Immunohistochemistry to identify EGFR mutations or ALK rearrangements in patients with lung adenocarcinoma

P. Hofman1,2,3,4, M. Ilie1,2,3, V. Hofman1,2,3,4, S. Roux5, A. Valenti6, A. Bernheim5,6, M. Alifano7, F. Leroy-Ladurie8, F. Vaylet9, I. Rouquette10, P. Validire11, M. Beau-Faller12, L. Lacroix13, J. C. Soria14,15 & P. Fouret5,16*

1Laboratory of Clinical and Experimental Pathology, Pasteur Hospital, CHU Nice, Nice; 2ERI-21, INSERM, Nice; 3EA4319, Medical School, Nice Sophia University, Nice, France; 4Human Tissue Bio bank Unit/CRB, INSERM, Nice; 5U985 Tumour Genetic, INSERM, Villejuif; 6Molecular Pathology, Biology and Pathology Department, Institut Gustave Roussy, Villejuif; 7Thoracic Surgery Department, CHU Hôtel-Dieu, APHP, Paris; 8Thoracic Surgery Department, Marie-Lannelongue Surgical Centre, Le Plessis-Robinson; 9Pneumology Department, HIA Percy, Clamart; 10Pathology Department, Rangueil Hospital-CHU Toulouse, Toulouse; 11Pathology Department, Institut Mutualiste Montsouris, Paris; 12Biochemistry and Molecular Biology Department, CHU Strasbourg, Strasbourg; 13Translational Research Laboratory; 14Medicine Department, Gustave-Roussy Institute, Villejuif; 15Paris XI University, Villejuif; 16Pierre et Marie Curie University, Paris, France

Received 25 August 2011; revised 3 October 2011; accepted 5 October 2011

Background: Immunohistochemistry has been proposed as a specific and sensitive method to identify EGFR mutations or ALK rearrangements in lung tumours.

Patients and methods: We assessed EGFR and KRAS by direct sequencing in 154 patients with lung adenocarcinoma. ALK rearrangements were assayed by FISH and RT-PCR. Immunohistochemistry was carried out and evaluated closely following published methods using recommended monoclonal rabbit or mouse antibodies.

Results: Thirteen of 36 exon 19 EGFR-mutated tumours (36%)—including 12 of 22 with p.Glu746_Ala750del (55%)—were positive with the 6B6 antibody that was raised against p.Glu746_Ala750del. One hundred eleven of 114 EGFR exon 19 wild-type tumours (97%) were negative with 6B6. Four of 21 exon 21 EGFR-mutated tumours (19%)—including 4 of 17 with p.Leu858Arg (24%)—were positive with the 43B2 antibody that was raised against p.Leu858Arg. One hundred twenty-two of 124 (98%) EGFR exon 21 wild-type tumours were negative with 43B2. Two of four ALK rearrangement (85%) were positive with the 5A4 antibody.

Conclusions: Immunohistochemistry is a specific means for identification of EGFR mutations and ALK rearrangements. It suffers, however, from poor sensitivity.

Key words: ALK, EGFR, immunohistochemistry, lung cancer, mutation

introduction

One of the clinical distinctions of lung cancer in never smokers (NS) is the observed response to tyrosine kinase inhibitors (TKI) that target the epidermal growth factor receptor (EGFR) [1]. Compared with ever smokers (ES), NS treated with EGFR TKI have higher response rates to treatment [2, 3]. The response to EGFR TKI is linked to constitutional activation of EGFR signalling in tumour cells [4]. Lung cancer in NS is characterised by a high frequency of activating EGFR mutations that are exclusive of KRAS mutations [5, 6].

Rearrangements of the anaplastic lymphoma kinase (ALK) have been identified in 2%–7% of all non-small-cell lung carcinoma [7–11]. ALK rearrangements are more frequent in NS or light ES with lung adenocarcinomas that are wild type...
Annals of Oncology

original articles

Volume 23 | No. 7 | July 2012
doi:10.1093/annonc/mdr535 | 1739

for both EGFR and KRAS [12]. Oncogenic fusion genes consisting of ALK and echinoderm microtubule associated protein like 4 (EMLA) encode chimeric proteins with constitutive kinase activity, which confers sensitivity to ALK TKI [7]. Inhibition of ALK signalling benefits most patients with ALK rearrangements [11].

