Pathology in non-small cell lung cancer: evolving scenario

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Contributions: (I) Conception and design: P Pisapia, G Troncone, U Malapelle; (II) Administrative support: None; (III) Provision of study materials or patients: All authors; (IV) Collection and assembly of data: All authors; (V) Data analysis and interpretation: None; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

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Abstract: In 70–80% of advanced non small cell lung cancer (NSCLC) patients the only available material for both morphological diagnosis and molecular biomarkers assessment for the better treatment choice is represented by small tissue samples (small biopsies or cytological specimens). In order to avoid inadequate results due to the low amount of nucleic acids input and the difficulty to obtain tissue re-biopsies, the so called “liquid biopsy” could play a relevant role in this setting of patients. Tumoral DNA can be extracted from different body fluids [urine, saliva/sputum, cerebrospinal fluid (CSF), pleural effusion (PE)] of the patient or from supernatant sample of cytological preparations. In this review we analyzed the different the different “liquid biopsies” approaches.

Keywords: Non small cell lung cancer (NSCLC); liquid biopsy; next generation sequencing (NGS); epidermal growth factor receptor (EGFR); tyrosine kinase inhibitor (TKI)

Received: 13 May 2019; Accepted: 20 May 2019; published: 05 June 2019.
doi: 10.21037/pcm.2019.05.04

View this article at: http://dx.doi.org/10.21037/pcm.2019.05.04

Introduction

Lung cancer represents the most leading cause of mortality for cancer worldwide (1). To date, for non small cell lung cancer (NSCLC) patients there is a significant improvement in the target therapeutic strategies (2-8). For this reason, the College of American Pathologists (CAP), the International Association for the Study of Lung Cancer (IASLC), and the Association for Molecular Pathology (AMP), the National Comprehensive Cancer Network (NCCN) and the American Society of Clinical Oncology (ASCO) guidelines, defined a number of genes to necessarily test in advanced NSCLC patients, including epidermal growth factor receptor (EGFR), anaplastic lymphoma kinase (ALK), ROS proto-oncogene 1 receptor tyrosine kinase (ROSI) and V-Raf murine sarcoma viral oncogene homolog B (BRAF) for treatment with tyrosine kinase inhibitors (TKIs) (9-11). In this evolving scenario, molecular predictive pathology plays a key role in the management of NSCLC patients (12). An important limitation in advanced NSCLC patients is represented by the low quantity of tissue specimens available for both morphological and molecular purposes (13). In particular, in a high percentage of these patients, the only material available to fulfill morphological and molecular requests is represented by small tissue biopsy and cytological samples, and in 30% of cases no tissue specimens are available (14-17). To overcome this limitation, a valid approach is the possibility to adopt liquid sources to assess the molecular status of these patients, the so called “liquid biopsies” (18-20). Among the different analytes that can be obtained from blood samples and the possibility to assess the molecular status of the different relevant biomarkers on other liquid specimens, to date the only approved analyte for TKI administration in NSCLC patients is represented by circulating tumor DNA (ctDNA) extracted from plasma to assess EGFR status (13,18). In particular, the analysis of ctDNA extracted from plasma is recommended in patients without tissue availability (insufficient tumor tissue or impossibility to obtain a tissue specimen) and to overcome problems relative to discomfort and risks of...
biopsies (13,18,21-26). An important limitation in this approach is represented by the low amount of ctDNA in the bloodstream (<0.5% of the total cell free DNA), that can be related to false negative results (13,14). Noteworthy, we demonstrated a high level of concordance between ctDNA and tissue results by using a next generation sequencing (NGS) approach with a small gene panel [SiRe®, Genedin (a spin-off of the Department of Public Health, University of Naples “Federico II”), Rome, Italy] that covers 568 clinical relevant mutations in 6 genes (EGFR, KRAS, NRAS, BRAF, PDGFRA and c-kit) (27-29). However, blood samples feature a number of analytes (e.g. circulating tumor cells, exosomes, platelet RNA and circulating tumor RNA), that showed an important role in NSCLC treatment decision making (30-33). In addition to blood samples, other fluids, that can be considered “liquid biopsies”, have the potential to give to molecular pathologists and clinicians the relevant information on the genetic assessment in NSCLC patients in order to administrate TKIs.

