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ORIGINAL ARTICLE

Efficacy of bronchoscopic biopsy for the detection of epidermal growth factor receptor mutations and anaplastic lymphoma kinase gene rearrangement in lung adenocarcinoma

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Abstract

Background: To explore the efficacy of bronchoscopic biopsy for the detection of epidermal growth factor receptor (EGFR) mutations and anaplastic lymphoma kinase (ALK) gene rearrangement in lung adenocarcinoma.

Methods: All patients with bronchoscopic biopsy-proven lung adenocarcinoma at the Peking Union Medical College Hospital from January 2009 to November 2011 were enrolled. Scorpion amplification refractory mutation system (ARMS) was used to detect EGFR gene mutations and fluorescence *in situ* hybridization (FISH) to detect ALK rearrangement. The correlation of immunohistochemistry (IHC) results with standard methods for EGFR mutation status and ALK rearrangement were checked.

Results: Bronchoscopic specimens were successfully used to detect EGFR mutation and ALK rearrangement with success rates of 85.2% and 71.3%, respectively, in non-small cell lung cancer patients. EGFR analysis by ARMS yielded a positive result in 35.8% (33/92) and positive ALK rearrangement was detected by FISH in 7.8% (6/77) of cases. It was more likely to be unsuccessful in patients with tumor cells less than 100/high power field and the ratio tumor numbers in 0–10%. In EGFR-IHC, the sensitivity and specificity of E746-A750 deletions were 73.3% (11/15) and 93.3% (70/75), respectively, and those of L858R were 93.3% (14/15) and 93.2% (69/74), respectively. In ALK-IHC, the sensitivity and specificity were 50% (3/6) and 100% (71/71), respectively.

Conclusions: Small bronchoscopic specimens could achieve higher successful detection rates via EGFR mutation and ALK gene rearrangement.

Introduction

Lung cancer is the leading cause of cancer-related mortality worldwide and the majority of patients have advanced disease at the time of diagnosis. Treatment of non-small cell lung cancer (NSCLC) has entered the age of target therapy according to tumor molecular markers.[1–3] Somatic mutations in the epidermal growth factor receptor (EGFR) tyrosine kinase receptor (TKI) can be targeted with small-molecular TKIs, such as gefitinib, erlotinib, and icotinib, and many studies have shown that TKI therapy in EGFR mutant NSCLC results in better response rates and longer progression-free survival compared with conventional chemotherapy.[4–6] Several studies have demonstrated a benefit in patients with NSCLC bearing anaplastic lymphoma kinase (ALK) rearrangements with improved progression-free and overall survival when crizotinib is given compared with traditional chemotherapy.[3,7] Therefore, the detection of EGFR gene mutations and rearrangement of the ALK gene is critical in pathological examination, especially in small tumor specimens, which are more convenient and practical than surgical specimens in patients with local advanced or metastatic NSCLC.

Genomic polymerase chain reaction (PCR) and direct sequencing methods have been widely used to detect EGFR mutations. It is well known that the direct sequencing method has poor sensitivity for the detection of EGFR

mutations in a mixed tissue of normal and tumor cells. In patients with local advanced or metastatic NSCLC, it is often difficult to obtain large tumor samples. Therefore, it is very important to use a more sensitive method for these small samples. The scorpion amplification refractory mutation system (ARMS) is a fluorescence-based method for specific detection of PCR production with an enhanced sensitivity of about 1% and is more commonly used for EGFR mutation detection in small tumor samples.[8,9] The standard method for detecting ALK rearrangement is fluorescence *in situ* hybridization (FISH).[10,11]EGFR mutation status and ALK rearrangement in lung adenocarcinoma by immunohistochemistry (IHC) using specific antibodies has frequently been reported; therefore,theref we determined the correlation of IHC results with standard methods for EGFR mutation status and ALK rearrangement.[12–16]

Biopsy by conventional bronchoscopes is one of the major methods for the diagnosis of lung cancer, and can easily be performed under moderate sedation in an outpatient setting. The specimens from this procedure are less well described, as is its efficacy in attaining sufficient tissue for molecular analysis in patients with lung adenocarcinoma. The rationale of this study was to assess the efficacy of biopsy by conventional bronchoscopes for obtaining sufficient tumor tissue of EGFR mutation and ALK rearrangement in patients with lung adenocarcinoma.

