Detecting ALK, ROS1 and RET Fusion Genes in Cell Block Samples

Abstract
Whether Cell block (CB) samples are applicable to detect anaplastic lymphoma kinase (ALK), c-ros oncogene 1 (ROS1) and ret proto-oncogene (RET) fusion genes in lung adenocarcinoma is still unknown. In this study, 108 cytological samples that contained lung adenocarcinoma cells were collected, and made into CB. The CB samples all contained at least 30% lung adenocarcinoma cells. In these patients, 48 harbored EGFR mutation. Among the 50 EGFR wild type patients who detected fusion genes, 14 carried EML4-ALK fusion (28%), 2 had TPM3-ROS1 fusion (4%), and 3 harbored KIF5B-RET fusion (6%). No double fusions were found in one sample. Patients with fusion genes were younger than those without fusion genes (p = 0.032), but no significant difference was found in sex and smoking status (p > 0.05). In the thirty-five patients who received first-line chemotherapy, patients with fusion gene positive had disease control rate (DCR) (72.7% VS 50%, p > 0.05) and objective response rate (ORR) (9.1% VS 4.2%, p > 0.05) compared with those having fusion gene negative. The median progression free survival (mPFS) were 4.0 and 2.7 months in patients harbored fusion mutations and wild type, respectively (p > 0.05). We conclude that CB samples could be used to detect ALK, ROS1 and RET fusions in NSCLC. The frequency distribution of three fusion genes is higher in lung adenocarcinoma with wild-type EGFR, compared with unselected NSCLC patient population. Patients with fusion genes positive are younger than those with fusion gene negative, but they had no significantly different PFS in first-line chemotherapy.

Introduction
Lung cancer is the leading cause of cancer death worldwide [1], non–small-cell lung cancer (NSCLC) accounts for about 85%. Along with the discovery of somatic epidermal growth factor receptor (EGFR) mutations, NSCLC patients with activating EGFR mutations benefit from EGFR-TKI therapy [2–4]. Since then, targeted therapies according to gene mutations lead a new trend in tumor therapy. Subsequently, more driver mutations are found in NSCLC, including many fusion gene mutations, such as anaplastic lymphoma kinase (ALK), ROS1 and RET.

Echinoderm microtubule associated protein like 4 (EML4)-ALK is the first targetable fusion gene to be identified in NSCLC [5]. The fusion is found about 2-7% in lung cancer [5–8]. Other genes which can fuse with ALK had also been found, including KIF5B and TFG [7,9,10]. In NSCLC never/light smokers without EGFR mutation...
the mutation frequency of EML4-ALK was 33% [11], and in lung adenocarcinoma patients with malignant pleural effusions having wild-type EGFR and measurable target lesions it was reported as 34% [12]. Many drugs that target EML4-ALK had been discovered, such as crizotinib, which was effective in ALK-rearranged NSCLC [13] and approved by US food and drug administration (FDA) in treating ALK-positive NSCLC. ROS1 was also reported to be a target of crizotinib [14,15], but its frequency only ranges from 0.7-1.7% [13,15–17] in lung adenocarcinoma. RET, as another fusion gene, is rarely detected in NSCLC, which is reported from 1-2% [18–20]. Several drugs (sunitinib, sorafenib, and vandetanib) that target RET fusions are effective [18,21].

Molecular typing is essential for NSCLC patients to select the optimal treatment. Although tumor tissue is the most valuable specimen for gene mutation detection, it is not always available especially for advanced NSCLC patients that are old aged and have inoperable tumor. In advanced lung cancer patients, 50% has malignant pleural effusions and 80% of the effusions can find tumor cells in microscope [22,23]. Therefore, this kind of cytological samples could be a surrogate to tumor tissues. In this study, CB samples were done and used to detect ALK, ROS1 and RET fusion genes, the relationship between clinicopathologic characteristics and the fusion genes were analyzed.

