Frequency of actionable alterations in epidermal growth factor receptor (EGFR) wild type non-small cell lung cancer: experience of the Wide Catchment Area of Romagna (AVR)

Elisa Chiadini¹, Matteo Canale¹, Angelo Delmonte², Claudio Dazzi³, Claudia Casanova³, Laura Capelli¹, Marita Mariotti², Maximilian Papi⁴, Alessandro Gamboni⁵, Maurizio Puccetti⁶, Sara Bravaccini¹, Alessandra Dubini⁷, Daniele Calistrì¹, Lucio Crinò², Paola Ulivi¹

¹Biosciences Laboratory, ²Department of Medical Oncology, Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST) IRCCS, Meldola, Italy; ³Medical Oncology Unit, S. Maria delle Croci Hospital, Ravenna, Italy; ⁴Oncology and Oncohematology Unit, Infermi Hospital, Rimini, Italy; ⁵Medical Oncology Unit, Infermi Hospital, Faenza, Italy; ⁶Pathology Unit, S. Maria delle Croci Hospital, Ravenna, Italy; ⁷Pathology Unit, Morgagni-Pierantoni Hospital, Forlì, Italy

Contributions: (I) Conception and design: P Ulivi; (II) Administrative support: None; (III) Provision of study materials or patients: M Puccetti, A Dubini; (IV) Collection and assembly of data: A Delmonte, C Dazzi, C Casanova, M Mariotti, M Papi, A Gamboni; (V) Data analysis and interpretation: P Ulivi; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Correspondence to: Paola Ulivi. Biosciences Laboratory, Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST) IRCCS, Via P. Maroncelli 40, 47014 Meldola, Italy. Email: paola.ulivi@irst.emr.it.

Background: Molecular diagnostics for non-small cell lung cancer (NSCLC) has become the standard of care for personalized treatment. Epidermal growth factor receptor (EGFR) mutation and EML4-ALK translocation represent the two most important alterations in first-line treatment decision-making. However, other potentially targetable alterations are also present.

Methods: One thousand consecutive NSCLC patients with EGFR wild type (wt) tumors diagnosed by routine molecular analysis were considered. KRAS, BRAF, ERBB2, PIK3CA, NRAS, ALK, MAP2K1, RET and DDR2 gene mutations were analyzed using the multiparametric Sequenom MassARRAY® platform. EML4-ALK and ROS1 rearrangements were also assessed by fluorescent in situ hybridization. HER4 status was determined by direct sequencing.

Results: Three hundred and forty-eight (34.8%), 31 (3.1%), 39 (4.4%), 14 (1.8%), 6 (0.7%), 16 (1.8%), 5 (0.6%) and 9 (0.9%) patients showed an alteration in KRAS, BRAF, ALK, ROS1, NRAS, PIK3CA, MAPK1/2 and HER2 genes, respectively. Of the 657 patients for whom all markers were determined, 318 (48%) patients had at least one alteration. Eight patients showed overlapping mutations, 4 KRAS mutation/EML4-ALK translocation, one KRAS mutation/ROS1 rearrangement, 2 KRAS/PIK3CA mutations, and one BRAF/PIK3CA mutations.

Conclusions: About 50% of our patients had a potentially targetable alteration, confirming the usefulness of a multiparametric approach for routine molecular diagnostics aimed at identifying potential therapeutic targets.

Keywords: Non-small cell lung cancer (NSCLC); targeted therapy; epidermal growth factor receptor (EGFR); multitarget analysis; Formalin-fixed paraffin-embedded samples (FFPE samples)
Introduction

Targeted therapy for non-small cell lung cancer (NSCLC) has transformed the outcome of patients carrying specific molecular alterations. In particular, epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs), such as gefitinib, erlotinib or afatinib, and anti-ALK agents, such as crizotinib, have changed the natural history of adenocarcinoma patients carrying specific EGFR mutations or EML4-ALK translocation/ROS1 rearrangements, respectively (1-5). Other potentially targetable alterations have been identified in lung cancer. Of these, BRAF and HER2 mutations are present in about 3% and 2% of patients with lung adenocarcinoma, respectively (6-8) and represent possible targets for therapy using anti BRAF ( vemurafenib or dabrafenib) or anti-HER2 (trastuzumab, dacomitinib, etc.) agents (8-11). Moreover, mesenchymal-epithelial transition factor (MET) alterations (mutation or amplification) would seem to identify a subset of patients who are more likely to respond to crizotinib (12,13). In addition, other potentially targetable alterations have been found in several genes, including NTRK1, PIK3CA, HER4, NRAS (6,14-18), and the frequency of these alterations differs in different ethnicities. The number of clinical trials aimed at analyzing the effect of targeted drugs specific for these different alterations is thus expected to increase enormously in the near future.

