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ANATOMICAL PATHOLOGY

EGFR mutation specific immunohistochemistry is a useful adjunct which helps to identify false negative mutation testing in lung cancer

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Summary

Mutations in *EGFR* guide treatment in non-small cell lung cancer (NSCLC). The most common mutations, exon 19 (delE746-A750) and exon 21 (L858R), can be identified by mutation specific immunohistochemistry (IHC). We present our prospective experience of universal reflex IHC and molecular testing in non-squamous NSCLC in the routine clinical setting. A total of 411 specimens from 332 patients were encountered over two years. Of these, 326 (98%) patients underwent EGFR IHC, 15 (5%) were positive for exon 19 deletions and 27 (8%) for exon 21 (L858R); 244 (73%) patients underwent molecular testing. Seventy-six mutations in 64 patients (19% of all patients encountered; 26% with sufficient material for testing) were identified. These comprised nine exon 18 (G719X) mutations, three also with exon 20 mutations; 24 exon 19 deletions, six also with exon 20 mutations; 23 exon 21 (L858R), three also with exon 20 mutations; and 8 exon 20 alone. All 15 exon 19 IHC positive patients were proven mutated (100% specificity, 63% sensitivity). Twenty-two of 27 exon 21 IHC positive cases were proven mutated while three patients had insufficient material for molecular testing (92% specificity, 96% sensitivity). The overall specificity and sensitivity of IHC for any EGFR mutation was 95% and 58%. Five patients initially thought to be wild type for *EGFR* but IHC positive underwent repeat molecular testing because of the discrepancy which confirmed the IHC result in three cases (60%). We conclude IHC is very specific but not sensitive. Whilst IHC cannot replace molecular testing, it is a useful adjunct which requires minimal tissue and identifies false negative molecular results which occurred in 5% of our patients with eventually confirmed *EGFR* mutations.

Key words: EGFR immunohistochemistry, EGFR inhibitors, erlotinib, gefitininb, lung cancer.

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INTRODUCTION

The presence of somatic mutations in the epidermal growth factor receptor gene (*EGFR*) identifies patients with non-small cell lung carcinoma who are likely to respond to specific tyrosine kinase inhibitors including gefitinib and erlotinib.[1–5] Pathogenic *EGFR* mutations are very rare in squamous cell carcinoma.[1–5] Therefore, the guidelines of the College of American Pathologists, International Association for the Study of Lung Cancer, and Association for Molecular Pathology, recommend molecular testing for the presence of activating mutations in the tyrosine kinase domain of *EGFR* (exons 18–21) in all patients with advanced or recurrent non-squamous non-small cell lung cancer (NSCLC).[6] The same guidelines also 'encourage' *EGFR* mutation testing in patients with early stage disease on the basis that, although treatment with tyrosine kinase inhibitors is currently only indicated in patients with locally advanced or metastatic non-squamous NSCLC, knowledge of the *EGFR* mutation status in all patients including those who are potentially operable may help plan treatment and has benefits from a laboratory workflow perspective.[6]

Although reflex testing of all non-squamous NSCLC would be desirable, molecular testing is expensive and in many jurisdictions mutation testing is only funded in the presence of advanced or metastatic disease. Standard molecular testing may require more material than is available in cytological or small biopsy specimens, particularly from a relatively inaccessible site such as lung. Furthermore, despite being considered the gold standard for identifying *EGFR* mutations, molecular testing may not identify all clinically significant *EGFR* mutations due to the limitations of the specific *EGFR* assays available or due to issues with quality assurance which may be encountered in the routine clinical setting for a variety of reasons. For example, particularly in low cellularity specimens, the presence of wild type *EGFR* from non-neoplastic cells may overshadow a clinically significant *EGFR* mutation and lead to a false negative result.[11,14]

The two most common mutations in *EGFR* are an in-frame deletion in exon 19 (delE746-A750) and a missense mutation in exon 21 (L858R) which together have been reported to account for up 80–90% of clinically significant *EGFR* mutations.[7,8] Because these two mutations result in two distinct mutant proteins, these can be detected by mutation specific immunohistochemistry (IHC) performed on formalin fixed,

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paraffin embedded tissue, using commercially available monoclonal antibodies.[9] *EGFR* mutation specific IHC has been reported to have a variable sensitivity ranging from 42.2% to 75.5%, but a very high specificity of up to 99.8%.[9–12] Therefore, IHC has been suggested either as a substitute for reflex molecular testing in the resource poor setting (particularly if molecular testing is not funded for localised disease) or as an adjunct to molecular testing, for example when insufficient material is available for analysis.

