB3500xl DNA Sequencer (Applied Biosystems).

Results

ALK fluorescence in situ hybridization

Ninety-six lung adenocarcinoma patients were included in this study, consisting of 37 female and 59 male patients. The mean age is 56.3 years (range, 24-83 years). Of these, 8 (8.33%, of 96) demonstrated an ALK rearrangement

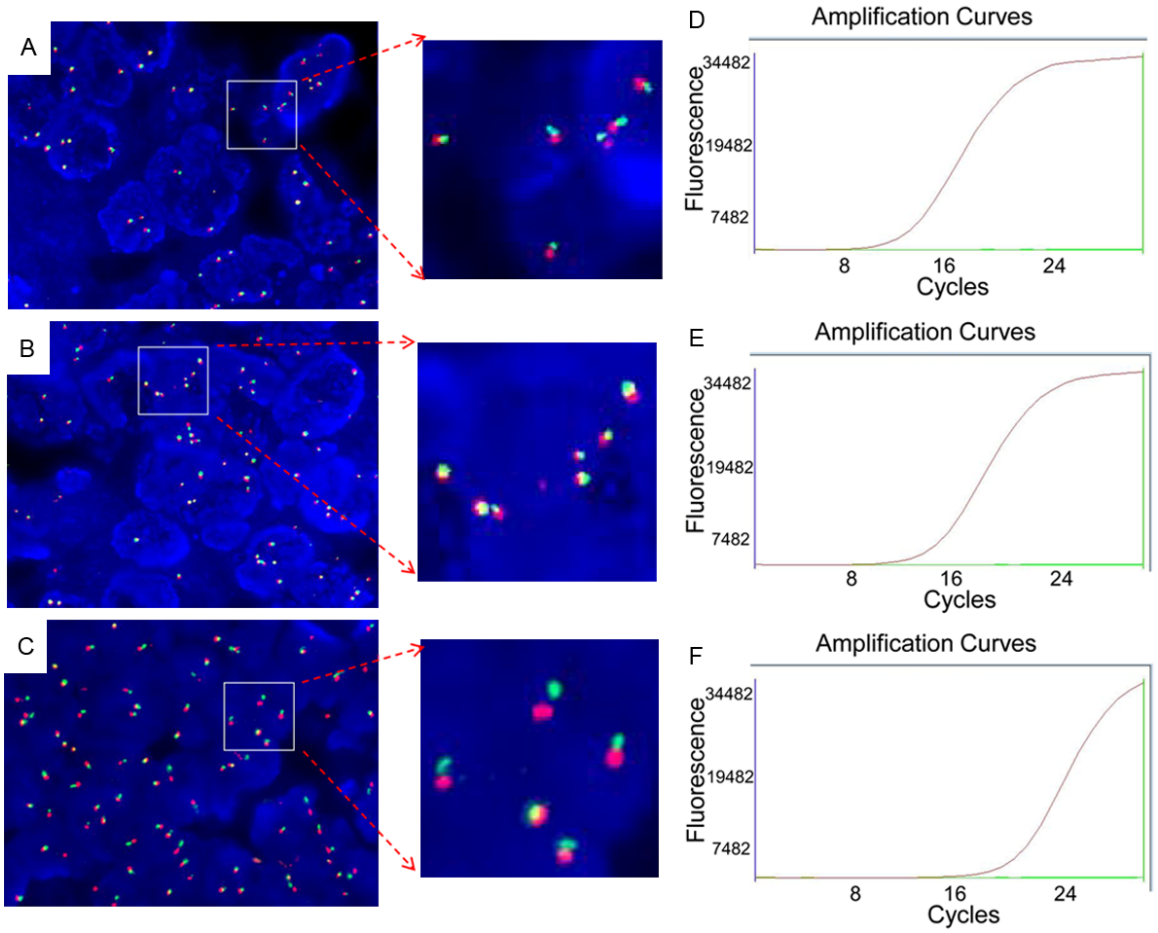

Figure 1. Pairwise detection of ALK fusion in lung cancer patients by FISH and real-time RT-PCR. A-C. FISH carried out with Vysis LSI ALK Dual color Break-Apart FISH probes detected ALK fusion as split red and green signals. FISH-negative cases showing intact two fused signals per nucleus. Original magnification X1000. D-F. RNA was extracted from the patients showed in the left panel, and then detected by real-time RT-PCR using. Graphs from real-time RT-PCR showing change in the reporter signal against PCR cycle number.

(FISH positive), and 88 cases were FISH negative (**Figure 1**; **Table 1**). The FISH-positive cases included 3 women and 5 men (**Figure 2**; **Table 1**). On FISH examination, split pattern and unbalanced rearrangement were observed in all cases.

ALK real-time RT-PCR

Using real-time RT-PCR, 10 cases (10.42%, of 96) were identified to have EML4-ALK rearrangement, including 4 cases of EML4-ALK fusion variants E13-A20, and 6 cases of variants E6-A20 (**Figure 2**; **Table 1**).

