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Technological advances in free-circulating tumour-derived DNA methylation analysis

Marcin Skalski[a], Jarosław Paluszczak*, [b]

Department of Pharmaceutical Biochemistry, Poznan University of Medical Sciences, Poland

* *Corresponding Autor:* Jarosław Paluszczak, Department of Pharmaceutical Biochemistry, Poznan University of Medical Sciences, 4 Święcickiego Street, 60-781 Poznań, Poland, phone: +48618546624, fax: +48618546620, email: paluszcz@ump.edu.pl

[a] https://orcid.org/0000-0003-3377-8313

[b] https://orcid.org/0000-0002-6187-7549

ABSTRACT

Cancers still constitute an important health burden, due to their high incidence and mortality. The discovery and clinical implementation of novel sensitive diagnostic markers could improve treatment outcomes. Circulating cell-free DNA (ccfDNA) offers great promise for the development of new molecular biomarkers. However, the analysis of ccfDNA methylation poses significant challenges due to ccfDNA fragmentation and low concentration in the blood. Techniques which have recently been developed for liquid biopsy studies have been adapted to the detection of trace amounts of ccfDNA. This mini-review focuses on recent technological advances which allow the discovery of new sensitive and specific liquid biopsy cancer biomarkers based on DNA methylation detection.

Keywords: cancer; biomarkers; liquid biopsy; circulating cell-free DNA; cfDNA; ctDNA; DNA methylation.

Introduction

Cancers are one of the main health burdens, with about 17 million new cases and 9.6 million deaths worldwide in 2018 [1]. Due to their high incidence and mortality, there is an urgent need for new diagnostic methods. The gold standards in cancer management, thus far, are tissue biopsy and protein-based biomarkers, but recently, rising enthusiasm for the development of so called „liquid biopsies" has been noted [2]. A liquid biopsy avoids invasive sampling because it relies on the analysis of cancer-derived components which circulate in the bloodstream, e.g. circulating tumour cells, exosomes or tumour-derived DNA, or which are present in other biological fluids, e.g. the saliva or urine. Importantly, a liquid biopsy is expected to overcome issues regarding tumour heterogeneity, since it should reflect the characteristics of virtually all cancer cell subclones, in contrast to tissue biopsies. This article focuses on recent methodological advances in the detection of DNA methylation in a liquid biopsy, which may potentially bring it closer to wider clinical use.

Characteristics of ctDNA

The field of liquid biopsy studies was significantly stimulated by the discovery of the presence of circulating cell-free DNA (ccfDNA) in the blood. ccfDNA is typically 80—200 bp in length, but ± 147 bp-long fragments are most prevalent, since this

is the average length of DNA participating in nucleosome formation. ccfDNA is released into biological fluids mainly as the result of apoptotic or necrotic cell death, but other mechanisms are also possible, e.g. active release. It can be found not only in the blood, but also in the urine, cerebrospinal fluid and saliva of healthy and cancer subjects. Physiologically, its blood concentration is low, but it can be elevated, e.g., in inflammatory diseases, after intensive physical training or during pregnancy [3]. Many studies investigated the applicability of the assessment of free-circulating tumour-derived DNA (ctDNA) in cancer diagnostics [4,5]. It is widely accepted that the blood concentration of cell-free DNA is significantly higher in cancer patients in comparison to healthy controls, and it depends on the cancer stage (tumour size and vasculature). The total concentration of ctDNA in the plasma ranges from undetectable amounts up to ~1000 ng/ml in advanced cancers [7]. Much is already known in terms of ctDNA isolation, storage and handling [3]. Apart from simple quantitation, ctDNA can be used in the clinic to detect cancer-specific mutations or assess ctDNA methylation pattern changes [2, 6, 7]. The analysis of the ctDNA methylation profile shows the greatest promise, because it allows for early detection and is considered to be tissue-specific in many cancers [8].

Methods of ctDNA methylation analysis

There is extensive data concerning methylation-based biomarkers in different cancers [4,8], but the number of commercially available tests is limited to just one, namely the FDA-approved Epi proColon® 2.0 CE (Epigenomics, Germany). It utilises the HeavyMethyl technique to detect the methylation of *SEPT9* in serum. The analysis of *SEPT9* methylation showed high sensitivity and specificity in the detection of colorectal cancer, although it did not outperform other available tests (e.g. FIT) when used in asymptomatic patients [9]. Nevertheless, the advantage of this type of assay lies in its non-invasive nature in comparison to troublesome faecal analysis or colonoscopy. Thus, blood-based liquid biopsies are preferable because of potentially high patient compliance.

The amount of ctDNA in the plasma is usually insufficient to analyse the methylation of more than a single gene per sample when using standard methods. However, reliable detection of most types of cancer usually requires the assessment of a panel of several genes. Thus, methods suitable for DNA methylation-based liquid biopsy should not only detect trace amounts of DNA, but also allow for the analysis of highly fragmented ctDNA in multiplexing formats. This means that as little as 7–10 pg of methylated DNA can be detected. Moreover, an amplicon length of up to 100 bp long is preferable in assays analysing short DNA fragments. ctDNA is always accompanied by some ccfDNA shed by other types of cells, thus the epigenetic background stemming especially from blood cells is always a possible problem. To minimise this risk, only genes whose methylation has been confirmed to be cancer-specific should be included in diagnostic panels. All of these factors need to be taken into account when designing assay conditions.

Several improved techniques for the sensitive detection of ctDNA methylation have been described recently (**Table 1**). They have been successfully implemented in the search for cancer biomarkers (**Table 2**). Due to the low concentrations of ctDNA in blood samples, the enrichment of target sequences, e.g. by the addition of carrier nucleic acid and/or by nested PCR, is frequently necessary in the first steps of the analysis. In this regard, the use of whole genome amplification of bisulfite-converted ctDNA does not seem to yield satisfactory results.

