The Use of Quantitative Real-time Reverse-Transcriptase PCR for 5’ and 3’ portions of ALK transcripts to detect ALK Rearrangements in Lung Cancers

Rui Wang¹,²*, Yunjian Pan¹,²*, Chenguang Li¹,²*, Haichuan Hu¹,², Yang Zhang¹,², Hang Li¹,², Xiaoyang Luo¹,², Jie Zhang¹,², Zhaoyuan Fang³, Yuan Li¹,⁴, Lei Shen¹,⁴, Hongbin Ji³, David Garfield⁵, Yihua Sun¹,²#, Haiquan Chen¹,²#

¹ Department of Thoracic Surgery, Fudan University Shanghai Cancer Center, Shanghai 200032, China
² Department of Oncology, Shanghai Medical College, Fudan University, Shanghai 200032, China
³ Laboratory of Molecular Cell Biology, Institute of Biochemistry and Cell Biology, Shanghai Institute for Biological Science, Chinese Academy of Science, Shanghai, China.
⁴ Department of Pathology, Fudan University Shanghai Cancer Center, Shanghai 200032, China
⁵ Promed Cancer Centers, Shanghai, China

*R. Wang, YJ. Pan, CG. Li contributed equally to this work and should be considered co-first authors.
#Correspondence should be addressed to Haiquan Chen (Email: hqchen1@yahoo.com) or Yihua Sun (Email: sun_yihua76@hotmail.com)

Address for correspondence:
Haiquan Chen, MD, Department of Thoracic Surgery, Fudan University Shanghai Cancer Center, 270 Dong-An Road, Shanghai 200032, China.
Telephone : 86-21-64175590-1707

Running Title: Detecting ALK-arranged lung cancers by Real-time RT-PCR
Key words: Lung cancer; ALK; FISH.

Conflict of Interest Statement:

No potential conflicts of interest were disclosed.
Translational Relevance

Approximately 3-7% of non-small cell lung cancers (NSCLC) harbor an ALK gene fusion, thus defining a unique tumor group that may be responsive to targeted therapy. However, the method for diagnosis of ALK fusions has not been standardized. The breakpoint in ALK consistently occurs at exon 20 and EML4, although other fusion partners can also drive a strong expression of the ALK kinase domain. We have developed a rapid and accurate method for diagnosing ALK rearrangement in lung tumors by detecting the 5' and 3' portions of ALK mRNA, using Quantitative Real-time RT-PCR. This method has high reproducibility, sensitivity and specificity, thus facilitating identification of ALK-rearranged NSCLCs that may respond to ALK inhibitors in clinical practice.
Abstract

Purpose: Approximately 3-7% of non-small cell lung cancers (NSCLC) harbor an ALK fusion gene, thus defining a tumor group that may be responsive to targeted therapy. The breakpoint in ALK consistently occurs at exon 20 and EML4 or other fusion partners, thus driving a strong expression of ALK kinase domain and resulting in an unbalanced expression in 5’ and 3’ portions of ALK transcripts. We have developed a rapid and accurate method by simultaneously detecting the expression in 5’ and 3’ portions of ALK mRNA.

Experimental Design: Quantitative Real-time Reverse-Transcriptase PCR (qRT-PCR) was used to examine expression levels of the 5’ and 3’ portions of ALK transcripts in 177 NSCLCs in which EGFR, KRAS, HER2 and BRAF mutations were absent. If unbalanced ALK mRNA expression was seen, ALK rearrangement was assumed to exist. ALK fluorescence in situ hybridization (FISH) was used to confirm the accuracy of qRT-PCR. RT-PCR and 5’ RACE coupling sequencing identified the fusion variants.

Results: Real-time RT-PCR showed excellent sensitivity and specificity (100% and 100%, respectively) for detection of ALK rearrangements in resected specimens. In addition, six novel ALK fusion variants were identified, including one KIF5B-ALK (E17;A20) and five EML4-ALK variants (E6a;A19, E6a/b ins 18;A20, E17b ins 39;A20, E10a/b, E13;A20 and E17 ins 65;A20).
**Conclusions:** Real-time RT-PCR is a rapid and accurate method for diagnosing ALK-rearranged lung cancers. Coupling of 5’ RACE to this method should further facilitate rapid identification of novel ALK fusion genes.
Introduction

ALK rearrangement represents a novel molecular target in a subset of non-small cell lung cancers (NSCLC) (1). Fusion of ALK with the upstream partner, EML4, was found in NSCLC in 2007(2). This occurred due to chromosomal inversion on chromosome 2p, resulting in formation of the EML4-ALK fusion oncogene. Subsequently, other fusion variants and fusion partners, such as TFG (3) and KIF5B (4), have been identified. Notably, ALK rearrangements occur almost exclusively in adenocarcinomas (ADC) (5). More recently, crizotinib (ALK inhibitor) has been recommended as first line systemic therapy for lung cancer patients with ALK rearrangements (6). However, the method for detecting the fusion gene has not been standardized.

