Detection of EML4-ALK Fusion Gene Expression in Circulating Tumor Cells (CTCs) Captured from Non-Small Cell Lung Cancer (NSCLC) Patients

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ABSTRACT

Detection of EML4-ALK fusion gene from the NSCLC tissue has become a routine practice to identify eligible patients for the targeted drug therapy. However, there are only few reports for the detection of this unique fusion gene from the blood. The aims of this study were to determine the feasibility of RT-PCR in detection of EML4-ALK rearrangement in blood draws of NSCLC patients. CTC was captured by micofabricated porous filter device; CTC platform designed to enrich CTCs by cell size and developed by our group previously. Twenty-five patients; 19 FISH-proven ALK positive, 6 ALK negative in primary tumor tissue were prospectively enrolled. RNA was extracted from the captured CTCs and RT-PCR was done to identify EML4-ALK fusion transcript. There were 25 stage IV NSCLC patients and CTCs were detected in 12 (48%); numbers of CTCs ranged from 1 to 12 CTCs/ml. More importantly, EML4-ALK fusion transcript was identified by RT-PCR (optimized for variant type 1 EML4-ALK fusion transcript) in tissue proven ALK+ patients; 11/19 (58%) including 4 patients treated with ALK inhibitor and 11/15 (73%) patients without ALK inhibitor. Furthermore, no single EML4-ALK fusion transcript was found in tissue proven ALK negative patients. We have proved the existence of EML4-ALK transcript using RT-PCR after the enrichment of CTCs from the peripheral blood draws in NSCLC patients. Being able to reliably detect EML4-ALK fusion transcript from enriched CTCs may allow us not only to identify EML4-ALK+ patients with simple blood draws but also to monitor the diseases along with the treatment.

INTRODUCTION

Circulating tumor cells (CTCs) are cancer cells in transit from the primary tumor to metastasis shedding into the blood vessels. The number of CTCs are considered having a prognostic value among various cancers such as breast cancer, lung cancer, prostate cancer and etc. [1, 2] and furthermore, studies regarding CTC's role as a surrogate biomarker for targeted drug therapy has been increasing [3, 4]. There are two kinds of CTC enrichment technologies. One is biological techniques which use cell surface marker to capture the cancer cells. These have been commonly used by cell biologists for a long time, but there are quite a number of cancer cells being negative for the specific markers. The other one is using physical characteristics between normal blood cells and cancer cells including cell size, density, deformability, and electric properties. Although the CTC counts are overall positively correlated with the cancer stage, CTCs are heterogeneous so that any single marker such as EpCAM or CK can't enumerate all the CTC populations and therefore, there are many EpCAM or CK negative CTCs in lung cancer. [5] CTC enrichment using its physical properties followed by molecular analysis may be the promising tool to cover marker negative CTCs.

EML4-ALK fusion was first discovered from a never-smoker lung cancer patient in 2007 [6], and the ALK fusion has been identified in 3% to 7% of non-small cell lung cancer (NSCLC) patients [6-8]. The drugs targeting the kinase activity of ALK were shown to improve the prognosis of NSCLC with ALK fusions [9]. The response rate to ALK tyrosine kinase inhibitors are as high as 50~60% [9-11]. Usually, additional tumor tissue is required to perform genetic tests for the drugs targeting specific molecular alterations such as EGFR mutation or ALK fusion other than conventional pathological examination. The list of targets related to oncogene addiction has been thoroughly researched and become the most wanted candidate for targeted drug treatment in NSCLC patients [12]. For example,

ROS1 fusion was proven to be a reliable marker of drug sensitivity [13], and RET fusion [14], MET exon 14 skipping [15, 16] and BRAF mutations [16] also have been candidates for targeted drugs. Also, the presence of T790M mutation from second biopsy in NSCLC patients with activating EGFR mutations is predictive to third generation EGFR tyrosine kinase inhibitor [17, 18]. However, some patients have difficulties in getting sufficient amount of tumor tissue for genetic tests due to limited accessibility, or high risk of complications such as bleeding or pneumothorax after the tissue acquisition. Vanderlaan et al reported that tumor biopsy failed in up to 8~10% of lung cancer patients at the time of diagnosis [18]. Considering the complication rate at the beginning of the diagnosis, we would expect that more complications can be occurred at the time of recurrence and moreover, the performance would be deteriorated as well. Therefore, the non-invasive technique to identify and monitor the molecular genetic changes can be one of the most important steps in treatment of NSCLC patient in the era of molecular targeted therapy.

The aims of this study were to determine the feasibility of reverse transcription polymerase chain reaction (RT-PCR) in detection of EML4-ALK rearrangement from captured CTCs in advanced NSCLC.

RESULTS

Spiking experiment and RT-PCR for EML4-ALK fusion detection

To detect of EML4-ALK fusion in blood, CTCs were isolated by using microfabricated porous filter as previously reported [19]. The filter was designed to enrich CTCs by cell size so that cancer cells in blood are difficult to pass this filter. To identify the recovery rate and feasibility of detection of fusion transcript, spike-in tests were performed. The recovery rate of EpCAM positive and CD45 negative cells was 40 ~ 50% for both of H3122 and H2228 cell lines (Figure 1A & 1B). The fusion gene was successfully detected with 10% of

RNAs from the recovered samples after spiking 1000 H3122 cells (having variant 1type of EML4-ALK fusion) in 1 ml of normal blood, but not after spiking 10 or 100 cells (Figure 1C). This suggest that lower limit of detection (LOD) of our assay is a range of 10~100 ALK fusion positive cancer cells per ml of blood. Therefore, we used 5 times more RNAs after RNA extraction from the cancer patient samples. Serial dilutions of H2228 tumor cells harboring EML4-ALK variant type 3 were also spiked and examined by RT-PCR analysis with primers for the variant type 3 EML4-ALK fusion. However, the primers could not detect the type 3 fusion even in a spike of 1000 H2228 cells (data not shown). We tested EML4-ALK variant type 1 only for patient-driven samples, but not type 3, based on the spike-in test result.

Study patients and capturing CTCs

There were 25 stage IV NSCLC study patients (female: 16, male: 9) and their characteristics were summarized in Table 1. The total of 19 patients had ALK fusion genes identified from their tumor tissues, 5 patients had EGFR mutations and 1 patient had triple negative tumor: EGFR (-), KRAS (-), ALK (-). Among the 19 patients with ALK fusion tumors, 4 patients were treated with ALK inhibitor (all being crizotinib) and the rest of 15 patients had never been treated with ALK inhibitor (1 patient who were on ceritinib for 14 days was considered as no treatment with ALK inhibitor) at the time of blood draws; 4 patients were treatment naïve and 11 patients had treatment with cytotoxic chemotherapy.

Capturing CTCs and detection of EML4-ALK fusion script with RT-PCR technique

CTC enrichment and counting showed EpCAM and Cytokeratin positive CTCs were detected in 4 (36%) out of 11 study samples and the numbers of CTCs ranged from 1 to 2 CTCs/ml (Table 1). The CTCs were mostly round in shape and the nucleus was

surrounded by EpCAM and Cytokeratin (Supplementary figure 1). Detected EML4-ALK variant 1; a band of 420bp in both of H3122 cells, positive control for EML4-ALK and the study patient were described in Figure 3A. A patient was defined as EML4-ALK positive, if at least one fusion transcript was detected among triplicate experiments. Sanger sequencing was done to make a final confirmation for the RT-PCR product. Also, fluoresce nce in situ hybridization (FISH) analysis of tumor specimens in ALK-positive patient; the transcript of EML4-ALK variant 1 for this study patient was also verified by RT-PCR as well. (Figure 3B, C)

Sensitivity and specificity of EML4-ALK RT-PCR test in enriched CTC

We calculated the sensitivity and specificity of EML4-ALK RT-PCR using enriched CTCs compared to the fluorescence in situ hybridization (FISH) result of tumor tissues (Table 2A). EML4-ALK fusion transcript was identified by RT-PCR (optimized for variant type 1 of EML4-ALK) from the enriched CTCs in tissue proven ALK+ patients; 11 out of 19 (58%) including 4 patients treated with ALK inhibitor and 11 out of 15 (73%) patients treated without ALK inhibitor (Table 2B). Interestingly, treatment naïve 4 patients with ALK fusion had positive results of CTC RT-PCR. If we excluded 4 patients on treatment with ALK-specific inhibitors (crizotinib), the sensitivity increased to 73% (11 out of 15) (Table 2B). Also, there was no single EML4-ALK fusion transcript was found from the retrieved CTCs in tissue proven ALK negative patients. Therefore, the sensitivity and the specificity of RT-PCR in detection of EML4-ALK fusion gene expression from the CTCs were 58% and 100%, respectively.