Despite the presence of numerous retrospective series, the role of *EGFR* mutation specific immunohistochemistry in the routine clinical setting remains to be clarified.[9–12] Put simply, because of its relatively poor sensitivity, can EGFR mutation specific IHC be justified when molecular testing (the current 'gold standard' for the identification of clinically significant mutations) is also routinely performed? Furthermore, is *EGFR* mutation specific IHC sufficiently specific when deployed in the routine clinical setting that positive staining could be used as the basis for major management decisions when insufficient material is available for molecular testing? At the time of writing there have been no published data on the utility of reflex EGFR IHC when applied prospectively in the routine clinical setting in parallel with molecular testing.

In this study we present our 2 year experience with the clinical deployment of reflex testing for *EGFR* mutation of all cases of non-squamous NSCLC encountered in all specimen types using both IHC and molecular testing and provide a rational argument for the inclusion of mutation specific immunohistochemistry in the routine laboratory workflow, either as a reflex test or in cases with insufficient material for molecular testing.

MATERIALS AND METHODS

Patients

To assess the specificity of immunohistochemistry before implementation of the approach, we initially validated the use of EGFR IHC on a retrospective tissue microarray (TMA) cohort of NSCLC undergoing surgery with curative intent. The demographic, histological, outcome and ALK IHC data have been previously reported.[13,14] Briefly, it comprised all patients undergoing lung cancer surgery with curative intent at the Royal North Shore Hospital, Sydney, Australia, between the years 2000 and 2010. The original histological slides and blocks had been reviewed independently and reclassified according to the International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society (IASLC/ATS/ERS) system[15] and the 7th edition 2009 Union for International Cancer Control/American Joint Committee on Cancer (UICC/AJCC) TNM staging system.[16] A TMA was constructed with dual 1 mm cores from all cancers. Cases which showed positive EGFR IHC (exon 19 and/or exon 21) then underwent confirmatory molecular testing performed by DNA mass spectrometry using matrix assisted laser desorption ionisation time-of-flight (MALDI-TOF) to assess the specificity of positive staining.

After mutation specific EGFR IHC was demonstrated to be highly specific in this retrospective cohort, we then prospectively implemented combined reflex IHC and molecular testing on all cases of NSCLC encountered during the period 1 January 2012 to 31 December 2013. Definite squamous cell carcinomas as defined by the IASLC/ATS/ERS system were excluded from further testing, but cases diagnosed as NSCLC, favour squamous carcinoma, using the classification did undergo testing. All patients with non-squamous NSCLC with sufficient material in at least one biopsy underwent both IHC and molecular testing.

Mutation specific EGFR immunohistochemistry

IHC for EGFR mutations was performed using two commercially available mutation specific rabbit monoclonal antibodies. One antibody is directed towards the exon 19 deletion delE746-A750 (clone 6B6, dilution 1:100; Cell Signaling Technology, USA). The other antibody is directed towards the EGFR exon 21 L858R point mutation (clone 43B2, dilution 1:50; Cell Signaling Technology).

IHC was performed using the Leica Bond III autostainer (Leica Microsystems, Australia). Slides were dewaxed in Bond Dewax solution (AR9222; Vision Biosystems, Australia) and hydrated in Bond Wash solution (AR9590; Vision Biosystems). Heat induced epitope retrieval was performed for 30 min at 97°C using the manufacturer's alkaline retrieval solution ER2 (VBS part no. AR 9640; Leica Microsystems). Slides were then incubated with the primary antibodies (concentration 1 in 100 for EGFR exon 19 and 1 in 50 for exon 21) for 30 min at room temperature. Antibody detection was performed using the biotin free Bond Polymer Defined Detection System (DS9713; Vision Biosystems) according to the manufacturer's protocol. Slides were then counterstained with haemtoxylin. In the routine diagnostic setting, IHC was reported by the pathologist or cytopathologist allocated to report the primary pathology of the case and was reported in the initial pathology report with a disclaimer statement that IHC is specific but not sensitive and that *EGFR* mutation analysis is considered the gold standard and will also be performed. For the purpose of this analysis, if there was discordant staining when IHC was performed on more than one specimen, the presence of a positive staining in any biopsy was considered a positive result. Staining was reported as either positive (if the reporting pathologist thought the pattern of staining represented genuine membranous or cytoplasmic expression of the protein in neoplastic cells, even if this expression was focal) or negative if there was no positive staining or the only staining present appeared non-specific. The pattern of staining for both EGFR exon 19 and 21 mutation specific IHC is illustrated in Fig. 1 and 2.

