SPECIAL REPORT

Next generation sequencing detection in archival surgically resected lung adenocarcinoma specimens harbouring the anaplastic lymphoma kinase fusion protein

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Abstract

Objective: To analyse clinical and molecular features in patients with surgically resected patients with lung cancer harbouring anaplastic lymphoma kinase fusion.

Methods: The retrospective study was conducted at Zhejiang Cancer Hospital, Hangzhou, China, and comprised data from November 2013 to August 2015 of lung cancer patients. Anaplastic lymphoma kinase, epidermal growth factor receptor, kirsten rat sarcoma viral oncogene, v-raf murine sarcoma viral oncogene homolog, REarranged during Transfection proto-oncogene, c-ros oncogene 1 receptor kinase, V-Erb-B2 avian erythroblastic leukaemia viral oncogene homolog 2 and mesenchymal epithelial transition factor were noted using next generation sequencing. Clinicopathological parameters were also investigated. All patients were followed up till August 10, 2017. Data was analysed using SPSS 22.

Results: Of the 19 patients, 15(79%) were non-smokers. Anaplastic lymphoma kinase rearrangements occurred in the acinar predominant in 6(31.6%), solid predominant 6(31.6%) and mucinous predominant 4(21%) adenocarcinomas. There was 1(5.2%) patient with epidermal growth factor receptor 21 G863D mutation. The 3-year disease-free survival rate in 5(26.3%) cases of anaplastic lymphoma kinase variant 1 was 5(100%), while in the 14(73.7%) cases of non-variant 1 group it was 9(64.3%) (p=0.257).

Conclusion: Anaplastic lymphoma kinase rearrangements did not tend to be accompanied with other driver genes. Difference between variant 1 and non-variant 1 patients was uncertain and needs to be further investigated.

Keywords: Lung cancer, ALK genotype, Next generation sequencing, Disease-free survival, Molecular characteristic.

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Introduction

Echinoderm microtubule-associated protein-like4 (EML4)-anaplastic lymphoma kinase (ALK) has been identified as one of driver genes in non-small-cell lung cancer (NSCLC) in preclinical experiments where inhibition of EML4-ALK was found to be leading to apoptosis of tumour cells expressing corresponding fusion protein.[1] EML4-ALK fusion transcript is found in approximately 4-5% of NSCLCs.[1] In most cases, ALK rearrangements are considered to be mutually exclusive with epidermal growth factor receptor (EGFR) and Kirsten rat sarcoma viral oncogene homolog (KRAS) mutations.[2] ALK inhibitors have shown to be more effective for lung cancer patients with ALK rearrangements compared to chemotherapeutic drugs. In comparison with standard firstline chemotherapy -- pemetrexed combined with platinum -- crizotinib is considered a superior choice for treating ALK-positive NSCLC patients.[3] The benefits of crizotinib include symptom improvement and better life quality.[4,5] Furthermore, patients with brain metastases treated with crizotinib had a significantly higher intracranial disease control rate compared to those treated with chemotherapy.[6]

Crizotinib has shown to be more efficient for patients with ALK variant 1 versus non-variant 1; median progression-free survival (PFS) of patients with ALK variant 1 treated with crizotinibi iwas significantly longer compared to those with ALK non-variant 1 (11.0 months vs 4.2 months, respectively; $p< 0.05$), which suggests that the efficacy of ALK inhibitors might be sensitive to the structurally diverse ALK kinase inhibitors, such as crizotinib and TAE684, varies in patients with dissimilar ALK fusion genes and EML4-ALK variants, and the differential sensitivity is related to the difference in protein of EML4-ALK expression cells.[7] The data could partly explain the phenomenon that ALK-positive tumours exhibit diverse responses to ALK inhibitors in clinical practices. Consequently, targeted therapy for patients with lung cancer harbouring ALK positivity should be further selected according to precise ALK genotype.[8] Moreover, it appears necessary to clear ALK genotype in lung cancer harbouring ALK fusion protein. It still remains unclear whether there is any difference among patients with resected lung cancer and different ALK fusion genes; especially with reference to disease free survival (DFS), as well as which patients are easier to relapse; those

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with ALK variant 1 or non-variant 1.