In this review, we focalized the attention on the advanced in the liquid biopsies approach, in particular paid attention on these other liquid sources different from blood samples.

Urine

The glomerular filtration represents a natural “centrifugation process” of plasma and allows, due to the low size of DNA fragments, the presence of tumor DNA in urine (20,34,35). A major advantage of urine samples is represented by the non-invasiveness in the collection than in blood samples (20). A major disadvantage is represented by the higher activity of both DNA and RNA hydrolyzing enzymes that contributed to degrade both analytes (36,37). Reckamp et al. firstly adopted in the TIGER-X trial, a phase 1/2 clinical study of a third generation TKI (rociletinib) in previously treated patients with a EGFR mutation in advanced NSCLC patients, urine sample to identify EGFR mutations (38). By using mutation enrichment PCR coupled with NGS detection, on n=60 urine samples with matched tissue specimens, the Authors showed a sensitivity of the EGFR exon 20 p.T790M, exon 21 p.L858R and exon 19 deletions of 72%, 75% and 67% when considering all urine volumes, and of 93%, 80% and 83%, respectively when a recommended urine volume of 90–100 mL was collected; the specificity was of 96%, 100% and 94% respectively (38). Of interest, the Authors emphasized the complementary role of urine and plasma with tissue. In particular, the authors identified in n=12 cases the EGFR exon 20 p.T790M (n=10 in urine and plasma samples, n=1 only in plasma and n=1 only in urine) previously undetectable or with inadequate results in tissue samples. Among these, n=9 showed a significant decrease of urine concentration of EGFR exon 20 p.T790M after 21 days from rociletinib administration (38). Li et al. analyzed n=160 urine, plasma and tissue samples from NSCLC patients, by digital droplet PCR (ddPCR), at various stages (39). On the overall, the Authors reported a high overall agreement between urine and tissue in particular in advanced stages (42% stages I/II vs. 93% stages III/IV) and between urine and plasma (75% stages I/II vs. 100% stages III/IV) (39). Hu et al. compared, by using ddPCR, urine samples with tissue specimens from n=213 NSCLC patients who underwent surgery (stages I–III) and with a known mutation for EGFR (n=111 EGFR exon 21 p.L858R and n=102 EGFR exon 19 deletions) (40). On the overall, only n=130 showed an EGFR positive result in urine samples (61%; 65.8% for EGFR exon 21 p.L858R and 55.9% for EGFR exon 19 deletions) (40). Interestingly, Authors demonstrated a reduction in urine of EGFR mutations in concordant cases after treatment (40). Zhang et al. performed ddPCR on n=160 urine samples, with matched tissue specimens, in advanced NSCLC patients (41). The authors showed an agreement on EGFR exon 21 p.L858R, EGFR exon 19 deletions and EGFR exon 20 p.T790M of 79%, 81% and 100% respectively (41). Considering all the EGFR mutations the positive predictive value (PPV) and negative predictive value (NPV) were 100% and 53.6% respectively (41). Wu et al. analyzed n=50 urine samples, in advanced (IIIB/IV) stage of disease with a NGS approach (42). The authors showed a concordance rate between tissue and urine of 70% (42). Wang et al. tested by a ddPBR approach n=200 urine and tissue samples from advanced stages NSCLC patients (43). The authors focused their attention on KRAS mutations, for prognostic purpose (43). On the overall, 78% (109/140) of cases showed KRAS mutation on both samples; no false positive results emerged in urine samples (n=60 negative cases for both samples) (43). The authors, also, emphasized that the identification and concentration of KRAS mutant DNA in urine is predictor of worse outcome (43). Similarly, Xie et al. analyzed n=150 (n=100 KRAS mutated cases and n=50 KRAS wild type cases) cases to investigate the potential role of urine analysis for prognostic purposes (44). Taking tissue results as gold standard, the overall agreement for the KRAS positive group was 77%; instead in the wild type group the concordance rate was 92% (44). The authors addressed an increasing number of KRAS positive cases in urine (before
tissue positive and urine negative) when serial urine analysis was performed (93%) (44). In Table 1 are summarized results on urine samples.