Several methods, including fluorescence in situ hybridization (FISH), reverse transcription-polymerase chain reaction (RT-PCR) coupled with direct sequencing and immunohistochemistry (IHC) are currently used for detecting ALK rearrangements in cytology or tissue samples. However, each technique is associated with specific strengths and weaknesses for screening. Current clinical trials of crizotinib use FISH as the diagnostic method for detecting ALK rearrangements (7). Unfortunately, FISH assay is a low-throughput assay requiring specialized technical resources and expertise and is thus not appropriate for detecting ALK rearrangements in large sample sizes. RT-PCR can define both ALK fusion partner and fusion variant, but cannot detect unknown ALK rearrangements. In addition, since there are a number of EML4-ALK variants and non-EML4-ALK fusions in NSCLCs, RT-PCR is not the optimal detection method. Previously, IHC seemed not to be economical or efficient (4). Unfortunately, ALK immune
reactivity is often weak and variable, making interpretation of results challenging. Recently, Exon Arrays examining expression levels of consecutive exons have been developed for detecting gene rearrangements (8). The breakpoint in ALK consistently occurs at exon 20 with the oncogenic effect of ALK rearrangement in NSCLC being due to EML4 or other fusion partners driving expression of ALK kinase domain (exon 20 to 29), with subsequent constitutive activation (5). In contrast, the remaining non-kinase region of ALK (exon 1 to 19) is not so expressed (8). Thus, we designed primer sets to amplify 5’ and 3’ portions of ALK mRNA. If unbalanced ALK mRNA expression was seen, ALK rearrangement was assumed to exist. Compared with other techniques, this assay seems to offer higher sensitivity, reduced complexity and a new method for detection.

**Materials and Methods**

**Specimen Collection**

A total of 654 frozen NSCLC tissue samples and their corresponding paraffin-embedded tumor tissues, including 617 (94.3%) ADCs and 37 (5.7%) adenosquamous carcinomas (ASCs), were collected from Oct. 2007 to Jun. 2011, with written informed consents from all patients. Tumor was obtained as previously described (9). The study was approved by The Committee for Ethical Review of Research at Fudan University Shanghai Cancer Center. Each case was reviewed and histological diagnosis confirmed according to World Health Organization criteria by two pathologists (L Shen and Yuan L). Four hundred and seventy-nine (73.2%) samples harbored known mutant kinases, including 400 EGFR, 53 KRAS, 16 HER2 and 10 BRAF, while 177 samples (26.8%) did not harbor any of the above mutations (“pan-negative” samples).
Exon Array

Exon array was performed as previously reported (8). mRNA was isolated using RNeasy Mini Kit (Qiagen, Valencia, CA, USA) as described by manufacturer’s protocol. rRNA was first removed with RiboMinus Human Transcriptome Isolation (Invitrogen) and cDNA obtained by Whole Transcript Sense Target Labeling. cDNA was fragmented and biotin-labeled using Whole Transcript Terminal Labeling (Affymetrix). Biotinylated targets were hybridized onto Affymetrix HuEX-1.0 exon array analysis (Affymetrix, Santa Clara, CA) following manufacturer's protocol. Arrays were scanned on GeneChip scanner 3000 7G.

Mutational Analyses

Mutational status of EGFR (exons 18-22), KRAS (exons 2, 3), HER2 (exons 18-21) and BRAF (exons 11-15) were assessed with standard reverse transcription-PCR-coupled direct sequencing. For detection of EML4-ALK fusions, primers were designed to amplify all known fusion variants, using cDNA, as described previously (10).

