Follow the trace of death: methylation analysis of cell-free DNA for clinical applications in non-cancerous diseases

“...DNA methylation signatures will be valuable tools to complement imaging-based technologies and allow concentrating the clinical examination on the most probable organ of origin.”

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Small amounts of tumor-derived DNA molecules can be found as circulating cell-free (ccf) DNA molecules that can be isolated from the serum/plasma of patients. The analysis of cell-free DNA bears great promise for the early detection of disease, monitoring of tumor burden and response to treatment as well as for the screening of disease recurrence or the development of resistance to genotype-driven treatments [1,2]. Furthermore, these so called ‘liquid biopsies’ are thought to be more representative of all macro- and microlesions present in the patient and the intraindividual molecular heterogeneity of the lesions might, therefore, be less of an issue to select the most appropriate treatment regimen. Other intensively investigated applications for the molecular analysis of ccfDNA include prenatal diagnostics [3] as well as graft host response [4].

While most of the recent efforts have focused on the analysis of genetic mutations to detect residual disease with high sensitivity and to follow the percentage of a known mutation under a genotype driven personalized treatment, analysis of DNA methylation patterns in cell-free DNA has a long standing tradition as an alternative biomarker for the same applications [5,6]. DNA methylation changes are widespread and frequent in cancer, are specific for tumor-derived molecules and in contrast to mutations they are normally confined to a relatively small region of the gene avoiding the need to adapt each assay to the specific profile of the patient.

Furthermore, a number of technologies based on (real-time) methylation-specific PCR (MSP, MethyLight, HeavyMethyl) have been developed, that allow the specific enrichment and amplification of these aberrantly methylated molecules enabling the development of commercial diagnostic tools. For example, the US FDA approved Epi *pro*Colon test analyzes methylation in the *SEPT9* gene in cell-free circulating DNA for the population-wide screening for colorectal cancer [7]. As DNA methylation changes precede often the development of cancerous lesions, analysis of DNA methylation in ccfDNA has also been shown to allow monitoring people at risk of progressing to cancer [8,9].

A number of well-powered blood- or tissue-based cohort studies have recently shown that DNA methylation changes are not only frequently observed in cancer, but also in a wide range of other complex diseases including metabolic, neurodegenerative, inflammatory and autoimmune diseases as well as in response to environmental stimuli such as smoking. However, changes occur with lower frequency and often smaller amplitude compared with the changes observed in cancer. Furthermore, as levels of cell-free DNA are relatively low in non-cancerous diseases and no specific mutations are present in these diseases, which can be used to detect the disease and track its evolution, the analysis of genetic variation and DNA methylation patterns in ccfDNA has attracted little interest in these diseases.

Jörg Tost

Laboratory for Epigenetics & Environment, Centre National de Génotypage, CEA-Institut de Génomique, 2 rue Gaston Crémieux, 91000 Evry, France
Tel.: +33 160 878 423
Fax: +33 160 878 485
tost@cng.fr

Driven by the technological evolution of next-generation sequencing (NGS)-based bisulfite sequencing, the laboratory of Dennis Lo showed in a pioneering work about 2 years ago that the genome-wide hypomethylation commonly observed in different cell types of patients with systemic lupus erythematosus can also be detected when analyzing the methylome of cell-free DNA [10]. The same group recently showed that DNA methylation analysis of tissue-specific DNA methylation markers in ccfDNA allows tracing back the tissue of origin of the circulating DNA [11]. Thus DNA methylation profiling of ccfDNA or analysis of DNA methylation patterns at loci with a known tissue specificity does add a level of precision to the analysis of cell-free DNA, which cannot be obtained by the analysis of genetic variation.

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Making use of tissue-specific DNA methylation patterns or signatures, several recent studies have now shown that DNA methylation patterns specific to the diseased organ can be detected in cell-free circulating DNA for a number of complex diseases, in which DNA molecules of dying cells are shed into the blood stream.

Methylation of the *PPAR-γ* promoter has previously shown to stratify fibrosis severity in liver biopsies of patients with non-alcoholic fatty liver disease (NAFLD) [12], a disease with increasing prevalence, which represents the majority of liver diseases in the western world and for which the degree of fibrosis is the best prognostic indicator [13]. A recent study now demonstrated that DNA methylation levels at specific CpGs of the *PPAR-γ* promoter are also elevated in circulating cell-free DNA in patients with NAFLD or alcoholic liver disease compared with controls and the degree of hypermethylation correlated positively with the fibrosis score [14]. The DNA methylation outperformed clinical biochemistry indices and showed a higher sensitivity and specificity compared with the widely used NAFLD fibrosis score [14]. Of note, a similar DNA methylation change has also been reported in peripheral blood mononuclear cells (PBMCs) of patients with hepatitis B associated liver fibrosis [15] and if this hypermethylation could also be detected in cell-free DNA, the analysis of DNA methylation patterns at the *PPAR-γ* promoter could be an exquisite tool for the detection and staging of liver fibrosis independent of its underlying cause. This could replace the need for invasive interventions such as liver biopsies, which are

