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Clinical results and importance of next-generation sequencing (NGS) in detecting targeted mutations in the treatment of metastatic Lung Cancer: Single center initial results

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Abstract

Objective: In Lung cancer (LC), which is one of the most deadly cancers, longer survival has been achieved with targeted agents. For this reason, it is important to find the patients who are suitable for targeted therapies. Next-generation sequencing (NGS) is a method that allows multiple genetic variants to be detected simultaneously by performing massive parallel DNA sequencing at the same time. We wanted to reveal the clinical effects and benefits of genetic variant analysis with NGS for our patients.

Material and Methods: Patients with stage IV non-squamous and not otherwise specified (NOS) non-small cell LC who underwent genetic variant analysis with NGS were included in the study, retrospectively.

Results: Total of the 51 patients, 41 (80.4%) were male and the median age was 64 (35-85) years. According to TNM, 21 (41.2%) patients were stage 4A, 30 (58.8%) patients were stage 4B and 39 (76.5%) patients had adenocarcinoma and 12 (23.5%) had NOS histology. NGS analyzes were performed in median 14 days (8-43) and determined 24 pathogenic variants in 17 (%25) patients: 9EGFR (%17,6), 6PIKC3A (%11,7), 5KRAS (%9,8), 2PTEN (%3,9), 1BRAF (%1,9), 1MET (%1,6) (7 of them concomitantly). Cytotoxic chemotherapy was recommended in 41, anti-EGFR agents in 8 (afatinib in 4, erlotinib in 4 patients) patients and anti-BRAF+MEK inhibitor agent (dabrafenib+trametinib) in 1 patient.

Conclusion: With the NGS, in just two weeks, both target and resistance genetic variants of our patients were detected at the same time and individualized treatments were applied. In this way, both time and cost were saved.

Key words: Lung neoplasm, DNA Mutational Analysis, DNA Sequence Analysis, ErbB Receptors.

Introduction

Lung cancer (LC) is the leading cause of cancer-related deaths worldwide (1). Histologically LC is classified primarily as small cell (15%) and non-small cell carcinomas (85%), whereas non-small cell lung cancers (NSCLC) are divided into two main groups as adenocarcinoma and squamous cell carcinoma (2). NSCLC, which accounts for the majority of LC, generally diagnosed in the advanced stage, while the 5-year survival rate is still low, around 16% (3). Unfortunately, the 5-year survival rate of stage IV patients treated with classical cytotoxic chemotherapy is <5% (3). However, it has been determined that this rate has increased to 15-25% by using targeted and immunotherapy agents (4-5).

Targeted therapies in LC have initiated with the detection of driver mutations such as *EGFR, ALK, ROS1* over a decade ago.

EGFR mutation is detected by 15-25% (more than 50% in Asian race), *ALK* mutation is around 2-6% and *ROS1* mutation is around 1% by conventional methods such as Real time Polymerase chain reaction (PCR), fluorescence in situ hybridization (FISH), or Sanger sequencing (SS). Anti-*EGFR*, such as gefitinib, afatinib, erlotinib and osimertinib, anti-*ALK* therapies such as crizotinib, alectinib, brigatinib, lorlatinib provided both the advantage of progression-free survival (PFS) and overall survival (OS) compared to cytotoxic chemotherapy in advanced LC (6). In addition due to oral administration, more manageable side effect profile, and rapid response, targeted agents have been superior to chemotherapeutics (7). Therefore, all updated guidelines such as the College of American Pathologists (CAP), International Association for the Study of Lung Cancer (IASLC), and the Association for Molecular Pathology (AMP) require mutation analyses of

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EGFR, ALK and ROS1 to all advanced non-squamous (adenocarcinoma, large cell carcinoma) and not otherwise specified (NOS) NSCLC (category 1). And they also recommended to detect mutations such as *MET, RET, HER2, BRAF* in a low level of evidence and to use targeted agents in appropriate patients (8). In spite of this information, it is now a known fact that detection of a mutation in step by step procedure by conventional methods such as FISH or SS causes both time and material wasting (9). Recently, with the completion of the cancer genome atlas project (TCGA), next generation sequencing method (NGS) has discovered. The most important advantage of this method over conventional methods is that it can perform multiple sequencing at the same time (massive parallel sequencing). Thus, more genetic variants can be detected at the same time, cheaper and faster. In addition, it enables the detection of variants using both tissue and liquid biopsies, thus reducing invasive interventions and also allowing the detection of genetic variants that are resistant to targeted agents (10).

