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Adequacy of endobronchial ultrasound-guided transbronchial needle aspiration samples processed as histopathological samples for genetic mutation analysis in lung adenocarcinoma

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Abstract. Phenotyping non-small-cell lung cancer is becoming increasingly important with the advent of molecular testing. Tumours harbouring somatic mutations in the gene that encodes for the tyrosine kinase domain of the epidermal growth factor receptor (EGFR) have been found to increase responsiveness to tyrosine kinase inhibitors. Endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA) is a minimally invasive technique for mediastinal node sampling. The available prospective data on EBUS-TBNA sample suitability for molecular profiling are currently limited. The aim of this prospective study was to evaluate the adequacy of EBUS-TBNA samples for EGFR and anaplastic lymphoma kinase (ALK) genetic mutation analysis in confirmed primary lung adenocarcinomas. We conducted a prospective analysis of 410 consecutive patients referred for EBUS-TBNA between 2010 and 2014. Rapid on-site cytological evaluation was not used. The samples were obtained using 21-gauge (21G) or 22G needles and were prepared as histopathological samples. A total of 91 samples were confirmed as lung adenocarcinomas and 80 of these samples were sent for EGFR mutation analysis. EBUS-TBNA had a diagnostic accuracy of 98.3% for malignancy. EGFR mutation testing was possible in 79/80 cases (98.75%). EGFR mutations were detected in 5/80 (6.3%) samples. ALK gene analysis, which became available during the study period, was requested and successfully performed in 21/21 samples (100%). The total combined genotyping success rate was 100/101 (99.0%). This UK study confirmed the high clinical utility of EBUS-TBNA samples processed as histopathological specimens for EGFR and ALK genotyping in primary lung adenocarcinoma. The needle gauge did not affect genotyping efficacy.

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Abbreviations: ALK, anaplastic lymphoma kinase; CK 5/6, cytokeratins 5/6; EBUS, endobronchial ultrasound; EBUS-TBNA, endobronchial ultrasound-guided transbronchial needle aspiration; ECOG, Eastern Cooperative Oncology Group; EGFR, epidermal growth factor receptor; EML4-ALK, echinoderm microtubule-associated protein-like 4-anaplastic lymphoma kinase; KRAS, Kirsten rat sarcoma; NICE, National Institute of Clinical Excellence; NSCLC, non-small-cell lung cancer; ROSE, rapid on-site evaluation; SD, standard deviation; TTF-1, thyroid transcription factor 1

Key words: lung cancer, endobronchial ultrasound, disease phenotyping, epidermal growth factor receptor, anaplastic lymphoma kinase

Introduction

Histological subtyping of non-small-cell lung cancer (NSCLC) has become an important aspect of lung cancer management owing to an expansion of treatment options for patients with specific tumour types. Historically, NSCLC was treated with platinum-based therapies, irrespective of histological subtype. With the advent of molecular testing to identify specific genetic mutations, there has been a move towards targeted therapies that are more effective when these mutations are present. Specifically, tumours harbouring somatic mutations in the gene which encodes for the tyrosine kinase domain of the epidermal growth factor receptor (EGFR) have been found to exhibit increased responsiveness to the tyrosine kinase inhibitors gefitinib and erlotinib (1-3). Furthermore, a phase III East Asia study comparing gefitinib with carboplatin/paclitaxel in never smokers/light smokers with adenocarcinoma demonstrated that patients with EGFR mutation-positive tumours achieved longer progression-free survival with gefitinib compared with those without EGFR mutations (4). Conversely, tumours with mutations of the Kirsten rat sarcoma (KRAS) viral oncogene, which are considered to be mutually exclusive with EGFR mutations, appear to exhibit resistance to gefitinib and erlotinib (5). Among NSCLC patients with rearrangements of the anaplastic lymphoma kinase (ALK) gene, ~5% have been shown to have improved progression-free survival when treated with the oral tyrosine-kinase inhibitor crizotinib (6). The National Institute of Clinical Excellence currently recommends the use of erlotinib for the first-line treatment of patients with locally

advanced or metastatic tumours harbouring somatic EGFR gene mutations (7), although crizotinib has not yet been recommended for patients with ALK-positive NSCLC (8).

To subcharacterise and genotype NSCLC, adequate histological tissue samples are required for diagnosis using standard morphological and immunohistochemical techniques, as well as molecular analysis. Since patients with NSCLC often present with advanced or metastatic disease, it is also important that the least invasive technique is used to obtain tissue for diagnosis.

Endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA) is a bronchoscopic invasive technique that is currently widely used for the staging and diagnosis of mediastinal lesions. The obtained samples provide one cell block and are smaller compared with those obtained via mediastinoscopy (historically considered as the 'gold standard' mediastinal sampling technique). However, EBUS-TBNA has a number of advantages when compared with mediastinoscopy: This technique is routinely performed as a day case procedure under conscious sedation (although it may also performed under general anaesthesia) and, as such, the morbidity and mortality rates are lower (9). This is of particular relevance for patients with poorer Eastern Cooperative Oncology Group (ECOG) performance status (10) who may otherwise be deemed unfit for more invasive sampling diagnostic techniques. In addition to improved patient comfort, using minimally invasive techniques is also more cost-effective (11).

Several studies have investigated whether samples obtained during EBUS-TBNA are sufficient for molecular analysis and have demonstrated high, although variable, adequacy rates (77.7-98.7%) (12-25). These studies were heterogeneous in terms of cytohistopathological techniques and sample size.

The aim of this single-centre UK prospective study was to evaluate the adequacy of biopsies obtained via EBUS-TBNA processed as histopathological specimens for EGFR genetic mutation analysis in confirmed primary lung adenocarcinoma with an intention to proceed to oncological therapy.

Materials and methods

Ethics approval. We conducted a prospective study of all consecutive patients referred to the Southmead University Hospital (Bristol, UK) a tertiary hospital providing EBUS service, for EBUS-TBNA between 2010 and 2014. The Local Research and Ethics Committee confirmed that, due to the observational nature of this study, and as this study was part of an ongoing service evaluation and standard of care for patients undergoing EBUS-TBNA, no specific ethics approval was required. All the results were double-reported by two lung histopathologists and were reviewed at multidisciplinary team meetings to determine the treatment plan.

Procedural and demographic data. Collected data included the age and gender of the patients referred for EBUS-TBNA, the number and location of nodal stations sampled, the number of needle passes per station, the gauge of the EBUS-TBNA sampling needle used, as well as the final diagnosis. The proportion of samples in which EGFR mutation analysis was requested and successfully performed was determined among patients with primary lung adenocarcinoma. The outcome of patients in whom molecular analysis of tumour samples was not performed was also recorded.

Procedure. EBUS-TBNA was performed as previously described, under light conscious sedation with midazolam and fentanyl using a convex probe ultrasound bronchoscope (Olympus BF-UC260FW; Olympus Corp., Tokyo, Japan) (26,27). The procedures were performed by three trained operators (ARLM, AJ and MJP). Mediastinal nodes or lesions were identified under ultrasound and sampling was performed using dedicated 21-gauge (21G) or 22G EBUS-TBNA needles (Olympus ViziShot, NA-201SX-4021 and NA-201SX-4022; Olympus Corp.) at the discretion of the operator. Lymph node size was measured during EBUS-TBNA. The number of needle passes per station sampled was determined by the operator, with a minimum standard of three passes. The samples were fixed in formalin baskets and sent for histopathological analysis, as our centre does not have a rapid-on-site evaluation (ROSE) cytopathology service (28).

Pathology techniques. The adequacy of the samples was histopathologically determined by the presence of lymphocytes indicating lymph node sampling. Immunohistochemistry was performed where clinically indicated and as determined by the pathologists. An immunopanel of thyroid transcription factor-1 (TTF-1), cytokeratins 5/6 (CK5/6) and p63 was used as recommended by International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society international multidisciplinary classification (29). Antibodies to CK5/6 and p63 were deemed to be consistent with squamous cell carcinoma (30,31). Antibodies to TTF-1 were also used, as TTF-1 is known to be expressed in ~75% of primary lung adenocarcinomas (32,33). DNA sequencing techniques were used to perform EGFR and, subsequently, ALK mutation analysis, in patients with confirmed primary lung adenocarcinoma who were considered suitable for oncological therapy (in practice, with an optimised ECOG performance status of 2). Molecular analysis was only performed on samples consisting of ≥50% malignant cells (or if 10x5-μm slides could be prepared) using pyrosequencing detecting the following mutations: Exon 18: c.2155G>A, p.(Gly719Ser); c.2155G>T, p.(Gly719Cys); c.2156G>C, p.(Gly719AIa); c.2159C>T, p.(Ser720Phe); exon 20: c.2294T>C. p(Val765Ala)c.2303G>T (p.Ser768Ile), c.2305G>T (p.Val769Leu), c.2369C>T (p.Thr790Met); and exon 21: c.2573T>G (p.Leu858Arg) and c.2582T>A (p.Leu861Gln).

