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DNA methylation signatures in circulating cell-free DNA as biomarkers for the early detection of cancer

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Detecting cell-free DNA (cfDNA) or circulating tumor DNA (ctDNA) in plasma or serum could serve as a "liquid biopsy", which would be useful for numerous diagnostic applications. cfDNA methylation detection is one of the most promising approaches for cancer risk assessment. Here, we reviewed the literature related to the use of serum or plasma circulating cell-free DNA for cancer diagnosis in the early stage and their power as future biomarkers.

circulating cell-free DNA, DNA methylation, diagnostic markers, cancer detection

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INTRODUCTION

"Liquid biopsy" has received enormous attention due to its obvious clinical implications for personalized medicine. It mainly contains circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA) detection in the blood from cancer patients. Liquid biopsy has great advantages by contrast of tissue biopsy: non-invasive (don't cause dissemination), real-time (track the dynamic changes of tumor), monitor the drug efficacy, highly sensitive and specific etc.

The cell-free DNA (cfDNA) includes both coding and noncoding genomic DNA (gDNA)which can be used to detect microsatellite instability, loss of heterozygosity (LOH), tumour-specific gene mutations, genetic polymorphisms, DNA methylation and integrity (size) (Schwarzenbach et al., 2011). The panel of DNA methylation also could be a type of biomarker for early cancer detection. In comparison to genetic alterations (such as mutations), usually distributed throughout the gene, DNA methylation alterations are concentrated in defined areas, often on the promoter, facilitating analysis. In a word, ctDNA is a new promising biomarker in oncology, with potential clinical applications for monitoring and for comprehensive molecular profiling of cancer (Figure 1).

POTENTIAL DNA METHYLATION BIOMARKERS FOR CANCER DETECTION

Unlike a few other biomarkers used for cancer detection, such as prostate specific antigen (PSA), ctDNA has come directly from a particular cancer—it cannot come from anywhere else—and benefited from technology advances, it can be analyzed at lower cost, and with better accuracy and easier to obtain than CTCs.

Tumor DNA can be released from primary tumors, CTCs, micrometastasis, or overt metastases into the blood of cancer patients. The majority of such ctDNA is derived from apo-

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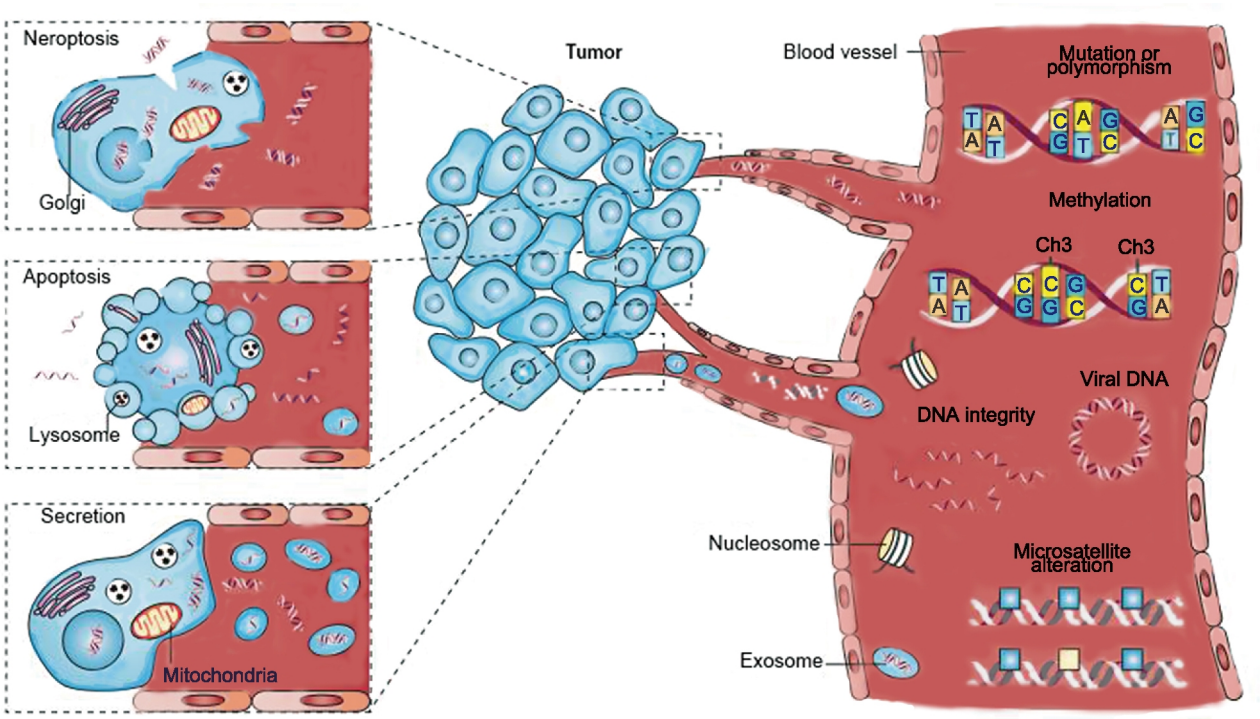

