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# RESULTS OF LIQUID BIOPSY STUDIES BY NEXT GENERATION SEQUENCING IN PATIENTS WITH ADVANCED STAGE NON-SMALL CELL LUNG CANCER: SINGLE CENTER EXPERIENCE FROM TURKEY

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## ABSTRACT

Several studies demonstrated the utility of plasma-based cell-free circulating tumor DNA (ccfDNA) in determination of mutations in non-small cell lung cancer (NSCLC). We aimed to report our results of next generation sequencing (NGS) using liquid biopsy in patients with NSCLC. Patients with advanced stage NSCLC were enrolled and their genomic profiling results were recorded. Next generation sequencing targeted panel includes 19 hot-spot genes. The plasma was separated from the peripheral blood sample and ccfDNAs were isolated for NGS. We performed genomic profiling in 100 patients (20 females and 80 males) with a median age of 59.3 (range 26-79). A second liquid biopsy was performed in eight patients who developed progressive disease after the first treatment. The study population had adenocarcinoma (AC) ($n$ = 73), squamous cell carcinoma (SCC) ($n$ = 14), or NSCLC-NOS (not otherwise specified) ($n$ = 13). In the SCC group, three of 14 patients had variants on *EGFR* and *MET* genes. In the AC and NSCLC-NOS groups, 39 out of 86 patients (45.3%) had variants. The most common one was in the *EGFR* gene ($n$ = 27, 31.4%) including seven mutations related to drug resistance and two were polymorphisms. Three patients had both driver and resistance mutations (*EGFR* T790M, $n$ = 2; *KRAS* exon 2 G12S and *MET* exon 14 E1012K, $n$ = 1). Fifteen patients (17.4%) had an activating *EGFR* mutation and eight patients (9.3%) had variants in the *KRAS* gene. We reported our results regarding genomic profiling related to treatment using liquid biopsy in patients with NSCLC. Advantages of this method are the non invasiveness and reproducibility.

**Keywords:** Liquid biopsy; New generation sequencing (NGS); Non-small cell lung cancer (NSCLC).

## INTRODUCTION

Non-small cell lung cancer (NSCLC) is an important cause of morbidity and mortality worldwide. Many patients with NSCLS are diagnosed at advanced stages and treated with targeted therapy and immunotherapy in addition to systemic chemotherapy [1]. The College of American Pathologists (CAP), International Association for the Study of Lung Cancer (IASLC) and Association for Molecular Pathology (AMP) recommended that EGFR, ALK, and ROS1 were necessary tests in advanced stage NSCLC patients whose tumors contain an element of adenocarcinoma (AC) in their 2018 updated testing guideline. Moreover, the results of the recent clinical data indicate those panels including *BRAF*, *MET*, *RET*, *ERBB2* and *KRAS*, should be used at a minimum [2]. Cell-free circulating tumor DNA (ccfDNA), which is released from the tumor into the systemic circulation, is used in the liquid biopsy [3].

Thompson *et al.* [4] and Schwaederlé *et al.* [5] demonstrated that next generation sequencing (NGS) of plasma-based ccfDNA can be used to assess mutations in NSCLC. Several recent retrospective and prospective studies also used plasma samples to decide targeted treatments [6,7]. The NGS used for the analysis of ccfDNA involves reading of the DNA strand 10,000-times using deep sequencing and allows determination of the type and frequency of a

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given mutation by bioinformatic analyses. It is possible to identify single base mutations, short insertions and deletions, wide genomic deletions, or rearrangements such as inversion and translocation (by amplifications) using NGS [8]. In the present study, we aimed to present our NGS results of liquid biopsy samples that is increasingly used in clinical practice for NSCLC that comprises several genetic alterations guiding therapeutic opportunities.

## MATERIALS AND METHODS

**Patient Selection.** A prospective review of 100 patients in Adana, Turkey with a diagnosis of advanced/metastatic NSCLC, whose physician requested clinical ccfDNA based genomic profiling from January 2017 to January 2019, were performed. All patients included in the analysis had metastatic or inoperable disease. A second liquid biopsy was performed in eight patients who developed progressive disease after the first treatment. All patients provided their written consent to the genomic profiling.