A case of serial monitoring during ALK inhibitor

CTCs were collected both at baseline and during an ALK-specific inhibitor, ceritinib from a patient (LC24). He was treated with cytotoxic chemotherapy, pemetrexed and cisplatin (PC) as a first-line treatment, and his disease progressed after the 6th cycle of PC. The first CTC was collected at that time and RT-PCR from the retrieved CTCs was positive for EML-ALK fusion before ceritinib treatment, but converted to negative result 2 months after the treatment of ceritinib. Computed tomography which was performed at that time also showed a partial remission.

DISCUSSION

This study showed the feasibility of RT-PCR as a diagnostic tool to identify EML4-ALK fusion gene after enrichment of CTCs from the simple peripheral blood draws. This assay might be utilized as a non-invasive tool to monitor the molecular genetic changes without an additional acquisition of tumor tissue in NSCLC patients. It has been quite a challenge to acquire additional tumor tissue during the course of treatment from the inoperable NSCLC patients due to lack of optimal measurable lesions and increasing complication rate. Crizotinib is an oral small-molecule tyrosine kinase inhibitor and targeted for ALK, MET, and ROS1 kinases. Detection of EML4-ALK fusion gene to confirm CTCs and use it as a surrogate marker for treatment may be the most important issues in the era of precision medicine in these days. Therefore, the most important aim of this study was to determine the feasibility of RT-PCR in finding EML4-ALK fusion genes which can be used as a treatment target and biomarker for the drug therapy. We could find out that EML-ALK fusion transcript could be detected with RT-PCR techniques after the enrichment of CTCs using microfabricated porous filter which was developed by our group[19]. We had 58% of sensitivity in detecting EML4-ALK fusion (variant 1) with treatment naiive patients. However, if we take the patients without history of ALK inhibitors,

the sensitivity became 73 %; 11 out of 15 patients treated without ALK inhibitor in this study. Furthermore, no single EML4-ALK fusion transcript was found in tissue proven ALK negative patients; specificity is 100%. Recently, Yohida et al [20] reported the frequency of EML4-ALK fusion gene as follows. There were 35 NSCLC study patients; variant 1 (19, 54%), variant 2 (5, 14%), variant 3a/3b (4, 12%) and others (variant 3, 5, 5', 5b, E13ins60; A20, E12ins51; A20, E17ins27; A20: 7, 20%). This result seemed to consist with our result as well. But it would be much better to support our data with other variants types as well even though they are considered to be the minor portions of the NSCLC patients.

We observed a serial sampling result with an ALK inhibitor in this study. There was a change from EML4-ALK positive CTCs to negative CTCs during the short period of treatment with ALK-inhibitors (8 weeks). This suggested that monitoring CTCs with their genetic profile would give us useful clinical insights in understanding tumor behavior. Pailler et al. also observed decreased number and different rearrangement patterns of ALK rearranged CTCs after the treatment [21]. Sometimes confirmation of CTCs with detection markers such EpCAM and CK using IF can be very difficult to apply in clinical practice due to EMT phenomenon and inconsistency of surface markers. Many studies showed that EpCAM is variously expressed in cancer cells and especially not expressed in CTCs undergo epithelial to mesenchymal transition (EMT) and from mesenchymal origin [22]. And CK is also heterogeneously expressed in tumor, and downregulated in breast cancer cells [23]. Cytokeratin also have critical disadvantage as a capture antibody due to its low specificity among the various types of cancer cells. We have experienced both false positive and false negative results by IF staining to visualize captured CTCs and also, there are always existing purity issues due to WBC contamination even there are more CTCs than positive staining result. Therefore, we all know that there are both WBC contaminations and more CTCs were captured even with negative IF staining result.

Therefore, we have tried the molecular diagnosis using RT-PCR which would overcome the relatively low sensitivity and specificity of IF staining method in detecting CTCs.

Although this study suggested that RT-PCR could identify the EML4-ALK fusion from CTCs, there several obstacles and limitations to overcome. We would like to address the limitations of this study as well. First, there are some controversial issues regarding the accuracy of RT-PCR and FISH in identifying EML4-ALK fusion gene from the enriched CTCs. we need direct comparison of RT-PCR with FISH in identifying EML4-ALK fusion gene from CTCs. Pailler et al reported that EML4-ALK fusion gene was identified with 100% sensitivity and specificity from the captured CTCs with FISH probes [21]. In this study, one patient showed negative ALK FISH but positive RT-PCR for primary tumor assessment [21]. Wu et al compared diagnostic accuracy between RT-PCR and FISH in tumor tissues, and they found that five (42%) among 12 samples with positive RT-PCR showed negative by FISH [24]. They also showed that two among nine cases with positive FISH were negative by RT-PCR. Extrapolating EML4-ALK fusion gene status between CTCs and primary tissue, we believe that both tools, RT-PCR and FISH for identification of EML4-ALK fusion gene still have complimentary role for each other. Second, we need to define significance of variable types of EML4-ALK fusion genes. We used one set of PCR primers, optimized for variant type 1 of EML4-ALK, and this primer set can detect both type 1 and type 2 of EML4-ALK fusion transcripts. The relative frequency of type 1 is reported as variable depending on ethnicity. Although Caucasian, Japanese [24]and Korean [25] population showed predominance of type 1, Chinese population showed predominance of type 3 [24]. Type 1 comprises 80% of all EML4-ALK fusion in Korean lung cancer patients [25], but our primer set cannot detect other types (including type 3, the second most common type) than type 1 and 2. Therefore, if RT-PCR was positive for EML4-ALK fusion gene from the retrieved CTCs, the PCR products were sequenced to confirm the type of

EML4-ALK fusion genes in this study. All sequenced PCR products were confirmed to have type 1 fusions. Considering 80% of type 1 EML4-ALK positives in Korean lung cancer patients, 73 % of the positives from 15 patients treated without ALK inhibitor suggest that the false negatives may be few in this study. We tried to figure out a variant type from primary tumors especially with negative RT-PCR results in CTC. Due to limitation of tissue availability, we tried RT-PCR from two primary tumors whose CTC RT-PCR were negative. One showed type 1 variant from primary tissue, and the other showed negative to type 1 variant detection (data not shown). We can speculate that some negative cases would result from other types of EML4-ALK fusions or non-EML4-ALK fusions. Multiplexed RT-PCR will help to detect various types of ALK fusions.

We tried to examine EML4-ALK fusion detection in blood. As we previously developed a microfabricated porous filter device based on CTC enrichment [19], we added previously known RT-PCR for EML4-ALK fusion detection[19, 26]. We set up many clinical processes from blood preparation and storage in the hospital to the molecular analysis at another site and performed this translational research as a prospective study. The result suggests that reverse transcription polymerase chain reaction (RT-PCR) detection of EML4-ALK rearrangement is feasible with enriched CTCs. Our study included four patients (LC11, 13, 14 and 17) who were treated with ALK inhibitors with variable treatment periods (23, 6, 12 and 24 months, respectively) and progressed to them. Another one case was treated with an ALK inhibitor for 2 weeks, and her CTCs showed positive result of RT-PCR. We could expect that EML4-ALK fusion gene positive tumor population would be decreased during the crizotinib treatment and so forth CTCs as well. RT-PCR technique could find the EML4-ALK fusion transcript on the negative IF staining of CTCs patient. However, we have very small subset of patients and further investigation with large-set of study patients is required to validate this interesting phenomenon. Therefore, we are also planning for

further investigation of detecting EML4-ALK fusion gene with RT-PCR method in a large set of patients who are very homogeneous and analyzing follow-up samples during the course of treatment.