Correlation of ALK FISH and real-time RT-PCR

As showed in **Table 1**, all eight (100%, of 8) patients detected as positively by FISH were real-time RT-PCR positive, while 86 (97.73%, of 88) ALK FISH negative cases were real-time RT-PCR negative. The total accordance rate between ALK FISH and ALK real-time PCR was 97.96%. As for the paradox No. 15 and No. 56 samples, we further send them for direct sequencing. In agreement with our real-time RT-PCR data, sequencing data showed EML4-ALK rearrangement.

Discussion

Although EML4-ALK positive carcinomas take up only 5% to 7% of all NSCLC cases, they represent a therapeutically important subcategory. NSCLC patients with EML4-ALK fusions showed an impressive responsiveness to the ALK tyrosine kinase inhibitor, crizotinib, in early phase clinical trial [6]. Therefore, identification

Table 1. ALK gene status among NSCLC patients detected by FISH assay and real time RT-PCR

Real time RT-PCR	FISH		Total
	Rearranged	WT	
Rearranged	8	2	10
WT	0	86	86
Total	8	88	96

Abbreviation: WT, wild type.

of appropriate patient population with reliable screening methods is the key to the overall success of tumor targeted therapies. Currently, several testing methodologies including FISH, IHC, and RT-PCR have been used for the EML4-ALK fusions examination. But each of them, inevitably, has its own advantages and disadvantages. Although FISH has been used to enroll patients in clinical trials, the optimal technique for large-scale screening in a clinical setting has not yet been determined. ALK immunohistochemistry has several desirable characteristics, such as low cost, timeliness of performance, relative ease. However, commercially available ALK antibodies have been reported to lack the sensitivity to detect the EML4-ALK fusion protein [16].

In this study, we introduced a novel real time RT-PCR and retrospectively compared it with FISH for the detection of ALK rearrangement in 96 lung adenocarcinoma. Using FISH as the standard procedure, we demonstrated that the novel real time RT-PCR is a highly sensitive (100%) method for the detection of the ALK rearrangement in lung adenocarcinoma. For two real time RT-PCR-positive but FISH-negative cases, their ALK rearrangements were confirmed by direct sequencing. The high sensitivity and specificity of the real time RT-PCR assay enables a reproducible, easy binary scoring system for evaluating ALK status.

Although a gold standard method for ALK-positive NSCLC had not been established, ALK positivity by FISH has been used as an eligibility criterion in clinical trials with crizotinib [6]. The break-apart probe allows detection of rearrangements, independent of the fusion partners or specific breakpoint. However, ALK gene alteration in NSCLC is an intrachromosomal rearrangement; thus, the split signal of the break-apart probe is relatively close, leading to difficult interpretation of ALK FISH in NSCLC. It has been reported that FISH as an initial screening tool did not detect all cases with ALK-positive NSCLC, because it can be misinterpreted as normal [17]. Similar to the FISH assay, IHC can detect ALK independent of the fusion partner. Due to the low level of EML4-ALK transcriptional activity, early studies with commercial ALK antibodies and standard protocol showed that IHC was specific but not sensitive for the detection of lung cancer with ALK rearrangement [17].

Multiplex RT-PCR system has been the usual screening strategy applied for ALK gene rearrangements [18, 19]. The presence of fusion transcripts as detected by real time RT-PCR provides direct evidence of chromosomal translocation. Therefore, it is desirable to establish a sensitive and accurate detection method for ALK fusion protein based on RT-PCR. In this study, we demonstrated that real time RT-PCR showed 100% of sensitivity, when compared with FISH results.

It has been reported that some of the ALK RT-PCR-positive tumors showed no break-apart FISH signals [17, 20]. In our study, two cases demonstrated false-negative FISH results. However, they showed positive for ALK by both real time RT-PCR and direct sequencing. Because the EML4 and ALK loci are mapped relatively close on chromosome 2p, the subtle changes in fluorescent signal, caused by intrachromosomal inversion in some positive cases, might be difficult to interpret and might lead to false-negative results. The limited probe separation in such cases reduces the sensitivity of FISH assay. One NSCLC patient with complex ALK rearrangement that was negative by FISH analysis has been reported to be crizotinib-sensitive [21]. Real time RT-PCR is a fast and sensitive method for detection of expressed known EML4-ALK fusion variants for which specific primers have been designed [18]. In this study, the sensitivity and specificity of real time RT-PCR for detection of ALK fusion were 100%. We found that only 2 of the 96 cases screened had inconsistent real time RT-PCR and FISH results. Two FISH-negative cases showed positive results by real time RT-PCR, which were confirmed by direct sequencing of the PCR products.

