CHAPTER THREE

Advances in Circulating Tumor DNA Analysis

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Abstract

The analysis of cell-free circulating tumor DNA (ctDNA) is a very promising tool and might revolutionize cancer care with respect to early detection, identification of minimal residual disease, assessment of treatment response, and monitoring tumor evolution. ctDNA analysis, often referred to as "liquid biopsy" offers what tissue biopsies cannot—a continuous monitoring of tumor-specific changes during the entire course of the disease. Owing to technological improvements, efforts for the establishment of preanalytical and analytical benchmark, and the inclusion of ctDNA analyses in clinical trial, an actual clinical implementation has come within easy reach. In this chapter, recent advances of the analysis of ctDNA are summarized starting from the discovery of cell-free DNA, to methodological approaches and the clinical applicability.

ABBREVIATIONS

ARMS amplification refractory mutation system
BEAMing beads, emulsion, amplification, and magnetics
CAPP-Seq cancer personalized profiling by deep sequencing
castPCR competitive allele-specific TaqMan PCR
cfDNA cell-free DNA
cffDNA cell-free fetal DNA
ctDNA cell-free circulating tumor DNA
CTCs circulating tumor cells
COLD-PCR coamplification at lower denaturation temperature-PCR
CRC colorectal cancer
DISSECT differential strand separation at critical temperature
dPCR digital PCR
ddPCR digital droplet PCR
EQA external quality assessment
gDNA genomic DNA
GE genome equivalent
iDES integrated digital error suppression
LNA locked nucleic acid
LOD limit of detection
LOH loss of heterozygosity
mCRC metastatic CRC

MRD minimal residual disease
MSI microsatellite instability
NGS next-generation sequencing
NSCLC nonsmall cell lung cancer
OS overall survival
PCR polymerase chain reaction
PFS progression-free survival
PNA peptide nucleic acids
QPCR quantitative polymerase chain reaction
SCNA somatic copy number alteration
SOP standard operating procedure
UV ultraviolet
RCA rolling circle amplification
TAm-Seq tagged-amplicon deep sequencing
TKIs tyrosine kinase inhibitors
UID unique identifier

1. INTRODUCTION

"*Written in blood—DNA circulating in the bloodstream could guide cancer treatment—if researchers can work out how best to use it*": this is what Nature featured in July 2014 [1]. In this chapter, the potential of cell-free circulating tumor DNA (ctDNA) was discussed with experts in the field. All experts agreed that the analysis of ctDNA is a very promising tool and might revolutionize cancer care with respect to early detection, identification of minimal residual disease (MRD), assessment of treatment response, and monitoring tumor evolution. Since then, many hurdles have been overcome while other issues have remained. On the one hand, technological improvements now allow the analysis of extremely rare alleles and a number of clinical trials have implemented ctDNA in their designs. Likewise, efforts are being made in order to establish benchmarks for the analysis of ctDNA, which is a crucial point for implementation in clinical routine. On the other hand, we lack a consensus on how to best use the available methods and the actual benefit for patients in terms of survival has yet to be proven. Finally, we still have much to learn about the biology and dynamics of ctDNA.

What exactly is the actual benefit of the so-called "liquid biopsy," i.e., the analysis of ctDNA from blood? Targeted therapies have dramatically improved response rates, survival and time to therapy failure in the last years, yet cancer is one of the most common causes of death worldwide [2]. Molecular profiling of tumors is a central element in the management of

many patients with cancer and is used to determine molecular targets at a single time point before treatment commences. Obtaining tumor material requires an invasive intervention, which not only carries some risks for patients, but also is a costly and time-consuming procedure. Moreover, a tissue biopsy only provides a snapshot of the molecular aberration in the tumor and may not be a true representation of the molecular profile. In addition to tumor heterogeneity at the time of diagnosis/biopsy, tumors are constantly evolving during the course of disease or under the selective pressure of a specific treatment. A liquid biopsy offers what tissue biopsies cannot—a continuous monitoring of tumor-specific changes during the entire course of the disease. In the last few years, it has been shown that ctDNA reflects the molecular landscape of a tumor and its metastases and therefore can reflect the efficiency and relevance of the chosen treatment specific for a molecular target or indicate the emergence of any resistance-conferring mechanisms. Furthermore, progression or recurrence can be predicted before it is clinically or radiologically obvious.