Here, we have proved the existence of EML4-ALK positive CTCs using RT-PCR after the enrichment of CTCs from the simple peripheral blood draw. Being able to reliably detect EML4-ALK fusion transcript from the enriched CTCs may allow us not only to find useful actionable targets and to monitor the diseases along the treatment with further investigation.

MATERIALS AND METHODS

Microfabricated porous filter device: CTC platform

Capturing CTCs was performed by microfabricated porous filter device which has been developed by our group previously [19, 26]. The principle of isolating CTCs was based on the theory that the most of the cancer cells have larger diameter than normal hematologic cells. Therefore, CTCs in blood would be remained on the platform of this micofabricated porous filter device while normal hematologic cells are easily passing through.

Spiking test with EML4-ALK positive cancer cells

Human NSCLC cell lines with EML4-ALK rearrangement, NCI-H3122 (H3122) and NCI-H2228 (H2228) were cultured in RPMI1640 medium supplemented with 10 % fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. For spike-in tests, 1 ml normal whole blood from healthy donors was spiked 10, 100, 500 and 1000 H3122/H2228 cells per 1ml of blood in triplicate. The whole process of CTC enrichment experiments was performed with 1 ml of blood samples as previously reported [19]. Whole blood samples spiked with the cancer cells were centrifuged for 20 minutes at 400 x g at

room temperature in the Ficoll-Hypaque solution (GE Healthcare, Piscataway, NJ), and then the PBMC layer was moved into a new tube. PBMCs were passed through the microfabricated porous filter by gravity. All the remaining cells on the filter were recovered into 1.5 ml Eppendorf tube by pipetting. The enriched cells were stained with the following three markers for CTC counting or used to extract total RNA for RT-PCR analysis. Captured cells were characterized by immunofluorescence staining; DAPI (4', 6-diamidino-2-phenylindole-2HCl) for nucleated cells, EpCAM or CK for cancer cells and CD45 for white blood cells (WBCs). For checking spike-in recovery rate, enriched H3122 and H2228 cells were counted under fluorescence microscope (Nikon Eclipse Ti-S, Nikon Corporation, Tokyo Prefecture, Japan).

Total RNA was extracted from the retrieved CTCs by using the Arcturus PicoPure RNA Isolation Kit (Life Technologies) according to the manufacturer's protocol. The purified RNA was eluted with 11 ul of nuclease-free water and the overall quality was assessed by electrophoresis and a NanoDrop-2000 (Thermo Fisher Scientific, Wilmington, DE, USA). 1 ul of the eluted RNA was used for WTA using the QuantiTect Whole Transcriptome Kit (Qiagen) according to the manufacturer's protocol. After whole transcriptome amplification, the RT-PCR for variant 1 of EML4-ALK fusion gene was performed in a volume of 20 ul containing 1X PCR buffer (including Mg^{2+}), 1mM dNTPs, 1U Taq DNA polymerase (TaKaRa), 1mM forward primer, 1mM reverse primer and 1ul of whole transcriptome amplified cDNA as template. Previously reported primers used for PCR amplification were the forward primers V3F (5'-GTCAGCTCTTGAGTCACGAGTT-3') and V1F (5'-GTGCAGTGTTTAGCATTCTTGGGG-3'), and the reverse primer ALK-R (5'-ATCCAGTTCGTCCTGTTCAGAGC-3') [19]. After an initial denaturation of 10 minutes at 95℃, 40 cycles of amplification were performed as follows: 95℃ for 30 seconds, 60℃ for

30 seconds, and 72℃ for 40 seconds. Quality of PCR products was checked by running in a 2% agarose gel.

Study patients

A total of 25 patients with NSCLC were prospectively enrolled in this study. All of the study patients were pathologically proven, and primary or metastatic tumor tissue was first screened for EGFR mutation and ALK fusion along with pathological examination. The schematic flow of capturing CTCs from the study patients as well as the final inclusion criteria were described in Figure 2. This study was approved by Institution Review Board of Seoul National University Hospital, Seoul National University College of Medicine (IRB No. 1209-029-424).

CTC enrichment and RNA extraction

5 to 10 ml of blood samples were drawn from the NSCLC patients and healthy donors. Acquired samples were collected in K_2EDTA anti-coagulants containing tubes and were processed within 5 hours. The samples from healthy donors were used as negative controls. CTC enrichment process from the study patients was performed with 5 ml of blood samples as previously reported [18]. PBMCs in 5 ml of blood samples were isolated by Ficoll-Hypaque density gradient centrifugation method and passed through the microfabricated porous filter. The enriched cells on the filter were retrieved and used for immunostaining or RNA extraction. RT-PCR was performed with 25 patients. Among them, 14 patents that have 10 ml of blood were in parellel analyzed by immunostaining. Because of limited amounts of blood, the other 11 samples that only has 5 ml of blood could not be analyzed by CTC counting.

EML4-ALK fusion detection by RT-PCR and Sanger sequencing

RT-PCR analysis was performed as described in spike-in tests. Only different thing is that 5 ul of the eluted RNA was used for WTA to increase the sensitivity. The DNAs in the expected PCR product size were cut from the gel and analyzed by Sanger sequencing to confirm the fusion site.

List of abbreviations

CTC, circulating tumor cell

FISH, fluorescence in situ hybridization

NSCLC, non-small cell lung cancer

RT-PCR, reverse transcription polymerase chain reaction

WBC, white blood cell

Competing Interest

We have the following interests. Co-authors, Du-Yeol Han and Byung-Hee Jeon are currently employed by Cytogen Inc. Co-authors, Eun-Hye Kim and Byung Chul Kim are former employee of Cytogen Inc.

Author contributions

PJK designed the study, analyzed the data and drafted the manuscript. KE-H carried out CTC enrichment and RT-PCR experiments, and drafted the manuscript. LD-W designed the study and performed a paper work for approval of ethical committee. KS set up RT-PCR and performed RT-PCR from tumor tissues. KB, KTM, KD-W, HDS provided study samples. HD-Y carried out CTC enrichment and RT-PCR experiments. JB-H designed the study and discussed with data. KBC designed the study, set up and supervised RT-PCR experiments, and drafted the manuscript. LS-H designed the study, provided study samples, performed a paper work for approval of ethical committee, analyzed the data and drafted the manuscript. All authors read and approved the final manuscript.

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Figure Legend

Figure 1: Spike-in test of CTCs and EML4-ALK RT-PCR using cell lines. A. Immunofluorescent images of recovered H3122 & H2228 cancer cells. H3122 & H2228 cancer cell (DAPI+, CD45-, EpCAM+) and leukocyte (DAPI+, CD45+, EpCAM-). All scale bars represent 5 μm. **B.** Overall efficiency after enrichment at two different cell loads: 10, 100 and 1000 H3122 & H2228 cells spiked. **C.** The mRNA expression of EML4-ALK fusion gene in spiked samples. The mRNA expression of fusion gene was measured by RT-PCR with variant type 1 primers. Upper and lower panels show the transcripts before and after enrichment, respectively. Beta-actin was used as a control for RNA integrity.

Figure 2: Schematic flow of capturing CTCs from the study patients.

Figure 3: RT-PCR detection of EML4-ALK positive NSCLC patients.

A. Identification of EML4-ALK variant 1; a band of 420bp was detected in both of H3122 cells, positive control for EML4-ALK and the study patient.

Expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a control.

A patient was defined as EML4-ALK positive, if at least one fusion was detected among triplicate experiments.

