

Identification of circulating tumor cells with EML4-ALK translocation using fluorescence *in situ* hybridization in advanced ALK-positive patients with lung cancer

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Abstract. Analysis of anaplastic lymphoma kinase (ALK) rearrangement in non-small cell lung cancer (NSCLC) is considered to be a useful tool when considering predictive biomarker detection for evaluating eligibility for targeted therapy. It is not always possible to perform a tumor biopsy in patients. Isolation and culturing of circulating tumor cells (CTCs) may be an alternative to tumor biopsies for the diagnosis of ALK rearrangement. Blood was collected from 22 patients with NSCLC harboring ALK rearrangement and was divided into two groups: One for immunofluorescence staining and the other for culture. Samples were filtered by size and cultured CTCs were analyzed for echinoderm microtubule-associated protein-like 4-ALK translocation using fluorescence *in situ* hybridization. CTCs positive for epithelial cell adhesion molecule and CTCs exhibiting ALK rearrangement were detected. Therefore, CTCs may be used as a potential alternative method to tissue biopsy for diagnosing ALK rearrangement. Additionally, this method may have clinical applications including serial blood sampling for the development of personalized cancer therapy based on individual genomic information.

Introduction

Lung cancer is the most common type of cancer and is the leading cause of cancer-associated mortality worldwide; non-small cell lung cancer (NSCLC) accounts for 80% of cases of lung cancer (1). Epidermal growth factor receptor

(*EGFR*) mutations have been detected in between 10 and 35% of patients with NSCLC, and tumors with *EGFR* mutations are sensitive to treatment with *EGFR* tyrosine kinase inhibitors (TKIs) (2). Anaplastic lymphoma kinase (ALK) rearrangement is another distinct subtype of lung cancer and is present in between 2 and 7% of NSCLC (3,4). Such tumors exhibit sensitivity to ALK inhibitors, including crizotinib and ceritinib (5,6). The majority of patients with NSCLC with ALK translocations are diagnosed in advanced stages of the disease (7). Therefore, it is usually difficult to obtain the tumor tissue required for diagnosing ALK using fluorescence *in situ* hybridization (FISH), which is considered to be the gold standard for diagnosis of ALK translocation. Additionally, secondary biopsies have become necessary to evaluate the mutation status of the remaining tumor tissue and to monitor the treatment responses (8). However, tumor tissue biopsy has its limitations; it is an unrepeatable and invasive procedure (9).

In previous studies, circulating tumor cells (CTCs) were detected in the blood of patients with lung cancer (10-14). These CTCs have been utilized for cancer diagnosis and genetic evaluation (15,16). CTC isolation technologies are divided into two categories: One is based on biological properties (cell-surface marker proteins) and the other is based on the physical properties (size, deformability, density and electric charge) (17-20). However, the isolation of CTCs remains challenging because of their low sensitivity. Although reverse transcription-polymerase chain reaction (RT-PCR) (21) and quantitative PCR (qPCR) (22) have been used for molecular characterization of CTCs, it has been technically difficult to detect RNA markers in CTCs. A CTC enrichment and culture platform was developed by CytoGen, Inc. for obtaining sufficient amounts of CTCs to be used for FISH and for genetic analyses, including genomics and transcriptomics (23).

In the present study, an investigation was performed into whether CTCs may be used to detect ALK rearrangement using FISH. Owing to the fact that only a limited number of CTCs may be obtained, a CTC culture method was developed to obtain sufficient CTCs for FISH analysis. Additionally, the effectiveness of ALK detection by FISH using cultured CTCs was evaluated.

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Materials and methods

Patients and samples. The present study was approved by the Institutional Review Board of The Catholic University of Korea, College of Medicine (XC15TIMI00240). Informed written consent was obtained from all the enrolled patients. Peripheral whole blood samples of 15 ml each were collected from 22 patients with NSCLC with ALK translocations and from 1 patient with NSCLC without an ALK translocation (patient no. 23). The ALK status had previously been confirmed using FISH analysis of biopsied or excised specimens. From each blood sample, 5 ml was used for CTC detection and enumeration using immunofluorescence staining, and the remaining 10 ml was used for CTC detection, culture and ALK diagnosis using FISH. Additional clinical and pathological information, including histological subtype, ALK positivity in biopsied samples, status of metastasis, smoking history, treatment history (chemotherapy or radiotherapy), use of crizotinib and current disease status, was documented.