Oncogenic drivers in NSCLC are considered mutually exclusive. Nevertheless, some studies have revealed that EGFR mutations and ALK rearrangements can concomitantly occur.[9-11] Both EGFR inhibitors and ALK inhibitors have durable effect on patients harbouring both gene alterations in the second line once the tumour has progressed.[9,10] EGFR mutations and ALK rearrangements may coexist in the same tumour cells.[10] Li et al. have reported no EGFR/KRAS mutation in seven EML4-ALK-positive patients.[12] Furthermore, Shaozhang et al. found no EGFR mutations in their patients; while KRAS mutation was observed only in one patient.[13] Moreover, a concurrent EGFR or KRAS mutation was detected in one among 200 cases of ALK-positive patients.[11]

The current study was planned to investigate clinical and molecular characteristics and prognostic value in postoperative patients with lung adenocarcinoma harbouring ALK fusion protein variant 1 or non-variant 1.

Materials and Methods

The retrospective study was conducted at Zhejiang Cancer Hospital, Hangzhou, China, and comprised data from November 2013 to August 2015 of lung cancer patients. After approval from the institutional ethics review committee, ALK-positive specimens were collected that were were diagnosed by immunohistochemistry (IHC) using a highly sensitive anti-ALK (D5F3) rabbit monoclonal primary antibody (Ventana Medical Systems Inc, Roche, Inc., Tuscon, AZ). They were analysed using OptiView Amplification Kit and an OptiView DAB IHC Detection Kit (Ventana Medical Systems Inc, Roche, Inc., Tuscon, AZ). All specimens were obtained from resected lung cancer tumours. Pathological diagnosis was based on the standard criteria defined by the World Health Organisation (WHO).[14] The breakdown of the stages was defined by the eighth edition of the Tumour, Node, Metastasis (TNM) classification for lung cancer.[15]

All stage IA patients with lung adenocarcinoma received no adjuvant chemotherapy; 1 stage IB patient received four cycles of pemetrexed plus cisplatin as adjuvant chemotherapy; and all stage IIA, IIB and IIIA patients received four cycles of adjuvant chemotherapy with gemzar combined with cispaltin or pemetrexed combined with cisplatin, and among them a patient with stage IIIA underwent adjuvant thoracic radiotherapy. Clinicopathological features including gender, age, stages, pathological types, smoking history and adjuvant treatment were noted.

Next-generation sequencing (NGS) was applied to detect ALK, EGFR, KRAS, v-raf murine sarcoma viral oncogene homolog (BRAF), Rearranged during Transfection proto-oncogene (RET), c-ros oncogene 1 receptor kinase (ROS1), V-Erb-B2 avian erythroblastic leukaemia viral oncogene homolog 2 (CerbB-2) and mesenchymal epithelial transition factor (MET).

For NGS, tissue deoxyribonucleic acid (DNA) was extracted using QIAamp DNA formalin-fixed, paraffin-embedded (FFPE) tissue kit (Qiagen) according to the manufacturer's instructions. DNA concentration was measured using Qubit dsDNA assay.

For NGS library preparation, DNA shearing was performed using Covaris M220, followed by end-repair, phosphorylation and adaptor ligation. Fragments 200-400 bp in size were selected by bead (Agencourt Ampure XP Kit, Beckmann-Kurt, California, USA) followed by hybridisation with capture probes baits, hybrid selection with magnetic beads and polymerase chain reaction (PCR) amplification. A bioanalyser high-sensitivity DNA assay was then performed to assess the quality and size of the fragments and the indexed samples were sequenced on Nextseq500 sequencer (Illumina, Inc., California, US) with pair-end reads.

Genetic profiles of all tissue samples were assessed by performing capture-based targeted deep sequencing using the 8-gene panel (Burning Rock Biotech Ltd.), covering 76kb of human genome. DNA quality and size were assessed by high-sensitivity DNA assay using a bioanalyser. All the indexed samples were sequenced on a NextSeq 500 (Illumina, Inc., USA) with pair-end reads.