Abbreviations: *M, 100bp size marker; H3122, positive control; A549, negative control*

B. Sequence of EML4-ALK variant 1; the chimeric transcript was identified by Sanger sequencing analysis.

C. FISH analysis of tumor specimens in ALK-positive patient; the transcript of EML4-ALK variant 1 for this study patient was also verified by RT-PCR as well.

Detection of EML4-ALK Fusion Gene Expression in Circulating Tumor Cells (CTCs) Captured from Non-Small Cell Lung Cancer (NSCLC) Patients

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ABSTRACT

Detection of EML4-ALK fusion gene from the NSCLC tissue has become a routine practice to identify eligible patients for the targeted drug therapy. However, there are only few reports for the detection of this unique fusion gene from the blood. The aims of this study were to determine the feasibility of RT-PCR in detection of EML4-ALK rearrangement in blood draws of NSCLC patients. CTC was captured by micofabricated porous filter device; CTC platform designed to enrich CTCs by cell size and developed by our group previously. Twenty-five patients; 19 FISH-proven ALK positive, 6 ALK negative in primary tumor tissue were prospectively enrolled. RNA was extracted from the captured CTCs and RT-PCR was done to identify EML4-ALK fusion transcript. There were 25 stage IV NSCLC patients and CTCs were detected in 12 (48%); numbers of CTCs ranged from 1 to 12 CTCs/ml. More importantly, EML4-ALK fusion transcript was identified by RT-PCR (optimized for variant type 1 EML4-ALK fusion transcript) in tissue proven ALK+ patients; 11/19 (58%) including 4 patients treated with ALK inhibitor and 11/15 (73%) patients without ALK inhibitor. Furthermore, no single EML4-ALK fusion transcript was found in tissue proven ALK negative patients. We have proved the existence of EML4-ALK transcript using RT-PCR after the enrichment of CTCs from the peripheral blood draws in NSCLC patients. Being able to reliably detect EML4-ALK fusion transcript from enriched CTCs may allow us not only to identify EML4-ALK+ patients with simple blood draws but also to monitor the diseases along with the treatment.

INTRODUCTION

Circulating tumor cells (CTCs) are cancer cells in transit from the primary tumor to metastasis shedding into the blood vessels. The number of CTCs are considered having a prognostic value among various cancers such as breast cancer, lung cancer, prostate cancer and etc. [1, 2] and furthermore, studies regarding CTC's role as a surrogate biomarker for targeted drug therapy has been increasing [3, 4]. There are two kinds of CTC enrichment technologies. One is biological techniques which use cell surface marker to capture the cancer cells. These have been commonly used by cell biologists for a long time, but there are quite a number of cancer cells being negative for the specific markers. The other one is using physical characteristics between normal blood cells and cancer cells including cell size, density, deformability, and electric properties. Although the CTC counts are overall positively correlated with the cancer stage, CTCs are heterogeneous so that any single marker such as EpCAM or CK can't enumerate all the CTC populations and therefore, there are many EpCAM or CK negative CTCs in lung cancer. [5] CTC enrichment using its physical properties followed by molecular analysis may be the promising tool to cover marker negative CTCs.

EML4-ALK fusion was first discovered from a never-smoker lung cancer patient in 2007 [6], and the ALK fusion has been identified in 3% to 7% of non-small cell lung cancer (NSCLC) patients [6-8]. The drugs targeting the kinase activity of ALK were shown to improve the prognosis of NSCLC with ALK fusions [9]. The response rate to ALK tyrosine kinase inhibitors are as high as 50~60% [9-11]. Usually, additional tumor tissue is required to perform genetic tests for the drugs targeting specific molecular alterations such as EGFR mutation or ALK fusion other than conventional pathological examination. The list of targets related to oncogene addiction has been thoroughly researched and become the most wanted candidate for targeted drug treatment in NSCLC patients [12]. For example,

ROS1 fusion was proven to be a reliable marker of drug sensitivity [13], and RET fusion [14], MET exon 14 skipping [15, 16] and BRAF mutations [16] also have been candidates for targeted drugs. Also, the presence of T790M mutation from second biopsy in NSCLC patients with activating EGFR mutations is predictive to third generation EGFR tyrosine kinase inhibitor [17, 18]. However, some patients have difficulties in getting sufficient amount of tumor tissue for genetic tests due to limited accessibility, or high risk of complications such as bleeding or pneumothorax after the tissue acquisition. Vanderlaan et al reported that tumor biopsy failed in up to 8~10% of lung cancer patients at the time of diagnosis [18]. Considering the complication rate at the beginning of the diagnosis, we would expect that more complications can be occurred at the time of recurrence and moreover, the performance would be deteriorated as well. Therefore, the non-invasive technique to identify and monitor the molecular genetic changes can be one of the most important steps in treatment of NSCLC patient in the era of molecular targeted therapy.

The aims of this study were to determine the feasibility of reverse transcription polymerase chain reaction (RT-PCR) in detection of EML4-ALK rearrangement from captured CTCs in advanced NSCLC.

RESULTS

Spiking experiment and RT-PCR for EML4-ALK fusion detection

To detect of EML4-ALK fusion in blood, CTCs were isolated by using microfabricated porous filter as previously reported [19]. The filter was designed to enrich CTCs by cell size so that cancer cells in blood are difficult to pass this filter. To identify the recovery rate and feasibility of detection of fusion transcript, spike-in tests were performed. The recovery rate of EpCAM positive and CD45 negative cells was 40 ~ 50% for both of H3122 and H2228 cell lines (Figure 1A & 1B). The fusion gene was successfully detected with 10% of

RNAs from the recovered samples after spiking 1000 H3122 cells (having variant 1type of EML4-ALK fusion) in 1 ml of normal blood, but not after spiking 10 or 100 cells (Figure 1C). This suggest that lower limit of detection (LOD) of our assay is a range of 10~100 ALK fusion positive cancer cells per ml of blood. Therefore, we used 5 times more RNAs after RNA extraction from the cancer patient samples. Serial dilutions of H2228 tumor cells harboring EML4-ALK variant type 3 were also spiked and examined by RT-PCR analysis with primers for the variant type 3 EML4-ALK fusion. However, the primers could not detect the type 3 fusion even in a spike of 1000 H2228 cells (data not shown). We tested EML4-ALK variant type 1 only for patient-driven samples, but not type 3, based on the spike-in test result.

Study patients and capturing CTCs

There were 25 stage IV NSCLC study patients (female: 16, male: 9) and their characteristics were summarized in Table 1. The total of 19 patients had ALK fusion genes identified from their tumor tissues, 5 patients had EGFR mutations and 1 patient had triple negative tumor: EGFR (-), KRAS (-), ALK (-). Among the 19 patients with ALK fusion tumors, 4 patients were treated with ALK inhibitor (all being crizotinib) and the rest of 15 patients had never been treated with ALK inhibitor (1 patient who were on ceritinib for 14 days was considered as no treatment with ALK inhibitor) at the time of blood draws; 4 patients were treatment naïve and 11 patients had treatment with cytotoxic chemotherapy.

