Molecular analysis of cultured CTC (circulating tumor cells) from lung cancer patients

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Abstract
Circulating tumor cells (CTCs) are cells that have shed into the bloodstream from primary tumor and thus rare. CTCs could provide better understanding of tumor metastasis and noninvasive monitoring of the disease progression. However the isolation and characterization has been a major technological challenge due to their rareness. Here, we suggest the CTC culture as an effective method to obtain ample number of CTCs, which can be used for molecular analysis of original tumor characteristics and further applications. We isolated and successfully cultured the CTCs from four lung cancer patients. We analyzed the cultured CTCs to detect preexisting ALK (anaplastic lymphoma kinase) fusion using real-time PCR method, and confirmed that cultured CTCs have maintained the molecular characteristics similar to those found in primary tumors. These results suggest that the isolation and culture of CTCs can be a substitute method for tumor tissue biopsy, and may provide clinical applications, including serial blood samplings for the personalized cancer therapy based on their genomic information.

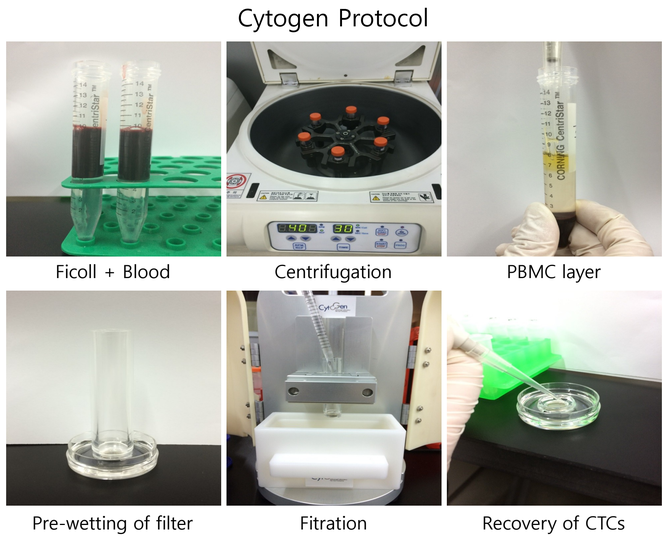
Introduction
Circulating tumor cells (CTCs) are present in the blood of cancer patients at low concentrations. It has been proposed that CTCs may be a prospective prognostic marker for cancer progression and survival in several types of cancer [1, 2] and a potential source of the metastatic tumor cells [3, 4]. Viable CTCs isolated from cancer patients can be a useful tool for identifying molecular targets and developing new cancer treatments. However, the isolation and characterization of CTCs are technically challenging due to their rareness and heterogeneity [5].
Lung adenocarcinoma is the most common subtype of lung cancer today. Recently, the treatment paradigm for advanced non-small cell lung cancer (NSCLC) has been transformed from conventional chemotherapy to targeted therapy based on molecular aberrations in primary tumor [6]. Now, it has been regarded as standard procedure to test lung carcinoma for the presence of *EGFR* mutation and *ALK* rearrangement upon diagnosis, in order to select patients for initial *EGFR* tyrosine kinase inhibitor (TKI) and *ALK* inhibitor therapy. However, the detection of such molecular abnormalities is complicated due to the difficulty in obtaining tumor material from repeated tissue biopsies [7]. Here, we were able to obtain sufficient amounts of CTCs through CTC cultures, and performed molecular analyses using cultured CTCs to confirm preexisting ALK rearrangement.

Materials and Methods

Blood collection
Blood samples (5-10 ml) from advanced NSCLC cancer patients

Primary culture of CTCs
Cytogen protocol [9]
Culturing in growth medium (RPMI-1640 medium supplemented with 10% FBS, 2% antibiotic-antimycotic) at 37°C, 5% CO_2 for 16-18 days

Cytogen Protocol



Immunofluorescence analysis
EpCAM (Cell Signaling), CD45 (Santa Cruz), DAPI staining

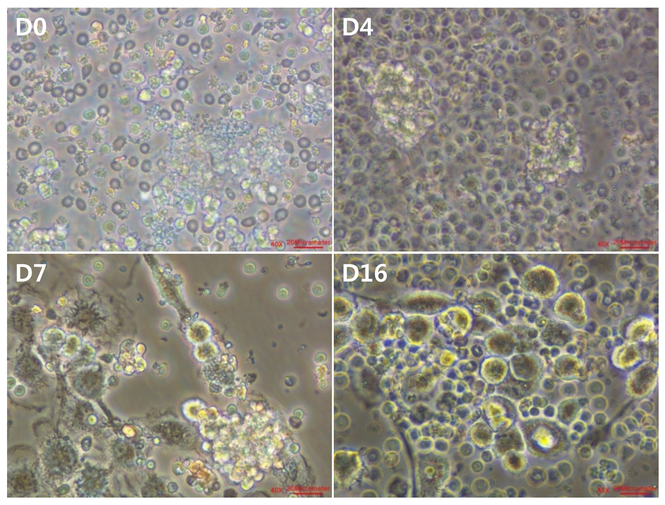
Immunocytochemistry
EpCAM (Cell Signaling)

FISH (Fluorescent in situ hibridization) for ALK rearrangement

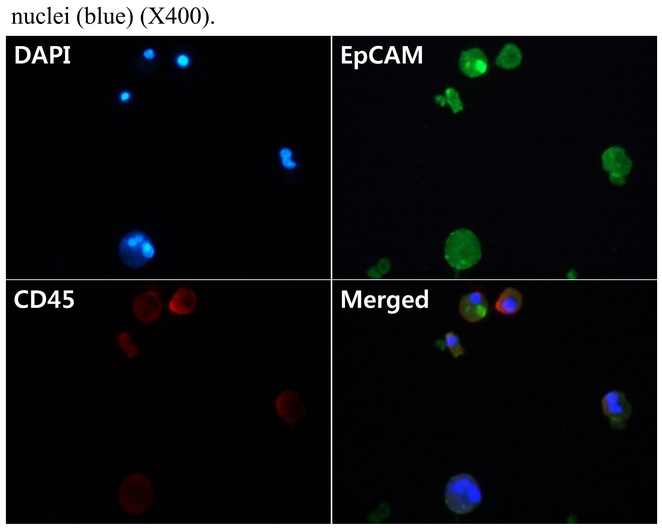
Quantitative real-time PCR for EML4-ALK fusion detection
AmoyDx EML4-ALK Fusion Gene Diagnostic Kit (Amoy Diagnostics Company Ltd.)

