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Short Communication

Lung cancer in the era of targeted therapy: Molecular and PD-L1 expression profiles of endobronchial ultrasound-guided transbronchial needle aspiration-diagnosed non-small cell lung carcinoma samples derived from standard of care testing in the West of Ireland

E. O'Connor, A. O'Keefe, S. O. Hynes, G. Callagy, D. Breen and A. M. Quinn*
University Hospital Galway, Newcastle Road, Galway, H91 YR71, Ireland.

ABSTRACT

Treatment of non-small cell carcinoma (NSCC) has transformed with the identification of molecular alterations with differing responses to targeted therapies. In lung cancer, these genetic changes are generally somatic, therefore are acquired during life and are present only in certain cells in the lungs. They can be present at different rates according to factors including geographical location, ethnicity, and smoking status. This study characterises the spectrum of molecular and immunological profiles of EBUS-TBNA-diagnosed NSCC samples in the West of Ireland. EBUS-TBNA-diagnosed NSCC reported by the Department for Anatomic Pathology at our institution over a 60-month consecutive period from January 2015 to December 2019 were studied to identify EGFR, ALK, BRAF, ROS1 and PD-L1 status. Over the 60-month period, the percentages of molecular alterations in NSCC were 10.3% EGFR, 6.3% ALK, 2% BRAF, 0% ROS1. In the most recent two years assessed, ALK translocations were identified in 10% (2019) and 11% (2018) of NSCC cases that underwent mutation analysis, which is surprisingly high. In patients with an ALK alteration, 67% had a light or never smoking history. 58.7% squamous carcinoma cases had PD-L1 expression of less than 1%, and 19.5% had PD-L1 expression above 50%. 36.8% adenocarcinomas expressed PD-L1 at less than 1% and 32% expressed PD-L1 at over 50% tumour proportion score. EGFR somatic lung carcinoma mutations were in line with international findings. Sensitising L858R substitutions in exon 21 and in-frame amino acid deletions in exon 19 accounted for 80% of single EGFR mutations. ALK translocation rates of NSCC in the West of Ireland appear notably high for a European population. Squamous carcinoma was more likely to have a low PD-L1 expression, which is unusual given the typical association with smoking and mutation burden.

KEYWORDS: adequacy, EBUS-TBNA, ALK, EGFR, PD-L1, non-small cell lung carcinoma, molecular, mutation, Ireland.

ABBREVIATIONS

Anaplastic lymphoma kinase (ALK)
Endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA)
Epidermal growth factor receptor (EGFR)
Fluorescent in-situ hybridization (FISH)
Immunohistochemistry (IHC)
National Comprehensive Cancer Network (NCCN)
Next generation sequencing (NGS)
Non-small cell carcinoma (NSCC)
Programmed death ligand-1 (PD-L1)
Tyrosine kinase inhibitor (TKI)
Standard deviation (SD)

*Corresponding author: AnneMa.Quinn@hse.ie

INTRODUCTION

Treatment of non-small cell carcinoma (NSCC) has transformed with the identification of molecular alterations with differing responses to targeted therapies [1] also known as personalised medicine. Mutations build up in critical genes that control cell growth, cell proliferation or repair of damaged DNA. Cells grow without regulation and this leads to tumour formation. In lung cancer, these genetic changes are generally somatic and therefore are acquired during life and are present only in certain cells in the lungs. Advanced lung cancers that harbour oncogenic mutations can be highly responsive to targeted therapies.

Epidermal growth factor receptor (EGFR) mutations are seen almost exclusively in adenocarcinomas and present at different rates according to factors including geographical location, race and smoking status [2]. In Caucasian populations the rates are lower and mutations are seen in approximately 10-15% of adenocarcinomas in Ireland and Europe [2, 3]. Sensitising EGFR mutations are the most common actionable driver mutations found in NSCC. They occur within EGFR exons 18-21, which encode a portion of the EGFR kinase domain. Mutations in exons 18, 19 and 21 are predictive of sensitivity to EGFR-targeted therapy whereas mutations in exon 20 such as T790M typically predict resistance to therapy. The most common activating EGFR mutations are the L858R substitution within exon 21 and exon 19 amino acid deletions [3].