The potential and benefit of ctDNA analyses are discussed extensively elsewhere [3–10]. The purpose of this chapter is to summarize recent advances in the analysis of ctDNA. First, we give a brief overview about the discovery of ctDNA and associated landmark developments. Second, we present the current knowledge of the biology of DNA. Third, we summarize preanalytical and analytical considerations and highlight some of the new methodological developments. Finally, we present recent data on the clinical utility of ctDNA analysis for the most common tumor entities. Owing to the vast amount of published data, we were not able to include all available studies. Moreover, although there are also many studies on the analysis of epigenetic changes in ctDNA, we mainly focused on genetic changes.

2. HISTORY AND LANDMARK DEVELOPMENTS

Although the structure of DNA was only first described by Watson and Crick [11], the presence of DNA in human plasma of healthy and sick individuals was already described by Mandel and Métais [12]. However, this finding did not gain too much attention and it took almost 30 years until the discovery was revived. After a long period of silence, Tan *et al.* reported about high levels of circulating DNA in patients with systemic lupus erythematosus (SLE) using both the diphenylamine reaction and gel-diffusion studies [13]. This observation finally drew attention to the fact

that cell-free DNA (cfDNA) circulates in plasma/serum. Moreover, in a pioneering work, Stroun and Anker demonstrated the spontaneous release of nucleic acids by living frog auricles, which helped promote further interest in this field [14]. Shortly after, Koffler *et al.* demonstrated increased levels of cfDNA in the serum of patients suffering from SLE or rheumatoid arthritis [15]. Steinman initially challenged these findings since he was unable to identify any DNA in plasma of healthy individuals [16]. He assumed that the appearance of cfDNA in the circulation is truly pathological; therefore, he suggested that only plasma should be measured [16]. Similar results were reported by Davis and Davis, who stated that serum is not suitable for the analysis of circulating DNA since genomic DNA (gDNA) is sporadically released into serum during the clotting process [17]. In 1977, Leon *et al.* first demonstrated a prognostic value of the cfDNA in rheumatoid arthritis patients [18]. High levels of cfDNA were commonly found in patients with more severe symptoms, who had active rheumatoid arthritis for less than 10 years, whereas patients with longer duration of disease showed lower levels of DNA [19]. In contrast, using a highly sensitive and specific radioassay, Cox and Gokcen found DNA as a normal constituent of both serum and plasma [20]. In the same year, Anker *et al.* postulated that human blood leucocytes spontaneously release DNA when incubated in vitro [21]. In the early 1980s, the value of circulating DNA concentrations in the diagnosis of pulmonary embolism (PE) or acute myocardial infarction was investigated [22–24], but these studies revealed discrepant results. Some of these landmark developments are displayed in Fig. 1.

2.1 ctDNA as a Potential Cancer Biomarker

The use of circulating DNA as a potential cancer biomarker was discovered in 1977 when Leon *et al.* reported elevated levels of cfDNA in the circulation of cancer patients (Fig. 1). In some patients, even a decrease of cfDNA after successful anticancer therapy was observed [19]. Using a radioimmunoassay for quantification, the absolute amounts of cfDNA were determined in the serum of 173 patients with various types of cancer and in 55 healthy individuals. The authors found significantly higher DNA levels in the serum of patients with metastatic disease, although no correlation between DNA levels and the size or location of the primary tumor could be seen. Those patients with decreasing cfDNA levels under therapy, however, showed shrinkage of tumor size and a reduction of pain. On the contrary, when cfDNA levels either increased or remained unchanged, a lack

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