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

Marcin Skalski¹, a, Jarosław Paluszczak*¹, b

¹ Department of Pharmaceutical Biochemistry, Poznan University of Medical Sciences, Poland

* Corresponding Author: 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

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
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.

**Acknowledgements**

**Conflict of interest statement**

The authors declare no conflict of interest.

**Funding sources**

This paper was supported by a grant from The National Centre for Research and Development (POIR.04.01.04-00-0003/17-00).
### References

1. Worldwide cancer statistics [Internet]. Cancer Research UK. 2019 [cited 2019 Jul 15]. Available from: https://www.cancerresearchuk.org/health-professional/cancer-statistics/worldwide-cancer.

2. De Rubis G, Krishnan SR, Bebawy M. Circulating tumor DNA — Current state of play and future perspectives. Pharmacol Res. 2018;136:35–44.

3. Bronkhorst AJ, Ungerer V, Holdenrieder S. The emerging role of cell-free DNA as a molecular marker for cancer management. Biomol Detect Quantif. 2019 Mar 1;17:100087.

4. Wang J, Han X, Sun Y. DNA methylation signatures in circulating cell-free DNA as biomarkers for the early detection of cancer. Sci China Life Sci. 2017 Apr;60(4):356–62.

5. Vlassov YV, Laktionov PP, Rykova EY. Circulating nucleic acids as a potential source for cancer biomarkers. Curr Mol Med. 2010 Mar;10(2):142–65.

6. Gai W, Sun K. Epigenetic Biomarkers in Cell-Free DNA and Applications in Liquid Biopsy. Genes. 2019 Jan;10(1):32.

7. Elazezy M, Joosse SA. Techniques of using circulating tumor DNA as a liquid biopsy component in cancer management. Comput Struct Biotechnol J. 2018 Oct 9;16:370–8.

8. Zeng H, He B, Yi C, Peng J. Liquid biopsies: DNA methylation analyses in circulating cell-free DNA. J Genet Genomics. 2018 Apr 20;45(4):185–92.

9. Song L, Jia J, Peng X, Xiao W, Li Y. The performance of the SEPT9 gene methylation assay and a comparison with other CRC screening tests: A meta-analysis. Sci Rep. 2017 Jun 8;7(1):1–12.

10. Guzzetta AA, Pisanic TR, Sharma P, Yi JM, Stark A, Wang T-H, et al. The promise of methylation on beads for cancer detection and treatment. Expert Rev Mol Diagn. 2014 Sep;14(9):845–92.

11. Olkhov-Mitsel E, Zdravic D, Kron K, van der Kwast T, Fleshner N, Bapat B. Novel multiplex MethyLight protocol for detection of DNA methylation in patient tissues and bodily fluids. Sci Rep. 2014 Mar 21;4:4432.

12. Fackler MJ, Bujanda ZL, Umbricht C, Teo WW, Cho S, Zhang Z, et al. Novel Methylated Biomarkers and a Robust Assay to Detect Circulating Tumor DNA