Materials and methods

Patients

We screened the data from all patients who received bronchoscopic biopsies at Peking Union Medical College Hospital from January 2009 to November 2011, and a subset of patients with biopsy-proven lung adenocarcinoma was identified for this study. All bronchoscope procedures were performed under moderate sedation in an outpatient setting. Biopsy samples were fixed in neutralized formalin solution and blocks were made in 24 hours. Experienced pathologists diagnosed pathology types, and an immunochemistry stain was performed to differentiate squamous cell carcinoma from adenocarcinoma, especially in cases of poorly differentiated carcinoma, and to determine the adenocarcinoma origin. A total of 2211 patients underwent bronchoscopy during the study period; 452 patients had a diagnosis of lung cancer; 108 patients were finally diagnosed with lung adenocarcinoma by tumor samples from bronchoscopic biopsies. The institutional review board at the Peking Union Medical College Hospital approved the study (No: S-494). Written informed consent was obtained from all patients. Clinical data was collected, including gender, age, smoking history, and clinical tumor node metastasis stage. Manifestations of the tumors under bronchospy were classifed as hyperplasia (i.e. apparent neoplasm) or infiltrated tumors (i.e. mucosal congestion and edema without apparent neoplasm).

Serial unstained 4 μm formalin-fixed paraffin embedded sections of the tissue specimens were prepared, and one section was used for hematoxylin and eosin (HE) staining, one for ALK-FISH, one for ALK-IHC, five for Scorpion-ARMS, and two for EGFR-IHC. HE staining slides were reviewed by an experienced pathologist for the total number and percentage of tumor cells in nucleic cells. Two investigators performed all of the procedures independently, and a third investigator reviewed any discordance.

Epidermal growth factor receptor (EGFR) mutation

The EGFR Scorpion Kit (DxS, Manchester, UK) was used to detect EGFR gene mutations in real-time PCR reactions.[8,9] Real-time PCR was performed using the Bio-Rad iQ5 real-time PCR system (Bio-Rad Laboratory, Hercules, CA, USA). Data were analyzed using Bio-Rad iQTM5 software (version 2.0). The cycle threshold (Ct) was defined as the cycle at the highest peak of the second derivative curve that represented the point of maximum curvature of the growth curve. Both Ct and maximum fluorescence were used for interpretation of the results. Positive results were defined as Ct ≤45 and maximum fluorescence intensity ≥30. When only the curve that indicated the wild type increased, the sample was considered wild type EGFR. When both wild and mutation-type curves increased, the sample was considered mutant-type EGFR.

Anaplastic lymphoma kinase (ALK) fluorescence *in situ* hybridization

Fluorescence *in situ* hybridization was performed on unstained 4 μm formalin-fixed paraffin embedded tumor tissue sections with the use of an ALK break-apart probe set (Vysis LSI ALK Dual Color, Break Apart Rearrangement Probe; Abbott Molecular, Rungis, France) using a paraffin pretreatment reagent kit (Vysis, Abbott Molecular). Assays were performed following the manufacturer's instructions. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole/Vectashield (Vektor Laboratories, AbCys, Paris, France).

Sections were analyzed with a Metafer slide scanning system (Metasystems, Altlussheim, Germany) under a 63 × oil immersion objective with a fluorescence microscope (M1, Zeiss, Stuttgart, Germany) equipped with appropriate filters, a charge-coupled device camera, and the FISH imaging and capturing software Metafer 4 (Metasystems). Signals were enumerated with the ISIS imaging system (Metasystems). Non-rearranged ALK showed fusion (orange signals) or very close apposition of the probes adjacent to the 3′ (red) and 5′

(green) ends of the gene. Rearranged ALK appeared as split 3′ and 5′ signals. Tumor tissues were considered ALK-FISH positive (ALK rearranged) if >15% of tumor cells showed split red and green signals and/or single red signals, according to previous publications.[3,7,10,11] Otherwise the samples were considered ALK-FISH negative.