Figure 1 (Color online) Cell-free nucleic acids in the blood (adapted from Schwarzenbach et al., Cell-free nucleic acids as biomarkers in cancer patients, 2011) (Schwarzenbach et al., 2011). ctDNA is mainly released by tumor cells in different forms in blood. Methylation, mutations, microsatellite alterations and LOH can be detected in ctDNA. The length of most of the cell-free DNA in healthy people is between 70 to 200 bp, but ctDNA, which from patients with tumors may have a length of 200 bp or even more than 1 kb.

ptotic and necrotic tumor cells. They release their fragmented DNA into the circulation (Swaminathan and Butt, 2006). CTCs liquid biopsy assay has high specificity and CTC demonstrates co-localization of signal as an integrated cell compared with ctDNA. However, several limitations still existed during CTCs liquid biopsy assay, such as low signal-to-noise in early-stage disease and heterogeneity effect (Ignatiadis et al., 2015). By contrast, ctDNA assay is disease-specific, treatment-specific and personalized. It is likely to be more sensitive to serve as a new type of non-invasive biomarker for early cancer detection. Unlike CTC capture, ctDNA enrichment doesn't depend on special equipment. Up to date, whether ctDNA could act as complementary or alternative biomarkers to CTCs remains unclear and needs further examination.

CtDNA methylation aberrant has been described and investigated for clinical applications in most cancer types. The ctDNA methylation is consistent among cancer patients compared with genetic alterations. With recent advances in sequencing related technology, ctDNA detectable in the body fluids of prostate cancer patients, may offer a solution and provide information regarding the diagnosis and prognosis of the patient, allowing appropriate and timely treatment to be administered. Several available ctDNA methylation detection methods are shown in Table 1. Currently, the most prevalent methods are MSP (methylation-specific PCR) and Q-PCR. It could be a challenge for researchers to select and implement appropriate methods for DNA methylation studies. Sergey et al. provides a concise guide for choosing a proper method for a specific project using a simple algorithm (Kurdyukov and Bullock, 2016). PCR-based approaches are based on inexpensive specialized equipment and could be implemented in a molecular genetics laboratory easily. It also has lower burden of false discoveries and the ability to confirm a large number of region of interests (ROIs) identified in genome-wide screening of a few samples. MSP is effective for detecting methylated or unmethylated alleles without quantification and that method is high sensitivity, low specificity but no quantitative. Bisulfite sequencing with NGS is rapidly becoming a more affordable option, and it is inevitable that this will become a standard technology based on DNA methylation profiling (Hernández et al., 2013).