Methods

Patients

We evaluated a cohort of 1,000 patients, all recruited from the Wide Catchment Area of Romagna (AVR), with histologically or cytologically confirmed advanced NSCLC classified as EGFR wt by routine diagnostic molecular analysis from January 2013 to December 2016. The clinical pathological characteristics of patients are reported in Table 1. The study was approved by our institutional Review Board and all patients gave written informed consent.

Biological samples

Formalin-fixed paraffin-embedded (FFPE) histological samples, cytological FFPEs (cell blocks) or cytological smears were available for molecular analysis. Biological samples were evaluated and selected by AVR pathologists. Tumor specimens comprising at least 50% tumor cells were selected and underwent DNA extraction.

EML4-ALK and ROS1 determinations

Selected FFPE histological or cytological sections and cytological samples were used to perform EML4-ALK and ROS1 determinations. FISH assay was performed using a break-apart ALK or ROS1 probe (Vysis LSI Dual Color, Break Apart Rearrangement Probe; Abbott/Vysis, Illinois, IL, USA. ALK and ROS1 rearrangements were scored as positive when ≥15% of tumor cells displayed split signals or isolated signals containing a kinase domain (red for ALK and green for ROS1), as previously described (19,20). Slides containing at least 50 tumor cells were considered evaluable and were read independently by two experts blinded to the patient’s history and histological findings.

Mutation analysis

Mutation analyses were centralized and performed in the Biosciences Laboratory of IRST IRCCS. KRAS, BRAF, ERBB2, PIK3CA, NRAS, ALK, MAP2K1, RET and DDR2 gene status was analyzed by Myriapod®Lung Status kit (Diatech Pharmacogenetics, Jesi, Italy) on MassARRAY® (SEQUENOM® Inc., San Diego, CA, USA). Exons 18 to 23 of the HER4 gene were evaluated by direct sequencing.

Statistical analysis

The chi-square test was used for group comparison of variables.

Results

Frequency of gene alterations

KRAS, BRAF and HER2 determinations were performed in the entire case series. Conversely, there was only sufficient biological to perform NRAS, PIK3CA, MAP2K1, ALK, RET and DDR2 mutation analysis in 901 patients, EML4-ALK evaluation in 889 patients and ROS1 determinations in 733 patients. Overall, characterization of all 11 markers was performed in 657 patients. HER4 mutation analysis was also carried out in 450 cases. Three hundred and forty-eight
Table 1 | Relation between the Different Alterations and the Clinical Pathological Characteristics of Patients

<table>
<thead>
<tr>
<th>Gene alteration</th>
<th>Total No. gene mutations</th>
<th>Gender (%)</th>
<th>Age, years (%)</th>
<th>Smoking habits* No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Female</td>
<td>Male</td>
<td>P</td>
</tr>
<tr>
<td>KRAS</td>
<td>348</td>
<td>132 (37.9)</td>
<td>216 (62.1)</td>
<td>0.950</td>
</tr>
<tr>
<td>BRAF</td>
<td>31</td>
<td>13 (41.9)</td>
<td>18 (58.1)</td>
<td>0.633</td>
</tr>
<tr>
<td>NRAS</td>
<td>6</td>
<td>4 (67.0)</td>
<td>2 (33.0)</td>
<td>0.204</td>
</tr>
<tr>
<td>PIK3CA</td>
<td>16</td>
<td>6 (37.5)</td>
<td>10 (62.5)</td>
<td>0.000</td>
</tr>
<tr>
<td>MAPK1/2</td>
<td>5</td>
<td>–</td>
<td>5 (100.0)</td>
<td>0.164</td>
</tr>
<tr>
<td>HER2</td>
<td>9</td>
<td>6 (66.7)</td>
<td>3 (33.3)</td>
<td>0.087</td>
</tr>
<tr>
<td>EML4-ALK</td>
<td>39</td>
<td>23 (59.0)</td>
<td>16 (41.0)</td>
<td>0.005</td>
</tr>
<tr>
<td>ROS1</td>
<td>14</td>
<td>9 (64.3)</td>
<td>5 (35.7)</td>
<td>0.053</td>
</tr>
</tbody>
</table>

*, percentages refer to the total number of patients with smoking habits information available.