PCR-based methods focus on site-specific analysis of one or several DNA regions [10,11]. One of the most interesting variants of quantitative methylation-specific PCR (qMSP) is cMethDNA. This modification of multiplex qMSP uses gene-specific standards and a two-step PCR procedure — enrichment of the target regions by multiplex-nested PCR followed by probe-based signal detection. It is relatively simple, but very robust — it allows for the sensitive detection of 1 methylated in 100,000 unmethylated copies. The examination of a panel of 10 differentially methylated genes using this approach allowed for highly sensitive and specific detection and monitoring of metastatic breast cancer [12]. On the other hand, next generation sequencing (NGS)-based methods simultaneously analyse hundreds or thousands

Table 1. Comparison of different DNA methylation-based techniques used in liquid biopsy studies

Method	Advantages	Disadvantages	Additional information	Amount of sample required	Number of analyzed regions per sample	Reference
ddMSP	High sensitivity High throughput	Multiplexing is challenging Relatively high cost	Allows for absolute quantitation	0.9 ml plasma	12 genes and 4 internal controls	[18]
cMethDNA	High sensitivity High throughput Relatively low cost	Complex assay design	Modification of qMSP, two sequential PCR reactions Multiplex reaction in preamplification step External control added	0.3 ml serum	10 genes	[12]
MCTA-seq	Very high number of analyzed genes Low sequencing depth required	Low throughput High cost	Low potential clinical usefulness due to complex analysis	2 ml plasma	Thousands of promoters adjacent to CpG tandems	[15]
qMSP	High throughput Relatively low cost	Limited number of multiplexed genes Challenging optimization of PCR conditions	One tetraplex reaction (3 test genes + reference gene on one sample)	35–70 ml urine	Up to 4 genes	[11]
			Several multiplex reactions on one sample	2-3 ml plasma	9 genes	[19]
QM-MSP	High throughput Relatively low cost	Complex assay design	Similar to cMethDNA but without external standards Results based on detected ratio of methylated vs unmethylated DNA	0.3 ml serum	Up to 12 genes	[20]

of regions [13, 14]. MCTA-Seq is a very interesting technique combining the sensitivity of PCR and the high throughput of NGS. The key idea of this technique is to use primers flanking CpG tandem repeats, which are found in many gene promoters, amplify them, and perform genome-wide analysis of the products. Such a workflow overcomes the problem of high DNA input required for NGS and the low ctDNA quantity in the bodily fluids [15]. However, the implementation of NGS-based techniques in diagnostic laboratories is problematic due to the high cost, thus this assay will rather be used for scientific investigations only.

Conclusions

We are currently experiencing a significant increase in the number of publications concerning ctDNA analysis [16]. Technological advances have made it possible to sensitively detect methylation in trace amounts of ctDNA. Unfortunately, knowledge about differentially methylated genes, coming from studies in large cohorts of patients, is still lacking. This problem can potentially be solved in the coming years thanks to the GRAIL consortium initiative [17]. Its aim is to create a Cell-free Genome Atlas, and thus fill almost all of the gaps in our understanding of ctDNA biology. Such data would be invaluable for any future applications. If successful, it will be a major breakthrough in the field of blood biomarker testing in precision medicine.

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Conflict of interest statement
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Table 2. Recent examples of liquid biopsy biomarkers based on the analysis of ctDNA methylation

Method	Sensitivity/ specificity	Cancer type	Genes analyzed	Clinical utility	Reference
qMSP	52–59%/ 95–96%	HNSCC	*SHOX2* *SEPT9*	Detection Prognosis (survival) Monitoring (recurrence)	[21]
qMSP	78%/84% (1 in 3 replicates) 73%/96% (2 in 3 replicates)	Colorectal cancer	*SEPT9*	Early detection	[9]
qMSP	63%/86%	Lung cancer	*CDO1* *TAC1* *SOX17*	Detection	[22]
qMSP, quasi-digital PCR	49–65%/ 88–94%	HNSCC	*SHOX2* *SEPT9*	Detection	[23]
Multiplex qMSP	72%/74%	Breast Colorectal Lung	*APC, FOXA1, RASSF1A*	Detection	[19]
cMethDNA	91%/96%	Breast	*AKR1B1, COL6A2, GPX7, HIST1H3C, HOXB4, RASGRF2, TM6SF1, ARHGEF7 TMEFF2, RASSF1*	Dectection Treatment response	[12]
methylBEAMing	NA	Glio-blastoma	*MGMT*	Treatment response	[24]
methylBEAMing	NA	Colorectal cancer	*EYA4, GRIA4, ITGA4, MAP3K14-AS1, MSC*	Prediction of response to regorafenib	[25]
Bisulfite sequencing	79.5–92.7%/ 85.2–92.8%	Lung cancer	9 regions	Detection	[26]
Targeted bisulfite sequencing	NA	Hepato-cellular carcinoma	10 markers 8 markers	Prognosis (survival) Treatment response	[27]
MCTA-seq	94%/89%	Hepato-cellular carcinoma	*RGS10 ST8SIA6 RUNX2 VIM* and 15 regions	Early detection	[15]
cfMeDIP-Seq	NA	Pancreatic cancer	Thousands of differentially methylated CpGs	Early detection	[28]

HNSCC – Head and Neck squamous cell carcinoma, MSP – methylation-specific PCR, NA – data not available, sensitivity – proportion of cancer case subjects who test positive for the biomarker, specificity – proportion of control subjects who test negative for the biomarker

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