Immunohistochemistry

The primary antibodies for EGFR mutation were E746-A750del and L858R, and for ALK rearrangement was D5F3 antibody (anti-rabbit, Cell Signaling Technology, Boston, MA, USA). The secondary antibody for both EGFR and ALK was polink-1 anti-rabbit and anti-mouse, respectively (Zhongshan, Jinqiao, China). After staining, we examined the slides under microscopy (×20) and recorded the location and intensity of staining, as well as the ratio of stained tumor cells to the total number of cells. The scores were assigned as follows: 0, no stained cells or weak staining intensity, with less than 10% tumor cells; 1+, faint or weak staining intensity, with more than 10% tumor cells; 2+, moderate, smooth cytoplasmic staining intensity; and 3+, strong, granular cytoplasmic staining intensity. Scores of 2+ and 3+ were defined as positive, and 0 or 1+ as negative.

Statistical analysis

SPSS version 17.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis, which included a non-parametric Wilcoxon's test to compare the differences between groups, and the coupled χ^2 test and consistency check to compare the differences between IHC and the standard methods. Kappa values were calculated: kappa > 0.75 indicated good consistency; 0.4 < kappa < 0.75 indicated fair consistency; and kappa < 0.4 indicated poor consistency. Statistical significance was defined as $P < 0.05$ (two-tailed).

Results

Patient demographics and disease characteristics

A total of 108 patients were finally diagnosed with lung adenocarcinoma by tumor samples from bronchoscopic biopsies, and the clinical and pathological characteristics of the 108 patients are summarized in Table 1. The median number of biopsies was four (range: 1 to 7); 100 (92.6%) patients had 3–5 biopsies.

Detection of EGFR gene mutation

Out of 108 patients diagnosed with lung adenocarcinoma, 92 patients (85.2%) had sufficient bronchoscopic biopsy specimens left for EGFR mutation detection; 16 did not have enough tissue left after clinical diagnosis. The difference in patients' clinical and pathologic characteristics was compared by a non-parametric Wilcoxon's test conducted between the successful and unsuccessful detection groups, and there were significant differences in the number and percentage of tumor cells (Table 2).

Table 1 Clinical and pathological characteristics of 108 patients with lung adenocarcinoma

Items	*n*	%
Gender		
Male	58	53.7
Female	50	46.3
Age		
<60	61	56.5
≥60	47	43.5
Smoking status		
No	70	64.8
Yes	38	35.2
TNM stage		
III	20	18.5
IV	88	81.5
Manifestation under bronchoscopy		
Hyperplasia	60	55.6
Infiltrate	48	44.4
Number of biopsies		
1~4	69	63.9
5~7	39	36.1
Total tumor cells		
0~99	11	10.2
≥100	97	89.8
Percentage of tumor cells		
0~10%	5	4.7
11~100%	103	95.3

TNM, tumor node metastasis.

Epidermal growth factor receptor analysis by Scorpions ARMS yielded a positive result in 33 (35.8%) of 92 samples tested; the 19 exon deletion was detected in 15 cases (16.3%), 21 exon L858R in 15 (16.3%), 21 exon L861Q in two (6.0%), 20 exon insertion in one (3.0%), and 20 exon S768I in one case (3.0%) (both 19 exon deletion and 21 exon L858R in 1 case). The difference in patients' clinical and pathologic characteristics was compared by a non-parametric Wilcoxon's test, and the EGFR gene mutation rate was higher in female patients and non-smokers, without statistic significance. The median overall survival in patients with the EGFR gene mutation and patients with EGFR wild type were 17.0 (95% confidence interval 14.6–24.2 months) and 14.0 months (95% confidence interval 12.2–16.5 months) respectively, and there was significant difference between two groups ($P = 0.036$).