Figure 1 | Frequency of gene alterations in the entire case series of EGFR wt patients.

(34.8%), 31 (3.1%), 39 (4.4%), 14 (1.8%), 6 (0.7%), 16 (1.8%), 5 (0.6%) and 9 (0.9%) patients showed an alteration in KRAS, BRAF, ALK, ROS1, NRAS, PIK3CA, MAPK1/2 and HER2 genes, respectively (Figure 1). Of the 657 patients in whom all the markers were determined, 318 (48%) showed at least one alteration. The different mutations found for each gene are shown in Figure 2. Eighty-four percent of KRAS mutations were found at codon 12, the majority (39%) being G12C alterations, while 10.3% of mutations involved codon 13. Around half of all BRAF mutations (54.8%) were V600E, whereas 45.2% were other exon 15 alterations or exon 11 mutations. In particular, 2 (14%) of the mutated patients with no V600E alteration harbored a different exon 15 mutation (D594G), while 12 (86%) showed an exon 11 alteration, 5 involving codon 466 (2 G466A, 2 G466E, one G466V) and 7 codon 469 (3 G469A, 1 G469E, 3 G469V). All NRAS mutations were at codon 61 (3 Q61K, 2 Q61L and one Q61R), whereas PIK3CA alterations were found in exon 9 (93.8%) in all but one patient (exon 20). Of the 5 patients carrying a MAPK1/2 mutation, 2 (40%) had a Q56P alteration whereas 3 (60%) showed a K57N substitution (Figure 2). Finally, all HER2-mutated patients had an exon 20 insertion. The only mutation found in HER4 gene was found in a former male smoker and located in exon 19 (G735V). No alterations were found in ALK, RET or DDR2 genes.

Eight patients showed overlapping mutations: concomitant KRAS mutation and EML4-ALK translocation (4 cases); KRAS mutation together with ROS1 rearrangement (1 case); concomitant KRAS and PIK3CA mutation (2 cases); and concomitant BRAF and PIK3CA mutation (1 case).

Gene alterations in relation to clinical pathological characteristics of patients

The relation between the different alterations and the clinical pathological characteristics of patients is reported in Table 2. EML4-ALK translocation was significantly correlated with gender, age and smoking habits (P=0.005, P=0.015 and P<0.001, respectively) and was more frequent in young, non-smoking females. ROS1 rearrangements were significantly correlated with gender and smoking habits (P=0.053 and P=0.002, respectively) but not with age. Moreover, KRAS mutations were significantly more common in current smokers (P<0.001), whereas NRAS mutations were only found in patients <70 years of age (P=0.032).

Of the 4 patients showing concomitant EML4-ALK
translocations and KRAS mutations (3 of whom were smokers), 2 were treated with first-line crizotinib and second-line ceritinib. One patient harboring a G12D KRAS mutation and with 70% fluorescent in situ hybridization (FISH) positivity initially showed stable disease with crizotinib but progressed after 5 cycles, and then again obtained stable disease with ceritinib, relapsing after 4 treatment cycles. Another patient with a G13S KRAS mutation and 50% FISH positivity obtained a partial response with crizotinib lasting 6 treatment cycles and another partial response with ceritinib lasting 3 cycles.

The only patient showing a concomitant KRAS mutation (G12V) and ROSI rearrangement was treated with second-line crizotinib but developed severe toxicity that led to treatment suspension before the clinical response could be evaluated.

**Discussion**

In the present study we report our results on a cohort of 1,000 consecutive NSCLC patients identified as EGFR wt by routine diagnostic molecular analysis performed at our...
Chiadini et al. Frequency of actionable alterations in EGFR wt NSCLC

