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Circulating cell-free nucleic acids and platelets as a liquid biopsy in the provision of personalized therapy for lung cancer patients

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ABSTRACT

Lung cancer is the predominant cause of cancer-related mortality in the world. The majority of patients present with locally advanced or metastatic non-small-cell lung cancer (NSCLC). Treatment for NSCLC is evolving from the use of cytotoxic chemotherapy to personalized treatment based on molecular alterations. Unfortunately, the quality of the available tumor biopsy and/or cytology material is not always adequate to perform the necessary molecular testing, which has prompted the search for alternatives. This review examines the use of circulating cell-free nucleic acids (cfNA), consisting of both circulating cell-free (tumoral) DNA (cfDNA–ctDNA) and RNA (cfRNA), as a liquid biopsy in lung cancer. The development of sensitive and accurate techniques such as Next-Generation Sequencing (NGS); Beads, Emulsion, Amplification, and Magnetics (BEAMing); and Digital PCR (dPCR), have made it possible to detect the specific genetic alterations (e.g. *EGFR* mutations, *MET* amplifications, and *ALK* and *ROS1* translocations) for which targeted therapies are already available. Moreover, the ability to detect and quantify these tumor mutations has enabled the follow-up of tumor dynamics in real time. Liquid biopsy offers opportunities to detect resistance mechanisms, such as the *EGFR* T790M mutation in the case of EGFR TKI use, at an early stage. Several studies have already established the predictive and prognostic value of measuring ctNA concentration in the blood. To conclude, using ctNA analysis as a liquid biopsy has many advantages and allows for a variety of clinical and investigational applications.

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1. 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 locally advanced or metastatic disease [1]. Over the last decade, several oncogenic driver mutations have been discovered in the histological subtypes of NSCLC, particularly adenocarcinoma, with mutations found in *KRAS* (32.2%), *EGFR* (11.3%), *ALK* (3.9%), *MET* exon 14 (4.2%), *BRAF* (7%), *PIK3CA* (2%), *ROS1* (2%), *HER2* (2%) and *RET* (1%), and amplification of *MET* (2.2%) and *HER2* (0.9%) [2,3]. Squamous cell carcinoma has also been associated with mutations in *PIK3CA* (12%), *PTEN* (10%) and amplification of *FGFR1* (20%) [3]. This molecular understanding has resulted in a treatment paradigm shift from "one treatment fits all" regimens, such as chemotherapy, to personalized treatment strategies developed on the basis of molecular alterations in tumor DNA [4]. The latter approach consists in screening for genetic aberrations in specific oncogenic signaling pathways, such as the RAS-RAF-MEK-ERK pathway. These pathways can be targeted to reverse the resulting uncontrolled growth, proliferation and survival when up-regulated [5]. These targeted therapies have been found to have a much higher clinical efficacy compared to standard therapy [6]. Genetic profiling of tumors has therefore become indispensable in the development of personalized therapies. Tissue biopsy is the gold standard for tumor genotyping, yet there are several limitations associated with mutational analysis in tissue. For example, lung cancer biopsies often provide limited, low-quality material, which is less suitable

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for molecular analysis [7]. Furthermore, single tumor biopsies do not always reveal the entire genomic landscape of tumor, heightening the need for personalized diagnosis and treatment [8]. As mentioned above, tissue biopsy remains the gold standard for assessing the mutational status of tumors. However, liquid biopsy, which allows for the analysis of several blood-based biomarkers, has emerged as a useful complementary technique.

This review examines the use of circulating cell-free nucleic acids (cfNA), consisting of both DNA (cfDNA) and RNA (cfRNA), as a liquid biopsy aiding in the provision of personalized therapies for lung cancer patients.

2. Liquid biopsy: cfDNA

The first identification of cfDNA in blood was reported by Mandel and Metais in 1948 [9]. Since then, cfDNA has found applications in many disciplines of medicine, but particularly in the evaluation of fetal DNA in the circulation of expectant mothers as a form of non-invasive prenatal (NIP) testing [10]. Liquid biopsy has also produced promising results in the field of targeted cancer therapy, given that the analysis of cfDNA and especially the fraction derived from tumor cells, namely circulating cell-free tumor DNA (ctDNA) enables the detection of specific genetic alterations as potential targets for targeted therapy [11]. CfDNA and ctDNA is released into the bloodstream through various mechanisms, including apoptosis, necrosis and even active shedding [12]. CtDNA is thought to be able to provide a broader picture of the genomic landscape of tumors throughout the body [13,14]. Murtaza et al. has demonstrated the potential of ctDNA in real-time sampling of multifocal clonal evolution [15]. The minimally invasive sampling required for liquid biopsy also offers the opportunity to detect the molecular changes that cancer cells undergo during treatment [13,16], and to monitoring tumor burden and the occurrence of relapse [17]. Several investigational studies have reported that ctDNA levels increase rapidly as the disease progresses, and decline in the case of resective surgery and/or successful medical therapy [14,16,18]. The low concentrations of ctDNA in blood, especially in the early stages of disease, and its potential as a liquid biopsy have prompted the development of specialized collection vessels, highly efficient isolation techniques and sensitive detection methods. Consequently, patients can be monitored closely and provided with personalized treatment regimens. Liquid biopsy not only allows for frequent sampling [19], but also provides an alternative to tissue biopsy among critically ill patients or when tissue specimens are limited or unavailable at the time of diagnosis. In the phase 3 IPASS and INTEREST studies, it was seen that only 42% and 31% of patients had tissue available or tissue could be obtained for molecular testing, respectively [20,21].