Molecular testing

Molecular testing of the prospective series was performed by a variety of methods at a number of different external centres, all of which held National Association of Testing Authorities (NATA) accreditation for testing. The techniques employed for molecular testing varied at different times depending on availability and cost. The approaches employed comprised MALDI-TOF polymerase chain reaction (PCR) using the Sequenom MassArray platform (Sequenom, USA), allele-specific PCR using the Cobas EGFR Mutation Test kit (Roche Molecular Systems, USA) and Sanger sequencing using 2 x bidirectional sequencing and direct mutation analysis for exons 18, 19, 20 and 21. If there were discordant results when molecular testing was performed on more than one specimen, the presence of a mutation identified in any biopsy was considered a positive result.

Molecular testing was not performed blinded as to the mutation specific IHC status and if the results were discrepant, repeat IHC and molecular testing was performed when sufficient material was available, preferably on a separate block or specimen. If the results of IHC and molecular testing were concordant, repeat molecular testing was not undertaken. A flow chart illustrating our approach to testing is provided in Fig. 3.

RESULTS

The retrospective cohort comprised 256 cases of surgically resected NSCLC. Using the IASLC/ATS/ERS classification system, 152 (59%) cases were classified as adenocarcinoma.[15] Positive staining was restricted to cases classified as adenocarcinoma, with six cases showing positive staining for EGFR exon 19 delE746-A750, and six cases showing positive staining for EGFR exon 21 L858R mutant protein. All 12 cases were proven to harbour the corresponding *EGFR* mutations using MALDI-TOF PCR. This arm of the study was not designed or intended to address the sensitivity of *EGFR* mutation analysis, but it did demonstrate that EGFR mutation specific IHC for both the exons 19 and 21 mutations was 100% specific in this cohort.

Having determined that EGFR mutation specific IHC was highly specific for *EGFR* mutation, we then proceeded to perform both mutation specific IHC and molecular testing on all cases of non-squamous NSCLC with sufficient material from 1 January 2012 to 31 December 2013.

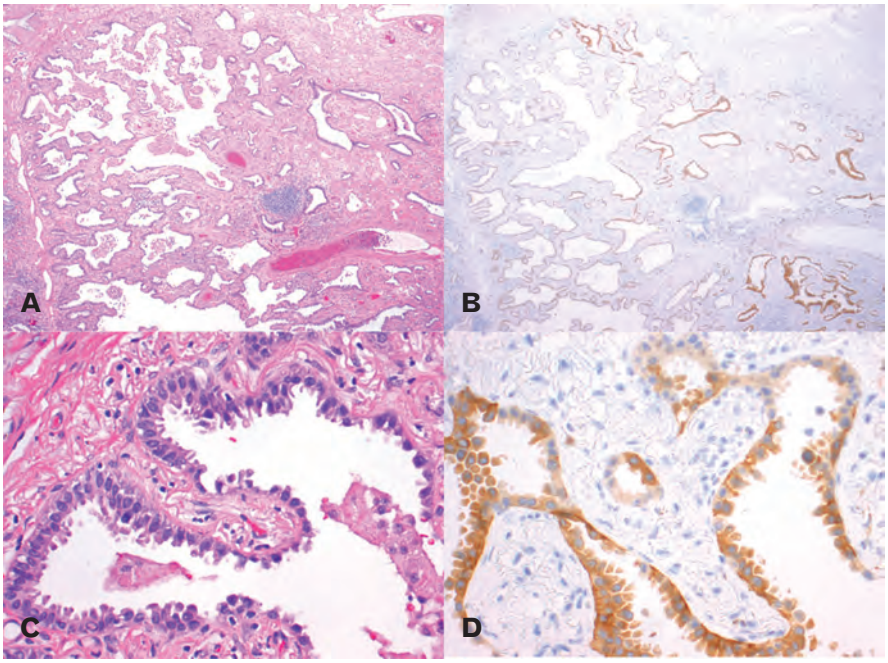

Fig. 1 (A–D) Serial H&E (A,C) and EGFR exon 19 IHC (B,D) stained sections. The malignant cells demonstrate intense cytoplasmic staining which is absent in non-neoplastic cells.

A total of 411 specimens from 332 patients with non-squamous NSCLC were encountered during this period. Of the 411 specimens, 222 (54%) were fine needle aspirations, 103 (25%) were core biopsies and 86 (20%) were excisions. Of the 332 patients, 158 (48%) were male and 174 (52%) were female with ages ranging from 29 to 94 years, and a median age of 68 years. Three hundred and twenty-six (98%) had sufficient material in at least one specimen for mutation specific EGFR IHC to be performed for both the exon 19 and 21 mutations. Forty-two (13%) showed positive staining for either the exon 19 or exon 21 mutation. Fifteen (5%) patients were immuno-positive for exon 19 del E746-A750, and 27 (8%) immuno-positive for exon 21 L858R. No patients were positive for both mutations on IHC.

