

Molecular Genetic Testing and Liquid Biopsy in Lung Cancer: Present and Future

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ABSTRACT

The genetic landscape of lung cancer has been expanded over decades with advancements in molecular genetic technologies. Despite improvements, the survival rate of lung cancer is still low. When diagnosed at an early stage, resection of tumor or lobectomy is possible, survival rate increases accordingly. Therefore it is crucial to identify diagnostic or predictive biomarkers and develop new technologies which can efficiently analyze these biomarkers. Since lung tumor tissue is difficult for sampling and requires invasive procedures, identification of non-invasive blood-based tumor biomarkers has become attractive recently. This review will summarize clinically significant key genetic biomarkers and focus on liquid biopsy which means analyzing of noninvasive biomarkers such as circulating tumor DNA (ctDNA), circulating tumor cells (CTCs), circulating miRNAs and exosomes.

Keywords: Lung cancer, genetic biomarkers, liquid biopsy

INTRODUCTION

Lung cancer is the second most common cancer type in the world, and its leading cause of cancer-related deaths in both sex. There are two main types of lung cancer. Small cell lung cancer (SCLC) accounts for about 10-15% of all lung cancers whereas non-small cell lung cancer (NSCLC) accounts for about 80-85%. It is estimated that 85% of all lung cancers are responsible for smoking. The survival rate is approximately 5 years despite medical care. Surgery, radiotherapy, chemotherapy and targeted therapy are the main therapeutic strategies. These treatments can reduce tumor growth but usually, relapse occurs. Genetic heterogeneity and tumor plasticity contribute to drug resistance and metastasis which both are responsible for mortality.

The survival rate of lung cancer is low; 5 years. This mainly because early diagnosis rate is still low, most cases are diagnosed with an advanced-stage when there is no effective curative treatment. Survival rate increases, when diagnosed at an early stage (Stage I and II) since resection of tumor or lobectomy, are possible at an early stage. Hence early diagnosis is very crucial. Currently available methodologies for using diagnosis have several limitations. Chest X-rays, for example, are not enough sensitive for lung cancer detection. More detailed type of chest x-ray, computed tomography (CT) is highly sensitive but specificity is low (1). It is therefore important to develop minimally-invasive or non-invasive methods for screening lung cancer.

Recent advances in molecular genetics technologies provide deeply understanding of tumor biology, response to treatment

and identification of diagnostic/prognostic biomarkers. Since lung tumor tissue is difficult for sampling and requires invasive procedures, identification of non- or minimally-invasive blood-based tumor biomarkers has become attractive recently. This review will summarize clinically significant genetic biomarkers and focus on liquid biopsy which means analyzing of noninvasive biomarkers such as circulating tumor DNA (ctDNA), circulating tumor cells (CTCs), circulating miRNAs and exosomes.

Key Diagnostic and Prognostic Genetic Biomarkers in Lung Cancer

Correlation between tumorigenesis and genetic alterations was first proposed by Nowell In 1976. Later on, progresses in the field of cytogenetics, molecular genetics and innovations in genomics technologies have demonstrated that cancer is driven by diverse genomic alterations. Especially, advances in sequencing technologies have revealed a genomic landscape of cancer. The first revolution began with first-generation sequencing era, which was used in Human Genome Project. The complete sequence of nucleotide base pairs of human DNA and mapping all of the genes were established by this project. By using first-generation sequencing technologies protooncogenes and 'driver' mutations have been identified. Driver mutations usually occur in genes of signaling proteins which are critical for the proliferation and survival of the cell and as a result, they cause a normal cell to transform into cancer. KRAS and TP53 mutations were earliest identified mutations in non-small cell lung cancers (NSCLC). However, first clinically significant mutations were identified in 2004 in epidermal growth factor receptor (EGFR). These mutations were

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Received: 21.02.2018 • **Accepted:** 09.04.2018

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detected specifically in tumor tissues of lung cancer patients who responded to tyrosine kinase inhibitor (TKI) treatment (2). EGFR mutation is the second most common mutation after KRAS in lung adenocarcinoma in America (about 15% of African Americans and Caucasians) and in Asian populations (nearly 60%). EGFR mutations usually occur in exon21 (L858R) and exon 19 (small insertions and deletions) and these mutations cause an activation of the oncogenic signaling pathway (3). Furthermore, these mutations cause tumor cells to be sensitive to EGFR TKIs such as first generation inhibitors gefitinib and erlotinib (4).