Method

Patients and CB Samples

108 patients with pleural, ascitic or pericardial effusions conducted EGFR mutation detection. They were all lung adenocarcinoma patients, in stage IV and had PS score 0-1. All patients had signed an informed consent for future molecular analyses. Patient follow-up was ended in 20th, December, 2013. The effusions (50 to 1200 ml) containing lung adenocarcinoma cells were collected from October 2012 to August 2013. Simply, the effusion was centrifuged at 2500 rpm for 3 minutes, the supernatant was removed and the precipitant was mixed with erythrocyte lysate for 10 minutes. After centrifuging at 2500 rpm for 3 minutes the precipitant was resuspended in normal saline solution and then was centrifuged again. The precipitant was packaged by mixing with warm agarose gel and had routinely dehydration before packaging in paraffin wax. Sections of 5 μm thick from the samples were used for hematoxylin and eosin staining and assessed by pathologists.

EGFR Mutations Detection

DNA was extracted from the 108 effusion samples or CB samples using tissue DNA kit and FFPE DNA kit (QIAGEN, Hilden, Germany) respectively. EGFR was examined using amplification refractory mutation system (ARMS) PCR method. The ARMS PCR procedure was as follows: 5 μl of 1 (effusion samples) or 2 ng/μl (CB samples) template DNA solutions was added to each reaction buffer and then [1] initial denaturation at 95°C for 5 min, [2] 15 cycles of 95°C 25 s, 64°C 20s, and 72°C 20s, [3] 31 cycles of 93°C 25 s, 60°C 35 s, and 72°C 20s was conducted before analyzing the results.

ALK, ROS1 and RET Fusion Gene Detection by ARMS PCR

CB samples were scraped into 1.5 mL tubes, and then total RNA was extracted using RNeasy FFPE kit (QIAGEN, Hilden, Germany). RNA was reversed to cDNA, added to reaction buffer and then ALK, ROS1 and RET fusion genes were detected using EML4-ALK, ROS1 and RET Fusion Gene Detection Kit (Amoydx, Xiamen, China) respectively by ARMS method as mentioned above. All the fusion positive samples were confirmed by DNA sequencing.

Statistical Analysis

The ORR, DCR, the relationship between fusion gene mutations and other clinical characteristics were evaluated by Pearson Chi-square test or Fisher’s exact test. Median PFS was analyzed by Kaplan–Meier method and compared between different groups using the log-rank test. The 2-sided significance level was set at P < 0.05. All data were analyzed using the Statistical Package for the Social Sciences version 17.0 software package (SPSS Inc., Chicago, Ill).

Results

The Quality of CB Samples

The CB samples were preserved between days to 10 months before cut into 5 μm thick sections, and then routinely stained by hematoxylin and eosin. Tumor cell content and pathological type were assessed by pathologists (Figure 1). All the samples were

![Figure 1](image-url). Cell block samples contain lung adenocarcinoma cells. CB samples of 5 μm thick sections from two patients were stained by hematoxylin and eosin. The lung adenocarcinoma cells in the pictures were marked by the black arrows.
confirmed to be lung adenocarcinoma, and the tumor cell content of each specimen was more than 30%.

**ALK, ROS1 and RET Fusion Types in the Fusion Positive Patients**

In the 108 patients, 48 (44%) had EGFR mutation. The characteristics of the 108 lung adenocarcinoma patients were listed in Table 1. They had no significant difference in age, sex and smoking status between patients with or without EGFR mutation. In the EGFR wild type patients 50 conducted fusion gene detection. Of these, 14 had ALK fusion (28%), 2 had ROS1 fusion (4%), and 3 had RET fusion (6%). PCR positive samples were all verified by DNA sequencing. The ALK fusions were: eight E(EML4) exon 13 with A (ALK) exon 20 fusions, four E20 with A20 fusions, one E18 with A20 fusion, and one E6 with A20 fusion. The ROS1 fusions were ROS1 exon 34 with TPM3 exon 8. The three RET fusions were all RET exon 12 with KIF5B exon n15.