Sequence data was mapped to the human genome (hg19) using BWA aligner 0.7.10. Local alignment optimisation, variant calling and annotation were performed using GATK 3.2, MuTect, and VarScan. Plasma sample was compared against its own white blood cells (WBCs) to identify somatic variants. Variants were filtered using the VarScan fpfilter pipeline, with loci with depth <100 being filtered out. At least 2 and 5 supporting reads were used for insertion or deletion of bases in the genome of organisms (INDELs) in plasma and tissue samples, respectively, while 8 supporting reads were used for single nucleotide variants (SNVs) in both plasma and tissue samples. According to the Exac, 1000 genomes, Single Nucleotide Polymorphism Database (dbSNP), Exome Sequencing Project (ESP) 6500SI-V2 databases, variants with population frequency over 0.1% were grouped as SNP and excluded from further analysis.[16-19] The remaining variants were annotated with ANNOVAR and SnpEff v3.6. DNA translocation analysis was performed using both Tophat 2 and Factera 1.4.3.

All patients were followed up till August 10, 2017. Survival time was calculated from the date of pathological diagnosis.

Data was analysed using SPSS 22. Overall data was screened and 3-year DFS was analysed using Fisher's test. Log-rank test was used to estimate and compare DFS. P<0.05 was considered statistically significant.

Results

Of the 19 patients, 10(52.6%) were males and 9(47.4) were females. The overall media age was 56 years (interquartile range [IQR]: 33-66 years), and 15(79%) were non-smokers (Table 1). ALK variant 1 was found in 5(26.3%) cases, variant 2 in 1(5.3%) case, variant 3 in 6(31.6%) cases, variant 5 in 2(10.5%) cases, and other variants in 5(26.3%) cases (Figure 1). ALK rearrangements occurred in the acinar predominant in 6(31.6%), solid predominant 6(31.6%) and mucinous predominant 4(21%) adenocarcinomas. The amplification of CerbB-2 and MET, and negative fluorescence in situ hybridization (FISH) detection were observed in 1(5.3%) patient (Figure 2). In 1(5.3%) patient, EGFR-21 G863D mutation was observed (Figure 3). There were no mutations

Table-1: Clinicopathological features of patients (n=19).

	n (%)
Gender	
Male	10(52.6)
Female	9(47.4)
Stage	
IA1/IA2/IA3	7(36.8)
IB 3(15.8)	
IIA	2(10.5)
IIB	3(15.8)
IIIA	4(21.1)
Pathological type,	
Lepidic predominant	1(5.2)
Acinar predominant	6(31.6)
Papillary predominant	2(10.5)
Micropapillary predominant	-
Solid predominant with mucin production	6(31.6)
Mucinous	4(21.1)
Smoking[a]	
Non-smokers	15(79.0)
Light smokers	-
Moderate smokers	1(5.2)
Heavy smokers	3(15.8)
Adjuvant treatment[b]	
Not received adjuvant treatment	9(47.4)
Neoadjuvant chemotherapy	1(5.2)
Adjuvant chemotherapy	10(52.6)
docetaxel combined with cisplatin	1(10)
gemcitabine combined with cisplatin	2(20)
pemetrexed combined with cisplatin	7(70)
Adjuvant radiotherapy	1(5.2)

a: Smoking history: Non-smokers, light smokers (≤10 pack-years), moderate smokers (11–30 pack-years), and heavy smokers (≥30 pack-years).

b: One patient received neoadjuvant and adjuvant chemotherapy; One patient received adjuvant chemotherapy followed by adjuvant radiotherapy.