Molecular analysis was performed on 251 patients (all of whom had IHC performed). Of these, 244 (74%) had sufficient material for complete sequencing of all four exons 18 to 21 and seven (2%) had sufficient material for partial sequencing of

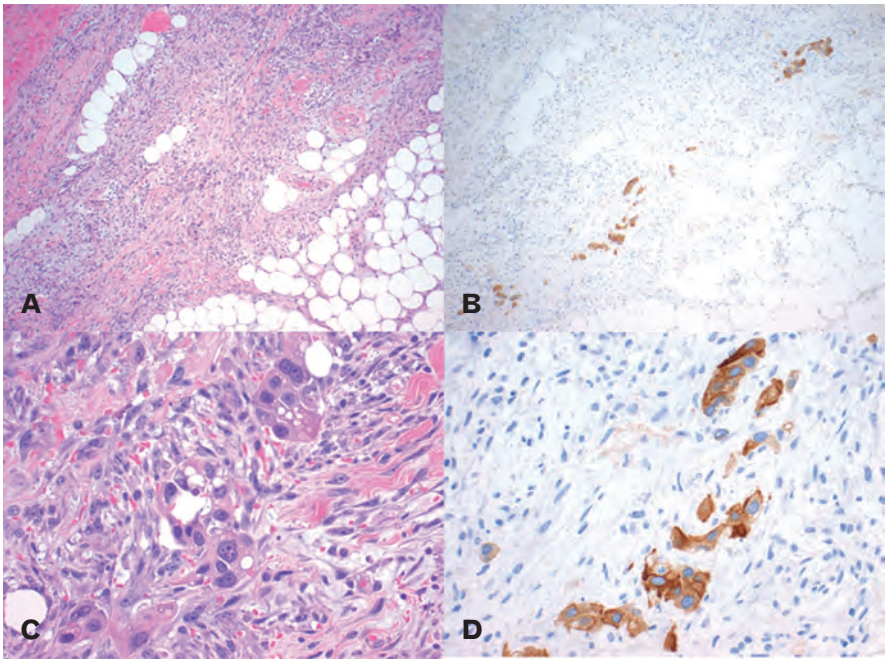

Fig. 2 (A–D) Serial H&E (A,C) and EGFR exon 21 IHC (B,D) stained sections. In this case the malignant cells demonstrate intense cytoplasmic staining. However, the fraction of neoplastic cells present in this case is very low (we would estimate less than 15% even in macrodissected areas). Although molecular testing was negative, even when performed three times on this case, the patient was offered treatment to which she responded. On the fourth time it was performed the presence of EGFR mutation was confirmed.

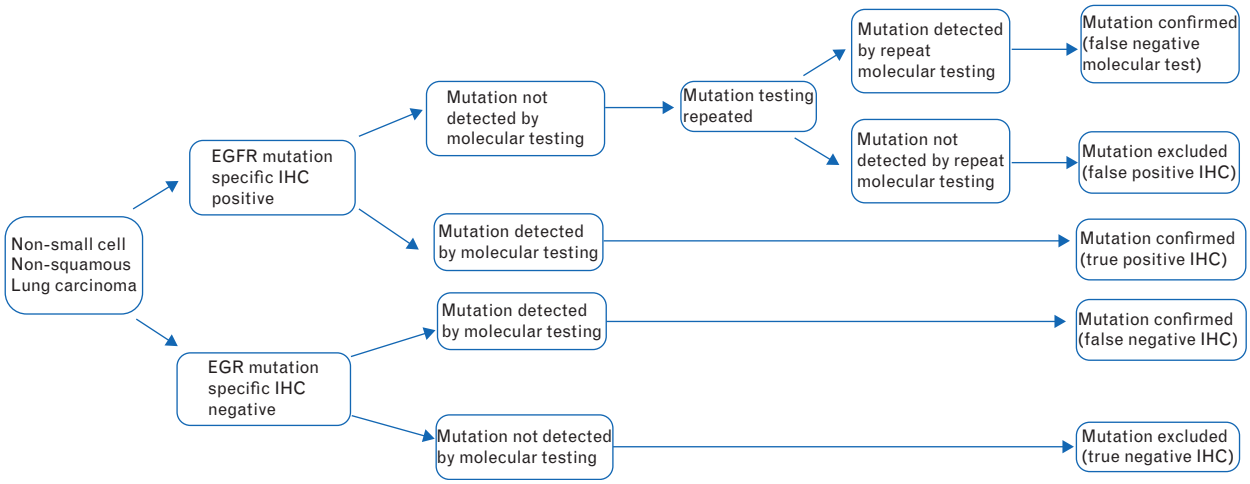

Fig. 3 Flow chart summarising our approach to combined IHC and molecular testing. If IHC and molecular testing were concordant, repeat testing was not undertaken. However, if IHC was positive and molecular testing was negative, repeat molecular testing was undertaken.