2.1. Practical approach to performing liquid biopsy in the clinic

2.1.1. cfDNA preparation

At present, EDTA tubes are most commonly used for blood collection. The anticoagulant in the tubes prevents clotting and acts as an indirect DNase I inhibitor [22]. CfDNA in blood is associated with a high turn-over due to the presence of nucleases, which means that plasma preparation must be performed within 1–2 h of blood sampling [23,24]. Plasma remains the matrix of choice, however, since higher cfDNA concentrations are observed in serum due to contamination by genomic DNA released by white blood cells during the clotting process [23]. Alternative blood collection tubes containing preservative reagents, such as Cell-Free DNA BCT® (STRECK tubes) and PAXgene Blood DNA tubes (Qiagen), have also been developed. The formaldehyde-free preservative reagents in these tubes not only stabilize nucleated blood cells, preventing the release of cellular genomic DNA, but also inhibit the nuclease-mediated degradation of cfDNA [25,26]. Toro et al. demonstrated that Cell-Free DNA BCT® tubes, in particular, make it possible to keep blood samples at room temperature for several days before plasma preparation, which has led to their application in NIP testing of fetal DNA and the analysis of ctDNA in cancer patients [26,27].

In order to ensure clinically meaningful sensitivity, it is essential to isolate cfDNA from plasma efficiently. Only then can the cfDNA yield be compared and normalized reliably when screening for the range of biomarkers associated with targeted therapies. CtDNA load has been reported to correlate with disease stage [28]. CtDNA can make up as little as 0.01% of the total cfDNA (minor allele frequency (MAF)) [29]. The fragment size profile of the cfDNA and ctDNA can differ. CfDNA primarily consists of uniform fragments, whereas the ctDNA fragment size depends on the cellular process causing its release into the circulation [30,31]. Jiang et al. reported that short DNA fragments preferentially carry tumor-associated aberrations [31]. Several kits have been developed to specifically isolate smaller fragments. Comparison in terms of their isolation efficiency, reproducibility and representation of smaller DNA fragments, has revealed that the QIAamp® circulating nucleic acid kit (Qiagen) performs best. Isolation kits designed to extract high-integrity genomic DNA from blood cells or virions, were less effective [32–34]. Our own research group compared the Qiagen kit with the Maxwell® RSC ccfDNA Plasma (Promega), which displayed similar results of cfDNA yield and ctDNA detection, where the Promega kit has the advantage of a fully automated protocol over the labor intensive Qiagen kit [35]. Standardization of ctDNA analysis requires a uniform centrifugation protocol as well as normalization of the amount of plasma input. Several protocols describe two sequential spins of blood samples (varying from 800 to 2000 g for 10–15 min) to separate plasma from buffy coat [36,37]. Page et al. described the need for a third bench top spin (1000 g for 5 min) to remove any remaining cells, platelets, and cellular debris [38]. High plasma input is necessary to generate sufficient ctDNA concentrations to be able to detect low MAF [32]. Besides high isolation efficiency, ctDNA analysis also requires methods with a low limit of detection (LoD).

2.1.2. ctDNA analysis

Several platforms have been developed to analyze ctDNA for the presence of particular biomarkers, including real-time quantitative PCR (qPCR); the Scorpion Amplification-Refractory Mutation System (ARMS); Beads, Emulsion, Amplification and Magnetics (BEAMing); digital PCR (dPCR); and Next-Generation Sequencing (NGS) (Table 1).

2.1.2.1. Real-time quantitative PCR. Real-time qPCR, consisting of both TaqMan based and Scorpion ARMS assays, are the most widely used platforms for the quantification of nucleic acids [48–51]. ARMS is a standard PCR method for detecting of single-base mutations and small deletions. It involves amplifying the target DNA using sequence-specific primers that only trigger this amplification when the target allele is contained within the sample. The presence or absence of a PCR product therefore indicates the presence or absence of the target [52]. Because this method is relatively insensitive in the detection of low levels of mutation, most likely due to primer competition, it is often combined with Scorpion technology, making it a qPCR-based technique. Scorpions consist of a PCR primer covalently linked to a probe, which in turn interacts with a quencher. The presence of a mutation can be detected by the Scorpion ARMS in a real-time PCR setting [40,53]. While qPCR is a standardized, relatively inexpensive, and technically straight-

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