With regard to EGFR mutation results detected by IHC in comparison with the ARMs method, the positive rate of deletion E746-A750 was 17.8% (16/92), and L858R was 21.4%

Table 2 Difference in patient characteristics between successful and unsuccessful detection groups for EGFR mutations

Items	Successful group ($n = 92$)	Unsuccessful group ($n = 16$)	χ^2 value	P
Gender				
Male	46	12	3.426	0.064
Female	46	4		
Age				
<60	55	6	2.753	0.097
≥60	37	10		
Smoke status				
No	60	10	0.044	0.834
Yes	32	6		
TNM stage				
III	19	1	1.874	0.171
IV	73	15		
Manifestation under bronchoscopy				
Hyperplasia	48	12	2.876	0.090
Infiltrate	44	4		
Number of biopsies				
1~4	56	13	2.454	0.117
5~7	36	3		
Total tumor cells				
0~99	4	7	23.132	0.000
≥100	88	9		
Percentage of tumor cells				
0~10%	1	4	17.652	0.0000
11~100%	91	12		

EGFR, epidermal growth factor receptor; TNM, tumor node metastasis.

Table 3 Difference in patient characteristics between successful and unsuccessful detection groups for ALK rearrangement

Items	Successful group ($n = 77$)	Unsuccessful group ($n = 31$)	χ^2 value	P
Gender				
Male	38	20	2.054	0.153
Female	39	11		
Age				
<60	45	16	0.419	0.517
≥60	32	15		
Smoke status				
No	49	21	0.163	0.686
Yes	28	10		
TNM stage				
III	17	3	2.252	0.133
IV	60	28		
Menifestation under bronchoscopy				
Hyperplasia	40	20	1.414	0.234
Infiltrate	37	11		
Numbers of biopsy				
1~4	51	18	0.639	0.424
5~7	26	13		
Total tumor cells				
0~99	0	11	30.421	0.000
≥100	77	20		
Percentage of tumor cells				
0~10%	0	5	13.022	0.000
11~100%	77	26		

ALK, anaplastic lymphoma kinase;TNM, tumor node metastasis.

(19/92). Taking Scorpions-ARMS as the standard method, the sensitivity of IHC for the 19 deletion was 73.3% (11/15), its specificity 93.5% (72/77), and its positive predictive value (PPV) 68.8% (11/16), while its negative predictive value (NPV) was 94.7% (72/76). There was no difference between IHC and ARMS for determining the presence of the 19 deletion based on a paired Chi-square test ($P = 1.000$). IHC detection of the 19 deletion had a fair consistency (kappa = 0.65). The sensitivity and specificity of IHC for 21L858R was 93.3% (14/15) and 93.5% (72/77), respectively, and the PPV and NPV were 73.7% (14/19) and 98.6% (72/73), respectively. The paired Chi-square test showed no significant difference between IHC and ARMS for detection of 21L858R ($P = 0.219$). Combining the results for 19 deletion and 21L858R, EGFR-IHC exhibited a sensitivity of 86.2% (25/29), a specificity of 87.3% (55/63), and a PPV and NPV of 75.8% (25/33) and 93.2% (55/59), respectively.

Detection of ALK rearrangement

For ALK rearrangement detection in NSCLC bronchoscopic specimens, 16 specimens were not sufficient, 11 were not qualified for FISH detection because there were less than 50 tumor cells in the bronchoscopic samples, and four specimens could not be calculated because of a weak or non-existent FISH signal. Therefore, the successful rate for ALK rearrangement FISH detection in bronchoscopic samples was 71.3% (77/108). Clinical and pathologic characteristics were compared between the successful and unsuccessful detection groups, and there was a significant difference in the number and percentage of tumor cells (Table 3). In the 77 samples successfully detected via FISH, positive ALK rearrangement was detected in six specimens (7.8%), while 71 were negative. Of the six patients, two were men and four women, aged from 40–60, all non-smokers, five had poorly differentiated tumors and one was unclear.

ALK rearrangement detection by IHC in comparison with the FISH method is shown in Table 4. The sensitivity and specificity of ALK-IHC was 50% (3/6) and 100% (71/71), respectively, and the PPV and NPV were 100% and 95.9%, respectively. There was no difference in the detection rates between ALK-IHC and FISH as determined by the paired Chi-square test ($P = 0.25$). The consistency was fair (kappa = 0.65).

Table 4 Comparison of IHC and FISH in detecting *ALK* gene rearrangement ($n = 92$)

	ALK FISH			
ALK-IHC	Positive	Negative	Could not be evaluated	Total (%)
0	0	47	10	57 (62.0%)
1+	3	24	4	31 (33.7%)
2+	3	0	1	4 (4.3%)
3+	0	0	0	0 (0)

ALK, anaplastic lymphoma kinase; FISH, fluorescence in situ hybridization; IHC, immunohistochemistry.