currently the gold standard technology for accurate diagnosis and staging of liver fibrosis, but associated with a high interventional risk as well as prone to the above-mentioned problems of sample heterogeneity. Similarly, DNA methylation at CpGs in *CUX2* and *REG1A*, very probably derived from dying cells of the exocrine pancreas, allowed detecting pancreatic cancer or pancreatitis with a much higher sensitivity compared with *KRAS* mutation analysis in ccfDNA, despite the relatively high abundance of this mutation in pancreatic cancer [16].

Absence of methylation at the insulin promoter is a specificity of insulin producing pancreatic β cells and the decrease of the DNA methylation degree at the *INS* promoter can, therefore, be used to monitor the proportion of β -cell derived DNA molecules in ccfDNA, which was found to be significantly elevated in patients with Type 1 diabetes [16,17]. The molecular analysis of the DNA methylation patterns at the *INS* promoter allowed for a perfect distinction between patients and controls in the analyzed cohort [16]. Furthermore, following allogeneic β -cell transplantation, quantitative analysis of unmethylated *INS* promoter fragments allowed to monitor the efficacy of the immunosuppressive treatment and might in the future allow adapting the treatment to prevent rejection of transplanted β cells. Complementing the analysis of the *INS* promoter, with other β -cell specific DNA methylation markers such as analysis of the *IAPP* promoter [18] could further increase the specificity and sensitivity of an assay for the detecting of T1D diabetes.

Brain related diseases are, in many cases, difficult to diagnose and monitor due to the frequent absence of non-invasive biomarkers. For brain cancers, mutation analysis of cerebrospinal fluid has, therefore, become the liquid biopsy of choice as ccfDNA from plasma/serum does often represent the tumor burden less well [19]. However, cerebrospinal fluid sampling remains an invasive procedure, which can only be justified under special circumstances. DNA methylation analysis of ccfDNA might provide a valuable option in some cases when the blood–brain barrier has been at least temporarily disrupted as recently demonstrated by the detection of unmethylated fragments of *MBP3* and *WMI* specific for oligodendrocytes in about 75% of patients with relapsing multiple sclerosis [16]. These DNA methylation patterns were not detectable in patients with stable disease emphasizing one more time the requirement of active cell death for the detection of disease-specific DNA methylation patterns in ccfDNA. Similarly, demethylation at the brain-specific CpG CG09787504 could be readily detected in ccfDNA of patients after traumatic or ischemic brain damage reporting on the rate of brain cell death [16].

Although the sample size was limited in most studies and no data from independent validation cohorts are yet available to replicate the performance of the analyzed loci, the studies demonstrate the potential of the analysis of DNA methylation patterns in circulating cell-free DNA for complex diseases. They provide a new avenue for the non-invasive detection and monitoring of progression of pathological conditions potentially replacing invasive procedures such as biopsies. Analysis of ccfDNA will provide useful data for the optimization of the timing of therapeutic intervention as well as monitoring of the therapeutic response to appropriate treatment regimens. The number of loci and CpGs per target region will probably need to be extended to increase the stability of the DNA methylation signature, as a higher discriminative power was already observed by including four to nine CpGs per analyzed region (instead of single CpGs) reducing interindividual phenotype-independent variation of DNA methylation levels while at the same time increasing the accuracy of the assignment of the tissue-of-origin [16]. Comprehensive analysis of the DNA methylation patterns of different tissues using unbiased technologies such as whole genome bisulfite sequencing (MethylC-seq), which are currently undertaken in large-scale efforts such as the RoadMap consortium [20], will allow the reliable identification of tissue-specific DNA methylation patterns. Furthermore, exhaustive mapping of DNA methylation in circulating cell-free DNA compartment in a large variety of diseases in large patient cohorts is required to evaluate the potential applications and more importantly authenticate the clinical validity of the biomarkers. Major efforts are

also required to standardize the sample acquisition as well as their processing and analysis, to obtain robust DNA methylation signatures that can be reproduced in different clinical settings. Nonetheless, the above-described studies constitute a proof-of-principle for a novel approach of detecting and monitoring complex diseases. Follow-up and replication studies will enable us to refine and optimize DNA methylation-based signatures in ccfDNA and ultimately allow the development of multidisease panels, which could complement biochemical assays for the noninvasive detection of complex diseases and determine the origin of physiological deregulation. If the published and future DNA methylation changes will be validated in larger and longitudinal cohorts, these DNA methylation signatures will be valuable tools to complement imaging-based technologies and allow concentrating the clinical examination on the most probable organ of origin. Death has shown us the way and might well improve life in the future.

