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Cell-free DNA in non-small cell lung cancer

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Lung cancer is the leading cause of cancer-associated deaths worldwide. Surgery is the standard treatment for early-stage non-small cell lung cancer (NSCLC). Advances in the knowledge of the biology of non-small cell lung cancer have revealed molecular information used for systemic cancer therapy targeting metastatic disease, with an important impact on patients' overall survival (OS) and quality of life. However, a biopsy of overt metastases is an invasive procedure limited to certain locations and not easily acceptable in the clinic. The analysis of peripheral blood samples of cancer patients represents a new source of cancer-derived material, known as liquid biopsy, and its components (circulating tumour cells (CTCS), circulating free DNA (cfDNA), exosomes, and tumour-educated platelets (TEP)) can be obtained from almost any body fluids. These components have shown to reflect characteristics of the status of both the primary and metastatic diseases, helping the clinicians to move towards a personalized medicine (1). This review focuses on the liquid biopsy component – circulating free DNA, its benefit for non-invasive screening, early diagnosis, prognosis, response to treatment, and real time monitoring of the disease in non-small cell lung cancer patients.

Keywords: cell-free DNA, non-small cell lung cancer, monitoring, liquid biopsy

INTRODUCTION

Lung cancer is the world's leading cause of cancer-related mortality. Approximately 80% of all lung cancer cases are non-small-cell-lung cancer (NSCLC) patients, the majority of whom present with a locally advanced or metastatic disease (2, 3). The majority of patients are diagnosed at late stages and have local or systemic advanced disease (stage III or IV) with 5-year survival rates of <5%. Over half of lung cancer patients die within one year of diagnosis (4, 5). Therefore, there is a great demand for new diagnostic and treatment options based on specific biomarkers, preferably detectable in tumour surrogate specimens derived by non-invasive procedures. The discovery of extracellular DNA circulating in blood, the so-called cell-free DNA (cfDNA), may greatly impact molecular diagnostics of lung cancer patients due to a simple, non-invasive access to genetic material

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detectable in the plasma and serum by sensitive molecular biology techniques (6).

Liquid biopsy: Cell-free DNA (cfDNA)

The term "liquid biopsy" qualifies different potential approaches for the detection of biomarkers found in circulating blood in cancer patients. Liquid biopsy analysis is a rapidly expanding field in translational cancer research and might be useful at different points of the diagnostic/therapeutic course of cancer patients, such as early diagnosis, estimation of the risk for metastatic relapse or metastatic progression (prognostic information), stratification and real-time monitoring of therapies, identification of therapeutic targets and resistance mechanisms (predictive information), and understanding the metastasis development in cancer patients (1).

Considering the fact that the lung parenchyma is highly vascularized, the interconnection between lung cells and peripheral blood is very close. The first identification of cfDNA in blood was reported by Mandel and Metais in 1948 (3, 7). Since then, cfDNA has found applications in many disciplines of medicine but particularly in the evaluation of foetal DNA in the circulation of expectant mothers as a form of non-invasive prenatal (NIP) testing (3, 8). Studies have demonstrated that circulating cfDNA exists at steady-state levels and increases, sometimes dramatically, with cellular injury or necrosis (9). In oncology, detection of cfDNA derived from tumours, also known as circulating tumour DNA (ctDNA), has been challenging for three primary reasons, which include: (1) discrimination of ctDNA from normal cfDNA, (2) the presence of sometimes extremely low levels of ctDNA, and (3) an accurate quantification of the number of mutant fragments in a sample (10).

Discriminating ctDNA from normal cfDNA is aided by the fact that tumour DNA is defined by the presence of mutations. These somatic mutations, commonly single base-pair substitutions, are present only in the genomes of cancer cells or precancerous cells and are not present in the DNA of normal cells of the same individual. This juxtaposition assures ctDNA exquisite biologic specificity as a biomarker. Accordingly, all the DNA sequencing methodologies that identify somatic variants could be used easily to identify ctDNA if tumour DNA fragments were abundant in the circulation of patients with cancer. Unfortunately, the detection of cfDNA derived from tumours carries substantial challenges, largely because ctDNA often represents a small fraction (1.0%) of the total cfDNA. Therefore, standard sequencing approaches like Sanger sequencing or pyrosequencing can only detect tumour- derived mutant fragments in patients with a heavy tumour burden and high levels of ctDNA (9–12).