Results

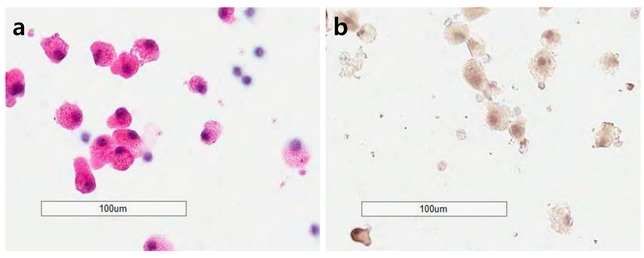
Representative images of CTC culture at day 0, 4, 7, and 16 (X400).



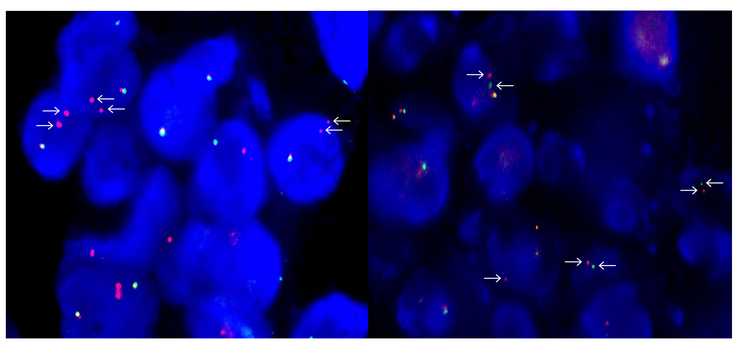
Immunofluorescent staining for EpCAM (green), CD45 (red), and nuclei (blue) (X400).



H&E staining (a) and Immunocytochemical staining for EpCAM (b) of cultured CTCs.



FISH (Fluorescent *in situ* hybridization) method for the detection of *ALK* rearrangement

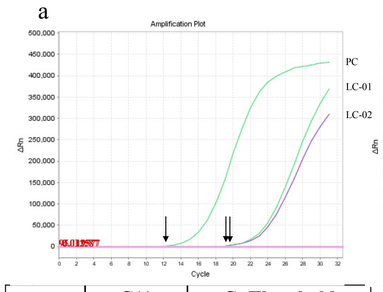


Isolated red pattern predominant

Frequent separate red and green pattern with occasional isolated red

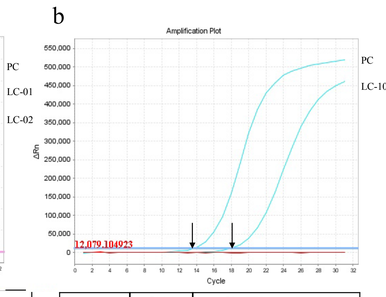
Real-time PCR analysis of cultured CTCs for ELM4-ALK rearrangement.

a



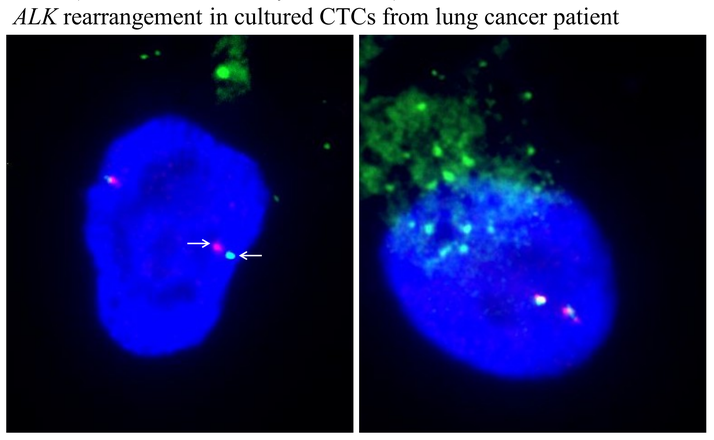
	C(t)	Ct Threshold
LC01	19.5491	2402.4
LC02	19.6349	2402.4
NC	ND	1963
PC	12.7681	1963
NTC	ND	1963

b



	C(t)	Ct Threshold
LC09	ND	12,079.1
LC10	18.15	12,079.1
NTC	ND	12,079.1
PC	13.79	12,079.1

FISH (Fluorescent *in situ* hybridization) method for the detection of *ALK* rearrangement in cultured CTCs from lung cancer patient



Separate red and green pattern

Conclusion
In our study, we showed that all FISH results in primary tumors were corresponding with real-time PCR results in cultured CTCs. Use of the cultured CTCs for molecular analysis has a merit of non-invasiveness and it could be easily repeated at different time-points during treatment to guide therapeutic decisions in a patient's treatment course.
For the successful application of this strategy to clinical practice, CTC culture conditions will be further optimized. In addition, further confirmative characterization methods, such as different cell marker staining and molecular profiling, should be developed for precise identification of cultured CTCs.

References
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