The so-called driver mutations are thought to be mutually exclusive, although there are some discrepancies with this theory. In cases without EGFR, BRAF or KRAS mutations, patients may have an activating ALK gene translocation. This occurs when ALK gene fuses to a component of other genes, most commonly echinoderm microtubule-associated protein-like 4 (EML4) gene. This results from an inversion on chromosome 2. ALK fusions are rare in Western populations, occurring in approximately 2-5% of adenocarcinomas, while present at a much higher rate in Asian non-smoking cohorts [4].

Patients with an EGFR mutation may respond to tyrosine kinase inhibitor (TKI) drugs such as erlotinib while ALK fusion predicts response to TKIs such as crizotinib [1, 5, 6].

NSCC tumour cells expressing programmed death ligand-1 (PD-L1) may respond to targeted immunotherapies [7, 8]. PD-1 is a transmembrane protein expressed on T-cells, B-cells, and NK cells. It is an inhibitory molecule that binds to the PD-L1 ligand which is expressed on the surface of many tissue types, including many tumour cells as well as haematopoietic cells. This interaction directly inhibits apoptosis of the tumour cell expressing PD-L1, promotes peripheral T-effector cell exhaustion and conversion of T-effector cells to regulatory T- cells [6].

In the clinical setting, endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA) is used as a staging and diagnostic tool for lung cancer and can be applied to the sampling of primary tumours or lymph nodes with potential metastatic carcinoma. EBUS allows minimally invasive sampling of hilar and mediastinal lymph nodes and has an established role in NSCC diagnosis and staging. Where appropriate, ancillary studies such as PD-L1 immunohisto/cytochemistry and mutation analysis are performed on samples received from EBUS-TBNA. Standard of care molecular tests include EGFR and BRAF mutation analysis, and screening for ALK translocations and ROS1 translocations. If the appropriate genetic alteration is present the tumour may respond to EGFR tyrosine kinase inhibitors, targeted ALK/ROS-1 inhibitors such as such as crizotinib, or BRAF inhibitor therapies.

This study characterises the spectrum of molecular and immunological profiles of EBUS-TBNA-diagnosed non-small cell lung carcinoma samples in the West of Ireland.

MATERIALS AND METHODS

This was a retrospective study. Ethical approval was attained from the local research ethics committee. The Apex laboratory information system at University Hospital Galway (UHG) was used to compile a list of all EBUS-TBNA-diagnosed NSCC cases reported by the Department for Anatomic Pathology over a 60-month consecutive period from January 2015 to December 2019. All NSCC reports was studied to identify tumour morphology, EGFR, ALK, BRAF, ROS1 and PD-L1 status as well as adequacy rates of sampling. Clinicopathological details were recorded from

the hospital digitalized patient information system, including age, sex, and tumour histomorphology. These were compared between groups.

EGFR status was determined using the cobas® EGFR Mutation Test (Roche P/N 06471463190). This assay covers 85% of known EGFR mutations. BRAF status was determined using the Roche BRAF/NRAS LSR (Roche P/N 07659962001) that covers 96.5% of known BRAF mutations. ALK expression was determined using the Ventana Anti-ALK (D5F3) antibody Kit (Ventana 06785042001) and confirmed using the Agilent IQ ALK FISH Breakapart Probe (Agilent G111600-8). ROS-1 status was determined using the Agilent IQ ROS-1 FISH Breakapart Probe (Agilent G111601-8).

RESULTS

In a 6-month period, 139 EBUS cytology requests were received, incorporating multi-sited specimens yielding 326 samples collected from the 139 cases. 266 samples were adequate in terms of sampling for cytological morphology evaluation (82%), while 60 samples (18%) contained insufficient material for diagnosis

EBUS-TBNA has a high diagnostic yield for molecular analysis in NSCC. 176/187 (94%) of samples sent for mutation analysis and 121/143 (84.6%) samples sent for PDL1 IHC were adequate.