cfDNA is extracellular DNA detectable in blood. The presence of cfDNA can be found in patients with malignant pathologies, but also in healthy individuals and in patients with non-malignant diseases, namely, erythematic lupus, rheumatoid arthritis, lung embolism, myocardium infarction, traumas, or invasive therapeutics practices (13). The size of the DNA released from dead cancer cells varies between small fragments of 70 to 200 base pairs and large fragments of about 21 kb and is longer than that of non-neoplastic DNA. In cancer patients the release of cfDNA may be the result of apoptotic and necrotic processes, which are characteristic of tumours with a high cellular turnover. The concentration of cfDNA in serum of cancer patients is about four times higher than that of healthy controls. According to the majority of the quantitative studies performed until the present time, cell-free circulating DNA is observed in healthy subjects at concentrations between 0 and 100 ng/ml of blood with an average of 30 ng/ml, whereas in cancer patients the concentration in plasma or in serum varies between 0 and 1000 ng/ml, with an average of 180 ng/ml (14).

Several driver mutations have been found in NSCLC, such as *EGFR* (epidermal growth factor receptor), *KRAS* (V-Ki-ras2 Kirsten rat sarcoma), *BRAF* (B-Raf proto-oncogene, serine/threonine kinase) or *HER2* (erb-b2 receptor tyrosine kinase 2). The detection of *HER2* mutations in cfDNA has not been evaluated (15).

In one study (15), the detection of *BRAF* mutations in cfDNA has been associated with lower PFS (progression-free survival) and OS (overall survival) in various types of tumours, such as melanoma ($P = 0.021$; 3.6 *vs.* 13.4 months for PFS and $P = 0.017$; 7 *vs.* 21.8 months for OS). However, to date the association between clinical outcomes of

NSCLC patients and the detection of *BRAF* mutations in cfDNA has not been investigated.

Another study has shown (16) that cfDNA for *EGFR* mutation detection could be an efficacious tool to predict the clinical outcomes of *EGFR* tyrosinekinase inhibitor (TKI) therapy. Patients were randomized to receive six cycles of gemcitabine/platinum plus sequential erlotinib or placebo. Blood samples at baseline, cycle 3, and progression were assessed for blood test detection rate, sensitivity, and specificity; concordance with matched tumour analysis ($n = 238$), and correlation with PFS and OS. Both the tissue and blood tests detect 41 *EGFR* mutations (including G719A/S/C in exon 18, deletions and complex mutations in exon 19, S768I, T790M, and exon 20 insertions, and L858R in exon 21). To be classified as *EGFR* mutation positive for this analysis, at least one activating mutation (exon 19 deletion, L858R, G719x, or L861Q) had to be identified in a sample.

Interestingly, the quantification of *EGFR* mutations has been demonstrated to correlate with early prediction of the clinical response to *EGFR* TKIs. Sixty-nine patients with *EGFR*-mutated tumours received erlotinib therapy and 21 negative control cases. Study shows that *EGFR* testing at baseline and serially at 4 to 60 days during tyrosine kinase inhibitor therapy revealed a progressive decrease in a semiquantitative index (SQI), starting from day 4, in 95% of cases. The rate of the SQI decrease correlated with per cent tumour shrinkage at two months ($p < 0.0001$); at 14 days, it was more than 50 in 70% of patients (rapid responders) (17).

The strength of cfDNA to predict the response to treatment has also been reported in a multivariate analysis, in which 246 advanced-stage NSCLC patients were screened for *KRAS* mutations in plasma before initiation of first-line chemotherapy. 17.5% presented with a *KRAS* mutation. OS was 8.9 months and PFS by intention to treat 5.4 months. Patients with a detectable plasma-*KRAS* mutation had a significantly shorter OS and PFS compared to the wild type (WT) patients (median OS 4.8 months versus 9.5 months, HR 1.87, 95% CI 1.23–2.84, $p = 0.0002$ and median PFS 3.0 months versus 5.6 months, HR 1.60, 95% CI 1.09–2.37, $p = 0.0043$). A multivariate Cox regression analysis confirmed the independent prognostic value of pm*KRAS* in OS, but not in PFS. The response rate to chemotherapy was significantly lower in the group of patients with a mutation compared to WT ($p < 0.0001$) (18).

Szpechcinski et al. (6) found significantly higher plasma cfDNA levels in NSCLC patients (mean 8.02 ± 7.81 ng ml^{-1}) than in subjects with a chronic respiratory inflammation (3.36 ± 1.80 ng ml^{-1}) and healthy individuals (mean 2.27 ± 1.51 ng ml^{-1}; $p < 0.0001$). Using real-time PCR, the authors measured plasma cfDNA concentrations before treatment in 50 resectable NSCLC patients, 101 patients with a chronic respiratory inflammation (chronic obstructive lung disease, sarcoidosis or asthma), and 40 healthy volunteers. They did not find significant differences in plasma cfDNA levels between patients with a chronic respiratory inflammation and healthy individuals.