**Targeted Multi-Gene Panel Testing.** Customized targeted multiple gene panel consisting of 19 genes (*AKT1*, *ALK*, *BRAF*, *DDR*, *ERBB2*, *ESR1*, *KIT*, *KRAS*, *MAP2K1*, *NRAS*, *NTRK*, *PDGFRA*, *PIK3CA*, *PTEN*, *ROS1*, *RICTOR*, *EGFR*, *MET*, *FGFR1* gene mutations, and *RICTOR*, *EGFR*, *MET*, *FGFR1*, *ERBB2* gene amplifications) was used for NGS. The ccfDNA was extracted from whole peripheral blood collected in 10 mL PaxGene (PreAnalytiX GmbH, Hombrechtikon, Switzerland) biological sample tubes. The results of NGS were obtained after the following steps: separation of plasma from the samples, isolation of ccfDNA from the plasma, target region enrichment in an appropriate quality and quantity, library preparation, clonal amplification and NGS steps. Afterwards, bioinformatic analyses were performed to determine the quality and variant analysis according to the clinical information of the patients to interpret the variants. All the workflow was carried out at Çukurova University AGEN TEM (Adana Genetic Diseases Diagnosis and Treatment Center), Adana, Turkey *via* the GeneReader NGS system (Qiagen GmbH, Hilden, Germany).

The isolation of ccfDNA was performed using the circulating cfDNA isolation Kit (QIAamp circulating nucleic acid kit; Qiagen GmbH) with the help of a vacuum system (QIAvac 24 Plus; Qiagen GmbH). The ccfDNA concentrations were determined using fluorometric DNA quantitation device (Qubit 3.0, Thermo Fisher Scientific, Waltham, MA, USA). The samples with adequate DNA concentrations were used for further laboratory workflow. The target enrichment of the region of interest was amplified by PCR (polymerase chain reaction). The bar coding and library preparation step was then performed. Thereafter, the samples were loaded into flowcells to be sequenced in the GeneReader NGS system (Qiagen GmbH).

**Bioinformatic Analysis and Interpretation.** The most complicated and difficult step is the accurate analysis of the large data from the sequenced NGS samples by an experienced medical geneticist and the team. The data with appropriate quality were selected before the data analysis. The selected data were compared with the reference genome data. The Human Genome Mutation Database (HGMD) (http://www.hgmd.cf.ac.uk/ac/index.php0, Catalogue of Somatic Mutations in Cancer (COSMIC) (https://cancer. sanger.ac.uk/cosmic), 1000 Genome Frequency and Ingenuity Knowledge Base databases (https://www.internationalgenome.org/1000-genomes-browsers), and SIFT, BSIFT (https://sift.bii.a-star.edu.sg/), PolyPhen-2 (http:// genetics.bwh.harvard.edu/pph2/dbsearch.shtml) and Clin Var (https://www.ncbi.nlm.nih.gov/clinvar/) *in silico* analysis were used for variant analysis. Then, selected variants were analyzed using bioinformatics tools to classify and evaluate them according to their clinical impacts, for potential influence on the treatment strategies, and to confirm the clinical diagnosis. The low quality variants were also assessed for the samples' status and clinical status of the patient. All the variants were evaluated in two steps: the first one was for its quality and possible effects independent of clinical diagnosis and mostly on the basis of quality control parameters such as for ward/reverse read balance and coverage. The second evaluation was performed for clinical diagnosis and possible effect on the sensitivity and/or the resistance to the treatment protocols.

## RESULTS

A total of 100 patients (20 females and 80 males) with NSCLC who were admitted to our clinic underwent targeted NGS from the liquid biopsy samples. All of the patients were of Turkish ethnic origin. The median age of the patients was 59.3 (range 26-79). While eight of the patients had a repeated genetic testing after treatment, other patients had only one test result. The histological diagnoses were made by the pathologists of our clinic. Seventy-three patients had AC, 14 had squamous cell carcinoma (SCC), and 13 had NSCLC-NOS (not otherwise specified). The study patients were grouped according to their histological lung cancer (LC) types as classified by the National Comprehensive Cancer Network (NCCN) in their targeted treatment recommendations. The demographic data and histologic LC types of the patients are listed in Table 1.

**The Potential Targetable Variants in the SCC Group.** Three of the 14 patients had variants: one patient had *EGFR* gene exon 21 driver and exon 20 resistance mutations together and the other two patients had a mutation in the *MET* gene exon 14 (Table 2).