The greatest technical challenge is to identify very low amounts of ctDNA in blood samples with variable amounts of cfDNA and choose the right panel of cancer-specific genomic aberrations. In the past three decades, DNA methylation was recognized as the promising biomarker for cancer detection (Feinberg, 2014; Laird, 2003). In contrast to DNA mutations, aberrant methylation of specific promoter regions can be consistent feature of cancer, which typically occur at a wide variety of sites (Chimonidou et al., 2014). Because of its consistency, ctDNA methylation is suitable for the design of broadly applicable clinical assays (Sun et al., 2015; Warton et al., 2016). Small differentially methylated reg-

Table 1 Available ctDNA methylation detected methods. (Adapted from Laird principles and challenges of genomewide DNA methylation analysis 2010) (Laird, 2010)[a)]

Pretreatment	Analytical step			
	Locus-specific analysis	Gel-based analysis	Array-based analysis	NGS-based analysis
Enzyme digestion	–	–	DMH MCAM MMASS	Methyl-seq MCA-seq
Affinity enrichment	MeDIP-PCR	–	MeDIP mDIP mCIP MIRA	MeDIP-seq MIRA-seq
Sodium bisulphite	MethyLight EpiTYPER Pyrosequencing	Sanger BS MSP MS-SNuPE COBRA	BiMP	RRBS BC-seq BSPP WGSBS

a) BC-seq, bisulphite conversion followed by capture and sequencing; BiMP, bisulphite methylation profiling; BS, bisulphite sequencing; BSPP, bisulphite padlock probes; COBRA, combined bisulphite restriction analysis; DMH, differential methylation hybridization; EpiTYPER assay is based on proven bisulfite-conversion biochemistry followed by PCR and a proprietary base-specific cleavage process; MCA, methylated CpG island amplification; MCAM, MCA with microarray hybridization; MeDIP, mDIP and mCIP, methylated DNA immunoprecipitation; MethyLight, a high-throughput methylation assay that utilizes highly sensitive and accurate fluorescence-based real-time PCR; MIRA, methylated CpG island recovery assay; MMASS, microarray-based methylation assessment of single samples; MS-SNuPE, methylation-sensitive single nucleotide primer extension; NGS, next-generation sequencing; RRBS, reduced representation bisulphite sequencing; -seq, followed by sequencing; WGSBS, whole-genome shotgun bisulphite sequencing.

ions (DMRs), such as CpG island shore methylation and large block (hypomethylated blocks), appear at an early stage. They have the potential value as early cancer detection biomarkers (Timp et al., 2014).

In comparison to genetic alterations such as mutations, which are usually distributed throughout the gene, DNA methylation alterations are concentrated in defined areas, often on the promoter, facilitating analysis.

DIAGNOSTIC VALUE OF THE METHYLATION SIGNATURE OF CELL-FREE CIRCULATING DNA

Circulating methylated DNA has been considered possible biomarkers for cancer detection, such as colorectal cancer, lung cancer, breast cancer, and pancreatic cancers (Pixberg et al., 2015). Several DNA methylation-based biomarkers are already commonly used in the clinic (Table 2), such as the testing of the DNA methylation status of *MLH1* for the diagnosis of Lynch Syndrome. Commercial products such as the Epi proColon test, analyzing methylation in the *SEPT9* gene for the population-wide screening for colorectal cancer (Church et al., 2014) and approved by the Chinese FDA in July 2015, and the Epi proLung (*SHOX2*) test (Ilse et al., 2014) are now available.

Panels with multiple genes can further improve the specificity and sensitivity of DNA methylation signatures and it has demonstrated as a stool-based screening test for colorectal cancer recently (Imperiale et al., 2014), where DNA methylation alterations were combined with mutation detection and which received the first US-FDA approval for a DNA methylation-based diagnostic test in 2014.