**Clinicopathologic Characteristics of the Gene Fusion Positive Patients**

The patients who harbored fusion gene mutation were listed in Table 2. In the EML4-ALK patients, 11 were under 60 and 8 were none or light smokers. The TPM3-ROS1 and two KIF5B-RET patients were under 60 years old and none-smokers, and one KIF5B-RET patient was a heavy smoker (30 pack-years) and under 60. There was no significant difference between the patients with and without any one of the fusion genes in sex, and smoking status (p > 0.05), but the patients with fusion gene mutations were younger than those without mutations (median age, 51 vs 61, p = 0.032).

**Clinical Outcome of Patients With and Without the Fusion Genes**

Thirty-five of the 50 patients received first-line chemotherapy in this hospital, including 29 carboplatin or cisplatin contained therapies, 2 single drug therapies and 4 TKI targeting EGFR therapies. In these patients, twenty-four did not carry any mutation of three fusion genes, eight were ALK fusion positive and three were RET fusion positive (Table 3). In the last follow-up, three patients did not get disease progression. ORR was 4.2% and 9.1% in patients without and with fusion gene mutation, respectively (p > 0.05); DCR was 50% and 72.7%, respectively (p > 0.05). The median PFS of the EML4-ALK positive patients was 4.2 (95% confidence intervals, 1.890-6.510) months vs 2.9 (95% CI, 1.658-3.942) months (p = 0.706) in the EML4-ALK negative patients and in either one of three genes positive patients it was 4.0 (95% CI, 2.605-5.395) months vs 2.7 (95% CI, 1.551-3.849) months (p = 0.371) in the non-positive patients (Figure 2). Although there was no significant difference between the two cohorts, the results showed a trend that patients with fusion genes had a better chemotherapy response than those without any one of fusion genes in chemotherapy.

**Discussion**

Cell block (CB) is a method to concentrate and preserve cells in fluid samples for long use. Compared with effusion smears, CB contains more cells to be identified and helps pathologists in decision making. It has been used routinely in pathological classification and also in gene detection. In certain cases it has an advantage to other conventional pathological methods [24]. In advanced-stage patients who cannot have their tissues dissected, CB samples could be an alternative selection. In this study, the authors detected ALK, ROS1 and RET fusion genes in EGFR wild type lung adenocarcinoma patients using cell block samples and analyzed the prevalence of the fusion genes and the relationship between clinicopathologic characteristics and fusion gene mutations.

Fluorescence in situ hybridization (FISH) is the primary method to detect ALK, ROS1 and RET fusions in NSCLC [14,25,26]. However, it is not wildly used in China due to its high spent, time consuming and also the interpretation of results. Immunohistochemistry (IHC) is another method to detect ALK fusion, but there is no standard procedure for all the labs and the same result could be explained differently by different pathologists. Soda showed us in his study that different technologies should apply to different samples, and multiplex RT-PCR was applicable for the fluid samples [27]. Here, we use a reverse-transcript polymerase chain reaction (RT-PCR) method-ARMS-to detect ALK, ROS1 and RET fusions in 50 CB samples. Wu [12] used RT-PCR and FISH to detect ALK fusion and they found a concordance rate of 85%, but they did not check cell block samples that were ALK fusion positive using FISH. Soda [27] reported in their research that PCR-based detection of EML4-ALK should have a higher analytic sensitivity compared with IHC or FISH. In this study, although we did not use FISH to conform the PCR results, we used DNA sequencing as a substitute. All the positive results using the PCR method were all conformed by DNA sequencing. We believe that the cell block samples could detect the three fusion genes using both RT-PCR and DNA sequencing.

We tested the quality of cell block samples from the points of malignant cell ratio and PCR controls, finding that they were...
qualified to do the gene detection. The fusion positive results were all validated by DNA sequencing and the specific variants were also given. The results indicate that cell block samples preserved at least 10 months could be used to detect fusion genes. EML4-ALK fusion gene detection using plural effusions had been reported by Wu et al. [12].