Saliva

Saliva contains a wide range of different proteins, nucleic acids, electrolytes, and hormones that derived from different organs (45). For this reason, saliva can represent a source for biomarkers assessment as shown by Streckfus et al., who detected in this specimen c-erbB-2 in breast cancer patients (46). In the experience by Wu et al., in addition to urine samples the Authors analyzed n=50 saliva samples (42). Interestingly, the authors showed a concordance rate between tissue and saliva of 74% (42). Pu et al. analyzed n=17 saliva samples, collected from NSCLC patients before and after surgery, by using an electric field–induced release and measurement (EFIRM) technology (47). As gold standard, the Authors adopted the Food and Drug Administration (FDA) approved Cobas assay on matched tissue samples (47). Of interest, all cases harbored an EGFR exon 19 deletion or EGFR exon 21 p.L858R and all wild-type cases were confirmed in saliva samples (47). Only n=1 EGFR exon 18 p.G719X was not found in saliva by the EFIRM assay, due to the absence of the specific probes (47). The Authors evidenced only n=1 false positive result (EGFR exon 21 p.L858R) on plasma sample (47). Hubers et al. performed on n=10 EGFR tissue mutated cases and n=20 without EGFR mutation (n=10 lung cancer patients EGFR wild-type cases and n=10 chronic obstructive pulmonary disease (COPD) patients), at least four different tests [Cycleave PCR, co-amplification at lower denaturation temperature-PCR (COLD-PCR), Pangaea Biotech SL Technology (PST), and high resolution melting (HRM)] (48). No false positive results were evaluated in saliva samples; moreover a sensitivity of 50% was the higher evidenced (48). In Table 2 are summarized results on saliva samples.

Cerebrospinal fluid (CSF)

The incidence of leptomeningeal metastases (LMs) in NSCLC patients is about 3% to 5% with an increasing in EGFR-mutated patients (49,50). Although lumbar puncture is an invasive procedure, CSF is a useful tool to obtain cell free DNA (cfDNA) in NSCLC patients with brain metastasis (20,51). Li et al. performed a NGS approach on n=26 CSF samples in EGFR mutated NSCLC patients with LM (52). In all cases the mutation was correctly evidenced in cfDNA extracted from CSF specimens (52). Additionally, mutations were found in matched precipitate of CFS and plasma samples (52). Interestingly, the detection rate of EGFR exon 20 p.T790M in CSF cfDNA was higher (7/23, 30.4%) than in plasma (5/23, 21.7%) (52). In another experience by the

<table>
<thead>
<tr>
<th>First author</th>
<th>N of urine samples (type of matched samples)</th>
<th>Technology</th>
<th>Concordance rate</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reckamp et al.</td>
<td>60 (tissue)</td>
<td>NGS</td>
<td>p.L858R (97.0%)<em>, del 19 (89.0%)</em>, overall (93.0%)*</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Li et al. (39)</td>
<td>160 (tissue)</td>
<td>ddPCR</td>
<td>p.L858R (97.0%)<em>, del 19 (89.0%)</em>, overall (93.0%)*</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Hu et al. (40)</td>
<td>213 (tissue)</td>
<td>ddPCR</td>
<td>p.L858R (65.8%), del 19 (55.9%), overall (61.0%)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Zhang et al. (41)</td>
<td>160 (tissue)</td>
<td>ddPCR</td>
<td>p.L858R (79.0%), del 19 (81.0%), p.T790M (100.0%)</td>
<td>80.0%</td>
<td>100.0%</td>
</tr>
<tr>
<td>Wu et al. (42)</td>
<td>50 (tissue)</td>
<td>NGS</td>
<td>70.0%</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Wang et al. (43)</td>
<td>200 (tissue)</td>
<td>ddPCR</td>
<td>–</td>
<td>70.0%</td>
<td>100.0%</td>
</tr>
<tr>
<td>Xie et al. (44)</td>
<td>150 (tissue)</td>
<td>ddPCR</td>
<td>–</td>
<td>77.0%, 93.0%**</td>
<td>92.0%</td>
</tr>
</tbody>
</table>