Quantitative Real-time PCR

mRNA was isolated by using RNeasy Mini Kit (Qiagen, Valencia, CA, USA) as described by manufacturer’s protocol. For mRNA analysis, an aliquot containing 2 μg of mRNA was transcribed reversely, using M-MLV reverse transcriptase and random primer (Invitrogen, Grand Island, NY, USA). Primers and conditions for qRT-PCR reactions are shown in Supplementary Table 1. qRT-PCR was done using SYBR Green PCR master mix (Applied Biosystems, Piscataway, NJ, USA) for a total volume of 10μl. Amplification protocol consisted of incubations at 95°C 10 min; 95°C 15s , 60°C 1 min, 40 cycles and detection by the ABI-Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA,
USA). Each sample was assayed in triplicate. Mean CT values of 5' or 3' portion of \textit{ALK} transcripts were calculated and the comparative CT method used to determine the quantity of target sequences in 3' portion of \textit{ALK} transcripts relative to 5' portion. Normalized expression value was calculated using the formula: 

\[ 2^{-\Delta\Delta CT = [(CT \text{ (3' portion of ALK)} - CT \text{ (B- actin))} - (CT \text{ (5' portion of ALK)}) - CT \text{ (B- actin))}]} \]

\textbf{Fluorescence in situ hybridization (FISH)}

\textit{ALK} rearrangements were detected in formalin-fixed, paraffin-embedded specimens by fluorescence in situ hybridization (FISH), using a commercially available break-apart probe for the \textit{ALK} gene (Vysis LSI \textit{ALK} Dual Color, Abbott Molecular, Abbott Park, IL, USA) in accordance with manufacturer's instructions. FISH was performed on the same tissue area. The probe hybridizes to band 2p23 on either side of the \textit{ALK} gene breakpoint. 5' \textit{ALK} signal was labeled with Spectrum Green (green), 3' \textit{ALK} signal with Spectrum Orange (orange). Criteria for probe signal interpretation in at least 100 interphase nuclei were as follows: i) separated green and orange signals or single red signal identified in at least 15% of tumor cells analyzed indicated rearranged \textit{ALK}; ii) overlapping of red and green signals (yellowish) indicated cells in which \textit{ALK} was not rearranged.

\textbf{5' RACE and RT-PCR}

Samples having high expression in \textit{ALK} kinase domain and positive for \textit{ALK} FISH, but negative for \textit{EML4-ALK} fusion genes, were subjected to 5'-RACE analysis. 5'-RACE was performed using SMARTer\textsuperscript{TM} RACE cDNA Amplification Kit (Clontech, CA, USA) as described by manufacturer’s protocol. The first tailed cDNA strand was obtained from 1 \mu g of total RNA. cDNA was amplified by PCR with a universal primer and ALK-SP1 primer (5'-
TCGTCCTGTTCAGAGCACACTTCAGG -3’) in ALK exon 22. Nested PCR amplification used the nested universal primer and ALK-SP2 primer (5’-GACACCTGGCCTTCAT ACACCTC-3’), which spans the ALK exon 21 region. Amplified PCR fragments were subjected to sequencing for identification of the fusion partner. KIF5B sequences were identified in one tumor (Case 93). RT-PCR was used to confirm the presence of KIF5B-ALK fusion transcripts in tumor and corresponding normal lung using the forward primer (5’-CAGAATTTCACAAGGAGGGAAG -3’) in exons 15 of KIF5B, and the reverse primer ALK-E20R (5’- TGCCAGCAAAGCAGTAGTTG -3’) in ALK exon 20. Amplified PCR fragments were sequenced for fusion partner identification.

**Immunohistochemistry (IHC)**

IHC staining was performed on 4 µm thick, formalin-fixed, paraffin-embedded tissue. Heat-induced epitope retrieval was performed with 1 mM EDTA, pH 9.0 (Maixin, Fuzhou, China). Slides were treated with 3% hydrogen peroxide for 15 minutes to block endogenous peroxidase activity. Mouse monoclonal anti-human CD246 (1:50 dilution, clone: ALK1, DAKO) was applied and incubated for 120 minutes. Slides were washed in PBS, and detected with horse radish peroxidase (HRP) conjugated anti-mouse Envision+ kit (DAKO), followed by counterstaining with hematoxylin. ALK IHC scores were assigned as follows: 3+, strong staining intensity in >5% tumor cells; 2+, moderate staining intensity in >5% tumor cells; 1+, faint or weak staining intensity in >5% tumor cells; and 0, no staining. IHC scoring was performed by two pathologists.

