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Driver Mutations in Non-Small Cell Lung Cancer: Utility of Liquid Biopsy

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Abstract: Non-small Cell Lung Cancer (NSCLC) has become the most prominent example demonstrating the importance of targeted therapy in cancer treatment. Up to 25% of patients with non-squamous NSCLC (nsNSCLC) harbor driver mutations which are responsible for the malignancy. For these patients, oral targeted drugs directed against the mutated gene yield better outcomes than chemotherapy or immunotherapy. Consequently, assessing the tumor for driver mutations has become standard of care in managing nsNSCLC. However, in 20% of lung cancer patients, the tissue biopsy is either unobtainable or insufficient to assay. In these circumstances, analyzing circulating tumor DNA (ctDNA) or circulating tumor cells (CTCs) from the blood can reveal the driver mutations. A prospective study was conducted with Biocept, to evaluate the clinical utility of a single gene assay using ctDNA and CTCs in patients with advanced NSCLC. Here, we report the comparison between biomarker expression of the tumor tissue and liquid biopsy of matched samples of 40 unique patients. *Methods*: Matched liquid biopsy and tumor tissue was analyzed from forty unique patients with stage III and IV NSCLC for EGFR, KRAS, BRAF, ALK, and ROS-1. Tissue was analyzed by Next Generation Sequencing (NGS), whereas peripheral blood samples for liquid biopsy was analyzed using circulating tumor DNA (ctDNA) for EGFR, KRAS, and BRAF mutations, and circulating tumor cells (CTCs) for ALK and ROS-1 mutations. *Results*: 80% of the patients (32/40) received both tissue biopsy and liquid biopsy analysis. The concordance between EGFR, KRAS, BRAF, ALK and ROS-1 between tumor tissue and liquid biopsy was 86%. Furthermore, liquid biopsy demonstrated a higher rate of conclusive results compared to tissue biopsy and led to a change in treatment for in 4 of the 40 patients. *Conclusion*: This works suggests that tissue and liquid biopsy can be complementary, and liquid biopsy can inform the course of treatment when a tissue biopsy is not available.

Keywords: Non-small Cell Lung Cancer, Liquid Biopsy, Cell-Free Tumor DNA, Circulating Tumor Cells, Driver Mutations

1. Introduction

Liquid biopsy utilizes peripheral blood to identify driver mutations in advanced cancers [1]. Such analyses may include assay of circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), exosomes or other analytes [1-5]. Next Generation Sequencing (NGS) of tissue and/or blood has become standard of care for first-line treatment selection in lung cancer. Such mutations serve as accurate biomarkers for sensitivity to oral targeted therapy, such as osimertinib, and provide outcome results superior to treatment with chemotherapy or immunotherapy [6-8]. The most common such driver (EGFR) was discovered in 2004 by Tom Lynch and associates at Dana Farber [9]. In 2016, the Food and Drug Administration (FDA) approved the first use of liquid biopsy in detecting EGFR mutations [3]. Given the importance of assessing the tumor for driver mutations, the National Comprehensive Cancer Network (NCCN) category 1 recommendation for patients with NSCLC is to test for epidermal growth factor receptor (EGFR), anaplastic lymphoma kinase (ALK), c-Ros oncogene-1 (ROS-1), met exon 14, RET, PD-L1, and proto-oncogene BRAF [7]. In instances

where a tissue biopsy cannot be obtained, liquid biopsy analysis can help inform the mutational status of the tumor. Liquid biopsy has additional advantages in that it is minimally invasive, less costly compared to tissue biopsy and has an improved turn around time. Furthermore, liquid biopsy also allows for serial monitoring of specific mutations that follow the course of disease and thus enables the physician to make a timely modification in therapy before potential symptoms arise [5]. These advantages have led to analysis of liquid biopsy becoming an essential tool in the management of advanced nsNSCLC. NGS is commonly used for molecular analysis of ctDNA, but when normal wild type DNA is present in large quantities, low frequency mutations may be difficult to detect. Recently, Biocept validated Target Selector, which is a platform that analyzes single gene copy mutations by Switch Blocker on the ctDNA, as well as genomic aberrations by FISH on the Circulating Tumor Cells (CTCs) [10]. The Switch Blocker technology suppresses the amplification of the wild type gene, leading to a high sensitivity gene detection assay. Here we report the results of a prospective study carried out by Biocept, where tissue biopsy NGS results were compared to liquid biopsy Target Selector results for EGFR, ROS-1, BRAF, KRAS and ALK, obtained from NSCLC Stage III and Stage IV patients.