Based on these findings, it has been proposed to use EGFR and ALK genetic analyses to guide treatment decisions in patients with advanced-stage lung adenocarcinoma. In order to increase the yield of genetic assays, investigators have proposed to use immunohistochemical assays, which are conveniently carried out on the formalin-fixed paraffin-embedded biopsy samples that are examined for pathological diagnosis. The results of several such studies support that immunohistochemistry is a sensitive and specific method to identify EGFR mutations [13–16] or ALK rearrangements [10, 17, 18] in paraffin-embedded lung adenocarcinoma specimens.

In this paper, we report the results of immunohistochemical assays to identify mutated EGFR or ALK rearrangements in patients with lung adenocarcinoma belonging to a cohort that was designed primarily to study lung cancer in NS. The higher prevalence of EGFR mutations or ALK translocations in NS compared with ES was expected to facilitate the evaluation of immunohistochemistry as a mean to assess genetic status.

methods

patients

All patients had been treated by surgery for lung adenocarcinoma. No patient had received chemotherapy before surgery. Patients belonged to two cohorts based on their smoking status. NS had a lifetime exposure of < 100 cigarettes. ES were matched with NS by centre, sex and stage. Formalin-embedded tumour samples were available for 154 patients, including 80 NS and 74 ES. This study was part of the Lung Genes (LG) project, which was approved by the Institut National du Cancer Review Board (Programme National d’Excellence Spécialisé Poumon).

sequencing of EGFR and KRAS

Genomic DNA was extracted from frozen samples containing at least 50% tumour cells (Qiagen, Courtaboeuf, France). Direct sequencing was carried out after on amplified EGFR exons 18, 19, 20, 21 (NM_005228.3) and KRAS codons 12 and 13 of exon 2 (NM_033360.2), respectively. Purified DNA was sequenced using BigDye® Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Sequencing reactions were analysed on 16-capillary ABI3130 or on 48-capillary 3730 DNA Analyzer® in both sense and antisense directions from two independent amplifications.

Sequences reading and alignment were carried out with SeqScape® software (Applied Biosystems). Sequencing data had been published for 46 NS [19].

FISH study

FISH to detect ALK rearrangements was carried out on paraffin-embedded sections using Vysis LSI ALK dual colour split probe (Abbott Molecular, Des Plaines, IL). Deparaffinised slides were immersed in pretreatment solution (Dako, Glostrup, Denmark) for 10 min at 95°C. After cooling, the pepsin solution was applied for 6–8 min at room temperature and then stopped in wash buffer. The ALK probe was heated for 5 min at 84°C and applied overnight at 44°C.Slides were washed with a stringent buffer for 10 min at 65°C, air-dried and counterstained with DAPI.

One hundred tumour cells were analysed for each case. The number of fluorescent signals within the nuclear boundary of each interphase tumour cell was counted using an AxioPhot-ZEISS fluorescent microscope at ×1000 magnification. Only nuclei with unambiguous signals were scored. The normal pattern of the ALK probe was seen as two yellow (or red and green overlapping) signals. The rearrangement was identified by split signals in > 15% tumour cells: only one yellow signal was detected and well separated green and red signals were seen. Samples were also deemed FISH positive in case of isolated 3’ signals (red) representing partial deletion of the 5’ part of ALK (green) in > 15% tumour cells [11]. The chromosome 2/ALK polysomy was detected if > 2 yellow signals were visible in one nucleus.

reverse transcription–polymerase chain reaction

RNA was extracted (Qiagen) and qualified using Agilent Bioanalyzer (Agilent, Massy-Palaiseau, France). Reverse transcription and PCR amplification was carried out using the published primers and protocol [11].

mutant EGFR immunohistochemistry

Immunohistochemistry was carried out using two mutation-specific rabbit monoclonal antibodies that were raised against peptides matching the E746-750 exon 19 15-bp deletion mutant sequence (clone 6B6; Cell Signalling Technology, Denver, CO) or the exon 21 L858R mutant sequence (clone 43B2; Cell Signalling Technology) of human EGFR [13].