**The Potential Targetable Variants in the AC And NSCLC-NOS Groups.** Thirty-nine of the 86 patients

**Table 1.** General demographics and histology.

| General Demographics | *n* | Histology | *n* |
|---|---|---|---|
| Patients | 100 | SCC | 14 |
| Median age (range) | 59.3 (26-79) | AC | 73 |
| Males/Females | 80/20 | NSCLC-NOS | 13 |

SCC: squamous cell carcinoma; AC: adenocarcinoma;
NSCLC-NOS: non-small cell lung cancer-not otherwise specified.

**Table 2.** Targetable mutations in squamous cell carcinoma patients.

| *n* | Activating Mutation(s) | Resistance Mutation |
|---|---|---|
| 24 | *MET* exon 14 I491T | |
| 29 | *MET* exon 14 V1014V | |
| 56 | *EGFR* exon 21 L858R | *EGFR* exon 20 T790M |

(45.3%) had at least one variant of the targeted multi-gene panel. Twenty-seven (31.4%) of the 86 patients had a variant in the *EGFR* gene, while seven had a resistance related mutation, two had a polymorphism, and three had both driver and resistance related variants (*EGFR* T790M, *n* = 2; *KRAS* exon 2 G12S and *MET* exon 14 E1012K, *n* = 1).

Fifteen patients (17.4%) had an activating *EGFR* mutation (Table 3). Eight patients (9.3%) had a *KRAS* gene variant. One patient had an isolated *ALK* mutation and another one had an *ERBB2* and *EGFR* gene variant in addition to an *ALK* mutation. While one patient had an isolated *MET* amplification, two patients had a concomitant *EGFR* resistance related variant. While two patients had an isolated ERBB2 gene variant, another two had EGFR variants and *ALK* mutation in addition to the *ERBB2* variant. One patient had variants in both *PDGFRA* and *KIT* genes. Another one had an isolated *BRAF* variant (Table 4). In the patients who experienced progressive disease after treatment, eight of them underwent a second liquid biopsy sampling after a median duration of 6 months (range 3-12) following the first one. In the second term of sequencing, a new onset *EGFR* T790M mutation, elimination of the target mutation, and a new onset of *BRAF* variant were detected. The characteristics and treatment protocols of these eight patients are summarized in Table 5.

**Table 3.** Detailed Molecular Findings of Adenocarcinoma and NSLC-NOS patients with detectable EGFR mutations

| Patient Number | Activating Mutation(s) | Resistance Mutation(s) | Polymorphism |
|---|---|---|---|
| 2 | EGFR Exon 19 A750P,<br>EGFR Exon 19 L747_E749 del | | |
| 4 | EGFR Exon 19 | | |
| 6 | EGFR Exon 21 L858R | EGFR Exon 20 T790M | |
| 7 | EGFR Exon 20 C797S<br>EGFR Exon 21 L858R | EGFR Exon 20 T790M | |
| 10 | | EGFR Exon 18 G721V | |
| 12 | EGFR Exon 20 S768I<br>EGFR Exon 21 L858R | | |
| 15 | EGFR Exon 21 L858R | | |
| 33 | | KRAS Exon 2 G12C | EGFR Exon 13 R521K |
| 38 | EGFR Exon 19 E746_A750del | | |
| 39 | EGFR Exon 18 G721S | | |
| 40 | | EGFR Exon 20 S784T | |
| 41 | | EGFR Exon 19 P753P | |
| 48 | EGFR Exon 19 T751_E758del | | |
| 50 | EGFR Exon 19 L747_P753delinsS | | |
| 53 | | EGFR Exon 20 A767_V469 duplication<br>ERBB2 amplification | |
| 55 | EGFR Exon 19 E746_A750del | | |
| 57 | EGFR Exon 19 E746_T751delinsA | | |
| 63 | | EGFR Exon 20 T790M<br>EGFR Exon 20 C797S | |
| 69 | EGFR Exon 18 I706S | | |
| 73 | | EGFR Exon 20 H773fs *53,<br>Met amplification | |

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|---|---|---|---|
| 78 | | EGFR Exon 20 K806I,<br>Met Exon 14 R988H,<br>Met Exon 14 Y989C | |
| 80 | | KRAS Exon 2 G12C | EGFR Exon 13 R521K |
| 81 | EGFR Exon 20 D807fs *90 | | |
| 84 | EGFR Exon 20 V786L | | |
| 85 | EGFR Exon 21 A864T | | |
| 86 | EGFR Exon 19 L747_T751del | KRAS Exon 2 G12S,<br>Met Exon 14 E1012K | |
| 88 | EGFR Exon 19 E746_A750delELRE A,<br>EGFR Amplification | | |