Collectively, we report that the real time RT-PCR is a sensitive and specific screening method to detect ALK rearrangement in lung cancer. Our study demonstrated that ALK RT-PCR-positive

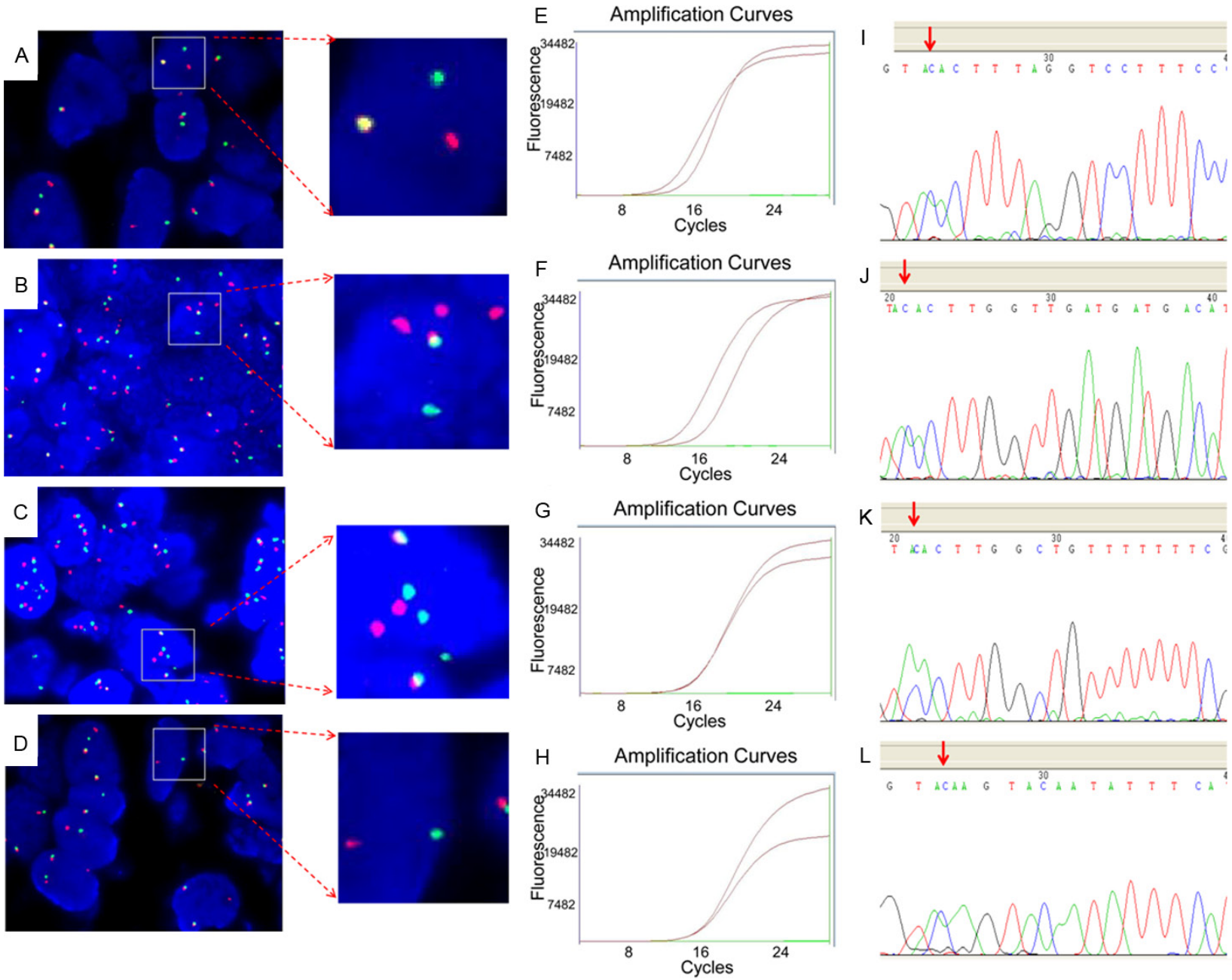
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Figure 2. Pairwise detection of ALK fusion in lung cancer patients by FISH, real-time RT-PCR and direct sequencing. A-D. FISH carried out with Vysis LSI ALK Dual color Break-Apart FISH probes detected ALK fusion as split red and green signals. A-D. FISH-positive cases representing split signals and isolated signals. Original magnification X1000. E-H. RNA was extracted from the patients showed in the left panel, and then detected by real-time RT-PCR using. Graphs from real-time RT-PCR showing change in the reporter signal against PCR cycle number. I-L. Direct sequencing showing ALK fusion in all the real-time RT-PCR positive cases.

but FISH-negative lung cancer did have rearrangement as confirmed by direct sequencing. Thus, this subgroup of patients should also benefit from ALK inhibitory therapy. Further clinical trials are required to address the predictive value of ALK real time RT-PCR in these patients.

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Disclosure of conflict of interest

None.

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