Although histological and cytological examinations are the gold standard in lung cancer diagnosis currently, patients are often confirmed diagnosis at late stages. Therefore, there is an urgent need to create new diagnostic methods to increase the early diagnostic rate, enhance the confirmed diagnostic rate, monitor potential disease progression to cancer in high-risk populations, and improve the differentiation of lung cancer from other lung diseases to avoid overtreatment of patients.

DNA methylation changes have been identified with its potential therapeutic importance (Hagood, 2014; Kabesch and Adcock, 2012). Wielscher et al. develop a four gene DNA methylation signature for the lung cancer detection and its differentiation from other lung diseases with a high risk of cancer development, such as *COPD* and *ILD*, in retrospective cohorts and provide a paradigm (Wielscher et al., 2015). It will be important to combine and compare the signature developed by Wielscher et al. with DNA methylation markers such as *SHOX2* and other previously reported markers as well as signatures that have been developed for the early detection and prognosis of lung cancer. The presented work is a first major step potentially allowing the detection of lung cancer at early stages, and more importantly the monitoring of at-risk populations for progression into lung cancer. The aberrant DNA methylation in exon 1 of $p16^{INK4a}$ in plasma DNA of these patients is identical to the alteration present in the corresponding carcinomas indicates that hypermethylated plasma DNA derives from the primary tumor (Silva et al., 1999).

Hypermethylation of promoter CpG islands is now recognized as an important and early event in carcinogenesis. Detection of methylated DNA in serum or plasma has been suggested as a marker for early cancer development. Promoter

Table 2 Some representative DNA methylation-based biomarkers used in the clinic

DNA methylation sites	Diseases	Related information	Reference
SEPT9	Colorectal cancer	Epi proColon test; Approved by the Chinese FDA in July 2015	(Church et al., 2014)
NDRG4, *BMP3*	Colorectal cancer	Panels with multiple methylated genes combined with mutation; Received the first US-FDA approval in 2014	(Imperiale et al., 2014)
SHOX2	Lung cancer	Epi proLung test; Approved by the Chinese FDA in July 2015	(Ilse et al., 2014)
HOXD10, *PAX9*, *PTPRN2*, *STAG3*	Lung cancer	Four gene DNA methylation signature	(Wielscher et al., 2015)
Exon 1 of $p16^{INK4a}$	Lung cancer	Aberrant DNA methylation in the primary tumor	(Silva et al., 1999)
Promoter of *RASSF1A*	Breast cancer	Hypermethylation among high-risk women	(Yazici et al., 2009)
Promoters of *GSTP1*, *EDNRB* and *APC*	Prostate cancer	Panels with multiple methylated genes	(Bastian et al., 2008; Goessl et al., 2001; Rogers et al., 2006)
MLH1	Lynch syndrome Endometrial cancer	Showed better performance characteristics than *BRAF V600E* mutation	(Bruegl et al., 2014; Pérez-Carbonell et al., 2010)

hypermethylation aberrant of *RASSF1A* in serum/plasma DNA may be common among high-risk women and present years before cancer diagnosis (Yazici et al., 2009). In conclusion, it is the first study to show epigenetic changes in plasma DNA of patients who gave blood years before breast cancer diagnosis. *RASSF1A* promoter methylation was more frequent in cases compared with population based controls.

CfDNA carrying tumor-specific alterations (circulating tumor DNA) has not been extensively investigated or compared with other circulating biomarkers (cancer antigen 15-3 (CA 15-3), circulating tumor cells) in breast cancer. CtDNA is an informative, inherently specific, and highly sensitive biomarker of metastatic breast cancer. Metastatic breast cancer ctDNA detection showed superior sensitivity to that of other circulating biomarkers and has a greater dynamic range that correlates with changes in tumor burden than CA 15-3 or CTCs (Dawson et al., 2013). CA 15-3 is a serum biomarker that is clinically useful in metastatic breast cancer detection whereas with a sensitivity of only 60 to 70% (Duffy et al., 2010). Changes in the levels of circulating nucleic acids have been associated with tumor burden and malignant progression.