Discussion

We demonstrated that bronchoscopic specimens could be successfully used to detect EGFR mutation and ALK rearrangement in NSCLC patients with success rates of 85.2% and 71.3%, respectively. Our results were lower than those published by Jurado *et al*., who conducted EGFR, Kirsten rat sarcoma mutation, and anaplastic lymphoma kinase gene rearrangement on 52 of 56 (93%) patients who underwent endobronchial ultrasound-guided-transbronchial needle aspiration.[17] The main reason for our lower results was the lack of availability of rapid on-site evaluation by a cytologist to confirm adequate tumor material. A strength of our study was that molecular analysis was attempted in all lung adenocarcinomas diagnosed by bronchoscopes as protocol. This stands in contrast to other studies based on selected cases in which testing was not performed in all cases.[18] Our results indicate that pathologists will be required not only to diagnose whether samples are benign or malignant, but also to evaluate whether they are suitable for molecular analysis. The reasons why a sample could be adequate for diagnosis but insufficient for molecular analysis include: small sample size, a sparse cellular sample resulting from tumor necrosis or sampling damage, contamination with blood or benign bronchial cells, or insufficient material triaged to the cell block. Using ALK rearrangement by FISH, the success rate was lower than for EGFR mutation. When we compared the tumor numbers and ratio between the successful and unsuccessful detection groups, the results showed that EGFR mutation and ALK by FISH are more likely to be unsuccessful in patients with less than 100/ high power field tumor cells and the ratio of tumor numbers in 0–10%. Shiau *et al*. also found that tumor cellularity was significantly associated with the rate of EGFR mutation test success.[19]

We also found that IHC staining with specific antibodies in bronchoscopic specimens had fair consistency to detect EGFR mutation and ALK rearrangement in comparison with standard methods. Compared with the standard method (Scorpion-ARMS), EGFR-IHC for small specimens showed good sensitivity and specificity with both greater than 80%, similar to results reported in previous studies.[12,13,20,21] The sensitivity of the L858R antibody was higher than E746-A750 (93.3% vs. 73.3%). The E746-A750 antibody recognizes an EGFR protein that results from a 15-bp deletion in exon 19, and the occurrence of non-15-bp (9, 12, 18 or 24-bp) deletions, which account for 35% of exon 19 deletions, reduces its sensitivity. Kawahara *et al*. reported that the E746-A750 antibody may not be useful for the identification of rare exon 19 deletion mutations, such as delL747-T751>P, delE746-T751, and delL746-T751.[12] This indicates the need to find new antibodies for additional mutation types to enhance the sensitivity of IHC-EGFR mutation detection. ALK rearrangement detection by IHC can be used as a screening method; there were no FISH positive results in patients with IHC scores of 0. Ultrasensitive automated IHC for detecting ALK status has now been developed and IHC is more economical and convenient than current standard methods of detecting mutations in tumors.[22–24]

Limitations of this study included a small sample size and the lack of specific comparison groups. In samples with low tumor volume or those that were sparsely cellular, mutations could be present but below the level of detection of individual tests, resulting in false negative findings; however, Khode *et al*. reported that comparison between cytology smears and concurrent formalin-fixed paraffin-embedded tissues from the same anatomic site had a concordance rate of 97%.[25] A direct comparison of the yield of testing in matched samples obtained from a surgical procedure would further validate the efficacy of bronchoscopic biopsy. Our initial aim, which was to determine the efficacy of bronchoscopic biopsy for the detection of EGFR mutations and ALK gene rearrangement in lung adenocarcinoma, was achieved in this study. As the next step, a prospective study of a large number of NSCLC patients with matched large tumor samples is likely to reveal the truth.

Conclusion

In conclusion, our results demonstrate that bronchoscopic specimens could achieve higher successful detection rates via EGFR mutation and ALK gene rearrangement. IHC with the E746-A750 deletion and L858R antibodies can be a convenient alternative detection method for EGFR gene mutations in small NSCLC bronchoscopic specimens.

Disclosure

No authors report any conflict of interest.

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