only selected exons. Of the 81 (24%) of 332 patients who were not sequenced, 39 (48%) had insufficient material in any biopsy for sequencing, 22 (27%) were encountered during a 3 month period when *EGFR* mutation was unavailable due to funding constraints and 10 (12%) had *ALK* gene rearrangement confirmed by reflex IHC followed by FISH testing and therefore did not undergo *EGFR* mutation testing because of their mutual exclusivity.[14] For 10 (12%) of patients, *EGFR* mutation testing was not performed due to pathologist preference or lack of adherence to protocol. The reasons for mutation testing not being performed are summarised in Table 1.

A total of 76 mutations from 64 patients were identified (19% of all patients encountered and 26% of all patients with sufficient material for molecular testing). Table 2 summarises the detected mutations. Briefly, nine patients harboured mutations in exon 18 (all of which were classified as G719X, connoting G719S, G719A or G719C mutations), of which three also harboured exon 20 mutations. Twenty-four were exon 19 deletions (6 also with exon 20 mutations). Twenty-three were exon 21 (all L858R) mutated (3 also with exon 20 mutations). Eight harboured mutations in exon 20 alone. The mutation data are summarised in Table 2.

Of the 15 patients who were immunopositive for exon 19 delE746-A750, all 15 were confirmed to have exon 19 deletions by molecular testing, giving a specificity of 100% for exon 19 IHC (15/15). A total of 24 exon 19 deletions were identified by sequencing, resulting in a sensitivity of exon 19 IHC of 63% (15/24).

Of the 27 patients who were immunopositive for L858R, 21 were confirmed to harbour an *EGFR* L858R exon 21 mutation by molecular testing. Of the five cases which did not have confirmed *EGFR* exon 21 mutations but which were immunopositive for L858R, three had insufficient material present for testing, giving a specificity of 92% (22/24). A total of 23 *EGFR* exon 21 mutations were identified by molecular testing, giving exon 21 IHC a sensitivity of 96% (22/23). It should be noted that only one EGFR exon 21 IHC positive case with ample material present for retesting was consistently mutation negative by Cobas test and Sanger sequencing and can be interpreted as an unequivocal false positive IHC stain, whereas the other case was hypocellular (estimated cellularity 15%) raising the possibility of false negative molecular testing given that it is very close to the 10% lower limit of cellularity for the Cobas detection system (Cobas CE-IVD package insert).

The overall sensitivity using exon 19 and 21 IHC for any mutations (exons 18–21) was 58% (37/64), whereas the overall specificity using exon 19 and 21 IHC for any mutations (exons 18–21) was 95% (37/39).

The total *EGFR* mutation rate amongst patients who underwent molecular testing in our unselected Australian population was 26% (64/244). Presuming that there may be some selection bias towards rebiopsying patients considered at high clinical risk for *EGFR* mutation, the absolute minimum rate of *EGFR* mutation in our unselected population, presuming that all 81 cases with insufficient material for EGFR mutation testing were wild type, is 19% (64/332).

There were three patients where mutation specific IHC was positive and initial molecular testing was negative. However, when molecular testing was repeated in view of this discrepancy, a corresponding mutation was identified, indicating initial false negative molecular testing. All three of these cases harboured the exon 21 (L858R) mutation. In one case, repeat testing had been performed on the same block by a different technique (Sanger rather than Cobas test), whereas in two cases repeat testing was performed on a different sample also with a different technique (Cobas rather than Sanger).