CTC detection and culture using the CytoGen, Inc. enrichment platform. From each patient, 15 ml blood was collected in acid citrate dextrose tubes and processed within 4 h. Blood (5 ml) was filtered according to size and used for immunofluorescence staining. Briefly, whole blood was separated by density gradient centrifugation (Ficoll-Paque; GE Healthcare Life Sciences, Little Chalfont, UK) and the peripheral blood mononuclear cells (PBMCs) were filtered through a high-density microporous (HDM) chip (CytoGen, Inc.) (24). The cells retrieved from the HDM chip were negatively selected using an immunomagnet [Dynabeads protein tyrosine phosphatase, receptor type C (CD45); Thermo Fisher Scientific, Inc., Waltham, MA, USA] to remove remaining white blood cells. Enriched CTCs were placed onto glass slides using Cytospin (Thermo Fisher Scientific, Inc.), fixed in 4% paraformaldehyde for 5 min at room temperature and kept at 4°C until further processing. The remaining blood samples for CTC culture were processed using a similar procedure, with the exception of immunomagnetic negative selection.

CTC enumeration by immunofluorescence staining. Cells were permeabilized with 0.2% Triton X-100 and then quenched with 0.3% H₂O₂. Following blocking with 1% bovine serum albumin (GE Healthcare Life Sciences, Logan, UT, USA) in PBS, the cells were incubated with mouse anti-epithelial cell adhesion molecule (EpCAM) antibody (1:100; Cell Signaling Technology, Inc., Danvers, MA, USA, cat. no. 2929). EpCAM signals were amplified using a Tyramide Signal Amplification system (Alexa Fluor 488-conjugated goat anti-mouse IgG; Thermo Fisher Scientific, Inc.), which was used according to the manufacturer's protocol. Cells were then incubated with rabbit anti-CD45 antibody (1:100; Cell Signaling Technology, Inc., cat. no. 13917) and Alexa Fluor 594-conjugated goat anti-rabbit secondary antibody (1:200; Invitrogen; Thermo Fisher Scientific, Inc., cat. no. A-11012). The slides were mounted using Fluoroshield with DAPI (ImmunoBioScience Corp., Mukilteo, WA, USA). Stained cells were observed and images were captured using a Nikon Eclipse Ti fluorescent microscope equipped with a 400X objective. CTCs were defined as EpCAM-positive and CD45-negative. For precise

identification of CTCs, PC9 cells (EpCAM-positive) and KG-1 cells (CD45-positive) were included as positive controls in each immunofluorescence staining.

Primary culture of CTCs. Enriched CTCs were collected, washed with PBS and cultured in 6-well Ultra-Low Attachment plates (Corning Incorporated, Corning, NY, USA) containing growth medium (Human Mesenchymal Stem Cell Growth Medium, Lonza Group, Ltd., Basel, Switzerland) at 37°C with 5% CO₂. The culture medium was replaced every 3-4 days with minimal disturbance to avoid cell loss. After ~21 days of culture, cell suspensions were fixed in 10% formalin and placed onto glass slides using a liquid-based slide processor (SurePath; BD Biosciences, Franklin Lakes, NJ, USA), according to the manufacturer's protocol.

ALK immunocytochemistry. A total of 4 randomly selected cases exhibited a good yield (viability >50% during CTC culture) following assessment using immunostaining for ALK. Immunocytochemical staining was performed using Benchmark Ultra (Ventana, Medical Systems, Inc., Tucson, AZ, USA) with a pre-diluted D5F3 clone (Ventana Medical Systems, Inc.), according to the manufacturer's protocol.

FISH analysis and interpretation. Echinoderm microtubule-associated protein-like 4 (EML4)-ALK translocation was assessed using Vysis ALK Break Apart FISH probe kit (Abbott Molecular, Inc., Des Plaines, IL, USA), which is a US Food and Drug Administration-approved test. It is designed to detect ALK in chromosome 2p23 in formalin-fixed tissue with two adjacent probes: One at the 3' end (orange) and one at the 5' end (green) of ALK. Pretreatment, protease digestion, overnight probe hybridization, post-washing and DAPI counterstaining were performed on each sample according to the manufacturer's protocol. For each test, ALK-positive NSCLC tissue was used as a positive control. Normal cells and tumor cells without ALK translocation typically exhibit two fusion signals, whereas cells with ALK translocation exhibit a characteristic ALK split pattern. Processed slides were automatically scanned using a BioView™ workstation (BioView; Abbott Molecular, Inc.) and a preliminary interpretation was made by the system.