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References

- Heitzer E, Ulz P, Geigl JB. Circulating tumor DNA as a liquid biopsy for cancer. *Clin. Chem.* 61(1), 112–123 (2015).
- Díaz LA Jr, Bardelli A. Liquid biopsies: genotyping circulating tumor DNA. *J. Clin. Oncol.* 32(6), 579–586 (2014).
- Wong FC, Lo YM. Prenatal diagnosis innovation: genome sequencing of maternal plasma. *Annu. Rev. Med.* 67, 419–432 (2016).
- Gielis EM, Ledeganck KJ, De Winter BY *et al.* Cell-free DNA: an upcoming biomarker in transplantation. *Am. J. Transplant.* 15(10), 2541–2551 (2015).
- How Kit A, Nielsen HM, Tost J. DNA methylation based biomarkers: practical considerations and applications. *Biochimie* 94(11), 2314–2337 (2012).
- Warton K, Samimi G. Methylation of cell-free circulating DNA in the diagnosis of cancer. *Front. Mol. Biosci.* 2, 13 (2015).
- Payne SR. From discovery to the clinic: the novel DNA methylation biomarker *mSEPT9* for the detection of colorectal cancer in blood. *Epigenomics* 2(4), 575–585 (2010).
- Tost J. DNA methylation signatures in circulating cell-free DNA for the monitoring of at-risk populations progressing to lung cancer. *EBioMedicine* 2(8), 798–799 (2015).
- Wielscher M, Vierlinger K, Kegler U, Ziesche R, Gsur A, Weinhausel A. Diagnostic performance of plasma DNA methylation profiles in lung cancer, pulmonary fibrosis and COPD. *EBioMedicine* 2(8), 927–934 (2015).
- Chan RW, Jiang P, Peng X *et al.* Plasma DNA aberrations in systemic lupus erythematosus revealed by genomic and methylomic sequencing. *Proc. Natl Acad. Sci. USA* 111(49), E5302–E5311 (2014).
- Sun K, Jiang P, Chan KC *et al.* Plasma DNA tissue mapping by genome-wide methylation sequencing for noninvasive prenatal, cancer, and transplantation assessments. *Proc. Natl Acad. Sci. USA* 112(40), E5503–E5512 (2015).

- 12 Zeybel M, Hardy T, Wong YK *et al.* Multigenerational epigenetic adaptation of the hepatic wound-healing response. *Nat. Med.* 18(9), 1369–1377 (2012).
- 13 Angulo P, Kleiner DE, Dam-Larsen S *et al.* Liver fibrosis, but no other histologic features, is associated with long-term outcomes of patients with nonalcoholic fatty liver disease. *Gastroenterology* 149(2), 389–397.e10 (2015).
- 14 Hardy T, Zeybel M, Day CP *et al.* Plasma DNA methylation: a potential biomarker for stratification of liver fibrosis in non-alcoholic fatty liver disease. *Gut* doi:10.1136/gutjnl-2016-311526 (2016) (Epub ahead of print).
- 15 Zhao Q, Fan YC, Zhao J, Gao S, Zhao ZH, Wang K. DNA methylation patterns of peroxisome proliferator-activated receptor gamma gene associated with liver fibrosis and inflammation in chronic hepatitis B. *J. Viral Hepat.* 20(6), 430–437 (2013).
- 16 Lehmann-Werman R, Neiman D, Zemmour H *et al.* Identification of tissue-specific cell death using methylation patterns of circulating DNA. *Proc. Natl Acad. Sci. USA* 113(13), E1826–E1834 (2016).
- 17 Akirav EM, Lebastchi J, Galvan EM *et al.* Detection of beta cell death in diabetes using differentially methylated circulating DNA. *Proc. Natl Acad. Sci. USA* 108(47), 19018–19023 (2011).
- 18 Olsen JA, Kenna LA, Spelios MG, Hessner MJ, Akirav EM. Circulating differentially methylated amylin DNA as a biomarker of beta-cell loss in Type 1 diabetes. *PLoS ONE* 11(4), e0152662 (2016).
- 19 De Mattos-Arruda L, Mayor R, Ng CK *et al.* Cerebrospinal fluid-derived circulating tumour DNA better represents the genomic alterations of brain tumours than plasma. *Nat. Commun.* 6, 8839 (2015).
- 20 Roadmap Epigenomics C, Kundaje A, Meuleman W *et al.* Integrative analysis of 111 reference human epigenomes. *Nature* 518(7539), 317–330 (2015).