In this study, we aimed to present the NGS results of our patients with advanced lung cancer and to show the effect of these results on our patient management.

Material and Methods

A total of fifty-one patients who were diagnosed as stage 4 non-squamous and NOS NSCLC between November 2018 and November 2019 at Atatürk University Medical Oncology and who underwent genetic variant analysis using NGS method were included in our study retrospectively.

Ethics committee approval was obtained from the ethics committee of Erzurum Ataturk University. All the procedures were performed according to the 1964 Helsinki declaration.

Sample and DNA Isolation

FFPE tumor specimens and liquid biopsy materials were collected from lung cancer patients referred to the medical genetics clinic. All patients had a clinical indication for molecular testing and were informed about the purpose of the molecular analysis by the treating physician.

DNA was isolated using the GeneRead™ DNA FFPE Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's protocol. The nucleic acid concentration was measured with the Qubit dsDNA HS Assay kits on the Qubit 2.0 Fluorometer (Thermo Fisher Scientific).

GeneReader Assay and Sequencing

In total, 40 ng of each DNA sample was used as a template for the QIAGEN GeneRead QIAact Lung DNA Panel UMI Kit according to the manufacturer's instructions. The GeneRead QIAact Lung DNA UMI Panel is designed to enrich specific target regions in select genes (*AKT1, ALK, BRAF, DDR2, EGFR, ERBB2/HER2, ESR1, FGFR1, KIT, KRAS, MAP2K1, MET, NRAS, NTRK1, PDGFRA, PIK3CA, PTEN, RICTOR, ROS1*). The amplicons in the panel cover multiple exons in 19 genes. -*Libraries were prepared using the QIAGEN GeneRead DNA Library Kit and an automated protocol on a QIAcube. PCR-enriched DNA and GeneRead libraries were qualified and quantified using a QIAGEN QIAxcel Advanced System. Emulsion PCR and bead enrichment steps were carried out using the GeneRead Clonal Amp Q Kit on a GeneRead QIAcube. Following clonal amplification, amplicon libraries were sequenced using the QIAGEN GeneRead Sequencing Q Kit and after an upgrade during the testing period, the GeneRead Advanced Sequencing Q Add-On on a GeneReader instrument (all protocols available on http://www.qiagen.com). QIAGEN Clinical Insight Analyze (QCI-A) software performed the secondary analysis of FASTQ reads generated by the GeneReader. Variants were imported into the QCI-interpret (QCI-I) web interface for data interpretation and report generation.

Results

Total of the 51 patients included in the study, 41 (80.4%) were male and 10 (19.6%) were female and the median age was 64 (35-85) years. 39 (76.5%) patients had adenocarcinoma and 12 (23.5%) had NOS histology. According to TNM, 21 (41.2%) patients were stage 4A, 30 (58.8%) patients were stage 4B. Metastasis of the patients were detected in 16 (31.4%) contralateral lobe, 13 (25.5%) bone, 6 (11.8%) cranial, 6 (11.8%) liver, 6 (11%, 8) adrenal, 4 (7.8%) pleura.

Mutation analysis with NGS was performed from tissue biopsy in 27 (52.9%) patients and liquid biopsy in 24 (47.1%) patients. As a result of these analyzes, no genetic variant was detected in 29 patients but 7 variants of uncertain clinical significance in 5 patients and 24 pathogenic variants in 17 patients were detected. Detailed results of uncertain clinical significance and pathogenic genetic variants are shown in Tables 1 and 2. Totally, 9*EGFR*, 6*PIKC3A*, 5 *KRAS*, 1*BRAF*, 2*PTEN*, 1*MET* pathogenic variants were determined. Seven of 24 pathogenic variants had concomitant mutations: *EGFR* + *PIK3CA* in 2 patients, *EGFR* + *KRAS* in 1 patient, *EGFR* + *PTEN* in 1 patient, *EGFR* + *MET* in 1 patient and *KRAS* + *PIK3CA* in 2 patients.