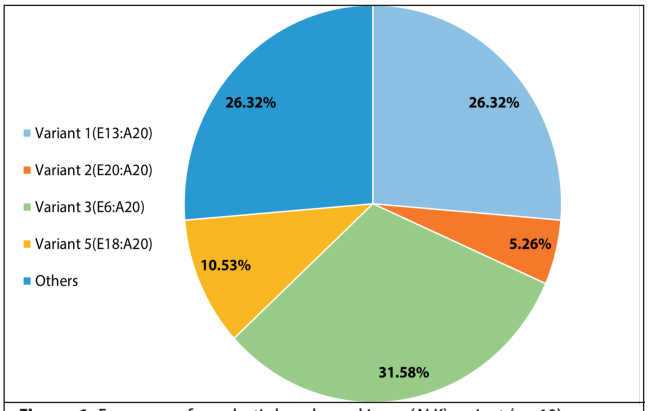

Figure-1: Frequency of anaplastic lymphoma kinase (ALK) variant (n=19).

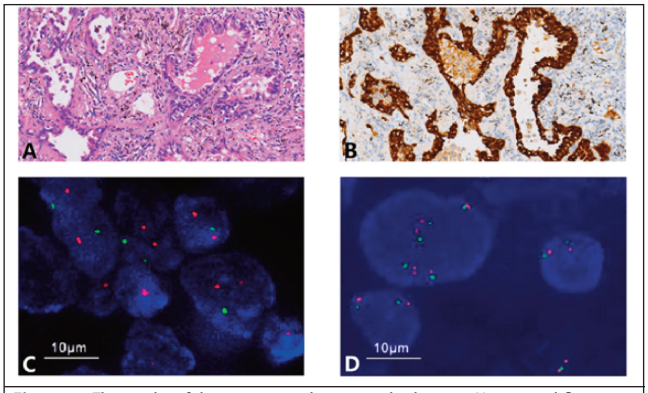

Figure-2: The results of the patients with tumours harbouring Her-2 amplification and c-Met amplification (FISH). (A) Hematoxylin-eosin (H&E) of the patients(200X), (B) Anaplastic lymphoma kinase(ALK) positive detected by immunohistochemistry (Ventana) (200X), (C) Her-2 amplification negative detected by fluorescence in situ hybridization(FISH), (D) c-Met amplification negative detected by fluorescence in situ hybridization(FISH).

Table-2: Clinicopathological features of patients (n=19).

No	Gene	Exon	Type	chr:pos	REF>ALT	AF
7	ERBB2	NA	amplification	17q12	NA	2.8%
7	MET	NA	amplification	7q31.2	NA	3.25%
18	EGFR	21	p.G863D	chr7:55259530	G>A	40.0%

ERBB2: Her-2, human epithelial growth factor receptor-2; MET: mesenchymal epithelial transition factor; EGFR: epidermal growth factor receptor

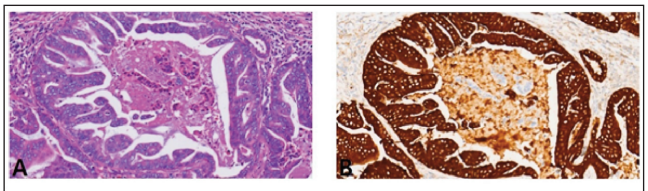

Figure-3: The patient with epidermal growth factor receptor (EGFR) 21 (G863D) mutation. (A) Haematoxylin-eosin (H&E) of the patients(200X), (B) Anaplastic lymphoma kinase(ALK) positive detected by immunohistochemistry (Ventana) (200X).

or amplifications of EGFR, KRAS, BRAF, RET, ROS1, CerbB-2 and MET in other patients (Table 2). There were no differences in terms of gender, tumour stage, pathological type and smoking history between patients with ALK variant 1 and non-variant 1 (p>0.05). No relapse was observed in the patients with ALK variant 1, while it was observed in 4(28.6%) patients with non-ALK variant 1; 3(75%) of them relapsed due to brain metastasis. The 3-year DFS rate in the ALK variant 1 group was 5(26.3%), while it was 14(73.7%) in non-variant 1 group it was 9(64.3%) (p=0.257) (Table 3; Figure 4).

Table-3: Clinicopathological features in patients with Anaplastic Lymphoma Kinase (ALK) variant 1 (n=5) and non-variant 1 (n=14) tumour.