ALK and ROS1 rearrangements are less commonly seen, <5% of lung cancers, firstly described in 2007 in lung adenocarcinomas (5-6). It was shown that Crizotinib, an inhibitor designed for a proto-oncogene receptor tyrosine kinase, Met, was found to respond in patients with ALK and ROS1 rearrangements in NSCLC as their ATP-binding sites share 77% amino acid identity. Although these genomic alterations are rare, they are commonly seen among non-smokers and seen almost solely in adenocarcinomas (7-8). Therefore, it is suggested that all patients with advanced lung adenocarcinoma should be assessed for ALK-ROS1 rearrangements and EGFR mutations regardless their smoking status (9).

Despite responded targeted therapy, relapse usually occurs after about one year following EGFR TKIs treatment, and a median of 8 and 19 months following after first-line targeted therapy with ALK and ROS1 alterations, respectively (8, 10). EGFR mutation (T790M) is the main cause of resistance. At the time of relapse, around 50-60% of patients acquire EGFR mutation. Other resistance mechanisms are activation of PIK3CA pathway, Met amplification and transformation of NSCLC to SCLC (small cell transformation) (11). Crizotinib resistance in ALK-rearranged patients mostly causes secondary ALK mutations. Mechanism of Crizotinib resistance in ROS1-rearranged patients is less well defined. However, there are some individual cases have been reported with ROS1 mutations (12-13).

Immune checkpoint blockade, in other words, immunotherapy, has great attention recently. Immunotherapeutic agents target proteins which keep T cell response under control during inflammation. It's well known that tumor cells can evade immune response by using several mechanisms, for example, upregulation of surface programmed death ligand-1 (PD-L1) which enables them to evade T cell-mediated response. There are approved immunotherapeutic agents for lung cancer treatment which include anti-PD-L1 and anti-programmed death-1 (PD-1) (14). Taube JM showed that tumor cell surface PD-L1 expression is associated with responsiveness to PD-1 blockade and there is a correlation between PD-L1 expression level and therapy response in patients with upregulated PD-L1 expression (15). Pembrolizumab was an approved therapy for only use in patients with 50% or more PD-L1 expression level based on randomized controlled trials (16). On the other hand, according to retrospective analyses, patients with EGFR mutations or ALK alterations demonstrate a low response to immunotherapy. Therefore, Pembrolizumab is approved only for ALK- or EGFR-negative patients. Efficacy of immunotherapy in ROS1-rearranged patients is less known.

Other significant oncogenic mutations are seen in BRAF, ERBB2, MET, RET and KRAS. These targetable alterations are still under clinical investigation in lung adenocarcinoma (12, 17-20). In addition, tumor suppressor mutations are suggested to have prognostic value. TP53 and RB1 mutations are suggested to have predictive roles for small cell transformation after EGFR TKI treatment in adenocarcinoma.

Blood-Based Biomarkers

Circulating tumor cells (CTCs)

Circulating tumor cells (CTCs) have been identified in blood circulation from cancer patients. Some tumor cells are thought to have left the tumor and joined into the vasculature or lymphatics. Therefore it is important to isolate them to have an information about their origin non-invasively. CTCs are extremely rare in the circulation, only between 5 and 50 CTCs per 5 ml of cancer patients' blood sample (21). Although they are rare, they have a potential to be used as a biomarker for tumor characterization, prognosis, monitoring cancer status and detection of recurrent (22). The presence of CTCs has been found to be related to poor outcome in metastatic NSCLC patients. A study showed that CTC number has a predictive role of overall survival (OS) in NSCLC. CTCs were collected before and after treatment of one cycle standard chemotherapy from 101 patients. The number of CTCs was higher in patients with stage IV NSCLC compared to stage IIIB or IIIA and number of over 5 CTCs per 7,5 mL were associated with shorter progression-free survival (PFS) and overall survival (OS) in patients with NSCLC (23). There is also a meta-analysis comprising 20 trials with 1,576 patients assessed the prognostic relevance of CTCs. CTCs were found to be associated with tumor stage and lymph node metastasis. Furthermore, there was a significant association between CTCs and shorter overall and progression-free survival (24).

A study of 56 patients showed that CTCs might be a predictor of recurrence after surgery in early-stage NSCLC. For CTC analysis, blood samples were collected before and one month after surgery. The mean number of CTCs was 3.16/10 mL before surgery and the number decreased to a mean number of 0.66 one month after the surgery. There was a significant association between the presence of CTCs after the surgery and early recurrence and a shorter disease-free survival (DFS) (25).