Preliminary assays showed no difference in staining intensity and proportion of labelled cells when the primary antibodies were incubated overnight or for 1 h, following the protocols described by Yu et al. [13] or Brevet et al. [16] and Simonetti et al. [15], respectively. The preliminary study included mismatching cases (e.g. immunohistochemistry negative with both incubation times and sequencing positive). We selected 1 h incubation time such that the procedure could be fully automated.

Deparaffinised slides were subjected to antigen retrieval by microwave boiling in 1 mol/l EDTA pH 9.0 (Dako) for 30 min. The staining procedure was carried out using an automate (Benchmark XT; Ventana Medical Systems, Roche Group Inc., Tucson, AZ). Intrinsic peroxidase activity was blocked by 3% hydrogen peroxide for 20 min. Goat serum (5%; Sigma, St Louis, MO) solution was used for blocking non-specific antibody binding. The primary antibodies were applied at the recommended 1 : 100 dilution. Slides were washed in PBS before incubation with labelled polymer-horseradish peroxidase anti-rabbit secondary antibody for 30 min at room temperature. For visualisation, theview DAB Detection kit was used according to the manufacturer’s instructions (Ventana Medical Systems, Faulquemont, France).

The intensity of staining as well as percentages of positive cells were assessed semi-quantitatively as previously described [13, 16]: 0 = no or faint staining in < 10% of tumour cells; 1 + = faint staining in > 10% of tumour cells; 2 + = moderate staining; 3 + = strong staining. Positive mutanspecific EGFR expression was considered as between 1 + and 3 + [13, 16]. Immunohistochemical staining in specimens was independently assessed by two pathologists (MI and PH) blinded to clinical and genotype data. When discrepancy between the two pathologists was noted, the slides were reviewed in order to obtain a consensus.

The level of discordance between the two pathologists was 3% (4/139) for the E746, A750 deletion antibody and 5% (7/137) for the L858R point mutation antibody.

ALK immunohistochemistry

Immunohistochemistry was carried out using the mouse monoclonal antibody 5A4, which was raised against a recombinant protein corresponding to a region which spans the tyrosine kinase catalytic domain.
and part of the C-terminus of NPM-ALK transcript (419–520 aa) (Abcam, Cambridge, UK).

An increased sensitivity was obtained using the intercalated antibody-enhanced polymer method as developed by Takeuchi et al. [17]. Briefly, slides were heated in Target Retrieval Solution (pH 9.0, Dako) for 40 min at 97°C. They were incubated at room temperature with Protein Block Serum-free Ready-to-Use solution (Dako) for 10 min. Then anti-ALK 5A4 antibody was applied for 30 min at a recommended dilution of 1:50. Slides were incubated at room temperature with EnVision FLEX + Mouse Linker (Dako) for 15 min. The immune complexes were then detected with the dextran polymer reagent.

The percentage of labelled tumour cells and intensity of staining was recorded for each specimen. Immunohistochemical staining in specimens was independently assessed by two pathologists (MI and PH) blinded to clinical and genotype data. No discordance was noted between the two pathologists.

statistical analysis
The frequencies of EGFR and KRAS mutations were compared using the two-tailed chi-square test.

results
clinicopathological characteristics
The 154 patients were predominantly women (88%). The median age was 65 years (interquartile range = 55–73 years). The stages according to UICC classification [20] were stage I for 87 patients, stage II for 19 patients and stage III for 48 patients. The adenocarcinomas comprised 119 mixed, 23 acinar, 5 papillary and 7 solid histological subtypes. NKX2-1 was expressed in 92% tumours.

EGFR mutations
EGFR status could be assessed in 151 tumours. Mutations were found in 62 cases (41%). They were more frequent (P = 0.0002) in NS (49 of 78, or 63%) than in ES (13 of 73, or 18%).