Results

Patient information. Included in the present study were 22 patients with NSCLC (Table I). All 22 had previously been diagnosed as positive for ALK rearrangement in tumor tissues using the Vysis ALK Break Apart FISH Probe Kit. ALK positivity in tissue biopsies was 41.2% (18-80%). There were 8 male patients (36%) and 14 female patients (64%). The mean age was 58.5 years (range, 32-82 years) and 8 patients (36%) had smoked previously. Among the 22 patients, 21 had mucinous carcinomas and 1 patient had an undifferentiated carcinoma. Remote metastases were observed in 20 patients (81%). All patients had received chemotherapy or radiation therapy, and 15 patients (68%) had additionally been treated with crizotinib. At the time of the present study, none of the patients had exhibited a complete response (0%). The disease was stable in 8 (36%), 3 exhibited partial responses (14%) and 11 had a progressive disease (50%).

Table I. Clinical characteristics of ALK-positive patients.

Characteristic	n
Total	22 (ALK-positive)
Sex	
Male	8
Female	14
Age, years	32-82 (mean, 58.5)
Smoking status	
Yes	8
No	14
Histological subtype	21 adenocarcinoma (21 mucinous carcinoma), 1 undifferentiated carcinoma
Metastasis	
Yes	20
No	2
Chemo- or radiation therapy	
Yes	22
No	0
Additional crizotinib treatment	
Yes	15
No	7
Current disease status	
Complete response	0
Stable disease	8
Partial response	3
Progressive disease	11

In total, 23 patients were analyzed (22 patients were ALK-positive, and 1 patient was ALK-negative).

CTC detection in patient blood. CTCs were defined as EpCAM-positive and CD45-negative cells (Fig. 1). CTCs were detected in 10/22 patients (45.5%) and the mean number of EpCAM-positive CTCs was 1.5 (1-8 cells) (Table II). CTC quantification in patient no. 2 was not possible because of the poor quality of the immunofluorescence staining.

Analysis of ALK FISH and ALK immunocytochemistry using cultured CTCs. ALK expression in cultured CTCs was analyzed using immunocytochemistry (Fig. 2) and FISH (Table II). FISH signals were detected in an mean of 56 cells (range 2-197). ALK rearrangement was observed in 16/22 patients (72.7%) and the mean number of positive cells was 3.2 (range, 0-13). Representative images of ALK rearrangement demonstrated using FISH are presented in Fig. 3.

Discussion

CTCs may be an indicator of cancer diagnosis, metastasis and prognosis (25), therefore focus has been placed on the clinical relevance of CTC detection and characterization (26). The characteristics of CTCs are variable because they undergo

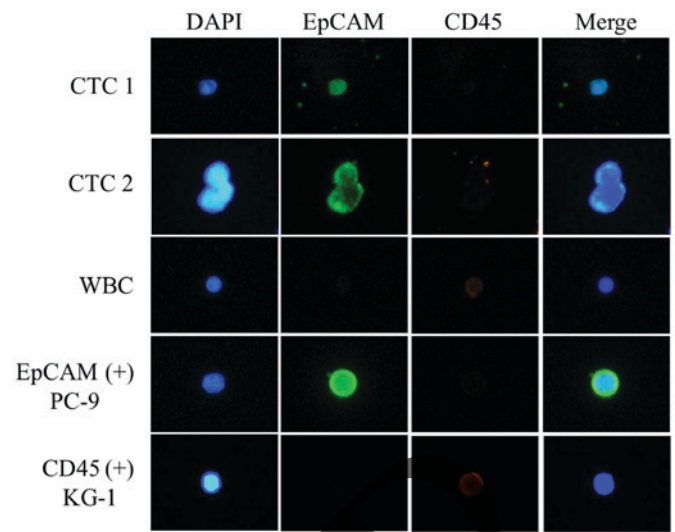


Figure 1. Immunofluorescence staining of circulating tumor cells from patients with lung cancer. EpCAM-positive and CD45-negative cells were identified. EpCAM, epithelial cell adhesion molecule; CD45, protein tyrosine phosphatase, receptor type C; CTC, circulating tumor cell; WBC, white blood cell.