NGS analyses were performed in median 14 days (8-43), and according to the data, standard cytotoxic chemotherapy was recommended in 41 patients, anti-*EGFR* agents in 8 patients (afatinib in 4 patients, erlotinib in 4 patients) and anti-*BRAF* + MEK inhibitor agent (dabrafenib + trametinib) in 1 patient. Table 2 shows the treatment agents applied to patients with pathogenic mutations in detail. In one patient, no variant could be detected by liquid biopsy, *ALK* mutation was detected by FISH and alectinib, which is an anti-*ALK* agent, was used. In order to detect resistance mutations, variant analysis with NGS was performed in 2 patients due to showing progression while using anti-*EGFR* agents and *EGFR* T790M mutations were detected in both of them.

Table 1. Uncertain significance genetic variants detected by Next-Generation Sequencing

Mutation	DNA Code	Protein	Allelic Fraction (%)	Community frequency (%)	Concomitant Mutation	DNA Code/Protein
PIK3CA	c.1173A>G	P1391M	21	0	-	-
ERBB2	c.1963A>G	P1655V	35	0	-	-
ERBB2	c.1963A>G	P1655V	33	0	-	-
BRAF	c.2128-5dupT		1,96	0	*PIK3CA*	c.1173A>G/ 1391M
BRAF	c.1860+66A>C		8,74	0	*EGFR*	c.1006+15G>A

Table 2. Pathogenic genetic variants detected by Next-Generation Sequencing

Mutation	DNA Code	Protein	Allelic Fraction (%)	Community Frequency (%) (gnomAD)	Concomitant Mutation	DNA Code/Protein	Treatment
EGFR	c.2235_2249del	p.E.746_A750del	4,22	0	-	-	erlotinib
EGFR	c.1787C>G	P596R	1,14	0	-	-	erlotinib
EGFR	c.2235_2249del	E.746_A750del	3,2	0	-	-	erlotinib
EGFR	c.2235_2249del	E.746_A750del	62	0	-	-	afatinib
EGFR	c.2235_2249del	E.746_A750del	0,8	0	*PIK3CA*	c.3140A>G/ H1047R	erlotinib
EGFR	c.2127_2129del	E.709_T710del	42	0	*PIK3CA*	c.1633G>A/ E545K	afatinib
EGFR	c.2237A>G	E.746G	1,06	0	*KRAS*	c.20T>G/ V7G	chemotherapy
EGFR	c.2573T>G	L858R	31	0	*PTEN*	c.697C>T/ R233	afatinib
EGFR	c.323G>A	R108K	0,45	0	*MET*	Amplifikasyon	afatinib
PIK3CA	c.1633G>A	E545K	8,58	0	-	-	chemotherapy
PIK3CA	c.1624G>A	E542K	17	0	-	-	chemotherapy
KRAS	c.34G>T	G12C	12	0	-	-	chemotherapy
KRAS	c.34G>T	G12C	54	0	-	-	chemotherapy
KRAS	c.35G>T	G12V	14	0	*PIK3CA*	c.1634A>C/ E545A	chemotherapy
KRAS	c.34G>T	p.G12C	17	0	*PIK3CA*	c.1633G>A/p.E545K	chemotherapy
PTEN	c.697C>T	R233	20	0	-	-	chemotherapy
BRAF	c.1799T>A	V600E		0	-	-	Dabrafenib+ trametinib

Discussion

The use of targeted agents in LC yields satisfactory results for both the patient and the clinician. These agents provide faster response and longer survival. In our study, a total of 10 druggable genetic variants were detected, 6 of which had concomitant mutations, and 8 of them were initiated with anti-*EGFR* and 1 with anti-*BRAF* agents. Cytotoxic chemotherapy was planned for 1 patient because of concomitant *EGFR* resistance mutation. And these target mutations were detected in median 14 (8-43) days, allowing first line using targeted therapies without the need for cytotoxic chemotherapy. Since some lung cancer patients do not have a single day to wait for treatment, it is seen that the rapid and simultaneous detection of target mutations by NGS is very beneficial for our patients.