Statistical analysis. All statistical analyses were performed using GraphPad Prism software, version 5 (GraphPad Software, Inc., San Diego, CA, USA). $P<0.05$ was considered to indicate statistically significant differences. Contingency table analysis was used with Fisher's exact test to calculate the diagnostic utility (sensitivity, specificity, negative predictive value and accuracy) of EBUS-TBNA for the presence of lymph node metastases. The prevalence of malignancy and proportion of confirmed primary lung adenocarcinoma samples in which EGFR mutation analysis was possible were also determined.

Follow-up. All patients with suspected malignancy with negative EBUS-TBNA samples were either referred for further

Table I. Final diagnosis for all patients (n=410).

Histological diagnosis	No. of patients
Malignancy[a]	216
Cyst	5
Sarcoidosis	92
TB	15
Benign/reactive nodes	73
Thyroid or parathyroid adenoma	2
Ectopic thyroid tissue	1
Pending clinical follow-up	2
EBUS-TBNA abandoned, therefore excluded	4

[a]Includes 7 samples that were negative on EBUS-TBNA (Table III). TB, tuberculosis; EBUS-TBNA, endobronchial ultrasound-guided transbronchial needle aspiration.

Table II. Histological diagnosis of all patients with EBUS-confirmed malignancy (n=209).

Histological diagnosis	No. of patients
Small-cell carcinoma	33
Adenocarcinoma	91
Squamous cell carcinoma	41
NSCLC-NOS	9
Lymphoma	11
Metastatic disease	18
Large-cell carcinoma	1
Carcinoid tumour	3
Poorly differentiated carcinoma	1
Indolent tumour	1

NSCLC-NOS, non-small-cell lung cancer not otherwise specified; EBUS, endobronchial ultrasound.

diagnostic investigations or were followed-up clinically for a minimum of 12 months with serial imaging.

Results

Diagnosis and confirmatory investigations. A total of 410 consecutive patients were referred for EBUS-TBNA during the study period (between 2010 and 2014). A total of 4 patients were excluded from the analysis, as EBUS-TBNA was abandoned due to patient-related factors. The median age of the patients undergoing EBUS-TBNA was 59.9 years and 230 patients were male. Table I shows the final diagnosis in all the patients and Table II shows the histological subtype of cancers diagnosed using EBUS-TBNA. The sensitivity, negative predictive value and accuracy were 96.8, 96.5 and 98.3%, respectively, for malignancy. Table III shows the investigations used to establish the diagnosis in the 7 patients who had false-negative EBUS-TBNA results, who were later confirmed to have a malignancy. Table IV shows the investigations used to confirm benign disease in patients with true-negative EBUS-TBNA results.

Table III. Investigations used to confirm diagnosis in patients with false-negative EBUS-TBNA results (n=7).

Investigation used to confirm diagnosis	No. of patients
CT-guided biopsy and mediastinoscopy	1
Repeat EBUS-TBNA	1
CT-guided biopsy[a]	2
Bone biopsy[a]	1
PET with elevated SUV[a]	2

[a]Radiological evidence of mediastinal adenopathy with diagnosis confirmed elsewhere and/or unfit or unsuitable for mediastinoscopy; therefore, the EBUS-TBNA results were false-negative. CT, computed tomography; EBUS-TBNA, endobronchial ultrasound-guided transbronchial needle aspiration; PET, positron emission tomography SUV, standardised uptake value.

Table IV. Investigations used to confirm benign disease in patients with true-negative EBUS-TBNA results (n=73).

Investigation used to confirm diagnosis	No. of patients
Mediastinoscopy	18
PET	5
Interval CT	24
Clinical follow-up	23
CT-guided biopsy	1
Liver biopsy	1
Resection	1

EBUS-TBNA, endobronchial ultrasound-guided transbronchial needle aspiration; PET, positron emission tomography; CT, computed tomography.

Table V. Outcome in patients whose samples were not sent for molecular analysis.

Outcome	No. of patients (%)
No treatment due to decline in performance status	6 (54.5)
Chemoradiotherapy	3 (27.2)
Radical radiotherapy	1 (9.1)
Surgery	1 (9.1)

The average size of the lymph nodes sampled was 2.1 cm [standard deviation (SD), 0.42 cm] and the average number of nodes sampled was 1.8 (SD, 0.94). There were three needle passes per station on average. One patient developed an urticarial rash after the procedure that was considered to be secondary to the fentanyl used for sedation. There were no other complications reported.

Table VI. Summary table of outcomes from previous studies.