There is a study presented in 2017 Multidisciplinary Thoracic Cancers Symposium. Blood samples of 48 patients were collected before, during, and after concurrent chemoradiation. 15 of 48 patients had a recurrence. No CTCs were detected in all patients following treatment but the number of CTCs increased in subsequent tests. This increase became detectable an average of 6 months before radiographically validation of recurrence. Although these results are promising they need further validation in larger patient cohorts.

Circulating tumor DNA (ctDNA)

Circulating tumor DNA (ctDNA) was first identified in 1977 but gained attention only recently as sequencing technologies have been advanced in the last decade. Researchers should be aware

of ctDNA is a different term from cell-free DNA (cfDNA). cfDNA comprises all cell-free DNA in circulation irrespective of their origin. However, ctDNA describes tumor-derived freely circulating DNA.

Cell-free DNA can be detected in all individuals at some level but tumor cell-derived ctDNA is proportional to the overall disease burden and therefore it is not always detectable (26). The amount of plasma ctDNA can vary from 0,01% to 90% of all cfDNA (27). The less the ctDNA ratio is, the more difficult it is to detect. A genotyping of cell-free DNA study showed that known EGFR and KRAS mutations are detectable in 100% of lung cancer patients with four or more metastatic sites and about 60% of those with a single metastatic site (28). Current technologies cannot detect ctDNA levels efficiently in early-stage disease (29). Therefore sensitive and reliable detection methods are required for clinical use. For lung cancer, EGFR activating mutations and EGFR TKI resistance mutation T790M are most studied mutations in ctDNA. Real-time PCR, digital droplet PCR (ddPCR) and NGS are methods currently used in routine, however, FDA approved plasma EGFR mutation test is cobas EGFR mutation test v2 (Roche Diagnostics, Indianapolis, IN, USA). Clinicians should be aware of false negative results. Because of the limited sensitivity of ctDNA mutation test, the FDA approval suggested a routine tissue biopsy and repeating the test in tumor tissue when a plasma assay is negative. Detection of either plasma or tissue EGFR mutations has the same degree of EGFR TKI response (9).

Studies showed that there is a high mutation concordance between ctDNA and tumor tissue. Therefore ctDNA is suggested to serve as a biomarker. A phase IV, open-label, single-arm study NCT01203917, evaluating first-line gefitinib, showed that EGFR mutation can be accurately detected with high concordance, specificity, and sensitivity by using ctDNA in advanced-stage NSCLC patients. Mutation concordance rate was 94.3% with a 95% confidence interval between 652 matched plasma and tumor samples in EGFR-positive NSCLC before treatment (30).

Another study found that EGFR mutation concordance rate was 92.9% with a sensitivity of 85.7% in matched serum and tumor samples obtained from 42 patients with advanced-stage NSCLC treated with gefitinib (31).

Two independent meta-analyses assessed the diagnostic accuracy of EGFR mutations in cfDNA and they found the sensitivity of 67.4% and 61% and specificity of 93.5% and 90% and respectively (32-33).

The ctDNA levels have been found higher in NSCLC patients compared to healthy subjects. (34-35). There was also an association between ctDNA levels and prognosis according to the study of Catarino et al. (6) High pretreatment ctDNA levels presented a lower mean survival time in NSCLC patients who received a first-line platin-based doublet chemotherapy in combination with a third-generation cytotoxic agent (36). Another study demonstrated a significant correlation between the increased concentration of plasma ctDNA and tumor progression following chemotherapy, advanced stage of the tumor and poor survival (37).

Newman et al. (23) introduced a sensitive technique for ctDNA quantifying. It is called cancer personalized profiling deep sequencing (CAPP-Seq). ctDNA was detectable in 100% of patients with stage II-IV and in 50% of patients with stage I NSCLC with a specificity of 96%, indicating its prognostic value. There was a significant association between ctDNA levels and tumor volume and discriminated between treatment-related and residual disease imaging changes. ctDNA levels provided an earlier predictive response than radiographic techniques (38).

The ctDNA analysis gives a clinician opportunity of monitoring minimal residual disease, possible tumor recurrence, and drug resistance. By regular ctDNA analysis during progression or tumor recurrence, new mutation can be earlier detected which cause resistance to first-generation inhibitors. However, there are some limitations need to be overcome. For example, a priori knowledge of the target gene of interest is required in most cases. Not all tissue-derived DNA mutations are expressed in ctDNA. Detection is difficult because of a high background of non-tumor cfDNA. Despite challenges, it's promising to be widely recognized in clinical practice in future.

