論　文　内　容　要　旨

Cell-Free DNA Analysis for EGFR mutations in Lung Adenocarcinoma Patients by Droplet Digital PCR

(デジタル PCR による遊離 DNA を用いた肺腺癌患者の EGFR 変異解析の検討)

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Background: Cell-free DNAs (cfDNA) analysis may provide a non-invasive diagnostic approach for lung adenocarcinoma patients. CfDNA, short double-stranded DNA derived from noncancerous and cancer cells, circulates in the bloodstream and carries the genetic information of the cancer cells, including tumor-specific mutations. As liquid biopsy, non-invasive approach for cancer diagnosis, cfDNA is one of the convenient blood-based biomaterials to be used. However, the low allele fraction (AF) of cfDNA derived from cancer cells is still challenging as it may affect the detection rate of mutation allele.

Recently, droplet digital PCR (ddPCR) has been developed with high sensitivity for mutation analysis. This study evaluated the utility of ddPCR for cfDNA analysis to examine its detection rate as liquid biopsy and compared the results with tumor samples for the correlation with the malignant grade.

Methods: Nineteen lung adenocarcinoma patients in Hiroshima University Hospital from 2010-2017 were enrolled in this study. All patients underwent biopsy and were diagnosed as lung adenocarcinoma. EGFR gene status in the biopsy specimen was already evaluated by clamp PCR (13 L858R, 3 E746-A750del, and 3 negatives). Tumor DNA (tDNA) samples were isolated from 19 formalin-fixed paraffin-embedded (FFPE) primary or metastasis site samples. Among them, 14 samples were obtained by transbronchial lung biopsy (TBLB) procedure and 5 samples were obtained by surgical procedure. All biopsy samples were taken before chemotherapy. Immunohistochemistry using EGF receptor L858R mutant-specific monoclonal rabbit antibody was conducted in 12 cases to evaluate the proportions of EGFR L858R-positive cells in each tumor. NCI-H1975 and Lc-Ad1 cell lines were used as positive and negative control, respectively. CfDNA was isolated from 17 serum samples taken at the time of diagnosis before starting chemotherapy. In addition, cfDNA was isolated from plasma samples of 4 healthy volunteers. CfDNA of healthy volunteers and placenta DNA were used for the detection limit of ddPCR assays analysis. CfDNA of healthy volunteers was used for the ddPCR false positive signal detection, while placenta DNA was used as negative control. NCI-H1975 and PC-9 cell lines were used as positive control for L858R and E746-A750del detection by ddPCR, respectively. Serial dilution of NCI-H1975 and PC9 cell lines with wild type human genomic DNA (placenta DNA) was performed to determine the detection limit of ddPCR assays. The detection limit was determined by the AF of the diluted cell line that was above that of cfDNA of healthy volunteers.

Results: Detection limit of AFs by ddPCR was 0.024% for EGFR L858R and 0.003% for EGFR E746-A750del. In tDNA samples, EGFR point mutation and deletion were detected

in 13/19 and 3/19 samples, respectively. In 17 cfDNA available cases, EGFR mutation and deletion were detected in 8/11 (61.5%) and 3/3 (100%) by ddPCR, respectively. In the remaining 3 negative cases, the tumor did not have EGFR L858R nor E746-A750del. Among cases where mutation was detectable in tDNA, AFs ranged from 5.7% to 71% (mean 32.7%) for EGFR L858R and from 7.5% to 34.7% (mean 19.4%) for EGFR E746-A750del. AFs of EGFR L858R mutation in cfDNA samples ranged from 0.04% to 5.12% (mean 1.3%) and EGFR E746-A750del ranged from 0.07% to 18.6% (mean 6.3%). Compared to tDNA, AFs of cfDNA were low.

The proportions of EGFR L858R-positive cells by immunohistochemistry in surgical samples ranged from 33.6% to 73.2% (mean 52.3%) and in TBLB samples ranged from 3.4% to 93% (mean 35.9%). AFs of tDNA by ddPCR and the proportions of EGFR L858R-positive cells by immunohistochemistry were relatively correlated ($r = 0.53$, $p = 0.1$, $N = 12$). However, no correlation was found between AFs of cfDNA and AFs of tDNA ($r = -0.11$, $p = 0.69$, $N = 17$) and between AFs of cfDNA and the proportions of EGFR L858R-positive cells ($r = -0.56$, $p = 0.09$, $N = 10$). In primary tumor biopsy samples by TBLB procedure, the case with the lowest proportion of EGFR L858R-positive cells (16.1%) had the highest AF of cfDNA (5.12%). In contrast, the case with the highest proportion of EGFR L858R-positive cells (67.7%) had no detectable mutated AF in cfDNA. No significant correlation was shown between AF rates of mutations in cfDNA with any clinicopathological factors, including outcome, tumor size, lymph node metastasis, distant metastasis, and stage. However, when AFs of cfDNA were divided into two groups, ≤0.1% and >0.1%, all patients with AF of cfDNA >0.1% had lymph node metastasis and distant metastasis.

Discussion: CfDNA derived from tumor cells is diluted with an abundant amount of cfDNA derived from normal cells, therefore, the AFs of mutation and deletion in cfDNA can be very low. Our study showed the ability of cfDNA analysis using ddPCR with high detection rate. The proportions of EGFR L858R-mutated cells in tissue samples relatively correlated with AFs of tDNA. However, no correlation was found between the AFs in cfDNA and tDNA. AFs of cfDNA may be associated with the characteristics of the tumor, such as lymph node metastasis and distant metastasis, instead of mutated cells number.

In conclusion, cfDNA analysis by ddPCR enables high detection rates for EGFR L858R and E746-A750del. Therefore, patients with lung adenocarcinoma could be benefited by cfDNA analysis using ddPCR as it may provide complementary data to the tumor biopsy for precision medicine.