	Variant 1 n (%)	Non-variant 1 n (%)	*p*-value	Chi-square
Gender,			0.628	0.434
Male	2(40)	8(57.1)		
Female	3(60)	6(42.9)		
Stage			0.275	5.125
IA1/IA2/IA3	1(20)	6(42.9)		
IB	1(20)	2(14.3)		
IIA	1(20)	1(7.1)		
IIB	2(40)	1(7.1)		
IIIA	-	4(28.6)		
Pathological type			0.461	4.388
Lepidic predominant	-	1(7.1)		
Acinar predominant	2(40)	4(28.6)		
Papillary predominant	1(20)	1(7.1)		
Micropapillary predominant	-	-		
Solid predominant with mucin production	-	6(42.9)		
Mucinous	2(40)	2(14.3)		
Smoking			1	0.434
Non-smokers	4(80)	11(78.6)		
Light smokers	-	-		
Moderate smokers	-	1(7.1)		
Heavy smokers	1(20)	2(14.3)		
3-year DFS	5(100%)	9(64.3%)	0.257	0.357

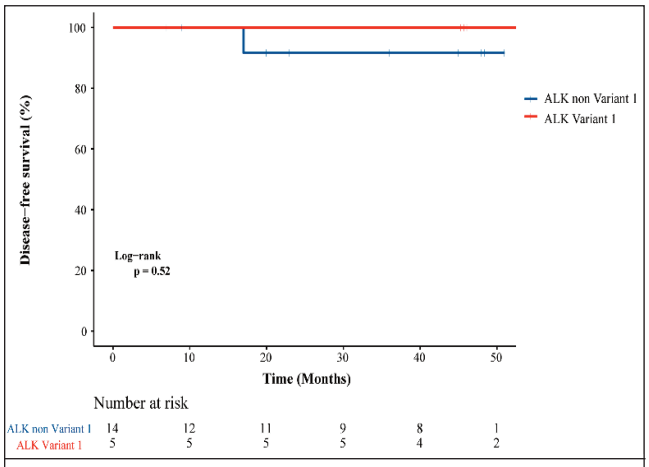

Figure-4: The Kaplan-Meier curve of disease-free survival (DFS) of anaplastic lymphoma kinase (ALK) variant 1 (n=5) and non-variant 1 (n=14) groups (p=0.519).

Discussion

The accuracy of Ventana (D5F3) IHC assay for detecting ALK rearrangement is nearly consistent with FISH in NSCLC patients.[20] In our cohort study, all specimens were collected from patients with positive ALK fusion protein tumour diagnosed using IHC (Ventana) technique. Furthermore, all ALK-IHC-positive specimens were confirmed by NGS. This suggested that application of IHC to detect EML4-ALK rearrangement could be preferentially recommended, while NGS may be considered in undefined cases. Reverse transcription PCR (RT-PCR) should be considered an alternative or supplemental method to detect ALK fusion oncogene in NSCLC patients.[21] Our fata regarding patient age and gender distribution is in line with Li et al.[11] Findings of Inamura et al.[22] and Sasaki et al[23] regarding different ALK variants are similar to our findings, which are different from the findings of another srudy.[7]

A study revealed that the acinar pattern is related to lung adenocarcinomas harbouring ALK rearrangements in Asian populations,[22] whereas in the Western patients, the signet-ring cell histology is more frequently reported.[24] The current study indicated that ALK rearrangement frequently occurred in the acinar predominant, solid predominant and mucinous predominant tumours. Additionally, the study supports the difference between pathological subtype of ALK-rearranged lung adenocarcinomas in Asian populations and in the Western patients. Moreover, most of our patients were non-smokers, which is consistent with earlier findings.[25]