Over a 60-month period there were 306 cases of NSCC diagnosed *via* EBUS-TBNA. This included adenocarcinoma (n = 182), squamous cell carcinoma (SCC) (n = 99), adenosquamous carcinoma (n = 8), NSCC not otherwise specified (NOS) (n = 7), large cell neuroendocrine carcinoma (n = 6) and poorly differentiated carcinoma (n = 4) (Table 1).

Oncogenic alterations: EGFR and ALK studies were conducted at our institution from 2015, while molecular studies for BRAF and ROS-1 were commenced in 2016. Molecular studies for EGFR/ ALK/BRAF/ROS1 were attempted on 187 samples over 60 months, of which 176 were adequate for testing (94%). This included adenocarcinoma (n = 148), squamous cell carcinoma (SCC) (n = 14), adenosquamous carcinoma (n = 7), NSCC not otherwise specified (NOS) (n = 3), large cell neuroendocrine carcinoma (n = 1) and poorly differentiated carcinoma (n = 3) (Table 1). Oncogenic gene alterations of ALK, EGFR, BRAF or ROS-1 were identified in 31 cases (Table 2). This included EGFR (n = 18), ALK translocation (n = 10), BRAF (n = 3), and ROS1 (n = 0). 2 of these cases subsequently developed resistance mutations, which included 1 ALK resistance mutation and 1 secondary EGFR mutation. Together, ALK and EGFR oncogenic alterations accounted for 29 of 31 (94%) oncogenic gene alterations detected. ALK translocations were detected by a combination of fluorescent in-situ hybridization (FISH), immunohistochemistry (IHC) and in certain cases next generation sequencing (NGS), while EGFR mutations were detached using the cobas® EGFR Mutation Test which is CE-IVD marked. The ROS-1assay was carried out using the Agilent IQ ROS-1 FISH Breakapart Probe Kit. The Roche BRAF/NRAS LSR assay was used for the identification of mutations in codons G466, G469, V600X and K601 mutations of the BRAF gene. The assay covers 96.5% of known BRAF mutations.

Of all NSCC that underwent molecular studies for each oncogenic alteration, the percentage of positive cases was EGFR (10.3%), ALK (6.3%), BRAF (2%), ROS1 (0%) over the 60-month period.

Histomorphology: Of the 31 cases with initial or pre-treatment oncogenic-driver alterations identified, there were 29 (94%) adenocarcinoma (18 EGFR, 9 ALK, 2 BRAF), 1 possible adenosquamous carcinoma (1 ALK), and 1 poorly differentiated carcinoma (1 BRAF).

Age distribution among NSCC: The 306 cases of EBUS-TBNA identifying NSCC comprised of 304 patients. The mean age (SD) of all patients with EBUS-TBNA-diagnosed NSCC was 68.2 (10.4). This included 176 males and 128 females. ALK-positive NSCC had a notably younger mean age (SD) of 55.4 (13.2), with the EGFR-positive NSCC mean age (SD) at 69.9 (7.8) and BRAF-positive NSCC at 74.5 (7.4) (Table 2).

ALK translocation-positive NSCC demographic: 10 patients were identified with an ALK translocation from 2015 to 2019. All 10 cases had an ALK translocation confirmed by both IHC and FISH (Table 3). This included 9 Irish patients and one patient originally from Germany. Smoking status was available for 9 cases. 4 were never

Table 1. Tumour morphology and PD-L1 status over 60 months 2015 to 2019.

Morphology	N=	Molecular studies successful	PD-L1 <1%	PD-L1 1-49%	PD-L1 >50%
Adenocarcinoma	182	148	25	21	22
SCC	99	14	27	10	9
Adenosquamous	8	7	0	1	1
NSCC NOS	7	3	0	1	1
Poorly differentiated	4	3	0	2	0
Large cell neuroendocrine	6	1	0	1	0
Total	306	176	52	36	33

Table 2. Mutation/translocations of NSCC per year.