Capturing CTCs and detection of EML4-ALK fusion script with RT-PCR technique

CTC enrichment and counting showed EpCAM and Cytokeratin positive CTCs were dete cted in 4 (36%) out of 11 study samples and the numbers of CTCs ranged from 1 to 2 CTCs/ml (Table 1). The CTCs were mostly round in shape and the nucleus was

surrounded by EpCAM and Cytokeratin (Supplementary figure 1). Detected EML4-ALK variant 1; a band of 420bp in both of H3122 cells, positive control for EML4-ALK and the study patient were described in Figure 3A. A patient was defined as EML4-ALK positive, if at least one fusion transcript was detected among triplicate experiments. Sanger sequencing was done to make a final confirmation for the RT-PCR product. Also, fluoresce nce in situ hybridization (FISH) analysis of tumor specimens in ALK-positive patient; the transcript of EML4-ALK variant 1 for this study patient was also verified by RT-PCR as well. (Figure 3B, C)

Sensitivity and specificity of EML4-ALK RT-PCR test in enriched CTC

We calculated the sensitivity and specificity of EML4-ALK RT-PCR using enriched CTCs compared to the fluorescence in situ hybridization (FISH) result of tumor tissues (Table 2A). EML4-ALK fusion transcript was identified by RT-PCR (optimized for variant type 1 of EML4-ALK) from the enriched CTCs in tissue proven ALK+ patients; 11 out of 19 (58%) including 4 patients treated with ALK inhibitor and 11 out of 15 (73%) patients treated without ALK inhibitor (Table 2B). Interestingly, treatment naïve 4 patients with ALK fusion had positive results of CTC RT-PCR. If we excluded 4 patients on treatment with ALK-specific inhibitors (crizotinib), the sensitivity increased to 73% (11 out of 15) (Table 2B). Also, there was no single EML4-ALK fusion transcript was found from the retrieved CTCs in tissue proven ALK negative patients. Therefore, the sensitivity and the specificity of RT-PCR in detection of EML4-ALK fusion gene expression from the CTCs were 58% and 100%, respectively.

A case of serial monitoring during ALK inhibitor

CTCs were collected both at baseline and during an ALK-specific inhibitor, ceritinib from a patient (LC24). He was treated with cytotoxic chemotherapy, pemetrexed and cisplatin (PC) as a first-line treatment, and his disease progressed after the 6th cycle of PC. The first CTC was collected at that time and RT-PCR from the retrieved CTCs was positive for EML-ALK fusion before ceritinib treatment, but converted to negative result 2 months after the treatment of ceritinib. Computed tomography which was performed at that time also showed a partial remission.

DISCUSSION

This study showed the feasibility of RT-PCR as a diagnostic tool to identify EML4-ALK fusion gene after enrichment of CTCs from the simple peripheral blood draws. This assay might be utilized as a non-invasive tool to monitor the molecular genetic changes without an additional acquisition of tumor tissue in NSCLC patients. It has been quite a challenge to acquire additional tumor tissue during the course of treatment from the inoperable NSCLC patients due to lack of optimal measurable lesions and increasing complication rate. Crizotinib is an oral small-molecule tyrosine kinase inhibitor and targeted for ALK, MET, and ROS1 kinases. Detection of EML4-ALK fusion gene to confirm CTCs and use it as a surrogate marker for treatment may be the most important issues in the era of precision medicine in these days. Therefore, the most important aim of this study was to determine the feasibility of RT-PCR in finding EML4-ALK fusion genes which can be used as a treatment target and biomarker for the drug therapy. We could find out that EML-ALK fusion transcript could be detected with RT-PCR techniques after the enrichment of CTCs using microfabricated porous filter which was developed by our group[19]. We had 58% of sensitivity in detecting EML4-ALK fusion (variant 1) with treatment naiive patients. However, if we take the patients without history of ALK inhibitors,

the sensitivity became 73 %; 11 out of 15 patients treated without ALK inhibitor in this study. Furthermore, no single EML4-ALK fusion transcript was found in tissue proven ALK negative patients; specificity is 100%. Recently, Yohida et al [20] reported the frequency of EML4-ALK fusion gene as follows. There were 35 NSCLC study patients; variant 1 (19, 54%), variant 2 (5, 14%), variant 3a/3b (4, 12%) and others (variant 3, 5, 5', 5b, E13ins60; A20, E12ins51; A20, E17ins27; A20: 7, 20%). This result seemed to consist with our result as well. But it would be much better to support our data with other variants types as well even though they are considered to be the minor portions of the NSCLC patients.

We observed a serial sampling result with an ALK inhibitor in this study. There was a change from EML4-ALK positive CTCs to negative CTCs during the short period of treatment with ALK-inhibitors (8 weeks). This suggested that monitoring CTCs with their genetic profile would give us useful clinical insights in understanding tumor behavior. Pailler et al. also observed decreased number and different rearrangement patterns of ALK rearranged CTCs after the treatment [21]. Sometimes confirmation of CTCs with detection markers such EpCAM and CK using IF can be very difficult to apply in clinical practice due to EMT phenomenon and inconsistency of surface markers. Many studies showed that EpCAM is variously expressed in cancer cells and especially not expressed in CTCs undergo epithelial to mesenchymal transition (EMT) and from mesenchymal origin [22]. And CK is also heterogeneously expressed in tumor, and downregulated in breast cancer cells [23]. Cytokeratin also have critical disadvantage as a capture antibody due to its low specificity among the various types of cancer cells. We have experienced both false positive and false negative results by IF staining to visualize captured CTCs and also, there are always existing purity issues due to WBC contamination even there are more CTCs than positive staining result. Therefore, we all know that there are both WBC contaminations and more CTCs were captured even with negative IF staining result.

Therefore, we have tried the molecular diagnosis using RT-PCR which would overcome the relatively low sensitivity and specificity of IF staining method in detecting CTCs.

Although this study suggested that RT-PCR could identify the EML4-ALK fusion from CTCs, there several obstacles and limitations to overcome. We would like to address the limitations of this study as well. First, there are some controversial issues regarding the accuracy of RT-PCR and FISH in identifying EML4-ALK fusion gene from the enriched CTCs. we need direct comparison of RT-PCR with FISH in identifying EML4-ALK fusion gene from CTCs. Pailler et al reported that EML4-ALK fusion gene was identified with 100% sensitivity and specificity from the captured CTCs with FISH probes [21]. In this study, one patient showed negative ALK FISH but positive RT-PCR for primary tumor assessment [21]. Wu et al compared diagnostic accuracy between RT-PCR and FISH in tumor tissues, and they found that five (42%) among 12 samples with positive RT-PCR showed negative by FISH [24]. They also showed that two among nine cases with positive FISH were negative by RT-PCR. Extrapolating EML4-ALK fusion gene status between CTCs and primary tissue, we believe that both tools, RT-PCR and FISH for identification of EML4-ALK fusion gene still have complimentary role for each other. Second, we need to define significance of variable types of EML4-ALK fusion genes. We used one set of PCR primers, optimized for variant type 1 of EML4-ALK, and this primer set can detect both type 1 and type 2 of EML4-ALK fusion transcripts. The relative frequency of type 1 is reported as variable depending on ethnicity. Although Caucasian, Japanese [24]and Korean [25] population showed predominance of type 1, Chinese population showed predominance of type 3 [24]. Type 1 comprises 80% of all EML4-ALK fusion in Korean lung cancer patients [25], but our primer set cannot detect other types (including type 3, the second most common type) than type 1 and 2. Therefore, if RT-PCR was positive for EML4-ALK fusion gene from the retrieved CTCs, the PCR products were sequenced to confirm the type of

EML4-ALK fusion genes in this study. All sequenced PCR products were confirmed to have type 1 fusions. Considering 80% of type 1 EML4-ALK positives in Korean lung cancer patients, 73 % of the positives from 15 patients treated without ALK inhibitor suggest that the false negatives may be few in this study. We tried to figure out a variant type from primary tumors especially with negative RT-PCR results in CTC. Due to limitation of tissue availability, we tried RT-PCR from two primary tumors whose CTC RT-PCR were negative. One showed type 1 variant from primary tissue, and the other showed negative to type 1 variant detection (data not shown). We can speculate that some negative cases would result from other types of EML4-ALK fusions or non-EML4-ALK fusions. Multiplexed RT-PCR will help to detect various types of ALK fusions.