Table 2 Clinical pathological characteristics of analyzed samples

<table>
<thead>
<tr>
<th>Variables</th>
<th>No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall</td>
<td>1,000</td>
</tr>
<tr>
<td>Age, years</td>
<td></td>
</tr>
<tr>
<td>&gt;70</td>
<td>431 (43.0)</td>
</tr>
<tr>
<td>≤70</td>
<td>569 (57.0)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>622 (62.0)</td>
</tr>
<tr>
<td>Female</td>
<td>378 (38.0)</td>
</tr>
<tr>
<td>Smoking habits</td>
<td></td>
</tr>
<tr>
<td>Current</td>
<td>379 (37.9)</td>
</tr>
<tr>
<td>Former</td>
<td>239 (23.9)</td>
</tr>
<tr>
<td>Never</td>
<td>145 (14.5)</td>
</tr>
<tr>
<td>Missing</td>
<td>237 (23.7)</td>
</tr>
<tr>
<td>Histotype</td>
<td></td>
</tr>
<tr>
<td>ADC</td>
<td>793 (79.3)</td>
</tr>
<tr>
<td>PDC</td>
<td>178 (17.8)</td>
</tr>
<tr>
<td>Other</td>
<td>29 (2.9)</td>
</tr>
<tr>
<td>Type of sample</td>
<td></td>
</tr>
<tr>
<td>Histological</td>
<td>639 (64.0)</td>
</tr>
<tr>
<td>Cytological</td>
<td>361 (36.0)</td>
</tr>
</tbody>
</table>

ADC, adenocarcinoma; PDC, poorly differentiated carcinoma.

...institute (IRST IRCCS). We demonstrated that about half of all the EGFR wt patients carried a potentially targetable gene alteration. The frequencies of alterations were as follows: KRAS, 35%; EML4-ALK, 4.4%; BRAF, 3.1%; ROS1, 0.7%; PIK3CA, 1.8%; MAPK1/2, 0.6%; and HER2, 0.9%. Such findings are in agreement with literature data (6,15,16,21). A slightly higher frequency of KRAS mutations was observed, possibly attributable to the fact that we considered a selected case series of EGFR wt patients in whom KRAS mutations were more frequent due to the mutual exclusivity of the 2 gene mutations. In accordance with previous authors (22), we observed a higher incidence of KRAS mutation and a high prevalence of G12C alterations in current smokers.

With regard to BRAF mutation, we saw that almost half of the mutated patients carried non-V600E alterations that were predominantly located in exon 11 at codons 466 and 469. It is known that V600 alterations predict sensitivity to anti-BRAF and anti-MEK combinations (23). Moreover, recent evidence suggests that non-V600 alterations may also be associated with sensitivity to such treatments (24). These results suggest that about 3% of EGFR wt patients could benefit from this type of targeted treatment. In agreement with other studies (7), no associations were observed between BRAF mutations and clinical pathological characteristics of patients. Around 6% of our patients harbored an EML4-ALK (4.4%) or ROS1 (1.8%) rearrangement, the majority of whom were predominantly young females who had never smoked, as described in other studies (19,25).

Other alterations that are potential targets for treatment are present in lung adenocarcinoma, e.g., 1.8% of our patients carried a PIK3CA mutation. Although there are still no drugs capable of inhibiting the growth of PIK3CA mutated cells, such mutations would seem to confer resistance to TKIs (26,27), making their determination of clinical importance.

We observed a slightly lower percentage of HER2 mutations with respect to that described in the literature (28) but similar (1.7%) to the findings of Mazières et al. (8) in a large case series. However, the relatively low sensitivity of the Sanger sequencing we used for the detection of HER2 mutations may partly explain our results. No significant associations were found between HER2 mutation and gender, age or smoking habits.

We also observed 8 patients with overlapping mutations, 4 of whom showed concomitant EML4-ALK and KRAS alterations. Of these, 2 underwent treatment with anti-ALK agents, one obtaining a partial response. In agreement with other authors, we have already seen that the presence of a KRAS mutation in patients with EML4-ALK translocation can confer resistance to treatment with crizotinib (29,30). Although the low number of double-mutated patients in our study does not permit us to draw any definitive conclusions about this, there were seem to be sufficient evidence to warrant KRAS status being taken into consideration in EML4-ALK translocated patients treated with anti-ALK agents.

In conclusion, although the frequency of single gene alterations in our study was low, about half of the patients with EGFR wt lung adenocarcinoma analyzed showed a potentially targetable alteration. Anti-ALK agents have already been approved for use as first-line treatment of EML4-ALK-translocated tumors. However, larger, randomized clinical trials are needed to verify the usefulness of targeted agents in tumors harboring other specific alterations.
Acknowledgements

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The study was approved by our institutional Review Board (No. 675 of 3.09.2013) and all patients gave written informed consent.

References