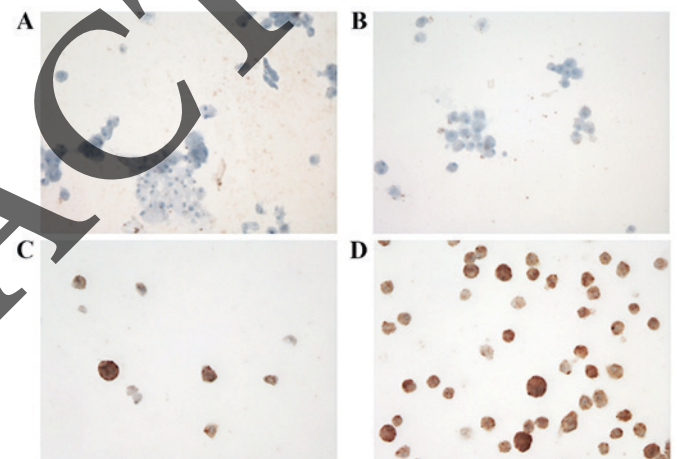


Figure 2. Immunocytochemical staining of CTCs. Cultured CTCs from blood from each patient: (A) no. 10, (B) no. 13, (C) no. 21 and (D) no. 22. Cultured CTCs from patient no. 10 revealed a high number of anaplastic lymphoma kinase (ALK)-positive CTCs; however, immunocytochemistry staining intensity was not markedly increased when compared with cultured CTCs from patient no. 13 which exhibited no ALK-positive CTCs. CTC, circulating tumor cells.

processes including epithelial-mesenchymal transition (EMT) to leave tumor tissue and enter the circulatory system (27). Additionally, cancer therapies are able to alter the characteristics of CTCs. This phenomenon is one of the major obstacles to CTC-based therapeutic approaches. In the pharmaceutical industry, the importance of anticancer drug development and patient-specific medication using CTCs has received much attention (28). However, these studies are limited by the changeable characteristics of CTCs under different environmental conditions (*in/ex vitro* vs. *in vivo*). Therefore, the accurate characterization of CTCs is essential for their clinical application.

When the CTCs are separated from cancer tissue, characteristics of the CTCs are altered throughout the EMT, which is

Table II. Detection and enumeration of CTCs from patients with non-small cell lung cancer with ALK arrangement.

Patient	Sex	Age, years	EpCAM ⁺ CTCs	ALK ⁺ CTCs	(ALK ⁺ CTCs)/ (FISH ⁺ CTCs), %	FISH ⁺ CTCs	Crizotinib	ALK in tissue biopsy, %
No. 1	F	35	5	3	5	58	Y	76
No. 2	F	82	Failed	0	0	16	Y	42
No. 3	M	59	0	0	0	3	Y	32
No. 4	F	54	2	4	31	13	N	48
No. 5	M	70	7	0	0	15	Y	54
No. 6	M	62	8	6	46	13	N	28
No. 7	F	76	5	13	9	149	N	31
No. 8	M	51	0	4	17	24	Y	21
No. 9	F	49	0	1	50	2	Y	33
No. 10	M	71	1	6	3	177	Y	37
No. 11	M	51	1	3	33	9	Y	23
No. 12	F	32	1	0	0	53	Y	18
No. 13	F	49	1	0	0	130	Y	27
No. 14	F	50	0	4	4	92	N	24
No. 15	F	51	0	0	0	4	Y	42
No. 16	F	63	0	1	1	88	Y	34
No. 17	F	56	0	1	25	4	Y	52
No. 18	M	71	1	3	50	6	N	49
No. 19	F	66	0	3	2	149	Y	34
No. 20	F	53	0	10	62	16	Y	60
No. 21	F	60	0	3	21	14	N	80
No. 22	M	76	0	5	3	197	N	73
Mean			1.5	3.2	5.7	56		42
Crizotinib treatment and ALK ⁺ CTCs patients (mean)				2.1	4.3	49.9	-	-
No crizotinib treatment and ALK ⁺ CTCs patients (mean)				5.4	7.9	69.1	-	-
No. 23	M	69	1	0	0	261	N	Negative

All patients underwent anticancer therapy. ALK⁺, detection of ALK rearrangement signal; FISH⁺, detection of FISH signal; CTC, circulating tumor cell; ALK, anaplastic lymphoma kinase; FISH, fluorescence *in situ* hybridization; F, female; M, male; Y, yes; N, no.