Study (year)	Patient no.	NSCLC type, n/total (%)	Feasibility of EGFR mutation analysis (%)	Prevalence of EGFR mutation (%)	ROSE	Processing	Size, mm (range)	(Refs.)
Nakajima *et al* (2007)	46	AdenoCa 46	43/46 (93.5)	11/43 (25.6)	N/A	Histology	N/A	(14)
Garcia-Olivé *et al* (2010)	185	AdenoCa 40/185 NSCLC-NOS 11/185	26/36 (72.2)	2/20 (10.0)	Yes	Histology	11 (9-17)	(13)
Schuurbiers *et al* (2010)	462	AdenoCa 35/462	27/35 (77.1)	2/27 (7.4)	N/A	Cytology, cell blocks	N/A	(16)
Nakajima *et al* (2011)	156	AdenoCa 127/156 (81.4) SCC 21/156 (13.5) Other 8/156 (5.1)	154/156 (98.7)	42/156 (26.9)	No	Cytology, needle washings	14 (4.8-33.4)	(15)
Santis *et al* (2011)	132	AdenoCa 94/132 (71.0) SCC 17/132 (13.0) Large-cell Ca 2/132 (1.5) Large-cell Ca 1/132 (0.07) NSCLC-NOS 18/132 (13.6)	126/132 (95.5)	13/126 (10.3)	Yes	Cytology	12±5	(12)
Navani *et al* (2012)	774	AdenoCa 68/119 (57.0) SCC 19/119 (16.0) Large-cell Ca 10/119 (8.0) NSCLC-NOS 22/119 (18.0)	107/119 (89.9)	7/107 (6.5)	N/A	Cytology, cell pellets	21	(20)
Esterbrook *et al* (2013)	394	AdenoCa 40/204 SCC 64/204 Small-cell Ca 43 Large-cell Ca 12 NSCLC-NOS 31	32/36 (88.8)	1/31 (3.1)	No	Cytology, cell blocks	N/A	(19)
Boulanger *et al* (2013)	82	AdenoCa 63/82 Large-cell Ca 19/63	80/82 (97.6)	4/80 (5.0)	No	Cytology, cell blocks	N/A	(18)
Folch *et al* (2013)	207	AdenoCa (84.1)	191/207 (92.3)	N/A	N/A	N/A	N/A	(17)
Jurado *et al* (2013)	205	AdenoCa 56/205	52/56 (92.9)	5/52 (9.6)	Yes	Cytology, cell blocks	N/A	(22)
Schmid-Bindert *et al* (2013)	106 biopsies (33 EBUS)	AdenoCa (45.0), SCC (30.0) NSCLC-NOS (8.0)	32/33 (96.7)	10/32 (31.2)	No	Histology	N/A	(24)
Kang *et al* (2013)	594	AdenoCa 104/202 (51.5) SCC 88/202 (43.6) Large-cell Ca 3 (1.5) NSCLC-NOS 4 (2)	69/74 (94.5)	16/74 (21.6)	No	Histology	14	(25)

NSCLC-NOS, non-small-cell lung cancer-not otherwise specified; SCC, squamous cell carcinoma; EGFR, epidermal growth factor receptor; ROSE, rapid on-site evaluation; N/A, data not available.

A total of 91 patients were diagnosed with primary lung adenocarcinoma by EBUS-TBNA. Of the 91 samples, 52 (57.1%) were obtained using a 21G needle and 39/91 (42.9%) using a 22G needle. EGFR mutation analysis was requested in 80/91 (87.9%) and ALK mutation analysis (which subsequently became available) was undertaken in 21/91 (23.1%) patients. Table V shows the outcomes of patients whose samples were not sent for molecular analysis.

All but one sample was considered to be sufficient for EGFR mutation analysis (79/80, 98.75%); the sample that was insufficient for mutation analysis was obtained using a 21G needle. EGFR mutations were detected in 5/80 (6.3%) samples. Of the 80 samples, 74 (92.5%) were negative for EGFR mutations. Of the 21 samples successfully tested for ALK mutations, all (100%) were found to be negative. In total, mutation testing for EGFR or ALK was successful in 100/101 EBUS-TBNA samples (99.0%).

Discussion

This single-centre prospective UK study demonstrated that EBUS-TBNA samples have a very high genotyping success rate for EGFR and ALK mutation analysis in primary lung adenocarcinoma when processed as histopathology specimens. The total genotyping success rate for EGFR and ALK combined was higher still (99.0%). EGFR mutations were detected in 6.3% (5/80) patients with primary lung adenocarcinoma, a mutation prevalence rate which is comparable to results from two other UK studies (6% in both), which included non-squamous NSCLC and all NSCLC types, respectively (19,20). Ethnicity was not a significant factor in our study, although higher rates of EGFR mutation positivity (≤51.4%) have been described in Asian populations (34). The diagnostic accuracy of EBUS-TBNA for malignancy was in keeping with expected results, confirming EBUS-TBNA was being performed to expected outcomes (9).