2. Materials and Methods

Forty patients (40) with a confirmed stage III or IV non-small cell lung cancer (NSCLC) were enrolled between August 2018 and September 2019. Patients underwent analysis of the peripheral blood at Biocept using Target Selector. The tumor tissue was analyzed by a local lab using NGS. All patients were tested for five driver mutations (EGFR, KRAS, BRAF, ALK, ROS-1) and PD-L1 expression using a pre-treatment blood sample. Written informed consent was obtained from each patient or their guardian.

Blood samples were collected in a proprietary CEE-Sure tube; a specialized tube developed by Biocept that contains preservatives. From a single tube, CTCs and ctDNA were analyzed. ctDNA was isolated and used for the detection of mutations and deletions (EGFR, KRAS, and BRAF) via Target Selector's Switch Blocker assay using a combination of RT PCR and sequence confirmation, whereas the CTCs were captured using a combination of a proprietary 10-antibody cocktail and microfluidic chamber. Cells were analyzed for gene rearrangements (ALK, ROS-1) using fluorescence in situ hybridization (FISH), while PD-L1 expression was studied using immunocytochemistry (ICC) of CTCs. A clinical report describing the assay results including any relevant driver mutation with associated guideline recommended or FDA approved treatment was issued to the ordering physician. Tissue biopsies were analyzed from a contemporaneous tissue biopsy and tested using NGS. Target Selector results from the liquid biopsy were compared to NGS results obtained from standard tissue biopsies in 32 patients which had matched tissue and liquid biopsy samples. Results were compared for rate of detection of key driver mutations using liquid biopsy vs. tissue biopsy, concordance between liquid biopsy and tissue biopsy to detect key driver mutations, number of cases in which liquid biopsy provided more information and impacted the treatment plan, and number of cases in which tissue biopsy could have been potentially avoided using liquid biopsy.

3. Results

Forty patients were enrolled in the study and underwent Biocept liquid biopsy. Of these, 32 had contemporaneous tissue biopsy. The age at diagnosis ranged between 43 to 90 years with median age of 63.5 years, male: female ratio was 1.22, 32 out of 40 (90% of patients) had a current or a previous history of smoking, 75% of patients had adenocarcinoma on histology while 85% had a stage IV disease (table 1).

Table 1. *Demographic data of patients included in the study.*

		Number	Percentage
Gender	Male	22	55%
	Female	18	45%
Age	Median 63.5 (range 43-90)		
Smoking history	Never Smoked	8	20%
	Former smoker	22	55%
	Current smoker	10	25%
Type	Adenocarcinoma	30	75%
	Squamous cell	5	12.5%
	Other	4	10%
	Unknown	1	2.5%
Stage	IV	34	85%
	IIIB	3	7.5%
	IIIA	3	7.5%

Out of 40 patients, 32 patients underwent tissue biopsy genotyping, 8 patients did not have a tissue biopsy for different reasons including insufficient tissue for NGS (4 patients), refusal of a tissue biopsy (2 patients), or the biopsy procedure was deemed too high of a risk (2 patients). All patients underwent liquid biopsy analysis by Target Selector. Complete genotyping for all driver mutations analyzed (EGFR, KRAS, BRAF, ALK, ROS-1) was achieved in 10/32 of tissue biopsies (31%), compared to 18/40 (45%) in the liquid biopsy group. In addition, four patients required a repeat tissue biopsy while liquid biopsy was repeated in two patients. Eleven tissue biopsies detected at least one positive driver mutation vs. 13 patients in the liquid biopsy group.