**Statistical analysis**

Data were analyzed using the Statistical Package for Social Sciences Version 16.0.
Results

Selection of primers for Quantitative Real-time PCR

Four out of 81 (4.9%) ADCs harbored ALK fusions by FISH. To confirm the chromosomal translocations leading to up-regulation of 3’ portion of ALK transcripts, samples were profiled on Affymetrix human Exon 1.0 arrays. Compared with expression in 5’ portion of ALK exons, four ALK-rearranged tumors showed higher expression level in 3’ portion (kinase domain). There were no significant differences in expression levels of ALK between 5’ and 3’ exons in 77 ALK wild type samples (Supplemental Figure 1). Based on these findings, qRT-PCR was applied to detect ALK rearrangements. Nine sets of primers were designed in 5’ portion of ALK mRNA (exons 4-18), and 5 sets in 3’ portion (exon 22-27), with qRT-PCR performed in 81 ADCs. Four sets of primers in 5’ portion and three in 3’ portion were eligible for detecting ALK expression. Results showed unbalanced ALK mRNA expression in only ALK fusion samples, which had a significantly higher expression in the 3’ than in the 5’ portion (Fig 1).

Screening of ALK rearrangements by qRT-PCR assay in “pan-negative” tumor samples

According to previous reports, EGFR, KRAS, HER2, BRAF and ALK mutations are almost always mutually exclusive. To validate feasibility in a larger sample size, we included 177 “pan-negative” cases for screening of ALK rearrangements by qRT-PCR. Patient characteristics are summarized in Supplemental Table 2. Of the 177 tumors, 40 (22.6%)
showed unbalanced expression in 5’ and 3’ portions of ALK mRNA and balanced expression in 137 of 177 (77.4%) (Fig.2). The normalized 3’ portion of ALK expression value ranged from 32.2 to 1573.7 (median value: 248.9) in samples with unbalanced and 0.45 to 11.8 (median value: 2.24) with balanced ALK expression. To confirm ALK rearrangements, all cases were subjected to ALK FISH and RT-PCR. Samples with unbalanced expression were confirmed positive for ALK FISH, while 137 tumors with balanced expression were negative (Fig.3). ALK fusions existed in 36 of 617 (5.8%) ADCs and 10.8% (4 of 37) of ASCs. Two cases with unbalanced ALK expression were originally misdiagnosed and classified as ALK FISH-negative (cases 81 and 93). Because tumor content in case 81 was lower than 10%, the FISH result was misinterpreted, the original FISH analysis re-reviewed and was incorrect. Case 93 was an ASC. The cause of mis-diagnosis was due to only the ADC component being positive for ALK FISH.

According to the relative expression in 3’ portion of ALK mRNA, NSCLCs could be divided into two subtypes: unbalanced ALK expression (relative expression in 3’ portion higher than 32), and balanced expression (relative expression in 3’ portion lower than 12). Even using 3’ portion of ALK expression value of 12 as a diagnostic criteria, qRT-PCR showed 100% sensitivity and specificity for detecting ALK-rearranged tumors. Diagnostic accuracy then was re-evaluated using two sets of primers (amplified 5’ and 3’ portions, respectively). High sensitivity and specificity for detecting ALK-rearranged tumors was achieved setting diagnostic criteria at 32 (Supplemental Table 3). Therefore, criteria for detecting ALK-rearranged tumors by qRT-PCR was felt best set at 12 when multiple sets of primers were applied and 32 when only two sets were used in the validation set. There were also
four cases found to be negative by RT-PCR but positive for FISH and qRT-PCR. Sensitivity of RT-PCR was 90 percent. ALK protein expression was detected in 90% of 40 ALK positive samples, which included scores of 0 (n=4), 1+ (n=6), 2+ (n=9) and 3+ (n=21) (Supplementary Figure 2). Relative expression levels in 3’ portion of ALK mRNA were significantly higher in samples with strong IHC staining than weak and moderate staining (665±106 vs 203.1 ± 67.7, p=0.002) (Table 1).

**Influence of tumor content on relative expression in 3’ portion of ALK transcripts**

Tumor content of snap frozen specimens and relative expression in 3’ portion of ALK mRNA are shown in Supplementary Figure 3. There was a trend for tumor content more than 50% having a higher relative expression in 3’ portion of ALK mRNA (328.7±374 vs 447.7±421.6), but without statistical significance (p=0.389). Of 7 ALK-positive samples with only 10% tumor content, the relative expression in 3’ portion of ALK mRNA was 51.6-685 (median: 101.1).