Mutations were found mainly in exon 19 (36 tumours, or 58%) and in exon 21 (21 tumours, or 34%). Mutations in exon 19 and mutations in exon 21 were exclusive. Five tumours harboured mutations in exons other than exon 19 or exon 21: two tumours in exon 18, two tumours in exon 20 and one tumour in both exon 18 and exon 20. All mutations were in frame.

The 36 mutations in exon 19 included 22 c.2235_2249 or c.2236_2250 deletions, p.Glu746_AlA750del mutation, 12 small indels (33%) and 2 small insertions. The 21 mutations in exon 21 included 17 c.2373 substitutions, p.Leu858Arg (81%), 2 c.2582 substitutions, p.Leu861Gln and 2 c.2582 substitutions, p.Leu861Glu.

KRAS mutations
KRAS status could be assessed in 151 tumours. Mutations were found in 36 cases (24%). They were more frequent (P < 0.0001) in ES (30 of 73, or 41%) than in NS (6 of 78, or 7.5%).

Mutations were found in codon 12 (33 tumours, or 92%) and in codon 13 (3 tumours, or 8%) of exon 2. KRAS mutations were exclusive of EGFR mutations.

ALK rearrangements
Tumours wild type for both EGFR and KRAS in NS were selected for assaying ALK rearrangements using FISH. Among 20 tumours that could be analysed, 4 (20%) displayed ALK rearrangements: 3 had split ALK 5’ and 3’ probe signals in > 50% cells and 1 had isolated 3’ signals in > 50% cells. Representative examples of these signals are shown in supplemental Figure S1 (available at Annals of Oncology online). Table 1 summarises the clinicopathological characteristics of the four patients with ALK rearrangement.

Ten tumours demonstrated chromosome 2 trisomy or polysomy, including one case with high-level polysomy (more than six copies).

EML4-ALK fusion transcripts
EML4-ALK fusion transcripts were found in 3 among 19 tumours wild type for both EGFR and KRAS that could be analysed by RT-PCR. Those three tumours had the ALK rearrangement as shown by the split ALK 5’ and 3’ probe signals. The fourth tumour with a rearrangement as shown by the 3’ isolated signal did not contain EML4-ALK fusion transcripts.

expression of EGFR proteins
Table 2 summarises the immunohistochemical data according to exon 19 or exon 21 EGFR status. Representative examples of immunohistochemical staining are shown in supplemental Figure S2 (available at Annals of Oncology online).

Using the described criteria [13, 16], 16 (9%) tumours were positive (1 + : 5 cases; 2 + : 7 cases; 3 + : 4 cases) with the 6B6 clone that was generated using a peptide containing the p.Glu746_AlA750del mutation, while 6 (4%) tumours were positive (1 + : 2 cases; 2 + : 3 cases; 3 + : 1 case) with the 43B2 clone that was generated using a peptide containing the p.Leu858Arg mutation.

Among 36 tumours harbouring exon 19 mutations, 13 (36%) were positive with 6B6, including 5 cases with 1 + positivity. Among 22 tumours harbouring the c.2235_2249 or c.2236_2250 deletion, p.Glu746_AlA750del mutation, 12 (54.5%) were positive with the 6B6 clone. Among 114 tumours

Table 1. Clinicopathological characteristics in four never smokers with ALK rearrangements

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Sex</th>
<th>Age (years)</th>
<th>TNM</th>
<th>Histological subtype</th>
<th>Differentiation</th>
<th>NNX2-1 expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>26</td>
<td>Female</td>
<td>77</td>
<td>IB</td>
<td>Mixed</td>
<td>Poor</td>
<td>Positive</td>
</tr>
<tr>
<td>66</td>
<td>Female</td>
<td>57</td>
<td>IIIa</td>
<td>Mixed</td>
<td>Poor</td>
<td>Positive</td>
</tr>
<tr>
<td>86</td>
<td>Female</td>
<td>47</td>
<td>IIIIB</td>
<td>Acinar</td>
<td>Intermediate</td>
<td>Positive</td>
</tr>
<tr>
<td>220</td>
<td>Female</td>
<td>46</td>
<td>IB</td>
<td>Solid</td>
<td>Poor</td>
<td>Positive</td>
</tr>
</tbody>
</table>
wild type for exon 19, 111 (97%) were negative with the 6B6 clone.
Among 21 tumours harbouring exon 21 mutations, 4 (19%) were positive with 43B2, including 1 case with 1 + positivity. Among 17 tumours harbouring c.2573 substitution, p. Leu858Arg mutation, 4 (23.5%) were positive with the 43B2 clone. Among 124 tumours wild type for exon 21, 122 (98%) were negative with the 43B2 clone.