One of these false negative molecular cases which was hypocellular and was consistently IHC positive is illustrated in Fig. 2. This adenocarcinoma had mutation testing repeated a total of two times from two separate specimens (pericardium and pericardial fluid) which remained negative for mutation. On the basis of the clinical impression of false negative molecular testing and true positive IHC, the patient was then offered treatment with gefinitinib for stage 4 lung cancer. Computed tomography (CT) and positron emission

Table 1 Reasons for mutation testing not being performed

Reason	No. patients	% without sequencing ($n = 81$)
Insufficient material	39	48%
Funding unavailable	22	27%
ALK translocation identified	10	12%
Lack of adherence to protocol	10	12%
Total	81	100%

Table 2 List of EGFR mutations from cohort of 332 patients

Case	Exon 18	Exon 19	Exon 20	Exon 21
1	G719X		S768I	
2	G719X		S768I	
3	G719X		S768I	
4	G719X			
5	G719X			
6	G719X			
7	G719X			
8	G719X			
9	G719X			
10		delE746 – A750		
11		delE746 – A750		
12		delE746 – A750		
13		delE746 – A750		
14		delE746 – A750		
15		delE746 – A750		
16		Deletion*	S768I	
17		Deletion*	S768I	
18		Deletion*	S768I	
19		Deletion*	S768I	
20		Deletion*	S768I	
21		Deletion*	S768I	
22		Deletion*		
23		Deletion*		
24		Deletion*		
25		Deletion*		
26		Deletion*		
27		Deletion*		
28		Deletion*		
29		Deletion*		
30		Deletion*		
31		Deletion*		
32		Deletion*		
33		Deletion*		
34			T790M	L858R
35			c.2310_2311insGGG:p.Asp770_Asn771insGly	
36			c.2320_232lins: p.His773_Val774ins	
37			T790M	L858R
38			T790M	L858R
39			S7681	
40			S7681	
41			Insertion*	
42			Insertion*	
43			Insertion*	
44			Insertion*	
45				L858R
46				L858R
47				L858R
48				L858R
49				L858R
50				L858R
51				L858R
52				L858R
53				L858R
54				L858R
55				L858R
56				L858R
57				L858R
58				L858R
59				L858R
60				L858R
61				L858R
62				L858R
63				L858R
64				L858R

* Insertion refers to the presence of any of the following exon 20 mutations: 2319_2320insCAC, H733_V744insH, COSM12377; 2310_2311insGGT, D770_N771insG, COSM12378; 2307_2308ins9GCCAGCGTG, V769-D770insASV, COSM12376; 2309_2310AC>CCAGCGTGGAT, V769_D770insASV, COSM13558; 2311_2312ins 9GCGTGGACA, D770_N771insSVD, COSM13428.

Deletion refers to the detection of any of the following deletions in exon 19: 2235_2249del15,E746_A750del, COSM6223; 2236_2250del15, E746_A750del, COSM6225; 2240_2257del18,L747_P753>S, COSM12370; 2240_2254del15, L747_T751del, COSM12369; 2239_2256del18, L747_S752del, COSM6255; 2239_2251>C, L747_T751>P, COSM12383; 2237_2251del15, E746_T751>A, COSM12678; 2237_2255>T, E746_S752>V, COSM12384; 2239_2248TTAAGAGAAG>C, E747_A750>P, COSM12382; 2239_2253del15, L747_T753del, COSM6254; 2239_2247del9, L747_E749del, COSM6218; 2235_2252>AAT, E746_T751>1, COSM13551; 2236_2253del18, E746_T751del, COSM12728; 2237_2254del18, E746_S752>A, COSM12367; 2238_2255del18, E746_S752>D, COSM6220; 2238_2248>GC, L747_A750>P, 12422; 2238_2252>GCA, L747_T751>Q, COSM12419; 2239_2258>CA, L747_P753>Q, COSM12387; 2240_2251del12, L747_T751>S, COSM12387; 2240_2251del12, L747_T751>S, COSM6210; 2233_2247del15, K745_E749del, COSM26038; 2253_2276del24, S752_I759del, COSM13556; 2235_2248>AATTC, E746_A750>IP, COSM13550; 2237_2252>T, E746_T751>V, COSM12386; 2235_2251>AATTC, E746_T751>IP, COSM13552, 2235_2255>AAT, E746_S752>I, COSM12385; 2237_2253>TTGCT, E746_T751>VA, COSM12416; 2237_2257>TCT, E746_T751>VA, COSM12416; 2237_2257>TCT, E746_P753>VS, COSM18427; 2238_2252del15, L747_T751del, COSM23571; 2239_2256>CAA, L747_S752>Q, COSM12403.

tomography (PET) scanning performed 3 months after commencing treatment demonstrated metabolic and radiological response and she continued to have stable disease 12 months after treatment initiation. Only during preparation of this manuscript (14 months after presentation) was *EGFR* mutation testing repeated a third time on archived material and she was confirmed to harbour a pathogenic mutation. Although the majority of clinical testing was performed using the Cobas test, two of three false negative molecular tests were from Sanger sequencing (a technique with less sensitivity in low cellularity specimens).