**Identification of novel KIF5B-ALK and EML4-ALK variants**

Previously unidentified were 6 ALK fusion variants, including one KIF5B-ALK and five EML4-ALK. Case 93 was positive for qRT-PCR and FISH but negative for RT-PCR, using primers spanning between exon 20 of ALK and reported EML4-ALK variants. Therefore, 5’RACE and sequencing were performed. A novel KIF5B variant and a fusion consisting of an in-frame fusion between exon 17 of KIF5B and exon 20 of ALK were found, confirmed by RT-PCR (Fig.4A).

A previously reported breakpoint of ALK gene occurred in exon 20 (2,4), one novel ALK
breakpoint (case 1523) being identified, an in-frame fusion between exon 6a of EML4 and exon 19 of ALK. This fusion point was different from those present in KIF5B-ALK and EML4-ALK previously identified (Fig.4B).

A novel isoform of EML4-ALK transcript with 53bp deletion in an original exon 10 was observed (case 988), mRNA splicing being referred to as exon 10b and the original exon 10 as exon 10a (Fig.4C). Also identified were 3 novel EML4-ALK variants with 18bp (case 1494), 39bp (case 981) and 65bp (case 737) insertions between EML4 and ALK exon 20. Extra base pairs (cases 1494 and 981) were mapped to ALK intron 19. cDNA would thus be expected to encode a fusion protein with an insertion of 6, 10, or 33 amino acids between EML4 and ALK sequences of the protein, respectively. Another novel isoform of EML4-ALK transcript with 58bp deletion in an original exon 17 was observed (case 981), with mRNA splicing being referred to as exon 17b (31bp) and the original exon 17 as exon 17a (69bp) (Supplemental Figure 4).

Discussion

We performed this study to evaluate the potential role of qRT-PCR as a diagnostic method for ALK-rearranged lung cancers. Its importance in NSCLCs relates to the apparently high predictive value of the gene translocation with regard to tumor sensitivity to ALK inhibitors. Fusion gene frequency is approximately 3%-7% (1), suggesting that over 90% of patients will not benefit from ALK inhibitors. Therefore, accurate and timely identification of such patients have important therapeutic implications.

We present a novel method for the diagnosis of lung cancers harboring ALK-rearrangements. By using a qRT-PCR assay to detect expression in 5’ and 3’ portions
of ALK transcripts, a cohort of 177 “pan-negative” lung cancers were screened, of which 40 (22.6%) samples harboring ALK arrangements were found. High sensitivity and specificity were achieved when using multiple sets of primers, selecting the relative 3’ portion of ALK expression value >12 as diagnostic. Therefore, qRT-PCR, alone, seems sufficient for diagnosis, pending finding new ALK fusion variants.

qRT-PCR offers high sensitivity, suggesting that the greater relative expression in 3’ portion of ALK mRNA has a high probability of representing an abnormal ALK gene. Currently, FISH, RT-PCR, and IHC are being evaluated for detecting ALK rearrangements. Although FISH is used for screening in clinical trials, it has limitations. As EML4-ALK results from a chromosomal inversion on chromosome 2p and because the two genes are normally only ~12 Mb apart, split signals may be subtle and easily overlooked (11). Previously, sensitivity varied between 93- and 98.6 percent (12-14). ALK rearrangements are infrequent and thus considered low-throughput so that FISH is not suitable for detecting ALK rearrangements in large sample sizes. Because FISH requires specialized technical resources, (such as fluorescence microscope) and expertise, it is not readily available. IHC is a readily available diagnostic tool, with three specified antibodies for ALK (ALK1 (15), D5F3 (12), 5A4 (17). Initial attempts to use ALK antibodies detecting ALK-rearranged tumors were disappointing (17). However, more sensitive ALK antibodies and techniques enhanced IHC signals so that IHC sensitivity has greatly improved. For example, the tyramide amplification method (using antibody ALK1) improves the ability to detect from 40% to 80% of specimens defined by FISH (12). By using IHC scoring, one report had a sensitivity of
90%, suggesting IHC can be used for screening in surgically resected specimens (15). We also used an ALK1 antibody (DAKO) in our initial screening attempts but some ALK rearranged tumors only displayed faint staining and a few wild type tumors were positive (Data not shown). These results showed that IHC may be promising but not optimal for screening.