We tried to examine EML4-ALK fusion detection in blood. As we previously developed a microfabricated porous filter device based on CTC enrichment [19], we added previously known RT-PCR for EML4-ALK fusion detection[19, 26]. We set up many clinical processes from blood preparation and storage in the hospital to the molecular analysis at another site and performed this translational research as a prospective study. The result suggests that reverse transcription polymerase chain reaction (RT-PCR) detection of EML4-ALK rearrangement is feasible with enriched CTCs. Our study included four patients (LC11, 13, 14 and 17) who were treated with ALK inhibitors with variable treatment periods (23, 6, 12 and 24 months, respectively) and progressed to them. Another one case was treated with an ALK inhibitor for 2 weeks, and her CTCs showed positive result of RT-PCR. We could expect that EML4-ALK fusion gene positive tumor population would be decreased during the crizotinib treatment and so forth CTCs as well. RT-PCR technique could find the EML4-ALK fusion transcript on the negative IF staining of CTCs patient. However, we have very small subset of patients and further investigation with large-set of study patients is required to validate this interesting phenomenon. Therefore, we are also planning for

further investigation of detecting EML4-ALK fusion gene with RT-PCR method in a large set of patients who are very homogeneous and analyzing follow-up samples during the course of treatment.

Here, we have proved the existence of EML4-ALK positive CTCs using RT-PCR after the enrichment of CTCs from the simple peripheral blood draw. Being able to reliably detect EML4-ALK fusion transcript from the enriched CTCs may allow us not only to find useful actionable targets and to monitor the diseases along the treatment with further investigation.

MATERIALS AND METHODS

Microfabricated porous filter device: CTC platform

Capturing CTCs was performed by microfabricated porous filter device which has been developed by our group previously [19, 26]. The principle of isolating CTCs was based on the theory that the most of the cancer cells have larger diameter than normal hematologic cells. Therefore, CTCs in blood would be remained on the platform of this micofabricated porous filter device while normal hematologic cells are easily passing through.

Spiking test with EML4-ALK positive cancer cells

Human NSCLC cell lines with EML4-ALK rearrangement, NCI-H3122 (H3122) and NCI-H2228 (H2228) were cultured in RPMI1640 medium supplemented with 10 % fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine. For spike-in tests, 1 ml normal whole blood from healthy donors was spiked 10, 100, 500 and 1000 H3122/H2228 cells per 1ml of blood in triplicate. The whole process of CTC enrichment experiments was performed with 1 ml of blood samples as previously reported [19]. Whole blood samples spiked with the cancer cells were centrifuged for 20 minutes at 400 x g at

room temperature in the Ficoll-Hypaque solution (GE Healthcare, Piscataway, NJ), and then the PBMC layer was moved into a new tube. PBMCs were passed through the microfabricated porous filter by gravity. All the remaining cells on the filter were recovered into 1.5 ml Eppendorf tube by pipetting. The enriched cells were stained with the following three markers for CTC counting or used to extract total RNA for RT-PCR analysis.

Captured cells were characterized by immunofluorescence staining; DAPI (4', 6-diamidino-2-phenylindole-2HCl) for nucleated cells, EpCAM or CK for cancer cells and CD45 for white blood cells (WBCs). For checking spike-in recovery rate, enriched H3122 and H2228 cells were counted under fluorescence microscope (Nikon Eclipse Ti-S, Nikon Corporation, Tokyo Prefecture, Japan).

Total RNA was extracted from the retrieved CTCs by using the Arcturus PicoPure RNA Isolation Kit (Life Technologies) according to the manufacturer’s protocol. The purified RNA was eluted with 11 ul of nuclease-free water and the overall quality was assessed by electrophoresis and a NanoDrop-2000 (Thermo Fisher Scientific, Wilmington, DE, USA). 1 ul of the eluted RNA was used for WTA using the QuantiTect Whole Transcriptome Kit (Qiagen) according to the manufacturer’s protocol. After whole transcriptome amplification, the RT-PCR for variant 1 of EML4-ALK fusion gene was performed in a volume of 20 ul containing 1X PCR buffer (including Mg^{2+}), 1mM dNTPs, 1U Taq DNA polymerase (TaKaRa), 1mM forward primer, 1mM reverse primer and 1ul of whole transcriptome amplified cDNA as template. Previously reported primers used for PCR amplification were the forward primers V3F (5’-GTCAGCTCTTGAGTCACGAGTT-3’) and V1F (5’-GTGCAGTGTTTAGCATTCTTGGGG-3’), and the reverse primer ALK-R (5’-ATCCAGTTCGTCCTGTTCAGAGC-3’) [19]. After an initial denaturation of 10 minutes at 95℃, 40 cycles of amplification were performed as follows: 95℃ for 30 seconds, 60℃ for 30 seconds, and 72℃ for 40 seconds. Quality of PCR products was checked by running in

a 2% agarose gel.

Study patients

A total of 25 patients with NSCLC were prospectively enrolled in this study. All of the study patients were pathologically proven, and primary or metastatic tumor tissue was first screened for EGFR mutation and ALK fusion along with pathological examination. The schematic flow of capturing CTCs from the study patients as well as the final inclusion criteria were described in Figure 2. This study was approved by Institution Review Board of Seoul National University Hospital, Seoul National University College of Medicine (IRB No. 1209-029-424).

CTC enrichment and RNA extraction

5 to 10 ml of blood samples were drawn from the NSCLC patients and healthy donors. Acquired samples were collected in K_2EDTA anti-coagulants containing tubes and were processed within 5 hours. The samples from healthy donors were used as negative controls. CTC enrichment process from the study patients was performed with 5 ml of blood samples as previously reported [18]. PBMCs in 5 ml of blood samples were isolated by Ficoll-Hypaque density gradient centrifugation method and passed through the microfabricated porous filter. The enriched cells on the filter were retrieved and used for immunostaining or RNA extraction. RT-PCR was performed with 25 patients. Among them, 14 patents that have 10 ml of blood were in parellel analyzed by immunostaining. Because of limited amounts of blood, the other 11 samples that only has 5 ml of blood could not be analyzed by CTC counting.

EML4-ALK fusion detection by RT-PCR and Sanger sequencing

RT-PCR analysis was performed as described in spike-in tests. Only different thing is that 5 ul of the eluted RNA was used for WTA to increase the sensitivity. The DNAs in the expected PCR product size were cut from the gel and analyzed by Sanger sequencing to confirm the fusion site.

List of abbreviations

CTC, circulating tumor cell

FISH, fluorescence in situ hybridization

NSCLC, non-small cell lung cancer

RT-PCR, reverse transcription polymerase chain reaction

WBC, white blood cell

Competing Interest

We have the following interests. Co-authors, Du-Yeol Han and Byung-Hee Jeon are currently employed by Cytogen Inc. Co-authors, Eun-Hye Kim and Byung Chul Kim are former employee of Cytogen Inc.

Author contributions

PJK designed the study, analyzed the data and drafted the manuscript. KE-H carried out CTC enrichment and RT-PCR experiments, and drafted the manuscript. LD-W designed the study and performed a paper work for approval of ethical committee. KS set up RT-PCR and performed RT-PCR from tumor tissues. KB, KTM, KD-W, HDS provided study samples. HD-Y carried out CTC enrichment and RT-PCR experiments. JB-H designed the study and discussed with data. KBC designed the study, set up and supervised RT-PCR experiments, and drafted the manuscript. LS-H designed the study, provided study samples, performed a paper work for approval of ethical committee, analyzed the data and drafted the manuscript. All authors read and approved the final manuscript.

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Figure Legend

Figure 1: Spike-in test of CTCs and EML4-ALK RT-PCR using cell lines. A. Immunofluorescent images of recovered H3122 & H2228 cancer cells. H3122 & H2228 cancer cell (DAPI+, CD45-, EpCAM+) and leukocyte (DAPI+, CD45+, EpCAM-). All scale bars represent 5 μm. **B.** Overall efficiency after enrichment at two different cell loads: 10, 100 and 1000 H3122 & H2228 cells spiked. **C.** The mRNA expression of EML4-ALK fusion gene in spiked samples. The mRNA expression of fusion gene was measured by RT-PCR with variant type 1 primers. Upper and lower panels show the transcripts before and after enrichment, respectively. Beta-actin was used as a control for RNA integrity.