First-line profiling of using broad, hybrid capture-based NGS testing for lung adenocarcinomas is a more comprehensive and efficient strategy compared to non-NGS testing.[26] A more precise and clinically useful classification for lung adenocarcinoma at the molecular level may be brought about by targeted NGS.[27] Traditional capillary-based single-gene sequencing using first-generation technique, known as Sanger sequencing, has been replaced by NGS since it allows massive parallel sequencing with lower cost and higher output.[28] Metastatic breast cancer patients harbouring human epidermal growth factor receptor 2 (HER2) positivity can clinically benefit from trastuzumab combined with chemotherapy. HER2 3+/FISH-positive NSCLC patients could also benefit from trastuzumab.[29,30] A durable response to crizotinib in ALK-negative NSCLC patients with de novo MET amplification has also been observed, indicating that crizotinib is also a MET inhibitor.[31] EGFR mutations and ALK translocations can coexist in part of NSCLCs, while coexistent prevalence of both increases in line with sensitivity of applied detection method for EGFR mutations. A study showed that EGFR and ALK alterations

concomitantly occurred in 4.4% (4/91) of ALK-translocated NSCLCs, while detection ratio of concomitant EGFR mutations and ALK translocations increased to 8.8%, 12.1% and 15.4% when applying additional peptide nucleic acid real-time PCR, NGS and mutant-enriched NGS in 91 ALK-translocated NSCLCs, respectively.[32] EGFR 21 (G863D) is very rare and it has even been reported in gastric cancer and thymoma.[33,34] In our study, amplification of CerbB-2 and MET was observed in only one patient, with very low and negative abundance for FISH detection. EGFR 21 (G863D) mutation was observed in another patient. Consequently, we assume that patients with ALK rearrangement rarely have other driver gene mutations or amplifications. This indicates that patients with advanced NSCLC harbouring ALK rearrangements may not need further detections for other driver genes alternations such as EGFR, MET, KRAS, ROS1, BRAF and RET, though parallel sequencing is still strongly recommended.

A study has suggested that overall survival for NSCLC patients with ALK positive is inferior compared to ALK-negative patients following surgical resection.[35] In addition, five-year risk of progression or recurrence is doubled for patients with ALK-positive tumours compared with ALK-negative tumours. In our cohort, there were no patients who relapsed in the ALK variant 1 group, while four patients relapsed due to brain metastasis in ALK non-variant 1 group. It can be deduced that patients with ALK non-variant 1 the relapse easily occurs, especially brain metastasis. Based on the limited sample size, there was no statistical significance in neither 3-year DFS rate nor DFS between the two groups, while improvement trends in 3-year DFS rate and DFS were observed in ALK variant 1 group. Further studies with larger sample size are necessary to verify these findings. A randomised phase II study has shown that adjuvant chemotherapy of pemetrexed plus carboplatin followed by gefitinib could improve DFS in postoperative stage IIIA-N2 NSCLC patients harbouring EGFR mutations.[36] Since ALK inhibitors have shown effectiveness in advanced NSCLC patients with ALK positivity, the application of ALK inhibitors in ALK-positive patients with surgically resected NSCLC is a potential therapeutic option to improve DFS and to lessen the risk of brain metastasis. Postoperative adjuvant crizotinib treatment for patients with lung adenocarcinoma patients harbouring ALK needs to be evaluated. Since patients with ALK variant 1 respond better to crizotinib compared to patients with non-variant 1, future studies should investigate whether ALK variant subtype could be used as a biomarker for population selection in ALK inhibitor adjuvant therapy for resected lung adenocarcinoma with ALK positivity. Studies with larger sample size are recommended to examine the role of ALK inhibitors in adjuvant treatments.

Conclusion

Patients with ALK rearrangement rarely had other driver gene mutations or amplifications. NSCLC patients harbouring ALK variant 1 tended to have better prognosis compared to those with ALK non-variant 1.

Disclaimer: None.

Conflicts of interests: None.

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Ethical conduct of research: This study was approved by the Medical Ethical Committee of Zhejiang Cancer Hospital and the ethics committee reference number is IRB-2016-86. The specimens were firstly obtained from Biological Sample Bank of Zhejiang Cancer Hospital which signed the written informed consent before surgery. Exempt written informed consent was also approved in this retrospective study by the Medical Ethics Committee of Zhejiang Cancer Hospital.

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