Year	Mutation analysis successful	ALK	EGFR	ROS-1	BRAF	KRAS	NRAS
2015	28	1	5	0	0	0	0
2016	36	0	6	0	0	6	0
2017	32	1	4	0	0	1	0
2018	32	4	2	0	2	1	0
2019	48	5	2	0	1	0	1
Total	176	11	19	0	3	8	1

Table 3. Demographics, smoking status, and tumour stage among ALK positive cases of NSCC.

Age/sex	ALK: FISH/IHC	Ethnicity	NSCC stage	Smoking status
60 M	Both	Irish	4	Never smoker
34 F	Both	German	3	Never smoker
51 M	Both	Irish	4	Never smoker
54 F	Both	Irish	3	Never smoker
43 M	Both	Irish	2	Ex-social smoker 20 years ago, 7.5 pack year history
46 M	Both	Irish	4	Ex-smoker, 10 pack year history
72 F	Both	Irish	3	Ex-smoker, "50 years ago"
54 M	Both	Irish	4	Ex-smoker
78 M	Both	Irish	4	Smoker, 30 pack year history
62 M	Both	Irish	4	No information provided

smokers. 1 patient was an ex-smoker of 20 years with a 7.5 pack year history. 1 was an ex-smoker with a 10 pack-year history. 1 was an ex-smoker of 50 years with no pack-year history determined. 1 was an ex-smoker with no further details to pack-year history. 1 was a current smoker with a 30 pack-year history. 9/10 cases had a stage 3 or 4 NSCC at time of diagnosis (Table 3).

EGFR positive NSCC: There were 19 NSCC with EGFR mutation(s) detected, comprising 18 patients. 15 cases had a single EGFR mutation and 4 cases had 2 EGFR mutations present. Of the cases with a single EGFR mutation, 7 had a sensitising exon 21 L858R mutation and 5 had a sensitising exon 19 deletion. The 3 remaining cases were: exon 20 S768I (resistance), exon 20 S768I (sensitising), and exon 18 G719X (sensitising). The 4 cases with 2 EGFR mutations were of: exon 19 deletion (sensitising) and Thr 790 Met (resistance), exon 21 L858R (sensitising) and Thr 790 Met (resistance), exon 21 L858R (sensitising) and Thr 790 Met (resistance), compound pG719C (sensitising) and pE709K (unknown significance).

PD-L1 status: PD-L1 IHC was attempted on 143 cases. 121 cases had PD-L1 status successfully determined (84.6% adequacy). 27 of 46 (58.7%) squamous carcinoma cases had an expression of less than 1%, and 9 of 46 (19.5%) had PD-L1 expression above 50%. 25 of 68 (36.8%) adenocarcinomas assessed for PD-L1 expressed less than 1% and 22 of 68 (32%) expressed over 50% by tumour proportion score (Table 1).

DISCUSSION

The frequency of ALK-positive gene alterations in this study is high compared to international epidemiological findings of a European population [1] and may be significant for the population cared for by this institution (West of Ireland). ALK translocations are normally seen in 2-5% of NSCC in Caucasian populations although they are recorded at higher rates in Asia. Over a 5-year period, 6.3% of EBUS-TBNA-diagnosed cases of NSCC had either an ALK translocation (n = 10) or an ALK resistance mutation (n = 1). 7% of adenocarcinomas or possible adenosquamous carcinomas tested over the 5-year period were positive for an ALK translocation or resistance mutation. In the most recent two years assessed, ALK translocations alone were seen in 10% (2019) and 11% (2018) of NSCC cases that underwent molecular analysis, which is surprisingly high.