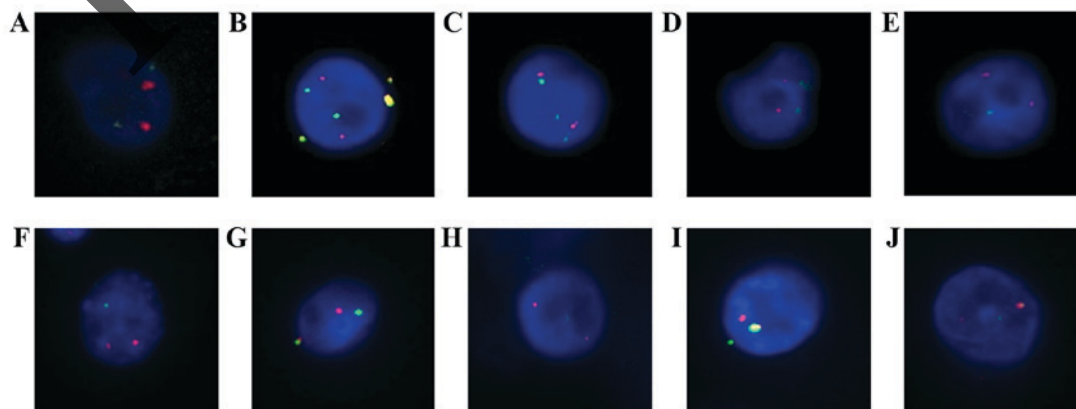


Figure 3. Detection of ALK rearrangements using FISH in cultured CTCs by Vysis ALK Break Apart FISH Probe kit. ALK-positive CTCs from patients with non-small cell lung cancer with ALK arrangement: (A) no. 4, (B) no. 6, (C) no. 7, (D) no. 8, (E) no. 9, (F) no. 14, (G) no. 17, (H) no. 18, (I) no. 19 and (J) no. 20. In (B), the fused yellow signals outside the cell were determined to be the noise signal.

a critical effect on the detection of CTCs because expression patterns of CTCs markers, including EpCAM, cytokeratin and vimentin, may be altered during the EMT process (13,29-32). In the present study, CTCs were isolated from the blood of patients with ALK rearrangement and were cultured to confirm whether the cultured cells maintain the ALK rearrangement. CTCs were identified using the CTC gold standard marker EpCAM. In 9 patients, EpCAM-positive CTCs were not identified in the blood, although ALK rearrangement was confirmed following the culture. We hypothesize that the phenomenon is due to the change in CTC properties caused by the EMT. ALK-rearranged CTCs consist of epithelial as well as mesenchymal types. Only CTCs with epithelial lineage may be detected using EpCAM staining (13,32). Therefore, it was assumed that the ALK-positive cultured CTCs in these 9 cases were mesenchymal.

Determining the ALK rearrangement status is crucial for prescribing treatment with ALK inhibitors including crizotinib, in patients with NSCLC (4,13). FISH, immunohistochemistry and RT-PCR have been used to detect ALK rearrangements (33). In the present study, ALK rearrangements were analyzed in patients with NSCLC who had been previously diagnosed as ALK-positive using tissue biopsies. ALK rearrangements were confirmed in CTCs of 16/22 patients.

The ALK inhibitor crizotinib has been used to treat ALK-positive patients with NSCLC. In total, >60% of such patients respond to crizotinib and the median progression-free time is 8-10 months (5). However, despite the initial response, in the majority of patients, the tumor relapsed because of crizotinib resistance. In previous studies, ceritinib has been identified to be an effective treatment option in crizotinib-resistant patients (6). Therefore, monitoring ALK-positive CTCs during crizotinib treatment using serial liquid biopsies may be a useful tool for making a decision on whether other ALK inhibitors, including ceritinib, are required.

The efficiency of CTC-based ALK rearrangement was tested using FISH and immunocytochemistry. The results from immunocytochemistry were unclear in certain patients. The scale of the present study is insufficient for accurate comparison and statistical analysis between ALK rearrangement of tissue biopsy and CTCs, therefore further study is required to reveal the association between ALK rearrangement in tissue biopsy and CTCs. However, on the basis of the results of the present study, it is suggested that FISH is a more effective and precise method for detecting ALK rearrangement in CTCs.

Analyzing ALK rearrangements in CTCs may be a useful method for predicting crizotinib resistance and for selecting additional inhibitor treatment in patients with NSCLC. The results of the present study demonstrated that detecting ALK rearrangements in CTCs using FISH may be considered as an alternative diagnostic method in patients from whom a tissue biopsy sample may not be obtained. This procedure is non-invasive and may, therefore, be repeated multiple times to monitor the drug response.

In the present study, the genetic associations between CTCs and tumor tissue of 22 patients with pre-diagnosed ALK rearrangement were verified. It is suggested that analysis of ALK rearrangement using FISH in CTCs be considered as an alternative method to tumor tissue biopsies where necessary. In addition, the following important insights into the use

of CTCs have been revealed: i) CTCs represent the genetic profile of primary tumor tissue; ii) culturing CTCs from liquid biopsies is an alternative to tissue biopsies for the detection of genetic variations in patients with cancer; iii) in patients with ALK rearrangement, CTCs may be helpful for monitoring the drug response and additional therapy selection.

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