In this study, we demonstrated that CB samples could be an option to substitute tissues to detect ALK, ROS1 and RET fusion genes in lung cancer patients. Patients with fusion gene mutation may have a better clinical response than those without mutations, which needed to be confirmed by a large sample study.

References

[1] Jemal A, Bray F, Center MM, Ferlay J, Ward E, and Forman D (2011). Global cancer statistics. CA Cancer J Clin 61, 69–90.

[2] Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, Harris PL, Haserlat SM, Supko JG, and Halausa FG, et al (2004). Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med 350, 2129–2139.

[3] Paez JG, Janne PA, Lee JC, Tracy S, Greulich H, Gabriel S, Herman P, Kaye FJ, Lindeman N, and Boggon TJ, et al (2004). EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science 304, 1497–1500.

[4] Pao W, Miller V, Zakowski M, Doherty J, Politi K, Sarkaria I, Singh B, Heelan R, Rusch V, and Fulton L, et al (2004). EGFR receptor gene mutations are common in lung cancers from “never smokers” and are associated with sensitivity of tumors to gefitinib and erlotinib. Proc Natl Acad Sci USA 101, 13306–13311.

[5] Soda M, Choi YL, Enomoto M, Takada S, Yamashita Y, Ishikawa S, Fujiwara S, Watanabe H, Kurasnsha K, and Hatanaka H, et al (2007). Identification of the transforming EML4-ALK fusion gene in non-small-cell lung cancer. Nature 448, 561–566.

[6] Koivunen JP, Mermel C, Zeijnnulah K, Murphy C, Lifshits E, Holmes AJ, Choi HG, Kim J, Chiang D, and Thomas R, et al (2008). EML4-ALK fusion gene and efficacy of an ALK kinase inhibitor in lung cancer. Clin Cancer Res 14, 4275–4283.

[7] Takeuchi K, Choi YL, Soda M, Inamura K, Bogashi Y, Harano S, Enomoto M, Takada S, Yamashita Y, and Satoh Y, et al (2008). Multiplex reverse transcription-PCR screening for EML4-ALK fusion transcripts. Clin Cancer Res 14, 6618–6624.

[8] Inamura K, Takeuchi K, Bogashi Y, Nomura K, Ninomiya H, Okui M, Satoh Y, Okumura S, Nakagawa K, and Soda M, et al (2008). EML4-ALK fusion gene is linked to histological characteristics in a subset of lung cancers. J Thorac Oncol 3, 13–17.

[9] Choi YL, Takeuchi K, Soda M, Inamura K, Bogashi Y, Harano S, Enomoto M, Hamada T, Haruta H, and Watanabe H, et al (2008). Identification of novel isoforms of the EML4-ALK transforming gene in non-small cell lung cancer. Cancer Res 68, 4971–4976.

[10] Takeuchi K, Choi YL, Bogashi Y, Soda M, Hatano S, Inamura K, Takada S, Ueno T, Yamashita Y, and Satoh Y, et al (2009). KIF5B-ALK, a novel fusion oncokine identified by an immunohistochemistry-based diagnostic system for ALK-positive lung cancer. Clin Cancer Res 15, 3143–3149.
[11] Shaw AT, Yeap BY, Mino-Kenudson M, Digumarthy SR, Costa DB, Heist RS, Solomon B, Stubbbs H, Admane S, and McDermott U, et al (2009). Clinical features and outcome of patients with non-small-cell lung cancer who harbor EML4-ALK. J Clin Oncol 27, 4247–4253.

[12] Wu SG, Kuo YW, Chang YL, Shih JY, Chen YH, Tsai MF, Yu CJ, Yang CH, and Yang FC (2012). EML4-ALK translocation predicts better outcome in lung adenocarcinoma patients with wild-type EGFR. J Thorac Oncol 7, 98–104.

[13] Kwak EL, Bang YJ, Camidge DR, Shaw AT, Solomon B, Maki RG, and Yang PC (2012). EML4-ALK translocation predicts better outcome in lung adenocarcinoma patients with wild-type EGFR.