**Table 4.** Detailed molecular findings of adenocarcinoma and non-small cell lung cancer-not otherwise specified patients other than *EGFR* mutations.

| # | Activating Mutation(s) | Resistance Mutation(s) | Polymorphism |
|---|---|---|---|
| 5 | | *KRAS* exon 2 G12C | |
| 33 | | *KRAS* exon 2 G12C | *EGFR* exon 13 R521K |
| 37 | | *KRAS* exon 2 G12V | |
| 49 | | *KRAS* exon 2 G12V (c.35G>T) | |
| 80 | | *KRAS* exon 2 G12C | *EGFR* exon 13 R521K |
| 86 | *EGFR* exon 19 L747_T751del;<br>*MET* exon 14 E1012K | *KRAS* exon 2 G12S | |
| 87 | | *KRAS* exon 2 G12D | |
| 90 | | *KRAS* intron 2 | |
| 14 | | *MET* amplification | |
| 73 | | *EGFR* exon 20 H773fs*53;<br>*MET* amplification | |
| 78 | | *EGFR* exon 20 K806I;<br>*MET* exon 14 R988H;<br>*MET* exon 14 Y989C | |
| 44 | *ALK* exon 29 K1491R | *ERBB2* exon 17 I655V | *EGFR* exon 13 R521K |
| 95 | *ALK* exon 22 C1156S | | |
| 31 | | *ERBB2* amplification | |
| 53 | | *EGFR* exon 20 A767_V469dup;<br>*ERBB2* amplification | |
| 79 | | *ERBB2* exon 17 I655V | |
| 58 | | | *PDGFRA* c.236G>A (G79D);<br>*KIT* c.2362-77G>A |
| 18 | *BRAF* exon 11 G469E | | |

**Table 5.** Second liquid biopsy results in adenocarcinoma and non-small cell lung cancer-not otherwise specified patients.

| # | First Liquid Biopsy | Second Liquid Biopsy | Prior Treatment |
|---|---|---|---|
| 2 | *EGFR* exon 19 A750P;<br>*EGFR* exon 19 L747_E749del | no variant detected | erlotinib |
| 10 | *EGFR* exon 18 G721V | no variant detected | paclitaxel + carboplatin |
| 13 | no variant detected | no variant detected | paclitaxel + carboplatin |
| 15 | *EGFR* exon 21 L858R | *BRAF* exon 15 R603* | erlotinib |
| 31 | *ERBB2* amplification | no variant detected | paclitaxel + carboplatin |
| 38 | *EGFR* exon 19 E746_A750del | no variant detected | erlotinib |
| 55 | *EGFR* exon 19 E746_A750del | *EGFR* exon 19 E746_A750del;<br>*EGFR* exon 20 T790M | erlotinib |
| 95 | *ALK* exon 22 C1156S | no variant detected | crizotinib |

## DISCUSSION

The treatment of NSCLC has become more successful and individualized using targeted protocols according to molecular subtypes. The earlier attempts with targeted treatments involved unselected patients and did not result in as good outcomes as anticipated. Regarding treatment with the epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) in unselected patients, while erlotinib increased median survival by 2 months in previously treated NSCLC patients [9], gefitinib did not improve survival in a similar population [10].

However, targeted treatment in patients selected according to molecular analyses resulted in unprecedented outcomes. For instance, a prospective study in patients with EGFR mutant LC, the response rate to targeted treatment was over 60.0% [11]. Crizotinib resulted in a similar success rate in LC patients with *ALK* rearrangements [12]. First line treatment with dabrafenib/trametinib in NSCLC patients carrying *BRAF* V600E mutations (seen in 2.0% of patients with NSCLC) resulted in a response rate of 64.0% and 10 months of progression-free survival [13]. The identification of the even rarer variants such as *MET* gene amplification and *ERBB2* mutations led to the discovery of new treatment pathways. In 2014, the NCCN recommended utilization of wider panels including *BRAF*, *ERBB2* (*HER2*), *MET*, *RET* and *ROS1* in addition to *EGFR* and *ALK*. The reason for their recommendation to widen the mutation panel was the ever-increasing success with novel treatments targeted to oncogenic driver mutations in comparison to cytotoxic and immune checkpoint inhibitor therapies.