qRT-PCR is rapid, simple, relatively high-throughput and inexpensive for detecting ALK rearrangement. This assay can be completed in 4 hours, with an easy-to-use protocol. Cost per patient is less than 10 dollars using SYBR Green PCR master mix (Applied Biosystems, USA). ALK FISH assays, however, require 2 days and at least $100 for each sample with the commercially available ALK probe (Vysis LSI ALK Dual Color, Abbott Molecular) and other FISH reagents. Furthermore, qRT-PCR can detect 16 samples with the 96-Well Detection System and 64 samples with the 384-Well Detection System by using two sets of primers each time. This relatively high-throughput assay is thus suitable for clinical screening.

Several factors, including loss of 5’ materials shown by FISH, polysomy, and tumor content can influence assay performance. Although expression level is very low or absent for wild type ALK, possibly loss of the 5’ ALK portion can lead to higher relative expression in the 3’ portion. In this study, four samples (cases 654, 781, 563, 636) had loss of 5’ material by FISH and showed high expression in the 3’ portion. Expression levels in 3’ portions of ALK mRNA were 78.4 (case 654), 103.2 (case 781), 879.2 (case 563) and 300 (case 636) -fold higher than the 5’ portion, respectively. Also, polysomy may influence performance of this method. In one study a few lung cancers harbored multiple copies of rearranged
chromosome (14). Thus, rearranged ALK mRNA expression level would be higher and more easily detected. However, we found no such cases. In addition, we demonstrated that Real-time RT-PCR is less prone to interference from non-tumor cells, with 10% tumor content being sufficient. Low tumor content samples, such as malignant pleural effusion and small biopsy samples, may be efficient and sensitive for detection.

Although our study demonstrates the promise of Real-time PCR for detecting ALK fusion, the inherently demanding nature of RNA may hamper its wide clinical application. Thus, careful sample processing for obtaining high quality RNA is important. Since reagents and instruments for Real-time PCR may be different, each laboratory trying to use this method should establish its own cut-off values. The enzyme might also sometimes decrease by using Oligo dT primers during reverse transcription, so that the 5' end is absent. Therefore, for obtaining more accurate results, random primers were used.

In conclusion, with wider clinical use of the ALK inhibitor, crizotinib, timely and accurate diagnosis of ALK arrangement NSCLCs are essential to identify those patients suitable for this target therapy. We believe that qRT-PCR assay is a preferred method for identifying such tumors in clinical practice.

Acknowledgements

We thank Qiong Lu, Shilei Liu, and Shihua Yao for their technical support.
Grant Support

This work was supported by the funds from National Natural Science Foundation of China(81101761, 81172218); Key Construction Program of the National “985” Project (985-YFX0102); Grants No. WJP1101 from Wu Jieping Foundation; and Grant from Shanghai Municipal Health Bureau.
References

5581-90.
Figure Legends

Figure 1: Relative expression in ALK 5' non-kinase region and 3' kinase region in lung adenocarcinomas (A). Schematic shows primers location on ALK (forward (↓) and reverse (↑)); (B), Relative expression of various ALK exon junctions in ALK wild type lung adenocarcinomas; (C), ALK kinase region expression higher than non-kinase region in ALK fusion samples. ALK expression value calculated using $2^{-\Delta CT}$ method: $2^{-\Delta CT} = 2^{[(CT (ALK \text{ kinase region})/ (ALK \text{ non-kinase region}) – CT (\beta\text{-actin}))]}$.

Figure 2: Detection of ALK fusions in 177 pan-negative lung tumors. Heatmaps of normalized exon junction expression values for ALK. Color scale illustrating level of expression is shown at bottom: red, higher values; green, lower values. Columns represent exon junction primer sets; rows represent lung tumors. ALK fusion samples showed significantly higher mRNA expression in ALK kinase region (3' portion) than non-kinase region (5' portion); ALK expression value calculated using $2^{-\Delta\Delta CT}$ method: $2^{-\Delta\Delta CT} = 2^{[(CT (3' \text{ portion of ALK mRNA}) - CT (\beta\text{-actin})) - CT (5' \text{ portion of ALK mRNA}) - CT (\beta\text{-actin})]}}$.