### 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                          | [21]      |
|                         |                          |             |                | Prognosis (survival)               |           |
|                         |                          |             |                | Monitoring (recurrence)            |           |
| qMSP                    | 78%/84% (1 in 3 replicates) 73%/96% (2 in 3 replicates) | Colorectal cancer | SEPT9 | Early detection | [9]        |
| qMSP                    | 63%/86%                  | Lung cancer | CD01 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 | Detection | 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 | RGST10 STB16A6 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.
in Metastatic Breast Cancer. Cancer Res. 2014 Apr 15;74(8):2160–70.
13. Han X, Wang J, Sun Y. Circulating Tumor DNA as Biomarkers for Cancer Detection. Genomics Proteomics Bioinformatics. 2017 Apr 1;15(2):59–72.
14. Volik S, Alcaide M, Morin RD, Collins C. Cell-free DNA (cfDNA): Clinical Significance and Utility in Cancer Shaped By Emerging Technologies. Mol Cancer Res. 2016 Oct 1;14(10):898–908.
15. Wen L, Li J, Guo H, Liu X, Zheng S, Zhang D, et al. Genome-scale detection of hypermethylated CpG islands in circulating cell-free DNA of hepatocellular carcinoma patients. Cell Res. 2015 Nov;25(11):1250–64.
16. Trigg RM, Martinson LJ, Parpart-Li S, Shaw JA. Factors that influence quality and yield of circulating-free DNA: A systematic review of the methodology literature. Heliyon. 2018 Jul 1;4(7):e00699.
17. Morrison C. Search for liquid biopsy grail points the way to drug discovery and development gems. Nat Rev Drug Discov. 2017 Jun;16(6):373–4.
18. Uehiro N, Sato F, Pu F, Tanaka S, Kawashima M, Kawaguchi K, et al. Circulating cell-free DNA-based epigenetic assay can detect early breast cancer. Breast Cancer Res BCR. 2016 19;18(1):129.
19. Nunes SP, Moreira-Barbosa C, Salta S, Palma de Sousa S, Pousa I, Oliveira J, et al. Cell-Free DNA Methylation of Selected Genes Allows for Early Detection of the Major Cancers in Women. Cancers. 2018 Oct;10(10):357.
20. Fackler MJ, Sukumar S. Quantitation of DNA Methylation by Quantitative Multiplex Methylation-Specific PCR (QM-MSP) Assay. Methods Mol Biol Clifton NJ. 2018;1708:473–96.
21. Schröck A, Leisse A, Vos L de, Gevensleben H, Dröge F, Franzen A, et al. Free-Circulating Methylated DNA in Blood for Diagnosis, Staging, Prognosis, and Monitoring of Head and Neck Squamous Cell Carcinoma Patients: An Observational Prospective Cohort Study. Clin Chem. 2017 Jul 1;63(7):1288–96.
22. Hulbert A, Jusue-Torres I, Stark A, Chen C, Rodgers K, Lee B, et al. Early Detection of Lung Cancer using DNA Promoter Hypermethylation in Plasma and Sputum. Clin Cancer Res Off J Am Assoc Cancer Res. 2017 Apr 15;23(8):1998–2005.
23. de Vos L, Gevensleben H, Schröck A, Franzen A, Kristiansen G, Bootz F, et al. Comparison of quantification algorithms for circulating cell-free DNA methylation biomarkers in blood plasma from cancer patients. Clin Epigenetics. 2017 Dec 1;9(1):125.
24. Barault L, Amatu A, Bleeker FE, Moutinho C, Falcomató C, Fiano V, et al. Digital PCR quantification of MGMT methylation refines prediction of clinical benefit from alkylating agents in glioblastoma and metastatic colorectal cancer. Ann Oncol. 2015 Sep 1;26(9):1994–9.
25. Amatu A, Schirripa M, Tosi F, Lonardi S, Bencardi-Kon, Bonazzina E, et al. High Circulating Methylated DNA Is a Negative Predictive and Prognostic Marker in Metastatic Colorectal Cancer Patients Treated With Regorafenib. Front Oncol. 2019;9:622.
26. Liang W, Zhao Y, Huang W, Gao Y, Xu W, Tao J, et al. Non-invasive diagnosis of early-stage lung cancer using high-throughput targeted DNA methylation sequencing of circulating tumor DNA (ctDNA). Theranostics. 2019 Apr 6;9(7):2056–70.
27. Xu R, Wei W, Krawczyk M, Wang W, Luo H, Flagg K, et al. Circulating tumour DNA methylation markers for diagnosis and prognosis of hepatocellular carcinoma. Nat Mater. 2017 Nov;16(11):1155–61.
28. Shen SY, Singhania R, Fehringer G, Chakravarthy A, Roehrl MHA, Chadwick D, et al. Sensitive tumour detection and classification using plasma cell-free DNA methylomes. Nature. 2018 Nov;563(7732):579–83.

Acceptance for editing: 2019-11-09
Acceptance for publication: 2019-12-30