These tests were performed step by step, therefore large tissue samples were inevitably needed [14]. Various clinical studies indicate that attainment of ccfDNA is an appropriate means of detecting mutations [15,16]. Recently, the advantages of plasma-based liquid biopsy such as accessibility, practical use and reproducibility, rendered it a good choice in patients with NSCLC. Tissue biopsies usually bear the difficulty of access and may provide insufficient samples for genetic testing due to the tumor heterogeneity. Furthermore, the need for a repeat biopsy after lack of response to treatment may be a cause of significant morbidity. The ASSESS study reported a high degree of concordance between plasma-based ccfDNA and tissue or cytology samples in NSCLC [17].

We aimed to report our NGS results using liquid biopsy samples in patients with metastatic NSCLC. The most common mutation was regarding the *EGFR* gene. Some of the patients had concomitant driver and resistance mutations. The second most commonly detected gene was *KRAS*. While the frequency of *EGFR* mutation in AC in the western populations was 19.2%; it was seen in 47.9% of Asian patients. The rates of *KRAS* mutations was 26.1% in the western and 11.2% in Asian populations [18]. Turkey lies between the western populations and Asia, and the frequencies of *EGFR* and *KRAS* mutations in the present study were 31.4% (17.4% activating) and 9.3%, respectively. The other targets of individualized treatment protocols include EML4-ALK fusion and variants of *MET*, *ERBB2*, *BRAF*, *PDGFRA* and *KIT*. The most common variants detected in patients with AC were in *EGFR* and *KRAS* genes. The two most commonly detected variants were also seen in patients with SCC in a diverse frequency according to their ethnic origin [19]. In the present study, *EGFR* and *MET* variants were also observed in patients with SCC. The activation of the MET signaling pathway is suggested to cause resistance to EGFR-TKI treatment. Crizotinib may provide successful outcomes in patients with *MET* overexpression [20]. Therefore, target mutations should be sought using liquid biopsy in patients with SCC. Despite the dramatic response to molecular targeted treatments, nearly every medication is associated with development of resistance. It is also important to understand the molecular pathways of NSCLC in order to understand mechanisms of resistance to medications [21].The understanding of resistance mechanisms against targeted treatments led to the development of second line treatment options specific to *EGFR* and *ALK* mutations. Moreover, the growing need for repeat biopsies in patients with LC is associated with a substantial morbidity risk. Furthermore, the paucity of centers for transthoracic biopsies in many healthcare systems cause a logistics problem. Therefore, the opportunity of following up patients with LC using blood-based tests is an important topic of interest in the technologically developing world [22,23].While the most common mechanism of resistance against first and second line EGFR-TKIs is the *EGFR* T790M mutation, *MET* and *ERBB2* amplification is responsible for a smaller proportion of resistance [24]. In the present study, we also aimed to investigate the causes of resistance in the eight patients with progressive disease after the first treatment, and found the following causes: new onset *EGFR* T790M mutation, erased target mutation and a new onset *BRAF* gene variant. The present study has some strong points as well as limitations. New onset variants that are potential targets of alternative treatment options and the disappearance of the previous mutations showed the necessity of repeated liquid biopsy studies. Liquid biopsy has a strong advantage in NSCLC because of the difficulties of repeated interventional procedures and accessibility problems. To the best of our knowledge, this study is the first to report on the real-world use of a comprehensive ccfDNA with clinical follow-ups and NGS platforms in the molecular diagnosis of patients with NSCLC in a Turkish population. The main limitation of working with the circulating tumor cell as a liquid biopsy

source is the fact that the sample obtained in patients with early stage cancer may not contain sufficient amounts of tumor-derived material and sometimes information from tumor tissue may be difficult to separate information from intact cells [25]. An example of this is *PDGFRA* c.236G>A (G79D), *KIT* c.2362-77G>A variants, which are not compatible with the diagnosis and clinical feature of a patient and was evaluated as a polymorphism. The second liquid biopsy sampling was performed only in a small number of cases and this was the other limitation of this study.

## CONCLUSIONS

In the present study we reported the results of targeted multigene panel NGS analysis of ccfDNA in Turkish patients with NSCLC. We demonstrated the feasibility of this advanced diagnostic method and identified mutations of resistance and potential treatment target. We suggest that the non invasive liquid biopsy will have a great clinical importance in the management of patients with NSCLC when obtained tissue samples are inadequate or repeat biopsy is difficult.

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