There were two cases which were IHC positive but negative for mutation analysis on both initial and repeat sequencing. One case which demonstrated diffuse strong positive staining arose in a female non-smoker of southeast Asian ethnicity. In the paraffin block, neoplastic cells were present in low cellularity (estimated cellularity 15%). The other case arose in a sarcomatoid carcinoma arising in a male heavy smoker and demonstrated only focal weak staining. Ample material was present in the paraffin block from this case.

When IHC was performed on more than one specimen, concordant staining was found in all but four patients. In three of these patients, the FNA showed negative staining for both *EGFR* mutations but the excision showed positive staining for *EGFR* exon 19 delE746-A750 mutation. In one case there were two FNA specimens, one which was negative by IHC and one which showed positive staining for the exon 19 mutation. All four cases were confirmed to harbour the corresponding mutations by molecular testing.

DISCUSSION

EGFR is a transmembrane receptor tyrosine kinase which is involved in the normal regulation of cell survival and cell development, and is frequently mutated in lung cancer.[17] Activating *EGFR* mutations are reported to occur in 10–35% of all lung cancers,[2,3,7,18] are more common in Asians, non-smokers and females, and occur rarely, if at all, in squamous cell carcinoma.[19,20] EGFR tyrosine kinase inhibitors, such as gefitinib and erlotinib result in improved progression free survival when compared to carboplatin/paclitaxel combinations in patients harbouring these mutations.[5] Therefore, identification of these mutations is mandatory in patients with advanced NSCLC with compatible (i.e., non-squamous) histology and may be beneficial and is 'encouraged' in patients with localised disease.[6] However, delivering this testing in the routine clinical setting is fraught with difficulties, including quality assurance, expense, delays in turnaround time and the need for a relatively large amount of tissue.

Clinically significant *EGFR* mutations typically affect the four exons encoding the tyrosine kinase domain (exons 18–21) and it is to these four exons that most clinically deployed assays are exclusively directed.[7,8] In our series in which there were 76 mutations, 32% were in exon 19, 30% in exon 21, 26% in exon 20, and 12% in exon 18.[1,7] These incidences are in keeping other reports where exon 19 (ΔE746-A750) and exon 21 (L858R) mutations dominate.[1,7] Presuming that all patients who did not have sequencing performed were wild type, the estimated minimum mutation rate of any *EGFR* mutation in our prospective cohort of 332 Australian non-squamous NSCLC was 19% (64/332). The mutation rate of any *EGFR* mutation amongst the sequenced population was 26% (64/244). These mutation frequencies are in line with the reported incidence of *EGFR* mutation in other series which ranges from 10–35%, with a significantly higher relative risk in non-smoking Asian populations.[2,3,18]

The common *EGFR* exon 19 deletions and exon 21 L858R point mutations identified by mutation specific immunohistochemistry and accounting for 47 of the 76 (62%) *EGFR* mutations in our series, are recognised as classical sensitising mutations which connote a good response to EGFR inhibition.[21] The *EGFR* exon 18 mutations detected in this study (all of which were classified as G719X, connoting G719S, G719A or G719C mutations), which accounted for 12% of mutations, are recognised as markers of response to tyrosine kinase inhibition, but it has been suggested that these mutations may respond less well to targeted therapy than exon 19 and exon 21 mutation. For example G719X mutations are associated with poorer overall response rates to gefitinib when compared to common EGFR mutations.[22]

It is interesting to note that 26% of all EGFR mutations detected in our series were in exon 20, in contrast to some previous reports where exon 20 mutations accounted for only 4–10% of all EGFR mutations.[23,24] It is possible that some of these patients with exon 20 mutations had previously been treated with tyrosine kinase inhibitors as this information was not routinely recorded, although we believe our high rate of exon 20 mutation is attributable to our approach of routinely testing for mutations in this exon (which is not universal practice). Most exon 20 mutations are associated with resistance or poor response to gefitinib and erlotinib treatment even when occurring in conjunction with a classical sensitising mutation (that is, they represent resistance mutations rather than sensitivity mutations).[22,25,26] For example Wu *et al.* found that insertions/duplications or deletions in individuals with T790M mutations had unfavourable responses to gefitinib, whereas patients with S768I mutations had a variable response.[25] In our cohort we identified four T790M and eleven S768I mutations.

It could be argued that failure to identify these exon 20 mutations has little clinical impact because they are usually resistance mutations which do not necessarily connote response to tyrosine kinases inhibition. Perhaps it could be suggested that failure to identify exon 18 mutations has less clinical impact than failure to identify the classical sensitising mutations in exon 19 and 21. However, we believe that the poor sensitivity of IHC which is only partly due to the inability to identify the exon 18 sensitivity mutations or exon 20 resistance mutations is such a major drawback that immunohistochemistry cannot replace molecular testing in the routine clinical setting.