Figure 3: mRNA from 177 pan-negative samples analyzed by qRT-PCR and FISH for detecting ALK rearrangement. ALK expression value calculated using $2^{-\Delta CT}$ method. Data were normalized to expression of 5' portion of ALK mRNA expression. The expression levels in 5' portion of ALK mRNA were used as a calibrator. Red arrow= split red-green signals indicative of ALK rearrangement, yellow arrow= touching red-green signals not indicative of ALK rearrangement.
Figure 4: Identification of novel variants of $KIF5B$-$ALK$ and $EML4$-$ALK$. All break points of $ALK$ transcripts allow resulting fusion to harbor kinase domain of $ALK$ (red); A, fusion between exon 17 of $KIF5B$ and $ALK$ exon 20 in case 93; B, exon 6a of $EML4$ fused to exon 19 of $ALK$; C, exon 13 of $EML4$ fused to exon 20 of $ALK$ with two $EML4$ splicing variants exon 10a and exon 10b in case 988.
Table 1 Correlation of qRT-PCR, FISH, RT-PCR and IHC

<table>
<thead>
<tr>
<th>Cases</th>
<th>3'/5'</th>
<th>FISH</th>
<th>RT-PCR</th>
<th>IHC</th>
<th>Pathol.</th>
</tr>
</thead>
<tbody>
<tr>
<td>782</td>
<td>49.5</td>
<td>P</td>
<td>V3a/b</td>
<td>-</td>
<td>A</td>
</tr>
<tr>
<td>981</td>
<td>64.8</td>
<td>P</td>
<td>E17b ins 39;A20</td>
<td>-</td>
<td>A</td>
</tr>
<tr>
<td>654</td>
<td>78.4</td>
<td>P</td>
<td>N</td>
<td>-</td>
<td>A</td>
</tr>
<tr>
<td>460</td>
<td>241.3</td>
<td>P</td>
<td>V1</td>
<td>-</td>
<td>A</td>
</tr>
<tr>
<td>1474</td>
<td>63.1</td>
<td>P</td>
<td>N</td>
<td>+</td>
<td>A</td>
</tr>
<tr>
<td>399</td>
<td>78.2</td>
<td>P</td>
<td>V3a/b</td>
<td>+</td>
<td>A</td>
</tr>
<tr>
<td>490</td>
<td>130.6</td>
<td>P</td>
<td>V3a/b</td>
<td>+</td>
<td>A</td>
</tr>
<tr>
<td>636</td>
<td>300.2</td>
<td>P</td>
<td>V1</td>
<td>+</td>
<td>A</td>
</tr>
<tr>
<td>667</td>
<td>556.4</td>
<td>P</td>
<td>V1</td>
<td>+</td>
<td>A</td>
</tr>
<tr>
<td>93</td>
<td>867.1</td>
<td>P</td>
<td>N</td>
<td>+</td>
<td>AS</td>
</tr>
<tr>
<td>834</td>
<td>32.2</td>
<td>P</td>
<td>V1</td>
<td>++</td>
<td>A</td>
</tr>
<tr>
<td>732</td>
<td>36.7</td>
<td>P</td>
<td>V2</td>
<td>++</td>
<td>A</td>
</tr>
<tr>
<td>988</td>
<td>39.6</td>
<td>P</td>
<td>E10a/b, E13;A20</td>
<td>++</td>
<td>A</td>
</tr>
<tr>
<td>788</td>
<td>51.6</td>
<td>P</td>
<td>V3a/b</td>
<td>++</td>
<td>A</td>
</tr>
<tr>
<td>1335</td>
<td>83.2</td>
<td>P</td>
<td>V1</td>
<td>++</td>
<td>A</td>
</tr>
<tr>
<td>1004</td>
<td>88</td>
<td>P</td>
<td>V1</td>
<td>++</td>
<td>A</td>
</tr>
<tr>
<td>781</td>
<td>103.2</td>
<td>P</td>
<td>V3b</td>
<td>++</td>
<td>A</td>
</tr>
<tr>
<td>1003</td>
<td>121.9</td>
<td>P</td>
<td>V1</td>
<td>++</td>
<td>AS</td>
</tr>
<tr>
<td>578</td>
<td>494.6</td>
<td>P</td>
<td>V1</td>
<td>++</td>
<td>A</td>
</tr>
<tr>
<td>81</td>
<td>101.1</td>
<td>P</td>
<td>N</td>
<td>+++</td>