Table 2. Immunohistochemical reactivity according to EGFR mutation in exon 19 and in exon 21

<table>
<thead>
<tr>
<th>Exon 19 Mutation</th>
<th>6B6 clone</th>
<th>43B2 clone</th>
</tr>
</thead>
<tbody>
<tr>
<td>p.Glu746_Ala750del</td>
<td>12 10 1</td>
<td>19</td>
</tr>
<tr>
<td>Indels</td>
<td>0 12 0 12</td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>13 23 1 33</td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>3 111 5 106</td>
<td></td>
</tr>
<tr>
<td>Exon 21 Mutation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p.Leu858Arg</td>
<td>0 17 4 13</td>
<td></td>
</tr>
<tr>
<td>p.Leu861Gln</td>
<td>0 2 0 2</td>
<td></td>
</tr>
<tr>
<td>p.Leu861Glu</td>
<td>0 2 0 2</td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>0 21 4 17</td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>16 113 2 122</td>
<td></td>
</tr>
</tbody>
</table>

*Was positive for both antibodies

One tumour harbouring the c.2236_2250del, p. Glu746_Ala750del mutation was positive with the 43B2 clone, whereas none of the tumours harbouring exon 21 mutations was positive with the 6B6 clone. None of the five tumours harbouring only mutations in exon 18 or exon 20 was positive with the antibodies.

expression of ALK protein

Table 3 summarises the results of FISH, RT-PCR and immunohistochemistry to identify ALK rearrangements in tumours wild type for both EGFR and KRAS.

Four samples displayed moderate staining in 70%–90% of tumour cells, while 16 other samples displayed no staining. The four samples displaying diffuse staining were deemed positive.

Among four tumours harbouring ALK rearrangement as determined by FISH, two were positive with the 5A4 clone. Among 13 tumours without ALK rearrangement that could be analysed with the 5A4 clone, 11 (85%) were negative.

Representative examples of immunohistochemical staining for ALK rearrangement are shown in supplemental Figure S3 (available at Annals of Oncology online).

discussion

We studied lung adenocarcinomas by means of immunohistochemistry with monoclonal antibodies that were reported to be specific and sensitive tools to identify EGFR or ALK mutations. The procedures were carried out by closely following published protocols including steps that were developed to increase sensitivity [13, 15–17]. The results were
analysed using previously reported criteria [13, 16]. The classification into positivity and negativity was straightforward as positive cases had a large proportion of reacting tumour cells, while no or minimal background staining could be seen in negative cases. In our hands, the specificity of immunohistochemistry could be considered good (from 85% to 98%), indicating that the antibodies were indeed able to specifically react in paraffin-embedded material with the peptides against which they were raised and that we used these antibodies with appropriate technical care. The sensitivity of immunohistochemistry, however, was clearly less than expected by previous reports [10, 13–18]: less than half of all the genetic alterations were identified by immunohistochemistry in our study. This was disappointing as genetic analyses of unselected patients with lung cancer yield few positive results.

The frequency of EGFR mutations was high (41% for the whole cohort and 63% for NS), consistent with the high prevalence of EGFR mutations that were recently reported in European [21] or Asian [22] NS with lung non-small-cell carcinoma. The frequency of ALK rearrangements (~20%) in NS with both wild-type EGFR and KRAS validates the selection of this subpopulation to increase the likelihood of finding ALK rearrangements [12]. Moreover, among the four patients with ALK rearrangements, the adenocarcinomas were of the solid subtype in one case and of the mixed subtype with low differentiation in two cases, which implies a substantial solid histological component. Our data are thus consistent with the histology seen in patients with ALK rearrangements [11].