Our EGFR mutation test failure rate (1/80, 1.25%) was lower compared with that described in previous studies, which reported failure rates of 10 and 12%, respectively (19,20). Several studies have investigated the feasibility of performing EGFR mutation testing on EBUS-TBNA samples and the data from these studies are summarised in Table VI. There is significant heterogeneity between these studies, but with overall high rates of adequacy. The results from our study compare favourably to these other studies in terms of mutation testing success rate and sample number.

There are important differences in the techniques used for handling EBUS-TBNA samples in these studies. Specifically, several of these studies used ROSE, which was not available in our institution. However, our very high sample adequacy rate suggests that ROSE may not be essential for delivering acceptable mutation success rates with a histopathological processing approach, although that would require further investigation. Our high diagnostic accuracy may also relate to the fact that we collected ≥3 samples, which has been shown to optimise yield (35).

Differing histopathological techniques have been used in previous EGFR mutation EBUS-TBNA sample feasibility studies. In our institution, the preferred method of analysis of EBUS-TBNA samples is processing using histopathological techniques (27). However, the majority of previous studies have used cell blocks or needle washings for cytopathological analysis. In the light of our data, we suggest that our EBUS-TBNA processing technique does not appear to negatively affect EGFR or ALK mutation analysis, although further studies investigating this specific area are required. Close collaboration and dialogue between local clinicians and pathologists remains of paramount significance in providing the optimal pathway for EBUS-TBNA sample analysis.

The majority of the studies on the feasibility of EGFR mutation testing in EBUS-TBNA samples have used the smaller 22G needle for sampling. Contrary to published data on malignancy phenotyping (27), both needle gauges (22G and 21G) achieved high success rates for EGFR mutation testing without significant differences, although the study may have been underpowered to show this, also considering the 98.75% success rate. The only sample insufficient for EGFR mutation analysis was obtained using a 21G needle. Needle gauge size and its effect on feasibility of mutation testing may be worthy of further investigation.

We acknowledge certain limitations to our study. First, this was a single-centre study, although our numbers compare favourably with other adenocarcinoma numbers in previously published studies. Second, we cannot discount the potential effect of tumour heterogeneity that may lead to samples being unrepresentative of the whole tumour; however, this is an issue in the interpretation of all EBUS-TBNA studies. Third, tumour EGFR mutation heterogeneity has also been described between primary metastatic sites, such as lymph nodes. However, this is considered to be rare and, similarly, is an issue in all such studies (36). Fourth, our study evaluated EGFR mutation testing in adenocarcinoma as per clinical practice at the time; thus, other NSCLC types were not included. However, we do not consider that this is likely to have significantly affected our results, since previous studies have demonstrated that EGFR mutations are almost exclusively observed in adenocarcinomas (37). In line with this fact, the guidelines published by the National Comprehensive Cancer Network do no recommend testing for EGFR mutations in patients with squamous cell carcinoma (38). Finally, our study was underpowered to evaluate the effect of needle gauge for a mutation testing failure rate of only 1.25%.

Our study demonstrated that EBUS-TBNA samples obtained without ROSE and processed as histopathology specimens are suitable for EGFR and ALK mutation analysis. This study is highly representative of clinical practice and the results are considered to be relevant for other EBUS-TBNA centres. Therefore, these data support the use of EBUS-TBNA as a tool to obtain genetically profiled diagnoses using a minimally invasive technique, which is known to be well tolerated by patients (39). Other studies have also demonstrated the feasibility of using EBUS-TBNA to identify other genetic mutations, including echinoderm microtubule-associated protein-like 4 (EML4)-ALK and KRAS viral oncogene mutations, with equally high adequacy rates (17,40). Testing for ALK was introduced in our institution in March, 2013. All 21 EBUS-TBNA samples have been found to be adequate for EML4-ALK mutation testing, with a 100% success rate. None of these samples were positive for the mutation. We would suggest that further larger prospective studies using ROSE are required in this area, but this also supports the idea that EBUS-TBNA samples analysed

by this technique are suitable for all mutation analyses. We anticipate that, as an increasing number of genetic mutations are identified, multigene mutation analysis will become more important in facilitating treatment plan individualisation.

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