*, advanced stages (III/IV); **, evaluation after serial urine analysis. ddPCR, digital droplet PCR; del, deletions; N, number; NGS, next generation sequencing.
### Table 2: Studies focusing on the adoption of saliva samples

<table>
<thead>
<tr>
<th>First author</th>
<th>N of saliva samples (type of matched samples)</th>
<th>Technology</th>
<th>Concordance rate</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wu et al. (42)</td>
<td>50 (tissue)</td>
<td>NGS</td>
<td>74.0%</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Pu et al. (46)</td>
<td>17 (tissue)</td>
<td>EFIRM</td>
<td>100.0%*</td>
<td>100.0%*</td>
<td>100.0%*</td>
</tr>
<tr>
<td>Hubers et al. (47)</td>
<td>10 (tissue)</td>
<td>Cycleave PCR</td>
<td>–</td>
<td>30.0%</td>
<td>100.0%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>COLD-PCR</td>
<td>–</td>
<td>40.0%</td>
<td>100.0%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PST</td>
<td>–</td>
<td>50.0%</td>
<td>100.0%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HRM</td>
<td>–</td>
<td>30.0%</td>
<td>100.0%</td>
</tr>
</tbody>
</table>

*, considering only EGFR exon 19 deletion and EGFR exon 21 p.L858R. COLD-PCR, co-amplification at lower denaturation temperature-PCR; del, deletion; EFIRM, electric field-induced release and measurement; EGFR, epidermal growth factor receptor; HRM, high resolution melting; N, number; PST, PangaeaBiotech SL Technology.

### Table 3: Studies focusing on the adoption of CSF samples

<table>
<thead>
<tr>
<th>First author</th>
<th>N of CSF samples (type of matched samples)</th>
<th>Technology</th>
<th>Concordance rate</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Li et al. (52)</td>
<td>26 (tissue)</td>
<td>NGS</td>
<td>100.0%</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Zheng et al. (53)</td>
<td>11 (tissue)</td>
<td>NGS</td>
<td>81.8%</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Ying et al. (54)</td>
<td>72 (plasma)</td>
<td>NGS</td>
<td>47.7%*, 32.7%**</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Kawahara et al. (56)</td>
<td>12 (tissue)</td>
<td>RT-PCR</td>
<td>–</td>
<td>87.5%</td>
<td>100%</td>
</tr>
<tr>
<td>Yang et al. (57)</td>
<td>30 (tissue)</td>
<td>ARMS-PCR</td>
<td>75.0%</td>
<td>67.0%</td>
<td>82.0%</td>
</tr>
<tr>
<td>Rong et al. (58)</td>
<td>5 (tissue)</td>
<td>ARMS-PCR</td>
<td>60.0%</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

*, considering EGFR activating mutations; **, considering all mutations. ARMS-PCR, amplification refractory mutation system-PCR; CSF, cerebrospinal fluid; N, number; NGS, next generation sequencing; RT-PCR, real time PCR.

same group, Zheng et al. analyzed n=11 CSF and plasma samples in ALK rearrangement positive patients (53). On the overall, the concordance rate was higher (9/11, 81.8%) in CSF samples than in plasma (5/11, 45.5%) (53). Ying et al. performed a capture-based targeted sequencing on n=72 matched CSF and plasma samples (54). The authors showed a higher mutation detection rate in CSF than plasma when considering either any mutations (81.5% vs. 62.5%) or EGFR mutations (58.3% vs. 44.4%) (54). In a detailed analysis on EGFR mutated cases, the Authors evidenced that EGFR activating mutations were identified in 51.4% of CSF samples and in 38.9% of plasma samples (54). Zhao et al. focused the attention on n=7 NSCLC patients who failed EGFR TKI treatment (gefitinib) (55). In particular, different from matched plasma samples, the authors showed the persistence of EGFR mutated clones in all CSF samples respect to plasma (2/7, 28.6%) due to the low penetration of gefitinib of blood-brain barrier (55). Kawahara et al. evidenced a high sensitivity, specificity and accuracy (87.5%, 100%, 91.7% respectively) for the detection of EGFR mutations, including EGFR exon 20 p.T790M, by using cobas® EGFR Mutation Test v2 (56). Yang et al. performed amplification refractory mutation system (ARMS)-PCR assay on n=30 lung adenocarcinomas with brain metastasis (57). The authors showed a PPV, NPV, sensitivity and specificity between tissue and CSF samples of 75%, 75%, 67% and 82% respectively; the concordance rate in EGFR mutated cases was 75% (57). Similarly, Rong et al. adopted ARMS-PCR on n=5 CSF of NSCLC patients with EGFR sensitizing mutations (58). In only n=3 (60%) cases the mutation was confirmed (58). In Table 3 are summarized results on CSF samples.