[14] Rikova K, Guo A, Zeng Q, Possemato A, Yu J, Haack H, Nardone J, Lee K, Reeves C, and Li Y, et al (2007). Global survey of phosphotyrosine signaling identifies oncogenic kinases in lung cancer. Cell 131, 1190–1203.

[15] Kohno T, Ichikawa H, Tani Y, Maekawa M, Okuda K, Yokota K, Hikosaka Y, Moriizama S, and Yano M, et al (2012). KIF5B/RET gene rearrangements in Japanese lung cancer. Lung Cancer 71, 4920–4931.

[16] Rikova K, Guo A, Zeng Q, Possemato A, Yu J, Haack H, Nardone J, Lee K, Reeves C, and Li Y, et al (2007). Global survey of phosphotyrosine signaling identifies oncogenic kinases in lung cancer. Cell 131, 1190–1203.

[17] Kimura H, Fujiwara Y, Sone T, Kunitoh H, Tamura T, Kasahara K, Nishio H, and Hiramoto M, et al (2012). KIF5B-RET fusion in lung adenocarcinomas through a comprehensive mRNA-based screen for tyrosine kinase fusions. Clinic Cancer Res 18, 6599–6608.

[18] Lipson D, Capelletti M, Yelenosky R, Otto G, Parker A, Jarosz M, and Curran JA, et al (2012). Identification of new ALK and RET gene fusions from colorectal and lung cancer biopsies. Nat Med 18, 382–384.

[19] Cavalli P, Riboli B, Generali D, Passalacqua R, and Bosio G (2006). EGFR genotyping in pleural fluid specimens in NSCLC patients. Lung Cancer 54, 265–266.

[20] Kimura H, Fujiwara Y, Sone T, Kunitoh H, Tamura T, Kasahara K, and Nishio H (2006). EGFR mutation status in tumour-derived DNA from pleural effusion fluid is a practical basis for predicting the response to gefitinib. Br J Cancer 95, 1390–1395.

[21] Sanz-Santos J, Serra P, Andreo F, Llatjos M, Castella E, and Monzo E (2012). Contribution of cell blocks obtained through endobronchial ultrasound-guided transbronchial needle aspiration to the diagnosis of lung cancer. BMC cancer 12, 34.

[22] Shaw AT, Solomon B, and Kenudson MM (2011). Crizotinib and testing for ALK. J Natl Compr Canc Netw 9, 1335–1341.

[23] Soda M, Isobe K, Inoue A, Maemondo M, Oizumi S, Fujita Y, Gemma A, Yamashita Y, Ueno T, and Takeuchi K, et al (2012). A prospective PCR-based screening for the EML4-ALK oncogene in non-small cell lung cancer. Clin Cancer Res 18, 5682–5689.

[24] Cai G, Wong R, Chhieng D, Levy GH, Gettinger SN, Herbst RS, Puchalski JT, Homer RJ, and Hui P (2013). Identification of EGFR mutation, KRAS mutation, and ALK gene rearrangement in cytological specimens of primary and metastatic lung adenocarcinoma. Cancer Cytopathol 121, 500–507.

[25] Cai W, Su C, Li X, Fan L, Zheng L, Fei K, and Zhou C (2013). KIF5B-RET fusion in Chinese patients with non-small cell lung cancer. Cancer 119, 1486–1494.

[26] Rodig SJ, Mino-Kenudson M, Dacic S, Yeap BY, Shaw A, Barletta JA, Stubbbs H, Law K, Lindeman N, and Mark E, et al (2009). Unique clinicopathologic features characterize ALK-rearranged lung adenocarcinoma in the western population. Clin Cancer Res 15, 5216–5223.

[27] Scagliotti G, Stahel RA, Rosell R, Thatcher N, and Soria JC (2012). ALK translocation and crizotinib in non-small-cell lung cancer: an evolving paradigm in oncology drug development. Eur J Cancer 48, 961–973.