The circulating methylated DNA has been applied as a biomarker in various forms of cancer, such as: Bladder methylation (Valenzuela et al., 2002), Breast cancer (Fiegl et al., 2005; Rykova et al., 2004; Sharma et al., 2010a; Sharma et al., 2010b), Cervical cancer (Ren et al., 2006), Colorectal cancer (deVos et al., 2009; Grützmann et al., 2008; He et al., 2010; Lecomte et al., 2002; Tänzer et al., 2010), Hepatocellular carcinoma (Chan et al., 2008; Wang et al., 2006; Wong et al., 2000), Lung cancer (An et al., 2002; Bearzatto et al., 2002; Liu et al., 2003; Ng et al., 2002; Ramirez et al., 2005), Ovarian cancer (Melnikov et al., 2009a; Müller et al., 2004), Pancreatic cancer (Liggett et al., 2010; Melnikov et al., 2009b), Prostate cancer (Bastian et al., 2005; Bryzgunova et al., 2008; Goessl et al., 2001; He and Bishop, 2016; Jernimo et al., 2002; Rouprêt et al., 2008; Sunami et al., 2009). Promising promoter hyper-methylation markers include: *EDNR* (Rogers et al., 2006), *GSTP1* (Bastian et al., 2008; Goessl et al., 2001) and *MDR* (Bastian et al., 2008) with sensitivity above 70% and specificity of 100% for the diagnosis of prostate cancer. This panel would likely include the assessment of methylation status in gene promoter regions within the *EDNR*, *GSTP1* and *MDR* genes.

Taken together, the panel of cfDNA methylation offers great promise for clinical research and medical diagnostics using information on DNA methylation status. However, there is a need to standardize sample processing and detection procedures, and to establish cut-off values or a range of values that can be used to guide diagnoses.

OPPORTUNITIES ON THE PATH TO CLINICAL UTILITY

Liquid biopsy overcomes tumor heterogeneity for personalization therapy and can be used for cancer detection before imagological diagnosis (Karampini and McCaughan, 2016; Ma et al., 2015). Genomic, epigenetic and transcriptomic levels may varies in the same patient who have metastatic or primary tumors. Despite the challenges in ctDNA clinical application, ctDNA detection has promise as a real-time repeatable method, giving its advantages of non-invasive, non-injurious, highly sensitive and specific. Also, ctDNA might be more promising biomarkers than protein biomarkers as they

may be more informative, accurate and specific. Current research evidence suggested that it is potential to use gene promoter methylation as a diagnostic biomarker and the quantification of ctDNA as a prognostic biomarker in cancer patients. In addition, advanced technology makes it possible to detect thousands of CpG sites, and whole-genome sequence, which can be applied to bisulfite converted ctDNA, can also be used for cancer detection (Mack et al., 2014; Pan et al., 2012). As identifying differentially methylated regions becomes easier, they are likely to be used as cancer biomarkers (Hoque et al., 2006; Powrózek et al., 2014; Shivapurkar and Gazdar, 2010; Warton et al., 2016). The emergence of personalized medicine will improve bio-banking procedures, which could facilitate the acquisition of clinical samples from more patients and controls. This would create more opportunities for cancer biomarkers discovery and improve the sensitivity and specificity. CfDNA carrying tumor-specific alterations (ctDNA) and aberrant DNA methylation has not been extensively investigated or compared with other circulating biomarkers in cancers. We believe that ctDNA assays may be used to personalize real-time treatment based on ctDNA or ctDNA methylation levels for cancer diagnosis, prognosis, and guidance for treatment in the future. The combination of DNA methylation markers, ctDNA concentrations and other genetic modifications (such as DNA integrity) may be informative with respect to cancer diagnosis.

Compliance and ethics *The author(s) declare that they have no conflict of interest. This manuscript is a review article and does not involve a research protocol requiring approval by the relevant institutional review board or ethics committee.*

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