Cancer is a genetic disease characterized by the uncontrolled growth of cells. Chromosome dissociation problems, replication errors or DNA damage, which cannot be corrected by repair mechanisms over the years, cause somatic mutations. These mutations are called driver mutations and give growth and survival advantages to certain cell groups by inhibiting apoptosis, accelerating cell proliferation (11). According to current data, the most important driver mutations in LC are *EGFR, ALK, ROS1, BRAF, KRAS, HER2, MET, RET, NTRK* (12). Detection of these mutations at the time of diagnosis and the use of targeted agents improve the quality of life and survival. Prior to NGS, target mutations allowed detection of a mutation in step by step procedure by conventional methods (13).

Although the recommended time is 2 weeks, in our country, the general approach is to detect *EGFR* mutation first by Real time PCR (mean 10 working days) and if negative results, *ALK* (10 working day) and *ROS* (10 working day) mutations are detected by FISH method, respectively. On average, treatment decisions for these three mutations were made within 3-4 weeks. However, there are situations where patients need urgent treatment, so our patients may have to take chemotherapy even if their mutations are detected in the first place. In addition to these 3 important mutations, many different mutations are detected by NGS method and results are obtained within 2 weeks on average (14). In our study, mutation analyzes were performed in median 14 days in accordance with the literature and treatment opportunities were provided to the patients. It is one of the most important advantages of NGS to introduce so many different driver and resistance mutations in such a short time.

The epidermal growth factor (EGF) family includes 4 receptor kinases: EGFR, EGFR2 (HER2 or ERBB2), HER3 (ERBB3), HER4 (ERBB4). When the activating ligand binds to these receptors, EGFR is active and sends intracellular growth, invasion, angiogenesis and anti-apoptotic signals (15). The most common *EGFR* mutations are detected in exon 19 and 21. In the Asian race, the mutation rate increases to 50% and in western it is around 15-20% (16-17). In our patient group, this rate is similar to western societies with 17.6%. Gefitinib, one of the first anti-*EGFR* agents, was first applied in the group of patients

who progressed after chemotherapy regardless of mutation status and the expected results could not be obtained (18). Thereafter, gefitinib and erlotinib were compared with chemotherapy in the *EGFR* mutant group and PFS prolongation was detected (11 months vs 6 months). According to these results, anti-*EGFR* agents has been approved in first-line treatment with *EGFR* mutant patients (19). In the following years, second-generation anti-*EGFR* agents, afatinib and dacomitinib, were introduced and showed superiority of PFS compared with gefitinib. The difference of these agents from the first generation is that they inhibit irreversible EGFR with HER2 and HER4. In addition, afatinib provided longer median OS versus chemotherapy in exon 19 mutant patients (20). Although targeted agents show very promising results at the beginning, they lose their effectiveness due to resistance mechanisms developed within the median 10-16 months. The most important resistance mutation is *EGFR* T790M, which is detected at around 50% and is much less at the time of diagnosis (21). In order to overcome this resistance mechanism, the third generation of anti-*EGFR* agent called osimertinib is used. The FLAURA study has shown that osimertinib provides significant PFS contribution to gefitinib and erlotinib in the first-line treatment in the *EGFR* mutant group (18.9 months vs. 10.2 months; 0.46; 95% confidence interval [CI], 0.37 to 95). 0.57; P <0.001) (22). In addition, ESMO 2019 published OS data for this study and showed that osimertinib provides longer OS with 38.8 months versus 31.8 months (p: 0.0462) (23). With this result, osimertinib can be considered to be rapidly displaced from second-line treatment to first line. In our study, 9 activating *EGFR* pathogenic variants were detected and afatinib was started in 4 and erlotinib was started in 4 of these patients. *KRAS* mutation is another common mutation in NSCLC and it is known to be around 25% on average. Although the survival of the KRAS mutant group was found to be shorter, targeted agents did not benefit. However, the *KRAS* mutation is thought to be a cause of intrinsic resistance to anti-*EGFR* agents (24). Despite the negative effects against anti-*EGFR* agents, recent studies have shown that *KRAS* mutation is associated with increased tumor-infiltrating lymphocyte, PD-L1 and tumor mutation burden and is thought to be a biomarker for immunotherapy agents (25). In our study, although 5 patients (9.8%) were *KRAS* mutated, 1 was found to be concomitant with activating *EGFR* mutation. In this patient, chemotherapy was given because of anti-*EGFR* resistance. If *EGFR* mutation was examined by conventional method and positivity was detected, anti-*EGFR* agents would be prescribed. As a result, treatment would be unresponsive and would cause unnecessary costs. However, better and more personalized treatments can be planned for patients because NGS can be detected at the same time in both targeted mutations and other mutations that are resistant to them.