In a meta-analysis of ten studies (19) regarding the diagnostic accuracy of cfDNA for lung cancer screening, the authors described a pooled specificity of 77% and sensitivity of 80%, and deduced that cfDNA alone is not suggested for lung cancer screening because of its rather low discriminative power. Studies were included in the meta-analysis if they provided both the sensitivity and specificity of circulating plasma DNA levels for the diagnosis between lung cancer and healthy controls. This meta-analysis included 752 lung cancer patients and 635 healthy controls. Lung cancer was confirmed by histological examination. According to the authors, the results of the circulating DNA assay should be interpreted in parallel with the results of conventional tests including cytological/histological examination and chest CT. The value of circulating DNA assay combined with conventional markers for lung cancer detection deserved further investigation.

The current evidence suggests that the diagnostic accuracy of quantitative analysis of circulating DNA is not lower than conventional serum biomarkers for lung cancer screening, at least. The value of circulating DNA assay in combination with conventional markers for lung cancer detection deserved further investigation.

Cell-free DNA in non-invasive screening, early diagnosis, and disease prognosis

Radiological imaging studies are only able to detect tumours when they are approximately 7–10 mm

in size and contain ~1 billion cells. As the volume of the tumour increases, the number of apoptotic and dead cells increases due to the increased cellular turnover (20). One of the aims of determination of total cfDNA is to use cfDNA as a marker for early cancer detection and disease prognosis.

Bettegowda et al. (21) evaluated the ability of ctDNA to detect tumours in 640 patients with various cancer types. The study showed that ctDNA is a broadly applicable, sensitive, and specific biomarker that can be used for a variety of clinical and research purposes in patients with multiple different types of cancer.

Ludovini et al. (22) demonstrated significantly higher plasma DNA levels in 76 lung cancer patients (mean 60.0 ± 99.8 ng ml/1) compared with 66 controls (mean 6.0 ± 8.8 ng ml 1, $P < 0.0001$). There were no significant differences in plasma DNA levels according to the NSCLC stage and histology.

Drift et al. (23) found a high circulating plasma DNA concentration at the time of diagnosis in NSCLC patients was a prognostic factor for poorer survival. The study included 46 untreated NSCLC patients, and 21 controls with a follow-up time of 6.5 years were analyzed. Median OS was significantly decreased for the patients compared to the controls (13.7 months (range 1.1–78.4) and 67.6 months (range 2.0–78.2), respectively; $p < 0.001$). The median DNA concentration of the patients who died ($n = 40$) was significantly higher compared to the patients that survived ($n = 6$) at the end of the follow-up (55 ng/ml versus 23 ng/ml, $p = 0.02$).

Circulating DNA may be used as a non-invasive biomarker requiring only a blood sample to refine the prognostic profile in NSCLC patients.

Cell-free DNA to monitor response and resistance to treatment

Another sought-after use of the cfDNA technology is the ability to follow patients over time for their response to treatment. Methods that specifically classify patients based on the presence of minimal residual disease are not currently available. In practice, clinical and pathologic criteria are used to predict patients who probably harbour residual disease. The leading system parameter utilized for this purpose is the TNM classification of malignant tumours (TNM) (15). cfDNA can be a useful biomarker to detect residual disease after surgery and may provide evidence to select patients who are likely to suffer recurrence.

Sozzi et al. (24) reported that the cfDNA measurement allowed discriminating patients with radically resected primary NSCLC (stages I–III) from healthy individuals, suggesting that the quantification of plasma DNA might represent a new approach to the monitoring of surgical procedures or to the assessment of the efficacy of chemo/radiotherapy. The present study reports the results of plasma DNA quantification by real-time quantitative PCR in the entire cohort of 1,035 volunteers enrolled in the early detection trial and investigated with low-dose spiral CT, based on a minimum follow-up of five years. Of the subjects, 956 remained cancer-free over the five years of the study, 38 developed lung cancer, and 41 developed other tumours. The clinical outcome of lung cancer patients was evaluated for a median follow-up period of 62 months. The study shows that at surgery, plasma DNA was higher in tumours detected at baseline (AUC-ROC, 0.80; $p < 0.0001$) and in stage II to IV tumours detected during the first two years of screening (AUC-ROC, 0.87; $p < 0.0001$). A longitudinal study of plasma DNA levels showed increased values approaching lung cancer diagnosis ($p = 0.001$). Higher plasma DNA was significantly associated with poorer 5-year survival ($p = 0.0066$).