However, we do believe that immunohistochemistry which is inexpensive, can be performed rapidly, is widely available, and requires minimal tissue, may have a role in the routine clinical setting as an adjunct to molecular testing. Although molecular testing is considered the 'gold standard' for the identification of *EGFR* mutations, this does not mean it is perfect and a major issue in the diagnostic laboratory is quality assurance of molecular testing. We believe that IHC can serve to add some level of quality assurance to molecular testing in the routine clinical setting primarily by decreasing the amount of false negative molecular testing. For example, we identified three cases where mutation testing was initially negative, but due to positive mutation specific EGFR IHC, repeat molecular testing revealed a mutation (in one case after molecular testing was completed four times and the patient had already responded to treatment). Put another way, three of 64 (5%)

patients with clinically significant *EGFR* mutations had false negative *EGFR* mutation testing and would not have been identified if EGFR immunohistochemistry had not been performed. Had it not been for our program of reflex EGFR IHC in addition to molecular testing, these patients would have been denied treatment with tyrosine kinase inhibitors. When we reviewed the circumstances of these false negative molecular tests, two could be attributable to relatively low cellularity specimens (but still within guidelines for the assays employed) but for one case no clear cause could be found.

Given the high specificity of EGFR IHC, we recommend that all patients with positive IHC results but initially negative molecular testing should undergo repeat testing with consideration being given to testing with another technique, on another biopsy or at another centre, and with particular care being given to quality assurance including tumour cellularity. Furthermore, if repeat molecular testing is negative but the sample is relatively hypocellular, the possibility of both false negative molecular testing as well as false positive IHC should be considered. In these cases we would recommend that treatment decisions be based on the outcome of both tests when interpreted with all other clinical and pathological features.

Our two patients with positive IHC but negative molecular testing illustrate this approach. One tumour was an adenocarcinoma present in very low cellularity but demonstrating intense strong positive staining which arose in a female nonsmoker with well differentiated adenocarcinoma (a classic phenotype associated with EGFR mutation). In this setting we would consider false negative molecular testing more likely and recommended a trial of targeted therapy if it can be accessed under local funding arrangements. In contrast, in the other case with positive IHC and negative molecular testing, there was ample material present for repeat testing and the tumour was poorly differentiated (non-small cell favour adenocarcinoma with sarcomatoid areas), arose in a Caucasian male heavy smoker and showed only focal weak staining by IHC. We would consider this a likely false positive of IHC and not recommend targeted therapy.

Another advantage of reflex EGFR IHC is that it requires minimal material and can often be performed on biopsies with insufficient material present for molecular testing. For example, in our series there were 38 patients (11.4% of all patients) who did not have sufficient material present for molecular testing. However 33 (87%) of these patients had sufficient material for EGFR mutation specific IHC. Three of these patients demonstrated positive staining. Because of the very high specificity of EGFR IHC (and the ongoing quality assurance provided by performing routine IHC in parallel with molecular testing) it is highly likely that these patients will harbour an *EGFR* mutation. At the very least, the positive staining for EGFR mutation specific IHC in these patients provides valuable information in the decision making process about whether to rebiopsy solely for molecular analysis and going further it perhaps could be used to justify a trial of targeted treatment without rebiopsy, provided there is confidence in the specificity of IHC in the local setting.

CONCLUSION

When deployed in the routine clinical setting, mutation specific IHC for exon 19 mutation is extremely specific but poorly sensitive (100% specificity, 63% sensitivity) whereas mutation specific IHC for exon 21 mutation shows lower specificity but much higher sensitivity (92% specificity, 96% sensitivity). When performed together in the routine clinical setting, EGFR mutation specific IHC with both antibodies has an overall sensitivity of 58% for detecting any EGFR mutations but a very high specificity of 95%. If IHC is strongly positive and molecular testing is negative, false negative molecular testing is more likely than false positive IHC.

We conclude that due to its relatively low sensitivity (including its inability to detect exon 18 or exon 20 mutations) EGFR mutation specific IHC cannot replace molecular testing in clinical practice. However, due to its low cost, ready availability, and very high specificity, it can play a valuable role as an adjunct to testing in the routine clinical setting either as a reflex test (as performed in our laboratory) or as a test only performed in low cellularity specimens where molecular testing may be unreliable or not possible. Not only does it help to identify false negative molecular testing which our study suggests may occur in at least 5% of *EGFR* mutated cases, it can also be performed on many biopsy specimens containing insufficient material for molecular analysis or in cases which do not meet local funding requirements for molecular testing.

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