Figure 2: Schematic flow of capturing CTCs from the study patients.

Figure 3: RT-PCR detection of EML4-ALK positive NSCLC patients.

A. Identification of EML4-ALK variant 1; a band of 420bp was detected in both of H3122 cells, positive control for EML4-ALK and the study patient.

Expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a control.

A patient was defined as EML4-ALK positive, if at least one fusion was detected among triplicate experiments.

Abbreviations: *M, 100bp size marker; H3122, positive control; A549, negative control*

B. Sequence of EML4-ALK variant 1; the chimeric transcript was identified by Sanger sequencing analysis.

C. FISH analysis of tumor specimens in ALK-positive patient; the transcript of EML4-ALK variant 1 for this study patient was also verified by RT-PCR as well.

Table 1: Characteristics of study patients

Patient No.	Age	Sex	Genetic changes	Treatment before CTC collection	Number of CTCs	RT-PCR
LC1	34	F	ALK	Op. / TC / CCRT	1	V1
LC 2	69	M	EGFR	Gefitinib	ND	-
LC 3	73	F	ALK	GP / Op. / CCRT / SRS	0	V1
LC 4	47	F	ALK	Naïve	0	V1
LC 5	61	F	EGFR	Gefitinib / Pem+CDDP	ND	-
LC 6	52	F	ALK	Op. / EP / RT	0	-
LC 7	35	M	ALK	GP	ND	-
LC 8	76	F	ALK	Naïve	0	V1
LC 9	60	M	ALK	Naïve	0	V1
LC10	58	F	EGFR	Gefitinib	ND	-
LC 11	71	F	ALK	RT / Gefitinib / Pem / Crizotinib	ND	-
LC 12	53	M	TN	GP	1	-
LC13	66	F	ALK	Crizotinib / GP / Pem / RT	ND	-
LC 14	44	F	ALK	GP / CCRT / GKS / Crizotinib / WBRT	ND	-
LC 15	55	F	EGFR	Gefitinib	ND	-
LC 16	48	F	ALK	Naïve	0	V1
LC 17	55	M	ALK	CCRT / Pem / Crizotinib	ND	-
LC 18	46	F	ALK	Gefitinib / Pem	0	V1
LC 19	68	F	EGFR	Gefitinib	ND	-
LC 20	50	M	ALK	Pem+CDDP	ND	-
LC 21	52	F	ALK	Pem+CDDP	ND	V1
LC 22	53	F	ALK	GP	ND	V1
LC 23	54	F	ALK	GP / CCRT / RT / Crizotinib; 2 weeks treatment	1	V1
LC 24	45	M	ALK	Pem+CDDP	2	V1
LC 25	42	M	ALK	Pem+CDDP	ND	-

CCRT, concurrent chemoradiotherapy; CDDP, cisplatin; EP, etoposide and cisplatin; GP, gemcitabine and cisplatin; Pem, pemetrexed; SRS, stereotactic radiosurgery; TC, paclitaxel and carboplatin; TN, triple negative (EGFR/KRAS/ALK:-/-/-); ND, not determined; -, not detected

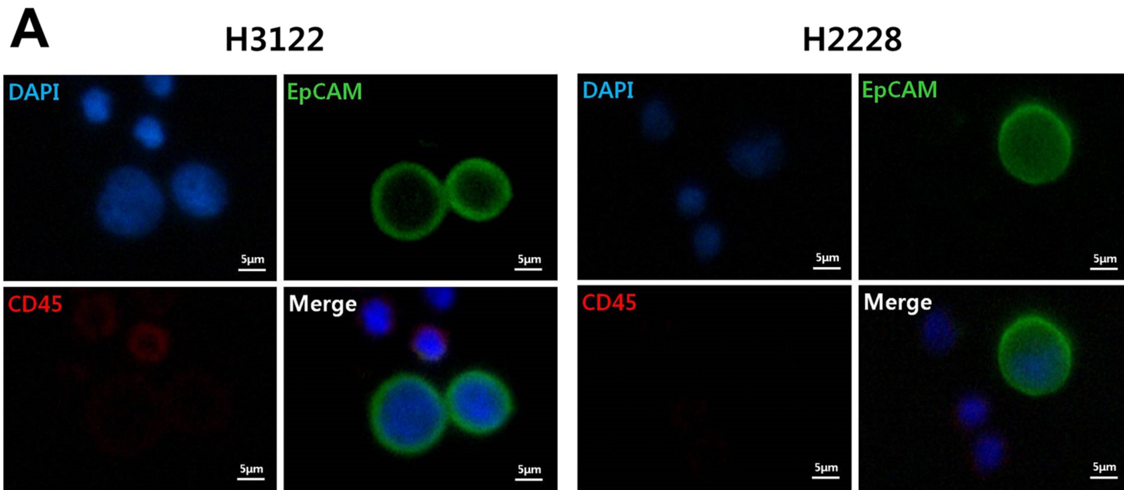
Table 2: Sensitivity and specificity of EML4-ALK RT-PCR in enriched CTC

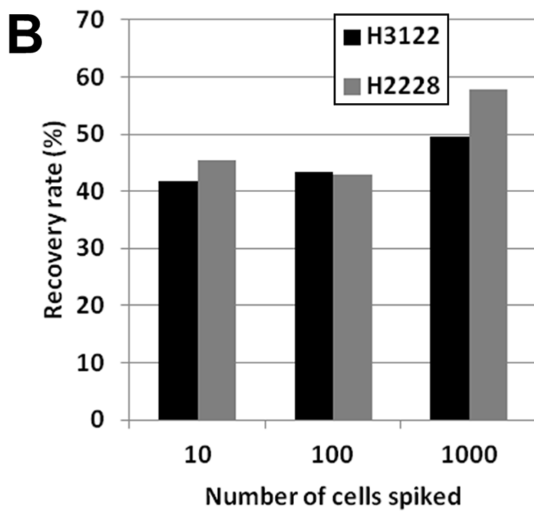
(A) All population

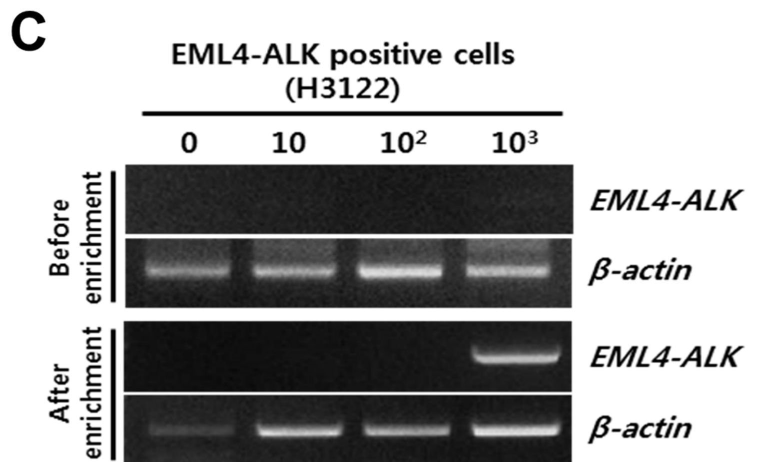
	ALK-FISH (+)	ALK-FISH (-)	Total
RT-PCR (+)	11 (58%)	0	11
RT-PCR (-)	8 (42%)	6 (100%)	14
Total	19	6	25