<td>A</td>
</tr>
<tr>
<td>1514</td>
<td>109.9</td>
<td>P</td>
<td>V1</td>
<td>+++</td>
<td>A</td>
</tr>
<tr>
<td>737</td>
<td>145.4</td>
<td>P</td>
<td>E17 ins 65;A20</td>
<td>+++</td>
<td>A</td>
</tr>
<tr>
<td>877</td>
<td>206.5</td>
<td>P</td>
<td>V2</td>
<td>+++</td>
<td>A</td>
</tr>
<tr>
<td>26</td>
<td>228.1</td>
<td>P</td>
<td>V1</td>
<td>+++</td>
<td>AS</td>
</tr>
<tr>
<td>245</td>
<td>248.9</td>
<td>P</td>
<td>V1</td>
<td>+++</td>
<td>A</td>
</tr>
<tr>
<td>1451</td>
<td>261.3</td>
<td>P</td>
<td>V1</td>
<td>+++</td>
<td>A</td>
</tr>
<tr>
<td>966</td>
<td>296.1</td>
<td>P</td>
<td>V2</td>
<td>+++</td>
<td>A</td>
</tr>
<tr>
<td>1228</td>
<td>393.4</td>
<td>P</td>
<td>V1</td>
<td>+++</td>
<td>A</td>
</tr>
<tr>
<td>1494</td>
<td>427.5</td>
<td>P</td>
<td>E6a/b ins 18;A20</td>
<td>+++</td>
<td>A</td>
</tr>
<tr>
<td>324</td>
<td>568.1</td>
<td>P</td>
<td>V2</td>
<td>+++</td>
<td>A</td>
</tr>
<tr>
<td>1523</td>
<td>685</td>
<td>P</td>
<td>E6a;A19</td>
<td>+++</td>
<td>A</td>
</tr>
<tr>
<td>584</td>
<td>714.1</td>
<td>P</td>
<td>E17;A20</td>
<td>+++</td>
<td>A</td>
</tr>
<tr>
<td>B56</td>
<td>731.5</td>
<td>P</td>
<td>V3a/b</td>
<td>+++</td>
<td>A</td>
</tr>
<tr>
<td>563</td>
<td>879.2</td>
<td>P</td>
<td>V1</td>
<td>+++</td>
<td>AS</td>
</tr>
<tr>
<td>1459</td>
<td>1144.1</td>
<td>P</td>
<td>V1</td>
<td>+++</td>
<td>A</td>
</tr>
<tr>
<td>591</td>
<td>1226.2</td>
<td>P</td>
<td>V3a/b</td>
<td>+++</td>
<td>A</td>
</tr>
<tr>
<td>964</td>
<td>1260.6</td>
<td>P</td>
<td>V2</td>
<td>+++</td>
<td>A</td>
</tr>
<tr>
<td>347</td>
<td>1341.8</td>
<td>P</td>
<td>V1</td>
<td>+++</td>
<td>A</td>
</tr>
<tr>
<td>B36</td>
<td>1422.8</td>
<td>P</td>
<td>V1</td>
<td>+++</td>
<td>A</td>
</tr>
<tr>
<td>1141</td>
<td>1573.7</td>
<td>P</td>
<td>V1</td>
<td>+++</td>
<td>A</td>
</tr>
</tbody>
</table>

P, positive; N, negative; *expression in 3’ portion of ALK mRNA relative to 5’ portion; Pathol., pathology; A: adenocarcinoma; AS: adenosquamous carcinoma.

V1,E13;A20, V2,E20;A20, V3a/b,E6a/b;A20.
Figure 2
Figure 3
Figure 4

A
KIF5B-ALK fusion transcript (Case 93)

B
EMI4-ALK fusion transcript (Case 1523)

C
EMI4-ALK fusion transcript (Case 988)
The Use of Quantitative Real-time Reverse-Transcriptase PCR for 5' and 3' portions of ALK transcripts to detect ALK Rearrangements in Lung Cancers

Rui Wang, Yunjian Pan, Chenguang Li, et al.

Clin Cancer Res  Published OnlineFirst July 12, 2012.

Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-12-0677

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2012/07/12/1078-0432.CCR-12-0677.DC1

Author Manuscript
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://clincancerres.aacrjournals.org/content/early/2012/07/12/1078-0432.CCR-12-0677. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.