3.1. Rate of Valid Tests

Liquid biopsy showed higher average rate of conclusive test results compared to tissue biopsy across all mutations (82% vs. 56% respectively; Figure 1). In fact, there were 5 cases where liquid biopsy provided more information than tissue biopsy, which ultimately resulted in liquid biopsy guiding treatment for 4 patients.

3.2. Concordance Between Liquid Biopsy and Tissue Biopsy on the Same Actionable Genes

Cases which yielded conclusive liquid and tissue biopsy results for every mutation were compared (table 2, Figure 2). Overall concordance between liquid biopsy and tissue biopsy was 86% across all five target mutations. Specificity of the liquid biopsy across all mutations was 94% while sensitivity was 50%.

3.3. EGFR, KRAS, BRAF

Among mutations tested using Target Selector method, 98% of liquid biopsy tests had conclusive results for those mutations. Concordance rate between Target Selector used on ctDNA from peripheral blood and NGS done on the tissue biopsy was 86%, 71%, and 93% for EGFR, KRAS, and BRAF mutations respectively. Specificity was >80% across all three mutations. Sensitivity was variable between different mutations; EGFR had 80% sensitivity, while KRAS and BRAF had a sensitivity of 25% and 50% respectively. Sensitivity in all three mutations was affected by the small sample size.

3.4. ALK, ROS-1

As mentioned above, a lower number of liquid biopsy conclusive test results was observed in mutations tested using Target Selector of CTCs (59% for both mutations). However, concordance between liquid and tissue biopsy was 92% and 90% for ALK and ROS-1 respectively. Specificity was high in both mutations (100% for ALK, 90% for ROS-1). As in mutations tested using ctDNA, sensitivity data for mutations tested using cell capture method was affected by small sample size.

3.5. PDL-1

A total of 17 patients had conclusive results for PDL-1 in both liquid biopsy using cell capture method and tissue biopsy. Tissue biopsy had higher conclusive results rate than liquid biopsy (84% vs. 65%). Overall concordance between liquid and tissue biopsy was 13/17; 76%, when compared to tissue biopsy, liquid biopsy had a specificity of 100% and a sensitivity of 0%.

3.6. Clinical Significance

In four patients, liquid biopsy detected mutations that were not discovered on tissue biopsy. In three patients, liquid biopsy detected T790M EGFR mutation which led to change treatment to osimertinib, while the fourth patient tested positive ROS-1 mutation on liquid biopsy that was not detected on tissue biopsy and was started on crizotinib as a result. In all four patients, treatment was changed based on the liquid biopsy results.

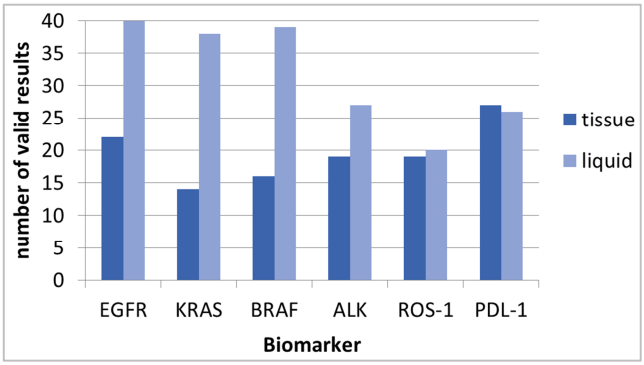

Figure 1. *Conclusive biomarker results (positive/negative) in tissue biopsy compared to liquid biopsy*. The rate of conclusive liquid biopsy test results was higher in mutations tested using ctDNA (EGFR, KRAS, BRAF) compared to tissue biopsy. On the other hand, liquid biopsy had lower conclusive results in mutations tested using cell capture method (ALK, ROS-1). * 40 patients underwent liquid biopsy (n=40), while a tissue biopsy was obtained in 32 patients (n=32).*

Table 2. *Detailed testing results for every target biomarker tested in both tissue and liquid biopsies for the 40 patients.*