Circulating microRNAs

There is growing attention to identifying non-invasive biomarkers as well as circulating microRNAs (miRNAs) for diagnosis, monitoring response to treatment. miRNAs are small non-coding RNAs, 19-22 nucleotides in length. They regulate gene expression in a negative manner through binding their target mRNAs. Dysregulation of miRNA expression was reported in several cancer types as well as lung cancer. miRNAs serve not only as a diagnostic biomarker but also as potential prognostic markers. Altered miRNA levels contribute to cancer formation and resistance to cancer treatment. Expression levels of miRNAs among lung cancer patients and healthy individuals were found significantly different in various studies. For example, in one study plasma samples from 100 early stage (I to IIIA) NSCLC patients and 100 healthy controls were screened for 754 plasma microRNAs and they identified a 24-miRNA expression panel which could distinguish lung cancer patients from healthy controls. When adding age, sex, and smoking status into this model, diagnostic power can be further enhanced (39). Another 6-miRNA expression panel has been shown to discriminate NSCLC patients from healthy individuals.

Serum or plasma miRNAs might be useful biomarkers not only for diagnosis but also for prognosis. A genome-wide serum miRNA expression analysis found 4 miRNAs (miR-1, miR-486, miR-499, and miR30d) have potential to be a predictor of overall survival in NSCLC patients. Patients harboring two or more high-risk miRNAs showed decreased survival compared to patients with one or no high-risk miRNA (40). Another study found that serum *miR-125b* expression was significantly associated with NSCLC stage and the high miR-125b level was a predictor of poor survival in a screening of 193 NSCLC patients (41). Although the studies are suggesting a potential role of circulating miRNAs as novel biomarkers, further validation is necessary for quantification of these miRNAs.

Circulating exosomes

Exosomes are nano-sized vesicles with a diameter of 30-150 nm. Exosomes are released from any cell and they can be found in various body fluids such as blood, urine, ascites or semen. It has been shown that tumor cells release higher amounts of exosomes than normal cells (42). Exosomes have different roles such as contributing tumor growth, metastasis, drug resistance and immunomodulation () through their cargo; DNA, proteins, lipids, mRNA, microRNA and other non-coding RNAs (43-46). These nanovesicles are stable in circulation, they are not degraded by RNase or proteinases which makes them suitable biomarker for clinical applications.

A study was carried out to elucidate potential roles of exosomes and their content in lung adenocarcinoma. This study showed that there is a significant difference in the level of total circulating exosome and miRNA levels between lung adenocarcinoma patients and healthy controls. It was also suggested that circulating exosomal miRNA have a potential to be a screening test for lung cancer since the patterns of tumor-derived miRNA and circulating exosomal miRNA were similar. However, there was no correlation between exosomal miRNA levels and the stage of disease (47)

Exosomal miRNAs are the most studied molecules. Unlike circulating plasma miRNAs, exosomal miRNAs are stable and protected from RNase degradation. the mir-21 level was found significantly high in NSCLC patients compared to healthy individuals (48).

Exosomes are not utilized in clinical practice currently, however, preliminary results demonstrated that there is a correlation between tissue and exosomal biomarkers. For example, a clinical case report of Taverna et al.(43) showed that plasma exosomes isolated from a chemo-naive 70 years old stage IV NSCLC patient harbor an EGFR activating mutation by a deletion in exon 19 (49).

CONCLUSION

Circulating tumor biomarkers, liquid biopsy, in other words, might be a useful test for diagnosis, predicting outcomes, monitoring disease status and treatment response in lung cancer patients. It is well known that lung cancer is most frequently diagnosed in an advanced stage. This is mainly because of a lacking screening test for the disease and therefore liquid biopsy can be a useful tool. Some of circulating biomarkers have already been recognized in routine clinical practice, for example, plasma EGFR mutation test. However present evidence for other biomarkers are not still sufficient to be included in routine tests. Further validation is needed with a larger cohort of patients in randomized clinical trials and longer monitoring. Combined assessment of these biomarkers could be a strategy to monitor dynamic changes during therapy.

There are still limitations of technologies to detect circulating biomarkers but there is a great effort in developing new sensitive and specific technologies. Advances in methodologies will provide not only identify and validate new biomarkers but also a new dimension to personalized care.

Peer-review: Internally reviewed.

Author contributions: Concept - S.E., S.O.B; Design - S.E., S.O.B; Supervision - S.O.B; Literature Search - S.E., S.O.B; Writing - S.E., S.O.B; Critical Reviews - S.O.B.

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

Financial Disclosure: The authors declared that this study has received no financial support

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How to cite:

Eroğlu S, Oğuzkan Balcı S. Molecular Genetic Testing and Liquid Biopsy in Lung Cancer: Present and Future. *Eur J Ther* 2018; 24(Suppl 1); S14–S18.