ALK alterations were only seen in adenocarcinomas and possible adenosquamous carcinomas. This is in keeping with international data [1]. In our study, ALK translocations are seen in a younger mean age than all other NSCC, again in keeping with international findings [9]. Patients with ALK translocations were more likely to be male. Examination of smoking status revealed 67% of cases had a light or never smoking history, with light smoking defined as less than 10-pack year history. There was only 1 current smoker identified at time of diagnosis of ALK-positive NSCC. 9 of the 10 cases were stage 3 or 4 NSCC at time of diagnosis. The patient ethnicity was either Irish (9) or German (1). For the geographic location and ethnicity of the study cohort, the ALK rates are unusually high, particularly in the most recent 2 years assessed.

The rates of EGFR NSCC somatic mutations audited were in line with international findings [10, 11]. Sensitising L858R substitutions in exon 21 and in-frame amino acid deletions in exon 19 accounted for 80% of single EGFR mutations in this population. L858R occurring as a single mutation is associated with sensitivity to EGFR tyrosine kinase inhibitors (TKI). EGFR exon 19 deletions are in-frame deletions also associated with EGFR TKI sensitivity. Most cases of NSCC treated with an EGFR TKI develop resistance 10-12 months after starting treatment. In this work, we noted that 3 cases with sensitizing mutations (2 with exon 21 L858R and 1 with exon 19 deletions) also harbored a resistance mutation of exon 20 T790M. This is known as a gatekeeper mutation and involves a threonine-to-methionine substitution in exon 20. This increases the affinity of mutant EGFR for ATP, thereby competitively inhibiting the binding ability of reversible EGFR TKIs [12]. The T790M mutation is the most common mechanism of acquired resistance to EGFR TKI therapy [13]. It accounted for 3 of 4 (75%) secondary EGFR mutations identified in this study. The age of EGFR-positive individuals was higher than for those harbouring an ALK fusion, which is not always typical as both alterations are associated with never/light smoking status, younger age and non-Caucasian race [3, 14].

BRAF-positive NSCC was detected in 2%, in keeping with expected findings. The prevalence of *BRAF*-mutated lung cancer is between 1.5 and 3.5% without any ethnic predilection [15, 16]. Although there were no ROS1 fusions identified in this study, the expected proportion would be 1-2% of NSCC [16].

Identification of patients who may benefit from immune checkpoint inhibition in NSCC is a key part of lung cancer work up. This is mainly done based on PD-L1 IHC expression. The NCCN guideline utilizes a cutoff of 50% tumour proportion score (TPS) for first line therapy and 1% TPS for second line therapy with pembrolizumab in NSCC [7]. The European Medicines Agency recommends the use of pembrolizumab as monotherapy for first-line treatment of metastatic NSCLC in adults whose tumours express PD-L1 with a ≥ 50% tumour proportion score (TPS) with no EGFR or ALK positive tumour mutation [17]. PD-L1 expression is the most widely used predictive marker to select patients most likely to respond to immunotherapy. Previous studies have shown that PD-L1 expression was significantly higher in SCC than adenocarcinoma [7]. PD-L1 expression is often increased in association with smoking history and high mutational load, therefore often high in SCC [7]. In our study, SCC was more likely to have a low PD-L1 expression. This was unusual and not expected. It could reflect a limited sample size. Testing for PD-L1 increased at our institution from 2018. Of the successful PD-L1 tests over the 60-month period of this study, 91 /121 occurred in 2018 and 2019, while the remaining 30 were spread among 2015-2017.

CONCLUSION

EBUS sampling has high adequacy for not only diagnosis (>80%), but also for further molecular and IHC ancillary studies in NSCC, with greater than 90% of samples with malignant cells found adequate for molecular analysis. The sampling technique (employed at UHG) involves 3 passes on average per node and extraction of all material into a cell block, without preparation of smears. This contributes to the adequacy of specimens. Careful immunocytochemical work-up during diagnostic classification helps to preserve material for subsequent testing. The proportion of ALK translocations in NSCC in the West of Ireland in recent years appears notably high for a European population. Further evaluation over the coming years will help establish if ALK trends remain high or balance out towards the expected rate of 2-5%. Evaluation of the ALK and PD-L1 rates in resections could be assessed to compare molecular profiles between early and later stage NSCC as resections are typically earlier stage NSCC.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to declare.

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