		Tissue Biopsy		
		positive	**negative**	**Undetermined / no result***
EGFR				
	positive	4	2	0
Liquid biopsy	negative	1	15	18
	Undetermined / no result	0	0	0
KRAS				
	positive	1	1	4
Liquid biopsy	negative	3	9	20
	Undetermined / no result	0	0	2
BRAF				
	positive	1	0	0
Liquid biopsy	negative	1	13	24
	Undetermined / no result	0	1	0
ALK				
	positive	0	0	0
Liquid biopsy	negative	1	12	14
	Undetermined / no result	0	6	7
ROS-1				
	positive	0	1	1
Liquid biopsy	negative	0	9	9
	Undetermined / no result	0	9	11
PDL-1				
	positive	0	0	2
Liquid biopsy	negative	4	13	7
	Undetermined / no result	6	4	4

* including patients who did not undergo tissue biopsy
** positive tissue PDL-1 expression was considered PDL-1 expression ≥ 50%

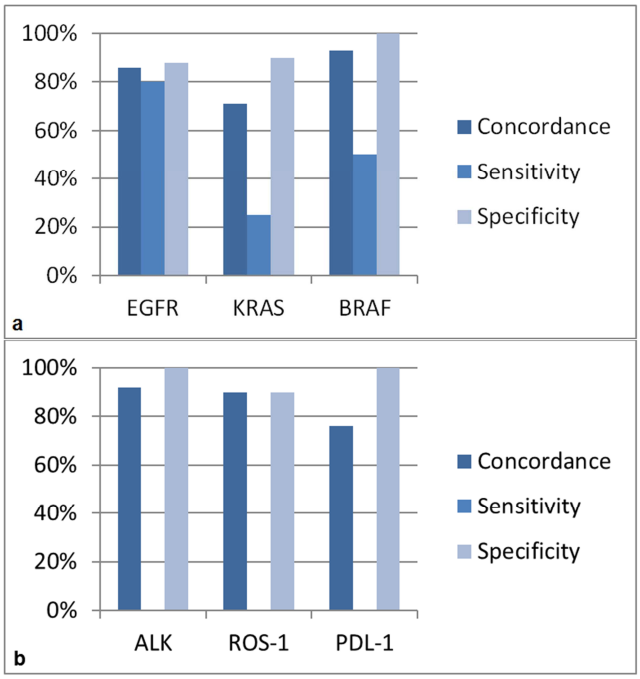

Figure 2. *Concordance, specificity, and sensitivity of liquid biopsy compared to tissue biopsy in tested biomarkers. Part a shows results of mutations detected using ctDNA while part b illustrates results for mutations detected using CTCs.*

4. Discussion

The most common type of lung cancer is NSCLC and about 2/3 of these present with advanced stage disease rendering systemic therapy as the dominant treatment option [7]. Prior to the introduction of immunotherapy and targeted therapy the median overall survival (OS) of patients with advanced NSCLC was approximately one year with a five-year survival rate of 5% [11]. The discovery of driver mutations and the associated targeted therapy has altered the natural history of this subgroup of patients [9]. The majority of driver mutations occur in the non-squamous subgroup-mostly adenocarcinoma nsNSCLC. First line treatment in this latter group consists of oral targeted therapy; more convenient and efficacious than conventional chemotherapy or immunotherapy [8, 12]. For patients without a molecular driver the outlook has also markedly improved with updated results using chemo immunotherapy approaching 2 years [15].

Immunotherapy works by altering the function of the immune system either by enhancing, activating, or suppressing normal function [14]. Most of these immunotherapy drugs act at the level of the T cell. While there are a multitude of pathways regulating T cell function there are two which dominate the clinical arena namely the PD-1 and CTLA-4 checkpoints. Inhibitory monoclonal antibodies that act to block inhibitory signaling are now in widespread use. For example, PD-L1, albeit a normal ligand is often overexpressed in the tumor microenvironment leading to inhibition of the T cell response.

