Review Article

Pathology in non-small cell lung cancer: evolving scenario

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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 (EGRF); tyrosine kinase inhibitor (TKI)

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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 (*EGRF*), anaplastic lymphoma kinase (*ALK*), ROS proto-oncogene 1 receptor tyrosine kinase (*ROS1*) 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*, *PDGFRα* 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 ddPRC 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

Table 1 Studies focusing on the adoption of urine samples

First author	N of urine samples (type of matched samples)	Technology	Concordance rate	Sensitivity	Specificity
Reckamp *et al.* (38)	60 (tissue)	NGS	–	p.T790M (93.0%), p.L858R (80.0%), del 19 (83.0%)	p.T790M (96.0%), p.L858R (100.0%), del 19 (94.0%)
Li *et al.* (39)	160 (tissue)	ddPCR	p.L858R (97.0%)*, del 19 (89.0%)*, overall (93.0%)*	–	–
	160 (plasma)		p.L858R (100.0%)*, del 19 (100.0%)*, overall (100.0%)*	–	–
Hu *et al.* (40)	213 (tissue)	ddPCR	p.L858R (65.8%), del 19 (55.9%), overall (61.0%)	–	–
Zhang *et al.* (41)	160 (tissue)	ddPCR	p.L858R (79.0%), del 19 (81.0%), p.T790M (100.0%)	80.0%	100.0%
Wu *et al.* (42)	50 (tissue)	NGS	70.0%	–	–
Wang *et al.* (43)	200 (tissue)	ddPCR	–	70.0%	100.0%
Xie *et al.* (44)	150 (tissue)	ddPCR	–	77.0%, 93.0%**	92.0%

*, advanced stages (III/IV); **, evaluation after serial urine analysis. ddPCR, digital droplet PCR; del, deletions; N, number; NGS, next generation sequencing.

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 2 Studies focusing on the adoption of saliva samples

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

*, 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

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

*, 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

Table 4 Studies focusing on the adoption of PE samples

First author	N of PE samples (type of matched samples)	Technology	Concordance rate	Sensitivity	Specificity
Liu *et al.* (64)	41 (tissue)	ARMS-PCR	76.2%	84.2%	90.9%
Lin *et al.* (65)	22 (tissue)	HRM	–	92.3%	100.0%
Kawahara *et al.* (66)	74 (tissue)	RT-PCR	–	44.4%	100.0%
Lee *et al.* (67)	32 (tissue)	RT-PCR	88.0%	89.5%	84.6%
	18 (tissue)		94.0%	–	–

ARMS-PCR, amplification refractory mutation system-PCR; HRM, high resolution melting; N, number; PE, pleural effusion; RT-PCR, real time PCR.

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).

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Footnote

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