Phosphatidylinositol 3-kinases (PIK3) play a major role in cell metabolism, migration, growth, and proliferation. PIK3 and AKT are important components of the EGFR pathway and induce oncogenesis and progression in LC. *PIK3CA* mutation is detected in 27% of glioblastoma, 25% of gastric cancer, 32% of colon cancer, and 1-4% of lung adenocarcinoma and the most common type of *PIK3CA* mutation is E545K (57.1%) (26-27). It is more common concomitantly with other mutations in LC cancer and the role of resistance to anti-*EGFR* agents is still controversial. However, in several studies, it was found that the survival of patients with concomitant *EGFR* and *PIK3CA* mutation with anti-*EGFR* agents was not different (28), *PIK3CA* c.1633G> A (p.E545K) mutation was found to be resistant to gefitinib in one trial (29). In our study, *PIK3CA* was found to be singular in 2 patients (3.9%) and concomitantly in 4 patients (7.8%). Two of the *PIK3CA* mutations were concomitantly with *EGFR* and c.1633G> A (p.E545K) was detected in one of them. Afatinib was applied instead of gefitinib in that patient due to possible resistance. It is thought that with the increasing use of NGS, the term known as class effect of drugs in oncology will lose its place to personalized medicine. The class effect is when an *EGFR* mutation detected any anti-*EGFR* agents can be given, but the concomitant mutations show us there is resistance to some of these drugs but not some the others. In this way, the clinician can determine which drug is given to the patient and get better results by NGS method.

Phosphatase and tensin homologously deleted in chromosome 10 (*PTEN*) acts as a very strong tumor suppressor, in case of mutation the tumor passes through the PI3K / mTOR / Akt pathway to an uncontrolled growth phase (30). According to TCGA, it is 15% positive in lung squamous carcinoma and 3% in adenocarcinoma and is generally accepted as a poor prognostic factor (31). On the other hand, in a study with 162 Korean NSCLC patients were included, *PTEN* mutation was detected in 4 (2.5%) cases by NGS method (32). In addition, in two separate studies, it was found that decreased *PTEN* expression caused resistance to gefitinib and erlotinib (33-34). In our study, *PTEN* mutation (R233) was present in 2 patients (3.9%), one of which was associated with *EGFR* mutation. In order to prevent possible resistance to erlotinib and gefitinib, second-generation anti-*EGFR* afatinib was given to the patient with this concomitant mutation.