The efficacy of this therapy in advanced stage NSCLC was demonstrated by the clinical trial Keynote 189 [13]. In this double blind, phase 3 trial, 616 patients with previously untreated advanced stage NSCLC were enrolled and were divided into either the placebo control group (cisplatin or carboplatin plus pemetrexed) or the experimental group (pembrolizumab with cisplatin or carboplatin plus pemetrexed). The PFS for immunotherapy versus control groups were 8.8 months and 4.9 months respectively [13]. OS at 12 months in the immunotherapy group to be 69.2% versus 49.4% in the control group. Updated median OS has been reported and remarkably is approaching two years [15]. FDA approved pembrolizumab in conjunction with pemetrexed and platinum-based chemotherapy as first line treatment for advanced stage NSCLC in 2018. Single agent immunotherapy efficacy has also been shown in patients whose tumors demonstrate high expression of PD-L1 TPS (tumor proportion score) >= 50%) [16]. Keynote 024 was a phase III study comparing first line chemotherapy to single agent pembrolizumab in patients with TPS >= 50%. This demonstrated that first line pembrolizumab monotherapy was superior to chemotherapy. The FDA subsequently approved pembrolizumab in patients with high PD-L1expression.

In NSCLC, targeted therapy directed against key driver mutations is now standard of care. The most important of these is in EGFR, commonly seen in patients with adenocarcinoma or non-squamous histology. These mutations are often found in nonsmokers, females, and those of Asian descent [17, 18]. Patients with EGFR mutations preferentially benefit from oral targeted therapy. The current targeted therapies used in patients with EGFR mutations include tyrosine kinase inhibitors (TKIs) osimertinib, gefitinib, erlotinib, dacomitinib, and afatinib [8].

The use of targeted therapy in EGFR mutant NSCLC was first reported in IPASS (Iressa-Pan Asian Study) which demonstrated a 12 month PFS of 24.9% for patients taking gefitinib versus 6.7% for patients taking standard chemotherapy [19]. The newest drug in this space, osimertinib, is now standard of care for EGFR mutant lung cancer. In the FLAURA trial, osimertinib demonstrated prolonged PFS versus a first generation TKI (18.9 months versus 10.2 months respectively) in patients with previously untreated metastatic NSCLC [12]. Updated results from FLUARA reported an impressive overall survival of 38.6 months in patients taking osimertinib versus 31.8 months in those taking the first generation TKI. Those patients given the third generation TKI also experienced less adverse events then those in the control group [20].

In addition to improved PFS and OS in patients with advanced stage NSCLC taking either immunotherapy or targeted therapy, these trials are also show improved quality of life (QOL) [21]. For example in Keynote 024 various methods to assess patient related outcomes (PROs) which were used to determine QOL. In these trials, it was consistently demonstrated that immunotherapy provided better QOL in patients versus chemotherapy treatment [16]. Trials with targeted therapy had comparable findings also demonstrating

Improved QOL for patients taking TKIs versus those on chemotherapy [22].

The critical significance of biomarkers in directing first line treatment selection cannot be overstated. The gold standard for detection of driver mutations is the tissue biopsy. The main challenge with tissue biopsy in lung cancer is the difficulty in obtaining sufficient tissue for pathology as well as molecular profiling including immunohistochemistry (IHC), and next generation sequencing (NGS) [23]. Tissue sufficiency becomes a challenge because advanced lung cancer patients are often elderly, infirm and have multiple medical co-morbidities. The lung is often subjected to biopsy and in these patients with chronic lung disease complications are more frequent. Some of the more common complications seen in patients with NSCLC include pneumothorax, dyspnea, and chest pain [24]. In a retrospective study with over 20,000 patients; 13,411 patients received diagnostic biopsy and 2,056 patients had post treatment biopsy; out of these, 8,973 patients were re-hospitalized for biopsy related complications within 30 days of the procedure taking place [24].

In patients treated with oral targeted therapy, resistance eventually develops, therefore it is critical that these patients undergo a repeat biopsy to characterize the mechanism of resistance. With the first and second generation oral TKI's, the major resistance mutation is T790M [25]. Osimertinib was developed to target this common resistance mutation. In addition, osimertinib has superior outcomes relative to first and second generation TKI's [12]. Unfortunately, resistance also develops in osimertinib treated patients and re-biopsy is essential. The patterns of resistance are more complex in this setting. These include C797S, Met amplification, and histologic transformation to SCLC (small-cell lung cancer) [26]. When patients on targeted therapy progress, we need to assay the tumor for resistance mechanisms. A less invasive approach, liquid biopsy, uses tumor DNA present in the plasma to identify biomarkers of resistance.