Nygaard et al. (25) found no significant correlation between the total cfDNA and the total tumour burden using Positron Emission Tomography (PET) in advanced NSCLC. The study included 53 patients who received first-line chemotherapy comprising carboplatin in combination with vinorelbine (one patient was additionally treated with bevacizumab in combination with chemotherapy; palliative radiotherapy was given if indicated) and PET/CT scan was performed and evaluated within a month before the blood sampling.

However, Newman et al. (26) found that ctDNA correlates with changes in the tumour burden. In their study, they assessed the sensitivity and specificity of CAPP-Seq for disease monitoring and minimal residual disease detection using plasma samples from five healthy controls and 35 samples collected from 13 patients with NSCLC. ctDNA

was detected in 100% of patients with stage II to IV NSCLC and in 50% of patients with stage I, with 96% specificity for mutant allele fractions down to ~0.02%. Monitoring levels of ctDNA by CAPP-Seq had the potential for measuring the tumour burden in early and advanced stage NSCLC. It was highly correlated with the tumour volume and distinguished between the residual disease and treatment-related imaging changes. The measurement of ctDNA levels allowed for an earlier response assessment than radiographic approaches.

Tissot et al. (27) extracted and quantified the cfDNA of 218 NSCLC patients before and after two or three cycles of platinum-based chemotherapy. Patients with high cfDNA concentrations (the highest tertile) at baseline had a significantly worse disease-free and overall survival than those with lower concentrations (the lowest and middle tertiles) (median overall survival 10 months (95% CI 10.7–13.9) versus 14.2 months (95% CI 12.6–15.8), respectively; $p = 0.001$). However, they did not find any association between the cfDNA concentration and the response to treatment.

"Liquid biopsies" are used to supplement the histological diagnosis of cancer and metastatic disease and in the future these assays may replace the need for invasive procedures. Applications include monitoring of the tumour burden, monitoring of the minimal residual disease, monitoring of tumour heterogeneity, monitoring of molecular resistance, and early diagnosis of tumours and the metastatic disease (20).

CONCLUSIONS

Personalized medicine in oncology relies on the customization of healthcare using molecular analyses. cfDNA is a promising biomarker for the detection and follow-up of NSCLC patients. In clinical practice, cfDNA may serve as an alternative for those patients who are unable to provide an accurate tissue-biopsy sample. It will also serve as an invaluable source of information, complimentary to imaging, during the period of the follow-up after surgery. Lastly, it will reduce the need for invasive sampling in the monitoring of cancer patients.

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NESMULKIŲ LĄSTELIŲ PLAUČIŲ VĖŽIO LAISVAI CIRKUOLIUOJANČIOS DNR DIAGNOSTINĖ IR PROGNOSTINĖ REIKŠMĖ

Santrauka

Įvadas. Plazmoje ar serume laisvai cirkuliuojanti DNR (cfDNR) kaip neinvazinis biožymuo, kuriam nustatyti reikalingas tik kraujo mėginys, gali parodyti sergančiųjų nesmulkių ląstelių plaučių vėžiu (NSLPV) ligos prognostinį profilį. Naujausi tyrimai rodo, kad cfDNR kiekybinės analizės diagnostikos tikslumas prilygsta įprastiniams kraujo biožymenims, naudojamiems plaučių vėžio atrankinei patikrai.

Tikslas. Straipsnio tikslas – apžvelgti diagnostinę ir prognostinę cfDNR reikšmę NSLPV atveju.

Metodai. Nagrinėtos publikacijos, susijusios su skystąja biopsija ir cfDNR, parašytos anglų kalba 2008–2017 m. Literatūra publikuota duomenų bazių PubMed ir Web of Science internetiniuose puslapiuose.

Rezultatai. Iš viso įtraukta 3 606 tiriamųjų. Išnagrinėta 10 prospektyvinių, dvi apžvalginės studijos ir viena metaanalizė. Straipsnyje pateikiama cfDNR nauda neinvazinei atrankinei patikrai, ankstyvajai diagnozei, prognozei, atsakui į gydymą, sergančiųjų NSLPV aktyviam stebėjimui.

Išvados. Remiantis atliktomis studijomis cfDNR pokytis turi būti interpretuojamas kartu su konvenciniais tyrimais, tokiais kaip citologinis ir (arba) histologinis ištyrimas, krūtinės ląstos kompiuterinė tomografija. Nerekomenduojama NSLPV patikrai naudoti tik cfDNR tyrimą, nes trūksta duomenų, kad rezultatai yra patikimi.

Raktažodžiai: laisvai cirkuliuojanti DNR, nesmulkių ląstelių plaučių vėžys, stebėjimas, skystoji biopsija