B-Raf (*BRAF*) is one of the most important protooncogenes and is detected between 2-4% in NSCLC, while it is around 3-7% mutant according to TCGA. The most common type of mutation is the *BRAF* V600E mutation, which is mostly detected in women and non-smokers (35). After the success of anti-*BRAF* treatments in malignant melanoma, and detection of *BRAF* mutant patients in LC have been found to have a shorter survival, it has been decided to use *BRAF* targeted therapy in lung cancer (36). *BRAF* and *MEK* inhibitor combination therapies have been tried since single-agent *BRAF* inhibitors did not provide superiority to chemotherapy in the first studies. In the Phase 2 study, dabrafenib was administered to previously treated patients in cohort A (78 patients), dabrafenib + trametinib to previously treated patients in cohort B (57 patients), and dabrafenib + trametinib to previously untreated patients in cohort C (36 patients). In the Cohort C, complete response was obtained in 2 patients and the overall response rate was 64%. In addition, median PFS was found to be 10.9 months and OS 24.6 in this cohort (37). FDA approval was obtained after these results and the guidelines were

included *BRAF+MEK* inhibitor therapy in the first-line treatment of NSCLC with *BRAF* V600E mutated. In our study, *BRAF* V600E mutation was detected in a non-smoking female patient and dabrafenib + trametinib treatment was planned.

The use of NGS, which was gradually increasing after finishing TCGA, was at a high cost in the early days, but over the years have been reduced to affordable prices (38). Detecting individual mutations by conventional methods seems to be more cost-effective at first, but the clinical results suggest the opposite. Pennel et al. found that the use of NGS in a health plan involving 1000,000 people was associated with both shorter time and significant cost savings than conventional methods (39). In our country, in fact, one-to-one comparative evaluation of *EGFR, ALK, ROS1* should be spent on average 100-150 USD (approximately 600 Turkish Liras) for conventional methods, while the cost of our NGS panel used in our hospital is 300-350 USD (approximately 1850 Turkish Liras) (according to the data obtained from the purchasing unit of our hospital). However, as the number of patients increases, it is expected that NGS costs will decrease. In addition, for example, if the *EGFR* mutation and concomitant resistance mutation cannot be detected by conventional methods at the diagnosis, the targeted agent to be administered may be ineffective. There are two similar examples in our patient group: gefitinib resistance EGFR mutant patients were detected with NGS and afatinib was applied. As a result, more money is given to the test, but no more money is unnecessarily spent on the whole treatment of the patient. In our country, the health insurance of individuals are covered by the state and have an important place in the budget of the state. Therefore, it is thought that the use of NGS method will be more beneficial for both the patients and the health service provider.

In addition to the positive features of the NGS method, there are also disadvantages (40): The first of these is the ability to detect error in continuously repeating sequence regions. However, when this error persists, it can be understood by the geneticist that it is a mistake. Another is that sometimes certain decisions about the effect of genes cannot be made due to the finalization of the resulting raw data through many software programs. For example, in our study, 2 *BRAF,* 2 *HER2*, 2 *PIK3CA*, and 1 *EGFR* mutations were identified as clinical uncertain significance variants. If these mutations were considered pathogenic, targeted agents could be added to the treatment of 4 of these patients. The last disadvantage is that, although it is gradually decreasing due to the increase in the use of NGS, false-negative results are seen in the liquid biopsy around 20-30%. In our study, we did not detect any *ALK* mutations with NGS, but we detected that one of our patients had *ALK* mutation by FISH method and we achieved a complete metabolic response at 3 months after alectinib use. Therefore, it is the role of the clinician to evaluate the results carefully and confirm them with another method if there is discordance.

The limiting points of our study were retrospective nature, small number of patients, and lack of response to treatment. However, the results of our article are important as there are very few studies showing the effect of NGS in clinical use.

Conclusion

LC is one of the deadly cancers. With the help of targeted agents, patients' survival is extended in a more comfortable way than chemotherapy. Therefore, it is the first duty of the clinician to determine the appropriate patients for the targeted therapies and to initiate the treatment. NGS, which have increased using in recent years, is a method that quickly and accurately identifies all targeted genetic variants and resistance variants at the same time. According to the results, we have shown, NGS has helped us to apply more personalized and more effective treatments to patients for achieving longer survival. In addition, the use of NGS method instead of conventional methods has saved time and cost for both patients and health service providers. For these reasons, it would be more beneficial to use NGS method wherever appropriate.

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