### Pleural effusion (PE)

Malignant pleural effusions (MPEs) are often observed in advanced NSCLC patients (59). Although thoracentesis is an invasive procedure, it is necessary for diagnostic, therapeutic and molecular purposes (60-62). Kimura et al. firstly reported the possibility to detect EGFR sensitizing mutation by analyzing DNA extracted from PE, and the
correlation with response (partial response in this paper) with TKI treatment (63). Liu et al. performed the EGFR analysis on n=41 matching metastatic pleural tumor tissues (MPTTs), MPE supernatant and MPE cell blocks (CBs) by using ARMS-PCR (64). On the overall, confronting the MPTT and MPE supernatant results, the EGFR mutation detection sensitivity and specificity in MPE supernatants was 84.2% and 90.9%, respectively (64). A higher sensitivity (94.7%) but lower specificity (81.8%) was observed when considering both MPE samples (64).

Lin et al. analyzed n=22 matched MPE supernatant, MPE cell pellet and tissue biopsy samples by using HRM (65). Considering tissue biopsy results as gold standard, no false positive results were evaluated among MPE supernatant samples (specificity 100%), whereas only n=1 false negative results was evidenced (sensitivity 92.3%) (65). A higher number of false negative results (4/22, 18.2%) was evidenced in MPE cell pellet samples (65). Similarly, Kawahara et al. analyzed n=74 (n=29 EGFR wild type and n=45 EGFR mutated cases) matched MPE supernatant, MPE cell pellet and tissue biopsy samples by using TaqMan Mutation Detection assay or fluorescence resonance energy transfer-based preferential homoduplex formation assay (F-PHFA) (66). The Authors showed a sensitivity and specificity on MPE supernatant of 44.4% and 100%, respectively (66).

Lee et al. performed the EGFR analysis by using a peptide nucleic acid (PNA) mediated PCR clamping on two groups of lung adenocarcinoma (ADC) patients (67). In particular, the Authors analyzed cfDNA extracted from PE in previously genotyped TKI-naïve patients (n=32; n=19 EGFR mutated patients and n=13 wild type cases) and TKI-acquired resistance patients (n=18) (67). In the first group a concordance between the two samples of 88% was obtained; in particular in cfDNA extracted from PE n=2 out of 19 EGFR mutations were missed and in the wild type group n=2 additional mutations were found (67). In the second group only n=1 EGFR mutation was missed in cfDNA extracted from PE. Of interest, in n=11 (61%) cases in addition to the initial EGFR sensitizing mutation, an additional EGFR exon 20 p.T790M was found (67). On the overall, considering the EGFR sensitizing mutations the concordance rate in this second group was of 94% (67). In Table 4 are summarized results on PE samples.

**Future perspectives and conclusions**

In addition to “body liquid biopsies”, another source of cfDNA for molecular purposes is represented by the supernatant after cytological preparations. This neglected material is normally discarded in cytological laboratories. However, several studies demonstrated the suitability of cfDNA extracted from supernatant obtained from cytological preparations for NGS analysis (68-74).

In conclusion, the so called “liquid biopsies” represent a valid material, not only in alternative to tissue based testing, to better define the molecular status of biomarkers in NSCLC patients, in order to define the best treatment choice (75,76).

**Acknowledgments**

None.

**Footnote**

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

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doi: 10.21037/pcm.2019.05.04