In our study, immunohistochemistry was carried out in surgical samples that may be less homogeneously preserved by fixation procedure than biopsy samples. This does not seem to be a confounding factor as the measures of sensitivity as sensitivities previously reported in immunohistochemical studies using surgical samples were acceptable for EGFR exon 19 mutation (79%) or exon 21 mutation (83%) [14] and excellent for ALK translocation (100%) [10]—proportions not very different from those recorded using biopsy samples [13, 15, 16, 18]. Moreover, the whole section of each sample in our series was carefully screened by both pathologists. Overnight incubation with primary antibodies may increase sensitivity of immunohistochemistry on surgical samples. However, in our preliminary study, there was no difference of immunolabelling results in EGFR-mutant cases, including mismatching cases, between 1 h and overnight incubation. The cut-off for positivity is also important. Cases with faint positivity in > 10% cells were all truly positive, validating the use of this low cut-off. It cannot be ruled out that few samples were not optimally fixed, thus preventing detection of fragile epitopes. Although suboptimal fixation of few samples could not explain the low sensitivity in the whole cohort, it could have been a major problem had the results of immunohistochemistry been used in the context of personalised medicine.

We report a sensitivity of 36% and 19% for immunohistochemistry when considering all exon 19 or 21 EGFR mutations, respectively. When restricting the analysis to the common mutations translating into peptides against which antibodies were generated, the sensitivity increased to 54.5% with 6B6 (p.Glu746_Ala750del) and to 23.5% with 43B2 (p.Leu858Arg). With one exception, the antibodies almost completely failed to recognise the other less common EGFR mutations, which accounted for 31% of exon 19 mutations and 19% of exon 21 mutations. The failure to recognise the less common EGFR mutations is evidence of specificity, but it may have contributed to low sensitivity. The 54.5% sensitivity reported here using B6 to detect p.Glu746_Ala750del is at the lower range of the confidence interval around the 69% sensitivity reported by Simonetti et al. [15] for exon 19 mutations. These authors also reported that most of the uncommon EGFR mutations in exon 19 were negative with 6B6. This is an unavoidable limitation of immunohistochemistry using currently available antibodies, as those less common mutations are able to activate EGFR signalling.

Our results with the ALK antibody also suggest a lack of sensitivity for immunohistochemistry as two of four rearrangements were missed by immunohistochemistry. It is easily explained by the fact that ALK rearrangements in lung cancer are usually associated with low levels of ALK expression in contrast to anaplastic lymphomas with ALK rearrangements where the protein is robustly detected by immunohistochemistry [23]. We selected the 5A4 mouse monoclonal antibody that was reported to give the best results among five monoclonal antibodies that were compared in a comprehensive study [17]. One could argue that an increase in sensitivity can be achieved using a higher affinity antibody such as the rabbit monoclonal antibody used by Mino-Kenudson et al. [18], although it is not currently commercially available.

RT-PCR also failed to identify one ALK rearrangement, presumably because not every ALK rearrangement leads to EML4-ALK fusion genes [11].

In conclusion, our results support that immunohistochemistry is a relatively specific means for identification of EGFR mutations and ALK rearrangements. In our hands, however, it suffers from poor sensitivity. As patients in our study were all treated by surgery, it is clear that the results of the genetic analyses would not have guided therapeutic decisions. Nevertheless, the surgical samples were very convenient to evaluate the actual performance of the antibodies in routinely fixed and paraffin-embedded lung tumours. We strongly recommend that laboratories should test the sensitivity of immunohistochemistry in their local conditions to be aware of its limitation in their routine practise.

acknowledgement


funding

This work was supported by Institut National du Cancer (Programme National d’Excellence Spécialisé Poumon) and by Association pour la Recherche sur le Cancer (SF20101201740).

disclosure

The authors declare no conflict of interest.

references