The utility of liquid biopsy in molecular characterization of lung cancer has been widely reported [27-29]. In a previous pooled retrospective analysis conducted with 81 patients who underwent driver mutation testing using liquid biopsy with or without tissue biopsy, oncogenic drivers detected in ctDNA result in treatment response in patients that was comparable to that seen in tissue irrespective of the variant allele fraction [28].

In the NILE (Noninvasive versus Invasive Lung Evaluation) study, the advantages of the liquid biopsy were again clearly demonstrated. 282 patients with previously untreated metastatic NSCLC were evaluated to test the non-inferiority of liquid biopsy against the standard tissue biopsy [29]. The results demonstrated that the use of liquid biopsy along with tissue biopsy increased detection of biomarkers by 48% [29]. The overall conclusion of this study was that liquid biopsy yielded similar results as standard tissue biopsies and should be considered as an alternative method of identifying biomarkers.

In our study we used the Biocept liquid biopsy to detect all the NCCN approved biomarkers. The Biocept assay uses ctDNA to detect EGFR, KRAS, and BRAF, and a cell capture technique for ALK, ROS-1, and PD-L1. We did not assay for RET or NTRK rearrangements, as these were not validated biomarkers at time of this study. We were able to successfully perform tissue biopsy on 80% of our patients (32/40), whereas 100% underwent successful liquid biopsy (4 tissue biopsies were insufficient to conduct genotyping, two patients refused tissue biopsy, and two patients were deemed too risky for tissue biopsy). This corroborates previous research that has shown tissue biopsy in advanced NSCLC is fraught, and insufficient tissue for critical molecular testing is a common clinical problem.

Rate of conclusive liquid biopsy test results (positive or negative) was higher in mutations tested using ctDNA method i.e. EGFR, KRAS, BRAF. On the other hand, liquid biopsy had lower conclusive results rate with mutations tested using a cell capture method. Thus, the liquid biopsy identified biomarkers from circulating tumor DNA more reliably than cell capture techniques, due to the paucity of circulating tumor cells. The converse was true for ALK, ROS-1, and PD-L1, where tissue biopsy was superior to the liquid biopsy.

The liquid biopsy can be obtained in all patients as it was in the 40 patients in this study. In addition, liquid biopsy provided treatment options for four of our patients which were not discovered by tissue biopsy. Our work corroborates the findings of the NILE study in that we detected driver mutations in tissue and liquid biopsy with a high concordance rate of 86%. These are complimentary tests and at our center they are often used together in advanced NSCLC patients.

5. Conclusion

Targeted therapy in NSCLC patients using identified biomarkers has become a viable means of personalizing cancer treatment. In this study we have shown supportive evidence that liquid biopsy of circulating tumor DNA (ctDNA) is sufficient to identify driver mutations with the same precision as standard tissue biopsy. In NSCLC patients where tissue biopsy is insufficient, or altogether unattainable (20%), liquid biopsy is a viable alternative. Continued research into biomarker screening in various cancer/tumor types may endorse liquid biopsy as an equally accurate, yet less-invasive, method as standard tissue biopsy.

Declarations

Funding

This study was funded by the Highmark Vital program.

Conflicts of Interest

The authors declare that they have no competing interests.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Code Availability

Not applicable.

Ethics Approval

This study did not require approval from the Institutional Review Board (IRB) as it does not meet the definition of human subject research according to the federal code of regulations: 45 CFR 46.102 (f).

Consent to Participate

Written informed consent was obtained from all participants of the study.

Consent for Publication

Not applicable as the manuscript does not include any identifiable information of the participants.

Authors' Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Khaled Alhamad, Herman Lo, Aaron C. Weidman, Zachary Otaibi, Ashish Sethi, and Suneera. The first draft of the manuscript was written by Robin Raquel Rodriguez, Andrew Friday, and Khaled Alhamad. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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