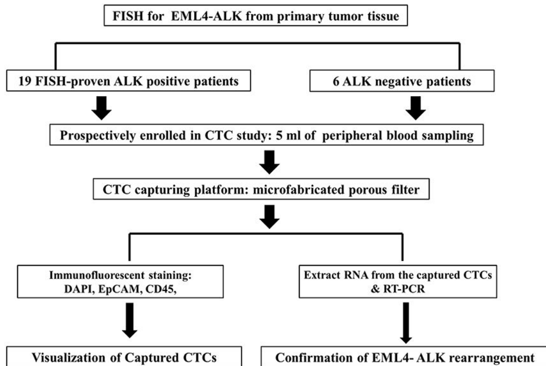
(B) When limited to no ALK inhibitor

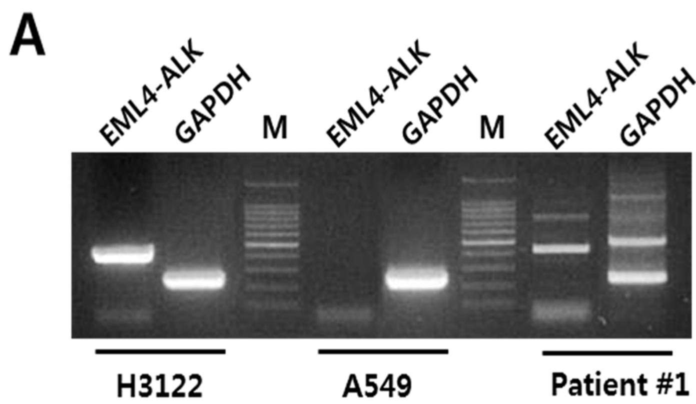
	ALK-FISH (+)	ALK-FISH (-)	Total
RT-PCR (+)	11 (73%)	0	11
RT-PCR (-)	4 (27%)	6 (100%)	10
Total	15	6	21

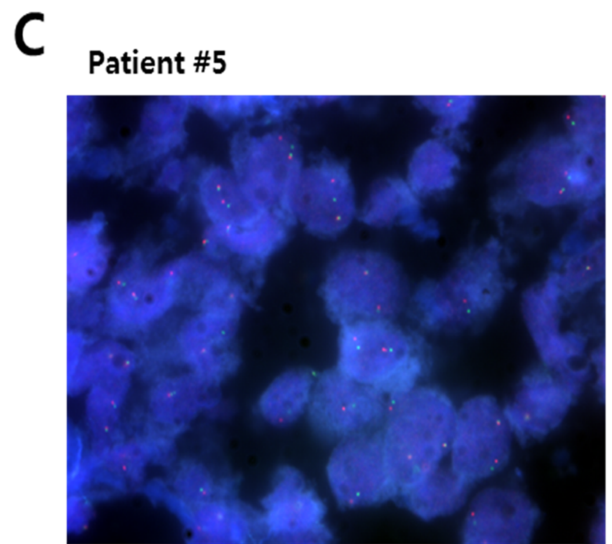

A
H3122
H2228
DAPI
EpCAM
CD45
Merge
5μm

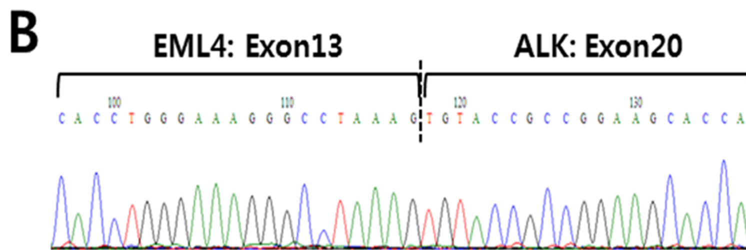

B
H3122
H2228
Recovery rate (%)
70
60
50
40
30
20
10
0
10
100
1000
Number of cells spiked


C
EML4-ALK positive cells
(H3122)
0
10
10²
10³
Before enrichment
After enrichment
EML4-ALK
β-actin
EML4-ALK
β-actin

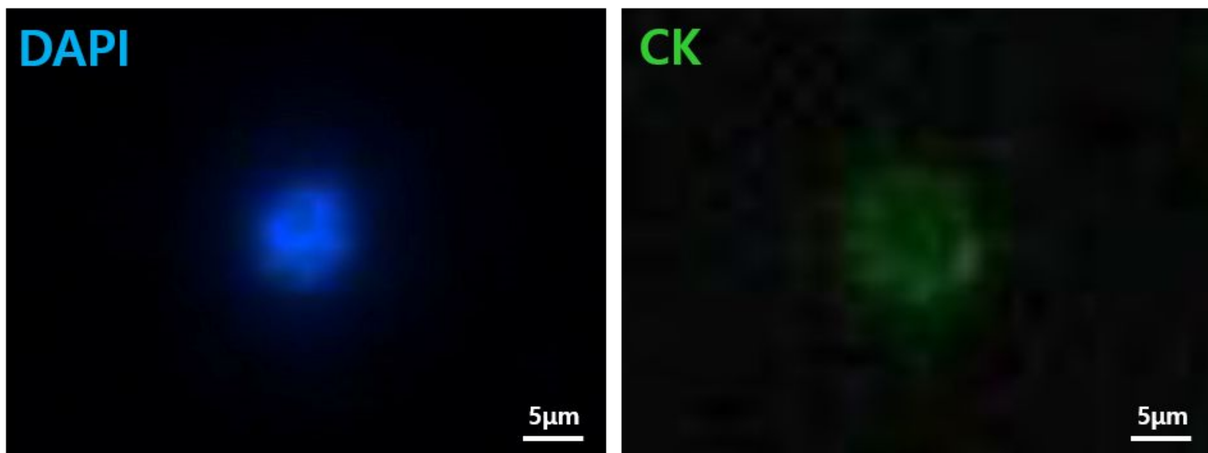

FISH for EML4-ALK from primary tumor tissue
19 FISH-proven ALK positive patients
6 ALK negative patients
Prospectively enrolled in CTC study: 5 ml of peripheral blood sampling
CTC capturing platform: microfabricated porous filter
Immunofluorescent staining:
DAPI, EpCAM, CD45,
Extract RNA from the captured CTCs
& RT-PCR
Visualization of Captured CTCs
Confirmation of EML4- ALK rearrangement

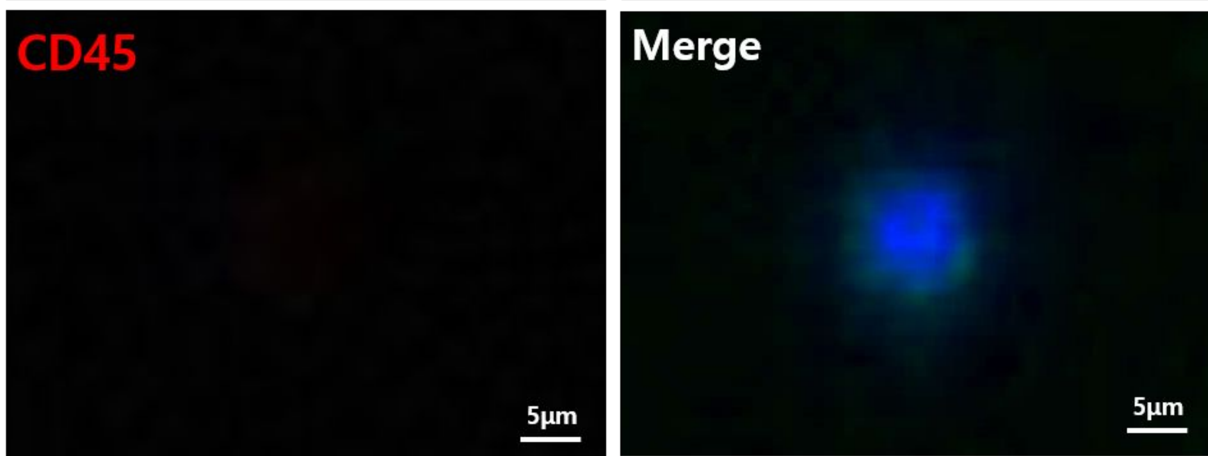

A
EML4-ALK
GAPDH
M
EML4-ALK
GAPDH
M
EML4-ALK
GAPDH
H3122
A549
Patient #1


C
Patient #5


B
EML4: Exon13
ALK: Exon20
CACCTGGGAAAGGGCCTAAAG
TGTACCGCCGGAAGCACCA

A LC001





B LC024 (one of total 2 CTCs)